Effectiveness of Sulforaphane in Increasing Drug Mediated Cytotoxicity Toward Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma

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Contents

ABSTRACT

Cancer is the leading cause of death in Canada with the head and neck cancer (HNC) ranked seventh in the rate of incidence. More than 90% of HNCs are squamous cell carcinomas (HNSCC) with an overall survival rate of 64.5%. One suggested cause for cancer treatment failure is the limitation of chemotherapy (CT) efficacy by its severe toxic side effects as the conventional treatment will cause damage to non-cancerous cells along with the cancer cells. Thus, reducing the dose of used CT while maintaining its efficiency is critical for improving the treatment outcome of HNSCC. Another possible cause for treatment failure is the presence of a subpopulation of cells inside the tumor that is highly treatment-resistant and able to cause recurrence termed cancer stem cells (CSCs). A plausible way to improve HNSCC treatment is to identify, isolate and target these CSCs.

The first aim of this thesis was to find if we can improve the efficacy of conventional chemotherapy; Cisplatin (CIS) and 5-Fluorouracil (5-FU), using a natural product Sulforaphane (SF), which is extracted from broccoli. We combined low doses of CT with SF in a dose concentration that can be achievable by oral ingestion of broccoli sprouts. This combined treatment was tested in-vitro on HNSCC cell lines SCC12 and SCC38 and on non-cancerous human cell line and primary cells for 3 days. Our results demonstrated that SF increased the cytotoxicity of CIS and 5-FU significantly by decreasing viability, proliferation, DNA repair after treatment and increasing apoptosis through activation of Caspase-dependent apoptosis pathway with no effect on non-cancerous cells. In conclusion, SF combined treatment can be a safe method to enhance chemotherapy and improve the patient's life quality.

The first step to target CSCs with any new treatment modality is to identify these cells and characterize them. The second aim of this thesis was to analyze the expression of CSCs cell markers CD44 and CD271 in HNSCC. The results showed that CD271+ cells are a subpopulation of CD44+ cells. In addition, CD44+/CD271+ cells have higher proliferation and growth rate, more treatment resistance and more tumorigenic in-vitro and in-vivo compared to CD44+/CD271- cells or the total cells population. These results suggest that CD271 is a more precise marker to identify HNSCC-CSCs compared to the widely used CD44.

Utilizing the data we collected from the first two parts of our project, we targeted the HNSCC-CSCs using SF. The third part of this thesis examined if the combination of SF with conventional CT, such as CIS and 5-FU, would increase its efficacy on CSCs as was reported for the total cellular population in HNSCC. The results demonstrated that SF increased the cytotoxicity of CIS and 5-FU toward HNSCC-CSCs both in-vitro and in-vivo, inhibited proliferation and tumorigenicity and prevented the elevation of the expression of stem cell (SC) related genes, such as BMI-1 and ALDh1A1, with conventional chemotherapy.

In summary, SF has a strong anti-cancerous effect. SF can augment the effect of CIS and 5-FU against HNSCC. Combining CD44 and CD271 cell markers are more reliable to isolate CSCs from HNSCC as compared to CD44 alone. Finally, SF proved to be a very promising anti-cancer stem cells therapy either alone or as a combination with conventional chemotherapy. This naturally derived chemical has great potential for future clinical applications.

RÉSUMÉ

Le cancer cause la majeure partie des décès au Canada. Le cancer de la tête et du cou (CTC) se situe en matière d'incidence au septième rang. Plus de 90% des CTCs sont des carcinomes épidermoïdes (CETC) avec un taux de survie global de 64,5%. De nombreux effets secondaires toxiques limitent l'efficacité de la chimiothérapie (CT) et contribuent principalement à l'échec des traitements. Les traitements chimiothérapiques conventionnels affectent en même temps les cellules saines et cancéreuses. Afin d'améliorer les résultats du traitement des CETC, nous devrions réduire la dose chimiothérapeutique tout en maintenant son efficience. À l'intérieur de la tumeur, une sous population des cellules souches cancéreuses (CSC) existe qui résiste hautement au traitement et provoque des récidives. Cette sous-population est associée à l'échec du traitement. Identification, l'isolation et le ciblage des CSC amélioreraient le traitement au CETC.

Le premier objectif de cette thèse était d'utiliser un produit extrait du brocoli, le Sulforaphane (SF), comme adjuvant au traitement conventionnel Cisplatine (CIS) et le 5-Fluorouracile (5-FU) et de déterminer son efficacité. Nous avons combiné de faibles doses de CT avec le SF à une concentration similaire à des quantités consommables de germes de brocoli. Ce traitement combiné a été testé in vitro sur les lignées cellulaires de CETC SCC12 et SCC38 ainsi que sur des lignées cellulaires humaines non cancéreuses et des cellules primaires pendant 3 jours. Le SF a significativement augmenté la cytotoxicité des traitements au CIS et du 5-FU. Le SF a diminué la viabilité, la prolifération, la réparation de l'ADN après traitement et a augmenté

l'apoptose par l'activation de la voie de l'apoptose dépendante de la Caspase sans effet sur les cellules non cancéreuses. En conclusion, le traitement combiné par SF pourrait améliorer la chimiothérapie et la qualité de vie du patient.

Pour toute nouvelle modalité de traitement pour cibler les CSC, la première étape consiste à leur identification et caractérisation. Le deuxième objectif de cette thèse était d'analyser avec deux méthodes d'analyse d'expression les marqueurs CD44 et CD271 dans les CSC et dans les cellules de CETC. Les résultats ont montré que les cellules +CD271 sont une sous-population de cellules +CD44. De plus, les cellules +CD44/ +CD271 ont un plus haut taux de prolifération, de croissance, de résistance au traitement et à former des tumeurs in vitro et in vivo par rapport aux cellules +CD44 / -CD271 ou à la population de cellules totales. Ces résultats suggèrent que le marqueur CD271 identifie plus précisément les CSC des CETC par rapport au marquage unique au CD44 largement utilisé.

En utilisant les données que nous avons collectées au cours des deux premières parties de notre projet, nous avons ciblé les CSC-HNSCC à l'aide de SF. La troisième partie de cette thèse a consisté à examiner un traitement combinatoire du SF avec les traitements conventionnels, CIS et 5-FU pour cibler les CSC. Nous voulions mesurer son efficience et comparer les résultats à ceux rapportés précédemment avec les cellules CETC. Nos résultats ont démontré que le SF augmentait la cytotoxicité du CIS et de 5-FU vis-à-vis les CSC des CETC à la fois in vitro et in vivo. Ce traitement inhibait la prolifération, la formation de tumeur et empêchait l'élévation

de l'expression de gènes liés aux cellules souches, tels que BMI-1 et ALDh1A1. Une amélioration par rapport aux chimiothérapies conventionnelles.

En résumé, le SF a un puissant effet anticancéreux. Chez l'humain, SF pourrait augmente l'efficacité des traitements conventionnelle au CIS et au 5-FU contre les CETC. La combinaison CD44 / CD271 est un marqueur plus fiable pour isoler les CSC du CETC par rapport à l'utilisation unique du CD44. Enfin, la thérapie par le SF seule ou combinée aux chimiothérapies conventionnelles s'est avérée très prometteuse contre les CSC. Ce produit chimique dérivé naturel a un grand potentiel clinique

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Originality & Author contributions:

This dissertation includes five manuscripts prepared by the candidate as the first author. Two are a literature review one is published in The Saudi Dental Journal and the other is manuscript has been submitted and under review in Current medical sciences. The other three manuscripts are original research articles. Two of them are published in Medical Oncology Journal and Carcinogenesis Journal and the last one is submitted and under review in British Journal of Cancer. In all these manuscripts, the PhD candidate made major contributions either in the design and experiments, performing the technical procedures, data collection and analysis, or writing the manuscripts. All the co-authors also played a significant role in work included in this Ph.D. thesis. A statement of the contribution of the candidate and the co-authors is provided below for each of these manuscripts.

Chapter two: Head and neck cancer management and cancer stem cells implication.

Osama A. Elkashty, Ramy Ashry, Simon D. Tran.

The candidate participated in determining the design of this work, reviewing the literature to select the appropriate papers, organizing topics and subtopics, and drafting the manuscript (90% of the work). Ramy Ashry assisted in in the study conception and design, acquisition of data (5% of the work). Simon D. Tran assisted in drafting of manuscript, critical revision and final approval of the manuscript (5% of the work). All authors reviewed the manuscript.

Originality: This work illustrates the current knowledge on the epidemiology, etiology, and management of head and neck cancer. Our review also covers in depth cancer stem cells (CSCs), their relations to head and neck cancer, and how CSCs affect cancer management and treatment

outcome. This manuscript has been published in The Saudi Dental Journal 2019; 31: 395-416. https://doi.org/10.1016/j.sdentj.2019.05.010.

Chapter three: Sulforaphane as a natural molecule in cancer prevention and treatment.

Osama A. Elkashty, Simon D. Tran.

The candidate participated in determining the design of this work, reviewing the literature to select the appropriate papers, organizing topics and subtopics, and drafting the manuscript (95% of the work). Simon D. Tran assisted in drafting of manuscript, critical revision and final approval of the manuscript (5% of the work). All authors reviewed the manuscript.

Originality: This work reviewed the current knowledge about Sulforaphane sources, its metabolism, and its cancer prevention and anti-cancer effect. This study also covered Sulforaphane's effect on cancer stem cells and the current and future human clinical applications in cancer. The study presented in this chapter has been submitted to Current Medical Sciences Journal and accepted with minor revisions.

Chapter five: Broccoli extract improves chemotherapeutic drug efficacy against head-neck squamous cell carcinomas.

Osama A. Elkashty, Ramy Ashry, Ghada Abu Elghanam, Hieu M. Pham, Xinyun Su, Camille Stegen and Simon D. Tran.

The candidate designed and performed all experiments and data analysis and wrote the manuscript (80% of the work). Ramy Ashry assisted in experiment design and proof-read the manuscript (5% of the work). Ghada Abu Elghanam assisted in experiment design and proof-read the manuscript (5% of the work). Hieu M. Pham assisted in designing and performing the proliferation experiments (4% of the work). Camille Stegen assisted in designing experiment and performing the flow cytometry analysis and proof-read the manuscript (2% of the work). Simon Tran designed and supervised the whole study and wrote and final approved the manuscript (4% of the work). All authors reviewed the manuscript.

Originality: This is the first study to test the combination of Sulforaphane with conventional chemotherapy, Cisplatin or 5-Fluorouracil, on the head and neck squamous cell carcinoma. Indeed, we were able to show that Sulforaphane can increase the cytotoxic effect of Cisplatin and 5-Fluorouracil against head and neck cancer with minimal to no toxicity on the non-cancerous cells. The reported results may be of great importance in the clinical settings as it allows for reduced doses of conventional chemotherapy with high efficacy. This manuscript has been published in the Journal of Medical Oncology, 2018 Aug 4;35(9):124. doi: 10.1007/s12032-018-1186-4.

Chapter six: Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas

Osama A. Elkashty, Ghada Abu Elghanam, Xinyun Su, Younan Liu, Peter J. Chauvin, Simon D. Tran.

The candidate designed and performed all experiments and data analysis and wrote the manuscript (80% of the work). Ghada Abu Elghanam assisted in the in-vivo experiments and data analysis (5% of the work). Xinyun Su and Younan Liu assisted in designing and performing treatment resistance experiments (5% of the work). Peter J. Chauvin assisted in obtaining and examining the human tissue (2% of the work). Simon Tran designed and supervised the whole study and wrote and final approved the manuscript (3% of the work). All authors reviewed the manuscript.

Originality: This study used two cancer stem cell markers, CD44 and CD271, to identify and isolate cancer stem cells from head and neck cancer cell lines. In this work we showed that CD271+ cells are actually a subpopulation of CD44+ cells. Also, CD44+/CD271+ cells possess higher cancer stem cells characteristics and higher treatment resistance compared to the CD44+/CD271- cells. These results suggest using CD271 marker as more precise marker than CD44 to isolate cancer stem cells from head and neck cancers. This manuscript has been published in in Carcinogenesis Journal 2019, bgz182, https://doi.org/10.1093/carcin/bgz182.

Chapter seven: Broccoli Extract Increases Drug-mediated Cytotoxicity Toward Cancer Stem Cells of Head and Neck Squamous Cell Carcinoma

Osama A. Elkashty, Simon D. Tran.

The candidate designed and performed all experiments and data analysis and wrote the manuscript (95% of the work). Simon Tran designed and supervised the whole study and wrote and final approved the manuscript (5% of the work). All authors reviewed the manuscript.

Originality: This study tested the effect of Sulforaphane either alone or combined with conventional chemotherapy, Cisplatin and 5-Fluorouracil on cancer stem cells in head and neck cancer for the first time. Our results suggest that Sulforaphane increased the cytotoxic effect of the conventional treatment against cancer stem cells and prevented their selection. Also, we showed for the first time that Sulforaphane combination with chemotherapy is relatively safe with non-cancerous stem cells as it did not affect its viability or function. This manuscript has been submitted and under review to British Journal of Cancer.

ABBREVIATIONS

ABC	ATP-binding cassette transporters
ABCB1	ATP Binding Cassette Subfamily B Member 1
Akt	Protein Kinase B
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APAF1	Apoptotic protease activating factor-1
ARE	Anti-oxidant response element
ATC	Amplifying transitory cell
BAX	Bcl-2-associated X
Bcl2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma extra-large
BMI-1	B cell-specific Moloney murine leukemia virus integration site 1
CCRT	Concurrent chemoradiotherapy
CD44	Cluster of differentiation 44
CD44v3	CD44 variant isoform 3
Cdc25B	Cell division cycle 25B
CDK1	Cyclin-dependent kinase 1
CICs	Cancer-initiating cells
CIS	Cisplatin
CML	Chronic myeloid leukemia

CRT	Chemoradiotherapy
CSCs	Cancer Stem Cells
CT	Chemotherapy
CYP450	Cytochrome P450 oxidase
DMEs	Drug-metabolizing enzymes
DR5	Death receptor 5
DTCs	Dithiocarbamate
EBRT	External Beam Radiation Therapy
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPV	Epstein-Barr virus
FACS	Fluorescence-activated cell sorting
Gli	Glioma-Associated Oncogene
GSH	Glutathione
GSK3	Glycogen synthase kinase-3
GST	Glutathione S-transferases
HDAC-1	Histone deacetylase 1
HIFs	Hypoxia-inducible factors
HIV	Human immunodeficiency virus
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HO-1	Hemeoxygenase-1

HPV	Human papilloma virus
HSV1	Herpes simplex virus type 1
H&E	Hematoxylin and Eosin
IAP	Inhibitors of apoptosis
IGRT	Image-guided radiation therapy
IL-8	Interleukin-8
IL-6R	IL-6 receptor
IMBT	Intensity modulated brachytherapy
IMRT	Intensity-modulated radiotherapy
ITC	Isothiocyanates
I.P	Intraperitoneal injection
KDR/flk-1	Kinase insert domain receptor/ Fetal Liver Kinase 1
Keap1	Kelch-like ECH-associated protein 1
LSCC	Laryngeal squamous cell carcinoma
MDR1	Multidrug Resistance Protein 1
MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
MRP1	Multidrug resistance-associated protein 1
NAC	N-acetylcysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGFR	Nerve growth factor receptor
NOD/SCID	Non-obese diabetic with severe combined immunodeficiency disease

NPSCC	Nasopharyngeal squamous cell carcinoma
NQO1	NAD(P)h Quinine oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OARs	Organs at risk
Oct4	Octamer-binding transcription factor 4
OPSCC	Oropharyngeal squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
PARP	Poly (ADP-ribose) polymerase
PET	Positron emission tomography
P-gp	Permeability glycoprotein
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PROM1	Prominin 1
PTCH-1	Patched-1
Ras	Rat sarcoma gene
ROCK	Rho kinase
ROS	Reactive oxygen species
RT	Radiotherapy
SC	Stem cell
SF	Sulforaphane
SHH	Sonic hedgehog
Smo	Smoothened

SOX2	SRY (sex determining region Y)-box 2
SP	Side population
STAT3	Signal transducer and activator of transcription 3
SXR	Steroid and xenobiotic receptor
TF	Transcription factor
TKIs	Tyrosine kinase inhibitors
TNFα	Tumor necrosis factor-alpha
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TSCs	Tumor stem cells
UGTs	UDP-glucuronosyltransferases
VEGF	Vascular endothelial growth factor
ZEB-1	Zinc finger E-box-binding homeobox 1
5-FU	5-Fluorouracil

Chapter 1 - Introduction:

1.1 Thesis outline

This thesis is prepared in a manuscript-based format according to the guidelines issued by McGill University regarding Doctoral Thesis preparation, and it consists of ten chapters. Chapter one includes a general introduction and thesis rationale. Chapter two reviewed the current knowledge on the epidemiology, etiology, and management of head and neck cancer with special attention to cancer stem cells. Chapter three includes the current information about Sulforaphane sources, its metabolism, and its cancer prevention and anti-cancer effect with emphasis on cancer stem cells and human clinical trials. Chapter two and three are both manuscripts of review papers and together comprise the literature review for the thesis topic. Chapter four includes the thesis-hypothesis and main objectives. Chapter five, six and seven are the original research manuscripts prepared by the candidate illustrating the effect of Sulforaphane either alone or combined with conventional chemotherapy, Cisplatin or 5-Flourouracil, on head and neck cancer and cancer stem cells. The manuscripts also report a more precise marker, CD271, to isolate cancer stem cells from head and neck cancer compared to the widely used CD44. Chapter eight contains the overall discussion and conclusions. Chapter nine includes the list of references cited in this thesis. Chapter ten is the appendix showing the articles published by the candidate during his Ph.D. studies.

1.2 Research Rationale

HNSCC is now the seventh most common cancer worldwide as it accounts for almost 4.6% of cancer cases in the world [1]. Despite the improvements in treatment modalities, the five-year survival rate for HNSCC has remained unchanged at about 50% over the past 30 years

[1,2]. One of the reasons for cancer treatment failure is that the efficacy of current standard CT is restricted partly due to the severe toxic/side-effects. Acute side effects are the most important limitation for CT, but recently there is growing evidence of higher rates of late toxicity side effects as well [3-5]. This includes the most widely used Cisplatin and 5-Fluorouracil. CIS forms DNA adducts which lead to induction of apoptosis in cancer cells [6] and the toxic side effects of CIS are dose-dependent, including nephrotoxicity, otological dysfunction, bone marrow suppression with hemolytic anemia, and neurotoxicity in the form of visual disturbances [7-9]. 5-FU inhibits the thymidylate synthase enzyme through its metabolite to inhibit cancer cells division [10] and the side effects of 5-FU range from the commonly occurring diarrhea, nausea, vomiting, neutropenia, and thrombocytopenia to the less common but more severe dermatologic effects, hand and foot syndrome, cardiotoxicity and neurotoxicity [11]. More research is needed into patient satisfaction and quality of life after receiving CRT for HNC [12,13].

Other reason for cancer treatment failure is related to the presence of a subpopulation of cells in the tumor called cancer stem cells which are suggested to have tumor-initiating potential, combined with the ability of self-renewal and multilineage differentiation [14,15]. CSCs possess several characteristics of normal stem cells [16,17]. For example, CSCs undergo self-renewal, maintain quiescence, show multipotentiality, and exert survival protein/anti-apoptosis proteins [16,17]. Another well-known characteristic of CSCs is their ability to expand their number by cell proliferation/survival and/or clone formation and differentiation [16,17]. CSCs may also show chemotherapy resistance, which causes a recurrence of cancers [18]. Thus, correct identification and isolation of these cells are the first steps to target them. The CD44+ population is shown to contain the CSC subpopulation, as the purified CD44+ cells from the primary tumors can give rise to tumors faster and by injecting less cell number in xenograft model compared to CD44- cells, and these xenograft tumors subsequently reproduce the original tumor heterogeneity observed in the primary tumor. CD44+ cell population has a greater capacity to handle oxidative stress and, as such, is more radioresistant [19]. On the other hand, in normal human oral epithelium, we can find a subpopulation of cells expressing a cell surface molecule designated as the CD271 antigen with stem cell–like properties as they [20,21].

Recently, this molecule was identified as a marker of CSCs in many tumors, such as human melanoma [22,23], esophageal carcinoma [24,25], and hypopharyngeal carcinoma [26]. Besides being expressed in discrete cells within the basal layer of normal oral epithelium, CD271 is also found in oral dysplasia and OSCC [27].

According to many studies, phytochemical is a potential source of therapeutics for diverse types of cancers and CSCs elimination [28]. Sulforaphane, which is obtained by hydrolysis of glucoraphanin, is the most characterized isothiocyanate compound which is found in high concentrations in cruciferous vegetables [29]. Approximately, 60%–80% of the metabolized glucoraphanin is converted to SF [30], with most broccoli variations contain between 0.1 and 30 µmol/g of glucoraphanin. Several studies in recent years have shown that SF has multiple biological activities, such as anti-inflammation, antioxidation and anticancer effects [31-33]. In addition, this compound is safe and associated with low toxicity [34], making it an excellent candidate as a chemotherapeutic agent [35]. SF has been demonstrated to target multiple pathways involved in cancer cells and can interfere at various levels of the carcinogenetic process either alone or in combination with other anticancer compounds. SF augmented the effect of imatinib and gemcitabine against CML cells and pancreatic cancer cells, respectively [36,37]. Georgios Kallifatidis in 2011 found that SF targeted the Notch pathway which inhibited the self-renewal ability of pancreatic CSCs. This effect was augmented by the combination of SF with Gemcitabine [36]. In 2012, Mariana Rodova reported that SF down-regulated the SHH pathway in pancreatic cancer also by inhibiting Smo, Gli1, and Gli2 [38]. Chia-Ming Liu in 2017 reported the inhibitory effect of SF on BMI-1 protein by up-regulation of miR-200c [39]. A diet containing three to five servings of broccoli per week is reported to be sufficient to decrease the risk of cancer development by almost 30%–40% [40]

This dissertation introduces the current knowledge about HNSCC treatment modalities and CSCs implications along a detailed review about Sulforaphane's anti-cancer effects. This is followed by an in-depth investigation on the effect of combining Sulforaphane with the conventional chemotherapy to target HNSCC cancer stem cells that was isolated using CD44 and CD271 markers.

Literature review:

Chapter 2 - Head and neck cancer management and cancer stem cells implication

2.1 Preface (connecting paragraph)

As we mentioned in the introduction section, cancer is one of the major causes of death worldwide and in Canada. Head and neck cancers are a group of cancers that arise in the head and neck region with 90% of those being squamous cell carcinoma. Head and neck cancer is considered one of the most prevalent cancers in Canada especially among men.

In this chapter, we reviewed the current knowledge on the epidemiology, etiology, and management of head and neck cancer. Our review also covers in depth cancer stem cells (CSCs), their relations to head and neck cancer, and how CSCs affect cancer management and treatment outcome.

The study presented in this chapter has been published in The Saudi Dental Journal 2019; 31: 395-416. https://doi.org/10.1016/j.sdentj.2019.05.010.

HEAD AND NECK CANCER MANAGEMENT AND CANCER STEM CELLS IMPLICATION

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Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Abstract

Head and neck squamous cell carcinomas (HNSCCs) arise in the mucosal linings of the upper aerodigestive tract and are heterogeneous in nature. Risk factors for HNSCCs are smoking, excessive alcohol consumption, and the human papilloma virus. Conventional treatments are surgery, radiotherapy, chemotherapy, or a combined modality; however, no international standard mode of therapy exists. In contrast to the conventional model of clonal evolution in tumor development, there is a newly proposed theory based on the activity of cancer stem cells (CSCs) as the model for carcinogenesis. This “CSC hypothesis” may explain the high mortality rate, low response to treatments, and tendency to develop multiple tumors for HNSCC patients. We review current knowledge on HNSCC etiology and treatment, with a focus on CSCs, including their origins, identifications, and effects on therapeutic options.

Keywords: Head and Neck cancer; Carcinoma, squamous cell; Cancer stem cells, Cancer treatment, Antineoplastic agents.

Abbreviation:

ABC, ATP-binding cassette transporters; ATC, Amplifying transitory cell; BMI-1, B cell-specific Moloney murine leukemia virus integration site 1; EGFR, Epidermal growth factor receptor; HIFs, Hypoxia-inducible factors; MDR1, Multidrug Resistance Protein 1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; TKIs, Tyrosine kinase inhibitors;

2.2 Introduction and epidemiology of head and neck cancer:

Head and neck cancers (HNC) are a group of cancers that arise in the oral cavity, pharynx, larynx, paranasal sinuses, nasal cavity, salivary glands, or head and neck lymph nodes [41]. HNC is the seventh most common cancer worldwide with around 600,000 new cases annually [1,42] and unacceptably high rates of mortality, especially in developing countries, reaching 300,000 deaths each year [43]. More than 90% of the HNC is head and neck squamous cell carcinoma (HNSCC), variant that originates from the mucosal lining epithelium of the upper aerodigestive tract [1]. Of interest, it was reported that around 20% of oral squamous cell carcinoma patients will develop an upper aerodigestive tract secondary tumor [44].

The estimate is that about two-thirds of the HNSCC cases occur in developing areas such as south and south-east Asia [45]. This great variation in the global prevalence of HNSCC can be seen with the prevalence rate of 5-8% of total cancer cases in Europe and America [46,47] compared to over 30% in India [48]. Historically, black HNSCC patients had poorer prognosis, higher recurrence, and mortality rates when compared to non-black patients [49]. This might be

due to their lower socioeconomic status, difficulties for health care access, delayed diagnosis, and lower rates of surgical intervention [50]. Such a difference in the rate of incidence was reversed in the USA with less black HNSCC patients when compared to non-black ones starting in 1990. This, in part, can be explained by the fast-rising incidence of Human papillomavirus (HPV)-HNSCC which also have a high incidence in whites in the USA [51].

HNC, and specifically oral cancer, was always described as a disease of old age with most epidemiological studies describing higher incidence in the age group of fifty to seventy years old [52]. There were reports that only 5% of HNC patients are in the age group from twenty-five to forty years old. However recently, there is an increase in HNC incidence in younger age groups [53]. This is partially related to the increase in smoking and usage of other drugs at young age [54], as well as the recently common sexually-transmitted HPV [53].

Generally, HNC is more common in men by a 2-5 fold compared to women in most countries [55], because of likely higher tobacco usage among men [56]. However, since the 1950s there was an increase in the incidence of HNC in females associated with the increased smoking among them [57]. In the USA, oral squamous cell carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) ratios between men to women are currently about 1.5:1 and 2.8:1, respectively [58]. In Canada, OSCC and laryngeal squamous cell carcinoma (LSCC) ratios between men to women are currently about 2.2:1 and 5.3:1, respectively [59].

We review in this paper the etiological factors behind this cancer and the current and future directions of treatment with special attention to the cancer stem cells hypothesis, its relation to head and neck cancer, and how it affects the line of treatment.

2.3 Etiology and pathogenesis:

Although tobacco, alcohol, and HPV are the primary HNC risk factors, the etiology of such neoplasm is multifactorial, and many additional causes have been recognized [60].

2.3.1 Tobacco

Tobacco usage is the main etiological factor behind HNC as about 90% of the diagnosed HNC patients reported a history of tobacco consumption [61]. It is reported that tobacco users have a 5-fold increased risk of developing oral cavity, oropharynx and hypopharynx cancers and a 10-fold increase in developing laryngeal cancers when compared to non-users [62]. There is a close correlation between cigarette smoking duration, intensity and frequency and HNC rate of development in patients [61]. In the same context, the risk of HNC development greatly decreases with increasing the duration of cigarette smoking cessation [63].

Another primary HNC risk factor, in particular for oral cavity cancers, is smokeless tobacco, such as snuff or chewing tobacco [64]. Individuals, who have used smokeless tobacco, have an estimated 80% increase in the risk of developing oral cavity cancer. Countries in which there is a popular use of smokeless tobacco (including betel quid or areca nut with added tobacco) have an attributable fraction of oral cavity cancer which is as high as 53% in India and 68% in Sudan, compared to 7% in the USA [65]. The frequent use of Shamma, Zarda, and Khat has assorted the HNC to be one of the commonest malignancies in Yemen [66].

2.3.2 Alcohol

Between 1% to 4% of HNC cases are attributable solely to alcohol consumption [67]. Hypopharynx cancer is the most common type of HNC types that is related to alcohol consumption [68]. Alcohol drinking works synergistically with tobacco use, imposing a remarkable multiplicative impact in increasing the risk of HNC, [69] to a level greater than 35-fold for individuals who daily consume two or more cigarettes packs along with more than four alcoholic drinks [70].

2.3.3 Occupation

Some epidemiological studies have drawn a link between industrial employment and increasing the risk to develop HNC. Industrial jobs such involving occupational exposures to wood dust, acid mists, asbestos or solvents and jobs related to textiles and leather manufacturing have higher incidence HNC rates [70]. Sinonasal undifferentiated carcinomas, a rare cancer of the nasal cavity and/or paranasal sinuses could be related to occupational exposures to chromium, nickel, and radium [45].

2.3.4 Solar exposure

Prolonged sunlight exposure is considered as a major risk factor of potentially premalignant disorder such as actinic cheilitis and lip squamous cell carcinomas that arise in the epithelial layer of the lower vermillion border [71]. There is a marked resemblance of the risk factors of lip cancer to those of skin cancer. However, the risk for cancer of the lower vermillion border was reported positively correlated with increased exposure to solar ultraviolet radiation and not related to skin cancer [72].

2.3.5 Immunologic diseases

Tumor immune surveillance is the process through which the immune system can specifically identify cancerous or precancerous cells, depending on their expression of tumor-specific antigens or cellular stress inducing molecules, and eliminate them before they can develop or progress [73]. An increased risk of HNC might be attributed to suppression of the immunity secondary to solid organ transplantation or Human immunodeficiency virus (HIV) infection. There is roughly 10-fold rise of lip cancer incidence, and a more modest 2-5-fold increase of HNC incidence at other sites, after solid organ transplantation [74]. In a retrospective study from Switzerland, there was a 3-fold increase in the development of carcinomas of the lip, mouth, pharynx, and lung in HIV-positive patients [75].

2.3.6 Viral infection

Chronic viral infections in human cells could encourage the mounting of multiple mutagenic onslaughts, initiating the cells transformation process, and ultimately giving rise to malignant disease. Transformed cells often exhibit chromosomal aberrations which may result from the integration of the viral genome into chromosomes of the host cell [76]. HPV is a very important risk factor for HNC as up to 15-20% of all HNC are closely related to high-risk HPV infection [77]. Furthermore, HPV-DNA can be found in up to 70% of OPSCC especially that is located at the tonsils [78]. It has been suggested that there is a possible interaction between tobacco consumption, alcohol use or HPV16 and Herpes simplex virus type 1 (HSV1) in OSCC development [79]. The most acceptable method to assess HPV tumor status is the surrogate marker p16 immunohistochemistry [80,81]. Overexpression of this surrogate marker is strongly associated with transcriptionally active high-risk HPV. Positive cases show a threshold of at least 70 percent nuclear and cytoplasmic expression with moderate to strong intensity.

Another oncogenic double-stranded DNA virus, besides HPV, is the Epstein-Barr virus (EBV) which is one of the human herpesvirus family capable to persist lifelong in the human body [79]. The oncogenic potential of EBV has been reviewed in a wide variety of benign and malignant tumors development, however, it was less correlated to HNC except for the strong association with nasopharyngeal squamous cell carcinomas (NPSCC) [82]. Interestingly, a study showed that nearly 60% of OSCCs were EBV genome positive [83], and another study correlated the poorer OSCC prognosis to the increased expression of EBV [84].

2.3.7 Premalignant Lesions

Oral squamous cell carcinoma (OSCC) can arise de novo or arise from pre-existing potentially malignant disorders such as oral leukoplakia, erythroplakia, oral submucous fibrosis, and lichenoid dysplastic lesions [85]. Oral lichen planus has a malignant transformation rate ranging from 1 to 5.8%, in particular the erosive form [86-88]. Other authors reported a strong association between OSCC and the erosive form of the lichen planus [89,90]. It has been reported that OSCCs originating from leukoplakic lesions have indeed a more favorable prognosis than those evolving de novo,[91]; however, a more recent study reported that the prognosis of these two groups of OSCCs is insignificantly different [92].

2.3.8 Genetic and familial factors

The high susceptibility of cancer development is closely related to various human genetic mutations and genetic polymorphisms [93]. A proto-oncogene is a normal gene that, due to mutations or increased expression, can become a tumor-inducing agent, i.e. an oncogene which encodes for an oncoprotein. Proteins that are encoded by proto-oncogenes, help to regulate cell

growth and differentiation; as they are often involved in signal transduction and execution of mitogenic signals [94]. A study in India reported the mutation in Rat sarcoma (Ras) gene is related to the development and progression of OSCC [95]. A more recent study reported CT120A gene as possible oncogene for HNSCC and its overexpression is associated with high tumor grades [96].

A tumor suppressor gene (anti-oncogene) is a gene that protects a cell from cancerous transformation. Usually, in combination with other genetic changes, when the tumor suppressor gene mutates leading to a loss or reduction in its function, the cell might progress to cancer. The loss of these genes may be even more important than the activation of proto-oncogene/oncogene for the formation of many types of human cancer cells [97]. Researchers had indicated that oral cancers may evolve through a series of mutations in tumor suppressor genes, especially p53 [98-100].

2.3.9 Other factors

Free radicals such as reactive oxygen species (ROS) are naturally formed in the body and play a crucial role in many normal cellular processes. However, at high concentrations, ROS can cause oxidative stress and be hazardous to the body damaging all major cellular components, including DNA, proteins, and cell membranes, and thus they may play a role in the development of cancer and other impaired health conditions [101]. ROS produced by tobacco consumption has have been correlated to HNC initiation and progression by either inducing genotoxicity and mutation, altering the salivary proteins and normal oral mucosa, or inducing inflammatory cells infiltration [102]. An epidemiological study conducted in Papua New Guinea strongly correlated the ROS to HNC development [103].

Antioxidants "free radical scavengers" are chemicals which interact with and neutralize these free radicals, thus preventing them from causing damage. The body capable of forming some of the antioxidants (endogenous) which it uses to neutralize free radicals. However, most of the antioxidants used by the body come from external (exogenous) sources, primarily the diet. Fruits, vegetables, and grains are rich sources of dietary antioxidants, and some dietary antioxidants are also available now as dietary supplements [104]. A study reported elevated oxidative stress and decreased antioxidant defense in patients with HNC [105].

2.4 Clinical presentation for HNSCC

Numerous signs and symptoms may be encountered depending on the location of the HNSCC. Tongue SCC usually presents as a deeply infiltrating ulcer with indurated growth, reducing its mobility. SCCs of buccal mucosa and floor of the mouth may present as either ulcers with raised indurated margins or exophytic lesions. SCC of the hard palate often presents a papillary exophytic growth rather than a flat or even an ulcerated one. On the other hand, soft palate and uvula SCC could appear as an ulcer with raised margins or as a fungating mass. Generally, the most common presenting features are ulceration, bleeding, localized pain plus referred ear pain, difficulty with speech, opening of the mouth or chewing, and neck swelling due to occasionally enlarged cervical lymph nodes [106,107].

Haemoptysis, dysphagia, odynophagia and quality change of voice are well-known signs and symptoms of the hypopharyngeal and supraglottic tumors. Voice hoarseness characterizes the glottic SCC. For the subglottic tumor, dyspnea and stridor frequently occur. Trachea SCC may bring about dyspnea, hoarseness, wheezing, cough and haemoptysis. SCC of the nasal or

paranasal sinuses may give rise to nasal fullness, nasal obstruction, epistaxis, paresthesia, rhinorrhea, and palatal bulge. Persistent non-healing nasal sore or ulcer, or in advanced cases, proptosis, diplopia, and lacrimation may evolve. The NPSCC patients are commonly presented with painless enlargement of upper cervical lymph nodes, blood-stained post-nasal drip, and serous otitis media due to Eustachian tube obstruction [106,107].

2.5 Treatment of HNC:

Tumor sub-site and tumor stage are the main factors affecting the choice of treatment modality for HNC patients. The performance status of each patient is another important aspect to take into consideration as treatment is often very intense with multiple side effects. Co-morbidity state in the HNC patients leads to poorer survival, irrespective of the choice of treatment [108]. HNC patients conventionally treated by either surgery, radiotherapy (RT), chemotherapy (CT), or combinations of these modalities. However, no worldwide standard mode of therapy exists [109]. The combined treatments can be delivered concurrently or in different temporal sequences. Recently, new targeted molecular therapies have shown very promising results [110,111].

A multidisciplinary approach is needed to decide the best treatment planning, and to assess posttreatment response. Surgeons, medical oncologists, and radiation oncologists, as well as dentists, speech/swallowing pathologists, dieticians, psychosocial oncology, prosthodontists, and rehabilitation therapists should be included in the decision team. A study reported that multidisciplinary tumor board affects diagnostic and treatment decisions in a significant number of patients specially with newly diagnosed head and neck tumors [112].

Furthermore, complex cases of head and neck cancer have better chances to be treated at high-volume centers, where expertise in each of previously mentioned disciplines can be found [113,114]. An analysis of outcomes from a large randomized trial (Radiation Therapy Oncology Group [RTOG] 0129) found that centers with high accrual to head and neck clinical trials reported significantly better five-year overall survival rate for their treated patients when compared with centers with historically low accrual (69 versus 51 percent) [115].

2.5.1 Surgical intervention:

2.5.1.1 Surgical removal oh HNSCC

Before radiotherapy was available as a treatment, surgery was the only treatment modality for HNC patients, then RT was suggested as a replacement [116]. However, this was not the case and the two treatment modalities were used together as combined treatment [117]. Over time, surgeons shifted their concerns from only removal of the lesion and promoted improved prognosis to also considering the preservation of organ function and cosmetic appearance, resulting in a continuous emerging of new techniques [118,119]. Surgical intervention in primary cancer treatment has changed, and it is rare now to perform surgical treatments for pharyngeal cancer as it can have an excellent prognosis with less invasive treatment modalities. However, in cases of treatment resistance or cancer recurrence, salvage surgery becomes mandatory [120] with, if possible, reconstructions with free flaps [121,122].

In the case of oral cavity primary cancers, surgery is still the main treatment option, and usually require a free-flap reconstruction with soft tissue if there are mandibular and bone resections [123]. Lower-stage OSCC is often treated with surgery alone while patients with higher

stages and poorer prognosis are treated with combined modalities [124]. In laryngeal cancers, small tumors which are only in the right or left vocal cords, are often treated with surgery while tumors that are in both vocal cords or spread beyond the vocal cords but still confined in the larynx are treated with External Beam Radiation Therapy (EBRT) alone, and tumors with spread beyond the larynx are treated with a laryngectomy followed by EBRT [125-129]. One of the landmarks in the development of new methods for larynx cancer treatment and surrounding organs preservation is the work done by the Department of Veterans Affairs Laryngeal Cancer Study Group [130]. They reported that induction CT and definitive RT can be effective in preserving the larynx compared to laryngectomy. There are, however, new forms of surgery which provide better organ-preserving capability such as; transoral laser microsurgery, transoral robotic surgery, and open partial laryngectomy which might increase the usage of surgery in primary laryngeal tumors [131].

2.5.1.2 Neck Dissection:

Prophylactic neck dissection is performed in some cases to remove any metastasized residual cancerous tissues in the cervical lymph nodes [109]. The original use of neck dissection was for a palliative treatment for HNC patients, but G.W. Crile at the beginning of the twentieth century [132] reidentified this procedure as a treatment for HNC, aiming to reduce the risk of regional lymph nodes recurrence [133]. Later on, H. Martin introduced the more modern form of neck dissection [134]. Starting from the 1960s, neck dissection became an integral part of surgical treatment in combination with RT, especially for patients with regional nodal metastasis [135].

With the preservation of organ function becoming more of an issue, there was a definitive change towards chemoradiotherapy (CRT) without neck dissection even with evidence of nodal metastasis. This debate about whether to use neck dissection or not in these patients and the possible effect on the prognosis, with or without RT, continued all through the 1990s [136,137]. During the last two decades, however, most studies have shown that there is no need to perform a planned neck dissection in patients with nodal metastasis who achieve a complete response after RT or CRT [138-140], and even if a neck dissection is deemed mandatory, a modified technique is recommended [141]. Parallel to this, neck dissection also has a new role as a diagnostic tool to detect micro-metastasis in the neck, considered as a prophylactic treatment preventing regional recurrence. This is usually used with OSCC due to its high incidence for micro-metastasis [142] and is commonly referred to as a staging, selective, or elective neck surgery [143,144].

According to the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS), neck surgery has three major types: radical, modified radical, and selective [145], and there are ongoing attempts to further develop this classification [146]. As an effort toward less extensive surgery, a new technique was introduced that only removes the regions of the neck that are most likely to be the site of metastasis. The sentinel node technique is regarded as an extremely selective neck dissection as it only dissects the gateway nodes [147,148]. This is a very promising technology and it may replace the conventional elective neck dissection in the near future [149].

Recently, new techniques have evolved to decrease the morbidity of the HNC patients after surgical treatment and to reconstruct the removed area. Techniques such as navigational

systems during surgery, stereolithographic models, robotic surgery, allotransplants, and tissue engineering are the future of reconstruction and soon will replace the conventional full thickness normal flaps [150].

2.5.2 Non-surgical treatment

The non-surgical treatment of HNC includes radiotherapy, either external or internal (brachytherapy), chemotherapy given for induction and/or concurrently, and pharmacological treatment.

2.5.2.1 External radiotherapy

About 40% of HNC patients undergo RT during their treatments. 60% of those patients will be receiving radiation as a definitive treatment, often in combination with surgery and CT [151]. External-beam radiotherapy or external radiotherapy is the conventional method for radiating HNC [152]. The usual method for EBRT is to deliver a photon beam from a linear accelerator. The ultimate goal is to deliver therapeutic radiation dosage to the tumor without affecting the surrounding tissues, especially tissues in the organ known for its vulnerability to radiation damage such as the spinal cord, the inner ear, and the salivary glands, also known as organs at risk (OARs). [153]. To help protect these organs at risk, a careful planning for the RT using 3-dimensional computed tomography-based imaging must be done [154,155]. RT is conventionally given in the form of fractions of 1.8 – 2.0 Gray (Gy), once daily, 5 days a week for a period of 6 or 7 weeks (making the total up to 70 Gy). In HNC patients' treatment, an accelerated schedule can be used, using six fractions per week, which appear to give improved

results compared to the five visits per week [156,157]. This accelerated schedule delivers the same radiation dosage in a shorter period, allowing less time for the tumor to recover [158,159].

To deliver adequate target volume coverage and to decrease the risk of RT-induced toxicity, there is a need for accurate delineation of the OARs in the treatment plan. To avoid subjective contouring variations between radiation oncologists in the definition of OARs anatomical sites and limits, contouring consensus guidelines have been developed and followed [160-162]. There is a risk of small changes in positioning the patient during RT due to weight loss, tumor volume changes, and changes in OARs, especially that the RT takes several weeks. Along with the fact that the patient is not fully immobilized during treatment might lead to high radiation doses to surrounding tissues. The new adaptive radiation treatment technique reduces this risk greatly when compared to the conventional radiation methods [163]. Another rapidly developing method to target HNC while preserving OARs is the use of proton beam radiation [164]. A well-known method for rescuing OARs is the use of intensity-modulated radiotherapy (IMRT) and image-guided radiation therapy (IGRT), which reduces the irradiation to the surrounding tissues while delivering curative high radiation dosage to cancer [153,165].

There is a strong debate on whether there should be pre- or postoperative EBRT in the last decades. One study showed that preoperative EBRT might be negative for surgery, particularly free-flap reconstructions, and this negative effect increased by increasing the time delay between the end of EBRT and surgery [166]. Another study reported that postoperative EBRT was associated with a higher risk of local recurrence [167]. Even though some authors supported preoperative EBRT, especially for the OSCC [168], most institutions use primary

surgery for small tumors, smaller than 6 mm, with postoperative EBRT considering the tumor stage, radicality, and histopathology [169].

While surgery may alter form and function, RT or chemoradiation treatment may cause acute effects such as mucositis, function alteration and dysphagia, fatigue, and airway edema. Long-term side effects may include severe dysphagia, osteoradionecrosis, aspiration pneumonia, or radiation fibrosis syndrome, which are directly related to radiation dose [170,171].

2.5.2.2 Brachytherapy

Brachytherapy, or internal radiation, means delivering the therapeutic radiation dose from encapsulated radionuclides within or close to a tumor [172]. This is done by using plastic tube catheters that release photon radiation and is implanted around the tumour, helping in delivering a high dose of radiation directly to the tumour without any beams passing through normal tissue. One important limitation of brachytherapy is that it is best suited for tumors with high accessibility for implantation of catheters. Some studies showed that smaller tumors could be fully treated with brachytherapy alone, while larger tumors, especially at the base of tongue, were better treated using a combination of EBRT and brachytherapy [173-175]. Innovative technologies in imaging and analysis, such as intensity modulated brachytherapy (IMBT), Magnetic resonance imaging (MRI), Computed tomography, and Positron emission tomography (PET) make brachytherapy more efficient and a safer method when compared to the conventional technique [175,176].

2.5.2.3 Chemotherapy and pharmacological treatment

Chemotherapy can be used as a palliative treatment alone, however, as a curative treatment it is always combined with RT which may be given before RT (as induction or neoadjuvant), alongside RT (as concomitant or concurrent), or in some cases after surgery (as adjuvant) [177]. The combination of RT and CT has been reported to decrease regional metastasis and improve survival rates while maintaining relatively low toxicity, especially in patients with advanced disease [178-180]. There is increasing use of a combined induction and concurrent chemoradiotherapy (CCRT) to reduce distant metastasis [181,182]. The current standard treatment of NPSCC is concurrent cis-Diammineplatinum(II) dichloride (Cisplatin) and RT followed by adjuvant CIS and 5-FU following the recommendation from the Intergroup 0099 study [45,183]. HNC patients with locally advanced, unresectable tumor are treated by CRT as a standard as long as the addition of CT is not indicated due to poor performance status or comorbid illnesses [184].

Acute side effects are the most important limitation for CT, but recently there is growing evidence of higher rates of late toxicity side effects as well [3-5]. More research is needed into patient satisfaction and quality of life after receiving CRT for HNC [12,13]. CIS has been reported to cause multiple tissue and organ toxicity due to its unspecificity along with the decrease in antioxidant defense system. CIS related toxic side effects include nephrotoxicity, hepatotoxicity, and cardiotoxicity [185]. 5-Fluorouracil, another gold standard CT for HNC, also have been reported to cause early and late side effects. These effects range from the common less severe diarrhea, nausea, vomiting, mouth sores, neutropenia and thrombocytopenia to the less common but life-threatening neurotoxicity and cardiotoxicity [186].

Since Bonner et al. (2006) reported an improved loco-regional control in advanced HNC patients treated with a concomitant combination of high-dose RT and cetuximab as compared to RT alone, there has been increasing awareness about the possible role of monoclonal antibodies in treatment [187,188]. Epidermal growth factor receptor (EGFR) is highly expressed in HNC and its overexpression is related to a poorer prognosis [189]. Cetuximab, an EGFR-targeting monoclonal antibody and the only targeted therapy to be routinely used in clinical practice for HNC, has been shown to significantly improve survival for HNC patients, especially with advanced and recurrent diseases [190]. Some of the side effects of cetuximab are the classic acneiform skin rash, hypomagnesemia, a risk for infusion reactions, and the less common anaphylactic reaction [191]. Another group of agents that have emerged recently are tyrosine kinase inhibitors (TKIs). These are a class of chemotherapeutics that act by blocking specific tyrosine kinases which are essential in cellular pathways promoting tumor growth, invasion, and metastasis [192]. The two most commonly studied TKIs are gefitinib and erlotinib [193]. These types of immune-related drugs are aimed at more specific treatments due to different responses in different patients [193,194].

2.6 Models of tumor heterogeneity

Mostly, the evolution of HNC occurs through the accumulation of several genetic mutations, which may be induced by environmental factors such as tobacco and alcohol abuse or persistent HPV infection [195]. However, it is not well understood how the alterations of multiple molecular and cellular pathways could yield the development and especially the recurrence of HNC. In general, there are two models aiming to clarify the development and maintenance of tumor growth and heterogeneity (Fig.1):

2.6.1 The stochastic model, also known as clonal evolution or clonal genetic model of cancer, is the traditional idea of carcinogenesis, where mutant tumor cells with a growth advantage when compared to the other cells are selected and expanded, considering that cells in the dominant population have a similar potential for recapitulating tumor growth [196]. In other words, malignant transformation originates from a randomized genetic mutation that might affect any cell. The mutant cell progeny, which attains a proliferative advantage with consequential genomic instability, accumulates more epigenetic and genetic events, causing selection of the more aggressive sub-clones with subsequent tumor evolution [197]. Different phenotypic and proliferative features of these sub-clones are responsible for the tumor heterogeneity. Such model proposes cancer as a disease of proliferation [198]. The major of the currently available therapeutic strategies is still based on this traditional model of carcinogenesis [199].

Due to conventional treatment resistance and tumor recurrence, researchers have focused on understanding the genetic changes directing a cell towards a malignancy and tumor behavior, without keeping an eye on the nature of cells that are affected by these mutations. Therefore, it is presently believed that a scant group of tumor cells, defined as cancer stem cells (CSCs), harbor the self-renewal potential and can give rise to a phenocopy of the genuine tumor [200].

2.6.2 The cancer stem cell model (The hierarchy model), is a cancer model suggesting that tumorigenesis is exclusively attributed to CSCs [201]. Such hypothesis is validated by the experimental findings that only a small number of tumor cells (i.e. CSCs) are capable of generating tumors upon serial transplantation in animal models [202-204].

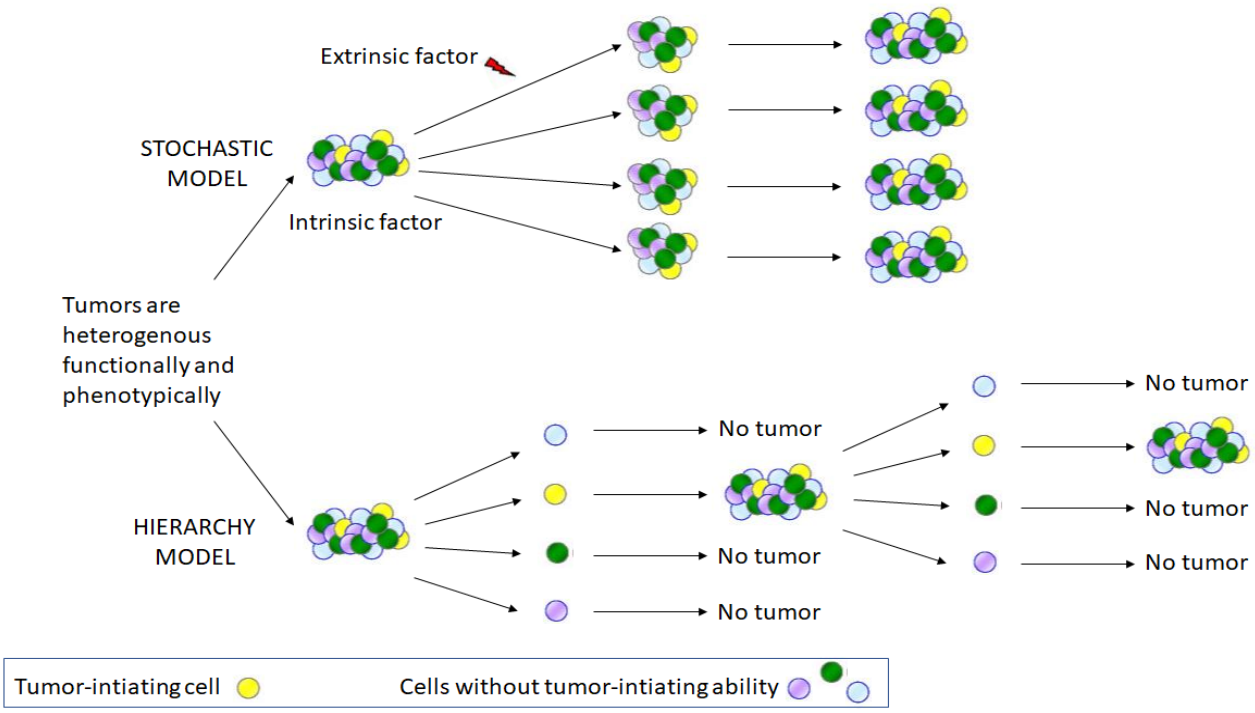

Figure 2.1 Models of tumor heterogeneity. Tumors are formed from cells that are heterogenous phenotypically and functionally. There are 2 most acceptable theories as to how this heterogeneity occur. According to the stochastic model, all tumor cells are biologically equal, however, intrinsic and extrinsic factors affect their behaviour causing this variability. This means tumor-initiating activity cannot be enriched by isolating cells based on intrinsic features. In contrast, the hierarchy model (Cancer stem cell model) hypothesize the existence of biologically different classes of cells each has its own function and behaviour. Only a subset of cells can start the tumorgenicity; these cancer stem cells possess two main criteria, self-renewal and multilineage differentiation, giving them the ability to form the bulk of the tumor. This model speculate that tumor-initiating cells can be identified and sorted based on intrinsic characteristics.

2.7 Cancer stem cells history:

Other than the well established two types of stem cells, adult and embryonic stem cells, the presence of a third type, termed as cancer stem cell (CSCs) was recently recognized [205]. There is increasing support for the concept that the majority of cells in bulk tumors are non-tumorigenic; having limited self-renewal ability (i.e. only a small subpopulation of cancer cells is

long-living with extensive self-renewal and tumor formation abilities). Other common names for CSCs are tumor stem cells (TSCs) or cancer-initiating cells (CICs) [206]. The consensus definition of a CSCs, being approved by the American Association of Cancer Research (AACR) workshop on cancer stem cell, is a cell within a tumor that has the capacity to self-renew and to deliver the heterogeneous lineages of cancer cells comprising the tumor, which would explain how CSCs could be responsible for driving tumorigenesis and tumor growth [207].

The concept that tumor growth depends on a subpopulation of stem cells, like in normal tissues, was suggested by Hamburger when he reported that only 1:1000 to 1:5000 cells isolated from a solid tumor was capable of forming colonies in-vitro [208]. Similarly, other published papers showed that only 1 to 4% of transplanted murine lymphoma cells were able to form colonies in the recipient mice [209,210]. There are two possible explanations for this observation. First, the tumor cells have a low potential for proliferation, making all cancer cells behave as CSCs. Second, there is only a small and identifiable subset of cells possess great proliferation capacity. Aiming to support the second hypothesis, Dick and co-workers successfully showed that human acute myeloid leukemia (AML) stem cells can be identified and isolated as CD34+CD38- cells from patient tissue samples [211]. According to this study, only CD34+CD38- cells were able to transfer AML from human patients to non-obese diabetic with severe combined immunodeficiency disease (NOD/SCID) mice while all others cellular phenotypes failed to do so.

After the identification of CSCs in AML, Al-Hajj and colleagues reported the presence of CSCs in solid tumors [199]. In this study, they found that only CD44+CD24-/low cells have the ability to form a tumor in immunocompromised mice while cells with other phenotypes were unable to form a tumor. In the past decade, other types of solid tumors have been reported to

contain CSCs such as in lung, colonic, prostatic, and pancreatic cancer [212-215]. In a landmark publication, Prince and collaborators reported the presence of highly tumorigenic, stem-like, cells in HNC [202].

Such model of tumorigenesis, which is exclusively based on the aberrant activity of CSCs, has been introduced to successfully explain the heterogeneous nature of many tumors in a more efficient way when compared to the stochastic model. According to the CSC theory, tumors are heterogeneous at the histological level (i.e. exhibiting areas of various differentiation degrees), at the genetic level (i.e. with areas showing different gene expression, yielding diverse immunohistochemical protein expression profiles), and at the proliferation level. Conclusively, tumor cells are heterogeneous, including HNC, at the functional level in terms of their capability of new tumors generation [216], as it has been postulated that the new tumor growth can only be initiated by a small tumor cells subpopulation harboring a distinctive phenotype and not by the tumor cells comprising the tumor bulk [217]. The proof of CSCs existence in HNC has also been validated by the similarity in the structure between well-differentiated tumors and their epithelium of origin. A well-differentiated OSCC can recapitulate the oral epithelium histological appearance and proliferation pattern. Well-differentiated tumor nests are usually arranged in three compartments of close resemblance to the normal epithelium: CSC basal compartment, amplifying transitory cell (ATC) compartment, and the innermost differentiated cell compartment. Such replica of the hierarchical proliferation pattern of non-tumor oral epithelia postulates the tumor growth maintenance by a single type of tumor cell, designated the CSC [216].

The frequency of CSCs varies from one cancer type to another and between different samples in the same tumor type. A previous study on AML reported that 1 in 10^6 cells can be called CSCs as it has self-renewal and tumor-forming capacity in nude mice [218]. In colon cancer, CSCs frequency has been reported ~ 2% [219]. In melanoma, there was great variation between the CSCs reported frequencies as it ranged between 0.1 - 41% [22,220]. There are multiple theories explaining this difference in CSC frequencies such as; cancer stage dependent, phenotypic switching between different tumor cells [221], or a consequence of the different definitions used by different researchers [222]. Since the gold standard method to detect CSCs is the in-vitro isolation followed by in-vivo formation of the tumor, this method may not detect cells with the ability to form the tumor in the original host but fail to do so in xenotransplantation.

In conclusion, CSCs are characterized by two main exclusive features in order to allow tumor formation, propagation, and maintenance. These features are: [A] differentiation, yielding heterogeneous progeny; and [B] self-renewal, maintaining an expanding a pool of stem cells [223].

2.8 Cellular origin of the cancer stem cell:

Different CSCs origins have been proposed wherein a subpopulation of self-renewing tumor cells is formed, giving rise to tumorigenesis. Normally, stem cells give rise to progenitor cells that can further divide into specialized or differentiated cells carrying out the specific body functions. It is controversial as to whether CSCs evolve from stem cells, progenitor cells, or differentiated cells in adult tissues, so this issue is currently under debate [207] (Fig.2).

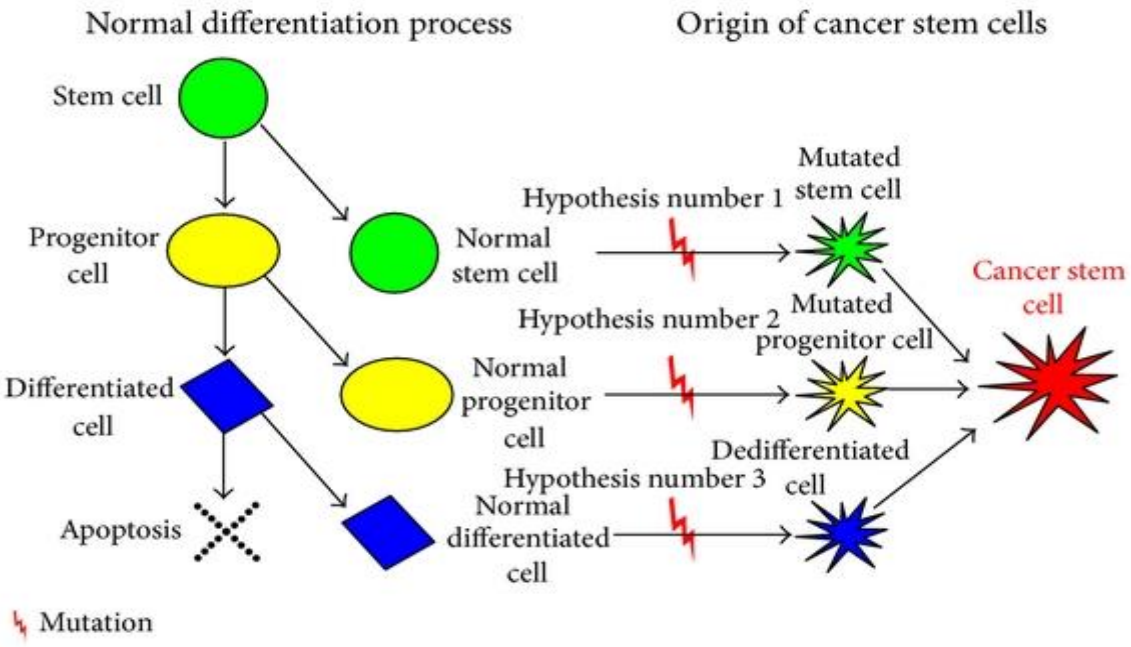

Figure 2.2 Hypothesis suggesting origin of cancer stem cells. In the process of normal differentiation, a cell differentiates to form two cells, differentiated and primitive. A terminally differentiated cell is formed from precursor progenitor cell and finally undergoes apoptosis. CSC may originate from a normal stem cell (Hypothesis number 1), a normal progenitor cell (Hypothesis number 2), or a normal differentiated cell (Hypothesis number 3) by genetic mutation which will activate self-renewal genes. This figure and figure legend were originally published in [224] under a Creative Commons license.

1. The First Hypothesis: Cancer stem cells arise from normal somatic stem cells (SCs), and it is the most accepted theory [225].

A close relationship between the build-up of genetic alterations and the malignant phenotypic progression of OSCC has been proposed [226]. As normal oral epithelial cells have a renewal rate of about 14-24 days, most of them do not exist long enough to accumulate the genetic changes necessary for OSCC development. It is estimated that three to six oncogenic events are needed for malignant transformation of the normal cell [227]. The hierarchical SCs structure present in human oral epithelia dictates that only long-time residents of oral epithelia

are the only cells capable of accumulating the necessary number of genetic changes needed for malignant transformation; for example, micro-environment control escape mutations [228].

Another reason supporting the origin of CSCs to be SCs is the fact that CSCs and normal SCs are endowed with self-renewal capabilities, and dysregulation of the self-renewal process is an early and indispensable step in carcinogenesis. Generally, the long-term survival of either normal or neoplastic tissue is dependent on its self-renewal capacity, whereas its overall size is maintained by the balance between the rates of cell proliferation and cell death across its various components [229]. In normal tissues, the number of SCs is kept under tight genetic regulation, yielding long-term maintenance of a constant tissue size [230]. In contrast, tumor tissues have escaped this homeostatic regulation, where the number of cells with the self-renewal capacity is constantly expanding, resulting in progressive tissue growth. Normal SCs already have self-renewal machinery that is known to be ready and activated, which means maintaining its activation is undoubtedly far simpler than de novo activation, through mutations, in the more differentiated cells that lack this self-renewal ability [15].

Because the size of neoplastic tissues is dependent on the number of cells able to self-renew, it is logic that a specific subset of oncogenes and/or tumor-suppressor genes affecting the self-renewal ability might be activated and/or disabled respectively in the oncogenesis process [231]. The best example, among cancer genes with direct control over self-renewal functions, is probably the B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) oncogene [232]. The Wnt, Notch, and Sonic hedgehog (SHH) pathways are classic examples among multiple signaling pathways that control BMI-1 function and implicated in oncogenesis. The findings that such pathways are pivotal self-renewal regulators in normal SCs and, at the same time, frequent

targets of activating mutations in cancer cells, propose that SCs and CSCs depend on a common set of signaling pathways controlling their numbers and stimulating their growth [233]. Henceforth, continued activation of proliferation pathways is not sufficient enough to endow cancer cells with unlimited growth potential [229]. It is also necessary to ensure activation of self-renewal pathways and/or inactivation of pathways that prevent self-renewal [233].

2. The Second Hypothesis: Cancer stem cells arise from normal progenitor cells.

Normal progenitor cells, being more abundant in adult tissues than SCs plus having a partial self-renewal capacity, can be a potential source of CSCs [215]. The tumor can sometimes originate from amplifying transitory cells (ATCs) in which their high proliferative rates may boost the risk of genetic mutations, and not exclusively in normal basal SCs. Through a reprogramming process, ATCs could attain remarkable self-renewal potentials, while preserving high proliferation rates without a complete loss of their differentiation capabilities [216].

It is proposed that the first set of early transforming mutations could accumulate in the SC compartment, and that the second set of late mutations, which might constitute the ultimate transforming event giving rise to cancer, might accumulate in more mature, downstream progenitors that originated as the progeny of mutated SCs [234]. In other words, mutated SCs might represent a reservoir population of pre-cancerous cells, whereas fully transformed progenitors might sustain the growth of the full-blown neoplastic mass [235].

3. The Third Hypothesis: Cancer stem cells arise from normally differentiated cells.

CSCs could originate from mature, differentiated cells through de-differentiation to become more stem cell-like. In this hypothesis, the de-differentiation process, as well as the

subsequent self-renewal of the proliferating cells, could be driven through the essential oncogenic genetic mutations [224]. The virtual lack of proliferative cells in the superficial strata of normal epithelium would assure the reprogramming hurdles for differentiated cells, requiring major molecular changes [216]. Given that identity maintenance is an indispensable requirement for a differentiated cell, its reprogramming can exclusively be accomplished by powerful modulators of the transcriptional and/or epigenetic machinery. In oncogenesis, more than one transcription factor (TF) participate in the reprogramming process. The downregulation of somatic genes involved in the conservation of a differentiated phenotype by c-Myc has been reported to be vital at an early stage, whereas other TFs such as octamer-binding transcription factor 4 (Oct4) and SRY (sex determining region Y)-box 2 (SOX2) are implicated in reprogramming at a later stage [236].

Differentiated cancer cells can acquire a CSC-like state through epithelial-mesenchymal transition (EMT) which is the liability of epithelial cells to attain polar, adhesive phenotype mesenchymal characteristics in response to specific environmental stimuli, in order to locally invade surrounding tissues and systemically disseminate to distant organs [216,237]. The activity of TFs such as Zinc finger protein SNAI1 (Snail) and Twist Basic Helix-Loop-Helix Transcription Factor 1 (Twist1) can promote EMT [238], where the polarity of epithelial cell is altered and E-cadherin protein expression is suppressed, among other actions [239]. As reported in breast, nasopharyngeal cancers, and HNSCC, EMT is engaged in the acquisition by differentiated cells of the SCs' properties, where Twist1 triggered BMI-1, another TF involved in SC self-renewal, and repressed E-cadherin expression [240-242]. Recently, researchers have immortalized epithelial cells in an effort to mimic the process of cancer development. They have reported that

immortalized epithelial cells showed signs of transformation from an epithelial phenotype to a spindle-shaped, more mesenchymal-like phenotype. In addition, these transformed cells expressed a higher capability to undergo self-renewal. These findings support the hypothesis that EMT could be a potential mechanism for epithelial cells de-differentiation (Zhao et al., 2010).

2.9 Cancer stem cells in head and neck cancers:

To date, flow cytometry/fluorescence-activated cell sorting (FACS) is the most commonly used technique to identify and isolate CSCs from different tumor types. Using cell surface antigens on HNC stem cells and tag them by fluorochrome-conjugated antibodies, Oncogenic researchers were able to identify these cells based on individual or a combination of markers. Generally, a variety of researches have stated cluster of differentiation 44 (CD44) as a CSC biomarker in breast, CNS, colon, prostate, and pancreas tumors [214,215,243]. Reategui et al [244] first discovered high levels of CD44 variant isoform 3 (CD44v3) expression in HNC tissues in comparison to normal ones. Despite the increased expression level of CD44v3 did not alter cell proliferation rate, a significant increase in cell migration was recorded. Defining CSCs in HNC was first based on CD44 expression (via flow cytometric analysis) as CD44bright and CD44dim populations. Prince et al [202] revealed the big difference between both populations to be so remarkable that only 5×10^3 CD44bright cells were capable of regenerating the tumor heterogeneity and demonstrating self-renewal function when transplanted into immunocompromised mice, whereas 5×10^5 CD44dim cells failed to form tumors.

A very interesting study, conducted by Wang et al [245] proved the intimate correlation between CD44v3, CD44v6, and CD44v10 isoforms and HNC lymph node metastasis with

advanced tumor volume status, perineural invasion plus decreased survival, and distant metastasis with the failure of RT, respectively. In-vivo studies utilized CD44 to assess the metastatic potential of CSCs in HNC, as they have shown that CD44high cells, rather than CD44low cells, resulted in lung lesions when injected in tails of NOD/SCID mice [246]. Since then, several studies have claimed that CD44 positive subpopulations, emanating from either HNC primary tissues or cell lines, exhibit a higher potential for proliferation, differentiation, migration, invasion, tumor-sphere formation, and resistance to chemotherapeutics [247-249].

CSCs have been shown to acquire a defense mechanism against ROS, enhanced by the CD44v9 isoform. Interaction of CD44v9 with xCT (a functional subunit of the cystine-glutamate transporter) promotes cystine uptake for the synthesis of reduced glutathione (GSH), which is the primary intracellular antioxidant. Therefore, tumor cells can avoid exposure to high levels of ROS, thus driving tumor growth and chemoresistance [250]. Patients with favorable responses to induction CCRT did not have a significant CD44v9 expression level in their HNC biopsy specimens, in comparison to CCRT non-responding patients, where CD44v9 positivity was considerably associated with poor prognosis along with advanced lymph nodal metastasis [251]. Recently, it has been suggested that a combination of CD44 with other markers, such as the cell adhesion molecule CD24, was more reliable in isolating HNC cancer stem cells when compared to using CD44 alone [252].

Several new cell surface antigens have recently been reported as potential markers for HNC stem cells. A study reported an increase in the expression of CD10 on HNC cells after RT or CT treatment [253]. In this study, CD10 used peptidase activation to generates peptides

supporting the proliferation of stem and progenitor cells. CD10+ cells isolated from HNC possess enhanced sphere formation in-vitro and tumor formation in-vivo, as well as showing a higher expression of the stem cell marker Oct3/4. Moreover, resistant HNC tumors show elevated CD10 expression that has been associated with local recurrence, distant metastases, and a higher histologic tumor grade [254]. Another recent study used sphere culture to enrich HNC stem cells for examining plasma membrane proteomics [255]. This group reported that CD166 (a transmembrane glycoprotein that mediates cell-cell adhesion) expressed significantly higher in spheroid cells compared with matched adherent cells. They also showed that, at low cell density, CD166hi HNC cells formed larger tumors than CD166lo cells after implantation in nude mice and were able to reproduce the heterogeneous tumor population, suggesting CSC behavior. Interestingly, CD166hi cells were localized at the tumor invasive front in HNC, which is a typical locale for CSCs.

CD133, also called prominin 1 (PROM1), is a surface cellular transmembrane, which was discovered as a normal hematopoietic SCs marker and later it has been identified as a putative CSC marker in brain, prostate, liver, lung, skin, and colorectal cancers [256]. Mizrak et al defined prominin as “molecule of the moment” in 2008 due to its importance in haematopoietic and CSCs identification and targeting [257].In the HEp-2 laryngeal cancer cell line, a minor subpopulation of CD133+ expression demonstrated sphere formation and self-renewal criteria of CSCs, plus the capacity to differentiate to phenotypically unique tumor daughter cells [258]. More recent studies have supported these findings, as CD133+ cells isolated from HNC cell lines have been suggested to display increased clonogenicity, proliferation, EMT phenotype, tumor-sphere formation, self-renewal, multilinear differentiation, and in-vivo tumorigenicity [259].

CD271 is known also as the low-affinity nerve growth factor receptor (NGFR) or p75 neurotrophin receptor. It plays a major rule in the nervous system as it controls functions such as cell survival [260], differentiation [261], and migration [262] of neuronal cells. Earlier, CD271 was reported as a squamous epithelial SCs marker in the larynx [263], oral cavity [20], and esophagus [25]. Recent studies reported CD271 as a CSCs marker in melanoma [22,23], esophageal carcinoma [24], and hypopharyngeal cancer [26]. Imai et al. were the first to speculate that CD271 is a marker of CSCs in HNC [26]. They reported high tumorigenicity in-vivo for CD271+ cells compared to the negative one and localization in the invasive front. Murillo-Sauca et al. also reported that CD271+ in HNC is more invasive with an enhanced capacity for metastasis to regional lymph nodes due to upregulation of Snai2/Slug [264]. In another study, they showed that CD271 a functional and targetable marker in HNC through monoclonal antibody [265].

Apart from cell surface antigens, functional activities of aldehyde dehydrogenase (ALDH) and ATP-binding cassette transporters (ABC transporters) have been used to identify and isolate HNC stem cells. ALDH is a large family of enzymes that control the transformation of aldehydes to carboxylic acids through oxidation and involved in converting retinol to retinoic acid [266,267]. Studies have reported that ALDH enriches for CSCs and is involved in EMT, self-renewal abilities, tumor formation, and resistance to chemotherapeutics [267,268]. The ALDH1A1 isoform is the most commonly reported to be responsible for enhanced ALDH activity in different types of CSCs, including HNC [269]. One study reported that as low as 500 ALDH + cells were able to create tumors, unlike the ALDH- cells [270]. Side population (SP) is a term describing a subset of cancer cells, that is considered CSCs, which possess the ability to efflux Hoechst DNA binding dye and

chemotherapeutic drugs using ABC transporters [271]. SP cells isolated from HNC are more tumorigenic, chemo-resistant and demonstrate self-renewal ability in-vivo [272-274]. Interestingly, a study reported an increase in the SP cells in HNC by the activation of EGFR, a receptor tyrosine kinase often overexpressed in HNC, and this phenotype was reversed by addition of EGFR inhibitor [275]. In another study, SP isolated form HNC metastatic cell lines had abnormal activation of Wnt/beta-catenin signaling as compared to non-SP cells [276].

2.10 Therapeutic implication of CSCs in HNC:

The CSC hypothesis has important implications regarding cancer therapy and may lead to new treatment strategies along with reviewing the conventional treatment paradigm. According to what we discussed earlier, within the diverse and heterogeneous cell population comprising the HNC mass, the small subpopulation of CSCs may be responsible for tumor recurrence, the initiation of metastasis because of high migration capacity, as well as resistance to both radio- and chemo-therapy. Intrinsic characteristics of CSCs such as an elevated level in ABC transmembrane proteins, a semi-quiescent state, and transformed apoptotic mechanisms limit susceptibility to cell death [272,277].

It is frequently suggested that CT resistance is related to accelerated drug transport and to drug metabolism [278,279]. Permeability glycoprotein (P-gp), a product of the gene ATP Binding Cassette Subfamily B Member 1 (ABCB1) or Multidrug Resistance Protein 1 (MDR1), is an ABC transporter associated with multidrug resistance, and it has been shown to induce the ability of resistance to multiple chemotherapeutic drugs [280]. The MDR1 gene encodes a P-gp transmembrane segment which function is the excretion of different drugs. Previous studies have

demonstrated that P-gp expression is correlated with the MDR of HNC [281-283]. Knocking down BMI-1 and CD44 have led to an enhanced chemo-sensitivity of CSCs in HNC.

Yaromina et al reported that therapeutic success after radiotherapy of human squamous cell carcinomas is inversely proportional to the percentage of CSCs within the tumor mass [284]. CSCs that survive the radiation are potentially responsible for recurrence, as they have the capacity for self-renewal and differentiate into the heterogeneous constituents of the tumor [201]. CSCs are inherently more radioresistant, by employing mechanisms which increase checkpoint activation and enhanced DNA damage repair responses [285]. However, increasing the radiation dose in HNC treatment will cause intolerable side effects that worsen the patients' life quality such as xerostomia [286]. This was explained by the effect of radiation on micro-niches of normal salivary SCs, often in close proximity to blood vessels in the salivary glands [287].

Central tumor hypoxia, which is found in the center of larger masses, may also provide a survival advantage to CSCs against chemotherapeutics or radiation [288]. Poor perfusion of larger tumor masses might help the enrichment of CSC phenotype by creating specific CSC niches in the same way the hypoxia maintains the pluripotency of embryonic SCs. Suboptimal blood flow will decrease the optimal distribution of chemotherapeutic agents to cancer cells as well as lowers the oxygen tension needed for free radical formation in response to radio- or chemo-therapy [289]. Overexpression of hypoxia-inducible factors (HIFs) in CSCs was correlated to radio-resistance in HNC [290]. Yang et al correlated the overexpression of HIF-1-α in CSCs with the induction of EMT which in turn increased mobility, as well as maintained their pluripotency [291].

During the surgical treatment of HNC, residual cancer cells may remain in the incisal margin, in the vicinity of the tumour, and in the adjacent tissues surrounding the tumor; those will be dealt with post-operatively or primarily with combined or primary radiotherapy. The CSCs model further emphasizes the great implication of safe margins during surgical intervention and demonstrates that the objective of revolutionary therapies must be the development of specific drugs against the CSCs of a tumor, which survive after the removal of the tumor bulk via conventional therapy modalities.

Because of what we discussed earlier, new strategies targeting CSCs are being under development to be used in combination with the traditional therapeutic means to prevent tumor relapse and to ensure a highly efficient and less toxic treatment for cancer (Fig.3). New techniques of targeting specific cell membrane growth factor receptors or downstream signaling pathway mutations are currently under investigation, especially in patients with metastatic tumors [292]. One of the most promising strategies for cancer treatment is inhibiting the key self-renewal signaling pathways (e.g. Wnt, SHH, Notch signaling pathways) that are aberrantly active in CSCs [293], introducing novel therapeutic approaches for HNSCC [294-296]. These new therapeutic techniques have a significant reduction in the CSCs, reducing its tumorigenicity, apoptotic resistance, and enhanced the sensitivity to CT [297,298].

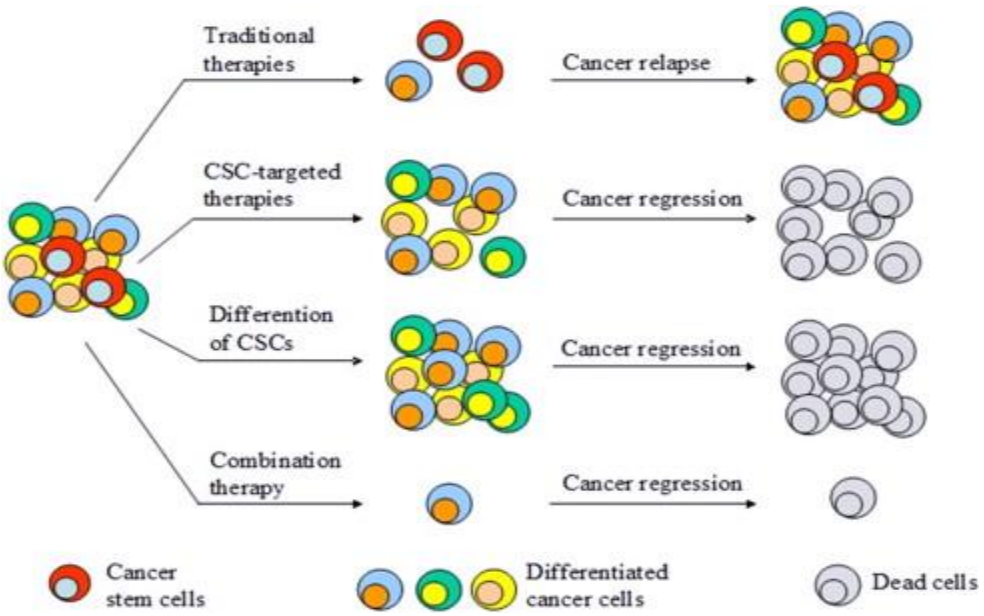

Figure 2.3 Therapeutic targeting strategies for CSCs. The traditional cancer therapies kill differentiated cancer cells but fail to target CSCs, resulting in cancer relapse. However, CSC-targeted therapies can eliminate or differentiate the CSCs, and the remaining and resulting differentiated cancer cells will die thereafter. But it is promising to combine CSC-targeted therapies and traditional therapies for depleting CSCs as well as killing differentiated cancer cells, this combination therapy may have the benefits of increased efficacy and quick action. This figure and figure legend were originally published in [299] under a Creative Commons license.

The markers used to isolate, identify and enrich CSCs are also ideal targets for cancer therapy [299]. DNA damage, caused by treatment with chemotherapeutic drugs, generates pro-apoptotic signals that are known to be suppressed by increased protein kinase B (Akt) phosphorylation, a mediator of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway [300,301]. In HNC, PI3k and Rho kinase (ROCK) have been suggested to mediate HA-CD44 promotion of CIS resistance; as simultaneous inhibition of both kinases reduced CIS resistance to a substantially greater degree than what was observed with inhibition of either enzyme alone. Also, the capacity of hyaluronic acid and CD44 to promote malignant tumor

phenotypes (such as abnormal proliferation, migration, and invasion) could be diminished in HNC cell line through the inhibition of these enzymes. Therefore, CD44 and its associated signaling molecules (i.e., ROCK and PI3K) have been introduced as innovated targets for the future development of novel therapies against HNC [302]. In another study, they reported that knockdown of CD44 increased the sensitivity of HNC cells to CIS [303].

Another approach favored targeting the drug-detoxify enzyme ALDH1A1 in HNC, Kulsum and his colleagues reported correlation between CIS resistance and elevated ALDH1A1 expression in HNC, which can be reversed by application of ALDH1A1 inhibitors [304,305]. Targeting ABC drug transporters, which in combination with other chemotherapeutic drugs, also offers a very powerful and selective strategy to eliminate CSCs [306]. Recent therapeutic strategies exploited the interdependence of CSCs and vascular endothelial cells (perivascular niche) in HNC to decrease the rate of tumor recurrence and distant metastasis [223].

Dysregulated apoptotic mechanisms (including impaired apoptotic machinery, increased DNA damage repair after CRT, and altered cell cycle checkpoint control) contribute to cancer development, progression, and CSCs resistance [307]. Therefore, induction of CSCs apoptosis through manipulating the apoptotic machinery reveals a great potential to eradicate CSCs for tumor therapy [299]. Several compounds have been introduced to induce apoptosis through targeting the intrinsic and extrinsic apoptosis pathways. For example, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that inhibits apoptosis by elevating the expression of survival factor [308]. Hexum et al. synthesized several bicyclic cyclohexenones capable for inhibiting NF-κB signaling by inhibiting NF-κB-induced interleukin-8 (IL-8) expression, thus exerting antiproliferative activity against lung adenocarcinoma epithelial

cell line, T cell lymphoblast-like cell line, and prostate carcinoma cell line [309]. Another interesting way to manage tumor progression is inducing the terminal differentiation of CSCs (Fig. 3) to lose their self-renewal property [310], by the means of either retinoic acids or drugs targeting tumor epigenetic changes [311].

Recently, phytochemicals and herbs have been suggested to be potential sources of therapeutics for CSC elimination, for example; resveratrol, curcumin, sulforaphane, and so forth [312].

2.11 Conclusion:

Head and neck cancers remain a frequent occurring disease associated with a high mortality rate. The etiology behind such cancer is multifactorial, however, temperance from smoking and alcohol remains the best way to prevent HNC. Aggressive surgical resection is the cornerstone of treatment, with increasing roles for both radiation and chemotherapy, especially for organ preservation. Cancer stem cells are a subpopulation of cells inside the tumor that cause treatment resistance and tumor recurrence which has special implications on the cancer treatment and progression.

Chapter 3 - Sulforaphane as a natural molecule in cancer prevention and treatment.

3.1 Preface (connecting paragraph)

According to recent studies, phytochemicals and herbs could be potential sources of therapeutics for cancer prevention and elimination as we mentioned in the second chapter. For example, resveratrol, curcumin, sulforaphane (SF), and so forth had been reported to suppress cancer and cancer stem cells. During the past few years, a number of studies have suggested that SF may have the potential to target the carcinogenic process through direct or indirect influences on several pathways, alone or in combination with other anticancer agents. Combination of SF with different cytotoxic drugs had an additive effect and strongly increased cell death and eliminated cancer stem cells characteristics including tumor-initiating potential, clonogenicity, spheroidal growth, and aldehyde dehydrogenase 1 (ALDH1) activity.

In this chapter, we reviewed the current knowledge about Sulforaphane sources, its metabolism, and its cancer prevention and anti-cancer effect. This literature review also covered Sulforaphane's effect on cancer stem cells and the current and future human clinical applications in cancer.

The study presented in this chapter has been submitted and accepted with minor modifications to Current Medical Sciences Journal

Sulforaphane As A Promising Natural Molecule in Cancer Prevention and Treatment

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Abstract

Tumorigenicity-inhibiting compounds have been identified in our daily diet. For example, isothiocyanates (ITCs) found in cruciferous vegetables were reported to have potent cancer-prevention activities. The best characterized ITC is sulforaphane (SF). SF can simultaneously modulate multiple cellular targets involved in carcinogenesis, including (1) modulating carcinogen-metabolizing enzymes and blocking the action of mutagens; (2) inhibition of cell proliferation and apoptosis induction; and (3) inhibition of neo-angiogenesis and metastasis. SF targets cancer stem cells through modulation of NF-κB, SHH, epithelial-mesenchymal transition, and Wnt/β-catenin pathways. Conventional chemotherapy/SF combination was tested in several studies and resulted in favorable outcomes. With its favorable toxicological profile, SF is a promising agent in cancer prevention and/or therapy. In this article, we discuss the human metabolism of SF and its effects on cancer prevention, treatment, and targeting cancer stem cells, as well as we provide a brief review of recent human clinical trials on SF.

Abbreviations:

APAF1, Apoptotic protease activating factor-1; CYP450, Cytochrome P450 oxidase; GSH, Glutathione; GST, Glutathione S-transferases; HDAC-1, Histone deacetylase 1; IAP, Inhibitors of apoptosis; ITC, Isothiocyanates; KDR/flk-1, Kinase insert domain receptor/ Fetal Liver Kinase 1; Keap1, Kelch-like ECH-associated protein 1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NQO1, NAD(P)H:quinine oxidoreductase 1; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine; TRAIL, Tumor necrosis factor-related apoptosis inducing ligand.

3.2 Introduction

Cancer is a cellular disease with multiple causes, genetic and/or epigenetic, leading to alteration in cellular homeostasis with the loss of control of cellular proliferation. It is a major cause of morbidity and mortality throughout the world as it is the second most frequent cause of death in Europe and the chief cause of death in old age [313]. Carcinogenesis is a multistep molecular process in which initially normal cells accumulate mutations in critical genes that disrupt the pathways controlling cell proliferation, apoptosis, differentiation, and senescence. It is generally divided into the stages of initiation, promotion and progression. The initiation stage is responsible for the unrestricted division and proliferation of cells mostly through genetic or epigenetic events. The promotion stage occurs after the initial cellular insult, when a chemical signal or event stimulates the expansion of the initiated cell into a clone of cancer cells which will acquire the appearance of a benign tumor with the cell mass remaining physically grouped and compact. Progression is the terminal stage of cancer when it acquires a malignant phenotype by cellular detachment from the tumor body to cause metastasis at distant sites [314]. One new emerging concept for cancer development is the “Hallmarks of Cancer” which constitute an organizing principle for rationalizing the complexities of neoplastic disease. These hallmarks are;

sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Two new hallmarks have been added recently for the original six; reprogramming of energy metabolism and evading immune destruction, which is called emerging hallmarks. Two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks; genomic instability and mutation, and tumor-promoting inflammation that cause the genetic alterations that drive tumor progression and support of multiple hallmark capabilities [229].

To be able to cure cancer, we need to fight it at all three stages or interfere with the hallmarks of cancer. Thus, a good chemopreventive agent should be able to modulate at all the different stages, not only the initiation stage. In this context, phytochemicals (biologically active compounds found in plants), such as the isothiocyanates (ITCs) from cruciferous vegetables are promising agents due to their ability to modulate multiple targets involved in the carcinogenetic process. Among ITCs, Sulforaphane (SF), which is obtained by hydrolysis of glucoraphanin was given special attention, as this can interfere at various levels of the carcinogenetic process [315]. In this review, we will discuss SF sources, metabolism, and its effects to inhibit, reverse, or delay the development of all the stages of carcinogenesis (Fig. 1). We will also highlight SF effect on the treatment-resistant cancer stem cells and how SF combined with conventional chemotherapy may increase the therapeutic effect.

Figure 3.1 position (Fig 3.1 can be found on page 79 of this thesis)

3.3 Broccoli as the main source of sulforaphane:

Cruciferae or Brassicaceae is a medium-sized and economically significant family of flowering plant including cruciferous vegetables. This plant family gets its name, Cruciferae, from the fact that they have flowers with four equal-sized petals in the shape of a 'crucifer' cross, while "Brassica" is the Latin term for cabbage [316]. Some of the commonly used cruciferous vegetables include broccoli, cabbage, mustard, Brussels sprouts, collard greens, cauliflower, turnips, and Chinese cabbage. Previous epidemiological studies have shown an inverse association between vegetable consumption, especially cruciferous vegetables, and the risk of cancer development; including breast, lung, prostate, and colorectal cancer [317-323]. A diet containing three to five servings of broccoli per week is reported to be sufficient to decrease the risk of cancer development by almost 30%–40% [40].

Like other vegetables, there are some nutrients and phytochemicals with cancer chemo-preventive properties in cruciferous vegetables. These include fiber, carotenoids, folate, and chlorophyll. However, cruciferous vegetables are unique in being rich with glucosinolates, sulfur-containing compounds which give these vegetables their pungent aromas and spicy (some say bitter) taste [324]. Glucosinolates are considered the driver behind cruciferous vegetables' chemo-preventive anti-cancer effect [316,325,326]. Glucosinolates can be hydrolyzed to give their biologically active compounds, including indoles and ITCs, either by the plants' enzyme myrosinase [327] or by the gastrointestinal microflora [328,329]. More than 100 glucosinolates have been identified in plants each has its unique hydrolysis products. Among them, glucoraphanin hydrolysis yields either sulforaphane (SF), an anti-cancerous compound, or nitrile, which has not been shown to exhibit any health benefits and maybe actually toxic to healthy cells [330]. Recent studies showed that SF or nitrile can be obtained through pH adjustments. A low

pH and ferrous ions favor the formation of nitrile; on the other hand, SF formation is favored by neutral pH [331].

Initially, SF was discovered in red cabbage and correlated to antimicrobial activity. Later it was found to induce the expression of phase II detoxification enzymes [331,332]. Natural SF can only be obtained through the hydrolysis of glucoraphanin by endogenous or exogenous myrosinase. An American study in 2004, which included 59 kinds of cruciferous vegetables, reported that seeds from most broccoli cultivars were a better source for glucoraphanin than the others [333]. Glucoraphanin is rapidly absorbed, metabolized, and excreted, with almost 80% of the ingested amount appearing in the urine within 12–24 h after consumption and/or administration [334]. Many factors may affect the bioavailability, and thus overall therapeutic benefit, of dietary SF, most importantly pharmacokinetic properties, genetic variation, and methods of food preparation [335]. Approximately, 60%–80% of the metabolized glucoraphanin is converted to SF [30], with most broccoli variations contain between 0.1 and 30 μmol/g of glucoraphanin. A study showed that young broccoli sprouts, such as 3-day-old broccoli sprouts, contain as much SF as 10–100 times larger quantities of mature vegetables with fewer quantities of indole glucosinolates, which are considered in some studies as potential tumor promoters especially in the post-initiation phase [336] while others consider it an anti-cancer agents [337].

SF has a rapid diffusion rate into the cells of the intestinal epithelium owing to its lipophilic nature and low molecular weight, which after it undergoes metabolism via the mercapturic acid pathway [338]. Myrosinase enzyme inactivation can be caused due to cooking and/or blanching (during freezing process) of cruciferous vegetables and has been shown to decrease the bioavailability of SF [339-341]. In general, most studies suggest that only about 30%–50% of the

initial administered dose is excreted due to the preparation processes [342,343]. A study showed that myrosinase activity can be lost by boiling for more than 1 min or steaming for more than 4–5 min [344].

3.4 Metabolisms of SF

Studies on the cell culture showed that SF is transported into cells by passive diffusion. Once inside the cell, it rapidly conjugates with intracellular thiols [345]. Glutathione, the most common intracellular thiol, conjugates to SF by an interchangeable bond with the induction of glutathione S-transferases (GST) [346-348]. This conjugate then undergoes sequential enzymatic modifications to form cysteinyl-glycine, cysteine and N-acetylcysteine (NAC) conjugates which are excreted in urine [345] (Fig.2).

SF intracellular accumulation is a rapid process, as was reported when tested with murine hepatoma cells that were exposed to 100 μM of SF for 30 minutes. The intracellular concentration of SF reached 6.4 mM, and 95% of the accumulated SF was SF-GSH conjugate [346]. GSH-conjugated SF is rapidly exported from cells partially through the membrane transporter multidrug resistance-associated protein 1 (MRP1) [349,350]. Since the conjugation of GSH with SF is a reversible process, continuous accumulation of intracellular SF necessitates a continuous presence of SF in the extracellular space to promote continuous cellular diffusion and bonding [345].

Sulforaphane metabolites are distributed throughout the body and accumulate in different tissues. Franklin and colleagues reported that after a whole body autoradiographic study in rats, high concentrations of ITC metabolites were detected in the gastrointestinal tract,

liver, kidneys, and blood [351]. Distribution of SF depends on the high degree of binding to GSH, and its capacity to drive passive diffusion [339,352]. There are few studies that have successfully measured the distribution of SF and its metabolites in humans, mostly because of analytical limitations. A study in humans reported that 74% (± 29%) of SF from broccoli extracts may be absorbed in the jejunum, and a small portion is returning to the intestinal lumen of the jejunum in the form of SF-GSH [338]. Another important biomarker in the determination of SF distribution is the SF metabolites plasma concentration, as it reflects the amount of SF exposed to the tissues [353]. In one study, more than 50% of total plasma SF metabolites were SF-GSH with free sulforaphane, although other metabolites, including SF-NAC, were present in quantifiable amounts [354]. Ye et al. have reported a rapid absorption and appearance of ITC and their metabolites in the plasma, serum, and erythrocytes of human subjects, with this level started to decline after first-order kinetics (indicating rapid distribution and/or metabolism) [355]. These 4 human subjects were given a single dose of 200 µmol broccoli sprouts ITC preparation, and the ITC plasma concentrations reached its peak between 0.943 and 2.27 µM withing 1 h post exposure, and a half-life calculated to be 1.77 h (±0.13 h). Another human study reported 2.4 µmol/L plasma concentration in 3 hours after simple ingestion of 40g of fresh broccoli sprouts [335]. Comparable results were obtained from a study on rats, after a single dose of (50 µmol) sulforaphane, a detectable level of SF metabolites was evident after 1 h, peaking around 20 µM at 4 h with a half-life of 2.2 h [356].

Subsequent tissue accumulation after the distribution is also an important characteristic in the context of SF and its ability to exert chemo-preventive and anti-cancer effects. In an in-vivo study using mice given 300 or 600 ppm SF, they recorded SF and SF-GSH plasma concentrations

at 124–254 nM and 579–770 nM, respectively [357]. This group also measured the SF and SF-GSH concentrations within the small intestine, which were between 3–13 nmol/g of tissue and 14–32 nmol/g of tissue, respectively. A study on the chemo-prevention of SF against breast cancer showed that consumption of a broccoli sprout preparation containing 200 µmol of SF gave a peak plasma concentration of 2.0 µM dithiocarbamate (DTCs), a sulforaphane metabolite, at 1 h after ingestion and mean epithelial-/stromal-enriched breast tissue DTC concentrations were 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue for the right and the left breast, respectively [358].

The major organ involved in the conversion of GSH conjugates into the corresponding N-acetyl-S-cysteine conjugates, which is a very important step for the subsequent excretion of ITC from the body, is the kidney [359]. It has been shown that SF excretion and its metabolites follows first-order kinetics, with most studies reporting clearance from the body within 72 h of administration [329,355]. In Ye et al. study, after a single dose of approximately 200 µM SFN, around 58.3% and 77.9% of the dosage was excreted in urine as SF equivalent in 8 hours and 72 hours, respectively [355]. Interestingly, the primary urinary metabolite, SF-NAC, showed similar growth inhibitory potencies with the human bladder cancer cells as SF utilizing same anti-proliferative mechanisms that have been reported with SF [360].

Figure 3.2 position (Fig 3.2 can be found on page 80 of this thesis)

3.5 Chemoprevention activities of SF (inhibition of initiation phase)

3.5.1 Inhibition of Phase I enzymes

Dietary and environmental pro-carcinogens need to be bio-activated by the drug-metabolizing enzymes (DMEs) into highly reactive intermediates carcinogens that can bind with

macromolecules [361]. DMEs are classified into two main categories: oxidative or conjugative. Oxidative enzymes such as; NADPH-cytochrome P450 oxidoreductase and cytochrome P450 oxidase (CYP450) with their electron transfer system are responsible for phase I reactions, whereas conjugative enzymes such as the UDP-glucuronosyltransferases (UGTs) are mediating phase II enzymes. Phase I enzymes often catalyze oxidation, reduction, and hydrolysis reactions and are involved in detoxification of chemicals and bio-activation of pro-carcinogens [362,363]. For instance, CYP2E1 activates carcinogens such as N-nitrosodimethylamine [364,365] and CYP1A2 caused the activation of 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine [366]. CYP450 enzymes are the principal oncogenic related phase I enzymes as it contributes to 66% of bioactivation of carcinogen [367]. Only six CYP450s; 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4 accounts for 77% of the reported activation reactions.

Several studies reported that SF can inhibit the activity of CYPs either by direct interaction with CYPs or by regulation of mRNA expression. For example, Maheo et. Al reported that SF dose-dependently inhibited the activities of CYPs 1A1 and 2B1/2 in rat hepatocytes, as determined by 7-ethoxyresorufin-O-deethylase and pentoxyresorufin- O-dealkylase, respectively, and it decreased CYP3A4 activity in human hepatocytes by regulating the mRNA levels [368]. Similar results were also found in human hepatocytes, as SF inhibited the genes' expression for CYP1A1, 1A2, and CYP3A4 [369]. Evidence also suggests that SF might inhibit CYP3A by acting as an antagonist for the human steroid and xenobiotic receptor (SXR), which is an essential TF regulating the expression of CYP3A genes [370].

3.5.2 Induction of Phase II enzymes

To minimize the damage from highly reactive metabolites, cells own innate protective mechanisms through the induction of phase II enzymes that are of major importance in detoxification of carcinogenic intermediates. These enzymes include, but not limited to, GST, NAD(P)H:quinine oxidoreductase 1(NQO1), and UGT [371,372]. Phase II enzymes are strong antioxidants with a relatively long half-life and can conjugate endogenous substrates such as GSH to phase I metabolites to stop the biotransformation and in turn enhance elimination and excretion [373]. Several studies reported the capability of SF in inducing a lot of Phase II detoxification genes such as; ferritin, epoxide hydrolase, glutathione peroxidase, glutamate cysteine synthetase, and GST [374-376]. In human, with HepG2 cells for example, SF was reported to increase mRNA of UGT family 1 member A1 (UGT-1A1) and GST alpha 1 (GST-A1) [366], NQO1 activity [377], and UGT1A1 protein along with bilirubin glucuronidation [378].

Genes of Phase II enzymes carry in their 5'-flanking region one or more DNA regulatory elements called anti-oxidant response element (ARE). ARE activation will lead to induction of the downstream genes expression [379]. The crucial activator for ARE is nuclear factor erythroid 2-related factor 2 (Nrf2) which in unstimulated cells is normally bound by its repressor Kelch-like ECH-associated protein 1 (Keap1). This bond between Nrf2 and Keap1 binds Nrf2 to Cullin 3-dependent ubiquitinase for subsequent ubiquitination and targeted proteasomal degradation [326]. In case of cellular stimulation by environment insult, the Nrf2-Keap1 complex is disrupted in the form of conformational changes, leading to a switch in ubiquitination from Nrf2 to Keap1, causing nuclear translocation of Nrf2 [326]. The nuclear Nrf2 will form complexes with other nuclear factors and binds with ARE to induce the transcription of phase II downstream targets [380].

SF has been reported to directly react with the thiol groups of Keap1 to form thionoacyl adducts [381-383]. This specific modification of Keap1 will release Nrf2 from sequestration, helping the subsequent activation of ARE-driven gene expression. This mode of action by SF was proven by experiments in Nrf2 knockout mice [375]. Thimmulappa et al. created a transcriptional profile of the small intestine of wild-type (Nrf2 + / +) and knockout (Nrf2 - / -) mice which received SF. Many genes were reported to be regulated by Nrf2, including the previously mentioned phase II xenobiotic metabolizing enzymes, as well as antioxidative and cytoprotective proteins that are highly significant in limiting cancer progression.

However, one new study has reported adverse effect of Nrf2 activation after lung cancer initiation in mouse models [384]. In this study, SF activation of Nrf2 was effective in preventing initiation of chemically induced lung cancer, on the other hand, this activation promoted progression of pre-existing tumors regardless of chemical or genetic etiology. Another study used transplacental and/or lactational exposure to dibenzo[def,p]chrysene (DBC) in a mouse model which produces T-cell lymphoblastic lymphomas during early adulthood, in addition to lung tumors later in life to assess the impact of maternal dietary SF on cancer risk in offspring. This study reported increased morbidity and no reduction in lung tumorigenesis in offspring born to mothers supplemented with dietary SF or broccoli sprout powders [385]. The researchers explained this higher mortality rate by immunotoxicity as a function of increased DBC exposure as the combination of SF with indole-3-carbinol showed lack of early mortality, reduced morbidity and tumorigenesis. These results suggesting that the use of SF or broccoli sprout supplements will need more detailed studies to obtain the optimal dose, schedule and duration especially during pregnancy.

3.5.3 Protection of DNA from chemical insults:

SF utilize multiple mechanisms to counteract the genotoxicity of several carcinogenic compounds. For instance, heterocyclic amines (HCA) exposure is linked with breast, colon, and prostate cancers development, and in vitro studies suggested that SF is a potent inhibitor of mutagenesis induced by HCA [386]. In another in vitro study, the co-treatment with SF and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) significantly reduced the formation of PhIP-DNA adducts in HepG2 human hepatoma cells. However, the same study suggested that SF effect was a preventive action rather than induction of DNA repair enzymes. This was proposed based on when PhIP-treated cells were post-treated with SF there was no decrease in the levels of PhIP-DNA adducts [377].

In MCF-10F human mammary epithelial cells, DNA adducts formation by the exposure to benzo(a)pyrene and 1.6-dinitropyrene was inhibited with SF treatment [387]. Comparable results were reported with human colorectal cells as SF protected DNA from the single-stranded breaks induced by benzo(a)pyrene [388], while in human liver cells, which express CYP2E1 and CYP1A2, SF inhibited the double-strand breaks caused by N-nitrosodimethylamine and 2-amino-3-methylimidazo [4,5-f] quinoline [365]. SF contrasted the genotoxicity on human lymphocytes that was induced by four different compounds: alkylating ethyl methanesulfonate, aneugen vincristine, oxidizing H2O2, and alkylating and oxidizing mitomycin C. This reduction was related to an enhanced apoptotic response with ethyl methanesulfonate and mitomycin C. In contrast, no increase in the fraction of apoptotic cells was found with SF combination when compared with the treatment with vincristine or H2O2 alone, suggesting a different mechanism for the

protective action of SF, such as the inhibition of cell proliferation or the induction of specific enzymes [389].

Results from in vivo studies provide conflicting results. In a classic two-stage carcinogenesis protocol (where effects on initiation are separated from effects on promotion), Gills et al. [390] reported that SF topical administration before and after 7,12-dimethylbenz(a)anthracene failed to decrease the percentage of tumor-bearing mice. In contrast, Kuroiwa et al. [391] reported that SF decreases the incidence of atypical hyperplasia in pancreatic ducts and the incidence of adenocarcinomas caused by N-nitrosobis(2-oxopropyl) amine injection in a hamster model of pancreatic carcinogenesis. These results suggest that SFN possesses an interesting antigenotoxic potential, however, research efforts need to expand toward the in vivo mechanisms.

3.6 SF as a potential drug for cancer therapy

3.6.1 Modulation of the Promotion Phase

3.6.1.1 Induction of cell cycle arrest

Several research groups have reported that SF can arrest cancer cells at G1 phase [392,393], S phase [394], or G2/M phase [395-397] in different cell lines. The main cell cycle phase when SF interfere is G2/M as this cell cycle arrest has been reported in PC-3 and DU-145 human prostate cancer cells [398,399], HCT-116, HT29, and Caco-2 human colon cancer cells [398,400,401], MCF-7 human breast cancer cells [395], U2-OS human osteosarcoma cells [402], KB and YD-10B human OSCC [403], even in non-solid tumors as acute lymphoblastic leukemia (ALL) [404] and in primary myeloma tumor cells [405].

The ability of SF to induce cell-cycle arrests was explained by different mechanisms. One of the theories explaining SF associated G2/M cell cycle arrest in PC-3 prostate cancer cells is the substantial decrease in the protein levels of cyclin B1, cell division cycle 25B (Cdc25B), and Cdc25C, and the subsequent accumulation of Tyr-15-phosphorylated (inactive) cyclin-dependent kinase 1 (CDK1) [398]. The latter event was due to activation of checkpoint 2 kinase. Confirmation of this hypothesis came from the significant reduction of SF-induced G2/M arrest after transient transfection of PC-3 cells with checkpoint 2 kinase-specific small interfering RNA duplexes [398]. Another suggested mechanism is inhibition of tubulin polymerization, resulting in mitotic arrest as was reported after SF treatment resulting in condensed chromosomes lacking equatorial metaphase alignment in MCF-7 cells [395], and aberrant and absent mitotic microtubules in F311 [406] and MCF-7 cells [395]. In KB and YD-10B human OSCC cells, the reported mechanism was the induction of p21, a potent cyclin-dependent kinase inhibitor, which led to a decrease in the cyclin B levels [403]. In ALL similar mechanism was reported with upregulation of p21 and inhibition of the Cdc2/Cyclin B1 complex [404]. These findings suggest that p21 induction might be one of several molecular mechanisms that SF utilize to induce cell cycle arrest. These findings suggest that SF can confront three of the hallmarks of caner; sustaining proliferative signaling, evading growth suppressors, and enabling replicative immortality.

3.6.1.2. Induction of apoptosis

Apoptosis, or programmed cell death, is an imperative and highly selective biological mechanism in multicellular organisms that play important role in the regulation of cell proliferation in both physiological and pathological conditions [407]. Apoptosis is commonly characterized by distinct morphological characteristic changes and energy-dependent

biochemical mechanisms. It is considered an essential part of various processes including, but not limited to, proper cell turnover, normal development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death. Deficient apoptotic system (either too little or too much) is considered a factor in many human pathological conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and multiple types of cancer [408].

SF has been reported to induce apoptosis in many different cancer cell lines. The first system used to show this was colon cancer cells [400,409,410]. This was followed by several types of cancers cells including; prostate tumor [392,411,412], medulloblastoma [413], mammary tumor [395], ovary tumor [414], pancreas tumor [397], leukemia [415,416], bladder tumor [393,394], melanoma [417], and myeloma [405].

Numerous molecular mechanisms have been suggested explaining the pro-apoptotic action of SF. In PNAC-1 pancreatic cancer cell line, SF induction of apoptosis was suggested to be caused by cleavage of caspase-8 which activates the death receptor pathway of apoptosis [397]. A similar mechanism was reported with HCT-116 colon cancer cells as SF induced activation of pro-apoptotic caspase-7 and caspase-9, independent of p53 expression [418]. In human bladder 5637 cancer cells, SF was correlated with the activation of caspase-8 and caspase-9, the initiating caspases that are essential in both extrinsic and intrinsic apoptotic pathways [419]. Park et al. also reported the SF ability to affect key molecular targets that are strongly involved in apoptotic pathways such as the downregulation of apoptosis repressors B-cell lymphoma 2 (Bcl2) and B-cell lymphoma extra-large (Bcl-XL) genes expression, the upregulation of pro-apoptotic Bcl-2-associated X (BAX), and proteolytic activation of caspase-3 [420]. In a more recent publication,

SF activated the caspase-3 dependent pathway through upregulation of BAX and p53 with downregulation of Bcl-2 in HEp-2 human epithelial carcinoma cell line [421] and OSCC KB and YD-10B cell lines [422].

Another study on DU145 prostate cancer cells reported the activation of BAX, the downregulation of the inhibitors of apoptosis (IAP) protein family, and the induction of apoptotic protease activating factor-1 (APAF1) after SF treatment [423]. In F3II mammary cancer cells, SF excreted its pro-apoptotic function by fragmentation of DNA repairing protein poly(ADP-ribose) polymerase (PARP) along the reduction in the expression of Bcl2 [395]. This mechanism with fragmentation of PARP has also been reported in SF-treated DU145 and PC-3 prostate cancer cells with increased release of histone-associated DNA fragments [412,423] and HT29 colon cancer cells [400].

Singh et al. proposed that the initiating signal of SF-mediated apoptosis is the formation of ROS with disruption of mitochondrial membrane potential causing elevation of oxidative stress and cytosolic release of cytochrome c via both death-receptor and mitochondrial caspase cascade pathways [31]. This was supported by the results from the Moon et al. study on tumor necrosis factor-alpha (TNFα) resistant leukemia cells as SF induction of apoptosis was through ROS-dependent activation of caspase-3 [424]. Singh and co-workers thus indicated the importance of the conjugation of SF with GSH, once inside the cells, during the metabolism, in order to deplete intracellular concentrations of GSH and hypothetically lower the oxidative stress threshold of cancer cells which was reported by Pham et al. with the human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 [397]. However, very high doses such as 40 µM SF with PC3 and LNCap cells induced disruption of mitochondrial membrane potential leading to the formation of

acidic vesicular organelles and autophagy causing inhibition in the release of mitochondrial cytochrome c and apoptosis [425]. Therefore, it has been suggested that ROS production after SF treatment has the ability to affect cell death in a different pathway to apoptosis [326]. From all these studies together, it is suggested that SF has an innate ability to modulate both extrinsic and intrinsic apoptotic pathways, via the production of ROS and regulation of gene expression.

Other suggested mechanisms include inhibition of NF-κB activity [411,426] leading to the decreased gene expression of NF-κB-regulated vascular endothelial growth factor (VEGF), cyclin D1, and Bcl-XL [427]. Inhibition of the expression of estrogen receptor alpha (ERα), giving SF the potential to inhibit cancer cell proliferation caused by aberrant hormone ER receptor expression [428]. SF showed the capability to enhance tumor necrosis factor-related apoptosis inducing ligand (TRAIL) activity in human osteosarcoma cells (Saos2 and MG63) and hepatoma cells [429,430]. This mechanism was explained to be activated through reactive oxygen species-mediated up-regulation of death receptor 5 (DR5). Taking together, these findings suggest that SF induce apoptosis in different cancer cells utilizing several apoptotic mechanisms, thus these mechanisms are not cell-specific.

SF was recently identified as a novel histone deacetylase (HDAC) inhibitor in multiple human cells such as; embryonic kidney 293 (HEK293) [431], HCT116 colon cancer cells [431], and three prostate epithelial cells (BPH-1, LnCaP and PC-3) [432]. HDAC inhibitors have been reported as apoptosis inducers through elevation of genes such as p21 and BAX [433], and they are well suited for cancer therapy as it induces cancer cell death at concentrations to which normal cells are relatively resistant [434]. At pharmacologically relevant levels, SF increased acetylated histones H3 and H4, and expression of p21Cip1/Waf1 [431,432]. Impact of SF on DNA

methylation and histone modifying enzymes on the regulation of genes commonly dysregulated during carcinogenesis was reviewed recently [435]. SF induction of apoptosis is opposing cancer's resisting cell death one of the hallmarks of cancer.

3.6.1.3 Induction of autophagy:

Autophagy is a catabolic process during which the cytoplasmic components, including organelles, such as mitochondria, are engulfed by the membranous vacuoles often called autophagosomes, which fuse with lysosomes where the contents are degraded by the lysosomal proteases, thereby maintaining the quality of cells [436]. Although there is no clear connection between autophagy and apoptotic cell death, in some systems autophagy seems to promote apoptosis. In some cases, apoptosis and autophagy can be seen in vivo in certain tissues, and both morphologies may be observed within the same cell [437].

SF has shown to induce autophagy by increasing expression of protein 1 light chain 3 (LC3) as well as upregulating its recruitment to the autophagosome in human PC-3 and LNCaP cells prostate cancer cells [425]. Treatment of cells with a specific inhibitor of autophagy (3-methyladenine) attenuated localization of microtubule-associated LC3 to autophagosomes but aggravated cytosolic release of cytochrome c and apoptotic cell death. In another study the combination of SF with bafilomycin A1, as an autophagy inhibitor, enhanced apoptotic effect of SF in breast cancer cell line MDA-MB-231 [438]. Relatively recent work shows SF induces a unique long non-coding RNA (LINC01116) in prostate cancer which significantly upregulated several genes including GAPDH (regulates glycolysis), MAP1LC3B2 (autophagy) and H2AFY (chromatin structure) [439].

3.6.2 Modulation of the Progression Phase

3.6.2.1. Inhibition of Angiogenesis

Angiogenesis is the biological process of forming new blood vessels, which is essential for the normal physiological conditions such as tissue growth, wound healing and remodeling; but it is also a critical process in tumor development since it provides the tumor with nourishment for growth, expansion, and metastasize [440]. Angiogenesis around tumors was observed over 100 years ago [441-443]. In 1968, it was hypothesized that tumors produce a diffusible 'angiogenic' substance [444,445]. However not until 1971 Folkman proposed that tumor growth and metastasis can be angiogenesis-dependent, thus blocking angiogenesis might be a suitable strategy to hinder tumor growth [446]. This possibility inspired a rigorous search for pro- and anti-angiogenic molecules [447]. Angiogenesis provides nutrition and oxygen supply to the intra-tumoral bulk and without it the tumor growth will be limited to 1-2 mm3 [29]. Tumor cells produce pro-angiogenic molecules which help in the progression of tumor angiogenesis such as VEGF [448].

SF was able to inhibit the formation of microcapillaries in-vitro with HMC-1 human microvascular endothelial cells through inhibition of VEGF. This was explained by inhibition of the expression of the VEGF receptor Kinase insert domain receptor/ Fetal Liver Kinase 1 (KDR/flk-1) at the transcriptional level along with inhibition of the Hypoxia-inducible factor-1 Alfa (HIF-1 α), and c-Myc [449]. This inhibitory effects of SF on angiogenesis was also reported in studies using human umbilical vein endothelial cells, as was shown by inhibited tube formation on Matrigel, [450,451] and bovine aortic endothelial cells [452]. SF inhibition of angiogenesis was also

demonstrated in-vivo. Intravenous administration of low doses of SF inhibited endothelial cell response to VEGF when used with a subcutaneous VEGF-impregnated Matrigel plug mouse model [452]. Treatment of human prostate cancer PC-3 cells with SF was correlated to the reduction of the expression VEGF through the inhibition of NF-κB activity [427]. Taking together, these findings indicate that SF interferes with all the crucial steps of neovascularization from proangiogenic signaling through endothelial cell migration to tube formation. These studies indicate that SF prevents angiogenesis induction, one of the hallmarks of cancer.

3.6.2.2 Inhibition of metastasis

Cancer metastasis or secondary neoplastic growth is one of the major barriers for cancer treatment. Cancer prognosis is mostly evaluated according to the degree of invasiveness of cancer and its ability to metastasize. Cancer metastasis pass through multiple sequential steps including escape of single metastatic cancer cells from the original tumor, intravasation and dissemination using blood and lymphatic vessels, anchorage in the microvasculature of the secondary site organ, expansion and proliferation at a new site [453-455]. The key physical barriers for cancer metastasis are the basement membrane and the extracellular matrix, thus the importance of proteolytic degradation used by malignant cancers to overcome these barriers [456-458]. It is a complex biological event correlated to individual characteristics of the cancer bearer such as; general conditions and the state of immune response, and precise features of cancer cells including location, size, and histological characteristics [454,455,458].

The attenuation effect of SF on cancer metastasis was demonstrated by inhibition of lung metastases induced by B16F-10 highly metastatic melanoma cells in C57BL/6 mice [459]. This

inhibition was presented by a significant reduction in pulmonary fibrosis markers and cellular proliferation markers, as well as an increase in the survival of animals bearing metastases. The mechanisms behind SF effects on metastasis was suggested to be due to the inhibition of the activation of matrix metalloprotease (MMP) 2 and 9, a family of endoproteinase with the capability to degrade most of the components of the extracellular matrix, permitting cell invasion and metastasis formation [460,461]. MMPs was shown to modulate certain neoplastic evolution promoting factors such as cytokines and growth factors [462]. In oral squamous cell carcinomas, SF was able to inhibit cellular migration and invasion in-vitro by the down-regulation of MMP-1 and MMP-2 [463], and it was reported to reduce axillary lymph node metastasis with KPL-1 human breast cancer cells after implanted in female athymic mice [464]. Recently, SF was reported to inhibit the migration and invasion of 95D and H1299 non-small cell lung cancer cells, which has relatively high metastatic potential, in-vitro and also it had anti-metastatic effect with xenografts in-vivo [465]. This effect was suggested to be due to decreasing miR-616-5p levels by histone modification which lowered the expression levels of the glycogen synthase kinase-3 (GSK3) leading to inhibition of GSK3β/β-catenin signaling pathway and epithelial-mesenchymal transition (EMT). SF can resist activating invasion and metastasis as one of the hallmarks of cancers.

3.7 SF as a cancer stem cells (CSCs)-inhibiting drug

CSCs can resist conventional treatment, such as radio- and chemo-therapy, due to enhanced DNA repair mechanism and higher levels of MDR proteins. The ablation fraction of tumor mass (fractional kill) is usually used as a measure for the efficacy of cancer treatments in the initial stages of testing and CSCs form a small proportion of the tumor, this may lead to the

selection of drugs that cannot affect the more resistant stem cells. The theory suggests that conventional cancer treatment will kill finally differentiated or differentiating cells, which form the major bulk of the tumor but lack the ability to generate new cells. A population of CSCs, which poses an unlimited proliferation ability and is responsible for the formation of tumor, could remain unaffected and cause relapse [15,466]. By targeting the population of CSCs, a better prognosis can be achieved with less morbidity.

A number of studies have reported the ability of SF to target CSCs either by direct or indirect mechanisms, alone or combined with other anticancer compounds [467]. As previously mentioned, sulforaphane can reduce NF-κB activity and nuclear translocation of the NF-κB subunit, thus decreasing the expression of NF-κB-regulated genes [426,427]. Kallifatidis et al. demonstrated the ability of SF to revoke the resistance of pancreatic CSCs to TRAIL by interfering with TRAIL-activated NF-κB signaling [468]. Specifically, SF inhibited the DNA binding capacity of transactivation-competent NF-κB dimers which was found in CSCs population, causing impairment to the expression of NF-κB target genes with antiapoptotic effects.

In pancreatic cancer, the SHH signaling pathway, which has a key role in embryonic development, is hyper-activated and contributes to the self-renew of pancreatic CSCs [469]. SHH pathway is activated when hedgehog binds to its receptor Patched-1 (PTCH-1) which will stop the inhibition of the transmembrane protein Smoothened (Smo) caused by PTCH-1, this will activate and translocate the Glioma-Associated Oncogene (Gli) family of TF inside the nucleus, leading to activation of target genes including Nanog, Oct4, VEGF, and Zinc finger E-box-binding homeobox 1 (ZEB-1) [467,470]. Gli1 and Gli2 are acetylated in the inactive state and require HDAC-1 for activation through deacetylation [471]. Also, a correlation between the SHH signaling pathway

and NF-κB signaling was drawn, as the overexpression of SHH is activated by NF-κB [472]. In an in-vitro model, SF modulated SHH pathway signaling by inhibition of Smo, Gli1, and Gli2 in pancreatic CSCs [38]. SF was also reported to inhibit the nuclear translocation and transcriptional functions of Gli1 and Gli2 in a dose-dependent manner. Li and co-workers reported that similar results one year after but in-vivo with primary pancreatic CSCs isolated from human pancreatic tumors implanted into the pancreas of mice [473]. One possible explanation of the SF inhibition of Gli was the modulation of HDAC1 activity as was reported in human cervical cancer cells [474].

EMT, which is of critical importance in tumorigenesis and metastasis [475], has been correlated to CSCs [476]. SF has also been reported to be able to downregulate EMT markers, including ZEB-1, Twist-1, and vimentin [477]. One of the suggested mechanism of that inhibition is SF suppressed the EMT process via COX2/MMP2,9/ZEB1, Snail, and miR-200c/ZEB1 pathways thus possessed the ability to suppress metastasis in human bladder cancer cells [478]. Several studies have also reported that SF may inhibit the pro-survival PI3K/Akt pathway, which has been considered as a master regulator in oncogenic cellular survival, growth, and resistance [32,414,479].

β-catenin, a dual function subunit of the cadherin protein complex involved in regulation and coordination of cell–cell adhesion and gene transcription with function as an intracellular signal transducer in the Wnt signaling pathway, has been shown to be vital for the self-renewal of CSCs and in the EMT process [480]. It has been reported that high ALDH activity is a marker for normal and malignant human mammary SCs [481]. In the human cervical carcinoma (HeLa) and hepatocarcinoma (HepG2) cell lines, SF had the ability to downregulate β-catenin [420]. Li et al. reported that SF was able to reduce the number of ALDH positive cells in breast cancer cell line

both in-vitro and in-vivo, as well as inhibit the Wnt/beta-catenin self-renewal pathway [482]. The mechanism was evaluated through western blotting analysis and β-catenin reporter assay, which showed SF to promote β-catenin phosphorylation and its subsequent degradation. That is why it was proposed that the inhibition of breast CSCs was a consequence, at least in part, of the downregulation of the Wnt/β-catenin self-renewal pathway [177].

MicroRNAs (miRNAs), a class of highly conserved small RNA molecules with the function of regulating gene expression [483], have been reported recently to play a major role during carcinogenesis in various types of cancers [484-486]. The genomic reorganization is usually located at a locus near miRNA clusters in different types of cancers. A pair of neighboring miRNAs has been reported to be deleted in human chronic lymphocytic leukemia [487], with loss or amplification of miRNA genes seen in other tumors [488]. On the other hand, the deletion or silencing of a gene encoding a miRNA that normally suppresses the expression of oncogenes will cause an increase in that protein expression and cancer development [489]. miRNAs such as miR-21 [490], miR-31 [491], miR-504 [492], miR-10b [493], let-7 [494], or miR-184 [492] were reported to be most commonly deregulated in OSCC. Recently, Liu et al. reported that SF targets cancer stemness and tumor-initiating properties in OSCC by inhibition of BMI-1 through induction of tumor suppressor miR-200c [39]. Treating T24 bladder cancer cells with SF led to upregulation of miR-200c causing inhibition of EMT and metastasis [478]. MiR-140 was reported to be upregulated with SF treatment in MCF10DCIS and MDA-MB-231 breast cancer cells, which affected the expression of SOX9 and ALDH1 [495]. In Human nasopharyngeal cancer cell lines Hone1, CNE1, CNE2 and Sune1, SF induced apoptosis in CSCs by inhibition of total STAT3 expression level and STAT3 phosphorylation by upregulation of miRNA-124-3p [496].

3.8 Combination of SF with conventional chemotherapy

As we mentioned above, SF may act as a CSCs-inhibiting drug and since conventional chemotherapeutic drugs may lack the ability to target the more resistant CSCs, SF can be used as a combination treatment to achieve better prognosis. Several studies have been conducted to test this hypothesis. Kallifatidis and coworkers, reported SF potentiated the cytotoxic effect of cisplatin (CIS), gemcitabine, doxorubicin, and 5-fluorouracil (5-FU) against pancreatic CSCs and CIS and taxol against prostate CSCs [36]. They found that SF combination to conventional CT inhibited the clonogenicity/spheroid formation capacity, as well as ALDH1 activity, indicating that SF targeted CSCs inside these tumors. In chronic myeloid leukemia (CML) CD34+CD38– and CD133+ cell populations possess the majority of stem cell activity, whereas CD34+CD38+ cells represent multipotent committed progenitor cells [497]. Lin and co-workers found that CD34+/CD38- cells have particularly high resistant to imatinib, an inhibitor which is the conventional treatment for leukemia, however, SF combination with imatinib sensitized CD34+/CD38– cells and induced apoptosis by inducing intracellular ROS [37].

In ovarian cancer, Chen and co-workers used both cisplatin-sensitive and cisplatin-resistant cell lines to test the effect of SF/CIS combination. They reported that the combined treatment can reduce cell viability of both cell lines in a time- and dose-dependent manner. Furthermore, SF enhanced cisplatin-induced apoptosis and G2/M phase arrest, thereby eliminating the resistance to CIS on ovarian cancer cells [498]. Other study reported that CIS alone might increase the cancer stem cell-like properties in gastric carcinoma cells via activating the IL-6 /IL-6 receptor (IL-6R) /signal transducer and activator of transcription 3 (STAT3) signaling.

However, SF combined treatment with CIS prevent this process by activating the miR-124, which directly targets the 3'-untranslated regions of the IL-6R and STAT3 [499].

Recently, our group showed that SF can be combined with CIS or 5-FU to enhance the cytotoxic effect against head and neck squamous cell carcinomas in a dose and time dependent manner [500]. SF increased apoptosis induction through the activation of Caspase-dependent apoptotic pathway without added cytotoxicity to non-cancerous cells. An interesting feature of SF is the ability to induce apoptosis in cells with p53 mutated or knocked-out. This allow it to overcome treatment resistance and enhance the efficacy of other chemotherapeutic agents as most of the conventional chemotherapeutic agents require the presence of intact p53 [501-503].

3.9 Human clinical trials of SF

Ever since SF showed the potential to induce phase II enzymes, many study groups have tried to explore the potential use of SF as a chemopreventive or therapeutic drug. Several commercially developed SF supplements are available nowadays; however, there is a difficulty in manufacturing a potent and bioavailable formula due to the intrinsic instability of SF molecule which interferes with this method of delivery [504]. To manufacture a sulforaphane-yielding supplement we need to retain both the glucoraphanin precursor and the myrosinase enzyme to allow metabolism and transformation to the bioactive isothiocyanate inside the body [505]. To the best of our knowledge, SF has not yet been investigated in humans in pure form, however, a number of phase I and II clinical trials on SF (from broccoli sprouts) are in progress or have been completed aiming to test its safety, tolerance, pharmacokinetics, and therapeutic benefit in healthy human subjects or in the oncogenesis field. Table 1 is listing some of the most prominent

clinical trials of SF. One recent human clinical trial used stabilized free sulforaphane for testing the effect on biochemical recurrence after radical prostatectomy in prostate cancer patients [506].

In the year 2000, Conaway and co-workers designed a study to compare the bioavailability of ITC from fresh and steamed broccoli [339]. Participants were prohibited from any food containing glucosinolates or ITCs for 48 hours then allowed to consume a single dose of 200 g of broccoli either fresh or steamed followed by the collection of blood and urine samples during the first 24 hours, which were analyzed for total ITC equivalent level using high-performance liquid chromatography. They reported that the average 24-hour urinary excretion of ITC equivalents amounted to 32.3 +/- 12.7% and 10.2 +/- 5.9% of the ingested ITC for fresh and steamed broccoli, respectively. These findings suggested that the bioavailability of ITC from fresh broccoli is almost 3 times higher than from steamed broccoli.

In 2005, Kensler and co-workers published a study on the effect of daily consumption of glucoraphanin and how it modulates aflatoxin bioavailability and disposition in residents of Qidong China [507]. Residents of Qidong are at high risk for the development of hepatocellular carcinoma, this is partially due to long-term exposure to aflatoxin-contaminated food, along with the airborne carcinogen, phenanthrene [508]. In Kensler study, two groups, each of 100 healthy adults, were selected: a group drank infusions of 3-day-old broccoli sprouts containing 400 µmol of glucoraphanin and the second group consumed no more than 3 µmol nightly for 2 weeks. Interestingly, there were no problems at all of safety or tolerance in their study. The study reported an inverse association between the level of SF metabolites, urinary levels of DTCs, and the carcinogen related markers, such as aflatoxin-DNA adducts and trans, anti-phenanthrene

tetraol, a metabolite of the combustion product phenanthrene. The same group in another study reported that SF increased the excretion of airborne pollutants in individuals consuming the broccoli extract beverage containing SF or glucoraphanin [509]. Although promising, this study's results displayed significant variability in the bioavailability levels of the active compound. Fahey et al. found similar results regarding the variation in bioavailability of SF between subjects in his study [510]. In their work, they administrated sulforaphane-rich broccoli sprout extract (BSE) to two distinct populations (Chinese and Baltimoreans) which gave bioavailability difference between individuals in both populations, ranging from 1% to 40%.

In 2007, Cornblatt et al. analyzed the bioavailability of SF in human tissues using human breast tissue as a model [358]. Eight women with scheduled reduction mammoplasty were registered for the study and they were given a broccoli sprout preparation that contains 200 μmol SF on average 50 min before the surgery. After the surgery they measured the level of DTCs in the removed breast tissue and the mean breast tissue DTCs concentration was found to be 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue for the right and the left breast respectively, putting in mind that the tissues were removed from the right breast first. In a recent intervention study, they measured the total SF metabolite concentration in plasma every hour in human subjects who consumed 40g fresh broccoli sprouts, which reached its peak after 3 hours (> 2 μM) [335].

In 2009, Riedl and co-workers published a clinical study on sulforaphane's induction of Phase II enzymes in the upper airway of human [511]. 65 participants were enlisted in this study and they consumed oral SF doses contained in a standardized broccoli sprout homogenate. They reported that Phase II enzymes such as glutathione-s-transferase M1 (GSTM1), GSTP1, NQO1, and hemeoxygenase-1 (HO-1) were all up-regulated in a dose-dependent manner with no

significant adverse events. These findings demonstrated the potential of using SF to reduce the inflammatory effects that accompany oxidative stress.

Oregon Health & Science University Knight Cancer Institute conduct a phase II clinical trial, from 2010 to 2013, aimed to investigate the effect of SF-enriched broccoli extract on recurrent prostate cancer [512]. In their study, 20 patients who were diagnosed with recurrent prostate cancer were given 200 μmol/day of sulforaphane-rich broccoli extracts for a period of 20 weeks. The primary endpoint was set as ≥50% PSA decline (prostate-specific antigen, which is usually elevated with prostate cancer) but only one patient reached this endpoint and seven patients experienced smaller PSA declines (<50%). However, it was found that the doubling time for PSA was significantly lengthened during the treatment (pre-treatment: 6.1 months; on-treatment: 9.6 months). Furthermore, 200 μmol/day SF was reported to be safe with no Grade 3 adverse event was observed.

Currently, an early phase 1 clinical trial is recruiting to test the effect of Avmacol®, a dietary supplement made from broccoli sprout and seed extract powder, in preventing recurrence in patients with tobacco-related HNSCC. This study aims to analyze the bioavailability of SF in this new drug as well as to determine the level of pharmacodynamic upregulation of Nrf2 target gene transcripts in the oral epithelium of patients after finishing the treatment for tobacco-related HNSCC, including high grade dysplasia, carcinoma in situ, or invasive carcinoma [513].

Table 3.1 position (table 3.1 can be found on page 75 of this thesis)

3.10 Conclusion:

Cancer is an extremely complex disease, with multiple genetic and molecular alterations which can be targeted with new pharmacological strategies. Considering the biological complexity in tumors, a promising strategy will be the use of non-specific agents, which can inhibit many targets concurrently. Phytochemicals represent an interesting source of multitarget compounds. Since 1992, after SF was reported as a strong inducer of phase II enzymes and chemopreventive agent, many studies followed and revealed that SF possess multiple chemotherapeutic abilities. These include proliferation inhibition, apoptosis, and cytodifferentiation induction, as observed in several in vitro and in vivo models. SF can induce terminal differentiation, generating cells with no or limited replicative capacity, leading to apoptosis [514]. Thus, differentiation may represent an alternative tactic to more conventional anticancer agents. Although there are several drugs for controlling cancer growth in humans, there are no drugs available that inhibit the metastasis of cancer cells specifically. This is because metastatic cancer cells can respond differently to radiotherapy or chemotherapy [459]. However, SF demonstrated high capability to inhibit metastases in different tumor models. Effectiveness is, however, not the only requirement for developing new chemopreventive or chemotherapeutic agents. Although larger-scale clinical trials are necessary, the completed human clinical trials with SF reported a favorable toxicological profile, no genotoxicity and high tolerability and safety in humans. To conclude, the recent literature has clearly demonstrated that Sulforaphane is a promising and safe chemopreventive molecule and a powerful future tool for fighting cancer.

Table 3.1: Summary of the most relevant human clinical trials on sulforaphane related to cancers, safety and bioavailability.				
References	**No. of Subjects**	**Intervention**	**Endpoints**	**Findings**
[339]	12	200 g fresh or steamed broccoli at a single dose	Compare metabolic fate of steamed vs. fresh broccoli	Bioavailability of ITC from fresh broccoli is approximately 3 times greater than from steamed broccoli
[507]	200	Hot water infusions of 3-day-old broccoli sprouts nightly containing 400 µmol glucoraphanin for 2 weeks	Determine whether broccoli sprouts can alter the disposition of aflatoxin and phenanthrene	Significant inter-individual differences in bioavailability; an inverse association between urinary levels of DTCs and urinary aflatoxin-DNA adduct.
[515]	12	21 doses of glucoraphanin or SF over 7 days (8 hours interval)	Evaluate safety, tolerance, and metabolism	No significant or consistent abnormal events (toxicities) associated with any of the sprout extract ingestions
[358]	8	Single dose of oral broccoli sprouts delivering 200 µmol SF at 1 hour pre-surgery	Evaluate whether Sulforaphane is bioavailable in human breast tissue	2 pmol/mg breast tissue was observed
[516]	3	Single dose of 68 g BroccoSprouts broccoli sprouts (approximately 105 mg SF)	Evaluate the effect of SF on HDAC activity in peripheral blood mononuclear cells (PBMC)	During the period 3–6 hrs. after ingestion, broccoli sprouts strongly inhibited HDAC activity in human PBMC.

[517]	22	400 g of broccoli per-week for over 6 months	Monitor global gene expression change in prostate gland before, during and after broccoli-rich diet	Broccoli consumption interacts with GSTM1 genotype to result in complex changes to signaling pathways associated with inflammation and carcinogenesis in the prostate
[511]	65	Dose-escalation from 25 g to 200 g of fresh broccoli sprouts	Determine the effect of SF on the expression of phase II enzymes	Safe and effective induction of Phase II enzymes in the upper airway of human subjects
[518]	6	Single and repeated intake of 300 ml of liquidized broccoli for 10 consecutive mornings	Pharmacokinetic study of SF	SF was rapidly absorbed, with peak plasma levels reached within 1.5 hour; plasma level declined rapidly to 50% of peak level within 3 hours, and then decreased to about 10-15% by about 8 hours, and thereafter remained fairly constant between 8 hours and 24 hours.
[519]	81	10 g and 5 g daily of broccoli sprout powder for 4 weeks	Determine whether broccoli sprout powder can reduce biomarkers of oxidative stress in type 2 diabetes	Significant decrease in oxidative stress index.

[512]	20	200 µmol/day of Sulforaphane-rich BSE for 20 weeks	Determine whether ≥50% reduction in PSA (prostate-specific antigen, which is often elevated in prostate cancer) can be achieved	No ≥50% reduction in PSA was observed; the doubling time for PSA is significantly lengthened under treatment; treatment with 200 µmol/day was safe with no Grade 3 adverse events.
[520]	98	200 µmol/day of BSE for 4-8 weeks	Evaluate if SF can prevent prostate cancer development and progression	Downregulation of AMACR and ARLNC1 genes (prostate cancer development), but no significant difference in HDAC activity or prostate tissue biomarkers
[521]	30	Constant dose of BSE dissolved in mango juice for 14 days	Determine the therapeutic benefit (measure proliferative rate) of sulforaphane and evaluate the ability of sulforaphane to modulate specific cytoprotective enzymes for breast cancer	Not published yet
[522]	17	50, 100, or 200 µmol/day of BSE administered orally for 28 days	Determine (1) adverse effects (2) visual and cellular changes in atypical nevi, (3) biodistribution, and (4) effect of sulforaphane on STAT-1 and -3 expression in melanoma	Oral BSE-SFN is well tolerated at daily doses up to 200 µmol and achieves dose-dependent levels in plasma and skin

[523]	49	High sulforaphane BSE (100 μmol sulforaphane) every other day for 5 weeks	Identify biological effects of sulforaphane on normal prostate tissue, and determine whether the consumption of BSE will inhibit growth of prostate cancer	BSE-rich broccoli soup affected gene expression in the prostate of men on active surveillance, consistent with a reduction in the risk of cancer progression.
[524]	54	BSE oral supplementation (250 mg of a broccoli seed extract containing GFN (BroccoMax™)) three times/day for 2–8 weeks	Determine (1) bioavailability, (2) effect of supplement on biomarkers of prognosis, and (3) effect on HDAC inhibition in breast Cancer	BSE supplementation for a few weeks is safe but may not be sufficient for producing changes in breast tissue tumor biomarkers.
[525]	70	SF caplets (Avmacol) in a daily dose for 12 weeks	Evaluate the protective effects of the nutritional supplement SF on doxorubicin-associated cardiac dysfunction in breast cancer	Not yet recruiting

Figures:

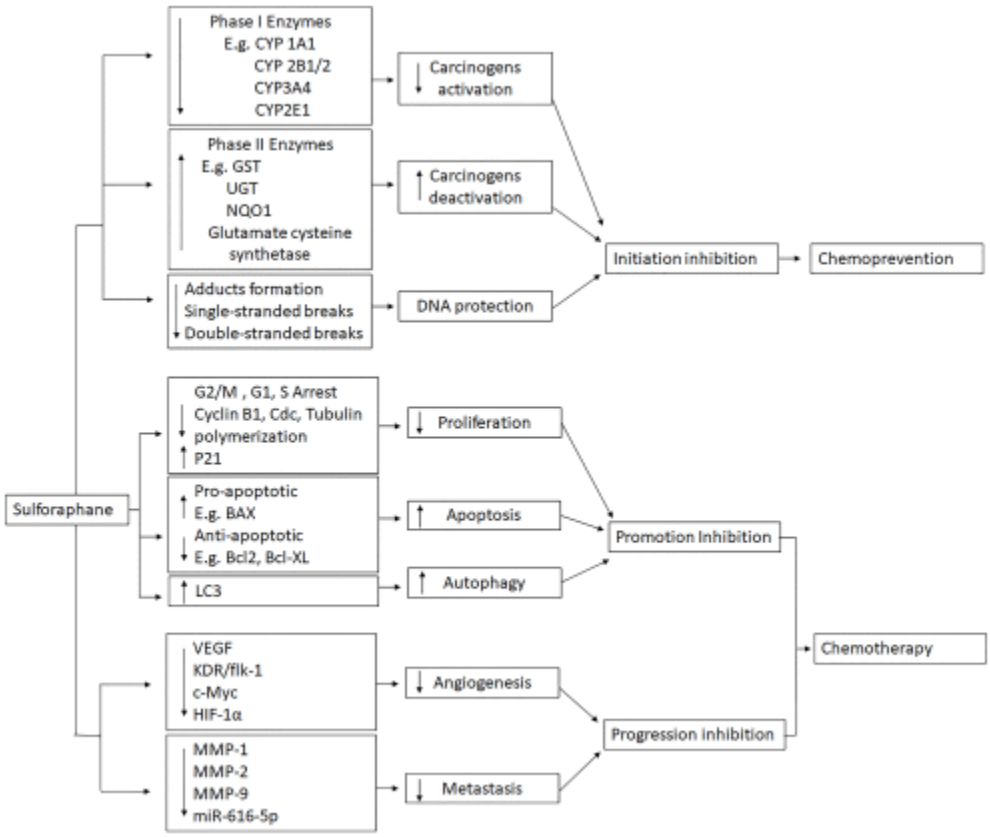

Fig 3.1 SF affects all three stages of the carcinogenetic process acting as both a chemopreventive and chemotherapeutic agent by modulating various molecular targets. The arrows reflect changes in protein levels/activities as well as gene expression

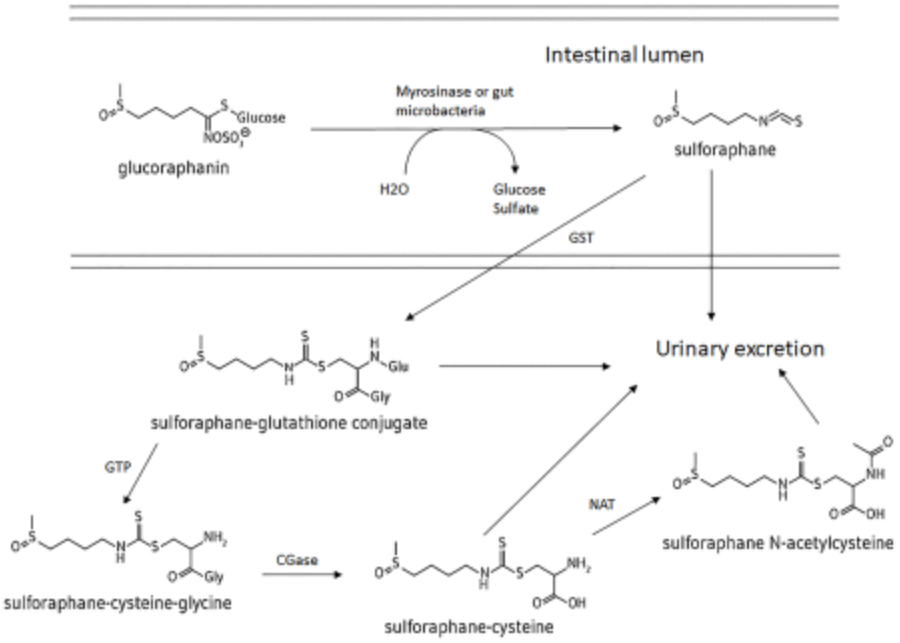

Fig 3.2 The conversion of glucoraphanin to sulforaphane, and its subsequent metabolism. Hydrolytic conversion of glucoraphanin to sulforaphane occurs by either the action of plant-derived myrosinase or the microbiota of the human colon. After rapid passive diffusion into the cells of the intestinal epithelium, sulforaphane undergoes metabolism via the mercapturic acid pathway. This process involves its initial conjugation with glutathione, rapidly catalyzed by GST enzymes. Then metabolized sequentially by γ-glutamyl-transpeptidase (GTP), cysteinyl-glycinease (CGase), and N-acetyltransferase (NAT). The conjugates are actively transported into the systemic circulation where the mercapturic acid (sulforaphane-NAC) and its precursors are actively secreted in urine

Chapter 4 - Thesis Hypothesis and Objectives

4.1 Study hypothesis

▪ The main hypothesis is based on the assumption that Sulforaphane can be used as co-treatment with the conventional chemotherapy to increase the cytotoxic effect against head and neck squamous cell carcinoma cancer stem cells without adding adverse effects on the non-cancerous tissues and cells. This implies that lower doses of chemotherapy can be achievable with the comparable or higher efficacy, especially in treatment-resistant cases, in the clinical settings of head and neck cancer treatment.

4.2 Objectives

To test the main hypothesis, this thesis has 3 main objectives:

▪ To evaluate the effects of using Sulforaphane as a co-treatment with Cisplatin or 5-Fluorouracil against head and neck squamous cell carcinoma.

▪ To isolate and characterize a pure cancer stem cells from head and neck cancer, as they are one of the causes of treatment failure and recurrence.

▪ Assess the effect of Sulforaphane either alone or combined with chemotherapy against head and neck squamous cell carcinoma cancer stem cells.

Body of the thesis:

Chapter 5 - Broccoli extract improves chemotherapeutic drug efficacy against head-neck squamous cell carcinomas.

5.1 Preface (connecting paragraph)

As we mentioned in the first chapter, head and neck cancer is ranked seventh in the rate of incidence worldwide and despite the improvement in treatment modalities, the five-year survival rate for head and neck squamous cell carcinoma has remained unchanged at about 50% over the past 30 years. One of the suggested reasons for treatment failure is the severe toxic side-effects of chemotherapy that limits its doses. The conventional chemotherapies used for head and neck cancers are Cisplatin and 5-Flourouracil. Cisplatin can cause serious side effects such as nephrotoxicity, otological disorders, bone marrow suppression, hemolytic anemia, and neurotoxicity. While 5-Flourouracil side effects range from the common mouth sores, loss of appetite, thrombocytopenia, and chemotherapy-induced acral erythema to the less common but more serious cardiotoxicity and neurologic effects. There is a rising need to develop methods capable of reducing the chemotherapy side effects and increase or maintain the cytotoxic effect on the cancer cells. Sulforaphane possess high anti-cancerous activity as described in chapter 3. Promising results have been reported using sulforaphane as combination therapy with other conventional treatment modalities against solid tumors.

In this chapter, we tested sulforaphane as a combination treatment with conventional chemotherapy; CIS and 5-FU. SF proved to possess anti-cancer activity and it increased the cytotoxic effect of CIS and 5-FU against head and neck squamous cell carcinoma. Our results suggested that this effect is due to stimulation of apoptosis through activation of Caspase-

dependent apoptotic pathway. This might allow the usage of lower dosages of chemotherapy which in turn will reduce the side-effects.

The study presented in this chapter has been published in the Journal of Medical Oncology, 2018 Aug 4;35(9):124. doi: 10.1007/s12032-018-1186-4.

Broccoli Extract Improves Chemotherapeutic Drug Efficacy Against Head-Neck Squamous Cell Carcinomas.

Running title: Sulforaphane Synergies Chemotherapy in Head and Neck Cancers

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5.2 Abstract

Purpose: The efficacy of cisplatin (CIS) and 5-fluorouracil (5-FU) against squamous cell carcinomas of the head and neck (SCCHN) remains restricted due to their severe toxic side-effects on non-cancer (normal) tissues. Recently, the broccoli extract sulforaphane (SF) was successfully tested as a combination therapy targeting cancer cells. However, the effect of lower doses of CIS or 5-FU combined with SF on SCCHN remained unknown. This study tested the chemotherapeutic efficacies of SF combined with much lower doses of CIS or 5-FU against SCCHN cells aiming to reduce cytotoxicity to normal cells.

Methods: Titrations of SF standalone or in combination with CIS and 5-FU were tested on SCCHN human cell lines (SCC12 and SCC38), and non-cancerous human cells (fibroblasts, gingival epithelial cells and salivary gland acinar cell line). Concentrations of SF tested were comparable to those found in the plasma following ingestion of fresh broccoli sprouts. The treatment effects on cell viability, proliferation, DNA damage, apoptosis and gene expression were measured.

Results: SF reduced SCCHN cell viability in a time- and dose-dependent manner. SF-combined treatment increased the cytotoxic activity of CIS by two-fold and of 5-FU by ten-fold against SCCHN, with no effect on non-cancerous cells. SF-combined treatment inhibited SCCHN cell clonogenicity and post-treatment DNA repair. SF increased SCCHN apoptosis and this mechanism was due to a down-regulation of BCL2 and up-regulation of BAX, leading to an up-regulation of Caspase3.

Conclusion: Combining SF with low doses of CIS or 5-FU increased cytotoxicity against SCCHN cells, while having minimal effects on healthy cells.

6 Keywords: Head and Neck cancer; Carcinoma, squamous cell; Sulforaphane; Drug therapy; Apoptosis; DNA damage.

5.3 Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is one of the most prevalent malignant neoplasms of the upper aerodigestive tract. SCCHN is now the seventh most common cancer worldwide, with over 500,000 new cases diagnosed and 380,000 deaths annually which is nearly 4.6% of all cancer cases [1,526]. Despite the improvements in treatment modalities, the five-year survival rate for SCCHN patients have remained unchanged at about 50% over the past 30 years [2,527] as 40 to 60% of SCCHN survivors suffer from relapse in the form of recurrences or metastases [528,529].

Resistance to standard surgical, radiation and chemical therapies continue to be a limiting factor in the treatment of SCCHN. One major factor in cancer treatment failure is because the efficacy of current standard chemotherapy, such as cisplatin (CIS) and 5-fluorouracil (5-FU), is restricted partly due to their severe toxic side-effects. CIS forms DNA adducts which lead to induction of apoptosis in cancer cells [6], while 5-FU inhibits the thymidylate synthase enzyme through its metabolite to inhibit cancer cells division [10]. These mechanisms have non-specific chemotherapeutic effects and thus affect both cancer and non-cancer (normal) cells. The toxic side effects of CIS are dose-dependent and can cause nephrotoxicity, bone marrow suppression with hemolytic anemia and neurotoxicity [7-9]. Similarly, the side effects of 5-FU include

dermatologic effects, hand and foot syndrome, neurotoxicity and cardiotoxicity [10]. Incidence of 5-FU associated cardiotoxicity is 7.6% with mortality rate between 2.2% and 13% [8]. Reducing the chemotherapeutic dose while maintaining its efficacy is critical to improve the treatment outcome of cancers and to decrease morbidity and mortality rates.

Recently, studies have highlighted the potential of phytochemicals as a source of therapeutics for certain forms of cancer [28]. Sulforaphane (SF) is the most characterized isothiocyanate compound and is found in high concentrations in cruciferous vegetables, such as in broccoli [29]. It has been demonstrated that SF has multiple biological activities such as anti-inflammatory, anti-oxidant and anti-cancerous [31-33]. In addition, SF has low toxicity [34], making it an interesting candidate as a chemotherapeutic agent. SF has been shown to target multiple pathways involved in cancer cells' functions when used in combination with other anti-cancer compounds. Specifically, SF increased the effect of imatinib and gemcitabine against chronic myeloid leukemia cells and pancreatic cancer cells, respectively [36,530]. However, the antioxidant ability of SF induced the expression of phase 2 metabolic enzymes, which may protect cells from reactive oxygen species [531]. This is a concern for many chemotherapeutic agents as they work through free radicals, so SF combination may reduce these drugs' efficacy. There are very few studies that examined the SF effect on head and neck squamous cell carcinomas and to our knowledge no one tested the effect of SF on the activity of conventionally used chemotherapy CIS and 5-FU as a combined treatment. We hypothesized that SF is a suitable agent to lower the doses of conventional chemotherapeutic drugs (such as CIS and 5-FU) without losing their efficacy. This would result in a reduction or even elimination of the severe toxic side effects associated with current chemotherapeutic drugs. This study examined the effects of

combining SF with low-dose chemotherapy against human SCCHN for the first time. We also determined the underlying mechanism of action of the SF combined chemotherapy.

5.4 Materials and methods

1. Cell culture

SCC12 and SCC38 cell lines were purchased from the University of Michigan and were used as models for SCCHN (Table 5.1). They were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Massachusetts, United States) supplemented with 1% non-essential amino acids (Gibco). Primary fibroblasts (FB) were isolated from human salivary glands and cultured in RPMI medium (Thermo Fisher, Massachusetts, United States) [532]. Both medias were supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic (Thermo Fisher). Gingival Epithelium Progenitors, Single Donor (HGEPs) were purchased from Cedar Lane Laboratories and were cultured in ready-to-use CnT-Prime medium (CELLnTEC, Switzerland) [533]. Immortalized normal human salivary gland acinar cell line (NS-SV-AC) was a gift from Dr. Azuma M (Tokushima University, Japan) and was cultured in KGM-2 (Lonza, Switzerland) supplemented with 2% Pen/Strep. All cell types were incubated in a humidified incubator at 37°C with 5% CO_2.

Table 5.1:

	UM - SCC12	**UM – SCC38**	**References**
Synonym	University of Michigan-Squamous Cell Carcinoma-12	University of Michigan-Squamous Cell Carcinoma-38	[534]

RRID	CVCL_7717	CVCL_7749	[534]
Primary tumor location	Larynx	Tonsillar pillar	[534-536]
Gender	Male	Male	[534-536]
Age	72 years	60 years	[536]
TNM stage	T2N1M0	T2N2aM0	[536,537]
Degree of tumor Differentiation	Moderate well differentiated SCC	Moderate well differentiated SCC	[536]
Doubling time	34 h	24 h	[538,539]
Chemotherapy resistance	Moderate	High	[540-542]
Radiotherapy resistance	High	Low	[536]

Table 5.1. Comparison between the two head and neck squamous cell carcinoma cell lines used in this study. Abbreviations: **RRID:** Research Resource Identifiers. **CVCL:** Cellosaurus (on-line knowledge resource on cell lines). **TP53:** tumor protein p53.

2. Cytotoxic agents

Sulforaphane (Cayman Chemical, Michigan, United States) was purchased as a solution in ethanol with purity ≥98% and stored at -20°C. Cisplatin (Cayman Chemical) was prepared in

phosphate-buffered saline to a 0.3 mg/ml stock and kept at 4°C protected from light. 5-Fluorouracil (Sigma Aldrich, Missouri, United States) was prepared in dimethyl sulfoxide (DMSO) to 50mg/ml stock. Final concentrations of the solvents in the working solution medium were 0.1% or less.

3. MTT assay

1 to 3 × 10^3 cells were seeded in 96-well plates according to cell type. Twenty-four hours later, they were treated with different concentrations of SF and/or chemotherapeutic agents and further incubated for 72hours. The medium was then removed and 10% solution of 5 mg/ml MTT in medium (Sigma Aldrich (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added to each well and incubated at 37°C for 2 hours. The medium was removed, and formazan was dissolved by adding DMSO to each well. The optical density was measured at 562/540 nm in an EL800 Microplate Reader (BIO-TEK Instruments, Vermont, United States). The assay was done in triplicates.

4. Colony-forming assay

Tumor cells were seeded at 1×10^5 cells per well in a 6-well tissue culture plates. Twenty-four hours later, the cultures were treated with SF 3.5 µM without CIS 0.5µg/ml or 5-FU 0.13 µg/ml and incubated for 72 hours. The cells were trypsinized, plated at a density of 400 living cells per well in 6-well tissue culture plates, and incubated for 10 days (changing the medium every 3 days). To determine colony formation, culture medium was removed, and colonies were fixed and stained with 1% crystal violet, 50% methanol in DDH_2O for 1 hour. The number of

colonies with >50 cells were counted under an inverted microscope and the percentage of cell survival was calculated.

To assess the cells ability to repair DNA, the previous technique was used but the cultures were treated with sub-lethal doses of SF (0.875 µM) and/or CIS (0.02 µg/ml), 5-FU (0.2 ng/ml) for 72hours.

5. Annexin V apoptosis detection

Post-treatment apoptosis was measured by using the PE-Annexin V Apoptosis Detection Kit (BD Bioscience, Ontario, Canada). Briefly, 1.5×10^5 cells were seeded per well in a 6-well plate for 24 hours and were then treated with SF and/or chemotherapeutic agents for 72 hours. Cells were detached using Accutase (Biolegend, California, United States), washed with annexin binding buffer, and then stained with PE annexin V and 7-AAD for 15 min in the dark at room temperature, cells were washed and resuspended in fresh buffer and analyzed by flow cytometry using a LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo vX (FlowJo LCC, Oregon, United States)

6. Evaluation of mRNA expression levels by quantitative real-time PCR (QPCR)

QPCR was used to detect changes in genes coding for BAX, Caspase3, and BCL2. Higher drugs concentrations were used in cells treatment to show the effect of treatment on the genetic level. Total RNA was extracted from SCCHN cells treated with SF 7 µM with or without CIS 2 µg/ml or 5-FU 13 µg/ml for 72 hours using TRIzol (Thermo Fisher Scientific). The first-strand cDNA was synthesized from 1 µg total RNA using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). For the quantification of gene amplification, QPCR was performed using

StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) in the presence of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Target sequences were amplified at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60-65°C annealing temperature for each gene for 1 min. The following gene-specific primers were used: GAPDH: (5'-GAGAAGGCTGGGGCTCATTT-3', 5'- AGTGATGGCATGGACTGTGG -3'), BCL2: (5'-CTGCACCTGACGCCCTTCACC-3', 5'-CACATGACCCCACCGAACTCAAAGA-3'), BAX: (5'-CGGGTTGTCGCCCTTTTCTA-3', 5'-TGGTTCTGATCAGTTCCGGC-3'), Caspase3: (5'-CTCGGTCTGGTACAGATGTCGA-3', 5'-CATGGCTCAGAAGCACACAAAC-3'). All assays were performed in triplicate and the expression was calculated on the basis of ΔΔCt method. The *n*-fold change in mRNAs expression was determined according to the method of $2^{-\Delta\Delta CT}$.

7. Statistical analysis

Data were presented as the means ± standard deviation (SD) of three independent experiments with comparable results. Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess significant differences between groups; *p*-values < 0.05 were considered statistically significant. GraphPad prism 6 software was used (GraphPad Software, California, United States)

5.5 Results

1. SF inhibited the growth of SCCHN cells

SCC12 and SCC38 cell lines were treated with various concentrations of SF alone. We found that SF inhibited the viability of both SCCHN cell lines to a similar extent (Fig 5.1a). The IC_{50}

of SF was 3.81 µM and 3.87 µM for SCC12 and SCC38, respectively. Morphological changes indicating early apoptosis as cellular swelling, pyknosis and formation of apoptotic bodies in cancer cells were observed at a concentration of 3.5 µM and it was more noticeable with 7 µM SF concentration (Fig S 5.1a). These inhibitory effects of SF increased over time, as demonstrated by the MTT assay (Fig 5.1b). These results indicated that SF inhibited SCCHN cell growth in a dose- and time-dependent manner.

2. SF increased the effects of chemotherapeutic drugs against SCCHN cells

SCC12 and SCC38 cells were treated with SF in combination with CIS or 5-FU; cell viability was analyzed by morphological inspection and MTT assay after 72 hours. The addition of SF to CIS more than doubled the cytotoxic effect on SCCHN cells, as compared to CIS alone, as the combined SF treatment with 0.5 µg/ml CIS had similar or even more inhibitory effect of 1 µg/ml of CIS alone. This effect was even greater in the SF+5-FU combined treatment as reduction in the cell viability was comparable to 10-fold higher doses of 5-FU alone. The combined SF with 0.013 µg/ml 5-FU had similar effect of the 0.13 µg/ml 5-FU alone and the same with 0.13 dose (Fig 5.2a&b). These results were observed in the both cell lines.

We found that the CIS treatment reduced the clonogenic ability of SCC12 and SCC38 to 64% and 60%, respectively, when compared to untreated (no drug) controls. SF reduced colony formation to 46% and 41% compared to untreated controls. The combined SF+CIS treatment further decreased colony formation to 25%. 5-FU also decreased the numbers of colonies formed to 50% and 38% in SCC12 and SCC38, respectively; however, the SF+5-FU combination further reduced the clonogenicity to 7% compared to controls (Figure 5.2c&d, Appendix table 5.1).

Related results were obtained when we tested the effects of SF on DNA repair post-treatment. SF, CIS, and 5-FU were administered at a concentration of 0.875 µM, 0.02 µg/ml and 0.2 ng/ml respectively based on dose–response experiments demonstrating that these concentrations were sub-lethal (Appendix figure 5.2a&b). CIS reduced clonogenicity to 75% and 77% for SCC12 and SCC38, respectively while SF reduced colony formation to 71% and 69% when compared to untreated controls. When combined, CIS+SF showed an additive effect and reduced colony formation to 24% and 22%. We had comparable results with 5-FU which reduced the clonogenicity to 77% and 70% for SCC12 and SCC38 respectively but when we used combined 5-FU+SF this reduction improved to 15% (Figure 5.2e&f, Appendix table 5.2). Taken together, our data showed that SF increased the drug-mediated cytotoxic effects on cellular viability, clonogenic ability and DNA damage in SCCHN tumors.

3. Sulforaphane has minimal cytotoxic effects on normal (non-cancerous) cells

We examined the toxicity of SF on non-cancerous cells. Human primary salivary fibroblasts (FB), human gingival epithelial progenitor cells (HGEPS), and a human salivary gland acinar cell line (NS-SV-AC) were treated with SF. Although SF had minimal toxic effect on FB and HGEPS, except when we used at a concentration 14 µM, with IC_{50} 23.46 µM and 23.32 µM respectively, we found a stronger toxic effect on the NS-SV-AC cell line with IC_{50} 6.36 µM but still higher than IC_{50} for SCCHN (Figure 5.3a). The morphological appearance of the tested cells did not change when less than 14 µM of SF was added (Figure S5.1b). Moreover, the difference between the combined treatment and the standalone effects of CIS or 5-FU on the tested cells, including NS-SV-AC, revealed no statistical significance (Figure 5.3b,c,d). This suggested that

normal (non-cancerous) mesenchymal and epithelial cells were not negatively affected by SF, while the viability of immortalized or malignant cells was reduced.

4. Sulforaphane increased drug-mediated cytotoxicity by induction of apoptosis

We then aimed to verify the induction by SF of apoptosis on cancer cells. SCC12 and SCC38 cells were treated with CIS or 5-FU with or without SF for 72 hours before being stained for annexin V and analyzed by flow cytometry. Single treatment with CIS induced early apoptosis in 12% and 8% of SCC12 and SCC38 cells, respectively. The combined treatment of SF+CIS increased the apoptosis to 20% (Figure 5.4a). Similarly, 5-FU as a standalone treatment induced apoptosis in 15% and 12% of the SCC12 and SCC38 populations. The combined treatment of SF+5-FU increased apoptosis to 20% and 24% (Figure 5.4b). This suggested that sulforaphane could reduce SCCHN cell numbers through the induction of apoptosis (Figure 5.4c,d).

5. Sulforaphane affected the regulation of pro- and anti-apoptotic genes

To better understand the enhancement of induction of apoptosis by chemotherapy in SCCHN cells through the addition of SF, we examined expressions of the genes that are critical for cell apoptosis in carcinoma. SCC12 and SCC38 cells were treated with SF, CIS, or 5-FU alone or in combination for 72 hours, followed by QPCR for the expression of the selected genes. Compared to the control group, BAX and CASP3 expression was significantly increased while the BCL2 was significantly decreased when 7 µM of SF was used. Similarly, the expression of BAX and CASP3 was increased while BCL2 was decreased significantly in the CIS and 5-FU treatments. However, when we used the combined SF+CIS or SF+5-FU treatments it elevated the expression

levels of BAX and CASP3 and reduced the expression level of BCL2 significantly when compared to CIS or 5-FU treatment alone. (Figure 5.5a,b).

5.6 Discussion

Squamous cell carcinoma of the head and neck is one of the most common malignant neoplasm. 60% of the reported cases for treatment present with locally advanced tumors and require combined modality therapy including surgery, radiotherapy, and chemotherapy [543]. One major reason for cancer treatment failure is the limited efficacy of the conventional chemotherapy by its severe toxic side effects. In this study, we presented an approach to decrease the chemotherapeutic dose while maintaining therapeutic efficacy by combining CIS or 5-FU with the low-toxicity, natural product sulforaphane.

Numerous studies reported the anti-neoplastic effect of SF against solid tumors such as breast tumors, hepatic tumors, brain tumors, pancreatic tumors, prostate tumors and skin tumors [29]. Recently, it was shown that SF has comparable cytotoxic effects on the squamous cell carcinoma of the head and neck [39,544,545]. Our results showed that SF decreased the SCCHN cell lines viability through increasing treatment dosage and duration. SF inhibitory effect on head and neck cancer cells is comparable to other types of cancers as the IC_{50} measured after 72 hours of treatment for SCC12 and SCC38 were very close to acute lymphocytic leukemia [545,546]. We used 3.5 µM SF dose for the rest of the experiment as this dose showed the first signs of apoptosis, was relatively safe to non-cancerous healthy cells and expected to be achieved by simply ingestion of fresh broccoli sprouts. Clarke reported SF 2.5 µM/L plasma concentration after 3 hours from ingestion 40 grams of fresh broccoli sprouts [335].

Our preliminary data suggest that SF can be used as a co-treatment to improve conventional chemotherapy against SCCHN. When we tested this hypothesis, we found that SF co-treatment decreased SCCHN cells viability two-fold more than CIS alone, and ten-fold more than 5-FU alone after 72 hours ($p<0.05$). This increase in cytotoxic effect can be used to reduce the conventional doses of CIS and 5-FU used in treatment and, in turn, reduce the dose-dependent side effects. The co-treatment with SF did not only affect the viability but also reduced the self-renewal ability of the SCCHN cells, as observed by measuring colony formation following a 72 hours treatment. The co-treatment significantly reduced the number of colonies formed when compared to the single treatment of CIS or 5-FU. Our results were comparable to those obtained by using SF against other types of cancers such as gastric carcinoma, pancreas and prostate cancers [36,547].

One of the causes for treatment failure is the ability of the cancer cells to evade the damage caused by the chemotherapy [548]. However, the synergetic effect of SF with CIS or 5-FU was noticeable in the inhibition of DNA repair after treatment. This was observed after treating SCCHN cells with a sub-lethal dose of CIS or 5-FU with or without SF for 72 hours, followed by a colony forming assay for 10 days. The co-treatment significantly decreased the clonogenic ability of the cells when compared to a single treatment. This indicated that the cells were unable to repair their damaged DNA after chemotherapy termination when SF was introduced. Our data demonstrated, for the first time, that the antioxidant properties of SF did not affect chemotherapy efficacy but actually increased the cytotoxic effects of chemotherapy on SCCHN cells.

One of the important criteria that make sulforaphane a suitable candidate for chemotherapy is the low toxicity on non-cancerous cells. We tested this by applying different

concentrations of SF on human primary fibroblasts, epithelial cells and a salivary acinar cell line for 72 hours followed by measuring cell viability. SF had minimal toxic effects on primary cells, except when administered in high doses. This was not the case with the acinar cell line which had a significantly lower IC_{50} when compared to the primary cells, but still higher than the SCCHN cells. This result could be because acinar cells were no longer normal (primary) cells since they were immortalized with the simian virus 40. This immortalization procedure likely led to expression of genes that were targeted by SF. We also tested the effects of the co-treatment on these cells, which showed comparable results; the co-treatment had no significant difference when compared to CIS or 5-FU treatment alone. This was observed in all of the tested cell types, including the acinar cell line. This observation was also reported in primary fibroblasts, endothelial cells, and immortalized 293 Kidney cells [36] and with a human gastric epithelial cell line (GES-1) [547].

The decreased SCCHN cell viability after using sulforaphane seemed to be caused by an increased induction of apoptosis. By using the annexin V assay, we found that SF treatment significantly increased early apoptosis in treated cancer cells. The combined treatment of SF and low doses of CIS or 5-FU led to increased apoptosis compared to using a single drug as a treatment. This was in agreement with reports by other groups [36,422].

It is suggested that various anti-cancer agents will stimulate different apoptotic pathways, including the death receptor–mediated pathway, the mitochondrial apoptotic pathway and the endoplasmic reticulum pathway [549]. While those pathways have different initiation mechanisms, they all have the same final phase in which the executioner caspases become activated [550]. The BCL2 proteins family is the center of regulation for Caspase3 – one of the

executioner caspases. Cellular apoptotic susceptibility with chemotherapy is regulated by the ratio between anti-apoptotic gene BCL2 and pro-apoptotic genes BAX, Bid and Bak [551]. In our study, QPCR results showed that SF increased chemotherapy-induced apoptosis utilizing the Caspase-dependent pathway by increasing the expression of Caspase3 through the up-regulation of BAX and down-regulation of BCL2. The combined treatment almost doubled BAX expression when compared to the single treatment. Comparable results were obtained via Western blotting by others [421,422]. Further investigations at the protein level changes should be made.

In summary, we demonstrated that SF did not decrease the cytotoxic effects of chemotherapy, but rather strongly enhanced their efficacy against SCCHN. The combined treatment efficiently increased apoptosis along with inhibiting clonogenicity and DNA repair without increasing the cytotoxicity in non-cancerous cells which will be of great clinical significant. The combined treatment may be of therapeutic benefit in the clinical settings in reducing the toxic side effects of chemotherapy and increasing its effect. Our data, combined with the works of others, suggest that SF can be used with lower doses of chemotherapy as co-treatments for the benefit of the patients.

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Compliance with Ethical Standards:

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Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Figures:

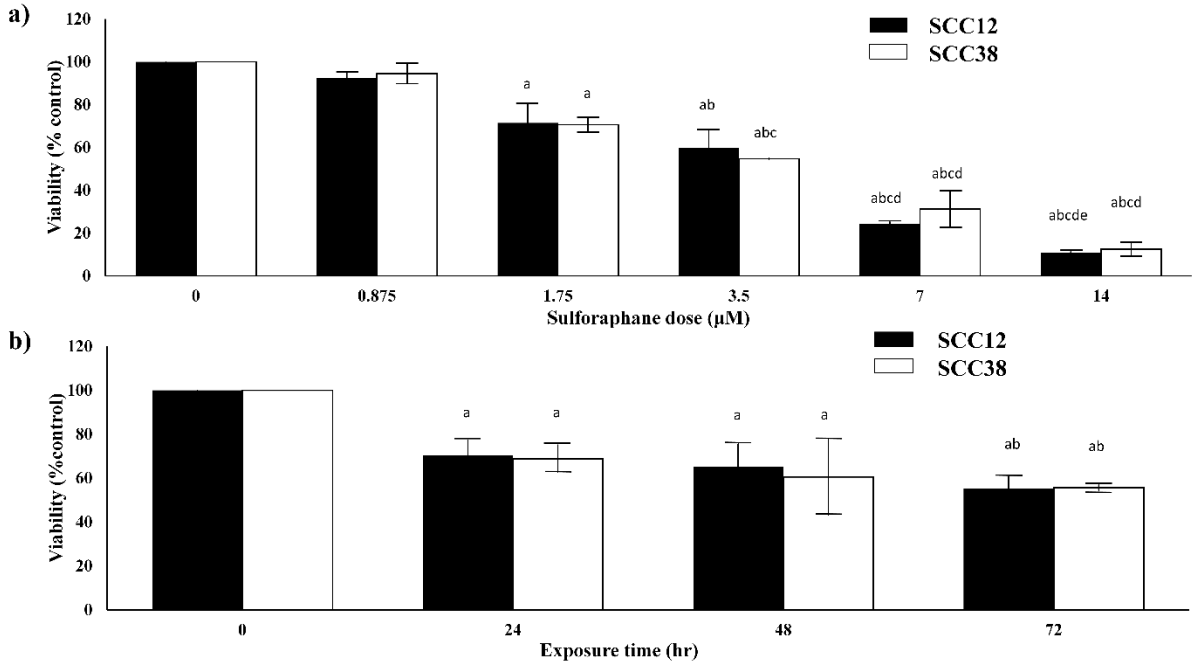


Fig 5.1 Sulforaphane affected SCCHN cell viability in a time- and dose-dependent manner. (a) SCC12 and SCC38 cells were treated with 0, 0.875, 1.75, 3.5, 7 and 14 μM of SF for 72 h. Cellular viability was measured in triplicate in three independent experiments by MTT assay. Data are presented as mean ± SD ("a" significance relative to 0 μM, "b" significance relative to 0.875 μM, "c" significance relative to 1.75 μM, "d" significance relative to 3.5 μM, "e" significance relative to 7 μM. $P < 0.05$). (b) SCC12 and SCC38 were treated with 3.5 μM of SF for the indicated times ("a" significance relative to 0 h, "b" significance relative to 24 h. $P < 0.05$)

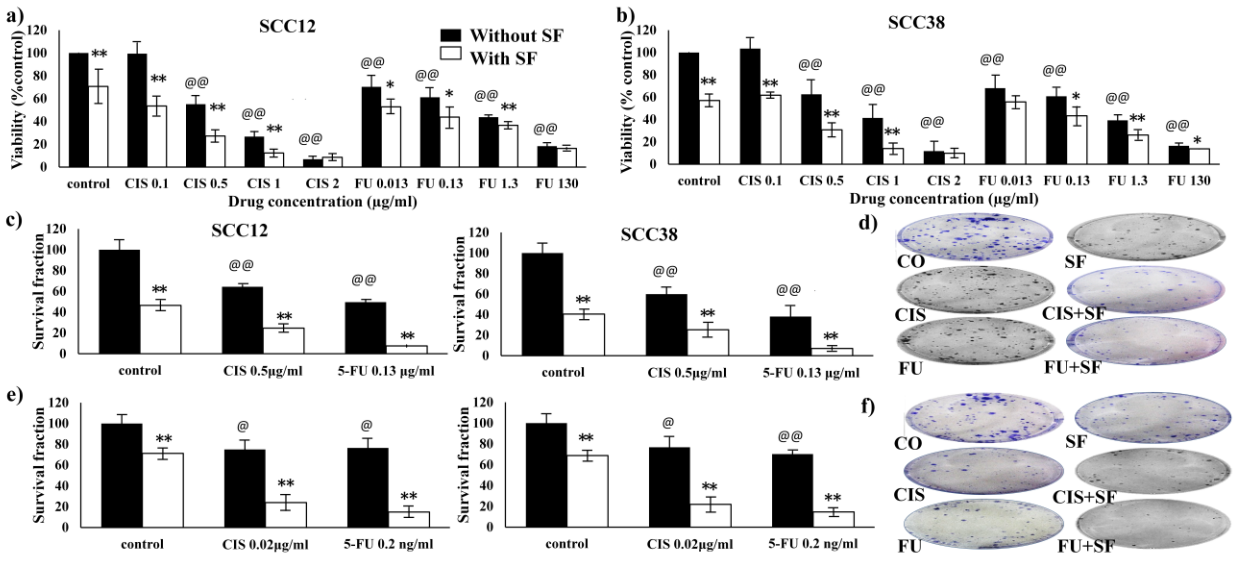


Fig 5.2 Sulforaphane synergized the effects of CIS and 5-FU against SCCHN cells. (a) SCC12 and (b) SCC38 cells were treated with 3.5 µM of SF with or without 0.1, 0.5, 1, 2 µg/ml of CIS or 0.013, 0.13, 1.3, 130 µg/ml of 5-FU for 72 h. Cellular viability was assessed using a MTT assay in triplicates in three independent experiments. Data are presented as mean ± SD (* $P < 0.05$ and ** $P < 0.01$ relative to treatment in the absence of SF, @@ $P < 0.01$ relative to control). (c) To verify the effects of SF on clonogenic cell division, SCC12 and SCC38 cells were pretreated with SF (3.5 µM) with or without CIS (0.5 µg/ml) or 5-FU (0.13 µg/ml) for 72 h before being seeded in 6-well plates for 10 days (400 cells/well). Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean ± SD (** $P < 0.01$ relative to treatment in the absence of SF, @@ $P < 0.01$ relative to control without treatment). Photographs of the fixed and stained colonies are presented on the (d) panel. (e) SCC12 and SCC38 cells were pretreated with sub-lethal doses of SF (0.875 µM) with or without CIS (0.02 µg/ml) or 5-FU (0.2 ng/ml) for 72 h and 400 cells per condition were seeded in 6-well plates for 10 days Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean ± SD (** $P < 0.01$ relative to treatment in the absence of SF, @ $P < 0.05$ and @@ $P < 0.01$ relative to control without treatment). Photographs of the fixed and stained colonies are presented on the (f) panel

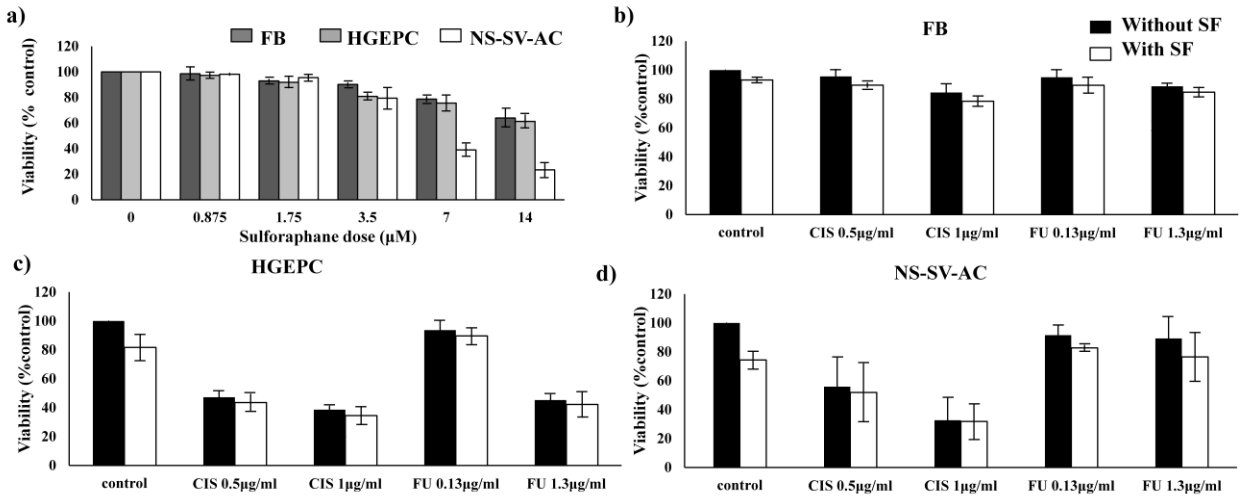


Fig 5.3 Sulforaphane had minimal to no effect on non-cancerous human cells. (a) Primary fibroblasts, primary gingival epithelial cells and a salivary acinar cell line were treated with 0, 0.875, 1.75, 3.5, 7 and 14 µM of SF for 72 h. The cell viability was evaluated in triplicate in three independent experiments by MTT assay. Data are presented as mean ± SD. (b) Primary fibroblasts, (c) primary gingival epithelial cells and (d) a salivary acinar cell line were treated with 3.5 µM of SF in the presence or absence of 0.5 and 1 µg/ml of CIS or 0.13 and 1.3µg/ml of 5-FU for 72 h, respectively. Viability were measured by a MTT assay in triplicates in three independent experiments. Data are presented as mean ± SD

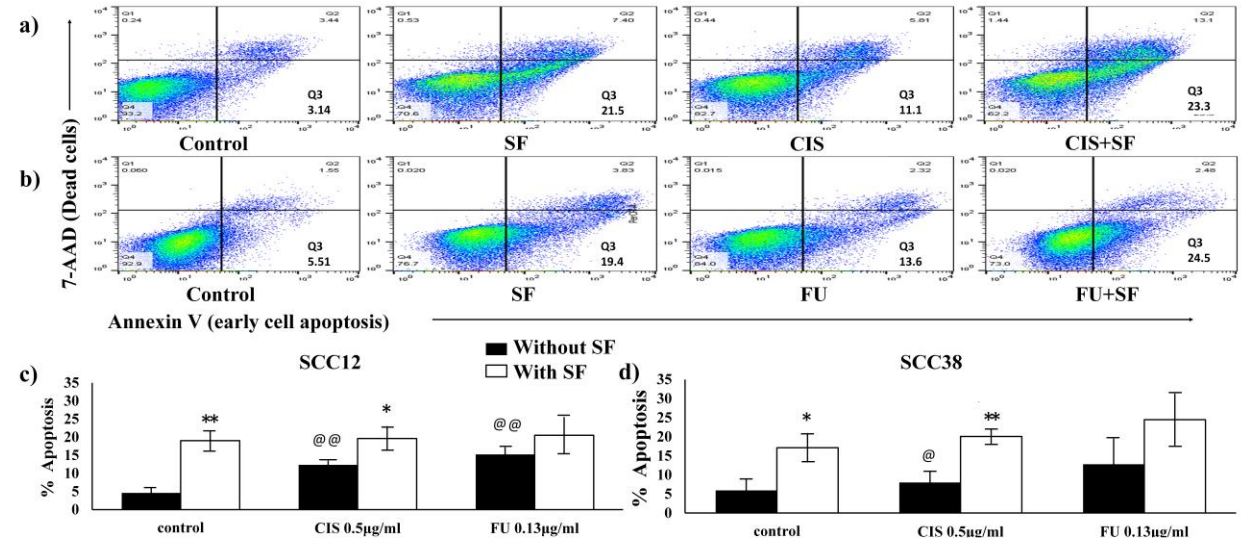


Fig 5.4 Sulforaphane increased drug-mediated cytotoxicity by inducing apoptosis. (a) SCC12 and (b) SCC38 were treated with 3.5 µM of SF with or without 0.5 µg/ml of CIS or 0.13µg/ml of 5-FU for 72 h.

The induction of apoptosis was assessed in triplicates in three independent experiments using annexin V/7AAD staining and flow cytometry. The data presented are gated on single cells. (c, d) The percentage of early apoptotic cells is presented as mean ± SD (** P < 0.01 compared with treatment in the absence of SF, @ P < 0.05 and @@ P < 0.01 relative to control without treatment)

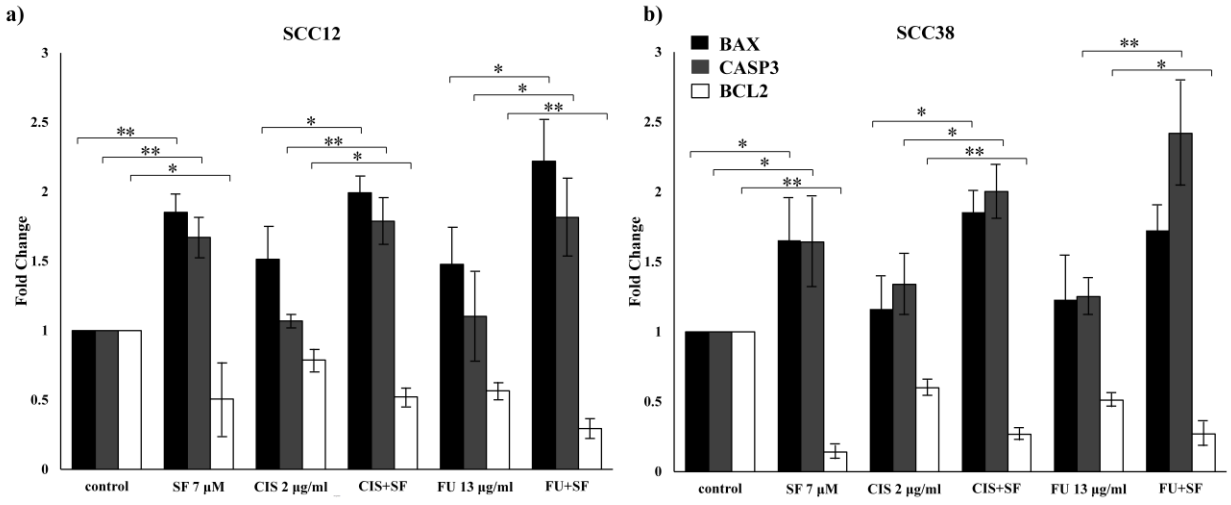
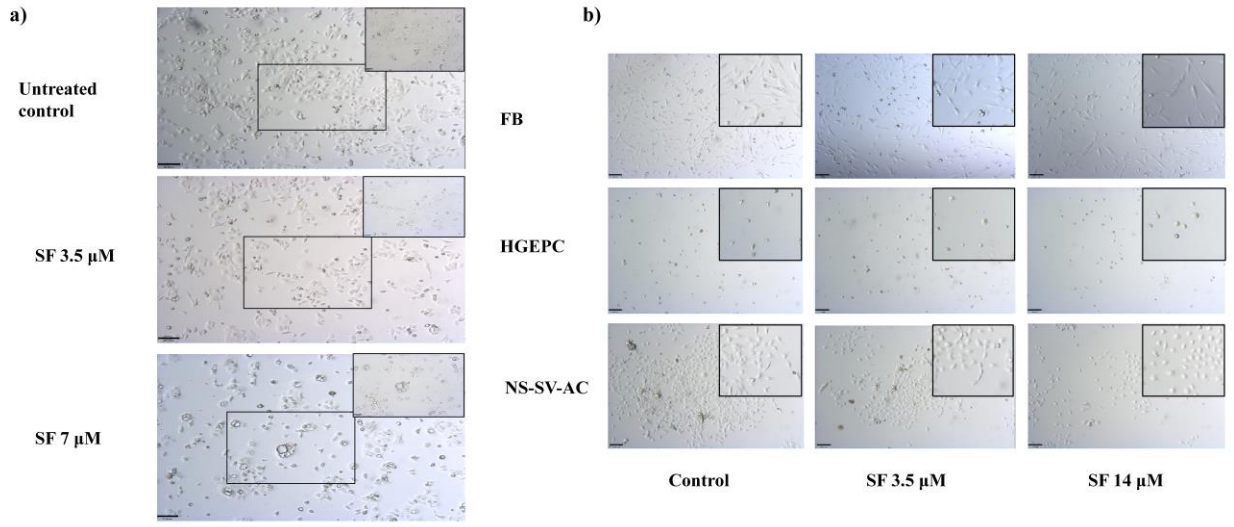


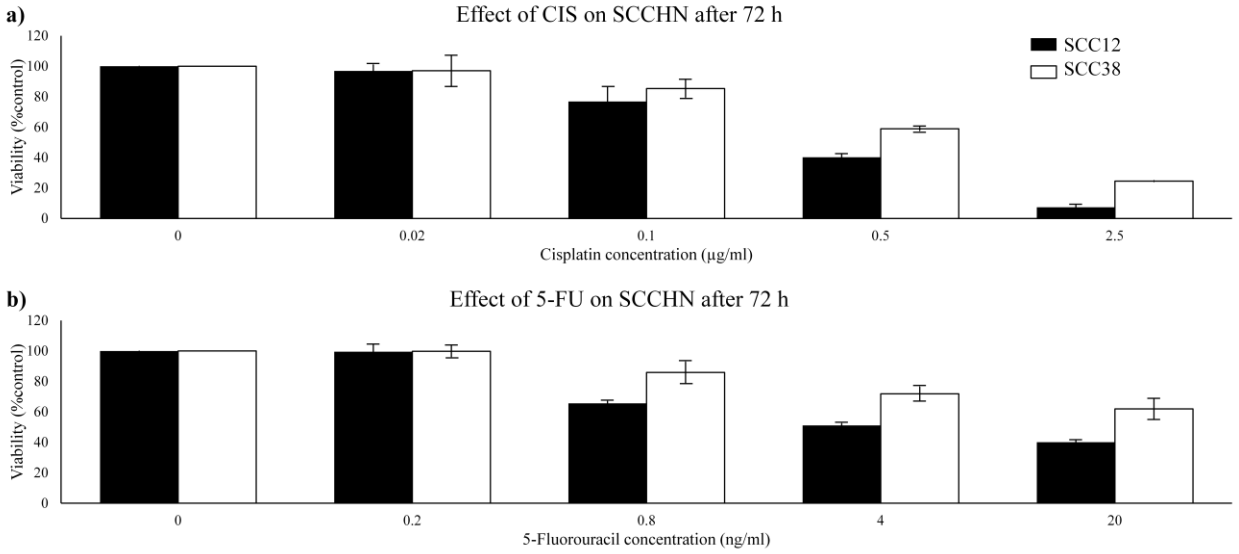
Fig 5.5 Sulforaphane mediated the up-regulation of pro-apoptotic and down-regulation of anti-apoptotic genes. (a)SCC12 and (b) SCC38 were treated with 7 µM of SF with or without 2 µg/ml of CIS or 13 µg/ml of 5-FU for 72 h. The expression of BAX, CASP3 and BCL2 was measured by QPCR and normalized to GAPDH expression. All assays were performed in triplicate in three independent experiments and were calculated on the basis of ΔΔCt method. Data represent mean ± SD (* P < 0.05 and ** P < 0.01 compared with treatment in the absence of SF)

Appendix Supplemental Figure 5.1 :



Appendix Supplemental Figure 5.1. Effect of increasing doses of SF on the morphology of cancerous and non-cancerous (healthy) cells. (a) Representative pictures of the changes in the morphology of SCC12 cells after SF treatment. Scale bar (5X magnification: 90 µm, 10X magnification: 43 µm). (b) Representative pictures of the changes in the morphology of SCC12 cells after SF treatment. Scale bar (5X magnification: 90 µm, inserts are 6X digital magnification).

Appendix Supplemental Figure 5.2:



Appendix Supplemental Figure 5.2. Measuring the sub-lethal dose for CIS and 5-FU. (a) SCC12 and SCC38 cells were treated with 0, .02, 0.1, 0.5 and 2.5µg/ml of CIS for 72 h. The cell viability was evaluated in triplicate by MTT assay. Data are presented as mean ± SD. (b) SCC12 and SCC38 cells were treated with

0, 0.2, 0.8, 4 and 20µg/ml of 5-FU for 72 h. The cell viability was evaluated in triplicate by MTT assay. Data are presented as mean ± SD.

	SCC12 (% CFUs)	SCC38 (% CFUs)
Untreated control	100 % ±9.36	100 % ± 8.96
SF	46.5 % ±4.54	40.6 % ±4.77
CIS	64.4 % ±1.83	59.9 % ±6.66
CIS+SF	24.7 % ±3.33	25.2 % ±6.32
5-FU	49.6 % ±2.34	38.1 % ±9.87
5-FU+SF	7.5 % ±1.11	7.1 % ±1.53

Appendix table 5.1. Sulforaphane increased the inhibitory effects of CIS and 5-FU against SCCHN cells clonogenicity. SCC12 and SCC38 cells were pretreated with SF (3.5 µM) with or without CIS (0.5 µg/ml) or 5-FU (0.13 µg/ml) for 72 h before being seeded in 6-well plates for 10 days (400 cells/well). Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean percentage of colony forming units compared to untreated controls ± SD.

	SCC12 (% CFUs)	SCC38 (% CFUs)
Untreated control	100 % ± 8.15	100 % ±8.72
SF	71.3 % ±5.2	68.8 % ±4.96
CIS	75 % ±8.56	76.8 % ±9.68
CIS+SF	24 % ±7.73	22 % ±6.99
5-FU	76.5 % ±9.12	70.2 % ±3.09
5-FU+SF	14.9 % ±4.87	14.7 % ±3.57

Appendix table 5.2. Sulforaphane increased the inhibitory effects of CIS and 5-FU against SCCHN cells DNA repair after treatment. SCC12 and SCC38 cells were pretreated with sub-lethal doses of SF (0.875 µM) with or without CIS (0.02 µg/ml) or 5-FU (0.2 ng/ml) for 72 h and 400 cells per condition were seeded in 6-well plates for 10 days. Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean percentage of colony forming units compared to untreated controls ± SD.

Chapter 6 - Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas.

6.1 Preface (connecting paragraph)

Head and neck squamous cell carcinoma is a major public health concern with poor prognosis and a 50% 5-year overall survival rate. This low survival rate is due to a number of factors, including local recurrence, distant metastasis, and therapeutic resistance. Recent data indicate the presence of cancer stem cells in many solid tumors, including HNSCC. As we presented in chapter two, the cancer stem cell theory suggests that a subpopulation of cells in the tumor possesses stem cell properties with the potential to self-renew and generate the entire heterogeneous tumor bulk in a unique 'hierarchic' pattern.

First step to target the treatment-resistant CSCs is to accurately isolate and characterize these cells from the total cell population. According to many studies, CD44 surface marker can be used to identify CSCs. The purified CD44+ cells from the primary tumors can give rise to tumors faster and by injecting less cell number in xenograft model compared to CD44- cells, and these xenograft tumors subsequently reproduce the original tumor heterogeneity observed in the primary tumor. Recently, CD271 was identified as a marker of CSCs in many tumors, such as human melanoma and hypopharyngeal carcinoma.

In this chapter, we used the two cancer stem cell markers, CD44 and CD271, to isolate a pure CSC population. CD271+ cells turned out to be a subpopulation of CD44+

cells. CD44+/CD271+ cells showed higher proliferation rates, self-renewal ability, treatment resistance, stem cells-related genes expression, and in-vivo tumorgenicity. Our work suggested that CD271+/CD44+ double staining is better method to isolate cancer stem cells compared to the commonly used CD44 alone.

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Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas

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Author contributions:

Osama A. Elkashty and Ghada Abu Elghanam conceived and carried out experiments and analyzed data, Xinyun Su, Peter J. Chauvin, and Simon D. Tran conceived experiments and analyzed data. Younan Liu carried out experiments. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Keywords: Head and Neck cancer; Carcinoma, squamous cell; Neoplastic stem cells; CD271; CD44; Tumor heterogeneity.

6.2 Abstract:

Head and neck squamous cell carcinoma (HNSCC) has poor five-year survival rate of 50%. One potential reason for treatment failure is the presence of cancer stem cells (CSCs). Several cell markers (particularly CD44) have been used to isolate CSCs but this remains a challenging

task to isolate a pure population in HNSCC. Recently, normal oral stem cells were isolated using CD271. We investigated the combined use of CD271 and CD44 to isolate an enriched CSCs followed by characterization in-vitro, in-vivo. and in patients' tissue samples.

Fluorescent-activated cell sorting was used to isolate CD44+/CD271+ and CD44+/CD271- from human HNSCC cell lines. Cell growth and self-renewal was measured with MTT and sphere/colony formation assays. Treatment-resistance was tested against chemotherapy (cisplatin and 5-flurouracil) and ionizing radiation. Self-renewal, resistance, and stemness-related genes expression was measured with qRT-PCR. In-vivo tumorgenicity was tested with orthotopic immunodeficient mouse model of oral cancer (N=50). Finally, we examined the localization of CD44+ and CD271+ in patients' tissue samples (N=10).

We found that CD271+ cells were a subpopulation of CD44+ cells in human HNSCC cell lines and tissues. CD44+/CD271+ cells exhibited higher cell proliferation, sphere/colony formation, chemo- and radio-resistance, upregulation of CSCs-related genes, and in-vivo tumorigenicity when compared to CD44+/CD271- or the parental cell line. These cell markers showed increased expression in patients with the increase of the tumor stage.

In conclusion, using both CD44 and CD271 allowed an enriched isolation of CSCs from HNSCC compared to CD44 alone. This pure CSCs will be more relevant in future treatment and progression studies.

6.3 Introduction:

Cancer is the leading cause of death in Canada, and it is responsible for over than 30% of all deaths annually [59]. Head and neck squamous cell carcinoma (HNSCC) is the seventh most

common cancer worldwide, as it accounts for over 500,000 new diagnosed cases and 380,000 deaths annually [526]. In Canada, 5850 new cancer patients were diagnosed with HNSCC and it was responsible for 1690 deaths in 2017 [59]. Despite the recent techniques for diagnosis and cancer treatment, the current prognosis for HNSCC is poor due to relapse in the form of local recurrence or metastasis. The five-year survival rate has remained around 50% for the last three decades [2,527].

One reason for cancer treatment failure is considered to be related to the presence of a subpopulation of cells in the tumor called “cancer stem cells” (CSCs), which are suggested to have tumor-initiating potential combined with the ability of self-renewal and multilineage differentiation [14]. Acute myeloid leukemia was the first malignancy that was discovered to contain CSCs; this was followed by the discovery in multiple tumor types, including lung, breast, brain, liver, pancreas and colon cancers [211,552]. CSCs share some of the characteristics of normal stem cells, such as the ability to undergo self-renewal, maintain quiescence, show multipotentiality, and exert survival/anti-apoptosis proteins [552]. In some tumors, CSCs were linked to chemo-resistance [553], radio-resistance [554], recurrence [555] and metastasis [556].

Cluster of differentiation 44 (CD44) is a transmembrane glycoprotein and a receptor for hyaluronic acid, an important component of the extracellular matrix, and a co-receptor for many growth factors and cytokines. The CD44+ cell population in cancer was shown to be CSC, as these purified CD44+ cells from the primary tumors gave rise to tumors faster and with injecting a lesser cell number in a xenograft model when compared to CD44- cells; these xenograft tumors subsequently reproduced the original tumor heterogeneity observed in the primary tumor. CD44+ cells can resist oxidative stress and, as such, is more radio-resistant [19]. CD44+ cells have

a greater ability to metastasize to regional lymph nodes in animal models [557]. Patients whose tumors had greater percentages of CD44+ cells had a significantly poorer clinical outcome [249]. Recent studies used CD44 surface marker expression as a sole marker for HNSCC-CSCs [558-560]. However, some studies demonstrated that CD44− cells can also initiate tumor in-vivo, form tumor-spheres and express treatment resistance, like CD44+ cells [561,562]. With different microenvironments, there will be some heterogeneity in the CSCs [563], and the CD44+ cells may not represent pure HNSCC-CSCs.

In normal human oral epithelium, we can find a subpopulation of cells with stem cell–like properties. These cells express a cell surface molecule, designated as the CD271+ cells [20,21]. Recently, this molecule was identified as a marker of CSCs in many tumors, such as human melanoma [23], esophageal carcinoma [24,25], and hypopharyngeal carcinoma [26]. Besides being expressed in discrete cells within the basal layer of normal oral epithelium, CD271 is also found in oral epithelial dysplasia and oral squamous cell carcinoma [27]. Oihana Murillo-Sauca has reported that CD271+ cells are a subpopulation the HNSCC and CD271 is a targetable marker to inhibit CSCs [265].

In the present study, we have used head and neck cancer cell lines to analyze the expression of CD44 and CD271 followed by isolation of CD44+/CD271+ and CD44+/CD271- subpopulations for further experiments. These populations were subjected to various molecular and cellular assays to determine whether CD271 has any significant contribution in the context of CD44 population towards defining CSC marker. We have clearly demonstrated that CD271+ cells comprised a purer CSCs subpopulation within the CD44+ cells, especially with regards to self-renewal, proliferation, treatment resistance and strong in-vivo tumorigenic capacity.

6.4 Materials and methods:

Human squamous cell carcinoma cell lines and patient tissue samples:

SCC12 (laryngeal SCC, RRID: CVCL_7717) and SCC38 (tonsillar SCC, RRID: CVCL_7749) cell lines were purchased from the University of Michigan and were used as models for HNSCC [500]. They were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% non-essential amino acids, 10% fetal bovine serum and 2% Antibiotic-Antimycotic (Thermo Fisher). All experiments were performed with mycoplasma-free cells.

A total of 10 oral squamous cell carcinomas were obtained from the Pathology Department of the McGill University Hospital Center. The sections were obtained from formalin fixed paraffin embedded (FFPE) tissue blocks. All tumor samples were intraoral and either located in the tongue, floor of the mouth, or gingiva. Six samples were from male patients and four from female patients. This study was performed with approval from the McGill Faculty of Medicine institutional review board (IRB study number A05-M62-05B). The specimens were obtained as an incisional biopsy or surgical resection for oral squamous cell carcinoma. Normal mucosa was obtained from blocks of the same patient but were free of dysplasia and distant from the primary tumor location (taken from the farthest margin of the surgical resection).

Flow cytometry and fluorescence-activated cell sorting (FACS):

Alexa Fluor® 700 Mouse Anti-Human CD44 (Clone G44-26) and PerCP-Cy™5.5 Mouse Anti-Human CD271 (Clone C40-1457) monoclonal antibodies for flow cytometry and sorting were obtained from BD Pharmingen. Tumor cells were harvested using Accutase™ Cell Detachment Solution (BD Bioscience) and resuspended as a single-cell suspension in staining buffer (1% FBS

in ice-cold PBS) with a final concentration $1x10^6$ cells/100µl. Cells were then incubated with the Fixable Viability Stain 450 (BD Bioscience) for 15 min on ice protected from light and washed twice in staining buffer to allow the exclusion of non-viable cells. Cells were blocked by incubation with Human TruStain FcX™ (Fc Receptor Blocking Solution, Biolegend) for 10 min then washed once with the staining buffer to block non-specific staining. Cells were then stained by the antibodies for CD44 and CD271 at a dilution of 1:20 for 30 min on ice protected from light and washed twice with the staining buffer. The samples were analyzed using LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo vX (FlowJo LCC). FACS of CD44+/CD271- and CD44+/CD271+ cells were performed using a BD FACSARIA III cells sorter (BD Bioscience). Only the highly positive stained cells were isolated as CD271+ cells. Cells incubated with the viability stain, blocking agent and the monoclonal antibodies and passed through the BD FACSARIA III cells sorter without sorting were used as the parent cell population. UltraComp eBeads™ Compensation Beads (Thermo Fisher) was used as control.

Immunofluorescent staining.

5 µm thick sections were cut on coated slides from FFPE tissue samples blocks. Slides were dewaxed with CitriSolv and rehydrated through graded alcohol. For antigen retrieval, they were immersed in 10% citrate buffer and treated in a water bath at 98°C for 15 min and then blocked with Power Block Universal Blocking Reagent (Biogenex) for 10 min followed by goat and donkey serum 5% for 1 hour to inhibit any potential non-specific binding. The slides were reacted with various prediluted primary antibodies overnight at 4°C in a humid chamber. The primary antibodies used in this study were: Mouse monoclonal anti CD44 (1:150, ab6124) and Rabbit monoclonal anti CD271 (1:200, ab52987) from Abcam. After 3 times washing in PBS, slides were

incubated with secondary antibodies (1:100) in the dark for 1 hour at room temperature. Secondary antibodies were Fluorescein (FITC) AffiniPure Donkey Anti-Mouse IgG (H+L) and Rhodamine Red™-X (RRX) AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoReserach). Then 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) was added for 3 min to label the nucleus of cells. Same tissue sections treated without the primary antibodies was used as negative controls and human skin tissue slides were used as positive control (photos not shown). Fluorescence pictures were taken by Leica DM4000 fluorescent microscope and the corrected total cell fluorescence (CTCF) using ImageJ software (NIH).

MTT assay

1.5×10^3 cells from CD44+/CD271-, CD44+/CD271+ , and unsorted parental cells were seeded in 96-well plates. After 1, 2, 3, 4, 5, 6 and 7 days, the medium was removed and 10% solution of 5 mg/ml MTT in medium (Sigma Aldrich (3-(4,5-Dimethylthoiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added to each well and incubated at 37°C for 2 hours. The medium was removed, and formazan was dissolved by adding DMSO to each well. The optical density was measured at 562/540 nm in EL800 Microplate Reader (BIO-TEK Instruments). The assay was done in triplicates and three independent experiments were carried out.

Colony-forming assay

CD44+/CD271-, CD44+/CD271+, and the unsorted parental cells were prepared as single cell suspensions and 400 cells/well were plated into a 6 well plate. Cells were allowed 2 weeks to form colonies under standard conditions, and the rate at which this occurred was recorded. To determine colony formation, culture medium was removed, and colonies were fixed and stained

with 1% crystal violet, 50% methanol in DDH_2O for 1 hour. The number of colonies with >50 cells were counted under an inverted microscope. The assay was done in triplicates and three independent experiments were carried out.

Sphere-forming assay:

CD44+/CD271-, CD44+/CD271+, and the unsorted parental cells were cultured overnight to eliminate dead cells. Next day, 5000 cells/500µl per well of 24 Ultra-Low Attachment Multiple Well Plate (Millipore Sigma) in DMEM-F-12 serum-free media (Gibco) reconstituted with 20 ng/ml of Epidermal Growth factor, 20 ng/ml of Basic Fibroblast Growth Factor, 0.5% N2 supplement (STEMCELL Technologies), 1% B27 supplement and 2% Antibiotic-Antimycotic (Thermo Fisher). The medium was added every 2–3 days. Formation of sphere-like structures was visible at 4–7 days and the photographs of groups were captured under Leica DM IL phase-contrast microscope (Leica Microsystems) using QICAM (QImaging) at 5× and 40× magnification at 14 days. All experiments were done in triplicate. Spheres were then collected by centrifugation and dissociated by trypsin (Thermo Fisher) to single cells. Cells were counted for each group using hemocytometer with Trypan blue staining to exclude the dead cells.

Drug resistance assay

Cisplatin (Cayman Chemical) was prepared in phosphate-buffered saline to a 0.3 mg/ml stock and kept at 4°C protected from light. 5-Fluorouracil (Sigma Aldrich) was prepared in dimethyl sulfoxide (DMSO) to 50mg/ml stock. Final concentrations of the solvents in the working solution medium were 0.1% or less. CD44+/CD271-, CD44+/CD271+, and the unsorted parental cells were seeded in 96-well plate at a density of 1500 cells/well and allowed to grow in normal

medium. After 24 h, the medium was replaced by 100 µl fresh medium containing Cisplatin (Cayman Chemical) at concentration of 0, 0.125, 0.25, 0.5, 1 or 2 µg/ml or 5-fluorouracil (Sigma Aldrich) at concentration of 0, 0.125, 0.5, 2, 8, or 32 µg/ml in triplicates and kept under standard culture conditions for another 72 h. Afterward, MTT assay was performed as mentioned above. Four independent experiments were carried out.

Radiation resistance assay

CD44+/CD271-, CD44+/CD271+, and the unsorted parental cells were subjected to ionizing radiation of 0, 2 or 4 Gray using RS200 X-ray biological irradiator (Rad source technologies). After that, 400 single live cells were seeded in 6 well plates and colony forming assay was continued as mentioned above. The assay was done in triplicates and three independent experiments were carried out.

Real-time qRT-PCR:

Total RNA was extracted from CD44+/CD271-, CD44+/CD271+ and the unsorted parental cells using TRIzol (Thermo Fisher Scientific). The first-strand cDNA was synthesized from 1 µg total RNA using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). For the quantification of gene amplification, QPCR was performed using StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) in the presence of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Target sequences were amplified at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 57.5-65°C annealing temperature for each gene for 1 min. The following gene-specific primers were used:

GAPDH: (5'-GAGAAGGCTGGGGCTCATTT-3', 5'-AGTGATGGCATGGACTGTGG-3'), BMI-1: (5'-TCCTTAACAGTCTCAGGTATCAACC-3', 5'-CACAGTTTCCTCACATTTCCA-3'), SMO: (5'-TGGTCACTCCCCTTTGTCCTCAC-3', 5'-GCACGGTATCGGTAGTTCTTGTAGC-3'), GLI1: (5'-TTGGAGAAGCCGAGCCGAGTATC-3', 5'-GAGTAGACAGAGGTTGGGAGGTAAGG-3'), NOTCH1: (5'-GCAGAGGCGTGGCAGACTAT-3', 5'-ACTTGTACTCCGTCAGCGTG-3'), SOX2: (5'-ACACCAATCCCATCCACACT-3', 5'-CAAACTTCCTGCAAAGCTCC-3') OCT4: (5'-CTCGAGAAGGATGTGGTCCG-3', 5'-GAAGTGAGGGCTCCCATAGC-3') and ALDH1A1: (5'-ATCAAAGAAGCTGCCGGGAA-3', 5'- GCATTGTCCAAGTCGGCATC-3'). All assays were performed in triplicate and the expression was calculated on the basis of ΔΔCt method. The *n*-fold difference in mRNAs expression was determined according to the method of $2^{-\Delta\Delta CT}$.

In-vivo tumor formation assay

The animal experiments were approved by the University Animal Care Committee at McGill University (Protocol #5330, www.animalcare.mcgill.ca). The total number of animals used was 50 NU/NU Nude (Crl:NU-Foxn1^{nu}) mice (Charles River). Mice were anesthetized with isoflurane (Isoba VetTM) (Schering Plough) (4% induction and 2% maintenance). Six to ten weeks old male mice were injected with either 1 X 10^3, 1 X 10^4 or 1 X 10^5 viable CD44+/CD271+, CD44+/CD271- or unsorted SCC12 cells in the side of the tongue, suspended in in 30 µl of normal saline using a 1-ml tuberculin syringe with a 30-gauge hypodermic needle. There were 5 mice per experimental group (5 mice x 6 experimental groups) and 5 mice in the control group. The mice were examined for tumor formation on the tongue every week, starting the first week from the day of injection and measured bidirectionally using a caliber, under gas anesthesia. Tumor size was calculated using the following formula: Volume = (width^2 * length)/2. Animals were sacrificed

after 32 days, and their tongues were collected, fixed in 10 % neutral buffered formalin and embedded in paraffin. Tumor formation was confirmed using H&E stained sections. Tumor sizes (in mm^3) from the $1X10^5$ cells injected group, as recorded every week, were compared between the three animal groups (CD44+/CD271+, CD44+/CD271-, and unsorted parental).

Statistical analysis

Data were presented as the means ± standard deviation (SD) of three independent experiments with comparable results. One-way analysis of variance (ANOVA) followed by post hoc Tukey's test were used to assess significant differences between three groups or more, while Student's t-test (Unpaired) was used between two groups. *p*-values < 0.05 were considered statistically significant and < 0.01 were considered extremely statistically significant. GraphPad Prism 8 software was used (GraphPad Software)

6.5 Results:

CD271+ cells are a subpopulation of CD44+ cells:

We assessed the prevalence of CD271 and CD44 expressions in the two tested human HNSCC cell lines using immunofluorescence staining and flow cytometry. The two cell lines were uniformly CD44+ with a negligible number of CD44- cells in SCC12 and SCC38 (less than 0.4% and 0.1% respectively). CD271+ cells were a subpopulation of CD44+ ones as it comprised 42.6% (±7.7) of the CD44+ cells in SCC12, and 23.1% (± 11.8) in SCC38 (Fig 6.1a&b). To validate the presence of the co-localization of CD271 and CD44 in HNSCC in human tissues, we used immunofluorescent staining from patient's oral squamous cell carcinoma tissue samples (Fig 6.1c). By using double anti CD44/CD271 staining, we detected a discrete expression of CD271

surface receptor on a distinct subpopulation of cells in normal oral epithelium (N=3) (Fig 6.1c). The dysplastic oral epithelium, as well as the well-differentiated oral SCC tumors, showed higher expression of CD271 in the "basal" aspect of the malignant epithelium (invasion front) compared to the normal epithelium while maintaining polarity (N=6). In more poorly differentiated tumors (N=3), CD271 expression was less organized with an increase in the CD271 expression, with the higher the tumor grade as shown by the measuring the CTCF of the CD271 staining (Fig 6.1d). Most importantly, since the basal half of the normal and basal two-thirds of dysplastic oral epithelium and most of the epithelial cell nests in HNSCC are CD44+, there was a co-localization of CD44 and CD271 expression in the normal and cancerous epithelial cells with CD271+ being a part of CD44+ cells.

CD44+/CD271+ cells have a higher growth rate in 2D and 3D culture conditions:

MTT assay was used to assess the proliferation of the isolated subpopulation in SCC12 and SCC38. CD44+/CD271+ cells showed higher proliferation rate compared to the CD44+/CD271- cells and the parent cell population in both tested cell lines. Furthermore, CD44+/CD271+ cells did not reach stationary growth phase until up to 5 days while the unsorted parental cells reached it after 4 days and the CD44+/CD271- cells reached a stationary phase after 3 days (Fig 6.2a).

CD271+ cells formed more colonies and in a shorter period of time when compared to CD44+/CD271- and the unsorted parental cells in SCC12 and SCC38 cell lines (Fig 6.2b&c). SCC12 cell line was selected for further experiments because it yielded more CD271+ cells.

The ability to grow in 3D conditions was tested by tumor sphere formation in a suspension culture. CD44+/CD271+ cells have significantly more spheres formation compared to CD44+/CD271- and the parental cell line (Fig 6.2d). Because we had different sizes of tumor-spheres, we collected the spheres, dissociated them and counted the living cells using trypan blue staining (Fig 6.2e). By dividing the cells number by spheres' number, we obtained an estimated number of cells we have per sphere. CD271+ cells had ~6729 cells/sphere while the CD271- cells had ~4949 cells/sphere and the parental population had ~5516 cells/sphere (Fig 6.2f). Because the cells' size is comparable, we can deduce that the spheres formed by CD271+ cells are bigger in size (Fig 6.2e&f).

CD44+/CD271+ cells possess higher resistance to chemotherapy and radiotherapy treatments:

To assess the chemo-resistance ability of the isolated cell populations, we exposed the cells to different concentrations of Cisplatin (CIS) and 5-Fluorouracil (5-FU) and calculated the inhibitory drug concentration that kills 50% of the cells (IC50). The drug resistance assay showed a statistically significant difference between the IC50 values of CIS in the three populations with four separate experiments. IC50 of CIS was 0.817 μg/ml for CD44+/CD271+, 0.375 μg/ml for CD44+/CD271- , and 0.496 μg/ml for the unsorted parental cell line (Fig 6.3a). The same trend of results was observed with 5-FU as the IC50 was 3.644 μg/ml, 0.766 μg/ml and 1.49 μg/ml with CD44+/CD271+, CD44+/CD271- and the unsorted parental cell line, respectively (Fig 6.3b).

CD44+/CD271+ cells showed more resistance to radiotherapy when compared to CD44+/CD271- cells and the unsorted parental cell line. The examined cell populations were exposed to 2Gy and 4Gy radiation doses then plated as single cells and allowed to form colonies

for 14 days. CD271+ cells formed significantly more colonies compared to the CD271- cells with 2Gy radiation and more than both CD271- and the total cells populations with 4Gy radiation (Fig 6.3c&d)

CD44+/CD271+ cells expressed higher levels of stem cell-related markers

qRT-PCR revealed that compared to CD44+/CD271- cells, the CD44+/CD271+ SCC12 cells have significantly higher expression levels of genes previously reported as self-renewal organizing genes, namely BMI1, SMO, and GLI1. While NOTCH gene expression was found expressed at the same level by both CD44+/CD271+ and CD44+/CD271- SCC12 cells (Fig 6.4a). Additionally, expression of stemness-related genes SOX2 and OCT4 were found to be higher in CD44+/CD271+ SCC12 cells compared to the CD44+/CD271- cells; however even with the higher expression of drug resistance related gene ALDH1A1 in CD44+/CD271+ SCC12 cells, the difference was not statistically significant (Fig 6.4b).

CD44+/CD271+ cells have higher in-vivo tumorigenicity in an orthotopic immunodeficient mouse model of oral cancer.

FACS sorted CD44+CD271+ cells, CD44+CD271- cells, unsorted parental SCC12 cells were implanted into the tongue of NU/NU Nude (Crl:NU-Foxn1^{nu}) mice (N=50) through a limiting dilution approach (Fig 6.5a). The results indicated that CD44+CD271+ cells have the greatest capacity to form tumors among these three cell populations (Table 6.1). CD44+CD271+ cells also have generated tumors with the most robust in-vivo tumor growth (Fig 6.5b). H&E staining was used to identify morphological differences between the three groups. Stained sections were observed, and digital images were captured with a light microscope. All formed tumors appeared

to have a typical SCC tumor morphology, well differentiated, with keratin pearl formation, cellular and nuclear pleomorphism and invasion into the surrounding skeletal muscle with no intercellular bridges and abnormal mitosis. (Fig 6.5c).

6.6 Discussion:

The inability to eradicate CSCs is among the most supported theories to explain cancer treatment resistance, recurrence, and metastases. Designing treatments targeting CSCs should decrease the mortality rates of cancers. To accurately identify CSCs in cancer is a crucial step and requires the fulfillment of three characteristics [223,564]. First, CSCs must express specific stem cell-related genes. Second, CSCs have a high self-renewal ability. And third, CSCs are tumorigenic and generate tumors in mice, even with a very few cell numbers. In this study, we extend the current understanding and characterization of CSCs in HNSCCs by demonstrating that within the CD44+ cell population in HNSCC, the CD44+/CD271+ cell subpopulation is an enriched CSC population. In addition, we demonstrated that CD44+/CD271+ cell subpopulation may play a major role in the development of treatment resistance of CD44+ cells, making these cells a more suitable target for therapy.

Our data are compatible with the theory that CSCs come from deregulated normal stem cells. In normal human oral epithelium, CD271 was used to identify a subset of cells in the basal layer of the epithelium that possessed stem cell-like characteristics [20]. These CD271+ cells showed a higher growth rate, had more colonies in-vitro, and formed cellular layers expressing key differentiation markers, comparable to culturing stratified epithelium on amniotic membranes. Because cells in the basal half of the oral epithelium express CD44, the CD271+ cells

are a subset of CD44+ cells in normal oral tissues. Our flow cytometry analysis and immunofluorescence staining showed co-localization of CD271 and CD44 in HNSCC cell lines and human tissue samples (Fig 6.1). Immunofluorescence staining showed that the number of CD271+ cells increased proportionally with tumor severity with more random organization (Fig 6.1c and d). This suggests increased cell proliferation and a loss of normal function, especially at the invasion front. Comparable results were obtained from hypopharyngeal cancer [26,565]. Thus, we suggest that CD44+/CD271+ cells in HNSCC possess many similar stemness characteristics as those CD44+/CD271+ cells found in the normal oral epithelium, but their number increased when the epithelium become more dysplastic.

Several studies have correlated the percentage of CSCs in cancer with patient prognosis. The higher percentage of CD44+ cells in HNSCC was found correlated to metastasis and recurrence [249]. Our data, along with what was reported in esophageal carcinoma [566], hypopharyngeal carcinoma [26], and oral carcinoma [567] suggest that the percentage of CD44+/CD271+ cells could be an indicator of prognosis (Fig 6.1c, d). Perineural invasion (PNI) was documented to be an indicator related to poor prognosis in HNSCC, as it was associated with a higher rate of recurrence and metastasis [568]. It was suggested that CD271 expression may cause PNI in HNSCC as it might become activated by nerve growth factors (NGF) in Schwann cell surrounding neurons [569]. Indeed, CD271 expression was correlated with PNI in melanoma [570], pancreatic cancer [571] and oral cancer [572].

Our results revealed that the growth rate of CD44+/CD271+ cells was much higher and reached a stationary phase much later when compared to CD44+/CD271- cells and the unsorted parental cells (Fig 6.2a). These results were similar to those reported by studies comparing

CD271+ cells to the CD271-negative cells in tongue carcinoma [573], oral carcinoma [265], hypopharyngeal carcinoma [26], gastric cancers [574], prostate cancer [575] and melanoma [23]. Self-renewal is a hallmark of CSCs, as these cells can give rise to new cells that will expand and proliferate. The colony-forming ability of a cell is used to measure its self-renewal characteristic and previous studies have used this assay as one of the methods to characterize CSCs in 2D culture [576,577]. Analogously in 3D suspension culture, the sphere-forming ability of cells is used to measure the self-renewal ability of different cancer cells [578]. Our results demonstrated that CD44+/CD271+ cells not only possessed both a greater colony forming and a sphere-forming ability when compared to the CD271- cells and their unsorted parental cells but also formed bigger spheres (Fig 6.2b to f).

Due to the increasing evidence that CSCs from several types of cancers possess more chemotherapeutic- and radiotherapeutic-resistance when compared to the non-CSCs population, cancer treatment strategies are being developed to target CSCs [562]. In HNSCC, CD44+ cells showed more resistance to oxidative stress and remained in higher numbers in xenografts following radiotherapy, when compared to CD44- cells [19]. In esophageal squamous cell carcinoma, CD271+ cells were found more resistant to the oxidative stress (which is a cytotoxic effect of cisplatin) [24,579]. Tolerance to reactive oxygen species was found increased in PC12 cells using neurotrophins in a CD271-dependent manner [580]. Also, CD271+ cells were more resistant to DNA-damaging agents in melanoma when compared to CD271- cells [581]. These studies support our findings that CD44+CD271+ cells were the subpopulation with the most resistance to oxidative stress in HNSCC. The exact mechanisms remain to be confirmed, but current studies suggest several mechanisms in CD271+ cells, such as the increased expression of

ALDH1A1, ALDH1A1-dependent activation of drug-efflux pump, ATP-binding cassette sub-family B member 1(ABCB1), and survival proteins (AKT and BCL2) [582].

CD44+/CD271+ cells showed higher expression of self-renewal related genes, namely BMI1, SMO and GLI1, when compared to CD44+/CD271- cells and the unsorted parental cell population (Fig 6.4). These finding suggest higher activation of the Hedgehog signaling pathway of self-renewal. There was also higher expression of stemness-related genes, SOX2 and OCT4, which is responsible maintain pluripotency [583]. Our results are in line with previous studies reporting that CD271+ cells expanded and possessed stem-like characteristics of the oral mucosa [20], esophagus [25] and esophageal cancer [566].

In addition to in-vitro assays using human HNSCC derived cell lines, we performed in-vivo tumorigenic assays by injecting these cells using a limiting dilution approach (table 6.1). Compared to CD44+/CD271- cells, CD44+/CD271+ subpopulation was found to have a higher tumor formation incidence and with a faster tumor growth rate when implanted at the same low injection cells number into immune-deficient mice. Other studies, which tested CD271 expression only, reported high tumorigenicity of CD271+ cells when compared to the negative subpopulation of other solid tumors such as hypopharyngeal carcinoma and oral cancer [565,584].

In conclusion, we demonstrated that using two cell markers CD44+/CD271+ was better than using CD44 alone. This was because CD271+ cells represented a subpopulation of CD44+ cells with increased tumorigenicity and treatment resistance in HNSCC. In the future, it should be possible to target CD271+ cells to eradicate HNSCC resistance [265]. Our findings support the

idea of CSCs role in the development of treatment resistance and tumor progression. Further studies on this subpopulation should be conducted for a better understanding of the pathogenesis and development of targeted cancer treatments.

Conflict of interest

All authors declare no potential conflicts of interest.

Acknowledgements

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Figures:

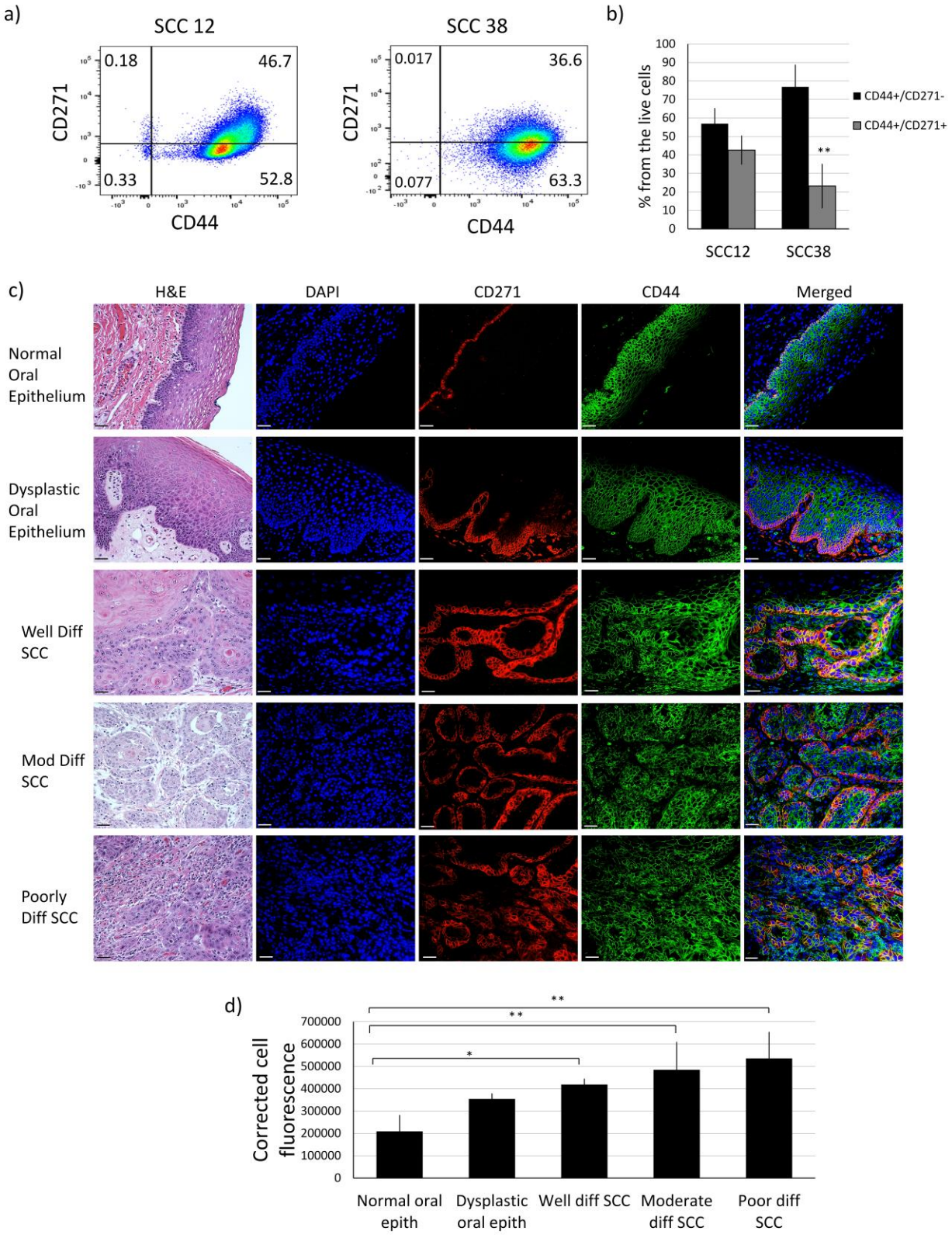
a)
SCC 12
SCC 38
CD271
CD44
0.18
46.7
0.33
52.8
0.017
36.6
0.077
63.3
b)
% from the live cells
CD44+/CD271-
CD44+/CD271+
SCC12
SCC38
c)
H&E
DAPI
CD271
CD44
Merged
Normal Oral Epithelium
Dysplastic Oral Epithelium
Well Diff SCC
Mod Diff SCC
Poorly Diff SCC
d)
Corrected cell fluorescence
Normal oral epith
Dysplastic oral epith
Well diff SCC
Moderate diff SCC
Poor diff SCC

Fig 6.1. CD271+ cells are a subpopulation of CD44+ cells. (a) SCC12 and SCC38 cells were examined for the expression of CD44 and CD271 markers using flow cytometry. (b) Percentage of CD44+/CD271- cells and CD44+/CD271+ cells in SCC12 and SCC38. (c) Human normal and oral SCC samples (N=10) were stained with H&E and monoclonal immunofluorescence antibodies against CD44 and CD271 for the assessment of the tumor grade and the localization of CD44+ and CD271+ cells (20X magnification. Scale bar: 37 µm). (d) Quantification of cell fluorescence of CD271+ from stained images in panel C with ImageJ software (n=3 to 6 per group). Data are presented as mean ± SD (* $P < 0.05$ and ** $P < 0.01$).

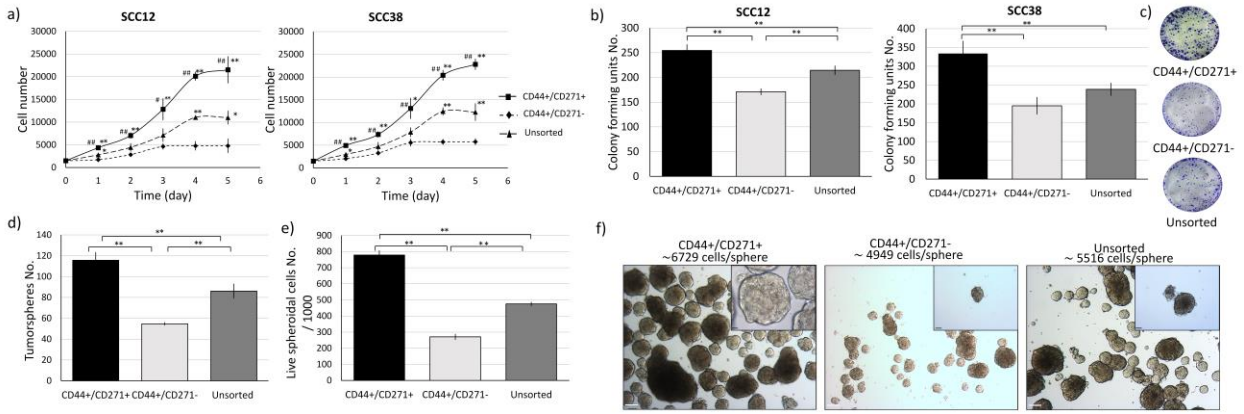

Fig 6.2. CD44+/CD271+ cells have higher growth rate in 2D and 3D culture conditions. (a) CD44+/CD271+, CD44+/CD271- and the unsorted parental cell population from SCC12 and SCC38 cell lines were seeded in 96-well plates and their cell growths were assessed using MTT assay (in triplicates from three independent experiments). Data are presented as mean ± SD (* $P < 0.05$ and ** $P < 0.01$ with CD44+/CD271-, $^{\#} P < 0.05$ and $^{\#\#} P < 0.01$ with parental cells). (b) Assessment of self-renewal characteristic in 2D culture with the colony forming assay. Single cells from each of the three cell populations from SCC12 and SCC38 were seeded in 6-well plates and allowed to form colonies for 14 days (400 cells seeded per well). Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean ± SD (** $P < 0.01$). Sample photographs of the fixed and stained colonies are presented on the (c) panel. (d) To assess self-renewal in 3D culture, tumor-sphere formation assay was used. Single cells from each of the three cell populations from SCC12 were cultured in anchorage-independent and serum-free culture conditions and allowed to form spheres for 14 days. Data are presented as mean ± SD (** $P < 0.01$). (e) The spheres were dissociated into single cells and counted in the presences of trypan blue stain. Cells numbers are presented as mean ± SD (** $P < 0.01$). (f) The spheres were counted, and photos were taken under a phase-contrast microscope with 5X (the main photos) and 40X (the inserts) magnification. Scale bar = 90 µM and 10 µM respectively.

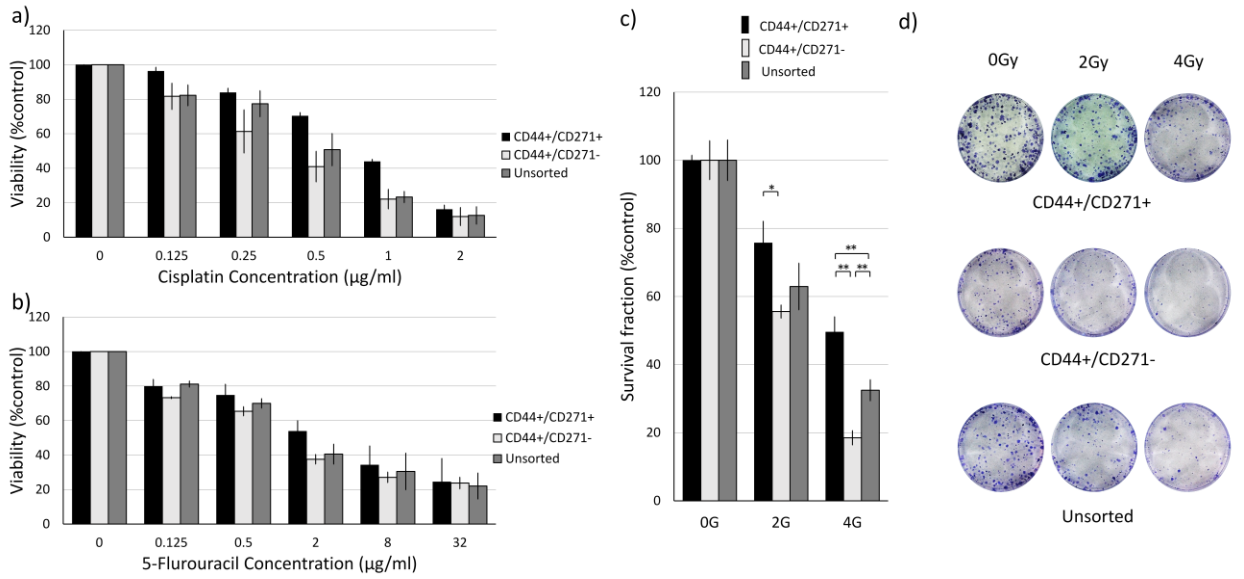

Fig 6.3. CD44+/CD271+ cells possess higher onco-treatment resistance. (a) CD44+/CD271+, CD44+/CD271-, and the unsorted parental cell population from SCC12 cells were treated with 0, 0.125, 0.25, 0.5, 1, and 2 µg/ml of CIS for 72 h. Cell viability was evaluated in triplicate by MTT assay. Data are presented as mean ± SD (b) The three cell populations from SCC12 were treated with 0, 0.125, 0.5, 2, 8, and 32 µg/ml of 5-FU for 72 h. Cell viability was evaluated in triplicate by MTT assay. (c) The three cell populations from SCC12 were exposed to 0, 2, or 4 Gy radiation doses then 400 single live cells were seeded in 6 well plate and allowed to form colonies for 14 days. Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean ± SD (*P < 0.05, ** P < 0.01). Sample photographs of the fixed and stained colonies are presented on the (d) panel.

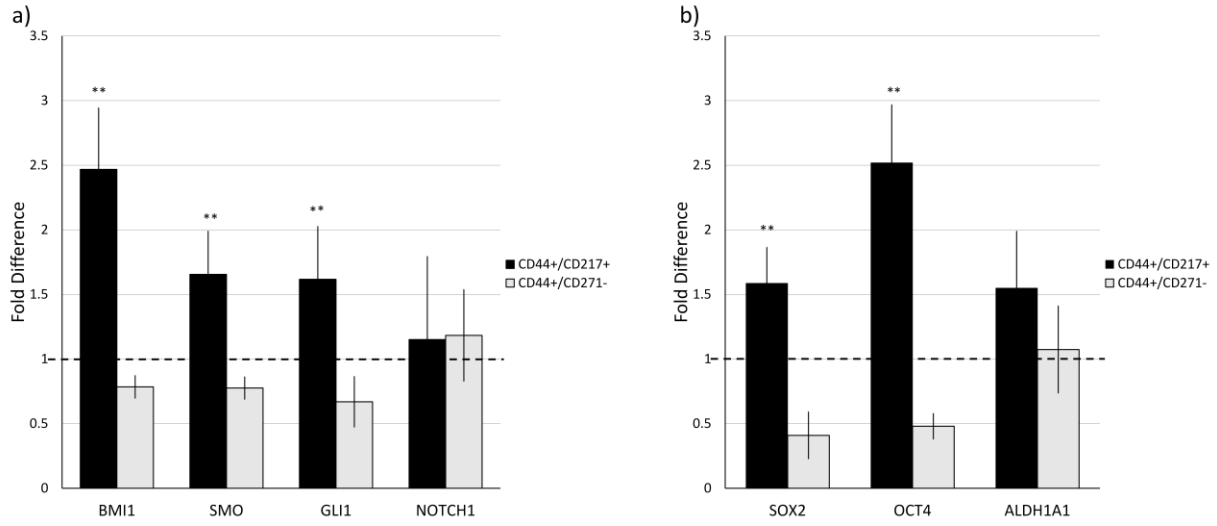

Fig 6.4. CD44+/CD271+ cells expressed higher levels of stem cell-related markers than CD44+/CD271- cells. Expression levels of CSCs self-renewal related genes; (a) BMI1, SMO, GLI1 and NOTCH1, (b) stemness-related genes; SOX2 and OCT4, and drug resistance-related gene ALDH1A1 were analyzed by quantitative RT-PCR. Y-axis shows the relative expression of the gene compared to GAPDH. The horizontal dashed line represents the relative genes expression level of the unsorted parental cell population. All assays were performed in triplicate in three independent experiments and were calculated on the basis of ΔΔCt method. Data represent mean ± SD (** P < 0.01).

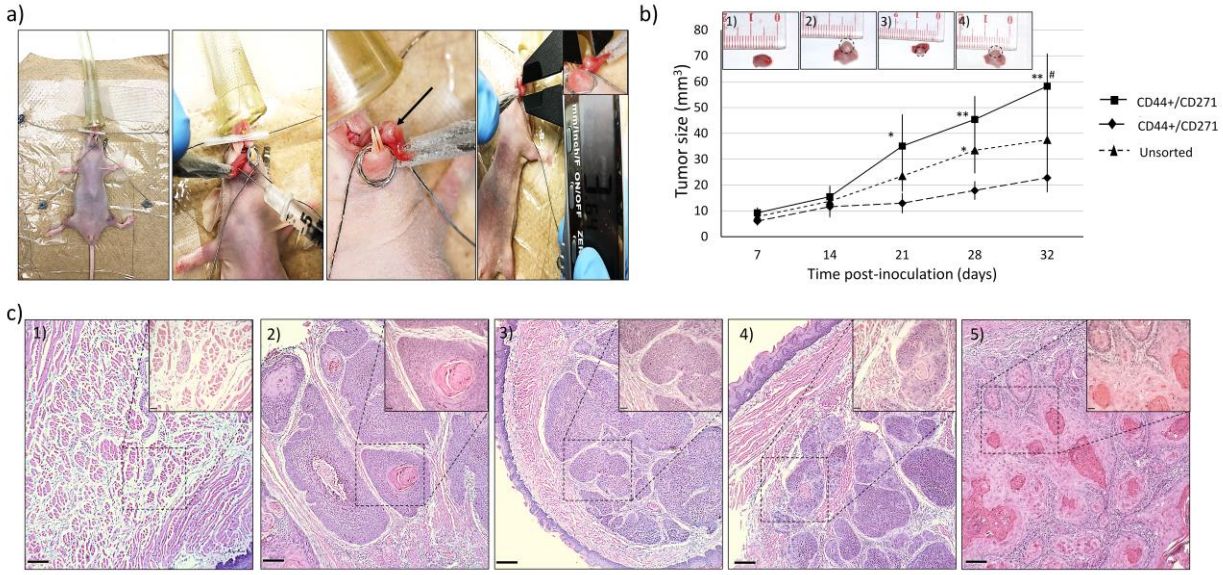

Fig 6.5. CD44+/CD271+ cells have higher tumorgenicity when compared to CD44+/CD271- cells. (a) CD44+/CD271+, CD44+/CD271-, and the parental cell population from SCC12 were injected in the tongue of NU/NU Nude (Crl:NU-Foxn1^{nu}) mice (N=50) and tumor formation and size were followed weekly. (b) Tumor volume (mm^3) was measured weekly after cancer cell inoculation. Data represent mean ± SD (* P < 0.05 and ** P < 0.01 compared to CD44+/CD271- and $^{\#}$ P < 0.05 compared to parental cells). (b1) Normal mouse tongue as negative control, (b2) tumor formation after 35 days with CD44+/CD271+ injection, (b3) tumor formation with CD44+/CD271- injection, (b4) tumor formation with the unsorted parental cell population injection. (c) Tumor formation following inoculation with the three cellular populations (hematoxylin and eosin staining). (c1) Normal mouse tongue as negative control, (c2) CD44+/CD271+, (c3) CD44+/CD271-, (c4) Unsorted parental cell population, and (c5) Well differentiated human HNSCC, used as a positive control (5X magnification. Scale bar: 150 µm, inserts 20X magnification. Scale bar: 37 µm).

Mice with tumor formed in relation to the number and types of cells injected.			
	1×10^3 cells	1×10^4 cells	1×10^5 cells
CD44+/CD271+	3/5 mice	5/5 mice	5/5 mice
CD44+/CD271-	0/5 mice	1/5 mice	3/5 mice
Unsorted parental cells	0/5 mice	2/5 mice	4/5 mice

Table 6.1. Tumorigenicity of CD44+/CD271+, CD44+/CD271-, and unsorted SCC12 cells in a nude mouse orthotopic xenograft model.

Chapter 7 - Broccoli Extract Increases Drug-mediated Cytotoxicity Toward Cancer Stem Cells of Head and Neck Squamous Cell Carcinoma.

7.1 Preface (connecting paragraph)

The recurrence and/or lack of response of certain tumors to radio- and chemotherapy has been credited to the presence of cancer stem cells. As we reviewed in the second chapter and showed in the sixth chapter, cancer stem cells are characterized by their capacity for self-renewal, their ability to introduce heterogeneity within a tumor mass, genomic instability, and their high resistance to both radiation and chemotherapy. Sulforaphane, type of isothiocyanate, is converted from glucoraphanin, a major glucosinolate in broccoli and broccoli sprouts. As discussed in the second chapter, Sulforaphane has been shown to not only be effective in preventing cancers, but also in inhibiting the growth of established tumors in animal models. We suggested using Sulforaphane as a co-treatment with Cisplatin and 5-Flourouracil to decrease the CSCs resistance and increase the effectiveness of the conventional treatments.

In this chapter, we isolated the head and neck cancer stem cells using CD44/CD271 double staining with FACS sorting. The effect of sulforaphane either alone or combined with conventional chemotherapy, Cisplatin and 5-Fluorouracil, has been evaluated on the isolated cellular population. Sulforaphane either alone or combined decreased cancer stem cells' viability, self-renewal and tumor formation ability in-vitro and in-vivo. Sulforaphane had minimal to no effect on the non-cancerous human stem cells and on the animal models. Our work suggested that sulforaphane can be used as a combination treatment with the conventional modalities to

enhance the cytotoxicity against cancer stem cells in head and neck cancers, while having minimal effects on healthy cells.

The study presented in this chapter has been submitted to British Journal of Cancer

Broccoli Extract Increases Drug-mediated Cytotoxicity Toward Cancer Stem Cells of Head and Neck Squamous Cell Carcinoma

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Running title: Sulforaphane and Chemotherapy in Cancer Stem Cells

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7.2 Abstract:

Background

Head and neck squamous cell carcinomas (HNSCC) are among the malignant neoplasms with poor prognosis. Treatment-resistant cancer stem cell (CSC) is one of the reasons for treatment failure. Considerable attention has focused on sulforaphane (SF), a phytochemical from broccoli, which exhibits anti-cancer properties. We investigated whether SF could enhance the

chemotherapeutic effects of Cisplatin (CIS) and 5-Fluorouracil (5-FU) against HNSCC-CSCs and the mechanisms of action.

Methods

FACS-isolated CSCs from SCC12 and SCC38 human cell lines were treated with SF alone or combined with CIS or 5-FU. Cell viability, colony and sphere-forming ability, apoptosis, CSC-related genes expression, and in-vivo tumor progression was assessed. Safety was tested on non-cancerous stem cells, and in-vivo.

Results

SF reduced HNSCC-CSCs viability in a time- and dose-dependent manner. SF-combination increased the cytotoxicity of CIS two-fold and of 5-FU ten-fold, with no effects on non-cancerous stem cells viability and functions. SF-combined treatments inhibited CSCs colony and sphere formation, and tumor progression in-vivo. Suggested mechanisms include stimulation of the Caspase-dependent apoptotic pathway, inhibition of SHH pathway, and decreasing the expression of SOX2 and OCT4.

Conclusions

Combining SF with lower doses of CIS or 5-FU enhanced drug cytotoxicity against HNSCC-CSCs, with minimal effects on healthy cells.

7.3 Introduction:

Head and neck squamous cell carcinoma (HNSCC) is the 7th most common malignancy worldwide, accounting for 580,000 new cases and over 380,000 deaths annually and is

representing approximately 6% of all cancer cases [1,526,585]. The current standard treatment of HNSCC is by multimodal approaches consisting of surgery, radiotherapy, and/or chemotherapy [586]. Despite advances in diagnostic tools and treatment modalities, HNSCC 5-years survival rate is about 50% [2,587]. Cancer stem cells (CSCs), also known as tumor-initiating cells, is a special subpopulation within the tumor [552]. When compared to the remaining tumor cells, CSCs are often more resistant to chemoradiotherapy and more tumorigenic [588]. Therefore, it is of great importance to develop strategies for targeting CSCs in order to improve HNSCC treatment outcomes.

Sulforaphane (SF), a phytochemical that exists in a large amount in cruciferous plants, has showed a promising anti-inflammatory, anti-oxidant, and anti-tumor effects [29,31-33]. Recent studies have proposed that SF exerts its anti-tumor effects through inhibiting both proliferation and cell cycle mechanisms, promoting apoptosis and protecting the precancerous cells from methylation [33,500]. However, its effect on cancer stem cells in HNSCC either alone or in combination with conventional chemotherapy remains poorly understood [505]. Therefore, our present study was designed to investigate whether SF could be a potent agent, to facilitate the chemotherapy efficacy of Cisplatin (CIS) and 5-Fluorouracil (5-FU) on HNSCC stem cells and to determine the mechanisms behind.

7.4 Material and Methods:

Cell culture

UM-SCC12 (laryngeal SCC, RRID: CVCL_7717) and UM-SCC38 (tonsillar SCC, RRID: CVCL_7749) human cell lines were purchased from University of Michigan in 2015 and used as

models for HNSCC [500]. These cell lines have been authenticated using STR analysis in 2019 at Genome Quebec. They were cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher, Waltham, Massachusetts, United States) supplemented with 1% non-essential amino acids. PDLSCs and DPSCs were isolated from extracted teeth and cultured in Minimum Essential Medium (MEM, Thermo Fisher) [589]. Both medias were supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic (Thermo Fisher). All cell types were mycoplasma-free and were incubated in a humidified incubator at 37°C with 5% CO_2.

Cytotoxic agents

Sulforaphane (Cayman Chemical, Ann Arbor, Michigan, United States) was purchased as a solution in ethanol with purity ≥98% and stored at -20°C. Cisplatin (Cayman Chemical) was prepared in phosphate-buffered saline (PBS) to a 0.3 mg/ml stock and was kept protected from light at 4°C. 5-Fluorouracil (Sigma Aldrich, St. Louis, Missouri, United States) was prepared in dimethyl sulfoxide (DMSO) to 50 mg/ml stock. The final concentrations of the solvents, either PBS or DMSO, in the working solution medium were 0.1% or less.

Fluorescence-activated cell sorting (FACS)

Flow cytometry and fluorescence-activated cell sorting was performed as previously described[590]. Briefly, Alexa Fluor®700 Mouse Anti-Human CD44 (Clone G44-26) and PerCP-Cy™5.5 Mouse Anti-Human CD271 (Clone C40-1457) monoclonal antibodies were obtained from BD Pharmingen. Tumor cells were harvested using Accutase™ (BD Bioscience, San Jose, Canada) and resuspended with a final concentration of $1x10^6$ cells/100 µl for the staining procedures.

FACS of $CD44^+/CD271^+$ cells were performed using a BD FACSARIA III cells sorter (BD Bioscience). UltraComp eBeads™ Compensation Beads (Thermo Fisher) were used as control.

MTT assay

1500 cells were seeded in 96-well plates and they were treated with different concentrations of SF and/or chemotherapeutic agents for 72 hours. The medium was then removed and 10% solution of 5 mg/ml MTT in medium (Sigma Aldrich) was added and incubated at 37°C for 2 hours. Formazan was dissolved by adding DMSO to each well after MTT removal. The optical density was measured at 562/540 nm in EL800 Microplate Reader (BIO-TEK Instruments, Winooski, Vermont, United States). For analyzing the effect of SF over time, the cells were treated with 3.5 µM SF and the same steps were followed daily for 4 consecutive days.

Colony-forming assay

$CD44^+/CD271^+$ cells were seeded at 1×10^5 cells/well in 6-well tissue culture plates. The cells were treated with SF and/or chemotherapeutic agents for 72 hours. Then, cells were detached, plated at a density of 400 single living cells/well in 6-well tissue culture plates, and incubated for 10 days while the medium was being changed every 3 days. The cell colonies were fixed and stained with 1% crystal violet, 50% methanol in DDH_2O for 1 hour. The number of colonies with >50 cells were counted under an inverted microscope.

Sphere-forming assay

5000 $CD44^+/CD271^+$ cells/500µl per well were seeded in 24 Ultra-Low Attachment Multiple Well Plate (Millipore Sigma, Burlington, Massachusetts, United States) in DMEM-F-12

medium (Thermo Fisher) reconstituted with 20 ng/ml of Epidermal Growth Factor, 20 ng/ml of Basic Fibroblast Growth Factor, 0.5% N_2 supplement (STEMCELL Technologies, Vancouver, Canada), 1% B27 supplement, and 2% Antibiotic-Antimycotic (Thermo Fisher). After 24 hours, SF and/or the two chemotherapeutic agents were added according to the concentrations in the graph (Fig 2). The medium was added every 2–3 days. Photographs of groups were captured at 14 days, using phase-contrast microscope.

For serial passage, single cells were obtained from Accutase-treated spheroids. Then, the same steps were followed as described above. Spheres were then collected by centrifugation and dissociated by Accutase to single cells to get the cells count.

Annexin V apoptosis detection

Post-treatment apoptosis was measured by using the PE-Annexin V Apoptosis Detection Kit (BD Bioscience). Briefly, 1.5×10^5 CSCs from SCC12 cell line were seeded per well, in a 6-well plate for 24 hours and were then treated with SF and/or the chemotherapeutic agents for 72 hours. Cells were detached using Accutase (Biolegend, San Diego, California, United States) then all procedures followed the manufacturer's protocol. Cells were analyzed by flow cytometry using LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo vX (FlowJo LCC).

Real-Time qRT-PCR:

Genes expression levels in $CD44^+/CD271^+$ cells from the SCC12 cell line after exposure to SF and/or chemotherapeutic agents for 3 days were measured as previously described[590]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Check the appendix for the gene-specific primers were used. The expression was

calculated based on ΔΔCt method. The *n*-fold difference in mRNAs expression was determined according to the method of $2^{-\Delta\Delta CT}$.

Western blot assay

$CD44^+/CD271^+$ cells from the SCC12 cell line were exposed to SF and/or chemotherapeutic agents for 3 days, then harvested using trypsin. A lysis buffer that consisted of 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 1% Na-Deoxycholate used to lysate the cells. After centrifugation at 15,000 g for 20 min, supernatants were recovered, and the protein content was quantified by the Pierce™ BCA Protein Assay Kit (Thermo Fisher). Protein samples (20–60 µg) were size-separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes. The blot was blocked by 5% skim milk and incubated with one of the following primary antibodies: anti-human BMI1, anti-BCL2 (Cell Signaling, Danvers, Massachusetts, United States), anti-SOX2, Anti-OCT4 and anti-β actin (Abcam, Cambridge, United Kingdom) overnight at 4°C. Horseradish peroxidase (HRP)- conjugated anti-goat or rabbit secondary antibody was then used. Antibody-bound proteins were detected by the spray on ECL (Zmtech Scientifique, Montreal, Canada) and ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, California, United States).

Osteogenic differentiation

DPSCs and PDLSCs were treated by 3.5 µM SF for 3 days, then the cells were collected and seeded in 6 well plates, 2×10^5 cells/well, and allowed to grow to 70% confluency in normal medium. Thereafter, the growth media were replaced with the osteogenic medium containing:

α-MEM supplemented with 1% Antibiotic/Antimycotic, 20% FBS, 2 mM Glutamine, 10^{-8} M dexamethasone sodium phosphate, 55 µM 2-Mercaptoethanol, 0.1 mM L-ascorbic acid and 2 mM beta-glycerophosphate. Control cells were cultured in normal growth medium. Both media were changed every 3 days. All cultures were allowed to grow for 21 days, then fixed and stained with Alizarin Red (Sigma). Photographs of all groups were captured using phase-contrast microscope at 5× magnification. Osteogenic quantification was done by unbinding the Alizarin Red stain using 10% (v/v) acetic acid followed by reading the absorbance at a wavelength of 405 using microplate reader.

Chondrogenic differentiation

DPSCs and PDLSCs were treated by 3.5 µM SF for 3 days, then the cells were collected as 5×10^5 cells in 15 ml polypropylene tubes. Cells were centrifuged, and the media were replaced with the StemXVivo Chondrogenic Base Media supplemented with StemXVivo Chondrogenic Supplement (R&D Systems, Minneapolis, Minnesota, United States) and 1% Antibiotic/Antimycotic. Control cells were cultured in normal growth medium. Every 3 days half of the medium was replaced by a new medium. All cultures were grown for 21 days, then the pellets were collected and frozen by OCT compound (Thermo Fisher), cryosectioned, and stained by Collagen Type II immunofluorescent staining. Photographs were captured using phase contrast microscope at 20× magnification. Chondrogenic quantification was done using ImageJ software (NIH).

In vivo assay and tumor xenografts

In the in-vivo experiment we used SF and CIS only, without 5-FU to decrease the number of mice. This animal research study was approved by the University Animal Care Committee at McGill University (Protocol #5330, www.animalcare.mcgill.ca) and conform to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The animals used in this study were 23 NU/NU Nude (Crl:NU-Foxn1^{nu}) mice (n = 5 in each group and n = 3 in the sham control group) (Charles River, Wilmington, Massachusetts, United States). All the mice were kept in clean conditions with soft food and water in the animal resource center at McGill University. Six to ten weeks old male mice received 1 X 10^4 $CD44^+/CD271^+$ SCC12 cells in the side of the tongue, suspended in 30 µl of normal saline using a 1-ml tuberculin syringe with a 30-gauge hypodermic needle, under general anesthesia with isoflurane (Isoba VetTM). After one week, mice bearing tumors were randomly divided into groups and different treatments were started. Mice were treated intraperitoneally (I.P) with the vehicle (normal saline) , SF (4 mg/kg), CIS (3 mg/kg) , or combination of SF and CIS every 3 days for a total of 6 doses [513]. The mice were examined weekly to measure the body weight and the tumor size bidirectionally using a caliber, under isoflurane gas anesthesia. Tumor size was calculated using the following formula: volume = $(width)^2$ * length/2. Animals were sacrificed after 49 days with CO2 inhalation, and tongues, livers, and kidneys were collected. Tumor formation and liver or kidney necrosis were assessed using H&E stained sections. Means of the body weights and tumor sizes as recorded every week were compared between the five animal groups.

Statistical analysis

Data were presented as the means ± standard deviation (SD) of three independent experiments done in triplicates with comparable results. One-way analysis of variance (ANOVA)

followed by post hoc Tukey's test were used to assess significant differences between three groups or more, while Student's t-test (Unpaired) was used between two groups. *p*-values < 0.05 were considered statistically significant and < 0.01 were considered extremely statistically significant. GraphPad Prism 6 software was used for the statistical analysis (GraphPad Software, San Diego, Canada).

7.5 Results:

Effects of sulforaphane on the viability and proliferation in HNSCC-CSCs

We exposed the isolated CSCs from the two cell lines to different concentrations of SF treatment which showed that SF can reduce the viability of HNSCC-CSCs in a dose-dependent manner (Fig 7.1a). The half maximal inhibitory concentration (IC50) of SF on CSCs was 5.54 µM and 5.13 µM for SCC12 and SCC38, respectively. The inhibitory effects of SF on cellular viability increased over time, as was shown by exposing the cells to 3.50 µM SF for different time periods (Fig 7.1b).

Adding 3.50 µM SF had a statistically significant increase in the inhibition of cell viability compared to the usage of CIS (Fig 1c) or 5-FU (Fig 7.1d) as single chemotherapy with almost every tested drug concentration. The effect is nearly doubled with CIS and increased ten times with 5-FU, especially at the lower chemotherapy concentrations.

By using 3.50 µM of SF alone, the clonogenic ability of the CSCs was reduced to 29%±10.1% and 24%±3.9% in SCC12 and SCC38, respectively, compared to control, which was comparable to using 0.5 µg/ml CIS. CIS as a single treatment, also reduced the clonogenic ability to 28%±2.4% and 19%±4.6% while 5-FU reduced it to 52%±6.8% and 38%±14% for SCC12 and

SCC38, respectively. However, the combined SF+CIS or SF+5-FU treatments completely prevented the colony formation (Fig 7.1e&f).

Effect of sulforaphane on self-renewal and apoptosis in HNSCC-CSCs

While single treatment with SF and to a more extent with CIS or 5-FU reduced spheroid formation, combined treatments inhibited spheroid formation most effectively (Fig 7.2a&c). The effect was not on the number only, but also on the size of the formed spheres; the combination treatments produced smaller spheres with less cell number (Fig 7.2b&d).

SF alone and to a more extent CIS or 5-FU inhibited secondary sphere formation, but the combined treatments had the most inhibitory effect on both, the number and the size of spheres (Fig 7.2e-f).

Single SF treatment induced early apoptosis in 46%±3.4% of CSCs compared to 32%±7.3% in the control group. The single treatment with CIS induced early apoptosis in 50.3%±2.4% in the CSCs, while the combined treatment of SF+CIS increased the apoptosis to 70.2%±11.1%. Similarly, 5-FU as a standalone treatment, induced apoptosis in 41.2%±6.4% in the CSCs and the combined treatment of SF+5-FU increased apoptosis to 60.3%±8.1% (Figure 7.2g&h). This suggested that sulforaphane could reduce HNSCC-CSCs numbers through the induction of apoptosis alongside inhibition of proliferation and self-renewal.

Effect of sulforaphane on HNSCC-CSCs genotype

By combining SF to CIS or 5-FU, there was a significant decrease in the expression levels of NOTCH1, SMO and GLI1 genes compared to using CIS or 5-FU alone. This led to inhibition in their downstream gene BMI1 too (Fig 7.3a).

The combined SF treatment decreased SOX2 expression significantly with both CIS and 5-FU compared to either chemotherapy alone. However, the reduction in expression of OCT4 was significant only with CIS combination treatment. Drug resistance and stemness-related gene ALDH1A1 expression was analyzed too. SF combination with CIS or 5-FU significantly reduced ALDH1A1 expression compared with either single treatment (Fig 7.3b).

Our results showed a significant decrease in BCL2 expression after combining SF to CIS or 5-FU, and although there was an increase in the expression of BAX with the combined treatment, it was not significant. Caspase3 expression was elevated with SF addition to CIS or 5-FU compared to using each chemotherapy alone (Fig 7.3c). qRT-PCR results were confirmed by western blotting to detect the changes at the protein level (Fig 7.3c).

Effect of sulforaphane on non-cancerous (healthy) stem cells

To examine the effect of SF alone or combined with each chemotherapy on non-cancerous human stem cells (nCSCs), periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) were used as models. SF alone did not show any significant toxicity on the nCSCs in low concentrations up to 3.50 µM (Fig 7.4a). Comparable results were obtained with the combined treatments; there was no significant difference between using CIS or 5-FU as a single treatment or after SF combination (Fig 7.4b&c).

There was no significant difference between the SF treated and untreated cells, in the ability of osteogenic differentiation in both types of tested cells (Fig 7.4d&e). We had similar results with chondrogenic differentiation in both PDLSCs and DPSCs (Fig 7.4f&g).

Effect of SF+CIS combination treatment in-vivo

To assess whether SF might influence the sensitivity of HNSCC-CSC xenografts toward chemotherapy, we transplanted $CD44^{+}/CD271^{+}$ cells from SCC12 cell line into the tongue of nude immunocompromised mice. Mice were I.P injected with vehicle (normal saline), SF, CIS or both agents together and tumor growth was measured weekly during a period of 49 days (Fig 7.5a). Compared to the control group, treatment with SF or CIS alone inhibited tumor growth and tumor volumes showed marked reduction by 59% or 54.5%, respectively. However, SF+CIS reduced the tumor volume by 73% at the end of the experiment. The significant difference in the tumor size was observed between the control group and the treatment groups after 14 days from treatment starting, and there was a significant difference between the combined treatment group and the other groups after 35 days (Fig 7.5b). Sulforaphane had no toxic effect on the mice either alone or combined with CIS, as determined by body weight compared to the sham group. There was an insignificant reduction in the body weight with the combined treatment during the administration period, however, the mice re-gained weight after treatment cessation. The control group showed a marked reduction in the body weight during the experiment (Fig 7.5c). There were no liver or kidney necrosis as shown in histological, H&E stained, sections in all mice groups (Fig 7.5d).

7.6 Discussion

Therapeutic efficacy of SF:

In a previous study, we demonstrate that SF can increase chemotherapeutic cytotoxicity of CIS and 5-FU toward HNSCC [500]. That was in line with other studies on oral cancers [403,422] and a variety of other types of cancers [36,547]. But little is known about the effect of the SF combined treatment on the HNSCC-CSCs. In our last published work, we suggested that $CD44^{+}/CD271^{+}$ is a suitable marker to isolate CSCs from HNSCC[590] and in this study we used these CSCs to examine the effect of SF/chemotherapy combination treatments. Our results showed that SF has a cytotoxic effect on HNSCC-CSCs, that elevate in both dose and time-dependent manner. Other studies on oral carcinoma [39] and other cancer types [468,482,591] reported comparable results. What we found new in our study, is that SF can be used as a combination treatment to enhance the toxicity of CIS and 5-FU against the more resistant CSCs in HNSCC. The usage of 3.50 µM of SF nearly doubled the effect of CIS and multiplied the effect of 5-FU by 10 times, especially at lower chemotherapy doses. This SF selected dosage is expected to be achievable in human bodies simply by oral ingestion of fresh broccoli sprouts. It was reported that following the ingestion of 40g of broccoli, SF plasma concentration reached 2.50 µM /L within 3 hours [335]. Remarkably, the SF cytotoxic effect was the same on the two selected cell lines, SCC12 and SCC38, even with the SCC38 being more chemo-resistant compared to SCC12 [540,542]. This suggests that SF effects CSCs in both chemo-resistant and chemo-sensitive HNSCC by the same mechanism making SF a promising tool in fighting tumors resistant for conventional chemotherapy.

Our results demonstrate that 3.5µM SF alone can reduce CSCs clonogenicity to the same extent as 0.5µg/ml CIS and more efficient than 1.3µg/ml 5-FU, however, the combination

treatments eliminated the clonogenic ability completely, either with CIS or 5-FU. Similar results were reported with Gemcitabine on pancreatic cancer, Taxol on prostate cancer [36] and CIS on gastric cancer [547]. Comparable results were obtained with sphere formation assay, as SF either alone or combined with CIS or 5-FU significantly reduced the number and size of primary and secondary spheres, compared to control or using each chemotherapy alone. The used SF dosage, 3.5 µM, was similar to the range, 0.5 to 10 µM, that used with other cancer types in similar studies, to inhibit tumor-sphere formation [36,482,591].

By using the annexin V/7-AAD assay, we found that SF treatment significantly increased early apoptosis in treated CSCs, which was equal to using 0.5µg/ml CIS and higher than 1.3µg/ml 5-FU. However, the combined treatment of SF and low doses of CIS or 5-FU, led to increased apoptosis compared to using a single chemotherapeutic drug or SF as a treatment. These results point out that SF might work with multiple mechanisms to target the CSCs, which could reduce the chance of developing resistance against its effect. SF induction of apoptosis on CSCs was reported also with pancreas and prostate cancer stem cells [36,468].

Our results demonstrated that SF+CIS combination has reduced the tumor size that is formed by the inoculation of HNSCC-CSCs in the tongue of immunocompromised mice, as compared to SF or CIS alone. During the period of the combination treatment, the mice had an insignificant reduction in the body weight compared to the sham group (no tumor burden), but the mice regained their weight after the treatment conclusion. This indicates that combination treatment is stressful for these mice but still tolerable. All tumor-bearing mice had decreased body weights compared to the sham group and it was highly significant with the control group (treated with saline only). This can be explained by the increase in the tumor size which caused

interference with the normal feeding process, even with the usage of soft food. SF biosafety was shown by H&E staining of the mice liver and kidney, as there was no necrosis with SF alone or combined with CIS. Several studies reported similar biosafety profile for SF combined with other drugs [36,592,593]. To our knowledge, we are the first to show that SF enhances the cytotoxicity of CIS and 5-FU toward HNSCC-CSCs.

Safety of SF on non-cancerous human stem cells:

Several studies demonstrated little to no toxicity of SF on non-cancerous human cells, including our recent study [39,421,500]. In the current study, however, we assessed the effect of SF on non-cancerous human stem cells viability and function. To our knowledge, it is the first time that SF combination with chemotherapy is tested on human stem cells. We demonstrated that SF does not affect the viability of human stem cells up to 3.50 µM, either alone or combined with CIS or 5-FU. In addition, the dose we selected for our experiments did not affect the differentiation function of stem cells, both osteogenic and chondrogenic. Several studies reported low doses of SF did not affect mesenchymal stem cells viability and protected it from carcinogens [594-596].

Molecular mechanism of SF-mediated targeting of HNSCC-CSCs:

Mechanistically, we recently demonstrated that SF enhanced the cytotoxicity of chemotherapy (CIS or 5-FU) against HNSCC by stimulation of Caspase-dependent apoptosis pathway [500]. In the current study, we report a comparable result with HNSCC-CSCs, as SF increased the apoptotic effect of CIS and 5-FU on CSCs by inhibition of BCL2. Numerous other molecular mechanisms have been suggested for the pro-apoptotic effect of SF, as the cleavage

of caspase-8 in pancreatic cancer [531] and fragmentation of DNA repairing protein poly (ADP-ribose) polymerase (PARP) while decreasing the expression of BCL2 in mammary, prostate and colon cancers [400,412,597].

Aldehyde dehydrogenase 1 (ALDH1) is a member of the aldehyde dehydrogenase family of cytosolic isoenzymes, which are highly expressed in many stem and progenitor cells [270]. Interestingly, $ALDH1^+$ HNSCC cells showed high self-renewal ability along with increased tumor formation, invasion, and treatment resistance [268]. The tumorigenic ability of CSCs is related to self-renewal, as it initiates tumor growth and spread [598]. It is suggested that dysregulation of self-renewal pathways in CSCs, such as; SMO, NOTCH1, and BMI1, is the cause of tumorigenicity and treatment resistance [232,598,599]. There are multiple studies reported that chemotherapies like CIS and 5-FU may cause selection of CSCs and increase the expression of self-renewal and drug resistance related genes, like BMI1 [600,601], or ALDH1A1 [36,602,603] which was also reported in our study. In our in vitro experiment, combined SF treatments prevented CIS and 5-FU induction of BMI1 and ALDH1A1 expression and enhanced downregulation of SMO, GLI1, and NOTCH1. Therefore, SF co-treatments might have contributed to the resensitization of CSCs to chemotherapeutic drugs. Interestingly, a similar effect was reported with other cancer types, either with gemcitabine or cisplatin [36,547].

It is suggested that octamer-binding transcription factor 4 (OCT4) is one of the best indicators for stemness and maintaining an undifferentiated state [604]. It was reported in a recent meta-analysis study, a close correlation between OCT-4 overexpression and poor overall survival of HNSCC patients [605]. SOX2 overexpression also was reported to affect invasion and metastasis induction in laryngeal squamous cell carcinoma [606]. Our results showed that SF

inhibited the expression of both SOX2 and OCT4 alone and significantly increased the inhibitory effect of CIS on both markers, while it had this effect with 5-FU on SOX2 only.

In conclusion, we demonstrated that SF strongly enhanced the cytotoxic effect of chemotherapy; CIS and 5-FU, against HNSCC-CSCs and prevented the elevation of self-renewal and drug-resistance related genes expression with conventional treatment modalities. The combined SF/chemotherapy treatments may be a promising option in clinical settings. Since our data combined with other studies suggest that SF could enhance the effect mediated by chemo- and radiotherapy, in vitro and in vivo, lower doses of these treatment agents might form successful treatment modalities when combined with SF.

Additional Information:

Ethics approval and consent to participate: The animal research study was approved by the University Animal Care Committee at McGill University (Protocol #5330, www.animalcare.mcgill.ca).

Consent for publication: Not applicable.

Data availability: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare no competing interests.

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Figures:

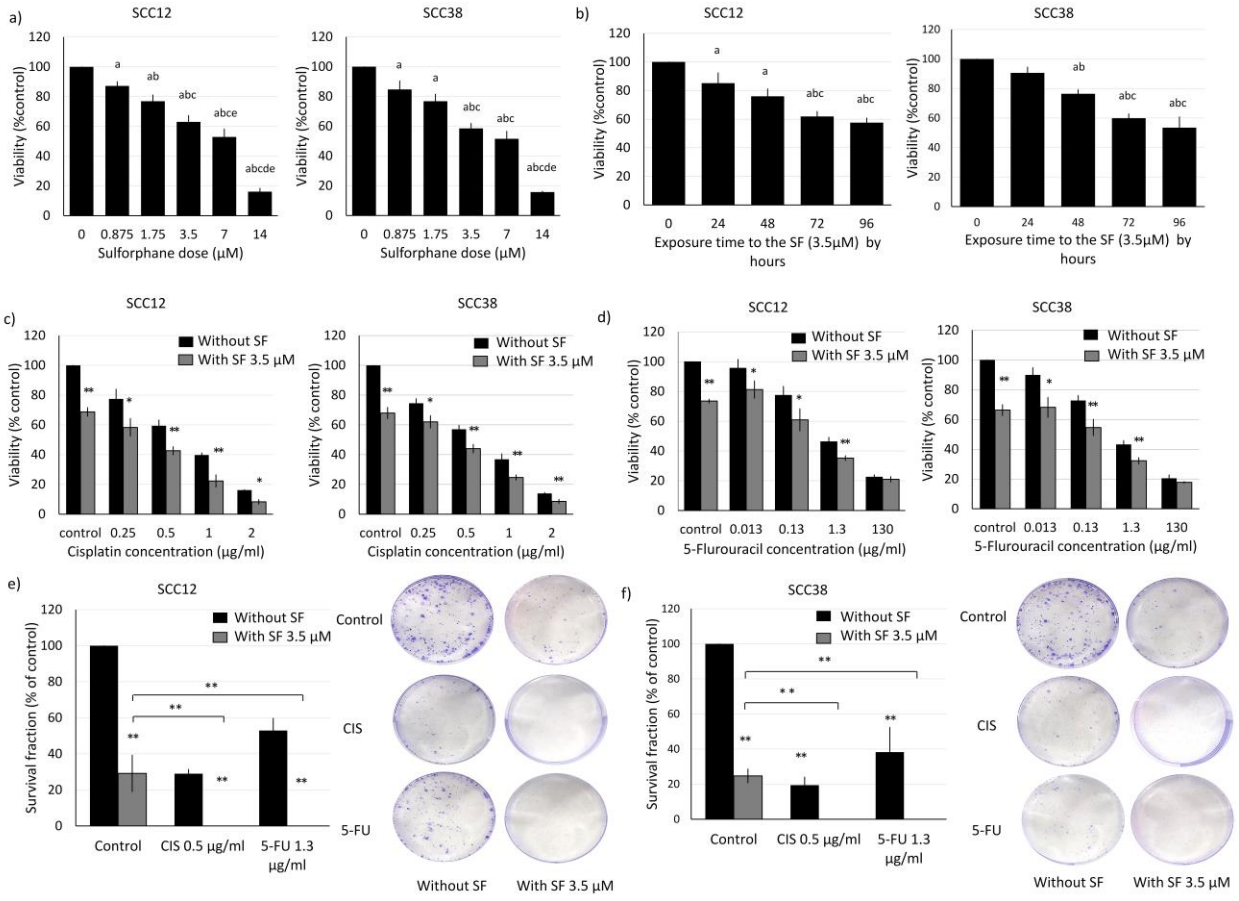

Fig 7.1 Effects of sulforaphane on the viability and proliferation in HNSCC-CSCs. (a) HNSCC-CSCs were treated with 0, 0.875, 1.75, 3.5, 7 and 14 µM of SF for 72 h. Data are presented as mean ± SD for N=3("a" means a p-value < 0.05 relative to 0 µM, "b" to 0.875 µM, "c" to 1.75 µM, "d" to 3.5 µM, "e" to 7 µM.).

(b) HNSCC-CSCs were treated with 3.5 µM of SF for the indicated times ("a" significance relative to 0h, "b" significance relative to 24h, "c" significance relative to 48h. P < 0.05). HNSCC-CSCs were treated with 3.5 µM of SF with or without 0.1, 0.5, 1, 2 µg/ml of CIS (c) or 0.013, 0.13, 1.3, 130 µg/ml of 5-FU (d) for 72 h. Data are presented as mean ± SD for N=3 (* P < 0.05 and ** P < 0.01 relative to treatment in the absence of SF). (e) HNSCC-CSCs were pre-treated with SF with or without CIS or 5-FU for 72 h before being seeded in 6-well plates for 10 days. Fixed and stained colonies containing > 50 cells were counted under an inverted light microscope. Data are presented as mean ± SD for N=3 (** P < 0.01). Photographs of the fixed and stained colonies are presented on the (f) panel.

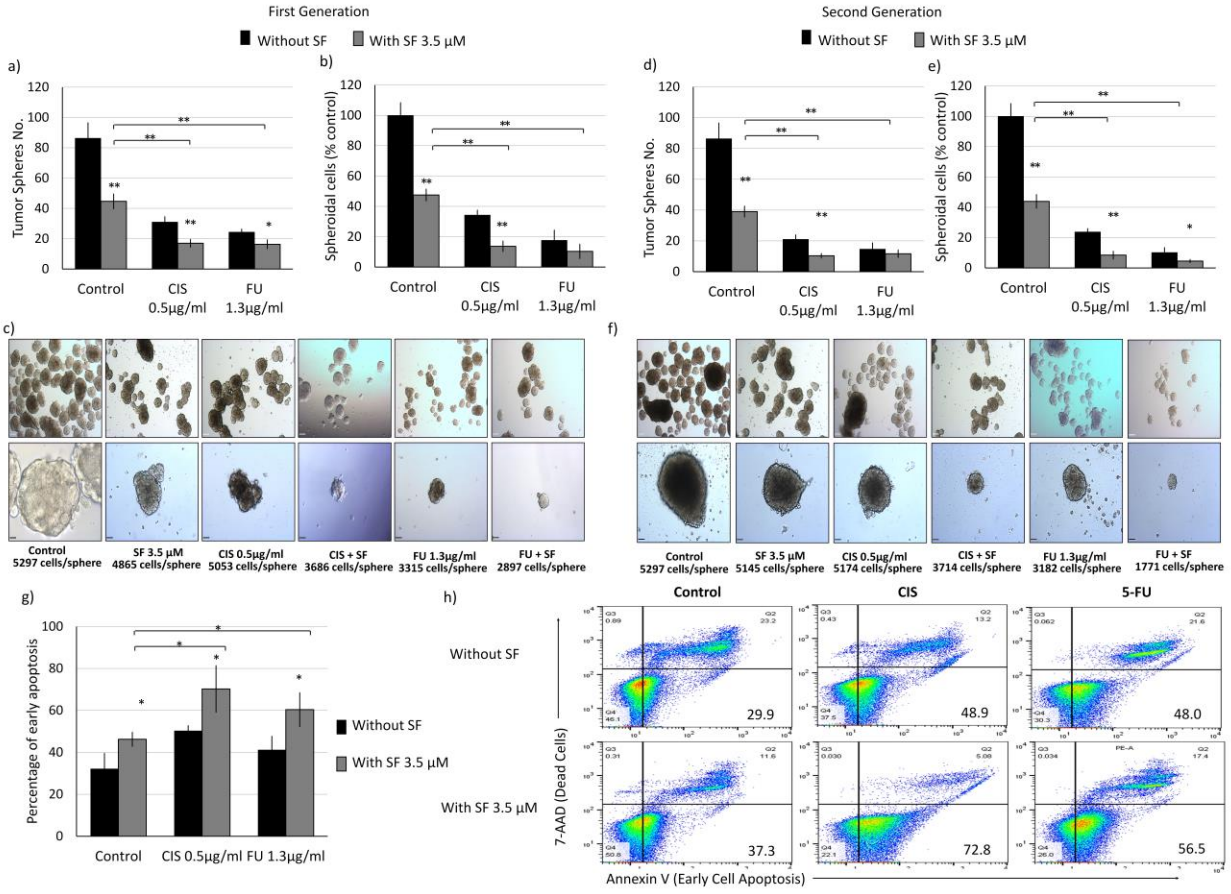

Fig 7.2 Effect of sulforaphane on self-renewal ability and apoptosis induction in HNSCC-CSCs. HNSCC-CSCs were seeded at clonal density (5000 cells/500µl per well) in ultra-low attachment plates for spheroid formation. Twenty-four hours later cells were treated with SF and/or CIS, or 5-FU and allowed to form spheres for 14 days. The spheres were counted (a), dissociated into single cells that were counted in the presences of trypan blue stain (b). (c) Representing photos taken under a phase-contrast microscope with 5X (the upper panel) and 40X (the lower panel) magnifications, a scale bar = 90 µM and 10 µM,

respectively, for the 1st generation. (d-f) Spheroids were dissociated to single cells and equal numbers of live cells were re-plated, and 14 days later, spheroids formation was quantified, photographed and dissociated to count the cells, this represents the 2nd generation. Data are presented as mean ± SD for N=3 (* P < 0.05, ** P < 0.01 relative to treatments in the absence of SF). (h) The percentage of early apoptotic cells is presented as mean ± SD for N=3 (* P < 0.05). (g) Flow cytometry graphs show the gating strategy as the vertical line represent the cutline for Annexin V staining and the horizontal line represents the cutline for 7-AAD staining.

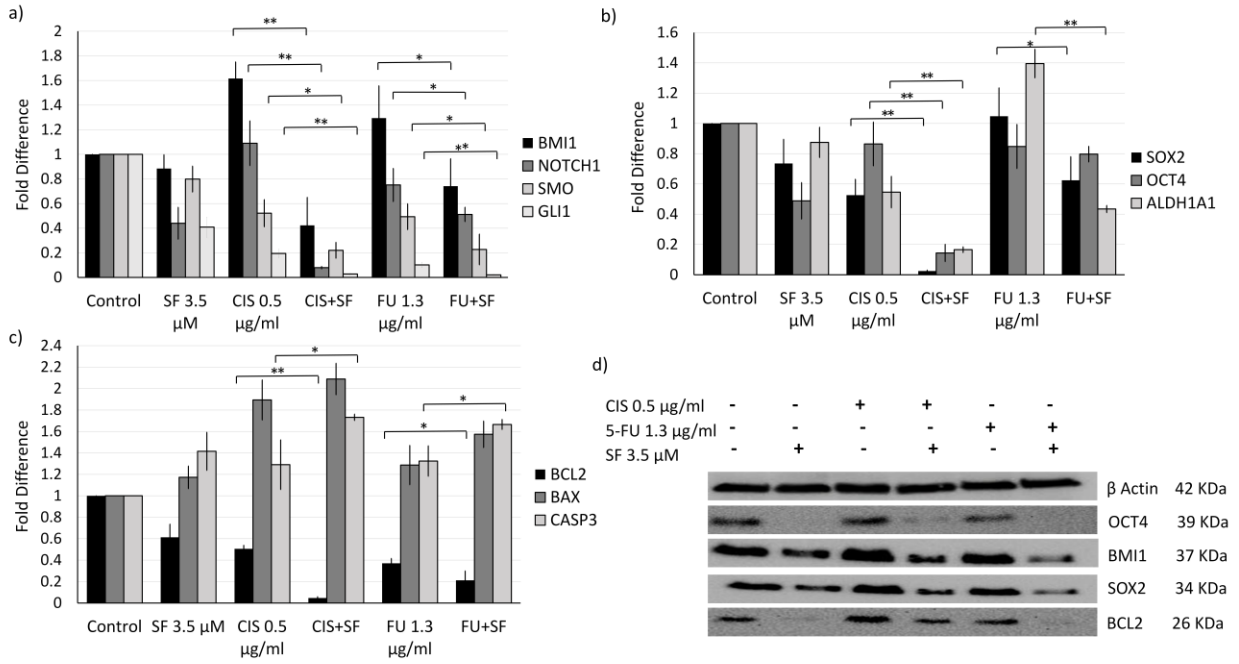

Fig 7.3 Effect of sulforaphane on the genotype of HNSCC-CSCs. HNSCC-CSCs were treated with 3.5 µM of SF with or without 0.5 µg/ml of CIS or 1.3 µg/ml of 5-FU for 72 h. The expression of (a) self-renewal related genes; BMI1, SMO, GLI1, and NOTCH1, (b) stemness and drug-resistance related genes; SOX2, OCT4, and ALDH1A1 and (c) apoptosis related genes; BAX, BCL2, and CASP3 was measured by qRT-PCR and normalized to GAPDH expression. Data represent mean ± SD for N=3 (* P < 0.05 and ** P < 0.01 relative to treatments in the absence of SF). (d) Proteins were harvested from the treated cells and expression of OCT4, BMI1, SOX2 and BCL2 proteins were analyzed by western blot. Expression of β-actin served as the loading control.

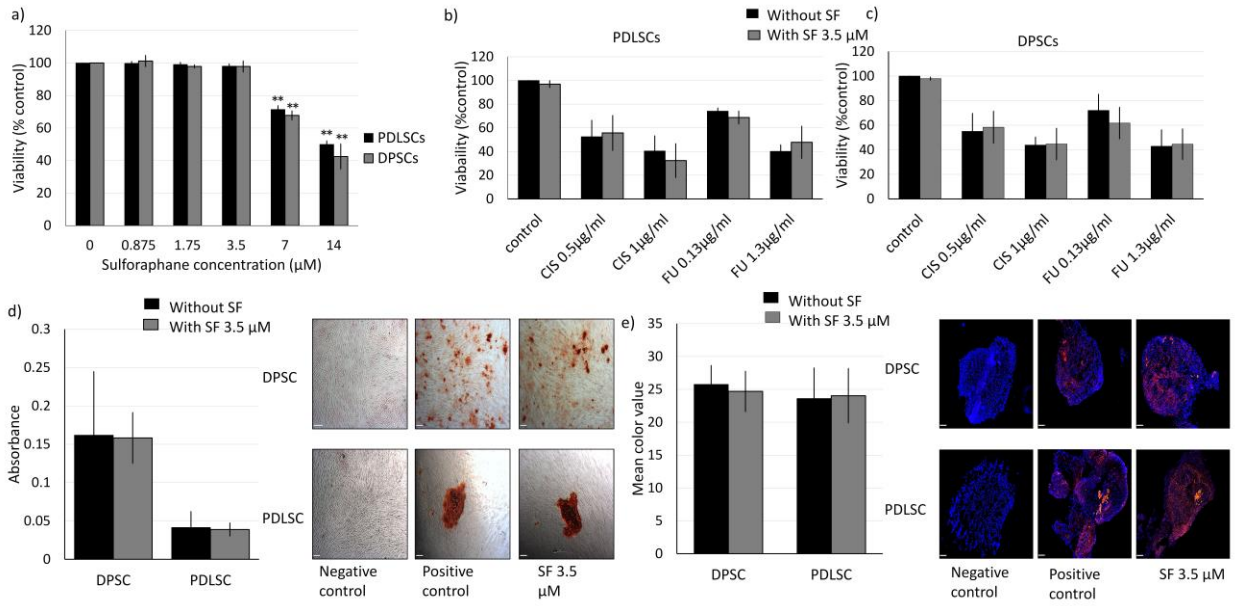

Fig 7.4 Effect of sulforaphane on non-cancerous stem cells. (a) Periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) were treated with 0, 0.875, 1.75, 3.5, 7 and 14 μM of SF for 72 h. Data are presented as mean ± SD for N=3 (** $P < 0.01$ compared control). (b) PDLSCs and (c) DPSCs were treated with 3.5 μM of SF in the presence or absence of 0.5 and 1 μg/ml of CIS or 0.13 and 1.3μg/ml of 5-FU for 72 h, respectively. Data are presented as mean ± SD. (d) PDLSCs and DPSCs were treated with 3.5 μM SF for 72 hours, induced to undergo osteogenic differentiation, then stained with Alizarin Red stain for identification and quantification. Photos were taken under a phase-contrast microscope with 5X magnification, scale bar = 90 μM. (e) PDLSCs and DPSCs were treated with 3.5 μM SF for 72 hours, induced to undergo chondrogenic differentiation, then cryosectioned and stained with Collagen Type II immunofluorescence staining, for identification and quantification. Photos were taken under a phase-contrast microscope with 20X magnification, scale bar = 47 μM.

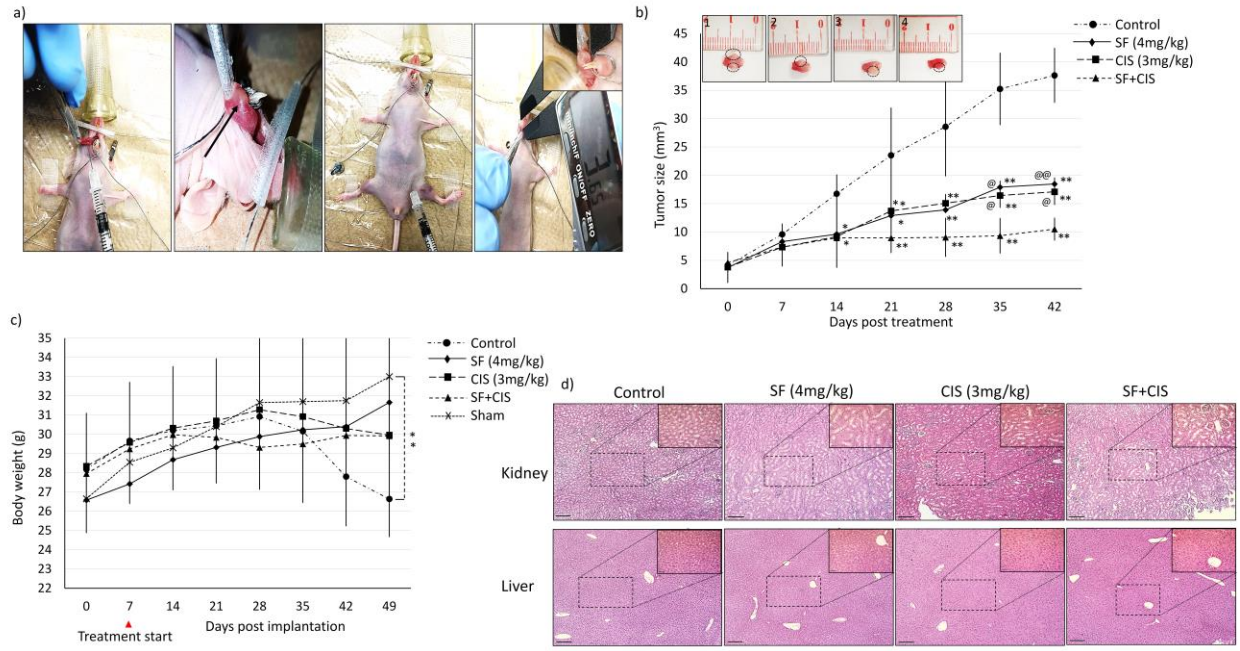

Fig 7.5 Effect of SF+CIS combination treatment in-vivo. (a) Intra-oral tongue xenografts of HNSCC-CSCs in nude immunocompromised mice were treated with IP injection of vehicle (normal saline), SF, CIS, or SF plus CIS (n=5/group), the tumor size and the bodyweight were monitored weekly. Black arrow indicates tumor formation after one week of tumor implantation. (b) Tumor volumes and (c) mice body weights were determined as described in "Materials and Methods" section. Data represent mean ± SD for N=5 (* $P < 0.05$ and ** $P < 0.01$ compared with control, @ $P < 0.05$ and @ $P < 0.01$ compared with combined treatment). (d) A representative H&E staining of kidney (upper row) and liver (lower row) tissues after treatments with SF and/or CIS or vehicle is shown with 5X magnification in the main photos and 20X magnification in the inserts; scale bar = 130 µm & 34 µm for the main photo and the insert, respectively).

Appendix:

Fluorescence-activated cell sorting (FACS)

Tumor cells were harvested using Accutase™ Cell Detachment Solution (BD Bioscience) and resuspended as a single-cell suspension in staining buffer (1% FBS in ice-cold PBS) with a final concentration of $1x10^6$ cells/100 µl. Cells were then incubated with the Fixable Viability Stain 450 (BD Bioscience) for 15 min on ice, protected from light and washed twice. Cells were blocked by

incubation with Human TruStain FcX™ (Biolegend) for 10 min, then washed once. Cells were incubated with the antibodies for CD44 and CD271 at a dilution of 1:20 for 30 min on ice, protected from light, then washed twice.

Real-Time qRT-PCR

The first-strand cDNA was synthesized from 1 µg total RNA using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher). For the quantification of gene amplification, qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (Thermo Fisher) in the presence of PowerUp SYBR Green Master Mix (Thermo Fisher). The following gene-specific primers were used:

GAPDH: (5'-GAGAAGGCTGGGGCTCATTT-3', 5'-AGTGATGGCATGGACTGTGG-3'), BMI-1: (5'-TCCTTAACAGTCTCAGGTATCAACC-3', 5'-CACAGTTTCCTCACATTTCCA-3'), SMO: (5'-TGGTCACTCCCCTTTGTCCTCAC-3', 5'-GCACGGTATCGGTAGTTCTTGTAGC-3'), GLI1: (5'-TTGGAGAAGCCGAGCCGAGTATC-3', 5'-GAGTAGACAGAGGTTGGGAGGTAAGG-3'), NOTCH1: (5'-GCAGAGGCGTGGCAGACTAT-3', 5'-ACTTGTACTCCGTCAGCGTG-3'), SOX2: (5'-ACACCAATCCCATCCACACT-3', 5'-CAAACTTCCTGCAAAGCTCC-3') OCT4: (5'-CTCGAGAAGGATGTGGTCCG-3', 5'-GAAGTGAGGGCTCCCATAGC-3'), ALDH1A1: (5'-ATCAAAGAAGCTGCCGGGAA-3', 5'- GCATTGTCCAAGTCGGCATC-3'), BCL2: (5'-CTGCACCTGACGCCCTTCACC-3', 5'-CACATGACCCCACCGAACTCAAAGA-3'), BAX: (5'-CGGGTTGTCGCCCTTTTCTA-3', 5'-TGGTTCTGATCAGTTCCGGC-3') and Caspase3: (5'-CTCGGTCTGGTACAGATGTCGA-3', 5'-CATGGCTCAGAAGCACACAAAC-3').

Chapter 8 - General discussion, conclusion and Future Directions:

8.1 Discussion and conclusion:

Cancer is the second leading cause of death worldwide just after cardiovascular diseases. While in Canada it is the leading cause of death as one in two Canadian will develop cancer in their life time and one in four will die from the disease. Head and neck cancer is one of the most prevalent malignant tumors across the world especially in developing countries such as India and Sudan. Despite the advanced improvement in diagnosis and treatment modalities for HNC, it still has an unacceptable high rate of mortality. One of the reasons behind HNC treatment failure is that the efficacy of the conventional chemotherapy is restricted because of its high toxicity to the non-cancerous tissues, making it very difficult to increase the used dosages for better results without adding more risk to the patient's health. The chemotherapy dose dependent side effects range from mild as mouth sores, loss of appetite, and thrombocytopenia, to the more sever cardiotoxicity, nephrotoxicity, and neurotoxicity with neurological disorders.

Recently, phytochemicals, biologically active compounds found in plants, have shown very promising results to function as anti-cancerous agents. Sulforaphane, the most characterized isothiocyanate compound found in cruciferous vegetables, has been reported to have an antioxidant, anti-inflammatory with chemopreventive and therapeutic effect against several cancer types either alone or as a combination with other drugs. However, its effect as a combined treatment with Cisplatin or 5-Fluorouracil on HNC still remains unknown.

In Chapter 5 of this thesis, we demonstrated that SF has a dose and time dependent inhibitory effect on HNSCC cells and this effect was associated with stimulation of apoptosis,

specifically the Caspase-dependent apoptotic pathway. Moreover, SF enhanced the cytotoxic effect of CIS by 2-fold and 5-FU by 10-fold when used as a combination treatment. Sulforaphane combination with CIS or 5-FU also increased the inhibition of colony formation and DNA repair compared to single treatment. Most importantly, that cytotoxic effect on the HNSCC cells was not accompanied by any toxicity to non-cancerous cells either SF as a solo treatment or as a combination with chemotherapy.

Another reason for HNC treatment failure is the recurrence or metastasis after treatment completion. Recently, recurrence and metastasis have been correlated to the presence of the more treatment-resistant cancer stem cells. These cells possess two main criteria; self-renewal ability with unlimited proliferation while maintaining undifferentiated state, and multilineage differentiation so it can give rise to the other types of cells that form the tumor heterogenicity. The first step to develop strategies or drugs that target CSCs is to correctly identify and isolate them. In Chapter 6 of this study, we reported that CD271+ cells are a subpopulation of CD44+ cells in HNSCC. This was evident in laryngeal and oropharyngeal squamous cell carcinoma cell lines and human oral squamous cell carcinoma tissues. We also demonstrated that using CD44/CD271 double immunostaining is a more precise method to isolate CSCs from HNSCC compared to the widely used CD44 alone. CD44+/CD271+ cells have more tumorigenic ability both in-vitro and in-vivo compared to CD44+/CD271- cells and might be responsible for the reported tumorigenicity of CD44+ cells in previous studies. CD271+ cells expressed more cancer stem cells related gene expression such as, BMI1, SMO, GLI1, SOX2 and OCT4 compared to CD271-.

Now since we know that SF can inhibit HNSCC and we have a suitable method to isolate CSCs from the cell lines we need to assess the effect of SF alone or combined with chemotherapy on the HNC-CSCs. Chapter 7 of this study demonstrated that SF has an inhibitory effect on HNC-CSCs in dose- and time-dependent manner. SF combination with the conventional chemotherapy elevated the cytotoxic effect of Cisplatin and 5-Fluorouracil on CSCs significantly and inhibited colony and sphere formation. We also reported different mechanisms for this effect as SF reduced the expression of stemness-related genes SOX2 and OCT4, inhibited self-renewal related gene BMI1 through inhibition of SHH pathway, and stimulated Caspase-dependent apoptotic pathway by inhibition of BCL2 gene expression. SF also prevented selection of cancer stem cells or elevation of stem cells related gene ALDH1A1 that was associated with CIS and 5-FU. Interestingly, SF either alone or combined with chemotherapy had no effect on the viability or function of non-cancerous human stem cells. Sulforaphane combination with Cisplatin significantly reduced the size of the xenograft tumors in the tongues of immunocompromised mice compared to Cisplatin or Sulforaphane alone. The combination treatment was tolerable with the mice with no significant loss of the body weight and no necrosis in kidney or liver.

This thesis preliminarily investigated the effect of SF combination with conventional chemotherapy to target HNSCC-bulk cells and CSCs and suggested the mechanism behind it. It provides the experimental basis for the future clinical application of this natural product in cancer treatment. By increasing the efficacy of the conventional chemotherapy and targeting CSCs we can reduce the conventional doses which will decrease the dose-dependent side effects and reduce the incidence of recurrence or metastasis which will improve the outcome of HNC and the life quality of the treated patients.

8.2 Future plan:

Investigating the underlying mechanisms of SF anti-cancerous apoptosis induction effect on HNSCC. SF cytotoxicity has been reported to be caused by complex, concurring, and multiple mechanisms as we mentioned in the third chapter. Among these mechanisms, the generation of ROS is important in promoting apoptosis and autophagy of target cells [607,608]. We will assess if the main drive behind the anti-cancer effect on HNC is the ROS by measuring of intracellular ROS using flow cytometry following staining with hydroethidine (HE) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), which have been shown to be somewhat specific for detection of O_2^- and H2O2, respectively [608,609]. We will then neutralize the ROS formation either by pretreatment with NAC, diphenyleneiodonium chloride and rotenone or after SF treatment using Sodium Azide, which is a scavenger of ROS. We will repeat our experiments to detect if ROS depletion will rescue the cells from the SF induced apoptosis.

Since SF has been reported to induce ROS in cancer cells, we will investigate the effect of SF on radiotherapy induced cytotoxicity. Our preliminary results (not published) showed that SF combination with radiotherapy enhanced the cytotoxic effect and prevented colony formation post-treatment significantly compared to using RT alone. We will investigate the mechanism behind these results and will assess the safety of this combined treatment modality on non-cancerous human cells and stem cells, specifically the effect on the gene and protein level.

Chapter 9 - Bibliography

1. Ferlay J, Soerjomataram I, Dikshit R*, et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer* 2015; **136**: E359-386.
2. Machiels JP, Lambrecht M, Hanin FX*, et al.* Advances in the management of squamous cell carcinoma of the head and neck. *F1000prime reports* 2014; **6**: 44.
3. Bentzen SM, Trotti A. Evaluation of Early and Late Toxicities in Chemoradiation Trials. *Journal of Clinical Oncology* 2007; **25**: 4096-4103.
4. Günen Yılmaz S, Bayrakdar İŞ, Bayrak S*, et al.* Late Side Effects of Chemotherapy and Radiotherapy in Early Childhood on the Teeth: Two Case ReportsErken Çocukluk Döneminde Alınan Radyoterapi ve Kemoterapinin Dişler Üzerine Geç Dönem Etkileri: İki Olgu Sunumu. *Turkish journal of haematology : official journal of Turkish Society of Haematology* 2018; **35**: 87-88.
5. O'Neill CB, Baxi SS, Atoria CL*, et al.* Treatment-related toxicities in older adults with head and neck cancer: A population-based analysis. *Cancer* 2015; **121**: 2083-2089.
6. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003; **22**: 7265-7279.
7. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney international* 2008; **73**: 994-1007.
8. Park SB, Goldstein D, Krishnan AV*, et al.* Chemotherapy-induced peripheral neurotoxicity: a critical analysis. *CA: a cancer journal for clinicians* 2013; **63**: 419-437.
9. de Jongh FE, van Veen RN, Veltman SJ*, et al.* Weekly high-dose cisplatin is a feasible treatment option: analysis on prognostic factors for toxicity in 400 patients. *British journal of cancer* 2003; **88**: 1199-1206.
10. Sonia Amin Thomas ZG, Sharvil Mehta, Kushal Patel. . Adverse effects of 5-fluorouracil: Focus on rare side effects. . *Cancer Cell & Microenvironment* 2016; **3**.
11. Sonia Amin Thomas ZG, Sharvil Mehta, Kushal Patel. Adverse effects of 5-fluorouracil: Focus on rare side effects. *Cancer Cell & Microenvironment* 2016; **3**.
12. McQuestion M, Fitch MI. Patients' experience of receiving radiation treatment for head and neck cancer: Before, during and after treatment. *Canadian Oncology Nursing Journal / Revue canadienne de soins infirmiers en oncologie* 2016; **26**: 11.
13. Furness S, Glenny AM, Worthington HV*, et al.* Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. *The Cochrane database of systematic reviews* 2010: Cd006386.
14. Finlan LE, Hupp TR. Epidermal stem cells and cancer stem cells: insights into cancer and potential therapeutic strategies. *European journal of cancer (Oxford, England : 1990)* 2006; **42**: 1283-1292.
15. Reya T, Morrison SJ, Clarke MF*, et al.* Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105.
16. Schulenburg A, Ulrich-Pur H, Thurnher D*, et al.* Neoplastic stem cells: a novel therapeutic target in clinical oncology. *Cancer* 2006; **107**: 2512-2520.
17. Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annual review of medicine* 2007; **58**: 267-284.
18. Al-Hajj M. Cancer stem cells and oncology therapeutics. *Current opinion in oncology* 2007; **19**: 61-64.
19. Diehn M, Cho RW, Lobo NA*, et al.* Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* 2009; **458**: 780-783.

20. Nakamura T, Endo K, Kinoshita S. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. *Stem cells (Dayton, Ohio)* 2007; **25**: 628-638.
21. Thompson SJ, Schatteman GC, Gown AM*, et al.* A monoclonal antibody against nerve growth factor receptor. Immunohistochemical analysis of normal and neoplastic human tissue. *American journal of clinical pathology* 1989; **92**: 415-423.
22. Boiko AD, Razorenova OV, van de Rijn M*, et al.* Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. *Nature* 2010; **466**: 133-137.
23. Civenni G, Walter A, Kobert N*, et al.* Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. *Cancer research* 2011; **71**: 3098-3109.
24. Huang SD, Yuan Y, Liu XH*, et al.* Self-renewal and chemotherapy resistance of p75NTR positive cells in esophageal squamous cell carcinomas. *BMC cancer* 2009; **9**: 9.
25. Okumura T, Shimada Y, Imamura M*, et al.* Neurotrophin receptor p75(NTR) characterizes human esophageal keratinocyte stem cells in vitro. *Oncogene* 2003; **22**: 4017-4026.
26. Imai T, Tamai K, Oizumi S*, et al.* CD271 defines a stem cell-like population in hypopharyngeal cancer. *PloS one* 2013; **8**: e62002.
27. Kiyosue T, Kawano S, Matsubara R*, et al.* Immunohistochemical location of the p75 neurotrophin receptor (p75NTR) in oral leukoplakia and oral squamous cell carcinoma. *International journal of clinical oncology* 2013; **18**: 154-163.
28. Xiao Z, Morris-Natschke SL, Lee KH. Strategies for the Optimization of Natural Leads to Anticancer Drugs or Drug Candidates. *Medicinal research reviews* 2016; **36**: 32-91.
29. Lenzi M, Fimognari C, Hrelia P. Sulforaphane as a promising molecule for fighting cancer. *Cancer treatment and research* 2014; **159**: 207-223.
30. Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cellular and molecular life sciences : CMLS* 2007; **64**: 1105-1127.
31. Singh SV, Srivastava SK, Choi S*, et al.* Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. *The Journal of biological chemistry* 2005; **280**: 19911-19924.
32. Shen G, Khor TO, Hu R*, et al.* Chemoprevention of familial adenomatous polyposis by natural dietary compounds sulforaphane and dibenzoylmethane alone and in combination in ApcMin/+ mouse. *Cancer research* 2007; **67**: 9937-9944.
33. Mi L, Wang X, Govind S*, et al.* The role of protein binding in induction of apoptosis by phenethyl isothiocyanate and sulforaphane in human non-small lung cancer cells. *Cancer research* 2007; **67**: 6409-6416.
34. Herman-Antosiewicz A, Xiao H, Lew KL*, et al.* Induction of p21 protein protects against sulforaphane-induced mitotic arrest in LNCaP human prostate cancer cell line. *Molecular cancer therapeutics* 2007; **6**: 1673-1681.
35. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by sulforaphane, a cruciferous vegetable-derived cancer chemopreventive agent. *Cancer research* 2005; **65**: 2035-2043.
36. Kallifatidis G, Labsch S, Rausch V*, et al.* Sulforaphane increases drug-mediated cytotoxicity toward cancer stem-like cells of pancreas and prostate. *Molecular therapy : the journal of the American Society of Gene Therapy* 2011; **19**: 188-195.
37. Lin L-C, Yeh C-T, Kuo C-C*, et al.* Sulforaphane Potentiates the Efficacy of Imatinib against Chronic Leukemia Cancer Stem Cells through Enhanced Abrogation of Wnt/β-Catenin Function. *Journal of Agricultural and Food Chemistry* 2012; **60**: 7031-7039.

38. Rodova M, Fu J, Watkins DN*, et al.* Sonic hedgehog signaling inhibition provides opportunities for targeted therapy by sulforaphane in regulating pancreatic cancer stem cell self-renewal. *PloS one* 2012; **7**: e46083.
39. Liu CM, Peng CY, Liao YW*, et al.* Sulforaphane targets cancer stemness and tumor initiating properties in oral squamous cell carcinomas via miR-200c induction. *Journal of the Formosan Medical Association = Taiwan yi zhi* 2017; **116**: 41-48.
40. Jeffery EH, Keck AS. Translating knowledge generated by epidemiological and in vitro studies into dietary cancer prevention. *Molecular nutrition & food research* 2008; **52 Suppl 1**: S7-17.
41. Daraei P, Moore CE. Racial Disparity Among the Head and Neck Cancer Population. *Journal of cancer education : the official journal of the American Association for Cancer Education* 2015; **30**: 546-551.
42. Jou A, Hess J. Epidemiology and Molecular Biology of Head and Neck Cancer. *Oncology research and treatment* 2017; **40**: 328-332.
43. Chaturvedi AK, Anderson WF, Lortet-Tieulent J*, et al.* Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013; **31**: 4550-4559.
44. Sankaranarayanan R. Oral cancer in India: an epidemiologic and clinical review. *Oral surgery, oral medicine, and oral pathology* 1990; **69**: 325-330.
45. Marur S, Forastiere AA. Head and neck cancer: changing epidemiology, diagnosis, and treatment. *Mayo Clinic proceedings* 2008; **83**: 489-501.
46. Tobias JS. Cancer of the head and neck. *BMJ (Clinical research ed)* 1994; **308**: 961-966.
47. Vigneswaran N, Williams MD. Epidemiologic trends in head and neck cancer and aids in diagnosis. *Oral and maxillofacial surgery clinics of North America* 2014; **26**: 123-141.
48. Shah SB, Sharma S, D'Cruz AK. Head and neck oncology: The Indian scenario. *South Asian journal of cancer* 2016; **5**: 104-105.
49. Ragin CC, Langevin SM, Marzouk M*, et al.* Determinants of head and neck cancer survival by race. *Head & neck* 2011; **33**: 1092-1098.
50. Dilling TJ, Bae K, Paulus R*, et al.* Impact of gender, partner status, and race on locoregional failure and overall survival in head and neck cancer patients in three radiation therapy oncology group trials. *International journal of radiation oncology, biology, physics* 2011; **81**: e101-e109.
51. Chaturvedi AK, Engels EA, Pfeiffer RM*, et al.* Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011; **29**: 4294-4301.
52. Fan Y, Zheng L, Mao MH*, et al.* Survival analysis of oral squamous cell carcinoma in a subgroup of young patients. *Asian Pacific journal of cancer prevention : APJCP* 2014; **15**: 8887-8891.
53. Al-Amad SH, Awad MA, Nimri O. Oral cancer in young Jordanians: potential association with frequency of narghile smoking. *Oral surgery, oral medicine, oral pathology and oral radiology* 2014; **118**: 560-565.
54. Gawecki W, Kostrzewska-Poczekaj M, Gajecka M*, et al.* The role of genetic factor in etiopathogenesis of squamous cell carcinoma of the head and neck in young adults. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery* 2007; **264**: 1459-1465.
55. Simard EP, Torre LA, Jemal A. International trends in head and neck cancer incidence rates: differences by country, sex and anatomic site. *Oral oncology* 2014; **50**: 387-403.
56. Thun M, Peto R, Boreham J*, et al.* Stages of the cigarette epidemic on entering its second century. *Tobacco control* 2012; **21**: 96-101.

57. Muscat JE, Richie JP, Jr., Thompson S*, et al.* Gender differences in smoking and risk for oral cancer. *Cancer research* 1996; **56**: 5192-5197.
58. Human papillomavirus-associated cancers - United States, 2004-2008. *MMWR Morbidity and mortality weekly report* 2012; **61**: 258-261.
59. Canadian-Cancer-Statistics-Advisory-Committee. Canadian Cancer Statistics 2018. Toronto, ON: Canadian Cancer Society; 2018. Available at: cancer.ca/Canadian-Cancer-Statistics-2018-EN (accessed 21-11-2018). Available from: http://www.cancer.ca/en/cancer-information/cancer-101/canadian-cancer-statistics-publication/?region=on#ixzz5XXikeMEh
60. Rettig EM, D'Souza G. Epidemiology of head and neck cancer. *Surgical oncology clinics of North America* 2015; **24**: 379-396.
61. Hashibe M, Brennan P, Benhamou S*, et al.* Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Journal of the National Cancer Institute* 2007; **99**: 777-789.
62. Vineis P, Alavanja M, Buffler P*, et al.* Tobacco and cancer: recent epidemiological evidence. *Journal of the National Cancer Institute* 2004; **96**: 99-106.
63. Schlecht NF, Franco EL, Pintos J*, et al.* Effect of smoking cessation and tobacco type on the risk of cancers of the upper aero-digestive tract in Brazil. *Epidemiology (Cambridge, Mass)* 1999; **10**: 412-418.
64. Secretan B, Straif K, Baan R*, et al.* A review of human carcinogens--Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish. *The Lancet Oncology* 2009; **10**: 1033-1034.
65. Boffetta P, Hecht S, Gray N*, et al.* Smokeless tobacco and cancer. *The Lancet Oncology* 2008; **9**: 667-675.
66. Abdul-Hamid G, Saeed NM, Al-Kahiry W*, et al.* Pattern of head and neck cancer in Yemen. *The Gulf journal of oncology* 2010: 21-24.
67. Anantharaman D, Marron M, Lagiou P*, et al.* Population attributable risk of tobacco and alcohol for upper aerodigestive tract cancer. *Oral oncology* 2011; **47**: 725-731.
68. Menvielle G, Luce D, Goldberg P*, et al.* Smoking, alcohol drinking and cancer risk for various sites of the larynx and hypopharynx. A case-control study in France. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP)* 2004; **13**: 165-172.
69. Hashibe M, Brennan P, Chuang SC*, et al.* Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2009; **18**: 541-550.
70. Blot WJ, McLaughlin JK, Winn DM*, et al.* Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer research* 1988; **48**: 3282-3287.
71. Khalesi S. A Review of Head and Neck Squamous Cell Carcinoma Risk Factors with More Focus on Oral Cancer. *J Dent & Oral Disord* 2016; **2**: 1032.
72. van Leeuwen MT, Grulich AE, McDonald SP*, et al.* Immunosuppression and other risk factors for lip cancer after kidney transplantation. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2009; **18**: 561-569.
73. Swann JB, Smyth MJ. Immune surveillance of tumors. *The Journal of clinical investigation* 2007; **117**: 1137-1146.

74. Piselli P, Serraino D, Segoloni GP*, et al.* Risk of de novo cancers after transplantation: results from a cohort of 7217 kidney transplant recipients, Italy 1997-2009. *European journal of cancer (Oxford, England : 1990)* 2013; **49**: 336-344.
75. Clifford GM, Polesel J, Rickenbach M*, et al.* Cancer risk in the Swiss HIV Cohort Study: associations with immunodeficiency, smoking, and highly active antiretroviral therapy. *Journal of the National Cancer Institute* 2005; **97**: 425-432.
76. Chang Y, Moore PS, Weiss RA. Human oncogenic viruses: nature and discovery. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 2017; **372**.
77. Kreimer AR, Clifford GM, Boyle P*, et al.* Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2005; **14**: 467-475.
78. Jelihovschi I, Bidescu AC, Tucaliuc SE*, et al.* DETECTION OF HUMAN PAPILLOMA VIRUS IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS: A LITERATURE REVIEW. *Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi* 2015; **119**: 502-509.
79. Acharya S, Ekalaksananan T, Vatanasapt P*, et al.* Association of Epstein-Barr virus infection with oral squamous cell carcinoma in a case-control study. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2015; **44**: 252-257.
80. Fakhry C, Lacchetti C, Rooper LM*, et al.* Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2018; **36**: 3152-3161.
81. Lewis JS, Jr., Beadle B, Bishop JA*, et al.* Human Papillomavirus Testing in Head and Neck Carcinomas: Guideline From the College of American Pathologists. *Archives of pathology & laboratory medicine* 2018; **142**: 559-597.
82. Maeda E, Akahane M, Kiryu S*, et al.* Spectrum of Epstein-Barr virus-related diseases: a pictorial review. *Japanese journal of radiology* 2009; **27**: 4-19.
83. Horiuchi K, Mishima K, Ichijima K*, et al.* Epstein-Barr virus in the proliferative diseases of squamous epithelium in the oral cavity. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* 1995; **79**: 57-63.
84. Gonzalez-Moles M, Gutierrez J, Ruiz I*, et al.* Epstein-Barr virus and oral squamous cell carcinoma in patients without HIV infection: viral detection by polymerase chain reaction. *Microbios* 1998; **96**: 23-31.
85. van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral oncology* 2009; **45**: 317-323.
86. Gonzalez-Moles MA, Scully C, Gil-Montoya JA. Oral lichen planus: controversies surrounding malignant transformation. *Oral diseases* 2008; **14**: 229-243.
87. Gonzalez-Moles MA, Gil-Montoya JA, Ruiz-Avila I*, et al.* Is oral cancer incidence among patients with oral lichen planus/oral lichenoid lesions underestimated? *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2017; **46**: 148-153.
88. Kaplan I, Ventura-Sharabi Y, Gal G*, et al.* The dynamics of oral lichen planus: a retrospective clinicopathological study. *Head and neck pathology* 2012; **6**: 178-183.
89. Barnard NA, Scully C, Eveson JW*, et al.* Oral cancer development in patients with oral lichen planus. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 1993; **22**: 421-424.

90. Silverman S, Jr., Gorsky M, Lozada-Nur F*, et al.* A prospective study of findings and management in 214 patients with oral lichen planus. *Oral surgery, oral medicine, and oral pathology* 1991; **72**: 665-670.
91. Bouquot JE, Weiland LH, Kurland LT. Leukoplakia and carcinoma in situ synchronously associated with invasive oral/oropharyngeal carcinoma in Rochester, Minn., 1935-1984. *Oral surgery, oral medicine, and oral pathology* 1988; **65**: 199-207.
92. Weijers M, Ten Hove I, Allard RH*, et al.* Patients with oral cancer developing from pre-existing oral leukoplakia: do they do better than those with de novo oral cancer? *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2008; **37**: 134-136.
93. Brunotto M, Zarate AM, Bono A*, et al.* Risk genes in head and neck cancer: a systematic review and meta-analysis of last 5 years. *Oral oncology* 2014; **50**: 178-188.
94. Todd R, Wong DT. Oncogenes. *Anticancer research* 1999; **19**: 4729-4746.
95. Saranath D, Chang SE, Bhoite LT*, et al.* High frequency mutation in codons 12 and 61 of H-ras oncogene in chewing tobacco-related human oral carcinoma in India. *British journal of cancer* 1991; **63**: 573-578.
96. Baltaci E, Ekizoglu S, Sari E*, et al.* CT120A Acts as an Oncogene in Head and Neck Squamous Cell Carcinoma. *Journal of Cancer* 2015; **6**: 1255-1259.
97. Mader C. The Biology of Cancer. *The Yale Journal of Biology and Medicine* 2007; **80**: 91-91.
98. Shin DM, Kim J, Ro JY*, et al.* Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis. *Cancer research* 1994; **54**: 321-326.
99. Poeta ML, Manola J, Goldwasser MA*, et al.* TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *The New England journal of medicine* 2007; **357**: 2552-2561.
100. Gonzalez MV, Pello MF, Lopez-Larrea C*, et al.* Loss of heterozygosity and mutation analysis of the p16 (9p21) and p53 (17p13) genes in squamous cell carcinoma of the head and neck. *Clinical cancer research : an official journal of the American Association for Cancer Research* 1995; **1**: 1043-1049.
101. Valko M, Leibfritz D, Moncol J*, et al.* Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology* 2007; **39**: 44-84.
102. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral oncology* 2001; **37**: 477-492.
103. Thomas SJ, MacLennan R. Slaked lime and betel nut cancer in Papua New Guinea. *Lancet (London, England)* 1992; **340**: 577-578.
104. Bouayed J, Bohn T. Exogenous antioxidants--Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxidative medicine and cellular longevity* 2010; **3**: 228-237.
105. Singh AK, Pandey P, Tewari M*, et al.* Free radicals hasten head and neck cancer risk: A study of total oxidant, total antioxidant, DNA damage, and histological grade. *Journal of postgraduate medicine* 2016; **62**: 96-101.
106. Adel K. El-Naggar JKCC, Jennifer R. Grandis, Takashi Takata, Pieter J. Slootweg. WHO Classification of Head and Neck Tumours. ed). International Agency for Research on Cancer, 2017.
107. Thompson LDR, Bishop JA. Head and neck pathology. In. Third edition. ed. (ed)^(eds). Elsevier: Philadelphia, PA, 2019.
108. Gourin CG, Boyce BJ, Vaught CC*, et al.* Effect of comorbidity on post-treatment quality of life scores in patients with head and neck squamous cell carcinoma. *The Laryngoscope* 2009; **119**: 907-914.

109. Argiris A, Karamouzis MV, Raben D*, et al.* Head and neck cancer. *Lancet (London, England)* 2008; **371**: 1695-1709.
110. Bonner JA, Harari PM, Giralt J*, et al.* Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *The Lancet Oncology* 2010; **11**: 21-28.
111. Dorsey K, Agulnik M. Promising new molecular targeted therapies in head and neck cancer. *Drugs* 2013; **73**: 315-325.
112. Wheless SA, McKinney KA, Zanation AM. A prospective study of the clinical impact of a multidisciplinary head and neck tumor board. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2010; **143**: 650-654.
113. Corry J, Peters LJ, Rischin D. Impact of center size and experience on outcomes in head and neck cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2015; **33**: 138-140.
114. Boero IJ, Paravati AJ, Xu B*, et al.* Importance of Radiation Oncologist Experience Among Patients With Head-and-Neck Cancer Treated With Intensity-Modulated Radiation Therapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2016; **34**: 684-690.
115. Wuthrick EJ, Zhang Q, Machtay M*, et al.* Institutional clinical trial accrual volume and survival of patients with head and neck cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2015; **33**: 156-164.
116. Colledge L. Treatment of Cancer of Pharynx and Larynx. *Br Med J* 1938; **2**: 167-168.
117. Gibson MK, Forastiere AA. Multidisciplinary approaches in the management of advanced head and neck tumors: state of the art. *Current opinion in oncology* 2004; **16**: 220-224.
118. Liu JC, Shah JP. Surgical technique refinements in head and neck oncologic surgery. *Journal of surgical oncology* 2010; **101**: 661-668.
119. Hernández-Vila C. Current prognosis and quality of life following surgical treatment for head and neck squamous cell carcinoma. *Plastic and Aesthetic Research* 2016; **3**: 203-210.
120. Wong RJ, Shah JP. The role of the head and neck surgeon in contemporary multidisciplinary treatment programs for advanced head and neck cancer. *Current opinion in otolaryngology & head and neck surgery* 2010; **18**: 79-82.
121. Patel RS, Goldstein DP, Brown D*, et al.* Circumferential pharyngeal reconstruction: history, critical analysis of techniques, and current therapeutic recommendations. *Head Neck* 2010; **32**: 109-120.
122. Burke MS, Kaplan SE, Kaplowitz LJ*, et al.* Pectoralis major myocutaneous flap for reconstruction of circumferential pharyngeal defects. *Annals of plastic surgery* 2013; **71**: 649-651.
123. de Bree R, Rinaldo A, Genden EM*, et al.* Modern reconstruction techniques for oral and pharyngeal defects after tumor resection. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery* 2008; **265**: 1-9.
124. Ettinger KS, Ganry L, Fernandes RP. Oral Cavity Cancer. *Oral Maxillofac Surg Clin North Am* 2019; **31**: 13-29.
125. Haigentz M, Jr., Silver CE, Hartl DM*, et al.* Chemotherapy regimens and treatment protocols for laryngeal cancer. *Expert opinion on pharmacotherapy* 2010; **11**: 1305-1316.
126. Cognetti DM, Weber RS, Lai SY. Head and neck cancer: an evolving treatment paradigm. *Cancer* 2008; **113**: 1911-1932.
127. Devlin JG, Langer CJ. Combined modality treatment of laryngeal squamous cell carcinoma. *Expert review of anticancer therapy* 2007; **7**: 331-350.
128. Pfister DG, Laurie SA, Weinstein GS*, et al.* American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer.

Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2006; **24**: 3693-3704.
129. PDQ-Adult-Treatment-Editorial-Board. Laryngeal Cancer Treatment (Adult) (PDQ®): Health Professional Version. [Accessed Mars 2018]: Available from: https://www.ncbi.nlm.nih.gov/books/NBK65746/
130. Wolf GT, Fisher SG, Hong WK*, et al.* Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. *N Engl J Med* 1991; **324**: 1685-1690.
131. Obid R, Redlich M, Tomeh C. The Treatment of Laryngeal Cancer. *Oral and Maxillofacial Surgery Clinics of North America* 2019; **31**: 1-11.
132. Crile G. III. On the Technique of Operations upon the Head and Neck. *Annals of surgery* 1906; **44**: 842-850.
133. Rinaldo A, Ferlito A, Silver CE. Early history of neck dissection. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery* 2008; **265**: 1535-1538.
134. Martin H, Del Valle B, Ehrlich H*, et al.* Neck dissection. *Cancer* 1951; **4**: 441-499.
135. Barkley HT, Jr., Fletcher GH, Jesse RH*, et al.* Management of cervical lymph node metastases in squamous cell carcinoma of the tonsillar fossa, base of tongue, supraglottic larynx, and hypopharynx. *American journal of surgery* 1972; **124**: 462-467.
136. Pellitteri PK, Ferlito A, Rinaldo A*, et al.* Planned neck dissection following chemoradiotherapy for advanced head and neck cancer: is it necessary for all? *Head Neck* 2006; **28**: 166-175.
137. Mendenhall WM, Villaret DB, Amdur RJ*, et al.* Planned neck dissection after definitive radiotherapy for squamous cell carcinoma of the head and neck. *Head Neck* 2002; **24**: 1012-1018.
138. Ferlito A, Corry J, Silver CE*, et al.* Planned neck dissection for patients with complete response to chemoradiotherapy: a concept approaching obsolescence. *Head Neck* 2010; **32**: 253-261.
139. Brown KM, Lango M, Ridge JA. The role of neck dissection in the combined modality therapy setting. *Seminars in oncology* 2008; **35**: 229-235.
140. Hamoir M, Schmitz S, Gregoire V. The Role of Neck Dissection in Squamous Cell Carcinoma of the Head and Neck. *Current Treatment Options in Oncology* 2014; **15**: 611-624.
141. Givi B, Andersen PE. Rationale for modifying neck dissection. *Journal of surgical oncology* 2008; **97**: 674-682.
142. Okura M, Aikawa T, Sawai NY*, et al.* Decision analysis and treatment threshold in a management for the N0 neck of the oral cavity carcinoma. *Oral oncology* 2009; **45**: 908-911.
143. D'Cruz AK, Vaish R, Kapre N*, et al.* Elective versus Therapeutic Neck Dissection in Node-Negative Oral Cancer. *New England Journal of Medicine* 2015; **373**: 521-529.
144. Coughlin A, Resto VA. Oral cavity squamous cell carcinoma and the clinically n0 neck: the past, present, and future of sentinel lymph node biopsy. *Current oncology reports* 2010; **12**: 129-135.
145. Robbins KT, Clayman G, Levine PA*, et al.* Neck dissection classification update: revisions proposed by the American Head and Neck Society and the American Academy of Otolaryngology-Head and Neck Surgery. *Archives of otolaryngology--head & neck surgery* 2002; **128**: 751-758.
146. Hamoir M, Leemans CR, Dolivet G*, et al.* Selective neck dissection in the management of the neck after (chemo)radiotherapy for advanced head and neck cancer. Proposal for a classification update. *Head Neck* 2010; **32**: 816-819.
147. de Bree R, Leemans CR. Recent advances in surgery for head and neck cancer. *Current opinion in oncology* 2010; **22**: 186-193.

148. Demir D. The Role of Sentinel Lymph Node Biopsy in Head and Neck Cancers and Its Application Areas. *Turkish archives of otorhinolaryngology* 2016; **54**: 35-38.
149. Sharma D, Koshy G, Grover S*, et al.* Sentinel Lymph Node Biopsy: A new approach in the management of head and neck cancers. *Sultan Qaboos University medical journal* 2017; **17**: e3-e10.
150. Yadav P. Recent advances in head and neck cancer reconstruction. *Indian journal of plastic surgery : official publication of the Association of Plastic Surgeons of India* 2014; **47**: 185-190.
151. Fraass B, Doppke K, Hunt M*, et al.* American Association of Physicists in Medicine Radiation Therapy Committee Task Group 53: quality assurance for clinical radiotherapy treatment planning. *Medical physics* 1998; **25**: 1773-1829.
152. Zackrisson B, Mercke C, Strander H*, et al.* A systematic overview of radiation therapy effects in head and neck cancer. *Acta oncologica (Stockholm, Sweden)* 2003; **42**: 443-461.
153. Dirix P, Nuyts S. Evidence-based organ-sparing radiotherapy in head and neck cancer. *The Lancet Oncology* 2010; **11**: 85-91.
154. Castadot P, Lee JA, Geets X*, et al.* Adaptive radiotherapy of head and neck cancer. *Seminars in radiation oncology* 2010; **20**: 84-93.
155. Mallick I, Waldron JN. Radiation therapy for head and neck cancers. *Seminars in oncology nursing* 2009; **25**: 193-202.
156. Overgaard J, Mohanti BK, Begum N*, et al.* Five versus six fractions of radiotherapy per week for squamous-cell carcinoma of the head and neck (IAEA-ACC study): a randomised, multicentre trial. *The Lancet Oncology* 2010; **11**: 553-560.
157. Mortensen HR, Overgaard J, Specht L*, et al.* Prevalence and peak incidence of acute and late normal tissue morbidity in the DAHANCA 6&7 randomised trial with accelerated radiotherapy for head and neck cancer. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2012; **103**: 69-75.
158. Johansson KA, Nilsson P, Zackrisson B*, et al.* The quality assurance process for the ARTSCAN head and neck study - a practical interactive approach for QA in 3DCRT and IMRT. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2008; **87**: 290-299.
159. Bourhis J, Overgaard J, Audry H*, et al.* Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. *Lancet (London, England)* 2006; **368**: 843-854.
160. Sun Y, Yu XL, Luo W*, et al.* Recommendation for a contouring method and atlas of organs at risk in nasopharyngeal carcinoma patients receiving intensity-modulated radiotherapy. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2014; **110**: 390-397.
161. Brouwer CL, Steenbakkers RJ, Bourhis J*, et al.* CT-based delineation of organs at risk in the head and neck region: DAHANCA, EORTC, GORTEC, HKNPCSG, NCIC CTG, NCRI, NRG Oncology and TROG consensus guidelines. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2015; **117**: 83-90.
162. De Felice F, Musio D, Tombolini V. Mastication structures definition in head and neck cancer. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2016; **118**: 419.
163. Surucu M, Shah KK, Roeske JC*, et al.* Adaptive Radiotherapy for Head and Neck Cancer. *Technology in cancer research & treatment* 2017; **16**: 218-223.
164. Leeman JE, Romesser PB, Zhou Y*, et al.* Proton therapy for head and neck cancer: expanding the therapeutic window. *The Lancet Oncology* 2017; **18**: e254-e265.
165. Wang X, Eisbruch A. IMRT for head and neck cancer: reducing xerostomia and dysphagia. *Journal of radiation research* 2016; **57 Suppl 1**: i69-i75.

166. Halle M, Bodin I, Tornvall P*, et al.* Timing of radiotherapy in head and neck free flap reconstruction--a study of postoperative complications. *Journal of plastic, reconstructive & aesthetic surgery : JPRAS* 2009; **62**: 889-895.
167. Gonzalez-Garcia R, Naval-Gias L, Roman-Romero L*, et al.* Local recurrences and second primary tumors from squamous cell carcinoma of the oral cavity: a retrospective analytic study of 500 patients. *Head Neck* 2009; **31**: 1168-1180.
168. Luukkaa M, Minn H, Aitasalo K*, et al.* Treatment of squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx--an analysis of 174 patients in south western Finland. *Acta oncologica (Stockholm, Sweden)* 2003; **42**: 756-762.
169. Genden EM, Ferlito A, Silver CE*, et al.* Contemporary management of cancer of the oral cavity. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery* 2010; **267**: 1001-1017.
170. Taberna M, Rullan AJ, Hierro C*, et al.* Late toxicity after radical treatment for locally advanced head and neck cancer. *Oral oncology* 2015; **51**: 795-799.
171. Stubblefield MD. Clinical Evaluation and Management of Radiation Fibrosis Syndrome. *Physical medicine and rehabilitation clinics of North America* 2017; **28**: 89-100.
172. Skowronek J. Current status of brachytherapy in cancer treatment - short overview. *Journal of contemporary brachytherapy* 2017; **9**: 581-589.
173. Mazeron JJ, Ardiet JM, Haie-Meder C*, et al.* GEC-ESTRO recommendations for brachytherapy for head and neck squamous cell carcinomas. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2009; **91**: 150-156.
174. Shibuya H. Current status and perspectives of brachytherapy for head and neck cancer. *International journal of clinical oncology* 2009; **14**: 2-6.
175. Kovacs G, Martinez-Monge R, Budrukkar A*, et al.* GEC-ESTRO ACROP recommendations for head & neck brachytherapy in squamous cell carcinomas: 1st update - Improvement by cross sectional imaging based treatment planning and stepping source technology. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 2017; **122**: 248-254.
176. Kovács G. Modern head and neck brachytherapy: from radium towards intensity modulated interventional brachytherapy. *Journal of contemporary brachytherapy* 2015; **6**: 404-416.
177. Choong N, Vokes E. Expanding role of the medical oncologist in the management of head and neck cancer. *CA: a cancer journal for clinicians* 2008; **58**: 32-53.
178. Iqbal MS, Chaw C, Kovarik J*, et al.* Primary Concurrent Chemoradiation in Head and Neck Cancers with Weekly Cisplatin Chemotherapy: Analysis of Compliance, Toxicity and Survival. *International archives of otorhinolaryngology* 2017; **21**: 171-177.
179. Argiris A, Haraf DJ, Kies MS*, et al.* Intensive concurrent chemoradiotherapy for head and neck cancer with 5-Fluorouracil- and hydroxyurea-based regimens: reversing a pattern of failure. *The oncologist* 2003; **8**: 350-360.
180. Mehanna H, West CM, Nutting C*, et al.* Head and neck cancer--Part 2: Treatment and prognostic factors. *BMJ (Clinical research ed)* 2010; **341**: c4690.
181. Brizel DM, Vokes EE. Induction chemotherapy: to use or not to use? That is the question. *Seminars in radiation oncology* 2009; **19**: 11-16.
182. Haddad RI, Posner M, Hitt R*, et al.* Induction chemotherapy in locally advanced squamous cell carcinoma of the head and neck: role, controversy, and future directions. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018; **29**: 1130-1140.

183. Al-Sarraf M, LeBlanc M, Giri PG*, et al.* Chemoradiotherapy versus radiotherapy in patients with advanced nasopharyngeal cancer: phase III randomized Intergroup study 0099. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 1998; **16**: 1310-1317.
184. Adelstein DJ, Li Y, Adams GL*, et al.* An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2003; **21**: 92-98.
185. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *European journal of pharmacology* 2014; **740**: 364-378.
186. Focaccetti C, Bruno A, Magnani E*, et al.* Effects of 5-fluorouracil on morphology, cell cycle, proliferation, apoptosis, autophagy and ROS production in endothelial cells and cardiomyocytes. *PloS one* 2015; **10**: e0115686-e0115686.
187. Sundvall M, Karrila A, Nordberg J*, et al.* EGFR targeting drugs in the treatment of head and neck squamous cell carcinoma. *Expert opinion on emerging drugs* 2010; **15**: 185-201.
188. Bonner JA, Harari PM, Giralt J*, et al.* Radiotherapy plus Cetuximab for Squamous-Cell Carcinoma of the Head and Neck. *New England Journal of Medicine* 2006; **354**: 567-578.
189. Ang KK, Berkey BA, Tu X*, et al.* Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer research* 2002; **62**: 7350-7356.
190. Vermorken JB, Mesia R, Rivera F*, et al.* Platinum-Based Chemotherapy plus Cetuximab in Head and Neck Cancer. *New England Journal of Medicine* 2008; **359**: 1116-1127.
191. Price KAR, Cohen EE. Current Treatment Options for Metastatic Head and Neck Cancer. *Current Treatment Options in Oncology* 2012; **13**: 35-46.
192. Bell RB, Leidner RS, Crittenden MR*, et al.* OX40 signaling in head and neck squamous cell carcinoma: Overcoming immunosuppression in the tumor microenvironment. *Oral oncology* 2016; **52**: 1-10.
193. Blaszczak W, Barczak W, Wegner A*, et al.* Clinical value of monoclonal antibodies and tyrosine kinase inhibitors in the treatment of head and neck squamous cell carcinoma. *Medical oncology (Northwood, London, England)* 2017; **34**: 60-60.
194. Sharafinski ME, Ferris RL, Ferrone S*, et al.* Epidermal growth factor receptor targeted therapy of squamous cell carcinoma of the head and neck. *Head Neck* 2010; **32**: 1412-1421.
195. Albers AE, Chen C, Koberle B*, et al.* Stem cells in squamous head and neck cancer. *Critical reviews in oncology/hematology* 2012; **81**: 224-240.
196. Nowell PC. The clonal evolution of tumor cell populations. *Science (New York, NY)* 1976; **194**: 23-28.
197. Campbell LL, Polyak K. Breast tumor heterogeneity: cancer stem cells or clonal evolution? *Cell cycle (Georgetown, Tex)* 2007; **6**: 2332-2338.
198. Shakib K, Soskic V. Stem cells in head and neck squamous cell carcinoma. *British Journal of Oral and Maxillofacial Surgery* 2011; **49**: S99.
199. Al-Hajj M, Wicha MS, Benito-Hernandez A*, et al.* Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 2003; **100**: 3983-3988.
200. Sampieri K, Fodde R. Cancer stem cells and metastasis. *Seminars in cancer biology* 2012; **22**: 187-193.
201. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. *Cancer research* 2006; **66**: 1883-1890; discussion 1895-1886.

202. Prince ME, Sivanandan R, Kaczorowski A*, et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**: 973-978.
203. Dalerba P, Dylla SJ, Park IK*, et al.* Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**: 10158-10163.
204. Yang ZF, Ho DW, Ng MN*, et al.* Significance of CD90+ cancer stem cells in human liver cancer. *Cancer cell* 2008; **13**: 153-166.
205. Lapidot T, Sirard C, Vormoor J*, et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648.
206. Wu X, Chen H, Wang X. Can lung cancer stem cells be targeted for therapies? *Cancer treatment reviews* 2012; **38**: 580-588.
207. Clarke MF, Dick JE, Dirks PB*, et al.* Cancer Stem Cells—Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer research* 2006; **66**: 9339-9344.
208. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science (New York, NY)* 1977; **197**: 461-463.
209. Park CH, Bergsagel DE, McCulloch EA. Mouse myeloma tumor stem cells: a primary cell culture assay. *Journal of the National Cancer Institute* 1971; **46**: 411-422.
210. Bruce WR, Van Der Gaag H. A QUANTITATIVE ASSAY FOR THE NUMBER OF MURINE LYMPHOMA CELLS CAPABLE OF PROLIFERATION IN VIVO. *Nature* 1963; **199**: 79-80.
211. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* 1997; **3**: 730-737.
212. Eramo A, Lotti F, Sette G*, et al.* Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell death and differentiation* 2008; **15**: 504-514.
213. Ricci-Vitiani L, Lombardi DG, Pilozzi E*, et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115.
214. Collins AT, Berry PA, Hyde C*, et al.* Prospective identification of tumorigenic prostate cancer stem cells. *Cancer research* 2005; **65**: 10946-10951.
215. Li C, Heidt DG, Dalerba P*, et al.* Identification of pancreatic cancer stem cells. *Cancer research* 2007; **67**: 1030-1037.
216. Gonzalez-Moles MA, Scully C, Ruiz-Avila I*, et al.* The cancer stem cell hypothesis applied to oral carcinoma. *Oral oncology* 2013; **49**: 738-746.
217. Margaritescu C, Pirici D, Simionescu C*, et al.* The utility of CD44, CD117 and CD133 in identification of cancer stem cells (CSC) in oral squamous cell carcinomas (OSCC). *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie* 2011; **52**: 985-993.
218. Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature immunology* 2004; **5**: 738-743.
219. Todaro M, Alea MP, Di Stefano AB*, et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell stem cell* 2007; **1**: 389-402.
220. dos Santos RV, da Silva LM. A possible explanation for the variable frequencies of cancer stem cells in tumors. *PloS one* 2013; **8**: e69131-e69131.
221. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? *Nature medicine* 2009; **15**: 1010-1012.
222. Zapperi S, La Porta CA. Do cancer cells undergo phenotypic switching? The case for imperfect cancer stem cell markers. *Scientific reports* 2012; **2**: 441.

223. Bhaijee F, Pepper DJ, Pitman KT*, et al.* Cancer stem cells in head and neck squamous cell carcinoma: a review of current knowledge and future applications. *Head Neck* 2012; **34**: 894-899.
224. Shah A, Patel S, Pathak J*, et al.* The Evolving Concepts of Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma. *The Scientific World Journal* 2014; **2014**: 8.
225. Costea DE, Tsinkalovsky O, Vintermyr OK*, et al.* Cancer stem cells - new and potentially important targets for the therapy of oral squamous cell carcinoma. *Oral diseases* 2006; **12**: 443-454.
226. Califano J, Westra WH, Meininger G*, et al.* Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2000; **6**: 347-352.
227. Hahn WC, Weinberg RA. Rules for making human tumor cells. *N Engl J Med* 2002; **347**: 1593-1603.
228. Calabrese C, Poppleton H, Kocak M*, et al.* A Perivascular Niche for Brain Tumor Stem Cells. *Cancer cell* 2007; **11**: 69-82.
229. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674.
230. Morrison SJ, Qian D, Jerabek L*, et al.* A genetic determinant that specifically regulates the frequency of hematopoietic stem cells. *Journal of immunology (Baltimore, Md : 1950)* 2002; **168**: 635-642.
231. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. *Nature* 2001; **411**: 349-354.
232. Allegra E, Trapasso S, Pisani D*, et al.* The role of BMI1 as a biomarker of cancer stem cells in head and neck cancer: a review. *Oncology* 2014; **86**: 199-205.
233. Dalerba P, Clarke MF, Weissman IL*, et al.* 7 - Stem Cells, Cell Differentiation, and Cancer. In: Abeloff's Clinical Oncology (Fifth Edition). Niederhuber JE, Armitage JO, Doroshow JH*, et al.*, (ed)^(eds). Content Repository Only!: Philadelphia, 2014; 98-107.e103.
234. Rossi DJ, Jamieson CHM, Weissman IL. Stems Cells and the Pathways to Aging and Cancer. *Cell* 2008; **132**: 681-696.
235. Jan M, Snyder TM, Corces-Zimmerman MR*, et al.* Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Science translational medicine* 2012; **4**: 149ra118.
236. Abollo-Jimenez F, Jimenez R, Cobaleda C. Physiological cellular reprogramming and cancer. *Seminars in cancer biology* 2010; **20**: 98-106.
237. Smith A, Teknos TN, Pan Q. Epithelial to mesenchymal transition in head and neck squamous cell carcinoma. *Oral oncology* 2013; **49**: 287-292.
238. Sanchez-Tillo E, Lazaro A, Torrent R*, et al.* ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene* 2010; **29**: 3490-3500.
239. Cano A, Perez-Moreno MA, Rodrigo I*, et al.* The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature cell biology* 2000; **2**: 76-83.
240. Morel A-P, Lièvre M, Thomas C*, et al.* Generation of Breast Cancer Stem Cells through Epithelial-Mesenchymal Transition. *PloS one* 2008; **3**: e2888.
241. Xia H, Cheung WK, Sze J*, et al.* miR-200a regulates epithelial-mesenchymal to stem-like transition via ZEB2 and beta-catenin signaling. *The Journal of biological chemistry* 2010; **285**: 36995-37004.
242. Widschwendter M, Fiegl H, Egle D*, et al.* Epigenetic stem cell signature in cancer. *Nature genetics* 2007; **39**: 157-158.

243. Singh SK, Hawkins C, Clarke ID*, et al.* Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396.
244. Reategui EP, de Mayolo AA, Das PM*, et al.* Characterization of CD44v3-containing isoforms in head and neck cancer. *Cancer biology & therapy* 2006; **5**: 1163-1168.
245. Wang SJ, Wong G, de Heer AM*, et al.* CD44 variant isoforms in head and neck squamous cell carcinoma progression. *The Laryngoscope* 2009; **119**: 1518-1530.
246. Davis SJ, Divi V, Owen JH*, et al.* Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. *Archives of Otolaryngology–Head & Neck Surgery* 2010; **136**: 1260-1266.
247. Sterz CM, Kulle C, Dakic B*, et al.* A basal-cell-like compartment in head and neck squamous cell carcinomas represents the invasive front of the tumor and is expressing MMP-9. *Oral oncology* 2010; **46**: 116-122.
248. Su J, Xu XH, Huang Q*, et al.* Identification of cancer stem-like CD44+ cells in human nasopharyngeal carcinoma cell line. *Archives of medical research* 2011; **42**: 15-21.
249. Joshua B, Kaplan MJ, Doweck I*, et al.* Frequency of cells expressing CD44, a head and neck cancer stem cell marker: correlation with tumor aggressiveness. *Head Neck* 2012; **34**: 42-49.
250. Nagano O, Okazaki S, Saya H. Redox regulation in stem-like cancer cells by CD44 variant isoforms. *Oncogene* 2013; **32**: 5191-5198.
251. Aso T, Matsuo M, Kiyohara H*, et al.* Induction of CD44 variant 9-expressing cancer stem cells might attenuate the efficacy of chemoradioselection and Worsens the prognosis of patients with advanced head and neck cancer. *PloS one* 2015; **10**: e0116596.
252. Han J, Fujisawa T, Husain SR*, et al.* Identification and characterization of cancer stem cells in human head and neck squamous cell carcinoma. *BMC cancer* 2014; **14**: 173-173.
253. Fukusumi T, Ishii H, Konno M*, et al.* CD10 as a novel marker of therapeutic resistance and cancer stem cells in head and neck squamous cell carcinoma. *British journal of cancer* 2014; **111**: 506-514.
254. Piattelli A, Fioroni M, Iezzi G*, et al.* CD10 expression in stromal cells of oral cavity squamous cell carcinoma: a clinic and pathologic correlation. *Oral diseases* 2006; **12**: 301-304.
255. Yan M, Yang X, Wang L*, et al.* Plasma membrane proteomics of tumor spheres identify CD166 as a novel marker for cancer stem-like cells in head and neck squamous cell carcinoma. *Molecular & cellular proteomics : MCP* 2013; **12**: 3271-3284.
256. Wu Y, Wu PY. CD133 as a marker for cancer stem cells: progresses and concerns. *Stem cells and development* 2009; **18**: 1127-1134.
257. Mizrak D, Brittan M, Alison M. CD133: molecule of the moment. *The Journal of pathology* 2008; **214**: 3-9.
258. Zhou L, Wei X, Cheng L*, et al.* CD133, one of the markers of cancer stem cells in Hep-2 cell line. *The Laryngoscope* 2007; **117**: 455-460.
259. Sun Y, Han J, Lu Y*, et al.* Biological characteristics of a cell subpopulation in tongue squamous cell carcinoma. *Oral diseases* 2012; **18**: 169-177.
260. Casaccia-Bonnefil P, Gu C, Chao MV. Neurotrophins in cell survival/death decisions. *Advances in experimental medicine and biology* 1999; **468**: 275-282.
261. Yan H, Schlessinger J, Chao MV. Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. *Science (New York, NY)* 1991; **252**: 561-563.
262. Sailer MH, Gerber A, Tostado C*, et al.* Non-invasive neural stem cells become invasive in vitro by combined FGF2 and BMP4 signaling. *Journal of cell science* 2013; **126**: 3533-3540.
263. Li X, Shen Y, Di B*, et al.* Biological and clinical significance of p75NTR expression in laryngeal squamous epithelia and laryngocarcinoma. *Acta oto-laryngologica* 2012; **132**: 314-324.

264. Chung MK, Jung YH, Lee JK*, et al.* CD271 Confers an Invasive and Metastatic Phenotype of Head and Neck Squamous Cell Carcinoma through the Upregulation of Slug. *Clinical Cancer Research* 2018; **24**: 674-683.
265. Murillo-Sauca O, Chung MK, Shin JH*, et al.* CD271 is a functional and targetable marker of tumor-initiating cells in head and neck squamous cell carcinoma. *Oncotarget* 2014; **5**: 6854-6866.
266. Sobreira TJ, Marletaz F, Simoes-Costa M*, et al.* Structural shifts of aldehyde dehydrogenase enzymes were instrumental for the early evolution of retinoid-dependent axial patterning in metazoans. *Proceedings of the National Academy of Sciences of the United States of America* 2011; **108**: 226-231.
267. Marcato P, Dean CA, Giacomantonio CA*, et al.* Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. *Cell cycle (Georgetown, Tex)* 2011; **10**: 1378-1384.
268. Yu CC, Lo WL, Chen YW*, et al.* Bmi-1 Regulates Snail Expression and Promotes Metastasis Ability in Head and Neck Squamous Cancer-Derived ALDH1 Positive Cells. *Journal of oncology* 2011; **2011**.
269. Yang L, Ren Y, Yu X*, et al.* ALDH1A1 defines invasive cancer stem-like cells and predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2014; **27**: 775-783.
270. Clay MR, Tabor M, Owen JH*, et al.* Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck* 2010; **32**: 1195-1201.
271. Dou J, Gu N. Emerging strategies for the identification and targeting of cancer stem cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2010; **31**: 243-253.
272. Zhang P, Zhang Y, Mao L*, et al.* Side population in oral squamous cell carcinoma possesses tumor stem cell phenotypes. *Cancer letters* 2009; **277**: 227-234.
273. Tabor MH, Clay MR, Owen JH*, et al.* Head and neck cancer stem cells: the side population. *The Laryngoscope* 2011; **121**: 527-533.
274. Yanamoto S, Kawasaki G, Yamada S*, et al.* Isolation and characterization of cancer stem-like side population cells in human oral cancer cells. *Oral oncology* 2011; **47**: 855-860.
275. Chen JS, Pardo FS, Wang-Rodriguez J*, et al.* EGFR regulates the side population in head and neck squamous cell carcinoma. *The Laryngoscope* 2006; **116**: 401-406.
276. Song J, Chang I, Chen Z*, et al.* Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling. *PloS one* 2010; **5**: e11456.
277. Clarke MF, Fuller M. Stem Cells and Cancer: Two Faces of Eve. *Cell* 2006; **124**: 1111-1115.
278. Morrison R, Schleicher SM, Sun Y*, et al.* Targeting the mechanisms of resistance to chemotherapy and radiotherapy with the cancer stem cell hypothesis. *Journal of oncology* 2011; **2011**: 941876.
279. Ogawa K, Yoshioka Y, Isohashi F*, et al.* Radiotherapy targeting cancer stem cells: current views and future perspectives. *Anticancer research* 2013; **33**: 747-754.
280. Becker M, Levy D. Modeling the Transfer of Drug Resistance in Solid Tumors. *Bulletin of Mathematical Biology* 2017; **79**: 2394-2412.
281. Chen KG, Jaffrezou JP, Fleming WH*, et al.* Prevalence of multidrug resistance related to activation of the mdr1 gene in human sarcoma mutants derived by single-step doxorubicin selection. *Cancer research* 1994; **54**: 4980-4987.
282. Ng IOL, Lam KY, Ng M*, et al.* Expression of P-glycoprotein, a multidrug-resistance gene product, is Induced by radiotherapy in patients with oral squamous cell carcinoma. *Cancer* 1998; **83**: 851-857.

283. Rabkin D, Chhieng DC, Miller MB*, et al.* P-Glycoprotein expression in the squamous cell carcinoma of the tongue base. *The Laryngoscope* 1995; **105**: 1294-1299.
284. Yaromina A, Krause M, Thames H*, et al.* Pre-treatment number of clonogenic cells and their radiosensitivity are major determinants of local tumour control after fractionated irradiation. *Radiotherapy and Oncology* 2007; **83**: 304-310.
285. Eyler CE, Rich JN. Survival of the Fittest: Cancer Stem Cells in Therapeutic Resistance and Angiogenesis. *Journal of Clinical Oncology* 2008; **26**: 2839-2845.
286. Toledano I, Graff P, Serre A*, et al.* Intensity-modulated radiotherapy in head and neck cancer: Results of the prospective study GORTEC 2004–03. *Radiotherapy and Oncology* 2012; **103**: 57-62.
287. Vissink A, Mitchell JB, Baum BJ*, et al.* Clinical Management of Salivary Gland Hypofunction and Xerostomia in Head-and-Neck Cancer Patients: Successes and Barriers. *International Journal of Radiation Oncology*Biology*Physics* 2010; **78**: 983-991.
288. Heddleston JM, Li Z, Lathia JD*, et al.* Hypoxia inducible factors in cancer stem cells. *British journal of cancer* 2010; **102**: 789.
289. Satpute PS, Hazarey V, Ahmed R*, et al.* Cancer stem cells in head and neck squamous cell carcinoma: a review. *Asian Pacific journal of cancer prevention : APJCP* 2013; **14**: 5579-5587.
290. Vlashi E, McBride WH, Pajonk F. Radiation responses of cancer stem cells. *Journal of Cellular Biochemistry* 2009; **108**: 339-342.
291. Yang M-H, Hsu DS-S, Wang H-W*, et al.* Bmi1 is essential in Twist1-induced epithelial–mesenchymal transition. *Nature cell biology* 2010; **12**: 982.
292. Marur S, Forastiere AA. Head and Neck Squamous Cell Carcinoma: Update on Epidemiology, Diagnosis, and Treatment. *Mayo Clinic proceedings* 2016; **91**: 386-396.
293. Takebe N, Harris PJ, Warren RQ*, et al.* Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. *Nature reviews Clinical oncology* 2011; **8**: 97-106.
294. Takahashi-Yanaga F, Kahn M. Targeting Wnt Signaling: Can We Safely Eradicate Cancer Stem Cells? *Clinical Cancer Research* 2010; **16**: 3153-3162.
295. Keysar SB, Le PN, Anderson RT*, et al.* Hedgehog signaling alters reliance on EGF receptor signaling and mediates anti-EGFR therapeutic resistance in head and neck cancer. *Cancer research* 2013; **73**: 3381-3392.
296. Stransky N, Egloff AM, Tward AD*, et al.* The Mutational Landscape of Head and Neck Squamous Cell Carcinoma. *Science (New York, NY)* 2011; **333**: 1157-1160.
297. Lim YC, Kang HJ, Kim YS*, et al.* All-trans-retinoic acid inhibits growth of head and neck cancer stem cells by suppression of Wnt/β-catenin pathway. *European Journal of Cancer* 2012; **48**: 3310-3318.
298. Zhao Z-L, Zhang L, Huang C-F*, et al.* NOTCH1 inhibition enhances the efficacy of conventional chemotherapeutic agents by targeting head neck cancer stem cell. *Scientific reports* 2016; **6**: 24704.
299. Han L, Shi S, Gong T*, et al.* Cancer stem cells: therapeutic implications and perspectives in cancer therapy. *Acta Pharmaceutica Sinica B* 2013; **3**: 65-75.
300. Igarashi M, Irwin CR, Locke M*, et al.* Construction of large area organotypical cultures of oral mucosa and skin. *Journal of Oral Pathology & Medicine* 2003; **32**: 422-430.
301. Fayard E, Xue G, Parcellier A*, et al.* Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. *Current topics in microbiology and immunology* 2010; **346**: 31-56.
302. Torre C, Wang SJ, Xia W*, et al.* Reduction of hyaluronan-CD44-mediated growth, migration, and cisplatin resistance in head and neck cancer due to inhibition of Rho kinase and PI-3 kinase signaling. *Archives of otolaryngology--head & neck surgery* 2010; **136**: 493-501.

303. Chen YC, Chang CJ, Hsu HS*, et al.* Inhibition of tumorigenicity and enhancement of radiochemosensitivity in head and neck squamous cell cancer-derived ALDH1-positive cells by knockdown of Bmi-1. *Oral oncology* 2010; **46**: 158-165.
304. Kulsum S, Sudheendra HV, Pandian R*, et al.* Cancer stem cell mediated acquired chemoresistance in head and neck cancer can be abrogated by aldehyde dehydrogenase 1 A1 inhibition. *Mol Carcinog* 2017; **56**: 694-711.
305. Visus C, Wang Y, Lozano-Leon A*, et al.* Targeting ALDH(bright) human carcinoma-initiating cells with ALDH1A1-specific $CD8^+$ T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2011; **17**: 6174-6184.
306. Lou H, Dean M. Targeted therapy for cancer stem cells: the patched pathway and ABC transporters. *Oncogene* 2007; **26**: 1357.
307. Signore M, Ricci-Vitiani L, De Maria R. Targeting apoptosis pathways in cancer stem cells. *Cancer letters* 2013; **332**: 374-382.
308. García MA, Carrasco E, Ramírez A*, et al.* Apoptosis as a Therapeutic Target in Cancer and Cancer Stem Cells: Novel Strategies and Futures Perspectives. In: Apoptosis and Medicine. (ed)^(eds), 2012.
309. Hexum JK, Tello-Aburto R, Struntz NB*, et al.* Bicyclic Cyclohexenones as Inhibitors of NF-κB Signaling. *ACS Medicinal Chemistry Letters* 2012; **3**: 459-464.
310. Soltanian S, Matin MM. Cancer stem cells and cancer therapy. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2011; **32**: 425-440.
311. Massard C, Deutsch E, Soria JC. Tumour stem cell-targeted treatment: elimination or differentiation. *Annals of Oncology* 2006; **17**: 1620-1624.
312. Efferth T. Stem cells, cancer stem-like cells, and natural products. *Planta medica* 2012; **78**: 935-942.
313. von Meyenfeldt M. Cancer-associated malnutrition: An introduction. *European Journal of Oncology Nursing* 2005; **9**: S35-S38.
314. Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nature reviews Cancer* 2003; **3**: 768-780.
315. Mithen R, Faulkner K, Magrath R*, et al.* Development of isothiocyanate-enriched broccoli, and its enhanced ability to induce phase 2 detoxification enzymes in mammalian cells. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2003; **106**: 727-734.
316. Higdon JV, Delage B, Williams DE*, et al.* Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacological research* 2007; **55**: 224-236.
317. Verhoeven DT, Goldbohm RA, van Poppel G*, et al.* Epidemiological studies on brassica vegetables and cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 1996; **5**: 733-748.
318. Voorrips LE, Goldbohm RA, Verhoeven DT*, et al.* Vegetable and fruit consumption and lung cancer risk in the Netherlands Cohort Study on diet and cancer. *Cancer causes & control : CCC* 2000; **11**: 101-115.
319. Voorrips LE, Goldbohm RA, van Poppel G*, et al.* Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. *American journal of epidemiology* 2000; **152**: 1081-1092.
320. Feskanich D, Ziegler RG, Michaud DS*, et al.* Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. *Journal of the National Cancer Institute* 2000; **92**: 1812-1823.
321. Giovannucci E, Rimm EB, Liu Y*, et al.* A prospective study of cruciferous vegetables and prostate cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American*

Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2003; **12**: 1403-1409.
322. Joseph MA, Moysich KB, Freudenheim JL*, et al.* Cruciferous vegetables, genetic polymorphisms in glutathione S-transferases M1 and T1, and prostate cancer risk. *Nutrition and cancer* 2004; **50**: 206-213.
323. Neuhouser ML, Patterson RE, Thornquist MD*, et al.* Fruits and vegetables are associated with lower lung cancer risk only in the placebo arm of the beta-carotene and retinol efficacy trial (CARET). *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2003; **12**: 350-358.
324. Drewnowski A, Gomez-Carneros C. Bitter taste, phytonutrients, and the consumer: a review. *The American journal of clinical nutrition* 2000; **72**: 1424-1435.
325. Bianchini F, Vainio H. Isothiocyanates in cancer prevention. *Drug metabolism reviews* 2004; **36**: 655-667.
326. Tortorella SM, Royce SG, Licciardi PV*, et al.* Dietary Sulforaphane in Cancer Chemoprevention: The Role of Epigenetic Regulation and HDAC Inhibition. *Antioxidants & redox signaling* 2015; **22**: 1382-1424.
327. Bell L, Wagstaff C. Glucosinolates, Myrosinase Hydrolysis Products, and Flavonols Found in Rocket (Eruca sativa and Diplotaxis tenuifolia). *Journal of Agricultural and Food Chemistry* 2014; **62**: 4481-4492.
328. Conaway CC, Krzeminski J, Amin S*, et al.* Decomposition rates of isothiocyanate conjugates determine their activity as inhibitors of cytochrome p450 enzymes. *Chemical research in toxicology* 2001; **14**: 1170-1176.
329. Shapiro TA, Fahey JW, Wade KL*, et al.* Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 1998; **7**: 1091-1100.
330. Matusheski NV, Jeffery EH. Comparison of the Bioactivity of Two Glucoraphanin Hydrolysis Products Found in Broccoli, Sulforaphane and Sulforaphane Nitrile. *Journal of Agricultural and Food Chemistry* 2001; **49**: 5743-5749.
331. Liang H, Yuan Q. Natural sulforaphane as a functional chemopreventive agent: including a review of isolation, purification and analysis methods. *Critical reviews in biotechnology* 2012; **32**: 218-234.
332. Prochaska HJ, Santamaria AB, Talalay P. Rapid detection of inducers of enzymes that protect against carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* 1992; **89**: 2394-2398.
333. West LG, Meyer KA, Balch BA*, et al.* Glucoraphanin and 4-hydroxyglucobrassicin contents in seeds of 59 cultivars of broccoli, raab, kohlrabi, radish, cauliflower, brussels sprouts, kale, and cabbage. *J Agric Food Chem* 2004; **52**: 916-926.
334. Cramer JM, Jeffery EH. Sulforaphane absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men. *Nutrition and cancer* 2011; **63**: 196-201.
335. Clarke JD, Hsu A, Riedl K*, et al.* Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacol Res* 2011; **64**: 456-463.
336. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* 1997; **94**: 10367-10372.

337. Prakash B, Amuthavalli A, Edison D*, et al.* Novel indole derivatives as potential anticancer agents: Design, synthesis and biological screening. *Medicinal Chemistry Research* 2018; **27**: 321-331.
338. Petri N, Tannergren C, Holst B*, et al.* Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. *Drug metabolism and disposition: the biological fate of chemicals* 2003; **31**: 805-813.
339. Conaway CC, Getahun SM, Liebes LL*, et al.* Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutrition and cancer* 2000; **38**: 168-178.
340. Rungapamestry V, Duncan AJ, Fuller Z*, et al.* Effect of cooking brassica vegetables on the subsequent hydrolysis and metabolic fate of glucosinolates. *The Proceedings of the Nutrition Society* 2007; **66**: 69-81.
341. Vermeulen M, Klopping-Ketelaars IW, van den Berg R*, et al.* Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. *J Agric Food Chem* 2008; **56**: 10505-10509.
342. Jones RB, Frisina CL, Winkler S*, et al.* Cooking method significantly effects glucosinolate content and sulforaphane production in broccoli florets. *Food Chemistry* 2010; **123**: 237-242.
343. Saha S, Hollands W, Teucher B*, et al.* Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects after consumption of commercial frozen broccoli compared to fresh broccoli. *Molecular nutrition & food research* 2012; **56**: 1906-1916.
344. Wang GC, Farnham M, Jeffery EH. Impact of thermal processing on sulforaphane yield from broccoli (Brassica oleracea L. ssp. italica). *J Agric Food Chem* 2012; **60**: 6743-6748.
345. Zhang Y, Tang L. Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. *Acta Pharmacologica Sinica* 2007; **28**: 1343.
346. Zhang Y. Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis* 2000; **21**: 1175-1182.
347. Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? *Carcinogenesis* 2009; **31**: 90-99.
348. Zhang Y, Kolm RH, Mannervik B*, et al.* Reversible conjugation of isothiocyanates with glutathione catalyzed by human glutathione transferases. *Biochemical and biophysical research communications* 1995; **206**: 748-755.
349. Zhang Y, Callaway EC. High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. *The Biochemical journal* 2002; **364**: 301-307.
350. Callaway EC, Zhang Y, Chew W*, et al.* Cellular accumulation of dietary anticarcinogenic isothiocyanates is followed by transporter-mediated export as dithiocarbamates. *Cancer letters* 2004; **204**: 23-31.
351. Brüsewitz G, Cameron BD, Chasseaud LF*, et al.* The metabolism of benzyl isothiocyanate and its cysteine conjugate. *Biochemical Journal* 1977; **162**: 99-107.
352. Herr I, Buchler MW. Dietary constituents of broccoli and other cruciferous vegetables: implications for prevention and therapy of cancer. *Cancer treatment reviews* 2010; **36**: 377-383.
353. Cramer JM, Teran-Garcia M, Jeffery EH. Enhancing sulforaphane absorption and excretion in healthy men through the combined consumption of fresh broccoli sprouts and a glucoraphanin-rich powder. *The British journal of nutrition* 2012; **107**: 1333-1338.
354. Al Janobi AA, Mithen RF, Gasper AV*, et al.* Quantitative measurement of sulforaphane, iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography-tandem electrospray ionisation mass spectrometry. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 2006; **844**: 223-234.

355. Ye L, Dinkova-Kostova AT, Wade KL*, et al.* Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clinica chimica acta; international journal of clinical chemistry* 2002; **316**: 43-53.
356. Hu R, Hebbar V, Kim B-R*, et al.* In Vivo Pharmacokinetics and Regulation of Gene Expression Profiles by Isothiocyanate Sulforaphane in the Rat. *Journal of Pharmacology and Experimental Therapeutics* 2004; **310**: 263-271.
357. Khor TO, Hu R, Shen G*, et al.* Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable. *Cancer research* 2006; **66**: 1316-1316.
358. Cornblatt BS, Ye L, Dinkova-Kostova AT*, et al.* Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis* 2007; **28**: 1485-1490.
359. Lock EA, Reed CJ. Xenobiotic metabolizing enzymes of the kidney. *Toxicologic pathology* 1998; **26**: 18-25.
360. Tang L, Li G, Song L*, et al.* The principal urinary metabolites of dietary isothiocyanates, N-acetylcysteine conjugates, elicit the same anti-proliferative response as their parent compounds in human bladder cancer cells. *Anti-cancer drugs* 2006; **17**: 297-305.
361. Lenzi M, Fimognari C, Hrelia P. Sulforaphane as a Promising Molecule for Fighting Cancer. In: Advances in Nutrition and Cancer. Zappia V, Panico S, Russo GL*, et al.*, (ed)^(eds). Springer Berlin Heidelberg: Berlin, Heidelberg, 2014; 207-223.
362. Iyanagi T. Molecular Mechanism of Phase I and Phase II Drug-Metabolizing Enzymes: Implications for Detoxification. In: International Review of Cytology. (ed)^(eds). Academic Press, 2007; 35-112.
363. Deng J, Zhao L, Zhang NY*, et al.* Aflatoxin B1 metabolism: Regulation by phase I and II metabolizing enzymes and chemoprotective agents. *Mutation research* 2018; **778**: 79-89.
364. Barcelo S, Gardiner JM, Gescher A*, et al.* CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. *Carcinogenesis* 1996; **17**: 277-282.
365. Barcelo S, Mace K, Pfeifer AM*, et al.* Production of DNA strand breaks by N-nitrosodimethylamine and 2-amino-3-methylimidazo[4,5-f]quinoline in THLE cells expressing human CYP isoenzymes and inhibition by sulforaphane. *Mutation research* 1998; **402**: 111-120.
366. Bacon JR, Williamson G, Garner RC*, et al.* Sulforaphane and quercetin modulate PhIP-DNA adduct formation in human HepG2 cells and hepatocytes. *Carcinogenesis* 2003; **24**: 1903-1911.
367. Rendic S, Guengerich FP. Contributions of Human Enzymes in Carcinogen Metabolism. *Chemical research in toxicology* 2012; **25**: 1316-1383.
368. Maheo K, Morel F, Langouet S*, et al.* Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. *Cancer research* 1997; **57**: 3649-3652.
369. Gross-Steinmeyer K, Stapleton PL, Tracy JH*, et al.* Sulforaphane- and phenethyl isothiocyanate-induced inhibition of aflatoxin B1-mediated genotoxicity in human hepatocytes: role of GSTM1 genotype and CYP3A4 gene expression. *Toxicological sciences : an official journal of the Society of Toxicology* 2010; **116**: 422-432.
370. Zhou C, Poulton EJ, Grun F*, et al.* The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Molecular pharmacology* 2007; **71**: 220-229.
371. Kensler TW. Chemoprevention by inducers of carcinogen detoxication enzymes. *Environmental health perspectives* 1997; **105 Suppl 4**: 965-970.
372. Vargas-Mendoza N, Morales-Gonzalez A, Madrigal-Santillan EO*, et al.* Antioxidant and Adaptative Response Mediated by Nrf2 during Physical Exercise. *Antioxidants (Basel, Switzerland)* 2019; **8**.

373. Chen Y, Shertzer HG, Schneider SN*, et al.* Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels. *The Journal of biological chemistry* 2005; **280**: 33766-33774.
374. Hu R, Xu C, Shen G*, et al.* Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (−/−) mice. *Cancer letters* 2006; **243**: 170-192.
375. Thimmulappa RK, Mai KH, Srisuma S*, et al.* Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray. *Cancer research* 2002; **62**: 5196-5203.
376. Wu L, Juurlink BH. The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats. *Journal of hypertension* 2001; **19**: 1819-1825.
377. Jiang ZQ, Chen C, Yang B*, et al.* Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers. *Life sciences* 2003; **72**: 2243-2253.
378. Basten GP, Bao Y, Williamson G. Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. *Carcinogenesis* 2002; **23**: 1399-1404.
379. Zhang Y, Gordon GB. A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. *Molecular cancer therapeutics* 2004; **3**: 885-893.
380. Dinkova-Kostova AT, Holtzclaw WD, Kensler TW. The role of Keap1 in cellular protective responses. *Chemical research in toxicology* 2005; **18**: 1779-1791.
381. Kensler TW, Egner PA, Agyeman AS*, et al.* Keap1-nrf2 signaling: a target for cancer prevention by sulforaphane. *Topics in current chemistry* 2013; **329**: 163-177.
382. Hong F, Freeman ML, Liebler DC. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chemical research in toxicology* 2005; **18**: 1917-1926.
383. Dinkova-Kostova AT, Holtzclaw WD, Cole RN*, et al.* Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99**: 11908-11913.
384. Tao S, Rojo de la Vega M, Chapman E*, et al.* The effects of NRF2 modulation on the initiation and progression of chemically and genetically induced lung cancer. *Mol Carcinog* 2018; **57**: 182-192.
385. Shorey LE, Madeen EP, Atwell LL*, et al.* Differential modulation of dibenzo[def,p]chrysene transplacental carcinogenesis: maternal diets rich in indole-3-carbinol versus sulforaphane. *Toxicol Appl Pharmacol* 2013; **270**: 60-69.
386. Shishu, Kaur IP. Inhibition of mutagenicity of food-derived heterocyclic amines by sulforaphane--a constituent of broccoli. *Indian journal of experimental biology* 2003; **41**: 216-219.
387. Singletary K, MacDonald C. Inhibition of benzo[a]pyrene- and 1,6-dinitropyrene-DNA adduct formation in human mammary epithelial cells bydibenzoylmethane and sulforaphane. *Cancer letters* 2000; **155**: 47-54.
388. Bonnesen C, Eggleston IM, Hayes JD. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer research* 2001; **61**: 6120-6130.
389. Fimognari C, Berti F, Cantelli-Forti G*, et al.* Effect of sulforaphane on micronucleus induction in cultured human lymphocytes by four different mutagens. *Environmental and molecular mutagenesis* 2005; **46**: 260-267.
390. Gills JJ, Jeffery EH, Matusheski NV*, et al.* Sulforaphane prevents mouse skin tumorigenesis during the stage of promotion. *Cancer letters* 2006; **236**: 72-79.

391. Kuroiwa Y, Nishikawa A, Kitamura Y*, et al.* Protective effects of benzyl isothiocyanate and sulforaphane but not resveratrol against initiation of pancreatic carcinogenesis in hamsters. *Cancer letters* 2006; **241**: 275-280.
392. Chiao JW, Chung FL, Kancherla R*, et al.* Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. *International journal of oncology* 2002; **20**: 631-636.
393. Shan Y, Sun C, Zhao X*, et al.* Effect of sulforaphane on cell growth, G(0)/G(1) phase cell progression and apoptosis in human bladder cancer T24 cells. *International journal of oncology* 2006; **29**: 883-888.
394. Tang L, Zhang Y. Dietary isothiocyanates inhibit the growth of human bladder carcinoma cells. *The Journal of nutrition* 2004; **134**: 2004-2010.
395. Jackson SJT, Singletary KW. Sulforaphane Inhibits Human MCF-7 Mammary Cancer Cell Mitotic Progression and Tubulin Polymerization. *The Journal of nutrition* 2004; **134**: 2229-2236.
396. Parnaud G, Li P, Cassar G*, et al.* Mechanism of sulforaphane-induced cell cycle arrest and apoptosis in human colon cancer cells. *Nutrition and cancer* 2004; **48**: 198-206.
397. Pham N-A, Jacobberger JW, Schimmer AD*, et al.* The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. *Molecular cancer therapeutics* 2004; **3**: 1239-1248.
398. Singh SV, Herman-Antosiewicz A, Singh AV*, et al.* Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. *The Journal of biological chemistry* 2004; **279**: 25813-25822.
399. Wang L, Liu D, Ahmed T*, et al.* Targeting cell cycle machinery as a molecular mechanism of sulforaphane in prostate cancer prevention. *International journal of oncology* 2004; **24**: 187-192.
400. Gamet-Payrastre L, Li P, Lumeau S*, et al.* Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer research* 2000; **60**: 1426-1433.
401. Wang M, Chen S, Wang S*, et al.* Effects of phytochemicals sulforaphane on uridine diphosphate-glucuronosyltransferase expression as well as cell-cycle arrest and apoptosis in human colon cancer Caco-2 cells. *The Chinese journal of physiology* 2012; **55**: 134-144.
402. Kim MR, Zhou L, Park BH*, et al.* Induction of G(2)/M arrest and apoptosis by sulforaphane in human osteosarcoma U2-OS cells. *Molecular medicine reports* 2011; **4**: 929-934.
403. Kim JH, Han Kwon K, Jung JY*, et al.* Sulforaphane Increases Cyclin-Dependent Kinase Inhibitor, p21 Protein in Human Oral Carcinoma Cells and Nude Mouse Animal Model to Induce G(2)/M Cell Cycle Arrest. *Journal of clinical biochemistry and nutrition* 2010; **46**: 60-67.
404. Suppipat K, Park CS, Shen Y*, et al.* Sulforaphane induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells. *PloS one* 2012; **7**: e51251-e51251.
405. Jakubikova J, Cervi D, Ooi M*, et al.* Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate, in multiple myeloma. *Haematologica* 2011; **96**: 1170-1179.
406. Jackson SJT, Singletary KW. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis* 2004; **25**: 219-227.
407. Reed JC. Apoptosis-targeted therapies for cancer. *Cancer cell* 2003; **3**: 17-22.
408. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology* 2007; **35**: 495-516.
409. Gamet-Payrastre L, Lumeau S, Gasc N*, et al.* Selective cytostatic and cytotoxic effects of glucosinolates hydrolysis products on human colon cancer cells in vitro. *Anti-cancer drugs* 1998; **9**: 141-148.

410. Jakubíková J, Sedlák J, Mithen R*, et al.* Role of PI3K/Akt and MEK/ERK signaling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription, G2/M arrest and cell death in Caco-2 cells. *Biochemical Pharmacology* 2005; **69**: 1543-1552.
411. Choi S, Lew KL, Xiao H*, et al.* D,L-Sulforaphane-induced cell death in human prostate cancer cells is regulated by inhibitor of apoptosis family proteins and Apaf-1. *Carcinogenesis* 2007; **28**: 151-162.
412. Singh AV, Xiao D, Lew KL*, et al.* Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts in vivo. *Carcinogenesis* 2004; **25**: 83-90.
413. Gingras D, Gendron M, Boivin D*, et al.* Induction of medulloblastoma cell apoptosis by sulforaphane, a dietary anticarcinogen from Brassica vegetables. *Cancer letters* 2004; **203**: 35-43.
414. Chaudhuri D, Orsulic S, Ashok BT. Antiproliferative activity of sulforaphane in Akt-overexpressing ovarian cancer cells. *Molecular cancer therapeutics* 2007; **6**: 334-345.
415. Fimognari C, Nusse M, Berti F*, et al.* Sulforaphane modulates cell cycle and apoptosis in transformed and non-transformed human T lymphocytes. *Annals of the New York Academy of Sciences* 2003; **1010**: 393-398.
416. Fimognari C, Nusse M, Cesari R*, et al.* Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. *Carcinogenesis* 2002; **23**: 581-586.
417. Misiewicz I, Skupinska K, Kasprzycka-Guttman T. Sulforaphane and 2-oxohexyl isothiocyanate induce cell growth arrest and apoptosis in L-1210 leukemia and ME-18 melanoma cells. *Oncology reports* 2003; **10**: 2045-2050.
418. Pappa G, Lichtenberg M, Iori R*, et al.* Comparison of growth inhibition profiles and mechanisms of apoptosis induction in human colon cancer cell lines by isothiocyanates and indoles from Brassicaceae. *Mutation research* 2006; **599**: 76-87.
419. Park HS, Han MH, Kim GY*, et al.* Sulforaphane induces reactive oxygen species-mediated mitotic arrest and subsequent apoptosis in human bladder cancer 5637 cells. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2014; **64**: 157-165.
420. Park SY, Kim GY, Bae SJ*, et al.* Induction of apoptosis by isothiocyanate sulforaphane in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells through activation of caspase-3. *Oncology reports* 2007; **18**: 181-187.
421. Devi JR, Thangam EB. Mechanisms of anticancer activity of sulforaphane from Brassica oleracea in HEp-2 human epithelial carcinoma cell line. *Asian Pacific journal of cancer prevention : APJCP* 2012; **13**: 2095-2100.
422. Cho NP, Han HS, Leem DH*, et al.* Sulforaphane enhances caspase-dependent apoptosis through inhibition of cyclooxygenase-2 expression in human oral squamous carcinoma cells and nude mouse xenograft model. *Oral oncology* 2009; **45**: 654-660.
423. Cho SD, Li G, Hu H*, et al.* Involvement of c-Jun N-terminal kinase in G2/M arrest and caspase-mediated apoptosis induced by sulforaphane in DU145 prostate cancer cells. *Nutrition and cancer* 2005; **52**: 213-224.
424. Moon DO, Kim MO, Kang SH*, et al.* Sulforaphane suppresses TNF-alpha-mediated activation of NF-kappaB and induces apoptosis through activation of reactive oxygen species-dependent caspase-3. *Cancer letters* 2009; **274**: 132-142.
425. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. *Cancer research* 2006; **66**: 5828-5835.

426. Jeong WS, Kim IW, Hu R*, et al.* Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway. *Pharmaceutical research* 2004; **21**: 661-670.
427. Xu C, Shen G, Chen C*, et al.* Suppression of NF-kappaB and NF-kappaB-regulated gene expression by sulforaphane and PEITC through IkappaBalpha, IKK pathway in human prostate cancer PC-3 cells. *Oncogene* 2005; **24**: 4486-4495.
428. Ramirez MC, Singletary K. Regulation of estrogen receptor alpha expression in human breast cancer cells by sulforaphane. *The Journal of nutritional biochemistry* 2009; **20**: 195-201.
429. Kim H, Kim EH, Eom YW*, et al.* Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant hepatoma cells to TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of DR5. *Cancer research* 2006; **66**: 1740-1750.
430. Matsui TA, Sowa Y, Yoshida T*, et al.* Sulforaphane enhances TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells. *Carcinogenesis* 2006; **27**: 1768-1777.
431. Myzak MC, Karplus PA, Chung FL*, et al.* A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer research* 2004; **64**: 5767-5774.
432. Myzak MC, Hardin K, Wang R*, et al.* Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. *Carcinogenesis* 2006; **27**: 811-819.
433. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *Journal of the National Cancer Institute* 2000; **92**: 1210-1216.
434. Ungerstedt JS, Sowa Y, Xu WS*, et al.* Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 2005; **102**: 673-678.
435. Tortorella SM, Royce SG, Licciardi PV*, et al.* Dietary Sulforaphane in Cancer Chemoprevention: The Role of Epigenetic Regulation and HDAC Inhibition. *Antioxid Redox Signal* 2015; **22**: 1382-1424.
436. Cuervo AM. Autophagy: Many paths to the same end. *Molecular and Cellular Biochemistry* 2004; **263**: 55-72.
437. Jia L, Dourmashkin RR, Allen PD*, et al.* Inhibition of autophagy abrogates tumour necrosis factor α induced apoptosis in human T-lymphoblastic leukaemic cells. *British Journal of Haematology* 1997; **98**: 673-685.
438. Kanematsu S, Uehara N, Miki H*, et al.* Autophagy inhibition enhances sulforaphane-induced apoptosis in human breast cancer cells. *Anticancer research* 2010; **30**: 3381-3390.
439. Beaver LM, Kuintzle R, Buchanan A*, et al.* Long noncoding RNAs and sulforaphane: a target for chemoprevention and suppression of prostate cancer. *The Journal of nutritional biochemistry* 2017; **42**: 72-83.
440. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249.
441. Goldmann E. THE GROWTH OF MALIGNANT DISEASE IN MAN AND THE LOWER ANIMALS,: WITH SPECIAL REFERENCE TO THE VASCULAR SYSTEM. *The Lancet* 1907; **170**: 1236-1240.
442. Algire GH, Chalkley HW, Legallais FY*, et al.* Vasculae Reactions of Normal and Malignant Tissues in Vivo. I. Vascular Reactions of Mice to Wounds and to Normal and Neoplastic Transplants. *JNCI: Journal of the National Cancer Institute* 1945; **6**: 73-85.
443. Ide AG. Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. *AJR Am J Roentgenol* 1939; **42**: 891-899.
444. Greenblatt M, Philippe SK. Tumor Angiogenesis: Transfilter Diffusion Studies in the Hamster by the Transparent Chamber Technique2. *JNCI: Journal of the National Cancer Institute* 1968; **41**: 111-124.

445. Ehrmann RL, Knoth M. Choriocarcinoma: Transfilter Stimulation of Vasoproliferation in the Hamster Cheek Pouch—Studied by Light and Electron Microscopy2. *JNCI: Journal of the National Cancer Institute* 1968; **41**: 1329-1341.
446. Gupta GK, Milner L, Linshaw MA*, et al.* Urinary basic fibroblast growth factor: A noninvasive marker of progressive cystic renal disease in a child. *American Journal of Medical Genetics* 2000; **93**: 132-135.
447. Nishida N, Yano H, Nishida T*, et al.* Angiogenesis in cancer. *Vascular health and risk management* 2006; **2**: 213-219.
448. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* 2005; **69 Suppl 3**: 4-10.
449. Bertl E, Bartsch H, Gerhauser C. Inhibition of angiogenesis and endothelial cell functions are novel sulforaphane-mediated mechanisms in chemoprevention. *Molecular cancer therapeutics* 2006; **5**: 575-585.
450. Bao Y, Wang W, Zhou Z*, et al.* Benefits and risks of the hormetic effects of dietary isothiocyanates on cancer prevention. *PloS one* 2014; **9**: e114764-e114764.
451. Asakage M, Tsuno NH, Kitayama J*, et al.* Sulforaphane induces inhibition of human umbilical vein endothelial cells proliferation by apoptosis. *Angiogenesis* 2006; **9**: 83-91.
452. Jackson SJ, Singletary KW, Venema RC. Sulforaphane suppresses angiogenesis and disrupts endothelial mitotic progression and microtubule polymerization. *Vascular pharmacology* 2007; **46**: 77-84.
453. Netland PA, Zetter BR. Tumor-Cell Interactions with Blood Vessels during Cancer Metastasis. In: Fundamental Aspects of Cancer. Goldfarb RH, (ed)^(eds). Springer Netherlands: Dordrecht, 1989; 84-97.
454. Liotta LA. Tumor invasion and metastases: role of the basement membrane. Warner-Lambert Parke-Davis Award lecture. *The American journal of pathology* 1984; **117**: 339-348.
455. Fidler IJ. Tumor Heterogeneity and the Biology of Cancer Invasion and Metastasis. *Cancer research* 1978; **38**: 2651-2660.
456. Liotta LA. Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer research* 1986; **46**: 1-7.
457. Liotta LA, Tryggvason K, Garbisa S*, et al.* Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 1980; **284**: 67.
458. Nicolson GL. Cancer metastasis. Organ colonization and the cell-surface properties of malignant cells. *Biochimica et biophysica acta* 1982; **695**: 113-176.
459. Thejass P, Kuttan G. Antimetastatic activity of Sulforaphane. *Life sciences* 2006; **78**: 3043-3050.
460. Chambers AF, Matrisian LM. Changing Views of the Role of Matrix Metalloproteinases in Metastasis. *JNCI: Journal of the National Cancer Institute* 1997; **89**: 1260-1270.
461. Stetler-Stevenson WG, Hewitt R, Corcoran M. Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. *Seminars in cancer biology* 1996; **7**: 147-154.
462. Haizhen Z, Bowen JP. Antiangiogenesis Drug Design: Multiple Pathways Targeting Tumor Vasculature. *Current Medicinal Chemistry* 2006; **13**: 849-862.
463. Jee H-G, Lee KE, Kim JB*, et al.* Sulforaphane Inhibits Oral Carcinoma Cell Migration and Invasion In Vitro. *Phytotherapy Research* 2011; **25**: 1623-1628.
464. Kanematsu S, Yoshizawa K, Uehara N*, et al.* Sulforaphane inhibits the growth of KPL-1 human breast cancer cells in vitro and suppresses the growth and metastasis of orthotopically transplanted KPL-1 cells in female athymic mice. *Oncology reports* 2011; **26**: 603-608.
465. Wang DX, Zou YJ, Zhuang XB*, et al.* Sulforaphane suppresses EMT and metastasis in human lung cancer through miR-616-5p-mediated GSK3beta/beta-catenin signaling pathways. *Acta Pharmacol Sin* 2017; **38**: 241-251.

466. Bouhassira EE. The SAGE Encyclopedia of Stem Cell Research. In. Second Edition ed. (ed)^(eds): Thousand Oaks, California, 2015.
467. Li Y, Zhang T. Targeting cancer stem cells with sulforaphane, a dietary component from broccoli and broccoli sprouts. *Future oncology (London, England)* 2013; **9**: 1097-1103.
468. Kallifatidis G, Rausch V, Baumann B*, et al.* Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. *Gut* 2009; **58**: 949-963.
469. Tang SN, Fu J, Nall D*, et al.* Inhibition of sonic hedgehog pathway and pluripotency maintaining factors regulate human pancreatic cancer stem cell characteristics. *International journal of cancer* 2012; **131**: 30-40.
470. Munoz P, Iliou MS, Esteller M. Epigenetic alterations involved in cancer stem cell reprogramming. *Molecular oncology* 2012; **6**: 620-636.
471. Canettieri G, Di Marcotullio L, Greco A*, et al.* Histone deacetylase and Cullin3-REN(KCTD11) ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. *Nature cell biology* 2010; **12**: 132-142.
472. Nakashima H, Nakamura M, Yamaguchi H*, et al.* Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer research* 2006; **66**: 7041-7049.
473. Li SH, Fu J, Watkins DN*, et al.* Sulforaphane regulates self-renewal of pancreatic cancer stem cells through the modulation of Sonic hedgehog-GLI pathway. *Mol Cell Biochem* 2013; **373**: 217-227.
474. Ali Khan M, Kedhari Sundaram M, Hamza A*, et al.* Sulforaphane Reverses the Expression of Various Tumor Suppressor Genes by Targeting DNMT3B and HDAC1 in Human Cervical Cancer Cells. *Evidence-based complementary and alternative medicine : eCAM* 2015; **2015**: 412149.
475. Iwatsuki M, Mimori K, Yokobori T*, et al.* Epithelial-mesenchymal transition in cancer development and its clinical significance. *Cancer science* 2010; **101**: 293-299.
476. Liu X, Fan D. The epithelial-mesenchymal transition and cancer stem cells: functional and mechanistic links. *Current pharmaceutical design* 2015; **21**: 1279-1291.
477. Srivastava RK, Tang SN, Zhu W*, et al.* Sulforaphane synergizes with quercetin to inhibit self-renewal capacity of pancreatic cancer stem cells. *Frontiers in bioscience (Elite edition)* 2011; **3**: 515-528.
478. Shan Y, Zhang L, Bao Y*, et al.* Epithelial-mesenchymal transition, a novel target of sulforaphane via COX-2/MMP2, 9/Snail, ZEB1 and miR-200c/ZEB1 pathways in human bladder cancer cells. *The Journal of nutritional biochemistry* 2013; **24**: 1062-1069.
479. Fresno Vara JA, Casado E, de Castro J*, et al.* PI3K/Akt signalling pathway and cancer. *Cancer treatment reviews* 2004; **30**: 193-204.
480. Valkenburg KC, Graveel CR, Zylstra-Diegel CR*, et al.* Wnt/β-catenin Signaling in Normal and Cancer Stem Cells. *Cancers* 2011; **3**: 2050-2079.
481. Ginestier C, Hur MH, Charafe-Jauffret E*, et al.* ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell stem cell* 2007; **1**: 555-567.
482. Li Y, Zhang T, Korkaya H*, et al.* Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2010; **16**: 2580-2590.
483. Kato M, Slack FJ. microRNAs: small molecules with big roles –C. elegans to human cancer. *Biology of the Cell* 2008; **100**: 71-81.
484. Yu S-L, Chen H-Y, Chang G-C*, et al.* MicroRNA Signature Predicts Survival and Relapse in Lung Cancer. *Cancer cell* 2008; **13**: 48-57.

485. Seike M, Goto A, Okano T*, et al.* MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. *Proceedings of the National Academy of Sciences* 2009; **106**: 12085-12090.
486. Iorio MV, Casalini P, Tagliabue E*, et al.* MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. *European Journal of Cancer* 2008; **44**: 2753-2759.
487. Calin GA, Dumitru CD, Shimizu M*, et al.* Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences* 2002; **99**: 15524-15529.
488. Calin GA, Croce CM. MicroRNAs and chromosomal abnormalities in cancer cells. *Oncogene* 2006; **25**: 6202.
489. Ventura A, Jacks T. MicroRNAs and Cancer: Short RNAs Go a Long Way. *Cell* 2009; **136**: 586-591.
490. Li J, Huang H, Sun L*, et al.* MiR-21 Indicates Poor Prognosis in Tongue Squamous Cell Carcinomas as an Apoptosis Inhibitor. *Clinical Cancer Research* 2009; **15**: 3998-4008.
491. Liu C-J, Tsai M-M, Hung P-S*, et al.* miR-31 Ablates Expression of the HIF Regulatory Factor FIH to Activate the HIF Pathway in Head and Neck Carcinoma. *Cancer research* 2010; **70**: 1635-1644.
492. Yang MH, Lin BR, Chang CH*, et al.* Connective tissue growth factor modulates oral squamous cell carcinoma invasion by activating a miR-504/FOXP1 signalling. *Oncogene* 2011; **31**: 2401.
493. Lu Y-C, Chen Y-J, Wang H-M*, et al.* Oncogenic Function and Early Detection Potential of miRNA-10b in Oral Cancer as Identified by microRNA Profiling. *Cancer Prevention Research* 2012; **5**: 665-674.
494. Yu C-C, Chen Y-W, Chiou G-Y*, et al.* MicroRNA let-7a represses chemoresistance and tumourigenicity in head and neck cancer via stem-like properties ablation. *Oral oncology* 2011; **47**: 202-210.
495. Li Q, Yao Y, Eades G*, et al.* Downregulation of miR-140 promotes cancer stem cell formation in basal-like early stage breast cancer. *Oncogene* 2013; **33**: 2589.
496. Li X, Zhao Z, Li M*, et al.* Sulforaphane promotes apoptosis, and inhibits proliferation and self-renewal of nasopharyngeal cancer cells by targeting STAT signal through miRNA-124-3p. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2018; **103**: 473-481.
497. Corbin AS, Agarwal A, Loriaux M*, et al.* Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *The Journal of clinical investigation* 2011; **121**: 396-409.
498. Chen H, Landen CN, Li Y*, et al.* Enhancement of Cisplatin-Mediated Apoptosis in Ovarian Cancer Cells through Potentiating G2/M Arrest and p21 Upregulation by Combinatorial Epigallocatechin Gallate and Sulforaphane. *Journal of oncology* 2013; **2013**: 9.
499. Wang X, Li Y, Dai Y*, et al.* Sulforaphane improves chemotherapy efficacy by targeting cancer stem cell-like properties via the miR-124/IL-6R/STAT3 axis. *Scientific reports* 2016; **6**: 36796.
500. Elkashty OA, Ashry R, Elghanam GA*, et al.* Broccoli extract improves chemotherapeutic drug efficacy against head-neck squamous cell carcinomas. *Med Oncol* 2018; **35**: 124.
501. Fimognari C, Nusse M, Lenzi M*, et al.* Sulforaphane increases the efficacy of doxorubicin in mouse fibroblasts characterized by p53 mutations. *Mutation research* 2006; **601**: 92-101.
502. Fimognari C, Lenzi M, Sciuscio D*, et al.* Combination of doxorubicin and sulforaphane for reversing doxorubicin-resistant phenotype in mouse fibroblasts with p53Ser220 mutation. *Annals of the New York Academy of Sciences* 2007; **1095**: 62-69.
503. Kaminski BM, Steinhilber D, Stein JM*, et al.* Phytochemicals resveratrol and sulforaphane as potential agents for enhancing the anti-tumor activities of conventional cancer therapies. *Current pharmaceutical biotechnology* 2012; **13**: 137-146.
504. Dosz EB, Jeffery EH. Commercially produced frozen broccoli lacks the ability to form sulforaphane. *Journal of Functional Foods* 2013; **5**: 987-990.

505. Houghton CA, Fassett RG, Coombes JS. Sulforaphane: translational research from laboratory bench to clinic. *Nutrition reviews* 2013; **71**: 709-726.
506. Cipolla BG, Mandron E, Lefort JM*, et al.* Effect of Sulforaphane in Men with Biochemical Recurrence after Radical Prostatectomy. *Cancer Prevention Research* 2015; **8**: 712-719.
507. Kensler TW, Chen JG, Egner PA*, et al.* Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2005; **14**: 2605-2613.
508. Egner PA, Chen JG, Wang JB*, et al.* Bioavailability of Sulforaphane from two broccoli sprout beverages: results of a short-term, cross-over clinical trial in Qidong, China. *Cancer prevention research (Philadelphia, Pa)* 2011; **4**: 384-395.
509. Kensler TW, Ng D, Carmella SG*, et al.* Modulation of the metabolism of airborne pollutants by glucoraphanin-rich and sulforaphane-rich broccoli sprout beverages in Qidong, China. *Carcinogenesis* 2012; **33**: 101-107.
510. Fahey JW, Wehage SL, Holtzclaw WD*, et al.* Protection of humans by plant glucosinolates: efficiency of conversion of glucosinolates to isothiocyanates by the gastrointestinal microflora. *Cancer prevention research (Philadelphia, Pa)* 2012; **5**: 603-611.
511. Riedl MA, Saxon A, Diaz-Sanchez D. Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. *Clinical immunology (Orlando, Fla)* 2009; **130**: 244-251.
512. Alumkal JJ, Slottke R, Schwartzman J*, et al.* A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer. *Investigational new drugs* 2015; **33**: 480-489.
513. Li Q-Q, Xie Y-K, Wu Y*, et al.* Sulforaphane inhibits cancer stem-like cell properties and cisplatin resistance through miR-214-mediated downregulation of c-MYC in non-small cell lung cancer. *Oncotarget* 2017; **8**: 12067-12080.
514. Suh N, Luyengi L, Fong HH*, et al.* Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. *Anticancer research* 1995; **15**: 233-239.
515. Shapiro TA, Fahey JW, Dinkova-Kostova AT*, et al.* Safety, Tolerance, and Metabolism of Broccoli Sprout Glucosinolates and Isothiocyanates: A Clinical Phase I Study. *Nutrition and cancer* 2006; **55**: 53-62.
516. Myzak MC, Tong P, Dashwood W-M*, et al.* Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Experimental biology and medicine (Maywood, NJ)* 2007; **232**: 227-234.
517. Traka M, Gasper AV, Melchini A*, et al.* Broccoli consumption interacts with GSTM1 to perturb oncogenic signalling pathways in the prostate. *PloS one* 2008; **3**: e2568-e2568.
518. Hanlon N, Coldham N, Gielbert A*, et al.* Repeated intake of broccoli does not lead to higher plasma levels of sulforaphane in human volunteers. *Cancer letters* 2009; **284**: 15-20.
519. Bahadoran Z, Mirmiran P, Hosseinpanah F*, et al.* Broccoli sprouts reduce oxidative stress in type 2 diabetes: a randomized double-blind clinical trial. *European Journal Of Clinical Nutrition* 2011; **65**: 972.
520. Zhang Z, Garzotto M, Davis EW*, et al.* Sulforaphane Bioavailability and Chemopreventive Activity in Men Presenting for Biopsy of the Prostate Gland: A Randomized Controlled Trial. *Nutrition and cancer* 2019: 1-14.
521. Study to Evaluate the Effect of Sulforaphane in Broccoli Sprout Extract on Breast Tissue: Available from: https://clinicaltrials.gov/ct2/show/study/NCT00982319
522. Tahata S, Singh SV, Lin Y*, et al.* Evaluation of Biodistribution of Sulforaphane after Administration of Oral Broccoli Sprout Extract in Melanoma Patients with Multiple Atypical Nevi. *Cancer prevention research (Philadelphia, Pa)* 2018; **11**: 429-438.

523. Traka MH, Melchini A, Coode-Bate J*, et al.* Transcriptional changes in prostate of men on active surveillance after a 12-mo glucoraphanin-rich broccoli intervention—results from the Effect of Sulforaphane on prostate CAncer PrEvention (ESCAPE) randomized controlled trial. *The American journal of clinical nutrition* 2019; **109**: 1133-1144.
524. Atwell LL, Zhang Z, Mori M*, et al.* Sulforaphane Bioavailability and Chemopreventive Activity in Women Scheduled for Breast Biopsy. *Cancer prevention research (Philadelphia, Pa)* 2015; **8**: 1184-1191.
525. Protective Effects of the Nutritional Supplement Sulforaphane on Doxorubicin-Associated Cardiac Dysfunction: Available from: https://clinicaltrials.gov/ct2/show/NCT03934905?term=sulforaphane&rank=1
526. Fitzmaurice C, Allen C, Barber RM*, et al.* Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. *JAMA oncology* 2017; **3**: 524-548.
527. Jemal A, Bray F, Center MM*, et al.* Global cancer statistics. *CA: a cancer journal for clinicians* 2011; **61**: 69-90.
528. Jemal A, Siegel R, Ward E*, et al.* Cancer statistics, 2008. *CA: a cancer journal for clinicians* 2008; **58**: 71-96.
529. Parkin DM, Bray F, Ferlay J*, et al.* Global cancer statistics, 2002. *CA: a cancer journal for clinicians* 2005; **55**: 74-108.
530. Lin LC, Yeh CT, Kuo CC*, et al.* Sulforaphane potentiates the efficacy of imatinib against chronic leukemia cancer stem cells through enhanced abrogation of Wnt/beta-catenin function. *J Agric Food Chem* 2012; **60**: 7031-7039.
531. Pham NA, Jacobberger JW, Schimmer AD*, et al.* The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. *Molecular cancer therapeutics* 2004; **3**: 1239-1248.
532. Schwarz S, Rotter N. Human salivary gland stem cells: isolation, propagation, and characterization. *Methods in molecular biology (Clifton, NJ)* 2012; **879**: 403-442.
533. Abdallah MN, Abdollahi S, Laurenti M*, et al.* Scaffolds for epithelial tissue engineering customized in elastomeric molds. *Journal of biomedical materials research Part B, Applied biomaterials* 2017.
534. Expasy.org. Available from: http://web.expasy.org/cellosaurus/
535. Brenner JC, Graham MP, Kumar B*, et al.* Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head Neck* 2010; **32**: 417-426.
536. Grenman R, Carey TE, McClatchey KD*, et al.* In vitro radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. *Cancer* 1991; **67**: 2741-2747.
537. Carey TE, Van Dyke DL, Worsham MJ. Nonrandom chromosome aberrations and clonal populations in head and neck cancer. *Anticancer research* 1993; **13**: 2561-2567.
538. Nagel R, Martens-de Kemp SR, Buijze M*, et al.* Treatment response of HPV-positive and HPV-negative head and neck squamous cell carcinoma cell lines. *Oral oncology* 2013; **49**: 560-566.
539. Pekkola-Heino K, Joensuu H, Klemi P*, et al.* Relation of DNA ploidy and proliferation rate to radiation sensitivity in squamous carcinoma cell lines. *Archives of otolaryngology--head & neck surgery* 1994; **120**: 750-754.
540. Bradford CR, Zhu S, Ogawa H*, et al.* P53 mutation correlates with cisplatin sensitivity in head and neck squamous cell carcinoma lines. *Head Neck* 2003; **25**: 654-661.

541. Martens-de Kemp SR, Dalm SU, Wijnolts FM*, et al.* DNA-bound platinum is the major determinant of cisplatin sensitivity in head and neck squamous carcinoma cells. *PloS one* 2013; **8**: e61555.
542. Wang L, Mosel AJ, Oakley GG*, et al.* Deficient DNA damage signaling leads to chemoresistance to cisplatin in oral cancer. *Molecular cancer therapeutics* 2012; **11**: 2401-2409.
543. Vermorken JB, Specenier P. Optimal treatment for recurrent/metastatic head and neck cancer. *Ann Oncol* 2010; **21 Suppl 7**: vii252-261.
544. Kim JH, Han Kwon K, Jung JY*, et al.* Sulforaphane Increases Cyclin-Dependent Kinase Inhibitor, p21 Protein in Human Oral Carcinoma Cells and Nude Mouse Animal Model to Induce G(2)/M Cell Cycle Arrest. *Journal of clinical biochemistry and nutrition* 2010; **46**: 60-67.
545. Jee HG, Lee KE, Kim JB*, et al.* Sulforaphane inhibits oral carcinoma cell migration and invasion in vitro. *Phytotherapy research : PTR* 2011; **25**: 1623-1628.
546. Suppipat K, Park CS, Shen Y*, et al.* Sulforaphane induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells. *PloS one* 2012; **7**: e51251.
547. Wang X, Li Y, Dai Y*, et al.* Sulforaphane improves chemotherapy efficacy by targeting cancer stem cell-like properties via the miR-124/IL-6R/STAT3 axis. *Scientific reports* 2016; **6**: 36796.
548. Al-Dimassi S, Abou-Antoun T, El-Sibai M. Cancer cell resistance mechanisms: a mini review. *Clinical and Translational Oncology* 2014; **16**: 511-516.
549. Ferreira CG, Epping M, Kruyt FA*, et al.* Apoptosis: target of cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2002; **8**: 2024-2034.
550. Cryns V, Yuan J. Proteases to die for. *Genes & development* 1998; **12**: 1551-1570.
551. Min K-j, Kwon TK. Anticancer effects and molecular mechanisms of epigallocatechin-3-gallate. *Integrative Medicine Research* 2014; **3**: 16-24.
552. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature reviews Cancer* 2008; **8**: 755-768.
553. Donnenberg VS, Donnenberg AD. Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. *Journal of clinical pharmacology* 2005; **45**: 872-877.
554. Baumann M, Krause M, Hill R. Exploring the role of cancer stem cells in radioresistance. *Nature reviews Cancer* 2008; **8**: 545-554.
555. Jiang X, Zhao Y, Smith C*, et al.* Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. *Leukemia* 2007; **21**: 926-935.
556. Brabletz T, Jung A, Spaderna S*, et al.* Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nature reviews Cancer* 2005; **5**: 744-749.
557. Chinn SB, Darr OA, Owen JH*, et al.* Cancer stem cells: mediators of tumorigenesis and metastasis in head and neck squamous cell carcinoma. *Head Neck* 2015; **37**: 317-326.
558. Irani S, Jafari B. Expression of vimentin and CD44 in mucoepidermoid carcinoma: A role in tumor growth. *Indian Journal of Dental Research* 2018; **29**: 333-340.
559. Alsheddi M, Aljuaid A, Mohammed D. Expression of stem cell marker CD44 in selected benign and malignant salivary gland tumors. *Saudi Journal of Oral Sciences* 2018; **5**: 80-83.
560. Mishra A, Sriram H, Chandarana P*, et al.* Decreased expression of cell adhesion genes in cancer stem-like cells isolated from primary oral squamous cell carcinomas. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2018; **40**: 1010428318780859.
561. Oh SY, Kang HJ, Kim YS*, et al.* CD44-negative cells in head and neck squamous carcinoma also have stem-cell like traits. *European journal of cancer (Oxford, England : 1990)* 2013; **49**: 272-280.

562. Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2008; **26**: 2839-2845.
563. Coulon A, Flahaut M, Muhlethaler-Mottet A*, et al.* Functional sphere profiling reveals the complexity of neuroblastoma tumor-initiating cell model. *Neoplasia (New York, NY)* 2011; **13**: 991-1004.
564. Sheng X, Li Z, Wang DL*, et al.* Isolation and enrichment of PC-3 prostate cancer stem-like cells using MACS and serum-free medium. *Oncology letters* 2013; **5**: 787-792.
565. Mochizuki M, Tamai K, Imai T*, et al.* CD271 regulates the proliferation and motility of hypopharyngeal cancer cells. *Scientific reports* 2016; **6**: 30707-30707.
566. Okumura T, Tsunoda S, Mori Y*, et al.* The biological role of the low-affinity p75 neurotrophin receptor in esophageal squamous cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2006; **12**: 5096-5103.
567. Soland TM, Brusevold IJ, Koppang HS*, et al.* Nerve growth factor receptor (p75 NTR) and pattern of invasion predict poor prognosis in oral squamous cell carcinoma. *Histopathology* 2008; **53**: 62-72.
568. Fagan JJ, Collins B, Barnes L*, et al.* Perineural invasion in squamous cell carcinoma of the head and neck. *Archives of otolaryngology--head & neck surgery* 1998; **124**: 637-640.
569. Tosaki T, Kamiya H, Yasuda Y*, et al.* Reduced NGF secretion by Schwann cells under the high glucose condition decreases neurite outgrowth of DRG neurons. *Experimental neurology* 2008; **213**: 381-387.
570. Chan MM, Tahan SR. Low-affinity nerve growth factor receptor (P75 NGFR) as a marker of perineural invasion in malignant melanomas. *Journal of cutaneous pathology* 2010; **37**: 336-343.
571. Zhu Z, Friess H, diMola FF*, et al.* Nerve growth factor expression correlates with perineural invasion and pain in human pancreatic cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 1999; **17**: 2419-2428.
572. Kolokythas A, Cox DP, Dekker N*, et al.* Nerve growth factor and tyrosine kinase A receptor in oral squamous cell carcinoma: is there an association with perineural invasion? *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 2010; **68**: 1290-1295.
573. Tong D, Sun J, Huang P*, et al.* p75 neurotrophin receptor: A potential surface marker of tongue squamous cell carcinoma stem cells. *Molecular medicine reports* 2017; **15**: 2521-2529.
574. Jin H, Pan Y, Zhao L*, et al.* p75 neurotrophin receptor suppresses the proliferation of human gastric cancer cells. *Neoplasia (New York, NY)* 2007; **9**: 471-478.
575. Khwaja F, Tabassum A, Allen J*, et al.* The p75(NTR) tumor suppressor induces cell cycle arrest facilitating caspase mediated apoptosis in prostate tumor cells. *Biochemical and biophysical research communications* 2006; **341**: 1184-1192.
576. Liu S, Dontu G, Mantle ID*, et al.* Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer research* 2006; **66**: 6063-6071.
577. Diamandis P, Wildenhain J, Clarke ID*, et al.* Chemical genetics reveals a complex functional ground state of neural stem cells. *Nature chemical biology* 2007; **3**: 268-273.
578. Krishnamurthy S, Dong Z, Vodopyanov D*, et al.* Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer research* 2010; **70**: 9969-9978.
579. Nor C, Zhang Z, Warner KA*, et al.* Cisplatin induces Bmi-1 and enhances the stem cell fraction in head and neck cancer. *Neoplasia (New York, NY)* 2014; **16**: 137-146.
580. Mi Z, Rogers DA, Mirnics ZK*, et al.* p75NTR-dependent modulation of cellular handling of reactive oxygen species. *Journal of neurochemistry* 2009; **110**: 295-306.

581. Redmer T, Walz I, Klinger B*, et al.* The role of the cancer stem cell marker CD271 in DNA damage response and drug resistance of melanoma cells. *Oncogenesis* 2017; **6**: e291.
582. Yang Y, Zhou W, Xia J*, et al.* NEK2 mediates ALDH1A1-dependent drug resistance in multiple myeloma. *Oncotarget* 2014; **5**: 11986-11997.
583. Shi G, Jin Y. Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem Cell Res Ther* 2010; **1**: 39-39.
584. Osman TA, Parajuli H, Sapkota D*, et al.* The low-affinity nerve growth factor receptor p75NTR identifies a transient stem cell-like state in oral squamous cell carcinoma cells. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2015; **44**: 410-419.
585. Heron M. Deaths: Leading Causes for 2016. *National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System* 2018; **67**: 1-77.
586. Elkashty OA, Ashry R, Tran SD. Head and neck cancer management and cancer stem cells implication. *The Saudi Dental Journal* 2019.
587. Gregoire V, Lefebvre JL, Licitra L*, et al.* Squamous cell carcinoma of the head and neck: EHNS-ESMO-ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2010; **21 Suppl 5**: v184-186.
588. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nature reviews Cancer* 2005; **5**: 275-284.
589. Bakkar M, Liu Y, Fang D*, et al.* A Simplified and Systematic Method to Isolate, Culture, and Characterize Multiple Types of Human Dental Stem Cells from a Single Tooth. *Methods in molecular biology (Clifton, NJ)* 2017; **1553**: 191-207.
590. Elkashty OA, Abu Elghanam G, Su X*, et al.* Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas. *Carcinogenesis* 2019.
591. Labsch S, Liu L, Bauer N*, et al.* Sulforaphane and TRAIL induce a synergistic elimination of advanced prostate cancer stem-like cells. *International journal of oncology* 2014; **44**: 1470-1480.
592. Rausch V, Liu L, Kallifatidis G*, et al.* Synergistic activity of sorafenib and sulforaphane abolishes pancreatic cancer stem cell characteristics. *Cancer research* 2010; **70**: 5004-5013.
593. Zhou W, Kallifatidis G, Baumann B*, et al.* Dietary polyphenol quercetin targets pancreatic cancer stem cells. *International journal of oncology* 2010; **37**: 551-561.
594. Alkharashi NAO, Periasamy VS, Athinarayanan J*, et al.* Assessment of sulforaphane-induced protective mechanisms against cadmium toxicity in human mesenchymal stem cells. *Environmental science and pollution research international* 2018; **25**: 10080-10089.
595. Li Q, Xia J, Yao Y*, et al.* Sulforaphane inhibits mammary adipogenesis by targeting adipose mesenchymal stem cells. *Breast cancer research and treatment* 2013; **141**: 317-324.
596. Zanichelli F, Capasso S, Cipollaro M*, et al.* Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect. *Age (Dordrecht, Netherlands)* 2012; **34**: 281-293.
597. Jackson SJ, Singletary KW. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis* 2004; **25**: 219-227.
598. Dave B, Chang J. Treatment resistance in stem cells and breast cancer. *Journal of mammary gland biology and neoplasia* 2009; **14**: 79-82.
599. Heiden KB, Williamson AJ, Doscas ME*, et al.* The sonic hedgehog signaling pathway maintains the cancer stem cell self-renewal of anaplastic thyroid cancer by inducing snail expression. *The Journal of clinical endocrinology and metabolism* 2014; **99**: E2178-E2187.

600. Xu ZY, Tang JN, Xie HX*, et al.* 5-Fluorouracil chemotherapy of gastric cancer generates residual cells with properties of cancer stem cells. *International journal of biological sciences* 2015; **11**: 284-294.
601. Nör C, Zhang Z, Warner KA*, et al.* Cisplatin Induces Bmi-1 and Enhances the Stem Cell Fraction in Head and Neck Cancer. *Neoplasia (New York, NY)* 2014; **16**: 137-W138.
602. Wintzell M, Löfstedt L, Johansson J*, et al.* Repeated cisplatin treatment can lead to a multiresistant tumor cell population with stem cell features and sensitivity to 3-bromopyruvate. *Cancer biology & therapy* 2012; **13**: 1454-1462.
603. Vishnubalaji R, Manikandan M, Fahad M*, et al.* Molecular profiling of ALDH1(+) colorectal cancer stem cells reveals preferential activation of MAPK, FAK, and oxidative stress pro-survival signalling pathways. *Oncotarget* 2018; **9**: 13551-13564.
604. Marynka-Kalmani K, Treves S, Yafee M*, et al.* The lamina propria of adult human oral mucosa harbors a novel stem cell population. *Stem cells (Dayton, Ohio)* 2010; **28**: 984-995.
605. Fan Z, Li M, Chen X*, et al.* Prognostic Value of Cancer Stem Cell Markers in Head and Neck Squamous Cell Carcinoma: a Meta-analysis. *Scientific reports* 2017; **7**: 43008.
606. Yang N, Hui L, Wang Y*, et al.* Overexpression of SOX2 promotes migration, invasion, and epithelial-mesenchymal transition through the Wnt/beta-catenin pathway in laryngeal cancer Hep-2 cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2014; **35**: 7965-7973.
607. Doudican NA, Wen SY, Mazumder A*, et al.* Sulforaphane synergistically enhances the cytotoxicity of arsenic trioxide in multiple myeloma cells via stress-mediated pathways. *Oncology reports* 2012; **28**: 1851-1858.
608. Xiao D, Powolny AA, Antosiewicz J*, et al.* Cellular responses to cancer chemopreventive agent D,L-sulforaphane in human prostate cancer cells are initiated by mitochondrial reactive oxygen species. *Pharmaceutical research* 2009; **26**: 1729-1738.
609. Liang J, Jahraus B, Balta E*, et al.* Sulforaphane Inhibits Inflammatory Responses of Primary Human T-Cells by Increasing ROS and Depleting Glutathione. *Front Immunol* 2018; **9**: 2584-2584.

Chapter 10 - Appendix: Articles published by the candidate during his Ph.D. studies

The first paper to be published from the thesis. Broccoli extract improves chemotherapeutic drug efficacy against head–neck squamous cell carcinomas (chapter 5). Hyperlink for the online and PDF version (https://link.springer.com/article/10.1007/s12032-018-1186-4)

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ORIGINAL PAPER

Broccoli extract improves chemotherapeutic drug efficacy against head–neck squamous cell carcinomas

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Abstract
The efficacy of cisplatin (CIS) and 5-fluorouracil (5-FU) against squamous cell carcinomas of the head and neck (SCCHN) remains restricted due to their severe toxic side effects on non-cancer (normal) tissues. Recently, the broccoli extract sulforaphane (SF) was successfully tested as a combination therapy to target cancer cells. However, the effect of lower doses of CIS or 5-FU combined with SF on SCCHN remained unknown. This study tested the chemotherapeutic efficacies of SF combined with much lower doses of CIS or 5-FU against SCCHN cells aiming to reduce cytotoxicity to normal cells. Titrations of SF standalone or in combination with CIS and 5-FU were tested on SCCHN human cell lines (SCC12 and SCC38) and non-cancerous human cells (fibroblasts, gingival, and salivary cells). Concentrations of SF tested were comparable to those found in the plasma following ingestion of fresh broccoli sprouts. The treatment effects on cell viability, proliferation, DNA damage, apoptosis, and gene expression were measured. SF reduced SCCHN cell viability in a time- and dose-dependent manner. SF-combined treatment increased the cytotoxic activity of CIS by twofolds and of 5-FU by tenfolds against SCCHN, with no effect on non-cancerous cells. SF-combined treatment inhibited SCCHN cell clonogenicity and post-treatment DNA repair. SF increased SCCHN apoptosis and this mechanism was due to a down-regulation of BCL2 and up-regulation of BAX, leading to an up-regulation of Caspase3. In conclusion, combining SF with low doses of CIS or 5-FU increased cytotoxicity against SCCHN cells, while having minimal effects on normal cells.

Keywords Head and Neck cancer · Carcinoma, squamous cell · Sulforaphane · Drug therapy · Apoptosis · DNA damage

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is one of the most prevalent malignant neoplasms of the upper aerodigestive tract. SCCHN is now the seventh most common cancer worldwide, with over 500,000 new cases diagnosed and 380,000 deaths annually which is nearly 4.6% of all cancer cases [1, 2]. Despite the improvements in treatment modalities, the 5-year survival rate for SCCHN patients has remained unchanged at about 50% over the past 30 years [3, 4] as 40–60% of SCCHN survivors suffer from relapse in the form of recurrences or metastases [5, 6].

Resistance to standard surgical, radiation, and chemical therapies continues to be a limiting factor in the treatment of SCCHN. One major factor in cancer treatment failure is because the efficacy of current standard chemotherapy, such as cisplatin (CIS) and 5-fluorouracil (5-FU), is restricted partly due to their severe toxic side effects. CIS forms DNA adducts which lead to induction of apoptosis in cancer cells

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REVIEW ARTICLE

Head and neck cancer management and cancer stem cells implication

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KEYWORDS
Head and neck cancer;
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Abstract Head and neck squamous cell carcinomas (HNSCCs) arise in the mucosal linings of the upper aerodigestive tract and are heterogeneous in nature. Risk factors for HNSCCs are smoking, excessive alcohol consumption, and the human papilloma virus. Conventional treatments are surgery, radiotherapy, chemotherapy, or a combined modality; however, no international standard mode of therapy exists. In contrast to the conventional model of clonal evolution in tumor development, there is a newly proposed theory based on the activity of cancer stem cells (CSCs) as the model for carcinogenesis. This "CSC hypothesis" may explain the high mortality rate, low response to treatments, and tendency to develop multiple tumors for HNSCC patients. We review current knowledge on HNSCC etiology and treatment, with a focus on CSCs, including their origins, identifications, and effects on therapeutic options.

Contents

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ORIGINAL ARTICLE

ORIGINAL ARTICLE

Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas

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Abstract

Head and neck squamous cell carcinoma (HNSCC) has a poor 5-year survival rate of 50%. One potential reason for treatment failure is the presence of cancer stem cells (CSCs). Several cell markers, particularly CD44, have been used to isolate CSCs. However, isolating a pure population of CSC in HNSCC still remains a challenging task. Recent findings show that normal oral stem cells were isolated using CD271 as a marker. Thus, we investigated the combined use of CD271 and CD44 to isolate an enriched subpopulation of CSCs, followed by their characterization *in vitro*, *in vivo*, and in patients' tissue samples. Fluorescent-activated cell sorting was used to isolate CD44+/CD271+ and CD44+/CD271− from two human HNSCC cell lines. Cell growth and self-renewal were measured with MTT and sphere/colony formation assays. Treatment-resistance was tested against chemotherapy (cisplatin and 5-fluorouracil) and ionizing radiation. Self-renewal, resistance, and stemness-related genes expression were measured with qRT-PCR. *In vivo* tumorigenicity was tested with an orthotopic immunodeficient mouse model of oral cancer. Finally, we examined the co-localization of CD44+/CD271+ in patients' tissue samples. We found that CD271+ cells were a subpopulation of CD44+ cells in human HNSCC cell lines and tissues. CD44+/CD271+ cells exhibited higher cell proliferation, sphere/colony formation, chemo- and radio-resistance, upregulation of CSCs-related genes, and *in vivo* tumorigenicity when compared to CD44+/CD271− or the parental cell line. These cell markers showed increased expression in patients with the increase of the tumor stage. In conclusion, using both CD44 and CD271 allowed the isolation of CSCs from HNSCC. These enriched CSCs will be more relevant in future treatment and HNSCC progression studies.

Introduction

Cancer is the second leading cause of death in the USA and the first cause in Canada, as it is responsible for over 30% of all deaths annually (1,2). Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide, as it accounts for over 580,000 new diagnosed cases in 2018 (3). In Canada, 5850 new cancer patients were diagnosed with HNSCC and it was responsible for 1690 deaths in 2017 (1). Despite recent advances for diagnosis and cancer treatment, the current prognosis for HNSCC is poor due to relapse in the form of local recurrence or metastasis. The 5-year survival rate has remained approximately 50% for the last three decades (4).

One reason for cancer treatment failure is considered to be related to the presence of a subpopulation of cells in the tumor called "cancer stem cells" (CSCs), which are suggested to have tumor-initiating potential combined with the ability of self-renewal and multilineage differentiation (5). Acute myeloid

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