Comprehensive Analysis of Genetic Mutations in HPV-Positive and HPV-Negative Oropharyngeal Squamous Cell Carcinoma: A Literature Review

Mai Atique

Faculty of Dental Medicine and Oral Health Sciences

McGill University, Montréal, Québec, Canada

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DEDICATION

This work is dedicated to my support system, my beloved parents *Ramadan* & *Ghada*, and to my brothers *Mohamed, BahaaElDin* & *Amro*. In that order.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
HNC	Head and neck cancer
HPV	Human papillomavirus
OPC	Oropharyngeal cancer
OPSCC	Oropharyngeal squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
TCGA	The cancer genome atlas
AJCC	The American joint committee on cancer
UICC	The union for international cancer control
MeSH	Medical subject headings
REC	Research ethics committee
REB	Research ethics board
WES	Whole genome sequencing
NGS	Next-generation sequencing
ISH	*In situ* hybridization
IHC	Immunohistochemical
RFLP	Restriction fragment length polymorphism
PCR	Polymerase chain reaction
OGM	Optical genome mapping
RPPA	Reverse phase protein array
PPI	Protein-protein interaction
MDSCs	Myeloid-derived suppressor cells
DAMPs	Damage-associated molecular patterns
NLR	Neutrophil-to-lymphocyte ratio
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
LSCC	Laryngeal squamous cell carcinoma
NSCC	Nasal squamous cell carcinoma
ICD-0	International Classification of Diseases for Oncology
GLOBOCAN	Global Cancer Observatory

WHO	World Health Organization
IARC	International Agency for Research on Cancer
INHANCE	International Head and Neck Cancer Epidemiology
TSNA	Tobacco-specific N-nitrosamines
HBV	Hepatitis B virus
HCV	Hepatitis C virus
EBV	Epstein-Barr virus
HHV4	Human herpesvirus 4
KSHV	Kaposi’s sarcoma-associated herpesvirus
HHV8	Human herpesvirus 8
MCPyV	Merkel cell polyomavirus
HTLV-1	Human T-cell lymphotropic virus type 1
OR	Odds ratio
ROS	Reactive oxygen species
PI3K	Phosphoinositide 3-kinase
RB1	Retinoblastoma
SNP	Single nucleotide polymorphism
HIV	Human immunodeficiency virus
p53	tumor protein p53
pRb	Retinoblastoma protein
PVs	Papillomaviruses
Bp	Base pairs
E	Early
L	Late
VLPs	Virus-like particles
ORFs	Open reading frames
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
OS	Overall survival
TORS	Transoral robotic surgery
g:Profiler	Functional enrichment analysis tool
HSPG	Heparin sulfate proteoglycan
HIF	Hypoxia-inducible factor
TNM	Tumor, node, metastasis

T	Extent of the tumor
N	Extend of spread to the lymph nodes
M	Presence of metastasis
LN	Lymph nodes
LCR	Long control region
RhPV1	Rhesus papillomavirus type 1
PCPV-1	Pygmy chimpanzee papillomavirus type 1
PAH	Polycyclic aromatic hydrocarbons
GSEA	Gene set enrichment analysis
ELA2	Neutrophil elastase 2
Qupath	Quantitative pathology
STATA	Statistics and data
DNA	Deoxyribonucleic acid
ASIR	Age-standardized incidence rate
AF	Attributable fraction
FDA	Food and drug administration
MALT	Mucosal-associated lymphoid tissues

ABSTRACT

Human papillomavirus (HPV) is linked with the high incidence of oropharyngeal squamous cell carcinomas (OPSCC). Although HPV-positive and HPV-negative OPSCC have distinct oncogenesis processes, the literature lacks a comprehensive summary about specific genetic alterations. Therefore, our study was done to compile, analyze, and interpret existing information on genetic mutations in HPV-positive and HPV-negative OPSCC to understand molecular characteristics of these tumors. The objectives of this research are to 1) conduct comprehensive literature review to identify and characterize genetic alterations involved in HPV-positive OPSCC, 2) conduct secondary analysis using The Cancer Genome Atlas (TCGA) public platform to validate the identified genetic alterations in HPV-positive OPSCC, 3) investigate biological processes using enriched pathways analysis, and 4) validate these pathways using large cohort of samples obtained from patients with OPSCC. We searched the literature in four bibliographic databases (Medline, PubMed, Web of Science and Scopus) for eligible studies based on MeSH (Medical Subject Headings) terms and keywords. Search terms included HPV, papillomavirus, head and neck cancer (HNC), head and neck squamous cell carcinoma (HNSCC), OPSCC, and pharyngeal cancer. Studies selected from the databases were imported into Rayyan software for identification and removal of duplicates and for screening titles and abstracts by three reviewers based on predefined eligibility criteria. List of the genetic alterations in HPV-positive and HPV-negative were extracted. A bioinformatician used the TCGA platform to validate the genetic alterations in HPV-positive and HPV-negative and their potential clinicopathological impact. Enriched analysis was done to identify the most relevant pathways and molecular markers using multiple software including g:Profiler, GSEA, Cytoscape and EnrichmentMap. A molecular candidate (Neutrophil Elastase/ELA2 antibody) involved with neutrophil infiltration was identified to be validated by immunohistochemistry reaction (IHC). Tissue slides were scanned in Aperio ScanScope® (Leica Biosystems) and quantified using QuPath (v0.2.3). Statistical analysis was done to compare the clinicopathological data with the protein expression using STATA (StataCorp LLC). It was identified 1556 studies and retained 38 studies for extraction, including studies that were published between 1995 and 2023 involving 8,311 HNC patients from 12 different countries. The 10 most common mutated genes that were identified from conducting the comprehensive literature review were *TP53* (n=22), *PIK3CA* (n=20), *PTEN* (n=16), *NOTCH1* (n=14), *RB1* (n=13), *FAT1* (n=13), *FBXW7* (n=12), *HRAS* (n=10), *KRAS* (n=10) and *CDKN2A* (n=10). These genes are involved with several biological processes such as cell cycle and

deoxyribonucleic acid (DNA) damage response, PI3K/AKT/mTOR signaling pathway, Notch signaling pathway and RAS/MAPK signaling pathway. From TCGA analysis, the most prevalent mutated genes in HPV-positive OPSCC were *PIK3CA, TP53, PTEN, NOTCH1, FAT1, RB1, FBXW7, HRAS, KRAS,* and *CDKN2A*. Pathways enriched analysis revealed alteration in inflammatory responses, specifically related with neutrophils infiltration. Validation was conducted in a cohort of patients with OPSCC, consisting of 12 metastatic and 40 non-metastatic cases, followed for over 10 years. Notably, metastatic HPV-positive OPSCC showed upregulation of the nuclear ELA2, while non-metastatic tumors exhibited weak to moderate expression. ELA2 protein was overexpressed in OPSCC samples, especially in recurrent OPSCC tumors.

RÉSUMÉ

Le virus du papillome humain (VPH) est associé à l'incidence élevée des carcinomes épidermoïdes oropharyngés (CEOP). Bien que les CEOP VPH positifs et négatifs aient des processus d'oncogenèse distincts, la littérature ne résume pas entièrement les altérations génétiques spécifiques. Notre étude a compilé, analysé et interprété les informations sur les mutations génétiques dans les CEOP VPH-positifs et VPH-négatifs pour comprendre leurs caractéristiques moléculaires. Les objectifs de cette recherche sont de 1) mener une revue complète de la littérature pour identifier et caractériser les altérations génétiques impliquées dans le CEOP VPH-positifs, 2) effectuer une analyse secondaire avec la plateforme publique *l'Atlas du génome du cancer* (TCGA) pour valider les altérations génétiques dans les CEOP VPH-positifs, 3) étudier les processus biologiques via une analyse de voies enrichies et 4) Valider ces voies avec une large cohorte d'échantillons de patients atteints de CEOP. Nous avons recherché dans quatre bases de données bibliographiques (Medline, PubMed, Web of Science et Scopus) pour trouver des études éligibles basées sur les termes et mots-clés MeSH (Medical Subject Headings). Les termes de recherche incluent le VPH, le papillomavirus, le cancer de la tête et du cou (CTC), le carcinome épidermoïde de la tête et du cou (CETEC), le CEOP et le cancer du pharynx. Les études sélectionnées ont été importées dans le logiciel Rayyan pour l'identification et la suppression des doublons ainsi que pour la sélection des titres et des résumés par trois examinateurs selon des critères prédéfinis. La liste des altérations génétiques chez les VPH positifs et négatifs a été extraite. Un bioinformaticien a utilisé la plateforme TCGA pour valider les altérations génétiques des VPH positifs et VPH négatifs ainsi que leur impact clinicopathologique potentiel. Une analyse enrichie a été réalisée pour identifier les voies et les marqueurs moléculaires les plus pertinents avec plusieurs logiciels, notamment g:Profiler, GSEA, Cytoscape et EnrichmentMap. Un candidat moléculaire (anticorps Neutrophil Elastase/ELA2) impliqué dans l'infiltration des neutrophiles a été identifié pour être validé par réaction immunohistochimique (IHC). Les lames de tissus ont été numérisées dans Aperio ScanScope® (Leica Biosystems) et quantifiées avec QuPath (v0.2.3). Une analyse statistique a comparé les données clinicopathologiques avec l'expression de la protéine en utilisant STATA (StataCorp LLC). 1 556 études ont été identifiées et 38 études ont été retenues pour extraction, y compris des études publiées entre 1995 et 2023 portant sur 8 311 patients CTC de 12 pays différents. Les 10 gènes mutés les plus courants identifiés lors de la revue de la littérature étaient *TP53* (n = 22), *PIK3CA* (n = 20), *PTEN* (n = 16), *NOTCH1* (n = 14), *RB1* (n = 13), *FAT1* (n = 13), *FBXW7* (n = 12), *HRAS* (n = 10), *KRAS* (n = 10) et

CDKN2A (n = 10). Ces gènes sont impliqués dans plusieurs processus biologiques tels que la réponse aux dommages du cycle cellulaire et de l'acide désoxyribonucléique (ADN), la voie de signalisation PI3K/AKT/mTOR, la voie de signalisation Notch et la voie de signalisation RAS-MAPK. D'après l'analyse TCGA, les gènes mutés les plus répandus dans les CEOP positifs pour le VPH étaient *PIK3CA, TP53, PTEN, NOTCH1, FAT1, RB1, FBXW7, HRAS, KRAS* et *CDKN2A*. L'analyse enrichie des voies a révélé une altération des réponses inflammatoires, spécifiquement liée à l'infiltration de neutrophiles. La validation a été réalisée dans une cohorte de patients atteints de CEOP, composée de 12 cas métastatiques et 40 cas non métastatiques, suivis pendant plus de 10 ans. Notamment, les CEOP métastatiques positifs au VPH ont montré une régulation positive de l'ELA2 nucléaire, tandis que les tumeurs non métastatiques présentaient une expression faible à modérée. La protéine ELA2 était surexprimée dans les échantillons CEOP, en particulier dans les tumeurs CEOP récurrentes.

PREFACE

This is a manuscript-based thesis written according to the updated standards established by McGill Graduate and Postdoctoral Studies for fulfilling the requirements of a Master's degree in the Dental Sciences-Thesis program. The manuscript follows the primary goal of this research which is a comprehensive literature review analyzing the genetic mutations in both HPV-positive and HPV-negative OPSCCs in order to understand the molecular characteristics of these tumors. Based on the standards of McGill University, the manuscript comprised a separate set of appendices and references list.

The first chapter includes an introduction to the topic, which is followed by a literature review providing the current knowledge in the field. The manuscript related to the thesis is presented in the third chapter as a standalone document, including the published tables, figures, supplementary materials, and references list. The fourth chapter ends the thesis with an overall conclusion and future directions. Lastly, a list of references used for the thesis writing is provided.

The manuscript involved a multidisciplinary team and the specific contribution from each author was provided in the next section.

CONTRIBUTION OF AUTHORS

Manuscript:

Genetic Mutations Associated with Inflammatory Response Caused by HPV Integration in Oropharyngeal Squamous Cell Carcinoma

Mai Atique, Master's Candidate, Faculty of Dental Medicine and Oral Health Sciences, McGill University, 2001 Avenue McGill College, Montréal, QC, H3A 1G1, Canada: Screened and selected studies, extracted data, assessed the quality of studies, and wrote the manuscript.

Isis Muniz, PhD, Faculty of Dentistry, Federal University of Paraíba, Cidade Universitária Campus I Castelo Branco III João Pessoa, Paraíba, 58051-900. Brazil, Postgraduate Program in Dentistry (PPGO): Screened and selected studies, validated, and reviewed the manuscript.

Fatemeh Farshadi, Master's Candidate, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Division of Experimental Medicine: Screened and selected studies, validated, and reviewed the manuscript.

Michael Hier, Professor, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Department of Otolaryngology - Head and Neck Surgery: Provided resources and contributed to review the manuscript.

Alex Mlynarek, Associate Professor, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Department of Otolaryngology - Head and Neck Surgery: Contributed to review the manuscript.

Marco Macarella, Assistant Professor, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Department of Otolaryngology - Head and Neck Surgery: Contributed to write the manuscript.

Mariana Maschietto, Professor, Department of Structural and Functional Biology, Institute of Biology, Universidade Estadual de Campinas (UNICAMP), Campinas 13084-225, SP, Brazil, Boldrini Children's Center, Campinas 13084-225, SP, Brazil: Contributed to the study design, data analysis, data visualization, and reviewed the manuscript.

Belinda Nicolau, Professor, Faculty of Dental Medicine and Oral Health Sciences, McGill University, 2001 Avenue McGill College, Montréal, QC, H3A 1G1, Canada: Provided feedback on manuscript structure and reviewed the manuscript.

Moulay A. Alaoui-Jamali, Professor, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Division of Experimental Medicine: Provided critical review and comments to improve the manuscript.

Sabrina Daniela da Silva, Associate Professor, Faculty of Medicine and Health Sciences, McGill University, 3755 Chem. de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada, Department of Otolaryngology - Head and Neck Surgery: Supervised all steps, contributed to the conceptual design, interpretation, and reviewed the manuscript (Corresponding Author).

1 INTRODUCTION

Cancer stands as the leading cause to death in the world imposing a substantial burden on individuals' health and the healthcare system[1]. It is expected that approximately 45% of Canadians will develop cancer during their lifetime, with an estimated fatality rate of 22%[1]. In 2020, cancer caused a higher proportion of total deaths (26.4%) compared to other leading causes, including heart disease (17.5%), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (5.3%), accidents (5.0%), and cerebrovascular diseases (4.5%)[1]. Quebec and eastern provinces have shown higher cancer mortality rates than Ontario and western provinces[1]. Among different cancer types, HNC affect over five million people worldwide each year and causing 370,000 deaths[2,3].

Aside from tobacco and alcohol consumption, HPV has been recognized as being linked to the increased incidence of HNC, mainly affecting the base of the tongue and tonsils[4]. This incidence can be seen in high income countries such as Canada, United States, Australia, and Sweden through which males below 60 years old are the most affected[5]. HPV is a group of viruses that have genetic material of circular double-stranded DNA that can integrate into host cell genome infecting skin and mucous membranes of humans[6,7].

HPV is the most known sexually transmitted virus which can be categorized into high-risk and low-risk types[8]. Persistent infections with high-risk HPV types result in genetic changes in the host cells[9]. The E6 and E7 oncoproteins drive genetic instability that affect different cellular processes by mainly inhibiting apoptosis and promoting cancer cell proliferation[9]. The current standard of care for both HPV-positive and negative HNC comprises surgical procedures associated with chemo-radiotherapy[4]. However, this strategy is linked with severe adverse effects[4]. Several studies are proposing de-escalation clinical trials for HPV-positive OPSCC patients aiming to improve the quality of life and avoid late toxicity while maintaining acceptable survival rates[10]. OPSCC often exhibits genetic changes, which may result in abnormal activation of signaling pathways that contribute to tumor development[11]. Several studies have discussed the specific genetic alterations involved in HPV-positive OPSCC. However, there is lack of a conclusive result. This thesis conducted a comprehensive literature review involving HPV-positive OPSCC to summarize our current understanding of the

epidemiology, risk factors, genetic predisposition, carcinogenesis with a particular focus on aberrant signaling pathways and immunomodulatory mechanisms.

2 LITERATURE REVIEW

This chapter presents a literature review about epidemiology and the primary risk factors such as tobacco, alcohol, and HPV infection associated with HNC. It describes the characteristics of HPV, carcinogenesis, genetic predisposition, and a brief explanation about inflammatory infiltration. This section's end shows the clinical impact of HPV-positive HNC aligned with the thesis's goals.

2.1 HNC definition

HNC is a group of malignancies that affect different parts in the head and neck region, including oral cavity, oro- naso- and hypopharynx, larynx, paranasal sinuses, and salivary glands[12,13]. HNC is a heterogenous group of diseases that comprises variety of subclassifications regarding location, aetiology and molecular findings[14]. However, more than 90% are classified as HNSCC[15-18]. The International Classification of Diseases for Oncology (ICD-O), introduced in 1976 established a topographical code, starting with "C," to identify the anatomical site, followed by three digits indicating primary location and subsite[19]. For instance the classification includes: a) oral squamous cell carcinoma (OSCC) (ICD-10: C06.9), involving tongue, lips, palate, and floor of the mouth, b) OPSCC (ICD-10: C10.8), which includes posterior and lateral pharyngeal walls, soft palate, tonsils, base and posterior one-third of the tongue, c) laryngeal squamous cell carcinoma (LSCC) (ICD-10: C32.8) that consists of carcinomas in the subglottis, glottis and supralottis, and d) nasal squamous cell carcinoma (NSCC) (ICD-10: D02.3) including nasal cavity and paranasal sinuses[18,20] (**Figure1**). The focus of this thesis is HPV-positive OPSCC.

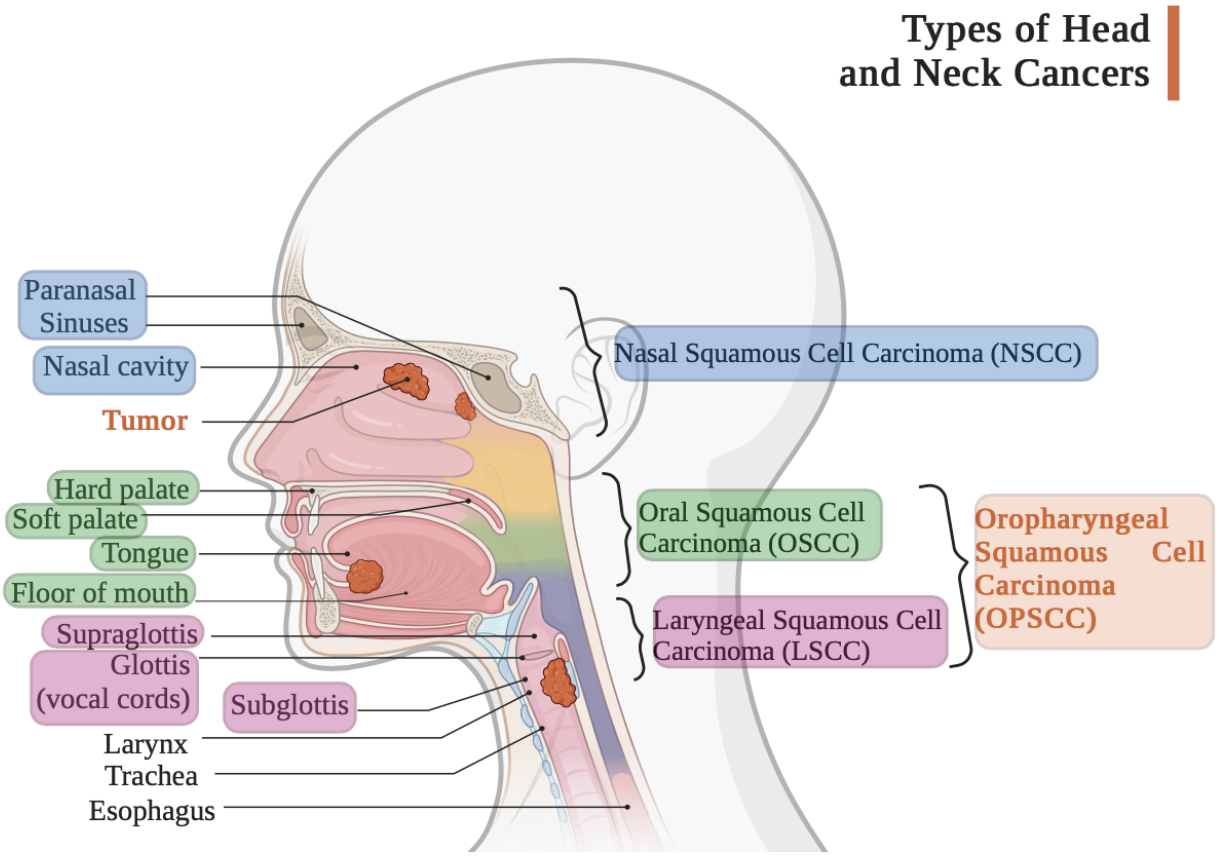

Figure 1: Different topographies of HNC. HNC is divided into OSCC (green), OPSCC (orange), LSCC (purple) and NSCC (blue) through which over 90% are SCC. Each color code refers to the parts associated with each SCC region. OPSCC affects posterior and lateral pharyngeal walls, soft palate, tonsils, base, and posterior one-third of the tongue. Figure created using biorender. *Abbreviations:* SCC: squamous cell carcinoma; OPSCC: oropharyngeal squamous cell carcinoma; OSCC: oral squamous cell carcinoma; NSCC: nasal squamous cell carcinoma; LSCC: laryngeal squamous cell carcinoma.

Besides ICD-O classification, the TNM (Tumor, Node, Metastasis) system is used to stage cancer based on tumor extension[21]. The system is used to classify malignancy and assist in prognostic cancer staging[21]. In 1968, the Union for International Cancer Control (UICC) introduced the initial TNM classification, then updated into further editions which were published by the American Joint Committee on Cancer (AJCC)[22]. Over time, the classification has undergone multiple updates[23]. In 2018, a new clinical and pathological TNM classification was published to address the distinct prognosis associated with HPV-positive OPSCC in the eighth edition[24-26] (**Table 1).**

Table 1: Clinical and pathological classification for HPV- positive OPSCC following the eighth edition UICC/AJCC TNM staging system.

HPV- positive OPSCC	Clinical Stage			Pathologic Stage		
Category	**T**	**N**	**M**	**T**	**N**	**M**
Stage I	T1,T2	N0: no regional LN's N1: ipsilateral LNs	M0	T1,T2	N0: no regional LNs N1: 1-4 LNs	M0
Stage II	T1,T2	N2: bilateral or contralateral LNs	M0	T1,T2	N2: greater than or equal 5 LNs	M0
	T3	N0: no regional LNs N1: ipsilateral LNs N2: bilateral or contralateral LNs	M0	T3,T4	N0: no regional LNs N1: 1-4 LNs	
Stage III	T4	Any N	M0	T3,T4	N2: greater than or equal 5 LNs	M0
	Any T	N3: greater than 6 cm LN(s)	M0			
Stage IV	Any T	Any T	M1	Any T	Any N	M1

Abbreviations: UICC = The union for international cancer control; AJCC = The American joint committee on cancer; TNM = Tumor, Node, Metastasis; HPV = human papilloma virus; OPSCC = oropharyngeal squamous cell carcinoma; T = extent of the tumor; N = extent of spread to the lymph nodes; M = presence of metastasis; LN = lymph nodes. Adapted from[27].

2.2 HNC descriptive epidemiology

The world's population is expected to reach around 9.2 billion by 2040 with an estimated 29 million cases new cases of cancer[28]. By 2030, it is predicted 30% increase in HNC cases reaching 1.08 million new cases diagnosed per year[29]. According to GLOBOCAN (Global cancer observatory) data, HNC ranks as the seventh most prevalent worldwide, with around 890,000 new cases diagnosed annually, representing around 4.5% of all cancer diagnoses[30-32]. Also, it leads to approximately 450,000 fatalities each year, accounting for 4.6% of all cancer-related deaths across the globe: ranking as the ninth most common cause of death[33].

According to Canadian Cancer Society, Canada registered 7,500 cases of HNC and 2,100 death cases in 2022[34]. Moreover, an estimated 38,000 cases of HNCs associated with HPV, specifically in the oropharyngeal area, occur per year[35]. Between 1998 and 2004, there was a

225% rise in HPV-positive OPSCC, while at the same time, a 50% decrease in cancers linked to alcohol and tobacco was observed[36]. By the year 2040, it is anticipated that HPV will emerge as the primary causal factor for OPSCC[36,37].

Around 4% of all cancers that occurs in the United States are caused by HNC; it was estimated 66,920 HNC new diagnosed cases and 15,400 HNC death cases by 2023[38]. HNC incidence is increasing in low income and high-income countries, and several etiological factors are behind this rise[39]. The incidence of HPV-positive OPSCC present both geographic and sex differences; through which studies have shown that age-standardized incidence rate (ASIR) is elevated in Europe and North America[34,35]. Despite male and female being affected by HPV-positive OPSCC, the ASIR showed higher rates in males than females; overall, male have two to four times higher incidence than female[34,35]. Worldwide, the attributable fraction (AF) or proportion of HPV-positive OPSCC has risen from 30.8% to 42.7%[34,35]. The highest incidence of HPV-positive OPSCC was registered in North America; ASIR (3.41 per 100,000 for male and 0.71 for female) with around 63% AF[35]. Prevalence of HPV-positive OPSCC in the United States (66.3%; CI 56.1-75.9) showed similar increasing patterns as in Canada[35-37]. The ASIR of HPV-positive OPSCC increased from 1.6 per 100,000 population in 1992 to 2.6 in 2009[38]. OPSCC cases were more prevalent in males than in females, with 1.5% change per year for male compared to 0.8% for female[39].

In relation to the mortality rates, the records showed higher OPSCC age-standardized deaths in male (0.89 per 100,000 population) than in female (0.17)[37]. In addition, high-income countries showed higher mortality rates (1.14 in male and 0.22 in female per 100,000 population) compared to low-income countries (0.80 in male and 0.51 in female per 100,000 population)[40].

2.3 HNC risk factors

Oncogenic viruses such as HPV, alcohol and tobacco consumption are considered the main risk factors for HNC[1]. Areca nut (betel quid) chewing is also associated with HNC in Southeast Asia and Asia-Pacific[41]. However, this section will focus on the main risks' factors associated with HNC development.

2.3.1 Tobacco consumption

According to World Health Organization (WHO) report in 2023, tobacco remains a significant public health issue, causing the deaths of more than eight million individuals per year[42,43]. Death cases of non-smokers in direct contact with tobacco smokers, also known as second-hand tobacco smoke, have reached around 1.3 million cases[42,43].

The relation between HNC and tobacco was first introduced by Abbe *et al.*, 1915[44]. Tobacco was officially classified as carcinogen for HNC in 1986 by the International Agency for Research on Cancer (IARC)[45,46]. The cumulative lifetime risk is linked to factors such as daily amount of tobacco, the duration and intensity of smoking including frequency of inhalations[47-49]. The International Head and Neck Cancer Epidemiology (INHANCE) consortium published a comprehensive pooled analysis from large case-control studies conducted in 2016 showed that smoking five to ten cigarettes daily (95% CI, 2.00-3.40) resulted in a two-fold increase in cancer risk compared to individuals who smoked zero to three cigarettes per day (95% CI, 1.21-1.90)[47,50].

There are various forms of tobacco use, including combustible types such as cigarettes, cigars, as well as smokeless products such as areca nut and chewing tobacco[51]. In 2022, the Food and Drug Administration (FDA) established a list of 93 carcinogens and potentially harmful constituents in tobacco smoke and tobacco products, including tobacco-specific N-nitrosamines (TSNA) and polycyclic aromatic hydrocarbons (PAH)[52,53]. Surveillance of the levels of tobacco carcinogens as well as regulatory actions are needed to ensure control of their levels so that potential reduced risks of cancer and other diseases may be achieved.

2.3.2 Alcohol consumption

At least 75% of HNC are associated with alcohol consumption[53,54]. Alcohol independently increases the risk of HNC from 1% to 4%[55,56]. Bruguere *et al*., showed that drinkers consuming 100–160g/day (equivalent to 12.5–20 units/day) presented a relative risk (adjusted for tobacco), of 13.5 for OSCC, 15.2 for OPSCC, and 28.6 for hypopharyngeal cancers[53,54]. However, the synergistic effect of alcohol is evident when it interacts with tobacco, accounting for 72% of

cases[56,57]. Smokers who consume two or more cigarette packs and more than four drinks of alcohol daily have a higher tendency to develop HNC by more than 35 times[46,49].

Once alcohol reaches the bloodstream, it undergoes frequent metabolic processes that results in toxic byproducts including hydroxyl and ethoxy radicals and acetaldehyde[58]. Acetaldehyde is known to be the carcinogen that triggers the malignant transformation[59]. The use of alcohol is usually linked to increased HNC risk, specially for alcohol users with slow ethanol metabolism[59]. HNC incidence may be reduced by alcohol cessation[60].

2.3.3 HPV infection

HPV is DNA virus form a distinct family called *Papovaviridae*[61,62]. HPV types are categorized into mucosal (infecting upper aerodigestive and anogenital tract) or cutaneous types (infecting skin)[63,64]. Mucosal virus types are contributing to HNC[63]. Based on the potential for malignant transformation, mucosal virus can be categorized into low-risk and high-risk types[64]. HPV can be further classified into alpha, beta, nu, mu and gamma[63] (**Figure 2)**.

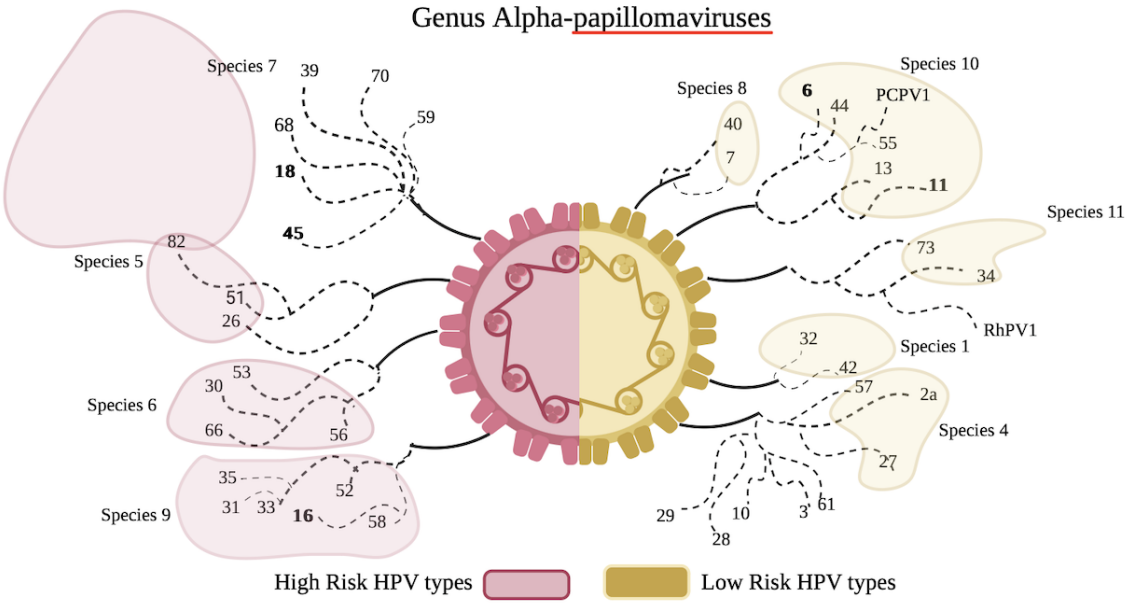

Figure 2: Alpha-papillomaviruses types. HPV genotypes within the alpha papillomavirus genus are categorized according to species. Those species housing high-risk HPV genotypes are denoted with pink color, while low-risk HPV genotypes are highlighted in yellow. Figure created using biorender. *Abbreviations:* HPV = human papilloma virus; PCPV-1 = pygmy chimpanzee papillomavirus type 1; RhPV1 = rhesus papillomavirus type 1. Adapted from[65].

HPV is the most common sexually transmitted disease worldwide affecting 50%-80% of sexually active persons is HPV[66]. In high-income countries, 72% of HNC cases are caused by HPV; whereas in low-income countries, HPV affects 13% of HNC cases[67]. Until now, the literature has discovered over 200 HPV subtypes[68-70] **(Figure 3)**. High-risk HPV types include types -16, -18, -31, and/or -33 causing almost 90% of HPV-positive HNC[71]. HPV infection is associated with different types of cancers, including anal cancer, cervical cancer, HNC, penile cancer, vaginal and vulvar cancer[71-73]. While cervical cancer rates have declined, likely due to vaccination and effective screening; the HPV-positive anogenital and HNC are on the rise[77,78].

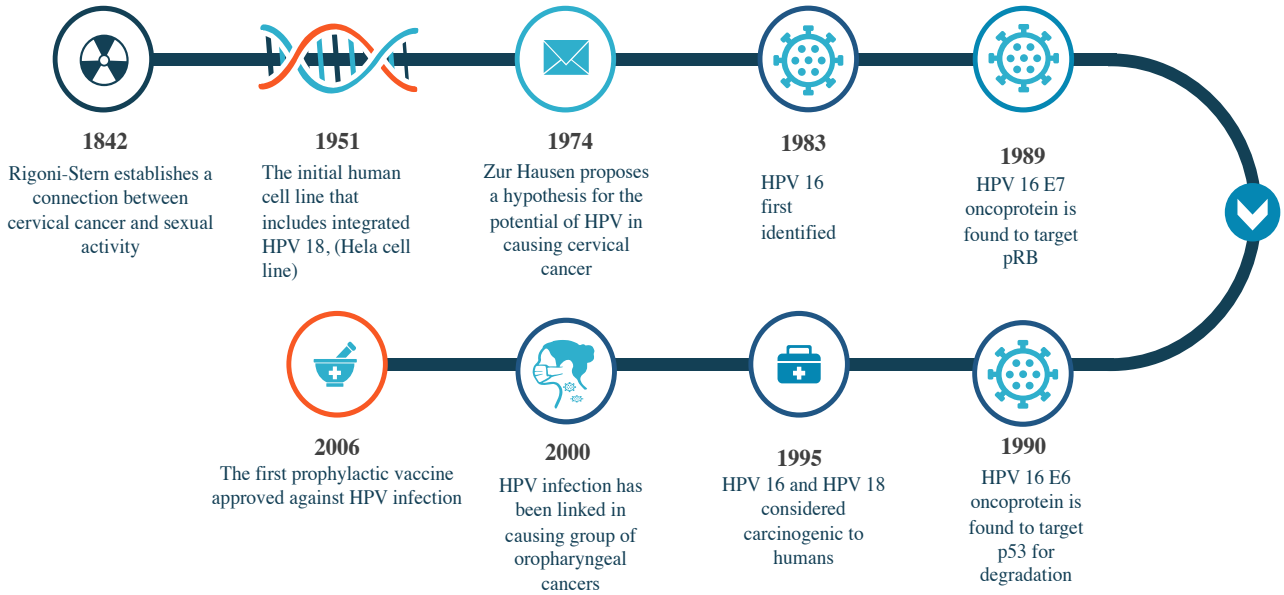

Figure 3: HPV from discovery to vaccination. HPV was identified as a major risk factor for developing cervical cancer in 1970's. Numerous HPV subtypes were further identified and categorized based on their genetic features during 1980's. In 2006, HPV vaccines were discovered with Gardasil and Cervarix being the most common. *Abbreviations:* HPV: human papilloma virus; E: early; pRb: retinoblastoma protein; p53: p53 protein. Adapted from[74].

2.3.3.1 HPV genome structure

Papillomaviruses are icosahedral DNA viruses that have a diameter of 52–55 nm; the viral particles consist of single double-stranded DNA containing approximately 8000 base-pairs

(bp)[67]. This DNA is associated with cellular histones and enclosed within a protein capsid made up of 72 pentameric capsomers[67]. All HPV subtypes comprises eight open reading frames (ORFs) divided into three regions: 1) The early (E) region, which is responsible for encoding proteins (E1–E8) essential for viral replication; 2) The late (L) region, which encodes the structural proteins (L1–L2) required for the assembly of viral particles (virions); 3) A predominantly non-coding section known as the long control region (LCR), essential for transcription and replication of viral DNA[67] (**Figure 4**). Within the capsid, there are two structural proteins, namely late (L1), making up 80% of the total viral protein, and L2; these proteins are encoded by the virus itself[67]. The expression of L1, either on its own or in conjunction with L2 lead to the production of virus-like particles (VLPs)[67] (**Figure 4**). The roles of the HPV proteins are summarized in the Table 2.

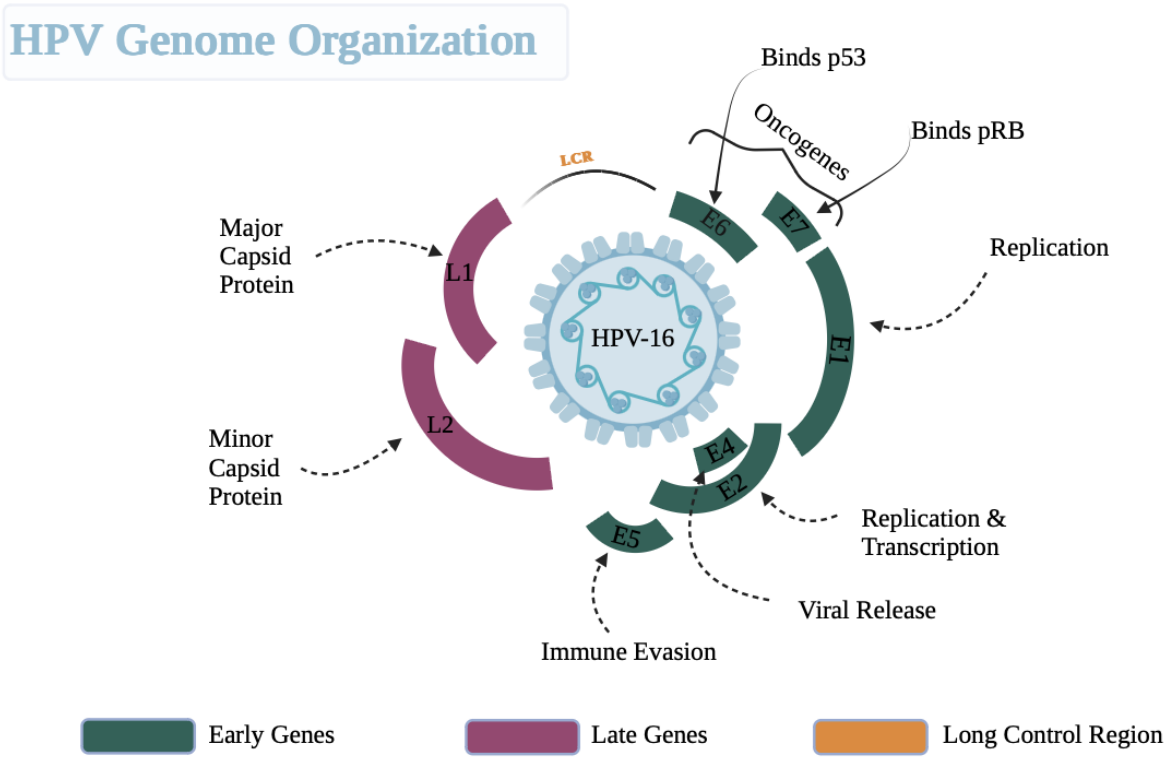

Figure 4: HPV genome organization structure. This is an illustration of HPV genome encoding eight oncogenes (E1, E2, E4, E5, E6, E7, L1 and L2). E6 and E7 are the main oncoproteins involved in the host cell cycle. The remaining 'early' genes, such as E1, E2, E4, and E5, encode other proteins involved with the viral DNA replication. The two 'late' genes, L1 and L2, are responsible for encoding proteins for viral capsid formation. Figure created

using biorender. *Abbreviations:* HPV = human papilloma virus; LCR = long control region; p53 = tumor protein p53; pRB = retinoblastoma protein; L = late; E = early. Adapted from[75].

Table 2: Description of the HPV proteins and their functions involving early region (E1-E8) and late region (L1 and L2).

Protein	**Function**
Early Region	
E1	Initiating DNA replication and transcription
E2	Regulates Viral Transcription and DNA replication by controlling ORFs E6 to E7
E3	Unknown
E4	Interacts with cytoskeleton proteins by altering the extracellular matrix cell
E5	Interacts with cellular proteins and downregulates major histocompatibility complex class 1 molecules.
E6	Degrades p53 oncoproteins
E7	Binds to the Rb oncoprotein
E8	Unknown
Late Region	
L1	Major viral capsid structural protein
L2	Minor viral capsid structural protein

Abbreviations: DNA = deoxyribonucleic acid; E = early; L = late; ORFs = open reading frames; p53 = tumor protein 53; Rb = retinoblastoma protein. Adapted from[76].

2.3.3.2 HPV carcinogenesis

HPV infection has been associated with several cancers, including cervical, vulvar, vaginal, anal, penile, and HNCs, more specifically oropharyngeal cancer (OPC)[77]. Traditionally, OPC was attributed to alcohol or tobacco use[78]. However, Gillison *et al*., (2000) showed the association between HPV infection and OPC[79].

For HPV to cause infection, the viral particles need to gain access to the keratinocytes located in the basal layer of stratified squamous epithelium[80]. This basal layer is adhered to the epithelia basement membrane, so for virions to reach the deeper layers of the epithelium, there must be a passage to enter through[81]. This passage requires destruction of epithelium that can be in the form of micro-abrasions that occur during direct physical or sexual contact[82].

The majority of OPSCCs associated with HPV tend to develop in the palatine and lingual tonsils, indicating a connection with the specialized histological composition of tonsil tissue[10]. The tonsil tissue is known to have reticulated epithelium that lines the crypts; disruption of the epithelial layer can lead to viral deposition in the absence of mucosal trauma[10]. HPV viral integration is increased when there is persistent infection[83] essential mechanism for OPSCC carcinogenesis[84].

Biological processes altered by HPV ranged from its most known targets, p53 and pRb inhibition dependent pathways, to signaling pathways such as EGFR and MAPKs[85,86]. HPV can also modify energy and cellular metabolism by targeting Akt, mTOR, and autophagy[86]. DNA damage is promoted by HPV to obtain an efficient viral replication[87]. So, critical pathways are implicated in HPV-related cancers include: 1) p53 and pRb pathways (regulating cell cycling), 2) PI3K–PTEN–AKT pathway (avoiding apoptosis); 2) EGFR pathway (facilitating growth factor signaling); 3) TGFβ pathway (mediating growth factor signaling); and 4) angiogenesis, inclusive of hypoxia-inducible factor (HIF)[85]. The outcome of HPV involvement in these pathways is related with genomic instability which ends into complete cellular transformation[88]. Understanding how HPV oncoproteins modify these biological processes may provide novel insights into the basic mechanisms of oncogenesis.

Table 3: Different characteristics of HPV-positive and HPV-negative OPSCC.

Parameter	HPV- Positive OPSCC	HPV- Negative OPSCC
Anatomical Site	Tonsils/ Base of the tongue (Oropharynx)	All sites (Floor of mouth, lateral and ventral surface of the tongue)
Histology	Non-keratinzed/ Basaloid SCC	Keratinized SCC
Gender	4-5-fold more common in men	2-3-fold more common in men
Age	Younger	Older
Race	Whites	Blacks
Smoking Consumption	50%-65% Smoking History	More than 90% Smoking History Risk increases with increasing tobacco use
Alcohol Consumption	Not Significant	Synergistic with tobacco in increasing risk
Sexual History	Strong association to the number of oral sex partners	Not Significant
Socioeconomic Status	Higher	Lower
Incidence	Increasing	Decreasing
Prognosis	Good	Poor
Survival	Improved	Worse/Unchanging
Staging	Stage 3-4	All stages (T1-4)
Distant Metastasis	Unusual sites: Brain, skin	Lung

Abbreviations: HPV = human papilloma virus; OPSCC = oropharyngeal squamous cell carcinoma; SCC = squamous cell carcinoma; T = extent of the tumor. Adapted from[12,89].

In HPV-positive HNC, there is a site specificity with the viral DNA frequently found in Waldeyer's tonsillar ring[90]. Waldeyer's tonsillar ring consists of adenoid, tubal, lingual and palatine tonsils[90] (**Figure 7**). This ring is characterized by specialized reticulated squamous epithelium infiltrated with lymphoid tissue[80]. This epithelium is a fenestrated, discontinuous basement membrane, believed to enable immune cells to reach oral antigens[91] and serve as inherent interruptions in epithelial barriers, potentially allowing HPV to reach the basal keratinocytes even without traumatic epithelial disruption[92] (**Figure 8**). These characteristics may explain the HPV location preferentially in the Waldeyer's regions[90].

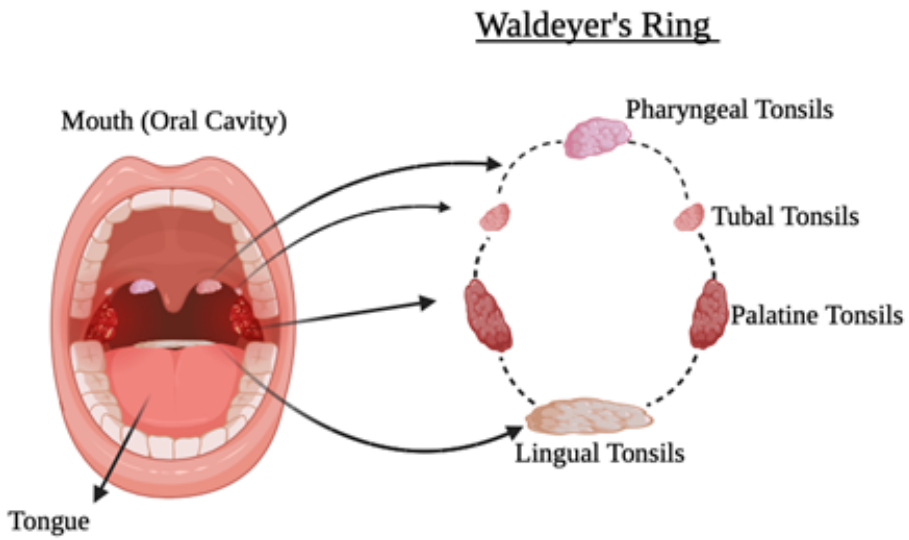

Figure 5: Waldeyer's ring. Waldeyer's ring consists of non-contiguous mucosal-associated lymphoid tissues (MALT) arranged in a circumferential configuration in the nasopharynx and oropharynx which consists of tonsils (pharyngeal, tubal, palatine and lingual), adenoids and lymphoid tissue. The ring contains lymphocytes immune cells helping in protecting the body against infections. Figure created using biorender. Adapted from[93]

HPV reaches the basal membrane, L1 binds to heparan sulfate proteoglycan (HSPG), and the capsid conformation will change[80]. These conformational changes allow the virus particles bind to the cell receptor and be internalized[80,94,95].

Basal epithelial cells are the first cells which get infected by HPV and infection undergoes complex stages which involves viral genome replication, gene expression and assembly of new viral particles[67]. Early viral proteins (E6 and E7) interfere with host cell regulatory mechanisms facilitating cell proliferation and preventing apoptosis, helping in establishing persistent infection within the host epithelial cells[71]. Interactions between E6/E7 proteins and p53 and pRb respectively contribute to dysregulation of key cellular processes, resulting in transformation of infected cells and development of HPV-positive cancers[71].

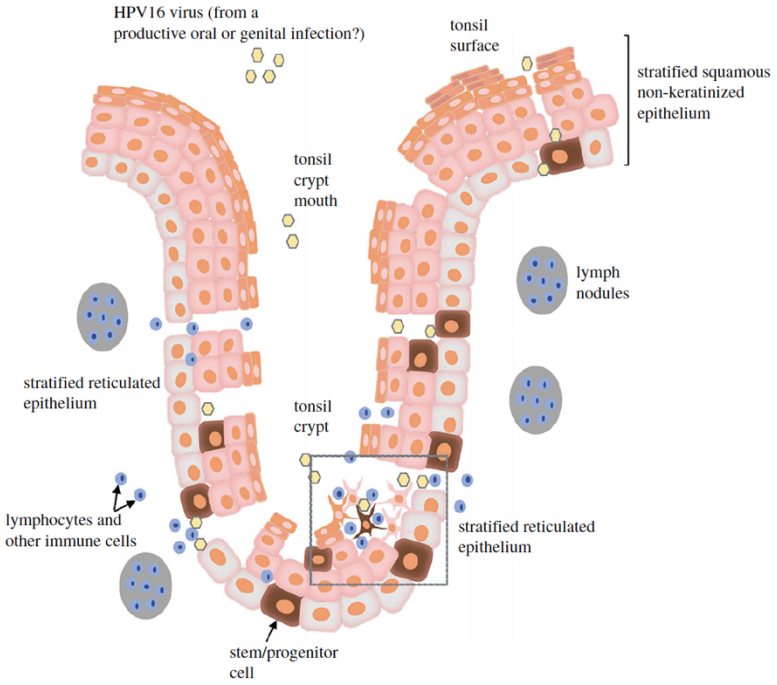

Figure 6: Epithelium of tonsils. Differences between the epithelial lining on the tonsil crypt surface (stratified squamous non-keratinized) and within the crypt (stratified reticulated). The boxed area highlights keratinocytes characteristic of the crypt reticulated epithelium, featuring long processes connecting with adjacent cells through desmosomes. The intercellular spaces are occupied by infiltrating lymphocytes. *Abbreviations:* HPV = human papilloma virus. Adapted from[96]

2.3.3.3 HPV-positive OPSCC inflammation mechanism

The connection between cancer and inflammation dates back almost 150 years when Rudolf Virchow observed a significant infiltration of leukocytes in tumor tissues[97]. Inflammation is linked to approximately 15%-20% of all human solid tumors[98]. However, there is a gap in understand the impact of inflammation on HPV tumor initiation and progression[98,99]. Liu *et al.,* examined the morphology of neutrophils and lymphocytes using large number of specimens involving OPC, normal oral mucosa, dysplasia, and carcinoma *in situ*[99]. The results indicated that samples from dysplasia, carcinoma *in situ*, and cancer were associated with higher inflammation[99]. The inflammatory process involves a complex cell-cell interactions and a cascade of events, including the participation of cytokines, chemokines, and the cells producing them, which collectively activate and regulate the inflammatory response[100-104]. HPV DNA analysis showed higher positivity of HPV16 in samples with chronic inflammation compared to mild and moderate inflammatory infiltration (P = 0.0009)[99] This suggests that inflammation might play a role in progression of HPV[99] and combination of HPV infection with inflammation could serve as a valuable marker for tumor prognosis[99].

2.4 Other risk factors

Other risk factors can be associated with HNC development. For instance, dietary factors, including inadequate nutrition and low folate intake showed to be a risk factor for HNC[12]. Reduced odds of HNC likelihood were observed in association with increased lean protein, vegetables, and fruits consumption (OR:0.53, 95CI: 0.39, 0.71)[105]. There was a positive association between laryngeal cancer and having sweets, processed and high-fat meats and fried foods (OR:2.12, 95%CI: 1.21, 3.72)[105]. In addition, poor oral hygiene occupational exposures to substances such as wood dust and asbestos, the infection with Epstein-Barr virus, Plummer-Vinson and Li Fraumeni syndrome, Fanconi anemia, dyskeratosis congenita, and sun exposure[12].

2.5 HNC treatment and prognosis

The prognosis in HNC is significantly influenced by the HPV status[106-108]. Treatment decisions are determined by the clinical and pathological stage of the cancer, encompassing surgery, radiation therapy, chemotherapy, immunotherapy, or a combination of these modalities[109,110]. Diagnosis of tumors in the head and neck region often occurs at advanced stages, leading to the standard use of chemotherapy in conjunction with radiotherapy[111]. However, this treatment approach is associated with considerable toxicity and severe side effect[112-114]. Currently, despite recent advancements in therapeutic discovery, patients with HNC continue to exhibit one of the lowest survival rates among cancer patients[115,116]. However, HPV-positive OPC is more sensitive to chemotherapy and radiotherapy compared with HPV negative HNC. Ang *et al*. reported a 3-year overall survival (OS) rate of 82.4% for HPV-positive OPC compared to lower OS rate of 57.1% for classical OPC[114].

The next chapter of the thesis presents the manuscript that directly aligns with the research objective of the master's study, focusing genetic alterations linked to the inflammatory response in HPV-positive OPC.

3 AIMS AND OBJECTIVES

The main goal of this thesis is to understand the molecular characteristics of HPV-positive and HPV-negative OPC, by examining the current databases on the genetic alterations of both.

To achieve this, the thesis aims to:

1- Analyse the databases of genetic mutations in HPV-positive and HPV-negative OPSCC by conducting an extensive literature review. This will enable identification of the role that genetic mutations play in the biological pathways that are involved in HPV-OPC carcinogenesis.
2- Validate the identified set of genes mutated using TCGA platform as a form of secondary analysis. Also, the identified biological pathways were validated using enriched gene pathway analysis and samples of HPV-OPC.

Manuscript I entitled "**Genetic Mutations Associated with Inflammatory Response Caused by HPV Integration in Oropharyngeal Squamous Cell Carcinoma**" addresses the first aim of this thesis by systematically reviewing the published papers on HPV-OPC genetic mutations. The specific objective of this manuscript is to identify the common genetic mutations in HPV-positive OPC which was clearly shown as the main result of this review (Manuscript 1) elucidated in the most common ten mutated genes *(TP53, PIK3CA, PTEN, NOTCH1, RB1, FAT1, FBXW7, HRAS, KRAS,* and *CDKN2A)*.

4 MANUSCRIPT

Genetic Mutations Associated with Inflammatory Response Caused by HPV Integration in Oropharyngeal Squamous Cell Carcinoma

Mai Atique 1,2, Isis Muniz 1,2,3, Fatemeh Farshadi 1,2, Michael Hier 1, Alex Mlynarek 1, Marco Macarella 1,
Mariana Maschietto 4,5, Belinda Nicolau 3, Moulay A. Alaoui-Jamali 2 and Sabrina Daniela da Silva 1,2,*

1 Department of Otolaryngology and Head and Neck Surgery, McGill University,
Montreal, QC H3T 1E2, Canada; mai.atique@mail.mcgill.ca (M.A.); isisamuniz@gmail.com (I.M.); fatemeh.farshadi@mail.mcgill.ca (F.F.); michael.hier@mcgill.ca (M.H.); alex.mlynarek@mcgill.ca (A.M.); marco.mascarella@mcgill.ca (M.M.)
2 Segal Cancer Centre and Lady Davis Institute for Medical Research, Departments of Medicine and Oncology, Sir Mortimer B. Davis-Jewish General Hospital, Faculty of Medicine, McGill University,
Montreal, QC H3T 1E2, Canada; moulay.alaoui-jamali@mcgill.ca
3 Graduate Program in Dentistry, Health Sciences Center, Federal University of Paraiba, Campus I,
João Pessoa 58051-900, PB, Brazil; belinda.nicolau@mcgill.ca
4 Department of Structural and Functional Biology, Institute of Biology, Universidade Estadual de
Campinas (UNICAMP), Campinas 13084-225, SP, Brazil; marianamasc@gmail.com
5 Boldrini Children's Center, Campinas 13084-225, SP, Brazil
* Correspondence: sabrina.wurzba@mcgill.ca

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Abstract: (1) Background: Head and neck cancer (HNC) ranks as the sixth most prevalent cancer in the world. In addition to the traditional risk factors such as alcohol and tobacco consumption, the implication of the human papillomavirus (HPV) is becoming increasingly significant, particularly in oropharyngeal cancer (OPC). (2) Methods: This study is based on a review analysis of different articles and repositories investigating the mutation profile of HPV-related OPC and its impact on patient outcomes. (3) Results: By compiling data from 38 datasets involving 8311 patients from 12 countries, we identified 330 genes that were further analyzed. These genes were enriched for regulation of the inflammatory response (*RB1*, *JAK2*, *FANCA*, *CYLD*, *SYK*, *ABCC1*, *SYK*, *BCL6*, *CEBPA*, *SRC*, *BAP1*, *FOXP1*, *FGR*, *BCR*, *LRRK2*, *RICTOR*, *IGF1*, and *ATM*), among other biological processes. Hierarchical cluster analysis showed the most relevant biological processes were linked with the regulation of mast cell cytokine production, neutrophil activation and degranulation, and leukocyte activation (FDR < 0.001; *p*-value < 0.05), suggesting that neutrophils may be involved in the development and progression of HPV-related OPC. (4) Conclusions: The neutrophil infiltration and HPV status emerge as a potential prognostic factor for OPC. HPV-infected HNC cells could potentially lead to a decrease in neutrophil infiltration. By gaining a better molecular understanding of HPV-mediated neutrophil immunosuppression activity, it is possible to identify a meaningful target to boost antitumor immune response in HNC and hence to improve the survival of patients with HNC.

Keywords: head and neck cancer; oropharyngeal squamous cell carcinomas; human papillomavirus; mutational profile; prognosis

1 Introduction

Head and neck cancer (HNC) is the sixth most prevalent cancer worldwide repre- senting more than 660,000 new cases and 325,000 deaths per year [1]. Risk factors driving the HNC landscape include alcohol, tobacco consumption, and infection with the human papillomavirus (HPV). HPV infection is emerging as a primary catalyst for a growing proportion of cancers of the tonsillar region, the base of the tongue, the soft palate, and the oropharynx, including oropharyngeal cancer (OPC) [2,3]. The diverse array of HPV types includes over 200 distinct serotypes, with HPV16 and HPV18 being the most prevalent oncogenic viral subtypes linked to OPC [4,5]. After initial infection, HPV can persist within host cell nuclei as an extrachromosomal episome, but can subsequently integrate into the host genome [6–8]. However, reported rates of HPV integration into the genome vary across studies. Data from The Cancer Genome Atlas (TCGA) indicate that HPV integrates in approximately 71% of virus-positive HNC cases and 83% of cervical cancer cases [8].

Beyond persistence and integration, HPV can profoundly influence tumor cell behavior, leading to distinct clinical outcomes in comparison to smoking-related counterparts [9,10]. This divergence is mirrored in the molecular mechanisms underpinning oncogenesis and specific mutations found in HPV-positive versus HPV-negative tumors [11,12]. Remarkably, HPV-related OPC as well as in anal and vulvar cancer represents a distinct molecular entity compared to its HPV-negative counterpart, demonstrating more favorable treatment responses and higher survival rates [13–15].

In 2017, the American Joint Committee on Cancer (AJCC) and the Union for Interna- tional Cancer Control (UICC) restructured the clinical staging system for patients. This effort involved the revision of the staging framework to incorporate genetic, histological, and prognostic variants, enabling the differentiation of prognostic disparities observed in HPV-related OPC [16–18]. It is observed that HPV-positive HNCs have fewer mutated genes compared to HPV-negative tumors, which tend to accumulate a higher number of mutations over time, leading to an increased mutational burden [12,19,20]. This article aims to provide a comprehensive evaluation of studies delving into the genetic profile of mutations in HPV-related OPC cases, alongside HPV-negative cases. Moreover, through the application of enrichment analysis and multiple validations using independent public datasets, the study aims to establish meaningful correlations between the identified genetic alterations, disruptions in relevant pathways linked to tumorigenesis, and the identified ge- netic alterations, pathways

disruptions relevant to tumorigenesis, and the multidisciplinary management of OPC in the context of the HPV status.

2 Materials and Methods

2.1 Study Selection

The comprehensive search strategy was done using the following databases: Medline, PubMed, Web of Science, and Scopus with the assistance of a librarian (up to 1 October 2023). The following Medical Subject Headings (MeSH) or "text words" were: HPV, human papillomavirus, papillomavirus, head and neck cancer, head and neck squamous cell carcinoma, oropharyngeal squamous cell carcinoma, pharyngeal cancer, survival, outcome, prognosis, prognostic, prognostic biomarkers, mutation, gene mutation, DNA mutation, DNA damage, and metastasis. Searches were performed in May 2023, with no restriction on the year of publication (Supplementary Table S1).

2.2 Inclusion and Exclusion Criteria

Inclusion criteria comprised articles in English that performed genetic analyses and comparisons between populations of HPV-related cases and HPV-negative OPC. Exclusion criteria were studies unrelated to HNC, animal and preclinical (in vitro) models, unrelatedto risk factors such as alcohol, tobacco, HPV 16–18, epigenetics, clinical trials, pediatric population, gene methylation, gene expression, copy number variation, another disease (not in cancer), another cancer type (not HNC), full text not available, reviews of the literature, case reports, conference abstracts, and letters to the editor.

2.3 Data Collection

Studies selected from the databases were imported into Rayyan software (https://rayyan.ai/terms) [21] for the identification and removal of duplicates and reading of titles and abstracts by three authors (MA, IM, and FF). The full text was retrieved for those studies where decisions could not be made based on the abstract and for those who presented the eligibility criteria. Data extraction from the studies included in this scoping review was summarized in a Microsoft Excel table (Microsoft 365). The following information was collected: authors, year of publication, impact factor, country, sample size, study type, molecular technique used, HPV status, and genes mutated. To identify mutated genes, genomic information was extracted directly from the reported data in each original article.

2.4 Technical Validation in Public Database

This research analyzed the mutation profile of OPC considering the HPV status. The TCGA public database was used to technically confirm the genetic mutations and the clinicopathological impact using the Head and Neck Squamous Cell Carcinoma database (TCGA, Firehose Legacy). Detailed descriptions of all other cohorts have been provided elsewhere [22–59] (Table 1). From the TCGA cohort, 115 samples were characterized as positive for HPV16 status, 74 being negative and 41 being positive. The data from this cohort were used to assess the influence of the genes on both overall survival and disease-free survival. The enriched analysis was done using multiple software including g:Profiler (https://biit.cs.ut.ee/gprofiler/, accessed on 1 August 2023), GSEA (http://software.broadinstitute.org/gsea/, accessed on 1 August 2023), Cytoscape (http://www.cytoscape.org/, accessed on 1 August 2023), and EnrichmentMap (http://www.baderlab.org/Software/EnrichmentMap, accessed on 1 August 2023).

Table 1. Characteristics of the published studies included in the analysis.

Author, Year	Journal Impact Factor	Country	Sample Size	Study Type	Molecular Techniques *
Harbison et al., 2018 [23]	19.477	USA	84	Cross-sectional	WGS, NGS
Chung et al., 2015 [24]	32.976	USA	252	Multicenter	NGS, ISH, IHC
Doerstling et al., 2023 [25]	4.322	USA	79	Retrospective	IHC, NGS
Dogan et al., 2019 [26]	7.316	USA	157	Retrospective	Target sequencing
Dubot et al., 2018 [27]	10.002	FRANCE	122	Retrospective	NGS
Gleber-Netto et al., 2018 [28]	6.921	USA	52	Retrospective	NGS, PCR, IHC
Gronhoj et al., 2018 [29]	4.638	DENMARK	114	Retrospective	NGS
Haft et al., 2019 [30]	6.921	USA	46	Retrospective	NGS
Koncar et al., 2017 [31]	4.711	USA	743	Retrospective	IHC, ISH, NG
Labarge et al., 2022 [8]	6.333	USA	12	Retrospective	WGS, OGM
Lim et al., 2019 [32]	13.312	KOREA	93	Multicenter	NGS
Qin et al., 2018 [33]	4.997	USA	36	Rettrospective	NGS.
Reder et al., 2019 [34]	5.972	GERMANY	24	Retrospective	NGS.
Reder et al., 2021 [35]	4.711	GERMANY	139	Retrospective	NGS.
Saba et al., 2020 [36]	3.240	USA	35	Retrospective	NGS
Wahle et al., 2022 [37]	5.08	USA	47	Retrospective	WGS, ISH, IHC
Stransky et al., 2011 [38]	63.832	USA	92	Retrospective	WGS
Williams et al., 2021 [39]	8.209	USA	703	Retrospective	NGS
Antonsson et al., 2016 [40]	2.532	AUSTRALIA	219	Case-control	NGS
Barten et al., 1995 [41]	4.548	GERMANY	37	Retrospective	PCR, IHC
Benzerdjeb et al., 2021 [42]	7.778	FRANCE	110	Cross-sectional	PCR, NGS
Chen et al., 2021 [43]	13.312	USA	489	Retrospective	ELISA
Chiosea et al., 2013 [44]	4.638	USA	75	Retrospective	NGS
Ekalaksananan et al., 2020 [45]	2.874	THAILAND	106	Case-control	PCR
Fallai et al., 2009 [46]	8.013	ITALY	78	Prospective	NGS, PCR
Farnebo et al., 2015 [47]	4.354	SWEDAN	169	Case-control	PCR–RFLP.
Hong et al., 2016 [48]	6.901	AUSTRALIA	202	Retrospective	Pyrosequencing
Cortelazzi et al., 2015 [49]	3.539	ITALY	76	Cross-sectional	PCR
De Carvalho et al., 2019 [50]	2.874	BRAZIL	25	Retrospective	PCR, WGS
Friedland et al., 2012 [51]	2.025	AUSTRALIA	60	Retrospective	PCR
Ghosh et al., 2013 [52]	2.435	INDIA	84	Prospective	NGS
Gross et al., 2014 [53]	41.376	USA	376	Prospective	PCR
Huang et al., 2019 [54]	11.205	USA	113	Retrospective	ISH, IHC, WGS
Licitra et al., 2006 [55]	50.739	ITALY	100	Retrospective	NGS, PCR, IHC

Table 1. *Cont.*

Author, Year	Journal Impact Factor	Country	Sample Size	Study Type	Molecular Techniques *
Mazurek et al., 2016 [56]	5.972	POLAND	200	Case-control	PCR
Saba et al., 2015 [57]	2.031	USA	8	Proof of concept	NGS
Sewell et al., 2014 [58]	13.801	USA	49	Prospective	RPPA
Shaikh et al., 2021 [59]	6.575	USA	2905	Retrospective	WGS, NGS, IHC

* WGS (whole-genome sequencing); NGS (next-generation sequencing); ISH (in situ hybridization); IHC (immunohistochemistry); RFLP (restriction fragment length polymorphism; PCR (polymerase chain reaction); OGM (optical genome mapping); RPPA (reverse-phase protein array).

2.5 Experimental Validation in Patients' Samples

Ethics and Patient Cohort

This study was approved by the Medical/Biomedical Research Ethics Committee (REC) of CIUSSS West-Central Montreal Research Ethics Board (REB, Montreal, QC, Canada) and informed consent was obtained from each patient.

Primary tumor samples were retrospectively collected from patients with OPC at the Jewish General Hospital, McGill University, Montreal, Quebec, Canada between 2010 and 2013 (with at least 10 years of follow-up). Patient demographics and survival out- comes were collected. HPV status was confirmed via p16 immunohistochemistry (IHC) as well as polymerase chain reaction (PCR). Detailed clinical information is provided in Supplementary Table S2. Disease-free survival was defined as the time from diagnosis to recurrence at any site or death. Recurrence was defined as the presence of local, re- gional, or distant disease after completion of treatment confirmed by microscopic exam. Strengthening the reporting of observational studies (STROBE Statement) was used to ensure appropriate methodological quality (http://www.strobe-statement.org/, accessed on 7 November 2023).

2.6 Immunohistochemistry

IHC staining was conducted at the Department of Pathology & Molecular Pathology Core Facility (Lady Davis Institute, Montreal, QC, Canada). Human Neutrophil Elas- tase/ELA2 Monoclonal Antibody (R&D Systems, Minneapolis, MN, USA, MAB91671R100;1:2000) was used to validate neutrophil infiltration. Tissues were examined using an Ape- rio ScanScope® slide scanner (Leica Biosystems, Buffalo Grove, IL, USA) and staining quantification was performed using QuPath (v0.2.3).

2.7 Statistical Analysis

All data were presented as mean SEM using the softw‗re GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). For statistical analysis, samples were categorized into two groups: (1) negative/weak and (2) moderate/strong positive cases. For frequency analysis in contingency tables, statistical analyses of associations between variables were performed by Fisher's exact test, and for continuous variables, the non- parametric Mann–Whitney U test. A p-value < 0.05 was considered significant.

3 Results

3.1 Overview of the Included Studies

Following the search protocol and screening strategy, 1556 manuscripts were identified. A total of 872 studies were published in English and 32 in different languages (including German, Chinese, Spanish, Hungarian, Russian, French, English, Japanese, and Czech). After the exclusion of 651 duplicate studies, the two reviewers also excluded 863 ineligible articles based on the title and abstract and an additional 41 articles based on the full-text assessment. Thus, 38 articles were included in the qualitative synthesis. The PRISMA flow diagram illustrates the search strategy and the number of studies found and retrieved (Figure 1).

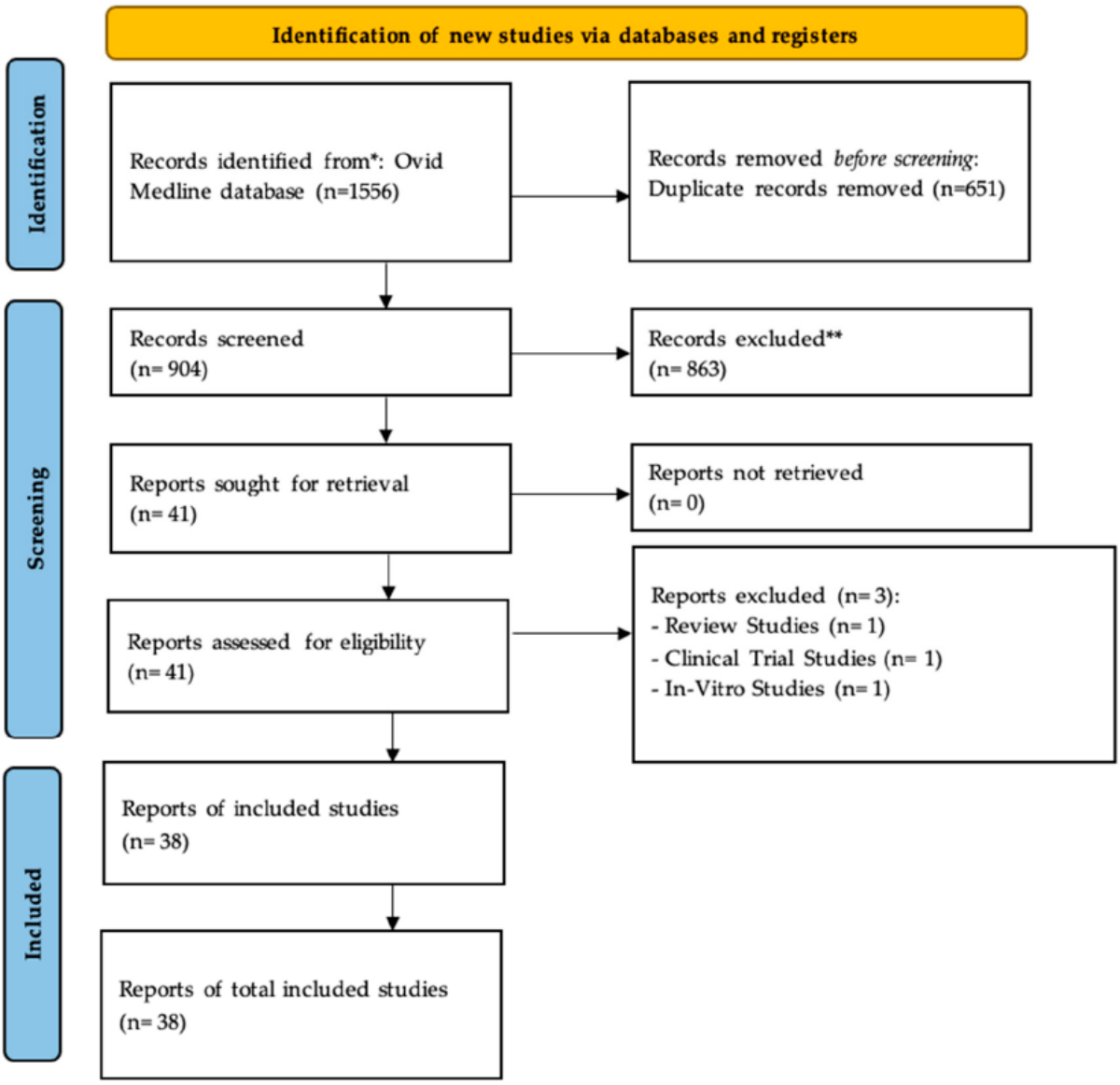


Figure 1. PRISMA flowchart highlighting the search strategy used to retrieve studies from the databases (Medline, PubMed, Web of Science, and Scopus). It identified 1556 articles and following the inclusion criteria, 38 articles were selected and included in this study. * Considered the number of records identified from each database or register searched. ** Number of records excluded by a human and automation tools [22].

The 38 studies included in this research were published between 1995 and 2023 and they involved 8311 HNC patients from 12 countries [23–59] (Table 1). Most studies were based on the retrospective cohort (n = 24). The most common country to lead the studies in mutational profile in HNC was the USA (n = 20/38). Two out of thirty-eight articles have included the list of gene mutations in both HPV-positive and negative cases. This study mainly focuses on retrieving data from HPV-positive patients to understand the alterations in cell pathways. Next-generation sequencing (NGS) (n = 10), PCR (n = 9), and p16 IHC staining (n = 8) were the most commonly used techniques, followed by other sequencing techniques such as whole-genome sequencing (WGS) (n = 5) and in situ hybridization (n = 3). In total, 330 genes were identified (Supplementary Table S1) and submitted to enriched analysis. As expected, *TP53* (n = 22) and *PIK3CA* (n = 20) genes were the most commonly mutated genes in HPV-related OPC cases.

3.2 Technical Validation—Common Gene Mutations in HPV-Positive HNC

In HPV-positive HNC, several genes were identified (Supplementary Table S1) and also confirmed as commonly mutated in the technical validation using the Head and Neck Squamous Cell Carcinoma database (TCGA, Firehose Legacy) (Figure 2). The data from this cohort were also used to assess the influence of the genes on both overall survival and disease-free survival (Figure 2B). The specific mutation landscape may vary to some extent depending on the tumor location and the HPV viral subtype (typically HPV16). However, the 10 most common mutated genes were *TP53* (n = 22), *PIK3CA* (n = 20), *PTEN* (n = 16), *NOTCH1* (n = 14), *RB1* (n = 13), *FAT1* (n = 13), *FBXW7* (n = 12), *HRAS* (n = 10), *KRAS* (n = 10), and *CDKN2A* (n = 10) (Figure 2A). Supplementary Figure S1 shows the identified genes in the 38 articles screened; different color codes were used to represent which genes were described from which article. It is important to consider that the most frequently mutated genes, such as *TP53*, *PIK3CA*, *CDKN2A*, *FAT1*, *CASP8*, and *HRAS*, can impact several pathways and biological processes, such as cell cycle, DNA damage response,

PI3K/AKT/mTOR signaling pathway, Notch signaling pathway, and *RAS/MAPK* signaling pathway.

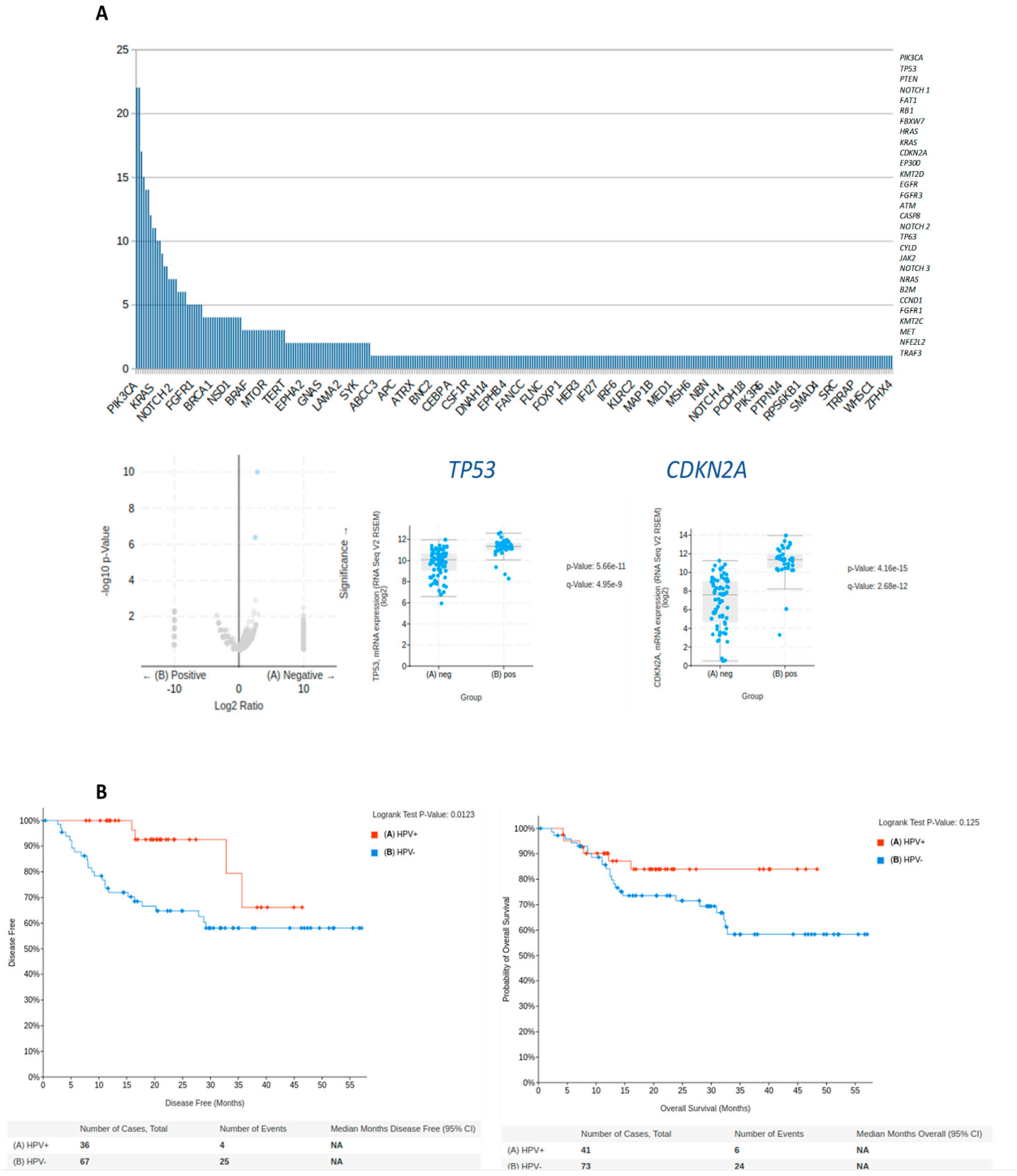


Figure 2. Comparison of mutation frequencies and gene expression profiles in HPV-positive and HPV-negative OPC. (**A**) In an analysis of all the investigated studies, HPV-positive OPC exhibited fewer mutations compared to HPV-negative tumors. The top bar graph illustrates the most prevalent mutated genes in HPV-related OPC, including *PIK3CA*, *TP53*, *PTEN*,

NOTCH1, *FAT1*, *RB1*, *FBXW7*, *HRAS*, *KRAS*, and *CDKN2A*. Using public data from the TCGA database, mRNA expression levels of *TP53* and *CDKN2A* exhibited significant differences between the two groups, highlighting their dis- tinct gene expression profiles in HPV-positive versus HPV-negative cases. (**B**) Significant differences were observed between HPV-positive and HPV-negative tumors in terms of overall survival and disease-free survival considering both genes using the Head and Neck Squamous Cell Carcinoma database (TCGA, Firehose Legacy). Among the 115 samples examined, 74 were identified as negative for HPV status, while 41 were confirmed as positive. Notably, HPV-positive cases exhibited enhanced overall survival rates compared with HPV-negative tumors.

3.3 Enriched Analysis of Mutated Genes

The list of all mutated genes was submitted to an enriched analysis. Gene ontology (GO) revealed 18 genes involved in the regulation of the inflammatory response *(RB1*, *JAK2*, *FANCA*, *CYLD*, *SYK*, *ABCC1*, *SYK*, *BCL6*, *CEBPA*, *SRC*, *BAP1*, *FOXP1*, *FGR*, *BCR*, *LRRK2*, *RICTOR*, *IGF1*, and *ATM*) (Figure 3). Hierarchical analysis revealed the biological processes most relevant were linked with the regulation of leukocyte migration, mast cell cytokine production, neutrophil degranulation, and leukocyte activation (FDR < 0.001; *p*-value < 0.05) (Figure 3C).

In order to provide experimental validation for the results from the enriched analysis that showed alteration in neutrophil activation and degranulation (Figure 3C), we selected a cohort of HNC patients to confirm the status of neutrophil expression (Supplementary Table S2). For the independent sample set, 52 paraffin embedded HNC tissue specimens from 12 patients who had lung metastasis (metastatic cases) and 40 patients who had negative lymph node status without recurrence or metastatic disease (good outcomes; non-metastatic cases) and were followed for at least 157 months were evaluated using IHC assays in a TMA. Most of the patients were male (59.6%), and the majority were aged over 50 years (84.6%) (Supplementary Table S2). First, before the antibody selection, we performed an additional analysis using the UMAP (Uniform Manifold Approximation and Projection) plot to provide an illustrative representation of gene clusters formed through the application of Louvain clustering on gene expression profiles across different immune cell types (Figure 4A). The table below the UMAP provides annotations and gene counts that connect to the core function of a neutrophil elastase (ELA2), also known as

polymorphonuclear leukocyte elastase, which is a serine protease belonging to the chymotrypsin family. This shows us the specificity of ELA2 for the neutrophil activity.

Immunohistochemistry staining was done in our patients' cohort (treated in a single institution) and it revealed elevated nuclear overexpression of ELA2 protein in metastatic HPV-related OPC. In contrast, weak to moderate expression was observed in non-metastatic tumors, and negative expression was detected in morphologically normal epithelial cells (Figure 4B). However, no statistically significant *p*-values were observed in the associations involving age ($p = 0.599$), sex ($p = 0.500$), tobacco consumption ($p = 0.087$), alcohol abuse ($p = 0.985$), lymph node stage ($p = 0.158$), locoregional recurrence ($p = 0.275$), and vital status ($p = 0.500$), but were for clinically advanced T stage ($p = 0.023$) (Supplementary Table S1).

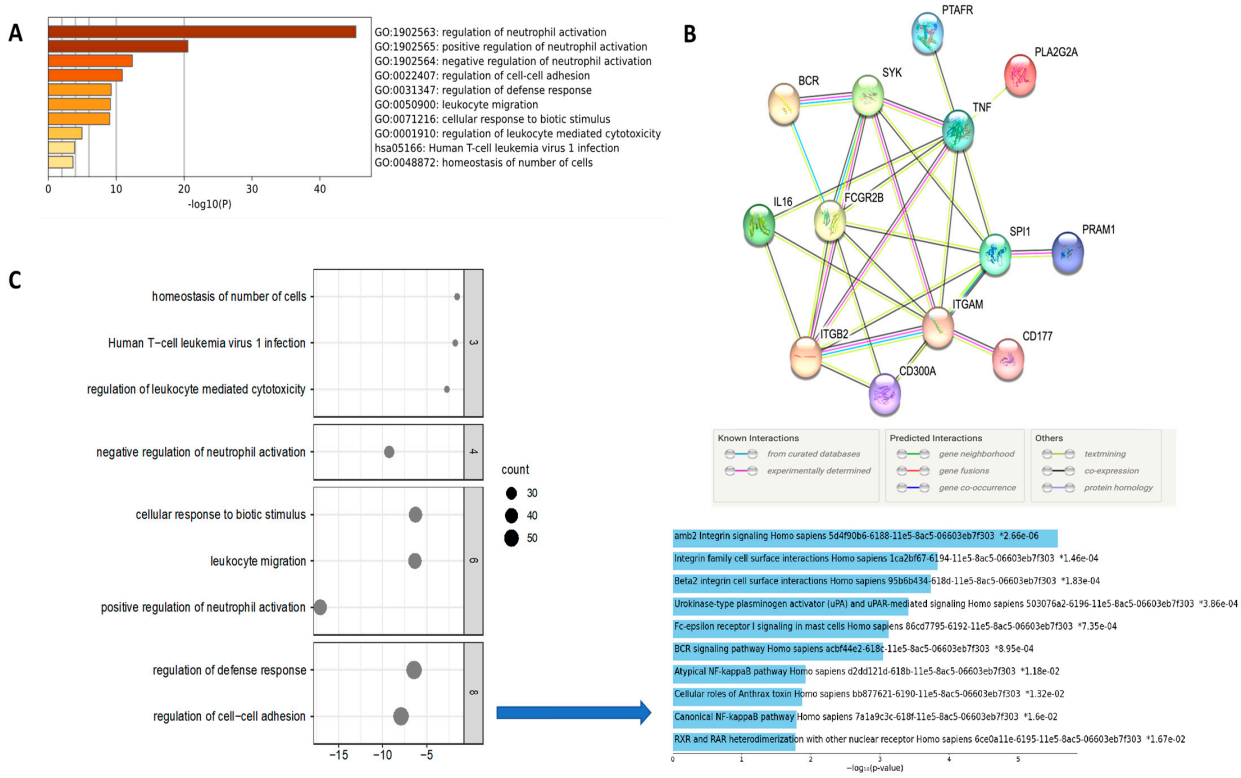
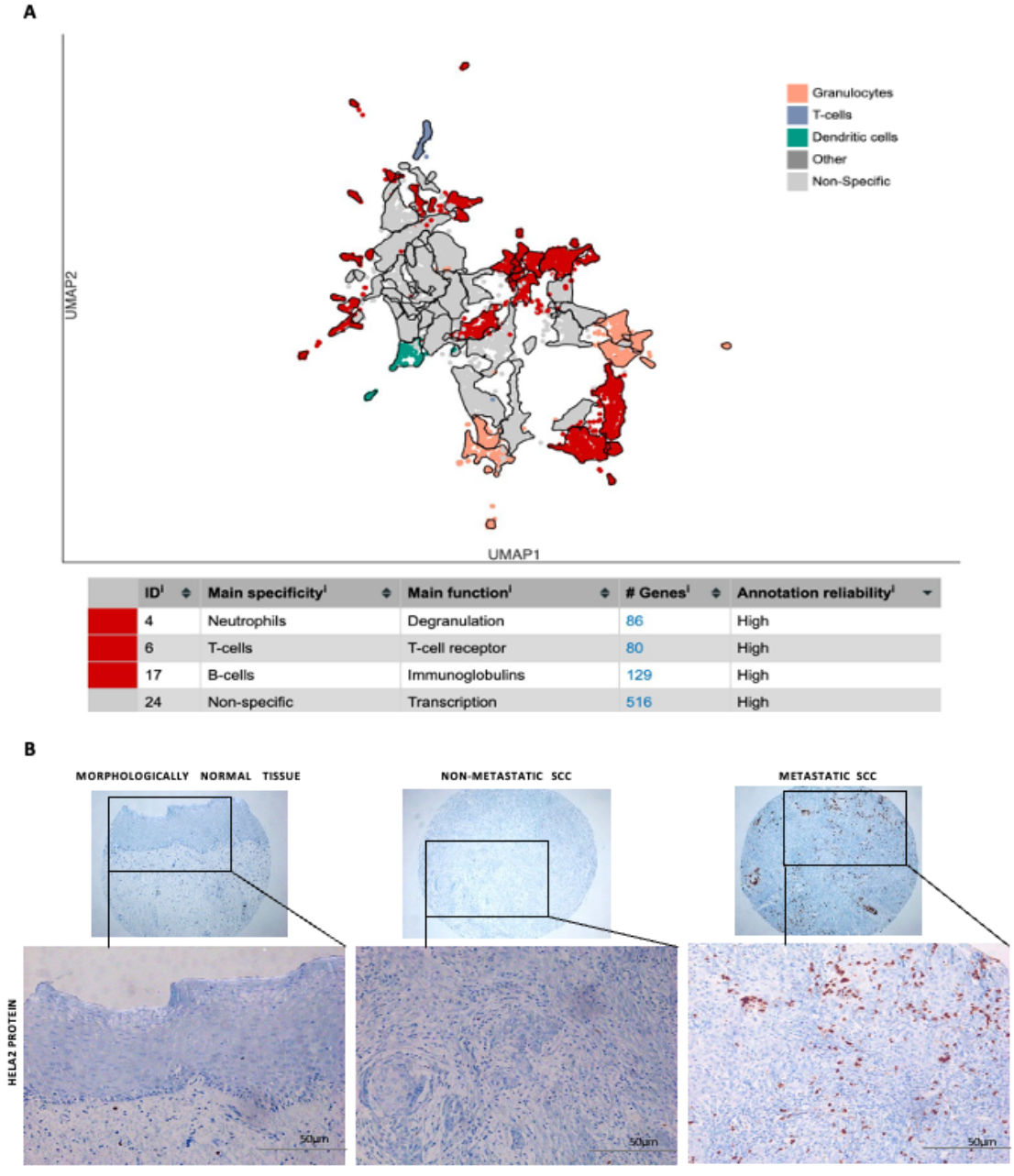


Figure 3. (**A**) Gene sets with low *p*-values and high enrichment scores identified a significant biological context in the regulation of neutrophil activation. (**B**) Protein–protein interaction (PPI) data are represented as nodes (proteins) and edges (interactions) to construct a network based on computational algorithms that integrate various sources of biological data. (**C**) Enrichment analysis showed functional categories and pathways overrepresented in the network related to the regulation of cell–cell adhesion, especially related to integrin signaling.



	ID	Main specificity	Main function	# Genes	Annotation reliability
	4	Neutrophils	Degranulation	86	High
	6	T-cells	T-cell receptor	80	High
	17	B-cells	Immunoglobulins	129	High
	24	Non-specific	Transcription	516	High

Figure 4. (**A**) The UMAP (Uniform Manifold Approximation and Projection) plot illustrates cell clusters created through Louvain clustering of ELA2 gene expression in various immune cell types. The table provides cellular annotations associated with the primary function of ELA2 identified and validated in our study. (**B**) Immunohistochemistry images for ELA2 protein in oral cancer and morphologically normal epithelial. A weak staining was observed in morphologically normal epithelial cells while a strong intensity of nuclear immunostaining was

detected in oral cancer samples, especially in the recurrent tumors. Graphs represent the ELA2 immunohistochemistry level (intensity) in normal, tumor, and metastatic lymph nodes. Original magnification: 50× (**top**) and 200× (**bottom**).

4 Discussion

The exponential increase of HPV-related OPC over the last two decades has gained attention. This subset of OPC is characterized by a distinct genomic mutational burden compared to its HPV-negative counterparts [2,60] (Figure 5). In this context, an in-depth exploration was conducted to delineate the mutation profile of HPV-related OPC patients, drawing from a comprehensive literature review spanning from 1995 to 2023 [23–59]. The genetic landscape showcased six prominent genes (*TP53*, *NOTCH1*, *CDKN2A*, *PIK3CA*, *HRAS*, and *PTEN*) exhibiting frequent mutations. These genes encode pivotal signaling molecules that underlie the pathogenesis of HNC [61]. Notably, *TP53* and *PIK3CA* emerged as pivotal players, with *TP53* being the most recurrently mutated gene in locally advanced HNC [61,62], and *PIK3CA* ranked as the most frequently mutated oncogene across human cancers [63].

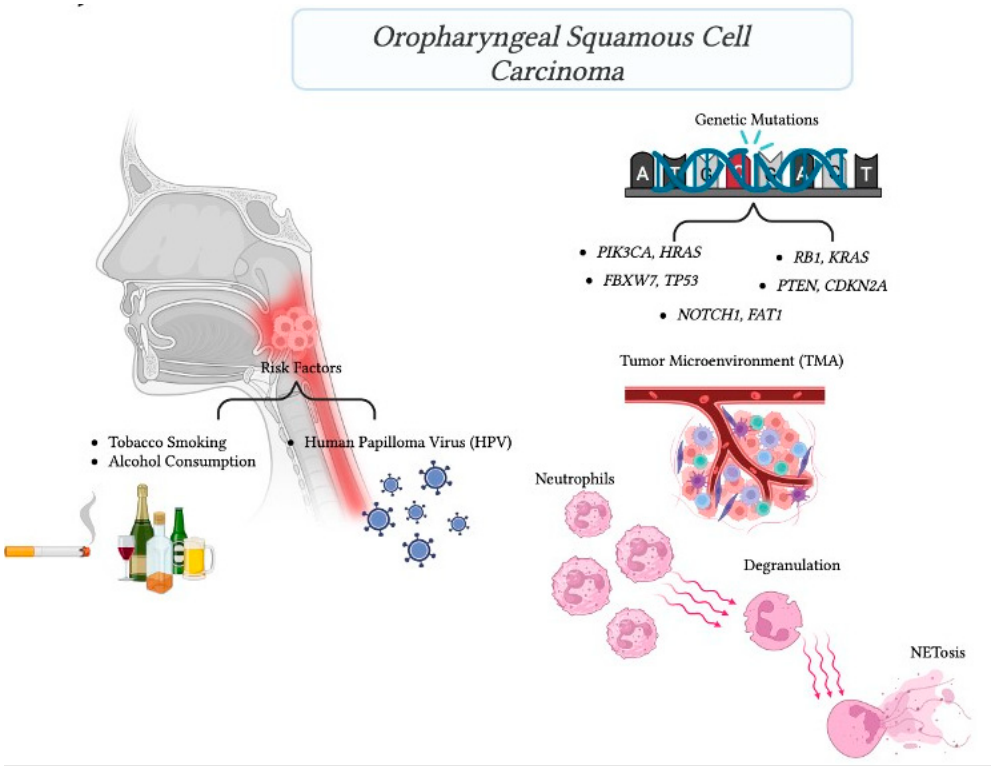


Figure 5. Squamous cell carcinoma comprises over 95% of head and neck cancers. Major risk factors include tobacco and alcohol. HPV is involved in 71% of oropharyngeal cancers. Specific key genetic mutations were associated with HPV-positive oropharyngeal cancer

(*PIK3CA*, *RB1*, *FBXW7*, *PTEN*, *NOTCH1*, *HRAS*, *KRAS*, *TP53*, *CDKN2A*, *FAT1)*. The intricate interplay between human papillomavirus (HPV) and mutations within the tumor microenvironment (TME) is complex. HPV infection can initiate a particular immune response, but tumors can also evolve and develop mechanisms to modify and escape the immune detection. A comprehensive understanding of these interactions is crucial for developing effective therapeutic strategies for HPV-associated tumors, including head and neck cancers. Figure created using BioRender.

The *TP53* gene encodes the tumor protein p53, functioning as a critical tumor suppressor that regulates cell division and reduces uncontrolled proliferation [64,65]. Intriguingly, *TP53* mutations in HPV-positive HNC have been linked to treatment resistance and poorer clinical outcomes. Meanwhile, mutations in the *PIK3CA* gene, responsible for encoding the PI3K catalytic subunit alpha (p110α), activate the PI3K/AKT/mTOR signaling pathway. This subset of *PIK3CA* mutations observed in HPV-positive HNC plays a pivotal role in tumorigenesis, potentially contributing to increased cell proliferation, tumor growth, and survival [66–68]. The dysregulation of these pathways collectively orchestrates the development and progression of HPV-positive HNC. In patients with HPV-negative HNC samples, it is commonly noted that there is a higher mutation load in comparison with HPV-positive tumors. Our working hypothesis is that the absence of the virus particles requires the acquisition of a larger set of mutated genes to facilitate cellular transformation. In contrast, within HPV-positive samples, the presence of the viral genome regulates the expression of specific genes that modify the cells toward malignancy. These genes are likely associated with cell-cycle regulation. However, a more comprehensive understanding of the functional repercussions of these mutations and their implications for targeted therapies and patient outcomes remains imperative.

Gene-enriched pathway analysis unveiled the predominant involvement of the inflammatory response in HPV-related OPC. Notably, 18 genes (*RB1*, *JAK2*, *FANCA*, *CYLD*, *SYK*, *ABCC1*, *SYK*, *BCL6*, *CEBPA*, *SRC*, *BAP1*, *FOXP1*, *FGR*, *BCR*, *LRRK2*, *RICTOR*, *IGF1*, and *ATM*) were intricately linked to the activation of neutrophils. The intricate interplay between the tumor microenvironment and immune cell subsets has emerged as the focal point of investigation in HNC research [69]. The presence of HPV infection often triggers a robust immune response, fostering chronic inflammation within the tumor microenvironment [70]. Remarkably, HPV-positive tumors display heightened immune cell infiltration compared to HPV-negative HNC. These infiltrating immune cells include various subsets of T cells (e.g., CD8+ cytotoxic T cells,

CD4+ helper T cells), natural killer (NK) cells, macrophages, and dendritic cells [71]. The neutrophils, which represent a pivotal component of the immune response, are intricately recruited to the tumor site through a complex interplay between tumor-derived chemokines and adhesion molecules, such as CXCL8/IL-8 and E-selectin [72–74]. Once they are established within the tumor microenvironment, neutrophils can polarize and assume distinct functional phenotypes, oscillating between a pro-inflammatory N1 phenotype and an immunosuppressive N2 phenotype [75,76]. This versatile plasticity is modulated by an interplay of chemokines, cytokines, and damage-associated molecular patterns (DAMPs) emanating from both tumor cells and the surrounding inflammatory milieu [72]. The interactions between neutrophils and other immune cell subsets, including T cells, dendritic cells, and myeloid-derived suppressor cells (MDSCs), sculpt the intricate landscape of the local immune response [77].

The relevance of neutrophils extends further, with a high-circulating neutrophil-to- lymphocyte ratio (NLR) emerging as a common feature in numerous cancer types, including HNC [78–80]. Interestingly, elevated neutrophils have been associated with chemotherapy and immunotherapy resistance in HPV-positive cancers. Neutrophil-derived factors, encompassing reactive oxygen species (ROS), cytokines, and extracellular traps (NETs), can exert a dual influence, promoting tumor growth, angiogenesis, and metastasis, while also suppressing adaptive immune responses. Moreover, neutrophils can influence the infiltration and functionality of tumor-infiltrating lymphocytes (TILs), thereby intricately modulating the overall antitumor immune response.

In HPV-positive cancers, the presence of NETs within the tumor microenvironment has gained attention, owing to their potential to foster tumor progression by inducing angio- genesis and evading the immune response [81,82]. The dynamic role of neutrophils, driven by their phenotype heterogeneity and functional plasticity [81,83,84], positions them as critical regulators of both pro-inflammatory and anti-immune responses [81]. Their context- dependent antitumor or pro-tumor activity depends on the molecular stimulus within the tumor microenvironment [81,83], where a delicate balance controls the equilibrium between these phenotypes [69]. In the specific context of HPV-related OPC, a high NLR has been associated with ad- vanced clinical stages and poorer survival rates [81,83,85–87]. Paradoxically, HPV infection could potentially suppress the recruitment of tumor-associated neutrophils (TANs) to HPV- related OPC [88]. The influence of TANs in promoting cancer progression stems from their ability to induce angiogenesis, release ROS, and generate reactive nitrogen species (RNS),

thereby inducing genotoxic effects upon tumor cells [83,87,89]. Furthermore, TANs secrete cytokines (IL-1β, TNF-α, IL-6, and IL-12) that foster a chronic inflammatory milieu, alongside arginase 1, which inhibits CD8 T cell function, contributing to an immunosuppressive environment [90]. Unraveling the intricate interactions between tumor cells, neutrophils, and the surrounding milieu represents an imperative avenue for research, promising the development of innovative strategies to impede cancer progression and metastasis.

Conversely, it is known that the most effective antitumor mechanism involving neutrophils is through antibody-dependent cell-mediated cytotoxicity (ADCC) [83]. Pro- inflammatory neutrophils can be activated to display a stronger antitumor phenotype through the molecular interaction with the granulocyte colony-stimulating factor (G-CSF), transforming growth factor-α (TNF-α), and/or by blocking transforming growth factor-β (TGF-β) [83]. These interactions culminate in the activation of a cytotoxic immune response directed against tumor cells [83]. However, the underlying mechanism by which tumor-derived signals reprogram neutrophils to undergo this functional transformation is poorly understood and warrants further investigation. Ultimately, a deeper understanding of the intricate interactions between neutrophils and HPV-related HNC will likely provide novel insights into their role within metastatic pathways, potentially identifying targetable mechanisms that modulate neutrophil phenotype.

5 Conclusions

In summary, the intricate involvement of neutrophils in the development and progression of HPV-related OPC has become increasingly apparent. The infiltration of neutrophils and the underlying HPV status hold significant promise as prognostic parameters for OPC. Notably, the presence of HPV infection within HNC cells may induce a decreasing effect on neutrophil infiltration. The outcomes from this study have paved the way for novel avenues of investigation, focusing on unraveling the intricate crosstalk between cancer cells and the immune infiltrate microenvironment. These dynamic interactions orchestrate changes in the neutrophil population, presenting opportunities to conceive innovative therapeutic strategies. The prospect of personalized immunomodulation emerges as a promising frontier to treat patients with HPV-related HNC. As future research will involve deep investigations of the complexities of these interactions, we are primed to uncover transformative interventions that hold the potential to enhance the prognosis and overall quality of life for individuals battling HPV-related HNC.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines12010024/s1, Table S1: Data extraction of the included articles in the scoping review. The 38 articles presented the list of HPV-positive (+) and HPV-negative (−) mutated genes. A total of 2 articles out of the 38 articles screened have included both lists of genes. However, the rest included HPV + mutated genes which this scoping review mainly highlights. A total of 330 genes were identified and submitted to enriched analysis. *TP53* (n = 22) and *PIK3CA* (n = 20) genes were the most mutated genes in HPV-related OPC cases. Table S2: Distribution of the OSCC cases according to demographic, lifestyle, and clinical variables. Figure S1: A list of all genes mutated with highlighting on the most cited genes, which are *TP53* (n = 22), *PIK3CA* (n = 20), *PTEN* (n = 16), *NOTCH1* (n = 14), *RB1* (n = 13), *FAT1* (n = 13), *FBXW7* (n = 12), *HRAS* (n = 10), *KRAS* (n = 10), and *CDKN2A* (n = 10). Different color codes representing the 38 articles screened show which gene was collected from which article. The most frequently mutated gene is *TP53* followed by *PIK3CA*. Genes are for cell survival and proliferation (*TP53*, *HRAS*, and *PIK3CA*), cell-cycle control (*CDKN2A*), cellular differentiation (*NOTCH1*), and adhesion and invasion signaling (*FAT1*), and tumor suppression (*FAT1*, *NOTCH1*, and *CDKN2A*).

Author Contributions: Conception and design: S.D.d.S.; Development of methodology: M.A., I.M. and F.F.; Acquisition of data: M.A., I.M. and F.F.; Analysis and interpretation (e.g., statistical analysis): S.D.d.S. and M.M. (Mariana Maschietto); Writing, review, and/or revision of the manuscript: M.A., I.M., F.F., M.H., B.N., M.A.A.-J., A.M., M.M. (Marco Macarella), M.M. (Mariana Maschietto) and S.D.d.S.; Administrative, technical, or material support: M.H., B.N., M.A.A.-J. and S.D.d.S.; Study supervision: S.D.d.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Medical/Biomedical Research Ethics Committee (REC) of CIUSSS West-Central Montreal Research Ethics Board (REB #2011-84, 10-153; February 2023).

Informed Consent Statement: The informed consent was obtained from each patient.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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5 DISCUSSIONS AND CONCLUSION

The following section provides a summary of the research, including its rationale, findings, strengths, and limitations. It also discusses future research directions and explores the implications of the results.

4.1 Summary of research

The primary goal of my thesis was to conduct a comprehensive literature review focusing on studies related to HPV-positive OPSCC to identify the most common genetic alterations across all examined studies. Using the information generated from this literature, other team members fulfill the objectives stated in the chapter aims and objectives. While I am the first author on the paper, I did not participate in the execution of other aims of the papers. Therefore, the discussion and conclusions on this chapter refers only to the literature review that I conducted. A literature review was conducted to identify the mutation profile of patients with HPV-positive OPSCC. This research identified ten most common mutated genes which were *TP53, PIK3CA, PTEN, NOTCH1, RB1, FAT1, FBXW7, HRAS, KRAS,* and *CDKN2A* as mentioned earlier.

HPV-positive cancers represent a significant global health challenge, emphasizing the critical need for a comprehensive understanding of the biological mechanisms driving tumor development. Further exploratory data analysis revealed the complex interplay between tumor cells, neutrophils, and the surrounding TME. n HPV-positive tumors, the viral oncoproteins E6 and E7 interfere with the proteins p53 and Rb involved in regulatory pathways within the cells by eliminating the need for various genetic changes induced by prolonged exposure to

cigarettes. Consequently, HPV-positive OPSCC exhibits a molecular-genetic profile distinct from cancers associated with smoking[111,112]. The presence of HPV in OPSCC defines a clinically unique form of HNSCC with significantly better clinical outcomes compared to its HPV-negative counterpart[113,114]. So, it is a consensus that HPV-driven OPSCC and OPSCC associated to tobacco and alcohol consumption represent biologically distinct entities[25,115-120] The information about the molecular profile of HPV-positive HNSCC might provide new opportunities to develop novel biomarkers for HPV diagnostics and innovative therapeutic approaches.

4.2 Strengths and limitations

Since this thesis was based on conducting a comprehensive literature review. Strength of this review depends entirely on the quality of included studies. That is in terms of the range and number of papers included, the broad focus captured most of the genetic mutations noted in HPV-positive and HPV-negative OPSCC.

This review encompasses various study designs, including retrospective cohorts, thus including a spectrum of papers covering the clinical as well as genetic and epidemiological perspectives. Additionally, the maximum interval of included published studies was up to 2023 ensuring the relevance and latest research findings. The result of our study that neutrophil infiltration and HPV status may have potential prognostic significance in OPSCC are clinically significant in predicting the patient's prognosis and helping in finding new treatment strategies.

The cumulative data of approximately 8300 patients across 12 countries within this review helped in highlighting the gaps in knowledge and further implying the potential directions towards future research studies.

Due to the reliance of the searching process on pre-existing literature, this research study may have limitations related to publication bias, which could result in an overrepresentation of studies with significant or positive findings. Studies' comparability and relevance may be impacted by changes in treatment modalities, technology, and diagnostic criteria over time, given the period from 1995 to 2023. A scoping review provides a broad overview, but its analysis may not be as in-depth as that of systematic reviews.

4.3 Implications for public health

This study pinpoints the key genetic changes associated with HPV-positive and HPV-negative OPSCC. This knowledge can support future investigations in developing drug screening methods and early detection tools. The fundament of this thesis can help educational health programs about risk factors and sexual behaviours as well as prevention and vaccination campaigns, including to avoid the future occurrence of HPV-related cancers.

4.4 Future directions

The results of this study open new avenues for future research projects to explore the functional outcomes of the identified genetic alterations aiming to evaluate their role in HPV-positive HNC development and progression. The use of preclinical and animal models could support future studies to potentially develop chemical compounds to target these genetic alterations identified in my thesis in order to perform target drug evaluation. The advent of new molecular genome-altering technologies such as CRISPR/Cas9 allows for genetic mutations to be removed or inserted in the germ line of a mouse faster and less expensively than previous methods. This technique could also be used to knockout or knockin the genes identified in my thesis to perform functional analysis. In addition, manipulations of gene expression with siRNAs and antisense oligonucleotides, allow for even greater exploration into genomics and systems biology enhancing the potential for drug discovery and personalized medicine. However, caution should be considered in interpreting *in silico* data to the relevance in clinical settings. It is recommended that results from *in silico* prediction algorithms should never be used as the sole evidence for clinical decision making.

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7 APPENDIX- Supplementary Material for Manuscript

Supplemental Tables & Figures

Supplemental Table S1: Data extraction of the included articles in the scoping review. The 38 articles present the list of HPV-positive (+) and HPV-negative (-) mutated genes. 2 articles out of the 38 articles screened have included both lists of genes. However, the rest included HPV + mutated genes which this scoping review mainly highlights. 330 genes were identified and submitted to enriched analysis. *TP53* (n= 22) and *PIK3CA* (n= 20) genes were the most mutated genes in HPV-related OPC cases.

AUTHOR, YEAR	HPV + GENES MUTATED	HPV - GENES MUTATED
1. R. Alex Harbison et al., 2018	KMT2D, FGFR3, CYLD, EP300, PIK3CA, RB1, PEG3, STAT3, TTSC2, B2M, CREBBP, FBXW7, FLT1, NCOR1, NSD1, PTEN, USP9X, BRIP1, NBN, NFE2L2, TACC3, ARID1B, ARID5B, DDR2, EPHA2, FANCA, KDM5C, LRRK2, MAP2K2, MAPK1, NOTCH3, PTPRD, SMAD2, SYK, TRAF3, TRRAP. FLT1, IDH2, AR, ASXL1, ATM, AXIN1, BAP1, BCR, CIC, ELF3, FANCA, FAT1, FLT4, GRIN2A, HDAC4, HIF1A, IFNGR1, KMT2D, LRP2, MAP3K5, MED12, MTOR, NBN, PIK3R6, RB1, SRC, TACC3, TRRAP, TSC2, XPO1. **RECURRENT OPSCCS:-** TP53, CASP8, FAT1, HLA-A, AJUBA, AND NSD1.	
2. Chung, C. H. et al, 2015	IK3CA, SOX2, MLL2 (KMT2D), RB1, BCL6, EP300, NOTCH1, PTEN, FGFR3, ASXL1, KLHL6, FBXW7, TP53, ATM, BRCA2, BRIP1 (BACH1), LRP1B, ATRX, KDM6A, BRCA1, BLM, JAK2, NF1, HRAS, MYC, ATR, FGF19, FGF3, FGF4, RICTOR	TP53, CDKN2A/B, FGF19, FGF3, FGF4, PIK3CA, CCND1, NOTCH1, LRP1B, SOX2, MLL2 (KMT2D), EGFR, KLHL6, BCL6, ATR, NFE2L2, NOTCH2, MYC, FGFR1, ATRX, JAK2, SMAD4, RICTOR, ZNF703, BRCA2, FOXL2, PRKDC, GPR124, KDM6A, APC.
3. Doerstling, S. et al., 2023	ATM, CCND1, CDKN2A, RB1, EGFR, FBXW7, FGFR1, FGFR2, FGFR3, IDH1, KRAS, NRAS, HRAS, NOTCH1 AKT1, MTOR, PIK3CA, PTEN, TP53, AR, ALK,, BRAF, BRCA1, BRCA2, CDK/RB PATHWAY (CCND1, CDKN2A, RB1), FGFR1-4, FLT3, JAK2, MET, MLH1, MSH2, MSH6, PI3K PATHWAY (AKT1, MTOR, PIK3CA, PTEN).	
4. Dogan, S. et al., 2019	TP53, SOX2, CDKN2A/2B, PIK3CA, TP63, KMT2D , NOTCH1, FAT1, 11Q13 GENE CLUSTER (FGF3/FGF4/FGF19/CCND1) , FOXA1, NOTCH PATHWAY GENES (NOTCH1, NOTCH2, NOTCH3, NOTCH4, EP300, FBXW7, SPEN, KDM5A), HISTONE MODIFIERS (KMT2D, CREBBP, KMT2C, EP300, KMT2A). NFE2L2, KEAP1, CUL3, EGFR, ERBB2, FGFR1, FGFR3, FOXA1, TERT, NKX2-1, FGFR1, PMA1P1, ERBB2, EGFR, YAP1, ATM, MYC, KEAP1, CASP8, CUL3. JAK3. MAP3K13, FOXA1, HRAS, CCND1,PIK3/AKT/MTOR PATHWAY. FAT1, RUNX1.	
5. Dubot, C. et al., 2018	CDKN2B, RB1,MDM2, RICTOR, PI3K, KRAS, NRAS, MAPK, AJUBA, SYNE2, USP9X, KDM6A, NSD1, LRP1B. CELL CYCLE PATHWAY (TP53, CCND1, CDKN2A), PI3K/AKT/MTOR PATHWAY (PIK3CA), TYROSINE KINASE RECEPTORS (EGFR, FGFR1), CELL DIFFERENTIATION (FAT1, NOTCH1).	
6. Gleber-Netto, F. O. et al., 2018	TP53, NOTCH1, CDKN2A, NOTCH2, PIK3CA, FAT1, FBXW7, KEAP1, NFE2L2, NSD1, TP63, EGFR, HRAS, CASP8, CCND1, TGFBR2	
7. Gronhoj, C. et al., 2018	APOB, BIRC6, SPTBN1, FAT2, KMT2A, FAT1, BPTF, TRIO, HERC2, KALRN, ZNRF3, BNC2, NOTCH2, FGFR2, SMAD2, AR, SIN3A, PTCH1, DNMT3A, ARHGAP35, F5, IGF1R, CATSPER1, IQGAP1, SFMBT2, MET, DUBP1, TENM2, TSC1, ARID5B, FAT2, FNDC1, BIRC6, PTPN14, QSER1, ALS2CL, PIK3CB, ARID2, NOTCH3, APOB, FGFR1, NF2, NRXN3, MYH9, PRPF8, FOXP1, PEX2, PBRM1, IPO7, SPTA1, TRIO, ABCG1, TJP2, EP300, RET, SLX4, AKT2, FN1, HCFC1, PCDH18, WHSC1, BPTF, CREBBP	

8. Haft, S. et al., 2019	PIK3CA, KMT2C, FBXW7, FGFR3, CREBBP, FAT1, NSD1, KMT2D, NOTCH1, CASP8, CLTCL1, EPHA2, HLA-A, HRAS, PDE4DIP, PTCH1, PTEN ZNF750, ADCY4, AJUBA, ATM, CTCF, CUL3, FANCA, MET, LRP1B, CASZ1, CYLD, EP300, KALRN, MACF1, ASXL3, CSMD3, DST, FLNC, HUWE1, KIAA1407, ASPM, DNAH5, FAM135B, HERC1, HFM1, LRRC37B, MAP1B, MUC4, MUC5B, POLR3A, TRAF3, SERPINB5, USP6, FLG	
9. Koncar, R. F. et al., 2017	ABL1, AKT1, ALK, APC, ATM, BRAF, BRCA1, BRCA2, CDH1, C-KIT, CMET, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KRAS, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, STK11, TP53, VHL.	
10. Labarge, B. et al., 2022	FLG, PIK3CA, MUC12, ZNF750, USE1, KMT2D, MUC6, TP63, SLITRK3, NLRC5, MORN1, EFNB2, HLA-A, KRTAP1-1, B2M, PXN, CDKN2A, RB1, TRAF3, PTEN.	
11. Lim, S. M. et al., 2019 Multicentric study	TP53, CDKN2A, CCND1, PIK3CA, KMT2C, FAT1, RELN, FAT4, CDKN2B, EGFR, KMT2D, NFE2L2, ADGRV1, NOTCH1, FAT2, CTTN, MYC, GNAS, EPHB4, ASXL3, CHD4, SOX2, ASXL1, KEAP1, AR, NOTCH2, KLHL6, TERT, KRAS, PTEN, MAP3K9, CDH9, CDH1, HPV	
12. Qin, T. et al., 2018	PDE4DIP, FAT1, NOTCH2, AHNAK, NUMA1, MKI67, ABCC3, ABCC1, PRKDC, TP53, MUC16, NRG1, TIAM1, NOTCH3, CASP8, CEP290, KLRC2, MAP3K1, NBAS, PTPRB	
13. Reder, H. et al., 2019	TP53, RB1, STK11, CDH1, HRAS, KRAS, NRAS, FAT1, PIK3CA, PIK3R1, PTEN, FANCA, FBXW7, CYLD, BCL6, TP63, TAF1, EP300, DDX3X, NOTCH1, JAK1, JAK2, PDGFRA.	
14. Reder, H. et al., 2021.	TP53, RB1, STK11, CDH1, HRAS, KRAS, NRAS, FAT1, PIK3CA, PIK3R1, PTEN, FANCA, FBXW7, CYLD, BCL6, TP63, TAF1, EP300, DDX3X, NOTCH1, JAK1, JAK2, PDGFRA.	
15. Saba, N. F. et al., 2020	PIK3CA, TP53, KMT2A, GNAQ, KDM6A, LAMA2, PTEN, DDX3X, BRCA1, BRCA2, ABCC2, CHD7, ERBB3, H3F3A, INPP4B, RB1, JAK2, NF1, PDGFRA, ALK, FGFR2, MAP2K2, MAPK1, MET, RET, ROS1, SOS1, FLT3, KIT, KRAS, PTEN, INPP4B, AKT1, AKT2, MTOR, PIK3R1, RPS6KB1	
16. Wahle, B. M. et al., 2022	PIK3CA, FGFR3, ZNF750, SYNE2, FLG, SYNE1, PLXNA1, PLEC, HERC1, ZFHX4, VPS8, USH2A, TRAF3, TACC2, RYR3, PTEN, OTOG, LAMA2, KIAA1109, JRK, FBN3, EP300, DNAH5, DNAH14,	
	CUX1, BIRC5, AK5, SMARCAL1, PIK3R1, IQCG, METTL24, FBXW7, B2M, NRAS, IFI27, HLA-B, FGF2, AKT1, FGF8	
17. Stransky, N. et al., 2011	TP53, CDKN2A, CASP8, FAT1, NOTCH1, PTEN, SYNE1, HRAS, PIK3CA, MED1, MLL2, TP63, IRF6, EZH2, SYNE2, NOTCH3, RIPK4, NOTCH2, DICER1, RB1.	
18. Williams, E. A. et al., 2021	PIK3CA, KMT2D, FBXW7, PTEN, KMT2C, TP53, BCL2L1, NOTCH1, RB1, HPV16	
19. Antonsson, A. et al., 2016	EVER1 & EVER2	
20. Barten, M. et al., 1995	P53 MUTATION	
21. Benzerdjeb, N. et al., 2021	TP53 MUTATIONS.	
22. Chen, Z. et al., 2021	FAT1 MUTATIONS.	
23. Chiosea, S. I. et al., 2013	PIK3CA, HRAS AND PTEN GENE MUTATIONS.	
24. Ekalaksananan, T. et al., 2020	P53 R282 GENE MUTATIONS.	
25. Fallai, C. et al., 2009	TP53 MUTATIONS.	
26. Farnebo, L. et al., 2015	DNA REPAIR GENES XPC, XPD, XRCC1, XRCC3 AND HPV, P53 MUTATIONS.	
27. Hong, A. et al., 2016	P53 MUTATIONS.	
28. Cortelazzi, B. et al., 2015	PIK3CA, PTEN, IGF1R, IGF1, IGF2, HER2, HER3.	
29. De Carvalho, A. C. et al., 2019	TP53 MUTATIONS.	
30. Friedland, P. et al., 2012	**EGFR, KRAS, BRAF.**	
31. Ghosh, A. et al., 2013	**PHF2 , FANCC, PTCH1 ALTERATIONS**	
32. Gross, A. M. et al., 2014	**TP53 MUTATIONS, CASP8**	
33. Huang, C. et al., 2019	NSD1, NOTCH1, TP53, CDKN2A, PIK3CA	
34. Licitra, L. et al., 2006	TP53 MUTATIONS.	
35. Mazurek, A. M. et al., 2016	KRAS, EGFR GENE MUTATIONS	

36. Saba, N. F. et al., 2015 Pilot Study	CDKM2C, SYK, WNT10B, CEBPA, MAP3K8, FGR, GATA1, OL4, PDGFA, CDKN2A, E2F2, TP53, PIK3CA, FGFR3, RB1, MET	
37. Sewell, A. et al., 2014	PIK3CA MUTATIONS	
38. Shaikh, H. et al., 2021	PIK3CA, KMT2D, TP53, LOH, KMT2C, CYLD, FBXW7, NOTCH1, RB1, PIK3CB, PTEN, B2M, NF1, ASXL1, KDM6A, PIK3R2, CHEK2, NSD1, FAT1, MAPK1, CREBBP. TERT, TP53, PIK3CA, RB1, NOTCH1, ARID1A, NF1, KMT2D, FAT1, LOH, ASXL1, KMT2C, CDKN2A, FBXW7, ATM, FGFR3, KDM6A, FANCM, EP300, B2M, KRAS.	TP53, TERT, PIK3CA, NOTCH1, FAT1, CDKN2A, LOH, KMT2D, RB1, KMT2C, ASXL1, NF1, EP300, ARID1A, FBXW7, NFE2L2, CYLD, NSD1, KDM6A, PBRM1, FGFR3, HRAS. TP53, TERT, CDKN2A, LOH, KMT2D, NOTCH1, PIK3CA, FAT1, ARID1A, ASXL1, KMT2C, NSD1, FBXW7, EP300, RB1, CREBBP, NF1, CYLD, KDM6A, HRAS, PTEN, NFE2L2.

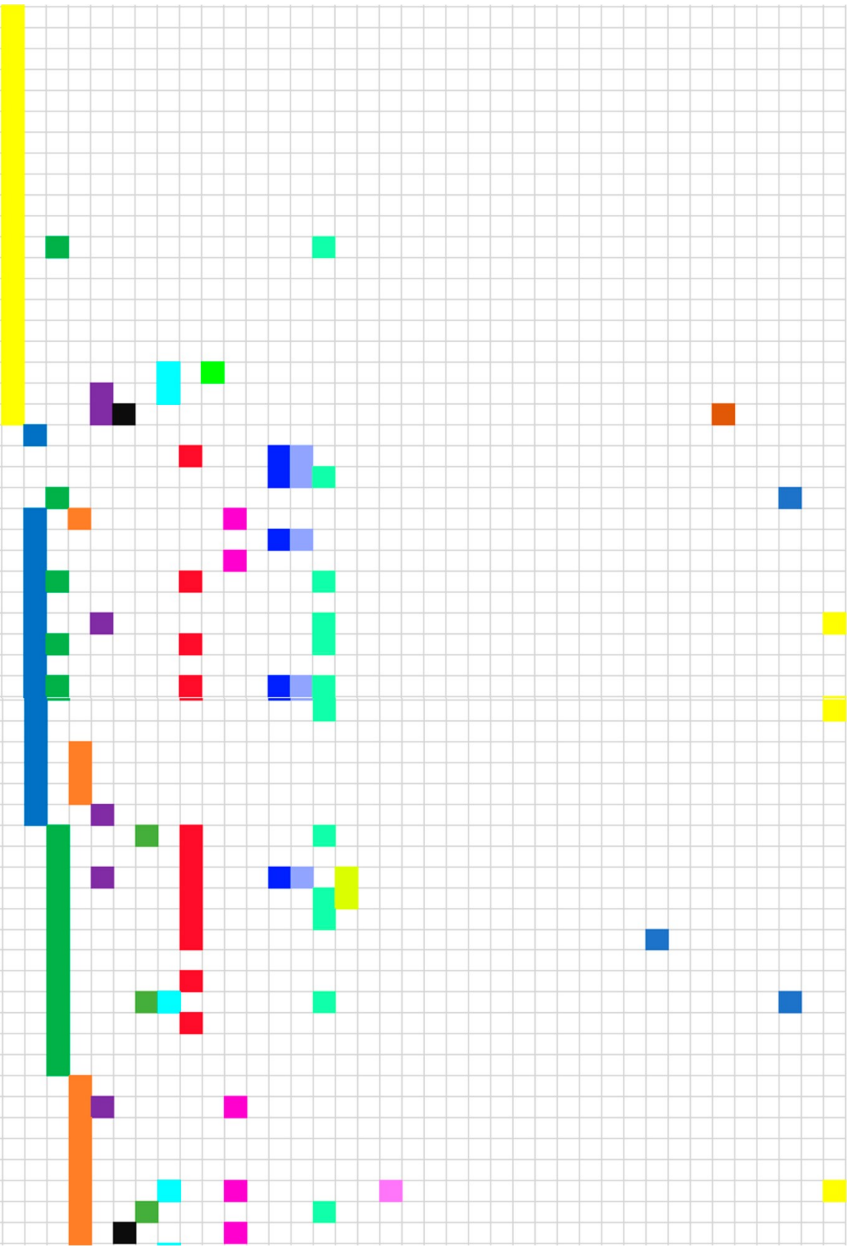
Supplemental Table S2: Distribution of the OSCC cases according to demographic, lifestyle, and clinical variables.

Variable	Category	Paraffin-embedded samples n(%)	HELA2 n (%)		
			Negative	Positive	*P-value*
Age	< 50 year	8 (15.4)	7 (16.7)	1 (10)	0.599
	≥ 50 year	44 (84.6)	35 (83.3)	9 (90)	
Gender	Male	31 (59.6)	26 (61.9)	5 (50)	0.500
	Female	21 (40.4)	16 (38.1)	5 (50)	
Smoking habit	No	23 (44.2)	21 (53.8)	2 (22.2)	0.087
	Yes	29 (55.8)	18 (46.2)	7 (77.8)	
Alcohol consumption	No	16 (37.2)	13 (37.1)	3 (37.5)	0.985
	Yes	27 (62.8)	22 (62.9)	5 (62.5)	
Clinical stage	T1+T2	32 (61.5)	**29 (69)**	**3 (30)**	**0.023**
	T3+T4	20 (38.5)	**13 (31)**	**7 (70)**	
Lymph nodes	N0	40 (76.9)	34 (81)	6 (60)	0.158
	N+	12 (23.1)	8 (19)	4 (40)	
Recurrence or metastasis	No	40 (76.9)	31 (73.8)	9 (90)	0.275
	Yes	12 (23.1)	11 (26.2)	1 (10)	
Status	Alive	45 (86.5)	37 (88.1)	8 (80)	0.500
	Dead	7 (13.5)	5 (11.9)	2 (20)	

Supplemental Figure S1: shows list of all genes mutated with highlighting on the most cited genes which are *TP53* (n=22), *PIK3CA* (n=20), *PTEN* (n=16), *NOTCH1* (n=14), *RB1* (n=13), *FAT1* (n=13), *FBXW7* (n=12), *HRAS* (n=10), *KRAS* (n=10) and *CDKN2A* (n=10). Different colour codes representing the 38 articles screened to show which gene was collected from which article. The most frequently mutated gene is *TP53* followed by *PIK3CA*. Genes for cell survival and proliferation (*TP53*, *HRAS* & *PIK3CA*), cell-cycle control (*CDKN2A*), cellular differentiation (*NOTCH1*), and adhesion and invasion signalling (*FAT*). Tumor suppressor Genes (*FAT1*, *NOTCH1* & *CDKN2A*).

	Gene Mutated	Total Articles	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
2	KMT2D	8																																						
3	FGFR3	8																																						
4	CYLD	5																																						
5	EP300	9																																						
6	PIK3CA	20																																						
7	RB1	13																																						
8	PEG3	1																																						
9	STAT3	1																																						
10	TTSC2	1																																						
11	B2M	5																																						
12	CREBBP	4																																						
13	FBXW7	12																																						
14	FLT1	1																																						
15	NCOR1	1																																						
16	NSD1	3																																						
17	PTEN	16																																						
18	USP9X	2																																						
19	BRIP1	1																																						
20	NBN	1																																						
21	NFE2L2	4																																						
22	TACC3	1																																						
23	ARID1B	1																																						
24	ARID5B	2																																						
25	DDR2	1																																						
26	EPHA2	2																																						
27	FANCA	4																																						
28	KDM5C	1																																						
29	LRRK2	1																																						
30	MAP2K2	2																																						
31	MAPK1	3																																						
32	NOTCH 1	14																																						
33	NOTCH 2	6																																						
34	NOTCH 3	5																																						
33	NOTCH 2	6																																						
34	NOTCH 3	5																																						
35	PTPRD	1																																						
36	SMAD2	2																																						
37	SYK	2																																						
38	TRAF3	4																																						
39	TRRAP	1																																						
40	HPV E6/E7	2																																						
41	TP63	6																																						
42	ZNF750	3																																						
43	HRAS	10																																						
44	KRAS	10																																						
45	TP53	22																																						
46	CDKN2A	10																																						
47	CCND1	5																																						
48	FAT1	13																																						
49	SYNE1	2																																						
50	USH2A	1																																						
51	ZFHX4	1																																						
52	MLL2	1																																						
53	EGFR	8																																						
54	MYC	3																																						
55	LRP1B	3																																						
56	PIK3CB	2																																						
57	CASP8	7																																						
58	FGFR1	5																																						
59	FLT1	1																																						
60	IDH2	1																																						
61	AR	4																																						
62	ASXL1	4																																						
63	ATM	7																																						
64	AXIN1	1																																						
65	BAP1	1																																						

66	BCR	1
67	CIC	1
68	ELF3	1
69	FLT4	1
70	GRIN2A	1
71	HDAC4	1
72	HIF1A	1
73	IFNGR1	1
74	LRP2	1
75	MAP3K5	1
76	MED12	1
77	MTOR	3
78	NBN	1
79	PIK3R6	1
80	SRC	1
81	TSC2	2
82	XPO1	1
83	HLA-A	3
84	AJUBA	2
85	NSD1	4
86	BRIP1	1
87	STK11	3
88	PIK3R1	3
89	PIK3CA	2
90	SOX2	3
91	BCL6	3
92	KLHL6	2
93	BRCA2	4
94	ATRX	1
95	KDM6A	4
96	BRCA1	4
97	BLM	1
98	JAK2	6
99	NF1	3
100	ATR	1
101	FGF19	2
102	FGF3	2
103	FGF4	2
104	RICTOR	2
105	FGFR2	4
106	IDH1	2
107	NRAS	6
108	AKT1	4
109	ALK	3
110	BRAF	3
111	FGFR4	1
112	FLT3	2
113	MET	5
114	MLH1	2
115	MSH2	1
116	MSH6	1
117	RUNX1	1
118	CDKN2B	3
119	FOXA1	1
120	SPEN	1
121	KDM5A	1
122	KMT2C	5
123	KMT2A	3
124	KEAP1	3



125	CUL3	2
126	ERBB2	2
127	TERT	3
128	NKX2	1
129	NKX1	1
130	PMA1P1	1
131	YAP1	1
132	JAK3	2
133	MAP3K13	1
134	MDM2	1
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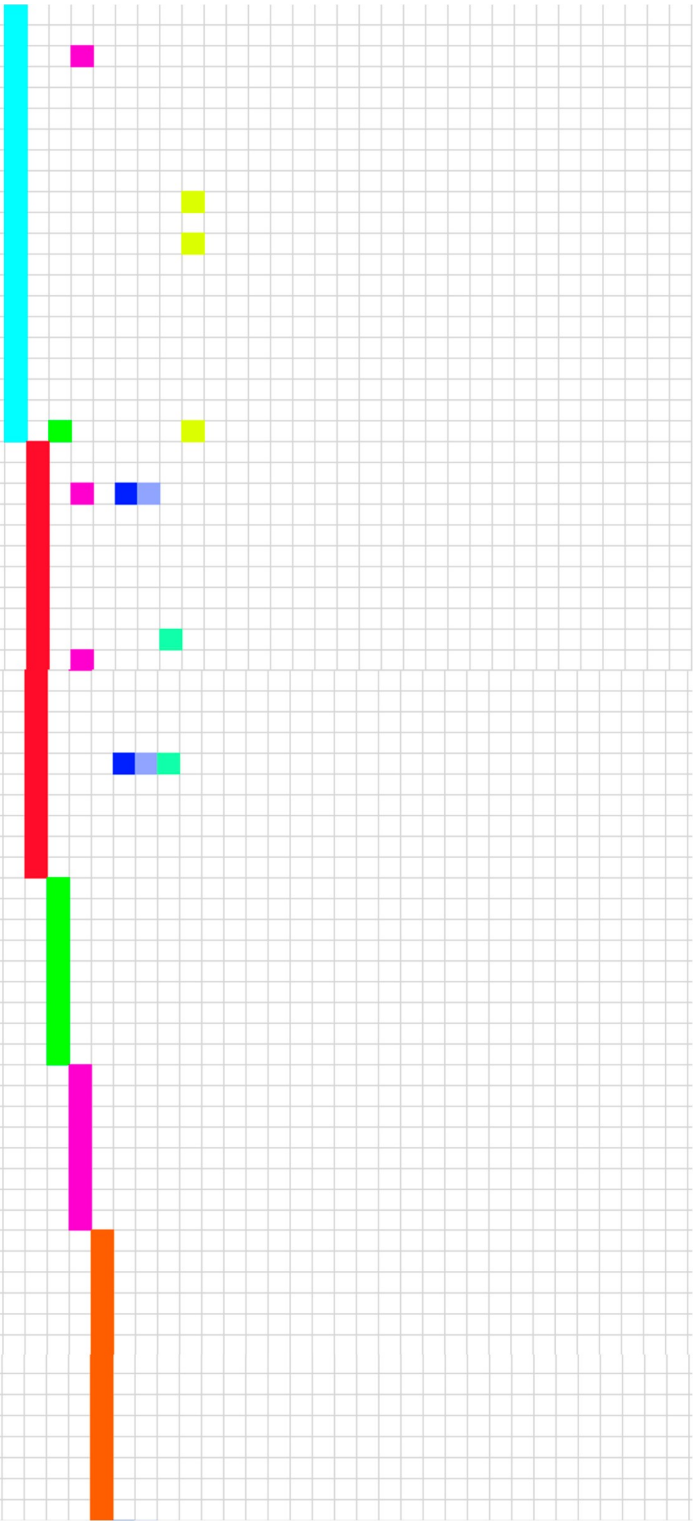

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