Characterization and Validation of PITX2 and TGM2 methylation in HPV-related Head and Neck Squamous Cell Carcinoma

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To my incredible partner “Mehdi”, thank you for smoothing this path, for the support and holding my hand in the most challenging situations, without you, it was never possible.

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Abstract

Head and Neck Cancer (HNC) is the seventh most common tumor in the world, with the main risk factors associated with cigarette smoking and alcohol consumption. However, human papilloma virus positive (HPV+) HNC cases are increasing significantly from 16.3% to 72.7% in the last three decades. Similar to other cancer types, HPV-driven tumors exhibit alterations in DNA methylation profile. In this study, DNA methylation was evaluated in HNC patients following their characterization regarding the main risk factors. Bisulfite-converted DNA from 24 patients were hybridized in the Human Methylation 450 BeadChip microarrays. Fluorescence signals were analyzed using GenomeStudio software with the methylation module v.1.9.0 (Illumina). To characterize the expression of genes containing differentially methylated regions (DMRs), the initial sample set was evaluated using Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Protein validation was validated by immunohistochemistry (IHC) reaction for the top two candidates (PITX2 and TGM2) using an independent cohort of 100 patients with HNC and the results were associated with clinicopathological outcomes. The analysis of tumor microenvironment (TME) components, the cell-cell interactions and the neighbor's spatial distribution were evaluated by imaging mass cytometry analysis (IMC) in patients diagnosed in early-clinical stage but with metastatic competence. As a result, we identified 2,655 DMPs located at 1,073 genes, being 384 hypomethylated and 2,271 hypermethylated CpG sites. Considering only the risk factors (high- risk *versus* low risk), it was identified 15 DMRs located at promoter regions of 12 genes. From the 12 genes (*ZNF323, FBXO39, SLFN12, TGM2, ZAP70, STK32B, HOXA2, LMO3, C1orf74, ST8SIA4, C12orf42,* and *PITX2*), we confirmed a concordant mRNA expression for eight genes (*ZNF323, FBXO39, TGM2, ZAP70, LMO3, C1orf74, C12orf42,* and *PITX2*). *LMO3, PITX2,* and *TGM2* were selected for further validation in a large cohort of patients' samples and PITX2 and TGM2 showed higher protein expression in HPV positive HNC cases, compared to the low-risk cases. Besides, higher expression was associated with tumor depth (P=0.0011) and advanced tumor stage (P=0.0042). This data was also independently validated in The Cancer Genome Atlas (TCGA) platform using 530 HNC samples. IMC showed that the TME impacts the biological behavior of the tumor comparing HPV-positive and HPV-negative, however its effect on clinical outcomes in HNC showed that PITX2 and TGM2 may play a critical role in various pathophysiological conditions, including inflammation and cancer. The development of epigenetic drugs to combat inflammation or tumors is predicated on immune system cells. During disease

progression, epigenetic modifications may influence transcription patterns (genes), which can either help with the clearance of pathogens or result in evasion by the pathogens. In this study, it was identified PITX2 and TGM2 as the main candidates in HPV-negative and HPV- positive HNC respectively. The role of these genes on tumor development and HNC progression will be assessed, and the relevant mechanisms will be investigated as potential new targets for the diagnostic, prognostic and therapeutic approaches for patients with HPV-positive HNC.

Resume

Le cancer de la tête et du cou (CHC) est la septième tumeur la plus courante dans le monde, les principaux facteurs de risque étant associés au tabagisme et à la consommation d'alcool. Cependant, les cas de CHC positifs au virus du papillome humain (VPH+) augmentent significativement, passant de 16,3 % à 72,7 % au cours des trois dernières décennies. Comme pour d'autres types de cancer, les tumeurs entraînées par le VPH présentent des altérations dans le profil de méthylation de l'ADN. Dans cette étude, la méthylation de l'ADN a été évaluée chez des patients atteints de CHC, suivant leur caractérisation par rapport aux principaux facteurs de risque. L'ADN converti par le bisulfite de 24 patients a été hybridé dans les microréseaux BeadChip de méthylation humaine 450. Les signaux de fluorescence ont été analysés en utilisant le logiciel GenomeStudio avec le module de méthylation v.1.9.0 (Illumina). Pour caractériser l'expression des gènes contenant des régions différentiellement méthylées (DMR), l'ensemble initial d'échantillons a été évalué en utilisant la réaction en chaîne de la polymérase quantitative en transcription inverse (RT-qPCR). La validation des protéines a été validée par réaction d'immunohistochimie (IHC) pour les deux principaux candidats (PITX2 et TGM2) en utilisant une cohorte indépendante de 100 patients atteints de CHC et les résultats ont été associés à des résultats clinicopathologiques. L'analyse des composants de l'environnement tumoral (TME), des interactions cellule-cellule et de la distribution spatiale des voisins a été évaluée par analyse de cytométrie de masse d'imagerie (IMC) chez des patients diagnostiqués à un stade clinique précoce mais avec compétence métastatique. En conséquence, nous avons identifié 2 655 DMP situés sur 1 073 gènes, étant 384 hypométhylés et 2 271 hyperméthylés sur les sites CpG. En considérant uniquement les facteurs de risque (risque élevé contre faible risque), 15 DMR situés dans les régions promoteurs de 12 gènes ont été identifiés. Parmi les 12 gènes (*ZNF323, FBXO39, SLFN12, TGM2, ZAP70, STK32B, HOXA2, LMO3, C1orf74, ST8SIA4, C12orf42* et *PITX2*), nous avons confirmé une expression concordante de l'ARNm pour huit gènes (Z*NF323, FBXO39, TGM2, ZAP70, LMO3, C1orf74, C12orf42 et PITX2). LMO3, PITX2* et *TGM2* ont été sélectionnés pour une validation supplémentaire dans un grand échantillon de patients et PITX2 et TGM2 ont montré une expression protéique plus élevée dans les cas de CHC positifs au VPH, par rapport aux cas à faible risque. De plus, une expression plus élevée a été associée à la profondeur de la tumeur (P=0,0011) et à un stade tumoral avancé (P=0,0042). Ces données ont également été validées

indépendamment sur la plateforme The Cancer Genome Atlas (TCGA) utilisant 530 échantillons de CHC. L'IMC a montré que le TME impacte le comportement biologique de la tumeur en comparant les cas positifs et négatifs au VPH, cependant son effet sur les résultats cliniques dans le CHC a montré que PITX2 et TGM2 peuvent jouer un rôle critique dans diverses conditions pathophysiologiques, y compris l'inflammation et le cancer. Le développement de médicaments épigénétiques pour combattre l'inflammation ou les tumeurs est fondé sur les cellules du système immunitaire. Durant la progression de la maladie, les modifications épigénétiques peuvent influencer les modèles de transcription (gènes), ce qui peut soit aider à l'élimination des pathogènes, soit entraîner une évasion par les pathogènes. Dans cette étude, PITX2 et TGM2 ont été identifiés comme les principaux candidats dans les cas de CHC négatifs et positifs au VPH respectivement. Le rôle de ces gènes dans le développement tumoral et la progression du CHC sera évalué, et les mécanismes pertinents seront investigués comme de nouvelles cibles potentielles pour les approches diagnostiques, pronostiques et thérapeutiques pour les patients atteints de CHC positif au VPH.

Format of the Thesis

This thesis is based on the guidelines provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. It consists of five main chapters: the introduction (chapter 1), the manuscript currently in preparation based on my MSc project (chapter 2), the general discussion (chapter 3), the conclusion and future directions (chapter 4), and the bibliography that mainly includes the references cited in the general introduction and general discussion (chapter 5).

Author Contributions

This manuscript is a collaborative work based on a multidisciplinary team. The experiment design and preliminary analysis for the first section of the manuscript was done in the laboratory of Dr Sabrina Wurzba in collaboration with Felipe Luz Torres Silva, Jefferson Muniz de Lima and Dr. Mariana Maschietto. Once I joined the team, I was responsible for immunohistochemistry analysis, image mass cytometry (IMC) evaluation, and all the bioinformatics analysis involved in the validation stage for testing the main hypotheses of this research. I was responsible for all the downstream analysis and the cell segmentation and machine learning part.

An image of another manuscript that we are working on was used in the general introduction (Figure 3) which was created by Megan Araujo with support of an online tool (biorender.com).

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List of Abbreviations

- ACTB: Actin Beta
- AJCC: The American Joint Committee on Cancer
- CAFs: Cancer-Associated Fibroblasts
- CD: Cluster of Differentiation (followed by numbers for specific markers)
- CTLA-4: Cytotoxic T-Lymphocyte-Associated Protein 4
- CTLs: Cytotoxic T Lymphocytes
- CNV: Copy-Number Variation
- DMPs: Differentially Methylated Positions
- DMRs: Differentially Methylated Regions
- ECs: Endothelial Cells
- ECM: Extracellular Matrix
- EBV: Epstein-Barr Virus
- E: Early
- FOXP3: Forkhead Box P3
- FFPE: Formalin-Fixed Paraffin-Embedded
- GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
- GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
- HE: Hematoxylin and Eosin
- HPV: Human Papillomavirus
- HPRT: Hypoxanthine Phosphoribosyltransferase
- HNC: Head and Neck Cancer
- ICAM1: Intercellular Adhesion Molecule 1
- ICIs: Immune Checkpoint Inhibitors
- IHC: Immunohistochemistry
- IFN-y: Interferon gamma
- IMC: Imaging Mass Cytometry
- iNOS: inducible Nitric Oxide Synthase
- L: Late
- LPS: Lipopolysaccharide

- LSCC: Laryngeal Squamous Cell Carcinoma
- MDSCs: Myeloid-Derived Suppressor Cells
- MSC: Mesenchymal Stem Cells
- NETs: Neutrophil Extracellular Traps
- NK: Natural Killer
- NSCC: Nasal Squamous Cell Carcinoma
- OPC: Oropharyngeal Cancer
- OPSCC: Oropharyngeal Squamous Cell Carcinoma
- OSCC: Oral Squamous Cell Carcinoma
- OS: Overall Survival
- PD-1: Programmed Death 1
- PD-L1: Programmed Death-Ligand 1
- PPARy: Peroxisome Proliferator-Activated Receptor gamma
- RFS: Relapse-Free Survival
- ROS: Reactive Oxygen Species
- SCC: Squamous Cell Carcinoma
- SMA: Smooth Muscle Actin
- TAMs: Tumor-Associated Macrophages
- TCGA: The Cancer Genome Atlas
- TGF-β: Transforming Growth Factor beta
- TILs: Tumor-Infiltrating Lymphocytes
- TNF-a: Tumor Necrosis Factor alpha
- TMA: Tissue Micro-array
- TME: Tumor Microenvironment
- UICC: The Union for International Cancer Control
- VEGF: Vascular Endothelial Growth Factor

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1 Chapter 1. Introduction

1.1 Cancer

Cancer is a subject of great interest in the scientific community due to the complex biology and multi-step of tumor development, resulting in a range of overlapping and sometimes conflicting theories. Recently, Hanahan and Weinberg (2000) in one of the most significant study in cancer biology, proposed six key hallmarks of cancer (**Figure 1**), including: 1. proliferative capacity without control, often due to mutations that activate growth-promoting pathways; 2. evasion of the mechanism that prevent excessive growth, such as tumor suppressor genes; 3. avoidance of the programmed cell death (apoptosis), allowing cancer cells to survive and accumulate genetic changes; 4. maintenance of the telomeres, the protective caps on chromosomes, allowing cells to divide indefinitely; 5. stimulation of angiogenesis to supply nutrients and tumor growth; and 6. ability to invade and spread to distant sites (metastasis) (1). This study mainly focused on the process by which normal cells undergo alterations that lead to the formation of cancer (2). Weinberg's further studies expanded the hallmarks of cancer into the molecular mechanisms underlying tumor initiation and progression (3). It was showed that genome instability and mutations play an essential role in shaping these hallmarks of cancer. This model relies on an evolutionary process that begins with single mutations in individual cells, leading to increase adaptability over neighboring cells, enabling them to grow and adapt to their environment (4) (5). Mutations that provide a selective growth advantage, and thus promote cancer development, are called driver mutations, and those that do not promote any selective advantages are called passenger mutations (6). Since the researchers gained a better understanding of cancer progression concepts, they included additional hallmarks such as deregulated cellular energetics, immune evasion, tumor-promoting inflammation, genomic instability, and reprogramming of energy metabolism (7).

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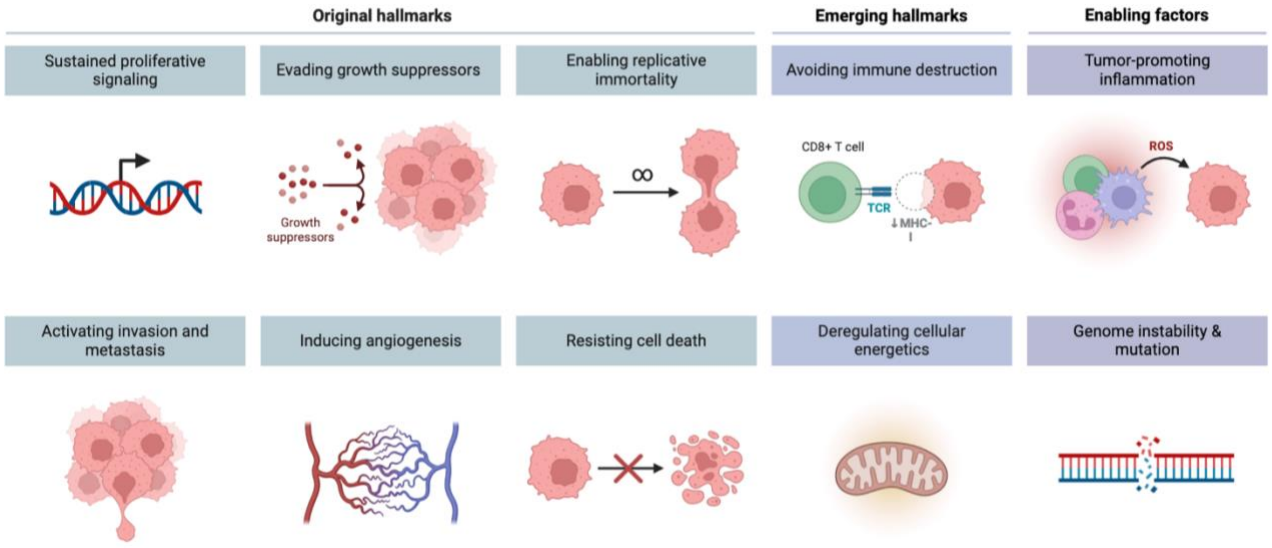

Figure 1. Overview of the hallmarks of cancer. These hallmarks include sustained proliferative signaling, which contributes with continuous cell growth, evasion of growth suppressors, activation of promoter signals towards invasion and metastasis, inducing angiogenesis, and resisting cell death (apoptosis). The emerging hallmarks include immune evasion and deregulation of the metabolism. Inflammation has a key role in promoting tumor growth, genome instability and mutation, that can lead to tumor progression and metastasis. The figure created by biorender.com.

This thesis highlighted three key factors that contribute to cancer development including: 1. epigenetics and risk factors associated with HNC; 2. tumor-promoting inflammation; 3. changes in the tumor microenvironment (TME) which create conditions that support cancer cells growth, survival, and dissemination.

1.2 Epigenetics

Epigenetics are changes in gene expression that are inherited and do not affect the DNA nucleotide sequence (8). However, the epigenetic definition extended to include stable, long-term variations in the cellular transcriptional profile that are not necessarily inherited through epigenetic events (9). Several lifestyle and environmental factors, such as tobacco smoke, alcohol consumption, polycyclic aromatic hydrocarbons, infectious pathogens, heavy metals exposition, and other

indoor and outdoor pollutants can modify disease risk and health outcomes through epigenetic pathways (10). Three central mechanisms are involved in regulating gene expression through epigenetic which include: 1. DNA methylation; 2. histone modification and 3. regulatory non-coding RNAs such as microRNAs that regulate expression at the post-transcriptional level (11). These mechanisms can affect the accessibility of genomic *loci* to transcription enzymes and regulate key cellular processes, therefore they are essential for cell growth, tissue differentiation and tumor development (12).

1.2.1 DNA methylation

DNA methylation is the most studied epigenetic mechanism involving the transfer of a methyl group onto C5 position of the cytosine to form 5-methylcytosine (13). The majority of DNA methylation occurs on cytosines that precede guanine nucleotide or CpG sites (14). DNA methylation in the CpG promoter regions is often associated with repression of gene expression by altering the conformation of DNA itself and local histone structures leading to a transcription silencing (15). Methylation can be didactyly explained as an on or off switch for gene expression as shown in **Figure 2**. In summary, flow of genetic information in biological systems from DNA>RNA>Protein is the central dogma in molecular biology. When a gene is actively expressed, the DNA can be transcribed into mRNA, which is then translated into a protein (16). If the gene is silenced or "off" through a DNA methylation for example, this can prevent the transcription and consequently the protein expression (**Figure 2**).

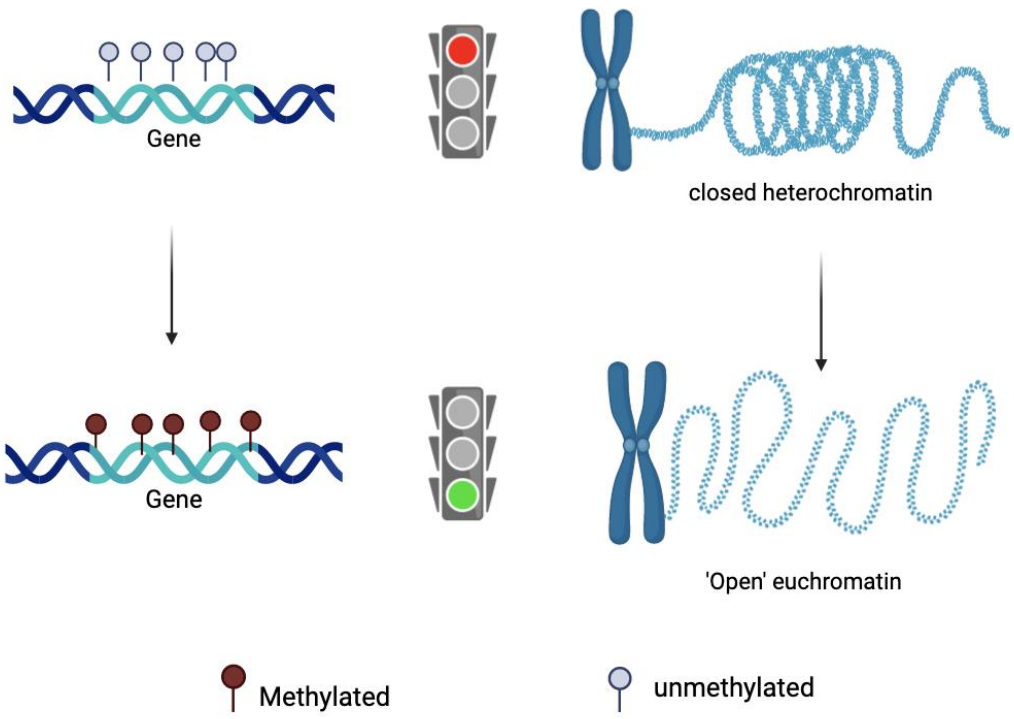

Figure 2. Unmethylated *versus* methylated promoter region. DNA methylation serves as a critical epigenetic mechanism for modulating gene expression and chromatin architecture. Unmethylated genes (left top) are often actively transcribed, while hypermethylation (left bottom) leads to gene silencing, as depicted by the transition from 'open' euchromatin to 'closed' heterochromatin (right). Figure created with biorender.com.

Broad changes of the epigenome are linked with cancer initiation and progression (17). Cancer cells show a global loss of CpG methylation, including regions with low density of CpG sites, repeat elements, retrotransposons, and laminin-associated domains (LADs) (18). This can result in both hypomethylation of the whole genome and hypermethylation of specific genes within the same cell. The alterations in the DNA methylation profile, influenced by various risk factors, can contribute to the tumor phenotype, including HNC progression (19).

1.2.2 Risk factors

There are several lifestyle and environmental risk factors associated with HNC development. Oncogenic viruses such as HPV, alcohol and tobacco consumption are considered the main risk factors for HNC (20). The other risk factors are poor oral hygiene, chronic mechanical irritation in case of oral cavity cancers, tobacco or betel nut chewing, and Epstein-Barr virus (EBV) in cases of nasopharyngeal carcinoma (21). However, this section will focus on the main risks' factors associated with HNC development.

1.2.2.1. Tobacco and alcohol consumption

Smoking is an independent risk factor for HNC (22). It is estimated that 90% of HNC is linked to tobacco use (23). The cumulative lifetime risk is linked to several factors such as daily amount of tobacco, the duration of smoking, and intensity of cigarette smoking, including the frequency of inhalations. Studies have hypothesized that covalent DNA modifications caused by tobacco can induce DNA damages through DNA methylation. Specifically, the alteration in the DNA methylation profile in promoter/regulatory region related to tobacco consumption are associated with cancer progression, lymph node invasion, metastasis and other processes (24) (25) (26).

In the same way, alcohol is the other major independent risk factor for HNC. For alcohol abuse, a case-control study using 11,221 cases and 16,168 controls showed that the population attributable risk for tobacco and alcohol was 72% for HNC, of which 35% was tobacco and alcohol combined (27). Recent studies have confirmed that the epigenetic changes induced by alcohol, including DNA methylation, are associated with tumorigenesis (28).

1.3 HPV+ HNC

HPV is a sexually transmitted infection (29), with approximately 200 different subtypes (30). HPV infection is associated with different types of cancers, including anal cancer, cervical cancer, HNC, penile cancer, vaginal and vulvar cancer (31) Globally, the number of reported cases of HPV+HNC has increased by 26.6% (32). In the United States, half of the HNC cases are linked to HPV infections [27]. In HNC the most common site affected by HPV is the OPC region (33). (These tumors are divided into two entities based on the presence or absence of HPV: HPV+ and HPV-

negative (HPV-) types. These two types differ phenotypically. Although the underlying process is still unclear, patients with HPV+OPC have a better radiotherapy response and better overall survival (34). (**Table 1**). In the United States, individuals diagnosed with HPV+OPC are often younger, have a higher socioeconomic status, and have less exposure to alcohol and tobacco compared with those diagnosed with HPV-HNC (35). (**Table 1**). The American Joint Committee on Cancer (AJCC/UICC) has added information about the presence or absence of HPV infection in HNC to complement the traditional TNM staging system (tumor, nodes and metastasis) since 2018 (36). The introduction of HPV vaccinations is expected to reduce the number of cases of OPC that are caused by HPV infection (37). It is also necessary to emphasize that the best strategy to fight against HNC is prevention, especially when the pathogenic agent is known and identifiable and the primary prevention tool such as HPV immunization is available. (38)

Table 1. Characteristics and comparison between HPV- negative and HPV+HNC.

Characteristics	HPV-Negative	HPV-Positive
Site (39)	All sites	Tonsil, base of tongue
Risk factors (40)	Tobacco/alcohol	Sexual behavior
Age (41)	65 years and higher cohorts	25-45 years (Younger cohorts)
Stage (42)	Variable, often diagnosed at later stages	Early T stage - advanced N stage
Histology (43)	Keratinized	Basaloid/poorly differentiated, non-keratinized
p16 (44)	Decreased expression, linked to worse outcomes	Increased expression, linked to better outcomes
Adaptive immunity (45)	Lower immunogenicity, reduced immune recognition	Higher tumor immunogenicity, improved immune recognition
Disease onset and progression	Altered tumor suppressor genes and signaling pathways (46)	Integration of HPV into host genome, action of E6 and E7 proteins (47)

TP53 gene (encodes p53) (48)	Frequently deleted or mutated, impaired tumor suppression	Rarely altered: p53 function eliminated by HPV E6 proteins
Molecular alterations	HRAS and CASP8 alterations, amplification of *RTK, PIK3CA, EGFR, HER2, FGFR1* (49)	Amplification of *PIK3CA, E2F1* oncogenes, *TRAF3* truncation, *FGFR2/3* mutation/fusion (50)
Retinoblastoma associated protein (RB1)	Inactivated by phosphorylation, leading to cell cycle dysregulation (51) (52)	Proteasomal destruction by HPV E7 protein, affecting cell cycle control (53)
Treatment response (54)	Reduced response to standard therapies and poor prognosis	Better response to standard therapies, improved prognosis
Angiogenesis (55)	Increased angiogenesis	Less reliant on angiogenesis for growth
Distant Metastasis	Higher tendency for distant metastasis Atypical distant metastasis pattern observed (56)	Lower propensity for distant metastasis Distant spread classically to lungs (57)
Treatment resistance (58) (59)	Often resistant to radiation and chemotherapy	More sensitive to radiation and chemotherapy
Genetic instability (60)	Higher degree of genetic instability	Lower genetic instability

Abbreviation: HPV: Human Papillomavirus, T stage: Tumor stage, N stage: Node stage,p16: Protein p16 (no further abbreviation), TP53: Tumor Protein p53, HRAS: Harvey Rat Sarcoma Viral Oncogene Homolog, CASP8: Caspase 8, RTK: Receptor Tyrosine Kinases, PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase Catalytic Subunit Alpha, EGFR: Epidermal Growth Factor Receptor, HER2: Human Epidermal Growth Factor Receptor 2, FGFR1: Fibroblast

Growth Factor Receptor 1, FGFR2/3: Fibroblast Growth Factor Receptor 2/3, E2F1: E2F Transcription Factor 1, TRAF3: TNF Receptor-Associated Factor 3, RB1: Retinoblastoma 1

1.3.1 HPV in the pathogenesis of HNC

HPVs are single double-stranded, circular and covalently closed DNA genome within a protein capsid (61). HPV comprises both high-risk and low-risk strains, each with distinct implications for cancer development (62). Among them, 12 strains are considered as 'high-risk' including HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and –59 (63). High- risk HPV types -16, -18, -31, and/or -33 are responsible for nearly (90%) of HPV-positive HNC infections (64). Among these strains, HPV16 is the most prevalent subtype contributing to 85-90% of the OPC cases (65).

The HPV genome integrates into the host's chromosome leading to carcinogenesis (66). The virus's oncoproteins E6 and E7 interfere with the function of the cellular tumor-suppressor protein p53 and pRB (67) (68). The inactivation of these proteins contributes with the dysregulation of key cellular processes related with cell-cycle control and differentiation, which leads to tumor initiation and progression (69). HPV+HNC not only shows a different aetiology as "classical" tobacco and alcohol associated HPV-HNC, but it also distinct in their epidemiology, pathogenesis, clinical presentation, molecular findings and more importantly prognosis and treatment response (**Figure 3**) (70).

Chronic HPV infection can contribute to HNC development through epigenetic changes (71). Literature shows that HPV+HNC has higher rates of hypermethylation compared to the negative ones(72). Viral HPV proteins E6 and E7 promote DNA methyltransferase 1 (DNMT1) activity, which suppresses *TP53*, *RB,* and *CDH1* gene expression, and induces the upregulation of cell cycle genes (73). The protein p16INK4a, coded by *P16* gene, involved in cell cycle progression, and is a surrogate marker for HPV infection in HNC, has differential methylation in its promoter region (74). In addition, miR-139-3p and miR-375, which are involved in tumor suppression and targeting viral oncoproteins have been linked with HPV+HNC (75). These findings suggest that HPV infections play a role in epigenetic modulation, though further studies are necessary to understand their impact in HNC development (76).

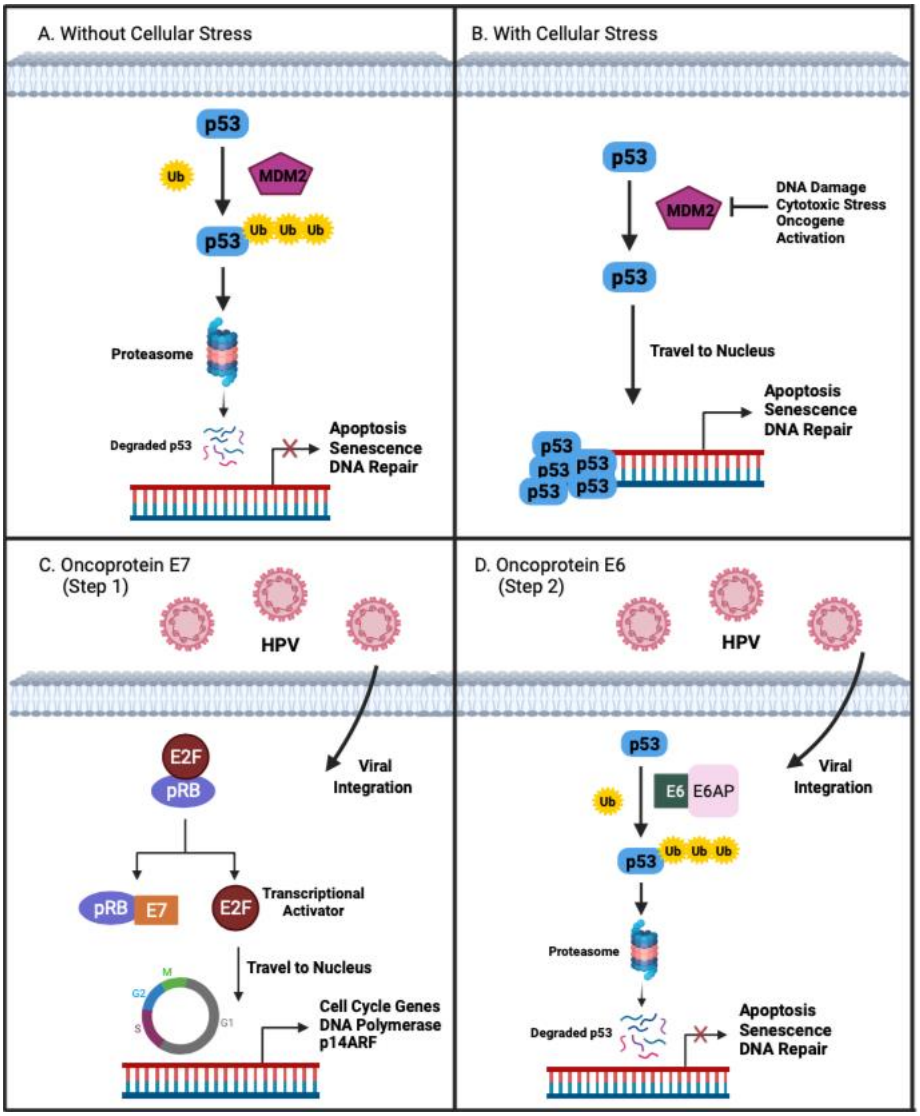

Figure 3. HPV infection induces tumorigenesis in patients with HNC. Under normal cellular conditions, p53 is ubiquitinated by MDM2 and degraded by proteasomes. When there is cellular stress, MDM2 is inhibited and p53 can travel to the nucleus to promote the transcription of genes involved in apoptosis, senescence, and DNA repair. In the presence of HPV infection, the virus integrates itself into the host cell. HPV oncogenic protein E7 sequesters pRB from E2F. E2F can then travel to the nucleus and initiate the transcription of cell cycle genes. p53 will try to mitigate these effects but it will be marked for degradation by HPV oncogenic protein E6 and ubiquitin ligase E6AP. Ub: Ubiquitin, p53: Tumor protein p53, MDM2: Mouse double minute 2 homolog, E2F: E2 factor, pRB: Retinoblastoma protein, HPV: Human papillomavirus, E7: HPV E7

oncoprotein, E6: HPV E6 oncoprotein, E6AP: E6-associated protein. Figure created with biorender.com.

1.4 The tumor microenvironment in HNC

The tumor microenvironment is composed of cancer cells, stromal tissue and the extracellular matrix (ECM) in a complex and dynamic interaction (77) (78). These interactions are determined by structural and biochemical properties of the ECM as well as by communication among the cells such as endothelial cells (ECs), cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSC), and a variety of different immune cells including lymphocytes and tumor-associated macrophages (TAMs) (**Figure 4)**. These multiple interactions with the tumor stroma determine not only cancer growth and metastasis but may also influence the drug sensitivity/resistance (79). The next topics will provide an explanation for each of these components.

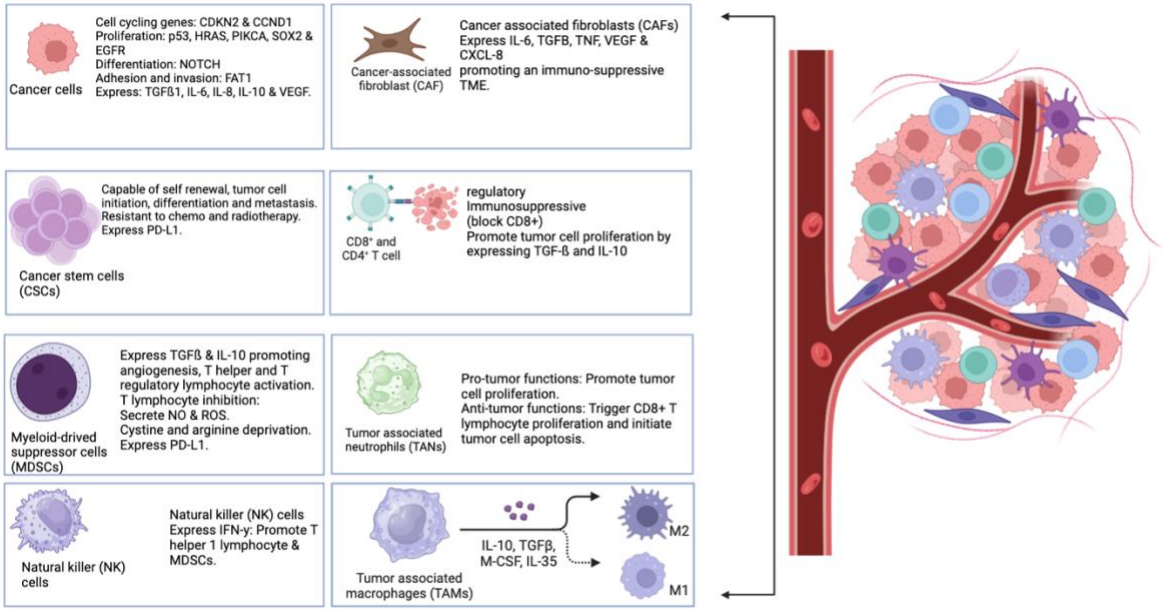

Figure 4. **The tumor microenvironment (TME) of head and neck cancer (HNC)**. The TME involves a complex extracellular matrix (ECM) and a variety of nonmalignant cells interspersed with cancer cells. The TME comprises of T lymphocytes, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, tumor-associated neutrophils

(TANs), and cancer-associated fibroblasts (CAFs), which are the main cellular components of the TME (80). Figure created with biorender.com.

1.4.1 Cancer-associated fibroblas (CAFs)

CAFs are the primary cell type found in the tumor stroma (81). These cells are responsible for creating a TME that promotes cell proliferation, angiogenesis, inflammation, invasion, metastasis, and drug resistance[(82). CAFs produce several growth factors, cytokines, and chemokines such as epidermal growth factor , (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), C-X-C motif chemokine ligands (CXCCLs): CXCL12 and CXCL14, CC chemokine ligands (CCLs): CCL5 and CCL7, and interleukins (IL) such as IL-6 and IL-17A to change the TME (83). They also produce matrix-metalloproteinases (MMPs), which remodeling and degrading ECM, ultimately promoting an invasive tumor phenotype (84) (85).

CAFs have a spindle-like shape and share similarities with mesenchymal and smooth muscle cells (86). Compared to normal fibroblasts, CAFs have different biological and morphological characteristics and are in a constitutively activated state (87). CAFs can be identified using different markers, including alpha-smooth muscle actin (a-SMA), fibroblast activation protein (FAP), fibroblast specific protein-1 (FSP-1), platelet-derived growth factor receptor alpha (PDGFR) and vimentin, which are indicative of their activated state (88). These cells can be derived from various types of progenitor cells, including resting resident fibroblasts, pericytes, endothelial cells, epithelial cells, adipocytes, and bone marrow-derived mesenchymal cells (BDMCs) (89). In HNC, a-SMA is the most commonly used marker to detect CAFs, which are frequently observed in the myofibroblast phenotype (90). Upregulation of a-SMA has been linked to poor prognosis in oral carcinoma (91). CAFs can also be identified by the absence of epithelial and endothelial markers, such as cluster of differentiation 31 (CD31) and cytokeratin (92). Several methods for targeting CAFs to inhibit tumor progression and enhance anti-tumor immunity have recently been reported (93). While preclinical studies have shown promise, to date they have been unsuccessful in human clinical trials (94).

1.4.2 Macrophages

Macrophages are a crucial type of immune cells that help maintain tissue balance and fight against pathogens (95). They are considered to be among the most important immune cells (96). Macrophages display remarkable flexibility, with the M1 and M2 states representing two extremes of activation (97). *In vitro* studies have shown that various cytokines can polarize macrophages towards another phenotype (98). These distinct phenotypes are regulated by different cytokine and chemokine (99). For instance, M1 macrophages produce pro-inflammatory cytokines and chemokines and are activated by interferon-y (IFN-y) and/or bacterial lipopolysaccharide (LPS) (100). They play a role in anti-tumor immunity, prevent proliferation, and have cytotoxic activity (101). In another hand, M2 macrophages are involved in tissue remodeling, angiogenesis, wound healing, and tumor progression (102). Various cytokines induce the M2 phenotype, which is characterized by an increased secretion of anti-inflammatory cytokines (103). TAMs are a significant portion of the macrophage population and play a major role in tumor cell growth, invasion, and metastasis. They promote tumor progression, angiogenesis, and suppress T cell immune response (104). TAM infiltration levels have been linked to poor outcomes in HNC, making it a potential prognostic marker (105). TAMs were previously considered a subpopulation of macrophages within the M2 phenotype, but it is now clear that they can adopt a wide range of activation states between M1 and M2, expressing both M2 and M1 markers (106). These markers include increased levels of interferon-(INF)-inducible chemokines CCL2, CCL5, CXCL9, CXCL10, and CXCL16 (M1), upregulated IL-10 (M2), arginase-1 (M2), peroxisome proliferator-activated receptor y (PPARy) (M2), TNF-a (M1), and MMP-9 (M1) (107).

1.4.3 CD8+ T cells

CD8+ T cells are a crucial component of the immune system (108). They express T-cell receptors (TCRs) that enable them to detect peptides presented by major histocompatibility complex 1 (MHC-I) (109). When exposed to an antigenic peptide, naïve T cells undergo a massive clonal expansion and differentiation process to become potent effector cells, also known as cytotoxic T cells (CTLs) (110). CTLs can destroy tumor cells by releasing cytotoxic mediators or triggering first apoptosis signal receptor ligand (FasL)-mediated apoptosis (111). Recent high-dimensional profiling technologies, such as single-cell RNA sequencing (scRNA-seq), spatial transcriptomics, mass cytometry (CyTOF), and proteomics have revealed three distinct functional states of tumor-infiltrating lymphocytes (TILs): naive, cytotoxic, and

dysfunctional (112). Although TILs with decreased effector function may show different degrees of exhaustion, they may still provide long-lasting responses to immune checkpoint inhibitors (ICIs) (113). Higher infiltration of CD8+ T cells has been linked to a better response to anti-programmed cell death protein 1 (PD1) and programmed death receptor ligand 1 (PD-L1) antibodies in patients with cutaneous HNC (114). Moreover, studies have shown that higher infiltration of CD4+ and CD8+ TILs are positively correlated with overall survival (OS), relapse-free survival (RFS), and better clinical outcomes (115).

1.4.4 Neutrophil

Neutrophils are critical component of the innate immune system, also known as polymorphonuclear leukocytes (PMNs) (116). They are the most abundant white blood cell population in circulation (117). Neutrophils and macrophages are the first responders to infections, pathogens, and injuries. (118).- The previous belief was neutrophils acted only as phagocytic cells by producing lytic enzymes and reactive oxygen species (ROS) (119). But recent studies have revealed that neutrophils can also create neutrophil extracellular traps (NETs) (120). NETs are formed following the activation of neutrophils and play an important role in the development of cancer, especially metastatic disease (121). This network is activated by releasing the cytotoxic cytosolic and granule proteins on a scaffold of decondensed chromatin, which occurs during a cell death process called NETosis (122). NETs are associated with several mechanisms linked to tumor metastasis and immune escape, including in HNC (123). Identifying and characterizing neutrophil subpopulations based on the expression of specific surface markers is challenging due to the lack of a unique biomarkers (124). So, researchers use various markers individually or in combination, such as CD11b, CD14, CD15, CD16, CD62L, and CD66b, to identify a similar subset of neutrophil population (125).

TANs in cancer display both pro- and anti-tumor properties (126). TANs also exhibit a phenotypic duality in the form of polarization states, similar to TAMs (127). These states have been identified as N1 and N2, where N1 presents anti-tumor and N2 has pro-tumor activity (128)Neutrophils acquire the pro-tumor N2 phenotype by increasing the expression of angiogenesis and invasion promoting factors such as CXCR4, VEGF, and MMP-9 and no expression of IFN-β (129). However, neutrophils can switch back to the cytotoxic N1 phenotype, which has high expression

levels of intercellular adhesion molecule 1 (ICAM1) and TNF-α and increased formation of NETs (130). This can occur in the presence of IFN-β or by blocking TGF-β (131). Targeting NETs could play a promising role in anti-cancer therapy, however, due to the complexity and multifaced roles of neutrophils within the TME, further investigation is needed (132).

1.4.5 Regulatory T-cells (Treg)

Treg are an immunosuppressive subset of CD4+ T cells characterized by the expression of the master transcription factor forkhead box protein P3 (FOXP3), cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD4, and CD25 (133). However, these markers are also expressed by effector T cells, which makes it difficult to distinguish between the two populations (134). Treg have a critical role in controlling immune response, as well as maintaining homeostasis, and regulating other immune cells:such as CD4 T helper cells, CD8 T-cells, B cells, NK cells, macrophages, and dendritic cells (135). Tregs can be divided into different subpopulations based on their location, origin, and expression profile of markers (136). At present, increasing evidence suggests that FOXP3+ T cells in humans, including suppressive and nonsuppressive subpopulations, have heterogeneous phenotypes and functions(136). For instance, Tregs that arise in the thymus are called natural regulatory T cells are important for maintaining self-tolerance (137). These cells constantly express the FOXP3 transcription factor . On the other hand, induced or adaptive Tregs, which can differentiate from conventional mature CD4+ T cells outside of the thymus, require activation in the presence of the cytokines IL-2 and TGF-β to upregulate FOXP3 (138). Their main function is to prevent inflammation (139). A systematic-review confirmed that increased levels of circulating Treg cells in peripheral blood can be a prognostic factor of survival in patients with oral cancer (140). Since the frequency and function of Tregs is related to tumor prognosis, researchers are studyng the effect of the therapy targeting Treg (141). New therapies such as the use of nanoparticles and the chimeric antigen receptor (CAR)-T cells bring to reality the possibilities of more efficiency, better outcome, and less toxicity in cancer treatment (142). However, further investigation involving these novel therapies are needed to provide a widespread use of engineered Tregs (143).

1.4.6 Myeloid-derived suppressor cell

A group of immature immune cells knowned as MDSCs have a role in regulating the immune response in several pathological conditions such as cancer and inflammatory diseases by inhibiting both adaptive and innate immunity (144). MDSCs are regulated by tumor-derived factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF, and IL- (145). They control the inflammatory microenvironment by depleting several amino acids (such as L-arginine, L-tryptophan, and L-cysteine), increasing the production of nitric oxide (NO), ROS, inducible NO synthase (iNOS), and arginase-1 (146). MDSCs also express PD-L1, which inhibits T cell activation, proliferation, and causes T cell apoptosis (147). Additionally, they regulate the activity of NK cells and the induction of immunosuppressive Tregs (148). MDSCs were initially found in the bloodstream of HNC patients as immature CD34+ cells that can suppress the activity of T cells (149). However, it is difficult to identify MDSCs based on surface markers due to the phenotypic diversity of the MDSC population (150). Different subpopulations of MDSCs express various myeloid markers, including CD11b, CD33, CD14, CD15, and CD16, but do not express HLA-DR (151). Although MDSCs were initially discovered for their immune-suppressive function in cancer, they have also been associated with other processes within the TME, such as promoting tumor angiogenesis inducing invasion in the ECM through the production of significant levels of MMPs (especially MMP-9), and most importantly, forming pre-metastatic niches (152).

1.4.7 Natural killer cell

NK cells are effector lymphocites that can rapidly identify and eliminate cells infected with a virus or cancer (153). These cells an be classified into two groups based on the surface markers they express, CD16 and CD56 (154). The majority of NK cells found in peripheral blood belong to the $CD^{56}dimCD^{16}bright$ subset, which is responsible for high natural cytotoxicity (155). The $CD^{56}brightCD^{16}dim$ subset produces higher levels of various immunomodulatory cytokines (156). NK cells secrete important cytokines including IFN-y and TNF-a, as well as other factors such as GM-CSF, IL-5, IL-8, IL-10, IL-13, CCL2, CCL3, CCL4, CCL5, and CXCL10 (157). In HNC, NK cells are important for tumor surveillance and prevent tumor growth, furthermore, NK cell infiltration has been associated with a favorable prognosis (157). HNC-infiltrating NK cells are susceptible to an array of immune evasion strategies regulated by tumors that must be overcome in order to induce the antitumor potential of NK cells (158). NK cell therapy in combination with conventional chemotherapy could potentially improve the outcome of oncology treatment (159).

1.4.8 Dendritic cells (DCs)

DCs are a heterogenous population of antigen presenting cells that infiltrate tumors and present tumor-derived antigens to naïve T cells (160). These cells generate primary signals that recruit T-cells, secondary signals that activate T-cell, signals that differentiate T-cells, and specific signals that direct attract T-cells to specific tissues (161). There are two types of DCs (cDC1 and cDC2) which activate anti-tumor immune responses by either presenting tumor antigens or secreting cytokines (162). In the TME, cDC1 facilitate CD8+ T-cells to fight tumor cells and secrete IL-12 to support T-cell function (163). Studies have shown that the cDC1 signature in the TME is related to higher TILs quantification scores and better patient survival (164).

The chapter 2 is the manuscript-related thesis project presenting these markers to characterize and understand the tumor phenotyping in HPV+ and HPV-HNC with distinctive metastatic competence.

1.5 Hypothesis and objectives

Hypothesis

The hypothesis of this study is that alterations in *PITX2* and *TGM2* gene expression may have a significant impact in the development and progression of HPV-related HNC and this can regulate critical biological mechanisms affecting the aggressive phenotype.

Objectives

1: Establish the clinical relevance of *PITX2* and *TGM2* in a large cohort of patients (n=200) including metastatic and non-metastatic samples to evaluate their impact to predict HNC progression.

2: Understanding the tumor microenvironment differences of patients with early-stage HPV+HNC and HPV-HNC with different metastatic competence.

2 Chapter 2: DNA Hypermethylation and Silencing of PITX2and TGM2 Correlated with Advanced Stage and Poor Prognosis in Young Patients with HPV Positive Head and Neck Cancer

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2.1 Abstract

Head and Neck Cancer (HNC) is the 6th most common tumor in the world, with the main risk factors associated with cigarette smoking and alcohol consumption. However, human papilloma virus positive (HPV+) HNC cases are increasing significantly from 16.3% to 72.7% in the last three decades. Recent studies have shown that HPV-driven tumors exhibit unique DNA methylation profile alterations. In this study, DNA methylation alterations were evaluated in HNC patients following their characterization regarding the main risk factors. Bisulfite-converted DNA from 24 patients were hybridized in the Human Methylation 450 BeadChip microarrays. Fluorescence signals were analyzed using GenomeStudio software with the methylation module v.1.9.0 (Illumina). To characterize the expression of genes containing differentially methylated regions (DMRs), the initial sample set was evaluated using reverse transcription quantitative polymerase chain reaction (qRT-PCR). Protein validation was validated by immunohistochemistry (IHC) reaction for the top two candidates (PITX2 and TGM2) using an independent cohort of 100 patients with HNC and the results were associated with clinicopathological outcomes. The analysis of tumor microenvironment (TME) components, the cell-cell interactions and the neighbor's spatial distribution were evaluated by imaging mass cytometry analysis (IMC) in patients diagnosed in early clinical stage but with metastatic competence. As a result, we identified 2,655 DMPs located at 1,073 genes, being 384 hypomethylated and 2,271 hypermethylated CpG sites. Considering only the risk factors (high- risk *versus* low risk), it was identified 15 DMRs located at promoter regions of 12 genes. From the 12 genes (*ZNF323, FBXO39, SLFN12, TGM2, ZAP70, STK32B, HOXA2, LMO3, C1orf74, ST8SIA4, C12orf42,* and *PITX2*), we confirmed a concordant mRNA expression for 8 genes (*ZNF323, FBXO39, TGM2, ZAP70, LMO3, C1orf74, C12orf42,* and *PITX2*). PITX2 and TGM2 were selected for validation in a large cohort of patients' samples and showed overexpression in HPV-negative and HPV- positive HNC respectively show This overexpression was associated with tumor depth (P=0.0011) and advanced tumor stage (P=0.0042). This data was also independently validated in The Cancer Genome Atlas (TCGA) platform using 530 HNC samples. Single-cell analysis of more than 209,302 cells revealed differences in immune landscapes between HPV+ and HPV-negative tumours with different metastatic competences. The analyses showed cellular neighbourhoods associated with metastasis in patients with HNC, which we leveraged to identify a unique population of PDL1, and NK cells associated with HPV+HNC. Our findings provide insight into the biology of HPV+HNC,

reinforcing the value of integrating spatial resolution to single-cell datasets to dissect the microenvironmental contexture of cancer.

Keywords: Head and neck cancer; DNA methylation; PITX2; TGM2; HPV; prognostic marker.

2.2 Introduction

Head and neck cancer (HNC) is the 6th most common cancer worldwide presenting 890.000 new cases and 450.000 deaths per year [1]. The actual incidence is expected to rise 30% by the year 2030 due to the exponential increase in human papillomavirus (HPV) infection [2]. Patients with HNC receive standard therapy based on surgery, radiotherapy, chemotherapy, immunotherapy, or combinations of these modalities [3]. These approaches do not consider that HNC differs from one patient to another and that the inter and intra-tumor heterogeneity can directly impact the treatment response [4]. Despite all the advances in surgical procedures and image-guided technologies, the outcomes of patients with advanced disease remain poor and the 5-years survival is stagnant at <50% [5]. Molecular and epidemiological research have identified a consistent set of risk factors linked with tobacco, alcohol consumption, and HPV infection leading to somatic mutations, epigenetic changes and ultimately to HNC formation [6]. Despite of the knowledge of these epidemiological associations, the epigenetic mechanisms underlying the role of these risk factors in HNC aetiology are poorly understood. Epigenetic modifications such as DNA methylation of genes related to cancer are key to detect cancer development as potential biomarkers [7]. Different types of cancers may have specific DNA methylation profiles which could impact on clinical outcomes [8]. DNA methylation is key to transcription silence and have critical role in the maintenance of cell stability [9]. Consequently, the DNA methylation is not only prognosis and predictive biomarker but also the target of therapy through inhibiting activity of DNA methyltransferase (DNMT1) enzymes such as 5-azacitidine and decitabine [10]. In this study, DNA methylation profiles were evaluated in HNC tumors following their characterization regarding the main risk factors and the impact on the tumor microenvironment.

2.3 Material and Methods

2.3.1 Study population

For methylation analysis, primary frozen samples from HNC patients with confirmed risk factors for tobacco and alcohol (n = 12) and non-risk patients (n = 12) followed up for 157 months (over 10 years) were surgically removed at the Department of Head and Neck Surgery (Jewish General Hospital - McGill University). For imaging mass cytometry (IMC) and immunohistochemistry (IHC), archival formalin-fixed and paraffin-embedded tissue (FFPE) blocks were obtained from

each patient and representative hemotoxylin and eosin (HE) slides taken from each tumor for histological confirmation. Approval for the study protocol was granted (CR17-44) following ethical review. Two pathologists reviewed the slides to select paraffin-embedded oral cancer specimens (**Table S1**). Tumor relapse was histologically confirmed, and patients were followed up after treatment. Eligibility criteria included previously untreated HNC patients submitted for treatment in the same institution without any distant metastasis at the diagnosis (M0). The tumor staging was re-classified according to the International Union Against Cancer (TNM) and grouped as early clinical stage (T1 + T2) or advanced clinical stage (T3 + T4) (165). The medical records were the main source to obtain detailed clinicopathological information. Strengthening the reporting of observational studies (STROBE Statement) was used to ensure appropriate methodological quality. All patient information and tissues were obtained after written informed consent and used in accordance with ethical guidelines.

Table 1. Clinical characteristic of samples submitted to DNA methylation profiling, immunohistochemistry, and imaging mass cytometry.

Sample ID	**Age**	**sex**	**Location**	**Smoke**	**Alcohol**	**T stage**	**N stage**	**HPV**	**risk factor**
C03	80	Male	Tongue	Yes	Yes	T4a	N0	Positive	Positive
C01	51	Female	Tongue	Never	Never	T2	N2c	Negative	Negative
C02	55	Male	Tongue	EX	Never	T-	N-	Negative	mixed
C04	67	Female	Buccal mucosa	Never	Never	T-	N-	Negative	Negative
C05	70	Female	Nasal cavity	Yes	Yes	T4a	N0	Positive	Positive
C06	71	Male	Buccal mucosa	Never	Never	T4a	N1	Negative	Negative

C08	50	Female	Tongue	EX	Never	T-	N-	Negative	mixed
C09	42	Male	Larynx	Yes	Yes	T-	N-	Positive	Positive
C010	73	Female	Tongue	Never	Never	T1	N0	Negative	Negative
C012	70	Male	Tongue	Yes	Yes	T2	N2b	Positive	Positive
C013	32	Male	Tongue	Never	Never	T3	N2b	Positive	mixed
C014	76	Female	Buccal mucosa	Never	Never	T4a	N2c	Negative	Negative
C015	49	Female	Tongue	Never	Never	T1	N1	Negative	Negative
C016	63	Male	Tongue	Yes	Yes	T3	N2b	Positive	Positive
C017	58	Male	Tongue	Never	Never	T-	N-	Negative	Negative
C018	72	Male	Pharynx	Never	Never	T4a	N2b	Negative	Negative
C020	69	Male	Floor of mouth	Never	Never	T3	N0	Negative	Negative
C021	60	Male	Larynx	Yes	Yes	TX	N1	Positive	Positive
C022	48	Male	Larynx	Yes	Yes	T2	N2c	Positive	Positive
C023	83	Male	Tongue	Yes	Yes	T2	NX	Positive	Positive
C024	62			Never	Never	T-	N-	Negative	Negative

2.3.2 DNA methylation analysis

DNA was converted using the EZ DNA Methylation kit (Zymo Research,USA) according to the manufacturer's recommendations. Bisulfite-converted DNA was hybridized in the Human Methylation 450 BeadChip microarrays (HM450K, Illumina, USA), following the Illumina Infinium HD methylation protocol, followed by images acquisition by the Illumina iScan SQ scanner (Illumina, USA). The fluorescence signals were interpreted with the GenomeStudio software [11]. (v.2011.1 with the methylation module v.1.9.0; Illumina, USA). Probes were annotated according to the Illumina annotation file using UCSC version hg19 of the human reference genome.

2.3.3 Copy-number variation (CNV) analysis

450k methylation array data were subjected to copy-number variation (CNV) analysis using the conumee Bioconductor software version 1.32.0 [12]. Each probed CpG is typically represented by two probes on the array (one for methylated and one for unmethylated). The methylation and unmethylated signal intensities are combined for the computation of CNV, and a ratio is created against healthy reference samples with a flat genome. The location of each chromosome is then used to plot this copy-number ratio on a graph. For normalization, a set of 71 control samples with a balanced copy-number profile from both male and female donors were used. We included genes that are often altered in HNC in our plots.

ChAMP package was applied to the dataset [13]. Using standard parameters in packages implemented in ChAMP, three samples were excluded due to not passing the QC parameters and XYS and multihit probes were excluded from further analysis. Signal intensities from type I and II probes were normalized using FunNorm[14]. Cellular compositions were corrected using the REfFreeEWAS package [15] as implemented in ChAMP, followed by comparison between groups. Differentially methylated positions (DMPs) and regions (DMRs) were characterized by comparing HNC cases positive and negative for risk factors.

2.3.4 qRT-PCR

To characterize and validate the expression of genes containing a DMR, the initial sample set was evaluated using qRT-PCR. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen, Carlsbad, CA, USA). qRT-PCR amplification was conducted using Power SYBR Green® Master Mix (Thermo Fisher, Carlsbad, CA, USA) and the quality control steps followed MIQE Guidelines [16] All reactions were performed in duplicate, and no-template reactions were used as a negative control. *HPRT* was selected as the most stable control gene from three endogenous genes tested (*GAPDH*, *ACTB*, *HPRT1*) using the geNorm algorithm [17].

Table 2. Primer details for amplification of specific DNA sequences: genes and orientation.

Gene	Orientation	Sequence
ZNF323	Forward	AGTTGGCATCAAAGCAAGAAATC
ZNF323	Reverse	CTGTGGCGTCTCTCTCCAG
ST8SIA4	Forward	GAAAGGCTGGCTCTTCAATCT
ST8SIA4	Reverse	ACCACTGACACATCTCGTTCT
C12orf42	Forward	CGGCCCGAGTAACACAGAG
C12orf42	Reverse	GCCCAGGAGTCTGCTTTGG
PITX2	Forward	TGTGGACCAACCTTACGGAAG
PITX	Reverse	ATGAGCCCATTGAACTGCGG
FAM89A	Forward	GAGAACGGCTTCTTCGATGAA
FAM89A	Reverse	GAGAACGGCTTCTTCGATGAA
RFX8	Forward	TCCTTAATGCTTTGGAAGGTGTT
RFX8	Reverse	CGCATAGTCTTAGCCATGTTGG
FBXO39	Forward	TCGACTCCCTCAGCTACATGA
FBXO39	Reverse	ACATGGTCTTTTTGAACTGGGG
SLFN12	Forward	AAGATAACCGTGTGATGCAGTT
SLFN12	Reverse	GGGGAGCAGGTAATGACGTATTT
TGM2	Forward	CAAGGCCCGTTTTCCACTAAG

TGM2	Reverse	GAGGCGATACAGGCCGATG
ZAP70	Forward	ACGCCAAGATCAGCGACTIT
ZAP70	Reverse	GGGTGCGTACCACTTGAGC
STK32B	Forward	CAACATAGCGACGGTAGTGAAA
STK32B	Reverse	CAGCTCATAGGCTGTGATG
HOXA2	Forward	CCECTGTCGCTGATACATTTC
HOXA2	Reverse	TGGTCTGCTCAAAAGGAGGAG
LMO3	Forward	CTGCTTTGCATGTCAGCTTTG
LMO3	Reverse	AACCTTCCTCGTAGTCCGTCT
C1orf74	Forward	GGGACTTATCTCTAGCAGTCTCC
C1orf74	Reverse	GGTATAGGGAACAGGATAGCCC
GAPDH	Forward	AATGAAGGGGTCATTGATGG
GAPDH	Reverse	AAGGTGAAGGTCGGAGTCAA
ACTB	Forward	GCACCCAGCACAATGAAG
ACTB	Reverse	CTTGCTGATCCACATCTGC
HPRT	Forward	GAACGTCTTGCTCGAGATGTGA
HPRT	Reverse	TCCAGCAGGTCAGCAAAGAAT

2.3.5 Tissue micro-array (TMA) construction

1.0 mm cores were extracted from previously microscopically defined HNC representative areas and matched morphologically normal epithelium from adjacent margins free of tumor with a Tissue Microarrayer® (Beecher Instruments, Silver Springs, MD, USA). Tissue cores were punched and arrayed in duplicate on a single recipient TMA paraffin block. Each core was spaced 0.2 mm apart. After cutting sections from the recipient block, the slides received a layer of paraffin to prevent oxidation and stored at −20 °C.

2.3.6 Immunohistochemistry (IHC)

IHC reaction was carried out on the TMA as we described [18]. In brief, the slides were incubated with primary antibodies diluted in PBS overnight at 4 °C using: anti-TGM2 (Thermofisher, USA, 1-2 µg/mL), anti-PITX2 antibody (Thermofisher, USA, 1:200), anti-p16 antibodies) (Roche Ventana 1:200) were used, following the manufacturer's protocols. Sections were incubated with secondary antibodies (Advanced TM HRP Link, DakoCytomation, Denmark) for half-hour followed by the polymer detection system (Advanced TM HRP Link, DakoCytomation) for half-hour at room temperature. Reactions were developed using a solution of 0.6 mg/mL of DAB (Sigma, St Louis, MO, USA) and 0.01% H_2O_2 and then counter-stained with hematoxylin. Positive controls were included in all reactions in accordance with manufacturer's recommendations. Negative control consisted in omitting the primary antibody and replacing the primary antibody by normal serum. IHC reactions were replicated on distinct TMA slides to represent different tissues levels in the same lesion. The second slide was 25–30 sections deeper than the first slide, resulting in a minimum of 300µm distance between sections representing 4-fold redundancy with different cell populations for each tissue.

2.3.6.1 IHC data acquisition

All TMA slides were scanned using an Aperio ScanScope CS (Leica Biosystems, Canada) whole slide scanner at 20X magnification, with a resolution of 0.25 µm/pixel. The resolution of all images was within the range 0.231–0.253 µm/pixel.

2.3.6.2 QuPath combination with StarDist

All tumor sections were analyzed in QuPath (version 0.2.3 University of Edinburg, UK) [19] based on a computerized digital image-processing system using the segmentation method StarDist [20]. After whole slide scan, an entire image was selected for analysis and imported into QuPath. The StarDist model was used for estimating positive tumor cells within the annotations. Positive tumor cells classifiers were trained, and quantification was based on the nuclear (p16) or cytoplams (TGM2 and PITX2) staining specificity of each marker. Data were extracted from QuPath, the ratio including percentages for each marker were further calculated in MS Excel by dividing the total number of positive tumor cells per each marker by the total number of tumor cells in the

corresponding H&E section. TensorFlow's [21] framework was used to enable the training and interference of deep neural networks.

2.3.6.3 Statistical analysis

Statistical analyses of associations between variables were performed by the Fisher's exact test (with significance set for $P<0.05$) and for continuous variables the non-parametric Mann–Whitney u test. All analyses were performed using the statistical software package STATA-13 (STATA Corporation, College Station, TX, USA).

2.3.7 Imaging mass cytometry (IMC)

IMC was done at the Rosalind and Morris Goodman Cancer Institute and the Quebec Cancer Consortium using the Fluidigm Hyperion Imaging Mass Cytometer System for laser-based cell ablation and imaging in their Single Cell and Imaging Mass Cytometry Platform (SCIMAP).The system had a 1μm resolution and gathered channel-specific signal data on a per-pixel basis This data helped record signal localization (corresponding to nuclear, cytoplasmic, and membrane) and intensity per individual cells. Cellular regions were identified by correlating with Ir191/193 which labels DNA, and CD45, e-cadherin, Pan-CK, smooth muscle actin (SMA) and vimentin for the cytoplasmic. The antibody panel including the target, clone information and metal isotype tag, is available in the **Table 3**. Conjugations of the metal to the antibodies were done using the Maxpar labeling kit (Fluidigm, USA). The concentration of the antibodies was adjusted by titration, ranging from 100 to 500 μg ml^{-1}, using Nanodrop (Thermo Scientific, USA).

Table 3. Antibody panel design and metal conjugation.

Metal	Target	Clone	Vendor	Cat #
106Cd	α-SMA	1A4	Abcam	ab240654
110Cd	Vimentin	D21H3	Cell Signal	46173SF
141Pr	CD11c	EP1347Y	Abcam	ab216655
143Nd	CD31	EP3095	Abcam	226157
144Nd	CD21	BU32	Biolegend	354902

146Nd	Ki-67	B56	BD	556003
148Nd	Pan-CK	C11	Fluidigm	3148020D
150Nd	PD-L1	SP142	Fluidigm	3150033D
151Eu	CD163	EdHu-1	Fluidigm	91H005151
155Gd	FoxP3	PCH101	Fluidigm	3155018D
156Gd	CD4	EPR6855	Fluidigm	3156033D
158Gd	E-Cad	24E10	Fluidigm	3158029D
159 Tb	CD68	KP1	Fluidigm	3159035D
161Dy	CD20	E7B7T	Cell Signal	92688SF
162Dy	CD8a	CD8/144B	Fluidigm	3162034D
165Ho	PD-1	EPR4877(2)	Fluidigm	3165039D
167Er	GranB	EPR20129-217	Fluidigm	3167021D
169Tm	Collagen I	Polyclonal	Fluidigm	3169023D
170Er	CD3	Polyclonal	Fluidigm	3170019D
173Yb	CD45RO	UCHL1	Fluidigm	3173016D
195Pt	Cell Seg Kit 1	N/A	Fluidigm	TIS-00001
196Pt	Cell Seg Kit 2	N/A	Fluidigm	TIS-00001
198Pt	Cell Seg Kit 3	N/A	Fluidigm	TIS-00001
191/193Ir	DNA	N/A	Fluidigm	201192A

2.3.7.1 Immune staining

FFPE TMA slides underwent deparaffinization and heat-mediated antigen retrieval using the Ventana Discovery Ultra auto-stainer platform (Roche Diagnostics) according to the manufacturer's instructions. FFPE slides were incubated at 70°C in pre-formulated EZ Prep solution (Roche Diagnostics), followed by incubation at 95°C in pre-formulated Cell Conditioning 1 solution (Roche Diagnostics) for a total run time of ~2.5 h. Slides were rinsed in 1× PBS and incubated for 45 min at room temperature in Dako Serum-free Protein Block solution (Agilent). An antibody cocktail containing metal-conjugated antibodies was prepared in Dako Antibody Diluent at optimized dilutions. Slides were stained with primary antibodies at 4 °C overnight and subsequently washed with 0.2% Triton X-100 and 1× PBS. A secondary antibody cocktail

containing metal-conjugated anti-biotin was prepared in Dako Antibody Diluent at the optimized dilution. Slides were incubated with anti-biotin for 1h at room temperature and subsequently washed with 0.2% Triton X-100 and 1× PBS. Slides were counterstained with Cell-ID Intercalator-Ir (Fluidigm) diluted at 1:400 in 1× PBS for 30 min at room temperature, rinsed for 5 min with distilled water, and air-dried prior to IMC acquisition.

2.3.7.2 IMC data acquisition

IMC image was obtained for each sample using the Hyperion Imaging Mass Cytometry™ (IMC™, Fluidigm, USA) after following the manufacturer's instructions for instrument calibration. Laser ablation was performed on the regions of interest at a frequency of 200Hz, resulting in a pixel size/resolution of 1 µm2. The raw data underwent preprocessing using the CyTOF software v7.0 (Fluidigm, USA) [22].

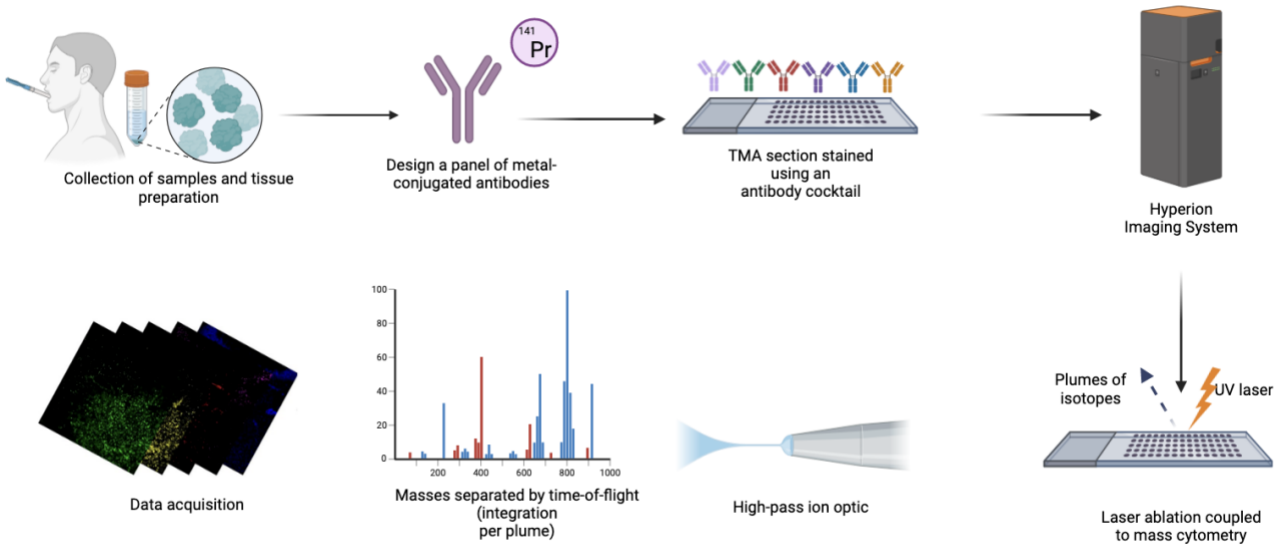

Figure1. Project design and sample processing. The workflow involves several stages including 1) Sample collection and tissue preparation; 2) Antibody panel design with specific antibodies conjugated with heavy metal isotopes; 3) Tissue staining involving tissue microarray (TMA) section stained with a cocktail of metal-conjugated antibodies, which enables the binding to specific cellular targets; 4) The hyperion imaging system was used to process the tissue samples;

5) Laser ablation feature coupled with mass cytometry for ionizing and analyzing the metal tag; 6) Ionized metal isotopes are generated and go towards the mass spectrometry component for ionization and analysis and data accusation. Figure created using biorender.com.

2.3.7.3 IMC data preprocessing and analysis

The images were analyzed following the protocol published by Windhager *et al*. [23]. The first step includes the quality control by manually reviewing the raw data using plugins like napari-imc [23] or ImageJ [24] for IMC. T. After this, the data undergoes preprocessing to extract multichannel images in TIFF format from the raw data. The raw. MCD (MathCad document) files were processed using the HistoCat++ V 3.0.0 [25], Initially, the raw files were converted into .tiff files. Following this, the data imported to enable medicine platform and a pretrained neural network was employed to segment the cells. The objective of this stage is to allow for deep learning segmentation, specifically using the DNA1 and DNA2 channels for the nuclear channel.

2.3.7.4 Artifact detection

An artifact annotation created using the enable medicine platform using the sensitivity method parameter "9" and the DNA1 and DNA2 channels for the nuclear channel. The clustering outcomes were evaluated to determine the presence of batch effects between samples and acquisition day, and the significant batch effects were corrected.

2.3.7.5 Clustering and dimensionality reduction

Cells were clustered using the Louvain method for graph-based modularity optimization provided by Enable Medicine ^{inc}. For the projection of multi-parametric data into a 2-dimensional space, the UMAP method was applied.

2.3.7.6 Cell–cell pairwise interaction analysis

To identify significant pairwise interaction and avoidance behaviours between cell types, we performed permutation tests of single-cell interactions as previously described [26]. Cells within a 6-pixel radius (6 μm) were considered interacting. Significant interaction was considered with $P \leq 0.01$.

2.3.7.7 Cellular neighborhood analysis

A spatial neighborhood graph was established using Enable Medicine's Spatial Neighbor Distance function [27] Following this, neighborhood cell type composition and mean marker intensities were aggregated.

2.4 Results

2.4.1 Study population

Methylation analysis combined with clinicopathological data from our cohort containing HPV+ (n = 171) vs. HPV negative cases patients (n = 20) with 15.7 (7.3%) months follow-up, identified a set of genes that are selectively overexpressed HPV+ compared to HPV- **(Table 4)**.

Table 4. The demographic characteristics of HNC patients that were included in the methylation study.

Characteristics	**HPV+ (n=15)**	**HPV- (n=20)**	**Validation cohort (n=136)**	**All patients (n=171)**	***P*-value**
Age (years)					
Mean (SD)	57.3 (14.6)	46.0 (14.7)	46.0 (14.4)	47.0 (14.6)	0.025
Gender					
Female	9 (60.0%)	13 (65.0%)	112 (82.4%)	134 (78.4%)	0.041
Male	6 (40.0%)	7 (35.0%)	24 (17.6%)	37 (21.6%)	
pT category					
pT1	NA	4 (20.0%)	21 (15.4%)	25 (16.0%)	0.001
pT2		5 (25.0%)	33 (24.3%)	38 (24.3%)	
pT3/4		11(55.0%)	82 (59.6%)	93 (59.6%)	
pN category					
pN0	NA	6 (30.0%)	74 (54.4%)	80 (51.2%)	0.001
pN1a		6 (30.0%)	29 (21.3%)	35 (22.4%)	

pN1b *pNx*		4 (20.0%) 4 (20.0%)	18 (13.2%) 15 (11.0%)	22 (14.1%) 19 (12.1%)	
Follow-up (months) *Mean (SD)*	14.5 (11.2)	15.7 (7.3%)	68.1 (30.9)	59.8 (34.5)	0.001

2.4.2 Methylation

DNA methylation levels in the tumor tissue from patients with and without exposition to risk factors (smoke, alcohol, and HPV) were associated with genes with a DMR located at the respective promoter region to evaluate the mRNA levels (**Figure 2**).

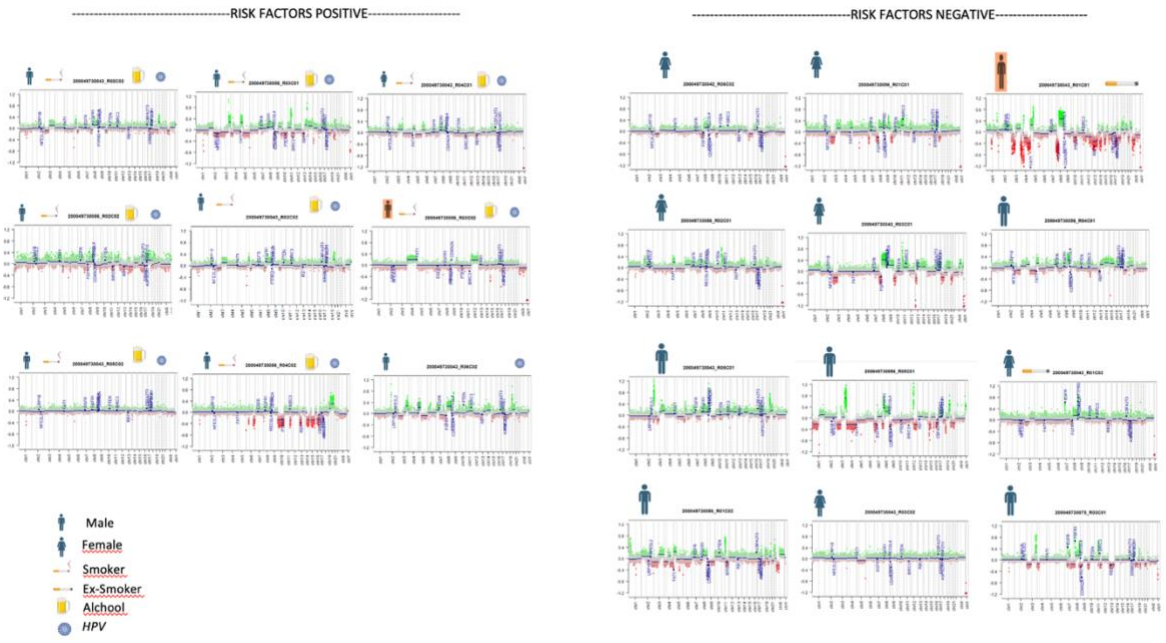

Figure 2. DNA methylation profiles and methylation levels observed in HNC with and without associated risk factors. The graphs show the DNA methylation at each CpG site in the different target regions.

Comparison between cases considering the risk factor exposition did not point to a DMP after multiple correction tests (ad*jP*≤0.05). It was identified CpG sites with a *P*-value <0.001 and methylation levels higher than 10% (**Supplementary Table 1**). There are 2,655 DMPs located at

1,073 genes, being 384 hypomethylated and 2,271 hypermethylated CpG sites. Considering DMRs, we found 12 hypermethylated and 3 hypomethylated in cases positive to risk factors, compared to the negative group (Table 5). The 15 DMRs are located at 17 genes (*ZNF323, FBXO39, SLFN12, TGM2, ZAP70, STK32B, STK32B, HOXA2, LMO3, C1orf74, ST8SIA4, C12orf42, PITX2, C17orf46, MIR1182,* and *RFX8*). To validate these findings, qRT-PCR in samples from HNC patients HPV+ and HPV- were performed, which confirmed the expression changes predicted by the methylation data for genes most importantly in *LMO3, PITX2, and TGM2* (**Figure 3**), where the expression profiles of each validated gene show agreement with the methylation changes (**Table 5**). *LMO3, PITX2,* and *TGM2*, showed consistent patterns of gene expression (**Figure 3**). TGM2 (HPV+) and PITX2 (HPV-) were choose as potential candidates for additional investigation based on the availability of the commercial antibodies.

Table 5. Differential methylation analysis results for genes in different chromosomal regions.

ID	Gene	chr	start	end	width	value	area	p.value	fwer	p.value Area	fwerArea
DMR_1	*ZNF323*	6	28303629	28304451	822	-2.54	32.99	1.37E-05	0.00	1.59E-03	0.24
DMR_2	*FBXO39*	17	6679254	6679781	527	1.97	23.68	4.65E-04	0.09	5.22E-03	0.57
DMR_3	*SLFN12*	17	33759484	33760527	1043	1.77	23.07	9.85E-04	0.16	5.84E-03	0.58
DMR_4	*TGM2*	20	36793608	36794002	394	1.76	21.11	1.55E-03	0.24	8.56E-03	0.66
DMR_5	*ZAP70*	2	98350651	98352002	1351	1.38	20.77	3.24E-03	0.41	9.35E-03	0.69
DMR_6	*STK32B*	4	5052526	5053596	1070	1.65	16.53	6.13E-03	0.49	2.45E-02	0.87

DMR_7	*HOXA2*	7	27142527	27143478	951	1.22	20.81	3.83E-03	0.50	9.26E-03	0.69
DMR_8	*LMO3*	12	16758692	16759391	699	1.74	13.91	9.81E-03	0.60	4.76E-02	0.95
DMR_9	*C1orf74*	1	209957601	209958343	742	-1.35	17.55	7.37E-03	0.60	1.88E-02	0.83
DMR_10	*ST8SIA4*	5	100238260	100239319	1059	1.47	16.16	9.55E-03	0.66	2.68E-02	0.88
DMR_11	*C12orf42*	12	103889516	103889960	444	1.58	14.24	1.29E-02	0.66	4.37E-02	0.94
DMR_12	*PITX2*	4	111544016	111544463	447	1.54	13.85	1.57E-02	0.71	4.84E-02	0.95
DMR_13	*C17orf46*	17	43339040	43339744	704	-1.32	14.57	1.70E-02	0.80	4.02E-02	0.94
DMR_14	*MIR1182*	1	231155632	231156359	727	1.22	14.65	1.77E-02	0.82	3.93E-02	0.94
DMR_15	*RFX8*	2	102090791	102091656	865	1.12	14.59	1.58E-02	0.83	4.00E-02	0.94

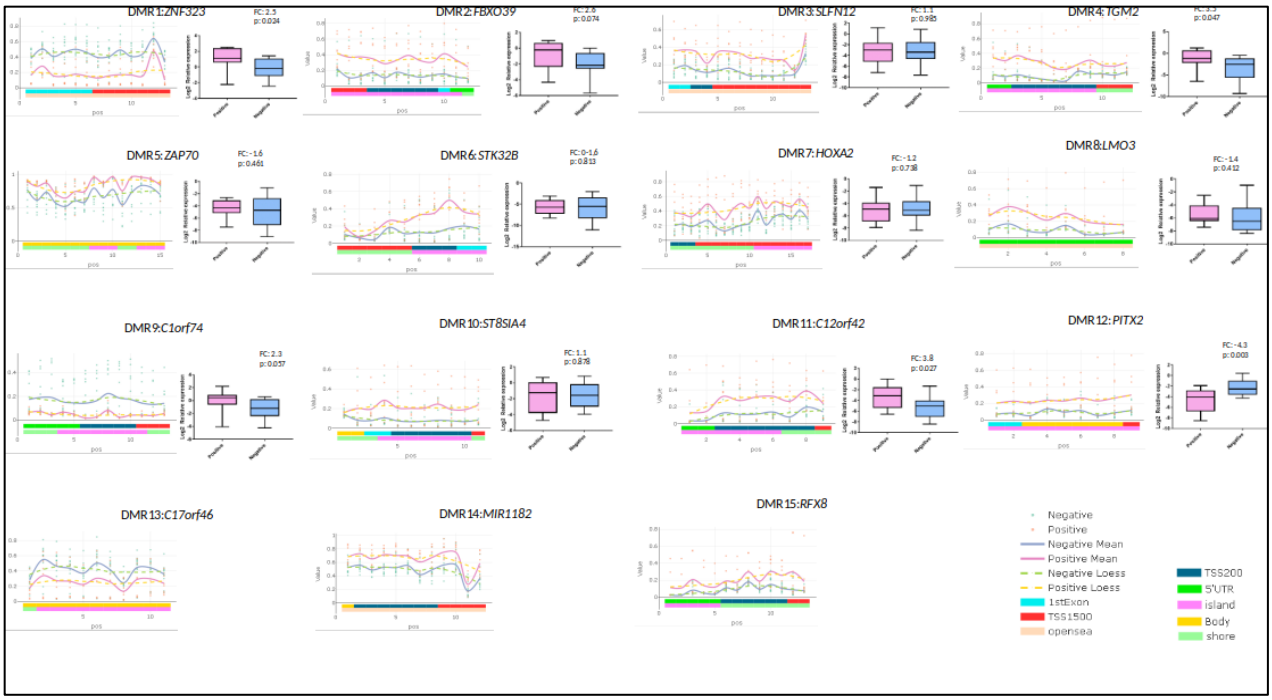

Figure 3. Validation of 15 differentially methylated regions (DMRs) in17 genes by qRT-PCR. Line graphs represent the expression profiles of each DMR with standard deviation indicated by error bars. box plots show detailed expression distribution. Identifiers above each plot correspond to specific DMRs and their associated genes. Color coding on the line graphs correlates with genomic features such as transcription start sites and CpG island regions. Box plots highlight expression fold changes, with significance denoted by p-values and fold changes (FC). this figure categorizes expression data into negative and positive deviations from the control mean.

2.4.3 Protein expression validated methylation data

Protein expression from two genes that presented agreement between DNA methylation and mRNA expression were evaluated in an independent cohort of cases. The analysis showed that in HPV-negative HNC cases, moderate levels of PITX2 expression were detected, whereas a down-regulation of TGM2 expression was seen, suggesting a potential inverse correlation with HPV status (**Figures 4B and 4C**). on the contrary, PITX2 showed weak expression in HNC cases, but specially down expressed in HPV+ cases; TGM2 was overexpressed in HPV+HNC.

Table 6. Demographic and clinical features of patients with P16 positive and negative HNC.

Feature	P16 Negative	P16 Positive	p-value
Age (years)	60.57 ± 10.54	64.08 ± 16.53	0.388
Sex (M/F %)	Male: 48.57%, Female: 51.43%	Male: 61.54%, Female: 38.46%	0.635
PITX-Positive %	0.69 ± 1.68	0.08 ± 0.12	0.202
HPV-Positive %	1.48 ± 1.58	3.72 ± 4.63	**0.015**
TGASE-Positive %	21.99 ± 20.03	24.94 ± 18.34	0.644

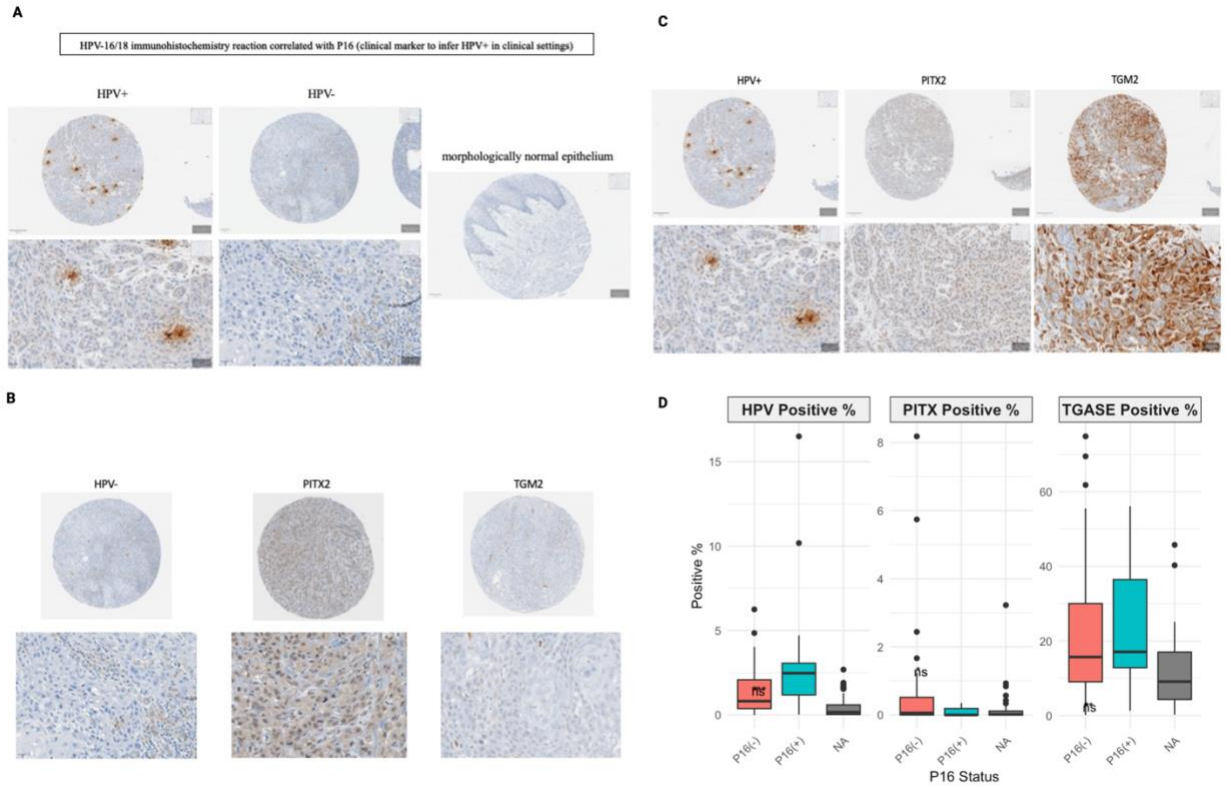

Figure 4. Validation of the protein expression of selected genes identified by DNA methylation in HNC patients with and without associated risk factors by immunohistochemical expression. A) TMA stained with HPV16/18 are showing the expression HPV+ in the left and HPV- in the right; a morphologically normal epithelium was used as control. **B**) First panel showed HPV- HNC case analyzed. Protein expression for PITX2 showed moderate to overexpression in HPV- HNC and TGM2 was down-regulated in HPV- HNC. **C**) First panel

showed the HPV+ case analyzed. Protein expression for PITX2 showed down-expression in HPV+ HNC cases and TGM2 was overexpressed in HPV+ HNC. Magnification of 200x; scale bar = 50um). **D**) Boxplots shows the percentage of positive expression for HPV-16/18, PITX2, and TGM2 markers expression categorized by P16 status. Median values and interquartile ranges are represented as individual points. The annotations 'ns' indicate no statistical significance in marker expression between P16(-) and P16(+) categories.

2.4.4 Image analysis identified distinct immune infiltration patterns within HNC samples.

In the heatmap analysis of normalized expression values **(Figure 5A**) different cellular markers and clusters of cells were shown. Whitin the enable medicine platform High expression of CD3 and CD4, clusters as helper T cell markers; High expression of CD20 and CD45RA, cluster as B cell markers; High expression of E-Cadherin and Pan-Cytokeratin, clusters as epithelial cell markers. High expression of Vimentin, aSMA, and CollagenI, clusters as fibroblast markers and High expression of CD14, CD163, and CD68, clusters as macrophage markers. Between the markers with their normalized expression values there was a significant overexpression of CD68 and CD163, suggesting a strong presence of these phagocytic cells, interestingly, helper T cells also showed a markedly higher expression of CD4 and FoxP3, which are typically associated with the regulatory functions of T cells.

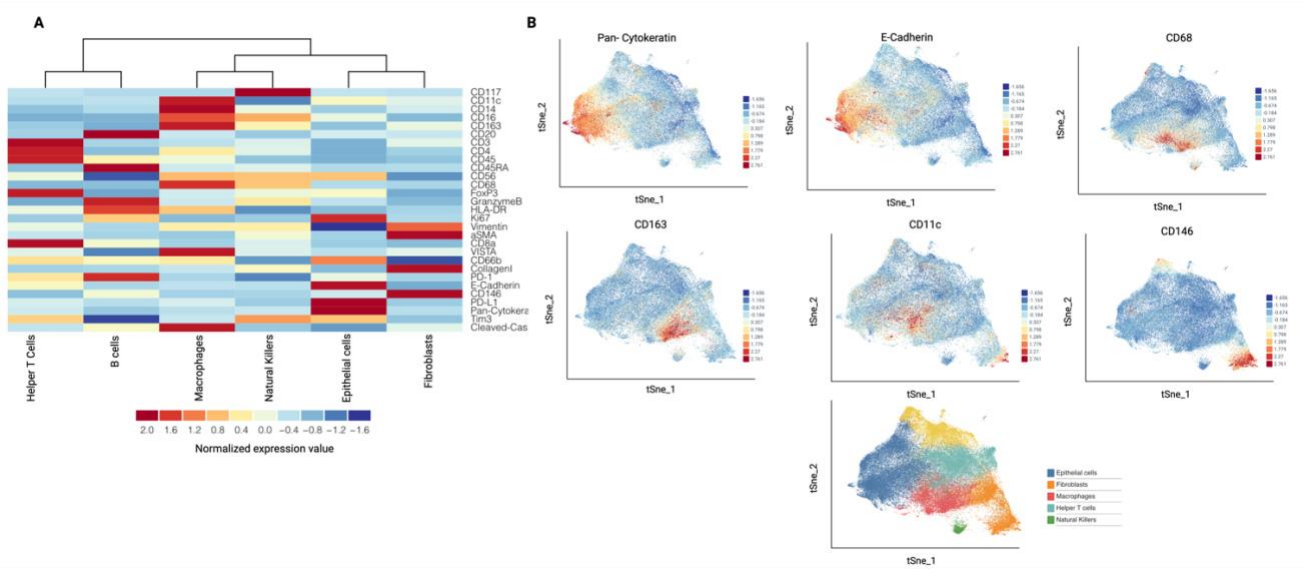

Figure 5. Unsupervised clustering. A) Heatmap provides a summary of the normalized expression values of marker across cell populations. Each row represents a different marker such as CD117, CD11c, CD14, CD163, CD20, CD3, CD45, CD56, Ki67. The colors range from red (high expression) to blue (low expression), shows the expression levels across the different cell types **B)** The t-SNE clustering offer a two-dimensional representation of the high-dimensional single-cell data, highlights the distribution and density of cells based on marker expression. Individual maps are provided for markers: Pan-Cytokeratin, E-Cadherin, CD68, CD163, CD11c, and CD146. Each point on a map represents a single cell, with the color that indicates the expression level of the marker from low (blue) to high (red). The lowermost t-SNE map shows the clustering of cells into distinct populations, color-coded by cell type: epithelial cells, fibroblasts, macrophages, helper T cells, and natural killers.

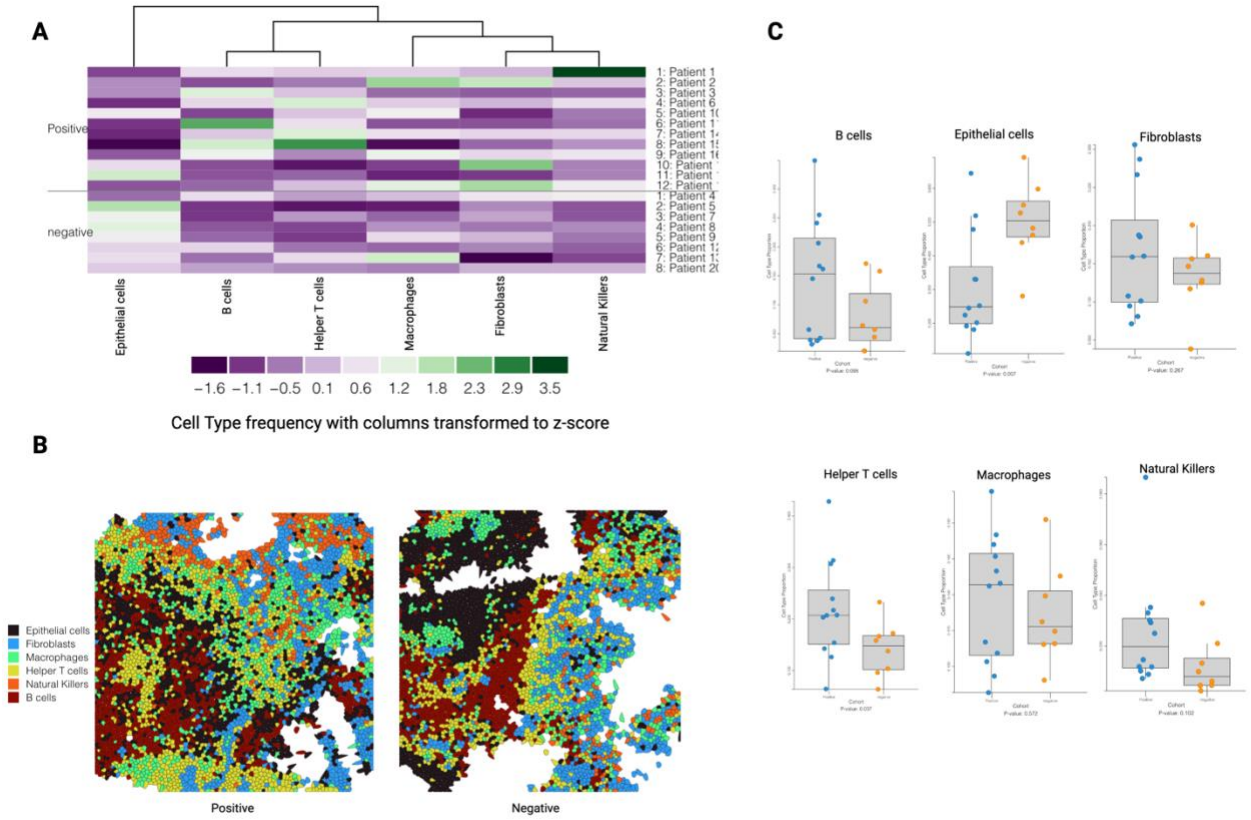

Figure 6. IMC showing the cell dynamics within the TME of the head neck cancer tissue. A) The heatmap shows the cell type frequencies in patient samples. The z-score transformation enables a direct comparison of cell type between samples categorized as 'positive' and 'negative' for HPV related HNC. **B)** Spatial distribution maps show the tissue compartmentalization of cell types in 'Positive' and 'Negative' patient groups. **C)** Box plots shows the variation and distribution of z-scores for cell frequencies among 'positive' and 'negative' groups.

In the HPV+ HNC there is a higher amount of macrophages and helper T cells compared to the negative condition, as indicated by the median values and the spread of the data points in their respective plots **(Figure 6C)** although the frequency of natural killers, B cells, and fibroblasts appears to be relatively consistent between the positive and negative conditions, with overlapping interquartile ranges and no significant shift in median values. Epithelial cells, while showing some variability, do not exhibit a marked difference between the two conditions.

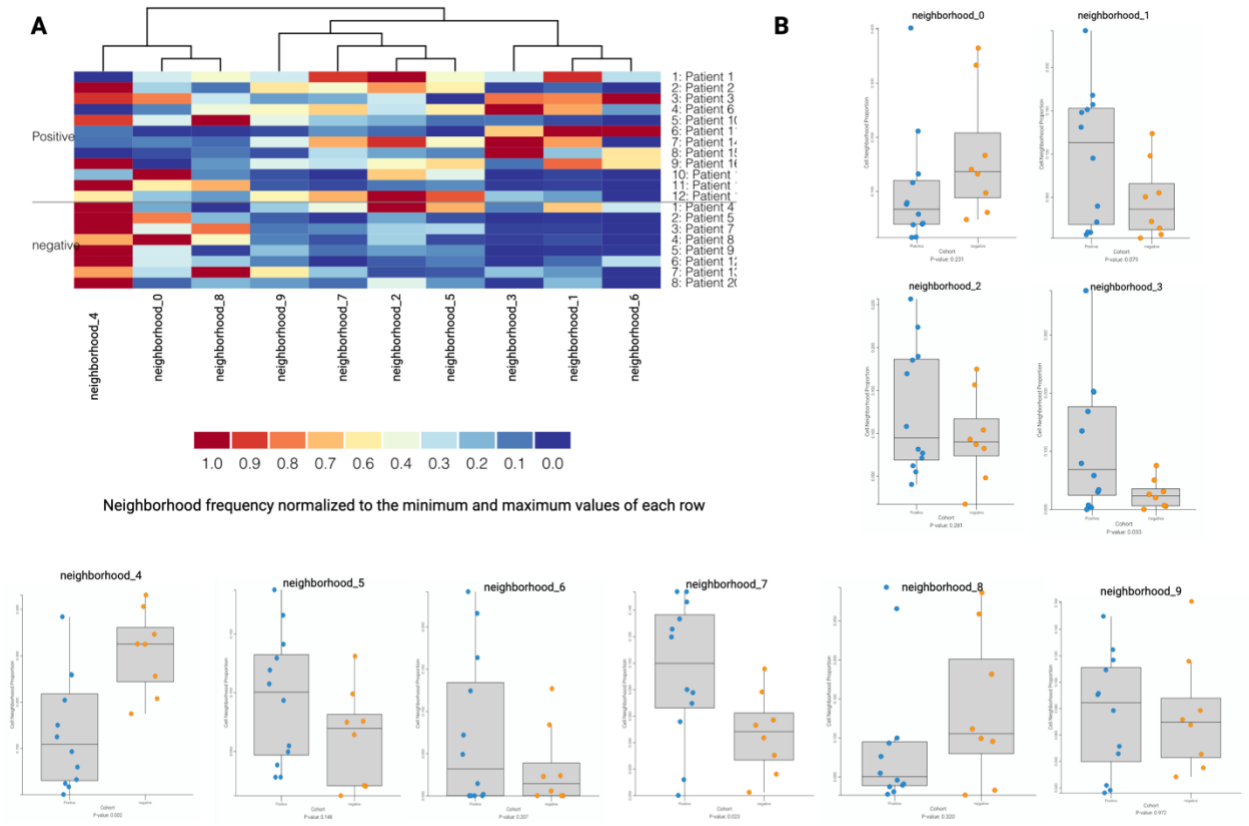

Figure 7. N**eighborhood analysis identifies cell populations associated with HNC phenotypes of interest from single-cell interaction. HNC cell phenotypes A) I**nteractions present in 20 HNC tumor images and three matched normal tissue images are represented as a heatmap in which the cell type in the row is significantly neighbored (red) or avoided (blue) by the cell type in the column. Significance was determined by permutation test ($P < 0.01$). **B)** Boxplots shows the distribution of interaction frequencies between different cell types in HNC tumors. Each boxplot corresponds to a specific neighborhood or interaction type and displays the median, interquartile range, and outliers of interaction frequency. The 'whiskers' of the boxplot extend to the extreme data points not considered outliers, and points are plotted individually to show the spread of the data beyond the quartiles. The statistically significant interaction between the frequencies of two cohorts is highlighted with p-values calculated using a Mann-Whitney U test to compare the distributions.

The boxplots across neighborhoods 0 to 9 and heatmap show variation in the distribution of the measured variable between the two cohorts. **(Figure 7 A and B).** Some neighborhoods show a statistically significant difference between the cohorts (as indicated by p-values), suggesting that these neighborhoods could be potential areas of interest, so the spatial neighborhood analysis was done for the next step to better understand and investigate the cellular interactions in TME.

2.4.5 Spatial neighborhood analysis reveals specific cellular interactions associated with tumor behavior.

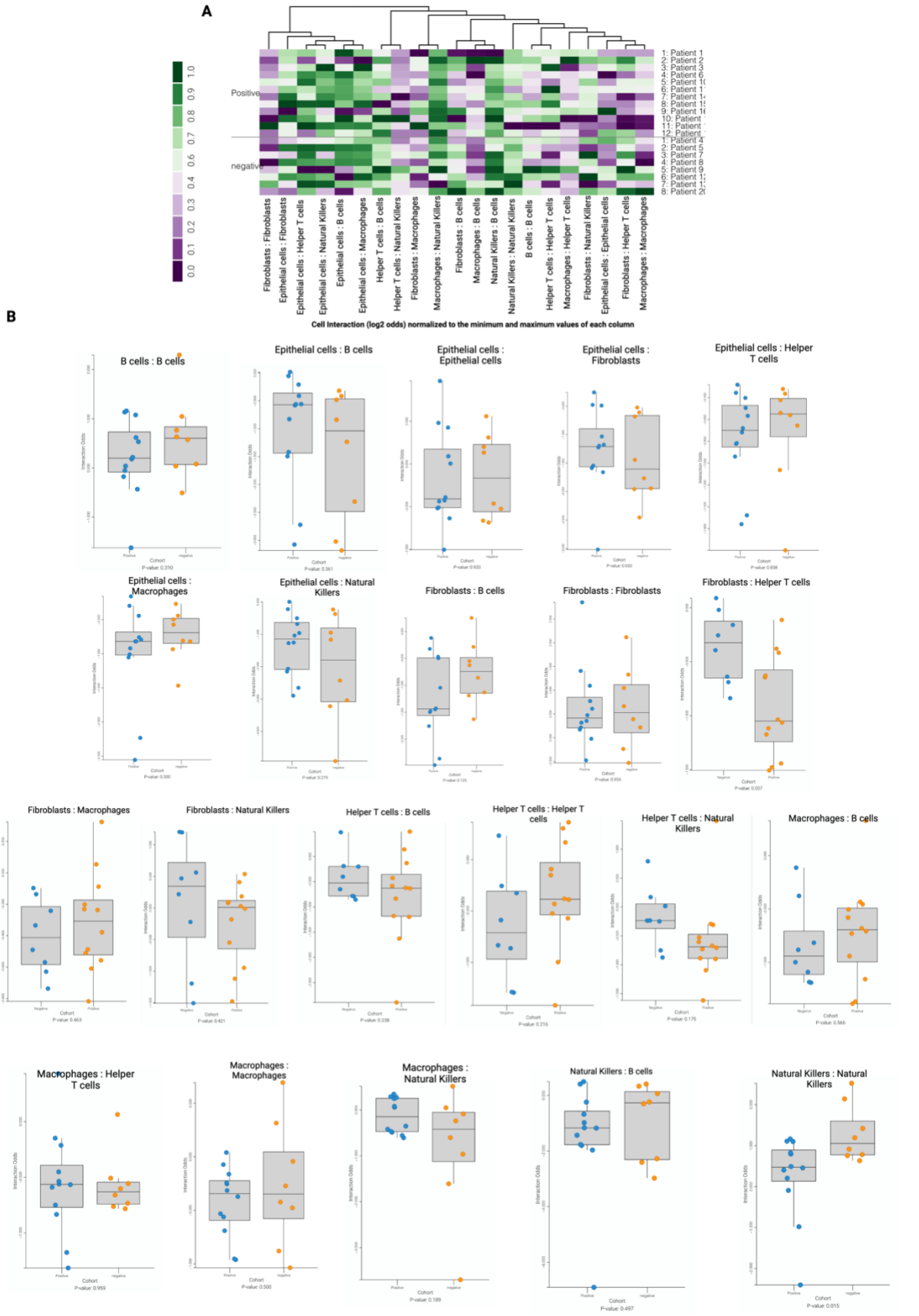
A
Positive
negative
Cell Interaction (log2 odds) normalized to the minimum and maximum values of each column
B
B cells : B cells
Epithelial cells : B cells
Epithelial cells : Epithelial cells
Epithelial cells : Fibroblasts
Epithelial cells : Helper T cells
Epithelial cells : Macrophages
Epithelial cells : Natural Killers
Fibroblasts : B cells
Fibroblasts : Fibroblasts
Fibroblasts : Helper T cells
Fibroblasts : Macrophages
Fibroblasts : Natural Killers
Helper T cells : B cells
Helper T cells : Helper T cells
Helper T cells : Natural Killers
Macrophages : B cells
Macrophages : Helper T cells
Macrophages : Macrophages
Macrophages : Natural Killers
Natural Killers : B cells
Natural Killers : Natural Killers

Figure 8. Inter- and intra-cellular interaction profiling A) This heatmap shows the ratios of cell-cell interactions in different patient samples, with an emphasis on the co-occurrence of various cell types within the 'positive' and 'negative' cohorts. Each row represents a specific cell interaction pair, with color intensity corresponding to the frequency ratio, normalized across all patient samples. **B)** The box plots show a detailed statistical analysis of selected cell-cell interaction ratios. The distribution of interaction ratios between various cellular pairs such as fibroblasts with themselves and with B cells, epithelial cells with natural killers, macrophages, and B cells, and B cells with themselves.

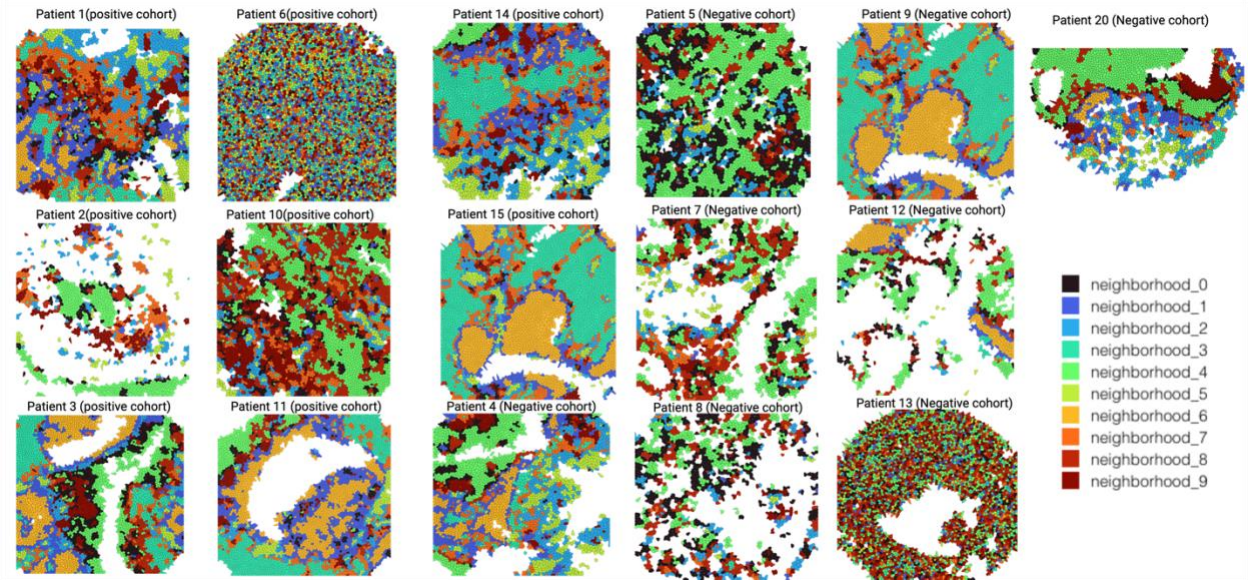

Figure 9. The spatial cellular neighbourhoods of HNC tumors. Representative Voronoi diagrams categorized with patients in P16+ cohorts and P16 negative cohort.

In addition to cell densities, cellular interactions can also play a crucial role in tumor control. We therefore determined which cellular interactions within the primary tumors were associated with metastases. immune populations from the IMC datasets were performed, generating 21 clusters. We determined which clusters interacted with each other and which clusters avoided each other. Focusing on interactions or avoidance that were significantly different between positive and negative patients, we could identify specific interactions or avoidance that could be associated with clinical outcomes.

Among the significant interactions associated with favorable outcomes P16 negative were a subset of natural killers- B cells interactions, and a subset of natural killers interacting with themselves. In contrast, in patients with P16 positive showed strong interaction in macrophages-natural killers. (**figure 8, 9**)

2.5 Discussion

Tobacco and alcohol consumption, as well as HPV infection, are known risk factors for HNC. The biological mechanisms involved in the development and progression of HNC are complex, Increased DNA methylation levels of tumor suppressor genes have been associated HNC. In our study, we used genome-wide DNA methylation data from HM450K to identify changes in DNA methylation in HNC patients and their potential association with primary cancer risk factors. We analyzed a well-defined cohort of HNC patients treated in a single center and identified a high frequency of gene hypermethylation, including *FBXO39, SLFN12, TGM2, ZAP70, STK32B, HOXA2, LMO3, C1orf74, ST8SIA4, C12orf42,* and *PITX2.* Previous works reported aberrant hypermethylation in gene promoters of HPV (+) oropharyngeal cancer cases, however, our analysis identified hypermethylation of new genes associated with HPV status, including two additional cadherin-associated genes (*TGM2* and *PITX2*) for which methylation levels were not previously described as deregulated in HPV (+) HNC. TGM2 is an enzyme that plays a role in cell differentiation, apoptosis, signal transduction, and wound healing. Recent studies suggest that TGM2 is involved in the clearance of apoptotic cells and that its suppression can lead to a pro-inflammatory phenotype. PITX2, on the other hand, is a transcription factor associated with inflammation in acute appendicitis and is linked to the progression of different cancers. PITX2 gene expression is known to facilitate invasion, proliferation, and metastasis in different types of cancer such as lung, ovarian, and colorectal cancer. DNA methylation at CpG island influences PITX2 expression and is associated with prostate recurrence. In our study, we investigated the prognostic value of PITX2 and TGM2 DNA methylation level, gene, and protein expression in HNC patients and its potential clinical impact. PITX2 and TGM2 may play a crucial role in the pathophysiology of various conditions, including inflammation and cancer. While further studies are needed to investigate the precise molecular mechanism underlying these findings, one can speculate that this methylation signature is able to integrate alterations induced by the multiple

exposures occurred in these patients (notably, heavy smoking, alcohol consumption and HPV infection), resulting in a better discriminator for survival. Developing epigenetic therapies to combat inflammation or tumors is based on immune system cells. Epigenetic modifications might affect transcription patterns during disease progression, which can either facilitate the clearance of pathogens or make it possible for the pathogens to evade the immune system. Understanding the relationships between the different immune effector pathways will permit improved immunomodulatory therapeutics, development of improved vaccines, and avoidance of unintended tissue damage. Our candidates should be tested in larger cohorts of patients with HNC, using multivariate statistical models, to improve the power and eventually provide a better marker for establishing more targeted treatments, especially when considering HPV status for de-escalation regimens to avoid long-term toxicity of standard-of-care treatment.

2.6 Ethics statement

This study was conducted in accordance with the Declaration of Helsinki (1964). All of the subjects included in this study provided their written informed consent. This study was approved by the Ethical Commission of CIUSSS-Montreal (CR17-44)

2.7 Funding

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2.9 Supplementary material

Table S1. CpG sites with a *P*-value <0.001 and methylation levels higher than 10%

chrom	strand	cdsStart	cdsEnd	name2
chr17	+	7993552	7997519	ASPSCR1
chr11	+	1,02E+08	1,02E+08	BIRC3
chr16	-	8894338	8904321	CBFA2T3
chr9	-	2196822	2197482	CDKN2A
chr7	+	5508697	5527331	EGFR
chr17	+	3785581	3788429	ERBB2
chr4	-	1,88E+08	1,88E+08	FAT1
chr8	-	3826990	3831496	FGFR1
chr2	-	1,41E+08	1,43E+08	LRP1B
chr2	-	1,78E+08	1,78E+08	NFE2L2
chr3	+	1,79E+08	1,79E+08	PIK3CA
chr10	+	8962370	8972522	PTEN
chr9	-	8317873	8733843	PTPRD
chr13	+	4887804	4905420	RB1
chr8	-	1,46E+08	1,46E+08	RECQL4
chr17	+	7823748	7836729	RNF213

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3 Chapter 3

3.1 Discussion

The incidence of HPV+ HNC is increasing rapidly and has exceeded cervical cancer to become the most common HPV-induced cancer in developed countries. Since patients with HPV + HNC respond very favorably to standard treatment, the emphasis has changed to reducing treatment intensity in order to improve patient's quality of life. Aberrant DNA methylation is an epigenetic hallmark of tumors and leads to tumor development and progression by silencing tumor suppressor genes and activating oncogenes. DNA methylation is a promising candidate for specific mechanisms by which environmental carcinogens and chronic inflammation can contribute to cancer development. Our study identified two main candidates associated with environmental risk factors is HNC: TGM2 and PITX2.

TGM2 is an enzyme belonging to the transglutaminase family that serves multiple functions, such as participating in cell differentiation, signal transduction, apoptosis, and wound healing. TGM2 is present in various cells, including macrophages. It has been known to aid in the clearance of apoptotic cells (efferocytosis), and recent findings suggest that defective efferocytosis is involved in the consequences of inflammation-associated illnesses. Suppressing TGM2 activity leads to a pro-inflammatory phenotype. The functional involvement of PITX2 in tumorigenesis remains undetermined. PITX2 gene expression is related to inflammation in acute appendicitis. PITX2 is a transcription factor during vertebrate embryogenesis, and irregular PITX2 expression is associated with the progression of various cancers. PITX2 and TGM2 may play a critical role in various pathophysiological conditions, including inflammation and cancer. The development of epigenetic therapeutics to combat inflammation or tumors is predicated on immune system cells. During disease progression, epigenetic modifications may influence transcription patterns (genes), which can either help with the clearance of pathogens or result in evasion by the pathogens.

4 Chapter 4

4.1 Conclusions

Aberrant methylation of DNA and inflammation can be induced by environmental carcinogens. Our results, based on genome-wide DNA methylation data from different cohorts, considered risk factors (smoking, tobacco and HPV infection) show that HPV infection greatly affects DNA methylation in HNC. Recognition of HPV + HNC with its favorable clinical outcomes drives the interest in de-escalating treatment to reduce side effects. However, inter- and intratumoral heterogeneity are the most significant challenge. Biomarkers are important for assigning patients to accurate risk subgroups. There is a need to integrate clinical, histopathologic, and molecular variations to develop a robust and clinically actionable paradigm that groups HPV+ HNC in accordance with these risk factors. Overall, this study provides new insight into epigenetic biomarkers that could help to improve risk stratification and treatment management, but further research is necessary to explore HPV status and subtypes in a large cohort of tumor samples.

4.2 Future Directions

The possibility of investigating whether monocyte polarization into M2-like macrophages can be induced by TGM2, a phenotype associated with promoting tumor growth, angiogenesis, metastasis, and immunosuppression, is planned to be explored further in future experiments. This may suggest a role of TGM2 in tumor progression. Additionally, it is hypothesized that a potential therapeutic strategy for invasive HNC could be the silencing of PITX2, and this hypothesis is planned to be explored in future experiments. We plan to conduct further experiments to investigate whether TGM2 is capable of inducing monocyte polarization into M2-like macrophages, a phenotype associated with promoting tumor growth, angiogenesis, metastasis, and immunosuppression. This may suggest the role of TGM2 in tumor progression. Additionally, we hypothesize that silencing *PITX2* could be a potential therapeutic strategy for invasive HNC, and we plan to explore this hypothesis in future experiments.

5 Chapter 5

5.1. Bibliography

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