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Human Papillomavirus Genotype and DNA Methylation of TP53, PIK3CA as Integrated Biomarkers for Oropharyngeal Carcinogenesis - A Review

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Summary
Head and neck squamous cell carcinoma (HNSCC), especially oropharyngeal squamous cell carcinoma (OPSCC), has recently been found to be significantly associated with human papillomavirus (HPV) infection. The aim of this review is to understand the genetic roles of HPV, methylation of TP53, and PIK3CA in the development of OPSCC. Research publication articles from PubMed and many other were searched using goggle search engine. Over fifty journals of international reputes were obtained, out of which more than forty-five gives reputable results information on HPV and methylation of TP53, PIK3CA integration in OPSCC. PIK3CA is the most frequently mutated gene in human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC). Prognostic implications of such mutations remain unknown. We sought to elucidate the clinical significance of PIK3CA mutations in HPV-associated OPSCC in patients treated with definitive chemo radiation (CRT). Recent landmark sequencing studies have demonstrated gene expression profiles and somatic mutations such as TP53, CDKN2A, PTEN, PIK3CA, EGFR, HRAS, FBXW7 and NOTCH1 in diverse anatomical sites of HNSCC. Head and neck squamous cell carcinoma (HNSCC) encompass a heterogeneous group of malignant neoplasms arising from the non-keratinizing epithelium of the upper aero digestive tract. Therefore, HPV, methylation of TP53 and PIK3CA are found to be indispensable Biomarkers in the development of OPSCC. Recent comprehensive large-cohort DNA methylation analyses at a genome-wide scale have revealed that there is an HPV-associated HNSCC subtype with increased DNA methylation. However, there is still room for elucidation of the mechanism of HPV and DNA methylation. A more detailed understanding of the molecular basis of this subtype might lead to the development of new therapeutic strategies, such as therapeutic de-escalation in this subtype.

Keywords: HNSCC- Head and Neck Squamous Cell Carcinoma, OPSCC- Oropharyngeal squamous cell carcinoma, HPV-Human papillomavirus.

Introduction

The molecular underpinnings of human papillomavirus (HPV)-related carcinogenesis in the context of head and neck squamous cell carcinoma (HNSCC) and focuses on HPV-positive oropharyngeal squamous cell carcinoma in areas for which specific data is available (Ferris & Westra, 2023). It covers the major pathways dysregulated in HPV-positive HNSCC and the genome-wide changes associated with this disease (Lim *et al.,* 2023). Head and neck squamous cell carcinoma (HNSCC) encompasses a heterogeneous group of malignant neoplasms arising from the non-keratinizing epithelium of the upper aero digestive tract (Sivasakthivel *et al.,* 2023). Anatomic subsites of HNSCC include the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx (Abuei *et al.,* 2023). Squamous cell carcinoma arising from these subsites collectively represents the sixth most common malignancy worldwide, accounting for

932,000 new cases and 379,000 deaths in 2015 (Tofanelli, 2020).

Over the past four decades, striking epidemiological trends have been observed in HNSCC (Yang *et al.,* 2023). Although the overall incidence of HNSCC has declined slightly, the relative contribution of each anatomic subside to the overall incidence of HNSCC has shifted dramatically (Bedard *et al.,* 2023). Incidence rates of tumours arising from non-oropharyngeal subsides (oral cavity, hypopharynx and larynx) have decreased while the incidence of oropharyngeal squamous cell carcinoma has steadily grown (Mazul *et al.,* 2023). These subset-specific epidemiological trends have been attributed to shifts in societal factors that have resulted in changes due to exposure to two divergent, but complementary classes of HNSCC. risk factors: (1) tobacco and alcohol consumption and (2) human papillomavirus (HPV) infection. Successful public health campaigns in high-income countries are largely credited with achieving population-level decreases in tobacco and alcohol consumption, with concomitant declines in tobacco-associated tumours such as non-oropharyngeal HNSCC and lung cancer. Trends toward sexual practices that increase the risk of contracting sexually transmitted pathogens, like HPV, have been linked to the rise in HPV-associated cancers including oropharyngeal HNSCC (OPSCC) and anal cancers (Davison, 2023). Currently, HPV-positive OPSCC cases are surpassing the incidence of HPV-positive cervical cancer (Ndon *et al.,* 2023).

Human papillomavirus is the most common sexually transmitted infection in the United States and the primary infectious cause of HNSCC (Chaudhary *et al.,* 2023). Although the spread of high-risk HPV infection is pervasive, the virus is cleared by most people within 18 months (Doorbar, 2023). It is believed that persistent infection is necessary for the development of HPV-associated malignancies (Kusakabe *et al.,* 2023). The oropharynx exhibits the strongest relationship to HPV. Indeed, HPV-positive oropharyngeal squamous cell carcinoma (OPSCC), is recognized as a distinct neoplastic entity with a unique molecular, histopathological, epidemiological, and clinical profile (Lim *et al.,* 2023). Patients with HPV-positive OPSCC diverge from the classical profile of HNSCC patients in that they are more often younger than 60 years in age and less likely to report a history of heavy tobacco and alcohol consumption (Brennan, 2023). HPV-positive OPSCC also exhibits marked sensitivity to treatment and a significantly better prognosis than HPV-negative HNSCC (Wang *et al.,* 2023). Observations which have led to the establishment of a staging system specific to this entity (Huber *et al.,* 2023) and shifts in treatment paradigms. These distinct epidemiological and clinical features are manifestations of the unique underlying biology of HPV-positive OPSCC (Ahn *et al.,* 2023). However, much of the research into the role of HPV in HNSCC was conducted before intimate links between HPV and OPSCC were widely recognized (Chakraborty *et al.,* 2023).

Human Papillomavirus

Papillomaviridae is an ancient clade of non-enveloped viruses with a circular double-stranded DNA genome (Coronado *et al.,* 2023). Within this family, approximately 200 human papillomavirus genotypes, or 'types', have been described based on viral genome sequence (Yao *et al.,* 2023). Twelve HPV types have been defined by the WHO as high-risk and exhibit high oncogenic potential (Gong *et al.,* 2023). At least 10 of these oncogenic HPV types (HPV16, 18, 31, 33, 45, 51, 52, 56, 58, and 59), as well as 6 low-risk HPV types (11, 32, 44, 53, 57, and 81), have been isolated from HNSCC tumours. HPV16 represents the primary viral cause of HNSCC and is identified in at least 87% of HPV-positive OPSCC (Zhu *et al.,* 2023). HPV18 and HPV33, the next most prevalent types, account for most of the remainder of HPV-positive HNSCC (Andrioaie *et al.,* 2023).

All papillomaviruses possess a genome of approximately 8 kilo base encoding 8 open reading frames (ORFs) that enable viral genome replication and viral particle assembly (Chowdhary *et al.,* 2023). ORFs of the HPV genome are organized based on the timing of expression with respect to the viral life cycle: early (E1, E2, E4, E5, E6, and E7) and late (L1

and L2) genes (Jönsson, 2023). The E1 viral helicase and E2 DNA-binding protein directly mediate viral genome replication, while E4, E5, E6, and E7 are accessory proteins that coordinate viral genome amplification and virulence (Ludwig *et al.,* 2023). The late genes L1 and L2 encode viral capsid proteins necessary for the final stages of virion assembly and mediate viral entry into future host cells (Ryabchenko *et al.,* 2023). The functional diversity of this limited set of ORFs is expanded through complex patterns of viral transcript splicing and stage-specific gene expression that is linked to host cell differentiation (Gimenez *et al.,* 2023).

Infection of HPV

The host cells for HPV infection are keratinocyte progenitors located in the basal layer of stratified squamous epithelia and adhered to the epithelial basement membrane (Yin *et al.,* 2023). Experimental models suggest that infection requires viral access to the basement membrane. In the epidermis and anogenital tract, HPV gains access to basal cells through micro-abrasions that occur during sexual or other direct physical contact (Trüeb & Dutra Rezende, 2023). Figure 1 shows the difference between Human Papillomavirus Positive OPSCC and HPV negative OPSCC.

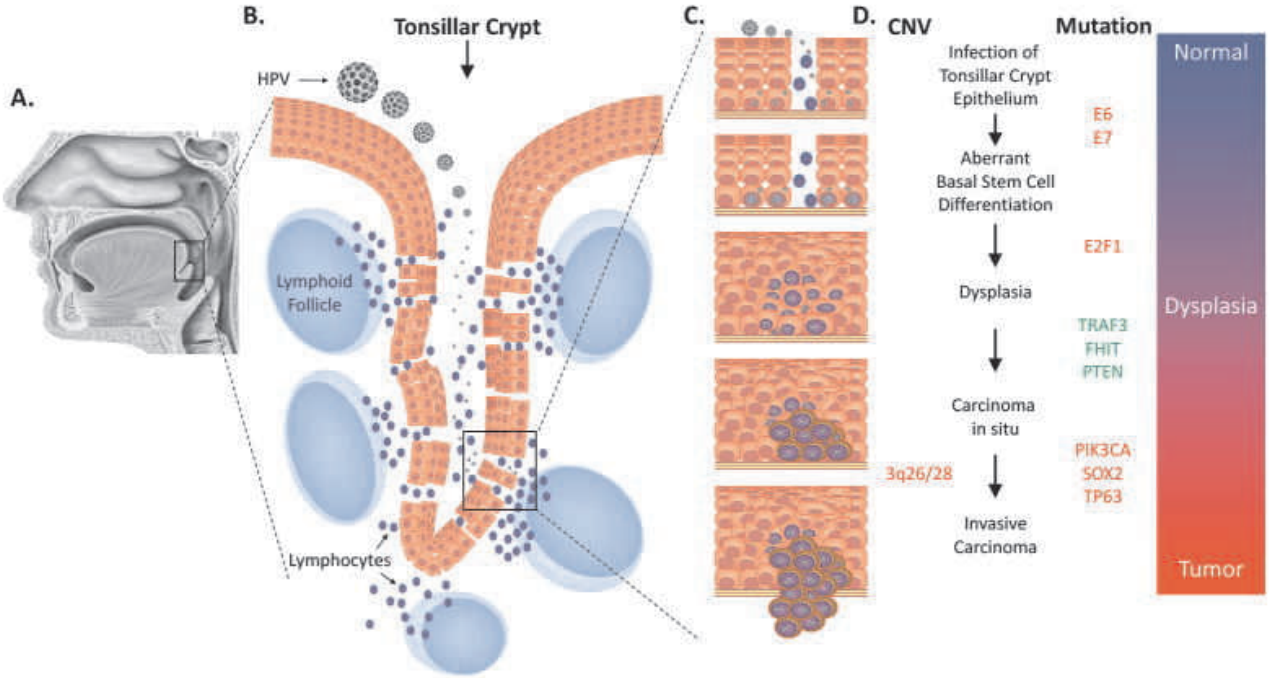

Figure 1: Difference between Human Papillomavirus Positive OPSCC and HPV Negative OPSCC

HPV status has widely been described as an independent predictor of improved overall and disease-free survival (OS and DFS) in OPSCC patients (Oka *et al.,* 2023). One of the first studies to demonstrate these improved outcomes of 74% reduction in risk of death from cancer among patients with HPV-related OPSCC was found compared with those with non HPV-related OPSCC (Lim *et al.,* 2023). A meta-analysis confirmed the data showing that patients with HPV-related OPSCC have a 28% reduce risk of death (meta HR: 0.72, 95% CI: 0.5–1) compared with non HPV-related OPSCC (Volpe *et al.,* 2023). Several phase II and III clinical trials in locally advanced HNSCC have also shown a survival benefit for patients with HPV-related cancers (Roof & Yilmaz, 2023). In addition, the RTOG 0129 study indicates that clinical factors, such as smoking history and stage, could also influence the prognosis of patients with HPV-related HNSCC (Ferris & Westra, 2023) . The combination of HPV status, tobacco smoking (pack-year) and tumour stage has shown to classify patients as having a low, intermediate, or high risk of death (Chen *et al.,* 2021): 3-year rates of OS were 93% (95% CI 88.3–97.7), 70.8% (95% CI 60.7–80.8) and 46.2% (95% CI 34.7–57.5), respectively. Additional analyses have indicated that patients with p16INK4a-positive OPSCC with T4 or

N2c-N3 disease have a high risk of disease progression, even if tobacco exposure is <10 pack years (Santaló Corcoy, 2020). It is estimated that >20% of patient with OPSCC have bad prognosis and this population need to be clearly identified (Farber *et al.,* 2023).

HPV Detection Technique

HPV testing is now mandatory for an accurate diagnosis and prognosis of patients with OPSCC (Paolini *et al.,* 2023). There are several available techniques such as p16INK4a IHC, detection of HPV-DNA by in situ hybridisation (ISH) or polymerase chain reaction (PCR), E6/E7 HPV-mRNA evaluation by ISH and reverse transcriptase– PCR (RT–PCR). Each technique yields different sensitivity and specificity profiles, but E6/E7 HPV-mRNA evaluation is considered the gold standard to confirm HPV causality since it detects oncogene transcriptional active HPVs. The p16INK4a expression is a surrogate marker of HPV involvement and it is the most widely implemented technique in the clinical setting (X. Li *et al.,* 2023). In OPSCC, p16INK4 shows high sensitivity >90% and moderate >80% specificity compared with HPV16 E6- mRNA expression (Andrioaie *et al.,* 2023). However, using p16INK4a IHC alone is questionable, because a subset of HPV-DNA and mRNAnegative HNSCCs show diffuse p16INK4a staining, indicating expression is not specific for HPV activity (Kylmä, 2023) (e.g. a mutation on Rb can also overexpress p16INK4a).

HPV Integration and HNSCC

The integration of HR-HPV DNA into the host genome has been considered an important biological step in the development of carcinogenesis in invasive cervical cancer and HNSCC (Chen *et al.,* 2023). Initial studies demonstrated that transcriptionally active integrated and/or episomal viral DNA in HNSCC cell lines was independent of viral copy number and integration sites. HPV integration can lead to host genomic instability, such as deletions, inversions, and chromosomal translocations (Lim *et al.,* 2023). A number of viral integration sites in the host genome were found in intergenic regions as well as cancer-associated genes such as TP63, ETS2, RUNX1, FOXA1 and ERBB2 (Patrizi, 2022). Moreover, viral integration into cellular genes was commonly identified in recurrent HPV16-positive OPSCC patients, and these cellular genes are related to cancer-associated signalling pathways or mechanisms (Li *et al.,* 2023). Integrated viral DNA copies could be in tandem. Viral DNA integration through the disruption of the viral E2 region leads to increased transcription of viral E6 and E7 (Thiruvengadam & Kim, 2023). Tumours with HPV DNA integration differ from HPV integration-negative tumours by different patterns of DNA methylation and gene expression profiles (Qin *et al.,* 2023). Recently, found that HPV 16 and HPV 18 integration events in cervical cancer and HNSCC were associated with local variations and genomic rearrangements based on the Pan-Cancer Analysis of Whole Genomes Consortium (Rodriguez *et al.,* 2023). HPV integration inducing genome instability is hypothesized to be a secondary genetic event in the carcinogenesis of HPV-associated HNSCC (Chakraborty *et al.,* 2023). HPV infection is associated with increased expression of the APOBEC genes APOBEC3A and APOBEC3B but exclusively with known driver genes such as TP53, CDKN2A and TERT (Xian *et al.,* 2023). These findings suggest a possible role of APOBECs in HPV-induced carcinogenesis, i.e., the activity of APOBECs as C-to-U RNA editing enzymes contributes to alterations in host genome expression, and APOBEC3A increases tumorigenesis in vivo (Warren *et al.,* 2022). In addition, as part of the immune defense system, APOBEC3A can sensitize cancer cells to cisplatin treatment by activating base excision repair and mediating the repair of cisplatin interstrand crosslinks (Petrilla *et al.,* 2023). These results suggest a role of impaired antiviral defence in driving the carcinogenesis of HPV-related HNSCC. HPV16 insertions also lead to the amplification of the PIM1 serine/threonine kinase gene in HNSCC cell lines (Wei *et al.,* 2022). The inhibition of PIM family kinases successfully decreased cell proliferation in vitro and in vivo in an HNSCC model (Liu *et al.,* 2023). Notably, viral integration can be found in both tumours that respond to treatment and recurrent tumours with more complex integration patterns in host gene (Jassim *et al.,*

2023). By analysing viral-host fusion transcripts, showed that the HPV-positive but HPV integration negative subgroup had better survival than the HPV integration-positive subgroup and HPV-unrelated HNSCC (Dong *et al.,* 2021). Moreover, HPV-positive but HPV integration-negative tumours had enhanced tumour infiltrates of immune cells and upregulated immune-related genes (Lim *et al.,* 2023). Consistently, another study indicated that HPV-related HNSCC can be subdivided into an immune cell enrichment phenotype and a phenotype with higher proliferation Thus, the enhanced immune profile in patients with HPV-positive but HPV integration-negative tumours may be attributed to better survival for these patients (Qin *et al.,* 2023). However, potential mechanisms for HPV integration-induced oncogenes (Lim *et al.,* 2023).

Genetic and Epigenetic Alterations

PIK3CA is the most frequently mutated gene in human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC). Prognostic implications of such mutations remain unknown (Lim *et al.,* 2023). We sought to elucidate the clinical significance of PIK3CA mutations in HPV-associated OPSCC in patients treated with definitive chemo radiation (CRT) (Bhatia & Burtness, 2023).

Recent landmark sequencing studies have demonstrated gene expression profiles and somatic mutations such as TP53, CDKN2A, PTEN, PIK3CA, EGFR, HRAS, FBXW7 and NOTCH1 in diverse anatomical sites of HNSCC (Park *et al.,* 2023). The Cancer Genome Atlas Network 2015). Importantly, diversity in the number of mutations and gene profiles was seen in patients with a history of tobacco use and between HPV-related and HPV-unrelated tumours (Eldridge *et al.,* 2023). The mutation rate of HPV-related tumors was almost half that of HPV-unrelated tumours (Saba *et al.,* 2023). Thus, two etiologies may result in the alteration of oncogenes and tumour suppressor genes that have tumorigenic effects involved in multistep biological processes (Latha *et al.,* 2023). HPV-related HNSCC harbours mutations in the oncogene PIK3CA encoding PI3K catalytic p110 subunit alpha, a loss of TRAF3 and the amplification of E2F1 (The Cancer Genome Atlas Network 2015) (van der Kamp et al., 2022). A recent comprehensive analysis on oral squamous cell carcinoma (OSCC) identified secondary genetic alterations, including PIK3CA, ZNF750 and EP300 as candidate cancer driver genes (Patten, 2023). APOBEC cytosine deaminase editing was associated with genomic mutation burden in HPV-related OSCC (Kleszcz, 2023). APOBEC-mediated cytosine deamination leading to PIK3CA mutations is involved in the tumorigenesis of HPV-driven tumours (Schrank *et al.,* 2023). As we discussed above, virus-host interactions, as seen by the interaction between HPV integration with APOBEC and others, may shape genomic alterations and facilitate tumorigenesis (Qualliotine *et al.,* 2023). Notably, PIK3CA mutations (2.6% to 19%) lead to the activation of the PI3K-AKT-mTOR1 signalling pathway necessary for the viral life cycle (Dong *et al.,* 2021). Moreover, both HPV-related and HPV-unrelated HNSCCs harbour PIK3CA mutations, and higher expression of PIK3CA in primary tumours is associated with tumour recurrence and chemo- and radio resistance (Kostopoulou *et al.,* 2022). Thus, inhibitors targeting the PI3K-AKT-mTOR pathway have been developed for cancer therapies (Jin *et al.,* 2023). However, the clinical response rates remain modest in these studies and warrant further investigation (Goodman *et al.,* 2023). Studies in patient-derived xenograft (PDX) models demonstrate that EGFR, KT1and CSMD1 copy number aberrations are related to the effect of PI3-kinase inhibition regardless of the status of PIK3CA mutation.

HPV and DNA Methylation

HPV is a small, circular, double-stranded virus that targets the basal layer of the epithelial cells in the head and neck region, HPV targets the oropharynx, especially the tonsils, and the base of the tongue (Ferris & Westra, 2023). There are more than 200 HPV types, which can be divided into high-risk and low-risk types based on their potential to induce cancer (Hinton *et al.,* 2023). Persistent high-risk HPV infections can progress to invasive cancer within 10 years, although the majority of these infections are cleared within 1 or 2 years (Reuschenbach *et al.,* 2023). In HNSCC, >90% of HPV-associated cases involve

HPV16, which is classified as a high-risk HPV (Lim *et al.,* 2023).

HPV16 is approximately 7900 bp in size. It exists in the nucleus of infected cells as a circular episome (Rossi *et al.,* 2023).. The proteins produced early during the infection are known as early proteins: E1, E2, E4, E5, E6, and E7 (B. Chen *et al.,* 2023). The proteins produced late during the infection are known as late proteins: L1 and L2. There is a long control region that codes no protein between the L1 stop codon and E6 AUG, and it contains the early viral promoter p97 (Jönsson, 2023). Another promoter p670 that is related to the late viral promoter exists in the E7 coding region (Jönsson, 2023). Based on these two promoters, HPV16 oncoproteins are generated. Among these HPV proteins, E2 inhibits the p97 promoter and results in inhibition of E6 and E7 (Evande *et al.,* 2023). Therefore, inhibition of E2, such as E2 disruption caused by HPV genome integration to human genome or DNA methylation of E2 binding site, causes the upregulation of E6 and E7 (Lim *et al.,* 2023). As mentioned herein, although HPV E6 and E7 are oncoproteins and inactivate p53 and RB respectively, these proteins also regulate the DNA methylation of the host genome (Kassab *et al.,* 2023).

For example, wild-type p53 negatively regulates DNA methyltransferase 1 (DNMT1) expression by forming a complex with specificity protein 1 (Sp.1) and chromatin modifiers on the DNMT1 promoter in lung cancer (Cirino *et al.,* 2023). In HPV-associated HNSCC, degradation of p53 is generally caused by the HPV E6 oncoprotein and DNMT1 is consequently upregulated (Chakraborty *et al.,* 2023). Additionally, the HPV E7 oncoprotein has been reported to form a complex with DNMT1 and DNMT1 is upregulated in HPV-associated OPSCC. and cervical cancer (Chakraborty *et al.,* 2023). Apart from E6 and E7, c-Myc (MYC) is also reported to recruit DNA methyltransferase 3 alpha (DNMT3A). and the MYC-associated genetic network is reported to be activated in HPV-associated HNSCC (Nakagawa *et al.,* 2021). The molecular mechanism of induction of DNA methylation in human papillomavirus (HPV)--associated head and neck squamous cell carcinoma (HNSCC) (Nakagawa *et al.,* 2021). Wild type p53 negatively regulates DNA methyltransferase 1 (DNMT1). HPV E6 oncoprotein cause degradation of p53 and it results in the upregulation of DNMT1 (left). HPV E7 oncoprotein forms a complex with DNMT1 and results in DNMT1 upregulation (middle) (Trejo Cerro, 2023). MYC is upregulated in HPV-associated HNSCC and recruit DNMT3B (right) (Chakraborty *et al.,* 2023).

Epigenetic Dysregulation in OPSCC

These biological differences between HPV-associated HNSCC and HPV-negative HNSCC are partly explained by the differences in mutation patterns (Lim *et al.,* 2023). Frequent mutations in several genes, such as TP53, CDKN2A, and PIK3CA, as well as in members of the NOTCH pathway, have been reported in HPV-negative HNSCC via genomic and transcriptomic approaches (Huang *et al.,* 2023). In HPV-associated OPSCC, p53 and RB are mainly inactivated by HPV E6 and E7 oncoproteins, respectively, therefore, somatic mutations in TP53 and CDKN2A are very rare (Kassab *et al.,* 2023). However, somatic mutations in PIK3CA, E2F1, and TRAF3 have been reported, and chromatin regulators, such as lysine methyltransferase2C (KMT2C), KMT2D, CREB-binding protein (CREBBP), and E1A-associated protein p300 (EP300), are also mutated in HPV-associated OPSCC (Nakagawa *et al.,* 2021). In addition, apolipoprotein B mRNA-editing catalytic polypeptide-like (APOBEC)-mediated mutagenesis, such as PIK3CA mutation and HPV genome mutation, has been reported in HPV-associated HNSCC (Lim *et al.,* 2023). The APOBEC3 signature is also displayed in HPV-associated HNSCC. Overall, HPV-associated OPSCC shows relatively fewer genetic alterations in cancer drivers than HPV-negative tumours at the exome level (Lim *et al.,* 2023).

Apart from mutations, epigenetic dysregulation is also a common pathological feature in human malignancy (Bhattacharya *et al.,* 2023). HNSCC samples have been characterized according to their patterns of DNA methylation, one of the critical epigenetic mechanisms that silence tumour suppressor genes in cancers (Qin *et al.,* 2023). In particular, it has been reported that viral infections can induce aberrant DNA methylation

during carcinogenesis and HPV-associated HNSCC tends to harbour a higher amount of aberrantly methylated DNA than HPV-negative HNSCC (Nakagawa *et al.,* 2021).

DNA methylation targeted therapy

Epigenetic-targeted therapy, especially targeting DNMTs, has the potential for tackling HPV-associated HNSCC (Nakagawa *et al.,* 2021). 5-azacytidine and 5-aza-2'-deoxycytidine are the most used, U.S. Food and Drug Administration-approved drugs (2004 and 2006, respectively) (Scholpa, 2023). These drugs are cytidine analogs that are incorporated into DNA, leading to covalent adduct formation and working as DNMTs inhibitors (Laranjeira *et al.,* 2023). They are used only for the treatment of some myelodysplastic syndrome and chronic myelomonocytic leukaemia cases, and their efficacy for solid cancers is under consideration, with clinical trials still ongoing (Molica *et al.,* 2023). One clinical trial is currently using 5-azacytidine for the treatment of HPV-associated and HPV-negative HNSCC. HPV-associated HNSCC tends to have higher methylation levels compare with HPV-negative HNSCC; thus, these drugs might be good candidates for treating HPV-associated HNSCC (Nakagawa *et al.,* 2021).

Conclusion

HPV induces DNA methylation in a complex manner during carcinogenesis. In this review, we provided an overview of DNA methylation, the relationship between DNA methylation and HPV-associated HNSCC, and how these mechanisms are related to the carcinogenesis of HPV-associated HNSCC, methylation of PIK3CA in HPV-associated OPSCC and Non-HPV-associated OPSCC, and methylation of TP53 in HPV-associated OPSCC and Non-HPV related OPSCC. Recent comprehensive large-cohort DNA methylation analyses at a genome-wide scale have revealed that there is an HPV-associated HNSCC subtype with increased DNA methylation. However, there is still room for elucidation of the mechanism of HPV and DNA methylation. A more detailed understanding of the molecular basis of this subtype might lead to the development of new therapeutic strategies, such as therapeutic de-escalation in this subtype.

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