

Epithelial-derived head and neck squamous tumourigenesis (Review)

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Abstract. Head and neck squamous cell carcinomas (HNSCCs), a heterogeneous group of cancers that arise from the mucosal epithelia cells in the head and neck areas, present great challenges in diagnosis, treatment and prognosis due to their complex aetiology and various clinical manifestations. Several factors, including smoking, alcohol consumption, oncogenic genes, growth factors, Epstein-Barr virus and human papillomavirus infections can contribute to HNSCC development. The unpredictable tumour microenvironment adds to the complexity of managing HNSCC. Despite significant advances in therapies, the prediction of outcome after treatment for patients with HNSCC remains poor, and the 5-year overall survival rate is low due to late diagnosis. Early detection greatly increases the chances of successful treatment. The present review aimed to bring together the latest findings related to the molecular mechanisms of HNSCC carcinogenesis and progression. Comprehensive genomic, transcriptomic, metabolomic, microbiome and proteomic analyses allow researchers to identify important biological markers such as genetic alterations, gene expression signatures and protein

markers that drive HNSCC tumours. These biomarkers associated with the stages of initiation, progression and metastasis of cancer are useful in the management of patients with cancer in order to improve their life expectancy and quality of life.

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Abbreviations: CSC, cancer stem cells; EBV, Epstein-Barr virus; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; lncRNA, long non-coding RNA; LSCC, laryngeal squamous cell carcinoma; miR or miRNA, microRNA; NPC, nasopharyngeal carcinoma; OSCC, oral squamous cell carcinoma; TNM, tumour, lymph node and metastasis; TSCC, tongue squamous cell carcinoma

Key words: HNSCC, biomarkers, HPV, miRNAs, oncogenes, lncRNAs, clinical trials

1. Introduction

Cancer is a complex disease that can be described as an uncontrolled and abnormal proliferation of cells. Most of the time these abnormal cells can spread and invade surrounding tissues, even migrating and reaching some other body parts by using bloodstream or lymphatic system (1).

Head and neck squamous cell carcinoma (HNSCC) is a type of complex malignant tumour originating from the mucosal epithelium in the mouth, pharynx, nose, throat and laryngeal region (2). HNSCC has unique anatomical structure and unpredicted tumour microenvironment (3). HNSCC originates from the flat squamous cells, a thin layer of tissue on the surface of head and neck structures (4). If this cancer is limited to the squamous cell layer is called carcinoma *in situ* and if HNSCC cancer cells proliferate beyond this cell layer and invade deeper tissues, it is classified as invasive (5).

Adoption and use of the tumour, lymph node and metastasis (TNM) cancer staging system (Fig. 1) allows clinicians and researchers to categorise complex tumours of the head and neck region in a defined way that can help in treatment plans and disease management (6).

Risk factors that can be linked with the development of HNSCC are excessive smoking of tobacco products (7) and their derivatives (8), regular and heavy consumption of alcohol (9), especially combined with tobacco use (10-12), human papillomavirus (HPV) infection commonly transmitted through oral sexual activity (13), and prolonged sun exposure. Other risk factors include gastroesophageal reflux disease and laryngopharyngeal reflux disease, as the back-flow of stomach acid into the upper airway, may contribute to HNSCC development. Finally, history of previous HNSCC can increase risk of developing another cancer disease in the same or a different region (2).

HNSCC treatment is currently based on radiation therapy (14), removal of cancer tumour (surgery), chemotherapy, targeted therapies and immunotherapy (15). Immunotherapy options for HNSCC include immune checkpoint inhibitors (ICIs), programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), cytotoxic T lymphocyte antigen 4 (CTLA-4), as well as natural killer cell receptors, such as those with inhibitory motives based on intracytoplasmic tyrosine. Additionally, there are oncolytic viruses capable of selectively infecting tumour cells with specific receptors, leading to their lysis without harming normal host cells (16). The typical approach to combination therapy involves using chemotherapy alongside radiotherapy. Additionally, there is a concurrent utilisation of chemotherapeutics and new compounds tailored to the molecular targets of specific cancer types in combination with radiation. Combination molecular targeted therapy often involves the simultaneous inhibition of EGF receptors with other targets and is a primary focus in combating cancer cell resistance to single epidermal growth factor receptor inhibition (17). All combinatorial therapies can improve response rates and survival outcomes in patients with HNSCC. There is the potential to repurpose anti-diabetic and anti-hypertensive drugs in the treatment of HNSCC as a means of reducing side effects and long-term usage of anticancer drugs or combinatorial therapies for patients. Pioglitazone, metformin and sodium-glucose co-transporter-2 inhibitors are among the anti-diabetic drugs that have been found to have positive effects in treating HNSCC. Additionally, atenolol, Hydralazine, Propranolol, candesartan and captopril are examples of anti-hypertensive agents that have been tested in clinical trials for HNSCC. The repurposed drugs not only enhance safety, tolerability and effectiveness when used in low doses, but are also suitable for long-term use, providing significant advantages over other drug classes in HNSCC treatment (18,19).

Despite the application of advanced treatments for treating HNSCCs (20), the overall 5-year survival rate has not shown significant improvement, and the number of individuals succumbing to HNSCC remains high (15). The problem lies in the fact that HNSCCs are typically detected at advanced stages, contributing considerably to high mortality rates associated with this disease (21). Methods to detect the presence of HNSCC at early stages and use of personalised therapy

can help in improving HNSCC treatment outcomes and this can be possible by the use of new biomarkers for this type of cancer (22). Biomarkers can be biological molecules found in body fluids including blood, cerebrospinal fluid, saliva, urine or tissues, which can indicate normal or abnormal conditions, disease state or assess the body's response to a certain treatment (23).

Current diagnosis and management of HNSCC is challenging as currently there is no single modern tool available that can accurately detect and predict the progression of this type of cancer (24). Identifying mechanisms of tumorigenesis and potential biomarkers will aid in early diagnosis, assist in selecting appropriate therapies for patients with HNSCC, and predict clinical outcomes.

The present review addressed significant gaps in the existing literature on the molecular mechanisms, evolution and novel biomarkers of HNSCC. Despite numerous studies, there is a noticeable lack of comprehensive reviews that integrate molecular approaches to the evolution of HNSCC and utilise multi-omics platforms for an in-depth analysis. Current literature tends to focus on specific aspects, such as genetic mutations or individual biomarkers. The present review aimed to take a broader perspective by integrating cancer evolution, molecular mechanisms and omics approaches to provide a comprehensive understanding of the mechanisms underlying the initiation, development and progression of HNSCC.

2. HNSCC tumorigenesis mechanisms; premalignant squamous evolution

Cancer often arises from a cell line that undergoes multiple mutations in cancer-related genes, such as tumour suppressor genes, oncogenes, or multiple epigenetic changes (25). Premalignant describes a condition that can turn into cancer. By definition, pre-malignancy is the presence of abnormal tissue that is considered a precursor to cancer and is linked with high risk of develop to cancer (26). HNSCC lesions containing driver mutations are often associated with clonal expansion and are observed more frequently in precancerous and malignant lesions than expected from the normal background mutation frequencies. The exact role of each driver mutation in the early initiation and development of cancer remains under investigation (27).

Early lesions in development in HNSCC are associated with oncogenic changes that generally lead to tumorigenesis, these lesions may be due to two main factors; firstly, transformation by a key initiating agent such as high-risk HPV (hrHPVs). HPVs enter through micro-abrasions or other breaches in the epithelial barrier, exposing the basal layer and primarily infect the basal epithelial cells by attaching to heparan sulphate proteoglycans, that induce conformational changes in the viral capsid proteins (L1 and L2), exposing additional receptor binding sites (such as $\alpha 6$ integrin), which aids in further binding and internalisation. After HPV enters the host cell through endocytosis, the L2 protein assists in the transport of the viral DNA to the nucleus, where the viral genome can be maintained episomally (as an independent circular DNA) or can be integrated into the genome (markedly more common in cancer). The viral genome replicates using the host's DNA replication machinery, with the assistance of

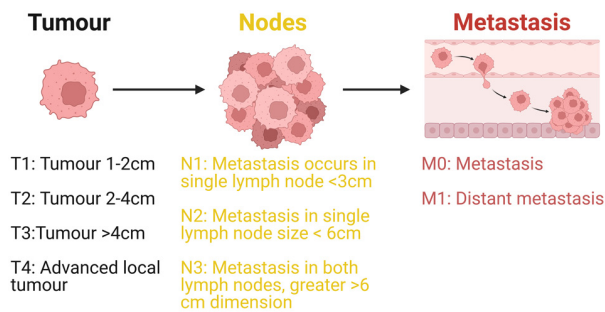


Figure 1. TNM classification system, a widely used staging for HNSCC; the system denotes the size and extent of the tumour. T signifies the size and width of the primary tumour, with higher numbers indicating more extensive or deeper growth within the tissue. T1-T4 indicate the size of the primary tumour, with larger numbers signifying greater tumour size area. N indicates the spread of cancer to nearby lymph nodes. N0 denotes the absence of cancer in nearby lymph nodes. N1-N3 indicate the presence of cancer and its size in lymph nodes with cancer. M designation indicates the presence of cancer metastasis, signifying the spread of cancer from the primary tumour to other areas of the body. M0 indicates no spread of cancer, while M1 indicates that the cancer has spread. Also, by using TNM stage system HNSCC can be categorised as stage I, II, III, or IV. The frequency of different types of head and neck cancer has variability depending on its association with HPV. Stage I or II indicates that the cancer is small and has not metastasised from the original site (head and neck cancer). Stage III or IV signifies that the cancer is larger and has spread to other parts of the body.

viral proteins E1, E2, E6 and E7 disrupting the normal cell cycle regulation by interacting with tumour suppressor proteins p53, CDKN2A and pRb, preventing apoptosis and promoting cell proliferation and transformation (28). Secondly, transformation of conventional HNSCC (HPV-negative). In non-HPV HNSCC, driver mutations can be caused by smoking, alcohol consumption and other environmental carcinogens, which are important risk factors. Exposure to these carcinogens causes DNA damage, genetic mutations and epigenetic changes that lead to early lesions such as leukoplakia and erythroplakia. Signs associated with these early lesions may include genetic changes in tumour protein P53 (TP53), CDKN2A, DNA methylation and dysplasia-expressing cell markers (29).

Normal tissue architecture and the pre-malignant micro-environment have a complex relationship that plays out a key role in natural selection and cancer evolution. Surrounding changes, such as fibroblast activation and remodelling of the immune microenvironment, can alter the strength of epigenetic changes caused by damage, by driver and passenger mutations. Environmental pressure on tumour cell transformation can be facilitated by the presence of growth factors or the introduction of normal cells that inhibit and force them to prevent invasion by creating an extracellular matrix. This microenvironment also regulates self-renewal in the epithelium (30).

Therefore, the evolution of HNSCC follows histologically ordered stages starting with pre-malignant epithelial cell hyperplasia, then dysplasia ranging from mild, moderate and severe, followed by carcinoma *in situ*, and finally to metastasis. In numerous cases, cancerous evolution from normal cells or the progenitor cell candidates, results in cancer stem cells (CSCs) with ability of self-renewing and proliferative properties after cancerous transformation (31).

In the following section, the potential biomarkers for HNSCC were discussed, which play roles in therapeutic

approach, diagnosis and predicting of the progression of premalignant stages of conventional squamous cancer cells, such as progression of cancers from pre-malignant to metastasis. Biomarkers of HNSCC are classified into the following categories based on the aetiology of the disease; HPV-associated oro-pharyngeal carcinoma biomarkers and conventional squamous carcinoma biomarkers.

3. HPV-associated oro-pharyngeal carcinoma biomarkers

These biomarkers are specifically associated with oropharyngeal carcinomas caused by HPV infection, particularly types 16 and 18. HPV is a non-enveloped double-stranded DNA virus with a circular genome of ~8,000 base pairs (32). It is a common sexually transmitted infection that can cause various diseases, including HNSCC. In particular, HPV primarily infects and replicates in epithelial cells, predominantly in the oropharynx (33). HPV can persist in the basal cells in a latent state without any symptoms for numerous years. The viral genome is maintained episomally and can reactivate under certain conditions. In numerous cases, the host immune system successfully clears the infection, often within 1-2 years. However, persistent infection with hrHPV types (such as HPV-16 and HPV-18) can lead to the integration of the viral DNA into the host genome, disruption of normal cellular functions and the development of malignancies. This makes HPV a prominent independent marker for HNSCC development (34). HPV 16 and 18 strains have recently become a powerful and widely used biomarker with excellent clinical outcomes as they are highly present in most HPV-related HNSCC cases (35). The oncoproteins from this virus play a significant role in cellular transformation and disruption of human cell cycle control (36). These viral proteins induce the degradation of key regulators such as TP53 and RB Transcriptional Corepressor 1 (RB1) proteins, resulting in the cell cycle's dysregulation and suppression of normal apoptosis (37). Evidence of viral DNA integration into cancer cells is crucial for the assessment of HPV status in oropharyngeal carcinomas (38).

In most HNSCCs from hrHPV-16 strains which can be used as biomarkers, NFE2L2 expression is reduced which in turn increases sensitivity to chemotherapy and radiotherapy (39).

Diagnostic implications of p16 expression and HPV testing in patients with HNSCC. P16, a cell cycle regulatory protein of CDKN2A gene, is always overexpressed in HPV-positive HNSCC. Currently, p16 is a commonly used immunohistochemical biomarker test for patients with HNSCC. In HPV-positive oral squamous cell carcinoma (OSCC), p16 overexpression results from the inactivation of the tumour suppressor protein retinoblastoma 1 (RB1) by the hrHPV E7 oncoprotein (40). Occasionally, p16 overexpression can also result from HPV-negative cases (41).

Analysis of overexpression of p16 protein through immunohistochemistry is widely used in determination of HPV infection. In some cases, most of patients with overexpression of p16 (P16⁺) are HPV-negative and also some of patients without p16 (P16⁻) are HPV-positive. Therefore, for improved practice, both p16 IHC and HPV testing is often used to ensure accurate classification of HPV-associated HNSCC (33).

Detection of P16 can be reliably used as high validity prognostic biomarker for HNSCC which is caused by HPV (42). In a research study where the prognostic relevance of the best biomarkers was compared to assess the status of HPV infection in HNSCC, it was found that CDKN2A, cyclin D1 (CCND1) and RB1 protein levels were significantly higher in HPV⁺ cases compared with negative controls as confirmed by PCR (42). HPV detection has potential in diagnosis and prognosis of cancer cells appearing in the head and neck area (39,43-45). Therefore, detecting HPV⁺ (45) and transcriptional products of E6 and E7, can serve as valuable diagnostic biomarkers for accurately identifying HPV-related HNSCC and will allow appropriate management and treatment of patients (33,39,42,44,46).

4. Conventional squamous carcinoma biomarkers

Conventional squamous carcinoma biomarkers that are associated with HNSCC are discussed in the following section.

TP53. Mutation on the TP53 tumour suppressor gene is a key factor in cancer development. Normally, when human cells are exposed to internal or external stresses (47), TP53 triggers either cell cycle arrest, cellular senescence or DNA repair and if DNA repair mechanism fails, cells undergo apoptosis (48). Determination of the role of the mutant TP53 in oral cancer using RNA sequencing (RNA-seq) showed that activation of endogenous TP53 through gain-of-function mutations in carcinogen-induced epithelial cells promotes resistance to anti-CD274 prevention immunity associated with increased IL17 signalling and a lack of exhausted CD8 T cells in the tumour microenvironment. These results support the use of TP53 as a biomarker to predict CD274 inhibitions in order to prevent HNSCC development (49).

In most cases, HNSCC with mutation in TP53 is strongly associated with invasive carcinomas and poor survival (50). Several studies have assessed these abnormalities of TP53 and it was found that TP53 has prognostic significance, indicating its potential as a marker for predicting outcomes in this type of cancer (51).

In an analysis of mutations in TP53 using next generation sequencing technology on samples of patients diagnosed with HNSCC of the pharynx, oral cavity and larynx, it was discovered that TP53 was the most commonly altered gene, indicating that TP53 has prognostic value for this type of cancer (52). In a comprehensive analysis of patients with HNSCC at different stages of the disease, at least one significant mutation of TP53 was identified in all tumour samples, consistent with previous studies that identified a somatic mutation of TP53 genes in smoking-related HNSCC (53).

Mutations in the TP53 gene disrupt its function, allowing cells with damaged DNA to survive and potentially become cancerous (47). This abnormality was frequently observed in HNSCC (54), and is associated with reduced survival (55). Hence, presence of mutations in TP53 can be used as a prognostic biomarker to provide valuable information for HNSCC (10,50,53,56).

RAS family of genes. The RAS family of genes comprises three highly conserved genes: KRAS proto-oncogene (GTPase),

NRAS gene NRAS proto-oncogene (GTPase) and HRAS proto-oncogene (GTPase), that were also known as Kirsten rat sarcoma viral oncogene homolog (KRAS), neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) and Harvey rat sarcoma viral oncogene homolog (HRAS), respectively (57). These genes encode proteins that act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. RAS proteins can bind to and activate downstream effector proteins in their active state, initiating a signalling cascade that promotes cell proliferation and division.

Abnormal activation of RAS genes typically occurs from mutations and epigenetic alteration preventing RAS proteins from functioning properly and cause uncontrolled cell proliferation, a hallmark of HNSCC (57,58). In HNSCC it was found that in samples with the KRAS variant, there may be an altered immune status as compared with those who do not have this mutation (59).

DNA from 180 HNSCC samples was analysed to determine whether or not oncogenes HRAS are associated with drug resistance; it was identified that when HRAS proto-oncogene, GTPase signalling is mutated, it triggers an increase of ERK signalling, which in turn renders cancer cells resistant to cetuximab treatment (60).

Utilizing large-scale genomic sequencing databases, it was revealed that HNSCC from various anatomical subsites may have genetic variations that disrupt RAS signalling in malignant cells (61). From The Cancer Genome Atlas database it was found that HRAS gene alterations-mutations are more frequently found in primary untreated HNSCC tumours compared with KRAS and NRAS genes' mutations. Additionally, it was demonstrated that most HRAS alterations can lead to aggressive form of cancer (61).

Detection of HRAS mutations can be used as a valuable biomarker in HNSCC and help in providing prognostic information (58). Most of the tumours with HRAS mutations have a high chance of recurrence (61). Therefore, detection of RAS mutations can be used in clinical settings and serves as a diagnostic and prognostic biomarker and will help to guide treatment choices which can improve the treatment outcomes in patients with HNSCC (62-64).

Phosphatase and tensin homolog (PTEN). PTEN is a tumour suppressor gene that plays a significant role in molecular biology by regulating cellular survival and proliferation (65). PTEN acts as a phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3), a signalling molecule that can activate the PI3K/AKT/mTOR signalling. By dephosphorylating PIP3 and PDK1, PTEN antagonises the PI3K/AKT signalling pathway and prevents its oncogenic effects (66).

The deactivation of PTEN in cancer can lead to activation of the PI3K/AKT pathway, which increases cell proliferation, inhibits apoptosis, activates cell cycle progression, survival, invasion and metastasis (67). In addition, PTEN can also lead to activation of other signalling pathways, such as the mTOR and NF- κ B pathways, which further contribute to tumorigenesis, oxidative stress and genomic instability in tumorigenesis development (68,69).

In experiments where human cell lines were used to model different stages of OSCC progression, PTEN levels in cancer

were found to be lower compared with normal cells. The decrease of PTEN expression level was consistent with the predicted downward trend of PTEN expression in successive stages of OSCC progression (68).

In silico analysis on the involvement of the PTEN in HNSCC found high target score for PTEN. PTEN was involved in proliferation and chemotherapy resistance, which were increased due to PTEN loss of function. These *in silico* studies revealed that PTEN could help as prognostic and predictive biomarker in HNSCC (70).

Loss of function mutation of PTEN expression is invariably linked with PI3K activation sustaining an important role in HNSCC tumorigenesis. This provides a framework for PTEN as a biomarker for targeted therapy (71). Therefore, alterations of PTEN can be used as prognostic biomarkers in cancer (68,69,72,73).

MKI67. MKI67 (Ki-67 enhancer marker) is a protein-coding gene located on human chromosome 10 (10q26.2) (74). MKI67 keeps specific mitotic chromosomes separated in the cytoplasm after nuclear envelope breakdown. MKI67 prevents chromosomes from collapsing into clumps of chromatin (75,76). MKI-67 expression normally is low in the G1 and early S phases but gradually increases to a maximum during mitosis (77). A study on the development of leukoplakia found that an increase in the MKI67 index can indicate a poor prognosis in leukoplakia (78).

MKI67 analysis in predicting HNSCC metastasis showed that MKI67 was an independent predictor of metastatic, recurrent disease and poor patient outcomes. This indicates that MKI67 has prognostic value regardless of the size of the primary tumour (79). MKI67 gene expression by quantitative PCR (qPCR) was performed in HNSCC samples in comparison with non-cancer control samples. The results of the experiment revealed that MKI67 was highly upregulated in HNSCC samples compared with control tissues. MKI67 upregulation was observed in all advanced tumour stages and lymph node status compared with early-stage HNSCC. Therefore, MKI67 expression can be used as a prognostic biomarker (80).

Higher MKI-67 index in premalignant lesions might indicate a higher risk of progression. A study evaluating the prediction value of MKI67 expression in patients with nasopharyngeal carcinoma (NPC) using immunohistochemistry found that MKI67 level was higher in tumours than in normal tissue. Thus, MKI67 can be employed as a predictive marker of HNSCC in a clinical environment, as its overexpression has been linked to poor overall survival in patients with NPC (81).

CCND1. CCND1 is a gene located on chromosome 11q13 and codes for protein that plays a crucial role in cell cycle progression (82). This protein belongs to the cyclin family of proteins that can bind and activate cyclin-dependent kinases (CDKs). CCND1 specifically binds to CDK4 and CDK6, forming active CDK4/6-CCND1 complexes that phosphorylate the retinoblastoma protein (pRb) a tumour suppressor that prevents cell cycle progression from the G1 to S phase (83). Phosphorylation of pRb inactivates its tumour suppressor function, allowing cells to enter S phase and replicate their DNA (84).

CCND1 overexpression is mostly observed in HNSCC and affects cell migration, angiogenesis and uncontrolled cell proliferation (85). CCND1 promotes tumorigenesis, which is associated with more aggressive form of tumour phenotype, poor prognosis and metastasis (86). OSCC with CCND1 overexpression was correlated with lymph node metastasis; thus, CCND1 is a prognostic biomarker for this type of cancer (82).

A cohort study analysed CCND1 in HNSCC as a prognostic biomarker and found that the CCND1 molecule is involved in cell cycle control. CCND1 overexpression was linked with several clinical and pathological stages of HNSCC tumour development and can be used as predictive biomarker (87).

CCND1 protein is often overexpressed in HNSCC; its elevated levels in premalignant lesions may indicate higher risk of progression to cancer. This was observed more frequently in numerous cases of HNSCC (85). By detecting the level of CCND1 protein in tissues, valuable prognostic information on HNSCC can be extracted (83,84,86,87).

CD274. CD274 cell surface protein, also commonly referred as PD-L1, is essential for controlling the immune response. It interacts with the immune system's PDCD1, also known as PD-1 receptor, causing immune system suppression and enabling cancer cells to avoid immune identification and elimination (88).

One way in which cancer cells resist the antitumour treatment is by overexpressing CD274 immune system reaction. In HNSCC, transmembrane protein CD274 is expressed in both haematopoietic and non-haematopoietic cancer cells. The intratumorally lymphocyte response is suppressed by the interaction of CD274 molecules with the PDCD1 receptor on T cells (89). Analysis of HNSCC tumour microenvironment found that tumour cells can utilise the PDCD1/CD274 axis to inhibit immune system. Furthermore, higher CD274 expression is linked with cancer metastasis (23).

An immunological analysis of CD274 role in a prospective cohort of oral precancer samples from 188 patients has shown that patients with oral precancer may exhibit characteristics of immune resistance. Hence, CD274 is a predictive biomarker for patients with oral precancer (90).

Analysis of CD274 and PDL2 expression in two well-controlled and independent cohorts of the primary head and neck tumour, reported that CD274 was observed as highly expressed mainly at the membrane surface and in the cytoplasm of the HNSCC tumour cells. CD274 proved to be useful as a valuable predictive biomarker for the overall survival rate (91).

The immunosuppressive impact of the PDCD1 receptor and its ligand CD274 was investigated using qPCR and western blot analysis of tissue from patients with HNSCC versus healthy individuals. The results revealed considerably greater mRNA expression of PDCD1 and CD274 in the tissues of patients with HNSCC compared with the healthy group. Increased expression of PDCD1 and CD274 could be a prognostic in HNSCC (92). Patients with HNSCC who exhibit high levels of CD274 expression typically have a poor prognosis and more aggressive form of disease (23,93).

CD274 is a potential theragnostic biomarker because it can predict therapy responsiveness in HNSCC disease such as the response to ICIs that target the CD274 pathway (50,91).

Notch Receptor 1 (NOTCH1). NOTCH1 is membrane receptor protein that belongs to the NOTCH receptor family (NOTCH 1-4) (94), is one of the important evolutionarily conserved signalling in the process of development of an embryo from zygote and tissue homeostasis. The NOTCH receptor signalling pathway involves complex steps that end up in regulation of important cellular functions, including proliferation, differentiation (95), cell-cell interactions, apoptosis and stem cell maintenance (96).

NOTCH1 regulates gene expression when its ligands (DLL1, DLL3, DLL4, JAG1 and JAG2) bind its receptor. This triggers arrangement of intracellular flagging pathways that lead to cleavage of the NOTCH1 intracellular space (NICD) (94). The NICD enter nucleus, where it works on transcriptional co-activator to regulate the expression of numerous NOTCH1-specific target genes, such as *HES1* and *HEY1* (97).

NOTCH1 analysis by exome sequencing from HNSCC samples found that the most frequently mutated gene was *NOTCH1*, which is mostly affected by copy number changes. In the same results, loss of heterozygosity at the *NOTCH1* gene locus was detected in two of three tumours with mutations that were analysed for copy number variations. These data from the sequencing experiment support the idea that NOTCH1 functions as a tumour suppressor gene in HNSCC. Moreover, those results emphasised the importance of evaluating the role of the NOTCH1 in cancer as a prognostic biomarker (98). NOTCH1 mutations are common and present in the majority of HNSCC; these changes may affect the normal function of NOTCH1, causing a loss of its tumour suppressor activities (95,97).

Single-cell RNA-seq was employed for analysis of specimens from patients with thyroid cancer who were undergoing a lobectomy of the thyroid compared with the non-malignant thyroid specimens collected from patients undergoing normal surgery (control). *NOTCH1* was the key target gene of *SATB2*, and the activation of the *SATB2*/NOTCH1 pathway was one of the reasons for the highly aggressive carcinogenicity of this subgroup of HNSCC (99). Alterations in NOTCH1 signalling or mutational status may provide prognostic information and potentially guide treatment decisions (95-97,99).

Telomerase reverse transcriptase (TERT). TERT is the catalytic subunit of telomerase (a special enzyme responsible for maintaining telomeres). In normal cells, the telomere length is shortened after each cell division, and this shortening is considered the biological unit that limits the lifespan of cells (100). In cancer cells, TERT enzyme is considered to counteract this shortening by adding DNA repeats to telomeres, allowing cancer cells to maintain their reproductive potential (101). TERT can perform reverse transcription, where it synthesises DNA from an RNA template called the enzyme internal RNA component (TERC), by adding TTAGGG repeats to the end of telomeres (101).

Upregulating TERT expression and telomerase activity, resulting in unlimited replication of cancer cells similar to embryonic or stem cells, has been shown to overcome replicative senescence in the vast majority of head and neck tumours (100). Pathways affecting telomerase reactivation are mutations in the DNA sequence controlling *TERT* gene expression and alternative telomere lengthening which use non-homologous recombination (101). TERT mutations lead

to continuous cell division which can cause development of HNSCC with aggressive features, poor clinical outcomes and shorter survival (102,103).

Telomerase activity evaluation from patients with HNSCC as a biomarker for evaluation and identification of recurrence during follow-up revealed that telomerase activity turned negative following surgical removal of cancerous tissues. In the same study, follow-up of most of the patients with HNSCC revealed a conversion of telomerase activity from negative to positive after a subsequent recurrence of HNSCC (104). Comparison of the rate of telomerase activity between HNSCC and healthy individuals revealed to be significantly higher in patients with cancer compared with controls (104).

In a meta-analysis study of TERT to assess its prognostic use in HNSCC it was found that most of cancer sample express high amount of TERT that is linked with treatments response. Therefore, TERT is a promising biomarker for prognosis of patients with HNSCC (103).

Epidermal growth factor receptor (EGFR). The human EGFR belongs to a family of transmembrane receptors called tyrosine kinases. Normally, EGFR responds to the binding of its natural ligands EGF and TGFA, which are present in the extracellular environment (105). EGFR initiates a signalling cascade within the cell, activating downstream effectors that are involved in cell proliferation (106,107).

In HNSCC, EGFR is often dysregulated, resulting in its overexpression or constitutive activation (108). This dysregulation of EGFR can occur through various mechanisms, including gene mutations and hormone signalling (107). Human EGFR alterations or mutations can be used as prognostic indicators for disease and therapeutic responses because they are directly correlated with worse clinical outcomes (109). Overexpression of EGFR was linked to reduced overall survival rate in patients with cancer (110). The EGFR-related cancer patient survival rate ranges from low to high as it was observed by numerous different research studies (111-113). Thyroid cancer cells consistently overexpress EGFR, which is associated with continuing cellular proliferation. These high expressions of EGFR in cancer cells appeared to have potential as a prognostic biomarker in HNSCC (114).

A meta-analysis has provided evidence that EGFR overexpression is common oncogenic mechanism in oral oncogenesis and metastasis. It was suggested that immunohistochemical analysis of EGFR should be integrated in routine prognostic evaluation in HNSCC (115).

Real-time analysis of EGFR in fluorescence-labelled fresh tissue from fresh biopsies of the oral cavity using a specific molecular contrast agent, revealed that EGFR is a biomarker for diagnosis of oral cancer, as it was found that >50% of patients with oral cancer overexpress EGFR in their cells. During carcinogenesis, the basal cells do not differentiate and continue to proliferate constantly in the epithelium. As a result, EGFR is mostly expressed in the cancerous epithelium and cells in the lining of an organ with abnormal changes are more likely to become cancerous (116).

Most of HNSCC cases exhibit EGFR overexpression which is linked with a poor cancer prognosis, increased tumour growth and therapy resistance (109). Therefore, EGFR can be widely used as a prognostic for HNSCC (108-110,117).

Vascular endothelial growth factor A (VEGFA). VEGFA is an angiogenic protein factor that plays a crucial role in increasing vascular permeability. Abnormal regulation of VEGFA can lead to initiation and development of different human malignancies (118). Downregulation of VEGFA stimulates the cell migration and proliferation of endothelial cells and leads to cancer development (119).

A research study conducted on patients with laryngeal squamous cell carcinoma (LSCC) shows overexpression of VEGFA in cancer cells was strongly linked with re-occurrence of cancer and metastasis (120). Thus, VEGF angiogenesis biomarkers can be used in identifying high-risk patients specifically in the early stage of development of HNSCC (120,121).

Increased VEGF level is linked with more advanced tumour stages and invasiveness. Specifically in laryngeal lesions, VEGFA expression has been observed to rise progressively from pre-malignant to metastasis stages (122). Another study suggests that VEGFA may serve as a significant predictive biomarker in laryngeal squamous cell cancer (123). VEGFA can be used in prognostic and diagnostic of HNSCC, aiding in prognosis and treatment decision-making for patients (119,121,124).

Squamous cell carcinoma antigen (SCCA). SCCA is a tumour-specific antigen which has two homologous isoforms, SCCA1 and SCCA2; they are coded by two genes, SERPINB3 and SERPINB4, respectively, located on chromosome 18 (18q21.3). SCCA belongs to the family of serine protease inhibitors. SCCA is frequently elevated in HNSCC and is used as a marker of tumour severity and recurrence (125).

A research study was conducted to determine the association of SCCA with clinical features in patients with HNSCC and analyse the clinical utility of serum SCCA in treatment. It was found that blood levels of SCCA were higher in HNSCC samples compared with normal controls (126).

In a follow-up analysis of patients with HNSCC, an SCCA test was performed during the examination and their results indicated that SCCA was higher in patients with cancer; therefore, SCCA could be a suitable predictive biomarker in cancer (127).

HNSCC samples were subjected to fine needle aspiration to determine the additional diagnostic value of SCCA. The experimental results revealed that the SCCA level was high in the nodes of HNSCC compared with control patients. Therefore, presence of SCCA in patients with cancer can be used as prognostic biomarker (126,128).

Lactate. Lactate is a product of glycolysis from pyruvate, especially without oxygen. Recently, it was found that lactate is a crucial regulator of the glycolytic phenotype and has been observed to promote tumour growth and metastatic spread in HNSCC (129).

Elevated lactic acid levels in HNSCC may indicate dependence on this altered metabolic pathway for energy production (130). Study of HNSCC biopsy from the primary site was conducted for quantification of lactate concentration using the luciferase bioluminescence technique. Most tumour lactate concentrations were found to be associated with subsequent metastasis HNSCC, thus rendering lactate a favourable prognostic biomarker (131).

Analysis of serum metabolites from salivary gland tumours by using the mass spectrometry method was compared with healthy controls. It was found that lactic acid levels were consistently higher in HNSCC samples than control samples (132).

An *ex vivo* study performed with the Proton HR-MAS MR method on oral squamous HNSCC tissues for metabolic abnormalities and signs of inflammation in OSCC showed a high level of lactic acid in OSCC compared with the controls; thus, lactic acid can be used as indicative biomarker for HNSCC (133).

CD44. CD44 is the surface glycoprotein of a cell, and has numerous functions including cell to cell adhesion, migration and signalling (134). A previous study has highlighted the involvement of a small subset of cancer cells called CSCs in HNSCC tumour initiation, development and resistance to treatments (135). CSCs in HNSCC are characterised by the presence of certain surface markers, such as CD44, PROM1, ALDH1A1 and ABCG2; their overexpression is generally linked with an unfavourable prognosis (136).

CD44 is involved in the Wnt/ β -catenin pathway, which functions in cell proliferation, differentiation and stem cell maintenance. Activation of this pathway may promote CSC properties and contribute to tumour growth and invasion in HNSCC (137).

An *in silico* study on HNSCC found that CD44 was a frequently observed CSC marker and is implicated in various tumour-related process including proliferation and metastasis (138). CD44 expression in patients with HNSCC appears to differ among different subsequent of squamous cancer on the oral cavity, pharynx or larynx (134).

A study on untreated patients with laryngeal cancer found that CD44 level is associated with lymph node metastases and resistance to treatment (139). Therefore, CD44 holds promise as a valuable biomarker on diagnosis and prognosis for HNSCC (138-141).

Epstein-Barr virus (EBV). EBV is a commonly occurring herpesvirus infecting humans and is mostly transmitted through contact with infected bodily fluids such as saliva or blood. The EBV genome is a linear dsDNA, ~172 kbp long (32,37). EBV causes cancer through expression of two important proteins, EBV-induced nuclear antigen 1 and latent membrane protein 1 (LMP1) that facilitate cell proliferation, hinder apoptosis and disrupt the normal functioning of the immune system (142).

Research conducted on determination of EBV status in HNSCC using an *in situ* hybridisation method found that infection with EBV can disrupt several intracellular signalling processes involved in cell cycle control. In the same study, it was reported that EBV LMP1 can inhibit CDKN2A expression and inhibit downstream effectors, including the transcription factors E2F4 and E2F5, and can promote cell proliferation. In addition, it was found that LMP1 was expressed in >50% of EBV-positive tumours with aggressive features (143).

In a case-control study, EBV DNA positivity detected by qPCR was higher in the laryngeal cancer samples suggesting that individuals who tested positive for EBV DNA virus in the laryngeal area had increased risk of developing laryngeal HNSCC cancer. Furthermore, in the same case-control study,

it was concluded that EBV DNA is positively linked with anti-apoptosis by BCL2 expression in their tissues (144).

A nomogram analysis incorporated EBV DNA as a new immunotropic marker to predict the survival rate in nasopharyngeal cancer. The results of nomogram analysis found that EBV can be recognised as a potential prognostic factor for HNSCC (145).

A meta-analysis on HNSCC found that EBV⁺ patients had higher probability of developing laryngeal cancer compared with healthy controls (146). Therefore, the presence of EBV can be utilised as a prognostic marker of HNSCC (147-149).

5. Non-coding RNA (ncRNA) biomarkers of HNSCC

In this section, both long non-coding RNA (lncRNA) and small RNA molecules that are not translated into a protein but have regulatory functions are discussed. lncRNAs are a varied class of ncRNAs that play important roles in biological processes and are linked to cancer formation and progression. Their presence in body fluids and link with tumour characteristics make them promising non-invasive biomarkers for cancer diagnosis and therapy (150-152). MicroRNAs (miRNAs or miRs) are short non-protein coding RNAs whose sequences are ~18-22 nucleotides in length. A wide range of miRNAs have been identified in humans, and numerous of them have the ability to target and regulate numerous genes. Abnormal expression of miRNAs has been observed in HNSCC, suggesting their involvement in the initiation and progression of this type of cancer (153). In this section, specific ncRNAs which have been identified as potential drivers of HNSCC and can be used as diagnostic, prognostic or predictive biomarkers, are included.

HOXA transcript at the distal tip (HOTTIP). HOTTIP is a lncRNA that exhibits a unique genomic arrangement where it is brought in close proximity to the HOXA genes through chromosomal looping (154). By binding to the WDR5 protein, HOTTIP forms a complex with the histone methyltransferase protein KMT2A (previously known as MLL). This complex, known as the WDR5-MLL complex, specifically targets the HOXA region, leading to H3K4 methylation and subsequent transcriptional activation of the HOXA locus. HOTTIP has been identified to have a key role in various cancers, including HNSCC (155,156).

Analysis of HOTTIP as a prognostic biomarker of HNSCC integrated with computational analysis, have consistently demonstrated elevated HOTTIP expression in tissues of individuals who developed HNSCC compared with those who did not have cancer (157).

HOTTIP expression was discovered to be related with various stages of cancer as well as patient survival, establishing it as an important prognostic marker. This indicates that high HOTTIP levels contribute to cancer growth, proliferation and progression. HOTTIP has a favourable correlation with survival and can be employed as a unique and important diagnostic and prognostic biomarker in HNSCC (158).

The involvement of HOTTIP in the initiation and drug resistance in oesophageal cancer was determined by using MTT assay. HOTTIP levels in the serum of patients with oesophageal cancer were detected by reverse transcription

(RT) PCR. Drug resistance was determined after treating Eca109 cells with various concentrations of Adriamycin for 24 h; the IC₅₀ was measured using MTT assay. The results revealed that serum levels of HOTTIP are higher in patients with HNSCC compared with controls; HOTTIP can activate the ABCG2 axis to cause drug resistance in cancer. The expression profile of HOTTIP can be documented as a prognostic marker in monitoring the response in treatment for patients with HNSCC (159).

Analysis of HOTTIP levels in patients with NPC using RT-qPCR combined with western blotting demonstrated that HOTTIP expression was extremely high in HNSCC cell lines. Knockdown of HOTTIP resulted in decreased expression of HOXA13, which ended up in the inhibition of the proliferation of cells and metastasis (154).

lncRNA HOTTIP can facilitate HNSCC formation by modulating the expression of HOXA13 in cancer cells. Therefore, it can be suggested that measuring the expression levels of HOTTIP could potentially be useful as biomarker in prognosis of HNSCC (154,157,160).

Metastasis associated lung adenocarcinoma transcript 1 (MALAT1). MALAT1 is a lncRNA that is upregulated in cancer. This ncRNA can interact with chromatin-modifying complexes and regulate expression of proto-oncogenes, genes involved in cell cycle progression and apoptosis. MALAT1 binds to miRNAs that regulate gene expression and by binding them, it indirectly regulates the expression of miRNA for tumour suppressor genes (161). MALAT1 is overexpressed in HNSCC tumours influencing the expression of genes associated with cell proliferation, angiogenesis and cancer invasion (162,163). MALAT1 levels were found to be elevated in tongue squamous cell carcinoma (TSCC), particularly in those with metastasis stages (164). Experimental studies involving the knockdown of MALAT1 in TSCC cells, demonstrated impaired cancer cell migration and proliferation abilities *in vitro*, as well as few metastases occurred *in vivo* (165).

PCR was used to investigate MALAT1 expression levels as a potential therapeutic target in HNSCC samples and healthy adjacent tissues. The Transwell cell migration assay and the MTT assay were used to assess cell proliferation, migration or invasion capabilities and apoptosis. MALAT1 was shown to be overexpressed in cancerous tissues relative to cells of healthy adjacent tissues. This high level of expression was connected to a lower survival rate in HNSCC. In the same study, MALAT1 knockdown resulted in a corresponding reduction in cancer cell proliferation, increased apoptosis and cell cycle arrest (166).

In HNSCC, MALAT1 is highly expressed in tumour cells and can be measured using simple methods such as blood tests. Overexpression of MALAT1 is associated with angiogenesis and invasion (162,163). Therefore, determination of levels of MALAT1 in HNSCC could be used as a therapeutic biomarker (164,167,168).

HOX transcript antisense RNA (HOTAIR). HOTAIR is a type of lncRNA that plays a role in regulating gene expression by interacting with the polycomb repressive complex 2 and lysine-specific demethylase 1 (169). HOTAIR promotes cancer by recruiting EZH2 and H3K27me3 to regulate CDH1,

inducing cancer proliferation, invasion and lower level of apoptosis (170). It is considered that HOTAIR can also induce cancer development by suppressing the expression of tumour suppressor genes (171).

HOTAIR level of expression is constantly increased in LSCC tissues compared with control ones (172). This upregulation is associated with advanced tumour stage, poor differentiation, metastasis and worse clinical outcomes (163). A meta-analysis discovered that patients with HNSCC have high levels of HOTAIR, which can speed up tumour growth and lead to the development of malignancies. HOTAIR expression, particularly in LSCC tissues, is strongly linked to the T phase, histopathological grades and the probability of lymphatic metastasis (170). To determine the role of HOTAIR in LSCC, PCR was used to assess its activity *in vitro* by using Hep-2 and Tu212 LSCC cell lines transfected by pHOTAIR or si-HOTAIR, and then survival and apoptosis were measured during the experiment. The results of the experiment revealed that HOTAIR gene expression was enhanced by pHOTAIR and oppositely suppressed by si-HOTAIR. In the same experiment, knockdown of HOTAIR resulted in significant inhibitory effect on cell division observed in Hep-2 and Tu212 cells; on the other hand, the opposite effect was observed in HOTAIR-overexpressing cells. Therefore, HOTAIR promotes proliferation and inhibits cell senescence of LSCC cells (173). HOTAIR can be employed as a potential prognostic biomarker for assessing the clinicopathological characteristics and prognosis of HNSCC (169,174).

Urothelial carcinoma-associated 1 (UCA1). UCA1 is a lncRNA that has been recently found to be upregulated in HNSCC (175). Its upregulation is associated with promoting tumour growth, invasion and metastasis through regulating proliferation genes, angiogenesis and epithelial-mesenchymal transition. Overexpression of UCA1 can increase the level of WNT6 and trigger subsequent responses of the WNT signalling pathway (176). UCA1 is also employed in regulating the level of the WNT/ β -catenin signalling pathway (177).

The levels of UCA1 were found higher in TSCC tissues, and there was a notable positive correlation with lymph node metastasis (178). HSCC specimens were examined and compared with adjacent normal tissues acquired from patients with proven HNSCC illness. UCA1 was overexpressed in all HSCC tumour cells. RT-qPCR was used to determine the comparative expression amounts of UCA1 in all pairs of HNSCC and their surrounding (normal) non-tumour cells. The overall level of UCA1 in HSCC tissues was markedly higher than in paired neighbouring normal tissues, showing that UCA1 is involved in the development of HNSCC (179).

Overexpression of UCA1 in HNSCC tissues exhibits correlation with tumour stage and prognosis, and has the capability to differentiate between HNSCC and benign lesions. UCA1 is easily accessible through blood, saliva and other bodily fluids. These characteristics indicate that UCA1 is used in prognosis and treatment determination of HNSCC (178,180).

Myocardial infarction-associated transcript (MIAT). MIAT is a lncRNA located on chromosome 22q12 (181). Initially identified for its involvement in myocardial infarction, MIAT has been found to play significant roles in development of various

cancers (182). MIAT is involved in initiation and progression of cancers such as thyroid cancer by binding to miR-324-3p and promoting PI3K/AKT signalling (183). MIAT was reported in other studies consistently supporting the notion that MIAT functions as an oncogene in HNSCC (184,185). A study focusing on LSCC discovered that MIAT expression level was notably elevated in almost all of LSCC samples (185).

To validate MIAT's role in the progression of papillary thyroid cancer, HNSCC tissues and cell lines were analysed using RT-qPCR. The investigation revealed that the expression level of MIAT was significantly higher in cancer tissue than in normal surrounding tissues. The same approach was used to assess the degree of MIAT expression in papillary thyroid cancer cell lines, which revealed that MIAT was overexpressed in all cancer cell lines as well. On the other hand, knocking down MIAT had the effect of decreased cancer cell progression and invasion (186). Thus, MIAT could be used in diagnostic or prognostic tests for HNSCC because its expression level can be measured with simple blood tests (187,188).

miR-21. miR-21 is a highly researched miRNA, particularly because it is consistently found to be overexpressed in numerous types of cancers (189). In numerous experiments, miR-21 level is constantly high in tumour tissues, serum, plasma, saliva and in blood of patients with HNSCC compared with healthy individuals (190,191). miRNA-21 overexpression in HNSCC is linked to oncogenic properties as it targets several tumour-suppressor genes including PTEN, TP53, TP63 and PDCD4. This miRNA plays a crucial role in cancer proliferation, invasion, metastasis and apoptosis (192). Elevated levels of miR-21 expression are considered as prognostic marker in HNSCC. It was also proved that its expression levels tend to decrease or become low after surgery (removal of cancer tissue) in patients with HNSCC with a favourable prognosis (193).

A pilot study that looked at salivary and serum miRNAs' association with OSCC development was conducted, where saliva and matched serum miRNAs were collected from patients with OSCC and healthy volunteers, with an equal proportion of smokers and non-smokers in each group. It was identified that miR-21 in saliva was among the most commonly overexpressed miRNAs in OSCC compared with healthy controls (194). Furthermore, miR-21 was shown to be statistically considerably more abundant in the OSCC saliva of smokers than non-smokers (controls) (194).

Meta-analysis research on the miR-21 level in patients with HNSCC found that miRNA can be used in clinical setting to evaluate the risk to develop head and neck tumours (195). Higher levels of miR-21 in patients are more likely linked to progression of HNSCC with worse clinical outcomes. Implementation of the method that can detect levels of miR-21 is crucial because it demonstrates significant promise in diagnosis and prognosis biomarker for HNSCC (193-196).

Let-7. Let-7 is a highly conserved family of miRNAs that usually regulate expression of genes by binding to the 3' untranslated region of target mRNAs, resulting in their degradation or repression of translation (197). Let-7 is often considered a tumour suppressor miRNA and its

under-expression (decreased levels) is associated with the loss of its tumour suppressor functions, allowing uncontrolled cell proliferation and avoidance of apoptosis (198).

In HNSCC, Let-7 miRNAs function as tumour suppressors by targeting important oncogenes such as KRAS and HMGA2 (199,200). It has been found that the Let-7 family has decreased levels in tumour tissues, serum and saliva of patients with HNSCC relative to healthy individuals. Furthermore, Let-7a expression levels have been associated to different clinical phases of the disease, with advanced laryngeal carcinoma exhibiting lower Let-7a expression than early-stage data. These data suggest that Let-7 miRNAs could be useful prognostic indicators for HNSCC (197).

Levels of the Let-7 miRNA family and CD274 in HNSCC were compared with controls. The results showed that Let-7 family miRNAs are lower in HNSCC and have a negative correlation with CD274 expression (201). *In vivo*, the analysis demonstrated that overexpression of Let-7a/b enhanced anti-cancer immunotherapy by CTLA4 blockade. Those findings indicate targeting the Let-7 family as a viable way to improve immune checkpoint treatment for HNSCC (201). Let-7 is a promising biomarker for HNSCC; it is considered as a stable biomarker that has the potential to revolutionise early detection and prognosis of the disease (198,202).

miR-375. miR-375 is a type of miRNA that works as a regulator of numerous cellular processes in organisms like cell differentiation, proliferation and apoptosis (203). Moreover, miR-375 targets and regulates several genes that control cell proliferation and survival, including Janus kinase 2 and insulin-like growth factor 1 receptor (204).

In HNSCC, miR-375 has been found to be downregulated, and this indicates its potential role and involvement in tumour development and progression. These low levels of miR-375 expression are linked with unfavourable prognosis, increased invasion of head and neck tumours, and higher chances of cancer metastasis (153,204).

A meta-analysis on HNSCC showed that miR-375 is a potential biomarker for HNSCC because the results from clinical samples revealed that miR-375 in plasma of HNSCC was significantly different when compared with controls, but did not differ by sex or age. Based on the analysis, it was suggested that miR-375 may be a diagnostic marker in HNSCC (205).

A research study on well-characterised oral squamous cell lines revealed a positive association between miR-375 and rearranged L-myc fusion transcription factor expression in chemo-resistant oral cancers. It was indicated that miR-375 influenced chemotherapy resistance (206). Therefore, miR-375 can be identified as a potential biomarker for HNSCCs. Measuring its gene expression level can help in detection, prognosis and therapy decision in HNSCC (205,206).

6. Epigenetic biomarkers

Epigenetic modifications are heritable, reversible changes in the way gene is expressed that do not result from changes in the sequence of DNA bases. Therefore, epigenetic processes change the phenotype without disturbing the DNA sequence (207). Epigenetic processes include DNA

methylation, histone post-translational modification, modification in chromatin structure and some effects from gene regulators such as ncRNA (208).

A meta-analysis demonstrated that methylation is involved in cancer initiation and progression. Methylation of repetitive sequences such as Long Interspersed Elements-1 (LINE), Alu and Sat- α can be used as prognostic biomarkers. Based on their findings, it is supported that patients with cancer with global hypomethylation in all of the populations studied have poor outcomes. From the point of view of clinicians, hypomethylation benchmark can be used, and pyrosequencing to detect LINE-1 in combination with other detectors of methylation levels in samples from a patient may be a useful way of predicting their patients' outcome. If low methylation is detected, a strengthening treatment, such as the use of postoperative radiotherapy, chemotherapy or some type of intervention treatment can be applied. Therefore, the level of global hypomethylation of DNA is an integral predictor of cancer (209). A research study showed that epigenetic mechanisms play a crucial activity in the initiation, progression and treatment of cancer. Methylation of repetitive sequences such as LINE-1 or tumour suppressors including DAPK, RASSF1 and ECA is very important in tumour progression. Furthermore, epigenetic changes associated with histone modification and chromatin remodelling can lead to open chromatin structure and transcriptional activation of HNSCC-related factors. Therefore, epigenetics modifications can serve as predictive and prognostic biomarkers for HNSCC (207).

7. Liquid biomarkers

Liquid biopsies are methods to detect and manage cancer through non-invasive procedures by analysing readily available body fluids including saliva, blood, spinal fluid and urine. In general, those fluids may contain various molecules released by tumour cells that contain important cancer information (210). This section will discuss the most important circulating molecules used in liquid biopsy technology and their role in patients with HNSCC.

Circulating tumour cells (CTCs). CTCs are represented by transient cancer cells from primary tumours or metastatic tissue that can enter nearby blood vessels and spread to distant sites. Their presence can alert presence of cancer and the possibility of metastases (211).

Currently, numerous tumour cell detection platforms and biochemical systems are widely accepted for CTC isolation, and the CellSearch system is the first FDA-approved platform (212).

Circulating tumour DNA (ctDNA). ctDNA is represented by cell-free DNA that is released into the blood by the degeneration of cancer cells through apoptosis and necrosis (213). Mutations in ctDNA can reflect the genetic makeup of the tumour, dead cells or the release of DNA fragments during cell division (214). Therefore, detection of ctDNA from samples of patients with cancer can provide valuable information on malignant transformation state and holds promise as the easy way to provide both predictive and prognostic information in patients with HNSCC (215).

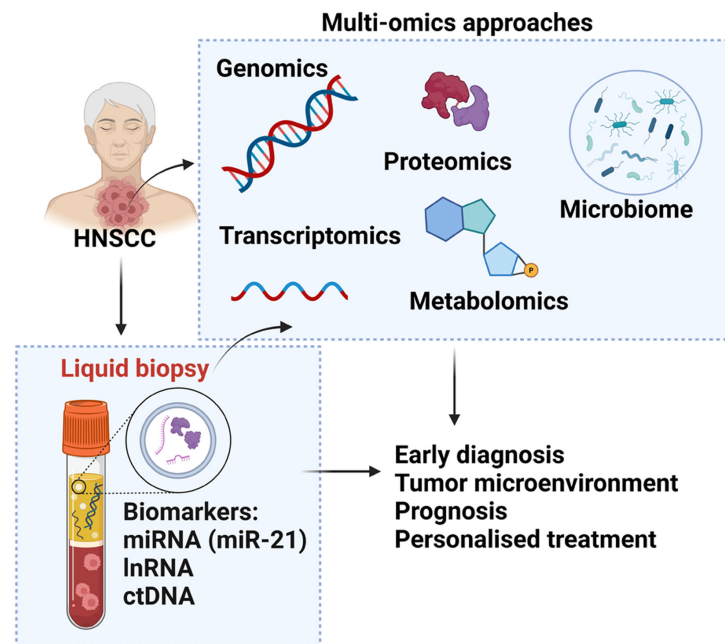


Figure 2. Multi-omics approaches available for patients with HNSCC. Genomics includes examining DNA sequences to detect genetic mutations, copy number variations and structural variations that contribute to tumorigenesis. The Transcriptomics approach focuses on the analysis of RNA sequences to explore gene expression patterns, alternative splicing events, and non-coding RNA profiles, offering insights into the functional consequences of genetic alterations. Proteomics entails studying proteins, their structures and functions to uncover post-translational modifications and protein-protein interactions that play crucial roles in cancer pathways. Epigenomics studies modifications such as DNA methylation and histone modifications, which control gene expression without changing the DNA sequence and play a crucial role in the development and progression of HNSCC. Metabolomics analyses all metabolites in the biological sample of HNSCC, offering insight into the metabolic state and changes in metabolic pathways related to this cancer. Microbiomics involves the study of all microbiomes associated with HNSCC, investigating their influence on cancer development and responses to treatment. HNSCC, head and neck squamous cell carcinoma.

Exosomes. Exosomes are a subset of extracellular membrane-bound vesicles that have a certain size range (30-150 nm); they are released by cells and can be detected in numerous body fluids (216). Exosomes are composed of a lipid bilayer containing cancer proteins, cancer miRNA and DNA that enable cell-to-cell communication. Moreover, the layer of lipid contributes to the tumour microenvironment and affects immune response in HNSCC (217). The exosomes in HNSCC may be a potential drug delivery vehicle for targeted therapy (218).

miRNAs. Circulating miRNA is a liquid biomarker in HNSCC including plasma-free miRNA, mRNA and lncRNA (219). miRNA expression in tumour cells can help to identify therapeutic targets or prognostic markers (220). A study to determine miRNA cycle stability in blood samples showed that plasma miR-196a and mi-196b expression was higher in HNSCC precancerous lesions when compared with normal controls (221). Therefore, measurement of tumour-derived miRNA in serum or plasma is a useful method for cancer diagnosis and treatment decision (219).

Overall potential of liquid biomarkers in HNSCC management. Liquid biopsies are an important tool for early detection of HNSCC, which can lead to improved patient outcomes and monitoring of treatment response and disease progression over time. Furthermore, liquid biomarker helps in the decision of using treatment strategies based on the specific genetic profile of the HNSCC detected by liquid biomarkers (222).

Current status of liquid-based biomarkers in HNSCC. Advancements in science and technology in this century have been made to adapt and use liquid biopsy as new alternative and non-invasive method for molecular characterisation of HNSCC. Liquid biopsies offer a promising method for monitoring patients with HNSCC and may guide treatment decisions. However, the development of liquid biopsies continues, and more studies are needed to confirm their accuracy and improve sensitivity, as well as to improve the special requirements that must be met before these platforms are recognised as alternative or complementary tests to the standard based on tissue biopsy (223).

8. Multi-omics platforms for patients with HNSCC

Currently, the standard method for the diagnosis of HNSCC is based on biopsy samples and histopathological interpretation. However, the current method does not describe all the characteristics of the cancer microenvironment and carcinogenesis processes. Multi-omics analysis is a comprehensive and integrated analysis of combined data derived from different omics techniques including proteomics, genomics, transcriptomics and metabolomics (224). Multi-omics analysis can provide a large database compared with a single analysis and provide important information about the pathogenesis of complex events, which will greatly contribute to the development of HNSCC diagnosis and treatment (225). The following are the currently available omics platform that can be used in HNSCC diagnosis and disease management (Fig. 2).

Genomic platforms: Genomics is the most studied and used field of omics. For patients with HNSCC it can involve whole genome sequencing, whole-exome sequencing, gene panels and copy number variations. The most common genomic indicators in initiation of HNSCC are caused by changes in CDKN2A, TP53, SMAD4, PIK3CA and KDM5C (226). In addition, activation of PIK3CA through mutations enhances the PI3K/AKT signalling pathway and mutations on NOTCH1, MET, STAT3, AKT, etc (95,227,228).

Epigenomic platforms: Nowadays, there are high-throughput and high-resolution DNA methylation analysis methods such as pyrosequencing and luminometric methylation assays that can analyse global DNA methylation levels in different HNSCC tumours (229). The most commonly detected indicators in epigenetics are DNA methylation and chromatin immunoprecipitation-sequencing for histone modifications. This epigenetic changes can lead to the silencing of tumour suppressor genes and the activation of oncogenes, facilitating tumorigenesis (230).

Transcription platforms: In HNSCC, transcriptomic analysis helps to find the differentially expressed genes between tumour and neighbouring normal tissues, revealing biomarkers and therapeutic targets. Available transcriptomic technologies include RNA-Seq and microarrays (231). Transcriptomic findings include altered RNA expression patterns of coding and non-coding genes, as well as alternative splicing events, offering insights into the functional consequences of these factors in HNSCC, for example, the detection of changes in miRNA and lncRNA levels.

Proteomics platforms: Generally, proteomics is used to quantify peptide abundance, modifications and interactions. Modern techniques such as mass spectrometry have revolutionised protein analysis and quantification and have recently been adapted for high-throughput analysis of thousands of proteins found in cells or body fluids (232). To efficiently investigate new protein biomarkers in HNSCC, a high amplification method is required for expression profiling and combining multiple biomarkers to increase discriminatory power (233). Example of protein indicators for initiation and development of HNSCC are changes in E-cadherin, EGFR, CD44, CyclinD1, PD-L1, PAI-1, XRCC1, RB, raptor proteins, cytokines and matrix metalloproteinases (234-237).

Metabolomics platforms involve the simultaneous evaluation of several types of small molecules including amino acids, fatty acids, carbohydrates or other products of cellular metabolism. Metabolic levels and ratios reflect metabolic function, and abnormal frequency disturbances often indicate disease (132). High-throughput LC-MS/MS analysis was performed on patients with HNSCC of all TNM to check the metabolic pathways of ESCC stages; the results provided evidence that cancer cell metabolites can be used to provide useful prognostic information about HNSCC development (238).

Microbiomics platforms: Microbes from oral cavity have been associated with development of HNSCC. Microbiome analysis platforms such as 16S rRNA-seq and metagenomic sequencing can be used to analyse disturbances in the human oral microbiome that may lead to imbalances and possible dysbiosis which are employed in the aetiology of HNSCC (239). It has been found that OSCC is associated with numerous oral

bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and some yeast types (240). The microbiome can be oncogenic, enhancing mucosal inflammation, immune effects and changes in cancer resistance or preventing the effectiveness of cancer treatment (241). Therefore, analysis of different microbial taxonomic groups may help to explore the role of the oral microbiome in initiation and progression of HNSCC (242). The presence of dysbiosis in HNSCC can be identified by conducting metagenomic sequencing of 16s RNA and shotgun sequencing. These bacteria can have an oncogenic effect on the mucosa, leading to enhanced tumor staging and grading, as well as impacting the response to chemotherapy treatments (241). The development of gingival squamous cell carcinoma might be influenced by the combined impact of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Prevotella intermedia* within the oral microbiome. This combination can trigger NF- κ B-mediated immune responses, potentially providing support for cancer survival, activating oncogenic pathways, and enabling cancer cell migration and invasion (242).

9. Clinical trials evaluating biomarkers for HNSCC in diagnosis, prognosis and treatment

Diagnosis, prognosis and treatment of HNSCC are particularly difficult due to complicated disease heterogeneity and a severe absence of validated novel biological markers with high clinical value, that may enhance patient quality of life and therapeutic strategies.

In the present review, clinical trials in which HNSCC biomarkers are the primary focus of several *in vitro* and *in vivo* studies aimed at determining the diagnostic, prognostic, or therapeutic value of biomarkers in HNSCC, were also included (Table I).

10. Current challenges in HNSCC and future directions

Currently, there is a deficiency in comprehensive omics research for HNSCC. While numerous individual omics studies have offered valuable insights, there is a lack of comprehensive omics studies that integrate data across numerous aspects, such as DNA, RNA, proteins, epigenomics, microbiomics and metabolites. Collaborative studies of this nature are crucial for gaining a full understanding of the complex biology of HNSCC.

Currently, massive validation of biomarkers is limited, despite the identification of numerous potential biomarkers, as only a few have been confirmed in large, independent cohorts. There is a need for comprehensive validation studies to establish the clinical usefulness of these biomarkers for early detection, prognosis and treatment response in HNSCC (243).

A great unresolved challenge in HNSCC is the significant heterogeneity in the cancer microenvironment and multidrug resistance mechanisms, contributing to variability in treatment response and resistance. It is essential to comprehend the underlying mechanisms of this heterogeneity and resistance to develop more personalised and effective treatment strategies (244). At present, the clinical and molecular data are not currently well integrated, therefore there is a requirement for research that combines clinical information with molecular

Table I. Clinical trials evaluating biomarkers for HNSCC in diagnosis, prognosis and treatment.

Identifier	Sample type	Biomarkers, Targeted	Purpose/Objectives
NCT05708209	Saliva	MALAT1, miR-124	The clinical trial aimed to determine useful and diagnostic accuracy of lncRNA MALAT1 as a potential salivary biomarker of oral squamous cell carcinoma as well as assessment of the salivary expression level of miRNA 124 which is targeted by MALAT1.
NCT02748707	Tumour tissues	TP53, PTGS2 (COX-2), EGFR	This clinical trial's objective is to study the effect of COX-2 inhibitor Celecoxib and EGFR tyrosine kinase inhibitor Erlotinib alone or in combination on molecular markers of apoptosis and angiogenesis.
NCT04305366	Saliva, blood, tissue	miRs	The main objective of this clinical trial study is to examine presence of miRNA markers in saliva, blood, FNA and tissue specimens in HNSCC patients and control. They evaluate whether these miRNA markers can provide prognostic or diagnostic clinical significance in management of this type of cancer.
NCT01487733	Tissue	HPV	The main objective of this clinical trial study is to analyse the association between biomarkers (HPV tumour status and ERCC1 expression) in patients with metastasis.
NCT03469544	Blood	HOTAIR	The main objective of this trial is quantitation of long non-coding RNA HOTAIR by Real time polymerase chain reaction in peripheral blood.
NCT03412058	Biopsy	CD274 (PD-L1)	To identify predictive factors of response to PD-1 and PD-L1 antagonists authorised for use in France in treatment of melanoma, NSCLC, or HNSCC.
NCT05904327	Plasma samples	HPV	The main objective is to investigate about circulating tumour HPV-DNA as a biomarker for HPV positive HNSCC.
NCT04085900	Blood and saliva	EBV	Objective of the study was to identify earlier detection biomarkers by test all participants for EBV associated biomarkers, including EBV-induced nuclear antigen 1-IgA, VCA-IgA, BNLF2b total antibodies (P85-Ab)

HNSCC, head and neck squamous cell carcinoma; miR or miRNA, microRNA; EBV, Epstein-Barr virus; HPV, human papillomavirus; EGFR, epidermal growth factor receptor; MALAT1, metastasis associated lung adenocarcinoma transcript 1; HOTAIR, HOX transcript antisense RNA.

discoveries to create algorithms that can assist with clinical decision-making for patients with HNSCC. Classification of biomarkers and their clinical significance in HNSCC is included in Table II.

Future directions. Generally, the future direction for HNSCC should focus on large-scale multi-omics, biomarker discovery and validations, use of new technologies for diagnosis and treatment development of targeted therapy and personalised treatment of patients with HNSCC.

11. Conclusions

In conclusion, the complex process of tumorigenesis in HNSCC was described and the important role of genetic

mutations, epigenetic modifications, dysregulated signaling pathways and biomarkers was highlighted. HNSCC development involves a series of stages from pre-malignant evolution that starts at the early stages of lesion development to the advanced stages of cancer invasion and metastasis. This complex interplay involves molecular events resulting in the transforming of normal epithelial cells into highly malignant phenotypes.

Identifying biomarkers for HNSCC is a dynamic and continuously evolving field in science, with promising implications for patient care. Biomarkers found in tissues and body fluids can indicate the presence of HNSCC, offering the potential for earlier and more accurate diagnosis. Furthermore, these biomarkers can provide prognostic information that could guide treatment decisions and predict patient outcomes.

Table II. Classification of biomarkers and their clinical significance in HNSCC.

First author/s, year	Marker	Category	Clinical application in HNSCC	(Refs.)
Solomon <i>et al</i> , 2020; Li <i>et al</i> , 2023	TP53	Predictive	Mutant Tp53 can predict individuals at high risk of developing HNSCC	(50,245)
Kovesi and Szende, 2003	MKI67	Predictive, prognostic	Presence of elevated MKI-67 index may indicate an unfavourable prognosis	(78)
Jasphin <i>et al</i> , 2016; Snietura <i>et al</i> , 2012	PTEN	Prognostic, predictive	PTEN level can be used as both prognostic and predictive marker after radiotherapy for high-risk HNSCC	(72,246)
Gioacchini <i>et al</i> , 2016	CCND1	Prognostic	The level of CCND1 expression is positively correlated with different stages of cancer	(84)
Murphrey <i>et al</i> , 2023; Bossi <i>et al</i> , 2012	EGFR	Predictive, prognostic	Determining the level of EGFR as predictive and prognostic role may help select the patients who can mostly benefit from accelerated treatment or personalised medicine	(106,247)
Li <i>et al</i> , 2019; Qiao <i>et al</i> , 2020	CD274 (PD-L1)	Prognostic, therapeutic	The level of CD274 expression is directly related to the clinical behaviour of HNSCC	(88,248)
Gisterek <i>et al</i> , 2006; Edirisinghe <i>et al</i> , 2023	VEGFA	Prognostic, therapeutic	VEGFA expression is usually correlated with stage and state of the tumour. This makes it a favourable biomarker in prognosis of HNSCC	(123,249)
Zhu, 2022	SCC-Ag	Prognostic	Squamous cell carcinoma antigen is always elevated in HNSCC compared with healthy cells	(125)
Kavitha <i>et al</i> , 2023; Gomez <i>et al</i> , 2020	CD44	Diagnostic, prognostic	CD44 is upregulated and correlated with clinicopathological characteristics of HNSCC	(134,135)
Roman and Aragonés, 2021; Augustin <i>et al</i> , 2020	HPV	Prognostic, theragnostic	HPV infection causes cancer through its oncoproteins E6 and E7. HPV-positive HNSCC has more favourable prognosis	(34,250)
Tan <i>et al</i> , 2017; Yoshizaki <i>et al</i> , 2023	EBV	Diagnostic, prognostic	Presence of EBV infection is closely associated with pathological characteristics of nasopharyngeal carcinoma	(251,252)
Yin <i>et al</i> , 2021; Shen, <i>et al</i> , 2019	HOTTIP	Prognostic	The level of HOTTIP in clinical samples can be implemented as indicative biomarker in HNSCC	(157,160)
Yang and Deng, 2014; Luo <i>et al</i> , 2018; Ye <i>et al</i> , 2021	MALAT1	Prognostic, theragnostic	MALAT1 promotes HNSCC progression, its level in samples may be used in diagnosis and treatment	(151,152, 253)
Cossu <i>et al</i> , 2019; Cantile <i>et al</i> , 2021	HOTAIR	Diagnostic, prognostic	In HNSCC, HOTAIR interactions interfere with different cellular processes during cancer evolution	(163,254)
Cossu <i>et al</i> , 2019; Wang <i>et al</i> , 2020	UCA1	Prognostic	Level of UCA1 may play an important role in different stages of cancer, as its level in metastasis stages is higher than that in the primary tumour	(163,255)
Song <i>et al</i> , 2019; Liu <i>et al</i> , 2019	MIAT	Prognostic	The level of MIAT in clinical samples can indicate progression of HNSCC	(183,184)
Shah <i>et al</i> , 2016; Mohammadi <i>et al</i> , 2021	miR-21	Theragnostic	Presence of miR-21 can be applied as theragnostic biomarkers for predicting of chemotherapy and radiotherapy response in HNSCC	(191,256)
Thomaidou <i>et al</i> , 2022	miR-200	Prognostic	The level of miR-200 family has been found to have the potential role of prognosis in patients with HNSCC	(153)
Yu <i>et al</i> , 2019; Ma <i>et al</i> , 2021	let-7	Prognostic, theragnostic	Determining the level of Let-7 miRNAs in clinical samples can help in prognosis and treatment planning because Let-7 is involved in tumour growth, differentiation and regenerative potential	(201,257)

HNSCC, head and neck squamous cell carcinoma; miR or miRNA, microRNA; EBV, Epstein-Barr virus; PTEN, phosphatase and tensin homolog; CCND1, cyclin D1; VEGFA, vascular endothelial growth factor A; UCA1, urothelial carcinoma-associated 1; MIAT, myocardial infarction-associated transcript.

To enhance HNSCC cancer management and personalised treatment in this new era of science and technology, it is crucial to discover more reliable novel biomarkers and incorporate them into clinical use. Multi-omics analysis can provide a large database compared with a single analysis and offer comprehensive insights into the heterogeneity of pathogenesis, significantly contributing to the development of new diagnostic tools and targeted therapies for HNSCC. By integrating multi-omics data, clinicians can improve understanding of the molecular underpinnings of HNSCC, leading to more effective treatment plans and improved patient management strategies.

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Authors' contributions

CAS performed literature review, wrote the original draft, and wrote, reviewed and edited the manuscript. CB, DAS, AKEN and GGP conceptualized the study, and wrote, reviewed and edited the manuscript. IM conceptualized and supervised the study, wrote the original draft, and wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

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