

miRNAs in head and neck cancer revisited

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Abstract

Background Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cause of cancer mortality in the world and the 5th most commonly occurring cancer. Tobacco smoking, alcohol consumption and human papilloma virus (HPV) infections have been associated with the occurrence of HNSCC. Despite advances that have been made in HNSCC treatment, smoking-associated HNSCC patients still exhibit a poor 5 year survival rate (30–50 %) and a concomitant poor quality of life. The major clinical challenge to date lies in the early detection of dysplastic lesions, which can progress to malignancy. In addition, there are currently no tools available to monitor HNSCC patients for early stages of local recurrences or distant metastases. In the recent past, micro-RNAs (miRNA) have been assessed for their role in cancer initiation and progression, including HNSCC. It is now well-established that deregulation of these single stranded, small non-coding, 19–25 nt RNAs can e.g. enhance the

expression of oncogenes or subdue the expression of tumor suppressor genes. The aims of this review are three-fold: first to retrieve from the literature miRNAs that have specifically been associated with HNSCC, second to group these miRNAs into those regulating tumor initiation, progression and metastasis, and third to discern miRNAs related to smoking-associated HNSCC versus HPV-associated HNSCC development.

Conclusions This review gives an overview on the miRNAs regulating the development of head and neck cancers. The ultimate establishment of miRNA expression profiles that are HNSCC specific, and miRNAs that orchestrate altered gene and protein expression levels in HNSCC, could pave the way for a better understanding of the mechanism underlying its pathogenesis and the development of novel, targeted therapies.

Keywords Head and neck squamous cell carcinoma (HNSCC) · microRNA (miRNA) · Human papilloma virus (HPV).

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1 miRNA characteristics

MicroRNAs (miRNAs) are non-coding regulatory RNA molecules of 19–25 nucleotides (nt) in length that have in recent years been encountered in all metazoans tested [1, 2]. Although, they account for a relatively small fraction of the expressed genome, the genes encoding miRNAs are located throughout the human genome in introns and exons of both coding and non-coding genes. miRNAs play a major role in maintaining tissue homeostasis by regulating many processes such as cellular proliferation, differentiation, migration, apoptosis, survival and morphogenesis [2–5]. It is estimated that the human genome may harbor up to 1,000 miRNAs [5–7]. Although miRNAs are not directly involved in encoding proteins, they are believed to control the expression of more than one third of all protein coding genes present within the

human genome. One miRNA can target and regulate mRNA transcripts of a large number of genes, while one mRNA can be targeted by multiple miRNAs. There are a number of mechanisms by which miRNAs can regulate the expression of target genes at the post transcriptional level, including enhancing mRNA degradation and inhibiting mRNA translation. miRNAs can inhibit mRNA translation through the assembly of an RNA-induced silencing complex that encompasses a number of proteins, including Argonaute [6–8].

Historically, miRNAs have been viewed as negative regulators of gene expression. Recent work by Vasudevan et al., has, however, shown that a small subset of miRNAs within the human genome can also enhance gene expression [7–9]. Specifically, they found that miRNA-369-3 can enhance the translation of *TNF α* mRNA when cells are grown under growth arrest conditions. They also found that miRNA Let-7 can up-regulate genes that govern and regulate cell cycle arrest [9]. In another study, miRNA-373 was found to initiate *E-cadherin* expression in prostate cancer [8–10]. For the purpose of this review, we have focused on miRNAs that have been associated with head and neck squamous cell carcinoma (HNSCC). We have extracted information from three databases, i.e., the miRBase (<http://www.mirbase.org/>), PhenomiR 2.0 (<http://mips.helmholtz-muenchen.de/phenomir/>) and the Head, Neck and Oral Cancer Database (<http://gyanxet.com/hno.html>). The aims of our review are threefold: first to document miRNAs that are specifically associated with HNSCC, second to group these miRNAs in those regulating tumor initiation, progression and metastasis, and third to discern miRNAs related to smoking-associated HNSCC versus HPV-associated HNSCC development.

1.1 Role of miRNAs in cancer

The role of miRNA in cancer has been reiterated and irrefutably established by many studies [reviewed in 9]. Several studies have shown that miRNA signatures (i.e., mRNA expression profiles) can be useful for classifying human cancers [reviewed in 6]. These studies have identified “cancer related miRNAs” through investigating expression profiles in matched normal and tumor tissues, as well as in body fluids. In addition, a vast number of studies has shown that miRNAs can play a role in regulating the expression of oncogenes and tumor suppressor genes, whereas others have shown that miRNA gene deletion or mutation can lead cancer initiation, progression and metastasis [9–12]. Classical examples of miRNAs that are down-regulated in cancer include miRNA-15 and miRNA-16 in chronic lymphocytic leukemia (CLL), the let-7 miRNA family in lung carcinoma and various other tumors [13–16], and miRNA-34 in neuroblastoma [17].

Calin et al., were the first to demonstrate that a differential expression of miRNAs may provide useful tools in the diagnosis and prognosis of human cancers [18] and they

subsequently reported altered expression of miRNAs during tumor initiation and progression [19]. Although, a direct causal relationship with cancer was not demonstrated, various other studies have subsequently successfully identified differentially expressed miRNAs in various neoplasms [9, 13, 20]. He et al., were amongst the first to report an oncogenic association of the miRNA 17–92 cluster in B cell lymphomas with an over-expression of the *c-MYC* oncogene [21] and Costinean et al. demonstrated a functional role of miRNA 155 in B-cell lymphoma using a transgenic mouse model [22]. Oncogenic miRNAs (Oncomirs) [23] were also found to affect the transformation of normal cells into tumor cells. As an example, miRNA-372 and miRNA-373 have been found to be able to deactivate the tumor-suppressive activity of the *p53* gene, thereby promoting cell proliferation and, ultimately, tumorigenesis [24]. The specific functional role of miRNAs in tumorigenesis has amply been investigated by either over-expression or knock-down of specific miRNAs, and these functional studies have paved the way towards its utilization in targeted cancer therapies [24].

1.2 Detection of miRNAs in body fluids

With the advances of molecular biological techniques and the increasing knowledge of tumor pathogenesis, biomarkers are now considered as an effective supplement, in conjunction to histological examination, for facilitating clinical decision making [25]. Extracellular miRNAs in serum, plasma, saliva and urine have recently been shown to be associated with various pathological conditions, including cancer [26]. Most of the circulating miRNAs are included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles or exosomes, rendering them inaccessible to degradation by RNases [27]. miRNAs in body fluids have already been exploited as potential biomarkers for malignancy based on its stability and relative ease of collection, isolation and detection [28]. Lawrie and colleagues [29] were the first to observe elevated levels of miRNA-21 in serum samples collected from large B-cell lymphoma patients, and almost simultaneously Mitchell et al. [30] demonstrated that circulating miRNAs isolated from serum or plasma can be used as diagnostic biomarkers to detect prostate cancer. More recently, other researchers have identified specific miRNAs (31, 200a and 125a) in saliva collected from oral squamous cell carcinoma patients [31, 32]. In addition, Weber et al., detected specific miRNAs in amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, plasma, pleural fluid, saliva, seminal fluid, tears and urine [28]. They also reported that the highest number of miRNAs (458) was detected in saliva and the lowest number (204) in urine. In addition, Liu et al. [31] found that after surgical resection of the primary tumor, the level of miRNA-31 in plasma decreased,

indicating that this miRNA may be useful in monitoring disease progression. These findings, however, need to be validated in large clinical trials before they can be applied in the clinic.

1.3 Isolation of miRNAs from tissues and body fluids

It is important to note that the methods of isolation and handling, including storage conditions, have a severe impact on the quality and quantity of miRNAs extracted from various biological matrices. Clearly, it is imperative to minimize handling and storage of the miRNA samples at room temperature [33], and optimized kits (miRNAeasy, Qiagen) are preferably used, as well as traditional Trizol liquid phase separation methods to isolate miRNA from various biological sources. One kit-based technique that has extensively been used is the mirVana miRNA Isolation Kit (Ambion, USA). Trizol/TRI-Reagent based extraction, followed by alcohol precipitation is, however, the most commonly used method to isolate both total RNA and miRNA from biological specimens. Although no single method has been shown to be superior, miRNAs extracted by the Trizol/TRI-Reagent are highly stable and, as yet, this method remains the “gold standard” for the isolation of miRNAs from cells and body fluids in a laboratory setting [34]. To evaluate the stability of miRNAs isolated from B lymphocytes (CLL), Mraz et al. performed a Real Time-PCR analysis on 29 miRNAs after storing the samples for 14 days at -80°C , with a follow-up after ~ 10 months of storage at -80°C [34]. They concluded that reproducible results can be achieved when miRNAs are stored at -80°C for long periods of time [34]. In addition, they emphasized the importance of working in a RNase-free environment.

2 Head and neck cancers

2.1 Epidemiology

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world based on its mortality, and approximately 900,000 new cases are diagnosed each year worldwide. In Australia alone 2,078 new cases are diagnosed every year (<http://www.cancer.org.au/about-cancer/types-of-cancer/head-and-neck-cancer.html>). HNSCC affects the nasal passages, sinuses, mouth, throat, larynx, swallowing passages, salivary glands and thyroid glands. Approximately 80 % to 90 % of HNSCCs are attributed to prolonged tobacco and alcohol use [35]. In addition, 30 % to 50 % of HNSCCs are associated with human papilloma virus (HPV) infections [36]. The most widely used predictors of survival for HNSCC are tumor stage at the time of presentation and, more recently, HPV genotypes present in the tumor in conjunction with tumor site and treatment regimen [37].

HNSCC is heterogeneous in nature and the early stages of the disease may not show any clinical symptoms. Thus, a large number of patients who arrive at the clinic present with late stages of the disease (stage III or IV) and, as a consequence, have a relatively poor prognosis. Despite advances in therapy, the average survival rates for smoking-associated HNSCCs are disappointing, with more than 50 % mortality within 5 years [38]. This high mortality rate is mainly due to the development of local recurrences, distant metastases and secondary primary tumors. The therapy is, therefore, complex and the prognosis is poor [38]. Early diagnosis of HNSCC holds the promise of an improved prognosis, but this promise is currently impeded by a lack of clinically validated biomarkers for reliable early detection and the paucity of targeted molecular therapeutics [38, 39].

2.2 miRNAs in head and neck cancers

Several investigators have identified miRNAs as potential biomarkers for HNSCC, but conclusive miRNA biomarkers have not yet been established for this disease [40]. The main impediment in identifying miRNAs that are of diagnostic and prognostic value for HNSCCs is attributed to its multifactorial etiology and wide heterogeneity [38]. Furthermore, the underlying mechanism of miRNA deregulation in HNSCC is not yet clear, although it has been reported that the miRNA processing machinery may be upregulated in HNSCC, i.e., Dicer and Drosha were found to be over-expressed in salivary gland tumors [41]. It has also been demonstrated that the expression of Drosha (miRNA processor) may affect the phenotype of squamous epithelial cells [42].

Extracellular miRNAs may be quite relevant as a source of biomarkers in HNSCC, especially for tumors that are located in the oral cavity and that secrete biomarkers into the saliva. Thus, investigating miRNA expression profiles in saliva could provide a snap shot of the biological and pathological processes which take place within a tumor in the oral cavity. miRNA expression profiling has also been used to detect pre-malignant lesions, highlighting its potential value as early diagnostic biomarkers. Oral leukoplakia is a clinical entity, defined as a white lesion in the oral mucosa which is resistant to being scraped off [43]. It is the most common pre-malignant oral lesion and 1 to 2 % of these lesions progressively undergo malignant transformation [43]. A current diagnostic dilemma lies in the fact that it is difficult to differentiate a progressive lesion from a non-progressive one. An invasive biopsy procedure is needed to assess histologic characteristics or molecular markers in order to identify a highly dysplastic lesion [44]. Cervigne et al. have reported a multi-miRNA prognosis predictor through the identification of miRNA expression profiles in progressive leukoplakias using consecutive progressive samples [45]. Of a predictive set of eight miRNAs, three

miRNAs, miRNA-345, -21 and -181b, were significantly associated with progressive squamous cell carcinoma.

miRNAs are increasingly being explored as putative prognostic biomarkers for HNSCC. Childs et al. used real-time PCR-based profiling of a panel of 236 unique miRNAs to compare the relative expression levels between tumor and adjacent normal tissues in a cohort of 104 HNSCC patients [46]. They reported that low absolute expression levels of miRNA-205 and Let-7d were significantly associated with a higher chance of a loco-regional occurrence and a shorter survival. Furthermore, the expression levels of these two miRNAs could be used to predict disease progression, independently of one another. Another study has reported the expression of miRNA-451 in HNSCC tumors as a strong predictor for relapse [47]. Additional recent evidence underscores the clinical utility of miRNAs as potential diagnostic biomarkers for HNSCC. The expression ratio of miRNA-221 to miRNA-375 could, for example, be used to discriminate HNSCC tumor tissue from normal tissue with a specificity of 0.93 and a sensitivity of 0.92 [48]. Furthermore, it has been reported that a high expression level of miRNA-205 can be used in discerning metastases [49]. The sample size ($n=19$) was small in this latter study and, thus, warrants further validation of this marker in a larger cohort. In a separate feasibility study, these authors reported significantly lower levels of miRNA-125a and miRNA-200a in saliva collected from oral squamous cell carcinoma patients compared to control subjects [32]. In a more recent study, they also reported elevated levels of miRNA-184 in plasmas of tongue squamous cell carcinoma patients [50]. Furthermore, the same authors found that the expression of miRNA-184 (anti-apoptotic function) in plasma decreased following surgical resection of the primary tumor. A better understanding of the molecular dynamics of miRNAs may be of help to design more efficient, targeted therapies, as was demonstrated by the successful treatment of HNSCC with epidermal growth factor receptor (EGFR)-specific antibodies [51]. Figure 1 provides a comprehensive overview of miRNAs documented in the literature that play a role in the transformation of normal epithelial cells into squamous carcinoma cells and, ultimately, metastatic cells.

2.2.1 miRNAs deregulated in smoking-associated HNSCC

Cigarette smokers are at an estimated 10-fold higher risk of developing HNSCC as compared to non-smokers [52]. This risk increases with increasing duration and extent of smoking [52]. In time, the risk may decrease with cessation of exposure, but the risk never reaches the level of non-smokers [53]. In addition, increased alcohol consumption has been identified as an independent risk factor for HNSCC [39]. When alcohol is used in conjunction with smoking, a synergistic effect enhances the risk of developing HNSCC [39].

Oral and laryngeal cancers are most commonly associated with tobacco use, whereas pharyngo-laryngeal cancers are more commonly associated with alcohol use [54]. Few studies have been aimed at investigating miRNA deregulation in HNSCC as a result of social habits such as areca (betel) nut chewing, tobacco chewing and alcohol ingestion. Through a multivariate analysis, Avissar et al. found that the expression of miRNA-375 increased with alcohol ingestion, and that its expression was higher in tumors of pharyngeal and laryngeal origin as compared to oral tumors [55]. Tsai et al. have linked areca nut extract exposure to an increased expression of miRNA-23a [56], and they have reported that miRNA-23a plays a vital role in inhibiting the expression of Fanconi Anaemia complementation G (*FANCG*, a gene that functions to prevent DNA double strand breaks), while promoting oral carcinogenesis [56]. A more recently published study has documented a correlation between miRNA expression profiles and cigarette smoking in lung carcinomas. Free radicals and oxidative compounds in tobacco smoke appear to down-regulate many miRNAs that are involved in tumor suppression [57, 58].

2.2.2 miRNAs deregulated in HPV-associated HNSCC

The incidence of HPV-associated HNSCC shows a distinct demographic pattern. Li et al. noted for example that 46 % of tonsillar carcinoma samples from Australian patients ($n=67$) were positive for HPV, whereas none of the tissue biopsies from Chinese patients ($n=16$) were HPV positive [59]. Kreimer et al. published a systematic review extracting information from 60 published studies on HPV found in HNSCC worldwide [60]. Their data suggest a higher prevalence of tumor site-specific HPV in studies from North America as compared to Europe and Asia. In addition, they noted that oropharyngeal SCCs had a higher HPV prevalence (35.6 %) than oral (23.5 %) or laryngeal (24 %) SCCs.

Clinical features, including prognosis and survival, of HNSCC patients are markedly affected by their HPV status [61]. Hellner et al. found that tonsillar cancers can be diagnosed through the presence of viral-encoded oncoprotein [62]. There is reasonable evidence now that SCCs that test positive for HPV have a far better prognosis than those that are HPV-negative. Lassen et al. documented that the overall 5-year survival rate after radiotherapy was 62 % in *p16* (surrogate marker for HPV)-positive patients compared to 26 % in the *p16*-negative patients [63]. Fischer et al. found that also in case of oropharyngeal SCCs, *p16*-positive patients had a higher (57.1 %) 5-year survival rate than *p16*-negative patients (26.8 %) [64].

The distinctive clinical features of HPV-positive carcinomas result from viral modulation of host miRNAs [61]. As of yet, few studies have been conducted to discern the

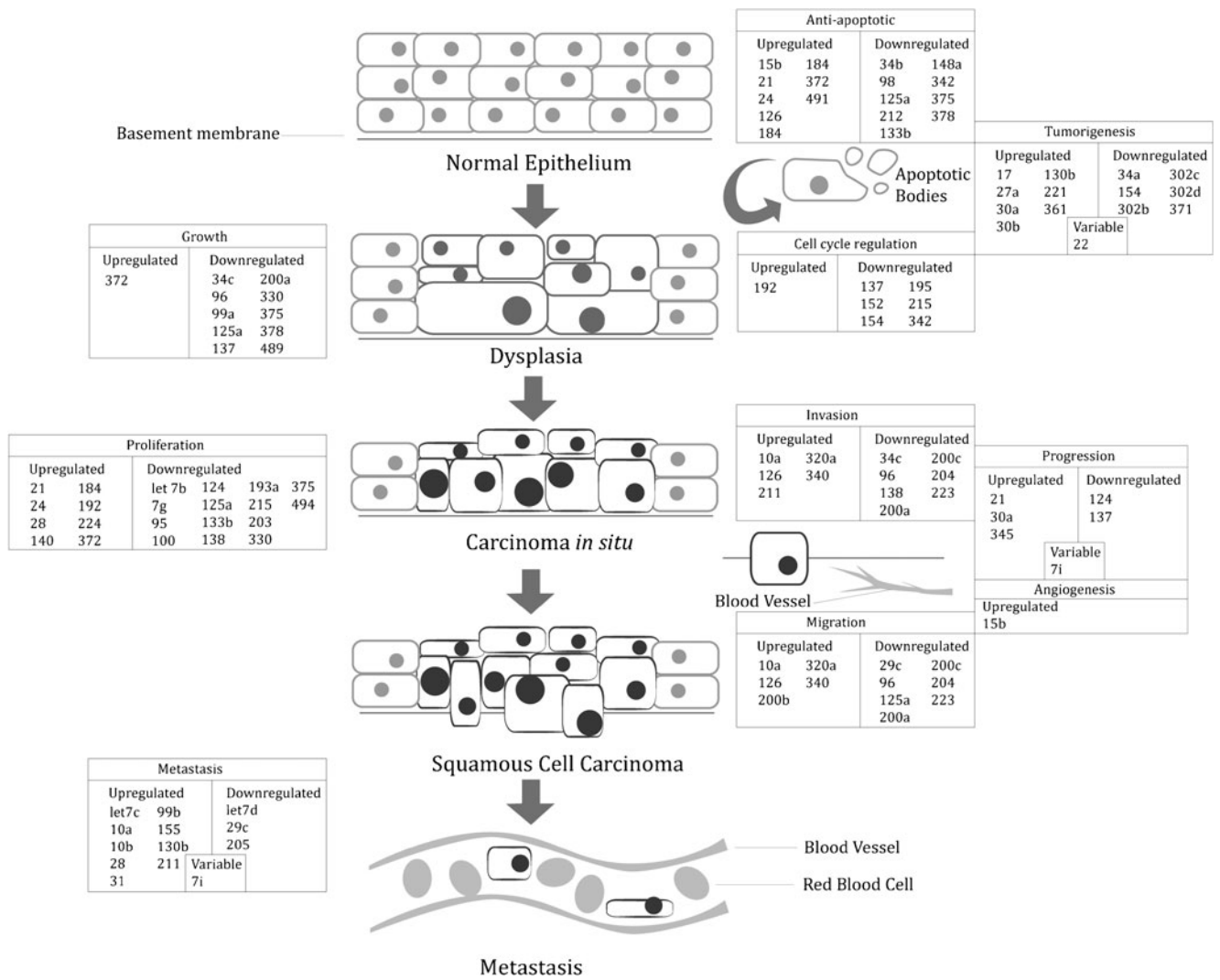


Fig. 1 miRNAs regulating the transformation of normal squamous epithelial cells into carcinoma cells, ultimately resulting in metastasis

miRNA expression profiles of HPV-infected HNSCCs as compared to its HPV-negative counterparts [65, 66]. Lajer et al. reported that HPV infection is closely associated with alterations in miRNA-127-3p and miRNA-363 levels in oral and pharyngeal SCCs [67]. By interfering with the E6-p53 and E7-pRb pathways, respectively, the HPV E6 and E7 oncoproteins may control the expression of the miRNA-15/16 cluster, the miRNA-7-92 family, miRNA-21, miRNA-23b, miRNA-34a and the miRNA-106b/93/25 cluster in host cells [68]. Wald et al. observed 12 differentially expressed miRNAs (up or down regulated) in HPV-positive HNSCC patients as compared to HPV-negative patients [65]. Figure 2 depicts the common miRNAs that are deregulated in HNSCC, either as result of smoking and drinking, or as a result of HPV infection. These data suggest that HPV infection is a risk factor for a subset of HNSCCs. The molecular pathways underlying HPV-negative HNSCCs remain to be elucidated.

HPV +		Smoking
Upregulated	Downregulated	Downregulated
33	29a	let7a,b,c,f
363	125a	26a
497	126	30a
Areca (betel nut)	127-3p	30c
Upregulated	142-5p	34a-c
23a	145	122a
Alcohol	155	140
Upregulated	181a	345
375	181b	Upregulated
	218	294
	221	
	222	
	379	

Fig. 2 miRNAs that are deregulated due to life style factors such as smoking, alcohol use, areca nut chewing and HPV infection

3 Conclusions

HNSCC is a complex disease with an overall poor prognosis. miRNAs regulate a myriad of biological processes, including cellular growth, differentiation, migration, apoptosis, survival and morphogenesis. With the emerging data supporting a central role of miRNAs in gene deregulation in HNSCC, the unravelling of miRNA expression profiles appears critical for the development of better diagnostic and prognostic tests. To this end, a number of studies have been aimed at investigating miRNA signatures for HNSCC surveillance, and many researchers have suggested to also employ this knowledge for the development of novel targeted therapies. The wide range of miRNAs that have been shown to be associated with the various stages of HNSCC development, however, requires additional studies aimed at deciphering the exact cellular pathways involved and, thus, identifying the exact targets for therapy.

Recent evidence from translational studies suggests that individual miRNAs and/or miRNA signatures may be useful for the diagnosis and prognosis of a vast array of human cancers. However, there are a number of issues to be considered when validating an individual cancer marker or a panel of markers for their use in the clinic, including a well-defined clinical question and a hypothesis to be tested, a well-defined sample cohort, a sound experimental design taking into consideration the modes of sample collection, miRNA isolation and quantification, data analysis, consideration of tumor heterogeneity as a variable, and robust high throughput technology platforms with a high-end bioinformatics pipeline. Developing and validating a panel of miRNAs that is associated with various subsets of HNSCC hold promise for a change in the way we diagnose this type of cancer, including an improvement in clinical outcome. Additionally, translating this knowledge into clinical management will ultimately be beneficial for the treatment of HNSCC patients.

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Conflict Of Interest None.

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