



MicroRNAs in head and neck squamous cell carcinoma: a possible challenge as biomarkers, determinants for the choice of therapy and targets for personalized molecular therapies

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Abstract: Head and neck squamous cell carcinoma (HNSCC) are referred to a group of heterogeneous cancers that include structures of aerodigestive tract such as oral and nasal cavity, salivary glands, oropharynx, pharynx, larynx, paranasal sinuses, and local lymph nodes. HNSCC is characterized by frequent alterations of several genes such as *TP53*, *PIK3CA*, *CDKN2A*, *NOTCH1*, and *MET* as well as copy number increase in *EGFR*, *CCND1*, and *PIK3CA*. These genomic alterations play a role in terms of resistance to chemotherapy, molecular targeted therapy, and prediction of patient outcome. MicroRNAs (miRNAs) are small single-stranded noncoding RNAs which are about 19–25 nucleotides. They are involved in the tumorigenesis of HNSCC including dysregulation of cell survival, proliferation, cellular differentiation, adhesion, and invasion. The discovery of the stable presence of the miRNAs in all human body made them attractive biomarkers for diagnosis and prognosis or as targets for novel therapeutic ways, enabling personalized treatment for HNSCC. In recent times the number of papers concerning the characterization of miRNAs in the HNSCC tumorigenesis has grown a lot. In this review, we discuss the very recent studies on different aspects of miRNA dysregulation with their clinical significance and we apologize for the many past and most recent works that have not been mentioned. We also discuss miRNA-based therapy that are being tested on patients by clinical trials.

Keywords: Head and neck squamous cell carcinoma (HNSCC); microRNAs (miRNAs); biomarkers; radioresistance; chemoresistance; metastasis

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Introduction

Head and neck cancer are the sixth leading cancer worldwide. About 90% of this cancer is known by squamous cell carcinomas (HNSCC) that emerge from the oral cavity, pharynx, or larynx and for this reason it is a heterogeneous pathology. The major etiologic agents involved in the carcinogenesis of HNSCC are tobacco, alcohol consumption and HPV infection (1-4). The most common

cause of mortality in HNSCC patients is high incidence of local recurrences (about 60% of cases) (5,6).

Despite advances in surgery, radiotherapy and chemotherapy, there has been little improvement in HNSCC survival in the last three decades and the clinical outcome has still remained poor. One of the major obstacles in current therapy of HNSCC is radio and chemoresistance (7,8). Mutation in tumor suppressor genes like

TP53 (about 70–80%) and *p16INK4a* (about 50–80%) are the most frequently detectable genetic alterations reported in HNSCC (9-12). Due to the high rate of genetic and molecular heterogeneity of HNSCC tumors, the diagnosis, prediction, and prognosis of this cancer is particularly challenging (13). An early diagnosis opens the door to future care and treatment but the lack of clinically validated biomarkers for early diagnosis indicates the need for better approaches to identify biomarkers to improve therapeutic efficacy.

miRNAs are one of the best promising biomarkers that are considered as an attractive tool for diagnosis and prognosis of cancer (14-17). They are the class of endogenous expressed small non-coding RNA (19–25 nucleotides) which play a significant role in post transcriptional regulation of genes through interaction with mainly 3'UTR of target mRNAs (18). These small molecules play a crucial role in many biological processes such as cellular development, differentiation, metabolism, proliferation, migration, and apoptosis (19,20).

Several evidences show that aberrant expression of miRNA can lead to cancer initiation, progression and metastasis including HNSCC cancer (18,21). Moreover, they can influence therapy response and clinical outcome in HNSCC (22). Therefore, the significant role of miRNAs cannot be ignored. In this review we will summarize the most recent knowledge and literature about the different implications of microRNAs in HNSCC tumorigenesis and their role to the development of effective targeted therapy.

miRNA biogenesis

The biogenesis of miRNA consists of two pathways. The main pathway for biogenesis and the most studied is “canonical miRNA biogenesis”, the second is called “non-canonical miRNA biogenesis” (23,24) (*Figure 1*).

In the canonical miRNA biogenesis, miRNAs are encoded in the genome as a monocistronic or as a cluster which containing several miRNAs. Later these clusters are transcribed to a polycistronic and processed to separate mature miRNAs (23). In most cases, biogenesis starts with transcription by RNA polymerase II (*Figure 1*, A1 panel). Then the primary transcripts with a stem loop structure (pri-miRNA) are modified by 5'capping, polyadenylation and RNA editing (25). The pri-miRNA is processed in the nucleus by microprocessor complex which containing RNaseIII endonuclease Droscha and the cofactor double-stranded RNA (dsRNA)-binding protein DGCR8

(DiGeorge syndrome critical region gene 8) and cleaved into a smaller precursor termed pre-miRNAs (26). The pre-miR is exported to the cytosol by the exportin 5 (export receptor) in a complex with RAN-GTP through the nuclear pore complex (27). In the cytoplasm the pre-miRNA is cleaved by the endonuclease Dicer and its binding factor TRBP (also known as TRBP2) and produce the miRNA duplex (guide and passenger strand). In the last process called RNA-induced silencing complex (RISC) loading, the miRNA duplex that is produced by Dicer delivered to a core member of AGO family which chooses only one strand as a mature miRNA (guide strand) and interact directly with it. The AGO protein with guide strand separates from the dicer and forms the RISC (23). The other strand of miRNA duplex (passenger strand) is usually released and degraded. However, recent studies show that sometimes passenger strands act as a miRNA and regulate target genes (28).

Besides the canonical biogenesis there are several classes of non- canonical miRNA biogenesis (23,24). Here we overview some of the common non-canonical miRNAs. One of the important classes of these miRNAs which originate from the introns are referred to mirtrons (*Figure 1*, A2 panel). These miRNAs are transcribed by RNA polymerase II and then spliced. After splicing, the lariat structures are debranched by Lariat debranching enzyme (LDBR) and folded to form pre-miRNA. These miRNAs are processed by Drosha independent, Dicer dependent mechanism. Then they enter the canonical miRNA biogenesis and they are transported to the cytoplasm by exportin 5 (29-32). Some of the non-canonical pre-miRNA derived from tRNA and snoRNA (*Figure 1*, A2 panel). Despite their expression level, AGO association and mRNA targets are still unclear (33-35).

miRNA function

The specific mechanisms of miRNA function consist of binding seed sequence of miRNA to 3'UTR of the target mRNA in a sequence specific manner. Therefore, they induce inhibition of mRNA translation or degradation and cleavage of mRNA (*Figure 1B*) (36). Several evidences show that some miRNAs bind not only to the 3'UTR of mRNA but also bind to the CDS (coding sequence) and 5'UTR of target mRNA (37). When miRNAs interact with promoter region can also activate translation or regulate transcription (38,39). Moreover, miRNAs are important for normal animal development (40). In addition, Extracellular miRNAs function as signaling molecules to mediate cell-

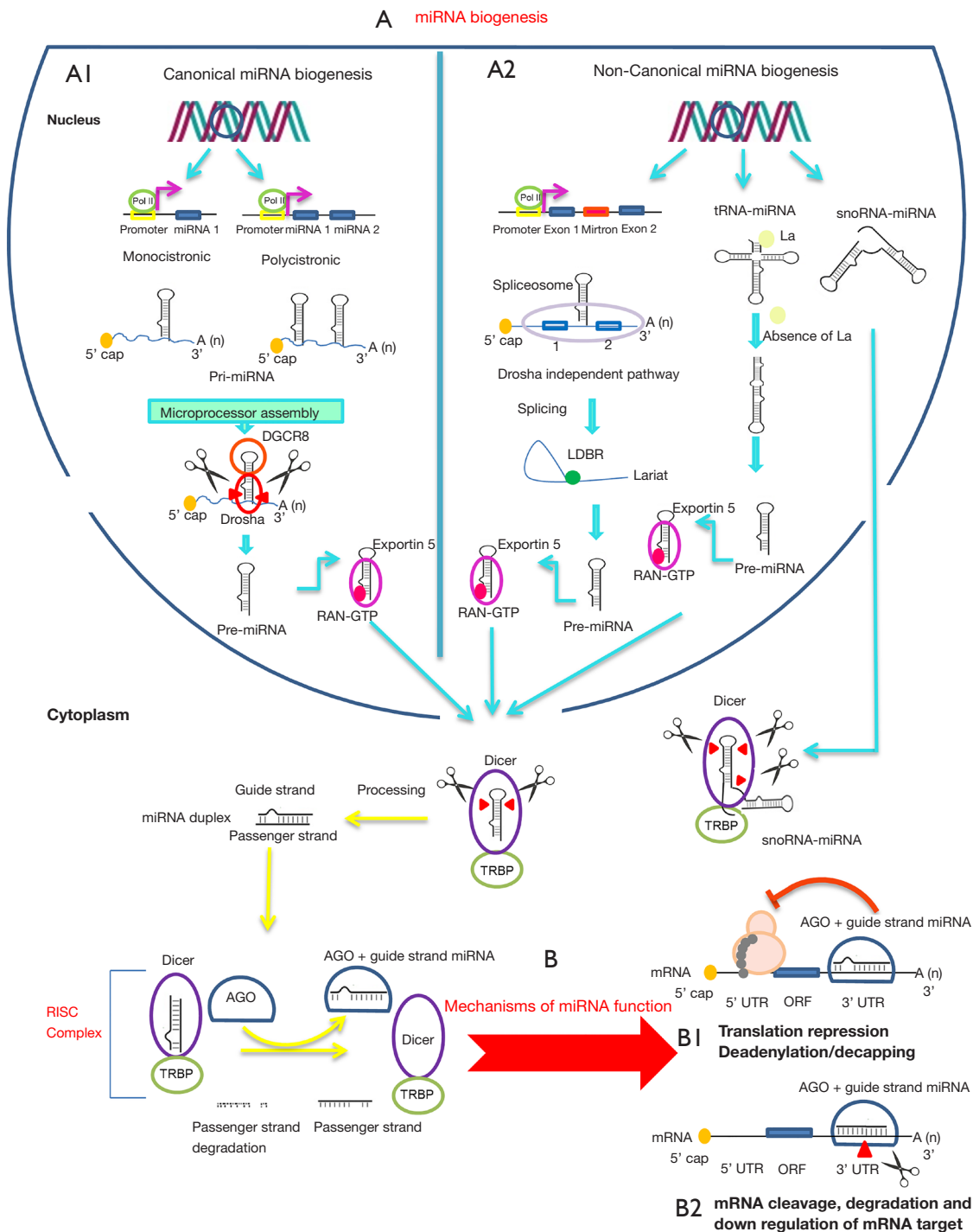


Figure 1 microRNA biogenesis and function diagram. (A) miRNA biogenesis that consists of two pathways. A1: canonical miRNA biogenesis that starts with transcription by RNA polymerase II and produce pri-miRNA. Then pri-miRNA is cleaved by Drossha and DGCR8 into pre-miRNA. Pre-miRNAs are exported from the nucleus into the cytoplasm by exportin-5 and Ran-GTP. In cytoplasm Dicer and TRBP generates miRNA duplex. In general, guide strand is incorporated into the RNA induced silencing complex (RISC). A2: noncanonical miRNA biogenesis. Mirtron, tRNA-miRNA and snoRNA-miRNA are common non-cononical miRNAs. In nucleus, they processed by different mechanism. Then in cytoplasm, they enter the canonical miRNA biogenesis. (B) Mechanisms of miRNA Function. Generally, miRNAs bind to 3'UTR of the target mRNA (B1) inhibit target mRNA translation (B2) mRNA degradation.

cell communication (38,41). Chang *et al.* reported that miR-92a is exported from endothelial cells through extracellular vesicles to macrophages to regulate their functions and enhance atherosclerotic progression (42).

MicroRNAs take part in the tumorigenesis process in HNSCC

By using microRNA expression profiling by microarray analysis, qRT-PCR method, and RNA sequencing, altered miRNA expression in HNSCC cancer has been investigated (1,43,44). miRNAs expression profile is also emerging among the best biomarkers for diagnosis and treatment of cancer, including HNSCC (45). It has been shown that downregulated miRNAs could function as tumor suppressors by negatively regulating oncogenes and overexpressed miRNAs could function as oncogenes by inhibiting tumor suppressor genes (*Figure 2*) (46,47). In the following sections, we overview some of the recent studies about the function of miRNAs in HNSCC cancer.

One of the most commonly observed miRNA deregulations in various human cancers including HNSCC, is upregulation of miR-21. The significant correlation between miR-21 overexpression and poor prognosis of HNSCC patients has been described. Therefore, miR-21 could be used as a prognostic biomarker in HNSCC patients (48,49). miR-21 regulates the expression of numerous target genes and these findings support the role of miR-21 as an oncomiR. In nasopharyngeal carcinoma (NPC), overexpression of miR-21, led to increased cell proliferation and suppressed apoptosis through direct targeting *PTEN/AKT* pathway (50). In oral squamous cell carcinoma, exosomes derived from cisplatin-resistant OSCC cells transferred miR-21 to OSCC parental cells and induced cisplatin resistance by targeting *PTEN* and *PDCD4* (51). Another study observed that sophocarpine (SC) is a chemotherapeutic agent which effectively inhibited proliferation, invasion, and migration of HNSCC cells through downregulation of miR-21 expression by blocking Dicer-mediated miR-21 maturation (52) (*Table 1*).

miRNAs and the relationship with TP53

As mentioned before, in HNSCC, *TP53* tumor suppressor gene is the most frequently genetic alteration (about 70–80%) that has been reported (10,83). A group of microRNAs whose expression associates with *TP53* mutation was identified (84). Between them, 12 miRNAs

whose expression correlates with recurrence-free survival and a group of 4 miRNAs that correlates with cancer-specific survival were determined. Among the 12 miRNA signatures, miR-96-5p emerges as an oncogenic miRNA with prognostic significance in HNSCC. miR-96-5p is one of the members of the cluster miR-96/182/183. It is located on chromosome 7q32.2. Data has shown that overexpression of miR-96-5p was significantly higher in the tumoral tissues in comparison with the non-tumoral tissues (53). This overexpression was significantly stronger in the subset of mutant p53 patients and predicts local recurrence development in HNSCC patients. Furthermore, overexpression of miR-96-5p led to increased cell migration and radio-resistance, chemotherapy resistance in HNSCC cells by directly targeting *PTEN* and activation of PI3K-AKT signaling pathway (53).

Another study has shown that highly expressed miR-205-5p, in HNSCC expressing mutant p53 proteins, targeted *BRCA1* and *RAD17* expression leading to inefficient DNA repair and increased chromosomal instability (56). Moreover, this miRNA is among 12 miRNA signatures previously cited and it had the best score in predicting the risk of recurrence in HNSCC (84). Intriguingly, high miR-205-5p expression in HNSCC peritumoral tissues was related to the early detection of minimal residual disease involved in tumor development (56). Surprisingly, in oral squamous cell carcinoma, miR-205-5p has been reported to suppress cancer cell migration and invasion by directly targeted and regulated the tissue inhibitor of met alloproteinases-2 (*TIMP-2*) gene and act as a tumor suppressor (85). Also, Childs and colleagues have reported that combined under-expression of miR-205 and let-7d showed negative correlation with the survival prognosis of head and neck cancer patients (86). This shows that miRNA expression patterns and biological activity of miRNA are highly specific to different cell-type, tissue context and cellular differentiation status (87).

Recently, it has been shown that long non-coding (lnc) MIR205HG expression was significantly higher in tumoral samples carrying mutant p53 than in tumoral tissues expressing wild-type p53 (88). Moreover, lnc-MIR205HG decreased endogenous miR-590-3p leading to increased cyclin B, cdk1, and YAP protein expression inducing cell proliferation and cell migration (88).

miRNAs as tumor suppressors

In contrast, some miRNAs act as a tumor suppressor by

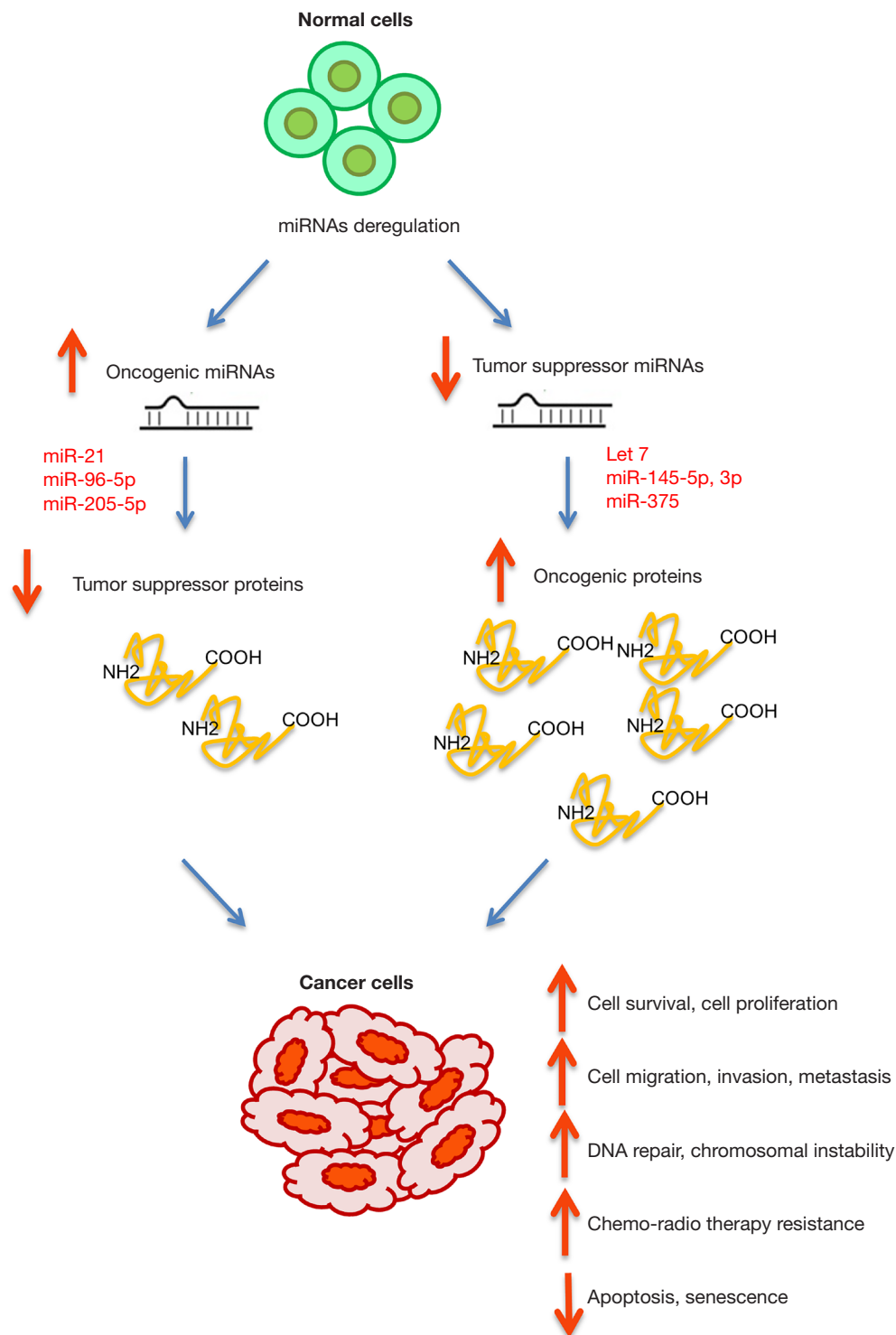


Figure 2 Mechanism of miRNAs deregulation and cancer development. Some miRNAs function as oncogenes or tumor suppressors. miRNAs deregulation may transform the normal cell into cancer cell. Therefore, the overexpression of oncogene miRNAs by negatively regulating tumor suppressor genes and down regulated tumor suppressor miRNAs by negatively regulating oncogenes may promote cancer development. The final outcome may lead to increased cell proliferation, invasion, metastasis, drug resistance and decrease apoptosis.

Table 1 Summary of oncogene and tumor suppressor miRNAs and proposed target genes in HNSCC

miRNAs	Target genes	References
OncomiRs		
miR-21	<i>PTEN</i>	(50,51)
	<i>PDCD4</i>	(51)
miR-96-5p	<i>PTEN</i>	(53)
miR-134	<i>PDCD7</i>	(54)
miR-155-5p	<i>ARID2</i>	(55)
miR-205-5p	<i>BRCA1, RAD17</i>	(56)
miR-361-3p	<i>OSR2</i>	(57)
miR-501-5p	<i>CLCA4</i>	(58)
miR-762	<i>PHLPP2, FOXO4</i>	(59)
miR-4295	<i>NPTX1</i>	(60)
Tumor suppressor miRs		
Let7 family	<i>Ras</i>	(61)
Let-7c	<i>IGF1R, HMGA2</i>	(62)
Let-7e-5p	<i>CCR7</i>	(63)
miR-30-5p	<i>EGFR</i>	(64)
	<i>MET</i>	
	<i>IGF1R</i>	
	<i>IRS1</i>	
miR-99a-3p	<i>VEGFA</i> (bioinformatic analysis), <i>STAMBP</i>	(65,66)
miR-125a-5p	<i>ERBB2, EIF4EBP1, TXNRD1</i>	(67)
miR-125b	<i>PRXL2A</i>	(68)
miR-140-5p	<i>PAK4</i>	(69)
miR-145-3p	<i>MYO1B</i>	(70)
miR-145/143	<i>MDM2</i>	(71)
miR-145-5p	<i>FSCN1</i>	(72)
miR-200 family	<i>QKI</i>	(73)
miR-141	<i>EGFR</i>	(74)
miR-204-5p	<i>Brd4</i>	(75)
	<i>JAK2</i>	(76)
	<i>SNAI2, SUZ12, HDAC1, JAK2</i>	(77)
miR-375	<i>KLF5</i>	(78)
	<i>IGF1R</i>	(79)
	<i>JAK2</i>	(80)
miR-381	<i>STC2</i>	(81)
miR-625	<i>SOX4</i>	(82)

targeting the oncogenes. Several studies have demonstrated that let-7 family is downregulated in HNSCC. Moreover, reports have claimed that let-7 targets a number of genes such as *RAS* (61), *HMG A2* (62) and other oncogenes that is shown in *Table 1*. As mentioned before, combined under-expression of miR-205 and let-7d were associated with poor survival of HNSCC (86).

Recently, data indicated that let-7e-5p expression was down-regulated in HNSCC tissues and in highly aggressive cells. let-7e-5p inhibited the progression and metastasis of HNSCC by targeting *CCR7* (63). Fadhil and colleagues showed that let-7a-5p and miR-3928 were significantly down regulated in saliva of HNSCC patients and salivary let-7a-5p significantly influenced by cancer staging and lymph node metastasis (89).

One of the tumor suppressor miRNAs in HNSCC is miR-145 that targets different genes. miR-143 and miR-145 form a bicistronic cluster in 5q32-q33 (90,91). Accumulating studies showed that both miRNAs are significantly downregulated in cancer tissues (70,92,93). Importantly, Zhang and colleagues identified that miR-143/miR-145 were activated by wild-type p53 consequently, they were involved in the p53 signalling pathway. Moreover, the authors have shown that high levels of miR-143 and miR-145 led to apoptosis of p53 wild-type HNSCCs cells and inhibit both *in vitro* and *in vivo* cellular proliferation by reducing *MDM2* and enhancing p53 activity (71).

Recently, in laryngeal squamous cell carcinoma (LSCC), the decrease of miR-145-5p increased *FSCN1* (fascin actin-bundling protein 1) protein level causing migration, invasion, tumor growth and EMT (epithelial-mesenchymal transition) (72). The authors identified low miR-145-5p/high *FSCN1* expressions were associated with poor prognosis and survival in LSCC patients (72). Recent study suggested that carboplatin (analogue of cisplatin) induced the expression of miR-145 in a dose-dependent manner and inhibited the expression of miR-145 direct targets. Furthermore, they showed that pifithrin- α inhibited the p53 in carboplatin-treated cells therefore, reduced miR-145 expression and the miR-145 direct targets (94).

A great number of studies reported that miR-375 act as an antitumor miRNA in HNSCC and this miRNA was strongly repressed in tumor tissues in comparison with normal tissues. Functional analysis showed that miR-375 target several oncogenes that are summarize in *Table 1*. Moreover, downregulation of miR-375 significantly correlates with cancer survival, poor prognosis, and distant metastasis (95,96). In oral squamous cell carcinoma, low

expression level of miR-375 was significantly associated with late stage disease, larger tumor size and increased invasion (97). Furthermore, another study in OSCC revealed the involvement of miR-375 in cancer progression via *KLF5* (78). In LSCC miR-375 expression can impede cell proliferation, migration, invasion and enhance apoptosis through *IGF1R* (79). Also, miR-21/miR-375 ratio is an independent prognostic factor in LSCC patients and this ratio is so specific for disease prediction (98).

Recently, antitumor immunotherapy has great potential to treat several cancers including HNSCC (99). Upregulation of programmed death 1 ligand 1 (*PD-L1*) in cancer cells and its ligation to PD-1 on T cells promote cancer cell escape from immune surveillance. Secretion of interferon- γ (CD4+ T cell effector cytokine and essential for PD-L1 expression) from natural killer (NK) cells activates the JAK2/STAT1 pathway and increases *PD-L1* expression. Wu and colleagues confirmed that miR-375 inhibited IFN- γ -induced PD-L1 surface expression in HNSCC cells via targeting *JAK2* (80). Consequently, miR-375 plays a potential role in enhancing T cell-mediated antitumor immune response against HNSCC by inhibiting PD-1/PD-L1 signaling (8075). *Table 1* summarizes some of the recent oncogene and tumor suppressor miRNAs in HNSCC (54,55,57-60,64-69,73-77,81,82).

Role of microRNAs in local recurrence and metastatization process in HNSCC

Local recurrences and metastasis are the most common cause of death in HNSCC patients. Therefore, the need to identify biomarkers for early detection of local recurrence and improve survival of HNSCC patients is critical. Migration and invasion are the primary step in the metastatic process. Epithelial cancer cells convert to migrant mesenchymal cells by going through an EMT process and these cells produce new tumors in the same location (recurrence) or different location (metastasis) (100). Therefore, in this process the expression levels of epithelial markers (E-cadherin) are downregulated and the mesenchymal markers (e.g., fibronectin, N-cadherin, vimentin) are upregulated (101). Several transcription factors such as ZEB1, ZEB2, Snail, Slug, and Twist, are involved in the EMT process and the expression levels of these factors are associated with the metastatic status (102). Many recent studies have shown that miRNAs participate in regulating of recurrence, EMT and metastasis (102,103).

Many studies demonstrated that miR-200 family members are downregulated in various cancers including HNSCC and are involved in the EMT process (104-106). The miR-200 family derived from two different chromosome regions: miR-200b/miR-200c/miR-429 cluster which is located on chromosome 1p33.36 and miR-141/miR-200a cluster which is located on 12p13.31 (107). miR-200c has found to negatively regulated the expression of *BMI1* and significantly inhibited EMT in malignant HNSCC by reducing the expression of *BMI1/ZEB1* (108). In another study, reciprocal correlation between miR-141/miR-200c and *ZEB1* was considered. The authors showed that the enforced expression of miR-141 and miR-200c significantly inhibited HNSCC cells migration and led to increased E-cadherin and decreased *ZEB1* expression (105). Recent study has suggested that forced expression of miR-200a/b/429 inhibited expression of *ZEB1/2* and decreased cell migration in OSCC cell lines by direct targeting *QKI* (quaking protein). *QKI* is a member of the signal transduction and activation of RNA (STAR) protein family and it is an important regulator of EMT that has been reported to increase during EMT and modulate EMT-related phenotypes (73).

Downregulation of miR-138 has been frequently reported in HNSCC (109-111). miR-138 showed to regulate the EMT via targeting *VIM*, *ZEB2* and *EZH2* genes and control the transcription activity of the E-cadherin in HNSCC. Taken together, this study suggested that miR-138 controls EMT (112). Reinforcing data has shown that ectopic transfection of miR-138 reduced the expression of both *RhoC* and *ROCK2* in tongue squamous cell carcinoma (TSCC) cells and led to decreased cell migration and invasion (113). Recently, in OSCC has been reported that the expression level of miR-138 is negatively correlated with metastasis. The lower expression of miR-138 is related to the higher rate of metastasis and poorer prognosis of the patients. Therefore, miR-138 is emerging as a promising biomarker and therapeutic target for OSCC with metastatic potential (109). Moreover, circulating miRNAs have been identified to be potential biomarker. In support of this, it was reported the positive correlation between decreased serum miR-138 and worse clinical outcome as well as shorter survival (114). Consequently, serum miR-138 might be a potential biomarker for detection and prognosis prediction of HNSCC (114).

Another interesting miRNA involved in carcinogenesis and tumor progression is miR-155-5p found to be involved in cell proliferation, migration and invasion (55,115,116).

In OSCC tissues and HNSCC cell lines, miR-155-5p has found significantly upregulated (55,117). This miRNA can regulate EMT by direct targeting *ARID2*. Downregulation of miR-155-5p by inhibitor led to increase the expression level of epithelial markers E-cadherin and decrease the expression level of mesenchymal markers vimentin and Snail (55). Furthermore, in another study a significant negative correlation was observed between miR-155-5p and E-cadherin expression suggesting its involvement in EMT in OSCC. High expression of miR-155-5p was associated with poorer disease free survival (115,118). *Table 2* summarizes some of the miRNAs that are involved in the EMT process in HNSCC.

miRNAs and local recurrence events

HNSCC cancers are characterized by a high incidence of local recurrences (6). Interestingly, a signature of 4 miRNAs (miR-96-5p, miR-21-3p, miR-141-3p and miR-130b-3p) was identified whose high expression in OSCC tumors are able to predict local recurrence development independently from the other clinical and pathological variables (120). The authors showed that inhibition of the four prognostic miRNAs expression in led to decrease proliferation rate by downregulation of *cyclin D1* (120). Furthermore, Ganci and colleagues identified 35 miRNAs which are deregulated in both tumor and peritumoral tissues, compared with normal matched samples (121). In particular, 4 miRNAs out of these 35 miRNAs (miR-21-3p, miR-21-5p, miR-96-5p and miR-429) predicted local recurrence independently from prognostic clinical variables (121). Therefore, the power of miRNA signature for prediction of local recurrence could not be ignored.

The impact of microRNAs in radioresistance and chemoresistance in HNSCC

The standard cure for HNSCC management consists of radical surgical removal of the tumor accompanied by the adjuvant radiotherapy which can be administered as monotherapy or with chemotherapy and personalized molecular therapy (122,123). Despite all this effort, half of the treated patients are relapsed and do not respond to subsequent therapeutic treatments (124). Distant metastasis formation is most frequent leading to poor prognosis and life quality (124). Furthermore, second primary cancers in the head and neck region (locoregional recurrence) and in other sites as lungs, gastrointestinal tract occur in about

Table 2 Summary of miRNAs that are involved in EMT process in HNSCC

miRNAs	EMT target genes	References
miR-138	<i>VIM, ZEB2, EZH2</i>	(112)
	<i>RhoC, ROCK2</i>	(113,114)
	<i>AKT1</i>	(110,111)
miR-145-5p	<i>FSCN1</i>	(72)
miR-155	<i>ARID2</i>	(55,117)
miR-200 family	<i>BMI1</i>	(108)
	<i>ZEB1/ZEB 2</i>	(104-106,119)
	<i>QKI</i>	(73)
miR-204	<i>SNAI2, SUZ12, HDAC1, JAK2</i>	(77)

30–50% of HNSCC patients, particularly in those that are HPV-negative (124).

At present, the principal anticancer drug for advanced HNSCC is cisplatin (CDDP), often coupled with radiotherapy (RT) (125,126). Resistance to chemotherapy and RT is associated with poor prognosis, sometimes there is either lack of tumor response or recurrences occur a few weeks after an initial complete response. In these cases, the outcome of patients is much poorer (127). Therefore, CDDP and RT resistance represent the biggest clinical problem in HNSCC patients showing recurrence and because it affects the probabilities of containment of the tumor and greatly affects patient quality of life.

The biological and molecular mechanisms that are the basis of resistance of the tumors to RT and chemotherapies enclosing alterations pathways of DNA damage response and double strand breaks repair, autophagy, apoptosis, proliferation, and angiogenesis. In the last years, many studies have reported that aberrant regulation of these cellular functions is often due to the dysregulation of the miRNA activities and/or expression. The potential of miRNAs to be employed as biomarkers to predict the post-therapy outcomes of HNSCC patients is very high and intriguing.

Signaling pathways that resulted to affect the resistance to the therapies are EGFR, PI3K/AKT, NF- κ B, RAS-MAPK, and TGF- β , as well as JAK-STAT signaling (128).

miRNAs and EGFR pathway

EGFR (epidermal growth factor receptor) is overexpressed in about 90% of HNSCC. High EGFR expression

correlates with poor prognosis of HNSCC patients and resistance to conventional RT. Diverse miRNAs that target *EGFR* transcript are downregulated in HNSCC patient tissues contributing to the development of cancer and the acquisition of the malignant phenotype (128,129). Recently, data have shown *EGFR* and hepatocyte growth factor receptor (*c-MET*) are directly downregulated by both miR-1 and miR-206 (130). The authors reported that miR-1 and miR-206 expression levels were significantly reduced in HNSCC cell lines and patient cancer tissues compared with matched non-tumoral tissues, while in parallel *EGFR* and *c-MET* were expressed at high levels (130). In human genome, miR-1/miR-133a and miR-206/miR-133b are present as miRNA clusters in 20q13.33 and 6p12.1, respectively. Also, miR-133a and miR-133b expression levels frequently downregulated in HNSCC specimens (130). The restoration of miR-1 and miR-206 expression in HNSCC cell lines determined the downregulation of *EGFR* and *c-MET* mRNA and the decrease of some oncogenic activities as proliferation, migration, and invasion, showing that these miRNAs act as tumor suppressors in normal cells of oral-pharynx-larynx mucosa (130). miR-1 and miR-206 have a tumor suppressor role also in other type of cancers such as lung cancer, colon cancer and glioblastoma (131-135).

Recently the miR-30 family has identified as an important regulator of signaling and tumor suppressor in HNSCC patients (64). The authors integrated miRNA and mRNA expression, copy number variation, and DNA methylation data from The Cancer Genome Atlas (TCGA), with a functional genome-wide screen revealing that miR-30 family is commonly repressed in HNSCC and showing

the inverse correlation with overexpression of predicted target mRNAs deregulated in HNSCC. Pivotal molecules involved in proliferation, differentiation, adhesion, and invasion were repressed and among them also *EGFR* and *c-MET* mRNAs (64). Interestingly the treatment with a miR-30a-5p mimic designed for targeted nanomedicine significantly inhibited HNSCC xenograft tumor growth and inhibited growth receptors EGFR and MET *in vivo* (64).

Recurrent/metastatic HNSCC positive to EGFR expression are currently treated with platinum-based chemotherapy plus the anti-EGFR monoclonal antibody (mAb) cetuximab (136,137). Interestingly, a recent conspicuous and in-depth meta-analysis study revealed important gene-signature (138) and miRNA-signature clusters associated with the treatment response and progression free survival of the patients (139). In particular, in recurrent/metastatic HNSCC patients in the phase II PANI01 trial, the association of Cluster3 gene-signature, representing predictive genes for anti-EGFR cetuximab treatment with seven selected miRNAs (miR-199a-3p, miR-199b-5p, miR-199a-5p, miR-130b-3p, miR-140-5p, miR-214-3p, miR-34a-5p) were significantly associated with panitumumab efficacy, another anti-EGFR-mAb (137). PANI01 tumor samples were analyzed using functional genomics to explore response predictors to anti-EGFR therapy (137).

miRNAs and PI3K/AKT pathway

The activation of EGFR-signaling can induce the downstream phosphorylation cascade of *PI3K* (phosphatidylinositol-3-kinase) and *RAS* (140). The activation of *PI3K* induces the AKT pathway that control different cellular function cell survival, apoptosis, DNA damage response and EMT (140). At the same time, the induction of *RAS* stimulates a tyrosine kinase cascade, Raf/MEK/MAPK that phosphorylates Myc, FOS, and Jun, some transcriptional factors that lead to cell proliferation (140).

Many miRNAs are resulted to target key components of PI3K and RAS pathways. Interestingly, with the aim to identify prognostic miRNAs for survival, Wu and colleagues performed *in silico* analysis from the miRNA expression profiles and clinical information of 481 HNSCC patients by TCGA dataset (141). They highlighted two miR-4652-5p and miR-99a-3p associated signatures, 8 hub gene and significant signaling pathways correlated with calcium, as well as cell proliferation, circadian entrainment,

EGFR, PI3K-Akt-mTOR, and P53 signaling pathways for estimating the prognosis of patients with HNSCC.

Let-7 miRNA family regulates the expression of *RAS* and several other cell-cycle related genes (61,142). Several HNSCC tissues showed a low expression of most miRNAs of the let-7 family (143). Recently, Manikandan and colleagues showed that let-7a, let-7d, let-7f, miR-16, and/or miR-143 were able to target 48 genes at different levels of the PI3K/Akt signaling pathway (144). Furthermore, the oncomiRs, miR-29b, miR-142-3p and miR-203 targeted 11 genes of the P53 signaling pathway. They hypothesized that the miRNA deregulated expression may simultaneously induce the constitutive activation of PI3K/Akt pathway and the suppression of the P53 pathway (144). In particular, the biological functions of miR-21 have been extensively investigated in many kinds of cancers, as well as in HNSCC. Its high expression represents a common feature of pathological cell growth, invasion, and chemotherapy resistance because among its target genes are present critical tumor suppressor such as phosphatase and tensin homolog (*PTEN*), programmed cell death protein 4 (*PDCD4*), hypoxia inducible factor-1 α (*HIF-1 α*) (48,145-148).

Next generation sequencing analyses of HNSCCs have identified frequent gene alterations including *TP53*, *CDKN2A*, *CASP8*, *FAT1*, *NOTCH1*, *HRAS*, *PIK3CA*, *MLL2*, and *FBXW7* (149). *TP53* is the most frequent target of genetic alterations in human cancers, with a prevalence of 40–80% in HNSCC (<http://www-p53.iarc.fr>). Whole-exome sequencing analyses reported that 58 *TP53* mutations occur in HNSCC with a frequency of 72% (Cancer Genome Atlas). The majority of the published results strongly indicate that *TP53* mutations produced p53 proteins with a gain-of-function activity contribute to enhance tumor progression, metastatic potential, and/or drug resistance of HNSCC (150). Previously it has shown that *TP53* mutations are associated with miRNA expression alteration in HNSCC (84). Some of the *TP53*-associated miRNAs predicting clinical outcome was also previously reported to be prognostic in HNSCC, as miR-151a-3p, miR-324-5p and miR-21 (151).

Interestingly, miR-96-5p is associated to *TP53* status and its high expression level, also associated with other miRNAs, resulted in prediction of local recurrence independently from other clinical co-variables either in tumors or in histologically tumor-free peritumoral tissue (53,84,120). In particular, it has shown that miR-96-5p in the mutant p53 HNSCC increased cell migration and it is involved in radio- and chemo-therapy resistance, at least in

part, by directly targeting *PTEN* transcript and maintaining high activation levels of PI3K-AKT pathway (53).

After many years of research, several groups have highlighted miR-34 as a key tumor suppressor miRNA whose target genes are involved in drug resistance mechanisms. A great number of studies reported that low levels of circulating miR-34 family members or tumor-specific miR-34 expression are associated with low response to chemotherapy (152-155). Re-expression of miR-34 in cancer cells was found to induce apoptosis, cell cycle arrest or senescence (156-158). miR-34a has found significantly repressed in HNSCC tissues and cell lines (159). Studies with tumor samples from HNSCC patients revealed an inverse relationship between miR-34a and *survivin* as well as miR-34a and *E2F3* transcription factor expression. The restoration of miR-34a levels in HNSCC cell lines strongly decreased *E2F3* and *survivin* levels and inhibited cell proliferation and angiogenesis (159).

miR-34 is emerging as a biomarker that could improve cancer precision medicine (160). To date, miR-34 is the only miRNA to have entered a clinical trial (ClinicalTrials.gov Identifiers: NCT02862145; NCT01829971). The delivery system with the best combination of efficacy, biodistribution and safety was the NOV340 technology consisting of ionizable liposome that forms a particle with a diameter of ~120 nm. This study was designed to investigate the biomarkers, pharmacodynamics, and pharmacokinetics of the liposomal micro-ribonucleic acid-34 (miR-34; MRX34) (161,162). Forty-seven patients including eleven cancer types are enrolled and the safety, maximum tolerated dose (MTD) and clinical activity of MRX34 were assessed (161). MRX34 successfully exhibited antitumor activity in the patients with refractory solid tumors but the clinical trial was withdrawn soon because of five immune related adverse events occurred (162). Furthermore, it was observed the unexpected accumulation of MRX34 in healthy tissues due to extravasation of nanocarriers. However, MRX34 in combination with dexamethasone pretreatment, showed a dose-dependent modulation of relevant target genes with a manageable toxicity profile in most of patients providing important information to improve miRNA-based cancer therapy.

The impact of miRNAs in cachexia

About 80% of patients with advanced cancer treated with radio and chemotherapy, including HNSCC patients,

are affected by cachexia (163,164). Cachexia is a complex multifactorial syndrome, impossible to treat with food supplements, characterized by the loss of mass and strength and density of skeletal muscle and by the loss of fat mass, with consequent progressive loss of body weight (165). Moreover, cachexia decreases the response to anticancer therapy, and consequently it is correlated with a poor outcome of the patients (165,166). Several groups focused their attention on the identification of molecular mechanisms that govern cachexia cancer-related to select disease biomarkers and new druggable targets. It is crucial to treat cancer cachexia in order to improve patient's quality of life to render anti-cancer therapy truly effective (167,168). Recently, miRNAs and other non-coding RNA are found to be involved in the pathogenesis of cancer cachexia (169,170). Animal cachectic models showed the association of miRNAs expression with the different features of cachexia (170,171).

In HNSCC there are still not many correlations between miRNA expression and cachexia as in other tumors (171). However, recently Powrózek and colleagues in a cohort of radiotherapy-treated HNSCC patients observed that low levels of miR-130a were associated to higher risk to be classified as cachectic, compared to those with high levels of miR-130a (172). Previously the cytokine TNF α has shown to be a target of miR-130a in cervical cancer cell lines (173). Accordingly, in HNSCC patients the low levels of miR-130a were associated with high plasma levels of TNF α confirming the key role of inflammation in the onset of cancer-associated cachexia (173). Furthermore, it was evaluated the role of circulating miRNAs in a cohort of advanced HNSCC patients treated with radio-chemotherapy (170). In the sera of patients miR-21 was found highly expressed and modulated by radio-chemotherapy treatment. These preliminary results are in line with other observations showing that microvesicles overexpressing miR-21 from lung and pancreatic cancer cell lines induced apoptosis of skeletal muscle cells by activating TLR7 signaling downstream of *JNK* (174). miR-21 oncogenic activities include increasing of cell proliferation, migration, invasion, metastasis, and resistance to chemotherapeutic treatments demonstrating to be an important biomarker of poor outcome in several solid and hematological malignancies (147,175).

For a wider and more complete reading regarding the involvement of miRNAs associated with cachexia during muscle and adipose tissue wasting in various types of drug-resistant tumors, it can be referred to specific reviews (170,171).

Extracellular microRNAs as biomarkers of HNSCC diagnosis and prognosis

miRNAs are very stable molecules and their expression can be measured in biofluids including plasma, serum, urine, and saliva. This has given more strength to the role of miRNAs as biomarkers in many processes related to HNSCC disease like radio- and chemotherapy resistance, cancer progression, and metastasis (176,177). In the recent years, powerful technologies and highly sensitive instruments have been developed to detect the presence of non-coding RNA in biofluids (178). Some examples of advanced techniques to detect nucleic acids used in liquid biopsy are beads, emulsions, amplification, and magnetics (BEAMing), Cancer Personalized Profiling by deep Sequencing (Capp-Seq), Safe-Sequencing System (Safe-SeqS), 'Tagged-amplicon deep Sequencing' (Tam-Seq), in addition to the next-generation sequencing (NGS) already in use in solid tumors (179,180).

miR-21 represents one of the most studied oncomiRs. Its high expression level and its oncogenic role has been reported in different tumors (181). miR-21 high expression was also reported in HNSCC cancer tissues and it was consistently reported to be higher in the plasma, serum-derived exosomes, and in the whole blood of patients compared to healthy controls (182-186). miR-21 expression has recently been reported to be a valuable tool for early detection of tumor recurrence after surgery or chemoradiotherapy and for monitoring patient outcome (183). A longitudinal study showed that the expression level of miR-21 purified by plasma of HNSCC patients was significantly reduced after two months of treatment in case of no relapse. On the other hand, plasma miR-21 did not show a decrease in treated patients who had developed recurrences during the follow-up period (183).

Unlike miR-21, miR-9 is considered to be a miR "tumor suppressor", in fact its lower expression in plasma was observed at recurrence or metastasis in nasopharyngeal carcinoma and in metastatic patients compared to patients without distant metastases (187). Human papillomavirus (HPV) is an etiological risk factor for HNSCC carcinogenesis and generally HPV positive HNSCC is more radiosensitive with a better prognosis than HPV negative HNSCC (188). miRNA expression patterns are found to be associated with HPV status in HNSCC patients (189). A group of HPV miRNAs were identified and among them miR-15a/miR-16/miR195/miR-497 family, miR-143/miR-145 and the miR-106-363 clusters

appear to have an important role in the pathogenesis of HPV (189).

Recent reports highlighted that HNSCC tumor microenvironment can regulate tumor response to radiation therapy throughout a crosstalk between tumor cells and the tumor microenvironment (190). Accordingly, it has been reported that miR-9 found enriched in HPV positive HNSCC exosome were able to enter into the macrophages inducing M1 macrophage polarization via downregulation of PPAR δ , a member of the ligand-activated nuclear receptor superfamily involved in inflammation and innate immunity (191). High miR-9 levels rendered HPV positive HNSCC more sensitive to the radiotherapy and further survival analysis suggested that increased level of miR-9 was positively correlated with improved prognosis (191).

About the therapy monitoring, several miRNAs are reported as biomarker from human HNSCC biofluids. For example, high expression of miR-142-3p, miR-186-5p, miR-195-5p, miR-374b-5p and miR-574-3p in the plasma correlated with worse outcome representing promising markers for prognosis and therapy monitoring in the plasma of HNSCC patients (192).

Interestingly, ten HNSCC- or radiation-associated miRNAs were detected in saliva samples (miR-93, miR-125a, miR-142-3p, miR-200a, miR-203, miR-213, let-7a, let-7b, let-7g and let-7i) (193). Furthermore, among them, miR-93 and miR-200a were significantly higher expressed 12 months post-radiotherapy than at baseline. Therefore, these miRNAs seem to be good candidate as biomarkers for the treatment monitoring post-radiation for HNSCC (193).

Recent study identified some miRNAs regulated in OSCC in comparison to a healthy adjacent tissue. The expression of the identified miRNAs in the circulation of same patients was checked (194). Among 48 differentially expressed miRNAs in tissue, 30 miRNAs were also found in the serum of the same patients. miR-32-5p was significantly found at high levels in tumor compared to healthy tissue and it was shown to be up-regulated in the serum. Therefore, this suggests a validated miRNA signature that can be used as potential non-invasive biomarkers of OSCC (194).

HNSCC, as previously mentioned, is typically characterized by a high rate of local recurrences. Biomarkers are able to early indicate the risk of incidence of relapse. Consequently, many research groups are looking for miRNA signatures that can be predictive. Citron

and colleagues, by using NanoString array in a cohort of 44 HNSCC patients, identified a signature of four miRNAs that classified the patients at high- or low-risk of recurrence (195). Targets of this signature were genes related EMT process and in particular belonging to druggable pathways (i.e., *SP1* and *TGFβ*) (195). SP1 and TGFβ inhibitors are already used in the clinical, thus the potential of the discovery of this miRNA signature has the immediate translational relevance.

Therefore, the discovery of miRNAs signature with a strong prediction power could give a great benefit to the HNSCC management not only for the diagnosis or the outcome of the disease but also during eventual clinical trials and the efficacy of the treatments.

Challenges for therapies and future aims

On June of 2020, the Food and Drug Administration approved nivolumab (OPDIVO, Bristol-Myers Squibb Co.) for patients with unresectable advanced, recurrent, or metastatic esophageal squamous cell carcinoma (ESCC) after prior fluoropyrimidine- and platinum-based chemotherapy (ATTRACTION-3 clinical trial: NCT02569242). Nivolumab and pembrolizumab, anti-PD-1 immune checkpoint inhibitors, were approved in 2016 for the treatment of patients with recurrent HNSCC refractory to platinum-based regimens (196,197). Immunotherapy is a novel option to treat HNSCC, designed to enhance the immune response to eliminate cancer cells. Thus, predictive biomarkers are urgently needed to classify the patients that may benefit from immunotherapy. Circulating miRNAs are already shown to have potential as predictive biomarkers for anti PD-1/PD-L1 treatment response in diverse cancer diseases (198,199). Recently, it was reported that let-7 family expression was significantly down-regulated in HNSCC, showing a negative correlation with PD-L1 expression (200). Furthermore, *in vivo* experiments showed that let-7a/b over-expression potentiated anticancer immunotherapy by CTLA-4 antibody treatment offering a novel potential strategy to enhance immune checkpoint therapy for HNSCC (200). To date, only two clinical trials in patients with recurrent/metastatic head and neck squamous cell carcinoma (HNSCC) are present that study the expression of circulating miRNA following treatment with immunotherapy as marginal aim of the project (ClinicalTrials.gov identifiers: NCT03843515 and

NCT03723967) (201,202).

From all the studies that have been cited and from many others that unfortunately has not been possible to comment, it is increasingly evident the involvement of miRNAs in pathways relevant to HNSCC cancerogenesis, metastasis, and resistance to treatment. The plethora of the *in vitro* and *in vivo* experiments that are collected by diverse researchers have proven that miRNAs may be useful for the early onset of tumor or the recurrence and for individualized treatment strategies. Although there are a very large number of scientific research articles in the literature, there is a considerable discrepancy on the real application of any miRNA-based biomarkers to the prognosis and treatment of HNSCC. In general, we could reflect that HNSCC is a very heterogeneous disease. Several *in vitro* data sometimes are weak, probably leading to inconsistent results among the studies. Hence, it is emerging the need to increase the number of prospective studies to give solidity to basic and translational research.

However, the combined use of these various miRNA signatures as biomarkers should provide a more personalized approach to clinical management of HNSCC patients which would ultimately lead to better disease outcomes.

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