Review of MicroRNA Proposed Target Genes in Oral Cancer.
Part II

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ABSTRACT

Objectives: Cancer is the product of alterations in oncogenes, tumour suppressor genes and most recently microRNA genes not as a single event or single change but rather as a multistep process. The role of microRNA genes in carcinogenesis is recently explored and appears to be an early event in the pathogenesis of this as well as other disease processes and occurs via gene regulation by their own products, the microRNAs. The purpose of this article was to review the literature concerning MicroRNA proposed target genes in oral cancer.

Material and Methods: A review of the available literature from 2000 to 2011 regarding the potential roles assumed by microRNAs in oral cancer was undertaken using PubMed, Medline, Scholar Google and Scopus. Keywords for the search were: microRNA and oral cancer and target genes, microRNA deregulation and oral cancer, microRNA and carcinogenesis in the head and neck/oral cavity. English language full length articles were reviewed.

Results: Several microRNAs deregulated in oral cancer have been functionally validated and their exact target genes have been identified. Furthermore the carcinogenesis pathways impacted by these alterations has been proposed for some of these microRNAs.

Conclusions: The expanding knowledge of specific roles of certain microRNAs is further contributing to our understanding of the complexity of tumour progression and behaviour. Consideration of this information and incorporation into treatment modalities through targeted therapy could potentially enhance our abilities to improve outcome especially when other established therapies have failed.

Keywords: oral cancer; head and neck cancer; microRNA; gene targeting; carcinogenesis tests.
INTRODUCTION

Cancer is the product of alterations in oncogenes, tumour suppressor genes and most recently microRNA genes not as a single event or single change but rather as a multistep process [1-6]. The role of microRNA genes in carcinogenesis is recently explored and appears to be an early event in the pathogenesis of this as well as other disease processes. MicroRNAs mediate gene expression at the posttranslational level by degrading or repressing target messenger RNAs (mRNA) or by translational inhibition of target genes. The possibility that microRNA involvement in cancer is simply a reflection of loss of normal cellular identity that naturally accompanies malignant transformation has been entertained. The identification though of microRNA genes in regions of the genome that are consistently involved in chromosomal rearrangements in cancer cells and do not contain oncogenes or tumour suppressor genes signifies a causative event in carcinogenesis [7-9]. The significance of microRNAs in cancer is accentuated by the fact that they can function as oncogenes by down-regulating tumour suppressor genes or as tumour suppressor genes by down-regulating oncogenes. MicroRNA genes’ up-regulation can be the result of amplification, deregulation of a transcription factor or demethylation of CpG islands in promoter regions. MicroRNA genes’ function as tumour suppressors is regulated by deletions, epigenetic silencing or loss of expression of one or more transcription factors [1,10-15].

The purpose of this article was to review the literature concerning MicroRNA proposed target genes in oral cancer.

MATERIAL AND METHODS

A review of the available literature from 2000 to 2011 regarding the potential roles assumed by microRNAs in oral cancer was undertaken using PubMed, Medline, Scholar Google and Scopus. Keywords for the search were: microRNA and oral cancer and target genes, microRNA deregulation and oral cancer, microRNA and carcinogenesis in the head and neck/oral cavity. English language full length articles were reviewed

MicroRNAs as oncogenes and tumour suppressor genes

The recognition that microRNAs can function as oncogenes came from studies on the microRNA-17 cluster that was found to be amplified in breast, stomach, lung, pancreatic cancers as well as B-cell lymphomas [16-25]. Another example is microRNA-21 that was first identified as a potential oncogene in glioblastoma and several other solid organ cancers (breast, prostate, stomach, lung) [19,26,27]. The function of microRNA-21 as an oncogene was further validated by demonstration of increased apoptotic cell death and decreased growth upon its inhibition in breast and glioblastoma cell lines. These studies support that microRNA-21 promotes tumorigenesis by suppressing apoptosis [26,27].

The first identification of microRNA function in tumour suppression originated with identification of loss of the locus of chromosome 13q14 where microRNA-15a and microRNA-16-1 are clustered in approximately one half of CLL (chronic lymphocytic leukemia) and other cancers such as prostate [28-30]. Finally the demonstration that the RAS oncogene is a target for the human let-7 family members has provided another potential role of microRNA genes as tumour suppressor genes [8,31]. The low expression of let-7 has been correlated with high expression of RAS in lung cancers, providing a mechanism by which microRNA function loss can be a promoter of tumorigenesis [32].

Several microRNAs were found to have specific alterations in oral cavity squamous cell carcinomas. Among these, several microRNAs have been functionally validated and their potential target genes have been identified. Some microRNAs have direct targets while others have indirect effect or target several genes.

Oral cancer related MicroRNAs potential target genes - direct interactions

MicroRNA-184 was identified to be among the 24 up-regulated mature microRNAs by at least 3-fold expression difference in laser microdissected cells from four tongue squamous cell carcinomas and paired normal controlled. The cellular response to microRNA-184 inhibition, demonstrated smaller and denser cells compared to control cultures. Proliferation assays showed reduced proliferation after transfection with microRNA-184 inhibitor compared with controls. In two of the cell lines (Cal27 and HN96) immunostaining for c-Myc showed that the c-Myc positive cells were decreased after transfection (34% versus 76% in Cal27 and 32% versus 78% in HN96). Furthermore in the transfected cell lines there was statistically significant difference in the percentage of apoptotic cells compared to the controls: Cal27: 10.64% versus 0.35%, HN21B: 7.05% versus 0.62% and HN96: 9.25% versus 0.78% (all P < 0.05, using Mann-Whitney U test). It was thus postulated that microRNA-184 acts as an oncogene by
inducing proliferation and inhibiting apoptosis potentially by targeting c-Myc [33]. Another study though has recently proposed a different explanation for the effects of microRNA-184 on epithelial cells and cancer cell lines, including Cal27 (an aggressive behaving cell line) via Akt signaling. Suppression of the Akt pathway that is associated with increased cell apoptosis and death was demonstrated with ectopic expression of microRNA-184 [34]. Additional studies to clarify these contradicting findings are required.

The function of microRNA-137 and microRNA-193a (studied together) as tumour suppressors was demonstrated in oral squamous cell carcinoma cell lines. It has been demonstrated that both microRNAs are silenced by tumour specific DNA hypermethylation using these cell lines. MicroRNA-137 when ectopically expressed in cell lines lacking its expression inhibited growth via Go-G1 arrest. MicroRNA-193a in contrast demonstrated same results by caspase-mediated apoptosis. After testing the potential targets based on computer predictions ([CDK6, E2F6, NCOA2/TIF2 for microRNA-137] and [E2F6, KRAS, MCL1, MYCN, PTK2/FAK for microRNA-193a]) the only statistically significant differences in protein levels and control transfectants revealed CDK6 as potential target for microRNA-137 and E2F6 for microRNA-193a [35].

Interestingly in a recent study microRNA-137 was found to induce cell cycle arrest at G1, inhibit invasion and target cell division cycle 42 (Cdc42) one of the best characterized members of the Rho GTPase family in colorectal cancer cells. Cdc42 has been shown to be up-regulated in several human cancers including head and neck. Consideration should be given to investigate Cdc42 as a target for microRNA-137 in oral cancers [36,37].

In the study by Wong et al. using laser-captured microdissected tissue from squamous cell carcinoma of the oral tongue microRNA-133a and microRNA-133b were identified to be deregulated compared to paired normal epithelial cells [33]. Reduction of proliferation rate and increase in apoptotic cells were noted when the cells from the tongue SCC cell line Cal27 were transfected. Computer target gene prediction proposed that pyruvate kinase type M2 (PKM2) transcription could be targeted by both microRNA-133a and microRNA-133b. In tumorigenesis replacement of the tissue specific PKM by PKM2 occurs and is believed to facilitate adaptation of cells to potentially low glucose and oxygen environment and facilitate tumour invasion. Up-regulation of PKM2 has been demonstrated in several tumours including colorectal, skin and gastric cancers [38-40]. The ability of tumour cells to adapt to microenvironment alterations is a key element for their survival and tumour progression. Ability to survive under hypoxia conditions or in the presence of chemotherapeutic agents by developing resistance is a major contributor to treatment failures and yet still poorly understood. Studies have shown that there is some contribution from microRNA deregulation to these adaptations by cancer cells as mentioned earlier. Partial regulation of the High Mobility Group A2 (HMGA2) protein, a member of the HMGA family, was identified to be controlled at least in part by miRNA-98 in cancer cell lines from the head and neck studied in hypoxia conditions. HMGA2 expression occurs predominately during embryogenesis. However, proteins from the HMGA family are implicated in differentiation, neoplastic transformation, integration and expression of viral genome. Studies of HMGA2 in breast cancer have shown association of increased expression with poor prognosis and metastasis. In oral squamous cell carcinoma the same protein has been implicated in acquisition of mesenchymal characteristics by the epithelial cells. MicroRNAs from the let-7 family were also found to contribute to changes of the protein expression during these hypoxia conditions [41-43].

**Oral Cancer related MicroRNAs potential target genes - complex interactions**

Cohen et al. demonstrated a DNA synthesis control involving microRNA-15a that affects cell cycle progression using a human tumour xenograft [44]. The xenograft involved injection of SQ20B tumour cells into limbs of female athymic nude mice. Protein kinase C alpha (PKCa) was identified as a key mediator of squamous cell carcinoma proliferation via activation of MAPK and negative regulation of microRNA-15a which inhibits cyclin E expression. These interactions lead to increased cell cycle proteins and thus enhanced DNA synthesis. In brief MAPK is shown to be required for DNA synthesis in SQ20B cells and activation initiates cell proliferation while growth is maintained via additional mechanisms. Cyclin E deregulation on the other hand has been associated with a number of highly aggressive tumours and poor prognosis, such as in laryngeal squamous cell carcinoma. Finally microRNA-15a is a DNA synthesis inhibitor. This feed forward loop involves protein kinase Ca that inhibits microRNA 15a that allows uninhibited cyclin E expression and thus promotes DNA synthesis [44-47].

Perhaps the most consistently deregulated microRNA that has been identified in malignant processes is microRNA-21. It is a well established oncogene that has been demonstrated to promote cell proliferation and suppresses apoptosis identified in various cancers.
including squamous cell carcinoma of the head and neck/oral cavity. Once it was realized that microRNA-21 transfected cells had a growth advantage over normal controls the potential targets were investigated. Several potential target genes have been suggested over the years. Zhu et al. demonstrated that microRNA-21 function as oncogene and its role in tumorigenesis is at least in part through regulation of the tumour suppressor gene tropomyosin 1 (TPM1) [48]. Two additional direct targets were identified in the same study: programmed cell death 4 (PDCD4) and maspin. Both these genes are implicated in cell invasion and tumour metastasis in breast cancers. It was soon realized that the actual role of microRNA-21 in tumorigenesis is accomplished by complex interactions with tumour/metastasis and suppressor genes [48]. A caspase activation that occurs with knockdown of microRNA-21, proposed by Chang et al., triggered the investigation of the potential role of microRNA-21 in the apoptotic pathway in head and neck cancers [49]. This was accomplished by studying the release of cytochrome c since it precedes activation of caspase. Inhibition of microRNA-21 caused decrease in cytochrome c release that taken together with the findings from transfection data suggests that the downstream targets of microRNA-21 are unlikely to be any of the major suppressor genes. The authors speculated that it would be more realistic that microRNA-21 inhibits several mRNAs and thus a cascade of events, that prevents apoptosis and thus increase in proliferation is initiated [26,49].

Using samples from tongue specimens and normal controls Li et al. were able to demonstrate a reverse correlation between two tumour suppressor genes and microRNA-21 [50]. Both TPM1 and PTEN (phosphatase tensin homologue) genes’ expression was inhibited by microRNA-21 in oral tongue cancers. TPM1 and PTEN immunohistochemistry staining was significantly different between cancer and adjacent normal tissues as well. Inhibition of microRNA-21 in tongue cancer cell lines reduced survival and anchorage dependant growth and induced apoptosis. Lastly, microRNA-21 was found to be an independent prognosticator of poor survival for tongue squamous cell carcinoma [50-52].

Ten oral squamous cell carcinoma (OSCC) cell lines and NHOK controls were used to study microRNA-125b and microRNA-100. Levels for both microRNAs were lower in OSCC cell lines than controls. Among the potential target genes altered by each microRNA, seven were studied further. KLF13, CXCL11, FOAX1 for microRNA-125b and LD1, EGR2, MMP13 and EGFR2 for microRNA-100. Transfection of OSCC cell lines with microRNA-125b resulted in the following interesting observations: KLF13 was down-regulated, CXCL11 and FOAX1 were up-regulated. Cell proliferation, along with modification of gene expression, is some important biological functions controlled by microRNA-125. KLF13 for example is a transcription factor and is involved in cell proliferation and differentiation of the heart in addition to development of B-cell and T-cell. Another interesting observation is that KLF13 is required for expression of cyclin D1 (and CCL5) that is known to be over expressed in human OSCC [53,54]. CXCL11 is a chemokine and a prominent ligant for CXCR3 that is involved in immunity, inflammation, and angiostasis and has been implicated in several cancers [55,56]. In an animal model CXCL11 increased survival and reduced metastasis [57]. FOXA1 a transcription factor has been shown to be involved in both events: growth stimulation and repression, while is responsible for expression of estrogen responsive genes. In prostate cancer FOXA1 over expression blocks metastasis and inhibits proliferation [58-60]. These observations may have therapeutic implications taking into consideration the function of the target genes altered by microRNA-125b.

MicroRNA-100 is down-regulated in head and neck cancers among others and transfection of OSCC lines modified the expression of several genes. EGFR3, a fibroblast growth factor receptor and predicted target for microRNA-100, is mutated in several malignancies including oral squamous cell carcinoma and is involved in development and proliferation [61]. ID1 a helix-loop-helix protein that inhibits transcription is over expressed in a variety of malignancies, including oral and esophageal squamous cell carcinoma and has been associated with metastasis in breast cancers [62-65]. MMP13, a member of the matrix metalloproteinase family of proteins, is responsible for activation of other MMP members and is altered in OSCC cells transfected with microRNA-100 and so is EGFR2 a transcription factor that is over-expressed in oral squamous cell carcinoma [66-70]. Both microRNA-125b and microRNA-100 have the ability to modify the expression of non target genes supporting their role of contribution to oral cancer via direct and indirect mechanisms.

Up-regulation of microRNA-24 has been observed in a number of cancers including OSCC [19,71,72]. In OSCC, specifically up-regulation of microRNA-24 appears to be associated with disease progression. The study by Liu et al. in 2010 using tissue samples and adjacent normal tissue confirmed the up-regulation of microRNA-24 in tongue squamous cell carcinoma, one of the most common subtypes of oral cancer [73]. It was further demonstrated that microRNA-24 expresses its
effects in part through targeting RNA-binding protein DND1, which in turn post-transcriptionally regulates its downstream genes, including cyclin-dependent kinase inhibitor 1B (CDKN1B). It has been shown previously that the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B) is up-regulated by DND1 at the post-transcriptional level [74].

Among the several important candidate targets for microRNA-24 predicted DND1 was of interest as DND1 has been shown to modulate microRNA activity by binding to the microRNA targeting sequences on the 3' UTR of the targeted mRNAs [74]. Therefore, DND1 post-transcriptionally regulates gene expression by inhibiting the microRNA access to the target mRNA. Previous studies demonstrated that DND1 is essential for the motility and survival of germ cells in zebrafish, and disruption of DND1 gene can induce testicular germ cell tumours in mice [75,76].

The group concluded that microRNA-24 is a multi-functional molecule regulator that regulates a variety of biological processes. One of its major roles in tongue cancer is regulating cell proliferation and apoptosis by targeting inhibitors of cyclin-dependent kinases (e.g., CDKN1B and CDKN2A). Furthermore, the results from the study suggested an intriguing mechanism for microRNA-24 to express its effects through targeting DND1, which in turn regulates a group of downstream genes at post-transcriptional levels [76].

MicroRNA-7 has been demonstrated to function as tumour suppressor in several cancers and was found to be down-regulated in oral tongue cancer cell lines [77-79]. Several proto-oncogenes are targeted by microRNA-7 including insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2) epidermoid growth factor receptor (EGFR), v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) and p21/CDC42/RAC1-activated kinase 1 (PAK1) [79-81].

MicroRNA-7 was initially studied by Liu et al. in 2009 in two oral cancer cell lines (UM1 and UM2) derived from the same patient that demonstrated though differences in aggressiveness [78]. The group demonstrated that UM1, the more aggressively behaving cell line, exhibited reduced microRNA-7 levels when compared to the less aggressive line UM2. Subsequent studies by Jiang et al. in 2010 using the same cell lines determined that insulin-like growth factor1 receptor (IGF1R) is a target of microRNA-7 in oral tongue squamous cell carcinoma cell lines [82]. It was further shown that down-regulation of IGF1R that in turn attenuated the insulin growth factor 1 (IGF1) induced activation of protein kinase B was mediated by microRNA-7. The activation of protein kinase B leads to reduced cell proliferation and cell cycle arrest and an increased apoptosis rate. The function of microRNA-7 on several key signaling molecules in several human cancers, including oral tongue cancer based on the findings from this study, indicates a contribution to carcinogenesis via multiple pathways and in various levels [82].

Dihydrofolate reductase (DHFR) gene was found to be a target for microRNA-205 which was consistently down-regulated in squamous cell carcinoma of the head and neck including the oral tongue compared to adjacent normal tissue. DHFR plays a critical role in folate metabolism and represents a target of methotrexate, a commonly used chemotherapy agent. DHFR activity is associated with the tumour suppressor gene p53 as both are targeted for degradation through the MDM2 ubiquitin ligase pathway. A specific MDM2 polymorphism, SNP309, has been correlated with poor prognosis in some cancers and early in head and neck cancer [83]. An additional link to these two pathways is through p14ARF that is frequently lost or mutated in these cancers. P14ARF activity results in increased degradation of DHFR that in turn results in resistance to folate antagonists such as methotrexate, in cell that have non functional, mutated p53 [84].

Two putative genes for microRNA-138 precursors, termed pre-microRNA-138-1 and pre-microRNA-138-2, have been predicted in mouse genome recently [85]. Their human homologs have been located on chromosome 3p21.33 and 16q13, respectively. Interestingly, loss of heterozygosity (LOH) at both chromosome loci has been frequently detected in HNSCC and appears to correlate with tumour progression (i.e., cervical lymph node metastasis) [86-88]. The study demonstrated that reduced microRNA-138 level is associated with enhanced metastatic potential in OSCC. The molecular mechanism(s) that underlie the effect of microRNA-138 on metastasis were further investigated by Jiang et al. in 2010 using bioinformatics-based prediction [89]. A total of 86 potential targets for microRNA-138 were identified [89]. Among those predicted targets, 3 of them are major players in the Rho GTPase signaling cascade. These targets are as follows: RhoC, 1 of the 3 Rho GTPases; ROCK2, a Rho-associated kinase and ARHGEF3, one of the guanine nucleotide exchange factors (GEFs). The Rho GTPase is a subfamily of the Ras superfamily. The members of the Rho GTPase family have been described as “molecular switches” that regulate cell shape, polarity and locomotion through their effects on many aspects of intracellular actin dynamics [90]. There are 3 Rho GTPases in human, RhoA, RhoB and RhoC, which share 85% amino acid sequence identity and exhibit distinct cellular functions [91]. RhoA plays key roles in the regulation of
actomyosin contractility as well as cell proliferation and survival. RhoB, which is localized primarily on endosomes, has been shown to regulate cytokine trafficking and cell survival. RhoC plays a major role in the regulation of actin cytoskeleton, cell shape, attachment and motility, which is highly relevant to cancer metastasis. Rho GTPases carry out these distinct functions by activating various downstream effectors, including Rho-associated kinases (such as ROCK1 and ROCK2). The activity of Rho GTPases is tightly controlled by several families of regulators, including guanine nucleotide dissociation inhibitors, GEFs and GTPase-activating proteins. These constitute the major players in the Rho GTPase signaling pathway [91]. Based on the bioinformatic analysis as well as the observed cellular changes associated with microRNA-138 noted by Liu et al. the group hypothesized that microRNA-138 regulates the cancer metastasis by targeting the Rho GTPase signaling cascade [77,78]. These findings were in agreement with previous studies demonstrating that the expression of RhoC is progressively increased as

Table 1. Commonly deregulated microRNAs in head and neck/oral cavity cancer (HNOCC) and their proposed target genes

<table>
<thead>
<tr>
<th>MicroRNAs deregulated in HNOCC</th>
<th>Proposed Target gene(s) additional mechanisms and findings</th>
<th>Author / Year / Journal</th>
</tr>
</thead>
<tbody>
<tr>
<td>microRNA-15a</td>
<td>PKCa (protien kinase c alpha) down regulates microRNA-15a that directly inhibits cyclin E</td>
<td>Cohen et al /2009 / Cancer Research</td>
</tr>
<tr>
<td>microRNA-103 and microRNA-107</td>
<td>PDCD4 (programmed cell death protein 4) TGFB1R3 (tumour growth factor receptor beta 3)</td>
<td>Ramdas et al. / 2008 / Head and Neck.</td>
</tr>
<tr>
<td>microRNA-205 and let-7</td>
<td>DHFR (dihydrofolate reductase) let-7 has been associated in oral cancers with decreased survival in cases with the KRAS-LCS6 genotype variant</td>
<td>Nakashima et al. / 2008 / Acta Otolar. Christensen et al. / 2009 / Carcinogenesis.</td>
</tr>
<tr>
<td>microRNA-125b and microRNA-100</td>
<td>KLF13, CXCL11 and FOXA1 and EGFR3 (epidermoid growth factor receptor 3)</td>
<td>Henson et al. / 2009 / Genes Chromosomes Cancer.</td>
</tr>
<tr>
<td>microRNA-24</td>
<td>DND1 (dead end 1, an RNA binding protein which in turn regulates a group of downstream genes at post-transcriptional levels)</td>
<td>Liu et al. / 2009/ FEBS letters.</td>
</tr>
<tr>
<td>microRNA-7</td>
<td>IGF1R (insulin-like growth factor 1 receptor)</td>
<td>Jiang et al./2010/Biochemical Journal.</td>
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tumours become more aggressively metastatic, and that RhoC expression promotes metastasis [92-94]. RhoC functions through direct interaction with its downstream signaling molecule, Rho associated kinases (e.g., ROCK1 and ROCK2), which in turn phosphorylate both a range of cytoskeletal proteins allowing for the generation of contractile forces and the ezrin family proteins that link the actin cytoskeleton to the plasma membrane. ROCK2 was identified as another direct microRNA-138 target gene in the Rho GTPase signaling pathway, and a highly conserved targeting site for microRNA-138 was identified in the 30 UTR of the ROCK2 mRNA. These results suggested that microRNA-138 regulates ROCK2 gene expression primarily by inhibiting translation. Metastasis requires passage across tissue boundaries, which is facilitated by increased cancer cell motility due to cytoskeletal remodeling. The Rho GTPases signaling cascade plays a central role in regulating cell adhesion, migration and the cytoskeleton [90]. Although Rho family gene mutations are relatively rare in tumours, over-expression of these genes are common events in cancer cells. The over-expression of key genes in Rho GTPases signaling cascade, including RhoC and ROCK2, has been frequently linked to enhanced metastatic potential in various cancer types [95-98]. A novel paradigm in which microRNA-138 regulates RhoC specific GTPase signaling cascade by targeting both RhoC and ROCK2 mRNAs concurrently and suppresses their expression at posttranscriptional levels was demonstrated in these studies. This provides further evidence that microRNA-138 functions as a metastasis suppressor gene in tongue squamous cell carcinoma and may serve as a novel target for patients with highly metastatic tumours (Figure 1). Table 1 provides the commonly deregulated microRNAs in head and neck/oral cavity cancer (HNOCC) and their proposed target genes for easy reference.

CONCLUSIONS

Extensive literature exists on microRNA and its role in carcinogenesis, with more evidence on specific roles of these molecules and their involvement in pathways known to be altered in this disease complex process. The expanding knowledge of specific roles of certain microRNA is further contributing to our understanding of the complexity of tumour progression and behaviour. Consideration of this information and incorporation into treatment modalities through targeted therapy could potentially enhance our abilities to improve outcome especially when other established therapies have failed.

ACKNOWLEDGMENTS AND DISCLOSURE STATEMENTS

The authors have no conflict of interest to declare.

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