International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: 6.614
Available Online at www.journalijcar.org
Volume 9; Issue 11 (A); November 2020; Page No. 23241-23246
DOI: http://dx.doi.org/10.24327/ijcar.2020. 23246.4602

Research Article

A GLANCE AT MICRORNAS AND THEIR TARGET GENE DEREGULATIONS INVOLVED IN ORAL SQUAMOUS CELL CARCINOMA

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ARTICLE INFO

Article History:
Received 13th August, 2020
Received in revised form 11th September, 2020
Accepted 8th October, 2020
Published online 28th November, 2020

Key words:
microRNAs, miRNAs .Oral cancer, Oral Squamous cell carcinoma biomarkers, oncogenic miRNAs, tumor suppressor miRNAs, miRNA gene polymorphisms, miRNA target gene polymorphisms.

ABSTRACT

MicroRNAs (miRNAs) have emerged as key players in many biological processes influencing malignant transformation of a normal cell. It has been proposed that an over or under expression of specific miRNAs can be utilized as potential marker for making diagnosis, predicting prognosis and streamlining therapeutic choices in Oral Squamous cell carcinoma (OSCC). However, more recently researchers are targeting beyond differential expression and digging into impact of Polymorphisms and Single Nucleotide Polymorphism (SNPs) in microRNA genes and even more interestingly the miRNA target genes. Aim of this review is to firstly summarize the differentially expressed miRNA in Oral cancer diagnosis and prognosis utilizing the information retrieved through article searched from PubMed. Secondly, to review the available data on microRNA gene and microRNA target gene polymorphisms and their impact on Oral Cancer and thirdly to identify any gaps which can be filled by future research. microRNAs have turned out to be promising potential biomarkers in Oral cancer, but larger prospective studies are required to validate particular targets and explore their clinical utility. Research on microRNA gene and microRNA target gene polymorphisms is still in its infancy and exploration of this area is likely to uncover interesting scientific information.

Sources of Data /Study Selection: An extensive literature search was done using PubMed database articles in English between 2004 and 2020. We used following terms for search: Oral cancer, Oral Squamous cell carcinoma, Head and Neck cancer and/or microRNA, miRNA profiling, differential expression, miRNA genes, miRNA Target gene SNPS. We summarized the existing literature by reviewing articles with main objective of finding the impact of differentially expressed miRNAs, miRNA gene polymorphisms and miRNA target gene polymorphisms on diagnosis, prognosis and therapeutic implications.After reviewing the abstracts, full texts of selected abstracts were retrieved.

INTRODUCTION

Oral cancer is a highly variable disease that evolves through multiple heterogeneous steps. The underlying complex mechanism involves multiple genomic and proteomic alteration[1]. Extensive research has been carried out to uncover the roles of genes which are involved in causing the oral cancer but very little has been done to explore the role of mi RNAs which control the expression of cancer genes[2]. We are in the phase of understanding how these novel gene regulators are involved in oncogenesis.

Micro RNA Discovery

The discovery of miRNA has challenged theory of Central Dogma, which is considered as back bone of molecular biology. It is based on transfer of genetic information from DNA transcribed to RNA which is translated to protein. This concept was prevalent for a very long time until it was discovered that there are many genes which are transcribed into small RNAs that do not translate into proteins, instead they bind mRNA and repress their translation. MiRNAs are the largest group of these non-coding RNAs. The first small RNA was discovered by Rosalind Lee in 1993 while she was

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working on *C. elegans*. She observed gene, lin-4 was silencing another gene lin-14. Furthermore, transcript of this gene shared complementarity with a sequence in 3′UTR of lin-14, the gene being silenced. Since lin-4 did not translate into a protein, she proposed these transcripts might be involved in silencing by RNA-RNA interactions.[3]

Mi RNAs are short highly conserved stable molecules present in abundance in both tissues and biological fluids.[4]In year 2000 first miRNA let 7 was discovered in humans.[5]Thereafter immense research has uncovered several important features linked to miRNA.The tremendous work done in this field has been reflected by a steep rise in miRNA based publications, with a huge number of original publications contributed by 84 countries.[6]

MicroRNAs Key players in cellular processes

Although miRNAs do not encode protein but are capable of regulating multiple protein coding genes. This unusual capability of miRNAs makes them a key player in cell proliferation, differentiation, apoptosis and tumorigenesis. These micromanagers of gene expression regulate nearly 60% of genes which encode proteins. Around 45,000 miRNA targets have been identified in 3′UTR of human genome.They reduce the protein levels of targetgenes by one of two mechanisms, which is either transcriptional silencing if there is incomplete complementarity or mRNA cleavage if there is perfect complementarity between miRNA and 3′ UTR sequence of target gene.[5]

MicroRNA Biogenesis

RNA Polymerase II or III transcribes miRNA into Pri-miRNA which are long primary transcripts. These Pri-miRNAs are then cleaved into 70-100 nucleotides Pre-miRNA by Drosha, an enzyme present within the nucleus that forms secondary hairpin loop structure. Pre-miRNAs are then translocated into cytoplasm by an enzyme exportin. Pre-miRNAs are further modified in cytoplasm into small double stranded RNA (ds RNA) molecules which contain a mature miRNA and its antisense strand. Helicase releases the dsRNA in the form of a duplex, and now mature miRNA is ready to be added to RNA induced silencing complex (RISC) and the antisense strand is degraded. After association with RISC, miRNA binds to 3′ UTR of the target mRNA to inhibit translation by sequestering the transcript mRNA.[5]

Distribution of MicroRNAs

MiRNA have been detected in various fluids including serum, urine, saliva, milk, semen, bronchial lavage, pleural fluid and cerebrospinal fluid. Yu li et al conducted a study to detect circulating miRNAs in serum and established that serum samples may serve as effective approach for detecting miRNAs making it a reliable method for development of miRNA biomarkers. MicroRNAs are stable in serum due to formation of ribonucleoprotein complex with Argonaute proteins and incorporation in to exosomes. They also concluded that when compared with plasma, serum is a better medium for miRNA detection because of absence of anticoagulants like heparin which tend to inhibit PCR.[7]

What makes miRNA very attractive are the facts that they are highly conserved across species, they can serve as attractive diagnostic and prognostic tools because they are abundantly expressed not only in tissue but also in serum, saliva and other body fluids.[4] Their Isolation and quantification is reproducible and miRNA therapeutics are currently in clinical trials.Most importantly they are mysterious and despite research still a lot is hidden and needs to be uncovered. Its presence in saliva is a very catching piece of information in terms of early detection of oral cancers by a noninvasive method.[8]

Role in Cancers

Over several decades mutations in protein coding genes were considered main driver of carcinogenesis until discovery of miRNAs. They act as important players in carcinogenesis because of their close proximity to chromosomal break points. They have been shown to regulate important biological pathways of cell proliferation and apoptosis. Research work has thrown light on the capability of the miRNA to function as oncogenes or sometimes as tumor suppressors. Calin et al elucidated for the first time association of altered miRNA expression with malignancies and later many clinical and experimental studies corroborated such associations.[9]The differential miRNA signatures may serve as important tools for cancer diagnosis and prognosis. Role of miRNA in indicating prognosis, determining the tendency of recurrence and metastasis and predicting the response to treatment have been observed in multiple studies. Most catching aspect of miRNA is the novel therapeutic applications that can be put forth to regulate the altered miRNA expression. Altered miRNA expression is seen either in the form of over expression or under expression, therefore, strategies can be employed to reduce or increase miRNA respectively.[10]In order to reduce miRNA expression, anti-microRNA oligonucleotides (AMO) are introduced which compete with endogenous miRNA. This technique is further improved by adding 2′-O-methyl and 2′-O- methoxyethyl to the 5 prime end of AMO which increases their effectiveness and stability. The advent of small molecule inhibitor against specific microRNA is a further advanced strategy for over expressed miRNAs. Strategies are also designed to improve expression of miRNAs which are under expressed and function as tumor suppressor genes. This can be achieved by introducing these miRNA through viral vectors which have very good gene transfer ability but this may cause immunological reactions and it may not be tumor specific. To reduce immunogenic reactions, miRNA are introduced through liposomal system but it has low transfer ability. [11]

Micro RNA involved in OSCC

The micro RNAs involved in OSCC can be reviewed under three important headings as shown in Figure 1. First and most extensively covered is the differential expression of certain miRNAs, second is polymorphisms in miRNA genes and third is polymorphism in miRNA target genes.miRNA gene polymorphism and target gene polymorphismsare still in their infancy and more research is needed.

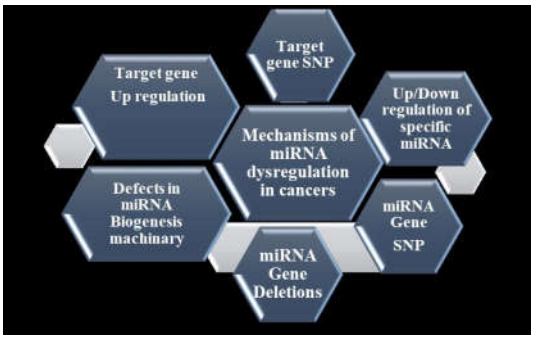

Figure 1 Mechanisms of miRNA associated dysregulation in oral cancer

MiRNA differential Expression Differential expression can be in the form of over or under expression**.** To evaluate the role of miRNA, 1168 miRNAs were analyzed in Oral Cancers via microarray. 46 miRNAs had altered expression with let-7a, let-7d, let-7f and miR-16 were under expressed while miR-29b, miR-142-3p, miR-144, miR-203, and miR-223 were over expressed in OSCC.[12] Tran et al conducted research on nine different head and neck cancer cell lines and investigated 261 mature miRNA genes on oligonucleotide array. They observed overexpression of 33 miRNA andunder expression of 22 miRNAs. They further identified that five up regulated miRNAs (miR-16, let-7b, miR-338-3p, miR-223, and miR-29a) have the potential to be used as biomarkers for oral cancer diagnosis.miR-16 and let-7b act as tumor suppressors and they have a low expression in many cancers and they downregulate oncogenes likeRAS and BCL2.[13] Soga et al conducted a research to identify differential miRNA expression in oral cancer and normal oral mucosa. On a micro RNA array of 768 miRNA sites, 177 miRNAs were differentially expressed. Out of these 177, an upregulation of more than 4 folds was observed in 12 miRNA (miR-31*, miR-31, miR135b, miR-193a-5p, miR-103, miR-224, miR-93, miR-200c, miR-183, miR-203, miR-21 and miR-223). The mechanism of these upregulations needs to be explored. Moreover three miRNAs (miR-489, miR-1291 and miR-483-5p) were down regulated in metastatic cancer suggesting a possible role in determining disease prognosis.[14]

Up regulated/over expressed miRNA (Oncogenic miRNAs)

Qin et al established that OSCC demonstrated over expression of miR-1290 which was associated with tumor metastasis, epithelial-mesenchymal transition and bad prognosis. They also identified that cyclin G2 (CCNG2) was a target of miR-1290 and its expression was reduced in OSCC. [15]Over expression of miR-29b, miR-144, miR-203, miR-142-3p, and miR-223 were observed by Manikandan and co investigators. These miRNAs target p53 signaling pathway genes which play a vital role in apoptosis, evasion of which is an important mechanism of carcinogenesis.[12]Another extensively studied miRNA is miR-21 which acts like an oncogene. Its targets include important genes including PTEN, a decreased expression of which promotes cell cycle progression, proliferation and inhibit apoptosis. [16]Target genes for mir 21 programmed cell death 4 (PDCD4) is an important player of invasion and metastatic potential of tumor.[17] *microRNA-184* acts as an oncogene by inducing proliferation and inhibiting apoptosis potentially by targeting c-Myc. Upon inhibition of *microRNA-184* cells became smaller and denser.[18]*microRNA-133a* and *microRNA-133b* enables cancer cells to survive in low glucose and oxygen conditions by replacing tissue specific PKM by PKM2. An up regulated level of PKM2 is observed in several tumors including colorectal cancers. [19, 20]

Down regulated /Under Expressed miRNA (Tumor Suppressor miRNAs)

Xiang Li and co-workers observed reduced expression of miR-144-3p in OSCCA cells. Tumor cell growth and infiltration was seen to be inhibited bymiR-144-3p in vivo and in vitro. Furthermore,miR-144-3p led to reduced Endoplasmic reticulum oxidoreduction-1-like (ERO1L) consequently decreasing function of signal transducer and activator of transcription 3 (STAT3) in tumors.[21]Zhang et al found that miR-4282 can be used as a biomarker in OSCC. miR-4282 acts by inhibiting LIN28B which is thought to be responsible for stabilizing mRNA in malignancy. LIN28B in turn down-regulated ZBTB2 leading to reduced tumor cell proliferation.[22]Cunjirigala and colleagues determined thatmiR-107 was under expressed in OSCC cells as compared to normal oral epithelial keratinocytes. They further demonstrated reduced OSCC cell growth when the cells were transfected with miR-107. TP53 regulated inhibitor of apoptosis1(TRIAP1) was found to be a direct target of miR-107.[23]Manikandan et al studied microRNA expression profiling in OSCC and observed that under expression of let-7a, let-7d, let-7f, and miR-16. These downregulated miRNAs target PI3K/Akt signaling which is responsible for limitless replication. They also found that miR-223 is linked to increasing tumor size and a late tumor stage whereas miR-1275 is associated with local lymph node metastasis.[12]Let-7, miR-155 and miR-146a under expression is associated with progression to metastatic tumors.[24]miR-375 is under expressed in over 91% OSCC and its low levels correlate with poor survival and distant metastases.[25]Kozaki et al demonstrated that *microRNA-137* and *microRNA-193a* are down regulated in OSCC and their potential targets are CDK6 and E2F6 respectively. Moreover, *microRNA-137* induces arrest of cell cycle at G1-S checkpoint stage in OSCC.[26] Fukumoto and colleagues established that *miR-26a* and *miR-26b* prevented tumor cell infiltration. *TMEM184B* gene is a target of *miR-26a/b.* Cellular migration and invasion can be inhibited by silencing it.[27]

Micro RNA gene polymorphisms in oral cancers

Polymorphisms in miRNA genes can have impact on function of miRNA in a number of ways including their biogenesis, stability and effect on target genes. Around 10 million SNPs are identified in Human genome involving both coding and non-coding regions of DNA. SNPs in coding region are well known for many human diseases by causing deletion, frame shift, non-sense or mis-sense mutation.[28]Recent research is directed towards identifying SNPS in non-coding regions and their impact on human diseases. Such SNPS will in turn modify Transcription Factor Binding sites or create new Transcription Factor Binding site. Theoretically speaking SNPs in miRNA genes may abolish or generate a target site but practically they are fine tuners instead of an on and off shut mechanism, thus a SNP may actually enhance or diminish binding to target site.[29] SNPS may also be present in pre miRNA but interestingly such SNPS are seen in less than 10% of pre miRNA and less than 1 percent of them are in the seed region.[27]Tandon et al conducted a study on 200 biopsy proven

cases of OSCC in Indian population to evaluate miR-499 A/G and miR-149 C/T polymorphism. They concluded that miR-499 A/G and miR-149 C/T polymorphisms maybe associated with OSCC.[30]Yin et al examined role of polymorphisms in miR-499 rs3746444, miR-196a2 rs11614913 and miR-146a rs2910164 in genetic predisposition to oral cancer in Chinese subjects. They observed Polymorphisms in miR-499 rs3746444 was linked to increased risk in male OSCC patients. C allele of miR-499 (rs3746444) had a statistically significant association with increased risk of OSCC. However, miR-196a2 rs11614913 and miR-146a rs2910164, polymorphisms failed to show a statistically significant associations with risk of OSCC.[31] Several studies have explored the relationships of SNPS in miRNA and head and neck cancers risk. Christensen et al found miR-196a2 rs11614913 polymorphism increase the risk of head and neck cancers.[32] Contrary to that Liu et al did not report any influence of miR-146a rs2910164 and miR-196a2 rs11614913 on head and neck cancer riskandinterestingly miR-499 rs3746444 variant resulted in reduced risk.[33]Song et al found that miRNA variants modify risk of HPV16-associated OSCC in nonsmokers.[34]

What makes any marker more exciting is its possible role in pre malignant condition. Variant allele of mir26a-1(rs7372209) increases risk of oral premalignant lesions (OPL) by more than 2 folds. Cases harboring *GEMIN3* rs197412 variant allele are found to have a significantly decreased risk of OPL risk whereas SNPs in *TRBP (*rs784567) and *GEMIN4 (*rs3744741) have a borderline significant association with risk of Oral precancerous Lesions.[35]Liu et al, examined impact of polymorphisms in pre-miRNA *hsa-mir-146a, hsa-mir-149, hsa-mir-196a2* and *hsa-mir-499* with risk of Head and Neck Squamous cell Carcinoma (SCCHN) in non-Hispanic whites. *Hsa-mir-499* polymorphism was the only one significantly associated with increased risk of SCCHN. They attempted to explore the effect of combined risk genotypes of all the four polymorphisms on risk of SCCHN and observed cases having variant alleles of all four genes had a 40% higher risk of SCCHN as compared to cases with no or single polymorphism. Young men who never smoked had the highest risk.Variant allele C of *pre-miRNA-146adecreases expression of both pre-miRNA-146a* and mature miRNA-146a; and *hsa-mir-146a* GC heterozygous genotype increases risk of papillary carcinoma of thyroid.[36]

Effective miRNA- MRNA Association

It is important to determine the right targets in effective miRNA-MRNA association. This begins by first of all identifying the critical binding match. Critical region for miRNA-MiRNA binding is the seed region (nucleotide 2-7 from 5′ UTR) of miRNA.[37] A base pairing of 8 nucleotide is ideal and more effective as compared to a 7 or 6 nucleotide base pairing and a 4 nucleotide base pairing would be completely nonfunctional. Effective binding means hydrolysis of mRNA phosphodiester bond and thus suppression of translation. For this absolute base pairing at nucleotide position 9-12 of mRNA is required. If there is additional base pairing between 3′ region of miRNA and MiRNA it is referred to as productive seed pairing. Once the match is confirmed, next step is to confirm conservation of the sequence in at least 4 species. The next step would be to confirm if the target is effective target. For this purpose the level of miRNA is altered and then the impact of altered level is analyzed on the mRNA level. This can be achieved by Dicer Knock out, Dicer is an important enzyme in miRNA biogenesis and knocking it out means no miRNA and there should be subsequent increase expression of miRNA in the absence of miRNA targeting which will confirm on arrays.

Mirna Target Gene polymorphisms

Genetic polymorphisms in miRNA target genes serve as important contributors in great genetic diversity seen in several human diseases including cancers. However these polymorphisms may be encountered in either pre-miRNA or within seed region of a mature miRNA. Interestingly, sometimes the miRNA has no polymorphism but it is an altered nucleotide sequence of miRNA binding site of the target gene upon which miRNA acts. Such SNPs are referred to as polymirts. Several researchers have attempted to explore the impact of polymirts on human diseases; growing data base and high throughput detection techniques has facilitated such detections.[38]

Saunders et al studied 3′ UTR of human genome from mammalian genome collection (National Centre for Biotechnology Information Build). Of 45,000 well annotated human transcripts 3′ UTR sequences of 18,000 genes were identified and 29,000 target sites were discovered of which 25,000 target sites were non- overlapping. Taking in to consideration the small size of target it is very likely for a SNP to have functional implications. Despite extensive research we are lacking clear understanding of miRNA-mRNA association. What we know is that this association involves 6-8 nucleotides base pairing between the two however on an average human 3′ UTR has a size of950 nucleotide. This makes it quite possible for one 3′ UTR to interact with multiple miRNAs. So a SNP in 3′ UTR target gene can have much more diverse implications for their ability to affect multiple miRNAs.[37]

Bhattacharya et al have grouped polymorphisms at miRNA target sites into four groups based on the impact of polymorphic allele on miRNA target gene. If it disrupts a conserved site, it is grouped 'D','N' if it disrupts non conserved site, 'C' if a new site is created and'O' if impact cannot be determined. They identified 22919 SNPs in miRNA target genes using two data bases miRecords and miRtasBase.[38]

The first miRNA target site SNP in KRAS impacting survival in Oral Cancer was discovered by Christensen et al .Let-7 microRNA binds to 3′ UTR of KRAS and induces its down regulation. A SNP at miRNA binding site of KRA Screates a variant allele KRAS LCS6. They conducted a population-based case–control study to examine prevalence of KRAS variant allele and its association with oral cancer risk and survival. They did not observe any association of variant allele with increased risk but individuals harboring variant allele had a reduced survival in oral cancer patients. This variant may be used as an additional prognostic marker for oropharyngeal squamous cell carcinoma.[39]Zhang et al discovered an increased risk tumor recurrence or a new primary with polymorphism rs3747238 in miRNA binding site of the SMC1B gene in early stage head and neck cancer.[40] An SNP is created in CASP3 gene at the miR-885-5p binding site as a result of alternative splicing or polyadenylation is linked to increased risk of primary HNSCC and recurrent primary tumor.[41]Wang Y et al discovered another SNP rs5030486 within the 3′ UTR of *TRAF6* which was found to increase the risk of OSCCA in

Chinese Han population and they suggested it may be used as a potential biomarker.[42]Table1 summarizes SNPs miRNA target genes in Head and Neck cancers and their impact.

In future researchers will focus on altered miRNA expression as a result of other non-coding RNA or RNA binding proteins competing with miRNA for target site binding. In oral cancer, RNA binding protein dead end 1 is targeted by miRNA-$_{24}$ which affects p53 network through its effect on cyclin dependent kinase inhibitor 1.[43]

Future research should focus onvalidating the markers that have already been discovered, identifying new markers for early diagnosis, disease severity, prognosis, response to treatment, targeted therapy to replace or block specifically deregulated miRNA via antisense and miRNA mimics through improved method of delivery via virus based particles and nanoparticles. This should be followed to evaluate the effect of targeted therapy in treating the cancer and preventing recurrence and metastasis.

Table 1 SNPs in miRNA binding sites of target genes in OSCC

SNP	Target gene	Effect	Authors
rs61764370	KRAS	Reduced survival	Christensen et al., 2009
rs3747238	SMC1B	Tumor recurrence	Zhang et al., 2010
rs1884056	BCL2	Tumor recurrence	Zhang et al., 2010
rs3809865	CASP3	Increases risk of primary and recurrent tumor	Sethi et al.,2014
rs3809865	Integrin *B3*	Increased risk of cancer	Wang et al.,2014
rs2675	Integrin *b5*	Cancer progression	Wang et al.,2014

SNP-Single nucleotide polymorphism, KRAS- Kristen Rat sarcoma, SMC1B-Structural maintenance of chromosome protein 1 b, BCL2-B-Cell lymphoma 2, CASP3-Caspase 3

CONCLUSION

It can be concluded through literature search that miRNA differential expression holds a future potential role **in predicting diagnosis and prognosis** of oral cancer. Moreover, polymorphisms in miRNA genes and miRNA target genes are exciting additions in the miRNA research, it can be inferred from the available data that they may serve as future prognostic tools but only limited work has been done in this area. Additional large scale studies into potential ability of these tools is expected to validate the existing data and fill the existing gaps.

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Study has not been presented any where
***Disclaimers*: None**

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