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Epigenetic Role of Onco-miRs in Oral Squamous Cell Carcinoma: A Clue to Modern Medicine

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Abstract Epigenetics has marked the beginning of a new era in the field of Genetics and Genomics. It can unravel mysteries about the intricate mechanisms underlying the process of development, differentiation and maturation of cells. Epigenetics may be considered as an "epicentre" of modern personalized medicine because of its influence over phenotypes which is driven by factors like nutrition, environment, exposure to toxic chemicals, etc. These risk factors are known to bring about a permanent change in the epigenetic profile of an individual. Thus, the epigenetic landscape defines the disease presentation and progression with a great precision. Oral cancer is one of the major causes for morbidity and mortality worldwide. Dysregulation of gene expression due to chromosomal aberrations have been extensively reported in the case of oral squamous cell carcinoma (OSCC). Recently, in addition to genetic processes, epigenetic mechanisms have also been shown to play a central role in the development and progression of disease. Exhaustive investigations in the field of cancer biology employing innovative technologies have led to the identification of biomarkers that are useful in characterizing oral cancers. miRNAs are regulatory elements which could exhibit oncogenic or tumor suppressor activity depending upon the cell type and its biological environment. Upregulation of miRNAs have been implicated in the tumor progression and invasion in OSCC. This review, will present a comprehensive note on the epigenetic role of Onco-miRs in the etiology of OSCC. A prelude on these onco-miRs as diagnostic markers and a potential target for treatment of OSCC will also be discussed briefly.

Keywords: *Epigenetics, miRNA, oral cancer, oncogenes, 3'-UTR*

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [23]. More than 0.2 million new cases of head and neck cancers are diagnosed in India each year. Oral cancer is the predominant form of HNSCC which accounts for about 30% of all cancer cases in the country. About 80,000 oral cancer cases are being diagnosed annually [49]. Oral squamous cell carcinoma is the most common and aggressive type of oral neoplasm which covers over 90% of all mouth malignancies [50]. Death due to OSCC is around 100,000 and has shown an increase over a period of time worldwide. The incidence of oral squamous cell carcinoma remains high, which is precipitated by a plethora of high risk factors like smoking, alcohol intake and tobacco chewing. Other risk factors such as chronic inflammation, viral infections, exposure to carcinogenic chemicals can contribute towards pathogenesis of OSCC [14]. Carcinogenesis is a process which has been extensively studied for several decades. Numerous genetic aberrations are involved in this process which is elucidated by *invitro* and *invivo* studies. Recently, the role of epigenetic factors in the development of cancer is widely debated. Studies have recognized and established "epigenetics" as the "hallmark of cancer". Epigenetics involves those heritable changes in gene expression that are not coded in the DNA sequence. They tend to occur more frequently than mutations in a gene and may persist for the entire life period of an organism or even for multiple generations [27]. Many questions about common complex genetic disorders like cardiovascular diseases, diabetes, neurological disorders, cancer etc., have been explained in terms of epigenetic mechanisms. Since epigenetic modifications are transgenerational, it is of great importance to study their effect on the genome. Epigenetic process also explains as how, two identical genotypes reared in the same environmental condition generates two different phenotypes.

2. Onco-miRs in Regulation of Gene Expression

MicroRNAs are small, noncoding, evolutionarily conserved, pleiotropically acting, single stranded RNA

molecule of about 22 nucleotides, known to regulate about 30% of mRNA translation and decay. More than 1,000 miRNAs are known to possibly control over 30,000 target genes. Basic cellular processes such as differentiation [37], proliferation [33], development [60], apoptosis [55], regulation of cell cycle [32] are influenced by miRNAs. More than 1898 unique mature miRNAs have been identified till date [14]. The regulatory mechanism involved in the translational inhibition involves an imperfect base pairing of miRNA with 3'-untranslated region (3'-UTR) of target mRNAs of protein coding genes which leads to the cleavage of homologous mRNA or translational inhibition [17]. miRNAs can be transcribed from intergenic regions independently (intergenic miRNAs) or cotranscribed along with the intron of a host gene (intragenic miRNA). Promoters specific for miRNA have also been described for intragenic miRNAs [43].

miRNA is transcribed by RNA polymerase II (Pol II) to generate a long primary transcript miRNA (pri-miRNA). The pri-miRNA is recognized and cleaved by RNase III enzyme Drosha and its binding partner Pasha yielding precursor miRNA (pre-miRNA). The pre-miRNA produced is about 70-90 nucleotides in length and has an imperfect stem loop hairpin structure. It is then exported to cytoplasm by exportin 5 in a Ran-GTP-dependent mechanism. The pre-miRNA is cleaved by a complex formed by Dicer and transactivation responsive RNA binding proteins (TRBP), resulting in a double stranded RNA duplex that contains both the mature miRNA strand and its complementary strand. The miRNA duplex is unwound by RNA helicase and the single stranded mature miRNA is incorporated into RISC (RNA-induced silencing complex). The miRNA then guides the RISC to complementary sites within target mRNA (Figure 1) [69,70,71].

3. Biogenesis of miRNA

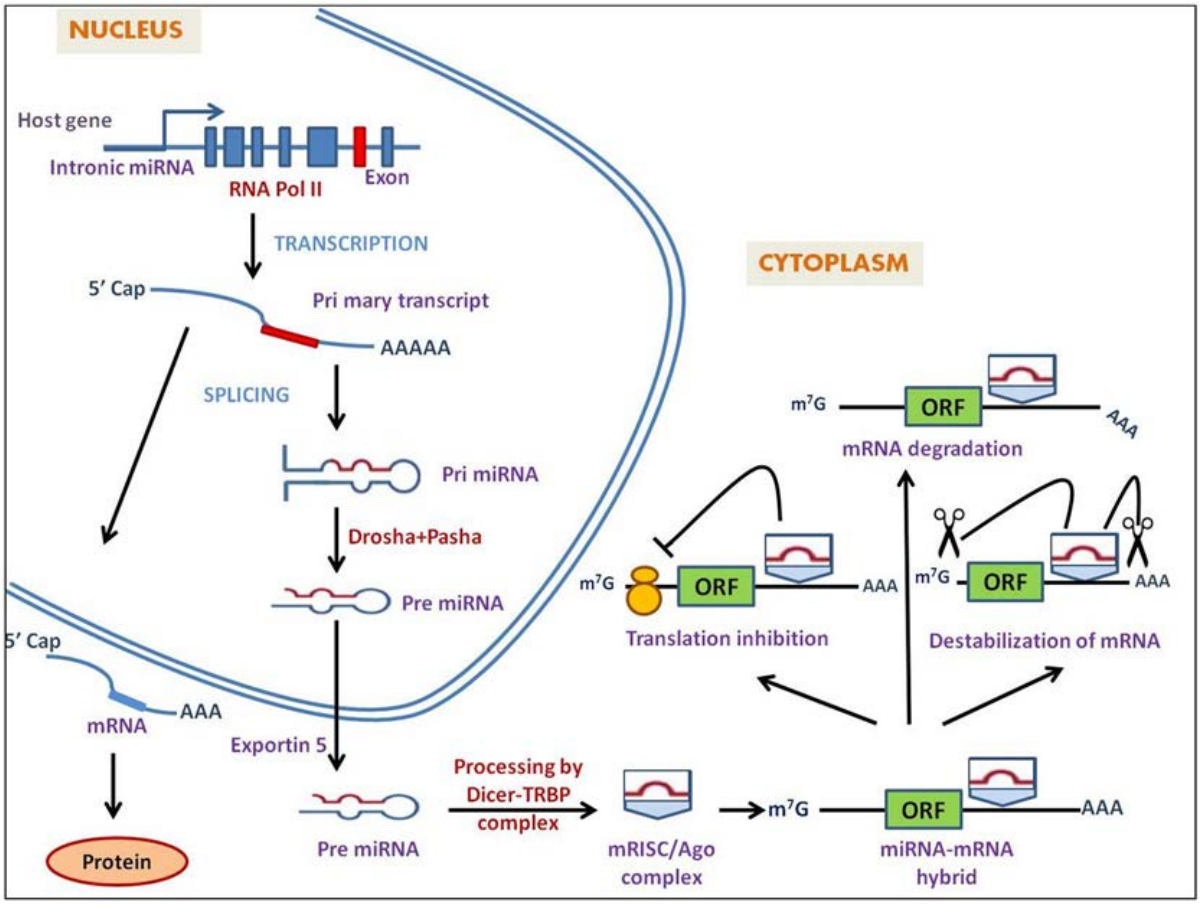

Figure 1. miRNA biogenesis and its possible functions

miRNAs could either act as oncogenic entities (onco-miRs) or tumor suppressors (tsMiRs) based on the cell type and the biological environment. The "oncomirs" or oncogenic miRs, promote tumor development by inhibiting tumor suppressor genes and or genes that control cell differentiation or apoptosis [10]. Since the miRNA patterns are tissue specific it is possible to discriminate carcinoma and normal cells based on their expression profile [56]. Transcriptional profiling studies on the differential expression of miRNAs in normal and cancer tissues have opened new avenues in the field of molecular diagnosis and prognosis [9,61]. These miRNA biomarkers can be used as surrogate markers for identifying tumor progression. These markers can be used to detect, categorize and predict the severity of the cancer type, wherein the profiles of specific miRNA expression seemed to be linked more aggressive form of cancer. Knocking down the oncogenic miRNAs may stunt the cancer growth and can serve as potential targets in treating cancer. In contrast, restoring the expression of tumor suppressor can also be a powerful approach towards cancer treatment.

Saito et al, in the year 2006 made an exciting discovery revealing the epigenetic mechanisms of miRNA. They found that the expression of miR-127 was found to be upregulated in cancer cell lines after treatment with 5-Aza-CdR, a methylation inhibitor and 4-phenylbutyric acid (PBA), a histone deacetylase inhibitor [47]. Both these compounds reduced DNA methylation levels and enable more open chromatin structures, which lead to the re-expression of miR-127 and other genes that had been silenced epigenetically in tumor cell lines [12]. This suggests that epigenetic mechanisms could control the expression of miRNAs. miRNAs exert their effect by a) epigenetically modulating enzymes which play a key role in methylation mediated silencing and chromatin remodelling [59] or by b) epigentically regulating themselves and also c) in a paracrine fashion via exosomes, microvesicles and protein complexes to influence tumor microenvironment [39]. They also promote the release of mediators which activate pro- or anti-cancer immune activity [3].

mir-21, -345, -181b, -184, -520g, -649, -518b, -146a [5,6], miR-24 [29], miR-181 [62,63], miR-30b [42], miR-31 [19,20], miR-494, miR-3651 [46], miR-155 [41] are a few micro RNAs whose expression is found to be increased in OSCC. Several miRNAs are being analysed and new miRNAs are being added to the list of potential oncomirs associated with the development and progression of OSCC. Detailing on the role of each miRNA would make this review more exhaustive, hence the epigenetic mechanisms involved in some of the common oncomirs in OSCC has been chosen for discussion.

4. Secretory miRNAs

There are several pathways by which miRNAs enter the blood stream, a) secreted as free miRNAs or b) encapsulated within microvesicles [65]. Significant amounts of extracellular miRNAs are enclosed in small membranous vesicles such as exosomes (multivesicular bodies), shedding vesicles and apoptotic bodies or packaged with RNA-binding proteins like high-density lipoprotein, Argonaute 2 (Ago2) and nucleophosmin 1 (NPM-1) [8] (Figure 2). Studies have also showed that the release of miRNAs into extracellular compartment is mediated by ceramide-dependent secretory exosomes. The level of ceramide is regulated by neutral sphingomyelinase 2 (nSMase2). Knockdown studies indicate a reciprocal relationship between the expression of nSMase2 and miRNAs [26]. Two other groups have identified Argonaute 2 a component of plasma miRNA-protein complex, which is responsible for the stability of non-vesicle associated plasma miRNAs [1,66]. Thus, the extracellular miRNAs remain protected from degradation by ribonuclease-abundant serum and other body fluids [7,16].

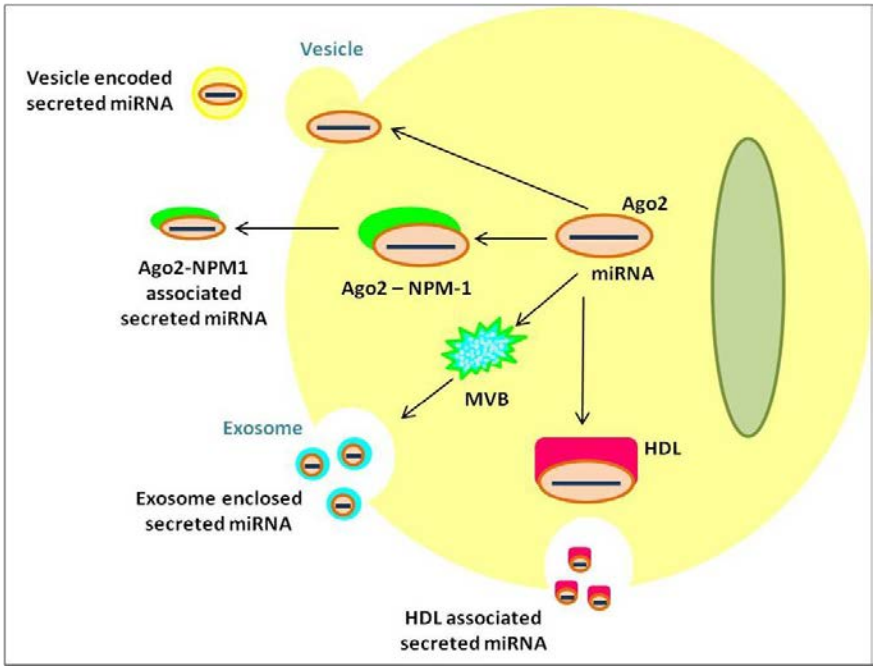

Figure 2. Active secretion of miRNA

Application of secretary miRNA as a novel biomarker in the diagnosis of OSCC is still in its juvenile stage. Park et al, [44], demonstrated the presence of salivary miRNAs and their association with OSCC. According to the study, two markers miR-125a and miR-200a, were found to be significantly low in OSCC patients when compared to healthy controls. Another study found five different miRNAs namely, miR-16, let-7b, miR-338, miR-223 and miR-29a to be strongly associated with high grade oral lesions [35]. An amalgamation of secretary miRNA profiles along with routine screening methods would improve the sensitivity of oral cancer detection.

4.1. miR-31

Micro RNA-31 has been shown to exert pleotrophic effect in several forms of cancer. miR-31 has been reported to activate hypoxia pathways by targeting the *FIH* (Factor inhibiting HIF1α) gene [31]. Exogenous introduction of miR-31 along with human telomerase reverse transcriptase (h*TERT*) into normal oral keratinocyte cells (NOKs) caused immortalization of oral

keratinocytes and the cell line was designated as M31OK3 [19]. High miR-31 expression is associated with high levels of VEGF and lower levels of E-cadherin in OPMD (oral premalignant disease) tissue. Although, the molecular mechanisms underlying miR-31 up-regulation in OSCC is still obscure, research on this miRNA has unravelled their putative role in tumor development.

A recent study showed that the expression of miR-31 was found to be higher in the early stages of tumor with the absence of lymph node metastasis, when compared to the late stage of OSCC [52]. Aberrant expression of miR-31 in the early stages enhances proliferation and tumorigenicity of OSCC by inhibiting the negative regulators of oncogenic pathways [34]. p16 is a tumor suppressor gene, whose loss of function has been associated with tumor development. This event confers malignant and metastatic potential of buccal SCC [11]. Pathway analysis disclosed the fact that p16 was negatively regulated by miR-31 [52]. Univariate and multivariate analysis performed to ascertain the level of expression in tumor subsite revealed higher levels of expression in buccal mucosa compared to tongue and gingiva. Upregulation of miR-31 was found in both tissue and blood samples of OSCC patients. The level of miR-31 in plasma of OSCC patients reduced remarkably after tumor resection, which is suggestive of the fact that this marker is tumor associated [34].

4.2. miR-21

miR- 21 has been found to be significantly over-expressed progressive leukoplakia and OSCC samples, implying a strong association with progression of oral carcinomas. Increased levels of expression could play an important role in malignant transformation. Expression level of miR-21 was found to exhibit 4- to 5-fold elevation in OSCC samples compared to normal tissue [15]. miR-21 is an oncogenic miRNA expressed in several carcinoma types and whose expression is found to vary with every tumor type. As with OSCC, the miR-21 expression was found to be in the tumor stromal fibroblast like cells and in intra tumor vessels [18]. It is strongly suggested that miR-21 induces cell proliferation and inhibit apoptosis by regulating the expression of target genes like PTEN (phosphatase and tensin homolog) and TPM1 (tropomyosin I) [54,68]. Dysregulation of programmed cell death 4 (PDCD4) is found in several cancer types. *PDCD4* is suggested to be a tumor suppressor and is known to play a critical role in apoptosis. Recent studies have reported that *PDCD4* may have an effect on transcription, translation and signal transduction pathways [28]. The level of PDCD4 protein expression was found to be less in the primary tumor samples from patients. Over-expression of this protein was found to reduce cell invasion [64] and under-expression resulted in enhanced cancer cell invasion [57]. MicroRNA-21 has been suggested to regulate PDCD4 by target prediction databases. Interaction of miR-21 could thus be a putative mechanism of PDCD4 regulation in OSCC.

Anti-sense miRNA are oligonucleotides which are employed to dissect the biological function of miRNAs. Knockdown studies employing anti-sense miR-21 oligonucleotide in CA-27 cell line reduced the expression of miR-21, which in turn resulted in growth inhibition. The effect of As-miR on enhancement apoptosis was confirmed through Annexin V and propidium iodide dual staining. Downregulation of miR-21 expression has been associated with sensitivity of CA-27 cells to cisplatin. Reports from several other studies have stated that oscillating levels of miR-21 in the cells could alter the potencies of other anticancer drugs [36,40]. As-miR and cisplatin produced a synergistic effect on the proliferation of CA-27 cells, which can be considered as a potential therapy for OSCC cases, as high dose of cisplatin is found to produce serious toxic effects like ‘nephro’ and ‘oto’ toxicity [58].

4.3. miR-24

OSCC cells were found to exhibit high miR-24 expression which is evident from the higher levels of miR-24 seen in the plasma of OSCC patients. Further, studies have also clarified the stability of miRNA in blood, shown to originate from tumor tissues [38]. miR-24 was found to be up-regulated in TSCC (tongue squamous cell carcinoma) which is considered to be the most common type of OSCC. Target prediction analysis revealed that highly conserved miR-24 binds to the 3’-untranslated region of RNA binding protein DND-1 (dead end 1) mRNA, which was found to regulate other downstream genes including cyclin-dependent kinase inhibitor 1B (CDKN1B) [29]. The miR-24 mediated downregulation of DND1 reduced the expression of CDKN1B, which was complemented by enhanced proliferation and reduced apoptosis of TSCC. Knocking down the miR-24 by anti-miR-24 LNA (locked nucleic acid knockdown probe specific to miR-24) resulted in the upregulation of DND1, followed by increased expression of CDKN1B. This was associated with a reduction in cell proliferation and increased apoptosis [30].

4.4. miR-221/222

miR-221 and miR-222 belongs to the same family of miRNA which is colocalized as a cluster in the short arm of chromosome X, sharing the same promoter regulatory region [13]. The expression ratio of miR-221: miR-375 showed high sensitivity of 92% and specificity of 93% for OSCC prediction [2]. Luciferase reporter assay validated *PUMA* (p53 up-regulated modulator of apoptosis) as a direct target of miR-222. miRNA is found to downregulate *PUMA*, thereby decelerating the apoptosis. UM1 cell line, derived from tongue squamous cell carcinoma cells (TSCC), with high invasive and migratory property was employed to study the role of miR-222. Antisense miR-222 transfection along with cisplatin treatment was found to inhibit the growth of UM1 cells by reducing proliferation and promoting apoptosis. The result is suggestive of the role played by miR-222 in regulating the expression of *PUMA* gene and provides novel rationales for combinational therapy [24].

4.5. miRNA as Metastatic Markers

Several studies have revealed the metastatic potential of miRNAs in various cell lines and animal models. miR-21 was found to be highly expressed in squamous cell carcinomas characterized by p53 mutations and metastasis. Increased expression of miR-21 mediated by mTOR and

Stat3 signaling augmented the invasive properties of mouse keratinocytes in vitro and in vivo [4]. In a study conducted by Kawakita et al, [25] using OSCC cell line, miR-21 was found to be associated with cancer invasion via Wnt/β-Catenin pathway. MiR-203, a human keratinocytes marker, has been implicated in cancer progression and metastasis [53,67]. A recent study by Severino et al, [51] have identified 6 miRNA markers associated with metastatic potential in OSCC, viz., miR-335, miR-296, miR-206, miR-23c, miR-1277 and miR-181d. In contrast, metastatic inhibitors such as miR-31 and miR-130b have also been reported in other cell lines. Identification of these metastatic tumor miR markers provides a new insight into the application of miRNAs as potential diagnostic tools which may assist in the evaluation of OSCC metastasis.

5. Conclusion

Circulating microRNAs concentration could serve as an excellent diagnostic source for screening cancer patients, monitor tumor dynamics and other pathological factors associated with OSCC cases. Conserved target motifs, seed sequences, 3′untranslated regions (UTR) are a broad spectrum of region in the genome targeted by miRNA. miRNAs exert strong control over gene expression thus making them powerful regulators of gene expression in numerous and complex cellular responses, including cancer cell invasion and metastasis. The metastatic potential of the primary tumor is determined by their genetic property. Biomarkers with the ability to differentiate cells with metastatic potential may impact management of neck disease, patient treatment and survival. As miRNAs significantly contribute to tumourigenesis, knowledge about the regulatory mechanisms driven by miRNAs in initiating and maintaining malignancy is essential to design effective strategies for cancer management. OSCC being a complex system, identification of the functional modules involved in such complex interactions between microRNA and their targets also becomes inevitable. To conclude, several cellular pathways in tumor progression may be affected by a single miRNA since they can target multiple mRNAs. Therefore, building a representative profile of miRNAs for each type of cancer would be of a great diagnostic value in molecular medicine.

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