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Research Article

miRNAs IN ORAL SQUAMOUS CELL CARCINOMA & CARCINOGENESIS: DIAGNOSTIC, THERAPEUTIC & PROGNOSTIC REVIEW

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ABSTRACT

Oral squamous cell carcinomas (OSCC) are one of the most frequent of all oral neoplasms in the world. In India also, OSCC is, a significantly frequent, cancer in men and in women. For OSCC, the five year survival rate has not shown any improvement , even in the last few decades despite significant improvements in therapy strategies.

Cancer tissue research revealed that there may be a link between changes occurring at molecular level and tissue level leading to evident malignant changes in the tissue and playing a pivotal role in disease progression. Previous research also shows that, somatic mutations could be useful as biomarkers to diagnose oral or other tumors along with serving as clear indicators for identifying early disease onset with saliva biochemical analysis, but, multiple biomarker candidates would be needed for the same.

Biomarkers would serve as objective indicators, to access the disease & its presence with genomic, proteomic, or metabolic expressions like nucleic acids, proteins, peptides, enzymatic changes, antibodies, metabolites, lipids, and carbohydrates obtained from body fluids. Thus, Biomarkers maybe useful for: evaluating the patient risk, patient assessment, recurrence detection.

According to an estimate it was found that over 30% of protein-coding genes get regulated by mi-RNAs, & thereby, mi-RNA indirectly control expression of those protein coding genes at the level of translation. Micro-RNAs are therefore, important in tumorogenesis owing to their proximity to chromosomal breakpoints and the dys-regulation of their expression levels that occur in many malignancies. Hence, if such aberrantly expressed mi-RNAs that disrupt the normal regulatory mechanisms in cancer cells are identified it would be an important first step towards elaborating the steps of mi-RNA - mediated oncogenic pathways. This, in turn, would significantly improve diagnosis, therapy, and prevention of the disease.

INTRODUCTION

Of all the malignancies occurring worldwide the incidence of oral cancers is approximately 2-4%. This suggests that, Oral squamous cell carcinomas (OSCC) are a group of lesions with a very high incidence from among all oral neoplasms, so much so that, 90% of all oral neoplasms are estimated to be OSCC. As reported by, M.Yakob *et al* & M. Maniknandan *et al* in 2012, OSCC accounted for 145,000 deaths worldwide, with less developed regions of the world sharing 77 % of the burden. In India too, OSCC is the leading cancer of men and a very common cancer of women. {1,2}

Various studies have shown that, the 5-year survival rate of oral cancer although 60-80%, if detected at, an early stage becomes as less as 20-40%, if OSCC is detected at the later stages (T-3 or T-4 stage). So, the 5-year survival rate decreases with later detection, emphatically indicating the necessity of early detection methods for increasing long-term patient survival. Also survival rate has been consistently poor for these patients in the past three decades despite improvements in therapy strategies. Thus, the key challenge is not only to to reduce the mortality and morbidity of this disease but also to develop strategies that help to identify and detect OSCC when it is at a very early stage, thereby, enabling an effective intervention and therapy to be done. Even today, the only reliable & scientifically credible, early detection techniques available are conventional clinical oral & histo-pathological examination.{2,3,9}

Tumors occurring on the lips, hard palate, upper and lower alveolar ridges, anterior two-thirds of the tongue, sublingual region, buccal mucosa, retromolar trigone and floor of the mouth are all referred to as Oral Cancer.

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Studies have shown that, a combination of environmental risk factors and genetic predispositions are causative for OSCC. Habits like smoking or chewing tobacco and alcohol drinking are very common , along with poor oral hygiene, poor diet and Human Papilloma Virus (HPV) infections , & when all these are combined with an individual's genetic predisposition they seem to have the ability to mutate oncogenes that are in charge of cell survival and proliferation ultimately leading to the disproportionately higher incidence of OSCC seen in India.{1,2}

OSCCs in non-smoking or no alcohol use individuals seem to have distinct disease characteristics showing a female predominance, higher average age, and a predilection for tumor to occur on the mandibular & maxillary alveolar ridge at diagnosis as observed by I Fukumoto *et al* .{11}

Other studies show that, clinically altered oral mucosa with premalignant lesions shows a higher oncogenic risk unlike normal oral mucosa and they include lesions identified as leukoplakia, erythroplakia, and leuko-erythroplakia .These are currently referred to as, potentially malignant lesions of the aero-digestive tract. The premalignant lesions, by definition, have cellular atypia & hence dysplasia of variable grades but do not show invasion of the underlying connective tissue. It is important to indicate that, the benchmark values that predict cancer occurrence are more a matter of debate, because, OSCC can arise, anywhere where epithelial dysplasia is detected irrespective of the cause of dysplasia. However, it is somatic mutations of tumor-specific DNA that are related to onset and development of malignancy from the premalignant lesions & those are detectable in cell DNA obtained from the saliva, plasma or other body fluids {1}

Recurrence is observed in 15-33% of patients & local recurrence is much more common than distant metastases, stressing importance of an improved diagnostic tool to predict which patients are most at risk for OSCC. recurrence.{1}

Carcinogenesis

Carcinogenesis, a complex multistep, multi-factorial process ocurring at the phenotype and genotype level & altering nuclear & cellular morphology & function leads to OSCC. Also, Cancer development is driven by the accumulation of genetic and epigenetic changes that disturb the homeostatic equilibrium between cell proliferation and cell death.

The molecular level changes that occur in carcinogenesis are:

1. cancer cell proliferation without external stimuli,
2. insensitivity to inhibitory growth signals,
3. evasion of apoptosis or cell death mechanisms and/or activation of anti-apoptotic genes,
4. unlimited replicative potential,
5. sustained angiogenesis,
6. invasion and metastasis ability,
7. genomic instability, and
8. Proto- oncogenes mutation caused by defects in DNA repair.

Research on cancer tissues has revealed that there may be a link between molecular level and tissue level changes that drive malignant changes in the tissue and play a pivotal role in disease progression.

Thus, the inference that can be drawn is that a study of the biological molecules involved in the molecular mechanism of carcinogenesis could provide valuable diagnostic data, i.e., of biomarkers, on the cancer disease process. Thus, the somatic mutations can be used as biomarkers to diagnose oral or other tumors.

Diagnosis of OSCC

Oral cavity is an easily observable area in order to do clinical examination, & even then, more often than not, Oral squamous cell carcinomas are surpassed without being diagnosed until, they reach, an advanced stage with grave symptoms or when they metastasize .Therefore the effectiveness of chemotherapy, radiotherapy and surgery becomes significantly reduced. Metastatic second primary tumors also develop by then & so further reduce the chances of, success of, multimodal therapeutic procedures .Ultimately, poor prognosis & a limited 5-year survival rate is all the patients can manage to achieve from treatment. Research & identification of biomarkers that can aid early diagnosis of OSCC, & those that can give an indication of quality of prognosis, and also of factors of significance for treatment response/overall survival is extremely essential {8}.

Methods of lesion detection and diagnosis are constantly being improved but there is still a requirement to minimize the essentiality for scalpel biopsy. Special scanning devices based on either infrared light or fluorescence may be helpful in this regard, as, they have the possibility of reducing patient apprehensions about repeated surgical biopsy & they may also allow to detect and diagnose in one step. Gene-based methods can also be used to determine changes in the mucous membrane of oral cavity that are indicative of cancer. Such methods initially used mRNA, and then micro-RNA, because RNA signatures for OSCC have been developed using surgically obtained tissue of previous biopsies. One more way to obtain markers of OSCC is , from body fluids, such as blood or saliva, but, they are showing variations , probably as a consequence of low RNA concentrations, leading to variable results . Coupled with these pitfalls there is also a limited follow-up of published RNA classifiers for OSCC along with the lack of standardized sample collection methods for RNA-based detection and diagnosis, ,thus, leading to slowing down of validation for clinical purposes.{10}

For almost three to four decades, changes in protein coding tumor suppressor genes and/or oncogenes have been thought to be the main drivers of tumor development. However, the recent discovery of thousands of genes that transcribe non-coding RNAs (including micro-RNAs) makes it obvious that cancer biology is even more complex than initially expected. Several layers of molecular regulators (e.g., mRNA, micro-RNA, and protein) are involved in the development and maintenance of cancerous phenotypes. As the progression of premalignant lesion into a cancerous tissue is often distinguished by the identification of cytogenetic changes such as the loss of chromosomal 3p region (a critical tumor suppressor gene region), resulting in a 3.8 fold higher risk of carcinogenesis, so, molecular regulators like, micro-RNAs, the 18-25 nucleotides long, non-coding RNA molecules, have recently gained significant attention as potential regulators and biomarkers for human carcinogenesis. {5}

micro-RNAs affect cell proliferation, apoptosis , and even chemotherapy resistance in OSCC patients as shown in the study by Janice M Yoshizawa & David T W Wong. Another important observation is that, micro-RNAs are shown in to be epigenetically regulated by DNA methylation in OSCC. There are distinct expression profiles of micro-RNAs in cancer cells which are due to being differentially expressed in cancer cells in comparison to normal cells & the same has been observed in the micro-RNA expression profiles between cells of OSCC& normal tissue cells. Many miRNAs in cancer cells exhibit the alteration in quantity of their expression level by many-fold ie. by tens to hundreds of times higher than their expression in normal cells along with a drastic change of level compared to that observed in expression levels of mRNAs. Thus, micro-RNAs can be potentially used as biomarkers to detect early-stage changes of oral cancer and probably advance to the development of mi-RNA-based cancer-treatment and therapies.{3}

Owing to these findings**,** next generation sequencing (NGS) of such RNAs are already being used to identify specific changes in circulating mi-RNAs of: lung, breast and nasopharyngeal cancer.

Similarly, 'Salivary biomarkers' can assist in monitoring the disease status of oral dysplasia patients eliminating or reducing the biopsies required in them for the purpose. Although, salivary diagnostics for OSCC is very promising due to the direct contact of saliva with premalignant or malignant lesions ,yet, no single bio-molecule has been shown to meet the real world requirement of high accuracy in identifying the onset of early disease .Thus, the need to use multiple biomarker candidates to achieve high accuracy and sensitivity in detecting OSCC seems essential. However, extensive & rigorous biomarker validation seems to be crucial to the acceptance of newly discovered biomarker candidates prior to adoption for clinical utilization. On the other hand, the advantage of using saliva is that, in saliva, tumor - specific DNA is positive in 100% of patients with oral tumors as reported by various studies. {6, 29}

Biomarkers

According to the National Institutes of Health (NIH), a biomarker is defined as a characteristic which can be objectively measured and evaluated as an indicator of: a normal biological process, a pathogenic process, or pharmaceutical - response to a therapeutic intervention.{7}

Thus, prior to its use in a clinical assay & health- risk assessment, verification and validation of a biomarker is essential {1}. Validation, in biomarker research, requires determination of, area under the Receiver Operating Characteristics (ROC) curve ie.AUC along-with, measurement of, the sensitivity and specificity of a marker. Sensitivity is the true-positive rate, which is described by the percentage of the total number of people with the disease that test positive and Specificity is the true-negative rate, which measures the proportion of individuals that test negative for the disease or actually do not have the disease. The AUC for a biomarker diagnostic test can range from 50%, which correlates to having no better insight than chance alone, to 100%, which denotes a perfect diagnostic test. {18,19}Thus, area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal) so making ROC curve, a fundamental tool for diagnostic test evaluation. Biomarkers, thus, define the distinguishing line of - the presence or absence of disease & function by identifying the underlying tissue changes in the disease process categorized as: genomic, proteomic, or metabolomic expressions. Micro-molecules that can function as biomarkers include: nucleic acids, proteins, peptides, enzymatic changes, antibodies, metabolites, lipids and carbohydrates. One or more of the body fluids ie blood, serum, plasma, body secretions (sputum, saliva), or excretions (stool, urine) can be used to obtain the biomarkers. Of significance is the fact that, the body fluids' sample for biomarker investigation can be obtained by noninvasive, minimally invasive or invasive methods. Hence, Nucleic acids (DNA/RNA) extracted from blood, saliva, oral exfoliative cells, or buccal smear cells can be instrumental in identifying mutations and can help to correlate and confirm the diagnosis, monitor the disease progression, or act as prognostic indicators in treatment.

Biomarkers can be used for

1. *Patient assessment in multiple clinical settings by* :
   - estimating disease risk, screening for occult primary cancer.
   - distinguishing benign from malignant findings
   - distinguishing one type of malignancy from another,
   - determining prognosis,
   - acting as predictors/screening, and
   - monitoring disease status.
2. *To either detect recurrence or determine progression/response to therapy*.
3. *To help in the determination of a patient's risk of developing oral cancer*: This is done with risk reduction strategies and monitored with effective & highly sensitive screening methods & tools using biomarkers. These strategies are efficient for high-risk groups & not for wholesale application to the entire population.1}

In saliva, Molecular signatures for the diagnosis of OSCC can be pursued at three levels which are sequential in occurrence as given below:

1. changes in the cellular DNA, which result in,
2. altered mRNA transcripts, leading to,
3. altered protein levels (intracellularly, on the cell surface or extracellularly){2}

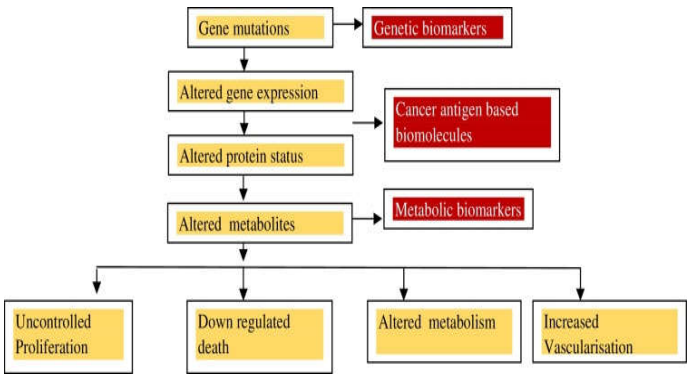

Figure 1 Levels of molecular signatures for diagnosis of OSCC

Table 1 Some salivary biomarkers & their uses

Salivary biomarker	use
1. L-phenylalanine	screening biomarkers and help in the early diagnosis and monitoring of OSCC
2. Cloning of an acidic lactase gene 2 , a proteomic biomarker	used to differentiate between squamous cell carcinoma and adenocarcinoma.
3. CD34 , angiogenetic marker	serves as an important predicting tool for recurrent cases of OSCC.
4. ILs & TNF	Regulate and Mediate inflammation and angiogenesis ,so, deregulation in their production facilitates tumor growth, invasion and metastasis
5. HPV (mainly type 16)	may be a causative factor, chiefly among persons who do not smoke or drink alcohol
6. cyclin D1 gene amplification	Is associated with with poor prognosis in OSCC.
7. Ki67 marker levels	High in the saliva of patients with OSCC
8. Mammary serine protease inhibitor (Maspin) levels	found to be decreased in the saliva of patients with OSCC.

Classification of biomarkers for OSCC

Biomarkers used in the diagnosis of OSCC can be classified into the following groups based on their biological function:

Cellular Biomarkers

1. Biomarkers of Cell cycle progression & proliferation eg: EGFR & Cyclin B1 .When these are over-expressed they indicate poor prognosis
2. Biomarkers of Tumor suppression & apoptosis eg:p53 & survivin .Their over-expression is usually indicative of aggressive tumor & poor prognosis
3. Biomarkers of Hypoxia eg: GLUT-1, EPOR .When these are over-expressed they also indicate poor prognosis
4. Biomarkers of Angiogenesis eg: VEGF & CD34 When positive they indicate poor prognosis & LN metastasis
5. Biomarkers of Cell adhesion & Matrix degradation eg: MMP-7,-9,-13,-14. When positive they too indicate poor prognosis

Humoral Biomarkers

1. Parathyroid hormone related proteins
2. Endothelins & their receptors eg: Endothelin
3. Inflammatory cytokines & chemokines eg: IL-6

Various Classifications of Cancer Biomarkers {10}

1. Based on biomolecules
   - DNA biomarkers
   - RNA biomarkers
   - Protein biomarkers
   - Glyco biomarkers.
2. Based on disease state
   - Prediction biomarkers
   - Detection biomarkers
   - Diagnosis biomarkers
   - Prognosis biomarkers.
3. Based on other criteria
   - Pathological biomarkers
   - Imaging biomarkers
   - In silico biomarkers

Recent advances in OSCC detection

Owing to the availability of proteomic technologies, such as mass spectrometry, liquid chromatography, and protein/peptide labeling technologies, it has become possible to detect the presence of low abundance molecules in the saliva proteome. Numerous studies have compared the proteomic profile of saliva of OSCC patients with that of OSCC-free controls wherein proteomic profile consists of proteins most of which are synthesized and subsequently secreted into the oral cavity by the salivary gland acinar cells. They observed & concluded that , the two proteomic profiles were different.

In 2008, 1166 salivary proteins were initially identified in a National Institute of Dental and Craniofacial Research (NIDCR)-funded project that sought to catalog and annotate the human salivary proteome. This project was an essential first step for saliva to be clinically useful in disease diagnosis and health monitoring.{1,19}

Similarly, the identification of aberrantly expressed mi-RNAs which disrupt the normal regulatory mechanisms in cancer cells was an important first step towards elucidating the details of mi-RNA - mediated oncogenic pathways.{15}

It implies, therefore that, understanding molecular oncogenic pathways based on the current genome-based approaches underlying OSCC could significantly improve diagnosis, therapy, and prevention of the disease.

Role of mi-RNAs as diagnostic biomarkers

Although it is known that, mi-RNAs circulate stably in different human body fluids, such as blood, saliva, urine and breath , & , can be accessible with non-invasive methods the role of some of them as potential **diagnostic biomarkers** has been noticed only recently by some researchers.{11}

The micro-RNAs in saliva have certain advantages as biomarkers compared with other salivary biomarkers like proteins, mRNAs, DNAs and bacterial products. These are:

1. aside from the distinctive function of mi-RNA as post-transcriptional regulator, mi-RNAs are stably present in saliva
2. the similarity between mi-RNA profiles of saliva and other body fluids imparts them a high availability as biomarkers for various human diseases.{14}

A correlation between the expression of specific micro-RNAs, and outcome of Squamous cell & Head and Neck Carcinomas & OSCC has also been reported. Thus, it was noted that , low levels of miR-375 correlated with poor survival and distant metastases whereas, high levels of miR-210 correlated with loco-regional recurrence in the study by Janice M Yoshizawa & David T W Wong.{11}

Micro-RNA as Biomolecule (mi RNA)

The small, endogenous, evolutionarily conserved, naturally abundant as well as relatively stable molecules of most biological and pathological process {4,5} about 22 nucleotides long , non-coding RNA molecules are micro-RNA.

Micro-RNAs are transcribed in the nucleus as a long, capped, polyadenylated precursor called primary micro-RNA (pre-microRNA) by an RNA polymerase II or III. This pre-micro RNA is subsequently processed by the ribonuclease (RNase) III called Drosha or the double-stranded DNA-binding Protein called DGCR8/Pasha producing a precursor micro-RNA (pre-micro RNA). Then the nuclear export receptor exportin 5/Ran GTP actively transports pre-micro RNAs to the cytoplasm. In the cytoplasm, Pre-miRNAs are processed by the RNase III endonuclease - Dicer along with the trans-activation-responsive RNA-binding protein (TRBP) and that produces a small double-stranded RNA structure of (22 nt) of micro-RNA. This duplex micro-RNA is unwound into mature single-stranded form and that integrates into the RNA-induced silencing complex (RISC), which escorts the complex into the complementary 3′UTR of the target mRNA.

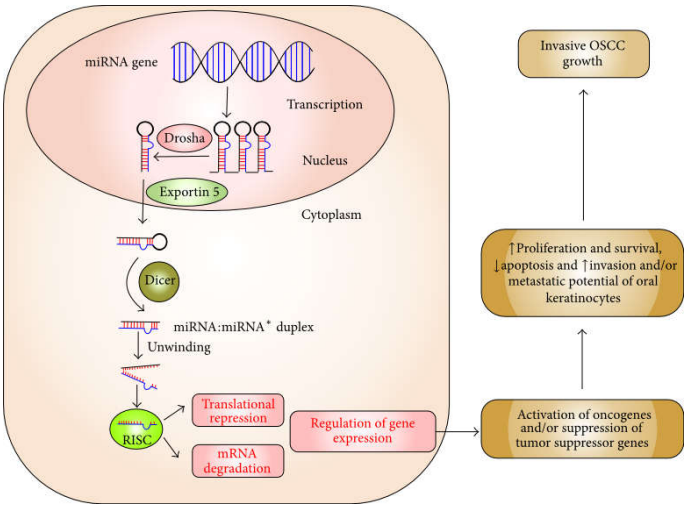

Figure 2 Transcription of miRNA

Two possible ways in which about 30% of protein-coding genes are regulated by micro-RNAs are:

a. by complementary binding of target mRNA or
b. by binding to imperfect complementary sites in the 3′ un-translated regions (3′UTR), ultimately controlling expression of these genes at the level of translation.

Hence, they function as post-transcriptional gene regulators & micro-coordinators of gene expression. By calculations of researchers, more than 45,000 micro-RNA target sites are present in the human 3′UTR and more than 60% of human protein-coding genes are probably regulated by multiple micro-RNAs rather than a single micro-RNA{8}. In many in vivo & in vitro studies, micro-RNA have proved to be effective tools to study the biology of diseases, thereby , indicating their great potential as novel diagnostic and prognostic biomarkers.

Role of Micro-RNAs in oral cancer

Micro-RNAs are important in tumorogenesis due to their proximity to chromosomal breakpoints and their dysregulated expression levels in many malignancies. Over-expression of certain micro-RNAs might result in the down-regulation of tumor suppressor genes, while under-expression of other micro-RNAs might cause oncogene up-regulation. Consequently, several studies evaluated the potential of micro-RNAs as diagnostic and prognostic biomarkers for cancers. {14,20}

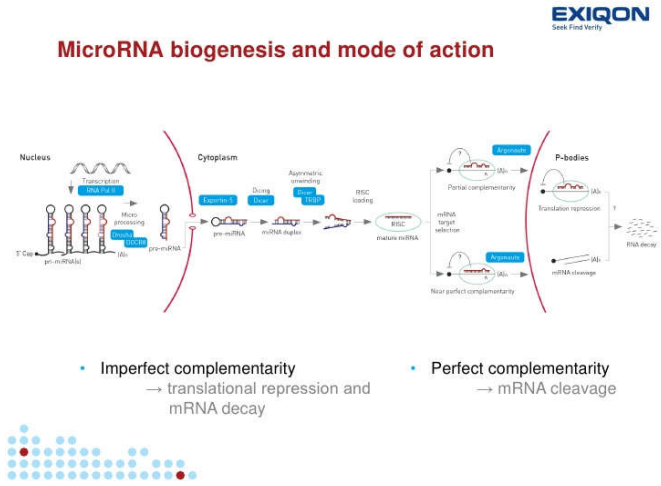

Figure 3 Mode of Action of mi-RNAs

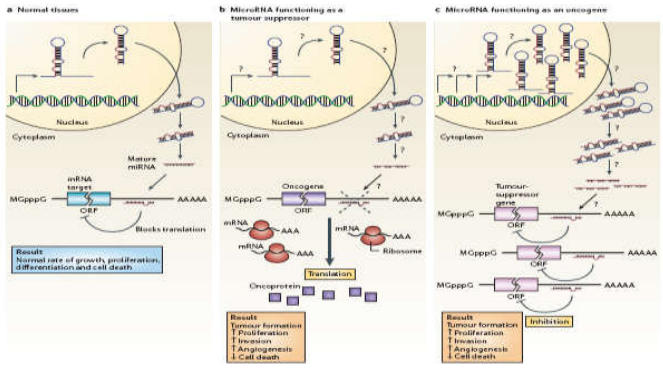

Figure 4 Micro-RNAs & Tumorogenesis

mi-RNAs in Tissues

In tissues, mi-RNAs have an advantage of being stable due to the shorter lengths that makes them less susceptible to degradation caused by chemical and/or physical environmental factors.

Since they can be easily isolated and measured from tissues and body fluids such as plasma, serum, saliva, milk, cerebrospinal fluids, so they are more useful.

Expression motifs of serum mi-RNAs are firmly linked to various diseases including cancer. Thus, interestingly, circulating mi-RNAs have many necessary features of ideal biomarkers.{13}

Also, a single mi-RNA can regulate expression and/or function of hundreds of target mRNAs and proteins and thereby regulate several biological processes (e.g., cell proliferation, differentiation, migration, apoptosis, and signal transduction) important for cancer development.

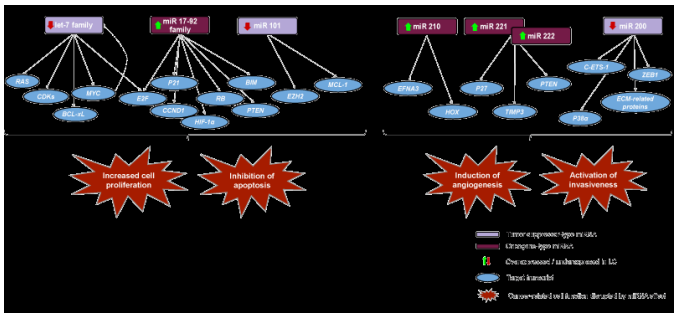

Figure 5 Multiple targets of single miRNA

Changes in protein coding tumor suppressor genes and/or oncogenes had been thought to be the main drivers of tumor development until recently. However, with the recent discovery of thousands of genes that transcribe non-coding RNAs (including mi-RNAs) it became obvious that cancer biology is even more complex than initially expected. It also showed that several layers of molecular regulators (e.g., mRNA, mi-RNA, and protein) are involved in the development and maintenance of cancerous phenotypes among which , study of mi-RNAs , has gained significant attention as potential regulators and biomarkers for human carcinogenesis.

Thus, in others malignancies, as well as in OSCC, mi-RNA is found to regulate several oncogenes and tumor suppressors, thereby controlling the growth, proliferation, metastatic attitude and drug- resistance also of a tumor . {5, 21}

Assessing miRNAs

The quantity of mi-RNAs can be easily estimated by various methods, such as Microarray, Hybridization, Deep-sequencing, qRT-PCR, and micro-beads analysis. Studies have shown that, two mi-RNAs (miR-125a, miR-200a) reduced in saliva of oral squamous cell carcinoma patients. {5}

Recent reports as by Y Yan *et al* & N Sethi *et al* have also identified cancer specific mi-RNA signatures in OSCC cell lines and tissue samples, such as down-regulation of other mi-RNAs like: miR-137, miR-193a, miR- 375, miR-145 and miR-222 and up-regulation of miR- 127, miR-21 and miR-10b. Thus, deregulated expression of mi-RNAs in OSCC and OSCC-derived cell-lines compared to their normal counterparts in these studies indicated their potential role in oral cancer development. Accordingly, several mi-RNAs have been shown to function either as tumor suppressors or as tumor promoters in OSCCs. In addition to their key biological functions in OSCC tumorogenesis, expression levels of several of mi-RNAs have been shown to correlate with clinico-pathological variables and to have a diagnostic and prognostic value in OSCC.{6,22}

As per studies published by Liu C J *et al* & by M Yakob *et al*, significantly lower levels of *miR-125a* and *mi-R200a* were found in the saliva from 50 OSCC patients as compared to 50 healthy control subjects. Simultaneously salivary *miR-31* was found to have increased significantly in patients with OSCC at all stages in their studies followed by a decrease in its level after the cancerous tissue had been excised. The increased *miR-31* in plasma & saliva, may also therefore, serve as powerful OSCC biomarkers of, prediction to disease and disease progression.{1,23}

Importance of salivary biomarkers therefore, is due to the fact that they can be used between biopsies to assist in monitoring the disease status of dysplasia patients. Although extensive and thorough biomarker validation is essential before any biomarker candidates can be tailored for clinical use, the results indicate that salivary diagnostics for OSCC is very promising due to the direct contact of saliva with premalignant or malignant lesions.

In this review, the oncogenic and tumor suppressive roles of mi-RNAs in OSCC development is highlighted and their potential value as diagnostic and prognostic markers for OSCC management is discussed.

Although, normal adult stem cells have a finite life span and eventually reach replicative senescence in culture. In tumoro-genesis the initial stage is, immortalization of such cells. Subsequently, a transformation process occurs in that cellular colony leading to the development of a frank neoplasm and then its further progression leads to fully invasive and metastatic malignancy. These alterations are accompanied by a series of molecular aberrances leading to the malignant transformation and clonal expansion of neoplastic cells. Immortalization or partial transformation of normal oral keratinocyte (NOK) has been achieved in vitro by transfection with the HPV16 E6 and E7 oncogenes, which inactivate p53 and Rb, respectively. This coupled with the expression of human telomerase reverse transcriptase (h*TERT*) gene that encodes the catalytic unit of telomerase for the maintenance of telomere length, causes an escape of NOK from replicative senescence & is acquired as an early event in oral carcinogenesis. Moreover, h*TERT* expression increases proportionately with the increase in severity of Oral premalignant disorder (OPMD).

In their study, Jin Park *et al* found that healthy saliva contains ~50 micro-RNAs. Like Anjii Min *et al*, they also found that two micro-RNAs, miR-125a and miR-200a, can discriminate oral cancer patients from control subjects ($P < 0.05$). Besides, according to them, the presence of micro-RNA in saliva has already been substantiated, and therefore, represents a third diagnostic alphabet in saliva, in addition to proteome and transcriptome analysis. {28}

To summarize, studies so far, have confirmed the importance of micro-RNA alterations in head and neck squamous cell carcinoma (HNSCC) or OSCC carcinogenesis..{5, 27}

miR-21

A well-studied mi-RNA, the miR-21, has been shown to be over-expressed and to regulate several biological functions in OSCC. Over-expression of miR-21 has also been observed in oral premalignant lesions (oral leukoplakia) compared to normal oral mucosa, indicating that alteration in miR-21 could be an earlier event in OSCC progression. Experimental data have demonstrated an oncogenic role of miR-21 in OSCC by promoting cell proliferation, invasion, anti-apoptosis, and chemo-resistance. These oncogenic functions were shown to be regulated by miR-21-mediated down-regulation of several established tumor suppressor molecules, including PTEN, programmed cell death 4 (PDCD4), tropomyosin, reversion-inducing cysteine-rich protein with kazal motifs (RECK), and dickkopf 2 (DKK2). In addition to the functional roles in OSCC cells, a growing body of evidence suggests that miR-21

might be important in the regulation of carcinoma associated fibroblasts (CAFs) induction and their activity. Also, higher stromal expression of miR-21 was associated with poor prognosis in OSCC. { 5,24,25}

miR-31

miR -31 and its passenger strand micro-RNA (miR-) have been shown to be up-regulated in oral leukoplakia (OLP) and OSCC and to have an oncogenic role in OSCC tumorogenesis. Experimentally, it repressed its target factor-inhibiting hypoxia-inducible factor (FIH) expression to activate hypoxia-inducible factor (HIF) under normoxic conditions & also affected several biological processes such as apoptosis, cell proliferation, migration, and epithelial-mesenchymal transition (EMT) in OSCC cells. It was shown to collaborate with human telomerase reverse transcriptase (hTERT) to immortalize normal oral keratinocytes (NOKs), indicating that it might contribute to early stage oral carcinogenesis .All these were seen to be mediated by the regulation of fibroblast growth factor 3 (FGF3) and RhoA expression levels. Salivary miR-31 (implicated in tumorogenesis) was appreciably superior in all stages of oral cancer, and salivary miR-31 was more copious than blood miR-31, representing the oral tumor origin of this biomarker.{5,27}

Similarly, miR-146a over-expressed in OSCC enhances OSCC tumorogenesis with, concomitant down-regulation of IL-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6), and NUMB.

Also, miR-134, miR=155 expression was up-regulated in HNSCC tissue specimens and cells compared to controls.{5}

1. Micro-R- 320 was down-regulated in OSCC-derived cell-lines & tissue due to hypoxia & promotion of tumor angiogenesis.
2. Down-regulation of miR-99a was observed in OSCC patient specimens and cell- lines, especially in OSCC patients with lympho-vascular invasion, suggesting a role for miR-99a in lympho-vascular invasion. In addition, miR-99a induces apoptosis of OSCC cells.(30)
3. MiR-218 has a tumor suppressive function but is epigenetically (DNA hyper-rmethylation) silenced in OSCC tissue specimens
4. miRNAs regulate .> EMT > EMT regulates tumor progression, invasion, and metastasis and acquisition of stem-like phenotype .
5. Studies have shown that micro-RNAs play a crucial role in the regulation of extracellular matrix (ECM) components, such as matrix metalloproteinases (MMPs) and integrins.
6. A miRNA cluster, miR-17-92, including miR-17, miR-19b, miR-20a, and miR-92a, was found to be significantly down-regulated in a more migratory OSCC-
7. miR-375, a tumor suppressor , was shown to be down-regulated in HNSCC

Distinct expression profile of micro-RNA in OSCC and oral premalignant tissue specimens compared to the normal controls offers the use of specific micro-RNA(s) signature for early stage diagnosis and prediction of OSCC prognosis {30}. Firstly, they are abundantly expressed in OSCC and control tissues and hence their isolation and quantification are convenient and reproducible.

Table 2 Tabular data on miRNA observations in various studies

MiRNA	OBSERVATION
1. let 7d	More in males
2. let 7a & let 7f	More in tongue OSCC than in gingivobuccal OSCC
3. miR 223 , miR 1275 & miR 29b	Significantly high in advanced tumors groups(III-IV & T3-T4) as compared to(I-II ;T1-T2)
4. miR -223	More in G1 than in G2 grade
5. miR142-3 p , miR 144 , miR 223 whose function is targeting genes enriched in the p53 signaling pathway, so,the inhibition of pathway may lead to evasion of apoptosis and defects in cell cycle checkpoints.	Significantly elevated in tumors with Nodal invasion
6. miR 1275 is borderline in change	
7. miR142-3 p	Lower in smokers than in Non smokers
8. miR -203	Significantly decreased in tumors with Nodal invasion
9. miR -203 & miR -223	Show differential expression in OSCC
10. Let-7 miRNAs	regulate the expression of *RAS & other genes involved in cellcycle & repress the cell divisions*
11. let-7a, let-7d, let-7f, miR-16, whose function is targeting genes enriched in the PI3K/Akt signaling thereby activating limitless replication potential.	Show reduced expression in OSCC
12. miR125-b	Lower in proliferation & radioresistence mechanism activation
13. miR125-a	It plays an important role in cell proliferation and can affect the genes involved in MAPK metabolism. The levels of miR-125 in saliva are reduced in patients of oral cancer compared to healthy individuals.
14. miR200-a	It is involved in tumor suppression and in early metastasis. The levels of miR-200 are also reduced in the patients of oral cancer compared to healthy individuals.
15. miR -31a	a tumor suppressor microRNA,studies show elevated miR-31 levels in all stages of squamous cell carcinoma patients and advocate the use of miR-31 as one of the earliest biomarker to detect OSCC

***Micro-RNAs as Tumor Suppressors in OSCC* {5}**

Several micro-RNAs have been shown to be down-regulated in OSCC.

Secondly, several OSCC-related micro-RNAs are secreted in bodily fluids such as serum, plasma, and saliva making them very useful for noninvasive clinical applications{5,25,28,29}

Problems in use of micro-RNAs as biomarkers in OSCC

Although the exploitation of micro-RNAs as biomarkers for dental diseases is promising, some constraints should be considered.

1. Owing to variable expression of candidate micro-RNAs identified through pilot studies, results of such studies remain non-valid.
2. As there is lack of suitable endogenous controls for normalization of salivary micro-RNAs, characterisation of micro-RNA patterns in body fluids such as serum, plasma, and saliva on a large scale remains limited & also future exploitation of micro-RNA datasets remains unclear.
3. Variations in, saliva collection influence the types of micro-RNAs obtained.
4. The development of suitable treatment and prevention methods for patients testing positive for the biomarkers in saliva is an important aspect that has been overlooked.
5. Unsupervised hierarchical clustering of the tumors and micro-RNAs, suggest that the naturally arising clusters do not represent the clinical tumor characteristics.
6. The micro-RNA expression is not pronounced in any specific direction, hence, confirming the molecular heterogeneity of OSCC.

Future Application of micro-RNA to Cancer Treatment

For micro-RNAs whose expression is reduced, re-introduction of mimics of such micro-RNAs that could restore the correct gene targets by modulation may be helpful. Conversely, for micro-RNAs whose expression is increased, the strategy should be aimed at their inhibition through the use of anti-micro RNAs or antagonists that can bind over-expressed micro-RNAs. These seem to be promising future applications of micro-RNA in cancer treatment.

However, micro-RNA-based therapies are still in preclinical and clinical development stages, but, with encouraging results.

Conflict of Interest: None

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