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PROXIMATE ANALYSIS OF PRE-IDENTIFIED MICRO RNAs DIRECTLY RESPONSIBLE FOR TONGUE, HEAD, NECK AND ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Squamous cell carcinomas have very high rate of incidence since recent past. Most of these carcinomas manifest very similar features. They include head & neck, tongue and most importantly oral squamous cell carcinoma with thick nodules or ulcer or erosion at the sites. In India 90-95% squamous cell carcinoma includes oral squamous cell carcinoma and head & neck squamous cell carcinoma. In recent years, it has become clear that miRNAs are involved in normal physiological processes, including cell proliferation, differentiation, apoptosis, as well as several pathological conditions such as tumours and regulation of gene expression. It is now well established that microRNAs are a family of small non coding RNAs of 21-25 nucleotides in length and transcribed by RNA polymerase II and III, evolving precursors that undergo a series of cleavage events to form mature micro RNA. In this study, around 20 pre-identified microRNAs have been taken to analyze their role in the progression or invasiveness or apoptotic in nature for oral, head & neck, glottis, tongue squamous cell cancer etc. MicroRNA assembles into RNA, induces silencing complex to activate the target RNA. The expression degree in progressive groups of microRNAs and target mechanism pathways are also the matter of concern over here.

Keywords: Oral squamous cell carcinoma, Head & neck squamous cell carcinoma, Responsible MicroRNAs, Putative functions, Target pathways, Expression degree.

1. INTRODUCTION

Among various types of malignancy, Oral Squamous Cell Carcinoma (OSCC) is most common oral malignancy, accounting for upto 80-90% of all malignant neoplasms of the oral cavity [1-2]. Head & neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and it also includes oral squamous cell carcinoma [3-4]. The incidence of oral cancer is highly variable throughout the world and it is accepted that oral cavity ranges from 6th to 9th most common anatomical location of cancer, depending mostly upon the specific region of some countries. The prevalence of OSCC is higher, reaching 10% of all cancers in Pakistan and 45% in India [2, 5-6]. It includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions, tongue (30-35%), gum (20-25%), floor of the mouth (5-7%), nasal palate (4-6%), salivary glands, chick lining (only 2-3%), gingival pallets [6-8]. Only the terms are interchangeably used for different aspects and the most frequent of these are OSCC, HNSCC, TSCC (Tongue Squamous Cell Carcinoma) [9]. TSCC is one of the most familiar forms of OSCC, with rapid progress and easy metastasis, which usually cause trouble of chewing, speech and ingurgitation [10-11]. Registration of this very fatal disease is not compulsory in India, so the true incidence and mortality may be higher, as many cases are unrecorded and loses follow up [12]. It has been predicted that India's incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035. This indicates that the death rate because of cancer will also increase from 680000 to 1- 2 million in the same period as predicted by one of the leading international research agencies [13]. Many of these diseases are directly related to classical etiologic factors like smoking and alcohol, areca or tobacco chewing, betel nut chewing like carcinogenic agents and human papilloma virus (HPV) plus poor oral hygiene [6,14-15]. According to the survey, till now OSCC malignancy tends to relapse or progress even after treatment within

5 years [16]. The overall survival rate for HNSCC, OSCC, TSCC patients remains dismal due to rapid metastasis and high regional relapse rate [17-19]. Thus, we can easily say that there is a great urgency and significance to identify molecular mechanisms and effective therapies for OSCC, HNSCC and TSCC.

MicroRNAs are small, non-coding, endogenously synthesized 16-29 nucleotides length RNAs, and are directly responsible for post-transcriptional regulation of messenger RNA (mRNA) expression [20-21]. In recent years, it has become clear that microRNAs are involved in normal physiological processes, cellular behaviors including cell-cycle regulation, cell proliferation, differentiation inflammation, stress response, migration, invasion, differentiation and apoptosis plus negatively regulate protein expression at the post-transcriptional or translational level [22-23]. Dysregulation of MicroRNAs are commonly observed in diverse ways including HNSCC, OSCC plus TSCC, and closely related to cancer progression, functioning as tumor oncogenes or suppressors, repressors, enhancers [10, 24-25]. MicroRNAs are transcribed individually or in combination [26-27] into long primary RNA transcripts (pri-MicroRNAs) by RNA polymerase II or III [27-30] which are further cleaved by a ribonuclease complex comprising two proteins, Drosha and Pasha. Drosha cleaves primary RNA transcripts into 70-100 nucleotide (nt) primary RNA transcripts that form secondary hairpin-loop structures which are specific to the targets [27, 31-32]. Primary RNA transcripts are translocated to the cytoplasm [27, 32-33]. Canonical microRNA production starts with long primary RNA transcripts (pri- MicroRNAs), which are cleaved by a ribonuclease complex comprising two proteins, Drosha and Pasha, to create ~60-70-nt precursor MicroRNAs (pre- MicroRNAs) [29, 34-35] and modified by Dicer into small double-stranded RNA molecules that contain the mature microRNAs, 16-29 nt in length [21] and its antisense strand [21, 27, 36] plays key roles in the regulation of target gene expression after transcription by bewitching in complementary base pairing with the 3′- UTR (untranslated region) of a target messenger RNA [37-39].

In this study, we have tried to interpret the previously published data of responsible microRNAs for OSCC, HNSCC, and TSCC in an ordered manner with their putative functions plus target pathways. Accumulating evidence suggests that there a lot of microRNAs which acts as enhancer like hsa-mir-31 [40], repressor like hsa-mir-210-3p [37], suppressor like hsa-mir-125b [3] etc. Silencing of some tumor suppressor genes plays inconsequential roles in proliferation, migration, evasion and promotion of OSCC, HNSCC, TSCC [41-43]. Hence, a more requisite understanding of the mechanisms of aberrant tumor suppressor, repressor plus enhancer gene expressions might be appropriate to get novel treatment strategies for ameliorating the prognosis of patients with OSCC.

The aim of this study is to clarify and summarize the relationship between mir 223 [44], mir 26a/26b family [45], mir 21 [46-48], mir 184 [49], mir 31 [50], mir 208b-3p [51], mir 3065-5p [51], mir 129-2-3p [51], mir 222-3p [52], mir 150-5p [52], mir 483-5p [53], mir 141 [54], mir 142-3p [55], mir 20 a [56], let 7 family [57], mir 155 [58], mir 204-5p [17], mir 210-3p [37], mir-125b [3], mir 211 [41] and OSCC, HNSCC and TSCC, which will contribute to find potential biomarker of oral squamous cell metastasis.

We hope this compendium will stimulate further research in this area, a fertile ground for study of microRNAs and how they are directly relatable to the sixth most common fatal disease with an estimated new cases 300,000 and 150,000 deaths annually [15] throughout the world.

2. MATERIAL AND METHODS

2.1. Preparation of data

In the very first step we have prepared a data set with the help of published data available from various types of research and review papers. Most importantly, we observed that there are a lot of experiments done on random microRNAs which cause different types of cancers. In this paper, we have analysed only 20 microRNAs which are directly responsible for oral squamous cell cancer, tongue squamous cell cancer, head & neck squamous cell cancer. This database have been prepared with the help of following mentioned research engines https://pubmed.ncbi. nlm. nih.gov, http://scholar.google.com/, https://www. sciencedirect.com/, https://www.scopus.com, http: //www.us.elsevierhealth.com/, https://clarivate .com /webofsciencegroup/solutions/web-of-science/.

2.2. Analysis of data

We have used an open source data machine learning and data visualisation tool named ORANGE (https:// orange.biolab.si/download/#windows). It is a component based visual programming software package for data visualisation, data mining and data analysis. The

components of orange are called widgets and they range from a simple data set visualisation, subset selection, pre processing to empirical evaluation of learning algorithm and prediction.

2.2.1. *Work flow presentation*

We constructed a workflow by dragging the widgets onto the canvas and connecting them by drawing a line from transmitting widget to receiving widget. The output widgets are on the right and the inputs are on the left. File widgets send the data to distribution widget, pivot table widget, differential expression and feature statistics widgets.

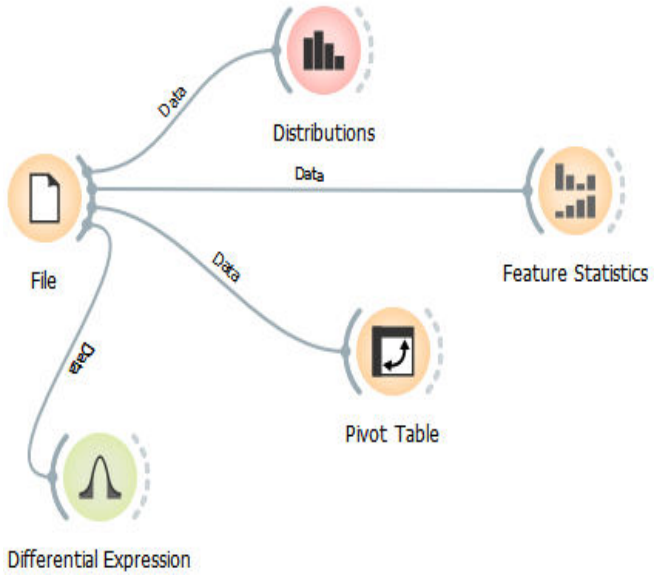


Fig. 1: Workflow done in ORANGE

2.2.2. *Distribution of the data*

The distribution of a set of data is the shape of the graph when all possible values are plotted on a frequency graph. One of the most common distributions is called normal distribution which is bell shaped. In addition, weibull or gamma distribution may be observed. Data distribution helps us to organise and display the data which includes box plot, bar chart, pie chart, histogram etc.

2.2.3. *Feature statistics and pivot table*

We have used Pivot table as an efficient widget to summarize the data from the data sheet or spreadsheet that includes sum, average and a statistical value which shows a meaningful way when it is together with feature statistics.

3. RESULTS

3.1. Putative actions of MicroRNAs

***mir 204-5p*:** It is a tumour suppressor for head & neck cancer (HNSCC), usually repressed in HNSCC and acts as a therapeutic target. Its decreased expression mainly suppresses epithelial mesenchymal transition (EMT) and STAT3 signalling pathway in *Homo sapiens.*

mir 223: It is a novel diagnostic biomarker for glottis, head & neck squamous cell cancer, acts as tumour suppressor and most importantly inhibits cell proliferation and cell apoptosis. It can be a potential therapeutic target for oral cancer.

mir 26a-26b family: 26a-26b family acts as repressor in tongue squamous cell carcinoma (TSCC). In case of TSCC, p21 activated kinase appears to be highly enriched when 26a-26b are underexpressed. So we can say that ample amount of 26a-26b family inhibits TSCC.

mir 21: It affects the carcinogenesis process by targeting the tumour suppressor genes thus plays an important role as a potential diagnostic prognostic and therapeutic biomarker for OSCC. Upregulated mir 21 shows in oral leukoplakia, controls hippo pathway and negatively coregulates the prognosis of oral cancer.

mir 184: Dysregulation of microRNAs causes carcinogenesis. Increased amount of mir 184 promotes cell proliferation and induced cell death by targeting sex determining region of Y chromosome in case of tongue squamous cell carcinoma.

mir 210-3p: This microRNA is highly carcinogenic in nature. In case of OSCC, mir 210-3p is highly expressed. So it can be used as prognostic and therapeutic biomarker. It promotes oral cancer angiogenesis.

mir 31: In both cases of small and large tumours present in lesions of OSCC patients, mir 31 shows high level of oncogenicity which enhance cell invasiveness, s phase percentage and tumour cell progression. It can be used as a diagnostic biomarker for OSCC.

mir 208b-3p: In case of oral leukoplakia, mir 208b-3p plays an oncogenic character expressed increasingly and can be used a histologic diagnosis as well as predictive biomarker for OSCC.

mir 3065-5p : It is a relatively novel microRNA which express decreasingly and thought to have a tumour repressive character which contributes to reduction of cell invasion, migration and progression in case of oral leukoplakia, the most common lesion of OSCC.

mir 129-2-3p: It is a novel micro RNA which express increasingly and thought to have a tumour repressive character which contributes to reduction of cell

invasion, migration and progression in case of oral leukoplakia.

mir 222-3p: mir 222-3p promotes cell proliferation, migration and progression acts increasingly by targeting cell cycle activator CDKN1b and suppressed the gene activation of OSCC.

mir 125-b: Decreased amount of mir 125-b can drive OSCC oncogenesis. Whereas increased amount of mir 125-b promotes cell division, migration and progression acts as suppressor in case of HNSCC.

mir 141: mir 141 promotes cell apoptosis of HNSCC and inhibits cell metastasis and proliferation. mir 141 appears to be a potential therapeutic target for HNSCC.

mir 483-5p: over expression of mir 483-5p promotes tumorigenesis of OSCC. It can be a novel prognostic, diagnostic biomarker for the patients of OSCC.

mir 142-3p: It has been found that mir 142-3p from oral cancer cells promotes growth of the tumour cell by eliminating its suppressive effect and it also affects the microenvironment of tumorigenesis of oral cancer.

mir 150-5p: The up-regulation of hsa mir 150-5p found in OSCC plasma only, and it gets decreased with tumour growth plus it acts as potential biomarker for early stage detection of OSCC.

mir 20a: In case of OSCC, the amount of mir 20a is an oncomiR which get increased and participates in cell proliferation and cancer progression. So it is a potential biomarker for diagnosis of oral squamous cell carcinoma.

Let 7 family: In case of OSCC and TSCC, let 7 family shows an aggressive role towards tumor forming or expression.

mir 155: It is a tumor promoter gene in OSCC and abnormal elevation can be shown in oral cancer tissues, acts as active prognostic biomarker.

mir 211: Cell promotion, invasion and migration ability is controlled by increased condition of mir 211 in case of OSCC. It shows oncogenic activity.

mir name	OSCC	HNSCC	TSCC	putative action	progressive group
mir 204-5p	0	1	0	tumor supressor	increased
mir 223	0	1	0	potential biomarker	decreased
mir 26a-26b	0	0	1	tumor supressor	increased
mir 21	1	0	0	tumor supressor	increased
mir 184	0	0	1	inhibitor	increased
mir 210-3p	1	0	0	repressor	increased
mir 31	1	0	0	enhancer	increased
mir 208b-3p	1	0	0	oncogenic	increased
mir 3065-5p	1	0	0	tumor supressor	decreased
mir 129-2-3p	1	0	0	tumor supressor	decreased
mir 222-3p	1	0	0	tumor supressor	increased
mir 125b	1	1	0	tumor supressor	increased
mir 141	0	1	0	tumor supressor	increased
mir 483-5p	1	0	0	promoter	increased
mir 142-3p	1	0	0	promoter	increased
mir 150-5p	1	0	0	promoter	increased
mir 20a	1	0	0	promoter	increased
let -7 family	0	1	1	enhancer	increased
mir 155	1	0	1	enhancer	increased
mir 211	1	0	0	promoter	increased

Fig. 2: Data sheet of preidentified micro RNAs

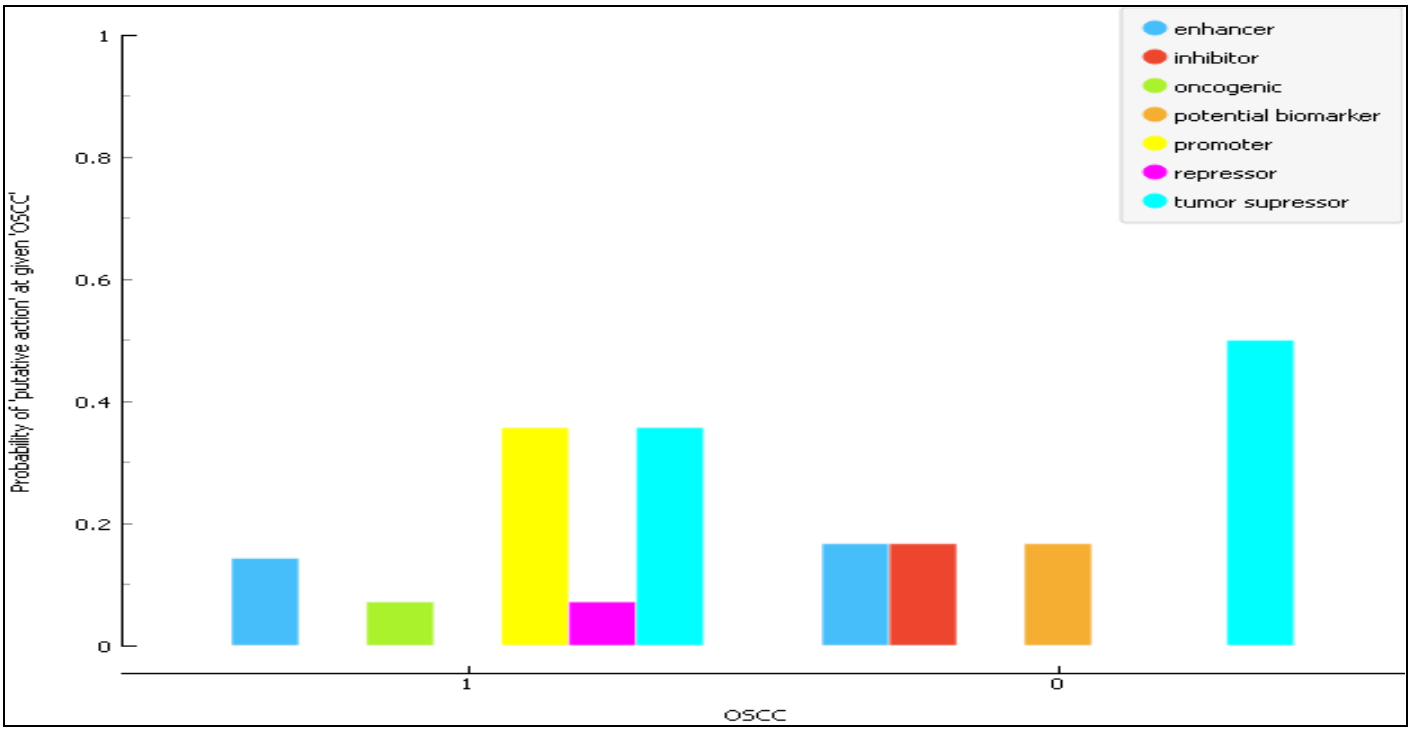
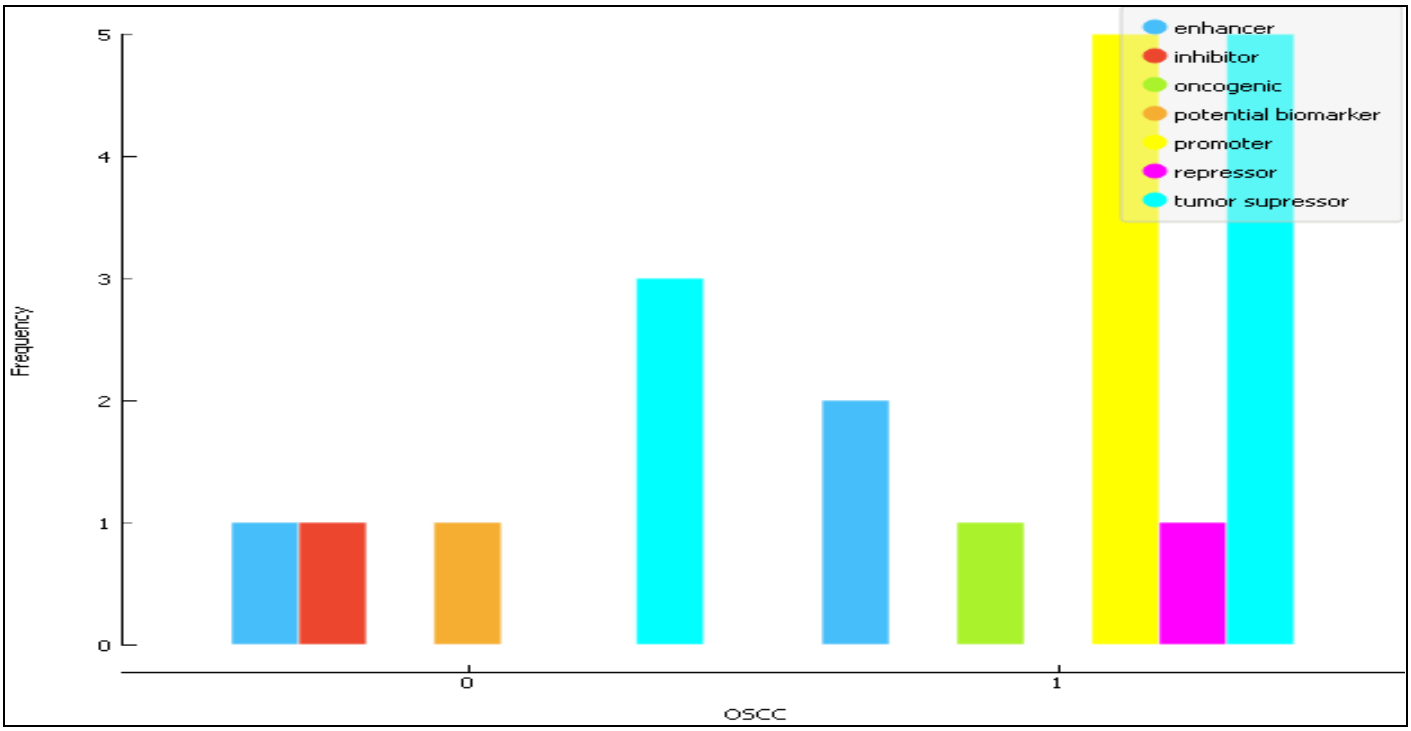
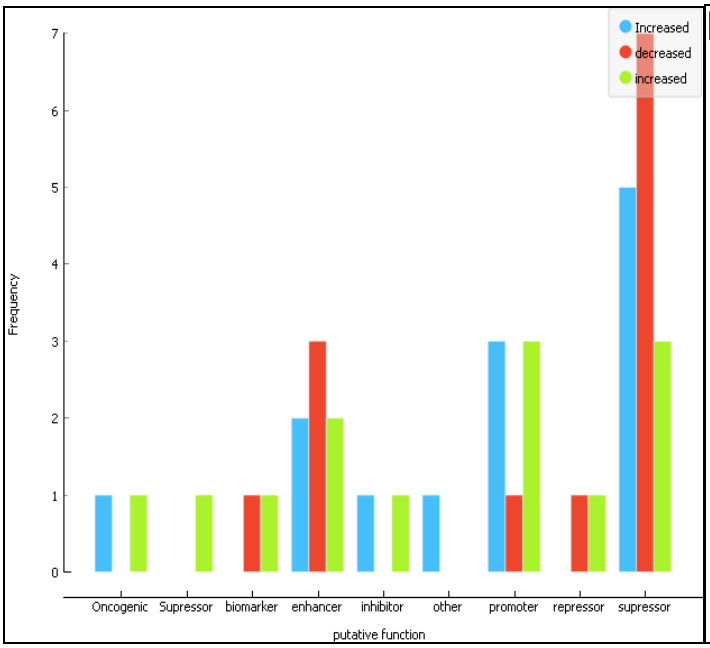


Fig. 3: The figure shows the discrete poisson probability distribution of several data in which putative function is the target variable and the outcome explained suppressor miR shows high frequency in case of OSCC.



Via probability distribution we can explain that tumour suppressor and promoter microRNAs show high potentiality towards Oral squamous cell carcinoma.

Fig. 4: The diagram shows jumbled up plotting between frequency of enhancer, inhibitor, tumour suppressor and OSCC



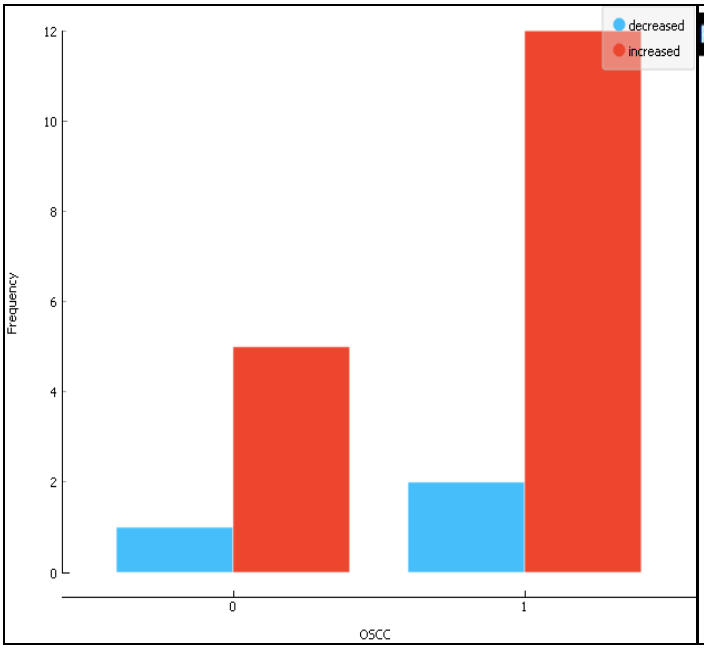
Pivot Table

Row feature: putative action
Column feature: HNSCC
Value feature: OSCC

			HNSCC		
		Aggregate	0	1	Total
putative action	enhancer	Count	2.0	1.0	3.0
		Count defined	2.0	1.0	3.0
	inhibitor	Count	1.0	0.0	1.0
		Count defined	1.0	0.0	1.0
	oncogenic	Count	1.0	0.0	1.0
		Count defined	1.0	0.0	1.0
	potential biomarker	Count	0.0	1.0	1.0
		Count defined	0.0	1.0	1.0
	promoter	Count	5.0	0.0	5.0
		Count defined	5.0	0.0	5.0
	repressor	Count	1.0	0.0	1.0
		Count defined	1.0	0.0	1.0
	tumor supressor	Count	5.0	3.0	8.0
		Count defined	5.0	3.0	8.0
	Total	Count	15.0	5.0	20.0
		Count defined	15.0	5.0	20.0

It can be observed that frequency of decreased conditioned suppressor and enhancer microRNAs is high, whereas frequency of putative function is medium for promoter biomarker microRNAs and low in oncogenic inhibitor micro RNAs. When the raw feature is putative action and column feature is HNSCC and value feature is OSCC, the pivot table data supports the diagram.

Fig. 5 & 6: The diagrams show a discrete distribution in which putative function is the target variable



Pivot Table

Row feature: putative action
Column feature: OSCC
Value feature: OSCC

		OSCC		
	Count	0	1	Total
putative action	enhancer	1.0	2.0	3.0
	inhibitor	1.0	0.0	1.0
	oncogenic	0.0	1.0	1.0
	potential biomarker	1.0	0.0	1.0
	promoter	0.0	5.0	5.0
	repressor	0.0	1.0	1.0
	tumor supressor	3.0	5.0	8.0
	Total	6.0	14.0	20.0

The frequency of occurring OSCC is high when the frequency of putative functions like suppressor, enhancer, promoter microRNAs gets increased.

Fig. 7 & 8: The diagram and pivot table shows progressive group distribution

4. DISCUSSION

The study describes the microenvironment of a cell which essentially includes microRNAs. Meticulous analysis of the research papers helped us to select 20 most important microRNAs in context of tongue, head & neck, and oral squamous cell carcinomas. The analysis revealed over-expression of 204-5p, mir 26a-26b family, mir 21, mir 184, mir 210-3p, mir 31, mir 208b-

3p, mir 222-3p, mir 125b, mir 141, mir 483-5p, mir 142-3p, mir 150-5p, mir 20a, let 7 family, mir 155, mir 211 or under-expression of mir 223, mir 129-2-3p, mir 3065-5p, mir 204-5p in the non and dysplasia of a cell ultimately progresses to oral squamous cell cancer, head & neck cancer or tongue squamous cell carcinoma. Indeed, the biological characteristics of some potential genes targeted by these above mentioned microRNAs have been found to connect with various critical biological processes related to oncogenesis and tumor suppression. Increased amount of mir 204-5p is thought to have a tumor suppressor effect for head & neck cancer. mir 204 is located at the genomic imbalanced 9q21.1-22.3 locus and mir 3065-5p is at 17q25.3 related with genetic aberration for HNSCC, OSCC, respectively [59,41]. Epigenetic alteration, for example, Hypermethylation of the promoter region of mir 129-2-3p has been reported to be an early event in oral carcinogenesis [51]. Expression level of oncogenic mir 208b-3p shows a good amount increase in progressed group and it acts as a potential biomarker of oral leukoplakia. In tumor development, particularly tumor angiogenesis, increased amount of mir 210-3p shows proangiogenic progress of squamous cells of oral region [37]. In healthy tissues, levels of mir 141 expression is higher than that of many tumors and decreased expression of mir 141 promotes tumor progression. It means mir 141 expresses weakly in head & neck squamous cell carcinoma tissues, and if its levels are elevated, it helps the patient to survive better [54]. Up-regulation of mir 211 and down-regulation of mir 125b can drive OSCC oncogenesis in a coordinated manner. In a stretch mir 125b down-regulated expression has been identified in HNSCC [3]. mir 211 acts as an active promoter of oncogenic squamous cells of oral region. In oral carcinoma, high expression of mir 211 and mir 21 has been associated with poor prognosis in patients. We found little evidence that increased amount of mir 483-5p and mir 211 acts as promoter and enhancer, respectively. Decreased amount of mir 125b contributes to oral malignancy [27]. Previous studies showed that reduced levels of let 7 are observed in the presence of increased expression of RAS protein [60]. This observation has led to the hypothesis that let 7 may inhibit growth of cancer through its effect on the RAS protein. Accordingly, in HNSCC, the presence of RAS protein was significantly associated with poor prognosis and increased tumor size [57]. In cancer development, the inhibitory effect of let 7a was further supported by findings of low amount of let 7a levels in HNSCC and lymph-node metastasis. mir 21 is mainly expressed for the development of tumor [47,61-62]. Increased amount of mir 21 has been observed in lung cancer, neuroendocrine tumor, gastric cancer, pancreatic cancer, breast cancer, glioblastoma, colon cancer, prostate cancer and bile duct cancer [47, 63]. mir 21 contains 22 nucleotides, processed from a 3400 nucleotides pre-transcript and is encoded by a single gene named VMP1 which is present on fragile site FRA17B on 17p23.2 that is overlapped with a transmembrane protein 49 (TMEM49) [47, 64-65]. The mir 21 regulates several cellular processes like cell proliferation, differentiation and apoptosis, tumor cell invasion, vascular infiltration and metastasis via targeting several genes such as tropomyosin 1, methyladenosine and programmed cell death genes in p53 mediated pathways and transforming factor beta pathway [66]. In case of oral leukoplakia, oral lichen planus presence of mir 21 and mir 31 are significantly high and downregulation in Oral sub mucous fibrosis (OSMF) and leukoplakia groups with OSMF [48]. mir 141 expresses in lower amount in many tumors than in healthy tissues and its increased expression inhibits tumor progression [54]. Mir 141 inhibits the development of prostate, gastric, colorectal and liver cancers by inhibiting cell proliferation, metastasis and cell apoptosis [67-69]. mir 141 is weakly expressed in head & neck cancer tissues and higher expression of this improves patients survival time [54]. Significantly increased amount of mir 20a and mir 150-5p serves as a potential biomarker and promoter for the genes responsible for OSCC. Corroborating previous findings shows mir 20a inhibits cell migration in oral cancer and thus it may be a prognostic biomarker for the disease [70] and also suggesting that mir 20a may act as oncogenic miRNAs (onco- mir) in OSCC. Various tumor related genes, such as HMGA2 [71], RB1CC1/FIP200 [72], SRCIN1 [73], STAT3 [74] AND PTEN [75], are targeted by mir 20a. Hence we conclude that mir 20a targeted therapy will strongly impact oral cancer [76]. Up-regulated circulating mir 223 shows tumor suppressor activity; i.e. tumor inhibiting miR and serves as a biomarker but also plays several beneficial role in pathology of oral cancer [44]. mir 184 plays an important role in many cancers like nasopharyngeal carcinoma [77], head & neck carcinoma [78] and neuroblastoma [79]. It has been reported that upregulated mir 184 in TSCC tissues shows significant role than the other non tumorous tissues and promotes the proliferation of cancerous squamous cells of tongue

[49]. mir 26a and mir 26b both are underexpressed in case of tongue squamous cell carcinoma. They repress the cell cycle of TSCC, migration, invasion and glycolysis while p21 activated kinase 1 shows high amount in the cell and promotes cell apoptosis [10]. Higher expression of mir 222-3p is correlated with OSCC tumor growth (40%) [80] and metastasis in tongue cancer by targeting matrix metalloproteinase 1 and manganese superoxide dismutase 2. Abberant decrease in cell invasion and migration has been reported dute to ectopic transfection of mir 222-3p [81]. If tissue and plasma consists of lymph node metastasis, mir 222-3p is down regulated and affects cell growth, invasiveness and apoptotic abilities by targeting to PUMA in OSCC [52].

5. CONCLUSION

In conclusion we successfully enlisted twenty micro RNAs which have been identified to be differentially expressed in OSCC, HNSCC and TSCC patients. The contemporary results indicate that OSCC, HNSCC, TSCC can potentially be detected and treated with relatively high specificity and sensitivity on the basis of microRNA levels. The panel of these microRNA studied showed potential as a novel putative biomarker for squamous cell carcinoma. In this study, we applied the concept of ORANGE data mining software on microRNA networks responsible for OSCC, TSCC and HNSCC. Hence, we conclude that amplification or overexpression of micro RNAs can control or downregulate tumor suppressors or other genes involved in cell progression and differentiation, thereby contributing to tumor formation by stimulating angiogenesis, invasion, proliferation and migration; *i.e.,* they show their nature as oncogenes. This study enriched our understanding about the role of micro RNAs as tumor suppressor/enhancer/promoter/ oncogenic for OSCC, TSCC or HNSCC development and will provide new insights into the probable treatment-related avenues.

Conflict of interest

None declared

6. REFERENCES

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