POTENTIAL MICRORNA SIGNATURE ASSOCIATED WITH POST-OPERATIVE TUMOUR RECURRENCE RISK IN SOUTH INDIAN ORAL SQUAMOUS CELL CARCINOMA PATIENTS IDENTIFIED BY NEXT-GENERATION SEQUENCING

Vinod Nair Sreekumar[1,2], Madhulaxmi M[3], Paramasivam Arumugham[4], Anna P Joseph[5], Ravindran Ankathil[6, 7*]

[1]Research Scholar, Saveetha Dental College and Hospital, Poonamallee High Road, Chennai, India
[2]Associate Professor, Department of Oral and Maxillofacial Surgery, P M S College of Dental Sciences and Research, Kerala University of Health Sciences, Trivandrum, India
[3]Professor, Department of Oral and Maxillofacial Surgery, Saveetha Dental College and Hospital, Poonamallee High Road, Chennai, India
[4]Research Scientist, Molecular Biology Laboratory, Saveetha Dental College and Hospital, Poonamallee High Road, Chennai, India
[5]Professor, Department of Pathology, P M S College of Dental Sciences and Research, Kerala University of Health Sciences, Trivandrum, India
[6]Jubilee Centre for Medical Research, Jubilee Mission Medical College and Research Institute, Thrissur, Kerala, India
[7]Senior Research Scientist, Central Research Laboratory, P M S College of Dental Sciences and Research, Kerala University of Health Sciences, Trivandrum, India
Corresponding author Email : rankathil@hotmail.com

Article History: **Received:** 18.04.2023 **Revised:**07.05.2023 **Accepted:** 16.06.2023

Abstract: *Oral squamous cell carcinoma (OSCC), one of the cancers originating from the epithelium lining the upper gastrointestinal system, is a serious global health issue. Despite improvements in diagnosis and novel tailored treatments, it is still considered one of the most prevalent cancers. Although systematic treatment methods, including surgery, radiation therapy and chemotherapy, have achieved reduced mortality and higher survivor rates, a significant proportion of OSCC patients undergoing oral treatment develop metastasis and recurrence.Therefore, it is essential to identify biomarkers for early diagnosis, prognosis prediction, treatment response and overall survival of OSCC. The current research aimed to identify the dysregulated miRNAs in cancerous tissues and plasma of OSCC patients compared to adjacent healthy tissue and plasma of normal control and also to identify whether the dysregulated miRNAs are present in the plasma of post-operative OSCC patients showing recurrence and no recurrence. For this study, blood samples, biopsies of normal and tumour tissue and FFPE blocks were taken from eleven OSCC patients with and without recurrence in the oral cavity and one normal control individual. A microRNA library was created after total RNA and microRNA isolation from these materials. This experimental study used next-generation sequencing (NGS) to identify the differentially expressed miRNAs in tissues and serum of OSCC patients and normal healthy control. The prominently dysregulated miRNAs were validated with qRT-PCR. By analysing the expression of their mRNA targets and performing a pathway analysis, we could narrowly concentrate on the expression mechanism of miRNA profiles linked with oral cancer recurrence. This research discovered differentially expressed miRNAs by next-generation sequencing (NGS) using paired tissue and serum samples from OSCC patients and matched controls. During tissue profiling, 317 miRNAs were discovered while 164 unique miRNAs were identified in serum plasma samples. When we examined the miRNA expression in tumours and healthy tissues, we discovered 22 differentially expressed miRNAs (11 miRNAs down-regulated and 11 up-regulated; false discovery rate 0.05). Our research revealed that specific miRNAs, including hsa-miR-142-3p, hsa-miR-32-5p, has-miR-21, hsa-miR-141, and hsa-miR-29c were up-regulated in tumour tissues more frequently as compared to normal healthy tissues. Meanwhile, hsa-miRNA 375, hsa-miRNA 199a-5p, hsa-miRNA 99a, let-7a, and hsa-miRNA 199a-3p were all found to be more frequently downregulated in tumour tissues. Out of these 22*

miRNAs, up-regulation of hsa-miR-32-5p, and down-regulation of hsa-miR-375 were identified in the pre-operative serum plasma of the same group of OSCC patients. Among these patients, 4patients with hsa-miR-375 downregulation developed early recurrence, 3 patients with miR-32-5p developed late recurrence and 4 patients with other miRNA dysregulation pattern showed no recurrence post-operatively till date. In this study, hsa-miR-375 and hsa-miR-32-5p appeared as the most predominant miRNAs associated with post-surgical recurrence. Our results suggest the potential of utilizing these circulating plasma miRNAs as noninvasive biomarkers associated with post-surgical recurrence risk in OSCC patients.

Keywords: *microRNA, oral cancer, circulating microRNA, recurrence risk, prognosis, NGS, qRT PCR, OSCC Recurrence, Next Generation Sequencing*

DOI: 10.48047/ecb/2023.12.10.717

INTRODUCTION

Head and Neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide, mainly comprises neoplasms of the oral cavity, pharynx, larynx, paranasal synapses, nasal cavity and salivary glands. Oral squamous cell carcinoma (OSCC) is a significant component of HNSCC, accounting for about 90% of all oral malignancies, representing a significant global burden with an estimated 300,373 cases per annum (Gupta et al 2016). According to Globocan 2018 report, ~ 120,000 new OSCC cases are detected yearly, ranking it the second most common cancer reported in India (Bray et al 2018; Ferlay et al 2018).Over 72,000 deaths in India are attributable to malignancies affecting the oral and maxillofacial region. Epidemiologic studies worldwide have implicated tobacco usage, alcohol consumption, and infection with viral agents, especially human papillomavirus (HPV), as the etiological factors contributing to the development of OSCC (Kreimer et al 2005; Mehanna et al 2010). Quite alarmingly, the incidence is surprisingly growing among young individuals too. In India, the widespread habit of smoking or grinding tobacco, and consuming alcohol, aside from inadequate oral hygiene, poor diet and HPV infection, has contributed to the disproportionately more significant number of OSCC (D'Souza and Kumar 2020).

Even after tremendous improvements achieved in OSCC treatment due to advances in surgery, radiotherapy, chemotherapy and targeted therapy, the 5-year overall survival of OSCC patients has not significantly improved in the last 20 years. (Slieker et al 2022). Only 50% of OC patients achieve a 5-year survival rate (Listl et al 2013). The 5-year survival has been reported to range from 80% for cases diagnosed with the localized disease to less than 30% for metastatic cases(Johnson et al 2011; van Lanschot *et al* 2020). Even after the implication of prognosis therapies like visual inspection and innovations in diagnosis and therapeutic strategies, late diagnosis has been documented to be causal to the rapid increase in the number and poor prognosis of OSCC (Leemans et al 2011). Non-implementation of the national OSCC screening program in India due to the lack of conclusive and specific biomarkers for early detection and progression surveillance of OSCC has added to the late-stage diagnosis of OSCC (D'Souza and Kumar 2020). The late-stage diagnosis often complicates the treatment of OSCC, causing difficulty in treating advanced OSCC due to lymph node metastasis and the frequent incidence of local recurrence and second primary tumours. A significant proportion of OSCC patients who have undergone surgical resection of the tumour will develop recurrence and metastasis. Therefore, the overall survival of oral squamous cell carcinoma (OSCC) remains unchanged mainly due to challenges in early diagnosis and the lack of reliable biomarkers for predicting treatment outcomes.

Identifying predictive pointers of excellent, moderate and poor prognosis is crucial for OSCC management. This demands the need to elucidate the mechanism underlying OSCC development, which may help optimize treatments and improve patient survival (Gao et al 2019). Identifying non-invasive biomarkers to determine the progression and risk of recurrence could immensely help OSCC patients. The development of recurrence is a major life-threatening event mainly associated with poor outcomes. Because of the molecular complexity of OSCC, it is challenging to identify OSCC patients who are likely

to develop recurrence after surgical removal of the tumour. Despite several clinical and pathological features being used to discriminate between low and high-recurrence risk patients, the current conventional prognostic factors are inadequate for OSCC. There is a lack of non-invasive and cost-effective biomarkers to identify the recurrence risk in OSCC patients. In addition to the anatomic extent of the disease, consideration of biological determinism reflected in the altered expression of biomarkers has become increasingly important in the personalized management of OSCC patients.

Recently, researchers have correlated noncoding RNAs, especially microRNAs (miRNAs) expressions and clinical parameters in cancers including OSCC, with recurrence and metastasis. MicroRNAs have significant roles in various bodily pathological and physiological activities (Liu et al 2018). Most importantly, miRNAs have been recognized for their multifaceted roles in the hallmarks of cancer, such as angiogenesis, epithelial-mesenchymal transition (EMT), cancer-stem cell biology, drug resistance etc. Many studies have supported the crucial involvement of miRNAs as tumour suppressors or tumour promoter genes in various cancers, including OSCC (Calin and Croce 2006; Langevin et al 2010; Wikhund et al 2011; Solomon and Radhakrishnan 2020). Wang et al (2021) reported that miRNAs are involved in the occurrence, progression and metastasis of OSCC and that the expression profiles of specific miRNAs were correlated with clinical stage, metastasis and patient survival. However, there are inconsistencies in the results across populations. Since miRNAs have been shown to correlate with tumour size and differentiation, they could be candidate biomarkers for estimating recurrence risk and clinical outcomes. It was hypothesized that dysregulated microRNAs might also be linked to postoperative disease recurrence and poor prognosis in OSCC patients. Since there is a dearth of information on the miRNAs signature associated with recurrence risk in South Indian OSCC, the present study aimed to investigate and determine the dysregulated microRNAs signature associated with recurrence risk in a group of South Indian OSCC patients who had undergone surgical resection of the tumour.

METHODOLOGY

The study was initiated after approval from the Institutional Research and Human Ethics Committee of Saveetha Institute of Medical and Technical Sciences, Chennai (SDC/Ph.D-01/19/09). The study materials included FFPE tumour tissue blocks, blood samples from OSCC patients, and normal healthy controls. Tissue and blood samples of patients recruited into the study were taken after their written consent, and their confidentiality was upheld. Clinical information was taken from the research participants' medical documents. It included demographic information, age at disease onset, histopathological subtype, stage, tumour grade, nature of treatment, details of first recurrence and disease outcome.

Verification of Samples for the study

- OSCC specimens from patients undergoing surgery were collected for microRNA expression profiling. The institutional multidisciplinary panel determined that each patient was qualified for surgical resection.
- Tissue specimens from the healthy control group for microRNA expression profiling were collected intraoperatively after verification by a pathologist.
- Blood samples (about 4ml) of both the groups (OSCC patients and Control group) were also collected for RNA extraction from serum samples, microRNA expression profiling and quantification.

Based on the TNM categorization for malignancy, OSCC tumours were graded. This describes the subject's level of cancer status. Squamous cell carcinoma was pathologically verified and confirmed in every included patient. For this study, the first and second groups included tumour and adjacent normal tissue specimens of OSCC patients who have already undergone primary surgical intervention with early

recurrence (4 patients) (less than 6 months after primary resection) and three with late recurrence (greater than 6 months after primary resection). The third group included tissues of four patients with no recurrence after primary surgical intervention, while the fourth group included healthy control subjects for comparison.

Total RNA extraction from FFPE block samples

H&E labelling was used to pinpoint the malignant and adjacent normal tissue sites. More than 70% of the tumour cells were in the malignant tissue, while the remaining 30% were in the surrounding healthy tissue. Microtomes prepared 5–20 microns thick cancerous and adjacent healthy tissue sections. The RecoverALL Total Nucleic Acid Isolation System for FFPE (Ambion, USA) was then used to recover total RNA from cancer and adjacent normal healthy tissues following the manufacturer's instructions. To quickly eliminate any remaining traces of paraffin, 4 samples of 20-micron-thick FFPE slices were treated with 1 ml of xylene. An extended protease K digestion of the tissues at 50°C was followed for DNAse I treatment. The isolated total RNA was eluted with RNAse-free water following several therapies.

Total RNA extraction from Blood plasma samples

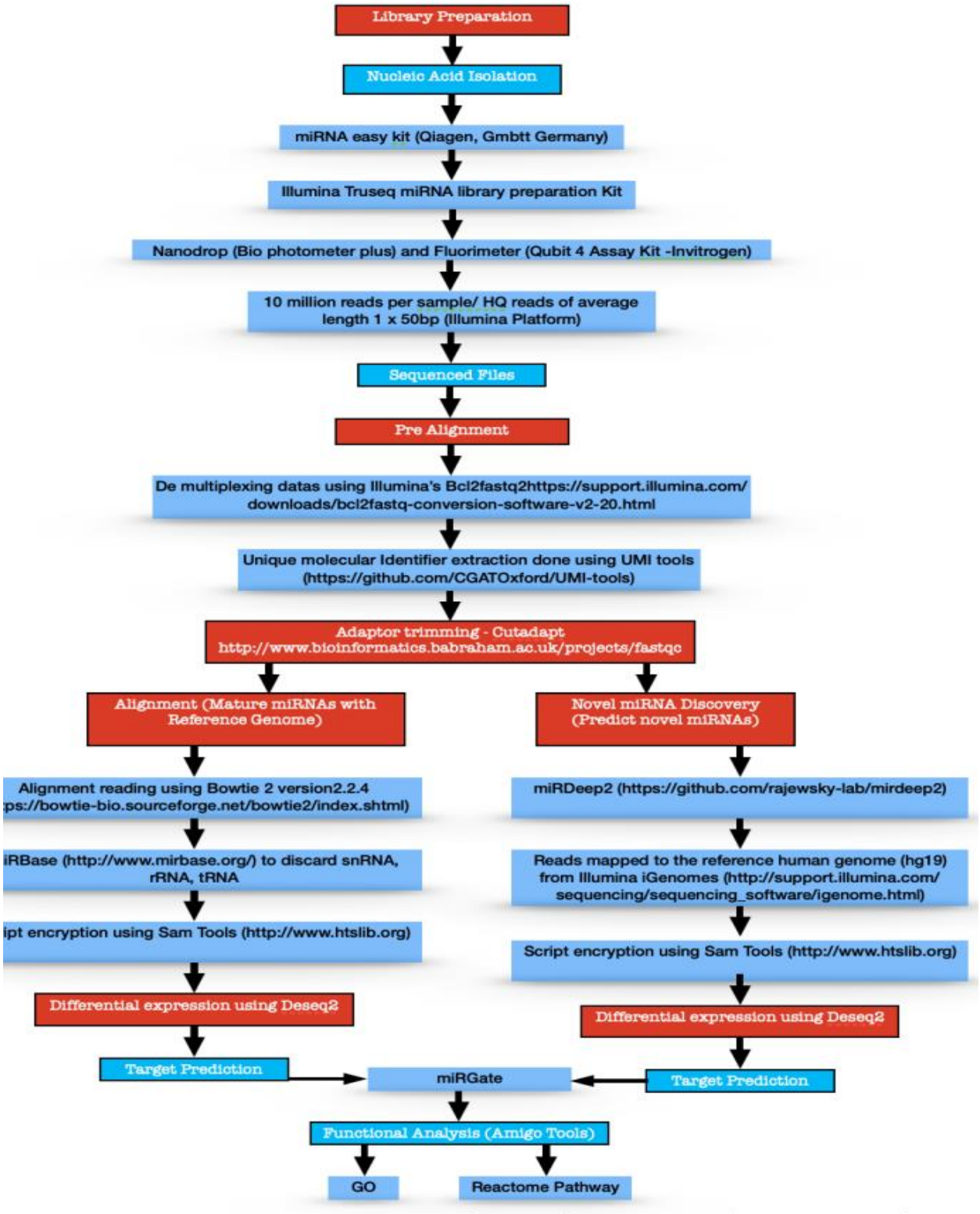
Blood samples from OSCC patients and control subjects were also collected and examined to determine whether the discovered putative miRNAs were present pre-operatively as well as post-operatively, as cell-free circulating miRNAs in their serum plasma samples. Blood samples of patients with OSCC were drawn into EDTA containers and prepared for plasma separation. Blood was centrifuged at 1600 g for 10 min at room temperature, and the supernatant plasma was transferred to a new container and kept at -80°C until use. Using the Trizol LS reagent and miRNeasy Mini Kit (Qiagen, Hilden, Germany), total RNA containing small microRNAs was isolated as directed by the manufacturer with the following modifications: Five hundred (500) ml of plasma sample was mixed with 1 millilitre of Trizol LS reagent. Following phase separation, the aqueous phase was added with 1.5 volumes of 100% ethanol before loading onto the miRNeasy column (Qiagen) following the manufacturer's directions. The RIN number was used to measure RNA integrity.

Pre- and post-surgical blood samples of these 11 OSCC patients and one control were also collected and subjected to plasma serum miRNA profiling. Out of these 11 patients, 4 showed early recurrence, 3 showed late recurrence and 4 showed no recurrence till date of writing. Circulating miRNA in the plasma serum of these 11 patients were analyzed. Blood samples from participants of these four groups (three patient groups and one control) were drawn using standard anterior neck venipuncture to obtain blood (4 cc), which was then centrifuged at 2200–2500 speed for 10 min at 4°C. Blood cells were isolated from the serum before freezing it at -80°C for microRNA analysis. While samples were kept at -80°C, the quantity and quality of extracted RNA were checked using Nanodrop (Bio photometer plus) and a fluorimeter (Qubit4). Only RNA with the following qualities was utilized for analysis: adequate amount (>2g), purity (A260/280 and A 260/230 between 2.0 and 2.2), and good quality (RIN>7). Samples were split into three groups, two of which were used for expression analyses, based on OSCC recurrence rates.

miRNA library creation and RNA extraction

Tissue samples (n=12) were processed in the Bullet Blender after being removed from the -80°C freezer. Total RNA was isolated using the commercially available column purification method of the Qiagen miRNeasy Mini Kit. DNase Kit without RNase, Qiagen), applied to the column per the directions provided by the manufacturer. Following the manufacturer's instructions, serum samples (n=7) were extracted using the MiRNEasy Serum/Plasma Kit (Qiagen). Using the 3' and 5' adapters, 1 ng of total RNA or small RNA from 300 µl of unquantified serum was concatenated to create single-stranded cDNA. Following that, the samples proceeded through 14 cycles of PCR at various temperatures (94°C for 30 seconds, 94°C for 15 seconds, 62°C for 30 seconds, 70°C for 15 seconds, and an extension phase). (last, at 70 °C).

Figure 1 (miRNA sequencing flow diagram)



The flow of research procedures for detecting the expression profiles of microRNAs in the tumour tissues, adjacent normal tissues and sera of patients with OSCC and normal healthy control is shown in Figure-1.

miRNA expression analysis using next-generation sequencing (NGS)

Each distinct library was processed for sequencing in a single-line cell lane using an index (5 min amplification). The amplified libraries underwent size selection and purification on a 6% agarose gel. After determining the amount and calibre of the miRNA libraries using the BioAnalyzer kit and the RNA Nano Lab Chip, samples were combined in a microtubule and sequenced on HiSeq.2500 device (Illumina Inc.) using the Illumina HiSeq v4 kit for a read cycle of 50bp (1x50) (Agilent Technologies, Santa Clara, CA, USA). Following the manufacturer's recommendations, the NEB Next multiplex RNA library production toolkit manufacturing procedure was utilized to create small RNA libraries from extracted RNA. They were quantified using the Qubit High Sensitivity Test. The samples were then combined, and sequencing was done on the Illumina Novaseq 6000.

miRNA library analysis and statistical interpretation

Sequence alignment from the libraries obtained in the preceding phase and miRNA quantification was performed using the sRNA Toolbox web service according to Rueda et al (2015). EdgeR1 using the GLM model and patient pairing samples was used to perform statistical analysis of differentially expressed miRNAs following Robinson et al (2010). miRNAs with a FDR<0.05 and FC>2.0 were considered as up-regulated; and FDR<0.05 and FC >0.50 were considered as down-regulated. The software R (3.2.2) and the Bioconductor tool DESeq were used to perform unsupervised hierarchical clustering of the most highly expressed miRNAs suggested by Anders & Huber (2010). To determine miRNA gene families, miRBase was employed following Kozomara et al (2011).

miRNAs target prediction and enriched pathways and GO Terms

miRBase targets (http://www.mRBase.org), www.mirbase.org, and http://pictar.mdc-berlin.de/ were used to identify the projected targets of the differentially expressed miRNAs in this work. The mirPath tool was used to project databases from the MicroT-CDS version 5.038. (version 3.0). We used the same method to recruit at least five miRNAs targeting this process or route after extracting keywords from KEGG molecular pathways and gene ontology (GO) (biological processes). p-values <0.05 were regarded as statistically significant

Statistical Analysis

SPSS (v23.0) statistical software was used for all statistical analyses. The ANOVA test compared the Ct delta values between the tumour and adjacent non-tumour tissues and control groups. To examine differences between various clinical types, the t-test and ANOVA tests were performed. At p <0.05, the difference was deemed statistically significant. Pearson's coefficient was used to determine correlation.

The Spearman's correlation test examined the connection and link between miRNAs in healthy blood, tumour tissues, and adjacent normal tissues and clinical prognosis. For a 2-fold change in miRNA expression levels, a Hazard Ratio (HR) was computed. The HRs of these miRNAs were less than 1. To identify the miRNAs differently expressed in blood samples and cancerousand adjacent non-cancerous tissues, paired t-tests were utilized. To compare miRNA expression in OSCC patients and age-matched healthy control, we used differential expression analysis (DEA) using Bioconductor package DESeq (1.2.0) and existing miRNA indices. p-adj <0.05 was defined as log2FC(1) for upregulated miRNAs and log2FC(1) for downregulated miRNAs. All two-sided p-values were considered statistically significant if they were <0.05. GraphPad Prism 5 demo programme and SPSS software were used for all statistical computations (GraphPad Software, San Diego, CA, USA).

RESULTS

We explained all the study details to the patients selected and obtained informed written consent before recruiting them into this study. The demographic and clinical details of the OSCC patients included in the present study are shown in Table-1. Figures 2 A to 2 F represent the demographic and clinical data of the recruited OSCC patients based on the distribution of gender (Fig. 2 A), betelnut chewing (Fig. 2 B), alcoholism (Fig. 2C), smoking (Fig. 2 D), recurrence rate (Fig.2 E) and histopathological differentiation (Fig.. 2 F). The distribution of OSCC patients according to AJCC pathological staging, T staging, oral site of involvement and lymph node metastases are shown in Figs, 3 – Figs. 7 respectively.

Table -1 Demographic and clinical data of OSCC patients

Age	49	53	36	56	61	55	36	64	59	38	57	48
Tobacco Habit	Smoker	Smoker	Ex-Smoker	Smoker	Non Smoker	Smoker	Smoker	Non Smoker	Ex-Smoker	Smoker	Smoker	Non Smoker
Alcoholism	Non Alcoholic	Alcoholic		Alcoholic	Alcoholic	Alcoholic	- -	Alcoholic	Alcoholic	Alcoholic	Alcoholic	Occasional
Betel Nut Chewing	Betel Nut Chewer	- -	Betel Nut Chewer	- -	Betel Nut Chewer	Betel Nut Chewer	- -	Betel Nut Chewer	Betel Nut Chewer	Betel Nut Chewer	Betel Nut Chewer	- -
Gender	MALE	MALE	FEMALE	MALE	MALE	MALE	MALE	FEMALE	MALE	MALE	MALE	MALE
Site of Involvement	BUCCAL MUCOSA	BUCCAL MUCOSA	BUCCAL MUCOSA	TONGUE	BUCCAL MUCOSA	PALATE	BUCCAL MUCOSA	BUCCAL MUCOSA	TONGUE	BUCCAL MUCOSA	ALVEOLUS	NORMAL HEALTHY MUCOSA
Histopathological Differentiation	**WELL**	**WELL**	**WELL**	**WELL**	**WELL**	**MODERATE**	**MODERATE**	**MODERATE**	**POOR**	**POOR**	**POOR**	
AJCC pathological Staging	STAGE II	STAGE II	STAGE II	STAGE II	STAGE II	STAGE II	STAGE III	STAGE III	STAGE III	STAGE IV	STAGE IV	- -
T staging	T2	T2	T2	T2	T2	T2	T3	T3	T3	T4	T4	- -
Lymph node metastases	N1	N0	N1	N0	N0	N1	N1	N2	N1	N2	N1	- -
Recurrence	RECURRENCE(Early)	RECURRENCE (Late)	NO RECURRENCE	RECURRENCE (Early)	RECURRENCE (Late)	RECURRENCE (Late)	RECURRENCE(Late)	RECURRENCE(Late)	RECURRENCE(Early)	RECURRENCE(Late)	RECURRENCE (Late)	- -
Prognosis	Good	Good	Good	Good	Poor	Good	Poor	Poor	Poor	Poor	Poor	- -

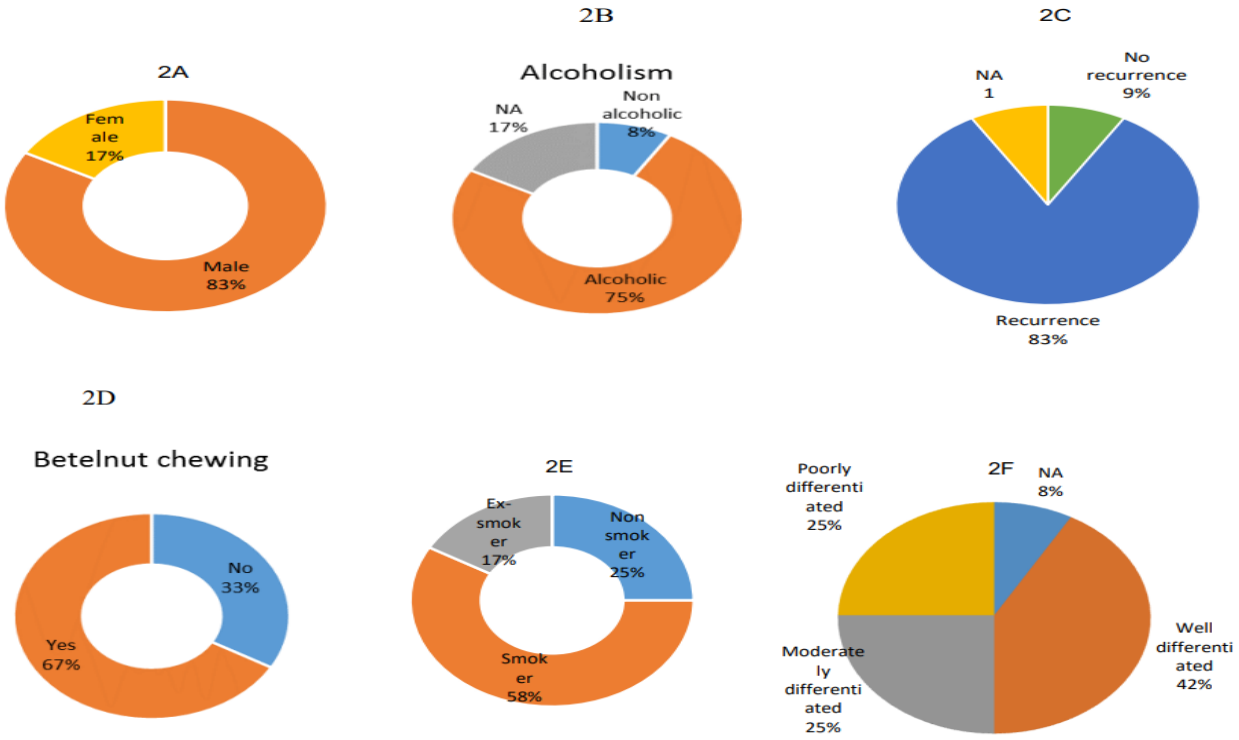


Figure 3 Distribution according to AJCC Pathological Staging

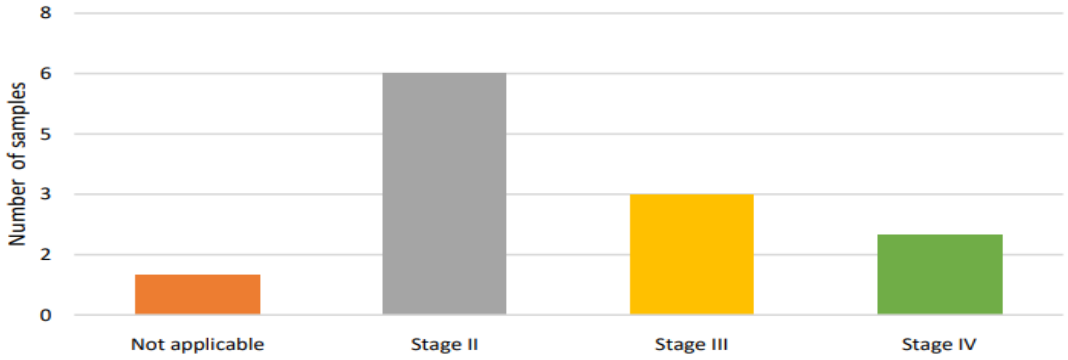


Figure 4 Distribution according to T Staging

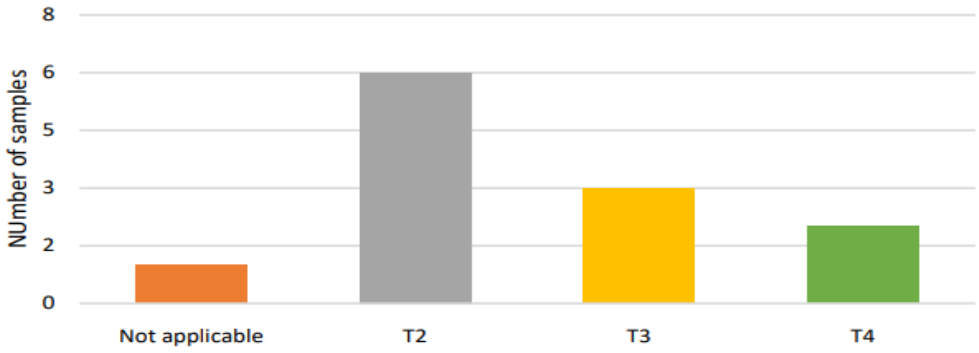


Figure 5 Distribution according to Site of Involvement

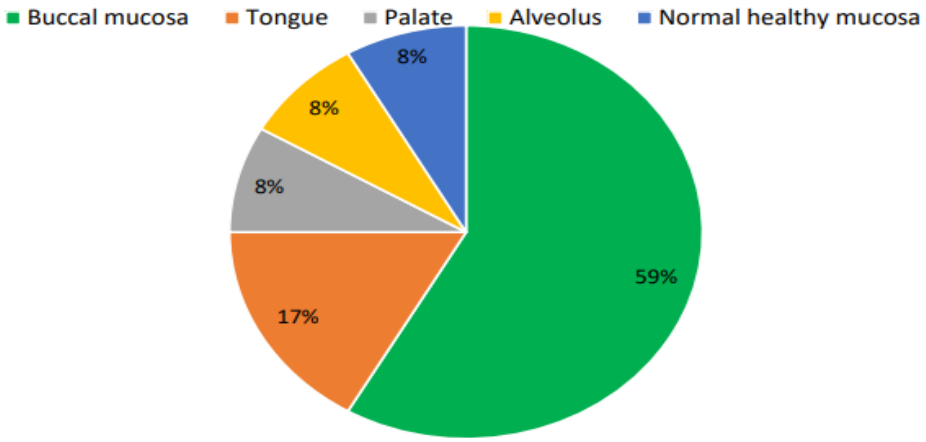


Figure 6 Distribution according to Lymph Node metastases

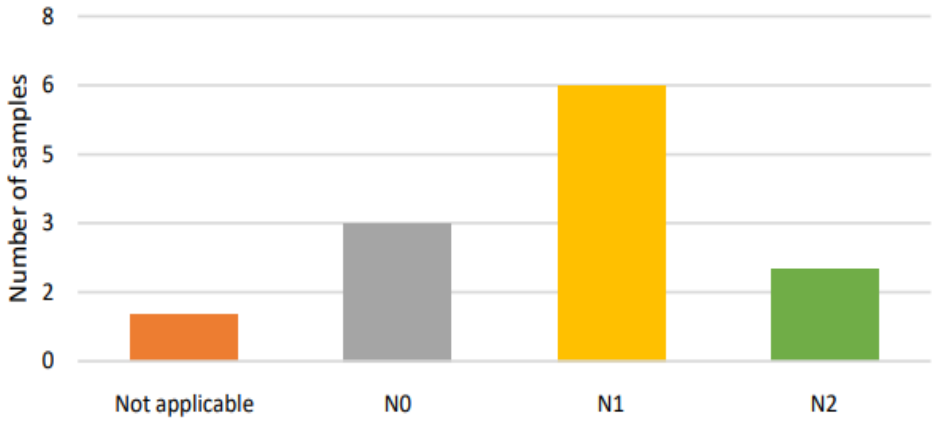


Figure 7 Distribution according to Recurrence

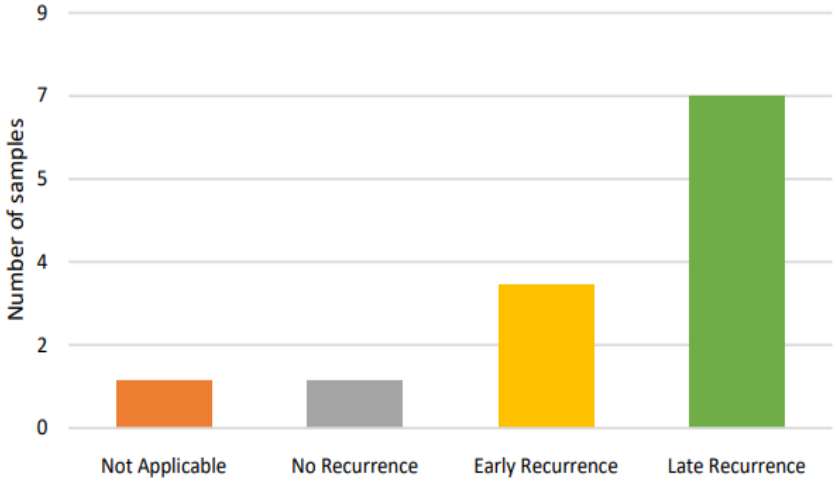


Table 3 miRNAs showing downregulated expression

Down Regulated MiRNA	Number of samples	Slide	OSCC
hsa-miR-375	4	Slide 6,7,5,21	OSCC 6,12,5,11
hsa-let-7a	1	Slide 2	OSCC 4
hsa-miR-199a-3p/hsa-miR-199b-3p	2	Slide 1,4	OSCC 18,7
hsa-miR-199a-5p	1	Slide 1	OSCC 9
hsa-miR-99a	1	Slide 2	OSCC19
hsa-miRPlus-E1016	2	Slide 9,3	OSCC 11,8

Table 2 miRNAs showing up regulated expression

Up Regulated miRNA	Number of samples	Slide	OSCC
hsa-miR-141	1	Slide 21	OSCC 11
hsa-miR-32-5p	3	Slide 9,3,2	OSCC 11,8,4
hsa-miR-21	2	Slide 1,4	OSCC 18,7
hsa-miR-142-3p	4	Slide 7,1,5,2	OSCC 12,9,5,19
hsa-miR-29c	1	Slide 6	OSCC 6

Principal component analysis of the 317 most differentially expressed miRNAs discovered in tissue samples from malignant and normal tissues, which revealed distinct expression patterns, demonstrated the sampling approach used during the discovery phase. Significant variations existed between the two study groups. After miRNA analysis and elimination, 317 miRNAs were discovered in tissues with incredibly low read counts (less than 3 reads per million in fewer than 50% of samples). There were 164 differentially expressed miRNAs found in the blood samples. Among these miRNAs found in the tissues and serum plasma of the same patient, we identified 138 aberrantly expressed miRNAs.

This critical miRNA screening step used next-generation miRNA expression profiles to find distinct miRNA expression patterns in cancerous and adjacent non-cancerous oral tissues. NGS resulted in the generation of 0.9 to 1.7 GB of raw data from each sample. We conducted quality checks and further analyzed the raw data. In malignant tissue, 560,054 valid readings were captured, while 3,300,900 valid readings were captured in adjacent non-cancerous tissue. The remaining valuable reads were assigned to the human miRNA precursor collection after filtering by rRNA, tRNA, snRNA and snoRNA. Malignant and adjacent non-cancerous cells yielded 41,225 and 203,889 reads, respectively. The 22 nt size class of these reads was the most common length in nearby malignant and non-cancerous tissue. Of the 940 known precursor miRNAs, 546 were found in malignant tissue and 364 in adjacent non-cancerous tissue compared to miRbase (15.0). We looked at the distinct genome-wide expression patterns of tissue

miRNAs across malignant and adjacent non-cancerous oral tissues using the Mann-Whitney test and fold-shifting of sequenced miRNAs.

Figure 8 volcano plot showing the relation between the logarithm of the p-values and the deltaLogMedianRatio (dLMR).

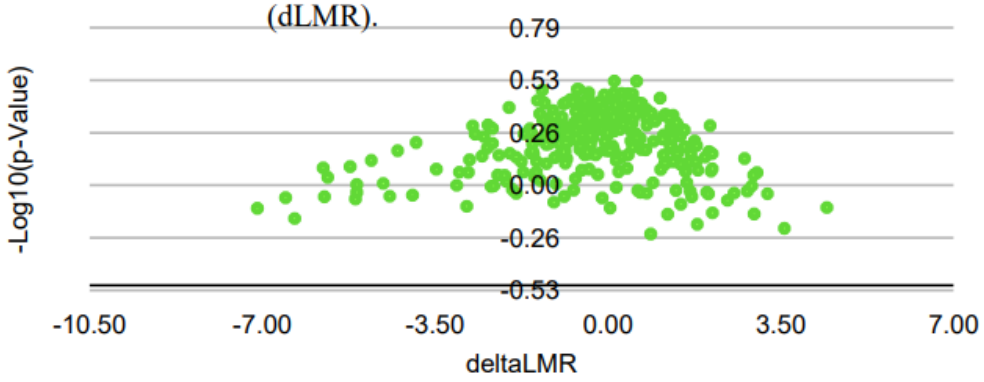


Figure 9 Graphical Representation of miRNA expression

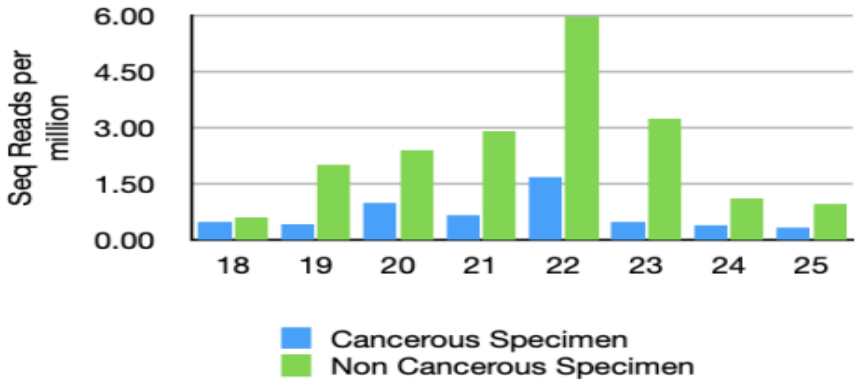
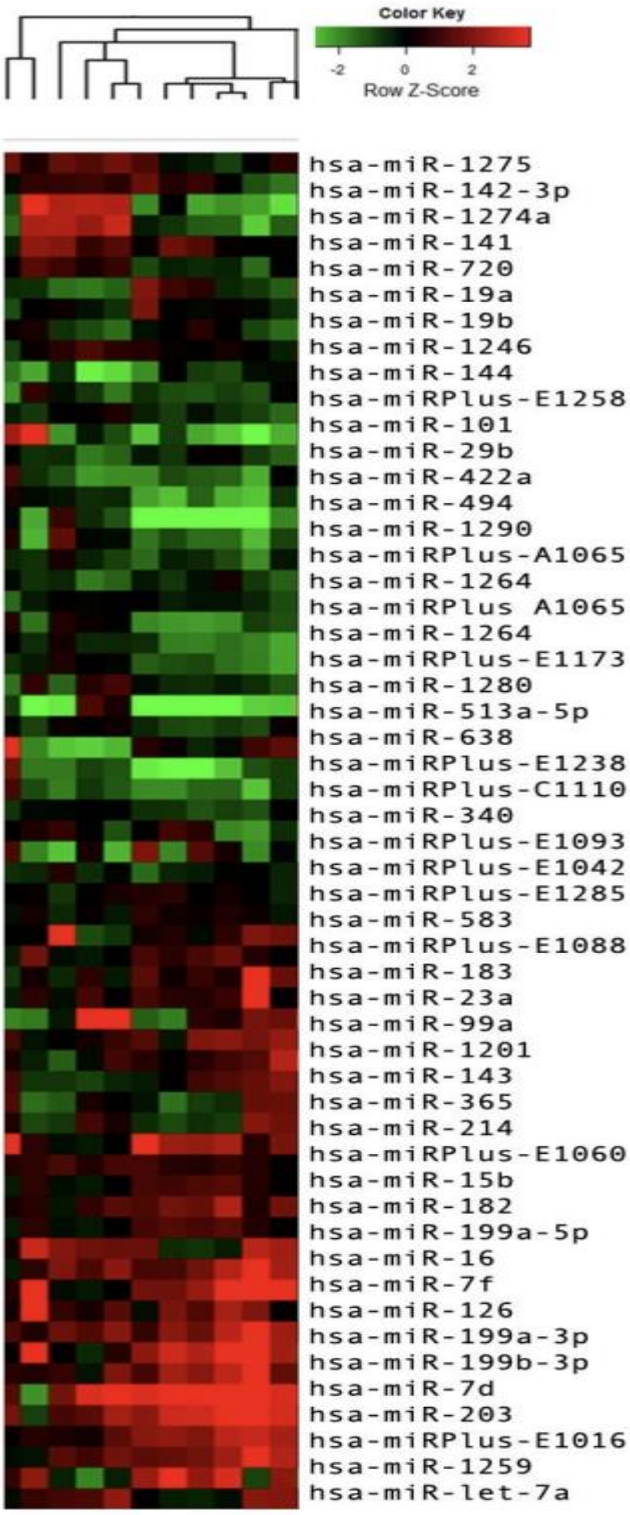


Table 4 Seq Reads per million

	Cancerous Specimen	Non Cancerous Specimen
18	0.50	1
19	0.43	2.00
20	1	2.40
21	0.67	2.90
22	1.69	5.98
23	0.48	3.25
24	0.40	1.10
25	0.34	0.97

Figure 10 Hierarchical Clustering of most frequently expressed miRNAs in OSCC patients



When analyzing the expression of miRNAs in tumours and healthy normal tissues, we found 22 miRNAs that were differentially expressed (11 miRNAs down-regulated and 11 up-regulated; false discovery rate [FDR] 0.05 and fold change [FC] less than 0.5 or greater than 2.0). Checking in The Gene Ontology (GO) revealed that these 22 differentially expressed miRNAs affected several significant biological functions, with 2 pathways being the most prevalent like p53 signalling pathway, PI3K/Akt signalling pathway. The most predominant microRNAs with up-regulated expression levels are shown in Table-2 and the most predominant microRNAs with down-regulated expression levels are shown in Table-3. Furthermore, we conducted a correlation study between the expression of these 22 miRNAs' fold changes in the normal /cancerous tissue and plasma serum, which revealed no link between expression (all $P > 0.05$). Additionally, we investigated the relationship between plasma serum levels and the production of these 22 miRNAs in normal and cancerous cells. Evidently, their expressions in plasma serum and tissues were related (all $P < 0.05$).

Validation of microRNA expression in clinical samples

Certain microRNAs, including miR-141, hsa-miR-29c, miRNA 375, miRNA 199a-5p, miRNA 99a, let-7a, and miRNA 199a-3p, that exhibited the biggest fold change (or least p-value) were chosen for validation in phase two. Nevertheless, real-time quantitative polymerase chain reaction (qRT-PCR), the most sensitive and exact approach, was validated. The most variable miRNAs were subjected to individual real-time quantitative polymerase chain reactions in a separate collection of OSCC tissue samples and matched adjacent normal tissue samples. Using real-time quantitative PCR, marker miRNAs (hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21, hsa-miR-141, hsa-miR-29c, hsa-miRNA 375, hsa-miRNA 199a-5p, hsa-miRNA 99a, let-7a, hsa-miRNA 199a-3p) were validated.

Validation

Real-time quantitative PCR was used to validate the miRNAs. Ten (10) ng of total miRNA and miRNA-specific loop primers were used in qRT-PCR. TaqMan miRNA technique was used for real-time PCR. In short, 1.33 µl of the RT product, 10 µl of the TaqMan Universal PCR Master mix, and 1 µl of the primer and probe mix were added simultaneously. The mixture was diluted with RNase-free water to a final volume of 20 µl. A 96-well plate was used to incubate the reaction at 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (CT) was used to determine the amount of miRNA expression, and the Ct technique was used to quantify miRNA expression levels relative to one another. GeNorm and NormFinder tools were used to choose endogenous controls for specific miRNAs, enabling precise normalisation of qRT-PCR data. In a second "in silico" validation, miRNA expression levels were examined using RNA-Seq data from oral malignant tissues obtained from the Cancer Genome Atlas (TCGA).

According to our study, expression levels of hsa-miR-142-3p, hsa-miR-32-5p, and hsa-miR-21 in serum plasma were considerably greater and the expression level of hsa-miRNA 375 in serum plasma was considerably lower in oral squamous cell carcinoma patients than in controls. Hsa-miR-32-5p, hsa-miR-21, and hsa-miRNA 375 showed statistically significant changes between particular tumour stages and controls.

DISCUSSION

Although OSCC remains one of the leading causes of mortality, effective methods for detecting and preventing the progression of OSCC remain elusive.Locoregional invasions, development of resistance to treatment, disease recurrence, and lymph node metastasis are the main reasons for the poor prognosis of OSCC patients (Mashouri et al. 2019). It is well documented that biomarkers are interacting, interdependent multipurpose parts of the biological system. Therefore, a prognostic model consisting of

multiple markers can increase the predictive power and better capture the clinical behaviour of cancer than a single marker. In concordance with this concept, the present study aimed to identify novel microRNA-based post-surgical prognostic models in OSCC to predict the recurrence risk of OSCC patients. Till now, qRT-PCR and miRNA microarray-based approaches have been utilized for performing miRNA expression analysis. In the present study, next-generation sequencing that has emerged as a cost-effective option, was utilized to study the miRNA expression in OSCC patients. To the best of available knowledge, this is the first study which investigated the differential expression profiles of miRNAs, simultaneously in the cancerous tissues and serum plasma of a group of south Indian OSCC patients using NGS and determined the dysregulated miRNA signature associated with post-surgical recurrence risk.

We identified unique signals of miRNA expression in cancer tissue and serum plasma samples from OSCC patients in the current investigation. OSCC cancer tissues are known to be controlled by several miRNAs that are known to be circulating and relevant as potential biomarkers. (Hudcova, et al. 2016). Ganci *et al* (2016) performed miRNA expression profiling on 74 OSCC and 38 normal tissues to identify and functionally characterize miRNAs that predict recurrence in OSCC. The expression of four prognostic miRNA signatures (miR-21-3p, miR-21-5p, miR-96-5p and miR-429) was able to predict recurrent risk independently from other clinical factors in OSCC. Among the 22 expressed miRNAs in the present study, the following miRNAs such as hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21, and hsa-miR 375 were identified to be the most frequent miRNAs that were differentially expressed in OSCC patients compared to normal tissues. Details of dysregulated miRNAs in the plasma serum of the same group of OSCC patients had been communicated separately (Sreekumar et al 2023) We found that the miRNAs hsa-miR-142-3p, hsa-miR-21, and hsa-miR-32-5p had abnormally high expression levels and hsa-miR 375 had abnormally low expression levels in OSCC patients. Also, it has been established that OSCC that expressed hsa-miR-375 , miR-142-3p and hsa-miR-32-5p, were the most predominant dysregulated miRNAs in the present study.

The study results showed that miR-142-3p was the most highly up-regulated miRNA in both the tissue and plasma serum samples of patients with OSCC. In the study on differential expression of 10 miRNA in 61 primary OSCC using miRNA microarray, Manikandan et al (2016) found up-regulation and down-regulation of a few miRNAs. However, miR-142-3p was the top up-regulated miRNA which was over-expressed in 87% of OSCC. High expression of miR-142-3p in the plasma has been reported to correlate with the worst outcome in OSCC patients (Summerer et al 2015). This represents a promising marker for prognosis and therapy monitoring in the plasma of OSCC patients. miR-142-3p has been implicated in targeting 11 genes of the p53 signalling pathway. It has been hypothesized that over-expression of miR-142-3p simultaneously induces the activation of P13K/Akt pathway and the suppression of the p53 signalling pathway.

The hsa-miR-32-5p was another predominant miRNA upregulated in tissues and sera of OSCC patients in the current study. In addition to being a prospective option for many solid cancers, serum hsa-miR-32-5p expression level could be a non-invasive diagnostic tool for predicting recurrence risk. Victoria Martinez et al (2015) identified that hsa-miR-32-5p was upregulated in tumour tissues and serum of OSCC patients and suggested the utility of hsa-miR-32-5p as a marker for in-invasive diagnosis of OCC patients. According to earlier investigations, hsa-miR-32-5p expression was elevated in the cancerous tissue and serum of patients with OSCC. With this information, it should not be surprising that some cancer-associated signalling pathways such as proteoglycans and p53 signalling that are more common in specific tissue types are regulated by hsa-miR-32-5p (Volinia et al 2006). A microRNA microarray study by Petronacci et al (2019) on 80 OSCC-affected patients and 8 healthy controls identified 80 dysregulated miRNAs (35 over-expressed and 45 under-expressed). Their results also indicated that altered expression of miR-497-5p and miR-4417 observed in OSCC, were related to the “proteoglycans in cancer” pathway. Hence, it is presumed that hsa-miR-32-5p, not singly, but synergically with other miRNAs might be acting to modulate disease recurrence. miR-32-5p has been reported (Qin et al 2023) to enhance cell proliferation, migration and invasion and epithelial mesenchymal transformation (EMT)

of OSCC by targetting Kruppel -like-factor 2 (KLF2), a tumour suppressor gene. KLF2 mediates the regulation of CXCR4 and promotes EMT which plays vital role in the infiltration and metastasis of OSCC. (Joseph et al 2018). Our results concord with Schneider et al (2018) who reported that miR-32-5p was upregulated in tissues and serum of OSCC patients. Although the specific mechanism of miR-32-5p in OSCC remains unknown, it could be a potential biomarker in OSCC.

In the present study, yet another microRNA upregulated in cancerous tissues and serum plasma of OSCC patients was miR-21. Overexpression of miR-21 has been reported to be linked to increased cellular proliferation, aggressive behaviour and metastasis because miR-21 targets multiple genes (Nguyen et al 2008; Wang et al 2009; Zheng et al 2018; Zhu et al 2008). The miRNA hsa-miR-21-5p has previously been more abundant in cancer tissue than in healthy surrounding tissue in OSCC patients (Yan, et al. 2008). Earlier, the existence of differentially expressed miRNAs with chemosensitivity and chemoresistance was reported by Yu et al (2010) in their study on cisplatin-sensitive and resistant TSCC cell lines. Furthermore, their study identified miR-21 as chemosensitive, while miR-214 and miR-23a were chemoresistant among the altered miRNAs (Yu et al 2009). A study by Chan et al (2014) reported that hsa-miR-21 overexpression is inversely associated with drug sensitivity in chemotherapy and progression-free survival. In a comparative analysis in parental and cisplatin-resistant cell lines Ghosh et al (2016) demonstrated that chemoresistance-specific miRNA signature modulates cancer stem-cell-like and EMT-type properties in OSCC. Since miR-21 regulates cell growth and proliferation by targeting PTEN, its overexpression was reported to be related to activating the Pi3K/Akt pathway and rapid cell growth (Yan et al 2017). This suggested that hsa-miR-21 is an essential biomarker of survival and response to treatment (Yan *et al* 2016). Gissi et al (2018) analysed the expression of a panel of miRNAs(miR-21, miR-375, miR-345 miR-1816, miR-146a, miR-649, miR-518b, and miR-191) in epithelial cells collected by oral brushing from OSCC from regenerative areas after OSCC surgical resection and from their normal distant mucosa, using qRT-PCR. A highly significant group difference was found for miR-21 and miR-191. This pilot study indicated that miRNAs could be eligible as target biomarkers for non-invasive OSCC diagnostic testing. Studies have demonstrated a negative correlation between hsa-miR-21 overexpression, progression-free survival and chemotherapeutic treatment susceptibility (Dioguardi, et al. 2022). Its overexpression is not a factor since anti-PTEN miR-21 is known to control cell proliferation and development. Jhadav et al (2022) evaluated the diagnostic accuracy of miR-21 in saliva and tumour tissue for presurgical assessment of lymph node metastasis in OSCC patients. They showed high diagnostic accuracy for assessment of cervical lymph node metastasis, Our findings imply that hsa-miR-21 plays a crucial role in developing OSCC malignancy and survival and treatment response.

In the present study, hsa-miR-375 was one of the miRNAs with the most predominant downregulated expression in OSCC tissue samples compared to healthy normal tissues with higher expression levels. Several researchers earlier discovered that hsa-miR-375 was downregulated in the tumour tissues of OSCC patients. The expression of miRNA hsa-miR-375 was reported to be around ten times less abundant in cancer tissue than in nearby healthy tissue in the study by Hudcova *et al* (2016). Moreover, there was a significant link between the variability of hsa-miR-375 expression in healthy and malignant tissues and the expression of this miRNA in the serum of OSCC patients. In the multicentric study by Yan et al (2016), the downregulation of microRNAs including miR-375 has been associated with OSCC recurrence in the plasma of OSCC. This study indicated that the plasma miRNA profile is altered in OSCC during its progression and can be used to monitor the likelihood of OSCC recurrence in patients after surgery. This implicates its role as a tumour-suppressive miRNA. In our analysis of the expression of miR-375 in the plasma serum from patients with OSCC, we found that hsa-miR-375 was significantly downregulated and highly correlated with early cancer recurrence. Furthermore, down-regulation of hsa-miR-375 was associated with a greater risk of lymph node metastasis and a shorter overall survival for OSCC patients. The matrix metallo protease13 (MMP13) expression is controlled by hsa-miR-375 levels, which results in more aggressive tumours and more significant metastatic activity (Osako et al 2016) On

the contrary, upregulation of hsa-miR-375 was reported to inhibit cell proliferation markedly, induce cell cycle arrest in the G0 / G1 phase, promote apoptosis ad induce radiosensitivity in OSCC cells (Zhang et al 2014). Hence, it is logical to presume that the downregulation of miR-375 in OSCC patients might promote recurrence markers by negatively regulating the above properties. This finding is interesting since down-regulated miR-375 may be a potential therapeutic target for OSCC patients. In the current tumour tissues and plasma serum study, other miRNAs such as hsa-miR-199a-p, hsa-let-7a-3p and hsa-miR-99a were down-regulated, whereas hsa-miR-29c and hsa-miR-141 were up-regulated in lesser frequencies. These miRNAs with dysregulated expression signatures may thus also be appealing candidates as markers for the non-invasive identification of OSCC patients.

The study by Schneider et al (2018) identified that the expression levels of miRNAs hsa-miR-21 and hsa-miR-375 were down-regulated in the serum of patients diagnosed with oropharyngeal squamous cell carcinoma and were highly associated with cancer recurrence. Gissi et al (2018) analyzed the expressions of a panel of miRNAs (miR-21, miR-375, miR-345, miR-181b, miR146a, miR- 649, miR-518b and miR-191 in epithelial cells collected by oral brushing from OSCC from regenerative areas after OSCC surgical resection and from their respective normal distant mucosa using real-time qRT-PCR, A highly significant group difference was found for miR-21($F = 6.58$, $p < 0.001$), miR-146a ($F = 6.94$, $p < 0.001$) and miR191 ($F = 17.07$ $p < 0.001$) Furthermore, altered expression of miR-146a and miR-191 was observed in regenerative areas after OSCC resection. This pilot study indicated that miRNAs could be eligible as target biomarkers for invasive OSCC diagnostic testing.

.Using microRNA microarray, Rajan et al (2021) profiled the mRNA expression in 30 oral tumour tissue and 18 normal samples. They identified a set of 105 miRNAs to be differentially expressed in OSCC, of which qRT-PCR validated a subset of 19 most dysregulated miRNAs. Among these, the up-regulation of miR-196a, miR-21, miR1237 and down-regulation of miR-204 and miR-144 classified a poor prognosis group of OSCC patients. Additionally, the miR-196a / miR-204 expression ratio emerged as the best predictor for disease recurrence and patient survival. In the study by Choubey et al (2023) in TSCC cell lines UMI, antisense miR-222 transfection combined with cisplatin treatment was found to reduce cell proliferation and promote apoptosis by downregulating the PUMA gene. The observation is interesting as it offers novel rationales for combinational therapy.

MicroRNAs induce post-transcriptional gene silencing by base pairing with a target mRNA and interfering with its protein translation (Liu et al 2013). MicroRNAs are increasingly recognized for their greater utility than mRNAs, as prognostic indicators owing to their stability within clinical samples and their robust expression patterns. Previous studies have cited the critical role of over-expression or down-regulation of specific miRNAs in modulating various components related to several carcinogenic mechanisms ranging from cell cycle alterations to angiogenesis and mechanisms of distant metastatic dissemination. Cyclins and cyclin-dependent kinases, transcription factors, signalling molecules and angiogenesis / antiangiogenic products have been recognized as specific targets of miRNAs (Pekarek et al 2023). According to Yang et al (2017), OSCC cells with abnormal miRNA expression evolve different capabilities for recurrence and metastases through epithelial-mesenchymal transition (EMT), an increase of migration and invasion, anoikis or gaining cancer stem cell (CSC) properties. As discussed above, some of the miRNAs differentially expressed are known to be directly involved in cancer development and progression. It was also revealed that several cancer-related pathways, especially proteoglycans and p53 signalling were among the most significantly related pathways. It has been documented that proteoglycans mediate many essential basic proteins associated with angiogenesis, tissue regeneration, cell growth and motility (Dituri et al 2022). Aberrant proteoglycan expression as a result of miRNA dysregulation makes cancer cells more aggressive (Espinoza-Sanchez & Gotte 2020;). Moreover, proteoglycans have been reported to participate in the receptor-ligand interaction between cancer cells and their environment, which may facilitate cell migration and increase circulating tumour cells (Ahrens et al 2020). Therefore, identifying the miRNAs dysregulation and understanding their role in the above processes will reveal the molecular mechanism of OSCC progression and yield novel

candidates for early detection of recurrence and metastasis in post-surgical OSCC patients. This was the main objective of the present study and it was achieved.

To sum up, we identified 22 miRNAs (11 up-regulated and 11 down-regulated) that varied in expression levels between healthy and malignant tissues in South Indian OSCC patients. Up-regulation of miRNAs such as hsa-miR-142-3p, hsa-miR-32-5p, has-miR-21 and down-regulation of miRNA hsa-miR - 375 in tumour tissues were identified as the most predominant and promising candidate biomarkers of post-surgical recurrence risk in OSCC. Same dysregulation pattern of these four miRNAs were found in the patient's plasma serum as well. When the miRNA expression levels in the plasma serum were compared between recurrence and recurrence-free OSCC patients and healthy control, four miRNAs (hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21 and hsa-miR – 375) showed differential expression level between the groups.Among these miRNAs, 4 patients with hsa-miR-375 downregulation developed early recurrence, 3 patients with miR-32-5p developed late recurrence and 4 patients with other dysregulated miRNAs showed no recurrence post-operatively till date. In this study, hsa-miR-375 and hsa-miR-32-5p appeared as the most predominant ones associated with recurrence This emphasizes the significance of up-regulated expression levels of, hsa-miR-32-5p, and down-regulated expression levels of hsa-miR - 375 in the serum as non-invasive diagnostic and recurrence risk predictive biomarker in post-operative OSCC patients. Down-regulation of has-miR-375 was predominant in early recurrence patients, Furthermore, OSCC patients with down-regulation of hsa-miR-375 and up-regulation of miR-32-5p, and hsa-miR-142-3p were associated with poor prognosis.

Reports on the microRNA expression profile in the tumour tissues and plasma sera of patients with OSCC globally demonstrated differences in expression profiles among populations. Hence, the results are consistent as well as inconsistent between other studies and the present study. This could be attributed to the racial/ethnic differences of OSCC patients, variation in the exposure of the study subjects to the OSCC risk factors, discrepancies in the sample size included and dissimilarities in techniques employed for miRNAs expression analyses.

Our results, though limited by a small sample size, suggest the potential of miRNAs expression profile as a molecular marker to predict OSCC diagnosis, prognosis and disease recurrence. This has important implications in the mechanistic explanation for the high recurrence risk in OSCC. However, the data must be considered with caution due to the relatively small size of the cohort. Further investigations including biological studies and clinical verification and validity of applicability to a larger patient group are warranted to strengthen these results. Because of this, it may be concluded that miRNA expression signatures can be employed in future research as survival markers and perhaps even as non-invasive biomarkers of OSCC development and post-surgical recurrence risk during follow-up. Furthermore, this microRNA expression signature profile aids the discovery of new targeted medicines and better patient care. Research progress in circulating miRNA detection and analysis will likely pave the way to developing novel minimally invasive or non-invasive biomarkers for OSCC. This information could be considered for developing novel therapeutic targets and agents for targeted therapy which could further enhance customized patient-centred treatment, in cases where other established therapies have failed.

Author contributions

Vinod Nair researched the literature, recruited the patients, performed the experiments, and wrote the original draft; Madhulaxmi and Arumugham Paramasivam critically revised the manuscript, Anna Joseph did the pathological confirmation and grading of tumours; Ravindran Ankathil conceived, designed and supervised the study and critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

None

FUNDING

None

REFERENCES

1. Gupta N, Gupta R, Acharya AK, Patthi B, Goud V, Reddy S, Garg A, Singla A. Changing Trends in oral cancer - a global scenario. Nepal J Epidemiol. 2016 Dec 31;6(4):613-619. doi: 10.3126/nje.v6i4.17255. PMID: 28804673; PMCID: PMC5506386.
2. Bray, F. et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries: A Cancer J. Clin. 68(6), 394–424. https://doi.org/ 10.3322/caac.21492 (2018).
3. J. Ferlay, M. Colombet, I. Soerjomataram, T. Dyba, G. Randi, M. Bettio, A. Gavin, O. Visser, F. Bray. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. European Journal of Cancer, 2018;103:356-387.
4. Kreimer, A.R.; Clifford, G.M.; Boyle, P.; Franceschi, S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: A systematic review. Cancer Epidemiol. Biomark. Prev. 2005, 14, 467–475.
5. Mehanna, H.; Paleri, V.; West, C.M.; Nutting, C. Head and neck cancer—Part 1: Epidemiology, presentation and prevention. BMJ 2010, 341, c4684.
6. D'Souza W, Kumar A, microRNAs in oral cancer: Moving from bench to bed as next generation medicine, Oral Oncology, Volume 111, 2020, 104916 ISSN 1368-8375, doi.org/10.1016/j.oraloncology.2020.104916.
7. F.J.B. Slieker, D.A.A. Rombout, R. de Bree, E.M. Van Cann. Local recurrence and survival after treatment of oral squamous cell carcinoma of the maxilla: A systematic review and meta-analysis. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology, 2022;133(6):626-638.
8. Listl, S., Jansen, L., Stenzinger, A., Freier, K., Emrich, K., Holleczek, B., Katalinic, A., Gondos, A., Brenner, H., & Survival Working Group, G. C. (2013). Survival of Patients with Oral Cavity Cancer in Germany. PLoS ONE, 8(1). https://doi.org/10.1371/journal.pone.0053415
9. Johnson NW, Warnakulasuriya S, Gupta PC, Dimba E, Chindia M, Otoh EC, Sankaranarayanan R, Califano J, Kowalski L. Global oral health inequalities in incidence and outcomes for oral cancer: causes and solutions. Adv Dent Res. 2011 May;23(2):237-46. doi: 10.1177/0022034511402082. PMID: 21490236.
10. van Lanschot, C.G.F.; Klazen, Y.P.; de Ridder, M.A.J.; Mast, H.; Ten Hove, I.; Hardillo, J.A.; Monserez, D.A.; Sewnaik, A.; Meeuwis, C.A.; Keereweer, S.; et al. Depth of invasion in early stage oral cavity squamous cell carcinoma: The optimal cut-off value for elective neck dissection. Oral Oncol. 2020, 111, 104940.
11. Leemans, C.R.; Braakhuis, B.J.; Brakenhoff, R.H. The molecular biology of head and neck cancer. Nat. Rev. Cancer 2011, 11, 9–22.
12. Gao, Y., Yuan, L., Pan, B. et al. Resilience and associated factors among Chinese patients diagnosed with oral cancer. BMC Cancer 19, 447 (2019). https://doi.org/10.1186/s12885-019-5679-0
13. Liu, H.; Lei, C.; He, Q.; Pan, Z.; Xiao, D.; Tao, Y. Nuclear functions of mammalian MicroRNAs in gene regulation, immunity and cancer. Mol. Cancer 2018, 17, 64.
14. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857-86
15. Langevin, S. M., Stone, R. A., Bunker, C. H., Grandis, J. R., Sobol, R. W., & Taioli, E. (2010). MicroRNA-137 promoter methylation in oral rinses from patients with squamous cell carcinoma of the head and neck is associated with gender and body mass index. Carcinogenesis, 31(5), 864-870.

16. Wiklund, E. D., Gao, S., Hulf, T., Sibbritt, T., Nair, S., Costea, D. E., Villadsen, S. B., Bakholdt, V., Bramsen, J. B., Sørensen, J. A., Krogdahl, A., Clark, S. J., & Kjems, J. (2011). MicroRNA Alterations and Associated Aberrant DNA Methylation Patterns across Multiple Sample Types in Oral Squamous Cell Carcinoma. PLoS ONE, 6(11).
17. Solomon MC, Radhakrishnan RA. MicroRNA's: the vibrant performers in the oral cancer scenario. Jpn Dent Sci Rev 2020;56:85-89.
18. Wang J, Lv N, Lu X, Yuan R, Chen Z, Yu J. Diagnostic and therapeutic role of microRNAs in oral cancer (Review). Oncol Rep (2021) 45(1):58–64. doi: 10.3892/or.2020.7854
19. Rueda A, et al. sRNA toolbox: an integrated collection of small RNA research tools. Nucleic acids research. 2015;43:W467–473. doi: 10.1093/nar/gkv555.
20. Robinson MD, McCarthy DJ, Smyth G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–140. doi: 10.1093/bioinformatics/btp616.
21. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11:R106. doi: 10.1186/gb-2010-11-10-r106.
22. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic acids research. 2011;39:D152–157. doi: 10.1093/nar/gkq1027.
23. Mashouri, L., Yousefi, H., Aref, A.R. et al. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Mol Cancer 18, 75 (2019). https://doi.org/10.1186/s12943-019-0991-5
24. Hudcova, K. et al. Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers. Tumour Biol 37, 12627–12633, https://doi.org/10.1007/s13277-016-5147-2 (2016).
25. Ganci F, Sacconi A, Manciocco V, Sperduti I, Battaglia P, Covello R, Muti P, Strano S, Spriano G, Fontemaggi G, Blandino G. MicroRNA expression as predictor of local recurrence risk in oral squamous cell carcinoma. Head Neck. 2016 Apr;38 Suppl 1:E189-97. doi: 10.1002/hed.23969. Epub 2015 Jun 24. PMID: 25532855.
26. Vinod Nair Sreekumar, M Madhulaxmi, Ravindran Ankathil, Paramasivam Arumugham. Identification of Differentially Expressed circulating miRNA by Next-Generation Sequencing in OSCC recurrence patients. Eur. Chem. Bull. 2023, 12(Special Issue 7), 2835-2846.
27. Manikandan M, et al. Oral squamous cell carcinoma: microRNA expression profiling and integrative analyses for elucidation of tumourigenesis mechanism. Mol. Cancer. 2016;15:28. doi: 10.1186/s12943-016-0512-8.
28. Summerer I, Unger K, Braselmann H, et al. Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. Br J Cancer 2015; 113(1): 76–82.
29. Victoria Martinez B, et al. Circulating small non-coding RNA signature in head and neck squamous cell carcinoma. Oncotarget. 2015;6:19246–19263. doi: 10.18632/oncotarget.4266.
30. Volinia S, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:2257–2261. doi: 10.1073/pnas.0510565103.
31. Chamorro Petronacci CM, Pérez-Sayáns M, Padín Iruegas ME, Suárez Peñaranda JM, Lorenzo Pouso AI, Blanco Carrión A, García García A. miRNAs expression of oral squamous cell carcinoma patients: Validation of two putative biomarkers. Medicine (Baltimore). 2019 Mar;98(13):e14922. doi: 10.1097/MD.0000000000014922. PMID: 30921188; PMCID: PMC6456104.
32. Qin S-Y, Li B, Chen M, Qin M-Q,Liu J-M, Lv Q-L. MiR-32-5p promoted epithelial-to-mesenchymal transition of oral squamous cell carcinoma cellsvia regulating the KLF2/CXCR4 pathway. Kaohsiung J MedSci. 2022;38:120–8.https://doi.org/10.1002/kjm2.12450
33. Joseph JP, Harishankar MK, Pillai AA, Devi A. Hypoxia induced EMT:a review on the mechanism of tumor progression and metastasis in OSCC. Oral Oncol. 2018;80:23–32

34. Schneider, A., Victoria, B., Lopez, Y. N., Suchorska, W., Barczak, W., Sobecka, A., Golusinski, W., Masternak, M. M., & Golusinski, P. (2018). Tissue and serum microRNA profile of oral squamous cell carcinoma patients. Scientific Reports, 8(1), 1-8. https://doi.org/10.1038/s41598-017-18945-z
35. Nguyen PT, Kudo Y, Yoshida M, Kamata N, Ogawa I, Takata T. N-cadherin expression is involved in malignant behavior of head and neck cancer in relation to epithelial-mesenchymal transition. Histol Histopathol. 2011;26(2):147-156.
36. Wang G, Mao W, Zheng S, Ye J (2009) Epidermal growth factor receptor-regulated miR-125a-5p – a metastatic inhibitor of lung cancer. FEBS J 276: 5571–5578
37. Zheng, Y., Xie, J., Jiang, F., Li, Y., Chang, G., & Ma, H. (2018). Inhibition of miR-21 promotes cell apoptosis in oral squamous cell carcinoma by upregulating PTEN. Oncology Reports, 40, 2798-2805. https://doi.org/10.3892/or.2018.6663
38. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 2008 Mar;18(3):350-9.
39. X.L. Yan, F.X. Huang, Q. Shao, Y.M. Huang, L. Deng, L.Q. Wu, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA, 14 (2008), pp. 2348-2360
40. Yu T., Wang X.Y., Gong R.G., Li A., Yang S., Cao Y.T., Wen Y.M., Wang C.M., Yi X.Z. The expression profile of microRNAs in a model of 7,12-dimethyl-benz[a]anthrance-induced oral carcinogenesis in Syrian hamster. J. Exp. Clin. Cancer Res. 2009;28:64. doi: 10.1186/1756-9966-28-64.
41. Chan JK, et al. The inhibition of miR-21 promotes apoptosis and chemosensitivity in ovarian cancer. Gynecologic oncology. 2014;132:739–744. doi: 10.1016/j.ygyno.2014.01.034.
42. Ghosh, R. D., Ghuwalewala, S., Das, P., Mandloi, S., Alam, S. K., Chakraborty, J., Sarkar, S., Chakrabarti, S., Panda, C. K., & Roychoudhury, S. (2016). MicroRNA profiling of cisplatin-resistant oral squamous cell carcinoma cell lines enriched with cancer-stem-cell-like and epithelial-mesenchymal transition-type features. Scientific Reports, 6(1), 1-15. https://doi.org/10.1038/srep23932
43. Yan Y, et al. Circulating miRNAs as biomarkers for oral squamous cell carcinoma recurrence in operated patients. Oncotarget. 2017;8:8206–8214.
44. Yan LX, et al. PIK3R1 targeting by miR-21 suppresses tumor cell migration and invasion by reducing PI3K/AKT signaling and reversing EMT, and predicts clinical outcome of breast cancer. International journal of oncology. 2016;48:471–484. doi: 10.3892/ijo.2015.3287.
45. Gissi DB, Morandi L, Gabusi A, Tarsitano A, Marchetti C, Cura F, et al. A Noninvasive Test for Micro-RNA Expression in Oral Squamous Cell Carcinoma. Int J Mol Sci. 2018;19:1789.
46. Dioguardi M, Spirito F, Sovereto D, Alovisi M, Troiano G, Aiuto R, Garcovich D, Crincoli V, Laino L, Cazzolla AP, Caloro GA, Di Cosola M, Lo Muzio L. MicroRNA-21 Expression as a Prognostic Biomarker in Oral Cancer: Systematic Review and Meta-Analysis. Int J Environ Res Public Health. 2022 Mar 14;19(6):3396. doi: 10.3390/ijerph19063396. PMID: 35329083; PMCID: PMC8948874.
47. Jadhav K B, Shah V, Chauhan N, Shah N, Parmar G. Expression of microRNA-21 in saliva and tumor tissue of patients with oral squamous cell carcinoma: a predictor of cervical lymph node metastasis. Oral Surgery Oral Medicine Oral Pathology Oral Radiology. (2022);133 (1) 60 – 69. DOI:https://doi.org/10.1016/j.oooo.2021.07.012
48. Hudcova K, et al. Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers. Tumour Biol. 2016;37:12627–12633. doi: 10.1007/s13277-016-5147-2.
49. Osako Y, et al. Regulation of MMP13 by antitumor microRNA-375 markedly inhibits cancer cell migration and invasion in esophageal squamous cell carcinoma. Int J Oncol. 2016;49:2255–2264. doi: 10.3892/ijo.2016.3745.

50. Zhang D, Ni Z, Xu X, Xiao J. 2014. MiR-32 functions as a tumor suppressor and directly targets EZH2 in human oral squamous cell carcinoma. Med Sci Monit. 20:2527–2535.
51. Rajan, C., Roshan, V. G., Khan, I., Manasa, V. G., Himal, I., Kattoor, J., Thomas, S., Kondaiah, P., & Kannan, S. (2021). MiRNA expression profiling and emergence of new prognostic signature for oral squamous cell carcinoma. Scientific Reports, 11(1), 1-12. https://doi.org/10.1038/s41598-021-86316-wM
52. Choubey, J., Wolkenhauer, O., Chatterjee, T. (2023). MicroRNA Biomarkers for Oral Cancer: A Meta-Analytic Review. In: Malviya, R., Sundram, S. (eds) Targeted Cancer Therapy in Biomedical Engineering. Biological and Medical Physics, Biomedical Engineering. Springer, Singapore. https://doi.org/10.1007/978-981-19-9786-0_19
53. Liu C.J., Tsai M.M., Tu H.F., Lui M.T., Cheng H.W., Lin S.C. miR196a overexpression and miR-196a2 gene polymorphism are prognostic predictors of oral carcinomas. Ann. Surg. Oncol. 2013;20:S406–S414. doi: 10.1245/s10434-012-2618-6.
54. Pekarek, L., Torres-Carranza, D., Fraile-Martinez, O., García-Montero, C., Pekarek, T., Saez, M. A., Rueda-Correa, F., Pimentel-Martinez, C., Guijarro, L. G., Diaz-Pedrero, R., Alvarez-Mon, M., & Ortega, M. A. (2023). An Overview of the Role of MicroRNAs on Carcinogenesis: A Focus on Cell Cycle, Angiogenesis and Metastasis. International Journal of Molecular Sciences, 24(8). https://doi.org/10.3390/ijms24087268
55. Yang X, Ruan H, Hu X, Cao A, Song L. miR-381-3p suppresses the proliferation of oral squamous cell carcinoma cells by directly targeting FGFR2. Am J Cancer Res. 2017;7(4):913–922.
56. Dituri F, Gigante G, Scialpi R, Mancarella S, Fabregat I, Giannelli G. Proteoglycans in Cancer: Friends or Enemies? A Special Focus on Hepatocellular Carcinoma. Cancers. 2022; 14(8):1902. https://doi.org/10.3390/cancers14081902.
57. Espinoza-Sanchez, N. A., & Goette, M. (2020, May). Role of cell surface proteoglycans in cancer immunotherapy. In Seminars in cancer biology (Vol. 62, pp. 48-67). Academic Press.