Section A-Research paper
ISSN 2063-5346

Identification of Differentially Expressed circulating miRNA by Next-Generation Sequencing in OSCC recurrence patients

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Abstract

Oral Squamous Cell Carcinomapatients' serum shows an abnormal expression of miRNAs, making them potential non-invasive diagnostic markers for mouth cancer. The researchers aimed to determine the functional significance of the differentially expressed miRNAs in serum samples from oral cancer patients and age-matched healthy individuals using next-generation sequencing (NGS).

Methods

We have categorised the patients with oral squamous cell carcinoma into four distinct groups: (a) OSCC with early recurrence; (b) OSCC with late recurrence; (c) OSCC with no recurrence; and (d) healthy control. We used NGS to detect miRNAs in the serum of OSCC patients.

Results

Gene Ontology (GO) annotations implicated miRNA with signalling pathways in peripheral nerve synapses, glutamatergic synapses, and cell morphogenesis, all of which play a pivotal role in the manifestation of cancer. Next Generation Sequencing in OSCC samples showed differential expression of four miRNAs: hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21, and hsa-miR 375, compared to an age-matched control case. We observed an up-regulation of hsa-miR-142-3p, hsa-miR-32-5p, and hsa-miR-21 alongside a down-regulation of hsa-miR 375.

Conclusion
We identified four miRNAs- hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21 up-regulated, and one down-regulated hsa-miR 375 as potential biomarkers for patients with OSCC. A larger sample size could validate these markers.
Keywords: Next-generation sequencing, NGS, Liquid biopsy, miRNA, oral cancer, OSCC, HNSCC

Introduction

Oral squamous cell carcinoma (OSCC) originates in the squamous cells lining the oral cavity, including the tongue, lips, gingiva, and lining mucosa. The focus of molecular biology research in OSCC is to comprehend the genetic and molecular changes that lead to the growth and advancement of this disease. Most studies focus on the genetic changes in OSCC, including mutations, amplifications, and deletions in specific genes.Molecular biologists investigate the role of oncogenes (genes that promote cancer growth) and tumour suppressor genes (genes that inhibit cancer growth) in OSCC. For example, the TP53 gene, which codes for a tumour suppressor protein, is frequently mutated in OSCC.Various molecular signaling pathways regulate cell growth, survival, and invasion, influencing OSCC. Researchers study signaling pathways such as the EGFR, PI3K/AKT, and Wnt/β-catenin pathways to understand their contribution to OSCC development and identify potential targets for therapy.

Epigenetic changes, including DNA methylation, histone modifications, and non-coding RNA expression, play a crucial role in OSCC. Researchers investigate the epigenetic alterations associated with OSCC to identify biomarkers for early detection and potential therapeutic targets.Scientists study the immune response in OSCC to understand how the tumour evades immune surveillance and to developimmunotherapeutic strategies to enhance anti-tumour immune responses.Molecular biologists search for molecular biomarkers that can aid in the early detection, prognosis, and prediction of treatment response in OSCC. These biomarkers may include specific genetic mutations, gene expression profiles, or epigenetic alterations.Based on the molecular alterations identified in OSCC, researchers explore targeted therapeutic approaches that inhibit or modulate the dysregulated molecules or pathways. For example, targeted therapies against EGFR, such as cetuximab, have shown promise in OSCC treatment.

Investigating the mechanisms of drug resistance in OSCC is crucial to overcome treatment limitations. Researchers study the molecular mechanisms underlying drug resistance, such as altered drug metabolism, efflux pumps, or genetic alterations, to develop strategies to overcome or prevent resistance.Precision medicine uses tumour genomic profiling to forecast treatment success (Bray, F. et al. 2018). The discovery of biomarkers has sparked research, identifying possible therapeutic targets and using bioinformatics tools for logical prediction. Numerous methods have identified microRNAs, a sizeable class of non-coding RNAs crucial to cellular functions and the manifestation of illness.

Single-stranded RNAs called microRNAs (miRNAs) are crucial in the control of gene translation. (Bartel 2009). In general, microRNAs function as translation-inhibiting negative regulators of gene expression by attaching to a partial complement sequence typically found in the 3' untranslated region (UTR) of the target mRNA. (Huntzinger et al, 2011) Solid tumours are one type of malignancy where changes in microRNA patterns and sequences are frequent. They are involved in

numerous dysregulated pathways in tumour cells, especially those linked to cancerous traits. (Hanahan & Weinburg. 2011; Ruan et al.2009) Some microRNAs have tumour-suppressive or oncogenic properties, which may influence tumours' development, growth, and spread (Grammatikakis et al. 2013). Additionally, miRNA transcript analysis could offer therapeutically useful molecular data for malignancy (Lee & Dutta. 2009). Nair SV et al. 2021) demonstrated that miRNA expression could identify various tumour subtypes and predict clinical outcomes. In most materials, including bodily fluids and formalin-fixed paraffin (FFPE) tissues, microRNAs are well conserved. These two factors have stimulated an ongoing hunt for miRNAs with potential use in diagnosis and prognosis. There is a potential link between OSCC recurrence and microRNAs. When there is a lack of understanding about the miRNA signature in OSCC, there is a high chance of it recurring. Indian OSCC patients have not undergone any studies on this issue, and no information is available. Therefore, it is urgent to carry out studies in this direction.

Researchers can use miRNA expression to identify various tumour subtypes, and in some instances, it can help to forecast clinical outcomes (Sreekumar VN. 2019). Additionally, miRNA transcript analysis could offer therapeutically useful molecular data for malignancy (Lee & Dutta. 2009). In most materials, including bodily fluids and formalin-fixed paraffin (FFPE) tissues, microRNAs are well conserved. These two factors have stimulated an ongoing hunt for miRNAs with potential use in diagnosis and prognosis. The chances of recurring are high when there is a need for more understanding of the miRNA signature in OSCC.

Non-coding single-stranded RNA molecules called microRNAs or miRNAs have a length of 20–22 bases. By starting translational suppression against or degrading their cognate mRNA targets, they control the expression of many genes. miRNAs have a variety of traits that make it possible to control various cellular processes at various phases of development (Sreekumar VN. 2016). Numerous studies show that tissue-specific miRNA regulation is involved in pathophysiological processes like cancer and metastasis. Blood, serum, urine, and other readily available materials can also identify miRNAs with little intrusion. Therefore, many clinical studies, particularly cancer research, aim to identify miRNAs and their associated target mRNAs (Manikandan M et al. 2016).

The rush to find new miRNAs and their target mRNAs is on as researchers try to uncover innovative targets or techniques in light of miRNAs' role in the emergence of oral cancer-a new method of cancer therapy. However, Microarray and RT-qPCR-based miRNA detection and profiling have drawbacks, such as poor sensitivity to miRNA expression, trouble predicting structural changes, and failure to discover novel miRNAs. Large-scale parallel sequencing techniques, like NGS, would be a superior solution for getting around this restriction and finding new infrequent miRNAs. Our current research uses next-generation Sequencing (NGS) to compare miRNA expression in paired PBMC samples from OSCC patients and matched controls. This experimental study aimed to use NGS to find miRNAs differentially expressed in PBMCs from patients with oral squamous cell carcinoma compared to age- and sex-matched healthy controls. By analysing the expression of their targets followed by pathway analysis, we specifically concentrated on the profile expression mechanism of miRNAs implicated in oral cancer. They were improving with maturity. Various biological processes, including cancer development and progression, have

implicated microRNAs (miRNAs) as essential regulators of gene expression. Researchers have studied miRNAs for their potential roles as prognostic markers and therapeutic targets in oral squamous cell carcinoma (OSCC) recurrence. This study will provide some insights into the involvement of miRNAs in OSCC recurrence.

Several studies have investigated the association between specific miRNAs and the recurrence of OSCC. Recurrent OSCC shows differential expression of specific miRNAs compared to non-recurrent cases. hsa-miR-21 and hsa-miR-375 have been dysregulated in recurrent OSCC and may serve as prognostic markers for recurrence.miRNAs play crucial roles in cancer recurrence by regulating various cellular processes in tumour growth, invasion, and metastasis. Specific miRNAs may promote or suppress OSCC recurrence by targeting genes involved in these processes. For instance, hsa-miR-21 targets tumour suppressor genes involved in apoptosis and cell cycle regulation, promoting OSCC recurrence.Modifying miRNA expression or activity has emerged as a potential therapeutic strategy for cancer treatment, including OSCC. The development of miRNA-based therapies, such as miRNA mimics or anti-miRNA oligonucleotides, holds promise for targeting specific miRNAs involved in OSCC recurrence. However, it is essential to note that translating miRNA-targeting therapies into clinical practice is still in its early stages and requires further research.

While miRNAs show promise as prognostic markers and therapeutic targets in OSCC recurrence, their integration into routine clinical practice is still ongoing. More research is required to confirm the practicality of particular miRNAs as predictive indicators and to establish standardised procedures for miRNA-centered diagnoses and treatments.Overall, researchers have implicated miRNAs in the recurrence of oral squamous cell carcinoma, and their dysregulation contributes to the underlying molecular mechanisms of recurrence. Additional research in this area could assist with identifying new biomarkers and treatment options for preventing and managing recurrent OSCC.

Materials and Methods

Study Population and Characteristics

The current study selected one healthy control patient, two OSCC patients with early recurrence, two OSCC patients with late recurrence, and two OSCC patients without recurrence.

Demographic Data

For the study, we identified one healthy individual with no history of chronic disease or acute illnesses who was age and gender-matched to the participant.The Saveetha Institute of Medical and Technical Sciences Institutional Human Ethical Committee approved this study (SDC/PH.D-01/19/09).Before including the subject in the study, the researchers explained all the details and obtained written informed consent. Blood samples were collected from the study and control groups and stored for further analysis.

RNA isolation and Sequencing

4 ml of blood from oral squamous cell carcinoma patients and their corresponding controls were taken in EDTA containers to find differentially expressed miRNAs. After performing density gradient centrifugation, we separated the peripheral blood mononuclear cells (PBMCs) from the samples. The miRNA easy kit (Qiagen, Gmbtt Germany) was used to extract RNA from PBMCs following the manufacturer's directions. The Nanodrop (Bio photometer plus) and Fluorimeter (Qubit4) monitored the RNA output while keeping the samples at -80°C. We split the samples into

three groups of two individuals, based on OSCC recurrence rates, to conduct expression experiments and a fourth group of healthy patients as control.

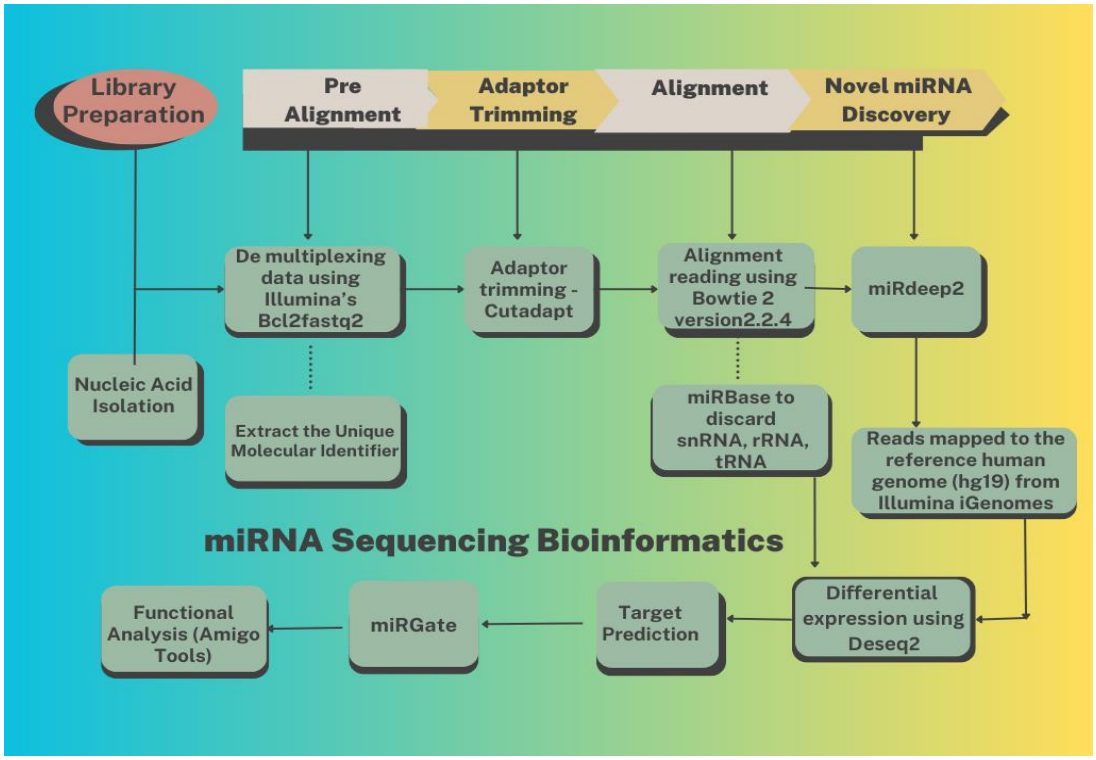

Figure 1
miRNA sequencing flow diagram

The Sequencing of samples resulted in the generation of 0.83 to 1.46 GB of raw data from each sample.

Table 1 NGS Analysis for Groups

Sl No	GROUP	PATIENT SAMPLES
1	OSCC with early Recurrence (<6months after initial treatment)	IA, IB
2	OSCC with late Recurrence (>6months after initial treatment)	IIA, IIB
3	OSCC with no Recurrence (5-year follow-up)	IIIA, IIIB
4	Healthy Control samples	IV

Each cohort only collected one sample, combining the RNA samples for each group. This combination method aimed to eliminate biological differences brought on by varying gene expression levels in samples from the three groups.

The NGS analysis of the miRNAs (Table 1) utilised Illumina's Novaseq 6000 technology. The Qubit BR Assay Kit (Invitrogen cat #Q10211) was used to quantify seven combined RNA samples.

The RNA sample's purity, content, and integrity were verified using QiAXPERT, and the RIN (RNA integrity number) was assessed on the strip station using a monitor strip (Agilent, Cat #5067-5576) for detection. The scale rates the RIN number from 1 to 10, with 1 indicating the worst possible condition and 10 indicating the best possible condition.

Statistical Analysis

We used DESeq2 and known miRNA reads to analyse the differential expression and evaluate miRNA expression between OSCC patients and age-matched healthy individuals. Consider log2FC (1) for up-regulated miRNA and log2FC (-1) for down-regulated miRNA when p-adj 0.05.

Library preparation and Sequencing

We followed the vendor's instructions to create small RNA libraries using the NEB Next Multiplex Small RNA Library Preparation Kit procedure (NEB, #E7560S) with the extracted RNA. They were measured using the Qubit High Sensitivity Assay (Invitrogen, Cat. #Q32852). We combined the libraries and used an Illumina Novaseq 6000 for Sequencing.

Pre-processing of data

FastQC was used to assess the raw data's accuracy. The FastQC utility examines the distribution of baseline quality scores, sequence quality scores, average baseline content per read, read-to-read GC distribution, and read length distribution. To demultiplex the resulting raw fastq sequences, use the CASAVA v1.8 route. The Cutadapt utility removed unwanted sequences like conjugate sequences, primers, poly-A tails, and other related sequences (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Structural RNA contamination, including ribosomal RNA and carrier RNA segments, was eliminated using Bowtie2.

Prediction of miRNAs

We matched quality-controlled reads to the reference human genome (Human Genome - hg19) and the baseline miRNA data, such as miRBase (http: /www.mirbase.org/), to discover differential gene expression patterns and find novel and known miRNAs. Reads that matched more than one place in the genome met known small RNA positions (such as snRNA, rRNA, or tRNA) or did not match the reference genome were all eliminated. Illumina iGenomes quality-checked sequences mapped the human reference genome (hg19).(http://support.illumina.com/sequencing/sequencing software/igenome.html). A piece of software called miRDeep2 allows both expression analysis and the discovery of new miRNAs. We used miRDeep2 to discover novel and well-known microRNAs.

Differential expression analysis

DESeq2 was used for a differential expression analysis based on the number of deep sequences (reads per million; RPM) for known miRNAs. We matched the sRNA sequences with the mature miRNA sequence from miRbase22. The reads were combined, and the expression values were quantified using integers. Up-regulators refer to positive values of log2 alterations (Log2fc 1), while down-regulators refer to negative values (Log2fc -1).

Functional analysis of target genes

The enrichment analysis of the collection of Genoa utilised the targets of the up-and down-regulated miRNAs as inputs to identify the potential roles of the differentially expressed miRNAs. The enrichment analysis contains Reaction Pathways and GO (biological processes, cellular components, and molecular activities). We considered the fundamental values for further research within each group. Statistically significant was considered to be a p-value of 0.05 or lower.

ISSN 2063-5346

NGS Result Interpretation (Figure 2)

Based on NGS data, the current research sought possible miRNA signatures connected to oral squamous cell carcinoma in PBMCs. On an Illumina Novaseq 6000, expression profiles for matched samples were created. We gathered more than 15 million counts for each sample. Reads were evaluated for quality using FastQC, and high-quality sequences were kept for further analysis using Cutadapt if trimming was required. Bowtie also refused low-quality sequence reads (Q 20) when used. Each pretreatment stage caused a 1-2 data loss. Using the same, we screened out other RNA sequences and contamination of 17 bases.

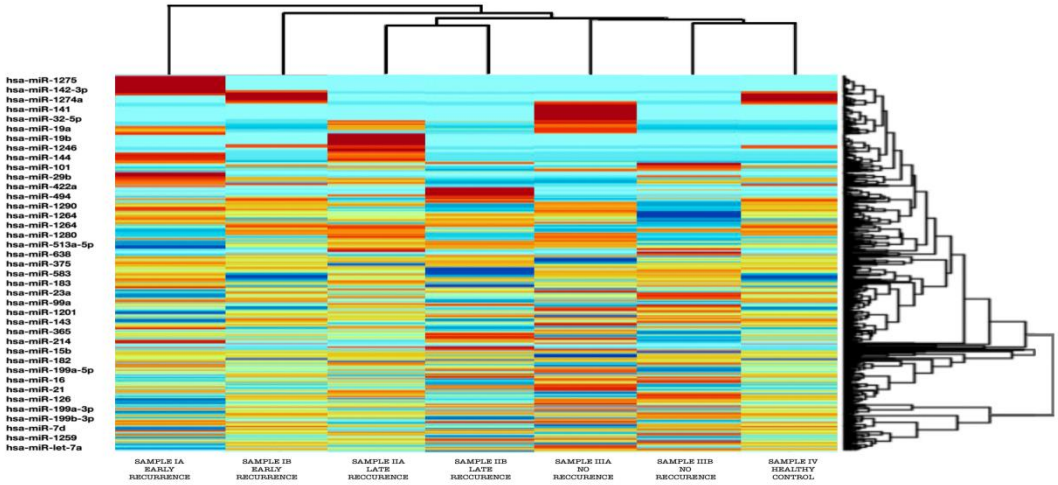

Figure 2: Heat map demonstrating Hierarchical Clustering of the miRNA

miRNA prediction, identification and quantification

The reference human genome [Human genome - hg19 from Illumina genomes (http://support.illumina.com/sequencing/sequencing_software/igenome.html)] and a miRNA database, miRBase (http://www.mirbase.org/), were used to align the reads. The 6 samples showed the top 4 predominant miRNAs, as seen in Table 2. We also identified hsa-miRNA-21 among them as the crucial regulator of oncogenic processes. Table 2summarises the distribution of known and novel miRNA profiling along with their targets.

Table 2 showed that only four miRNAs exhibited significant differential expression with a p-adjusted value (FDR) of ≤0.05.The Bioinformatics & Evolutionary Genomics group at Ghent University has developed a free Venn diagram analysis tool. These four miRNAs have a fewcommon target genes. Additionally, two online web servers, miRDB (https://mirdb.org) and miRWalk, were used to predict the targets of the four miRNAs with differential expression. The target numbers of up-regulated miRNAs ranged from 419 to 2001 and down-regulated miRNA 375 is 3104. (Table 2).

Table 2 Expression Profiles of miRNA

miRNA	Expression Profiling	Targets	False Discovery Rate
hsa-miR-142-3p	Up regulated	419	4.63E-15
hsa-miR-32-5p	Up regulated	2001	4.89E-26

miRNA	Expression Profiling	Targets	False Discovery Rate
hsa-miR-21	Up regulated	893	3.61E-02
hsa-miR 375	Down Regulated	3104	2.18E-11

Functional Annotations of mRNAs (Targets of miRNA)

Functional annotation is a method of recognising functional elements along the sequence of a given genome. By analysing the pathways or biological processes that are more prevalent in the target genes, we can obtain valuable information about the function of microRNAs. A single miRNA could regulate numerous genes and vice versa. We used the miRNet web server to perform functional annotation on selected genes predicted as miRNA targets.GO annotations regarding molecular functioning and Reactome pathway were not observed significantly for miRNA-192. We observed a significant up-regulation in biological processes, specifically in regulating localised translations. Cellular components exhibited a high up-regulation similar to that observed with the transferase complexes. However, the functional analysis for the down-regulated miRNAs- miR-375, demonstrated that the target genes were involved in a variety of positive regulation processes like cell morphogenesis, nervous system development, neuron differentiation. The cellular component analysis identified a significant downregulation of the cell's leading edge and cytoplasmic vesicles. We observed the downregulation of glutamatergic synapses. GO molecular functioning demonstrated an association of the down-regulated miRNA with purine ribonucleoside binding, cytoskeletal protein binding, and protein kinase activity, while the Reactome pathway analysis demonstrated an association with signalling pathways involving Fc-gamma Receptor activation, antimicrobial peptides and gene expression.

Figure 3

Venn Diagram Depicting Target Genes for the Three Differentially Expressed Up-regulated miRNA. Each block shows the different types of miRNA and the number of targets. (i) hsa-miR-142-3p indicates 419 targets, (ii) hsa-miR-32-5p indicates2001 targets, and (iii) hsa-miR-21 indicates893 targets.

Discussion

Our study identified four differentially expressed miRNAs (Figure 3) (hsa-miR-142-3p, hsa-miR-32-5p, hsa-miR-21, and hsa-miR 375) associated with OSCC patients compared to healthy individuals. Samples of Oral squamous cell cancer patients in our study showed downregulated expression levels of miR-375, while healthy individuals exhibited higher expression levels of this miRNA. Other studies have found that hsa-miR 375 is downregulated in various types of cancer, supporting its role as a tumour suppressor. hsa-miR-375 expression was associated with a greater risk of lymph node metastasis and a shorter overall survival for OSCC patients. In OSCC cells, upregulation of hsa-miR-375 causes growth inhibition and cell cycle arrest in the G0/G1 phase, increases apoptosis, and improves radiosensitivity. In oral carcinoma tissues, hsa-miRNA-375 (hsa-miR-375) is highly downregulated and contributes to the development of mouth cancer. In various types of cancer, miR-375, which is a widely recognised microRNA that suppresses tumours, is frequently reduced. Previous studies have shown that the expression of hsa-miR-375 plays a role in OSCC recurrence. Literature reveals its much lower in the tissues and plasma of OSCC patients than in healthy people (M.Y. Siow et al, 2014, H.M Jung et al, 2013, L Jia et al 2015). In tissue samples, nonprogressive premalignant lesions had a considerably greater expression level of hsa-miR-375 than did progressive premalignant lesions. After the recurrence of OSCC, all samples showed a reduction in the expression of hsa-miR-375 (T Harris et al, 2012, JW Yan et al, 20). For High-risk individuals,we can use circulating and salivary hsa-miR-375 as a valuable tool to detect and diagnose pre-cancerous growths (S. Hu et al, 2008).

Literature search revealsin several cancer types, hsa-miR-142-5p has been identified as a cancer-promoting miRNA (S Iizumi et al, 2021; F Islam et al, 2018; Xu and Wang, 2018; L. Liu et al, 2017). Our research found that hsa-miR-142-5p was one of the up-regulated miRNAs in OSCC samples. hsa-miR-142-5p also plays a major role as an oncomiR. While both mimics and inhibitors affect miRNAs, mimics significantly boost transfection levels of miRNA expression, while inhibitors obstruct miRNA activity by utilising an antisense nucleic acid molecule.

Oral Squamous Cell Carcinomarecurrence patient's samples showed dominant up-regulation of hsa-miR-32-5p in their serum, indicating that these miRNAs could also serve as promising markers for non-invasive diagnosis of OSCC patients. Our research interpretation will aid healthcare professionals can use it for non-invasive diagnostic techniques validated for a broader range of patients. As mentioned before, several differentially expressed miRNAs have a direct involvement in the development and advancement of cancer. Finding numerous cancer-related pathways significantly enriched in different tissue types is not unexpected. Proteoglycans, the p53 signalling, and cancer-related pathways were among the most regulated (Volinia, S. et al, 2006).

Our study's findings showed that hsa-miR-21 was also significantly upregulated in OSCC when compared to healthy control samples (Yu E. H et al, 2017). Several studies have shown that the expression of hsa-miR-21 is correlated with smoking, while a different study has shown that the expression of hsa-miR-21 has elevated in alcoholics and pan masala chewers (Singh P et al, 2018; Arantes LM et al, 2017). Zhang et al. showed that nicotine increases hsa-miR-21 via the EMT transforming growth factor beta (TGF-β) pathway (Zhang Y et al, 2014).

Early identification and diagnosis of cancer require appropriate and non-invasive tumour biomarkers. Gene expression regulators, such as miRNAs, have been found to play a role in the growth of tumours. The release of miRNAs from damaged cells and their entry into the circulatory

system, which includes the blood and other bodily fluids, has also been validated by recent investigations. Moreover, researchers showed that plasmamiRNAs persist in a form that resists plasma RNase activity, indicating that detecting stable and intact miRNAs in plasma is possible. Researchers have verified many miRNAs as biomarkers for cancer detection. Additional in-vitro or in-vivo research is required to verify the significance of these miRNAs. Based on the literature search, according to the functional annotations and Reactome pathway analysis, one can assume that the miRNA identified as differentially expressed is causing tumour progression by affecting signalling pathways related to peripheral synapses and cell morphogenesis.

Conclusion

In this research, we found out that the blood sample of patients with OSCC recurrence patients showed differential expression of four miRNAs compared to age-matched healthy controls and recurrence-free patients likehsa-miR 142-3p, hsa-miR 32-5p, hsa-miR 21 and hsa-miR-375. Down-regulation of hsa-miR 375 is predominant in early recurrence patients. Patients with downregulation of has-miR-375 and up-regulation of has-miR-142-3p had poor prognoses. The bioinformatics analysis showed their role in OSCC pathogenesis and growth and may act as a prognosis or diagnostic biomarker. Further analysis is required to validate these findings in more subjects for their alleged role in OSCC as diagnostic and prognostic biomarkers.

Author Contribution Statement

Vinod Nair Sreekumar: Conceptualization, Methodology, manuscript reviewing and editing. Madhulaxmi M, Paramasivam Arumugham: Data curation, manuscript writing and editing. Ravindran Ankathil: Supervision and guidance in doing and writing manuscripts.

Funding

There was no external funding involved in this research.

Ethical Declaration

Saveetha Dental College (SDC/PH.D-01/19/09) granted ethics approval for the study, which followed the guidelines of the Declaration of Helsinki. Before participating in the study, all individuals provided written informed consent.

Conflict of interests

The authors declare that they have no competing interests.

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