Evaluation and Comparison of Plasma miRNA-31 in Oral Squamous Cell Carcinoma

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Abstract: *Background/Purpose*: Oral Squamous cell carcinoma is sixth most common cancer with considerable morbidity and mortality. The microRNAs (miRNAs) are set of short RNAs involved in regulating the expression of protein coding genes. They are up or down-regulated in carcinogenesis and in oral cancer. The miRNA-31 (miR-31) is increased in oral cancer.

Objective: To evaluate and compare the expression of miRNA-31 in plasma of Oral squamous cell carcinoma and control subjects.

Materials and Methods: Case control study was carried out in 25 cases of oral squamous cell carcinoma subjects and 25 normal control subjects. The level of miRNA-31 in blood plasma was evaluated by miRNA easy kit (quagen) and miRNA-based qRT-PCR. The fold change was observed and compared between OSCC and controls.

Results: The plasma level of miRNA-31 was significantly increased in OSCC patients compared to controls ($p<0.001$). The patients with moderately differentiated, grade 4 OSCC patients showed significant increase in fold change compared to control, well differentiated and grade 3 OSCC ($p<0.001$).

Conclusion: Our results indicate that plasma miR-31 may be used as an adjuvant biomarker the detection of OSCC patient.

Keywords: Oral Squamous cell carcinoma (OSCC), microRNA-31 (miRNA-31/miR-31), microRNA, Plasma, Diagnosis, Prognosis.

INTRODUCTION

Oral cancer is a one of the commonest malignant tumor of the oral cavity, with incidence ranked at sixth place of all malignancy worldwide and first in India [1,2]. The surgical excision with chemotherapy and radiotherapy is the treatment for oral cancer [3,4]. Treated patient has poor five year survival rate and in advanced stage it is much poorer survival rate [5]. Reconstruction of surgically excised region is still a challenge [4,5].

The patients quality of life, self esteem and psychology is negatively affected due to therapy induced maxillofacial malformation, dysphagia, and dysarthria of patients [5]. The early diagnosis with early treatment would reduce these complicatins and improves the prognosis [4,5]. In recent years there were many studies to identify early novel diagnostic markers, such as tumor DNA, exosomes and miRNAs were done [6-9]. These molecular methods may help in prevention as well as early detection there by reducing the complications [7,8]. So, the need for effective early diagnosis method and markers still exists [6-8].

The microRNAs (miRNAs) are class of small, shorter, non-coding RNA of 19-24 nucleotides. They regulates the expression of protein coding genes [10,11]. These miRNAs can post-transcriptionally regulate mRNA expression, thus leads to the post-transcriptional regulation of gene expression and influence almost all cellular pathways [10-12]. The disregulated miRNAs plays an important part in initiation, proliferation, invasion, metastasis, apoptosis, epithelial-mesenchymal transition (EMT), chemo-

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E-ISSN: 1929-6029/22

resistance, radio-resistance and cell cycle arrest of oral squamous cell carcinoma [12-14]. The up/down regulation of miRNA was reported in serum, saliva, tumor samples of oral cancer compared to normal control. It has been reported to be useful in early diagnosis, prognosis, recurrence, monitoring the treatment progress and the development of effective treatments [14-16].

The microRNA-31 (miR-31) is located on chromosome 9p21.3. It is one of the early mammalian mRNAs detected [17,18]. It functionally acts via interaction with 3'UTR constituting the RNA- induced silencing complex thus silencing the targeted gene [17]. The miRNA-31 is found have a role in cancer pathogesnesis and in OSCC. It is reported that miRNA-31 may modulate many target genes including fibronectin type III domain containing 5 (FNDC5), tensin 1 (TNS1), special AT-rich sequence-binding protein-2 (SATB2), E2F2, large tumor suppressor kinase 2 (LATS2), AT-rich interaction domain 1A (ARID1A), and hypoxia-inducible factor-1 (FIH-1) to bring changes in cacinogenesis [19-22]. The miRNA-31 is usually upregulated with increased expression levels in serum, saliva, urine, and oral tissue in oral carcinogenesis [23-29]. It was found to be increased in plasma of Primary and recurrent OSCC and different grades of OSCC [22,23,26]. So it can be used as an effective adjuvant diagnostic and prognostic biomarker oral cancer. The aim of the study was to evaluate and compare the expression of miRNA-31 in OSCC and normal control subjects.

MATERIAL AND METHODS

Experimental Design

The case control study design was carried out with new oral squamous cell carcinoma patients and controls reporting to Departments of dentistry, vinayaka mission's medical college hospital, karaikal, Puducherry, India.

Subjects

The study included 25 healthy controls and 25 oral squamous cell carcinoma subjects. The basic demographic, medical history, clinical staging and histopathological typing were recorded. The control group had 25 healthy people with 18 male and 7 female aged 41 to 70 years with a median of 54.3 years. Among the 25 patients 18 male and 7 females, aged between 44 to 75 years with median age of 55.4 years. Histopathologically, of the 25 patients 18 were diagnosed with well differentiated Squamous cell carcinoma while 7 were having moderately differentiated squamous cell carcinoma. Regarding the clinical stage, out o f25 patients 15 were in stage 3 whereas 10 were in stage 4.

Plasma Sample Collection, Storage and RNA Isolation

The peripheral blood specimen (5ml) was collected in EDTA coated tube from each subject for the study. It was processed within 4hr duration. The Plasma and blood was separated by centrifugation at 3000 rpm for 10 min at - 4C. The separated plasma was stored in 1.5 mL RNase free tubes at -80C. The 400 ml of Plasma from each sample was centrifuged at 1200 g for 10 min at 4C prior to miRNA extraction. The miRNA was extracted from plasma samples using miRNeasy plasma/serum kit (Qiagen, Germany) according to manufacturer's protocol. This miRNeasy procedures yields higher quality and quantity of miRNA, easy to perform and reduce the chances of contamination with salt or phenols that interfere with further processing miRNA for cDNA synthesis and RT-PCR.

cDNA Synthesis and Real-Time PCR

The miRNA CURY LNA RT kit (Qiagen, Germany) was used for the cDNA synthesis as per manufacturer's protocal. For the miRNA-based qRT-PCR tests, each PCR reaction was carried out in triplicates using SYBR Green Master Mix as per manufacturer's protocol (Applied Biosystems, Germany). Each reaction required a volume of 20 µl, which included 1 µl cDNA, 2.0 µl universal primer, 2.0 µl PCR primers, and 10 µl of 2x SYBR Green PCR Master Mix. The RNase-free water was used for the reaction. The denaturation at 94 °C for 30 sec was followed by 40 cycles at 55 °C for 30 sec, followed by melt curve analysis in the PCR amplification reaction. The RT-PCR systems were used to execute the reaction (Quant studio-5, Applied Biosystems). The change in fold expression of miR-21 was determined by computing delta det act method. In real-time quantitative PCR, the Ct mean cycle threshold is projected as the total number of cycles needed to produce a fluorescent signal that crosses the threshold value.

Statistical Analysis

The Graph Pad Prism version 6.01 was used to do all of the statistical analysis. The expression level of

Table 1: Gender and Mean Age Distribution of Control and Oral Squamous Cell Carcinoma (OSCC) Cases

Gender	Control		Cases	
	Number of sample	Mean age	Number of sample	Mean age
Male	18	54.26	18	52.63
Females	7	57.42	7	60.27
Total	25	54.3	25	55.4

Table 2: Distribution of Oral Squamous Cell Carcinoma (OSCC) Cases Based on Clinical Staging (STAGE), Histopathological Grading (WDSCC &MDSCC) and Gender

Gender	WDSCC	MDSCC	STAGE III	STAGE IV
Male	11	7	10	8
Female	7	-	5	2
Total	25		25	

WDSCC=well-differentiated squamous cell carcinoma.
MDSCC= moderately- differentiated squamous cell carcinoma.

plasma miR-21 was compared and tested with a t-test and one way ANOVA multiple comparison using Tukey's multiple comparisons test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The study included 25 healthy controls and 25 patients with pathologically confirmed oral squamous cell carcinoma. The Table **1** represents the distribution of age and gender of control and oral squamous cell carcinoma cases.

The Table **2** shows the distribution of OSCC cases with regard to histopathological grades and clinical tumour stages along with gender.

miRNA-21 Expression

The study evaluated expression of plasma miRNA-31 in Oral squamous cell carcinoma and normal control. we measured miRNA-31 levels in the plasma of patients with squamous cell carcinoma and controls using RT-PCR. Expressions were calculated with reference to the fold change as compared to housekeeping gene.

The control and squamous cell carcinoma subject's miRNA-31 was compared using t-test. The miR-31 fold change expression between control and oral squamous cell carcinoma shows significant difference at 0.001 levels. The fold change is three fold higher than control samples (Figure **1**).

The miR-31 expression between controls, well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma was compared using one way ANOVA multiple comparison using Tukey's multiple comparisons test. It shows significant difference at 0.001 levels in the fold change between control and WDSCC, control and MDSCC. Whereas the WDSCC and MDSCC showed a statistically significant difference of 0.001 level (Figure **2**).

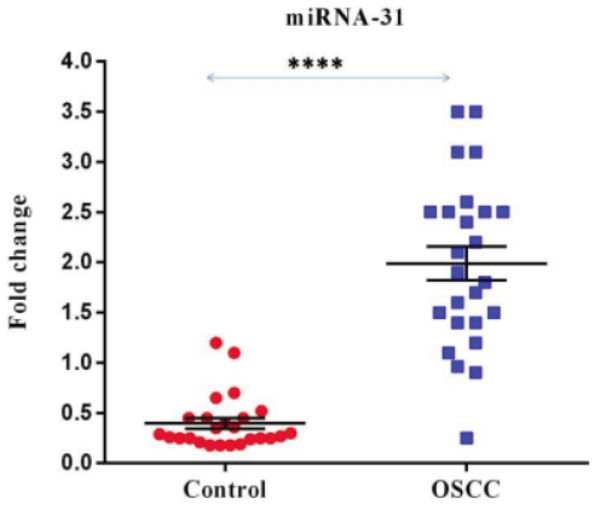

Figure 1: Expression of miRNA-21 in control and Oral squamous cell carcinoma (OSCC).

**** P < 0.001.

The miR-31 expression between control, clinical stage-III squamous cell carcinoma and stage-IV squamous cell carcinoma was compared using one way ANOVA multiple comparison using Tukey's

multiple comparisons test. It shows significant difference at 0.001 levels in the fold change between control and stageIII OSCC, control and Stage 4 OSCC. The stage-III and stage-IV OSCC did not show statistically significant difference in miRNA-31 expression (Figure **3**).

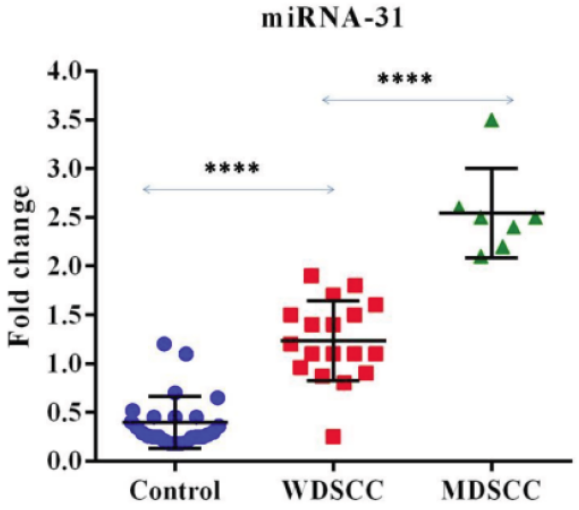

Figure 2: Expression of miRNA-21 in control, well-differentiated squamous cell carcinoma (WDSCC), moderately- differentiated squamous cell carcinoma (MDSCC).

**** P < 0.001.

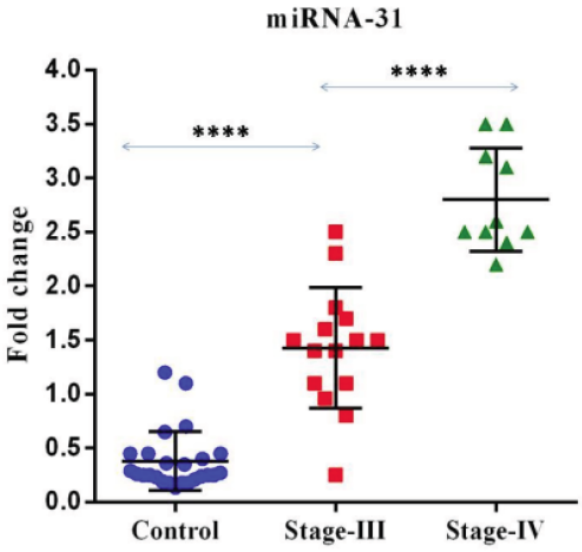

Figure 3: Expression of miRNA-21 in control, Stage-III oral squamous cell carcinoma, Stage-IV oral squamous cell carcinoma.

**** P < 0.001.

DISCUSSION

The miRNAs were found to be involved in all human metabolic activity. In disease status there is a disregulation of various miRNA. Similar abnormal regulations of miRNA were found and associated with oral cancer and other malignant tumours [10,11]. Many studies reported that plasma miRNAs were stable, resistant to RNase degradation and reproducible during their extraction process [15]. It is easy to collect by relatively non-invasive process and can be used for diagnosis and follow-up of diseased person [16]. The miRNA-31 is over-expressed in incremental folds in many carcinomas from early to late stage and in recurrence. The expression was reduced with surgical tumour resection, chemotherapy and radiotherapy treatment [15,18].

In present study we evaluated expression of plasma level of miRNA-31 in Oral squamous cell carcinoma and normal subjects, using RT-PCR. The result showed significant increased difference in fold changes in miRNA-31 of OSCC with that of the normal control (P<0.0001).

Liu *et al*. [23,24] reported that miRNA-31 in plasma was significantly increased in OSCC patients compared to the control subjects. He also reported that there was significant reduction in miRNA-31 expression after tumor resection in 88% (38/43) of the OSCC patients. The present study also reported similar increase in OSCC patients along with various grades and stages of OSCC. We didn't study the levels after the tumour resection.

Schneider A *et al.* [25] reported a increase in fold of miRNA-31 between oral squamous cell carcinoma and normal controls in tissues and serum. Kumari *et al.* [26] reported the use of salivary miRNA-31 in diagnosis and biopsy of oral squamous cell carcinoma. Similar increase in fold change was noted in our study can reflect the role of miRNA-31 in oral carcinogenesis.

Association between TNM stages was studied by Siow *et al.* [27] showed that miRNA-31 was significantly associated with TNM staging (P<0.05). Our study concurred with this finding and we found a significant difference between StageIII & Stage IV OSCC (P<0.001).

Uma *et al.* [28] reported a incremental fold of expression of miRNA-31 in normal, oral premalignant lesions with varying degree of dysplasias. Our study results show as the tumour severity increases as in well differentiated and moderately differentiated squamous cell carcinoma and TNM stage III & stage IV tumours, there is increased the expression of miRNA-31 significantly.

Kolokythas *et al.* [29] reported increase in miRNA-31 with increasing tumour size, stage and histopathological grades. Our study reported that there is increase in miRNA-31 with increasing clinical TNM staging and histopathological grading respectively.

CONCLUSION

We conclude from our results and earlier studies that there is a significant role of miRNA-31 in oral carcinogenesis, increasing severity, grades and stages of tumour. The increased level of miRNA-31 can be used as an adjuvant marker with biopsy for early detection of oral cancer and recurrence screening.

ACKNOWLEDGEMENT

We would like to thank the all the staff members of Central Research Laboratory, Vinayaka Mission's Medical College and Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Karaikal, Puducherry, India for their help in molecular procedures.

ETHICAL CONSIDERATION

The institutional ethical committee approval was obtained before starting the study. The confidentiality of the information obtained was maintained.

RESEARCH ETHICS AND CONSENT

The Informed consent was explained and obtained from every participant. The research was carried out according to the rules and ethical codes specified in the Declaration of Helsinki

FUNDING

The study did not receive any grant from any public, commercial, or not-for-profit sectors.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to data analysis, drafting, and revising of the paper and approved this work.

CONFLICT OF INTEREST

The author declared that they have no conflict of interest.

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Received on 28-10-2022 Accepted on 09-12-2022 Published on 30-12-2022

https://doi.org/10.6000/1929-6029.2022.11.23