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Expression of MicroRNA-21 in Plasma of Squamous Cell Carcinoma of Oral Cavity

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ABSTRACT: The study aim was to evaluate the expression of miRNA 21 and to compare miRNA-21 in plasma of patients with squamous cell carcinoma of oral cavity. The squamous cell carcinoma of oral cavity is commonest malignancy in world and commonest in India with considerable mortality and morbidity. It poses a major challenge to health of individual and society. The diagnosis at early stage helps to reduce mortality and morbidity and has a better prognosis. Multiple molecular and biochemical markers are studied for diagnosis, prognosis and recurrence prediction. One of the recently studied marker is miRNAs. The microRNAs (miRNAs) are small, tiny, non-coding RNAs that are observed to be up or down-regulated in patients with Oral cancer and play a role in all levels of carcinogenesis. One of the common miRNA is miRNA-21 and is upregulated in oral carcinomas. A case control study with 30 patients of SCCO and 30 controls of healthy subjects was done. The miRNA-21 levels were evaluated in blood plasma of cases and controls. The miRNA-21 fold change was observed and compared with SCCO and controls. The plasma miRNA-21 level was significantly increased between SCCO cases and controls. Further, the patients with moderately differentiated and Stage-4 SCCO showed increase in fold change of miRNA-21 significantly compared to well-differentiated and Stage-3 SCCO patients. We concluded from the study that the plasma miR-21 levels clearly differentiate between the normal healthy individual and SCCO cases. Further it also differentiates the Squamous cell carcinoma- well differentiated and Squamous cell carcinoma- moderately differentiated. It also differentiates stage-3 and stage-4 SCCO. So miRNA-21 may be of help as added prognostic and diagnostic marker for SCCO.

Keywords: Squamous Cell Carcinoma of Oral cavity (SCCO), Plasma, microRNA-21(miRNA-21/miR-21), microRNA.

INTRODUCTION

The most common malignant neoplasms in head and neck region is oral cancer (Bray *et al.,* 2015). Globally it is ranked as sixth common neoplasm and in India it first common malignancy (Bray *et al.,* 2015; Waranakulasurya, 2009). The common treatment for the oral cancer is extensive surgical excision with chemotherapy and radiotherapy (McCullough *et al.,* 2010). However, the overall survival rate in five year was reported as 65% and there is more poor survival rate in advanced stage. Though there are many efforts for reconstruction still it prognosis has not improved much (Montero *et al.,* 2015; Fang Chuan *et al.,* 2019).

The patient psychology and quality of life is seriously affected and impacted by cancer treatment induced malformation of maxillofacial region, dysarthria and the dysphagia (Fang Chuan *et al.,* 2019; Valdez *et al.,* 2018). The early diagnosis with recent molecular technology would help in early detection and prevention. So that early treatment at the early stage would help in improving prognosis and reducing complications. There exists a requirement for more effective method and markers for early diagnosis (Radhika *et al.,* 2016; Santhosh *et al.,* 2016; Payne *et al.,* 2018).

The microRNAs (miRNAs) are class of small, short length and non-coding RNAs that contain nucleotides of 19-24 base pair. The gene coding proteins expressions are regulated by miRNA. These miRNAs operates by binding to 3′ untranslated region (UTR) of

target gene mRNA and promotes the degradation or translational repression of the mRNA, this causes the gene expression by post-transcriptional regulation (Schickel *et al.,* 2008; Vlachos *et al.,* 2015; Inui *et al.,* 2010). The miRNAs have a role in oral squamous cell carcinomatous initiation, proliferation, apoptosis, invasion, epithelial-mesenchymal transition (EMT), metastasis, chemo-resistance, radio-resistance and cell cycle arrest. The dis-regulated expression of miRNA is expressed in tumor tissue, serum and saliva samples. It has clinical significance in early detection, treatment monitoring, prognosis and newer drug and treatment development (Wu *et al.,* 2011; Deng *et al.,* 2008; Wiemer 2007).

The miRNA 21 is one of the first and most studied miRNA marker. The miRNA-21 gene is present in the fragile site of FRA17B (16). It is commonly found increased in various types of cancers and in Oral squamous cell carcinomas (OSCC) as well (Kumarswamy *et al.,* 2011; Qi *et al.,* 2015). Earlier studies suggests that miR-21 acts by suppressing the tumour suppressor genes such as tropomyosin 1 alpha (TMP1), Phosphatase and tensin homolog (PTEN) and Programmed Cell Death 4 (PDCD4). The miRNA-21 has a role in tumors drug resistance. The miRNA-21 is reported to be up-regulated in blood plasma of OSCC in primary tumor, recurrent tumor as well as different grades of tumor (Sheedy *et al.,* 2010; Reis *et al.,* 2010). The study aim was to evaluate the expression of miRNA 21 and to compare miRNA-21 in plasma of patients with squamous cell carcinoma of oral cavity.

MATERIAL AND METHODS

Experimental design: The case control study was done newly reported and diagnosed cases of squamous cell carcinoma of oral cavity and controls.

Subjects: The study involved 30 controls of healthy subjects and 30 patients with pathologically confirmed squamous cell carcinoma of oral cavity with basic regional, medical information, tumour stage and histological grade type were recorded. The study group had 20 stage-3 and 10 stage-4 OSCC. It had 23 well-differentiated squamous cell carcinoma- well differentiated type (SCCWD) and 7 squamous cell carcinoma- moderately- differentiated type (SCCMD).

Collection and storage of plasma sample and RNA isolation: The 5 ml of blood was drawn in EDTA coated blood collection tubes. The blood centrifuged in 4°C for 10 minutes at 3000 rpm and the plasma was separated and stored at -80°C for total RNA isolation. Later 400ml of plasma from every sample was centrifuged at 4°C for 10 min at 1200 g before RNA extraction. The miRNeasy plasma/serum kit (Quagen, Germany) was used for extraction of the miRNA from plasma samples as per protocol by manufacturer.

Synthesis of cDNA and qRT-PCR: Using the microRNA extraction kit (miRNA CURY LNA RT (Qiagen, Germany) the synthesis of cDNA was done as per manufacturer protocol. The qRT-PCR for miRNA was done. Each reaction PCR was done in triplicates using SYBR Green Master (Applied Biosystems, Germany). Individual reaction had 20 µl volume comprising cDNA (1 µl), Universal primer (2 µl). PCR primer (2 µl) and SYBR Green PCR Master mix (10 µl). The RNase-free water was employed for qRT-PCR reactions. The 94 °C for 30 sec denaturation was followed by 40 cycles at 55 °C for 30 sec. The analysis of melt curve was done for amplification reaction of qRT-PCR. The Quant studio-5 Real time PCR system was used to execute these reactions. The Delta CT (threshold cycle) method was used to determine the fold change in expression of miR-21. In qRT- PCR, the mean threshold cycle (CT mean) value is the number of total reaction cycles required to generate a signal fluorescence in which the threshold value was crossed.

Statistical Analysis: The Graph Pad Prism version 6.01 was used for statistical analysis. The results were analysed by t-test and multiple comparisons test by one way ANOVA.

RESULTS

The 30 healthy controls of with 7 female and 23 males aged 41 to 75 years with a mean of 57.46 years and 30 patients of 7 female and 23 males of 40 to 80 years with mean of 56.2 years with pathologically confirmed SCCO were included for study (Table 1). The all 30 patients, histopathologically 23 were of WDSCC & 7 were of MDSCC and Clinically 20 were stage-3 and 10 were stage-4 in tumour staging (Table 2).

miR-21 expression: The expression of plasma miRNA in SCCO and normal control subjects were evaluated with inter-group comparison. The miRNA-21 levels in the plasma measured by calculating the fold changes between control and cases by qRT-PCR.

The miRNA-21 of SCCO cases and controls were compared by t-test. There was 2-3 times increase in fold change between control and cases at significant level of $P<0.001$ (Fig. 1).

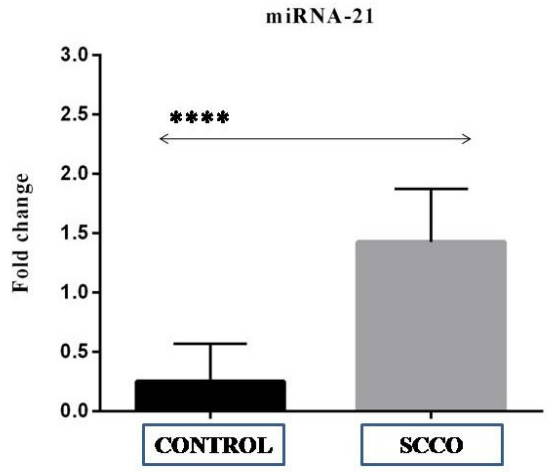
Table 1: Squamous cell carcinoma of oral cavity (SCCO) cases and Control subjects with sex and mean age distribution.

Sex	Control subjects		Case subjects	
	Number of controls	Mean age	Number of cases	Mean age
Male	23	56.26	23	53.73
Females	7	61.42	7	64.28
Total	30	57.46	30	56.2

Table 2: The clinical staging (STAGE), histopathological grading (SCCWD & SCCMD) and sex distribution of squamous cell carcinoma of oral cavity (SCCO) cases.

Sex	SCCWD	SCCMD	STAGE-3	STAGE-4
Male	16	7	15	8
Female	7		5	2

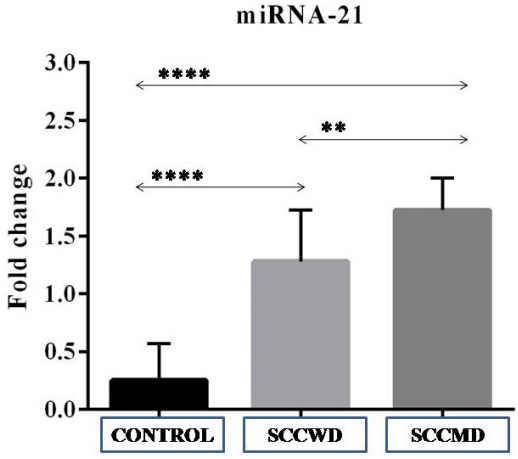
SCCWD=Squamous cell carcinoma well-differentiated type
SCCMD= Squamous cell carcinoma moderately-differentiated type



**** P < 0.001

Fig. 1. Control and squamous cell carcinoma of oral cavity (SCCO) with fold change expression of miRNA-21.

The controls, SCCWD and SCCMD were compared for miR-21 fold change expression by Tukey's ANOVA multiple comparison test. The control and SCCWD, control and SCCMD had significant difference in the fold change (P < 0.001). Whereas, the SCCWD and SCCMD had significant difference in fold change at P< 0.05. (Fig. 2).



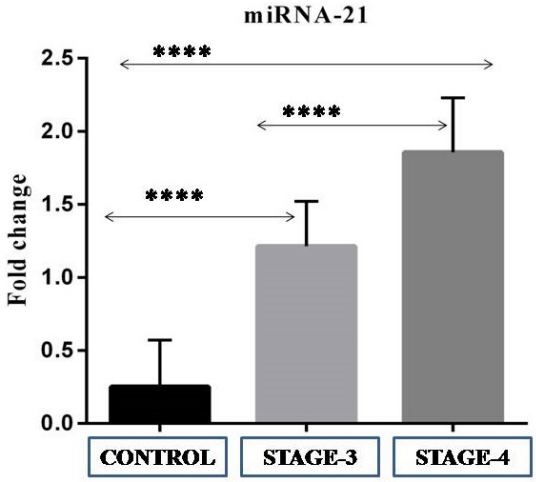
**** P < 0.001, ** P < 0.05

Fig. 2. The control, Squamous cell carcinoma well-differentiated type (SCCWD), Squamous cell carcinoma moderately- differentiated type (SCCWD) fold change expression of miRNA-21.

The control, clinical stage-3 and stage-4 SCCO with miR-21 fold change expression was compared by Tukey's ANOVA multiple comparison test. The control and stage-3, control and Stage-4 SCCO miR-21 fold change had differed significantly at P < 0.001. There was no significant difference in miR-21 fold change between stage-3 and stage-4 SCCO (Fig. 3).

DISCUSSION

The abnormal regulation of miRNA is observed with squamous cell carcinoma of oral cavity and other malignant tumours. Earlier reports had shown that miRNAs in plasma are resistant to degradation by enzymes, stable and repeatedly reproducible during molecular separation procedures (Alami *et al.,* 2021; Glinge *et al.,* 2017).



**** P < 0.001 or < 0.0001

Fig. 3. Control, Stage-3 and Stage-4 Squamous cell carcinoma of oral cavity fold change expression of miRNA-21.

Plasma miRNA collection is simple, easy and non-invasive may be useful for diagnosis, follow-up and reccurence screening. There is a sequential increase in over-expression of miRNA-21 from early to late stage malignant tumor. It is also over-expressed in recurrent tumor. This over-expression reverted to normal level after tumour resection and treatment (Si *et al.,* 2013; Narasimhan *et al.,* 2018).

The expression and observation of miRNA-21 in blood plasma of squamous cell carcinoma of oral cavity and controls was evaluated in present study. The study result had significant difference in expression of miRNA-21 between cases and control subjects. Similar, results were obtained by Schneider *et al.,* (2018) in their study with serum and tissue of oral squamous cell carcinoma and normal controls.

It was reported by Mahmood *et al.,* (2019) that there was an increased expression of serum miRNA-21 with increasing histological grades and size of the tumor. We also found similar high incremental relative miRNA-21 levels in squamous cell carcinoma and also there was significance difference among well and moderately differentiated SCCO, stage-3 and stage-4 SCCO to controls. Yu *et al.,* (2017) reported significantly increased expression of miRNA-21 from in centre of the tumour of periphery of the tumour and well differentiated tumours to poorly differentiated tumours. Similar results were observed in our study between, well and moderately differentiated SCCO, stage-3 and stage-4 SCCO to controls which signify indeed there is increased miRNA-21 over-expression as tumour size and severity increases.

Zahran *et al.,* (2015) reported a sequential increase in fold change over-expression of miRNA-21 in normal subjects, oral premalignant lesions with varying degree of dysplasias. Present study though not exactly similar

but showed a similar result of as the tumour severity, grade and stage increases the expression of miRNA- 21.

CONCLUSIONS

The Present study and earlier reports suggest that there is a close association of miRNA-21 with that of progress of SCCO as with increasing histopathological grades and clinical stages of tumour carcinogenesis. The over expression of miRNA may be utilized as a marker for early detection of SCCO as well as recurrence screening. The present study result concludes that plasma miR-21 levels clearly differentiate between the normal healthy individual and SCCO cases. Further it also differentiates the well differentiated and moderately differentiated Squamous cell carcinoma. It also differentiates stage-3 and stage-4 SCCO. So miRNA-21 may be used as adjuvant screening diagnostic marker and prognostic marker in SCCO.

FUTURE SCOPE

Further studies with larger samples in tissues, cell lines and blood may be needed to understand the importance of miRNA-21 in carcinogenesis which may help in early diagnosis, reccurence screening and treatment of the oral squamous cell carcinomas.

Research Ethics and Consent. The Institutional ethical committee clearance was obtained prior to commencing the study. The study was explained and informed consent was received from each subject.

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Conflict of Interest. None.

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