(International Print/Online Journal)

SJIF IMPACT FACTOR: 5.565
PUBMED-National Library of Medicine ID-101739732

ISSN (Print): 2209-2870
ISSN (Online): 2209-2862

International Journal of Medical Science and Current Research (IJMSCR)
Available online at: www.ijmscr.com
Volume 5, Issue 6 , Page No: 447-454
November-December 2022

IJMSCR

Clinico-Pathological Significance and Expression Portrait of miR-26a In Oral Squamous Cell Carcinoma

[1]Dr. Priya N S, [2]Dr. Ramakant Nayak, [3]Dr. Kishore Bhat
[4]Dr. Manohar Kugaji, [5]Ms. Meenaz Sangolli, [6]Dr. Prashanth R
[1]Professor, [2]Professor & Principal, [3]Professor & HOD, [4]Research Assistant, M.Sc. (Biotech), PhD
[5]Research Assistant, M.Sc. (Genetics), [6]Reader
[6]Department of Oral & Maxillofacial Surgery, [1]Department of Oral Pathology,
[3,4,5]Department Of Molecular Biology And Immunology,
[2]Department of Oral Pathology & Microbiology
[2,3.4.5]Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Belagavi.
[1,6] V S Dental College & Hospital, Bangalore, K R Road, VV Puram, Bangalore-560004

***Corresponding Author:**
Dr. Priya N S
Professor, Department of Oral Pathology, V S Dental College & Hospital
K R Road, V V Puram, Bangalore-560004

Type of Publication: Original Research Paper
Conflicts of Interest: Nil

Abstract

Background: Oral cancer is a multiphasic disease process with enormous impact on the lives of human beings. Its development, that is carcinogenesis is a complex event and is modulated by dual influence of environmental and genetic factors. In the recent past, the phenomenon of epigenetics is linking to the process of carcinogenesis leading to the application of miRNA profiles in the diagnosis and prognosis of OSCC (Oral Squamous cell carcinoma).

Aims and Objectives: The study aims to quantify the tissue expression of miR-26a in OSCC and to correlate its expression portrait with the clinico-pathological parameters.

Material and methods: Real time PCR was used to quantify miR-26a expression in 50 tissue samples of OSCC. Based on the mean values, it was categorized into high and low expressers.

Results: miR-26a was downregulated in OSCC and the expression levels of miR-26a was correlated significantly with lymph node status (p=0.023)

Conclusion: Our study indicates the role of miR-26a as a tumor suppressor gene in OSCC.

Keywords: miR-26a, miRNA expression, Oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC), with its expanding incidence and prevalence, with its divergent clinico-pathological dimensions and with its catastrophic behaviour, is considered to be of concern in recent years. The environmental factors, genetics and effectively epigenetics play a pivotal role in shaping and regulating the genotypic and phenotypic characteristics, behaviour and prognosis of OSCC. "Epigenetic Phenomenon" is a novel thread, linking to the process of carcinogenesis in OSCC.

Amongst the epithelial malignancies of the oral cavity, OSCC is the thirteenth most common cancer in the world.(1) The diagnostic methods in OSCC focusses on the cellular and phenotypic alterations and the treatment planning for various stages of OSCC depend chiefly on the standard histological evaluation which remains stable for the past decades. Molecular characterization of OSCC using molecular

markers like miRNAs could successfully supplement histopathology. [2]

miRNA is a gene-specific regulator, considered to be one of the most recent entrants in the category of epigenetic profiling and are involved in many essential biological activities such as cellular differentiation, proliferation, and apoptosis and thus, their deregulation can affect normal cell growth and even participate in the event of carcinogenesis in OSCC. [3]

Alterations in the expression patterns of miRNA is a common finding in OSCC tumorigenesis and altered miRNAs play a critical role in the initiation and progression of cancer functioning either as an oncogene or as tumor suppressor.[4] Understanding intricacies of cancer pathogenesis and variability of clinical and pathological parameters may lead the research direction towards miRNA- based therapeutics.

miR-26a is found to be significantly downregulated in OSCC, suggesting that, it may act as tumor suppressor and play a critical role in cancer cells, mediating oncogenesis and metastasis. However, functional role of miR-26 in OSCC is still unknown. [5]

Fukumoto I et al, investigated the functional role of miRNA-26a/b and identified its down regulation in OSCC in a PCR based analysis. Also suggested that restoration of these miRNAs inhibited cancer cell migration and invasion. [6]

There are research gaps with respect to the miR-26a studies in OSCC. With this regard, the purpose of the study is to quantify the tissue expression of miR-26a in OSCC and to correlate the expression portrait with the clinico-pathological parameters.

Material And Methods: 50 tissue samples from OSCC patients, collected from 2019 to 2021 were included in the study.

Total RNA was isolated using RNeasy FFPE Kit (Cat no: 73504, Qiagen, Hilden, Germany) from formalin-fixed, paraffin-embedded (FFPE) tissue sections as per manufacturer's instructions. Purified RNA was eluted by using RNase free water into 1.5 ml collection tube. Prime script RT Reagent kit (RR037A, Takara, Kusatsu, Japan) was used for cDNA synthesis following the manufacturer's directions. qPCR for miRNA-26a and U6 gene was performed by using 2X TB green Premix Ex Taq II (RR820A, Takara, Kusatsu, Japan) that includes Ex Taq HS DNA polymerase, dNTP mixture, Mg2+, Tli RNase H, and TB Green. The reaction mixture consisted of 12.5 µl of 2X TB green Premix Ex Taq II, 1 µl each of the primers, 2 µl of cDNA and 8.5 µl molecular grade water. Thermal cycling conditions were performed as: 95 ºC for 3 minutes, followed by 40 cycles of 95 ºC, 62 ºC and 72 ºC for 20 seconds each. U6 was used as the reference gene. Stem loop RT primers were used for cDNA synthesis. All the primer sequences referred were as mentioned in the **Table 1**.

Normalization and data analysis: The real-time PCR results were quantified and recorded as threshold cycle numbers (Ct), normalized against an internal control (U6), and relative expression of miR-26a was calculated using Livak method [9].

Statistical analysis: Statistical analyses was performed using SPSS version 25.0 Software to assess any statistical significant differences between the clinico-pathological parameters and the miR-26a levels. The correlation between the expression of miR-26a and clinico-pathological parameters were assessed with chi-square test. Level of significant was kept at 5% and values of $p < 0.05$ were considered significant.

Results:

Downregulation *of miR-26a in OSCC using real time RT PCR:*

The expression levels of miR-26a in 50 samples of OSCC tissues were detected by SYBR Green based I based stem loop real time RT-PCR. We found that miR-26a expression was significantly decreased in OSCC when compared with normal tissues. The real-time PCR results were recorded as threshold cycle numbers (Ct) and were normalized against a reference gene, internal control (U6). Fold change in the gene expression was calculated by Livak method. Fold change value of less than 1 was considered down regulated whereas fold change value of more than 1 was considered as upregulated.

Out of 50 tissue samples of OSCC, 45 samples showed down regulation of miR-26a expression and 5 samples showed upregulation and were excluded for the statistical analysis.

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Correlation of miR-26a expression with Clinico-pathologic parameters of OSCC patients:

The OSCC patients were divided into two groups high expressers (n=34) and low expressers (n=11) based on the mean level of miR-26a. The relationship between the miR-26a expression levels and clinico-pathological parameters of OSCC are summarized in **Table II.**

The study group consisted of patients less than 60 years (80%) and patients more than 60 years (20%) High downregulation of miR-26a was seen in 28 patients in less than 60 years category and 6 patients in more than 60 years category. **(Graph -1).**

Male patients were (64.4%) and females (35.6%) in the study group. 22 male patients and 12 female patients presented with high downregulation of miR-26a expression **(Graph -2).**

The commonest location involved by OSCC was buccal mucosa (44.4%) followed by Tongue (20%), Gingivobuccal Sulcus (GB Sulcus) (17.8%), alveolus (8.9%), RMT (Retro molar Trigone) (6.7%) and FOM (Floor of the mouth) (2.2%)

Predominantly, patients had tobacco habit (53.3%) followed by areca nut (33.3%) and smoking habit (8.9%). 4.4 % had sharp teeth in association with the lesion.

Tumor grading was based on differentiation of tumor cells. 29.9% were well differentiated, 46.7% were moderately differentiated and 11.1 % were poorly differentiated. Early invasive tumor type was observed in 13.3%.

Lymphocytes were prominently seen in the stroma of OSCC patients in 71.1%. Neutrophils in 4.4 %, Plasma cells and Eosinophils in 2.2 %. Combination of lymphocytes and plasma cells were seen in 17.8% and lymphocytes and neutrophils in 2.2%.

Out of 50 patients, 57.8% showed negative lymph nodes and 42.2% showed positive lymph nodes.

In the 19 cases of OSCC with lymph node metastasis, 11 (72.7%) presented as high expressers and 8 (32.4%) as low expressers. In the 26 cases of OSCC without lymph node metastasis, 23 (67.6) presented as high expressers and only 3 (27.3%) as low expressers (p=0.023, Chi- Square test, **Graph -3**).

There was no correlation between the miR-26a expression levels with age, (p=0.382) gender distribution (p=0.610) and tumor grading (p=0.307). The expression levels of miR-26a was correlated significantly with lymph node status (p=0.023).

Table 1: Primer sequence of miR-26a and control gene U6

Name	Primer	Sequences (5'-3')	Reference
U6	RT Primer Real-time PCR	GGAACGCTTCACGAATTTG Forward: ATTGGAACGATACAGAGAAGATT Reverse: GGAACGCTTCACGAATTTG	07
miR-26a	RT primer Real-time PCR	GCTGTCAACGATACGCTACCTAACGGCAT GACAGTGTCAGCCTA Forward: CTGTCAACGATACGCTAC Reverse: GTAATCCAGGATAGGCTG	08

Table 2: Association between miR-26a expression portrait and Clinico-pathological Parameters in OSCC

Clinico-pathological Parameters	No of cases	miR-26a	p- value

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		High expressers	Low expressers	
Age				
≤60 years	36	28	8	0.382
≥61 years	9	6	3	
Gender				
Males	29	22	7	0.610
Females	16	12	4	
Location				
Alveolus	4	3	1	
Buccal Mucosa	20	17	3	
FOM	1	0	1	-
GB Sulcus	8	5	3	
RMT	3	3	0	
Tongue	9	6	3	
Habits				-
Areca nut	15	11	4	
Sharp teeth	2	1	1	
Smoking	4	3	1	
Tobacco	24	19	5	
Pathologic Grading				
WD	13	9	4	0.307
MD	21	16	5	
PD	5	4	1	
Early invasive	6	5	1	
Inflammation				-
Lymphocytes	32	24	8	
Neutrophils	2	1	1	
Plasma cells	1	1	0	
Eosinophils	1	1	0	
Mixed	9	7	2	
Lymph node status				**0.023**
Positive	19	11	8	
Negative	26	23	3	

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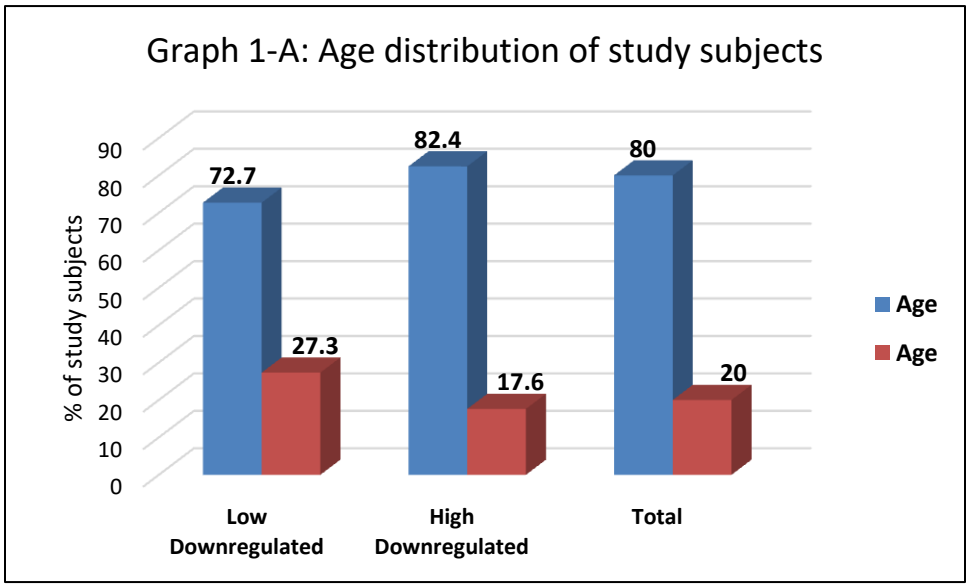
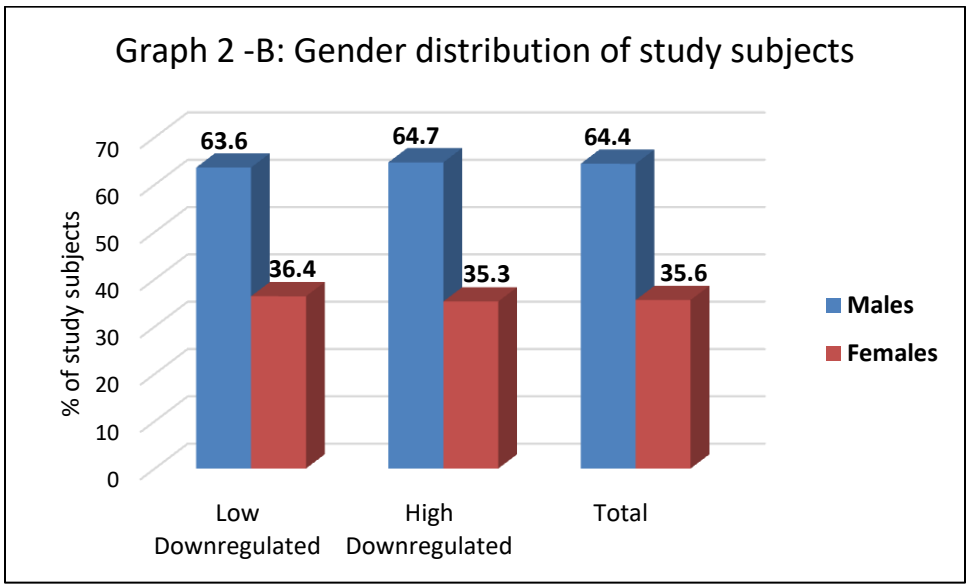
Graph 1: Association between Age and level of downregulation



Graph 2: Association between Gender and level of downregulation



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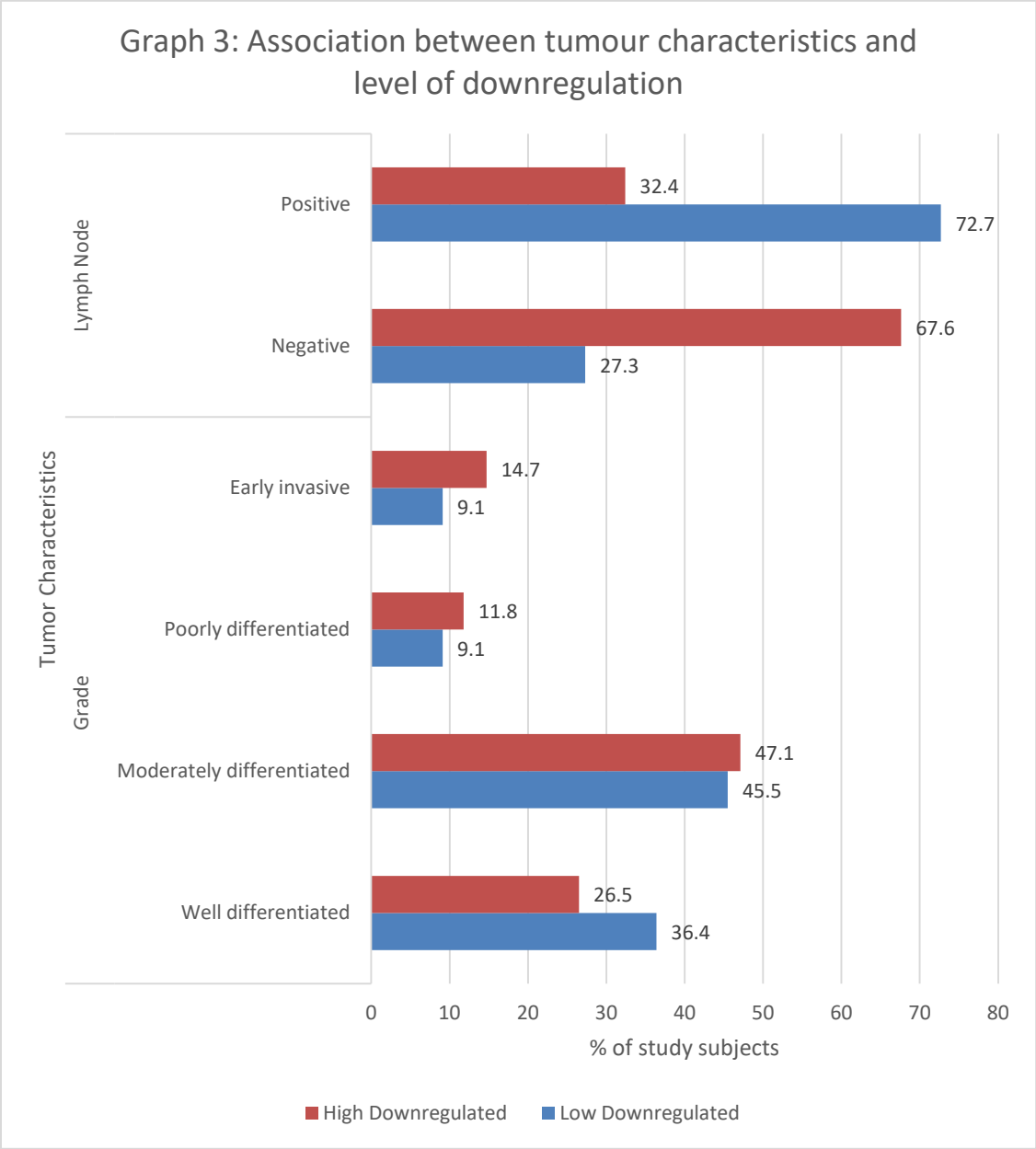
Graph 3: Association between tumour characteristics and level of downregulation



Discussion

OSCC is turning out to be a capricious disease striking challenges to diagnostician due to its erratic behaviour and also causing lethal impact on the way of life to the patients. Considering the inflating incidence of OSCC, it is an essential need to find potential ways to battle out cancer effects and utility of miRNA profiling is fetching as a promising biomarker in the direction of cancer therapeutics.

miRNA screening have been performed in several cancers in large scale and have shown unique expression profiles in cancers and consistently down regulated in breast cancer (Li et al, 2013 & 2014 nasopharyngeal carcinoma (Lu et al, 2011) and hepatocellular carcinoma (Zhao et al, 2014). [6]

miR- 26 is a family of miRNAs comprised of miR-26a-1, miR-26a-2 and miR-26b, located on chromosomes 3, 12 and 2 respectively. The expression of miR-26 has been found to be specific to different biological processes and many investigations have shown that miR-26 expression is disordered in various types of tumors. It is involved in tumorigenesis and acts either as tumor suppressive gene or oncogenic gene. [10]

It acts as tumor suppressor and miR-26a and miR-26b are downregulated in OSCC and its

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overexpression inhibits cell cycle, migration, invasion and glycolysis, while promoting cell apoptosis. [11]

A study by Fukumoto et al, 2015 has shown that miR-26a and miR-26b were significantly downregulated in OSCC tissues, suggesting that miR-26a and miR-26b may act as tumour suppressors. [6] Our study results were similar in comparison to this study.

Expression of miR-26a was positively correlated with MEG3 expression in clinical specimens and decreased MEG3 expression was associated with poor overall survival in TSCC patients (Ling-Fei Jia, 2014). The study also concluded that miR-26a was significantly correlated to tumor size and lymph node metastasis of TSCC. [12] In our study, significant correlation between miR-26a expression and lymph node status was observed. A recent study showed that the levels of miR-26a/b was robustly down-regulated in oral mucosal biopsies, serum and saliva in OLP (Oral Lichen Planus) patients compared with healthy control. It was suggest that vitamin D/VDR-induced miR-26a/b take protective functions in OLP via both inhibiting apoptosis and impeding inflammatory response in oral keratinocytes. [13]

Function role of miR-26a is still unknown and considering the relatively stable expression of miRNA's in tissue specimens, we investigated miR-26a expression in OSCC tissues in correlation with the clinico-pathological characteristics. Very few studies are centred upon expression of mir-26a and its correlation with clinico-pathological characteristics in OSCC. Our study is an attempt to explore the dysregulation and phenotypic components of OSCC.

Conclusion

miRNAs are emerging as reliable molecular markers holding a significant clinical potential as diagnostic and prognostic markers. Differential expression of miRNAs and its interrelationship with tumor characteristics may enable understanding the complex cancer biology and pathways involved in cancer progression. Also, manipulation of its function by miRNA inhibitors is contemplated as the novel approach for cancer treatment strategies.

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