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DOWN-REGULATED MICRORNA-133A IN ORAL SQUAMOUS CELL CARCINOMA AND ORAL SUB-MUCOUS FIBROSIS: NON-INVASIVE BIOMARKER

SINGH P[1], SRIVASTAVA AN[1*], SHARMA R[2], JAIN A[3] AND ANWAR M[1]

1: Department of Pathology, Era's Lucknow Medical College & Hospital, Era University, Lucknow

2: Department of Biosciences, Integral University, Lucknow

3: Department of Oral Pathology and Microbiology, Sardar Patel Post Graduate Institute of Dental and Medical Sciences, Lucknow

***Corresponding Author: Dr. Anand Narain Srivastava (Director Research), Department of Pathology, Era's Lucknow Medical College & Hospital, Era University, Lucknow**

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ABSTRACT

Background

Circulating miRNA-133a (miR133a) is a biomarker identified in various types of cancer. In this study, we evaluated the viability of miR-133a levels in oral sub-mucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) in serum samples for screening in OSCC and OSMF patients.

Methods

The relative expression level of miR-133a was evaluated by quantitative real time RT-PCR (qRT-PCR) in the serum of 20 OSCC, 20 OSMF patients and 40 healthy subjects.

Result

The result of the study revealed the down-regulated expression of miR-133a in OSMF and OSCC patients as compared to healthy subjects.

Conclusion

miR-133a expression was lost in OSCC and OSMF tissues and hence it acts as a tumor suppressor marker in oral cancer along with higher stability, specificity, sensitivity and

reliability. More studies are needed to validate it as an early diagnostic and prognostic biomarker in oral pre-cancer and cancer.

Keywords: Micro RNA-133A, oral sub-mucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC)

INTRODUCTION

It has been known that genetics and oral health are closely related (1).Study of the multiple disorder affecting craniofacial tissues have attracted the scientists towards the role of inflammation in infection and pain, the changes arise from a mutated gene and the consequences of depressed immunity (2).

Oral cancer is among the sixth most frequent cancer worldwide, with approximately 3,00,000 new cases and 1,30,000 deaths annually worldwide (3).It is the leading reason of cancers in India and South-East Asia (4).It is the most common malignant epithelial neoplasm that affect the oral mucosal region (5). The incidence rate of OSCC shows variation in different parts of the world and it ranges from 2-10/1, 00,000 in a year. Tobacco chewing (khaini, gudakhu, guthka andpaan masala) and some other tobacco related products are responsible for the oral cancer in the Indian subcontinent (6). The transformation of tobacco exposed normal oral mucosa to malignant lesions results in the carcinoma of oral region.In the early,1950s oral sub-mucous fibrosis was introduced and it is a malignant disease predominantly observed in Asian descent. It is a severe progressive disease and its clinical features depend on the stage of the disorder.It is a globally accepted Indian disease.The main histological features of this disease is fibrosis that mainly affects the major parts of the oral cavity, pharynx and upper third of the oesophagous (7). Among other malignant oral lesions,it shows the highest rate of transformation of malignancy. Whereas,with the follow-up of 17 years, it shows the 7.6% malignant transformation rate (8).Recently, the discovery of MicroRNAs has opened new opportunity in cancer biology, they are non-coding 21-25 ntd.sequences, that involve in the regulation of protein coding gene expression at the post-transcriptional level by targeting the 3'UTR region of specific mRNAs to either inhibit or degrade these mRNAs (9). An ample of evidences proposes that miRNAs are found to be altered in various types of cancers and play important roles in initiation, development and metastasis of cancers (10).Some miRNAs control the oncoproteins in a negative pattern in a normal cell and accordingly their altered expression patterns linked with several types

of cancer, and notably due to their stability in blood and other body fluids, these miRNAs act as a promising biomarker of cancer (11,12).miR-133a has been reported as a tumor suppressor in colorectal cancer (13), bladder cancer, prostate cancer (14), breast cancer (15),ovarian cancer (16).A TSCC cell study also revealed reduced expression of miRNA-133a in these cells in comparison to normal epithelial cells (17).

Besides these study, there is no evidence illustrated about the association between miR-133a and OSCC. Hence, the present study reveals miR-133 as a tumor suppressor to inhibit metastasis and cancerous properties in cells of OSCC. It was also postulated that miR-133a deregulation helps in the metastasis of patients of OSCC. These findings proved miR-133a as a novel therapeutic biomarker for early detection of OSMF and OSCC patients.

MATERIALS AND METHODS

Subjects and sample collection

In the present study, we have enrolled 20 OSMF patients, 20 OSCC patients and 40 healthy controls (Table-I). The blood samples were collected from OSCC patients attending from Surgical Oncology Department from King George's Medical University (K.G.M.U.), Lucknow, while the blood samples of OSMF and healthy controls were collected from the Department of Dentistry, Era's Lucknow Medical College and Hospital (ELMC&H), Lucknow, between february 2015 to february 2017.Informed consent was taken from all participants for sample andongoing data collection. The study was approved by Ethics Committee of ELMC&H. The inclusion and exclusion criteria have mentioned below for all the patients who have enrolled in the present study

Inclusion Criteria: All the fresh diagnosed cases of OSCC, OSMF and healthy controls have been considered in the study.

Exclusion Criteria:

1. Any patient who was in terminal stage of a disease and therefore not operable.
2. Patients who have AIDS or any other known Immunodeficiency disorder.
3. Cases of SCCs that either has or had in past any other malignancy.

Collection and storage of serum samples

Blood samples were collected in clot activator tube from each subject before their surgery.

All the information on basic demographical and clinical parameters has been taken from all the participants. According to American Joint Committee on cancer (AJCC), staging of tumors have been done on the basis of histology type, grade of tumor and cancer

stages. After the collection of blood samples, it was kept for 45 minutes to allow clotting, for serum isolation. Then it was processed according to Qiagen manufacturers (Germany) protocol. The blood was centrifuged at 3000 rpm (REMI) at 4^0c for 10 mins, additionally; the supernatant was again re-centrifuged for 10 mins at 13500 rpm at 4^0c to remove other contaminants such as erythrocytes. It has been done to yield better quality of RNA for further processing of total RNA isolation. The Serum was stored in -80^0C deep freezer for further steps of RNA isolation.

RNA isolation

According to manufacturer's instructions, Qiazol reagent (Qiagen, Germany) have been used for the extraction of total RNA isolation. Manufacturers have suggested taking 200 µl of serum sample for RNA extraction. For this purpose, the miRNeasy mini Kits (Qiagen, Germany) and miRNeasy serum/plasma (Qiagen; Germany) were used to extract miRNAs from samples of serum. The miRNeasy procedures are helpful in the removal of contamination of salts or phenols, which interferes at various steps of isolation.

cDNA synthesis (Reverse Transcription)

For the cDNA synthesis, total RNA samples were1.5 µl HiSpec buffer, 4 µl Nucleic Mix, 2 µl miScript master mix 2 µl and RNase free water10.5 µl. The total volume of final mixture was 20 µl. The reverse transcription PCR reaction condition was 95^0C for 5 mins and 37^0C for 60 mins and then held at 4°C.

Real-Time PCR

After synthesis of cDNA, for the miRNA based qRT-PCR assays, according to manufacturer's instruction, each PCR reaction was performed in triplicates using SYBR Green Master Mix (Qiagen, Germany). Each reaction was carried out in a volume of 25 µl, containing 1 µl cDNA, 2.5 µl universal primer, 2.5 µl PCR primers along with 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix were mixed with RNase free water (Table 2). The PCR amplification reaction included denaturation at 94^0C for 15 seconds followed by 40 cycles at 55^0C for 30 sec and 70^0C for 30 sec. The reaction was run in the 7900 Sequence Detection System 2.3 (Applied Biosystem). The Ct mean cycle threshold is predicted as a total number of cycles to generate a fluorescent signal to cross threshold value in real time quantitative PCR (Applied Biosystem). The miRNA-133a has not attained its threshold value.

Statistical analysis

All statistical analysis was done using Excel and SPSS Software.

RESULTS

Repressed expression of miRNA-133a in oral cancer

We evaluated the expression levels of miR-133a in serum of oral squamous cell carcinoma patients.Patient characteristics are shown in table I.RNA was extracted from serum sample and miRNA expression level of miR-133a were determined by qRT-PCR. The expression level of miRNA-133a was significantly lower in oral cancer as compared to healthy subjects (Fig 1).

Repressed expression of miRNA-133a in Oral sub-mucous fibrosis

We estimated expression levels of miR-133a in serum of oral sub-mucous fibrosis patients. Extraction of RNA was done and miRNA expression level of miR-133a was determined by qRT-PCR. The expression level of miRNA-133a was significantly reduced in OSMF as compared to healthy individuals.

Table I: Case Summary of OSCC/OSMF

Sex	Frequency	Percentage
Male	37	92.5
Female	3	7.5
OSCC		
Yes	20	50.0
OSMF		
Yes	20	50.0
Clinical Grade		
I-II	19	47.5
III-IV	21	42.5
Tobacco Chewers		
Yes	38	95.0
No	2	5.0
Smoking		
Yes	13	32.5
No	27	67.5
Pan Masala		
Yes	26	65.0
No	14	35.0
Alcohol		
Yes	8	20.0
No	32	80.0

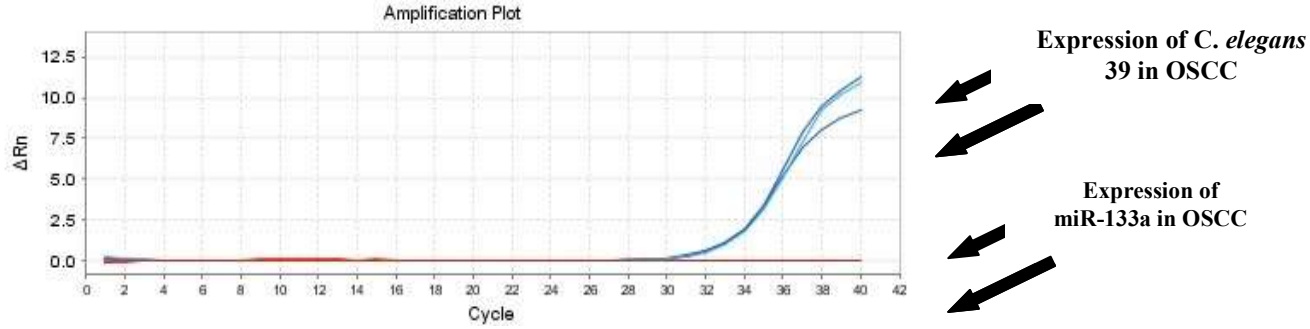

Figure-1: Expression of miRNA-133a and C. *elegans* 39 (Internal control) in OSCC

DISCUSSION

Tumor invasion and metastasis is the leading reason of mortality in patients with solid cancer but our knowledge about its molecular and cellular mechanisms is very limited. Clinicians control tumor metastasis in the application of clinical practice by the discovery of biomarkers that help in the monitoring of tumor invasion and metastasis. OSCC is a malignant disease and the incidence of its new cases increased in developing countries (18). A distinct feature of cancer cells is a deregulation of gene expression. It has been postulated that miRs play an important role in the deregulation of mRNA expression in several types of cancer (19). These miRmarkers are also helpful in the evaluation of risk of occurrence and predicting survival of the patients. The common methods for analysis of expression of miRs were real time PCR, Northern blot, microarray profiling and the bead based methods in either blood or tissue specimens (20,21). Since there is a limited knowledge in expression profile of circulating miRs in OSCC and OSMF. We have performed expression profile of miR-133a in OSCC and OSMF serum samples as compared to healthy controls. To eliminate invasive procedure like tissue resection. We considered serum sample for miR-133a expression analysis by qRT-PCR.

miR-133a, a myomiR, present in muscle. It is highly conserved in musculatures of flies in mice and humans. In the human genome (10), miR-133a forms clusters in three different chromosomal regions -6p 12.2, 18q11.2 and 20q13.33 (22). miR-133a involve in the regulation of actin related genes like tropomyosin2 (TPM2),tropomyosin3 (TPM3), fascin actin-budding protein1 (FSCN1) (23,24) and moesin (MSN),which regulate earliest differentiation of myogenic stem cells into myoblasts that results in muscle growing, regeneration and maintenance after the injury or stress period of skeletal muscle (13).

Many recent studies have been reported about miR-133a as a tumor suppressor in several types of malignancies mainly in gastrointestinal system (25, 26), Yoshino et al.(2011) and Uchida et al.(2013) revealed that miR-133a increased cell apoptosis and suppressed cell proliferation and migration of bladder cancer (27,28).Qiu et al.(2014) reported that miR-133a was found to be suppress cell cycle progression in gastric cancer (29). Additionally, in order to identify the functional significance of circulating miRNA-133a-3p in serum samples of OSCC and OSMF, our experimental data of qRT-

PCR then showed decreased expression of miRNA-133a-3p in OSCC and OSMF cells, that was well matched with the expression level in other types of malignancies of human such as bladder, lung, colorectal, esophageal, gastric and OSCC tissues (24,27,30,31). All the data have not attained its threshold value and hence miR-133a-3p has been considered as tumor suppressor biomarker in OSCC and OSMF patients. There is an urgent need more study about tumor suppressive expression of miRNA-133a- 3p in OSCC and OSMF to diagnose these cancers at their early stage.

CONCLUSION

There are a large number of evidences proposed about the significance of deregulations of miRs in the progression of human malignancies that has shed light on novel therapeutic, diagnostic and prognostic properties for different types of cancers including OSCC. The aim of our study was to find out the expression level of miR-133a-3p in OSMF and OSCC to diagnose it at their early stage. We have noticed that this miR-133a has not attained threshold value in OSMF and OSCC and normal control miRNA has attained its threshold value, hence it has been considered miR-133a may act as tumor suppressor miRNA and its deregulation helps in the progression of OSCC and OSMF. However, further investigation is necessary to be conducted on the larger sample size to predict it as tumor suppressor miRNA in OSMF and OSCC for early diagnosis, therapeutics and prognosis of this disease.

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