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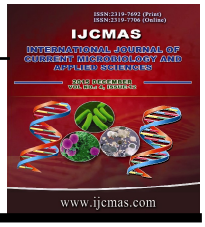

Original Research Article

Expression Analysis of Salivary Microrna-31 in Oral Cancer Patients

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Keywords

Oral Carcinoma, Saliva, MicroRNA-31, Taqman real time-qPCR

A B S T R A C T

Oral carcinoma is the 6th most common cancer in the world. Micro RNAs are small non-coding single-stranded RNAs. They have been shown to be capable of altering mRNA expression; thus some are oncogenic or tumor suppressive in nature. The salivary microRNA-31 has been proposed as a sensitive marker for oral malignancy since it was abundant in saliva more than in plasma. A total of 55 whole saliva samples were collected from 35 cases diagnosed with OC their ages and gender matched with 20 healthy subjects. Taq ManqRT PCR was performed for RNA samples. Mean age was 52.23+13.73 years in cases (range: 17-70 years) with male predominance represented 69%. Risk of smoking and alcoholism was highly significant. The median fold change of miR-31was significantly higher in patients group than in control group, 19.634 versus 1.962 ($P<0.001$). However, the correlation between age of patients and miR-31 fold change was non-significant negative correlation ($r = - 0.236$, $P>0.05$). Median miR-31 fold change was 19.63 in smokers and 21.12 in drinkers. Salivary miR-31 appeared to have significantly elevated in OC patients which point to its potential application as a biomarker for early detection and postoperative follow-up.

Introduction

Oral carcinoma (OC) is one of the most prevalent malignancies worldwide, approximately 263,900 new cases and 128,000 deaths in 2008 (Jemal *et al.*, 2011). Approximately 90% of all oral malignancies are oral squamous cell carcinoma (OSCC) and represents about 3.5% of all malignant tumors in the western societies (Ferlay *et al.*, 2010). The Established etiological factors are cigarette smoking and heavy alcohol abuse; however, a growing group of patients, including young adults and women, have no known tobacco or alcohol exposure have been emerged, therefore; possible viral etiologic factors such as oncogenic human papilloma virus (HPV) have been proposed (Rosebush *et al.*, 2011). The low survival rates and morbidity can be attributed to the late diagnosis (Peacock *et al.*,2008). Hence, several new trends have been emerging that have successfully addressed this problem among which salivary RNAs are noteworthy (Li *et al.*, 2004). The 5-years survival rate for OSCC has remained around 50%, one of

the lowest of the major cancers (Hardisson *et al.,* 2003). Diagnosis of OSCC is currently based on biopsy test, which is an invasive method. There is a need for developing a noninvasive screening tool (biomarker test) for early detection of OSCC. Biomarkers are molecular signatures that are unique to a certain disease (e.g., oral cancer).

The discovery of micro RNAs (miRNA), ~22-nucleotide-long non-coding RNA molecules, has revolutionized the understanding of the modulation of gene expression, nearly 700 micro RNAs have been identified in humans (miRBase: http://microrna. sanger.ac.uk/), a number that is rapidly growing and expected to reach ≥1,000 (Blenkiron *et al.,* 2007). Highly ubiquitous and largely conserved across species, micro RNAs regulate gene expression post transcriptionally by base pairing, usually imperfectly, to the 3□-untranslated region of a cognate mRNA (Bartel, 2004). The interaction of a micro RNA with a target mRNA transcript results either in translational repression of the mRNA or in its direct degradation (Cowland *et al.*,2007). Micro RNAs play important roles in regulating various cellular processes such as cell growth, differentiation, apoptosis, and immune response (Stadler*et al.,* 2008). Salivary microRNA-31 (miR-31) has been proposed as a sensitive marker for oral malignancy since it was abundant in saliva more than in plasma. After excision of OC salivary miR-31 was remarkably reduced, indicating that most of the upregulated salivary miR-31 level came from tumor tissues. Salivary miR-31 was significantly increased in patients with OC at all clinical stages including small tumors (Liu *et al.,* 2012).

The aim is to identify a clinically applicable diagnostic tool for early detection and post-operative follow up using non-invasive methods.

Materials and Methods

Patients

This study is approved by the Committee of ethical standards in the Faculty of Medicine, Al-Nahrain University and underwent to the terms of ethical considerations of the Iraqi Ministry of Health. Thirty five cases newly diagnosed clinically and the diagnosis was confirmed histopathologically by two independent pathologists with OC their ages and gender matched with controls attended to maxillofacial surgery clinic of Ghazi Al-Hariri for Specialized Surgery Hospital in Baghdad were enrolled in this study during the period from April 2014 till April 2015. Thirty five OC and 20 apparently health subjects were collected.

Saliva Samples

Up to 5 mL of unstimulated whole saliva samples from each subject was collected in a 50-mL centrifuge tube. The samples were centrifuged at 3000xg for 15 min at 4□C to spin down exfoliated cells, and the supernatant was transferred into microcentrifuge tubes followed by a second centrifugation at 12,000xg for 10 min at 4□C to completely remove cellular components as saliva supernatant samples. Samples were stored at -80□C until further processing (Michael *et al.*,2010).

Saliva RNA Extraction

Three hundred microliters of saliva supernatant were used for total RNA extraction using AccuZol^{TM} Kit (Cat # K-3090) according to the manufacturer's guideline (Bioneer, Korea). The concentration and purity of the extracted

total RNA was quantified by the use of NanoDrop™ 2000 Spectrophotometer instrument following the manufacturer instructions (Thermo Scientific, USA). The ratio of A260/A280 of wave length absorbance is calculated, a ratio of ~2.0 is generally accepted as "pure" for RNA. Then samples were treated with DNase enzyme prior to reverse transcriptase-PCR (RT-PCR) to remove the trace amounts of genomic DNA from the eluted total RNA by using RQ1 RNase-Free DNase Kit (Cat # M6101) according to manufacturer's instructions (Promega, USA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) synthesis for miR-31 was performed by using AccuPower® RocketScript™ RT PreMix Kit (Cat # K-2101) according to manufacturer's instructions (Bioneer, Korea) which contain all components for first-strand cDNA synthesis. Mastermix was prepared using 10µl of total RNA (100ng/µl), 1µl of specific RT-miR-31 primer (10 pmol) (5'-GTTGGCTCTGGTGCAGGGTCCGA GGTATTCGCACCAGAGCCAACAGCTA T-3'), and 9µl of DEPEC water. The 20µl reactions were incubated in an Applied Biosystems 9700 Thermocycler in a 96 well plate for 1 hour at 50□C (RT step), then 95□C for 5 min (heat inactivation step). The samples were stored at -20 °C until next step. The same protocol was done for cDNA synthesis of GAPDH (reference gene) with using random hexamer primer as the reverse primer.

Stem Loop Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The stem loop RT-qPCR was used in quantification of miR-31 expression analyses (that normalized by reference gene GAPDH) using quantitative Real-Time PCR technique, according to method described by (Kramer, 2012). The cDNA samples of miR-31 were used to prepare the qPCR master mix by using AccuPower® Plus DualStar™ qPCR PreMix Kit (Cat # K-6600) that dependent on TaqMan® probe FAM dye detection of gene amplification in Real-Time PCR system was done according to manufacturer's instructions (Bioneer, Korea). Mastermix was prepared using 5µl of miRNA-31 cDNA template (100 ng/µl), 2.5µl of (10 pmol from both forward and reverse miR-31 primers) (*miR-31F:* 5'-GTTTAGGCAAGATGCTGGC-3'; *miR-31R:* 5'-GTGCAGGGTCCGAGGT-3'; *miR-31probe*: FAM- TTGGCTCTGGTGCAGG-MG), 2.5 µl of 20 pmolTaqMan® prob, and 27.5 µl of DEPEC water. The 50µl reactions were incubated in a Miniopticon Real-Time PCR Thermocycler in a 96 well plate at 95□C for 5 min, followed by 45 cycle of 95□C for 15 sec and 60□C for 30 sec. The same protocol was done for the reference gene GAPDH (*F*: 5'- TCAGCCGCAT CTTCTTTTGC-3'; *R:* 5'- TTAAAAGCA GCCCTGGTGAC-3';and *probe:* FAM-ACCAGCCGA GCCACATCGCTC-TAMRA)

Data Analysis qRT-PCR

Threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. One of the experimental samples is the calibrator such as (Control samples), to determine the relative expression of a target gene in the test sample and calibrator sample using reference gene(s) as the normalizer, the expression levels of both the target and the reference gene(s) need to be determined using RT-qPCR. The data results of RT-qPCR for target and reference gene were analyzed by the relative quantification gene expression levels (fold change). Relative quantification by the ΔCT method using a

reference gene (Livak and Schmittgen, 2001), this method uses the difference between reference and target CT values for each sample. Different samples using the steps:

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref, calibrator) – CT (target, calibrator)

Second, normalize the CT of the reference gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

Statistical Analysis

It was assessed using Mann Whitney U test, Correlation between the different parameters was calculated by the spearman test and P values of $P<0.0001$ and $P<0.05$were considered highly significant and significant respectively.

Results and Discussion

Median fold change was higher in patients group than in control group, 19.634 versus 1.962, according to Mann Whitney U test showed that this difference was statistically highly significant ($P<0.001$) as shown in table (1).

According to Shapir-Wilk test for normality, there was a significant deviation of miR-31 fold change from the normal distribution in control and patients group ($P<0.05$) as in table (2).

The receiver operator characteristic (ROC) curve analysis revealed that the cutoff value for miR-31 was 6.623. Any patient had a value equal or more than 6.623 will be regarded as having a malignant tumor, correlation between age of patients and miR-31 fold change was studied using Spearman rank test that demonstrated a non-significant negative correlation ($r = - 0.236$, $P>0.05$), as shown in Figure (1).

By using Mann Whitney U test the median fold change was 19.193 and 23.336 in male and female patients respectively. Median miR-31 fold change in patients with SCC was (21.485) and was highest in grade III tumors (31.58), meanwhile was lowest in patients with lateral border of tongue (17.21). Median miR-31 fold change was 19.63 in smokers and 21.12 in drinkers as shown in table (3).

The clinical behavior of OSCC might be associated with tumor recurrence and poses a major challenge for early detection; thus, more sensitive and specific biomarkers are urgently needed for patients with oral cancerous and oral precancerous lesions as well as for postoperative fellow-up. Saliva, unlike other body fluids in which oral tissues are continually immersed, may provide more direct information regarding the disease status of the oral mucosa. Therefore, a search for novel biomarkers in the secretome of saliva may benefit patients with OC (Nagler, 2009).

MicroRNAs (miRNAs) are short non-coding RNAs, characterized by their regulatory role in cancer and gene expression, increasing evidence has suggested important roles for various miRNAs in carcinogenesis and that single miRNA is able to target multiple mRNAs transcripts and thereby potentially affect several important cellular pathways involved in tumorigenic processes,

therefore; the signature patterns of representative miRNAs may hold meaningful diagnostic value for early detection in cancer (Dalmay, 2008; Bartel, 2009.

Several studies have found that miRNAs in body fluids are stable and can resist degradation at high and low temperatures, in strong acids and bases, and by RNase, this is likely because free miRNAs in bodily fluids are wrapped up by proteins or stored within vesicles called exosomes (Li *et al.*,2004; Chen *et al.*,2011). Patel *et al.* (2011)found that the expression levels of salivary miRNAs were stable and those levels are reproducible within subjects. Concretely with above studies, measuring salivary miRNAs in patients with OSCC by qRT-PCR seems to be a promising approach to identify new biomarkers in saliva (Patel *et al.*, 2011).

The current results demonstrate that median fold change of miR-31 was markedly higher in patients group than in control group, 19.634 versus 1.962; this difference was statistically highly significant indicating its strong correlation with OC. These findings are in concordance with (Lajer *et al.*, 2011; Liu *et al.*, 2012; and Siow*et al.*, 2014). Liu *et al.*, (2012), found that salivary miR-31 was significantly increased in patients with OC at all clinical stages and that the miR-31 was more abundant in saliva than in plasma suggesting salivary miR-31 was a more sensitive marker for oral malignancy in addition, after excision of oral carcinoma salivary miR-31 was remarkably reduced indicating that most of the upregulated salivary miR-31 came from tumor tissues. Despite the fact that multiple sources may contribute to salivary miR-31, the result that salivary miR-31 was much higher than that of plasma implied a significant local contribution of miR-31 to the saliva contents. The results of present work point to a potential application of salivary miR-31 as a biomarker for early detection and postoperative follow-up of oral carcinoma. Association of miR-31 expression with OSCC, socio-demographic and clinicopathological parameters is essential for the better understanding of how miRNAs are involved in OSCC development. Although the investigated miR-31 here had previously been implicated in various cancers, yet their functions are largely unknown especially in OSCC(Liu *et al.*,2012).

Based on Spearman's test, age and gender of the patients group in the present work demonstrated a non-significant negative correlation with miR-31 expression. These results are in accordance with a recent study by Siow *et al.*, (2014). In relation to tumor subsite, the lowest median expression of miR-31 was in patients with lateral border of the tongue (median=17.21), the median was in highest level in grade III differentiation (median=31.58), followed by grade I differentiation (median=21.49). Current results were in agreement with Siow *et al.*, (2014), regarding both tumor subsite and the tumor grade differentiation.

MicoRNA-31 may play a role in the early stages of cancer development, as have been reported by previous studies such as enhancing early stage events functions like proliferation and tumorigenicity of OSCC cancer cells largely by inhibiting negative regulators of oncogenic pathways when expressed aberrantly (Liu *et al.*, 2010A). Liu *et al*, (2010B) demonstrated that miR-31 has been involved in regulation of lung cancer cell. These findings suggested that miR-31 may play a role in the different stage of oral carcinogenesis.

Table.1 Descriptive Parameters related to miR-31 Fold Change in Patients and Control Group

Group	Median	Mean	SD*	SE**	Minimum level	Maximum level	Skewness	Kurtosis	P-value
Patients	19.634	26.379	16.656	2.815	6.856	67.150	0.780	-0.460	P<0.00
Control	1.962	2.344	1.504	0.336	0.756	6.395	1.610	2.416	1

SD; Standard deviation; DE; Standard error

Table.2 Shapiro-Wilk Test for Normality

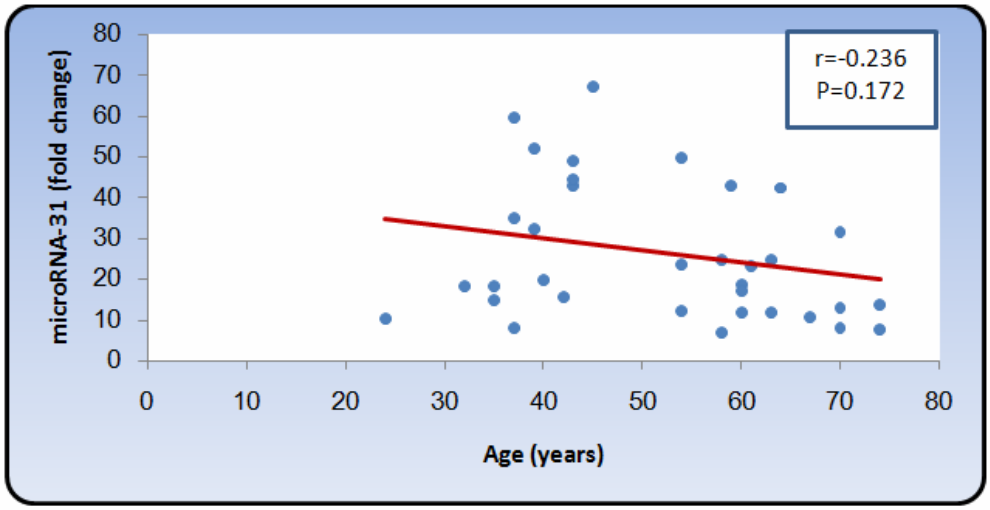
Group	Statistic	No.	P-value
Patients	0.898	35	0.004
Control	0.819	20	0.002

Table.3 Median Fold Change of MicroRNA-31 Associated with Different Variables in Patients Group

Variables	No.		Median fold change	P-value
Gender	Female	11	**23.336**	0.683^{NS}
	Male	24	19.193	
	Total	35	19.634	
Histological subtypes	SCC	26	**21.485**	-
	Others	9	12.882	
	Total	35	19.634	
Tumor location	Floor of the mouth	5	32.22	-
	Tonsils	5	17.24	
	Lateral border of tongue	14	**17.21**	
	Maxilla	4	22.23	
	Mandible	1	18.75	
	Parotid	2	33.88	
	Cheek	3	15.69	
	Lower lip	1	44.27	
	Total	35	19.63	
Tumor grade	Well differentiated grade I	24	21.49	-
	Moderate differentiated grade II	4	13.84	
	Poor differentiated grade III	7	**31.58**	
	Total	35	19.63	
Smoking	Smoker	21	**19.63**	0.946^{NS}
	Non-smoker	14	20.80	
	Total	35	19.63	
Alcoholism	Drinker	10	**21.12**	0.596^{NS}
	Non-drinker	25	19.63	
	Total	35	19.63	

NS= Not significant

Figure 1 Spearman Rank Correlation Between Age of Patients and miR-31 Fold Change



Coincidentally risk factor habits as smoking and alcohol consumptionhave median miR-31 expression nearly equal between positive and negative cases in the patients group; reiterating the fact that, these risk factors are independent effectors which are may be different from molecular point of view than the oncological role ofmiR-31 as an oncomir which effect of cell tumorigenesis, larger sample size with sufficient number of each habit group would be necessary to address this relationship.

In conclusion, salivary miR-31 appeared to have significantly elevated in oral carcinoma patients which point to its potential application as a biomarker for early detection and postoperative follow-up of oral carcinoma.

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