The validity of salivary microRNAs (hsa-miR-200a, hsa-miR-125a and hsa- miR-93) as oral squamous cell carcinoma biomarker

Shaimaa H. Mudhir, B.D.S., M.Sc. [(1)]
Raja H. Al-Jubouri, B.D.S., M.Sc., Ph.D. [(2)]
Ban A. Abdul Majeed, M.B.Ch.B., M.Sc., Ph.D. [(3)]

ABSTRACT

Background: Oral squamous cell carcinoma represents the vast majority of oral cancer it is a common malignant tumor with an increasing incidence. Around the world, the 5 year mortality rate of oral cancer is about 50%. Thus novel biomarkers for early detection oral squamous cell carcinoma are needed. The level of three salivary microRNAs namely hsa-miR-200a, hsa-miR-125a and hsa- miR-93 were measured in saliva of patients with oral squamous cell carcinoma and compared their levels in saliva of healthy control subjects to determine their potential as oral cancer biomarker.

Materials and methods: The level of these three microRNAs was measured by using revers transcription, preamplification and quantitative PCR.

Results: Only miR-200a present in a significantly lower level ($p<0.05$) in the saliva of oral squamous cell carcinoma patients than in control. miR-200a was the strongest parameter (most affected by disease status) in the context of differentiation between OSSC and healthy controls (having the highest ROC area of 0.781 which is significantly higher than the area associated with equivocal test). Coming next in order of importance in the context of case-control differentiation was normalized CT values for hsa-miR-93, which has a reasonably high ROC (0.650), but failed to show statically significance differences, $P>0.05$.

Conclusions: The detection of miRNAs in saliva can be used as noninvasive and rapid diagnostic tool for the diagnosis of oral cancer.

Key words: Saliva, miRNA-200a, miRNA-125a, miRNA-93, OSCC biomarkers, Real time PCR. (J Bagh Coll Dentistry 2014; 26(3):66-71).

الخلاصة

الخلفية : سرطان الخلايا الحرشفية للفم يمثل الغالبية العظمى من سرطان الفم هو ورم خبيث مع حدوث زيادة في معدلات الاصابة في جميع أنحاء العالم ، اذ إن معدل الوفيات 5 سنوات من سرطان الفم هو حوالي 50٪. وبالتالي هناك حاجة إلى مؤشرات حيوية جديدة للكشف المبكر عن سرطان الخلايا الحرشفية للفم. لقد تم قياس مستوى ثلاثة microRNAs اللعابية وهي miR-200a, miR-125a, miR-93 في اللعاب من المرضى الذين يعانون من سرطان الخلايا الحرشفية للفم و مقارنة مستوياتها في اللعاب من مجموعة اصحاء لتحديد كفائتها كعلامات بيولوجية لسرطان الفم. المواد و الطرق : تم قياس مستوى هذه microRNAs باستخدام النسخ العكسي(Revers transcription)، تضخيم (preamplification) والقياس الكمي (Real-time PCR).

النتائج : مستوى miR-200a اقل بكثير ($p>0.05$) في لعاب المرضى سرطان الخلايا الحرشفية للفم مقارنة مع مجموعة الاصحاء. وكان miR-200a المعلمة الاقوى (الأكثر تضررا من حالة المرض) في سياق التمايز بين OSSC و مجموعة الاصحاء (وجود أعلى منطقة ROC من 0.781 وهو أعلى بكثير من المنطقة المرتبطة) . المقبلة القادمة في الترتيب من حيث الأهمية في سياق الحالات والشواهد في التمايز هو miR-93 ، والتي لديها ROC مرتفعة بشكل معقول (0.650) ، لكنه فشل في اظهار ثابت فروق ذات دلالة ، $P > 0.05$.

الاستنتاجات : يمكن استخدام ال miRNAs في اللعاب كأداة تشخيصية واسعة وسريعة لتشخيص سرطان الفم.

INTRODUCTION

Oral cancer, predominantly Oral Squamous Cell Carcinoma (OSCC), involving any part of the oral cavity affects over 300 000 people worldwide annually.[(1)]

Saliva is considered as a mirror of body health and is composed of variety of analyses from systemic sources that reach the oral cavity through various pathways. The role of saliva as a diagnostic tool has advanced exponentially over the past decade. The ability to measure a wide range of molecular components in saliva and compare them with serum coupled with the easy and non-invasive method of collection has made it feasible to study microbes, chemical and immunological markers.

(1)Ph.D. student. Department of Oral Diagnosis, College of Dentistry/ Baghdad University.
(2)Professor. Department of Oral Diagnosis, College of Dentistry/ Baghdad University.
(3) Professor, Department of Pathology and Forensic Medicine, College of Medicine/ AL-Nahrain University.

MicroRNA (miRNA) are small, highly conserved, single stranded of about 22 nucleotides in length, non-coding RNA molecules involved in the regulation of gene expression. It is predicted that miRNA account for 1-5% of the human genome and regulate at least 30% of protein-coding genes [(2)].

MicroRNAs are transcribed by RNA polymerases II and III, generating precursors that undergo a series of cleavage events to form mature microRNA.

The conventional biogenesis pathway consists of two cleavage events, one nuclear and one cytoplasmic. However, alternative biogenesis pathways exist that differ in the number of cleavage events and enzymes responsible. How microRNA precursors are sorted to the different pathways is unclear but appears to be determined by the site of origin of the microRNA, its sequence and thermodynamic stability.

The regulatory functions of microRNAs are accomplished through the RNA-induced silencing complex (RISC). MicroRNA assembles into RISC, activating the complex to target messenger RNA (mRNA) specified by the microRNA. The degree and nature of the complementarity between the microRNA and target determine the gene silencing mechanism, slicer-dependent mRNA degradation or slicer-independent translation inhibition [(3)].

Three important observations early in the history of miRNAs suggested a potential role in human cancer. Firstly, the earliest miRNAs discovered in the roundworm *C. elegans* and the fruit fly *Drosophila* were shown to control cell proliferation and apoptosis[(4)]. Their deregulation may therefore contribute to proliferative diseases such as cancer. Secondly, when human miRNAs were discovered, it was noticed that many miRNA genes were located at fragile sites in the genome or regions that are commonly amplified or deleted in human cancer [(5)]. Thirdly, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal tissues [(6)]. Dysregulation of miRNA expression profiles has been demonstrated in most tumors examined [(7,8)]. However, the specific classification of miRNA as oncogenes or tumor suppressors can be difficult because of the intricate expression patterns of miRNAs.

MATERIALS AND METHODS

Twenty seven patients with oral squamous cell carcinoma were recruited at the Maxillofacial surgery clinic of Ghazi Al- Hariri Hospital, Al-Kadhimia, Al-Ramadi and Al-Yarmouk Teaching Hospital, also 27 healthy control subjects with age and sex matched to the study group were participate in this study.

Unstimulated whole saliva samples (for all patients and controls) were collected between 8 a.m and 11a.m. Subjects were asked to refrain from eating, drinking, smoking or oral hygiene procedures at least 1 hour before collection[(9)]. Consents from controls and patients were taken. Saliva samples were centrifuged at 2600rpm for 15 minutes at 4□C. The supernatant was removed from the pellet and treated with SUPERase (RNase inhibitor). For each 400µl of saliva supernatant 20µl of SUPERase were added. The saliva samples were then kept at -80º C until the time of RNA extraction [(1)] .

1. Saliva RNA extraction

Steps were conducted following the instruction leaflet of mirVana miRNA extraction and according to the manufacturer **(Ambion, USA)**.

Two hundred microliters of the supernatant saliva were used for RNA extraction by using the mirVana miRNA isolation kit according to the instructions of the manufacturer.

2. Reverse transcription:

Steps of the procedure were conducted according to the kit leaflet and according to the manufacture instructions of TaqMan® MicroRNA RT Kit (Applied Biosystems, USA).

3. Preamplification reaction

Steps of the procedure were conducted according to the kit leaflet and according to the manufacture instructions TaqMan® PreAmp Master Mix Protocol (Applied Biosystems, USA).

4. Real-time PCR reaction

Steps of the procedure were conducted according to the kit leaflet and according to the manufacture instructions (Applied Biosystem, USA)**.**

Statistical analysis of Data

1. Statistical Packages for Social Sciences-Version 20 (SPSS-20) was applied to analyze demographic criteria of study and control groups. Data were arranged as frequencies and the Chi-square extracted P value was taken as significant when < 0.05.

2. Real-time PCR data analysis

After the end of experiment the qRT- PCR machine displayed the data as **CT (Cycle Threshold) value** for each sample, CT value corresponds to the number of amplification cycles required for the fluorescent signal to exceed the background level. This means that CT levels are inversely proportional to the amount of products in the sample, i.e. a low CT value means a high expression of the miRNA and vice versa. Moreover, in this study miRNAs with a CT value above 40 cycles are considered non-expressed [(11)].

a. The data included:

- CT values for hsa-miR-200a for OSCC and healthy controls group.
- CT values for hsa-miR-125a for OSCC and healthy controls groups.
- CT values for hsa-miR-93 for OSCC and healthy controls groups.

b. Normalization of data:

For each array the mean expression value was calculated, without prior removal of CT values ≥ 35, and thereafter divided with each individual miRNAs CT value [(11)]**.**

RESULTS AND DISCUSSION

Salivary hsa-miR-200a level in OSCC and healthy controls groups.

Salivary miR-200a was present at lower level in saliva of oral squamous cell carcinoma patients than in healthy controls, this in agreement with Park *et al.* [1] who found miR-200a has been reported to be differentially expressed in head and neck cancer cell lines and other cancer cells [12-14]. Interestingly, in the present study, miR-200a was present at lower level in saliva of OSCC patients compared to healthy controls in contrast to Jiang et al. and Tran et al. [12,14] who found that miR-200a is present at higher levels in various oral squamous cell carcinoma lines. This discrepancy could be due to observing cell-free state of miRNAs compared with the ones in living cells. Because the supernatant saliva is the cell free phase of saliva, some of the miRNAs in supernatant saliva are likely byproducts of cell death. It is possible that cancer specific miRNAs undergo a more rapid degradation and/ or have a shorter half-life during the death, similar to the degradation of regulatory mRNA [1].

The result of the present study is also in agreement with Wiklund *et al.* [15] who found that miR -200a was present at lower levels in oral rinse from oral squamous cell carcinoma patients compared to healthy controls; however they observed no change in the level of this miRNA in saliva. For this reason miR-200a was suggested to function as putative tumor suppressors [16].

Salivary hsa-miR-125a level in OSCC and healthy controls group

This study revealed that salivary miR-125a was up-regulated in OSCC group with no significant difference in the level of between OSCC and healthy controls groups. This suggests that miR-125a may play an oncogenic role. In contrast Park *et al.* [1] found that saliva miR-125a was significantly different between the two groups and was present at lower level in OSCC patients than in healthy controls. Similarly Kozaki *et al.* [17] found that miR-125a was down-regulated in OSCC cell lines.

The oncogenic role of miR-125a was demonstrated by Zhang *et al.* [19] who found that miR-125a translationally arrests mRNA of the p53 tumor suppression. The basis of this activity is the high degree of sequence homology since the 3'-UTR of p53 harbors a sequence motif that is identical to the seed sequence (nucleotides 2-7 from the 5'- end) of miRNa- 125a. This finding added miRNA-125a to the growing list of miRNA with oncogenic targets [17-19].

Reversely Deo *et al.* [10] revealed that miR-125a may function as tumor suppressor for breast cancer with human antigen R (HUR) as a direct and functional target.

Through transient transfection studies, miR-125a with its homolog miR-125b have been shown to reduce ERBB2 and ERBB3 oncogenic proteins levels in SKBR3 cells, a human breast cancer cell lines [20]

If miRNAs are drivers of oncogenic and tumor suppressor pathways, this would expect to find miRNAs mutations can also be causative of the disease [21]. As a matter of fact single nucleotide polymorphisms (SNPS) associated with mature miR-125a has been reported by Duan *et al.* [22]**.** It seems that a comprehensive study should include sequencing of the miRNA as well to rule out the presence of polymorphisms and point mutations in miRNAs sequences before establishing an exact role in OSCC tumorgenesis [22]**.**

Salivary hsa-miR-93 level in OSCC and healthy controls groups

The results of this study showed that miR-93 was up-regulated in patients with OSCC compared with healthy controls. Although this up regulation did not reach a significant level (probably due to the sample size) it still relates this miRNA to the OSCC formation, probably in an association with other miRNAs, this in agreement with Park *et al.* [1] who found that salivary miR-93 was not significantly different ($p=0.17$) between OSCC and healthy controls groups.

Angela *et al.* [23] found that the miR-106b-25 cluster which is comprising from (miR-106b, miR-93 and miR-25) were up-regulated in all cell lines of head and neck cancer, because all these miRNAs are located in intron 13 of the MCM7gene and this region are frequently amplified in HNSCC [23-26]. They also suggested a biological significance of the miR-106b-25 cluster (miR-106b, miR-93 and miR-25) in HNSCC indicating that over-expression of this cluster might play an oncogenic role, perhaps mediated by E2F1 activation and impaired TGF-β signaling, ultimately resulting in uncontrolled proliferation, dysregulated cell cycling and increased invasiveness.

Fang *et al.*, demonstrated that miR-93 function as oncogene through enhancing tumor cell survival, blood vessel formation and tumor metastasis by targeting LATS2. They suggest that miR‑93 can potentially target a great number of genes, some acting directly on tumorigenesis and angiogenesis. Others may only indirectly affect tumorigenesis and angiogenesis [27]

Fang *et al.* [28] examined the function of miR-93 in angiogenesis and tumor formation. In vivo studies revealed that miR-93-expressing cells induced blood vessel formation, allowing blood vessels to extend to tumor tissues in high densities. Angiogenesis promoted by miR-93 in return facilitated cell survival, resulting in enhanced tumor growth.

Table 1: The case-control difference in mean normalized CT values of 3 selected miRNA

Variables	Case-control comparison		Sig.
	Controls	Cases (OSCC)	P (t-test)
Normalized CT value for hsa-miR-200a			<0.001
Range	(0.91 - 1.11)	(0.98 - 1.12)	
Mean	1.01	1.06	
SD	0.05	0.04	
SE	0.009	0.008	
N	27	26	
Normalized CT value for hsa-miR-125a			0.34[NS]
Range	(0.93 - 1.15)	(0.88 - 1.09)	
Mean	1.01	0.99	
SD	0.05	0.05	
SE	0.01	0.009	
N	26	27	
Normalized CT value for hsa-miR-93			0.1[NS]
Range	(0.79 - 1.12)	(0.82 - 1.1)	
Mean	0.99	0.95	
SD	0.08	0.07	
SE	0.015	0.014	
N	27	27	

Table 2: ROC area under the curve for 3 tested miRNA in the context of discrimination between cases with OSCC and healthy controls.

Variables	AUC	P
Normalized CT value for hsa-miR-200a	0.781	<0.001
Normalized CT value for hsa-miR-93	0.650	0.06[NS]
CT value for hsa-miR-93	0.613	0.16[NS]
CT value for hsa-miR-200a	0.575	0.35[NS]
CT value for hsa-miR-125a	0.553	0.51[NS]
Normalized CT value for hsa-miR-125a	0.536	0.66[NS]

Table 3: Linear correlation coefficient

Controls	Normalized CT value for hsa-miR-200a	Normalized CT value for hsa-miR-125a
Normalized CT value for hsa-miR-125a	r=0.111 P=0.59[NS]	
Normalized CT value for hsa-miR-93	r=-0.74 P<0.001	r=-0.717 P<0.001

Cases	Normalized CT value for hsa-miR-200a	Normalized CT value for hsa-miR-125a	Normalized CT value for hsa-miR-93
Normalized CT value for hsa-miR-125a	r=0.381 P=0.05[NS]	r=1 P<0.001	r=-0.799 P<0.001
Normalized CT value for hsa-miR-93	r=-0.843 P<0.001	r=-0.799 P<0.001	r=1 P<0.001

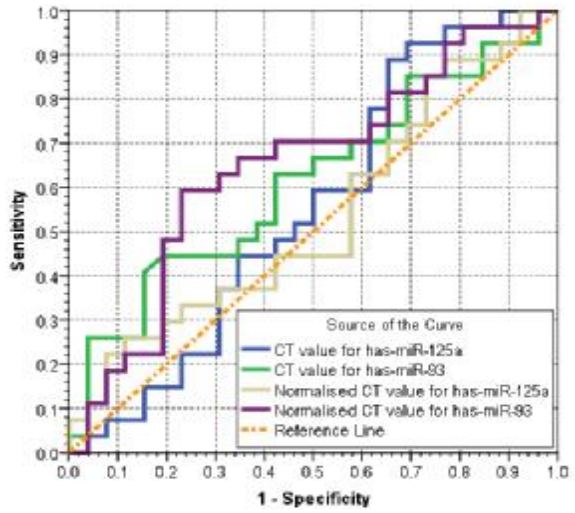

Figure 1: ROC curve showing comparing the area under the curve for hsa-miRNA-125a and hsa-miRNA-93 in the context of discrimination between cases with OSCC and healthy controls. (Higher CT values are in favor of controls).

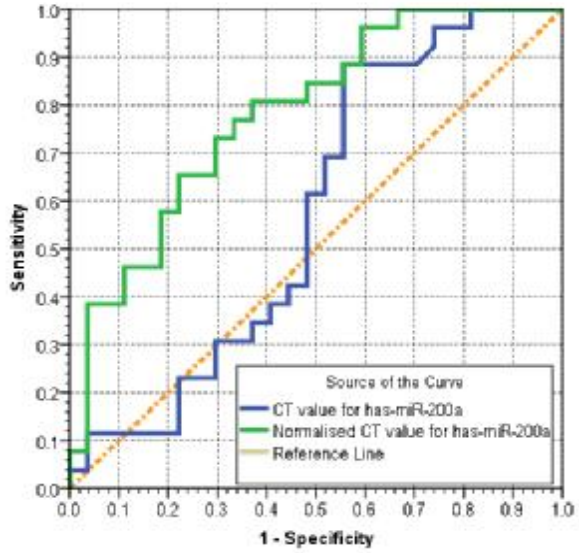

Figure 2: ROC curve showing comparing the area under the curve for hsa-miRNA-200a in the context of discrimination between cases with OSCC and healthy controls. (Higher CT values are in favor of cases).

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