Salivary microRNAs (hsa-miR-200a, hsa-miR-125a and hsa- miR-93) in relation to age, gender and histopathological parameters.

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ABSTRACT

Background: MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate gene expression by targeting specific mRNAs. The main objective of this study was measure the level of salivary (hsa-miR-200a, hsa-miR-125a and hsa- miR-93) in both oral squamous cell carcinoma and healthy controls to asses the association of them with age, gender and tumor grade

materials and methods 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 healthy controls by using reveres transcription, preamplification and quantitative PCR also the general information from each patient including the age, sex and tumor grade were recorded.

Results: Salivary miR-200a was down regulated while miR-93 was up regulated in saliva of females with OSCC compared to females of healthy control, also there was a weak and statistically insignificant positive linear correlation between tumor grade and hsa-miR-200a CT values (r=0.223).However each tumor grade group had a mean normalized CT value which was higher than that of controls with statistically significant differences, P<0.05. The results suggest that circulating miRNAs may be a biological marker of aging and tumor grade. More studies should be done to validate these results.

Conclusions: Both miR-200a and miR-93 could be used as biomarkers for poorly differentiated and aggressive cancer

Key words: Saliva, miR-200a, miR-125a, miR-93, OSCC, tumor grade, Real time-PCR. (J Bagh Coll Dentistry 2014; 26(4):120-125).

الخلاصة

خلفية : microRNAs هي الرنا غير المكودة الصغيرة التي تنظم التعبير ما بعد النسخ الجيني من خلال استهداف mRNAs. كان الهدف الرئيسي من هذه الدراسة قياس مستوى (miR-200a, miR-125a, miR-93) في لعاب كل من مرضى سرطان الخلايا الحرشفية للفم ومجموعة الاصحاء ودراسة علاقة كل منها مع التقدم في العمر والجنس ودرجة الورم 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 مجموعة الاصحاء باستخدام النسخ العكسي (Revers transcription) ، التضخيم preamplification و القياس الكمي Real-time PCR أيضا سجلت المعلومات العامة من كل مريض بما في ذلك السن والجنس و درجة الورم.

النتائج : يكون مستوى miR-200a اقل في حين miR-93 اعلى مستوى في اللعاب للإناث من مرضى سرطان الخلايا الحرشفية للفم مقارنة بالإناث من مجموعة الا صحاء، وأيضا كان هناك وجود علاقة خطية إيجابية ضعيفة وتكاد لا تذكر إحصائيا بين درجة الورم و قيم CT لـ miR-200a (r =0.223) ، ولكن كان لكل مجموعة درجة ورم ومعدل قيم CT المطبعة أعلى من مجموعة الاصحاء مع فروق ذات دلالة إحصائية ، P < 0.05 . تشير النتائج إلى أن تعميم miRNAs قد يكون علامة بيولوجية لتقدم العمر ودرجة الورم الصف. وينبغي أن يتم المزيد من الدراسات للتحقق من صحة هذه النتائج.

الاستنتاجات : كلا miR-200a و miR-93 يمكن ان تستخدم كمؤشر حيوي لسرطان الخلايا الحرشفية للفم ذات صفة سيئة التباين والعدوانية.

INTRODUCTION

Oral Squamous Cell Carcinoma is a common human malignant tumor with an increasing incidence. Oral squamous cell carcinoma has long been considered to be a tumor of the elderly and has been seen only sporadically before the third decade of life. The association of oral cancer with aging could be resulted from prolonged exposure to environmental carcinogens such as chemicals, radiation and viruses which are important promoting factors in the development of oral cancer [(1)]. Accumulative effects of these carcinogens through out prolonged exposure of the life in elderly patients may explain the increased incidence with aging [(2)].

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microRNAs, a family of an average 22 nucleotide long are non coding miRNA which play an important role in gene regulation. They are important in many biological and cellular processes including development, differentiation, cell cycle control and oncogenesis [(3)]. A significant amount of miRNA have been found in extra cellular human body fluids including blood plasma, urine, saliva and semen [(4-7)].

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 [(8)].

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

[9]. Thirdly, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal tissues [10].

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.

The general information was taken from each patient including the name, age and sex. Patients consents for participation in the study were also taken. A group of apparently healthy individuals with age and sex matching to patients served as a control group.

Un stimulated 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.[11]

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 -80 □c until the time of RNA extraction.[5]

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.[12]

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 [13].

RESULTS AND DISCUSSION

Salivary hsa-miR-200a, hsa-miR-125a and hsa-miR-93 levels in relation to gender and age

The present study revealed a statistically significant difference in saliva level of miR-200a and miR-93 between females of OSCC and healthy controls (p=<0.001, 0.016) respectively, whereby miR-200a was down-regulated in saliva of females with OSCC while miR-93 was up-regulated in saliva of females with OSCC.

These miRNAs (miR-200a, miR-125a and miR-93) did not show any statistical difference between males of OSCC and healthy controls groups.

However, these differences should raise the possibility of hormonal influences to be responsible for them. This necessitates more studies to be conducted to relate hormones to miRNAs expression in OSCC. Aging is a highly complex process where over time the accumulation of cellular and molecular damage leads to the functional decline of tissues and organs that eventually increase disease susceptibility and mortality. Although aging can be influenced by environmental factors also play a definitive role in regulating lifespan. In particular, modulation of gene expression in model

organisms has been shown to affect longevity.[14,15] It has been shown that miRNAs are differentially expressed with age in mouse brain, liver and skeletal muscle; However, the expression pattern appear to be tissue specific [16-18]. Also in human, miRNAs expression showed change with human age in peripheral blood mononuclear cells (PBMCs) [19]. Specifically it was found that the majority of miRNAs are downregulated with age and 9 age-associated miRNAs significantly decreased in abundance in older individuals (mean age 64) compared to young individuals (mean age 30) [20]

In considering the relation to age, only miRNA 125a was associated with a significant difference between age groups in the control group whereby it was up regulated in individuals <50 years and down regulated in those >65, indicating a change in expression with age table 3.15, However this finding was not observed in OSCC patients. It appears that the malignant process itself has some degree of modification on miRNA expression that overrides the affect of age. While miR-93 showed increased level with age with no significant difference between different age groups in both OSCC and healthy control groups, this result in agreement with Zhang X *et al.* [21] who found that miR-93 had an age-related increase.

Salivary hsa-miR-200a, hsa-miR-125a and hsa-miR-93 levels and tumor grade

Taking into consideration tumor grades, the results of the present study revealed wide variation. Generally speaking, taking all grade groups from one side and control group from the other side, the liner correlation did not give any significant statistical differences for all the studied miRNAs. miRNA-200a was significantly down regulated in all grades of OSCC (higher CT values) in comparison with control group (lower CT values). This is in agreement with Mongroo and Rutgi [22] who found similar results and suggested that miR-200 family could be considered as putative tumor suppressor and that they could definitely serve as a biomarker mainly in poorly differentiated malignancies. Although miR-125a showed the same changes i.e. down regulation in all grades, and in steady increase with advancing grade, the differences were not significant when compared to control. While miRNA-93 was associated with up regulation in different grades although in a non significant manner. However, the difference between the expression in poorly differentiated group significantly differed from that of the control, suggesting its usefulness as a biomarker to predict higher grades of OSCC.

Table 1: The case-control difference in mean Normalized CT values of 3 selected miRNA stratified by gender

	Case-control comparison		
	Controls	**Cases (OSSC)**	**P (t-test)**
A) Female			
Normalized CT value for hsa-miR-200a			<0.001
Range	(0.91 - 1.07)	(1 - 1.12)	
Mean	1	1.07	
SD	0.04	0.04	
SE	0.011	0.011	
N	15	12	
Normalized CT value for hsa-miR-125a			0.77[NS]
Range	(0.93 - 1.08)	(0.91 - 1.09)	
Mean	1	1	
SD	0.04	0.05	
SE	0.009	0.014	
N	15	13	
Normalized CT value for hsa-miR-93			0.016
Range	(0.9 - 1.12)	(0.82 - 1.05)	
Mean	1	0.93	
SD	0.07	0.07	
SE	0.017	0.02	
N	15	13	
B) Male			
Normalized CT value for hsa-miR-200a			0.1[NS]
Range	(0.95 - 1.11)	(0.98 - 1.12)	
Mean	1.02	1.05	
SD	0.05	0.04	

SE	0.015	0.01	
N	12	14	
Normalized CT value for hsa-miR-125a			0.16[NS]
Range	(0.95 - 1.15)	(0.88 - 1.05)	
Mean	1.02	0.98	
SD	0.07	0.04	
SE	0.021	0.011	
N	11	14	
Normalized CT value for hsa-miR-93			0.96[NS]
Range	(0.79 - 1.08)	(0.83 - 1.1)	
Mean	0.97	0.97	
SD	0.09	0.07	
SE	0.025	0.019	
N	12	14	

Table 2: The mean normalized CT values of 3 selected miRNA by tumor grade

	Controls	**Well differentiated OSCC**	**Moderately differentiated OSCC**	**Poorly differentiated OSCC**	**P (ANOVA trend) for tumor grade**
Normalized CT value for hsa-miR-200a					0.1[NS]
Range	(0.91-1.07)	(0.98- 1.12)	(1 - 1.12)	(1.09 - 1.1)	
Mean	1	1.05	1.05	1.09	
SD	0.04	0.04	0.04	0.01	
SE	0.011	0.015	0.01	0.005	
N	15	9	13	3	
P (ANOVA) = <0.001					
Well differentiated x Control = 0.011					
Moderately differentiated x Control = 0.004					
Poorly differentiated x Control = 0.002					
r=0.223 P=0.28[NS]					
Normalized CT value for hsa-miR-125a					0.47[NS]
Range	(0.93-1.08)	(0.95- 1.09)	(0.88- 1.08)	(1 - 1.03)	
Mean	1	0.99	1	1.01	
SD	0.04	0.04	0.05	0.01	
SE	0.009	0.015	0.013	0.008	
N	15	9	14	3	
P (ANOVA) = 0.8[NS]					
Well differentiated x Control = 0.39[NS]					
Moderately differentiated x Control = 0.58[NS]					
Poorly differentiated x Control = 0.84[NS]					
r=0.266 P=0.19[NS]					
Normalized CT value for hsa-miR-93					0.17[NS]
Range	(0.9- 1.12)	(0.82- 1.07)	(0.83 - 1.1)	(0.87- 0.92)	
Mean	1	0.96	0.95	0.89	
SD	0.07	0.07	0.07	0.02	
SE	0.017	0.023	0.02	0.013	
N	15	9	14	3	
P (ANOVA) = 0.16[NS]					
Well differentiated x Control = 0.33[NS]					
Moderately differentiated x Control = 0.2[NS]					
Poorly differentiated x Control = 0.041					
r=-0.24 P=0.24[NS]					

Note: r (The linear correlation coefficient) was calculated between tumor grade and normalized CT values

Table 3: The mean normalized CT values of 3 selected miRNA by age group stratified by case-control group membership

	Age group (years)			P (ANOVA trend)
	<50	50-65	>65	
A) Control group				
Normalized CT value for hsa-miR-200a				0.97[NS]
Range	(0.91 - 1.03)	(0.95 - 1.11)	(0.98 - 1.01)	
Mean	0.99	1.02	0.99	
SD	0.05	0.05	0.01	
SE	0.02	0.012	0.008	
N	6	18	3	
r=-0.145 P=0.47[NS]				
Normalized CT value for hsa-miR-125a				0.06[NS]
Range	(0.98 - 1.08)	(0.93 - 1.14)	(1.01 - 1.15)	
Mean	1	1	1.07	
SD	0.04	0.05	0.07	
SE	0.015	0.011	0.043	
N	6	17	3	
r=0.231 P=0.26[NS]				
Normalized CT value for hsa-miR-93				0.21[NS]
Range	(0.91 - 1.1)	(0.79 - 1.12)	(0.86 - 0.99)	
Mean	1.01	0.99	0.94	
SD	0.07	0.08	0.07	
SE	0.028	0.019	0.042	
N	6	18	3	
r=-0.113 P=0.57[NS]				
B) Cases group				
Normalized CT value for hsa-miR-200a				0.58[NS]
Range	(1.03 - 1.1)	(1 - 1.1)	(0.98 - 1.12)	
Mean	1.05	1.05	1.07	
SD	0.03	0.04	0.04	
SE	0.016	0.011	0.013	
N	4	12	10	
r=0.128 P=0.53[NS]				
Normalized CT value for hsa-miR-125a				0.5[NS]
Range	(0.95 - 1.03)	(0.88 - 1.09)	(0.94 - 1.08)	
Mean	0.98	0.99	1	
SD	0.03	0.05	0.05	
SE	0.012	0.016	0.015	
N	5	12	10	
r=0.092 P=0.65[NS]				
Normalized CT value for hsa-miR-93				0.3[NS]
Range	(0.87 - 1.02)	(0.82 - 1.1)	(0.83 - 1.07)	
Mean	0.97	0.96	0.93	
SD	0.06	0.07	0.08	
SE	0.027	0.021	0.024	
N	5	12	10	
r=-0.205 P=0.31[NS]				

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