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Original Research Article

Post-chemotherapy miR-146a expression and its prognostic potential in oral cancer patients

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Abstract

Purpose: *To determine miR-146a expression level after chemotherapy in oral cancer patients, and its prognostic value.*
Methods: *Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used for the determination of miR-146 expression levels. Based on the results, the analysis of the miR-146a expression in oral cancer patients was performed by drawing ROC curve to provide information on the prognostic value of miR-146a. The survival of the patients was monitored over a period of 5 years. The patients were categorized into high- and low-expression groups, and multivariate Cox regression analysis method was adopted to provide a more comprehensive analysis of individual risk factors influencing the prognosis of oral cancer.*
Results: *The miR-146a expression level in patients after chemotherapy was lower than that in patients before they received chemotherapy ($p < 0.05$). The specificity of using miR-146a to predict oral cancer was 76.83 %, the sensitivity 69.44 %, and the area between the curve and x-axis 0.78. In contrast, the survival level was significantly greater in high-expression patients ($p < 0.05$).*
Conclusion: *The independent risk parameters for buccal carcinoma are drinking, smoking, chronic leukoplakia, and miR-146a.*

Keywords: *Oral carcinoma, MiR-146a, Clinical effectiveness, Prognostic potential*

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INTRODUCTION

Oral cancer represents a generic term for malignant tumors taking place in the oral cavity. Its incidence has remained high in recent years, and it has become the sixth most prevalent cancer in the world. Its pathological characteristic is mostly squamous epithelial carcinoma, i.e., mucosal mutation [1]. Oral cancer has been on the rise among young people in recent years. In the year 2021, 300,000 cases of oral cancer have occurred around the world, with survival of 50-60% over a 5-year period, and a possibility of approximately 150,000 deaths. Oral cancer is distributed in about 32% of the oral mucosa, 22% of the tongue, 11% of the lower lip, 11% of the palate, 8% of the vestibule, 5% of the alveoli, 5% of the floor of the mouth, and 3% of the gums [2]. At present, the clinical treatment of oral cancer is the same as that of many other types of cancer,

and it involves mainly surgery and chemotherapy. In the study carried out by Ketabat [3], it was reported that although surgical resection could be performed in the treatment of oral cancer, surgical resection caused permanent disfigurement, physical weakness, and severe dysfunction. Chemotherapy and radiotherapy produce obvious toxicity. These two treatment methods seriously impact the health and quality of life of cancer patients. Significant advancements have been achieved in the treatment of oral cancer with chemotherapy and radiotherapy, but the prognosis of the disease has not improved significantly over the past five decades. Therefore, the development of new treatment approach is of great importance both for treatment of oral cancer and for prolonging the lives of the patients. Besides, timely detection of early-stage tumors remains a priority and a major challenge for the medical community.

MicroRNAs (MiRNAs) act as endogenous molecules that adjust and control the expression of target genes. Moreover, they are involved in the regulation of a range of pathophysiological functions [4]. Many miRNAs are abnormally expressed during the progression of cancers [5-7]. Previous studies have found that miR-146a is closely related to the progression of hepatocellular carcinoma, non-small cell lung cancer and gastric cancer [8-10]. But there is no clear evidence whether it has a connection with laryngeal carcinoma.

Therefore, the expression of miR-146a in oral cancer was determined. Its value in predicting and prognosis of oral cancer patients was studied, in order to explore new potential diagnosis and treatment targets for clinic.

METHODS

Patients

A total of 82 oral cancer patients who were diagnosed by doctors at Urumqi Stomatological Hospital and received routine treatment from March 2015 to February 2018 (a period of three years) were chosen as the research group in this study. In addition, healthy subjects were selected. A control group containing 72 healthy persons was used for comparative study. The study group had a male-to-female ratio of 51:31, with a mean age of 42.4 ± 11.2 years. The control group had a male-to-female ratio of 40:32, with mean age of 41.7 ± 10.9 years. This study was approved by the ethical committee of Urumqi Stomatological Hospital, and followed the guidelines of Declaration of Helsinki [11].

Inclusion and exclusion criteria

Inclusion criteria

The ages of the patients involved in the study ranged from 30 to 70 years. They were all diagnosed and treated at Urumqi Stomatological Hospital, and they fully met the diagnostic criteria for oral cancer, with complete case data. All patients fully agreed and actively cooperate with all arrangements made by the hospital's medical staff.

Exclusion criteria

Patients who died during treatment, and those who had vital organ failure and damage or cardiovascular and cerebrovascular diseases, as well as patients suffering from other cancers, were excluded. Patients with diabetes, fatty liver, stroke, heart disease, comorbidities with other autoimmune diseases; mental diseases, central nervous system diseases, speech disorders, and visual disorders, were also excluded. Moreover, pregnant patients and those with hearing disorders, taste disorders, and other diseases that may directly or indirectly affect the results in this research were excluded.

Processing of blood samples

Fasting venous blood was collected in the morning, preserved at 4 °C for half an hour, and the serum specimen was obtained after the blood was centrifuged for 10 min at 3000 rpm. The extraction and storage of the supernatant were conducted at -80°C.

Equipment and chemicals

TRIzol (number JMS12279) was bought from Jiaozuo Lu Feifan Biotechnol. Co. Ltd, while SYBR Green Master Mix no. 11201ES03 was produced by Shanghai Zeyi Biological Technology Co. Ltd. Kit for miRNA reverse transcription (number AB-4366596) was supplied by Shanghai Rongweida Industrial Co. Ltd., USA, while ABI StepOne Plus fluorescent qPCR equipment (number 4376598) was bought from Guangzhou Besaico Biotechnology Co. NanoDrop 2000 spectrophotometer ND2000(c) was produced by Thermo Fisher Scientific. High-velocity centrifuge H1600 was product of Shanghai Saifu Biological Technology Co. Ltd. Low temperature (-80℃) refrigerator (number ARCTIKO-86) was purchased from Senxi Technology Co. Ltd. The primer sequence of miR-146a was produced by Shanghai Shenggong Biological Engineering Co. Ltd.

Table 1: Primer sequences used for PCR

Variable	Upstream	Downstream
U6	5'-TCTCTGCTCCTCGTTCGA-3'	5'-GCGCCCATACGACCAAATC-3'
miR-146a	5'-TGTTGCTGAAGAGCTCTGGTAC-3'	5'-TCGTCTGATGATGTCGAATGTAC-3'

Detailed information on the primer sequences is presented in Table 1.

Determination of mRNA expression

Total RNA was extracted from freshly-separated intravenous serum at a temperature of 8 °C using TRIzol kit strictly in line with the kit protocol. The amount RNA and its level of impurity were measured employing UV spectrophotometry. The RNA absorbance was between 1.8 and 2.1. If the extracted RNA failed to satisfy this criterion, it extraction was done for a second time. After extraction, the integrity of the RNA was examined via electrophoresis on a denaturing agarose gel at a concentration of 1 %. The reaction system for total RNA was reconfigured. The researchers correctly followed the instructions of the miRNA reverse transcription kit in the reverse-transcription of the miRNA under specific conditions, in order to synthesize its corresponding cDNA which was preserved at -20 °C for fresh-keeping. The fluorescent RT-PCR was carried out employing an ABI StepOne Plus fluorescent quantitative PCR instrument. The reaction volume was 12.33 uL, and DEPC water was added to make it up to 20 uL. The PCR conditions were: 95 ℃ for 5 min, 95 ℃ (45 s), 60 ℃ (60 s), and 72 ℃ (45 s). The internal reference employed for the reaction was U6. The experiment was conducted at least thrice, and the results of the relative mRNA expressions were calculated with $2^{-\triangle\triangle ct}$ procedure.

Patient follow-up

A three-year detailed follow-up on the health of the two groups was conducted through telephone calls and outpatient medical records, on the 4th, 8th and 12th months of each year.

Determination of main outcome predictors

The miR-146a levels in subjects suffering from oral cancer were monitored, and its predictive potential was determined.

Secondary observation indicators

The subjects were separately assigned to high- and low-miR-146a categories, among whom the grouping condition was the miR-146a expression levels (the grouping did not consider age, gender, location and other undifferentiated conditions). After grouping, the patients were closely observed and their 5-year survival and physical health status were accurately recorded. Thereafter, independent factors that affected the patients' prognosis were collated and analyzed using multivariate Cox regression analysis.

Statistical analysis

The data collected by the researchers were statistically analyzed using the SPSS20.0 method. GraphPad 7 was utilized to plot the graphs, while KS test was employed to investigate the distribution of the data. Normally-distributed data are presented as mean ± standard deviation (mean ± SD). Paired *t*-test was used for comparison within groups. Count data are expressed as number and percentage [n (%)], and they were analyzed with chi-square test. Independent sample *t*-test was used for between-group comparisons; paired *t*-test was employed for within-group comparisons, while the Kaplan-Meier approach was utilized to assess the 5-year survival of the compared patients. The predictive potential of miR-146a in buccal carcinoma was determined using ROC curves. Multi-factor Cox regression analysis was performed to identify risk factors impacting prognosis. Differences were considered significant when $p < 0.05$.

RESULTS

Patient profile

There were no statistical differences between the clinical data of the study and control groups in terms of gender, BMI, age, marital status, nationality, place of birth and residential area. This information was comparable between the two groups ($p > 0.05$), However, the differences in bad habits such as drinking, smoking, eating of betel nut, and duration of bad habits were all statistically significant ($p < 0.05$) (Table 2).

Expression levels of miR-146a in the sera of oral cancer patients

The results indicated that miR-146a expression level in serum before chemotherapy was 6.73 ± 1.02, while the serum miR-146a expression level after chemotherapy was 1.29 ± 0.84 ($p < 0.05$) (Figure 1)

Table 2: Clinical data [n (%)]

	Study group (n=82)	Control group (n=72)	χ^2 or t	P
Age	42.4±11.2	41.7±10.9	0.392	0.696
Gender			0.258	0.612
Male	50 (60.98)	41 (56.94)		
Female	32 (39.02)	31 (43.06)		
BMI （kg/m^2）	22.13±0.35	22.19±0.31	1.119	0.265
Marital status			0.028	0.866
Married	71 (86.59)	63 (87.50)		
Unmarried	11 (13.41)	9 (12.50)		
Nationality			0.644	0.422
Han	53 (64.63)	42 (58.33)		
Minority	29 (35.37)	30 (41.67)		
Place of residence			0.699	0.403
City	51 (62.20)	40 (55.56)		
Rural	31 (37.80)	32 (44.44)		
Smoking history			62.800	0.001
Yes	65 (79.27)	11 (15.28)		
No	17 (20.73)	61 (84.72)		
Drinking history			5.694	0.017
Yes	43 (52.44)	34 (35.82)		
No	39 (47.56)	38 (55.17)		
Edible areca catechu			54.570	0.001
Yes	69 (84.15)	18 (25.00)		
No	13 (15.85)	54 (75.00)		

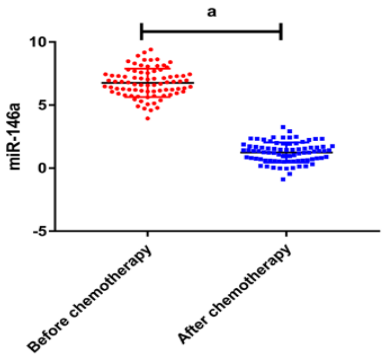

Figure 1: Serum levels of miR-146a. Comparison of the serum values of miR-146a in patients before and after chemotherapy showed that the difference (*a*) had a clear statistical significance ($p < 0.05$)

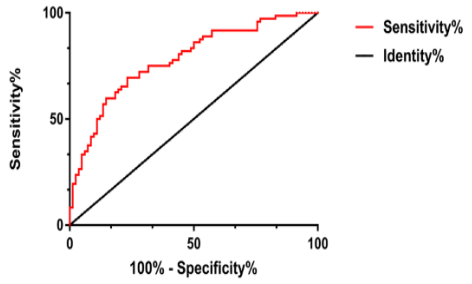

Figure 2: Specificity and predicted sensitivity of miR-146a for prediction of oral cancer based on ROC curve

Table 3: ROC curve data

Variable	MiR-146a
AUC	0.780
Std. Error	0.037
95% CI	0.707~0.853
P-value	<0.001
Cut-off	6.050
Sensitivity (%)	69.44
Specificity (%)	76.83

Potential of miR-146a for prediction of oral cancer

Analysis of the ROC curve in Figure 2 led to the conclusion that miR-146a had a specificity of 76.83% and a sensitivity of 69.44% in forecasting oral cancer when the cut-off value was 6.05, and the area of the curve enclosing the x-axis was 0.78. These results are presented in Table 3 as well as Figure 2.

Five-year survival level of patients

In this research, the results were categorized into 2 groups based on the median value of the expression level of miR-146a. There were 41 patients in the miR-146a high-expression group, and 41 patients who expressed low levels of miR-146a. The value of miR-146a in high-expression group was not less than 1.293, and miR-146a value in the low-expression group was lower than 1.293. The results showed that 22 patients in the high expression group died. Hence, it was calculated that, for patients in the high expression group, the five-year survival was around 46.34 %. In contrast, the group that

expressed low miR-146a had a total of 14 deaths within five years. With regard to the low-expression group, the 5-year survival was calculated to be approximately 65.85 %. This shows that the low-expression group had markedly higher survival level than the high-expression patients over the five-year period, with a difference of about 20%. Therefore, the mortality rate of cancer patients during the five-year period was related to the miR-146a expression. There was a close relationship between the two parameters (p < 0.05). In 5 years, 46.34 and 65.85 % of patients survived in the high- and low- miR-146a expression groups, respectively (Figure 3).

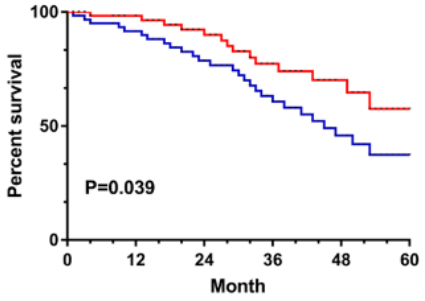

Figure 3: The 5-year survival of subjects in high- and low-expression groups. ***Note:*** Red curve = low expression group; dark curve = high expression group

Univariate Cox analysis results

Based on the median miR-146a levels, the results were categorized into 2 groups: 41 cases of miR-146a high-expression group and 41 cases of low-expression group. The miR-146a value in high-expression patients was ≥ 1.293, while the miR-146a value in low-expression patients was <1.293. The clinical data of the two groups were analyzed based on single factors. The results showed that age, gender, area of residence, and marital status were comparable. However, differences in smoking, drinking, erythema, leukoplakia, betel nut chewing, and miR-146a were all statistically significant (p < 0.05) (Table 4).

Multivariate Cox analysis results

Results from multivariate Cox regression analysis showed clearly that independent risk factors for patient prognosis did not include differences in age and gender, while chronic diseases such as leukoplakia and erythema, bad habits, drinking and smoking, and miR-146a-specific manifestations were independent risk factors for patient prognosis (Table 5).

DISCUSSION

Smoking and drinking are considered to be two of the chief culprits in oral cancer. Alcohol is an organic solvent which promotes the dissolution and absorption of carcinogens in tobacco. In recent years, head and neck cancer has become the seventh most common malignancy worldwide. Annually, more than 600,000 new cases are diagnosed. At the same time, the incidence of oral cancer is on the rise, and oral cancer accounts for half of the head and neck cancer cases. In addition, according to surveys, there is uneven distribution of head and neck

Table 4: Univariate Cox analysis

Clinicopathological features	High expression group (n=41)	Low expression group (n=41)	χ^2 or t	P
Age/years old				
<30	18 (43.90)	16 (39.02)	0.201	0.654
≥30	23 (56.10)	25 (60.98)		
Gender				
Male	22 (53.66)	23 (56.10)	0.049	0.824
Female	19 (46.34)	18 (43.90)		
Smoking				
Yes	35 (85.37)	21 (51.22)	11.040	0.001
No	6 (14.63)	20 (48.78)		
Leukoplakia and erythema				
Yes	11 (26.83)	29 (70.73)	15.810	0.001
No	30 (73.17)	12 (29.27)		
Drinking				
Yes	31 (75.61)	13 (31.71)	15.890	0.001
No	10 (24.39)	28 (68.29)		
miR-146a	1.39±0.84	1.03±0.75	2.047	0.043

Table 5: Multivariate Cox analysis

Factors	Univariate Cox			Multivariate Cox		
	Exp (B)	95CI%	Sig.	Exp (B)	95CI%	Sig.
Age (≥30VS<30)	0.258	0.183~0.712	0.003			
Gender (Male VS Female)	1.375	0.582~1.927	0.845			
Smoking (Yes VS No)	0.954	0.442~1.965	0.832	2.844	1.432~4.533	0.028
Leukoplakia and erythema (Yes VS No)	6.124	3.043~12.422	0.002	3.128	1.135~9.346	0.017
Drinking (Yes VS No)	0.132	0.037~0.225	0.001	2.628	1.244~5.739	0.029
miR-146a (≥1.290VS＜1.290)	0.965	0.543~1.859	0.923	1.021	1.013~1.137	0.015

cancer in the world. South America has the highest morbidity. In particular, the morbidity in Brazil has been increasing at an alarming rate in recent years. According to conservative estimates by relevant medical staff and scientific researchers, there were 16,340 new cases in 2016, out of which about 30% occurred in Brasilia, the capital of Brazil [12]. In addition, the incidence of head and neck cancer is also associated with gender and age. The incidence in men is higher than that in women, and it increases with age [13]. Due to its high incidence and aggressiveness in recent years, the identification of a key biomarker for oral cancer is particularly important for disease detection and comprehensive treatment.

It is known that miRNA is able to degrade target genes and inhibit their translation, thereby accomplishing post-transcriptional gene silencing. Studies have shown that miRNAs [14] affect and regulate almost 30% of the protein encoded in the body. They inhibit or promote tumor development by adjusting target genes. A study has revealed that miR-146a exerts anti-tumor effect [15]. However, there is no specific research showing whether miR-146a has anti-tumor effect in oral cancer.

This research specifically compared the basic clinical information of oral cancer patients and discovered differences in unhealthy habits such as smoking, drinking, and eating betel nut. Gupta *et al* believed that oral cancer should be a disease of multifactorial origin, i.e., risk factors and effects vary considerably in different populations [16].

Currently known risk factors include various forms of smoking, betel nut chewing, alcohol abuse and underlying chronic diseases such as chronic erythema and leukoplakia. These known risk factors are generally consistent with the results of this research. In this study, it was observed that the serum miR-146a was significantly lower in healthy individuals than in patients with oral cancer. After chemotherapy, a significant reduction in miR-146a could be seen. This suggests that miR-146a may be a potential diagnostic and therapeutic target for laryngeal cancer. The ROC curve and the coordinate x-axis constituted an area of 0.780. This value represents higher specificity and sensitivity, and can be used as a clinical prediction and diagnostic index for oral cancer.

The study has demonstrated that 5-year survival of patients who expressed high miR-146 levels (46.34 %) was significantly better than that of miR-146a low-expression group (65.85 %). These results imply that miR-146a could be employed as a predictor of survival. Based on Cox multivariate analysis, it can be inferred that smoking, drinking, chewing betel lang, chronic leukoplakia, and miR-146a are independent risk factors that affect the prognosis of patients.

In addition, according to the studies conducted by Osazuwa-Peters and other researchers, it is evident that at least 75 % of head and neck cancers are inextricably linked to the consumption of tobacco and alcohol. It is estimated that the frequency of buccal cancer in smokers and alcoholics is 30 – 48 % higher than that in the normal population [17].

This preliminary study has demonstrated the clinical potential of miR-146a in prediction of oral cancer. However, there are still certain limitations in the study. This research did not conduct basic cell experiments and rat experiments. Therefore, more in-depth experimental analysis is needed to further confirm the results of this research and provide more valuable clinical information. In addition, quitting unhealthy habits such as smoking, alcoholism, and chewing betel lang can effectively reduce the incidence of oral cancer.

CONCLUSION

This study has shown that miR-146a has a high expression level in oral carcinoma patients. Habits such as drinking, smoking, chronic leukoplakia and erythema are independent risk

factors for oral cancer. This finding provides valuable reference information for clinical practice.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this study was done by the authors mentioned in this manuscript, and all liabilities related to the content of this article will be borne by the authors. YS and LL designed the research, drafted the manuscript. collected and analysed the experimental data. WH critically revised the intellectual content of the manuscript. All authors read and approved the final manuscript.

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