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Study of expression levels and clinical significance of miR-503 and miR-375 in patients with esophageal squamous cell carcinoma

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Abstract. – OBJECTIVE: This study aims to investigate the expression levels and clinical significance of miR-503 and miR-375 in patients with esophageal squamous cell carcinoma.

PATIENTS AND METHODS: 40 cases of cancer tissues and adjacent tissues in patients with esophageal squamous cell carcinoma were collected from March 2017 to September 2017 in the Department of Gastroenterology in the First Affiliated Hospital of Henan Polytechnic University. qRT-PCR was used to analyze the expressions of miR-503 and miR-375 in cancer tissues and adjacent normal tissues. The association between their expressions and the gender, age, degree of tumor differentiation, TNM staging, presence or absence of lymph node metastasis, and other clinicopathological characteristics of patients with esophageal squamous cell carcinoma was analyzed.

RESULTS: The results showed that the expression level of miR-503 in esophageal squamous cell carcinoma tissues (6.83±2.14) was significantly higher than that in adjacent tissues (2.45±1.13). The expression level of miR-375 in esophageal squamous cell carcinoma tissues (3.75±1.06) was significantly lower than that in adjacent tissues (7.45±1.13). The expression levels of miR-503 and miR-375 in esophageal squamous cell carcinoma were related to the existence of lymph node metastasis, degree of differentiation of esophageal squamous cell carcinoma and TNM staging ($p<0.001$). There was no correlation between the expressions of miR-503 and miR-375 and the age, gender and tumor size of patients ($p>0.05$).

CONCLUSIONS: miR-503 was highly expressed in esophageal squamous cell carcinoma and miR-375 was lowly expressed in esophageal squamous cell carcinoma. miR-503 and miR-375 were closely related to the lymphatic metastasis, degree of differentiation and TNM staging of the tumor.

Key Words
miR-503; miR-375; esophageal squamous cell carcinoma; TNM staging.

Introduction

Esophageal squamous cell carcinoma, a common malignant digestive tract tumor, originates from esophageal epithelial cells. As the fifth most prevalent malignancy in the world, statistics show that more than 310,000 people die of esophageal squamous cell carcinoma every year, and the disease is affected by many factors such as unhealthy diet and food contamination[1]. For the treatment of patients with esophageal squamous cell carcinoma[2], a combination of surgical resection, radiotherapy and chemotherapy is generally used clinically. However, the increased incidence and mortality of esophageal squamous cell carcinoma are affected by the disease condition and surgical plan[3]. According to clinical data[4], patients with early esophageal squamous cell carcinoma have good surgical outcomes, and the efficacy of surgery is poor for patients with intermediate and advanced esophageal squamous cell carcinoma. Therefore, patients with complete tumor eradication only account for one-fifth of the total number of patients undergoing esophageal cancer resection. The prognosis of patients with esophageal squamous cell carcinoma is poor, and the five-year survival rate is less than 20%. At present, the condition and postoperative prognosis of patients with esophageal squamous cell carcinoma are confirmed by monitoring changes

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of specific serum markers. However, in recent years, studies[5,6] have found that biological serum markers are unstable, which may cause certain experimental deviations in results. Therefore, screening for better tumor markers becomes a new hot spot for monitoring the development of esophageal squamous cell carcinoma.

MicroRNAs (miRNAs)[7,8] are a class of endogenous non-coding RNA with regulation function, which involved in cell proliferation, differentiation, and apoptosis all the time and are expressed in many tissues. Some miRNAs are considered to be tumor-specific diagnostic markers and new therapeutic targets. According to the expression of miRNAs in different tumors, they can be divided into oncogenes[9] and tumor suppressor genes[10]. However, the expression of the same miRNA in different tumors is different, and the effects may also be different. It has been reported that[11] miR-375 was lowly expressed in liver cancer, gastric cancer, pancreatic cancer, and other diseases, but it was highly expressed in canceration tissues of breast cancer patients. The expression level of miR-503 in tumor tissues such as liver cancer, uterine cancer, and renal cancer showed a downtrend, while the expression level in the renal cancer tissue showed an upward trend[12]. However, there are few reports on the expressions of miR-375 and miR-503 in esophageal carcinoma. This study focuses on the expressions of miR-503 and miR-375 in patients with esophageal cancer squamous cell carcinoma, and the analysis of the relationship between their expressions and clinicopathological parameters in patients with esophageal squamous cell carcinoma. The study also explores new clinical monitoring indicators for esophageal squamous cell carcinoma.

Patients and Methods

Specimen Collection

40 cases of cancer tissues and adjacent normal tissues in patients with esophageal squamous cell carcinoma were collected in the Department of Gastroenterology in The First Affiliated Hospital of Henan Polytechnic University from March 2017 to September 2017. There were 24 males and 16 females with an age range of 45 to 80 years. The average age was (50.09±9.45) years. The general clinical data of esophageal squamous cell carcinoma patients in this study are shown in Table I.

Exclusion and inclusion criteria were as follows. (1) Only patients admitted to The First Affiliated Hospital of Henan Polytechnic University were included. (2) Specimens were collected after surgery and confirmed as esophageal squamous cell carcinoma tissue or adjacent normal tissue by the Pathology Department. (3) Subjects who had received radiotherapy or chemotherapy, and subjects with a family history of genetic diseases, blood diseases, and history of drug use were excluded. Before collecting the specimens, all patients were informed in advance. They agreed to be involved in the study and signed the consent form. This study was approved by the Ethics Committee of the Second People's Hospital of Jiaozuo City.

Table I. General clinical data of esophageal squamous cell carcinoma patients in this study (n=40).

Factor		[n (%)]
Gender		
	Male	24 (60.00)
	Female	16 (40.00)
Age		
	≤50	18 (45.00)
	>50	22 (55.00)
Smoking situation		
	Yes	30 (75.00)
	No	10 (25.00)
Canceration site		
	Upper esophagus	14 (35.00)
	Central esophagus	10 (25.00)
	Lower esophagus	16 (40.00)
Tumor size (cm)		
	≤5	27 (67.50)
	>5	13 (32.50)
Degree of tumor differentiation		
	Mid-low	18 (45.00)
	Well	22 (55.00)
TNM staging		
	I-II	20 (50.00)
	III-IV	20 (50.00)
Lymph node metastasis		
	Yes	25 (62.50)
	No	15 (37.50)
Depth of invasion		
	Intramucosa	24 (60.00)
	Submucosa	16 (40.00)

Main Reagents and Instruments

MiRNA reverse transcription kit (MSK Biotechnology Co., Ltd., Wuhan, China), miRNA fluorescence quantification kit (MSK Biotechnology Co., Ltd., Wuhan, China), TRIzol RNA extraction kit (Jianglai Bio), ultraviolet spectrophotometer (Koromee Scientific Instrument Co., Ltd., Shanghai, China), quantitative real-time polymerase chain reaction (qRT-PCR) instrument

Table II. MiR-375, miR-503 and their internal reference primer sequences.

Groups	Forward primers	Reverse primers
miR-503	5'-TAGCAGCGGGAACAGTT-3'	5'-GTGCAGGGTCCGAGGT-3'
miR-375	5'-CGCGGTTTGTTCGTTCGGCTC-3'	5'-ATCCAGTGCAGGGTCCGAGG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

(Bio-Rad, Hercules, CA, USA), miRcute microRNA isolation equipment (Macy Instruments Co., Ltd., Shanghai, China). miR-375, miR-503 and their internal reference primers were synthesized by Beckman Culture. The primer sequences are shown in Table II.

Experimental Steps of Detecting MiR-503 and MiR-375

The fresh esophageal squamous cell carcinoma tissues and adjacent normal tissues after surgical resection in The First Affiliated Hospital of Henan Polytechnic University were immediately frozen in liquid nitrogen. 15 mg of frozen esophageal squamous cell carcinoma tissues and 15 mg of frozen adjacent normal tissues were fully ground. TRIzol RNA kit was used to extract total tissue RNA and ultraviolet spectrophotometer was used to measure RNA concentration. The reagent required for reverse transcription of mRNA was dissolved at room temperature, and then put on ice. 1 μL of random primers per 1 μg of total RNA was add. The final concentration of primers was 10 μM, and the total volume was not more than 13 μL. They were incubated at 65°C for 10 min, and immediately placed on ice. After complete cooling, the reverse transcription reaction was started. Reaction system: 1 μl of M-MLV, 1 μl of Olig(d T), 0.5 μl of RNA enzyme inhibitor, 1 μl of dNTPs, and RNAse free water made up to 15 μl. The mixture was incubated at 37°C for 60 min. 1 μl of cDNA was extracted at 85°C for 5 s to prepare a PCR reaction system: 2.5 μl of 10×PCR buffer, 1 μl of dNTPs, 1 μl of the forward and 1 μl of reverse primer, 0.25 μl of Taq DNA Polymerase, and ddH2O made up to 25 μl. The mixture was put at 94°C for 1 min. There were 30 cycles of 94°C for 30 s, 56°C for 30 s, and 70°C for 30 s. PCR was performed under the condition of 70°C for 5 min. The relative amount of target gene was calculated from the outcome parameters. The $2^{-\Delta\Delta Ct}$ method was used to quantify the target gene.

Statistical Analysis

Statistical analysis was performed with SPSS 17.0 software (IBM). All quantitative data were expressed as ±s. The *t*-test was used to compare the differences of miR-503 and miR-375 levels between esophageal cancer patients and healthy controls. The relationship between the expressions of miR-503 and miR-375 in patients with esophageal squamous cell carcinoma and clinical parameters was analyzed using the Chi-square test. When $p<0.05$, the difference was statistically significant.

Results

Expressions of MiR-503 and MiR-375 in Esophageal Squamous Cell Carcinoma Tissues and Adjacent Normal Tissues

The results of the experiment showed that the expressions of miR-503 in canceration tissues and adjacent normal tissues of esophageal squamous cell carcinoma patients were (6.83±2.14) and (2.45±1.13). The expression of miR-503 in esophageal squamous cell carcinoma tissues was significantly higher than that in adjacent normal tissues. The expression of miR-375 in canceration tissues and adjacent normal tissues of esophageal cancer patients were (3.75±1.06) and (7.45±1.13). The expression of miR-375 in esophageal squamous cell carcinoma tissues was significantly lower than that in adjacent normal tissues. The results are shown in Table III.

Table III. Expression of miR-503 and miR-375 in esophageal squamous cell carcinoma tissues and adjacent normal tissues.

Groups	Esophageal squamous cell carcinoma tissues (n=20)	Adjacent normal tissues (n=20)	*t*	*p*
miR-503	6.83±2.14	2.45±1.13	8.094	<0.001
miR-375	3.75±1.06	7.45±1.13	10.680	<0.001

Relationship Between Expression of miR-503 and Clinicopathologic Characteristics of Patients With Esophageal Squamous Cell Carcinoma in the Study Group

The relative expression of miR-503 in esophageal squamous cell carcinoma tissues was significantly correlated with the degree of pathological differentiation, TNM staging, invasion depth, and lymph node metastasis (p<0.001), but it had no significant correlation with gender and age (p>0.05). The results are shown in Table IV.

(1) In stage I-II and III-IV of the study group (clinical TNM staging), the relative expressions of miR-503 were (4.93±1.10) and (8.73±1.28). The relative expression of miR-503 in esophageal squamous cell carcinoma tissues in the stage III-IV was significantly higher than that in the stage I-II (p<0.001).

(2) In the study group, the well-differentiated and mid-low-differentiated relative expressions of miR-503 in the pathological sites were (4.83±2.32) and (8.83±2.57). The relative expression of miR-503 in the mid-low-differentiated group was significantly higher than that in the well-differentiated group (p <0.05).

(3) The relative expressions of miR-503 in patients with lymph node metastasis and patients without lymph node metastasis were (8.24±1.25) and (5.42±1.63). The relative expression of miR-503 in patients with no lymph node metastasis was significantly lower than that in patients with lymph node metastasis.

(4) The relative expressions of miR-503 in the esophageal intramucosal and submucosa of esophageal squamous cell carcinoma patients were (5.62±1.73) and (8.04±1.25). The relative expression of miR-503 in the intramucosal was significantly lower than in submucosal (p<0.05).

Correlation Between Relative Expression of MiR-375 and Clinicopathologic Characteristics of Esophageal Squamous Cell Carcinoma Patients in the Study Group

The relative expression of miR-375 in esophageal squamous cell carcinoma tissues was significantly correlated with the degree of pathological differentiation, TNM staging, invasion depth, and lymph node metastasis (p<0.001), but it had no significant correlation with gender and age (p>0.05). The results are shown in Table V.

(1) The relative expressions of miR-375 in stage I-II and III-IV were (5.71±1.16) and (2.04±1.05). The relative expression of miR-375 in esophageal squamous cell carcinoma tissues in the stage III-IV was significantly lower than that in the stage I-II (p<0.001).

(2) In the study group, the well-differentiated and mid-low-differentiated relative expressions of miR-375 in the pathological sites of patients were (6.24±1.02) and (1.26±0.97). The relative

Table IV. Correlation between relative expression of miR-503 and clinicopathologic characteristics of esophageal squamous cell carcinoma patients in study group (n=20).

Clinicopathologic characteristics	N.	MiR-503	*t*	*p*
Gender			0.206	0.839
Male	12	6.75±2.32		
Female	8	6.94±1.42		
Age			0.413	0.684
≤50	9	6.98±2.12		
>50	11	6.68±1.05		
TNM staging			7.120	<0.001
I-II	10	4.93±1.10		
III-IV	10	8.73±1.28		
Lymph node metastasis			4.064	<0.001
Yes	15	8.24±1.25		
No	5	5.42±1.63		
Degree of differentiation			3.621	0.002
Mid-low	8	8.83±2.57		
Well	12	4.83±2.32		
Depth of invasion			3.586	0.002
Intramucosa	10	5.62±1.73		
Submucosa	10	8.04±1.25		

Table V. Correlation between relative expression of miR-375 and clinicopathologic characteristics of esophageal squamous cell carcinoma patients in study group (n=20).

Clinicopathologic characteristics	N.	miR-375	*t*	*p*
Gender			0.058	0.955
Male	12	3.76±0.41		
Female	8	3.74±1.10		
Age			0.620	0.543
≤50	9	3.72±1.05		
>50	11	4.03±1.16		
TNM staging			7.417	<0.001
I-II	10	5.71±1.16		
III-IV	10	2.04±1.05		
Lymph node metastasis			3.618	0.002
Yes	15	2.24±1.75		
No	5	5.26±1.02		
Degree of differentiation			10.900	<0.001
Mid-low	8	1.26±0.97		
Well	12	6.24±1.02		
Depth of invasion			8.624	<0.001
Intramucosa	10	5.49±1.01		
Submucosa	10	2.01±0.78		

expression of miR-375 in the well-differentiated group was significantly higher than that in the mid-low-differentiated group (p<0.001).

(3) The relative expressions of miR-375 in patients with lymph node metastasis and patients without lymph node metastasis were (2.24±1.75) and (5.26±1.02). The relative expression of miR-375 in patients without lymph node metastasis was significantly higher than that in patients with lymph node metastasis.

(4) The relative expressions of miR-375 in the esophageal intramucosal and submucosa of esophageal squamous cell carcinoma patients were (5.49±1.01) and (2.01±0.78). The relative expression of miR-375 in intramucosal was significantly higher than that in submucosal (p<0.001).

Discussion

Due to its high incidence in the world, esophageal squamous cell carcinoma is defined as one of the six malignant digestive tract tumors[13]. The cause of esophageal squamous cell carcinoma is insidious and complicated. Early esophageal squamous cell carcinoma patients have no evident clinical symptoms, resulting in missed diagnosis and misdiagnosis. Most of the esophageal squamous cell carcinomas at diagnosis are in an advanced stage. It has a tremendous impact on the postoperative prognosis of patients. The conventional diagnosis is based on the pathological staining biopsy of patients[14]. This method has the risk of esophageal infection in patients, and some patients have a certain degree of conflict with the method. The study about serum markers that had been put into clinical practice[15] showed that the serum markers that we found at present were not sensitive to esophageal squamous cell carcinoma. They were of little clinical significance for esophageal squamous cell carcinoma to make rapid and corresponding expression changes with the incidence of esophageal cancer patients. Therefore, finding tumor markers with high sensitivity and accuracy for esophageal squamous cell carcinoma has become a research hotspot in the clinical stage of esophageal squamous cell carcinoma.

miRNAs have special structures, and they can be classified into tumor suppressor genes and oncogenes according to their functional properties. Compared with the instability of serum markers at this stage[16], miRNAs are more suitable as monitoring indicators for tumor diagnosis and tumor invasion and deterioration since it is stable in tumor tissue or serum. In this study, real-time fluorescence quantitative PCR was used to detect the expression of miR-503 and miR-375 in esophageal squamous cell carcinoma tissues and adjacent tissues of patients. Gene chip technology was used to detect different expressions of miR-503 in esophageal squamous cell carcinoma

tissues and adjacent tissues in the early study of Siravegna et al[17]. In comparison, PCR technology has rapid detection and lower cost. While, the gene chip technology is expensive and is rarely used in the diagnosis of diseases directly. It is not suitable for routine clinical diagnosis of patients with esophageal squamous cell carcinoma. The findings of Wu et al[18] showed that miR-503 was lowly expressed in healthy adjacent tissues, while it was highly expressed in esophageal squamous cell carcinoma tissues. The degree of differentiation was increased, which promoted the speed of invasion and deterioration of esophageal squamous cell carcinoma. This is consistent with the results of this work in which the expression of miR-503 in the tissues of patients with esophageal squamous cell carcinoma was significantly higher than that in adjacent patients. The expression of miR-375 was quite different from that of miR-503. In other words, the expression of miR-375 in adjacent normal tissues was significantly higher than that in esophageal squamous cell carcinoma tissues. This is consistent with Wu et al's[19] opinion of studying esophageal squamous cell carcinoma through semi-quantitative RT-PCR. When they increase the expression concentrations of miR-375 in esophageal squamous cell carcinoma tissues, they can inhibit the proliferation and differentiation of esophageal squamous cell carcinoma to some extent. Szczyrba et al[20] regarded miR-375 as a regulatory target of tumor gene, and the expression of miR-375 was appropriately increased or decreased through multiple tumor signal channels. When the miR-375 concentration was decreased, the degree of tumor differentiation was low. When the miR-375 concentration was increased, the degree of tumor differentiation became higher. Therefore, we concluded that miR-375 was indeed involved in the differentiation and reproduction of tumor cells as a target gene. This opinion is in agreement with the studies mentioned above, in which miR-503 and miR-375 are the oncogenes and tumor suppressor genes of esophageal squamous cell carcinoma, respectively.

However, there were still some limitations in this study, such as fewer patients included in the study. More tissues of patients with esophageal squamous cell carcinoma with different pathological characteristics should be collected for the study. Then, the clinical significance of the expressions of miR-503 and miR-375 in the canceration tissues should be analyzed. The study also did not evaluate the prognosis of recurrence and survival after esophageal squamous cell carcinoma operation. The regulation mechanism of miR-503 and miR-375 in patients with esophageal squamous cell carcinoma has not been deeply studied. However, according to the data of patients in the experimental group, we will follow-up them on a regular basis and analyze the results after the test, so as to improve the study.

Conclusions

We found that the expression of miR-503 in esophageal squamous cell carcinoma tissues was significantly higher than that in adjacent normal tissues. From the median expression of miR-503, miR-503 was highly expressed in patients with esophageal squamous cell carcinoma. The expression of miR-375 in esophageal squamous cell carcinoma tissues was significantly lower than that in adjacent normal tissues. From the median expression of miR-375, miR-375 was highly expressed in patients with esophageal squamous cell carcinoma. The relative expressions of miR-375 and miR-503 were significantly correlated with the degree of pathological differentiation, TNM staging, and lymph node metastasis, but there was no significant correlation with gender and age. Since miR-503 and miR-375 are oncogenes and tumor suppressor genes of esophageal squamous cell carcinoma, respectively, it was presumed that the growth and invasion of esophageal squamous cell carcinoma can be controlled by regulating the expressions of miR-375 and miR-503 in patients with esophageal squamous cell carcinoma. miR-375 and miR-503 could be used as clinical monitoring indicators of esophageal squamous cell carcinoma.

Conflict of Interests

The authors declare that they have no competing interests.

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