



Expression of MicroRNA-224 in Cholangiocarcinoma and Its Clinicopathological Significance

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Abstract

MicroRNAs (miRNAs) are widely involved in the regulation of various pathological and physiological processes. One of the miRNAs, namely miR-224 expression has been found aberrant in a number of human cancers; however, its expression and role in cholangiocarcinoma have not been studied. In the present study, we investigated the difference of miR-224 expression in cholangiocarcinoma and adjacent normal tissues and explored its clinicopathological significance. Methods: The expressions of miR-224 in 30 cases of cholangiocarcinoma and its adjacent normal tissues were analyzed by RT-qPCR. The relationship between miR-224 and clinicopathological data was analyzed by statistical analysis. Results: The expression levels of miR-224-5p in cholangiocarcinoma were significantly higher than those in adjacent normal tissues ($P < 0.0001$). The expression level of miR-224-5p in cholangiocarcinoma was correlated with the pathophysiological behavior of tumor. That is, the relative expression levels of miR-224-5p in patients with lymph node metastasis were significantly higher than those in patients without lymph node metastasis ($P < 0.05$); The relative expressions of miR-224-5p in stage III and IV patients were significantly higher than those in stage I and II ($P < 0.05$). Conclusion: MicroRNA-224 is a potential tumor promoter in cholangiocarcinoma, which may regulate the occurrence and development of cholangiocarcinoma and be used as a molecular marker for the diagnosis and treatment of cholangiocarcinoma.

Cholangiocarcinoma is a highly invasive malignant tumor derived from cholangiocyte with a high mortality rate in hepatobiliary surgery. In recent decades, its morbidity and mortality have been increasing year by year [1]. Due to the insidious onset, rapid progression, difficulty in diagnosis, insensitivity to conventional radiotherapy and chemotherapy, low resection rate, and high recurrence rate, most patients with cholangiocarcinoma were diagnosed at an advanced stage and had lost the opportunity for radical surgery [2]. Therefore, there is an urgent need to find a new molecular marker that can evaluate the progression of cholangiocarcinoma, the prognosis of patients and the possibility of targeted therapy to improve the quality of life or postoperative survival rate.

MicroRNA (miRNA) is a non-coding short RNA with a size of about 21-25 nucleotides. It is widely involved in the regulation of various pathological and physiological processes, plays an important role in cell proliferation, apoptosis, differentiation, and is closely related to the occurrence and progression of tumors [3]. In recent years, a number of studies have shown that a variety of microRNAs are expressed abnormally in cholangiocarcinoma and act as oncogenes or anti-oncogenes [4]. As a subtype of microRNA, miR-224 has been found to have abnormal expression in a variety of malignant tumors and involved in the occurrence or progression of tumors [5,6]. However, there is no report about its expression and its clinical pathological significance in cholangiocarcinoma. Therefore, this study explored the differential expression of miR-224 in cholangiocarcinoma tissue and adjacent tissues and its clinicopathological significance, in order to provide new ideas for the prognosis of cholangiocarcinoma, and new targets for the precision medical treatment.

Materials and Methods

Patients

A total of 30 patients with distal bile duct cancer who underwent surgical treatment from September 2015 to October 2017 in the Department of Hepatobiliary and Pancreatic Surgery of Yantai Yuhuangding Hospital affiliated to Medical College of Qingdao University. The age distribution of patients was 58-73 years old, with an average age of 65.30 ± 4.324 years, and the ratio of male to female 3:2. None of the selected patients had received previous radiotherapy or chemotherapy.

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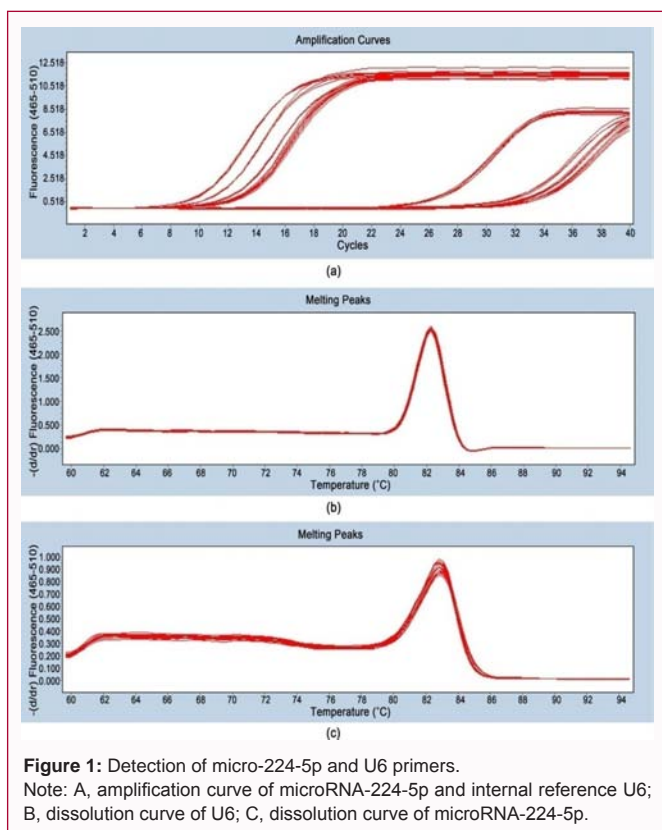
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All of them and their families had signed informed consent before surgery and agreed to use their postoperative tissue specimens for this study which was approved by the Hospital Medical Ethics Committee.

General information

All patients were definitively diagnosed finally by paraffin pathological Examination with complete clinical and pathological data. Among the patients, the postoperative pathology showed that there were 10 cases of moderately or poorly differentiated and 20 cases of well-differentiated, including 16 cases with positive labeled lymph nodes and 14 cases with negative.

Pathological staging and surgical variables

All patients were staged according to the 8th edition of the distal cholangiocarcinoma staging system proposed by the American Joint Committee on Cancer (AJCC): stage 0 is $TisN_0M_0$, stage I is $T_1N_0M_0$, stage II is $T_1N_1M_0$, $T_2N_0M_0$, $T_2N_1M_0$, $T_3N_{0-1}M_0$, stage III is $T_{1-3}N_2M_0$, $T_4N_XM_0$, stage IV is $T_XN_XM_1$ (Tis indicates carcinoma in situ. T_1 refers to the depth of tumor invasion is less than 5mm. T_2 and T_3 respectively indicate that the invasion depth is 5-12 mm and greater than 12 mm. T_4 indicates that the tumor invades celiac artery trunk, superior mesenteric artery, and/or common hepatic artery; N_0 indicates that no regional lymph nodes were found to metastasize., N_1 and N_2 refer to 1-3, greater than or equal to 4 regional lymph node metastases, respectively; M_0 means no distant metastasis, M_1 means distant metastasis).

Among the 30 patients, there were 21 patients in stage I and II, and 9 patients in stage III and IV. Among them, 23 patients underwent conventional pancreas to duodenectomy, a patient underwent pylorus-preserved pancreas to duodenectomy, 2 patients underwent biliary-jejunostomy, 3 patients underwent palliative tumor local excision plus biliary enter stomy, a patient underwent laparotomy and

T-tube drainage.

Experimental reagents or Chemicals

Trizol solution was obtained from Shanghai Pufei Biotech, reverse transcription primers and microRNA PCR primers were from Guangzhou Ruibo Biotech, and reverse transcriptase kit was from Promega Company of the United States.

Main instruments

F6/10 ultra-speed homogenizer form FLUKO Company, Nanodrop2000/2000C spectrophotometer from Thermo Corporation, LightCycler480 Real time PCR instrument form Swiss Roche.

Total RNA extraction

The tissue samples to be ground were taken out and cut to a size of about 3mm × 3mm × 3mm on dry ice with a sterile blade. Place the samples in EP tubes containing 1 mL of Trizol Lysis. After grinding, centrifugation was performed for 3 min. The supernatant was pipetted into new EP tubes. 200μL of chloroform was added to each tube, and the EP tubes were inverted by hand for 15s, and left standing at room temperature for 10 min. After 10 min, the mixture was centrifuged for 15 min, the supernatant was removed to new EP tubes, equal volume of pre-cooled isopropanol was added, and the mixture was stand at 4°C for 10 min. The supernatant was removed after centrifugation. 1mL 75% ethanol was used to wash the precipitate, the mixture was centrifuged for 5min, after discarding most of the supernatant, and the mixture was centrifuged again for 5min. The precipitation was dried at room temperature after removing the supernatant. When the RNA precipitation is substantially transparent, RNase-free water was added until is completely dissolved, the concentration and quality of the extracted RNA were determined by spectrophotometer. The extracted total RNA was stored at -80°C.

Reverse transcription to obtain cDNA

200 μL of RNase-free water was added per 1 nmol of the primer, vortexed and fully dissolved, and then centrifuged instantaneously to prepare the primer storage solution which having a final concentration of 5 μm. 1 μL of 5 μm RT primer storage solution and add 79 μL of RNase-free water were added to prepare 62.5 nM RT primer working solution. PCR primers were used at a concentration of 5 μm. Then, 2 μL of reverse transcription primer, 2.0 μg of total RNA and RNase-Free water were added into the PCR tubule to 11 μL. After mixing and centrifuging, the mixture was placed at 70°C for 10 min. Then the mixture was placed in an ice-water mixture immediately to anneal the reverse transcription primer and template. The above mixture was mixed evenly in an ice bath to prepare a reaction system of 25μL. The above system was reacted in a 42°C water bath for 1 h after centrifuging briefly. Then the RT enzyme was inactivated in a water bath at 70°C for 10 min, and the obtained RT product-cDNA was stored at -20°C.

RT-qPCR detection

The reaction system (12 μL systems) was configured in the following proportion: 6.0 μL of SYBR premix ex taq, 0.5 μL of upstream primer, 0.5 μL of downstream primer, 1.0 μL of template, 4.0 μL of RNase-Free water. In addition, RNA PCR reaction system(12 μL) included 6.0μL of SYBR premix ex taq, 0.3 μL of primer mix, 0.6 μL of template, 5.1μL of RNase-Free water. Performing Real-Time PCR and making melting curves. The primer sequences were as follows:

MiR-224-5p RT Primer Sequence:

5'-CTCAACTGGTGTCTGGAGTCCGGCAATT

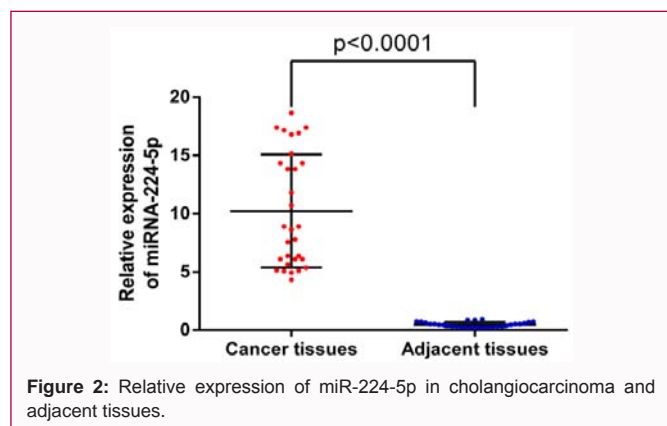


Figure 2: Relative expression of miR-224-5p in cholangiocarcinoma and adjacent tissues.

cholangiocarcinoma tissues were constructed (Figure 1A). The dissolution curves of miR-224-5P and U6 (Figure 1B,1C) are all single-peak which suggested that the products of miR-224-5P and U6 are both single.

Expression of miR-224-5p in cholangiocarcinoma tissues and adjacent tissues. The gene expression levels of miR-224 were significantly higher in the human cholangiocarcinoma samples (10.23 ± 0.8861) compared with the adjacent non-tumorous tissue samples (0.4734 ± 0.04018), as determined by RT-qPCR assay. $P < 0.0001$, suggesting that the difference between the two is statistically significant (Figure 2), (expanding $2^{-\Delta Ct}$ by 10^6 times for drawing).

The relationship between the expression of miR-224-5p and the clinicopathological features in patients with cholangiocarcinoma. According to RT-qPCR, the relationship between the expression of miR-224-5p and the clinicopathological features (age, sex, differentiation, lymph node metastasis, pathological stage) was analyzed. There was a significant correlation between the expression level of miR-224-5p and lymph node metastasis in patients. The relative expression of miR-224-5p in cancer tissues with lymph node metastasis was significantly higher than that without lymph node metastasis ($P < 0.05$). In addition, the expression level of miR-224-5p was found to be closely correlated with the pathological stage of patients with cholangiocarcinoma. The relative expression level of miR-224-5p in stage III and stage IV patients was significantly higher than that in stages I and II ($P < 0.05$). There was no significant correlation between the relative expression of miR-224-5p and clinicopathological factors such as age, sex and tumor differentiation ($P > 0.05$). (Table 1), (expanding $2^{-\Delta Ct}$ by 10^6 times for data analysis).

CAGTTGAGAACGGAAAC-3',

Upstream primer sequence: 5'-ACACTCCAGCTGGGCAAGTCACTAGTGTTCCGTT-3',

Downstream primer sequence: 5'-TGGTGTCGTGGAGTCG-3';

U6:Upstream primer sequence:5'-CTCGCTTCGGCAGCAC-3',

Downstream primer sequence: 5'-AACGCTTCACGAATTTGCGT-3'.

U6 was used as an internal reference to normalize the miRNA levels. The fold changes in gene expression of miRNA-224 were calculated using the Livak $2^{-\Delta Ct}$ method, where Ct is the crossing threshold value and $2^{-\Delta Ct}$ is the fold change in gene expression relative to a reference gene.

Statistical method

All data are presented as mean \pm standard deviation. Statistical analyses were performed using Graph Pad Prism software, version 6. Differences between the groups were analyzed by Chi-Square test. $P < 0.05$ was considered statistically significant.

Result

Detection of miR-224-5p and internal reference U6 primers. The amplification curves of miR-224-5P and U6 in some

Discussion

In recent years, the research of microRNAs in the field of oncology has become more and more extensive. MiRNAs bind to the 3' end of target mRNA by base-pairing principle and participate in post-transcriptional gene regulation. Its expression errors are closely related to the pathogenesis of many malignant tumors, and have a wide range of biological effects [7]. There are characteristic microRNA expression profiles in tumor tissues which are different from normal tissues, and the expression changes of these microRNAs

Table 1: Comparison of miR-224-5p Expression Levels in Cancerous Tissues of Patients with Cholangiocarcinoma with Different Clinicopathological Features.

Clinical pathological features	Number of cases n	Relative expression level of miR-224-5p	P value
age			
≤60岁	17	10.51 \pm 1.175	0.4237
>60岁	13	9.127 \pm 1.253	
gender			
male	18	10.44 \pm 1.092	0.7794
Female	12	9.918 \pm 1.547	
Differentiation			
Medium and poorly	10	10.97 \pm 1.902	0.5624
Highly	20	9.858 \pm 0.9587	
Lymph node metastasis			
+	16	14.05 \pm 0.8406	<0.0001
-	14	5.862 \pm 0.2627	
TNM staging			
Phase I, Phase II	21	8.520 \pm 0.9665	0.0017
Phase III, Phase IV	9	14.22 \pm 1.094	

can all be determined in the laboratory. Therefore, microRNAs may become a new molecular marker for tumor diagnosis or disease progression and a molecular target for judging tumor prognosis, which will benefit the diagnosis and treatment of tumor [8].

Studies have shown that many miRNAs are found to be abnormally expressed in tumors, in which miR-224 has received much attention. As a member of the microRNA family, miR-224 has been found to have significant abnormal expression and function as oncogenes or anti-oncogenes in a variety of human tumors such as liver cancer [9], gastric cancer [10], colorectal cancer [11], pancreatic cancer [12], diffuse large B-cell lymphoma [13], prostate cancer [14], breast cancer [15], cervical cancer [16], non-small cell lung cancer [17] and so on. Determinating its expression changes may become a new and important means for early diagnosis and prognosis of malignant tumors.

In this study, we selected the surgically resected cholangiocarcinoma tissue and adjacent non-tumorous tissues and detected the relative expression levels of miR-224 by RT-qPCR. The results showed that the expression level of miR-224 was significantly up-regulated in cancer tissues compared with adjacent tissues, suggesting that miR-224 plays a role as a potential oncogene in cholangiocarcinoma.

Combined with the clinicopathological data of patients, the relationship between the expression of miR-224 and clinicopathological features of patients was analyzed by statistical methods. The results showed that the expression level of miR-224 was not correlated with the age, gender and tumor differentiation of patients. However, its level in patients with lymph node metastasis was significantly higher than that without lymph node metastasis. And the later the pathological stage in patients, the higher the expression level of miR-224. The experimental results suggest that high expression of miR-224 may have a negative effect on the prognosis of patients with cholangiocarcinoma. Therefore, it is speculated that miR-224 may regulate the occurrence, progression, migration and invasion of tumors, which may provide a theoretical basis and reference for the prognosis of patients and a new target for precise treatment of cholangiocarcinoma.

Of course, this study also has limitations such as fewer patient cases and lack of follow-up. In addition, the precise value of miR-224 in the prognosis of patients with cholangiocarcinoma cannot be further evaluated.

At present, the research on microRNA-224 is not thorough enough. This study laid the foundation for the study of the regulation mechanism and signaling pathway of microRNA-224 and cholangiocarcinoma. The specific target genes involved in the regulation of microRNA-224 and the detailed molecular mechanisms involved in the biological function of cholangiocarcinoma require further improvement or exploration.

Conclusion

The above results reveal that the expression levels of miR-224-5p in cholangiocarcinoma were significantly higher than those in adjacent normal tissues. The expression level of miR-224-5p in cholangiocarcinoma was correlated with the pathophysiological behavior of tumor. MiR-224 may play a role in the process of occurrence, progression, invasion and migration of cholangiocarcinoma. These findings suggest that miR-224 is being assessed as a potential new

target in the treatment and a novel biomarker for diagnosis and prognosis prediction of cholangiocarcinoma.

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