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Expression and prognostic significance of *miR-375* and *miR-221* in liver cancer

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Abstract. The purpose of this study was to investigate the expression of *miR-375* and *miR-221* in liver cancer, and examine the correlations with pathological parameters and prognosis. We collected tumors and tumor-adjacent normal tissue from 70 patients with liver cancer admitted to the Department of General Surgery of Zhejiang Hospital. The expression of *miR-375* by RT-qPCR was significantly lower in liver cancer tissues than that in the tumor-adjacent normal tissues, and the low expression was correlated with the lymphatic metastasis and TNM stage. By contrast, the expression of *miR-221* was significantly higher in liver cancer than that in the tumor-adjacent tissues, and the high expression was correlated with the lymphatic metastasis and TNM stage. The overall 5-year survival rate of patients was 12.9% (9/70). Single-factor survival analysis revealed that *miR-375* and *miR-221* were the factors affecting the overall survival rate of liver cancer (P<0.05) and multivariate survival analysis by Cox proportional hazards model showed that *miR-375* and *miR-221* were the independent factors affecting the survival time of patients with liver cancer. Low expression of *miR-375* and high expression of *miR-221* are closely correlated with the occurrence and development of liver cancer, especially lymphatic metastasis and TNM stage. Thus, *miR-375* and *miR-221* can serve as reference biomarkers for guiding the treatment of liver cancer and for estimating prognosis.

Introduction

Liver cancer is the most common primary malignant tumor in the liver and ranks 5th among malignant tumors and 3rd in the mortality rate (1). Various factors can cause liver cancer, including excessive alcohol consumption, viral hepatitis, and chronic liver inflammation caused by non-alcoholic hepatic steatosis (2-4). Chronic inflammation in the liver can lead to recurrent injuries and proliferation of hepatic cells, further activating oncogenes as well as liver cancer-associated signal pathways and deactivating the tumor suppressor genes (5,6).

MicroRNAs (miRNAs) are non-coding single-stranded small RNAs 18-25 nucleotides in length. miRNAs can exert gene regulatory functions at the translational level, and play key roles in development, proliferation, differentiation, apoptosis, and carcinogenesis (7). Over 1,000 miRNAs are encoded in the human genome, which have the ability to regulate the expression of 60% of protein-encoding genes (8). miRNAs can act on the 3'-untranslated region (UTR) of target mRNAs, resulting in the abnormal downregulation of target genes (9).

Reports have described the abnormal expressions of multiple miRNAs in hepatic cells and peripheral serum of liver cancer patients (10). Among these miRNAs, *miR-375*, which is located on the genetic regions of cryba2 and Ccdc108 on 2q35, can inhibit the transcription and translation of the oncogene astrocyte elevated gene-1 (AEG-1), exerting the anti-carcinoma function in liver cancer cells (11). *miR-221*, which is located on the P11.3 region of the X chromosome, is closely correlated with tumors because the expression of *miR-221* is significantly elevated in many malignant tumor cells (12). *miR-221* can regulate the cell cycle, differentiation and apoptosis, as well as participate in the occurrence and development of tumor by adjusting the expression of p27, p53 upregulated modulator of apoptosis (PUMA), Bcl-2 modifying factor (BMF), c-kit, and DNA-damage-inducible transcript 4 protein (DDIT4) (13).

In a previous study, the differences in miRNA expression in liver cancer cells and normal liver cells were compared, and the results confirmed that upregulated expression of *miR-221* and downregulated expression of *miR-375* are involved in the occurrence and development of liver cancer by regulating the cell proliferation, cycle, apoptosis, migration and invasion, as well as clone formation (14,15). However, to the best of our knowledge, currently, there are no reports on the expressions of *miR-375* and *miR-221* directly in liver cancer tissues along with the pathological parameters and prognosis of liver cancer.

In the present study, we used quantitative RT-(q)PCR to determine the expression levels of *miR-375* and *miR-221* in liver tumor and tumor-adjacent normal tissue. We analyzed the correlation of *miR-375* and *miR-221* expressionwith clinicopathological parameters and prognosis of liver cancer in combination with clinical data.

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Table I. Primer sequences used for qRT-PCR.

Gene	Primer sequences
miR-375	F: 5'-GGCTCTAGAGGGGACGAAGC-3'
	R: 5'-GGCAAGCTTTTTCCACACCTCAGCCTTG-3'
miR-221	F: 5'-CAAGGAATCATGTATGCTGTAG-3'
	R: 5'-AGGATGACATTACACCTTATCTC-3'
U6	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'
	R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'

Materials and methods

Human tumor tissue. We collected frozen tumors and tumor-adjacent normal tissue from 70 patients with liver cancer who were admitted to the Department of General Surgery of Zhejiang Hospital for treatment between January 2008 and December 2010. These tumors were diagnosed as liver cancer through pathological examinations. All 70 patients received surgical treatment for the first time and had no chemotherapy history. This cohort had 38 males and 32 females, and the age range was 25-78 years with a median age of 45 years. The acquisition of samples was approved by the Clinical Ethics Committee of Zhejiang Hospital and all enrolled patients or their family signed the written informed consent. The 70 patients received postoperative follow-up for 5 years and the follow-up rate reached 100%. Recording of the survival time started from the 1st day after operation, and ended on the date of death of the patient or the last day of follow-up. Statistical analysis was carried out with the month as the unit.

Quantitative RT-PCR. Two samples (~50 mg each) were used from each frozen tumor and tumor-adjacent normal tissue. Total RNA was extracted according to the instructions of the RNA extraction kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Concentration and purification of total RNA were detected using ultraviolet-visible spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan), and extracted RNA was classified as qualified when the ratio of A260/A280 was between 1.8 and 2.0. Then, cDNA was generated by reverse transcription according to the instructions in the reverse-transcription kit. With the cDNA as template, the expression of *miR-375* and *miR-221* was detected according to the method given in the instructions of the RT-PCR kit with *U6* RNA as internal reference. Synthesis of primer, reverse-transcription kit, and real-time fluorescent quantitative PCR kit (Takara Bio, Dalian, China). Primer sequences of *miR-375*, *miR-221* and *U6* are shown in Table I, and reaction conditions were: 95˚C for 10 min, 95˚C for 15 sec, 60˚C for 1 min; 40 cycles of amplification. Ct value was obtained, and the relative expression was calculated using the method of $2^{-\Delta\Delta Ct}$. Calculation was carried out according to the formula: ΔCt (target gene) = Ct (target gene) - Ct (reference gene).

Statistical analysis. Data processing was performed using SPSS 17.0 software (International Business Machines Corporation, Armonk, NY, USA). Measurement data are presented as mean ± standard deviation and t-test was used for intergroup comparison. For countable data, Chi-square test was used for intergroup comparison. Single-factor survival analysis was carried out using the Kaplan-Meier method, the log-rank method was used to identify the difference in survival curve, and multivariate survival analysis was carried out using the Cox proportional hazards model. $P \leq 0.05$ indicates that the difference has statistical significance.

Results

Expression of miR-375 and miR-221 by RT-qPCR. We extracted RNA from the liver tumor and normal adjacent tissues and examined the expression of *miR-375* and *miR-221*. Compared with the tumor-adjacent normal tissues, the expression of *miR-375* was significantly decreased in liver cancer (Fig. 1). By contrast, the expression of *miR-221* was significantly elevated in liver cancer (Fig. 1).

Correlation of miR-375 and miR-221 expression with pathological parameters of liver cancer. Based on the expression levels of *miR-375* and *miR-221* in the 70 liver cancer tissues, the samples were divided into the *miR-375* high-expression (≥2.135), *miR-375* low-expression (<2.135), *miR-221* high-expression (≥1.795), and *miR-221* low-expression (<1.795) groups. No statistically significant differences were identified in the comparisons of factors such as age, sex and smoking history between the two groups (Table II). According to the clinical materials, we analyzed the correlations between the expression of *miR-375* and *miR-221* and the pathological parameters. Chi-square test showed that the abnormal expression of *miR-375* and *miR-221* correlated with the occurrence of metastasis and TNM staging (tumor-node-metastasis), but was not correlated with sex, age and tumor size (Table II).

Survival of patients with liver cancer. The 70 liver cancer patients were followed-up for 5 years. At that point, there were 9 patients alive and 61 patients dead due to further progression of liver cancer. The overall 5-year survival rate was 12.9% (9/70) and the mortality rate was 87.1% (61/70).

Single-factor analysis of the patient prognosis. We next analyzed the Kaplan-Meier survival curves of 70 liver cancer patients with expression of *miR-375* and *miR-221* (Fig. 2). Patients with high *miR-375* expression and low *miR-221* expression had a better survival prognosis. Differences in the curves of overall survival rate were analyzed using the log-rank test (Table III). According to the single-factor survival analysis, statistical significance was identified in the effects of *miR-375* and *miR-221* on the overall survival rate of liver cancer (Table III).

Multivariate analysis of expression of miR-375 and miR-221 with survival. Correlation of *miR-375* and *miR-221* expression with overall survival rate of liver cancer patients were analyzed via multivariate survival analysis by Cox proportional hazards model. Expression levels were all substituted into the formula, in which the substitution level was set as 0.05 and the deletion level was set as 0.1. In the factors affecting the survival of patients with liver cancer, the regression coefficient of *miR-375* was negative, indicating a relatively long survival

Table II. Correlation of *miR-375* and *miR-221* expression with pathological parameters.

		miR-375			*miR-221*		
Clinical data	Case	High expression (case, %)	Low expression (case, %)	P-value	High expression (case, %)	Low expression (case, %)	P-value
Male	38	12 (31.6)	26 (68.4)	>0.05	23 (60.5)	15 (39.5)	>0.05
Female	32	11 (34.4)	21 (65.6)		22 (68.8)	10 (31.2)	
Age ≥50 years	41	14 (34.1)	27 (65.9)	>0.05	25 (60.9)	16 (39.1)	>0.05
Age <50 years	29	9 (31.0)	20 (69.0)		20 (68.9)	9 (31.1)	
Tumor size ≥5 cm	44	15 (34.1)	29 (63.6)	>0.05	26 (59.1)	18 (40.9)	>0.05
Tumor size <5 cm	26	8 (30.8)	18 (69.2)		19 (73.1)	7 (26.9)	
Invasion and metastasis	48	12 (25.0)	36 (75.0)	<0.05	37 (77.1)	11 (22.9)	<0.05
No invasion or metastasis	22	11 (50.0)	11 (50.0)		8 (36.4)	14 (63.6)	
Stage (I-II)	42	18 (42.9)	24 (57.1)	<0.05	23 (54.8)	19 (45.2)	<0.05
Stage (III-IV)	28	5 (17.9)	23 (82.1)		22 (78.6)	6 (21.4)	

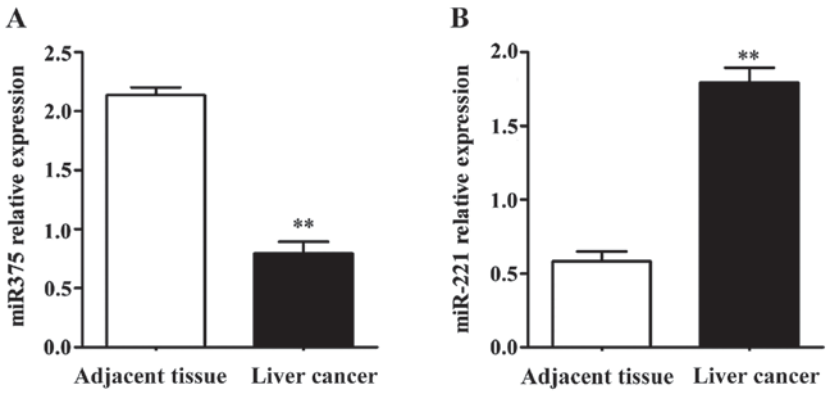

Figure 1. Expression of *miR-375* and *miR-221* in specimens by RT-qPCR. Compared with the tumor-adjacent normal tissues, the expression of *miR-375* was significantly decreased in the liver cancer tissues, but the expression of *miR-221* was obviously elevated. Expression of (A) *miR-375* and (B) *miR-221* in tissue sample. Compared with the tumor-adjacent normal tissues, **P<0.01.

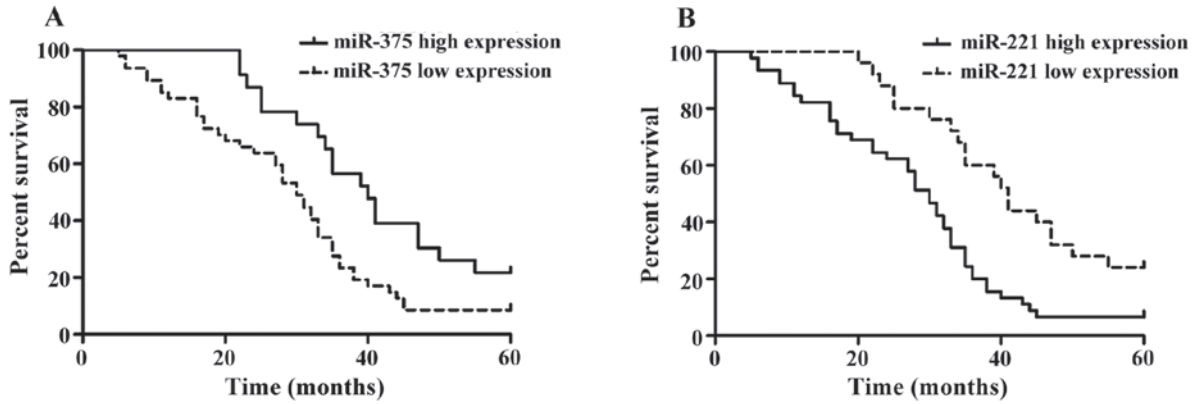

Figure 2. Expression of *miR-375* and *miR-221* and Kaplan-Meier survival curves of patients with liver cancer. (A) Kaplan-Meier survival curves of liver cancer patients with a high and low expression of *miR-375*. (B) Kaplan-Meier survival curves of liver cancer patients with a high and low expression of *miR-221*.

time of liver cancer patients with a high expression of *miR-375* (Table IV). However, the regression coefficient of *miR-221* was positive, indicating a relatively short survival time of liver cancer patients with a high expression of *miR-221* (Table IV).

Discussion

As a common malignant tumor, liver cancer is characterized by a relatively high mortality rate due to the lack of effective early diagnosis and treatments (16). Recent studies on the

Table III. Single-factor analysis of *miR-375* and *miR-221* expression with overall survival.

Group	Case	5-year survival cases	5-year survival rate (%)	Wald (log-rank)	P-value
miR-375				7.033	<0.05
High expression	23	5	21.7%		
Low expression	47	4	8.5%		
miR-221				11.23	<0.05
High expression	45	3	6.7%		
Low expression	25	6	24%		

Table IV. Multivariate survival analysis of *miR-375* and *miR-221* expression with overall survival rate by Cox proportional hazards model.

Variate	B	SE	Wald	P-value	RR (95% CI)
miR-375	-1.010	0.827	4.755	0.014	0.153 (0.035-0.837)
miR-221	0.748	0.203	4.870	0.012	1.743 (1.004-3.772)

effect and action mechanism of miRNAs have identified key roles in the occurrence and progression of tumors, changes in the microenvironment of tumor and tumor-associated domestication of immune cells. Thus, miRNAs can be used, not only in the diagnosis and prognosis of tumors, but also as a target in the treatment of the tumor (17).

At present, there are several studies on the correlation between *miR-375* expression and tumors. As biomarkers of liver cancer, miR-25, *miR-375* and let-7f can distinguish liver cancer cells, particularly when *miR-375* was used alone to detect liver cancer cells, with 96% specificity and 100% sensitivity (18). An *in vitro* study showed that *miR-375* expression is significantly decreased in a liver cancer cell strain. High expression of *miR-375* can suppress the proliferation and migration of tumor cells and induce cell cycle arrest and apoptosis (14). Furthermore, it was shown that *miR-375* can weaken the migration and invasion capability of liver cancer cells via inhibiting the expression of AEG-1 (11). Recent studies have reported a high expression of *miR-221* in liver cancer cells, which can promote the growth and proliferation of liver cancer cells by regulating cell differentiation (19). *miR-221* suppresses the activities of p27 and p57 by acting on cell cycle-dependent kinase, further increasing the number of cells in the S phase of cell cycle and facilitating the progression of the cell cycle in liver cancer cells (20). Additionally, *miR-221* can also interfere with the mTOR (mammalian target of rapamycin) signaling pathway via suppressing the DDIT4, thus inducing the occurrence and progression of tumors (21).

To investigate the expression of *miR-375* and *miR-221* in liver cancer, we found that *miR-375* expression was elevated in liver cancer, but *miR-221* expression was decreased. Further studies in combination with the clinicopathological characteristics of patients showed that the expression of *miR-375* and *miR-221* were correlated with the metastasis and TNM staging of patients, but not correlated with the sex, age and tumor size. We also found that *miR-375* and *miR-221* significantly affected the overall survival time of liver cancer patients. Analysis by multivariate Cox proportional hazards model showed that *miR-375* and *miR-221* affected the survival time of liver cancer patients and they were the independent indexes for estimating the prognosis of liver cancer patients. High expression of *miR-375* can serve as an indicator for excellent prognosis, while a high expression of *miR-221* is an indicator for poor prognosis.

Many studies have shown that *miR-375* and *miR-221* are closely correlated with multiple kinds of tumors. Expression of *miR-375* in non-small cell lung cancer is positively correlated with its prognosis (22). In esophageal squamous carcinoma can exert a tumor-suppressing effect by inhibiting the expression of insulin-like growth factor 1 receptor (23). Expression of *miR-221* is significantly upregulated in liver cancer cells and can promote the growth and metastasis of tumor cells through p27 and c-kit (21). *In vitro* studies showed that the upregulated expression of *miR-221* and downregulated expression of *miR-375* are identified in liver cancer cells (14,15). Mechanistic studies confirmed that these miRs can regulate proliferation, cell cycle, apoptosis and migration (14,15).

In conclusion, a low expression of *miR-375* and high expression of *miR-221* are closely correlated with the occurrence and development of liver cancer, particularly the lymphatic metastasis and TNM staging. Thus, *miR-375* and *miR-221* can serve as reference biomarkers for guiding the treatment of liver cancer and estimating the prognosis.

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