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 overall survival rate 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 Ccld108 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 11q13 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 expression with clinicopathological parameters and prognosis of liver cancer in combination with clinical data.
Table I. Primer sequences used for qRT-PCR.

| Gene   | Primer sequences                               |
|--------|-----------------------------------------------|
| miR-375 F: 5'-GGCTCTAGGGGACGAAAGC-3' | R: 5'-GGCAAGCTTTTCCACACCTAGGGCTTG-3' |
| miR-221 F: 5'-CAAGGAAATCATGTATGCTTAG-3' | R: 5'-AGGATGACATTACCTTATCTC-3' |
| U6     F: 5'-GCTTTCGAGCAGCATATATCAAAT-3' | R: 5'-CGCTTCACAGAATTTGGGTGTCAT-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^-ΔCt. 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≤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.
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     |      |                       |                          |                 |         |
| High expression | 23  | 5                     | 21.7%                    | 7.033           | <0.05   |
| Low expression | 47  | 4                     | 8.5%                     |                 |         |
| miR-221     |      |                       |                          |                 |         |
| High expression | 45  | 3                     | 6.7%                     | 11.23           | <0.05   |
| 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) |

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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