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Clinical significance of microRNA-155 expression in hepatocellular carcinoma

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Abstract. The present study aimed to evaluate the expression of microRNA-155 (miR-155) in hepatocellular carcinoma (HCC) and adjacent normal tissues, and assess its correlation with clinicopathological characteristics of this tumor type. miR-155 expression was detected in 40 HCC tissue samples and 40 samples of adjacent tumor-free tissue using fluorescent reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The association between miR-155 expression, clinicopathological features and 1-year relapse-free survival (RFS) in HCC and adjacent normal tissue samples was analyzed. RT-qPCR results revealed that, in 25 cases (62.5%), miR-155 expression levels were significantly increased in HCC tissues compared with the expression levels observed in pericarcinomatous tissues (P<0.05). miR-155 expression was observed to be significantly correlated with vessel invasion, Edmonson classification and clinical stage (P<0.05). However, miR-155 expression was not significantly correlated with gender, age, tumor size, tumor number, hepatitis B virus DNA copy number, cirrhosis or concentration of α-fetoprotein (P>0.05). A positive correlation was observed between late TNM classification of malignant tumor stage and 1-year RFS (P<0.05). Patients exhibiting high miR-155 expression levels were observed to exhibit a lower 1-year RFS than that of patients with reduced expression of miR-155 (48 vs. 73.3%), however this difference was not statistically significant (P=0.105). Additionally, correlations were observed between miR-155 expression and reduced differentiation, increased invasiveness and late stages of HCC. The current results demonstrated that miR-155 may be involved in the tumorigenesis of HCC and may be associated with clinical characteristics of HCC patients. Additional studies are required to clarify the mechanism of miR-155.

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Introduction

Hepatocellular carcinoma (HCC) is a major malignant tumor, which currently represents a significant threat to human health. Worldwide, HCC is the fifth most common malignant tumor observed in males, and the seventh most common amongst females. Furthermore, HCC is ranked second and sixth in terms of mortality for males and females, respectively (1). Approximately 50% of all novel HCC cases were diagnosed in China in 2008 (2). In China, liver cancer is the second largest cause of mortality in terms of malignant tumors. In 2008, only 30% of patients in Western countries and >10% of patients in Asia were eligible for curative therapies, including surgery orthotopic liver transplant or local ablation. Palliative treatments include transcatheter arterial chemoembolization and sorafenib (3). However, the majority of HCC patients are diagnosed at a late stage, due to the lack of highly specific and sensitive methods for the diagnosis of HCC in its early stages (4). Thus, ~80% of patients with HCC in China are diagnosed at a late stage, and the situation with regard to the diagnosis and treatment of liver cancer remains a significant issue (5). Previous studies have investigated the mechanisms underlying the occurrence and development of HCC; however, as yet, these mechanisms remain to be fully elucidated (6,7). Therefore, investigation of the pathogenesis of HCC, as well as exploration of novel diagnostic markers and therapeutic targets, have important significance for improving the diagnosis and treatment of HCC.

The first microRNA (miRNA) was identified in *Caenorhabditis. elegans* in 1993. miRNAs are small, single stranded non-coding RNAs of ~18-25 nt in length (8). miRNAs combine with the 3'-untranslated region of target messenger RNA (mRNA), resulting in translational repression or mRNA degradation. miRNAs are involved in various physiological and pathological processes, including cell proliferation, differentiation, growth, development and tumorigenesis (9). microRNA-155 (miR-155) is located on the third exon of the B-cell integration cluster (BIC) gene of human chromosome 21 (10). BIC does not contain an open reading frame and its overexpression may promote the abnormal

proliferation of cells. Experimental evidence has indicated that miR-155 is overexpressed in a number of types of neoplastic disease (11-13). miR-155 has a significant role in carcinogenesis, primarily as an oncogene (14).

The present study identified that miRNA-155 has a number of associations with the clinicopathological parameters of tumors. Additionally, miR-155 has an effect on the assessment of disease severity and estimate of prognosis of tumors. A study revealed that increased expression of miR-155 was associated with TNM classification of malignant tumor stage (TNM), lymph node metastasis and proliferating cell nuclear antigen positivity in breast cancer (15). Papaconstantinou *et al* (16) identified that miR-155 expression was associated with clinical stage and poor prognosis in pancreatic cancer. Additionally, Shibuya *et al* (17) identified that miR-155 expression was associated with lymph node metastasis in colorectal cancer. A further study revealed that there were significant associations between miR-155 expression and the development of digestive tract cancer (18). However, the association between the clinicopathological features of HCC and miR-155 expression remains to be elucidated. In addition, with the increase in the recurrence rate of HCC, an association between miR-155 expression levels and early recurrence following surgery remains to be elucidated. The current study will present an in-depth investigation of these outstanding questions, by analyzing the expression of miR-155 in HCC tissues and its clinical significance.

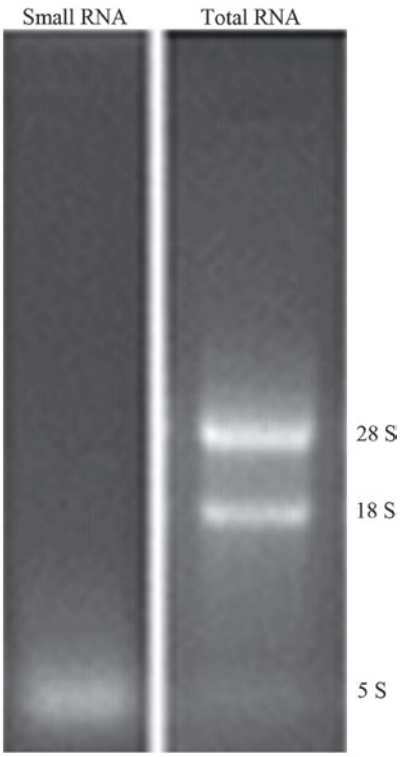

Figure 1. Agarose gel electrophoresis of RNA. Small RNA exhibited a bright 5 S band, indicating successful miRNA extraction.

Materials and methods

Specimens. The present study was conducted at the Department of Oncology, The First Affiliated Hospital of Jinan University (Guangzhou, China) and the Department of Oncology, The Affiliated Hospital of Guangdong Medical College (Zhanjiang, China). A total of 40 samples of cancerous and 40 samples of pericarcinomatous (3-5 cm from the tumor edge, confirmed by histopathology) tissue were removed from patients exhibiting HCC during surgery between January and December 2012. The patients had not received any other anticancer treatments prior to surgery. The present study was conducted in accordance with the Declaration of Helsinki (19) with approval from the Ethics Committee of Jinan University (Guangzhou, China). Written informed consent was obtained from all participants. The follow-up deadline was 31 December 2012; the shortest follow-up period was 3 months and the longest was 12 months.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To quantitate mRNA expression, total RNA was extracted from clinical samples and NPC cell lines using a cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan). The isolated total RNA was reverse transcribed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara Bio, Inc.) for miR-155, according to manufacturer's instructions. The sequence-specific forward primers for mature miR-155 and U6 internal control were 5'-ACACTCCAGCTGGGTTAATGCTAATCGTG-3' and 5'-CTCGCTTCGGCAGCACA-3', respectively (Takara Bio, Inc.). qPCR was performed using SYBR Premix ExTaq™ II (Takara Bio, Inc.) in a LightCycler 480 system (Roche Diagnostics, Indianapolis, IN, USA). The parameters of the PCR reaction were as follows: 94˚C for 2 min, 1 cycle; 94˚C for 20 sec, 60˚C for 34 sec for 40 cycles. All RT-qPCR reactions were performed in triplicate. Relative expression was calculated using comparative cycle threshold (Ct) values. The U6 small nuclear ribonucleoprotein was used as the inner reference gene for miR-155. miR-155 relative expression was calculated using $2^{-\Delta Ct}$, $\Delta Ct = Ct(miR\text{-}155) - Ct(U6)$. Relative quantification of miR-155 expression in cancer versus pericarcinomatous tissues was calculated using the $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = \Delta Ct(experimental\ group) - \Delta Ct(control\ group)$.

Statistical analysis. Data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Results of measurement data that were not normally distributed are expressed as the median and interquartile range (25-75%). Comparisons of the expression of miR-155 in cancerous and pericarcinomatous tissues were performed using the Wilcoxon signed rank test. miR-155 expression was compared across a diverse group of clinicopathological features using the Mann-Whitney test. Survival curves were constructed using the Kaplan-Meier method and evaluated using the Log-Rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Confirmation of successful miRNA extraction. Small fragments of RNA (<200 bp) were acquired using the miRcute miRNA Isolation kit. Electrophoresis revealed a bright 5 S band, indicating successful miRNA extraction (Fig. 1).

PCR results indicate miRNA expression. miR-155 expression was detected using SYBR Green fluorescence RT-qPCR. The amplification curves of the target and reference genes

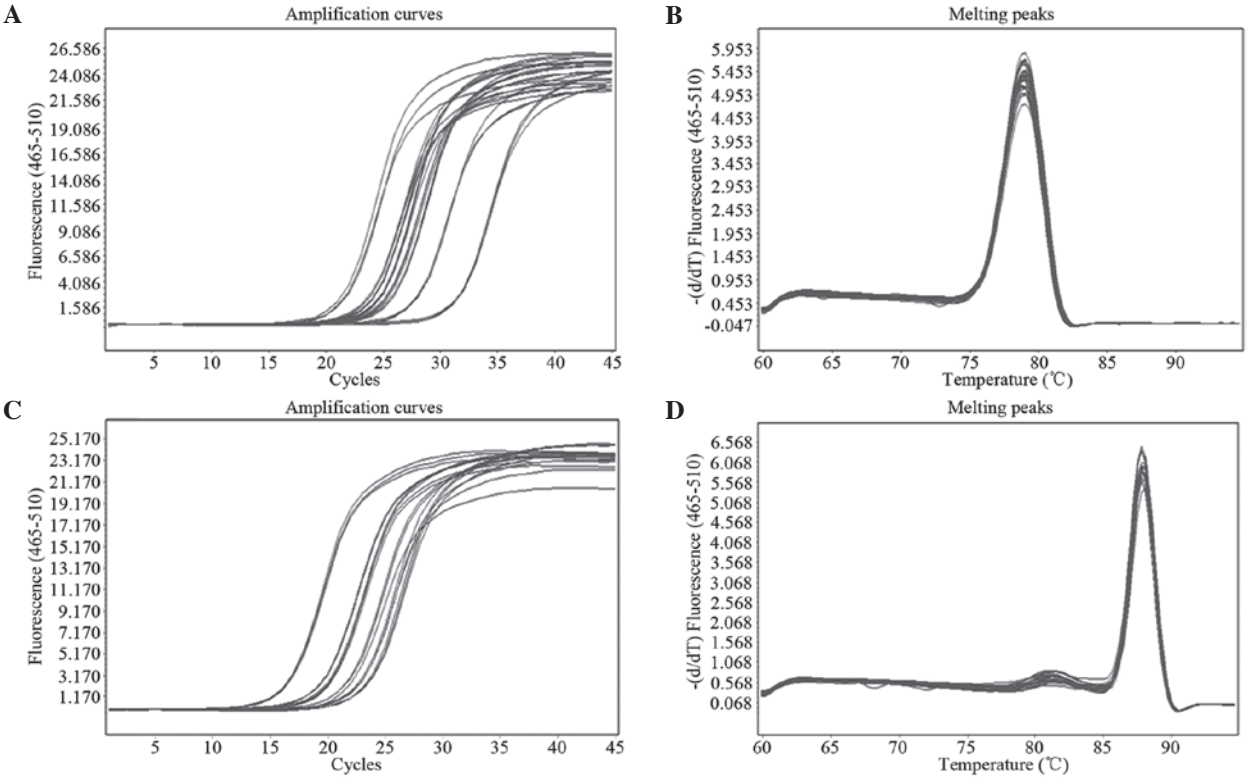

Figure 2. Amplification and dissolution curves. (A) Amplification curve of miR-155; (B) dissolution curve of miR-155; (C) amplification curve of U6 and (D) dissolution curve of U6. miR-155; microRNA-155; d/dt, derivative with respect to t.

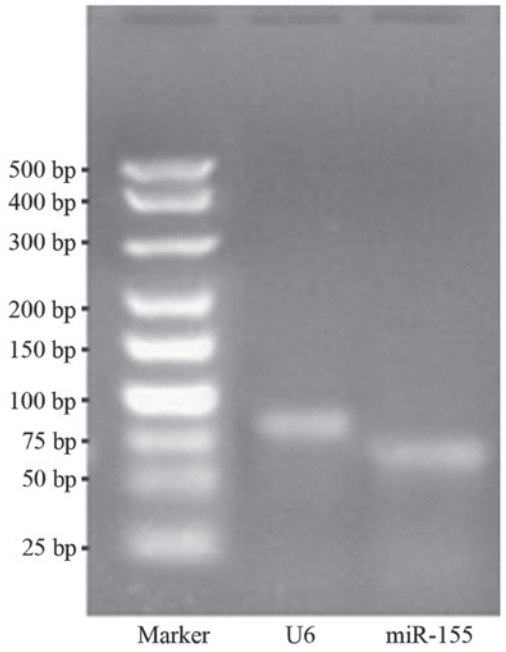

Figure 3. Polymerase chain reaction products evaluated using agarose gel electrophoresis. Two clear cataphoresis bands were visualized at 100 and 75 bp, consistent with the theoretical values for the reference gene U6 (94 bp) and target gene miR-155 (70 bp). U6, U6 small nuclear ribonucleoprotein.

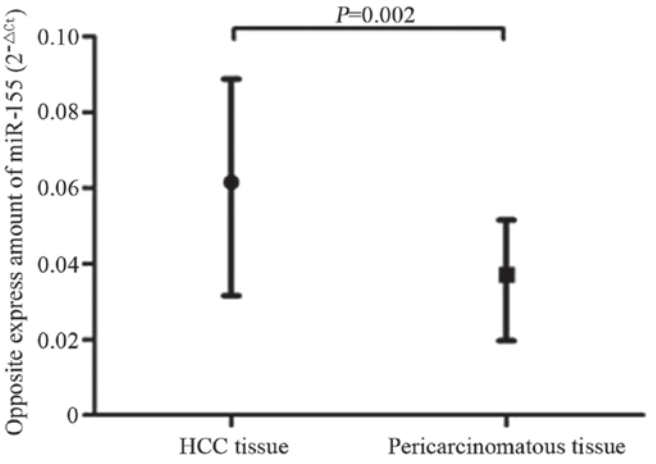

Figure 4. Increased miR-155 expression in HCC tissue. Opposite express quantities of miR-155 in HCC and pericarcinomatous tissue. HCC, hepatocellular carcinoma; miR-155, microRNA-155. Values are expressed as the mean and interquartile range.

were smooth, indicating that the amplification system was suitable for the reaction and the experiment conditions were correct. Thus, suggesting good experiment repeatability. The dissolution curve demonstrated narrow and sharp peaks and no impurity peaks, and the various melting temperatures identified correspond with distinct gene products. The dissolution temperature for each gene product was comparable in the various samples, confirming the high specificity of the amplified product (Fig. 2).

PCR product analysis with agarose gel electrophoresis. There were two clear cataphoresis bands visualized at 100 and 75 bp, which were consistent with the theoretical values for the reference gene U6 small nuclear ribonucleoprotein (94 bp) and target gene miR-155 (70 bp). Additionally, no other impurity

Table I. Analysis of miR-155 expression levels in HCC and pericarcinomatous tissue.

Tissue group	Cases, n	Relative expression levels of miR-155[a]	P-value
HCC	40	0.061 (0.032-0.089)	0.002
Pericarcinomatous	40	0.037 (0.019-0.052)	

[a]Values are expressed as the median (25th-75th percentile). HCC, human hepatocellular carcinoma; miR-155, microRNA-155.

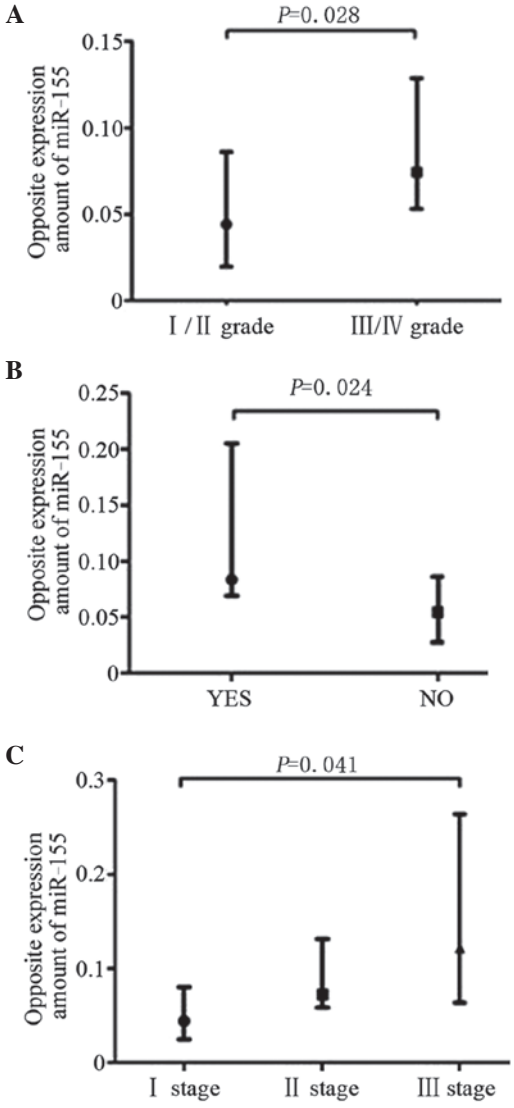

Figure 5. Association between miR-155 expression and (A) Edmonson grade, (B) vascular invasion and (C) American Joint Committee on Cancer TNM classification of malignant tumor stage. miR-155, microRNA-155. Values are expressed as the mean and interquartile range.

bands were observed, indicating that experimental conditions and primer design were correct (Fig. 3).

miR-155 is upregulated in HCC clinical specimens. In HCC tissues, miR-155 expression was upregulated in 62.5% (25/40) of samples, compared with that of pericarcinomatous tissue samples. The mean level of upregulation was a 2.570-fold increase [95% confidence interval (CI); 1.928-3.212; Table I].

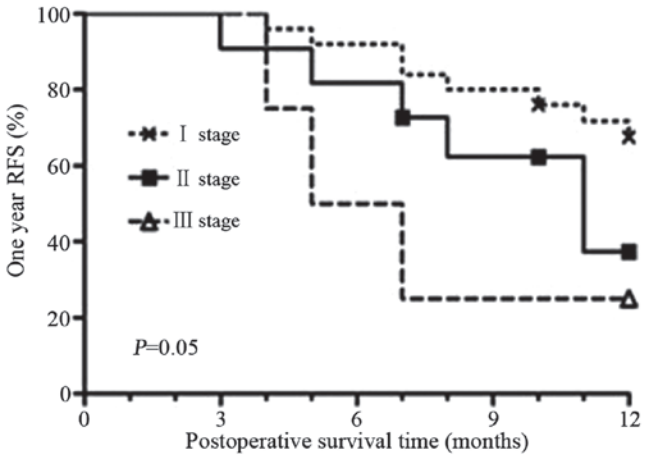

Figure 6. Association between the TNM classification of malignant tumor stage of patients with hepatocellular carcinoma and 1-year RFS. RFS, relapse-free survival.

Thus, miR-155 was significantly upregulated in HCC tissues compared with adjacent normal tissues (P=0.002; Fig. 4).

Association between miR-155 expression levels and clinicopathological parameters of HCC. An association was observed between miR-155 expression levels and Edmonson grade of HCC tissue, vascular invasion and clinical stage (P<0.05). However no association was observed between miR-155 expression and the gender, age, tumor size, tumor number or α-fetoprotein levels of patients (P>0.05; Table II; Fig. 5).

Association between miR-155 expression levels and 1-year relapse-free survival (RFS). Following surgery, the 40 patients who had exhibited HCC were followed-up for 1 year. Three of these patients were lost to follow-up during the follow-up period, resulting in a follow-up rate of 92.5%. During follow-up, there were 17 cases of tumor recurrence, therefore total 1-year RFS was 57.5%. The 40 patients evaluated in the present study were divided into high and low miR-155 expression groups. miR-155 expression levels in HCC tissue were classified as high when observed expression levels were >1-fold greater than the expression levels observed in pericarcinomatous tissue. By contrast, when expression levels in HCC tissue were observed to be <1-fold that observed in percarcinomatous tissue, a classification of low expression was applied. The Kaplan-Meier method was applied for prognostic analysis of single factors using the log-rank test. The result of this analysis revealed that there was a positive correlation between late TNM stage and reduced 1-year RFS (P=0.05). The percentage of patients with

Table II. Association between miR-155 expression and clinicopathological parameters of human hepatocellular carcinoma.

Clinicopathological parameters	Cases, n	Relative expression levels of miR-155	P-value
Gender			0.567
Male	35	0.062 (0.029-0.089)	
Female	5	0.058 (0.019-0.097)	
Age, years			1.000
>50	18	0.055 (0.035-0.113)	
≤50	22	0.062 (0.027-0.087)	
Edmonson grade			0.028[b]
I/II	23	0.044 (0.019-0.086)	
III/IV	17	0.074 (0.053-0.129)	
Tumor size, cm			0.887
>5	14	0.055 (0.029-0.095)	
≤5	26	0.062 (0.033-0.087)	
No. of tumors			0.617
1	30	0.060 (0.039-0.087)	
≥2	10	0.062 (0.049-0.133)	
Vascular invasion			0.024[b]
Yes	6	0.083 (0.069-0.205)	
No	34	0.054 (0.027-0.086)	
Cirrhosis			0.325
Yes	23	0.062 (0.021-0.086)	
No	17	0.061 (0.042-0.132)	
Malignant tumor stage[c]			0.041[b]
I	25	0.044 (0.025-0.080)	
II	11	0.072 (0.058-0.131)	
III	4	0.122 (0.063-0.264)	
Hepatitis-B virus DNA, copies/ml			0.051
>500	17	0.074 (0.059-0.099)	
≤500	23	0.044 (0.021-0.074)	
α-fetoprotein, ng/ml			0.086
≥400	15	0.044 (0.029-0.069)	
<400	25	0.074 (0.036-0.099)	

[a]Values are expressed as the median (25th-75th percentile); [b]indicates statistically significant results and [c]American Joint Committee on Cancer TNM classification. miR-155, microRNA-155.

high miR-155 expression achieving 1-year RFS was reduced compared with that of patients with low miR-155 expression (48.1 vs. 73.3%), however this difference was not statistically significant (P=0.118; Table III; Fig. 6).

Discussion

The occurrence of primary liver cell cancer is a chronic process, which involves a number of steps, including the inactivation of anti-oncogenic processes, the activation of oncogenes and the abnormal regulation of various cell signaling pathways (20). At present, a key area of liver cancer research is elucidation of the association between miR-155 expression and HCC. The present study revealed that miR-155 expression levels were enhanced in HCC tissues, consistent with previous findings by Hu *et al* (21). The present study also revealed an association between miR-155 expression levels and Edmonson grade (grade III/IV), vascular invasion and clinical stage (P<0.05), which was consistent with the findings of Song *et al* (22) in breast cancer. However, the results of the present study were inconsistent with the findings of Han *et al* (23), where an association between miR-155 expression and HCC tissue differentiation was observed, as the present study did not compare the differential expression in high and low HCC differentiation groups. In the present study, Edmonson grades I/II were classified as one group and grades III/IV were classified as another group. High expression of miR-155 and high Edmonson grade demonstrated a significant association (P<0.05). These results suggested that high expression levels of miR-155 may be associated with a high degree of malignancy and invasion in HCC. According to

Table III. Single factor analysis of influencing factors of 1 year RFS.

Clinicopathological parameters	Cases, n	1-year RFS, %	Log-rank test	
			χ^2	P-value
Gender			1.588	0.213
Male	35	60.0		
Female	5	40.0		
Age, years			0.819	0.374
>50	18	67.0		
≤50	22	50.0		
Edmonson grade			1.808	0.181
I/II	23	65.0		
III/IV	17	47.1		
Tumor volume, cm			0.965	0.324
>5	14	50.0		
≤5	26	61.5		
No. of tumors			1.988	0.168
1	30	63.0		
≥2	10	40.0		
Vascular invasion			2.709	0.100
Yes	6	33.0		
No	34	61.8		
Hepatocirrhosis			3.620	0.067
Yes	24	46.0		
No	16	75.0		
Malignant tumor stage[a]			6.004	0.050[b]
I	25	68.0		
II	11	46.0		
III	4	25.0		
Hepatitis-B surface antigen, copies/ml			2.196	0.141
>500	17	47.0		
≤500	23	65.2		
α-fetoprotein, ng/ml			1.316	0.257
≥400	15	47.0		
<400	25	64.0		
Postoperative chemotherapy			0.298	0.592
Yes	16	62.5		
No	24	54.2		
microRNA-155			2.632	0.118
Overexpression	25	48.0		
Low expression	15	73.3		

[a]American joint committee on cancer TNM classficiation; [b]indicates statistically significant results. RFS, relapse-free survival.

these data, miR-155 appears to be involved in tumor progression through the inhibition of multiple tumor suppressor genes, such as sex-determining region Y-gene related high-mobility-group box gene (24,25) and suppressor in cytokine signaling 1 (26), thus promoting proliferation and invasion in HCC. Surgical resection is the typical initial treatment for primary liver cancer (27). This previous demonstrated that the rate of HCC surgery recurrence rate was significantly increased within 1 year of surgery and was accompanied by poor prognosis, compared with the recurrence rate in subsequent years. Therefore, recurrence of HCC within 1 year following radical surgical resection is defined as early recurrence (28,29). A previous study (29) demonstrated that the total relapse rate in the first year directly following surgery was 43.3%, and HCC recurrence in subsequent years was 42.5%, which was consistent with the findings of Sun *et al* (29). Cai *et al* (30) retrospectively analyzed HCC

recurrence in 110 patients who had undergone radical surgical resection, and found that the formation of portal vein thrombus was a risk factor for early recurrence. Single factor analysis indicated that the postoperative 1-year RFS of HCC patients exhibiting vascular invasion was reduced compared with that of patients with HCC and no vascular invasion (33.6 vs. 61.8%), however a statistically significant difference was not indicated (P=0.10) and these findings were inconsistent with the results of a previous study (30). A problem with the present study was that the sample size of patients with HCC and vascular invasion was markedly smaller than the sample with HCC and no vascular invasion. Furthermore, the present study demonstrated that the 1-year RFS of late TNM stage patients was reduced (P=0.05).

There is a close association between miR-155 expression levels and HCC prognosis (31,32). Huang *et al* (33) demonstrated that there was an association between miR-155 expression levels and 5-year RFS of patients with HCC following radical surgical resection. The 5-year RFS of patients expressing high levels of miR-155 was reduced compared with patients exhibiting low levels of miR-155 (hazard ratio=2.002; 95% CI, 1.324-3.027). It remains to be fully elucidated whether miR-155 is associated with early recurrence of HCC. In the present study, it was observed that 1-year RFS of patients exhibiting high expression of miR-155 was reduced compared with patients exhibiting low expression levels of miR-155 (48.1 vs. 73.3%), however this difference was not statistically significant (P=0.105). Therefore, miR-155 expression levels may not be the sole predictor for early recurrence of HCC. Therefore, although miR-155 was overexpressed in HCC patients'tissues, it may not be the only predictor for early recurrence of HCC. This study suggeststhat miR-155 is related to the clinical characaticals of HCC.And it may be a novel diagnostic marker and potential therapeutic target in HCC.

Acknowledgements

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