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Original Article

Prognostic effect of IL-6/JAK2/STAT3 signal-induced microRNA-21-5p expression on short term recurrence of hepatocellular carcinoma after hepatectomy

Chengzhan Zhu[1*], Mao Zhang[1*], Jie Hu[2], Haoran Li[1], Shihai Liu[3], Tianxiang Li[1], Liqun Wu[1], Bing Han[1]

[1]*Department of Hepatobiliary and Pancreatic Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, P. R. China;* [2]*The Third Xiangya Hospital of Central South University, Changsha, Hunan, P. R. China;* [3]*Medical Animal Laboratory, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, P. R. China.* [*]*Equal contributors.*

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Abstract: Aim: To investigate the putative role of interleukin (IL)-6/Janus Kinase (JAK) 2/Signal transducers and activators of transcription (STAT) 3 signaling pathway in hepatocellular carcinoma (HCC) short term recurrence (STR), and whether the pathway promotes HCC progression through microRNA-21-5p (miRNA-21). Methods: Immunohistochemistry was performed to evaluate the protein expression of IL-6, JAK2 and STAT3. Real-time PCR was used to evaluate the miRNA-21 expression. We also analyzed the correlation of IL-6, JAK2 and STAT3 protein expression with miRNA-21. Clinicopathological variables, including prognosis, were compared between low and high-expressing groups of miRNA-21. Results: miRNA-21 expression was significantly increased in the HCC tumor tissue compared to the non-tumor tissue. IL-6 and STAT3 high expression was significantly correlated to high miRNA-21 expression in HCC tumor tissues. Patients with high miRNA-21 expression have more frequent early recurrence. The 6 month overall survival and disease-free survival rate of the miRNA-21 high groups were 72.1% and 30.8%, respectively. Moreover, high miRNA-21 expression was correlated with disease-free survival (DFS) ($P < 0.05$) and overall survival (OS) ($P < 0.05$). Multivariate analysis revealed that miRNA-21 and STAT3 high expression were independent prognostic factors of DFS and OS. The area under the ROC curve (AUC) of miRNA-21 and STR, DFS, OS was 0.951 ($P < 0.001$), 0.847 ($P < 0.001$), 0.844 ($P < 0.001$), respectively. Conclusions: miRNA-21 expression, induced by IL-6/JAK2/STAT3 signaling pathway, was increased in the early recurrence of HCC patients and indicated poor prognosis. Expression analysis of miRNA-21 revealed that it may be a valuable prognostic biomarker for HCC.

Keywords: Hepatocellular carcinoma, IL-6, JAK, STAT, microRNA, short-term recurrence

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, and the incidence is increasing [1]. Despite great advances in surgical management of the disease, the recurrence rate of HCC remains high even after curative hepatectomy and the long-term prognosis remains unsatisfactory. Moreover, HCC patients with early recurrence will experience early death [2]. Although great efforts have been made to explore suitable biomarkers for the prediction of the prognosis after resection of HCC, little research is focused on the early recurrence of HCC.

The interleukin (IL)-6/Janus Kinase (JAK) 2/Signal transducers and activators of transcription (STAT) 3 signaling pathway associates with the development of HCC. IL-6 can stimulate Janus-activated kinases (JAKs) and STAT3 phosphorylation, promoting cancer cell survival. The activated STAT3 will induce diverse cellular process through transcription of target genes, including Bcl-2, cyclin D1, and MMP2 [3]. Inhibition of STAT3 signaling was shown to block the anti-apoptotic activity of IL-6 in human HCC cells [4]. Moreover, IL-6/JAK2/STAT3 signaling pathway was shown to promote HCC progression through the maintenance and proliferation of HCC initiating cells [5]. Furthermore, STAT3 regulated the migration and invasion of a stem-

Table 1. Clinicpathologic characteristics of patient samples

Characteristic	Number of cases
Age (y)	
≤50	16
>50	34
Gender	
Male	40
Famale	10
AFP (ng/L)	
≤400	36
>400	14
Tumor number	
Single	48
Multiple	2
Tumor size (cm)	
≤5	37
>5	13
Tumor margin (cm)	
≤2	28
>2	22
Pathologic differentiation	
High	10
Middle and Low	40
Vascular invasion	
Yes	6
No	44
Capsule invasion	
Yes	14
No	33
Liver cirrhosis	
Yes	41
No	9
Virus infection	
Hepatitis B	50
Hepatitis C	0
Recurrence states (at follow-up)	
Yes	16
No	0
Expression of miRNA-21	
High	26
Low	24

AFP, α-fetoprotein; miRNA-21, microRNA-21-5p.

like subpopulation through microRNA-21-5p (miRNA-21) in HCC [6]. Targeting STAT3 may limit HCC progression through the regulation of miRNA-21 and its targets PTEN, RECK and PDCD4 [6]. The STAT3/miRNA-21 axis can also promote epithelial-mesenchymal transition inducing metastasis in several types of cancer [7, 8].

miRNA-21 was found to be overexpressed in most types of cancer [9], involved in tumor-development pathways related to cell proliferation, apoptosis, angiogenesis, cell invasion, inflammation and genetic instability [10]. miRNA-21 is also a therapeutic target in multiple epithelial cancers such as colorectal cancer [11] and renal cancer [12]. miRNA-21 is upregulated in HCC, and anti-miRNA-21 resulted in the loss of viability in the majority of HCC cell lines and robust increase of caspase activity, apoptosis, and necrosis [13]. miRNA-21 maybe a therapeutic target and also reverse drug resistance in several types of cancer [14, 15], including the resistance to interferon-α or 5-fluorouracil in HCC [16]. In addition, high miRNA-21 expression predicted poor prognosis in HCC [17]. However, there has been no report about whether the IL-6/JAK2/STAT3 pathway induces miRNA-21 expression in HCC of short term recurrence.

We first measured the IL-6, JAK2 and STAT3 protein expression with immunohistochemistry (IHC) and measured miRNA-21 gene expression with PCR. We investigated the correlation of miRNA-21 expression with IL-6/JAK2/STAT3 pathway. Then we investigated whether the pathway promotes HCC progression of short term recurrence (STR) through downstream targeting of miRNA-21. We also researched whether miRNA-21 expression can independently provide useful information in regards to the prediction of outcome in HCC.

Materials and methods

Patients and follow-up

A total of 50 consecutive patients with initially resectable HCC, who underwent hepatectomy at the Affiliated Hospital of Qingdao University between January 2011 and December 2012, were enrolled in this study. All patients were followed-up every month during the first half year and at least every 3-4 months thereafter. The follow-up period ended on 30 May 2016. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University, and written informed consent was obtained from all patients.

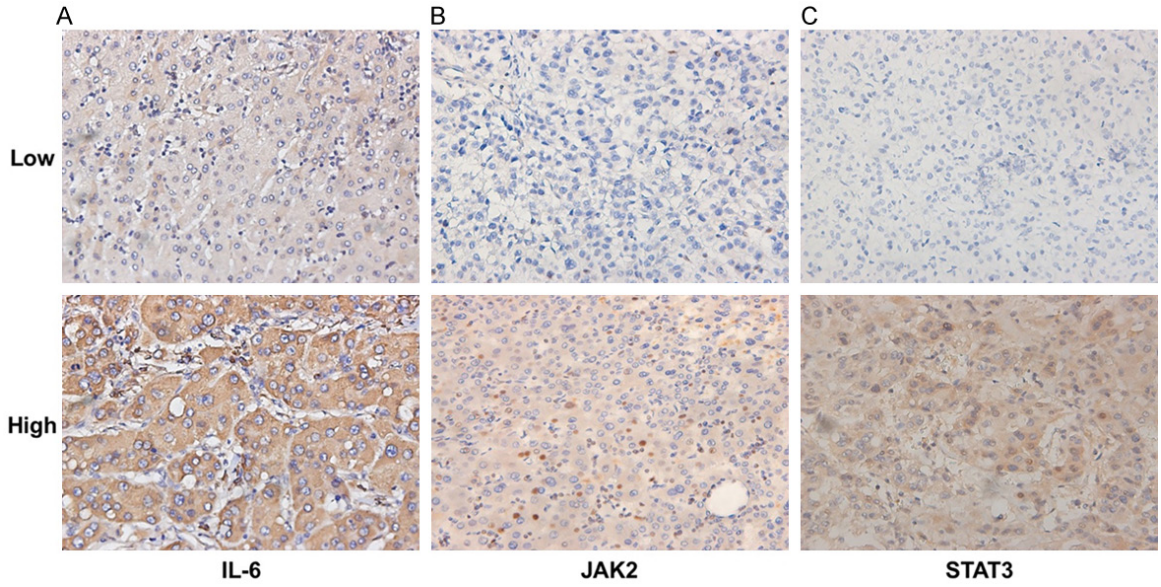

Figure 1. IHC staining of IL-6 (A. Magnification × 400), JAK2 (B. Magnification × 400), and STAT3 (C. Magnification × 400) in tumor tissues.

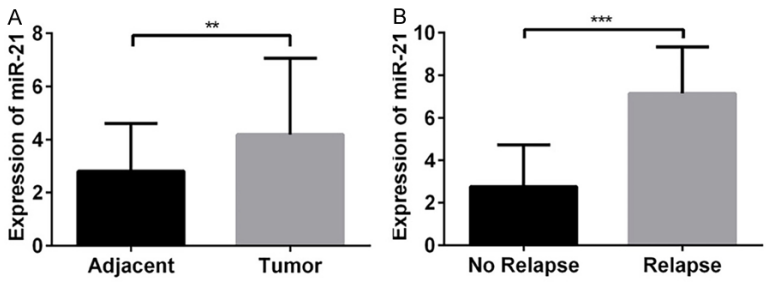

Figure 2. Expression of miRNA-21 in HCC tumor and adjacent nontumor tissue (A); Expression of miRNA-21 in short term relapse (≤6 months) and no relapse groups (B).

Real-time fluorescent quantitative PCR

The cancer tissue was ground up in liquid nitrogen, then the total miRNA was extracted and enriched with miRNA Extraction Kit (CWBio, Beijing, China) and miRNA first strand cDNA synthesis Kit (CWBio, Beijing, China) according to the instruction. The first chain cDNA corresponding to miRNA was synthesized according to the instructions of the miRNA cDNA first chain synthesis kit (CWBio, Beijing, China).

Real-time fluorescent quantitative PCR was done with the miRNA real-time fluorescent quantitative PCR detection kit (CWBio, Beijing, China). Using The hsa-U6 (Nolan Biomedical, Shanghai, China) was used as an internal control. The upstream primer sequence of hsa-miRNA-21 is 5'-TTT CTT GCC GTT CTG TAA GTG-3', the downstream sequence is 5'-TGG ATA TGG ATG: GTC AGA TGA A-3', the U6 probe sequence is 5'-ATT GGA ACG ATA CAG AGA AGA TT-3'. The reaction conditions were: 95°C 10 min, 95°C 15 s, 54°C 20 s, 74°C 20 s, with a total of 40 cycles, in a 25 μL PCR reaction system. The solution curves were plotted within the range of 75~95°C. The value of ΔC (T) was calculated with opticon 3 software. The target gene expression was expressed with $2^{-\Delta\Delta CT}$. The average value of each sample was obtained in 3 independent experiments.

Immunohistochemistry analysis

Formalin-fixed, paraffin-embedded sections of 50 surgical specimens were used for protein analysis performed with assistance of a pathologist in our hospital. Primary rabbit monoclonal anti-JAK2 antibody (Sigma-Aldrich, Germany; dilution at 1:100), primary rabbit polyclonal anti-STAT3 antibody (Sigma-Aldrich, Germany; dilution at 1:100), were used for detecting protein expression in HCC tissues. Unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 98°C for 24 min in a microwave. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature to

Table 2. Correlation between miRNA-21 and clinicopathologic characteristics

Variables	miRNA-21 expression level	
	Spearman correlation	*p*-Value
Age (y)	-0.316	0.025*
Gender	0.120	0.406
AFP	0.064	0.658
Tumor number	-0.008	0.955
Tumor size (cm)	0.113	0.434
Tumor margin (cm)	-0.197	0.171
Pathologic differentiation	0.180	0.211
Vascular invasion	-0.138	0.339
Capsule invasion	0.113	0.433
Liver cirrhosis	-0.346	0.014*
Virus infection	0.074	0.610
IL-6	0.318	0.024*
JAK2	0.038	0.791
STAT3	0.367	0.009*

*P < 0.05, statistically significant. miRNA-21, microRNA-21-5p; AFP, α-fetoprotein; IL-6, interleukin-6; JAK2, Janus Kinase 2; STAT3, Signal transducers and activators of transcription 3.

remove endogenous peroxidase activity. All antibodies were incubated for 1 h at room temperature. Sections were then washed three times for 5 min in phosphate-buffered saline, and then stained with horseradish peroxidase-tagged secondary antibody labeled with anti-rabbit polymers (Sigma-Aldrich, Germany) for 1 h at room temperature, and washed three times for 5 mins in phosphate-buffered saline. Finally, sections were developed in 3, 30-diaminobenzidine (Sigma-Aldrich, Germany) and counterstained with hematoxylin. For IL-6, JAK2 and STAT3, immunoreactions detected in human placenta were used as the positive control.

Statistical analysis

All statistical analyses were performed with statistical software SPSS version 18.0, (SPSS, Chicago, IL). Correlation between miRNA-21 gene expression and IL-6, JAK2 and STAT3 protein expression were calculated by Spearman relation. Experimental results are expressed as the mean ± standard deviation. The chi-square test or Student's t test was used to compare values between the two groups. Overall survival (OS) and disease-free survival (DFS) curves were generated using the Kaplan-Meier method, and the differences were compared using log-rank test. Univariate and multivariate analysis were performed based on the Cox proportional hazard regression model to compute a hazard ratio (HR). Receiver operating characteristic (ROC) curves were established for discriminating the prognosis of HCC patients, and the area under the curve (AUC) was calculated. A two-sided *P* value < 0.05 was considered significant.

Results

The expression of IL-6/JAK2/STAT3 and miRNA-21 in HCC tissues

The basic characteristic of the 50 consecutive patients is shown in **Table 1**. The IL-6/JAK2/STAT3 proteins in HCC were examined by IHC, and the miR-21 in HCC were examined by real-time PCR.

Representative IHC staining of IL-6, JAK2, and STAT3 in tumor tissues is shown in **Figure 1**. The IL-6/JAK2 is expressed in cytoplasm, but STAT3 is expressed in both cytoplasm and nucleus. According to the staining intensity and area, we divided the patients into high and low groups based on the expression of IL-6, JAK2 and STAT3, respectively.

The miRNA-21 was more highly expressed in tumor tissues compared with adjacent normal tissues (3.99 ± 2.95 vs. 2.69 ± 2.26, P < 0.001, **Figure 2A**). Patients were divided into a high-level group (n = 26) and a low-level group (n = 24) by the mean miRNA-21 level (4%) in the tumor tissue as cutoff value.

Previous studies have shown that STAT3 induced miRNA-21 expression in some cancer cells. We analyzed the correlation of miRNA-21 expression with IL-6, JAK2 and STAT3 protein expression in HCC tissues. There was a significant correlation between miRNA-21 and IL-6 expression (R = 0.318, P = 0.024), and also with miRNA-21 and STAT3 expression (R = 0.367, P = 0.009). There was no correlation between miRNA-21 and JAK2 (**Table 2**).

The clinicopathological characteristics and miRNA-21 expression are shown in **Table 2**. There was no significant association between gender, AFP, tumor size, tumor number, tumor margin, pathologic differentiation, vascular invasion, capsule invasion, and expression of

Table 3. Clinicpathologic characteristics of patients in the short term recurrence group and no recurrence group

Variables	Short term recurrence				
	No	Yes	*t-test*	X^2	*p*-Value
Age (y)	58.38 ± 11.26	54.38 ± 10.70	1.19		0.239
Gender (M/F)	27/7	13/3		0.023	0.880
Tumor number	1.03 ± 0.17	1.06 ± 0.25	-0.547		0.587
Tumor size (cm)	4.24 ± 2.57	5.63 ± 3.30	-1.632		0.109
Tumor margin (cm)	8.12 ± 8.38	6.51 ± 4.84	0.855		0.397
Pathologic differentiation (low/high)	29/5	11/5		1.861	0.172
Capsule invasion	8/34	7/16		2.118	0.146
Vascular invasion	6/34	3/16		0.009	0.925
Liver cirrhosis	31/34	13/16		1.015	0.314
Virus infection	30/34	13/16		0.441	0.507

AFP, α-fetoprotein.

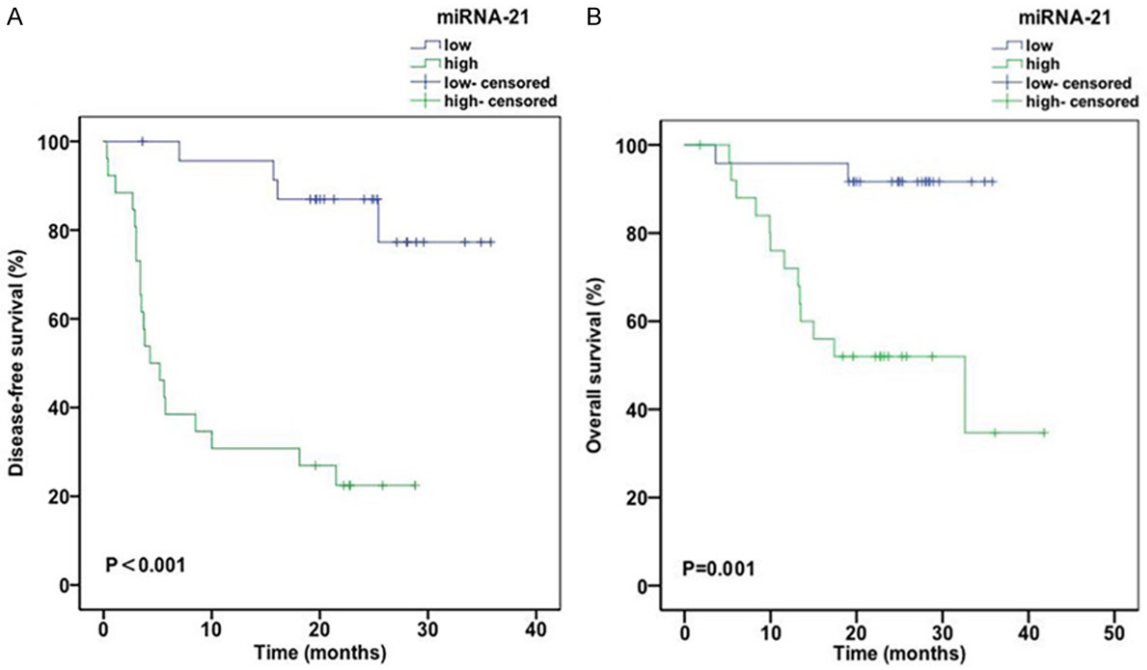

Figure 3. Expression of miRNA-21 correlated with DFS (A) and OS (B); The correlation between miRNA-21 and HCC patients' DFS/OS was further evaluated. The results showed that patients with high expression of miRNA-21 had poorer DFS (P < 0.001) and OS (P = 0.001).

miRNA-21. However, low expression of miRNA-21 correlated with liver cirrhosis (R = -0.346, P = 0.014) and advanced age (R = -0.316, P = 0.025).

The association of clinicopathologic characteristics and miRNA-21 expression with STR

The basic characteristic of the patients in the short term relapse and no relapse group is shown in **Table 3**. The STR of HCC patients is unrelated to clinicopathological characteristics. However, patients with high miRNA-21 expression had more frequent STR; the relative expression of miRNA-21 was significantly higher in the short term relapse group (≤6 months) than in no relapse group (7.16 ± 0.54 vs. 2.81 ± 034, P < 0.001, **Figure 2B**).

Association of miRNA-21 expression with HCC patient outcome

The 6 month survival rates were 72.1% and 95.8% for miRNA-21 high and low expression

Table 4. Univariate and multivariate analyses of various prognostic parameters in patients with HCC Cox-regression analysis for DFS

	Univariate analysis			Multivariate analysis		
	P-Value	HR	95% CI	*p*-Value	HR	95% CI
miRNA-21	< 0.001*	1.329	1.179-1.500	0.007*	1.289	1.071-1.551
IL-6	0.011*	13.820	1.822-104.804	0.147	5.511	0.549-55.287
JAK2	0.118	2.326	0.807-6.705			
STAT3	< 0.001*	8.269	2.840-24.076	0.003*	9.029	2.167-37.623
Age (y)	0.106	0.510	0.226-1.153			
Gender	0.506	1.440	0.492-4.218			
AFP	0.231	1.686	0.718-3.963			
Tumor number	0.989	1.014	0.136-7.537			
Tumor size (cm)	0.519	1.336	0.554-3.226			
Tumor margin (cm)	0.009*	0.292	0.116-0.740	0.063	0.219	0.044-1.088
Pathologic differentiation	0.531	1.371	0.511-3.677			
Vascular invasion	0.132	0.214	0.029-1.592			
Capsule invasion	0.512	1.344	0.556-3.254			
Liver cirrhosis	0.023*	0.358	0.147-0.870	0.821	1.165	0.311-4.365
Viral infection	0.601	0.751	0.256-2.199			

*P < 0.05, statistically significant. HCC, hepatocellular carcinoma; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; miRNA-21, microRNA-21-5p; IL-6, interleukin-6; JAK2, Janus Kinase2; STAT3, Signal transducers and activators of transcription 3; AFP, α-fetoprotein.

Table 5. Univariate and multivariate analyses of various prognostic parameters in patients with HCC Cox-regression analysis for OS

	Univariate analysis			Multivariate analysis		
	P-Value	HR	95% CI	*P*-Value	HR	95% CI
miRNA-21	< 0.001*	1.384	1.186-1.614	0.001*	1.441	1.158-1.793
IL-6	0.054	3.481	0.980-12.363	0.424	1.842	0.412-8.236
JAK2	0.920	1.054	0.382-2.907			
STAT3	0.002*	5.021	1.766-14.276	0.015*	4.463	1.330-14.983
Age (y)	0.181	0.500	0.181-1.381			
Gender	0.475	1.720	0.388-7.629			
AFP	0.113	2.357	0.816-6.808			
Tumor number	0.463	2.146	0.279-16.487			
Tumor size (cm)	0.705	1.248	0.397-3.925			
Tumor margin (cm)	0.119	0.402	0.128-1.266			
Pathologic differentiation	0.077	2.688	0.899-8.040	0.241	2.050	0.617-6.811
Vascular invasion	0.330	0.039	0.000-26.582			
Capsule invasion	0.211	1.931	0.688-5.423			
Liver cirrhosis	0.114	0.411	1.137-1.238			
Virus infection	0.914	0.921	0.206-4.118			

*P < 0.05, statistically significant. HCC, hepatocellular carcinoma; OS, overall survival; HR, hazard ratio; CI, confidence interval; miRNA-21, microRNA-21-5p; IL-6, interleukin-6; JAK2, Janus Kinase 2; STAT3, Signal transducers and activators of transcription 3; AFP, α-fetoprotein.

patients, respectively. Moreover, 6 month DFS in miRNA-21 high expression patients was 30.8% compared with 95.7% in low expression patients. Patients with miRNA-21 high expression had significantly shorter DFS (median 10.83 months, log-rank P < 0.001) and OS

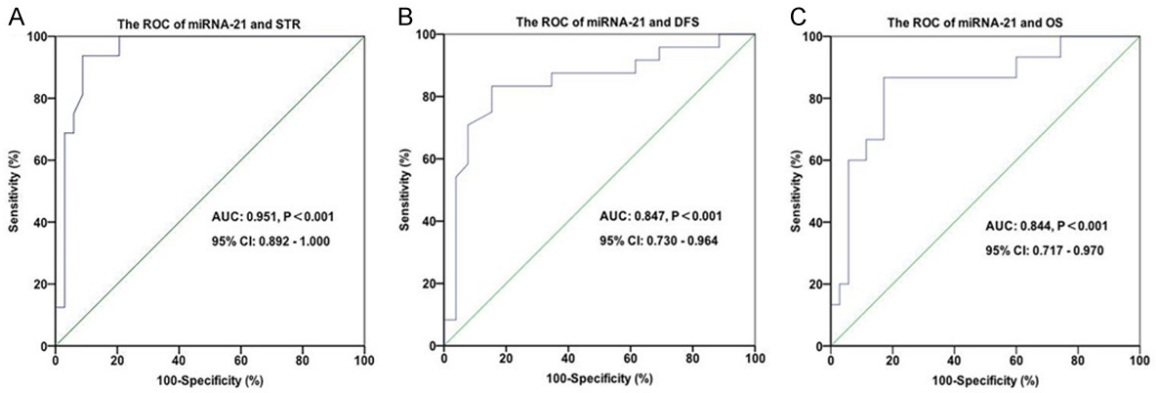

Figure 4. ROC curve analysis determines good sensitivity and specificity for the miRNA-21 level for discriminating relapse or survival. A. The mRNA levels of miRNA-21 yielded an AUC of 0.951 with 93.8% sensitivity and 91.2% specificity in discriminating whether STR occurred. B. The mRNA levels of miRNA-21 yielded an AUC of 0.847 with 83.3% sensitivity and 84.6% specificity in discriminating DFS. C. The mRNA levels of miRNA-21 yielded an AUC of 0.844 with 86.7% sensitivity and 82.9% specificity in discriminating OS.

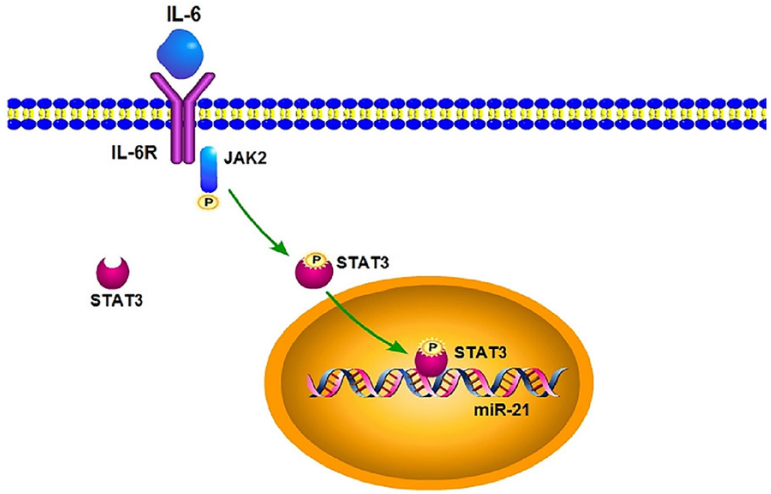

Figure 5. IL-6/JAK/STAT Pathway and miR-21. IL6 binds to IL6R and activates receptor-associated JAK dimerization. Activation of JAK leads to phosphorylation and dimerization of STAT3, translocation to the nucleus, activation to promote miRNA-21 expression.

rates (mean 25.30 months, log-rank P = 0.001) compared with low-expression patients (**Figure 3A**, **3B**).

Univariate analysis revealed that miRNA-21, IL-6, STAT3, tumor margin, and liver cirrhosis were prognostic factor in DFS (**Table 4**). Multivariate analysis revealed that miRNA-21, STAT3, and tumor margin were independent prognostic factors in DFS (**Table 4**). Moreover, univariate and multivariate analysis revealed that miRNA-21 and STAT3 were independent prognostic factors in OS (**Table 5**).

Moreover, the AUC of miRNA-21 and STR was 0.951 [95% confidence interval (CI), 0.892-1.000; P < 0.001], with 93.8% sensitivity and 91.2% specificity, respectively (**Figure 4A**). The AUC of miRNA-21 and DFS was 0.847 (95% CI, 0.730-0.964; P < 0.001), with 83.3% sensitivity and 84.6% specificity, respectively (**Figure 4B**). The AUC of miRNA-21 and OS was 0.844 (95% CI, 0.717-0.970; P < 0.001), with 86.7% sensitivity and 82.9% specificity, respectively (**Figure 4C**).

Discussion

IL-6, one of the major cytokines in the tumor microenvironment, is overexpressed in almost all types of tumor. High expression of IL-6 will induce systemic immune stimulation and a tumor microenvironment that will promote tumorigenesis by regulating multiple signaling pathways, including apoptosis, proliferation, and survival. Therefore, blocking IL-6 or inhibiting its associated signaling is a potential therapeutic strategy for the treatment of cancers. Serum IL-6 has been associated with prognosis in HCC patients [18]. The JAK2/STAT3 pathway is one of the downstream pathways that plays an important role in tumor cell proliferation, survival, inva-

sion, and immunosuppression [19]. In addition, JAK/STAT3 signaling also promotes cancer through inflammation, obesity, stem cells, and the pre-metastatic niche [19]. Newly identified roles of JAK/STAT3 in tumors are important targets for potential therapeutic strategies for cancer. However, the involvement of numerous cellular processes may cause adverse side effects. Finding a downstream target gene of STAT3 may provide more selective and promising therapeutic strategies. A previous study showed that the anti-cancer agent Icaritin could suppresses HCC initiation and malignant growth through the IL-6/JAK2/STAT3 pathway [20].

As the IL-6/JAK2/STAT3 signaling pathway associates with the development of HCC [21-23], we investigated the role of the pathway in HCC recurrence, and the correlation of the pathway and downstream target of miRNA-21 in promoting HCC progression. In a previous study, STAT3 was identified to bind to the conserved binding sites in the promoter of miRNA-21 by scanning the flanking sequences of microRNA transcription start sites in a mammary epithelial cell line [24]. STAT3 expression was also positively correlated with miRNA-21 expression levels in colon adenocarcinoma [24]. Inhibition of STAT3 by siRNA or by a pharmacological inhibitor (JSI-124) could reduce miRNA-21 expression levels in mammary epithelial cell line or myeloma cells [24, 25]. In our study, we found that miRNA-21 expression was significantly positively correlated with IL-6 and STAT3 expression in HCC tissue, which indicates that IL-6/JAK2/STAT3 pathway may promote HCC progression through promoting miRNA-21. Moreover, the miRNA-21 expression was found to be negatively correlated with age and liver cirrhosis. The circulating miRNA-21 has been reported as a diagnostic/prognostic biomarkers of the aging process [26]. But little is known about the miRNA-21 and age of patients. It was reported that higher levels of circulating miRNA-21 may be a candidate markers to discriminate hepatorenal syndrome from acute tubular necrosis in liver cirrhotic patients [27].

miRNA-21 plays an essential role in regulating biological behaviors, promoting cell proliferation [28], and inhibits apoptosis by suppressing several tumor suppressor genes [29, 30]. We found that the expression of miR-21 in HCC tissues was significantly higher than that in normal liver tissues adjacent to the tumor. That is same as in other reports [29, 31]. We have defined short-term recurrence as recurrence within 6 months after hepatectomy in our previous studies [32]. HCC with short term recurrence has stronger metastatic ability and unique biological behavior. The recurrence-related factors such as the tumor margin, tumor diameter, and differentiation in the short term relapse and no relapse groups were not significantly different. However, we found that miR-21 is highly expressed in the short term recurrence group, which indicates that the high metastatic ability of this subtype of tumor is closely related to miR-21.

In our study, miRNA-21 was closely related to the survival of DFS and OS in HCC. The risk factors for recurrence of liver cancer in this study were miR-21, IL-6, STAT3, tumor margin and liver cirrhosis; and miR-21, STAT3 and tumor margin were independent risk factors. miRNA-21 and STAT3 were risk factors for death in patients with HCC, and they were also independent risk factors. A limitation of our study is that it focused only on the phenomenon of relevance, and no further explanation of the underlying mechanism such miRNA-21 target genes or pathway was studied and discussed.

In conclusion, the IL-6/JAK2/STAT3 pathway activation in tumor microenvironments promoted miRNA-21 expression (**Figure 5**) which was significantly overexpressed in the HCC short term recurrence group and associated with poor prognosis. The expression analysis of miRNA-21 revealed that it may be used as a valuable prognostic biomarker for HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bing Han, Department of Hepatobiliary and Pancreatic Surgery, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266003, Shandong, P. R. China. Tel: +86-18661808308; Fax: +86-532-82911323; E-mail: wfkevinhan@163.com

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