Overexpression of miR-21 Is Associated With Recurrence in Patients With Hepatitis B Virus–Mediated Hepatocellular Carcinoma Undergoing Liver Transplantation

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ABSTRACT

Liver transplantation (LT) is the best treatment option for hepatitis B virus (HBV)–mediated hepatocellular carcinoma (HCC). Nevertheless, recurrence is the most important issue after LT. The aims of the present study were to evaluate the relation of dysregulated expression of microRNAs (miRNAs) in recurrence formation in HBV-mediated HCC cases. A total of 42 HBV-mediated HCC patients were evaluated in this study.

Among 21 miRNAs, the expression level of miR-106a and miR-21 were higher and miR-143 and miR145 were lower in patients with HCC compared with noncancerous liver tissues ($P = .0388$, $P = .0214$, $P = .0321$, and $P = .002$, respectively). Compared with nonrecurrent patients, the expression level of miR-21 was 3.54-fold higher and miR-145 was 2.42-fold lower in patients with recurrence during the 5-year follow-up ($P = .004$ and $P = .032$; respectively). In addition, according to multivariate Cox regression analysis, the overexpression of miR-21 was found to be a prognostic indicator in HBV-mediated HCC patients ($P = .002$).

In conclusion, we show a significant association between high expression of miR-21 and recurrence in HBV-mediated HCC. Therefore, up-regulation of miR-21 could serve as a promising prognostic marker for HCC.

HEPATOCELLULAR carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide [1]. The most common causes of HCC formation are known to be chronic infection with hepatitis B (HBV) and hepatitis C viruses or liver cirrhosis. Liver transplantation (LT) is the best treatment option for HCC and HBV infections, which account for the most frequent reason of LT [2]. Recurrence of HCC after LT is the most important cause for treatment failure [3]. However, there are no markers used to determine the recurrence potential in this cancer.

miRNAs, which are small, noncoding RNAs with a length of 20 to 24 bases, are involved in post-transcriptional regulation [4]. miRNAs regulate the biological behaviors of tumors, including proliferation, migration, and invasion in solid cancer. Studies have shown that miRNAs act as tumor suppressors or oncogenes during carcinogenesis in HCC [5]. For instance, miR-122, miR-139, miR-26, and miR-140 have been reported to be associated with overall survival of patients with HCC [6]. The expression profiles of microRNAs may be different in tumors of HCC with different causes [7]. MiR-224, miR-224-3p, and miR-452 have been more frequently reported in hepatitis C virus–induced HCC as compared to healthy controls and HBV-induced liver failure [8]. miR-375, miR-92a, miR-10a, miR-223, miR-423, miR-23b, miR-23a, miR-342-3p, miR-99a, miR-122a, miR-125b, miR-150, and let-7c have been determined as novel, noninvasive biomarkers in HBV-mediated HCC [9]. Although there is an increasing amount

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of evidence about the importance of miRNAs in HCC, the data about miRNA in the recurrent tissues during the follow-up period is limited [10–12]. To date, miR-3199-2, miR-4732, miR-22, miR-139, miR-210, and miR-550a-1 were found to be associated with a high risk of recurrence in HCC [12]. However, the prognostic value of miRNAs in predicting the risk of recurrence in patients with HBV-related HCC has not been fully elucidated.

In the present study, we investigated the expression levels of 21 individual miRNAs that are related to migration, invasion, and proliferation in HBV-related HCC specimens and adjacent nontumor tissues. Also, we evaluated the potential of miRNAs to predict HCC recurrence and prognosis after LT.

MATERIALS AND METHODS

Patient Selection

In this study, we evaluated 42 patients who were treated with LT for HBV-related HCC between 2006 and 2015 at Uludag University. None of the patients received any neoadjuvant therapy. After LT, the patients were seen for assessment of tumor recurrence using serum high-resolution multidetector computed tomography. The present study was approved by the Medical Ethics Committee of Uludag University. The normal tissue of 42 patients was used as control group.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Tumor and normal specimen of 42 patients were collected from the pathology archive at Uludag University. RNA was isolated from each sample using miRNeasy FFPE kit (Qiagen, Germantown, Md, United States) according to the manufacturer's protocol. RNA integration was verified using a spectrophotometer (Beckman Coulter, Inc, Fullerton, Calif, United States). Complementary DNA was synthesized form RNA using the RT2 miRNA First Strand Kit (Qiagen). Twenty-one miRNAs that play the roles of migration, invasion, and proliferation in cancer were analyzed in 42 paired HCC tumor and nontumor tissues using A LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). SNORD48 was used as a control to normalize the expression of miRNAs.

Relative mRNA expression levels of each miRNAs were calculated using the $2^{-\Delta\Delta C_T}$ method. qRT-PCR amplification reaction was performed on a LC480 (Applied Biosystems, Foster City, Calif, United States).

Statistical Analysis

The expression profiles of each miRNA were determined using an RT2 Profiler PCR Array Data Analysis (https://www.qiagen.com/kr/shop/genes-and-pathways/data-analysis-center-overview-page/). The cutoff values for each miRNA were analyzed using the receiver operating characteristic curves (SPSS). The effect of the dysregulation of miRNAs on the prognosis was evaluated by using a χ^2 test from SPSS 23 (IBM, Armonk, NY, United States). Disease-free survival (DFS) curves were determined using the Kaplan-Meier method from MedCalc 12.4.0 statistical software (MedCalc Software, Ostend, Belgium). Figures were obtained using GraphPad Prism 6 (GraphPad Software Inc, San Diego, Calif, United States) The 95% confidence intervals for all values were calculated using the associated estimated standard errors. Here, $P < .05$ was considered significant.

Table 1. The Expression Profiles of 21 Individual miRNAs in HBV-Mediated HCC Tissues Compared With Normal Liver Tissues

miRNAs	Control Groups $2^{-\Delta\Delta C_T}$	Tumor Groups $2^{-\Delta\Delta C_T}$	Fold Regulation	*P* Value
miR-106a	2.283505	3.504982	2.5349*	.0388†
miR-10b	0.609733	0.719766	1.1805	.095625
miR-122	1.2933	2.0574	1.5908	.2376
miR-139	1.248331	3.613867	2.9182	.02983
miR-143	0.761851	0.263816	−2.591*	.021645†
miR-145	0.527137	0.214694	−3.611*	.0020†
miR-147	0.503914	0.403818	−1.241	.581101
miR-148b	1.273893	3.189293	1.1823	.2363
miR-155	0.508305	0.651175	1.1223	.457338
miR-181	0.609733	0.719766	1.1805	.095625
miR-195	0.450625	0.315038	−1.43	.223734
miR-19a	2.618517	3.742468	1.4293	.132745
miR-21	1.892115	7.032316	3.7166*	.02139†
miR-210	0.474832	0.628394	1.1252	.1726
miR-214	0.614506	0.302876	−2.029	.438
miR-22	2.614506	5.302876	2.0225	.46784
miR-221	2.283505	3.504982	1.4175	.41645
miR-223	0.293873	0.381923	1.1033	.1823
miR-24	0.450625	0.215038	−2.099	.41645
miR-26a	2.272059	1.360764	−1.139	.0091
miR-27	1.203384	2.191384	1.8211	.2837

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction.
*Evaluated with the independent sample T-test using RT2 profiler PCR array data analysis.
†Significant at $P < .05$.

RESULTS

The present study included 32 male and 10 female unrelated patients who were diagnosed with HBV-mediated HCC. Patients ages ranged from 17 to 68 years old (median age at diagnosis was 41 ± 1.12). Median tumor size was 4.21 cm (range 2.3-4.9 cm). Recurrence was observed in 5 patients within the follow-up period.

We analyzed the expression profiles of 21 different miRNAs in 42 tumor tissues and 42 normal liver tissues by using qRT-PCR. As shown in Table 1, six miRNAs were down-regulated and fifteen miRNAs were up-regulated in HBV-associated HCC compared with the matched adjacent nontumoral samples. Among these miRNAs, miR-143 (2.99-fold, $P = .0321$) and miR-145 (3.61-fold, $P = .002$) were significantly decreased and miR-106a (2.5-fold, $P = .0388$) and miR-21 (3.7-fold, $P = .0214$) were significantly increased in tumor tissues compared to nontumor tissues (Fig 1).

In the 42 studied HCC patients, the median follow-up time was 65 months (37-119 months). According to the recurrence status within the follow-up period, patients were divided into 2 groups as the high-risk group and the low-risk group. Baseline demographic features of the patients such as sex, age, or tumor localization of the 2 groups did not

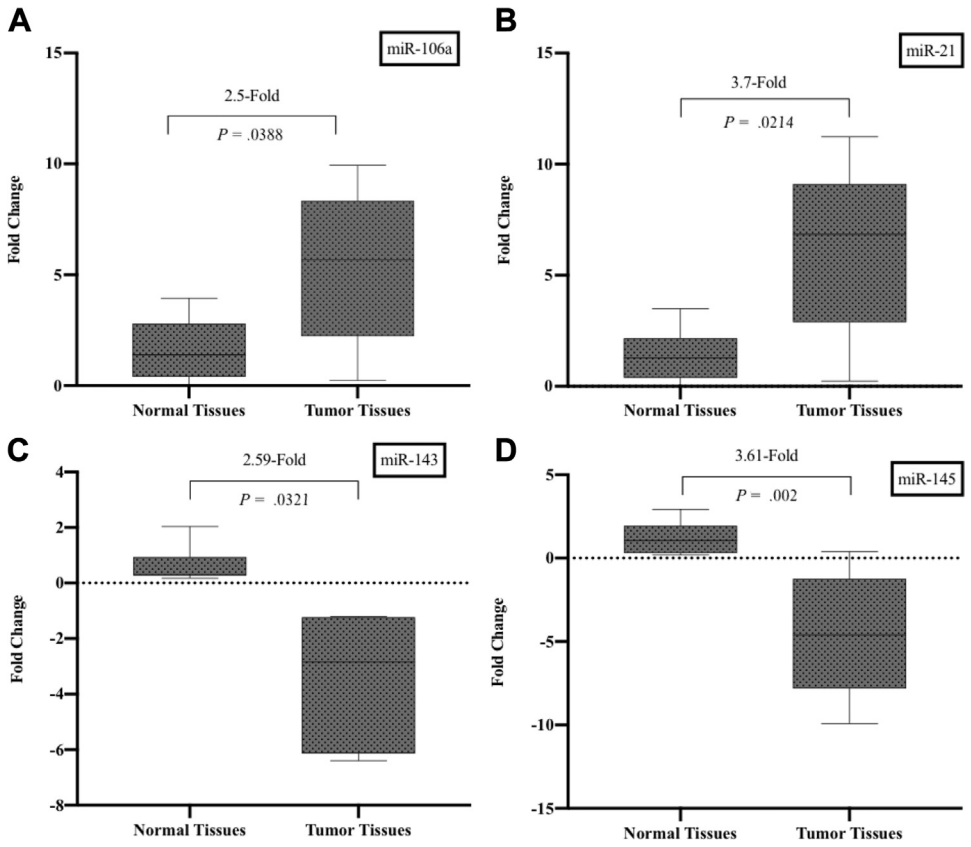

Fig 1. (**A**) The higher expression level of miR-106a, (**B**) higher expression of miR-21, (**C**) lower expression of miR-143, and (**D**) lower expression of miR-145 are detected in HBV-mediated HCC than in adjacent nontumorous tissues.

differ significantly ($P > .05$) (Table 2). To determine the relation between miRNAs with HCC recurrence, we analyzed 21 different miRNAs in the primary tissues of HBV-mediated HCC with recurrence within the follow-up period (high-risk group) compared with primary tissues of HBV-mediated HCC without recurrence (low-risk group). We observed that the increased expression of miR-21 (3.54-fold, $P = .004$) and the decreased expression of miR-145 (2.42-fold, $P = .032$) were significantly significant in the high-risk group (Fig 2). During the follow-up time, 5 cases (11.9%) developed recurrence, including 5 (100.0%) cases with high miR-21 expression levels and 3 (60.0%) cases with low miR-145 expression levels. Kaplan-Meier curves showed that patients with high miR-21 expression levels had a relatively shorter DFS time than those with low miR-21 expression levels ($P = .002$) (Fig 3). The lower expression of miR-145 was also associated with short DFS ($P = .036$). In addition, to define the risk factors associated with a

Table 2. The Effect of miRNAs Expression Levels on Prognosis

Variables	HR	Lower	Upper
miR-21 high vs low	3.019	0.219	6.939
miR-145 low vs high	1.120	0.293	2.958
Age	1.364	0.283	1.834

Cox regression analysis of DFS in HBV-mediated HCC.
Abbreviations: CI, confidence interval; DFS, disease-free survival; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio.
*Significant at $P < .05$.

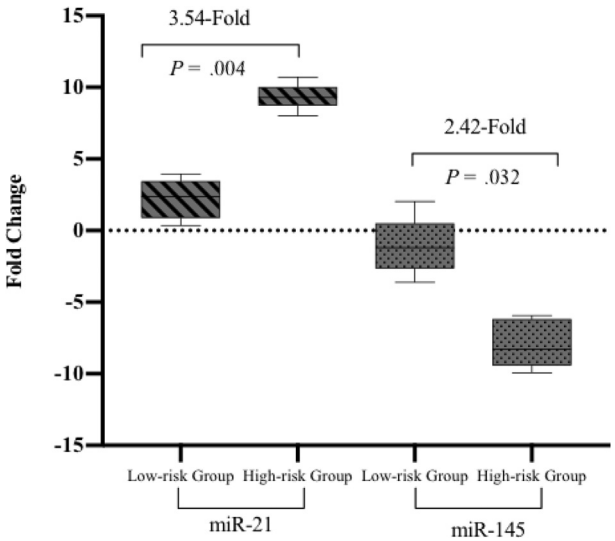

Fig 2. Dysregulations of miR-21 and miR145 were associated with recurrence in HBV-mediated HCC patients.

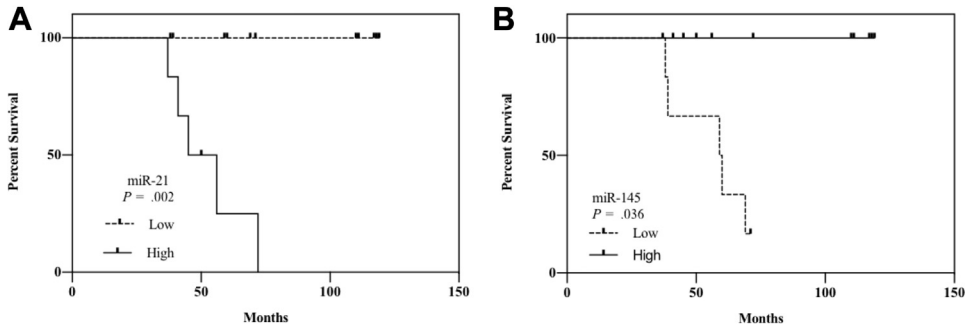

Fig 3. Kaplan-Meier curves of **(A)** miR-21 and **(B)** miR-145 expressions in HBV-mediated HCC.

recurrence for HBV-mediated HCC, the expression profiles of miR-21 and miR-145 and age were evaluated using multivariate Cox regression. The multivariate analysis showed that a higher expression level of miR-21 was a prognostic indicator for the recurrence probability of patients with HBV-mediated HCC patients ($P = .002$).

DISCUSSION

Recently, studies have identified several miRNAs for HCC diagnosis and prognosis, but these studies were performed in different population with a multitude of different underlying HCC [12–14]. Furthermore, the expression of miRNAs may differ in tumors from patients with different ethnic origins [7]. For example, down-regulation of miR-26a was indicated to be associated with recurrence in Chinese patients with HBV-mediated HCC. On the other hand, the lower expression of miR-26a was not related with a poor prognosis of HBV-mediated HCC in American cohort of patients and the white population [15]. Similarly, miR-26a was not found to be significantly significant in our cohort with HBV-mediated HCC and also recurrence. miR-210, miR-22, and miR-139 have been most frequently reported to be associated in studies on virus-mediated HCC [6]. However, miR-210, miR-22, and miR-139 were insignificant in our study cohort. We examined the differences in miRNA expression patterns between primary tumors of recurrent and nonrecurrent HBV-mediated HCC patients. In this study, we explored the role of miRNAs in HCC. We detected miR-143 and miR-145 were decreased and miR-106a miR-21 were increased in 42 patients with HBV-mediated HCC compared with normal liver tissues. Additionally, a close association between dysregulation of miR-21 and miR-145 and the formation of recurrence has been observed in HBV-mediated HCC. miR-145 expression profile in HCC remains controversial. Some studies demonstrated increased expression, whereas decreased expression was demonstrated in others. Zhao et al indicated that the expression of miR-145 was significantly decreased in the tumor tissue of patients with HBV-associated HCC compared with nontumor distal tissue [16]. However, there was no correlation between a poor prognosis and the lower expression of miR-145 in this study [16]. Our results showed that miR-145 was down-regulated in HBV-associated HCC tumors as compared with noncancerous tissues. Moreover, miR-145 was significantly significant in our cohort with recurrent tissues. miR-21 acts as an oncogene though direct repression of activity phosphatase and tensin homolog gene and promotes metastasis in tumor [17]. Numerous studies have shown that miR-21 is highly expressed in various tumors, including HCC [18–20]. Moreover, studies showed that miR-21 expression was associated with disease progression in patients with HCC [18,19]. We determined that miR-21 was up-regulated in HBV-mediated HCC tumors and it was associated with formation of HCC recurrence after LT. Additionally, according to a multivariate Cox regression model of evaluated miRNAs, age, sex, tumor localization, and tumor size, only high miR-21 expression level was found to be an independent risk factor for predicting the recurrence in HBV-mediated HCC.

The current study has several limitations. We investigated only 21 miRNAs that play specific biological roles by using a qRT-PCR. Although the current study included a homogeneous and carefully selected group of patients, its limitation was the relatively small group of patients who were analyzed.

In conclusion, we demonstrated that HBV-mediated HCC patients with high expression levels of miR-21 were associated with tumor recurrence. miRNA-21 is an independent risk factor in predicting the prognosis of patients with HCC after LT. Using this marker, a new scoring system can be designed to detect recurrence in HBV-mediated HCC patients following LT.

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