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ORIGINAL ARTICLE

MicroRNA-224 Expression and Polymorphism Predict the Prognosis of Hepatitis B Virus-Related Hepatocellular Carcinoma Patients After Liver Resection

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SUMMARY

Background: **Hepatocellular carcinoma (HCC) is one of the common lethal types of tumors all over the world. Overexpression of mircoRNA-224 (miR-224) has been reported to act as a potential biomarker for HCC patients. The goal of our study was to assess the prognostic impact of the expression and polymorphism of miR-224 in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) patients after liver resection.**
Methods: **A total of 62 cases of HBV-positive HCC patients, 17 HCC patients without HBV, and 13 healthy cases were enrolled in this study. Blood leukocyte miR-224 level were determined by qRT-PCR. Genotyping analysis of miR-224 rs188519172 was performed using an allele-specific PCR assay. All patients were undergoing partial liver resection and the prognostic values of miR-224 rs188519172 polymorphism for tumor development, survival rate, and liver injury after liver resection were examined.**
Results: **When we compared the blood leukocyte miR-224 level between HCC patients and healthy cases, we found that it was significantly increased in HCC patients. By subgroup analysis, it demonstrated that miR-224 expression was significantly increased in the HBV positive group compared with the HBV negative group. miR-224 rs188519172 AG + GG phenotype was significantly associated with severe liver injury after liver resection and patients carrying miR-224 rs188519172 AG + GG phenotype have a higher risk of cirrhosis and lower overall and disease-free survival rate. Meanwhile, the combination of miR-224 rs188519172 AG + GG phenotype and AFP value could improve the prognosis assessment of HBV related HCC.**
Conclusions: **miR-224 rs188519172 polymorphism is an indicator of liver injury and a novel prognostic biomarker for tumor development and survival of HBV related HCC patients after liver resection.**
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KEY WORDS

liver resection, HCC, microRNA, polymorphism, prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the second leading cause of cancer death in China [1]. In spite of techniques such as local ablation having been rapidly developed, liver resection is still considered the most effectual treatment methods for HCC. However, the short-term and long-term relapse free survival (RFS) after resection remains unsatisfactory mainly because of the high recurrence

rate [2]. The leading causative factors of HCC include chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, exposure to aflatoxin-contaminated food and alcohol consumption. Chronic HBV carriers have a 5 - 15-fold increased risk of HCC compared with the general population [3]. Also, HBV infection is a greater risk factor for tumor recurrence after curative treatment for HCC [4]. A number of molecular epidemiological studies have demonstrated that genetic and environmental factors both critically modulate the progression of HBV-related HCC [4-6]. Therefore, it is urgent and necessary to identify such factors as biomarkers which could predict recurrence, metastasis, and prognosis for patients with HCC to improve the individual treatment.

MicroRNAs (miRNAs) are a recently discovered class of small noncoding RNAs that are implicated in many physiological and pathological responses as post-transcriptional repressors of gene expression. Mature miRNAs can specifically bind to 3' UTRs of target cellular mRNA in turn triggering mRNA degradation or inhibition of translation [7]. Animal experiments and clinical studies have indicated that microRNAs play important roles in development of various cancers [8,9]. With the expected advances in understanding the functions of microRNAs, many microRNA biomarkers have been evaluated as novel tools in the diagnosis and monitoring of cancers [9].

Genetic variants presented in miRNA genes or genes involved in processing mechanisms may inhibit miRNA expression [10]. Moreover, SNPs located in some specified sites may weaken the affinity between miRNAs and their target mRNAs [11]. Therefore, SNPs in certain microRNA sites might affect the individualized differences of their expression or functions, and, therefore, predict the prognosis of certain diseases. In this study, we demonstrated that miR-224, which was closely related to the regulation of cell proliferation, migration, and invasion in a variety of cancer cells, was significantly up-regulated in serum samples of HBV-related HCC patients after liver resection. Notably, miR-224 rs188519172 polymorphism was associated with the treatment outcome and prognosis of patients in a prospective cohort of patients with HBV-related HCC after liver resection.

MATERIALS AND METHODS

Clinical subjects

A total of 62 cases of HBV-positive HCC patients who underwent liver resection surgery in Linyi Central Hospital were included in this study. Blood biochemistry, coagulation function, chest X-ray, and electrocardiogram were performed to assess the patient's bodily functions. Transabdominal ultrasound, CT angiography, multi-slice spiral computed tomography and a three-dimensional reconstruction system were used to ascertain the size and location of the lesion. Liver function was evaluated according to the Child-Pugh grading. For detecting the expression patterns of miR-224, 17 HCC patients who underwent liver resection surgery without HBV infection and 13 healthy cases, were used in this study as controls. The blood samples were collected before and after operation from all the patients to monitor the liver function. Blood samples from the healthy cases were collected at the same time. HCC tissue samples were obtained from all the included patients. The study was approved by the ethics committee of the Linyi Central Hospital, and informed consent was obtained from all patients before participation. All the experiments were carried out according to principles of the Helsinki Declaration.

RNA extraction

Blood leukocytes were isolated by Ficoll (Amersham Pharmacia Biotech, Sweden) density gradient centrifugation from blood samples of the patients before the operation, together with the blood samples from the healthy volunteers. The total RNA was extracted from blood leukocytes and tissues samples using the Qiagen miRNeasy® Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Then the RNA was stored at -80°C until analysis.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

qRT-PCR analyses for miRNAs were performed using TaqMan miRNA assays (Ambion) in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reverse transcription reactions were performed using the following parameters: 16°C for 30 minutes, 42°C for 30 minutes, and 84°C for 5 minutes. PCR reactions were performed using the following parameters: 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, and then 60°C for 30 seconds. U6 small nuclear RNA was used as endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle method. Relative expression was calculated using the comparative threshold cycle (Ct) method.

Genotyping

For the miR-224 rs188519172 polymorphism we used allele-specific PCR according to the previous report [11]. Briefly, two different PCR reactions are performed with one or the other allele specific primer. The primers used were a common forward:
5'CCTCAAGAATCCTCCTCACT3'
and a reverse for the G-allele:
5'GTGGTTCCGTTTAGTAGATGAC3'
and a reverse for the A-allele:
5'GTGGTTCCGTTTAGTAGATGAT 3'.

Statistical analysis

The results are expressed as means ± SD from at least 3 separate experiments performed in triplicate. The differences between groups were determined using two-tailed

Student's *t*-test, using SPSS software (Armonk, NY, USA). p-values of less than 0.05 were considered significant. The Chi-square test or Fisher's exact test was used to analyze the relationship between the miR-224 rs188519172 polymorphism and the clinicopathological features.

RESULTS

miR-224 was increased in HBV related HCC patients

Detailed demographics for all the subjects, including 62 HBV-related HCC patients, 17 HBV negative HCC patients, and 13 healthy volunteers, are listed in Table 1. There were no differences in gender, age, smoking, and drinking, among the three groups. Indicators for tumor development, including tumor size, stage, and lymphatic metastasis, did not differ between the HBV positive and negative HCC patients. Blood leukocytes from HBV-related HCC showed the highest miR-224 expression, followed by HBV negative HCC groups, and the healthy cases showed significantly decreased miR-224 expression compared with the HCC subjects (Figure 1A). Additionally, miR-224 expression was assessed in tissues of HCC, together with the non-tumor liver tissues (Figure 1B). HCC tissues showed significantly increased miR-224 expression compared with non-tumor tissues, and HBV infection could significantly induce the miR-224 expression in HCC patients. To further investigate the diagnostic value of miR-224 expression in HCC, ROC curves were constructed. Blood leukocyte miR-224 discriminated HCC patients from healthy control patients with an AUC of 0.755 (95% CI = 0.612 - 0.898). As a control, the serum AFP yielded an AUC of 0.723 (95% CI = 0.584 - 0.861) (Figure 1C). These results indicated that miR-224 might act as a biomarker for the diagnosis of HBV related HCC patients.

Association of miR-224 rs188519172 polymorphism with liver injury in HBV related HCC patients after liver resection

SNP rs188519172 is located on the miR-224 gene and might play an important role in miR-224 expression and function [11]. In this study, we initially found that miR-224 rs188519172 polymorphism was related with the liver injury in the HBV related HCC patients after liver resection. Of these patients, serum ALT levels at 24 hours post-resection were significantly raised compared with the baseline. Meanwhile, serum ALT levels were significant higher in the AG + GG genotype group, compared to the AA group at 24 hours post-resection, whereas their differences before surgery did not reach statistical significance (Figure 2A). Similar results were also seen in AST, TBIL, and DBIL (Figure 2B - D). The above data indicated that miR-224 rs188519172 polymorphism correlated with the severity of hepatic injury in the HBV related HCC patients after liver resection.

Association of miR-224 rs188519172 polymorphism with prognosis of HBV-related HCC patients

Previous studies have demonstrated that severe liver injury in the early-phase after hepatectomy might promote tumor growth and provide a favorable environment for tumor progression and invasion [12]. Therefore, we next explored the association of miR-224 rs188519172 polymorphism with the prognosis of HBV-related HCC patients. As shown in Table 2, there was a significant correlation between AG + GG genotype and cirrhosis, whereas the correlation did not reach statistical significance with the stage, tumor size, and lymphatic metastasis. Additionally, Kaplan-Meier curve and log-rank test showed that patients with AG + GG phenotype had an unfavorable overall survival prognosis (Figure 3A) and a shorter disease-free survival (Figure 3B). Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 3. By log-rank analyses, AG + GG phenotype, stage classification, lymph node metastasis and tumor size were identified as potential predictors of DFS and OS (Table 3). Multivariate analysis showed that miR-224 rs188519172 polymorphism was independently associated with OS and DFS (Table 4). The above data indicate that AG + GG genotype of miR-224 rs188519172 was associated with a worse prognosis in HBV-related HCC patients.

Combined analysis of AFP value and miR-224 rs188519172 AG + GG genotype improve the assessment of prognosis in HCC patients

As one of the most important tumor markers, AFP has been proven by many studies to predict the prognosis of HCC, independently or in combination with other factors [13,14]. Hence, the combination of AFP value (at the cutoff value of 20 ng/mL) and miR-224 rs188519172 AG + GG genotype as predictor for the prognosis were subsequently tested. Patients were divided into four groups: AFP^{high}AG + GG, AFP^{low}AG + GG, AFP^{high}AA and AFP^{low}AA. Patients having AFP^{high}AG + GG had a significant poorer overall survival rate than the other three groups, whereas the differences among AFP^{low}AG + GG, AFP^{high}AA, and AFP^{low}AA groups did not reach statistical significance (Figure 4A). Similar results were also seen in DSF analysis (Figure 4B). Meanwhile, for two-year mortality, the AFP^{high}AG + GG displayed an AUC value of 0.874 by ROC curve analysis, while AFP^{high} and AG + GG displayed AUC values of 0.742 and 0.727, respectively (Figure 4C). These clinical results suggested that the synergistic effect of AFP value and miR-224 rs188519172 AG + GG genotype might improve the assessment of prognosis in HCC patients.

DISCUSSION

HCC usually arises in the setting of cirrhosis or bridging fibrosis in HBV-associated chronic liver disease, and this pathogenesis is usually regulated by various

Table 1. Demographics and clinical features of the HCC patients and the healthy controls.

		HC (n = 13)	HCC patients				P3c value
			HBV- (n = 17)	P1a value	HBV+ (n = 62)	P2b value	
Gender	Male	9	12	0.936	45	0.807	0.871
	Female	4	5		17		
Age	> 55	6	10	0.491	34	0.568	0.770
	≤ 55	7	7		28		
Somking	Yes	7	13	0.193	41	0.402	0.417
	No	6	4		21		
Drinking	Yes	12	14	0.427	55	0.702	0.485
	No	1	3		7		
Tumor size	> 5		5		27		0.293
	≤ 5		12		35		
Stage	I/II		6		30		0.337
	III/IV		11		32		
Lymphatic metastasis	Yes		6		34		0.153
	No		11		28		
Cirrhosis	Yes		8		41		0.151
	No		9		21		

a - P_1 was calculated by comparing the difference between HBV- HCC patients with the healthy control group.
b - P_2 was calculated by comparing the difference between HBV+ HCC patients with the healthy control group.
c - P_3 was calculated by comparing the difference between HBV- HCC patients with HBV+ HCC group.

Table 2. Genotype distributions of miR-224 rs188519172 polymorphism in HBV-related HCC patients.

	miR-224 rs188519172 polymorphism			
	AA	AG + GG	p-value	OR (95% CI)
Stage				
I or II	23	7	0.146	2.248 (0.747 - 6.764)
III or IV	19	13		
Lymphatic metastasis				
Yes	20	14	0.098	0.390 (0.126 - 1.208)
No	22	6		
Tumor size				
> 5	15	12	0.071	0.37 (0.124 - 1.107)
≤ 5	27	8		
Cirrhosis				
Yes	23	18	0.006	0.135 (0.028 - 0.154)
No	19	2		

miRNAs [15]. Many studies have reported that miR-224 was one of the most commonly over-expressed miRNAs that affect diverse crucial cellular pathways in HCC pathogenesis [16-18]. Shi et al. analyzed 26 independent full-text studies retrieved from public databases holistically, finding that miR-224 was significantly increased in most of the datasets [16]. Lin et al. reported that serum miR-224 was significantly higher in early-

Table 3. Univariate 5-year overall survival and disease-free survival analysis for clinical characteristics and miR-224 rs188519172 polymorphism in HBV positive HCC patients.

Parameters	Overall survival		Disease-free survival	
	p	HR (95% CI)	p	HR (95% CI)
Age > 55 year	0.443	1.231 (0.724 - 2.094)	0.004	0.438 (0.248 - 0.773)
Stage III or IV	0.001	4.951 (2.732 - 8.971)	0.001	0.269 (0.152 - 0.478)
Lymph nodes metastasis	0.001	7.842 (0.091 - 0.718)	0.001	0.168 (0.082 - 0.344)
Tumor size > 5 cm	0.001	0.173 (3.482 - 17.66)	0.001	0.225 (0.121 - 0.418)
Cirrhosis	0.013	2.120 (1.174 - 3.828)	0.008	0.450 (0.250 - 0.812)
rs188519172 AG + GG	0.045	0.855 (0.296 - 1.863)	0.016	0.502 (0.287 - 0.879)

HR - Hazard ration.

Table 4. Multivariate cox regression analyses miR-224 rs188519172 polymorphism for overall survival and disease-free survival analysis of the HBV positive HCC patients.

Parameters	Overall survival		Disease-free survival	
	p	HR (95% CI)	p	HR (95% CI)
Age > 55 year	0.541	0.808 (0.408 - 1.600)	0.415	0.780 (0.395 - 1.542)
Stage III or IV	0.049	0.457 (0.203 - 1.032)	0.019	0.430 (0.212 - 0.873)
Lymph nodes metastasis	0.010	0.255 (0.091 - 0.718)	0.004	0.255 (0.102 - 0.640)
Tumor size > 5 cm	0.001	0.173 (0.078 - 0.383)	0.001	0.187 (0.084 - 0.414)
Cirrhosis	0.464	1.310 (0.636 - 2.698)	0.370	0.694 (0.474 - 1.083)
rs188519172 AG + GG	0.006	0.378 (0.188 - 0.759)	0.004	0.370 (0.188 - 0.728)

HR - Hazard ration.

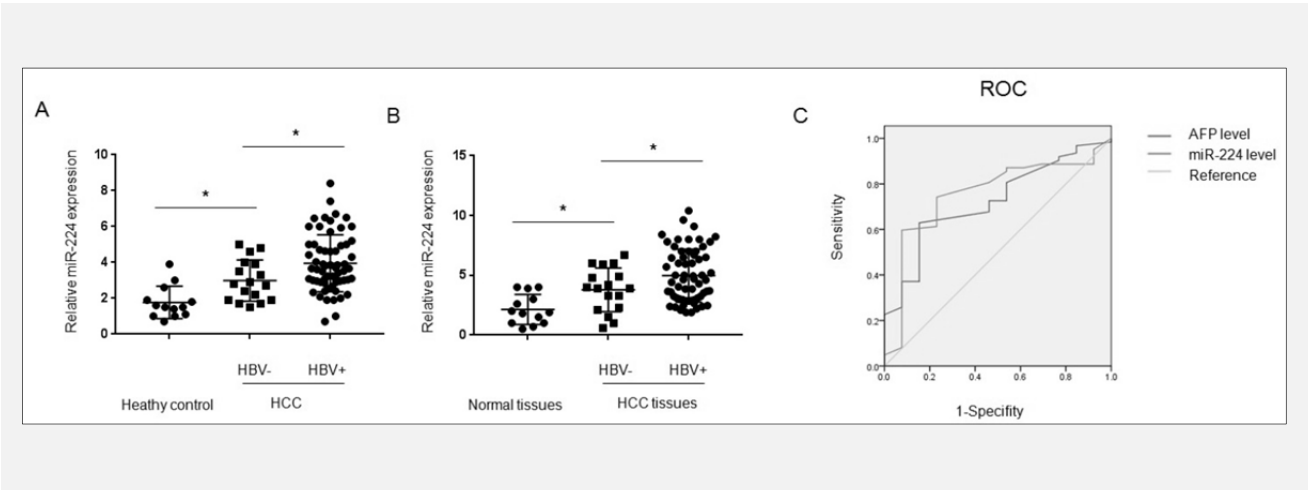

Figure 1. miR-224 expression is enhanced in blood leukocytes of HCC patients and HCC tissues.

(A) qPCR results comparing miR-224 levels in blood leukocytes among HBV negative HCC patients, HBV positive HCC patients, and healthy controls. (B) qPCR results comparing miR-224 levels between HBV negative and HBV positive HCC tissues and normal tissues. (C) The ROC curve for blood leukocyte miR-224 level and serum AFP level in relation to the HCC patients. * p < 0.05; ** p < 0.01; *** p < 0.001. qPCR, quantitative polymerase chain reaction.

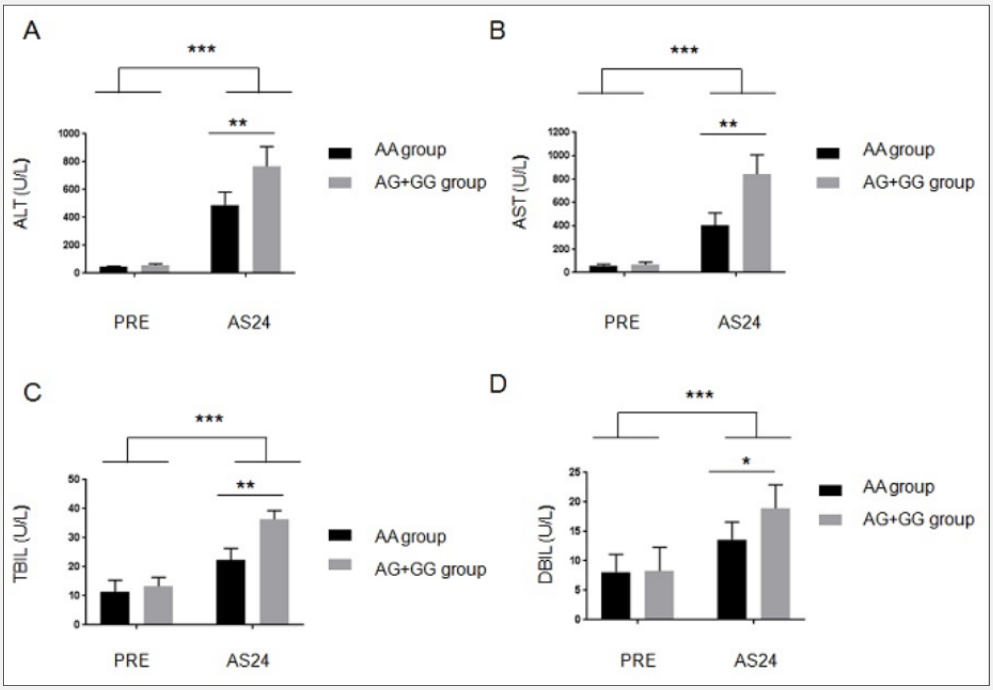

Figure 2. Association of miR-224 rs188519172 polymorphism with liver injury in HBV related HCC patients after liver resection.

Serum (A) ALT, (B) AST, (C) TBIL, and (D) DBIL levels before and after partial hepatectomy of HCC patients with different genetic polymorphisms at miR-224 rs188519172 polymorphism SNP site. PRE: before hepatectomy; AS24: 24 hours after hepatectomy. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

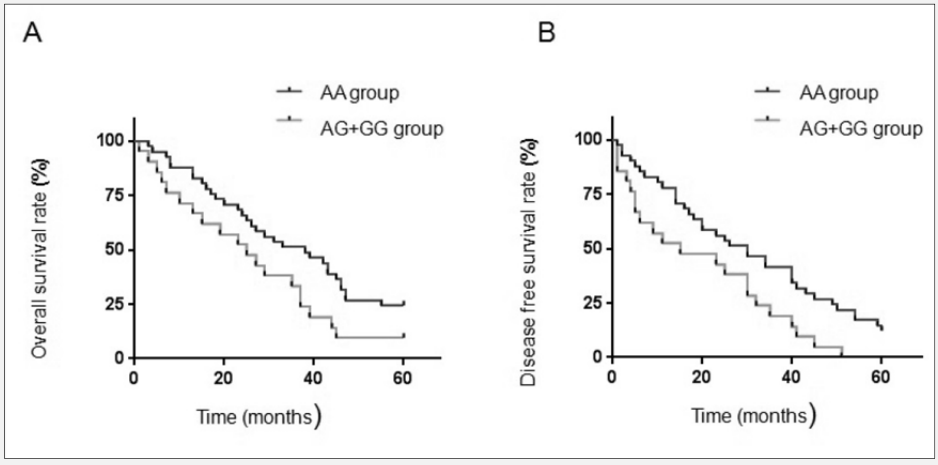

Figure 3. Association of miR-224 rs188519172 polymorphism with the prognosis in HBV related HCC patients after liver resection.

(A) overall survival and (B) disease free survival of patients with different genetic polymorphisms at miR-224 rs188519172 polymorphism SNP site. AG + GG: miR-224 rs188519172 genotype is AG or GG; AA: miR-224 rs188519172 genotype is AA.

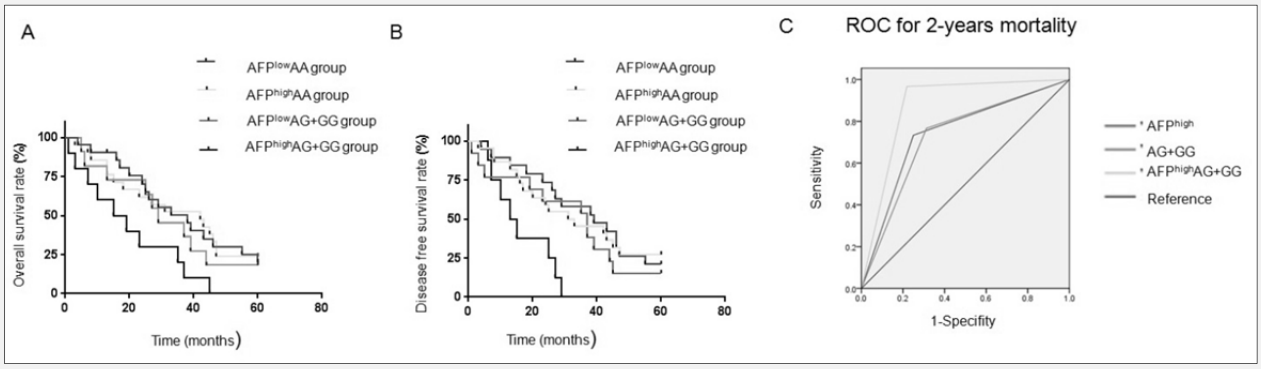

Figure 4. Combined analysis of AFP value and miR-224 rs188519172 AG+GG genotype improve the assessment of prognosis in HCC patients.

(A - B) Kaplan-Meier curves for time to (A) OS and (B) DFS of patients according to combined AFP value and miR-224 rs188519172 AG + GG genotype. (C) The ROC curve for AFP value and miR-224 rs188519172 AG + GG genotype. AFP^{high}: AFP value > 20 ng/mL; AFP^{low}: AFP value < 20 ng/mL; AG + GG: miR-224 rs188519172 genotype is AG or GG; AA: miR-224 rs188519172 genotype is AA.

stage HCC, indicating that it might act as a biomarker for detection of hepatocellular carcinoma at an early stage [17]. Okajima et al. demonstrated that miR-224 expression was significantly higher in HCC plasma, tissues, and cell lines, and this expression pattern can reflect the tumor dynamics [18]. Further, several *in vitro* and *in vivo* experiments suggested that miR-224 level might positively regulate the development of HBV associated HCC [19,20]. As a confirmation and expansion, the present study found that the blood leukocyte miR-224 level was higher in HCC patients as compared with healthy control cases. By subgroup analysis, it demonstrated that miR-224 expression was significantly increased in the HBV positive group compared with the HBV negative group. Together with the previous evidence, our results indicated that there might be tight association between miR-224 and pathogenesis of HBV related HCC.

The highlight of our research was that we found the miR-224 rs188519172 polymorphism had a statistically significant association with the clinical feature and prognosis of HBV related HCC patients. Patients carrying miR-224 rs188519172 AG + GG phenotype have a higher risk of cirrhosis and lower overall and disease-free survival rate. In multivariate Cox analysis, miR-224 rs188519172 AG + GG phenotype remained an independent prognostic factor. Patients in the miR-224 rs188519172 AG + GG group had a 0.378-fold decrease in their risk of death from any cause and a 0.370-fold decreased risk of recurring cancer, compared with patients in the miR-224 rs188519172 AA group. Furthermore, a combined survival analysis of miR-224 rs188519172 polymorphism and the AFP value revealed that AFP^{high} AG + GG phenotype predicted the poorest OS and DFS rate. All these results demonstrated that miR-224 expression and polymorphism might be an independent or combined indicator of HBV related HCC patients and a novel prognostic biomarker for tumor development and survival rate after liver resection.

It is noteworthy that our results also indicated that some patients might have more severe liver injury after hepatectomy because they carry the miR-224 rs188519172 AG + GG phenotype. The role of miR-224 in liver injury is rarely studied and is controversial. Yu et al. demonstrated that miR-224 could play a self-protective or adaptive role in APAP-induced liver injury [21]. In contrast, Hung et al. found that miR-224 promotes liver injury by down-regulating the glycine N-methyltransferase gene [20]. Varied results occur under distinct liver injury models possibly because they have different targets. Polymorphisms in miRNA genes could potentially alter various biological processes by influencing the processing and/or target selection of miRNAs [22].

CONCLUSION

Our results suggested that the AG + GG phenotype enhances the miR-224 ability to promote liver injury, and the exploration of specific molecular mechanisms is ongoing in our team. Additionally, as we know, liver injury is the major complication in the early-phase after hepatectomy, which could not only promote tumor growth but also provide a favorable environment for tumor progression and invasion [23]. Whether miR-224 plays a key role in liver injury caused tumor develop-

ment is also an attractive question that should be further elucidated.

Author Contributions:
Chaoyu Wu performed the majority of the experiments with assistance from Jian Zhang, Gongen Tang, and Yanmei Xu. Yanmei Xu and Cuihong Lu collected the clinical characteristic from the patients. Yun Li designed this subject and wrote the manuscript.

Declaration of Interest:
The authors declare that they do not have any potential conflicts of interest to disclose.

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