

A Preoperative Measurement of Serum MicroRNA-125b May Predict the Presence of Microvascular Invasion in Hepatocellular Carcinomas Patients



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Abstract

The high recurrence rate remains a major problem that strongly influenced the prognosis of hepatocellular carcinoma (HCC) patients who received hepatectomy. The presence of microvascular invasion (MVI) is regarded as the most important risk factor that contributes to the postoperative recurrence. Our previous study has hinted that serum microRNA-125b (miR-125b) was associated with MVI. The aim of the present study was to identify whether serum miR-125b can serve as a biomarker to reliably predict microvascular invasion (MVI) preoperatively. MiR-125b was quantified in 108 HCC patients' serum before they received surgery by quantitative real-time PCR (qRT-PCR). Our results revealed that MVI was associated with relapse free survival (RFS) of postoperative HCC patients; surgical margin width was associated with postoperative RFS in MVI present patients, but not in the patients without MVI. Multivariate analysis revealed that miR-125b, tumor size and AFP were the independent predictive factors associated with MVI in this cohort ($P = .001, .001, .003$, respectively). The probability of the predictive accuracy of miR-125b was 76.95% (51.32% specificity and 87.50% sensitivity), which was almost equal to the classifier established by combination of AFP and tumor size (78.82% probability, 65.63% specificity and 84.21% sensitivity). Furthermore, the combination of tumor size, AFP and miR-125b yielded a ROC curve area of 86.68% (72.37% specificity and 84.38% sensitivity). Our study indicated that serum miR-125b can be used to predict MVI of HCC patients before they received hepatic resection. Therefore, miR-125b can potentially guide individualized treatment, which helps HCC patients, with or without MVI, to benefit from different surgical approach.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the second most common cause of cancer mortality. An estimated 782,000 new liver cancer cases and 745,000 cancer deaths occurred worldwide [1]. Liver resection (LR) and orthotopic liver transplantation (OLT) are the best radical treatments and well perceived as a curative treatment for HCC in cirrhotic patients with good functional liver reserves [2]. However, the high recurrence rate of HCC in the remnant liver, which reach an incidence of more than 20% at 1 year and 70% at 5 years [3], remains one major obstacle that is strongly

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influenced the long-term survival of patients with HCC who have undergone hepatectomy. The presence of microvascular invasion (MVI) is regarded as the most important risk factor that is significantly associated with recurrence within two years after surgery [4,5]. Unfortunately, MVI can currently be detected only by postoperative histological examination, which greatly limits the usefulness for preoperative assessment of prognosis [6], let alone when patients are given nonsurgical treatments, such as radiofrequency ablation, ethanol injection or transcatheter arterial chemoembolization. And, the incidence of MVI is greater than 20% in resected HCC patients [7]. Currently, there are no effective predictors with the ability to predict MVI effectively before hepatic resection. Certain serum factors, such as AFP [8] and PON1 [9], have been identified as predictors of MVI preoperatively, but the effectiveness or convenience was far from satisfaction. Identification preoperative predictors of MVI were able to provide satisfactory reference information for clinicians to select appropriate surgical and/or therapeutic strategies for patients with HCC.

MicroRNAs (miRNA) are small non-coding RNAs and have crucial functions in human diseases, including cancer. Due to its high stability and detectability in blood plasma or serum, miRNAs constitute a novel class of non-invasive biomarkers [10]. In recent years, there are many studies investigating the possible ability of miRNAs serve as diagnostic or prognostic biomarkers in human cancers, including HCC [11,12]. miR-125b has been demonstrated to suppress the proliferation and metastasis of human liver cancer cell [13,14]. However, only a few studies have focused on identifying the predictive value of circulating miR-125b in the serum of patients with HCC [15]. Our earlier study hinted that the expression of serum miR-125b was associated with microvascular invasion [16], however, additional studies are needed to reaffirm. In this study, we detected the level of miR-125b in a cohort of 108 HCC patients' serum in order to validate its value of predicting MVI and its ability to serve as a biomarker. Our study suggested that preoperative serum miR-125b can serve as a useful biomarker that helps to reliably predict the presence of MVI before HCC patients received surgery.

Materials and Methods

Patients With HCC

One hundred eight patients with HCC who underwent hepatectomy from April 2012 to October 2013 were included in this study. The including criteria were as follow: (1) did not receive any preoperative treatment, such as ablation, TACE, radiotherapy, chemotherapy or targeted therapy, (2) preoperative liver function was Child-Pugh A degree, (3) treated with curative surgical liver resection (microscopically surgical margin free of tumor), (4) intraoperative blood loss was less than 600 ml and no intraoperative or postoperative blood transfusion, (5) single lesion or synchronism multiple primary lesion and no satellite nodules were proven by postoperative pathology, (6) liver failure or death did not happened within 30 days postoperatively.

All serum samples were collected before the patients had received surgery at Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS), between April 2012 and October 2013 and stored at -80°C refrigerator. This study was approved by ethics committee approval from cancer hospital CAMS, and all the participants signed written informed consent forms.

miRNA-Specific Quantitative Real-Time RT-PCR

Serum samples from 108 HCC patients were analyzed by using miRNA-specific quantitative real-time RT-PCR. miRNA was isolated using a mirVana PARIS kit (Ambion). Megaplex RT reactions and

pre-amplification reactions were run according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Let-7d was used as an internal control for normalization [17]. Real-time PCR was performed using the StepOne Plus Real-time system (Applied Biosystems, Foster City, CA, USA) and fold changes in gene expression were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [18]. The mean miRNA level from three real-time quantitative PCR experiments was calculated for each case.

Follow-Up

Patients were followed-up at 3-month intervals for the first 2 years and at 6-month intervals thereafter. During the follow-up visit, alpha-fetoprotein (AFP), liver function, chest x-ray, enhanced computed tomography (CT) and/or enhanced magnetic resonance imaging (MRI) were performed. If inner-hepatic-recurrence was suspected, the lesion was confirmed by hepatic digital subtraction angiography (DSA) and/or contrast-enhanced ultrasound (CEUS). The recurrence status was determined as described before [16]. Relapse free survival was defined as the time interval from the date of surgery to the time of initially detected recurrence/progression or censored on the last follow-up. The last follow-up was May 2015. Until then, 55 patients developed recurrence.

Survival Analysis

Based on MVI and surgical margin width, the Kaplan-Meier estimator was used to calculate the median survival time of the relapse free survival (RFS) and to describe the survival curve. A comparison of survival analysis was performed and the *P* value was calculated by the log-rank test. According to pathologically proven MVI status, the patients were divided into 2 groups: MVI present group and MVI absent group. The surgical margin was divided into the following 2 categories as the subgroups: wide surgical margin group (WSM group) with a negative surgical margin historically measured as greater more than 10 mm in width, narrow surgical margin group (NSM group) with a negative surgical margin historically measured less than 10 mm in width.

Statistical Analysis

SPSS 19.0 software was used for the statistical analysis. The *P* value was bilaterally tested, and values less than 0.05 were regarded as statistically significant. The patients were divided into groups based on the median values of continuous variables or discrete variables. The significance of differences between each pair of groups that created was assessed by the chi-square test and fisher exact test. Student *t* test was used to compare the continuous variables. Univariate and multivariate logistic regression analysis were done to evaluate the association of the miRNA signature and clinicopathological data to MVI, respectively. The *P* values were calculated using the Wald test.

Logistic regression analysis was performed to analyze various combinations of clinical parameters and miR-125b. Using the independent predictors of MVI according to multivariate analysis, the receiver operating characteristic (ROC) curves were created and the area under the curve (AUC) was used to determine the feasibility. The Youden's Index was used to identify the optimal cut-off point. As defined, the corresponding sensitivity and specificity was showed.

Results

The Level of Serum miR-125b was Correlates With MVI and Envelop Invasion in 108 HCC Patients

We divided the 108 patients into two groups based on the median value of the expression level of miR-125b. As shown in Table 1, low

Table 1. Correlation Between the Level of Serum miR-125b and Clinicopathologic Features in 108 HCC Patients Underwent Hepatectomy

	All Cases	miR-125b Expression Level		P Value
		Low Expression (n = 54)	High Expression (n = 54)	
Age				
≥60	38	21	17	.420
<60	70	33	37	
Gender				
Male	89	45	44	.800
Female	19	9	10	
Tumor size				
≥5cm	43	24	18	.236
<5cm	66	30	36	
Tumor multiplicity				
Single	97	49	48	.750
Multiple	11	5	6	
MVI				
Present	32	23	9	.003
Absent	76	31	45	
Envelope invasion				
Present	63	38	25	.011
Absent	45	16	29	
Histological grade				
Well	14	5	9	.396
Moderate	78	42	36	
Poorly	16	7	9	
BCLC				
A	72	33	39	.221
B	36	21	15	
Viral hepatitis				
Negative	4	1	3	.435
HBV	98	49	49	
HCV	6	4	2	
Cirrhosis				
Present	91	48	43	.186
Absent	17	6	11	
AFP (ng/ml)				
≥400	25	12	13	.820
<400	83	42	41	
GGT (U/ml)				
≥55	49	21	28	.176
<55	59	33	26	

expression of miR-125b was significantly correlated with MVI and envelope invasion ($P = .003$ and $P = .011$, respectively). No significant association was found between miR-125b and other clinic-pathological features, such as the patient's age, gender, tumor size, tumor multiplicity, histological grade and BCLC stage, viral hepatitis, cirrhosis, AFP and GGT ($P > .05$).

MVI was Associated With Relapse Free Survival of PostOperative HCC Patients

As shown in Table 2, association of MVI and the other clinical parameters were analyzed with univariate analysis. Tumor size, BCLC stage, AFP and envelop invasion were significantly associated with MVI, whereas other features, including age at diagnosis, gender, differentiation, tumor multiplicity, cirrhosis, viral hepatitis and GGT were not.

According to the presence or absence of MVI, 108 HCC patients were divided into two groups. The Kaplan-Meier curves of relapse free survival (RFS) according MVI was plotted in Figure 1, the group of patients without MVI had a longer RFS than the group with MVI, the median RFS was 9.07 month and 30.73 month, respectively ($P = .000$).

The Prognostic Value of the Width of the Surgical Margin in HCC Patients

As shown in Figure 2A, 57 patients had a wide surgical margin (>10 mm), 51 patients had a narrow surgical margin (≤10 mm)

Table 2. Correlation Between MVI and Clinicopathologic Features in 108 HCC Patients Underwent Hepatectomy

	MVI Present (n=32)	MVI Absent (n=76)	P Value
Age (years)	53.88±11.69	53.64±11.26	.924
Gender (Male/Female)	26/6	63/13	.838
Tumor size (cm)	6.53±3.92	3.93±2.10	.001
Tumor multiplicity (Single/Multiple)	29/3	68/8	1.000
Envelope invasion (Present/Absent)	26/6	37/39	.002
Differentiation (Well/Moderate/Poorly)	1/26/5	13/52/11	.140
BCLC (A/B)	14/18	58/18	.001
Viral hepatitis (Negative/HBV/HCV)	1/29/2	3/69/4	.960
Cirrhosis (Present/Absent)	27/5	64/12	.983
Ln (AFP)*	5.56±3.37	3.29±2.48	.001
Ln (GGT)*	3.95±0.79	3.87±0.71	.569

* Ln (natural logarithms): the primitive value of AFP and GGT was skew distribution in the groups, the logarithmic transformation was performed to make the value fitted Gaussian distribution.

based on a microscopic examination. The surgical margin width was significantly different between the groups ($15.61 ± 3.5$ mm vs. $2.61 ± 2.15$ mm). The median relapse-free survival time in patients with a wide surgical margin (>10 mm) and a narrow surgical margin (≤10 mm) were 30.73 months and 22.97 months, respectively. There was no significant difference among the groups ($P = .129$, Figure 2B).

The Kaplan-Meier curves were also used to analyze the association of the surgical margin width and RFS in the subgroup of patients with or without MVI, respectively. As shown in Figure 2C and D, a wide surgical margin status provided a favorable RFS time in the patients with MVI: the median relapse-free survival time for the narrow (n = 15) and the wide (n = 17) margin groups were 8.03 months and 16.50 months, respectively. The difference was significant ($P = .007$, Figure 2C). On the other hand, the surgical margin status did not influence the RFS in patients without MVI: the median relapse-free survival time for the narrow (n = 36) and the wide (n = 40) margin groups were 33.67 months and 30.73 months, respectively. The difference was not significant ($P = .761$, Figure 2D). Our results revealed that surgical margin width was associated with postoperative relapse free survival in MVI present HCC patients, but not associated with postoperative relapse free survival in MVI absent HCC patients.

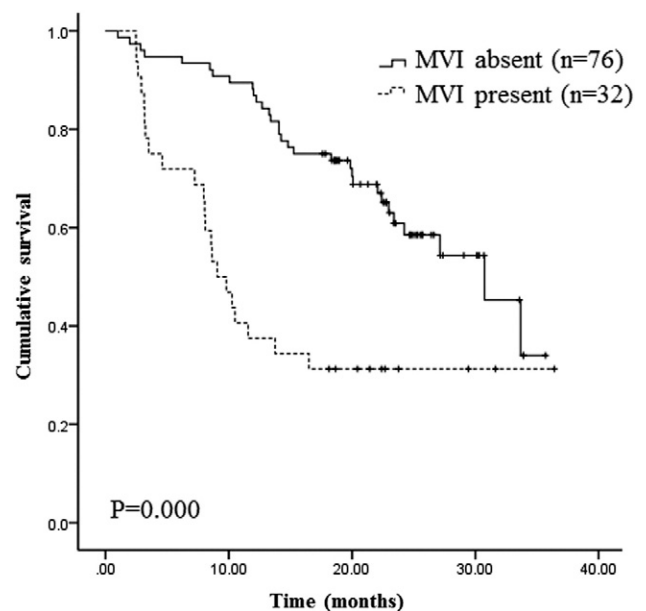


Figure 1. Microvascular invasion (MVI) was associated with relapse free survival.

A

	MVI Absent	MVI Present
Narrow surgical margin (≤10 mm)	36 (33.33%)	15 (13.89%)
Wide surgical margin (>10mm)	40 (37.04%)	17 (15.74%)

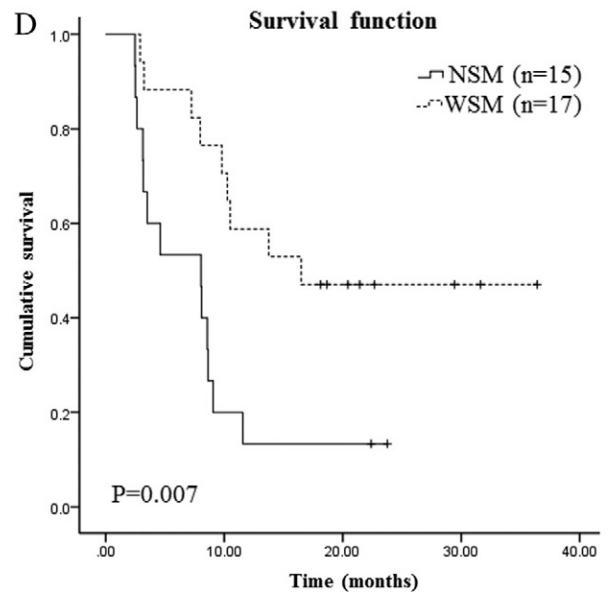
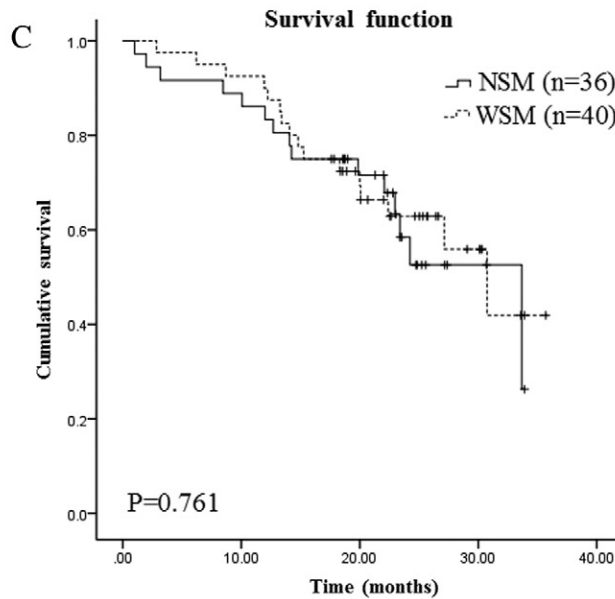
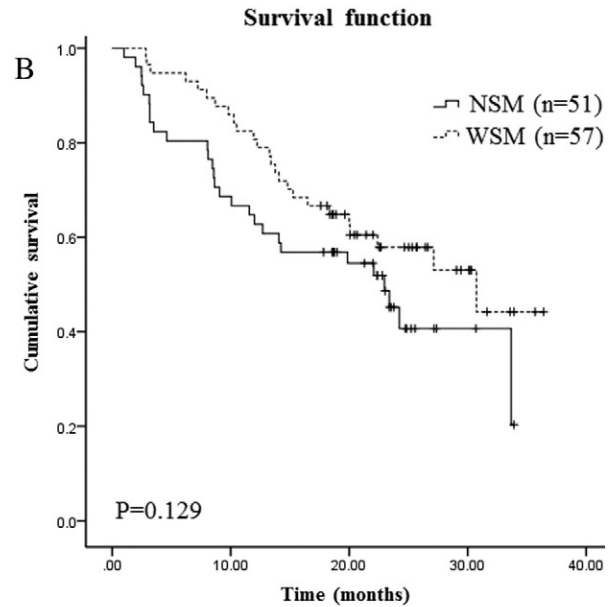


Figure 2. Association of the width of the surgical margin and relapse free survival by Kaplan-Meier curves and the log-rank test. A, According to the surgical margin width, the case number in each subgroup was showed in the table. B, 108 patients with narrow surgical margin (NSM) or wide surgical margin (WSM). C, In the subgroup of HCC patients without MVI, patients with narrow surgical margin or wide surgical margin. D, In the subgroup of HCC patients with MVI, patients with narrow surgical margin or wide surgical margin. MVI: microvascular invasion. NSM: narrow surgical margin. WSM: wide surgical margin.

The Classifier of miR-125b for Predicting the Presence of MVI of Preoperative HCC Patients

As positive postoperative MVI is a strong predictor of recurrence for HCC patients after resection, we wanted to identify pre-operative factors that are independently associated with MVI. Multivariate analysis revealed that miR-125b, AFP and tumor size were the independent predictive factors associated with MVI (Table 3). Then, the discriminative power of these factors in predicting MVI of HCC patients before operation was verified. To evaluate the predictive value, the ROC curve was used to analyze the sensitivity and specificity. As shown in Figure 3, the ROC curve of miR-125b showed an AUC of 76.97% (87.50% sensitivity and 51.32% specificity), which was almost equal to the classifier established by combination of AFP and tumor size

(78.82% probability, 84.21% sensitivity and 65.63% specificity). Furthermore, the combination of AFP, MVI and miR-125b yielded a ROC curve area of 86.68% (84.38% sensitivity and 72.37% specificity). The results demonstrated that the combination of AFP, MVI and miR-125b was a much more powerful discrimination tool in predicting MVI of HCC patients before they received operation.

Discussion

Many risk factors may contribute to the postoperative recurrence, including AFP, satellite nodal, tumor size, MVI and TNM stage [19]. Among them, the presence of MVI has been reported to be the most significant independent risk factor affecting relapse-free survival following curative resection and/or liver transplantation [20–23], the

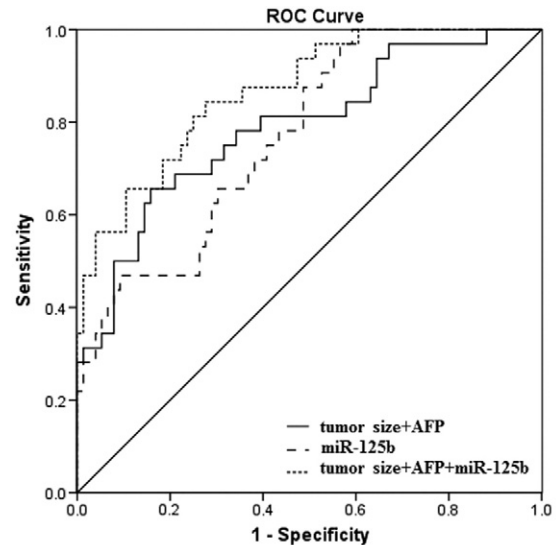
Table 3. Univariate and Multivariate Cox Proportional Hazards Regression Analysis of MVI in Relation to miR-125b and Clinical Parameters of 108 HCC Patients

Variable	Logistic			
	Univariate Analysis		Multivariate Analysis	
	HR (95%CI)	P Value	HR (95%CI)	P Value
miR-125b	0.409 (0.263-0.635)	.000	0.371 (0.211-0.654)	.001
Tumor size	1.449 (1.193-1.761)	.000	1.522 (1.181-1.960)	.001
AFP	1.301 (1.121-1.510)	.001	1.348 (1.104-1.645)	.003
Envelope invasion	4.568 (1.688-12.356)	.003		
BCLC	4.143 (1.725-9.949)	.001		
Differentiation	1.743 (0.778-3.902)	.177		
GGT	1.181 (0.670-2.081)	.566		
Hepatitis	1.216 (0.313-4.717)	.778		
Cirrhosis	1.012 (0.325-3.154)	.983		
Tumor multiplicity	0.879 (0.218-3.553)	.857		
Gender	0.894 (0.307-2.606)	.838		
Age	1.002 (0.966-1.039)	.923		

odds ratio (OR) reach the level as high as 28.40 [8]. In the present study, the RFS rates at 1 year for HCC patients with or without MVI were 62.5% (20/32) and 13.2% (10/76), respectively, which are consistent with the previous study [24]. Our data combined with the results of other studies indicated that MVI in HCC patients could provide a more consistent and reliable prediction of poor prognosis.

In addition, the invasion of the microvasculature is regarded as the main way to spread cancer cells both in the hepatic circulation and the systemic circulation before liver resection and/or liver transplantation in patients with MVI [25]. And, the incidence of MVI was closely related to the distance from the tumor capsule of the primary HCC [26]. A previous study concluded that MVI was found beyond 1 cm from the tumor capsule of the primary tumor in only a few HCC patients [5]. Accordingly, a more than 1-cm resection margin might be adequate for the majority of patients to reduce tumor recurrence. However, preserving non-tumorous liver parenchyma is also an important consideration, especially in cirrhotic liver, for decreasing the incidence of postoperative liver failure and improving the chance of performing multidiscipline-team treatment in case of tumor recurrence. A recent study demonstrated anatomic resection could achieve better survival than non-anatomic resection in HCC patients with MVI [23]. On the contrary, anatomic resection and non-anatomic resection brought similar survival in patients without MVI, whereas non-anatomic resection could preserve more remnants liver [24]. In this cohort, the RFS of the wide margin group was better than the narrow margin group, but the differences did not reached statistical significance ($P = .129$). However, the RFS for patients with MVI was significantly better in the wide margin group than the narrow margin group, despite the small sample size (wide/narrow, 17/15). The result implied that a wide surgical margin is more advantageous for HCC patients with MVI. On the other hand, the RFS for patients without MVI was no significant difference between the wide and narrow margin group, implying that a narrow resection margin could be benefit for HCC patients without MVI and could preserve as much non-tumorous liver parenchyma as possible. All of these indicated that the subgroup of HCC patients with or without MVI can benefit from different surgical approach.

Since MVI is a histopathologic diagnosis, surgical specimens examination is still the only accurate method for assessing MVI until now [27]. It cannot be confirmed prior to the surgery. This naturally leads to questions about how to identify the existence of MVI preoperatively. Although previous studies showed that diffusion-weighted imaging (DWI) of MR was useful in predicting MVI for HCCs [28] and



	HCC (n=108)		
	tumor size +AFP	miR-125b	tumor size+AFP+miR-125b
Sensitivity	84.21%	87.50%	84.38%
Specificity	65.63%	51.32%	72.37%
AUC	78.82%	76.97%	86.68%

Figure 3. Receiver operating characteristic curve analysis for predicting the presence of microvascular invasion of HCC patients preoperatively. ROC curves for the combination of tumor size and AFP, miR-125b and the combination of tumor size, AFP and miR-125b in total 108 patients, respectively. The sensitivity, specificity and AUC were indicated below the ROC graph.

infiltrative tumor margin in CT scan had a significant correlation with MVI [29], detection of MVI using preoperative radiological imaging still remains a difficulty even using the latest imaging procedures, such as ultrasonography, CT and MRI. Some other studies demonstrated that tumor size and high AFP level [8,30], and histological grade [31] were associated with higher rates of MVI, but using clinical data to identify MVI accurately is also far from satisfaction. In our study, tumor size and preoperative AFP level were independent risk factor associated with MVI, which are consistent with previous reports. But the predictive accuracy and efficiency of these two factors was dissatisfaction. In recent years, miRNAs have been proposed as novel diagnostic tools for classification and prognostic stratification of HCC. Here, when a third parameter, serum miR-125b, was added to the classifier that established by combined tumor size and AFP to predict MVI preoperatively, the probability was further improved from 78.82% to 86.68%. The results demonstrated that the combination of tumor size, AFP and miR-125b was a much more powerful discrimination tool in predicting MVI preoperatively and may provide clinicians with an opportunity to make a more accurate predictive diagnosis of MVI.

In summary, given the fact that MVI has a pivotal impact on recurrence and surgery plan selection of HCC patients, determining an accurate preoperative prediction of MVI is essential needed. Our study found that the combined classifier of tumor size, serum miR-125b and preoperative AFP level is a reliable tool with sufficient accuracy in predicting MVI in HCC patients before they received hepatic resection. But the results of our study have some limitations

due to its retrospective, single-center design and limited sample size. More randomized controlled trials with a large sample size are needed to further confirm the results.

Disclosure of Potential Conflicts of Interest

There are no conflicts to disclose.

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