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ORIGINAL RESEARCH

Identification of Cholangiocarcinoma Associated with Hepatolithiasis via the Combination of miRNA and Ultrasound

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Background: Identification of cholangiocarcinoma (CCA) associated with hepatolithiasis (HL) is difficult. There is no effective method to discriminate CCA associated with HL (HL-CCA) from HL currently.

Objective: To explore the value of clinical data, ultrasonic characteristics and miRNA expression level in the identification of HL-CCA.

Methods: Thirty-one patients with HL-CCA in Huazhong University of Science and Technology Union Shenzhen Hospital were enrolled in the observation group, while 40 patients with HL alone were included in the control group. The clinical data, ultrasonic characteristics, and miRNA expression level of the two groups were recorded and analyzed to explore the potential indicators for the identification of HL-CCA.

Results: The accuracy of ultrasound in the diagnosis of HL-CCA was low (54.84%). Multivariate logistic regression analysis showed that liver abscess (P=0.021), indistinct border demarcation (P=0.015), non-homogenous echotexture (P=0.019), missed portal vein around lesion (P=0.032), miRNA-21 (P=0.018) and miRNA-221 (P=0.009) were the potential indicators for the identification of HL-CCA. The combined diagnosis based on logistic regression contained liver abscess, border demarcation, echotexture, portal vein around lesion, miRNA-21 and miRNA-221. The results showed that the accuracy of combined diagnosis identifying HL-CCA was the most accurate (AUC=0.911), which was significantly greater than the AUC of miRNA-21 or miRNA-221 individually (P<0.05), with a sensitivity and specificity of 77.42% and 97.50%, respectively.

Conclusion: Patients with HL-CCA show high incidence of hepatic abscess and elevated miRNA-21 and miRNA-221 expression level. The ultrasonic features are more likely to show indistinct border demarcation, non-homogenous echotexture, and missed portal vein around lesion. The combined diagnosis is more accurate in the identification of HL-CCA.

Keywords: cholangiocarcinoma, hepatolithiasis, microRNA, differential diagnosis, combined diagnosis, ultrasound

Cholangiocarcinoma (CCA) associated with hepatolithiasis (HL) is a malignant tumor originating from the bile duct.[1,2] The early symptoms of CCA associated with HL (HL-CCA) are not obvious, and they are easily confused with biliary inflammation caused by gallstones.[3,4] Accurate diagnosis of HL-CCA is challenging and it is usually at an advanced stage when diagnosed, which indicates a worse prognosis and treatment outcomes. In addition, studies have reported an increased incidence of concurrent CCA in patients with HL.[5–7] Hence early identification of HL-CCA is of great importance. Detection of HL-CCA is dependent on imaging

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modalities, such as ultrasound, CT, and MRI. However, it is difficult to differentiate CCA from fibrosis in HL since prolonged affected liver segments often become fibrotic and scarred. Ultrasound is the primary imaging modality for hepatobiliary diseases. But in the cases of HL-CCA, clinicians tend to rely on the characteristics of HL to attribute infiltration features to inflammation of the bile duct wall. Even if the tumor is developed at the middle and advanced stages, it is difficult to distinguish concomitant HL-CCA and HL only by ultrasound.[8] Tumor markers such as CA19-9, CEA are commonly used indicators for the identification of benign and malignant liver tumors, but their roles in the identification of HL-CCA is controversial.[9,10] To date, there is no effective method to differentiate concomitant CCA in HL. In recent researches, the changes in miRNA may be associated with the development of tumors, and their association with CCA has gradually been confirmed. Correa-Gallego et al[11] analyzed the miRNA by deep sequencing technology and found that the expression levels of miRNA-21 and miRNA-221 in patients with CCA were significantly higher than in normal people. However, the role of microRNA in the identification of HL-CCA remains unclear. This study analyzed the clinical data, ultrasonic features and miRNA expression level in the patients with HL-CCA, and explored the value of different indicators in the differential diagnosis of HL alone and HL-CCA, aiming to find an appropriate identification method to further help the management of HL-CCA.

Materials and Methods

Participants

Seventy-one patients with HL who were admitted to Huazhong University of Science and Technology Union Shenzhen Hospital from January 2010 to June 2018 were recruited. Inclusion criteria were as follows: (1) Patients underwent surgical treatment and the pathological results were completely preserved. (2) Complete physical examinations, blood routines, tumor markers, and ultrasound examinations were performed and the results were preserved within 1 week before surgery. Patients combined with other malignant tumors or severe cardiovascular and cerebrovascular diseases were excluded in this study. According to the postoperative pathology, patients were divided into the observation group if HL-CCA was confirmed, while they were divided into the control group if HL alone was confirmed. The study was approved by the ethics committee of Huazhong University of Science and Technology Union Shenzhen Hospital (NO. 103004). All patients included in the study had a detailed understanding of the research content and signed informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Research Methods

Data Collection

Patients' clinical data were collected in the study, including age, gender, family history of malignancy, liver or back pain, liver fibrosis, liver abscess, cirrhosis, portal hypertension, cholangitis, secondary bile duct stricture, and history of hepatitis B. The serological indicators, including serum alkaline phosphatase (ALP), alanine aminotransferase (AST), aspartate aminotransferase (ALT), glutamyl transpeptidase (GGT), total bilirubin (TBIL), carcinoembryonic antigen (CEA), carbohydrate antigen (CA19-9) were detected by Hitachi automatic biochemical analyzer 7060 (Hitachi, Yokohama, Japan).

Ultrasound Examination

In the fasting state, the patient was placed in the supine position and exposed to the upper abdomen. Ultrasound was performed using Resona 7 ultrasound diagnostic system (Mindary, Shenzhen, China) and the Acuson S2000 ultrasound system (Siemens, Erlangen, Germany). The ultrasonic characteristics of the lesion area were recorded in detail, including diameter, location, shape, border demarcation, echo density, echotexture and posterior attenuation. Meanwhile, the situation of intrahepatic bile duct dilatation and portal vein around lesion were observed.

miRNA Detection

Before surgery, 5 mL-fasting venous blood sample of each patient was collected. The sample was centrifuged at 4°C and temporarily stored in a refrigerator at −80°C. RNA was extracted from plasma samples and reversely transcripted to cDNA. The reverse transcription results were detected using a 7300-type real-time PCR instrument (Applied Biosystem, USA), and the relative concentrations of miRNA-21, miRNA-34c, miRNA-200b, and miRNA-221 were recorded.

Statistical Methods

All data were processed using Statistical Product and Service Solutions (Chicago, IL, USA) software (version 22.0) and plotted by R package version 3.6.2 and MedCalc version 12 (MedCalc Software, Ostend, Belgium). The

categorical variables were expressed in number (percentage), and the chi-square test and Fisher's exact test were used for comparison. The numerical data conforming to the normal distribution were expressed as , and independent sample *t*-tests were used for comparison. The numerical data that did not meet the normal distribution were expressed as median (interquartile range), and Mann–Whitney *U*-tests were used for comparison. The association of potential variables with the risk of HL-CCA was performed using multivariate logistic regression analysis. ROC curves were established to evaluate the accuracy of potential indicators for identifying HL-CCA. Statistical significance was defined as 2-tailed P<0.05 for all tests.

Results

Ultrasonic and Pathological Features of HL-CCA

In this study, 40 patients with HL (Control Group) were accurately diagnosed by ultrasound. The ultrasound images of HL were mainly characterized by fine-like, spot-like round or clump-like hyperechoic mass with irregular shape in the liver. The gallstones were mostly located in the left lobe of the liver. The hyperechoic mass caused by gallstones in HL were distributed along the intrahepatic bile duct. It often merged with a dendritic expansion of intrahepatic bile duct, and was located in the dilated bile duct. The diagnostic accuracy of ultrasound for HL-CCA was low. In this study, only 17 of 31 patients with HL-CCA (Observation group) were correctly diagnosed (54.84%). The ultrasonic features of HL-CCA were mainly characterized by irregular clump-like hyperechoic mass in the liver, and the border demarcation between the masses and the bile duct wall were indistinct. The mass was displayed in isoechogenicity or mixed echogenicity, and it often surrounded the gallstone which showed a hyperechoic mass. At the bile duct truncation, the mass often protruded into the lumen. The portal vein around lesion of HL-CCA were hazy or missed. Pathological examination showed that there were 15 cases of papillary carcinoma, 14 cases of tubular adenocarcinoma, 2 cases of mucinous adenocarcinoma (Figure 1).

Comparison of the Clinical Data Between the Observation Group and Control Group

Compared with the clinical data between the observation group and control group, the proportions of liver abscess and cirrhosis in the observation group were close to 20%, which were greater than those in the control group (P<0.05). The remaining clinical data were similar in the two groups (P>0.05, Table 1).

Comparison of the Ultrasonic Characteristics Between the Observation Group and Control Group

Compared with the ultrasonic characteristics between the observation group and control group, the proportions of indistinct border demarcation, non-homogenous echo texture, missed or hazy portal vein around lesion in the observation group were higher than those in the control group (P<0.05). The remaining ultrasonic characteristics were similar between the two groups (P>0.05, Table 2).

Comparison of the Laboratory Indicators Between the Observation Group and Control Group

The expression of miRNA-21 and miRNA-221 in the observation group was higher than those in the control group (P<0.05), while the remaining indicators were similar in the two groups (P>0.05, Table 3).

Associations of Differentiated Indicators with the Risk of HL-CCA

Multivariate logistic regression analysis was performed to further analyze the differentiated indicators between the two groups to explore the potential identification value for HL-CCA. It revealed that except the cirrhosis, the liver abscess (P=0.021), indistinct border demarcation (P=0.015), non-homogenous echotexture (P=0.019), missed portal vein around lesion (P=0.032), miRNA-21 (P=0.018) and miRNA-221 (P=0.009) were the potential indicators for the identification of HL-CCA (Figure 2).

Accuracy Analysis of the Diagnosis for HL-CCA

The accuracy of the potential indicators identifying HL-CCA independently was not high. The specificity of liver abscess was high (97.50%) but the sensitivity was very low (22.58%), indicating that it would cause a large number of missed diagnosis. The specificity of the border demarcation, echotexture and the sensitivity of the portal vein around lesion were less than 60%, suggesting that the border demarcation, echotexture and portal vein around lesion were not suitable for the identification of HL-CCA independently. The AUC of miRNA-221 identifying

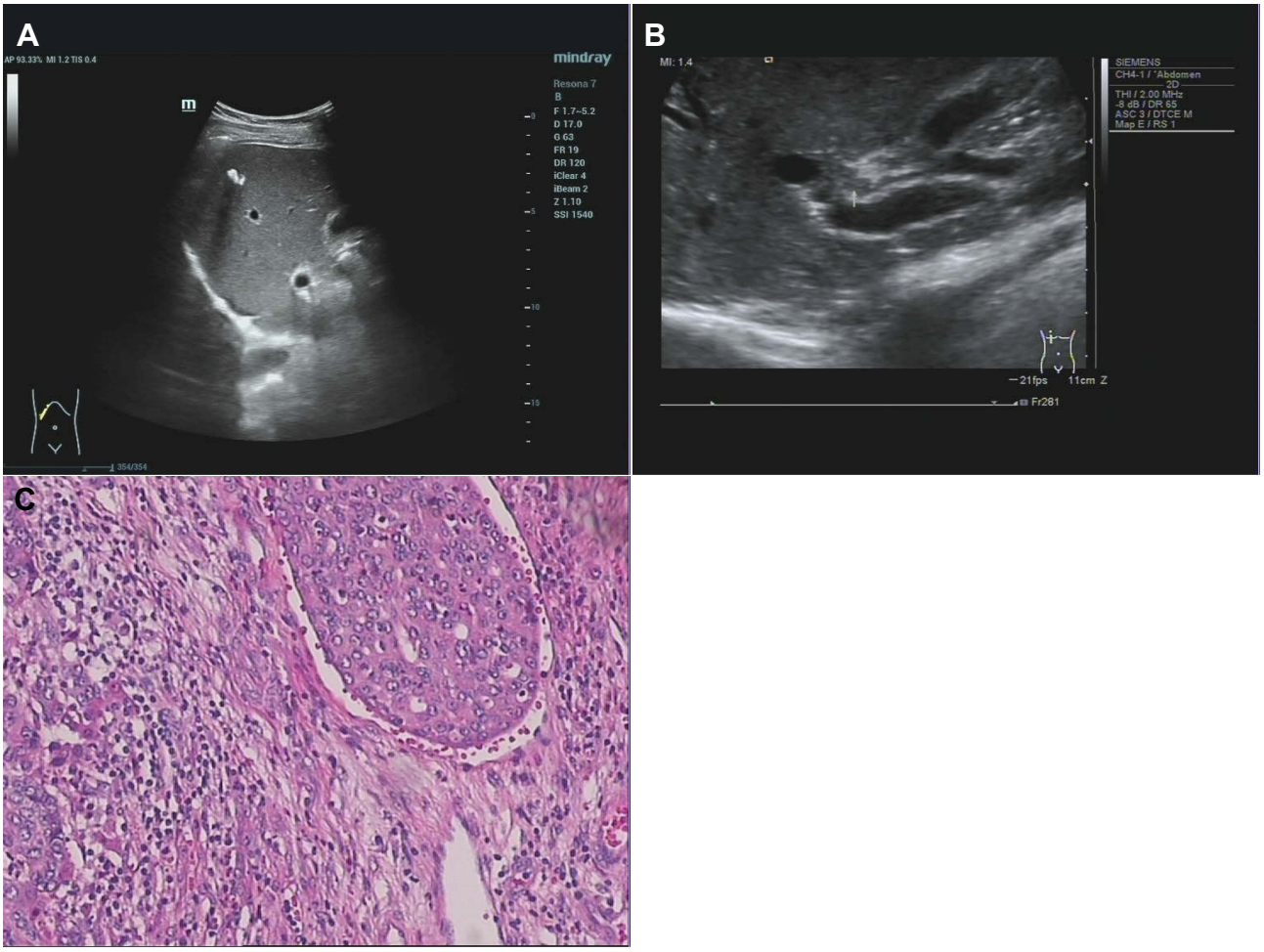

Figure 1 Ultrasonic and pathological features of HL-CCA. (**A**) Ultrasonic feature of HL. (**B**) Ultrasonic feature of HL-CCA. (**C**) Hematoxylin-eosin staining of HL-CCA (magnification 400×). The cancerous tissues are arranged in a cord-like, nest-like shape, and some are glandular structures.

HL-CCA was below 0.8 (greater than miRNA-21), suggesting that it was also not suitable for identification. These results revealed that the identification of HL-CCA was very difficult, and the individual identification of potential indicators could not achieve an ideal accuracy. This study combined liver abscess, border demarcation, echotexture, portal vein around lesion, miRNA-21 and miRNA-221 based on logistic regression model in order to improve the identification accuracy. It found that the accuracy of the combined diagnosis was the highest (AUC=0.911), which was significantly greater than the AUC of miRNA-21 and miRNA-221 ($P<0.05$). The best diagnostic point was 0.48, and the sensitivity and specificity was 77.42% and 97.50%, respectively (Table 4 and Figure 3).

Discussion

The incidence of CCA was about 5–13%.[12] HL-CCA can be detected at any stage, including the evaluation, treatment, or follow up of HL. HL is a known risk factor for CCA, which has been well documented. In cases of HL-CCA, there are no specific symptoms other than the clinical manifestation of HL. Hence, the diagnosed accuracy of HL-CCA is low.[13] Currently, early diagnosis of HL-CCA in clinical setting is still challenging even though there have been advances in diagnostic modalities and various efforts to identify it in early stages.[14]

It has been known that miRNAs are involved in almost all life activities of cells including cell proliferation, differentiation and apoptosis.[15,16] Recent studies have shown that the miRNA expression level may be related to cancers.[17,18] Therefore miRNAs are very promising diagnostic, prognostic biomarkers and therapeutic targets.[19] Profiling of miRNA has been explored as an invasive procedure for the detection of cancer. Wang et al[20] found that the miRNA profile differentiated patients with

Table 1 Comparison of Clinical Data Between the Observation Group and Control Group

	Observation Group (n=31)	Control Group (n=40)	t/X^2 Value	P value
Age (years)	53.82±21.18	51.92±19.73	0.390	0.698
Gender (Male/Female)	14/17	21/19	0.376	0.540
Smoking [n(%)]	9 (29.0%)	14 (35.0%)	0.284	0.594
Alcoholism [n(%)]	5 (16.1%)	7 (17.5%)	0.032	0.859
Liver or back pain [n(%)]	25 (80.6%)	33 (82.5%)	0.040	0.841
Cholangitis [n(%)]	19 (61.3%)	23 (57.5%)	0.104	0.747
Hepatitis B [n(%)]	17 (54.8%)	20 (50.0%)	0.164	0.686
Family history of malignancy [n(%)]	2 (6.5%)	1 (2.5%)	–	0.577*
Secondary bile duct stricture [n(%)]	15 (48.4%)	16 (40.0%)	0.149	0.699
Liver fibrosis [n(%)]	5 (16.1%)	5 (12.5%)	–	0.735*
Liver abscess [n(%)]	7 (22.6%)	1 (2.5%)	–	0.018*
Cirrhosis [n(%)]	6 (19.4)	1 (2.5%)	–	0.038*
Portal hypertension [n(%)]	3 (9.7%)	0 (0%)	–	0.308*

Note: *Indicates Fisher's exact test.

Table 2 Comparison of Ultrasound Characteristics Between the Observation Group and Control Group

		Observation Group (n=31)	Control Group (n=40)	t/X^2 Value	P value
Diameter (cm)		1.62±0.59	1.38±0.52	1.819	0.073
Lesion location	Liver right lobe Liver left lobe Hepatic portal	5 18 8	7 23 10	0.025	0.988
Lesion echo	Hypoechoic Isoechoic Hyperechoic Mixed echoic	2 5 16 8	3 6 26 5	–	0.501*
Lesion shape	Regular Irregular	13 18	26 14	3.753	0.053
Border demarcation	Clear Indistinct	8 23	22 18	6.100	0.014
Echo texture	Homogeneous Non-homogenous	9 22	23 17	5.717	0.017
Posterior attenuation	No Yes	9 22	6 34	2.064	0.151
Intrahepatic bile duct dilatation		21	34	2.980	0.084
Portal vein around lesion	Missed Hazy Clear	8 10 13	4 7 29	6.928	0.031

Note: *Indicates Fisher's exact test.

pancreatic adenocarcinoma from healthy people. Moreover, the combination of miRNAs and CA19-9 was more effective in discriminating carcinoma.[21] Because miRNAs are involved in the tumorigenesis processes, the up-regulation of onco-miRNAs or the down-regulation of tumor suppressor miRNA can be utilized as prognostic indicators. Studies have revealed a significant correlation between elevated miRNA expression and OS.[22] The up-regulation of onco-miRNA leads to anti-apoptosis, proliferation and etastasization while the downregulation of tumor suppressor miRNA leads to cancer spreading, which may lead to therapeutic possibilities.

Table 3 Comparison of the Laboratory Indicators Between the Observation Group and Control Group

Variables		Observation Group (n=31)	Control Group (n=40)	t/X^2/U Value	P value
ALP (U/L)		110.3 (87.4, 141)	102.3 (89.2, 113.6)	479	0.102
ALT (U/L)		24.3 (15.2, 38.0)	22.5 (14.3, 31.8)	520.5	0.248
AST (U/L)		29.1 (16.3, 36.5)	18.5 (14.5, 31.0)	479	0.102
GGT (U/L)		29.4 (24.6, 37.8)	26.1 (18.7, 33.8)	463.5	0.070
TBIL (μmol/L)		14.63±7.83	11.63±6.82	1.723	0.089
CEA (ng/mL)		2.17±0.93	1.83±0.88	1.575	0.120
CA19-9 (kU/L)		20.14±8.12	17.52±6.28	1.534	0.130
miRNA	miRNA-21	1.481±0.896	1.036±0.573	2.617	0.011
	miRNA-34c	0.043±0.021	0.037±0.025	1.074	0.287
	miRNA-200b	0.164±0.381	0.119±0.481	0.427	0.671
	miRNA-221	1.223±0.791	0.815±0.612	2.421	0.018

Abbreviations: ALP, alkaline phosphatase; AST, alanine aminotransferase; ALT, aspartate aminotransferase; GGT, glutamyl transpeptidase; TBIL, total bilirubin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen.

However, despite this interesting perspective, critical obstacles that often involve the delivery of miRNA-targeting agents must still be overcome before transition to clinical applications. There are numerous preclinical data but few clinical trials on the use of miRNAs nowadays.[23] This study compared the clinical data, ultrasonic features, and miRNAs expression level of patients with HL alone and HL-CCA in order to find an accurate method for identifying HL-CCA.

Clinical and Laboratory Indicators of HL and HL-CCA

The present study revealed that patients with HL-CCA had differences in the incidence of liver abscess and cirrhosis

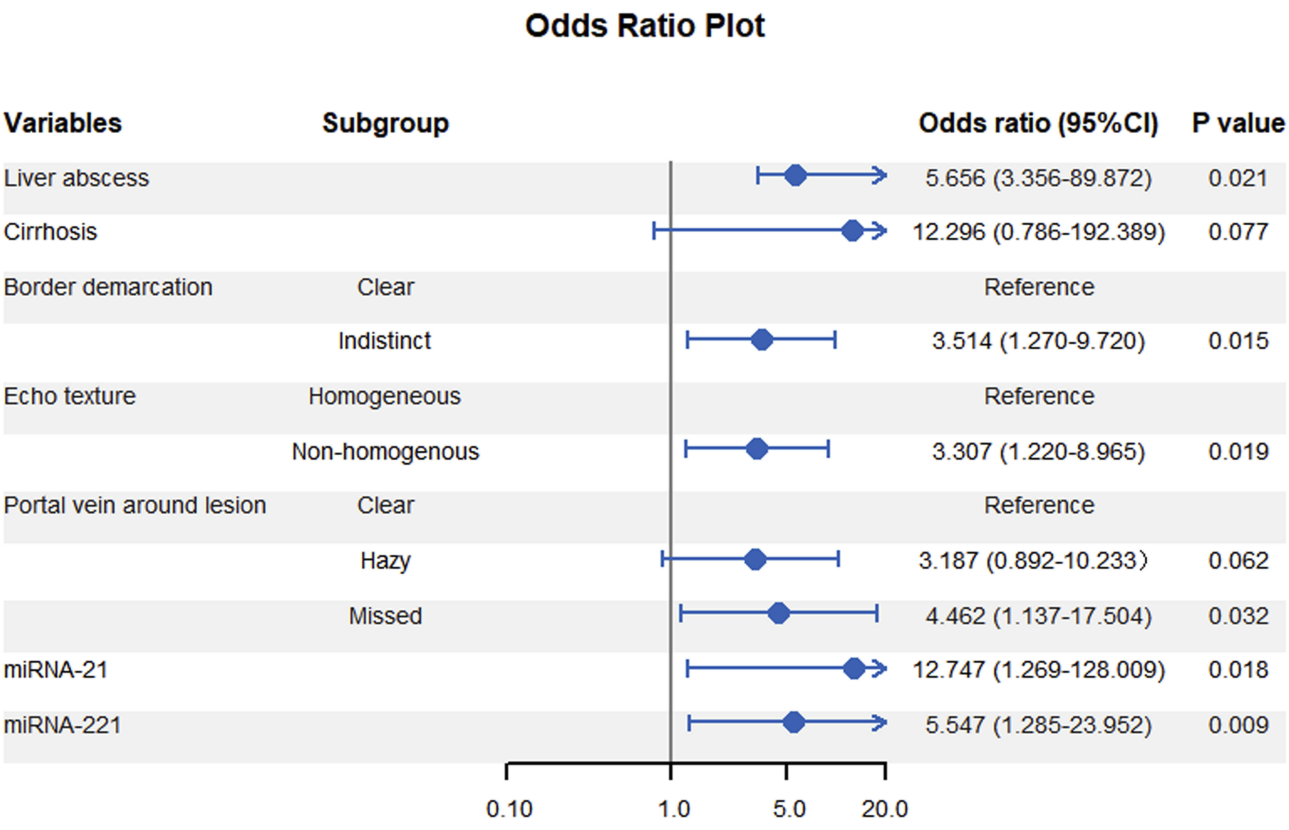

Figure 2 Forest plot of the logistic regression analysis for the influence of potential variables on HL-CCA.

Table 4 ROC Analysis of Potential Indicators for Differential Diagnosis of HL-CCA

	AUC	95% CI	Cut off Point	Sensitivity (%)	Specificity (%)
Liver abscess	–	–	–	22.58 (7/31)	97.50 (39/40)
Border demarcation	–	–	–	74.19 (23/31)	55.00 (22/40)
Echo texture	–	–	–	70.97 (22/31)	57.50 (23/40)
Portal vein around lesion	–	–	–	58.06 (18/31)	72.50 (28/40)
miRNA-21	0.610*	0.487–0.723	0.56	96.77	30.00
miRNA-221	0.767*	0.651–0.859	1.32	54.84	95.00
Combination	0.911	0.819–0.965	0.48	77.42	97.50

Note: Compared with the combination, *P<0.05.
Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval.

compared to the patients with HL alone. Multivariate regression analysis revealed that liver abscess was an independent risk factor for HL-CCA. It may be because that the gallstones block the bile ducts and cause cholestasis. The local inflammation promotes necrosis and liquefaction of the liver lobes to form liver abscess.[24] When associated with CCA, the incidence and severity of bile duct obstruction are worsened. Compared with HL alone, the possibility of liver abscess in HL-CCA is increased. However, not all patients with HL-CCA accompanied liver abscesses, and the ROC analysis of liver abscess in identifying HL-CCA indicated a low sensitivity.

Studies have analyzed the laboratory indicators of patients with CCA and found that hepatobiliary injury caused by CCA can cause the rise of laboratory indicators such as ALP, GGT and TBIL. However these indicators are also increased in HL.[25] In this study, the TBIL, ALP, and GGT in patients with HL-CCA were only slightly higher than in patients with HL (P>0.05). It is worth noting that we did not find any difference in serum CEA and CA19-9 between the two groups. It maybe indicated that the laboratory indicators and the common tumor markers such as CEA and CA19-9 were limited in the differentiation of HL-CCA and HL alone.

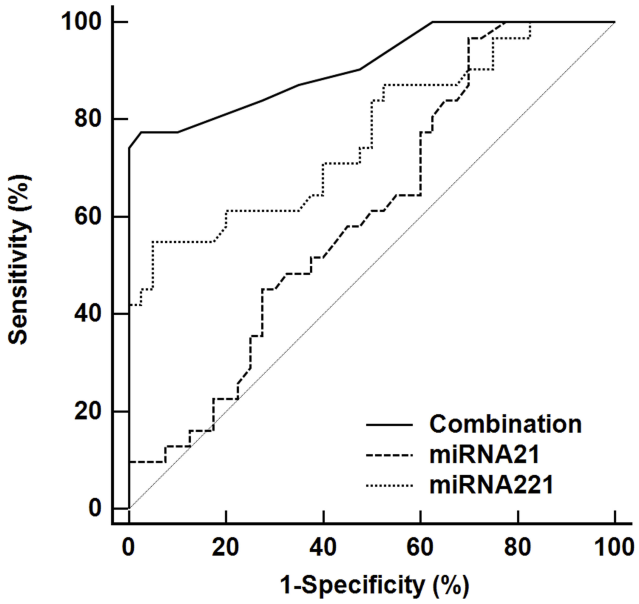

Figure 3 ROC analysis of miRNA-21, miRNA-221 and their combination in the differential diagnosis of HL-CCA.

Comparison of Ultrasonic Characteristics Between HL and HL-CCA

In this study, compared with HL alone, the ultrasonic features of patients with HL-CCA showed an indistinct border demarcation, non-homogenous echotexture, and a high proportion of missed portal vein around lesion. These ultrasonic features are independent risk factors for HL-CCA. However, the ROC analysis revealed that the sensitivity and specificity of the border demarcation, echotexture and portal vein around lesion are not high, suggesting that it is difficult to diagnose HL-CCA merely rely on ultrasonic features.

Identification of CCA by miRNAs

It is well known that miRNAs can participate in cell proliferation, differentiation, etc., and directly regulate the expression of protooncogene and tumor suppressor gene. miRNA-21 has been shown to be overexpressed in many types of tumors (lung, stomach, liver, breast, etc.).[26–29] It has become a tumor marker for tumor staging, treatment and prognosis. The study by Huang et al[30] has found that miRNA-21 can enhance the invasion and metastasis of CCA cells, suggesting that it may play an important role in the invasion and metastasis of CCA. Volinia et al[31] reported that miRNA-21 is significantly overexpressed in human CCA cells and plays the role of an oncogene. Meng et al[32] found that miRNA21 was overexpressed in CCA

even at an early stage. Inhibition of miRNA-21 can reduce the proliferation and invasion of CCA cells.

miRNA-221 has also been widely reported in various tumors. It has been found that miRNA-221 detected in tumor tissue or serum may be used as a diagnostic marker for malignant tumors and used to predict tumor aggressiveness and prognosis. Hence, the changes of miR-221 may indicate the presence of malignant tumors.[33,34] According to the study of Correa-Gallego et al,[12] miR-221 may be a potential marker for the diagnosis of CCA. And miRNA-221 silencing can inhibit the increase of tumor value or promote its apoptosis, which provides the basis for the study of miRNA-221 as a therapeutic target.

The results of the present study revealed that the levels of miR-21 and miR-221 in patients with HL-CCA were significantly higher than those in patients with HL alone. Both of them could be used as independent risk factors for HL-CCA. However, the ROC analyses revealed that when miR-21 or miR-221 was used as a diagnostic indicator individually. Their specificity or sensitivity was limited to identify HL-CCA.

Combined Diagnosis Can Improve the Diagnostic Efficacy of HL-CCA

ROC analysis of the risk factors for HL-CCA revealed that the clinical symptoms, ultrasonic features, and miRNA expression level had different degrees of deficiencies in diagnosing HL-CCA. In this study, a logistic regression model was used to establish a combined diagnosis model. The accuracy of the combined diagnosis was significantly increased (AUC=0.911), which was significantly higher than the AUCs of each indicator. It indicated that when combined with liver abscess, miR-21 & miR-221 levels and ultrasonic features, the diagnosis of HL-CCA is the most accurate. The best diagnostic point for the combined diagnosis was 0.48, with a sensitivity and specificity of 77.42% and 97.50%, respectively. It indicated that the SPSS software could used to establish the combination model to determine the probability of patients with HL-CCA after recording the data of liver abscess, miR-21, miR221 levels and ultrasonic features. The patient with HL is more likely to develop CCA if the probability is >0.480.

Limitation and Prospective

Although many target genes directly regulated by miRNA-21 and miRNA-221 have now been predicted, few have been confirmed in clinical setting. The application value of miRNA-21 and miRNA-221 in the diagnosis, treatment and prognosis of HL-CCA needs to be further explored. In addition, there are some differences in the results of different methods for detecting miRNA. This study plan to establish a CCA database based on the data from multi-center hospitals to further explore the combined model in clinical setting.

Conclusion

This study compared the clinical data, ultrasonic characteristics and miRNA expression level in patients with HL alone and HL-CCA. Patients with HL-CCA have high incidence of hepatic abscess and elevated miR-21 and miR-221 expression levels. The ultrasonic features are more likely to show indistinct border demarcation, non-homogenous echotexture, and missed portal vein around lesion. The combination of these indicators can more accurately discriminate HL-CCA from HL.

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Disclosure

The authors report no conflicts of interest in this work.

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