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Original Paper

The Plasma LncRNA Acting as Fingerprint in Hilar Cholangiocarcinoma

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Key Words
Cancer • Sensitivity • Specificity • Risk score • Circulating

Abstract
Background & Aims: Current studies have indicated that long non-coding RNAs (lncRNAs) could act as tumor biomarkers for disease diagnosis and prognosis prediction. In this study, we mainly focused on determining the expression of circulating lncRNAs in patients suffering for hilar cholangiocarcinoma (HC), aiming to reveal the potential lncRNA as a fingerprint. ***Methods:*** A total 12 lncRNAs were previously proven to be aberrantly expressed in HC tumor tissues. All of the 12 lncRNAs were selected as candidate targets for subsequent circulating lncRNA assay. The candidate lncRNAs were validated by qRT-PCR arranged in training and validation sets. The risk score analysis was employed. Data was presented with receiver operating characteristic curve (ROC). ***Results:*** Circulating PCAT1, MALAT1, and CPS1-IT1 were significantly increased in plasma samples of HC patients in both the training set and validation set. Through ROC analysis, we found that the three plasmatic lncRNAs presented the area under ROC curve value (AUC) as 0.784, 0.860, and 0.677. Further combination with the three factors indicated a higher power (AUC, 0.893; sensitivity, 85.5%; specificity, 93.2%). ***Conclusion:*** This was the first time to reveal the potential circulating fingerprints for predicting HC. PCAT1, MALAT1, and CPS1-IT1 may act as novel early diagnosis biomarkers for predicting HC.

Introduction

Cholangiocarcinoma accounts for approximately 10% of primary hepatic tumors and is divided into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) [1, 2]. Nearly 80%–90% of cholangiocarcinomas are of extrahepatic origin and are divided into perihilar (Klatskin tumors) and distal tumors based on their location [3, 4].

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Another subgroup is Hilar cholangiocarcinoma (HC). Despite recent improvements in cholangiocarcinoma treatment, surgical resection and transplantation is still the main therapy, as this can cure early-staged patients [5, 6]. Unfortunately, there are no specific biomarkers or unique clinical manifestations for cholangiocarcinoma, meanwhile, HC is typically characterized by advanced stage diagnosis. Therefore, novel diagnostic or therapeutic targets that underlie HC initiation and progression should be explored to better diagnose and cure HC.

HC patients have prognoses and limited therapeutic regimens [7, 8]. Thus, improving the diagnostic efficacy of HC is urgent and necessary, especially regarding biomarkers. Classic humoral cholangiocarcinoma markers such as carbohydrate antigen (CA)-199 and CA-125 are now regarded as insensitive and unspecific [9, 10]. As gene expression patterns are greatly altered in HC, it is urgent to identify new epigenetic biomarkers for HC patients.

With the progression of whole-genome sequencing technology, it has been found that <2% of the transcribed mammalian genome is protein-encoding, while the rest is non-coding RNA [11-13]. Long non-coding RNAs (lncRNAs) are one type of non-protein encoding transcript, and are >200 bp in length [14, 15]. Recently, many lncRNAs have been reported to play important roles in tumorigenesis, cancer progression and drug resistance [16-18]. Furthermore, evidence of circulating lncRNAs as biomarkers is rapidly increasing and has been applied to human diseases [19, 20]. Researchers have recently identified that circulating lncRNAs might act as biomarkers for the early diagnosis of multiple human cancers, including hepatocellular carcinoma, gastric cancer, and prostate cancer [21, 22]. Most previous studies have focused on the promoting or inhibiting roles miRNAs might have on tumorigenic processes of malignancies such as HC [23]. However, more and more researchers have seen the potential of miRNAs as biomarkers for diagnosing, predicting, and monitoring the prognosis of HC patients. However, the potential predictive ability of lncRNAs in HC has not been fully investigated.

In this study, we identified lncRNAs that were differentially expressed in the tumor tissues of HC patients. In total, 12 lncRNAs (CCAT2, Sox2ot, UCA1, TUG1, AFAP1-AS1, CCAT1, NEAT1, PCAT1, PANDAR, MALAT1, H19, and CPS1-IT1) that were previously reported to have altered expression in HC were enrolled as candidate diagnostic makers. We hypothesized that these candidate lncRNAs might be released into circulation during HC initiation or progress and could be used to diagnose and monitor HC. Additionally, we aimed to determine lncRNAs that acted as fingerprints for the early identification of HC.

Materials and Methods

Samples and screening phase

All plasma samples were obtained from patients diagnosed with HC between July 2012 and June 2017 at Qilu Hospital of Shandong University and Binzhou Medical University Hospital. Control samples were from healthy volunteers without any health problems during their health check-ups at the aforementioned hospitals. This research protocol was approved by the Shandong University and Binzhou Medical University ethical review boards. Written informed consent was obtained from every participant. Peripheral blood samples of patients were collected before surgery. Blood samples were collected in a vacuum cube, followed by centrifugation at 5, 000 rpm for 15 min. All samples were stored at -80°C until analysis. The relevant clinical data of all patients were available. All patients were diagnosed by histological examination. Detailed clinical information of patients and healthy controls are summarized in Table1.

The screening phase was divided into a training set and a validation set. Paired samples, including 20 HC patients and 20 healthy controls, were enrolled while the remaining 120 patients and 120 controls were used as the validation cohort.

Training set

All lncRNA candidates were tested in an independent cohort of 20 plasma samples obtained from patients. All patients were pathologically diagnosed with HC at the Qilu Hospital of Shandong University and Binzhou Medical University Hospital. Expression levels of these candidates were analyzed in all samples, and the comparative $2^{-\Delta\Delta Ct}$ method was used for data presentation, and Student's t-test was used to analyze differences between patients and healthy controls.

Validation set

A case-control study was designed to validate differences in the relative expression levels of selected biomarker candidates in 120 HC patients and 120 healthy controls (the validation cohort).

Quantitative real-time PCR (qRT-PCR)

Three to five tubes of blood, each tube containing approximately 10 mL of blood, were used for plasma isolation. All tubes were EDTA-anticoagulation tubes. After centrifugation, we obtained approximately 20–30 mL of plasma for RNA extraction. Total RNA containing small RNAs was extracted from plasma using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The purity and concentration of RNA samples were determined from OD260/280 readings using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was determined by 1% formaldehyde denaturing gel electrophoresis. For mRNA detection, total RNAs (500 ng) were reverse transcribed using the reverse transcription kit and the Oligo-dT approach (with poly-A) or random hexamer primer method (without poly-A) (Takara, Tokyo, Japan). The primers used for RT-PCR are presented in Table 2. The SyberFreen technique was used for qRT-PCR, and was performed by using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA).

Risk score analysis

Risk score analysis was performed to evaluate associations between plasma lncRNA expression levels. The upper 95% reference interval of each lncRNA value in controls or the non-metastasis group was set as the threshold to code the expression level of the corresponding lncRNA for each sample as 0 and 1 in the training set. A risk score function (RSF) to predict HC was defined according to a linear combination of the expression level for each lncRNA. For example, the RSF for sample i using information from three lncRNAs was:

Table 1. The clinicopathological information in HC patients and healthy controls. a: Chi-square test

Characteristics	HC	Control	P value[a]
All cases	120	120	
Age			
<60	85	88	0.666
≥60	35	32	
Gender			
Male	91	90	0.910
Female	29	30	
Differentiation grade			
Well	33		
Moderate	61		
Poorly	26		
Tumor Size(cm)			
≤5cm	89		
>5cm	31		
Tumor Origination			
Left	66		
Right	49		
Bilateral	5		
TNM stage			
I-II	41		
III	79		
Metastasis			
Yes	98		
No	22		

Table 2. Primers for Quantitative real-time PCR

Gene name		Sequence
CCAT2	Forward Primer	AGACAGTGCCAGCCAACC
	Reverse Primer	TGCCAAACCCTTCCCTTA
Sox2ot	Forward Primer	GCTCGTGGCTTAGGAGATTG
	Reverse Primer	CTGGCAAAGCATGAGGAACT
UCA1	Forward Primer	TTTGCCAGCCTCAGCTTAAT
	Reverse Primer	TTGTCCCCATTTTCCATCAT
TUG1	Forward Primer	TAGCAGTTCCCCAATCCTTG
	Reverse Primer	CACAAATTCCCATCATTCCC
AFAP1-AS1	Forward Primer	ATGGGGTAACTCAAAAAGCCTG
	Reverse Primer	GCAGCAATTCAGAGCCAGTC
CCAT1	Forward Primer	TCACTGACAACATCGACTTTGAAG
	Reverse Primer	GGAGAAAACGCTTAGCCATACAG
NEAT1	Forward Primer	CCTGTCCGGCACCTCATAG
	Reverse Primer	GGCCTTTAGGCAGGCTCTT
PCAT1	Forward Primer	AATGG CATGAACCTGGGAGGCG
	Reverse Primer	GGCTTTGGGAAGTGCTTTGGAG
PANDAR	Forward Primer	CTGTTAAGGTGGTGGCATTG
	Reverse Primer	GGAGGCTCATACTGGCTGAT
MALAT1	Forward Primer	AGCGGAAGAACGAATGTAAC
	Reverse Primer	GAACAGAAGGAAGAGCCAAG
H19	Forward Primer	ATCGGTGCCTCAGCGTTCGG
	Reverse Primer	CTGTCCTCGCCGTCACACCG
CPS1-IT1	Forward Primer	CACAGATGATCCACGGCG TT
	Reverse Primer	GCGTGCATCAATGACACTTCA
GAPDH	Forward Primer	GGAGCGAGATCCCTCCAAAAT
	Reverse Primer	GGCTGTTGTCATACTTCTCATGG

rsfi=∑3j-1Wj.sij. In the above equation, sij is the risk score for lncRNA j on sample i, and Wj is the weight of the risk score of lncRNA j. The risk score of three lncRNAs was calculated using the weight by the regression coefficient that was derived from the univariate logistic regression analysis of each lncRNA. Samples were ranked according to their RSF and then divided into a high risk group, representing HC patients, and a low risk group, representing control individuals or non-metastatic patients. Frequency tables and receiver operating characteristic (ROC) curves were then used to evaluate the diagnostic effects of the profiling and to find the appropriate cutoff point, and to validate the procedure and cutoffs in the validation set.

Statistical analysis

Data are presented as mean (S.E.M.). Student's *t*-test and Mann-Whitney unpaired analysis of variance were used to evaluate statistical differences between patients and controls. Analysis of area under the ROC curve (AUC) was used to estimate the effectiveness of lncRNAs for predicting HC. Statistical analyses were performed using STATA 9.2 and GraphPad Prism software. In all cases, $P<0.05$ was considered significant. All *P* values were two-sided.

Results

Candidate lncRNA expression in HC plasma samples

Candidate lncRNAs were screened using the PubMed database through searching the terms "cholangiocarcinoma" and "lncRNA." This obtained 12 candidate lncRNAs: CCAT2, Sox2ot, UCA1, TUG1, AFAP1-AS1, CCAT1, NEAT1, PCAT1, PANDAR, MALAT1, H19, and CPS1-IT1. We first detected these 12 candidates in plasma samples from 20 HC patients and healthy controls (training set). Three of the 12 lncRNAs were detectable and significantly different in the plasma samples from HC patients and healthy controls. The remaining lncRNAs were filtered out. For example, UCA1, AFAP1-AS1, and H19 were in too low abundance for further investigation, while CCAT2, Sox2ot, TUG1, CCAT1, NEAT1, PCAT1, and PANDAR were not significantly different between patients and controls (Fig. 1). Thus, three lncRNAs (PCAT1, MALAT1 and CPS1-IT1) were further investigated in the validation set.

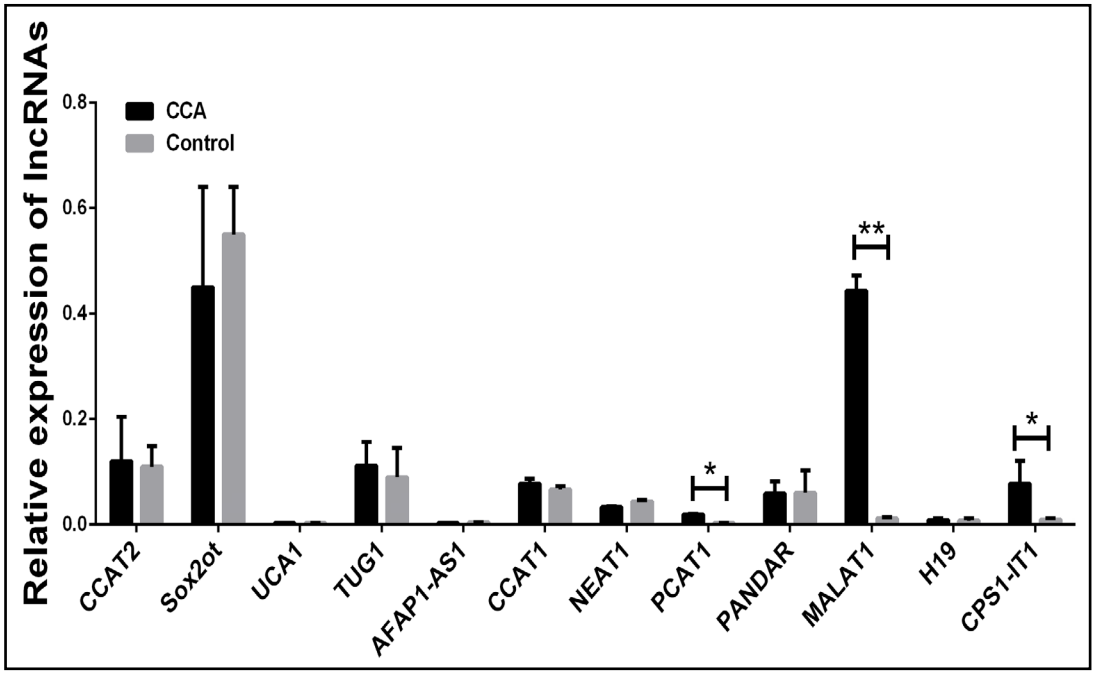


Fig. 1. Relative expression of the candidate lncRNAs. Total 20 paired plasma from HC patients and 20 controls were used in RT-qPCR analysis. Data was presented as mean ± SEM. Data was analyzed with Student's t-test. *indicated $p<0.05$ and ** indicated $p<0.01$.

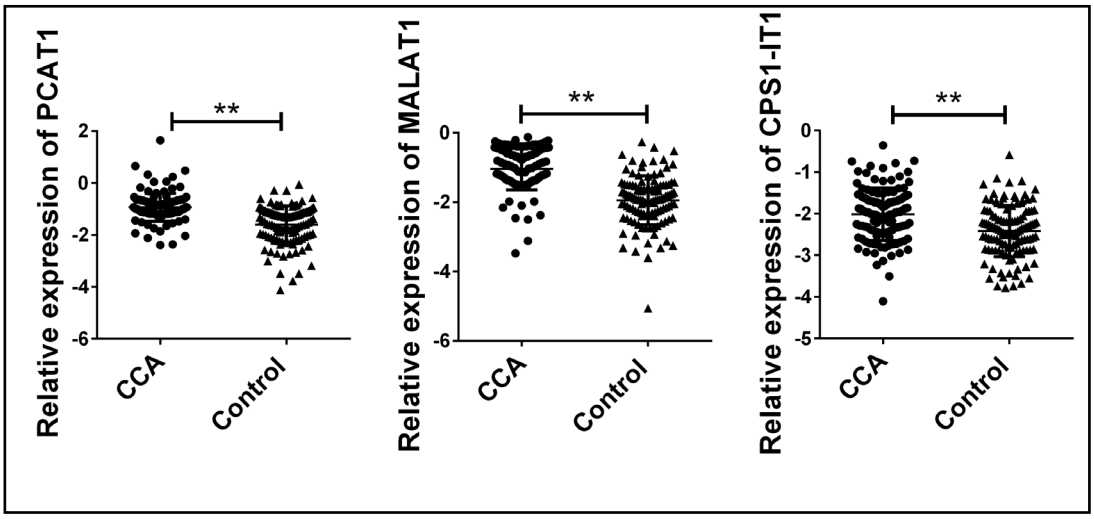


Fig. 2. Validation of lncRNAs expression in a larger sample size. Plasma from 120 HC patients and 120 controls were enrolled. Data were presented as plot of the median and range of log-transformed relative expression level and was analyzed with Student's t-test. ** indicated p<0.01.

Increased PCAT1, MALAT1 and CPS1-IT1 levels in the validation set

We next examined the expression of these three lncRNAs in the larger validation cohort of 120 cases and controls. As shown in Fig. 2, we found that PCAT1, MALAT1, and CPS1-IT1 levels were consistent with the results of the training set: increased expression and stable abundance.

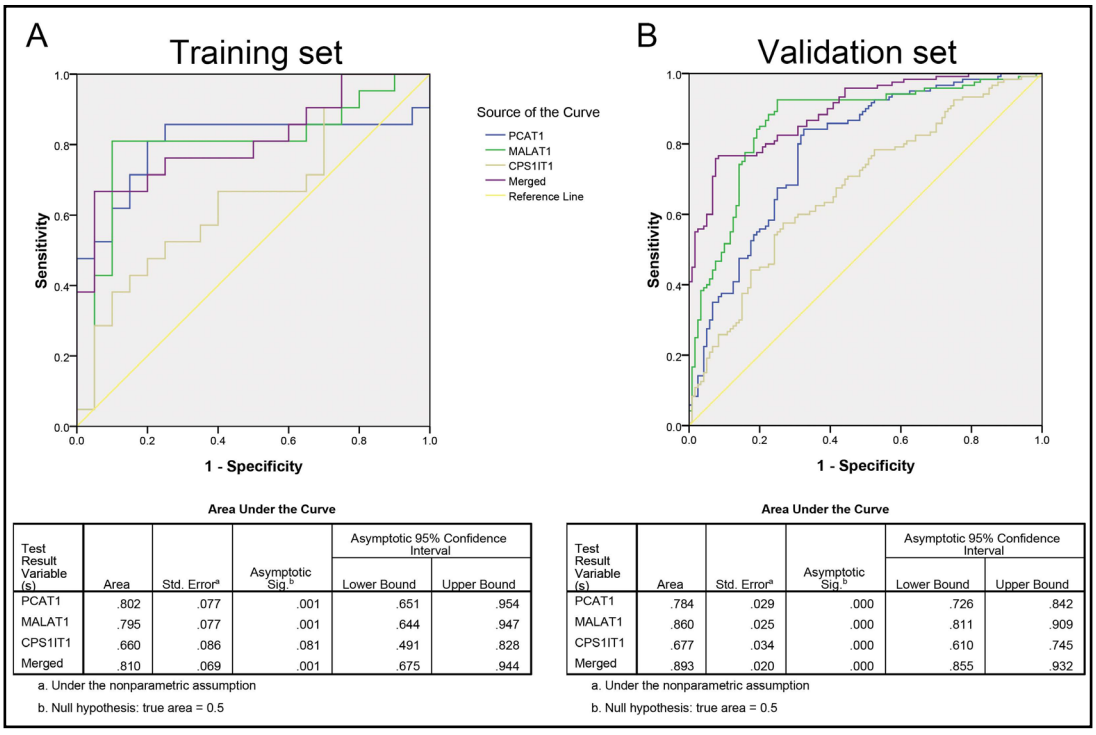
Table 3. Risk score analysis of in HC and cancer-free control plasma samples. [a] PPV, positive predictive value.[b] NPV, negative predictive value

Score	0-7.883	7.8833-11.525	PPV [a]	NPV[b]
Training set			0.800	0.800
HC	4	16		
Control	16	4		
Validation set			0.808	0.783
HC	23	97		
Control	94	26		

Risk score analysis for a potential HC fingerprint

A risk score formula was applied to evaluate the diagnostic value of the three lncRNAs. First, we divided the control group and case group into a training set according to the upper 95% credibility interval (95% CI) in the control group. The risk score was calculated based on the logistic regression analysis. All plasma samples were then divided into a high risk group which indicated the high possibility of being in the HC group, and a low risk group, representing predicted controls. We defined the cut-off value as the maximal value of sensitivity + specificity. The positive predictive value (PPV) and negative predictive value (NPV) were both calculated as 80.0% in the training set. We further applied the same value to calculate the risk score of samples in the validation set, for this analysis the PPV and NPV were 80.3% and 78.3%, respectively (Table 3).

ROC curve analysis was used to evaluate predictive diagnostic values of lncRNAs for HC. The AUC of the training set of three lncRNAs (PCAT1, MALAT1 and CPS1-IT1) signature were 0.802, 0.795, and 0.660, respectively, while the combination of the three factors possessed a moderate ability to discriminate between HCC patients and controls with an AUCe of 0.810 (Fig. 3A). The AUC of the validation set three lncRNAs signatures were 0.784, 0.860, and 0.677, respectively, and the combination of the three factors possessed a moderate ability to discriminate between HC patients and controls with an AUC of 0.893 (Fig. 3B).



Area Under the Curve

Test Result Variable (s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
PCAT1	.802	.077	.001	.651	.954
MALAT1	.795	.077	.001	.644	.947
CPS1IT1	.660	.086	.081	.491	.828
Merged	.810	.069	.001	.675	.944

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Area Under the Curve

Test Result Variable (s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
PCAT1	.784	.029	.000	.726	.842
MALAT1	.860	.025	.000	.811	.909
CPS1IT1	.677	.034	.000	.610	.745
Merged	.893	.020	.000	.855	.932

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Fig. 3. ROC analysis of the three potential biomarkers for HC by using risk score analysis. A: ROC analysis of the three potential biomarkers for HC in training set, merged indicated the combination of three lncRNAs. B: ROC analysis of the three potential biomarkers for HC in validation set, merged indicated the combination of three lncRNAs.

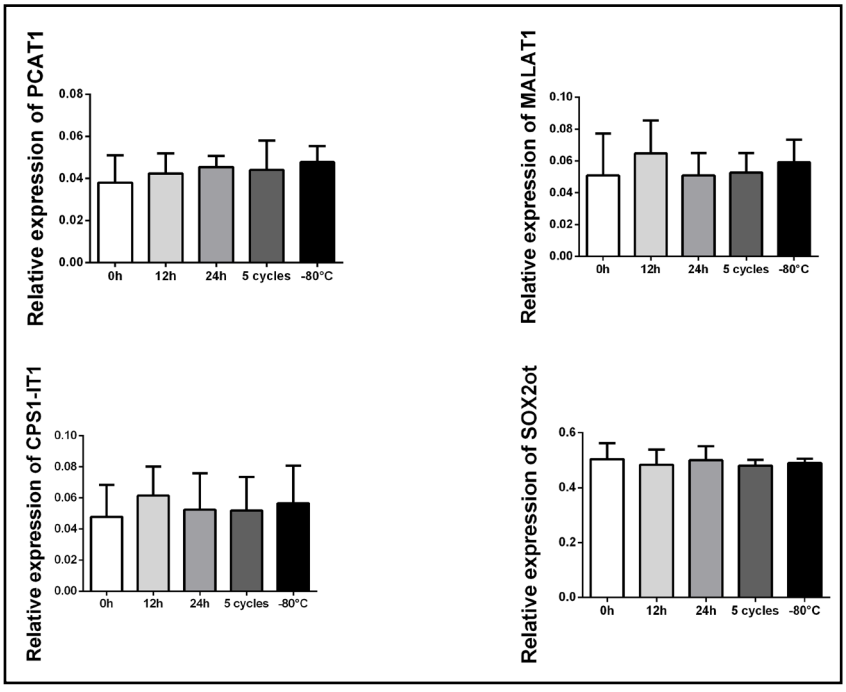


Fig. 4. Stability detection of lncRNAs in human plasma samples. The comparative $2^{-\Delta\Delta Ct}$ method algorithm was used for data presentation, and the Student's t-test was used to analyze the difference. Sox2ot was used as the positive control based on the abundant endogenous expression. Data was presented as mean ± SEM.

Stability detection of lncRNAs in human plasma samples

We next amplified the three lncRNAs in five healthy controls. We incubated human plasma obtained from three healthy controls at room temperature for 0, 12, and 24 h and subjected it to five freeze/thaw cycles before detecting the product by qRT-PCR. The relative expression of PCAT1, MALAT1, and CPS1-IT1 normalized to GAPDH are presented in Fig. 4, indicating that all the three lncRNAs were detectable in human plasma. Sox2ot was used as the positive control based on its abundant endogenous expression. We found that the expression level of the three lncRNAs were altered, indicating that PCAT1, MALAT1, and CPS1-IT1 were stably expressed and detectable in human plasma.

Discussion

Despite the number of diagnostic advances and therapeutic strategies that have been achieved for HC in recent years, the overall survival rates of HC patients is very poor due to recurrence and metastasis [24]. Due to the lack of effective preventive measures and obvious symptoms, HC patients are usually diagnosed at advanced clinical stages with local and/or distant metastases [25]. Therefore, it is of great importance to identify efficient biomarkers that offer diagnostic, prognostic, and therapeutic strategies. The etiology and mutational pattern between intra- and extra-hepatic cholangiocarcinoma types are different. In this study, we only focused on HC.

The exploration of HC biomarkers has been undertaken by numerous researchers for decades. These studies have been revealed significantly elevated miR-21 in patients with intrahepatic HC, showing high discrimination ability between patients and healthy controls. Interestingly, serum miR-21 levels have been found to decrease after surgical resection. Furthermore, serum levels of miR-483-5p and miR-222 were able to discriminate between PSC and HC patients [26]. Not only miRNAs, but also lncRNAs have been identified as potential biomarkers for predicting the occurrence or development of human malignant tumors. For example, circulating serum Linc00974 has been proven to be a biomarker for the early screening of hepatocellular carcinoma [27]; however, such clinical studies on circulating lncRNAs in HC have not been adequately performed.

In this study, there were a total of 12 lncRNAs that were included as biomarker candidates based on their previously reported functions. These specific lncRNAs were differentially expressed in HC tissues, and therefore might present altered levels in patients' serum samples. Previous studies have speculated that aberrantly expressed circulating lncRNAs might be derived from tumors. Thus, we examined the expression of the 12 lncRNAs in patients and healthy controls. By employing a multiple screening phase including training and validation sets, we found that only PCAT1, MALAT1, and CPS1-IT1 were differentially expressed in HC (increased). Upregulated PCAT1 in HC tumor tissues acts as a competing endogenous for miR-122, increasing levels of glycogen synthase kinase 3β and decreasing β-catenin levels in whole cell lysates and nuclear fractions, indicating that PCAT1 silencing inhibited Wnt/β-catenin-signaling, promoting cell invasion [28, 29]. LncRNA MALAT1 also promotes HC cell proliferation and invasion by activating the PI3K/Akt pathway [29]. For CPS1-IT1, CPS1, and CPS1-IT1 overexpression was correlated with increased CA19-9 positivity and lymph node metastasis [30]. Additionally, upregulation of CPS1 and CPS1-IT1 has a negative impact on the overall survival rate of HC patients. By employing the risk score analysis, we found that the combination of the three factors presented a high sensitivity and specificity with an AUC of 0.893, indicating that PCAT1, MALAT1, and CPS1-IT1 might serve as a signature for predicting HC.

Conclusion

In summary, we identified three lncRNAs, PCAT1, MALAT1, and CPS1-IT1, as the potential biomarkers for the tumorigenesis prediction of HC in this study. Due to the sample size limitation, our study were a preliminary work, a deeper investigation regarding the potential mechanism of the three lncRNAs in the occurrence or development of HC remains to be explored in the future.

Disclosure Statement

The authors declare that no conflict of interest exist.

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