Original Paper

HULC and Linc00152 Act as Novel Biomarkers in Predicting Diagnosis of Hepatocellular Carcinoma

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Key Words

Hepatocellular carcinoma • Long non-coding RNAs • HULC • Linc00152 • Biomarker

Abstract

Background/Aims: The alterations of long non-coding RNAs (lncRNAs) are related to multiple diseases. They can be detected in plasma as biomarkers for the diagnosis of multiple diseases. In this study, we aimed to determine the expression of circulating lncRNAs in human, which may be promising biomarkers for the diagnosis of hepatocellular carcinoma (HCC). *Methods:* Eight lncRNAs were chosen as candidates on the basis of the literature to evaluate the diagnostic value and accuracy of the plasma lncRNA profiling system. The candidate lncRNAs were validated by qRT-PCR arranged in the training and validation sets. Additional double-blind testing was performed in 20 patients clinically suspected of having HCC. *Results:* Circulating HULC and Linc00152 were significantly up-regulated in plasma samples of HCC patients during training set and validation set. Areas under the receiver operating characteristic (ROC) curves of the validated two lncRNAs signature were 0.78 and 0.85, respectively. Combination of HULC and Linc00152 possessed a moderate ability to discrimination between HCC and control with an area under ROC value of 0.87 while the combination of AFP was 0.89 with a positive correlation with tissues expression. *Conclusions:* Our results suggest that both plasma levels of HULC and Linc00152 achieve a fine diagnostic accuracy in diagnosing ontogenesis and metastasis of HCC and may act as novel biomarkers for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed cancer in men and seventh in women around the world, and the mortality of HCC

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ranks as a second in former and as a sixth in latter. The half of new cases of death occurs in China every year [1-3]. The prognosis of patients with HCC is poor and once HCC invades other organs and tissues or spreads distantly, the clinical outcome may be even worse [4-5]. Therefore, early detection of HCC is of great importance. The diagnosis of HCC mainly depends on imaging techniques, which are less efficient for detecting early HCC [6]. Plasma tumor marker detection is an ideal method for screening patients at an early stage and prefiguring their prognosis. At present, plasma alpha fetoprotein (AFP) is the most widely used tumor marker for HCC, however, AFP is limited by its low sensitivity and specificity [7]. Thus, looking for some new tumor markers with high sensitivity and specificity to detect the tumorigenesis and recurrence of HCC independent or as a supplement of AFP is badly needed. Cell-free nucleic acids (cfNA), such as DNA, mRNA and microRNA can be detected in the plasma of patients with cancer. A number of studies have implicated that changes in the levels of cfNA was linked to tumorigenesis and progression [8, 9].

Long non-coding RNAs (lncRNAs) are one type of non-protein coding transcripts with more than 200 nucleotides in length. LncRNAs are important regulatory molecules at every level of cellular physiology, alterations of which are related to multiple diseases, including cancers [10]. Some lncRNAs can be detected in plasma of both healthy people and certain patients. LncRNAs have been reported as biomarkers for the diagnosis of multiple diseases [11, 12], including cancers, such as cancers of colorectum, prostate and bladder [13-15]. Some previous studies showed that many lncRNAs were up-regulated or down-regulated significantly in tumor tissues compared with non-tumor tissues [16-20]. However, the aberrant expression of these lncRNAs in HCC has not been clarified yet. Although several other studies showed that some lncRNAs were up-regulated or down-regulated in HCC [21-23], their expression in plasma of HCC patients has not been investigated.

In the present study, we chose eight lncRNAs (UCA1, TUG1, CCAT1, MEG3, Linc00152, HULC, MALAT1 and GAS5) as plasma biomarker candidates on the basis of the literature. Relative expression levels of these candidates in plasma samples were analyzed in a small cohort of HCC patients and healthy controls, 32 patients with chronic hepatitis were regarded as disease controls. To identify a novel biomarker in predicting the diagnosis of HCC, a case-control study was designed to validate the significantly different relative expression of potential biomarkers in plasma samples of a large cohort of HCC patients and healthy controls.

Materials and Methods

Study design

Discovery phase. For the purpose of indentifying plasma lncRNAs as potential biomarkers, we searched candidate databases of lncRNAdb, LNCipedia, and LncRNA Disease. Previous studies showed that UCA1, TUG1, CCAT1 and Linc00152 were up-regulated obviously in tumor tissues compared with non-tumor tissues [16-19], while MEG3 was down-regulated significantly in tumor tissues. Regulations of all these lncRNAs in HCC have not been clarified yet. Several other studies have demonstrated that HULC and MALAT1 were overexpressed in HCC, while GAS5 was down-regulated in HCC [21, 22]. Nevertheless, the expression of these lncRNAs in plasma of HCC patients has not been performed. Thus, we selected eight lncRNAs (UCA1, TUG1, CCAT1, MEG3, Linc00152, HULC, MALAT1 and GAS5) as candidates for this research.

Training set. All candidates were tested in an independent cohort of 48 plasma samples from 24 HCC patients who underwent hepatic resections at the First Affiliated Hospital of Nanjing Medical University and 24 healthy controls. The expression levels of these candidates were analyzed in all plasma samples, and comparative delta-Ct method algorithms were used to analyze the difference between HCC patients and health controls. We found the expression of three lncRNAs among the eight candidates significantly differed between the 2 groups. Then, we selected these lncRNAs as potential biomarker candidates for further investigation.

Validation set. A case-control study was designed to validate the obvious difference of relative expression levels of the selected potential biomarkers candidates in another independent cohort of plasma

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samples from 66 HCC patients who underwent hepatic resections at the First Affiliated Hospital of Nanjing Medical University and 53 healthy controls. We found two lncRNAs of the three candidates were up-regulated significantly in HCC patients. Then, we analyzed the relationship between the two lncRNAs expression and the clinicopathological features of HCC patients, including age, gender, tumor size, differentiation grade, tumor number, tumor capsular, TNM stage and metastasis. Finally, we used receiver operating characteristic (ROC) curve to examine the diagnosis value of these two lncRNAs. The overflow was presented in Fig. 1.

Patient samples and clinical specimens

Study data were obtained from patients with HCC who underwent hepatic resections between August 2011 and September 2013 at Liver Transplantation Center of the First Affiliated Hospital, Nanjing Medical University (Nanjing, PR China). Controls were from healthy volunteers without any health problems during their health check-ups at the First Affiliated Hospital of Nanjing Medical University. The research protocol was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical
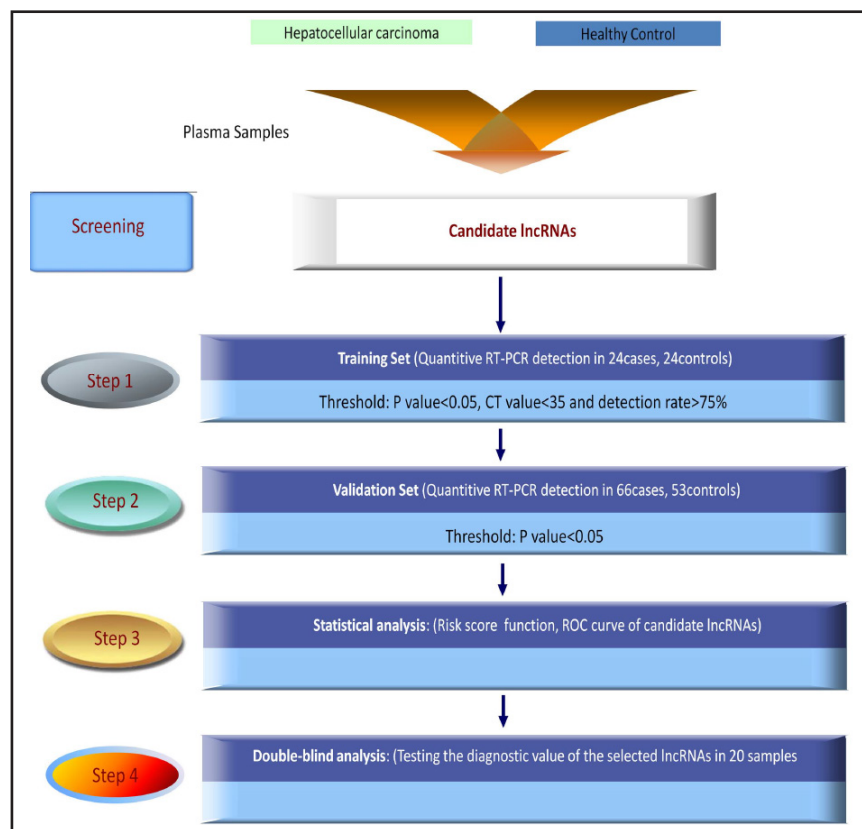
Fig. 1. Overview of the workflow.

Table 1. The primer of eight lncRNAs candidates and 5S rRNA

	Forward primer （5'--3'）	Reverse primer （5'--3'）
UCA1	CTCTCCATTGGGTTCACCATTC	GCGGCAGGTCTTAAGAGATGAG
TUG1	TAGCAGTTCCCCAATCCTTG	CACAAATTCCCATCATTCCC
CCAT1	CATTGGGAAAGGTGCCGAGA	ACGCTTAGCCATACAGAGCC
MEG3	GCCAAGCTTCTTGAAA GGCC	TTCCACGGAGTAGAGCGAGTC
Linc00152	CTCCAGCACCTCTACCTGTTG	GGACAAGGGATTAAGACACACA
HULC	ATCTGCAAGCCAGGAAGAGTC	CTTGCTTGATGCTTTGGTCTGT
MALAT1	AAAGCAAGGTCTCCCCACAAG	GGTCTGTGCTAGATCAAAAGGCA
GAS5	GGAGAGGGAGCCTGAGAAACG	TTACAGGGCCTCGAAAGAGTCC

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University. Written informed consent was obtained from every participant. Peripheral blood samples and tissues samples of patients were collected before surgery. Blood samples were collected in a separate vacuum cube, followed by centrifugation at 3,000 rpm for 10 min. All samples were stored at −80°C until further analysis. The relevant clinical data of all patients were available. All patients were diagnosed by histological examination.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were obtained from plasma samples using TRIzol reagent as described by the manufacturer (Invitrogen Life Technologies Co, Carlsbad, CA, USA). For mRNA detection, total RNAs (500ng) were reverse transcribed using the reverse transcription kit (Takara, Tokyo, Japan). The quality of RNA samples was assessed by a UV spectrophotometer (Bio- Rad, Hercules, CA, USA), and the 260/280nm absorbance ratio of samples were limited to 1.8-2.0. qRT-PCR was performed in 384-well plates by use of an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA) instrument according to the manufacturer's instructions. The qPCR reaction mixture consisted of 2.5 μl of SYBR® Premix Ex Taq™ (Perfect Real Time) (2×) (Takara: DRR081A TaKaRa, Japan), 0.5 μl of primers, 0.1 μl of ROX Reference Dye (50×),1 μl of the cDNA solution and nuclease-free H_2O at a final volume of 5 μl. 5S rRNA was used as an internal control while the cel-miR-39 was conducted as the external control. The primer of eight lncRNAs candidates and internal control were presented in Table 1. All samples were performed for four times. A melt-curve analysis was carried out at the end of the amplification to verify the non-specific amplification.

Statistical analysis

The results of qRT-PCR and other variables were presented as mean (S.E.M.).The Student's t-test and Mann-Whitney unpaired test analysis of variance were used to evaluate statistical differences between HCC patients and controls. Analysis of area under the ROC curve (AUC) was used to estimate the effectiveness of lncRNAs for prediction. Statistical analysis was performed using STATA 9.2 and GraphPad prism software. In all cases, P<0.05 was considered to be significant. All *P* values were two-sided.

Results

Expression of eight lncRNAs candidates in HCC in Training phase

As presented in Fig. 2 and Table 2, high expression levels of HULC and Linc00152 were observed in HCC patients compared with controls, while low expression level of UCA1 in HCC patients was identified. No statistical significance was demonstrated for the expression levels of TUG1, CCAT1 MEG3, MALAT1 and GAS5 in patients with HCC in contrast to controls.

Expression of selected lncRNAs candidates for HCC in validation phase

In validation phases, our results indicated that the relative expression levels of HULC and Linc00152 were up-regulated in HCC patients compared with controls. However, the

Table 2. The relative expressions of eight lncRNAs candidates for HCC comparing control in Training phase. *indicates statistically significant

|	HCC	control	*p* value
UCA1	0.02043 ±0.004120	0.04351 ±0.008908	0.0239*
TUG1	0.003672 ±0.0008270	0.01192 ±0.006560	0.3323
CCAT1	0.6556 ±0.2022	0.6857 ±0.1943	0.9152
MEG3	0.003363 ±0.0009886	0.001408 ±0.0002988	0.0997
Linc00152	0.8670 ±0.2069	0.002608 ±0.001010	< 0.0001*
HULC	0.9695 ±0.1615	0.003385 ±0.0006782	< 0.0001*
MALAT1	0.003637 ±0.0008856	0.005556 ±0.001307	0.2239
GAS5	0.006502±0.001176	0.004210 ±0.0007896	0.1021
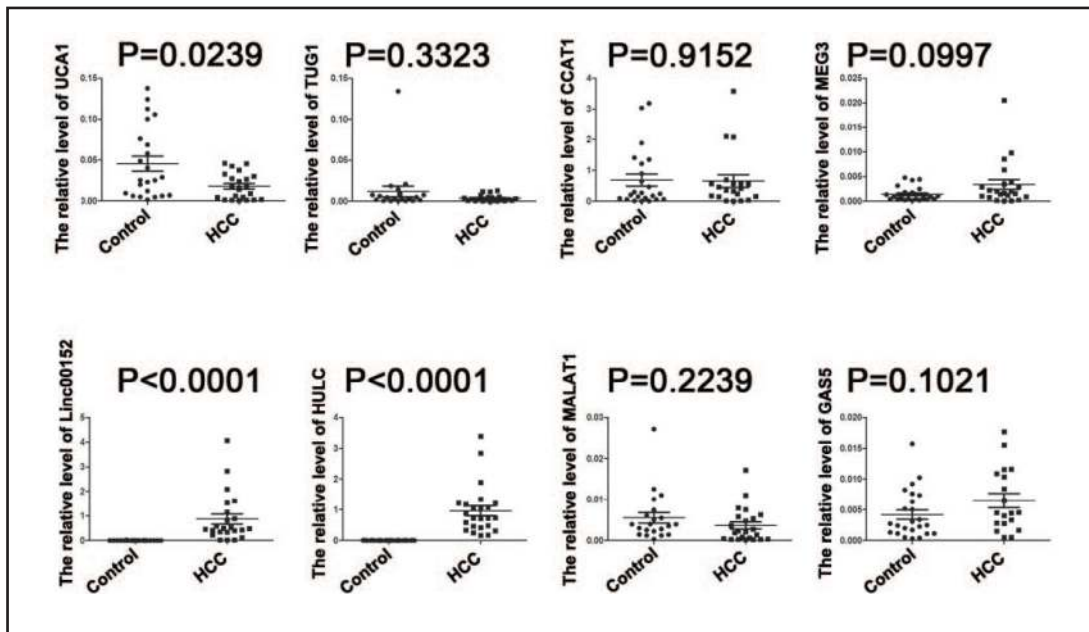
Fig. 2. HULC, Linc00152 and UCA1 were confirmed as the candidate lncRNAs in training set analysis. Increased level of HULC, Linc00152 and UCA1 were confirmed by RT-PCR in groups. Data were presented as plot of the median and range of log-transformed relative expression level.
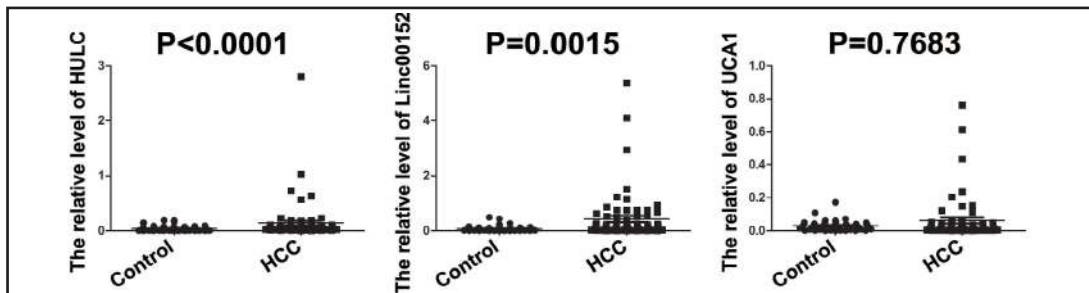

Fig. 3. HULC and Linc00152 were confirmed the up-regulation in validation set. Increased level of HULC and Linc00152 were confirmed by RT-PCR in groups. Data were presented as plot of the median and range of log-transformed relative expression level.

expression level of UCA1 in HCC patients showed no statistical significance when comparing with controls (Fig. 3).

Clinicopathological data analysis of HCC patients

The median value was set as the cut off value to classify the expression levels of HULC and Linc00152. As presented in Table 3, all clinicopathological data were divided into positive and negative groups according to different grouping criteria and were analyzed by chi-squared test or Fisher exact test. Tumor size, differentiation grade, TNM stage were grouped by 5 cm (the threshold of indicating small HCC), edmondson-Steiner grading, TNM Classification of Malignant Tumors (7th edition) as well as the current drug treatments(including HBC Antiviral therapy and Chemotherapy) and underlying disease (CH or other disease) was calculated. The clinicopathological relevance analysis of HCC patients demonstrated that the HULC expression significantly correlated with tumor size and tumor capsular, while the expression of Linc00152 was significantly related to differentiation grade, tumor size, TNM stage and tumor capsular.

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Table 3. The clinicopathological relevance analysis of HULC and Linc00152 relative expression levels in HCC patients. Clinicopathological data of 66 HCC patients were analyzed. For the relative expression of HULC and Linc00152, the median expression level was used as the cut off value. Data were analyzed by chi-squared test. *indicates statistically significant

Feather		HULC			Linc00152		
	Patients	Low	High	p value	Low	High	p value
Age(years)				0.1395			1.0000
<60	34	14	20		17	17	
≥ 60	32	19	13		16	16	
Gender				0.7569			0.2149
Male	53	26	27		24	29	
Female	13	7	6		9	4	
Tumor size (cm)				0.0253*			0.0005*
< 5	30	20	10		22	8	
≥ 5	36	13	23		11	25	
Differentiation grade				0.7288			< 0.0001*
Well	8	4	4		6	2	
Moderate	33	15	18		24	9	
Poorly	25	14	11		3	22	
Tumor number				0.7569			0.7569
Solitary	53	27	26		27	26	
Multiple	13	6	7		6	7	
Tumor capsular				0.0169*			0.0025*
Incomplete	15	3	12		2	13	
Complete	51	30	21		31	20	
TNM stage				0.1780			0.0066*
I	34	17	17		22	12	
II	16	11	5		9	7	
III	15	5	10		2	13	
IV	1	0	1		0	1	
Antiviral therapy				0.1395			1.0000
Yes	34	14	20		16	18	
No	32	19	13		17	15	
Chemotherapy				0.1395			
Yes	34	14	20		16	18	
No	32	19	13		17	15	
Underlying disease				0.9780			0.8800
Chronic hepatitis	51	10	41		18	33	
Others	15	3	12		5	10	
Metastasis				0.3553			0.0531
Yes	5	1	4		0	5	
No	61	32	29		33	28	

Receiver operating characteristic (ROC) curve analysis for predicting the diagnosis of HCC
ROC curve analysis was used to evaluate the predicting diagnosis value of lncRNAs for HCC. Areas under the ROC curves of the validated two lncRNAs signature were 0.78 and 0.85, respectively. Combination of HULC and Linc00152 possessed a moderate ability for discrimination between HCC patients and controls with an area under the ROC curve of 0.87, while the area was equal to 0.89 when they combine with AFP (Fig. 4).

Double-blind test
Another 20 plasma samples were tested in a double-blind fashion to validate the predictive ability of the two lncRNAs. We used the same risk score formula to analyze the

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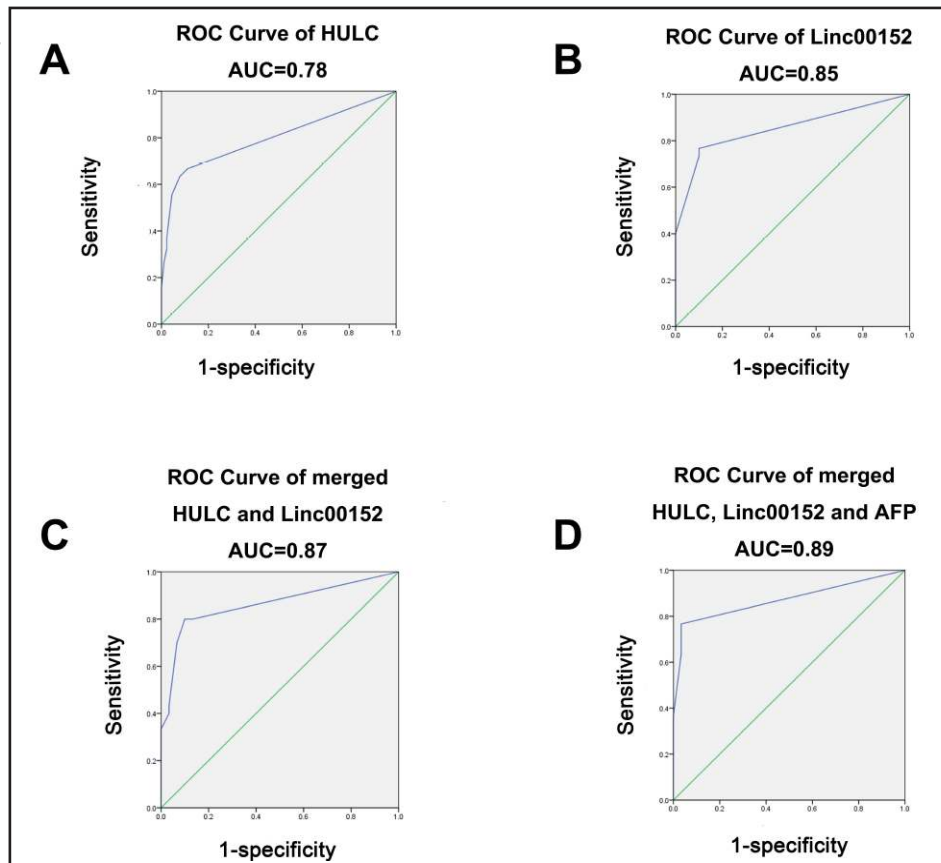**Fig. 4.** ROC analysis of the two potential biomarkers for HCC as well as the combination of AFP.

Fig. 5. Tissues correlated HULC and Linc00152 up-regulated specific in plasma of HCC. (A) Pearson correlation analysis of HULC and Linc00152 expression level between circulation and tissues. (B) The expression level of Linc00152 and HULC in HCC, CH and control group. Data were presented as plot of the median and range of log-transformed relative expression level. *indicated P<0.05.
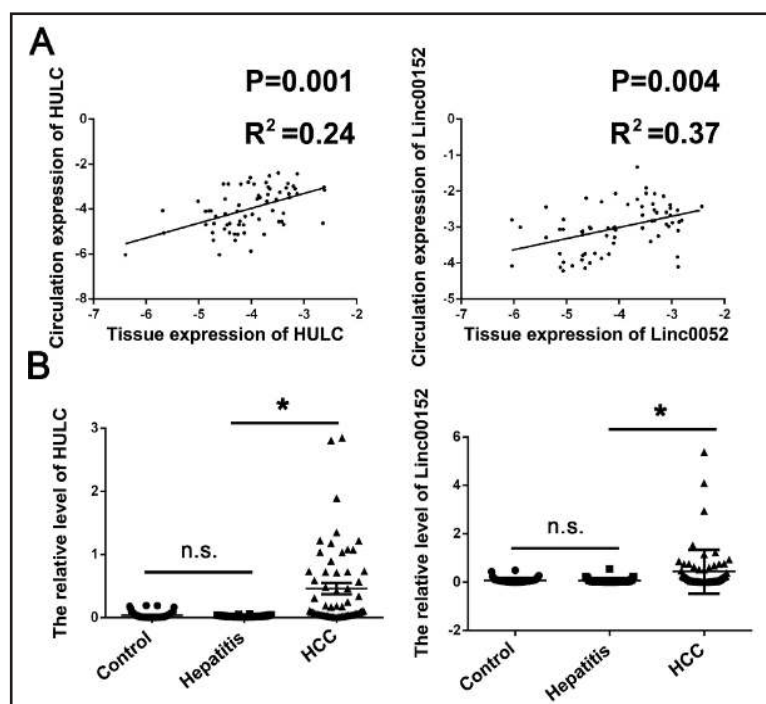
expression of the 2 lncRNAs in those plasma samples and classified them into a high-risk group and a low-risk group. On the basis of the pathologic diagnosis, the accuracy rate of the two-lncRNA profile as HCC signature was 81.9% (nine of eleven HCC cases were identified accurately).

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Fig. 6. The products of the amplification fragment were detected by agarose electrophoresis.
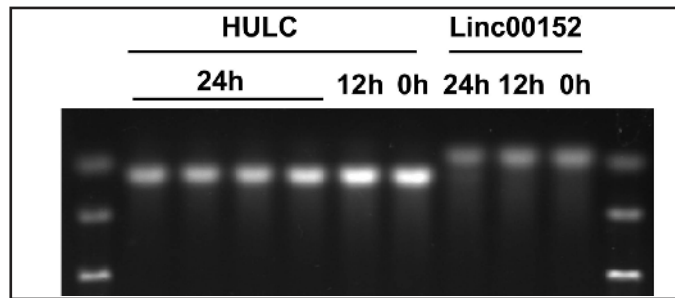
Specificity and stability detection of endogenous lncRNAs in human plasma

We further analyzed the expression of the two lncRNAs in the corresponding tissues of the same patients we included. Pearson correlation analysis showed a positive correlation of HULC and Linc00152 between their circulation expression levels and tissues expression levels (Fig. 5A). Since most patients investigated in our study were HBV positive, we enrolled another 32 patients suffering from chronic hepatitis (CH) as disease control group. The expression levels of Linc00152 and HULC were detected in these patients. As presented in Fig. 5B, there was no significant difference for the expression of the two factors between the control group and CH group, while an aberrant expression was obtained when comparing with the HCC group.

We next amplified the two lncRNAs in healthy controls, and detected the product of amplification by agarose electrophoresis. The bands presented in Fig. 6 indicated that both the two lncRNAs were detectable in human plasma. We further incubated human plasma obtained from three healthy controls at room temperature for 0 h, 12 h and 24 h. All of the process had minimal effects on the concentration of the two lncRNAs, demonstrating that these lncRNAs were sufficiently stable in human plasma.

Discussion

Due to the difficulty in early discovery, diagnosis and treatment, HCC is the most common malignant tumor with a higher mortality and an extremely low 5-year survival rate in the world [1, 24]. AFP, the main plasma tumor marker for HCC diagnosis and screening, has a potential capacity to screen early stage patients but with low sensitivity [7]. Furthermore, its specificity for HCC diagnosis is also not well because AFP can often rise in the absence of HCC, such as chronic hepatitis and cirrhosis [25-27]. Therefore, we aimed to seek a newly potential tumor marker with relative higher sensitivity and specificity or as a supplement of AFP for HCC.

Many researchers have demonstrated that lncRNAs play a very important role in tumor occurrence, invasion, and metastasis and can be considered as biomarkers in many human cancers [13-15, 28]. Some previous studies have shown that many lncRNAs were up-regulated or down-regulated dramatically in tumor tissues in contrast to non-tumor tissues [16-20]. However, the regulation of these lncRNAs in HCC remains largely unknown. Several previous reports presented that some lncRNAs were overexpressed or down-regulated in HCC [21-23]. Nevertheless, due to their characteristic of unstable expression and easily degradation in plasma, the expression of these lncRNAs in plasma of HCC patients has not been investigated. Thus, we chose eight lncRNAs (UCA1, TUG1, CCAT1, MEG3, Linc00152, HULC, MALAT1 and GAS5) as plasma biomarker candidates on the basis of the literature in this study.

Firstly, we detected the levels of these plasma lncRNAs from a small cohort of HCC patients and healthy controls- by qRT-PCR. The relative expression of these lncRNAs was calculated by use of delta-Ct method algorithms. The plasma levels of HULC and Linc00152 significantly increased in HCC patients, while the plasma level of UCA1 obviously decreased in HCC patients. Then, we conducted a case-control study to validate the outcome of training phase. Levels of HULC, Linc00152 and UCA1 were detected in a large cohort of HCC patients

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and healthy controls. As shown in Fig. 2, plasma levels of HULC and Linc00152 increased dramatically in HCC patients as well as in training phase. However, no significant difference of UCA1 level was observed between the two groups. Besides, the expression of HULC significantly correlated with tumor size and tumor capsular. Moreover, the expression of Linc00152 was significantly associated with differentiation grade, tumor size, TNM stage and tumor capsular. These findings suggested that HULC and Linc00152 might play an important role in tumor occurrence, tumor growth and tumor invasion. Apparently, higher levels of HULC and Linc00152 implicated a higher risk of tumor occurrence, growth and invasion of HCC. Accordingly, we hypothesized that these two lncRNAs might act as biomarkers in predicting the occurrence, growth, or metastasis in HCC. ROC curve analysis showed the evidence for great value of HULC and Linc00152 in predicting the diagnosis of HCC. Taken together, the results demonstrated that both of HULC and Linc00152 reached a well AUC and the combination of HULC, Linc00152 and AFP got the highest AUC in predicting oncogenesis, tumor growth, and tumor metastasis. As a result, HULC and Linc00152 can act as novel biomarkers in predicting the diagnosis of HCC, and combination of HULC, Linc00152 and AFP get the highest prediction value of HCC.

In summary, we firstly identify that plasma HULC and Linc00152 were up-regulated dramatically in HCC patients. Both HULC and Linc00152 plasma levels indicate a significant predication of tumor growth and metastasis of HCC, the combination of HULC, Linc00152 and AFP would achieve better diagnostic accuracy. Thus, plasma HULC and Linc00152 can serve as potential noninvasive biomarkers for predicting the diagnosis of HCC. Furthermore, HULC and Linc00152 may be applied as a potential target for HCC treatment. However, this research is limited by the small sample size. Further large-scale studies are needed to confirm these findings.

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Disclosure Statement

The authors declared no conflict of interest.

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