Int J Clin Exp Med 2018;11(11):12088-12096
www.ijcem.com /ISSN:1940-5901/IJCEM0074110

Original Article

Plasma lncRNAs acting as fingerprints in predicting hepatocellular carcinoma from HBV positive chronic hepatitis

Li Gong*, Xi Zhu*, Wei Zhang, Huihui Ni, Weihua Yin, Maoying Fu

*Department of Infectious Disease, The First People's Hospital of Kunshan Affiliated with Jiangsu University, Suzhou, China. *Equal contributors.*

Received February 6, 2018; Accepted July 14, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: Current studies have identified that circulating (either from plasma or serum) long non-coding RNAs (lncRNAs) present a potential ability for predicting occurrence or prognosis of multiple human malignant tumors. The present study aimed to screen potential lncRNAs as fingerprints for occurrence of hepatocellular carcinoma in patients with chronic hepatitis (CH). The lncRNA microarray was applied to screen potential biomarkers for HCC from CH patients. Each group contained three individual plasma samples. Multi-stage validation and risk score formula detection was used for validation. Eight dysregulated lncRNAs were obtained after Venny analysis. Further validation in a larger cohort, including 200 HCC patients, 100 CH patients, and 200 healthy controls, confirmed that increased nc-HOXC8-143, XLOC_000667, and AK123675 might be potential biomarkers for predicting early progress of HCC with an area under curve (AUC) of 0.821, 0.660, and 0.728, respectively. The merged AUC of the three factors was 0.907. This study also identified that circulating levels of three lncRNAs were associated with poor post-surgery prognosis of HCC patients.

Keywords: Hcc, long non-coding RNAs, AUC, chronic hepatitis, HBV

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, closely correlated with hepatitis B virus (HBV) infections [1, 2]. HCC is defined as a primary tumorigenesis in the liver, mainly in patients suffering from chronic liver cirrhosis or hepatitis B or C [3, 4]. The tumor gradually spreads to hepatocytes and, in advanced stages, metastasizes to other organs, such as the lungs and brain [5, 6]. HCC has become very common, causing death and affecting more than 500,000 people in the world. Poor outcomes of HCC are, in large part, due to the lack of timely diagnosis and frequent intrahepatic metastasis [7]. Indeed, vascular invasion, as a representative of intrahepatic metastasis, is a major cause of tumor recurrence within 2 years after resection [8]. To date, alpha-fetoprotein (AFP) measurements have been widely used in clinical practice. However, a recent study has shown that this assessment lacked adequate sensitivity and specificity for effective surveillance and diagnosis of HCC [9]. Therefore, seeking effective early biomarkers for diagnosis and targets for therapy is essential for diagnosis and treatment of HCC.

The human genome contains about 20,000 protein-coding genes, accounting for less than 2% of genome sequence [10]. Long non-coding RNAs (lncRNAs) are an important kind of non-coding RNA transcripts that range from 200 nt to 100 kb without protein-coding capacity [11]. Recently, many investigators have reported that lncRNAs play critically important roles in biological regulation, occurrence, and development of disease [12, 13]. It has been widely recognized that lncRNAs play crucial roles in the regulation of multiple biological processes, including proliferation, differentiation, apoptosis, tumorigenesis, and metastasis [14, 15]. An increasing number of circulating lncRNAs have been demonstrated to be dysregulated in plasma or serum, demonstrating their high potential as powerful and noninvasive tumor markers

Table 1. Clinic pathological analysis of HCC patients, CH patients, and cancer-free control samples

	HCC	CH	Control	*P* value
N	200	100	200	
Age Mean (SE) year	56.11 (10.2)	57.09 (9.3)	57.29 (10.3)	0.52[a]
Sex (male/female)	125/75	68/32	126/74	0.22[b]
Tumor Number				
Solitary	160			
Multiple	40			
Differentiation grade				
Well	66			
Moderate	100			
Poorly	34			
Tumor Size (cm)				
≤3 cm	118			
>3 cm	82			
TNM stage				
I-II	128			
III	72			
Metastasis				
Yes	122			
No	78			

[a]Student's t-test. [b]Chi-squared test.

[16, 17]. For example, Tang et al. reported that three lncRNAs (RP11-160H22.5, XLOC_014172, and LOC149086) were upregulated in HCC relative to cancer-free controls. Furthermore, XLOC_014172 and LOC149086 have been confirmed to be markedly increased in metastatic HCC [18, 19]. However, little evidence has been found for predicting HCC patients from HBV positive chronic hepatitis.

The present study aimed to characterize the genome-wide lncRNAs expression profile, in plasma from HCC patients and CH patients. Comparing with cancer-free controls, this study used lncRNAs microarrays to identify a panel of plasma lncRNAs that might serve as a novel biomarker for diagnosis of HCC.

Materials and methods

Samples and screening phase

The present study enrolled a total of 200 patients diagnosed with HCC. They were pathologically diagnosed in The First People's Hospital of Kunshan, Affiliated with Jiangsu University, between 2012 and 2016. This study also enrolled 100 patients suffering from HBV induced CH. All HCC patients and CH patients were confirmed with HBV infection. Patients with other malignant disorders, previous history of cancers, radiotherapy, and chemotherapy were excluded. The 200 cancer-free control subjects were confirmed without any malignant disorders or congenital diseases. This study was approved by the Institutional Ethics Committee of Jiangsu University. All research was performed in compliance with government policies and the Helsinki Declaration. Experiments were undertaken with the understanding and written consent of each subject.

Clinicopathological relevance analysis of all 500 individuals is summarized in **Table 1**. All 200 HCC patients enrolled in this study were clinically and pathologically diagnosed. There were no significant differences in distribution of age and sex between cancer/CH patients and cancer-free controls. The screening phase was divided into a training set and validation set. Twenty pairs of samples, including 20 patients with HCC, 20 patients with CH, and 20 healthy controls were enrolled, while the validation set contained two cohorts.

Training set

All candidates were tested in an independent cohort of 20 plasma samples obtained from patients. Expression levels of these candidates were analyzed in all samples and comparative $2^{-\Delta\Delta Ct}$ method algorithms were used to analyze differences between patients and health controls.

Validation set

A case-control study was designed to validate obvious differences of relative expression levels of selected potential biomarker candidates in another two independent cohorts, including 200 HCC patients, 100 CH patients, and 200 healthy controls. These were named as cohort I and II, respectively.

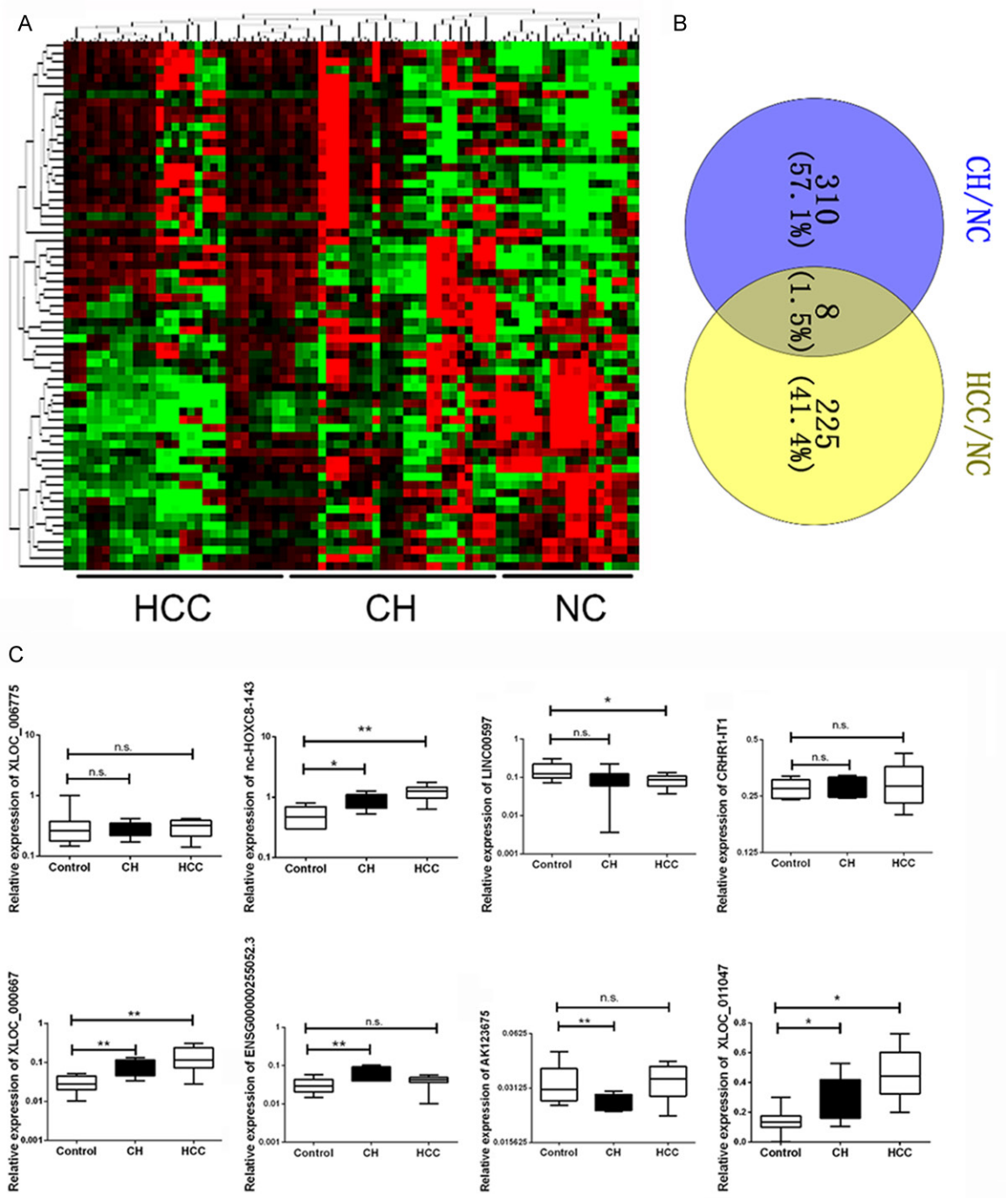


Figure 1. High throughput screening of circulating lncRNAs in HCC and chronic hepatitis (CH) patients. A. Hierarchical cluster analysis of lncRNA microarray detected in plasma from three HCC patients, three CH patients, and three controls. B. Venny analysis of dysregulated lncRNAs in HCC group and Dysplasia group, comparing with controls. C. Expression of 8 candidate lncRNA expression in 200 HCC patients, 100 CH patients, and 200 controls. Data are presented as mean ± SEM. Data was analyzed with Student's t test. *indicates $p<0.05$; **indicates $p<0.01$, n.s. indicates no significance.

Quantitative real-time PCR (qRT-PCR)

Total RNA was obtained from plasma samples using TRIzol Reagent, as described by the manufacturer (Invitrogen Life Technologies Co, Carlsbad, CA, USA). For mRNA detection, total RNAs (500 ng) were reverse transcribed using a reverse transcription kit (Takara, Tokyo, Japan). The quality of RNA samples was assessed by a UV spectrophotometer (Bio-Rad,

Table 2. Risk score analysis

Score	0-4.883	4.883-10.612	PPV[a]	NPV[b]
Training set			0.95	0.95
HCC	1	19		
CH	19	1		
Validation set			0.89	0.87
HCC	21	179		
CH	175	35		

[a]PPV, positive predictive value. [b]NPV, negative predictive value.

Hercules, CA, USA) and the 260/280 nm absorbance ratio of samples was limited to 1.8-2.0. QRT-PCR was performed using ABI Prism 7900 HT (Applied Biosystems, CA, USA).

Risk score analysis

Risk score analysis was performed to evaluate association between the concentrations of 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 corresponding lncRNAs for each sample as 0 and 1, in the training set. A risk score function (RSF) to predict TNBC group 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: 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 derived from univariate logistic regression analysis of each lncRNAs. Samples were ranked according to their RSF. They were then divided into a high-risk group, representing TNBC patients, and a low-risk group, representing predicted control individuals or non-metastasis patients. Frequency tables and ROC curves were then used to evaluate the diagnostic effects of profiling, to find the appropriate cut-off point, and to validate the procedure and cut-offs in the next validation sample set.

Statistical analysis

Venny online tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html) was employed in screening increased lncRNAs in the HCC group and CH group. All data are expressed as mean ± SEM. Statistical analysis was performed with Student's t-test for comparison of two groups and analysis of variance (ANOVA) for multiple comparisons. The statistical significance of microarray results was analyzed in terms of fold change using Student's t-test. False discovery rate (FDR) was calculated to correct the *P*-value. Fold changes ≥4 or ≤0.25 (P<001) were used as threshold values used to screen differentially expressed lncRNAs. Risk score analysis was applied in calculating the potential ability for predicting the ESCC receiver operating characteristic curve, created by STATA 10. Kaplan-Meier survival curves were plotted and log rank test was conducted. The significance of various variables for survival was analyzed by the Cox proportional hazards model in multivariate analysis. Statistical analysis was performed using STATA 10 and presented with GraphPad Prism 5.0 software. Results are considered statistically significant at P<0.05.

Results

Aberrant lncRNA expression profile in ESCC and dysplasia patients

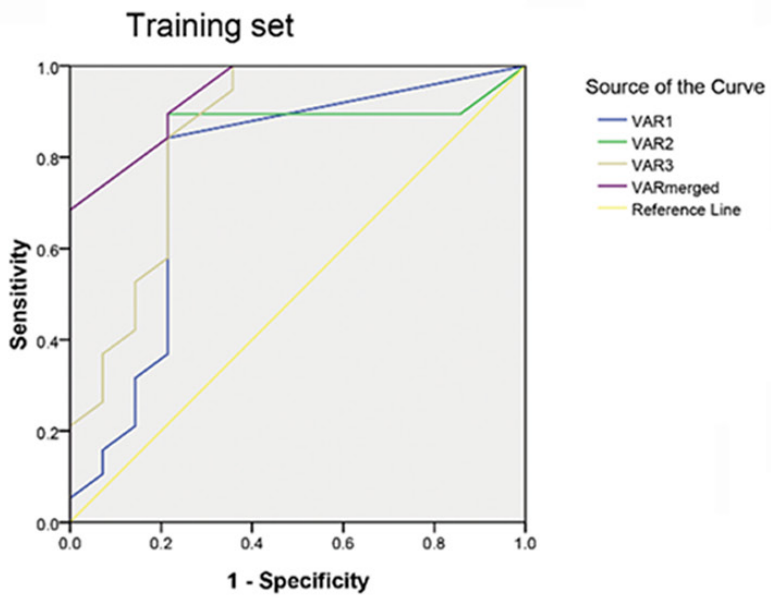
Human LncRNA Array v3.0 was used to detect lncRNAs derived from plasma of 3 patients with HCC, 3 patients with chronic hepatitis, and 3 cancer-free controls. Clustering analysis revealed an aberrant different expression lncRNA profile in these three groups (**Figure 1A**). A total of 233 lncRNA transcripts were specifically dysregulated in HCC patients and 318 lncRNAs in dysplasia patients, comparing to the control group. Of these, only 8 lncRNAs were selected, based on aberrant increased levels in both HCC patients and dysplasia patients with a cutoff of 4/0.25 (**Figure 1B**).

Multiple phase validation

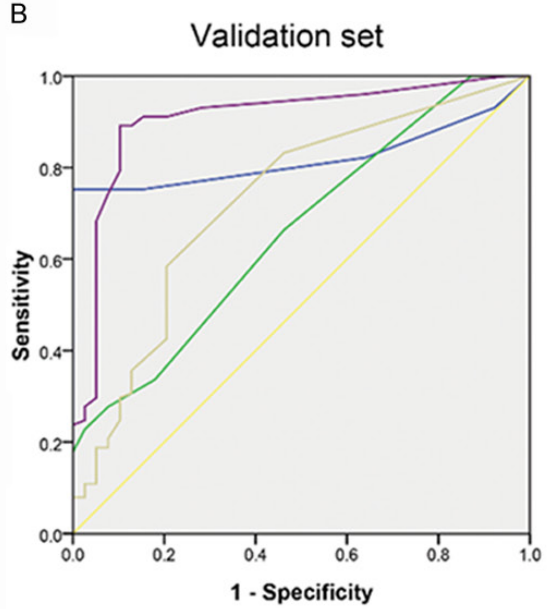
These 8 selected lncRNAs were further amplified in a larger sample, including 200 HCC patients, 100 CH patients, and 200 healthy controls. Only three lncRNAs (nc-HOXC8-143, XLOC_000667, and AK123675) were validated as significantly upregulated in both HCC patients and CH patients, comparing to the control group, while the remaining 5 lncRNAs presented a disqualification. Results are presented in **Figure 1C**.

To assess the diagnostic value of the three lncRNAs profiling system, a risk score formula

A



B



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
VAR1	.767	.093	.010	.584	.950
VAR2	.874	.068	.000	.741	1.007
VAR3	.855	.073	.001	.713	.998
VARmerged	.942	.037	.000	.870	1.014

The test result variable(s): VAR1, VAR2, VAR3, VARmerged has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

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
VAR1	.821	.035	.000	.752	.889
VAR2	.660	.050	.003	.562	.758
VAR3	.728	.049	.000	.632	.824
VARmerged	.907	.031	.000	.847	.967

The test result variable(s): VAR1, VAR2, VAR3, VARmerged has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 2. ROC analysis. Receiver operating characteristics (ROC) curves were drawn with the data of plasma lncRNAs separately, merged from 200 patients with HCC, 100 CH patients, and 200 healthy controls. Merged indicates the combination of three lncRNAs.

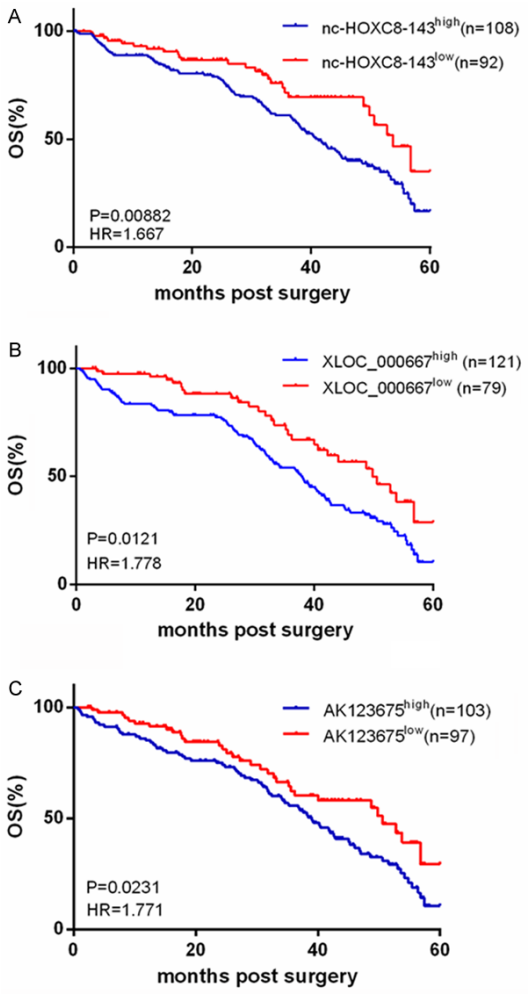


Figure 3. Prognosis predicting ability of circulating lncRNAs. Five-year survival analysis, reflecting the diagnosis value of these three indices on prognosis, was carried out using a Kaplan-Meier curve according to the follow up data. The upper 95% CI in control group was used as cutoff. A: nc-HOXC8-143; B: XLOC_000667; C: AK123675.

was used to calculate the risk score function for cases and control samples. Taking the three lncRNAs as candidates, 60 individuals were chosen in the three groups mentioned above (each group contained 20 individuals), randomly, as the training set. Expression of the three lncRNAs was calculated through risk score analysis. Based on risk score analysis, the optimal cutoff value (Value=4.883) was obtained with the value of sensitivity + specificity considered to be maximal. Plasma samples were then divided into a high-risk group, representing the possible HCC group, and a low-risk group, representing predicted controls. Positive predictive and negative predictive values were 95% and 95%, in the training set, respectively. Similarly, when the same cutoff value was applied to calculate the risk score of samples in the larger validation sets (the rest samples in each group), positive predictive and negative predictive values were 89% and 87% (**Table 2**).

ROC curve analysis was used to evaluate the predicting diagnosis value of lncRNAs for HCC from CH patients. Areas under the ROC curves (AUC) of the validated three lncRNAs signature were 0.767, 0.874, and 0.855, respectively, in the training set. Combination of the three factors possessed a moderate ability for discrimination between HCC patients and controls, with an area under the ROC curve of 0.942. Further validation set analysis revealed AUC of 0.821, 0.660, 0.728, and 0.907, respectively (**Figure 2**).

Circulating levels of lncRNAs associated with poor prognosis of HCC patients

Five-year survival analysis, reflecting the diagnosis value of these three indices on prognosis, was carried out using a Kaplan-Meier curve, according to the follow up data. All patients were sub-divided into high and low expression groups, according to 95% CI in the normal control group. All three lncRNAs indices were bad prognosis indicators for post-surgery HCC patients. Of these, significant survival differences were found in three lncRNAs: nc-HOXC8-143 (P=0.00882, HR=1.667), XLOC_000667 (P=0.0121, HR=1.778), and AK123675 (P=0.0231, HR=1.771) (**Figure 3**).

Stability detection of lncRNAs in human plasma

Next, this study amplified the three lncRNAs in five healthy controls and detected the product of amplification by agarose electrophoresis. The bands presented in **Figure 4** indicate that all three lncRNAs were detectable in human plasma. Human plasma obtained from three healthy controls was incubated at room temperature for 0 hours, 12 hours, 24 hours, and 48 hours, then treated with frozen-thawing for 3 cycles. It was found that neither the expression level of the three lncRNAs was alternated, indicating that nc-HOXC8-143, XLOC_000667, and AK123675 were stably expressed and detectable in human plasma.

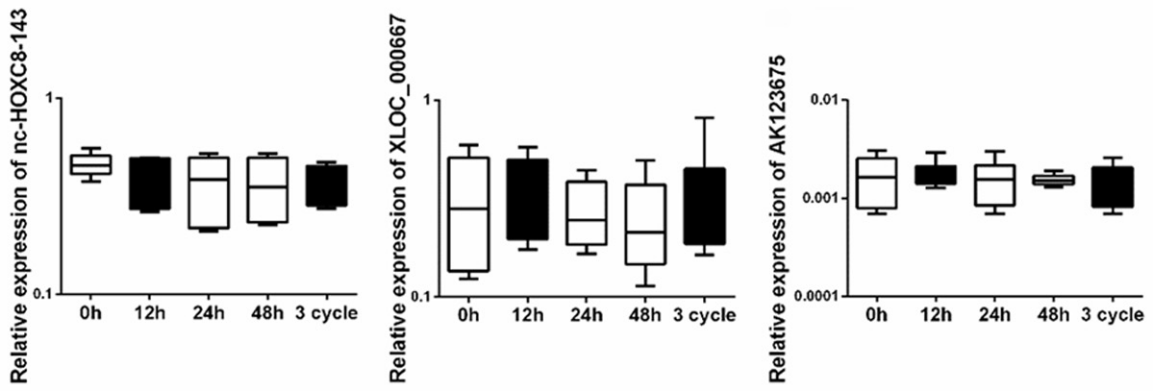


Figure 4. Stability detection of three lncRNAs in human plasma.

Discussion

The current study comprehensively analyzed lncRNA expression in HBV-related HCC, identifying three lncRNAs that might be fingerprints for HCC. Compared with previous reports, the present study had several advantages. First, HBV positive CH patients and primary HCC were treated as control groups to identify lncRNAs that were specifically differentially expressed in HBV-related HCC. Second, based on the development of HCC in a Chinese Han population, the biomarker for HCC from CH patients might be more effective. Third, compared with other reports, the present sample size was larger, perhaps providing better statistical efficacy.

Biomarkers are a vitally important part of clinical diagnosis and treatment. Many studies have focused on identifying effective biomarkers (especially blood biomarkers) because they are easy to obtain, do not require invasive methods, and have a lower economic burden. To date, the serum concentration of AFP is the most frequently used marker for diagnosis of HCC. However, increasing data has revealed that the sensitivity and specificity of AFP are not sufficient for effective diagnosis [20]. Therefore, there is an urgent need for more effective biomarkers for HCC. Researchers have identified multiple factors that might be potential fingerprints. However, most of these were conducted by comparing expression of these lncRNAs in a case-control study. The control group was healthy volunteers. This kind of biomarker might be useful for distinguishing HCC patients from healthy humans. However, the HCC patients were developed from HBV positive patients and most of them were suffering with CH or liver cirrhosis. Biomarkers for predicting HCC from patients with CH or liver cirrhosis might be useful for early diagnosis of HCC.

Generally, lncRNAs have been considred junk RNA and transcription noise. Recent evidence, however, has proven that lncRNAs are involved in various biological and pathological processes [14, 21, 22]. The present study found that 3 of the 8 candidate lncRNAs could be detected in HCC patients, CH patients, and healthy individuals, indicating that some lncRNAs might have extreme tissue specificity. Using qRT-PCR, it was demonstrated that expression levels of 3 cancer-associated lncRNAs varied significantly between the plasma of HCC patients, CH patients, and healthy controls. Additionally, risk score analysis revealed that three lncRNAs demonstrated great ability to discriminate between HCC patients and CH patients, while also predicting poor prognosis.

In conclusion, this study identified three lncRNAs, nc-HOXC8-143, XLOC_000667, and AK123675, as potential biomarkers for occurrence of HCC. These factors may serve as both diagnosis and prognosis biomarkers for HCC, especially for patients with an HBV infection background.

Disclosure of conflict of interest

None.

Address correspondence to: Maoying Fu, Department of Infectious Disease, The First People's Hospital of Kunshan Affiliated with Jiangsu University, Suzhou, China. Tel: +86-512-57559009; Fax: +86-512-57559009; E-mail: fumaoying66@yeah.net

References

[1] Wang TH, Wu CH, Yeh CT, Su SC, Hsia SM, Liang KH, Chen CC, Hsueh C and Chen CY. Melatonin suppresses hepatocellular carcinoma progression via lncrna-cps1-it-mediated hif-1alpha inactivation. Oncotarget 2017; 8: 82280-82293.

[2] Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q, Qin L, Wu X, Zheng Y, Yang Y, Tian W, Zhang Q, Wang C, Zhang Q, Zhuang SM, Zheng L, Liang A, Tao W and Cao X. Identification of mirnomes in human liver and hepatocellular carcinoma reveals mir-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 2011; 19: 232-243.

[3] Yong KJ, Gao C, Lim JS, Yan B, Yang H, Dimitrov T, Kawasaki A, Ong CW, Wong KF, Lee S, Ravikumar S, Srivastava S, Tian X, Poon RT, Fan ST, Luk JM, Dan YY, Salto-Tellez M, Chai L and Tenen DG. Oncofetal gene sall4 in aggressive hepatocellular carcinoma. N Engl J Med 2013; 368: 2266-2276.

[4] Arzumanyan A, Reis HM and Feitelson MA. Pathogenic mechanisms in hbv- and hcv-associated hepatocellular carcinoma. Nat Rev Cancer 2013; 13: 123-135.

[5] Rajyaguru DJ, Borgert AJ, Smith AL, Thomes RM, Conway PD, Halfdanarson TR, Truty MJ, Kurup AN and Go RS. Radiofrequency ablation versus stereotactic body radiotherapy for localized hepatocellular carcinoma in nonsurgically managed patients: analysis of the national cancer database. J Clin Oncol 2018; JCO2017753228.

[6] Gautheron J and Luedde T. A novel player in inflammation and cancer: the deubiquitinase cyld controls hcc development. J Hepatol 2012; 57: 937-939.

[7] Kang Z and Xiao E. Expression of cluster of differentiation 151 prior to and following transcatheter arterial chemoembolization therapy in patients with hepatocellular carcinoma and its association with clinicopathological characteristics. Oncol Lett 2018; 15: 1133-1142.

[8] DeWaal D, Nogueira V, Terry AR, Patra KC, Jeon SM, Guzman G, Au J, Long CP, Antoniewicz MR and Hay N. Hexokinase-2 depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin. Nat Commun 2018; 9: 446.

[9] Notarpaolo A, Layese R, Magistri P, Gambato M, Colledan M, Magini G, Miglioresi L, Vitale A, Vennarecci G, Ambrosio CD, Burra P, Di Benedetto F, Fagiuoli S, Colasanti M, Maria Ettorre G, Andreoli A, Cillo U, Laurent A, Katsahian S, Audureau E, Roudot-Thoraval F and Duvoux C. Validation of the afp model as a predictor of hcc recurrence in patients with viral hepatitis-related cirrhosis who had received a liver transplant for hcc. J Hepatol 2017; 66: 552-559.

[10] Fatica A and Bozzoni I. Long non-coding rnas: new players in cell differentiation and development. Nat Rev Genet 2014; 15: 7-21.

[11] Batista PJ and Chang HY. Long noncoding rnas: cellular address codes in development and disease. Cell 2013; 152: 1298-1307.

[12] Yu H, Xu Q, Liu F, Ye X, Wang J and Meng X. Identification and validation of long noncoding rna biomarkers in human non-small-cell lung carcinomas. J Thorac Oncol 2015; 10: 645-654.

[13] Yang J, Lin J, Liu T, Chen T, Pan S, Huang W and Li S. Analysis of lncrna expression profiles in non-small cell lung cancers (nsclc) and their clinical subtypes. Lung Cancer 2014; 85: 110-115.

[14] Tang J, Zhuo H, Zhang X, Jiang R, Ji J, Deng L, Qian X, Zhang F and Sun B. A novel biomarker linc00974 interacting with krt19 promotes proliferation and metastasis in hepatocellular carcinoma. Cell Death Dis 2014; 5: e1549.

[15] Tang J, Jiang R, Deng L, Zhang X, Wang K and Sun B. Circulation long non-coding rnas act as biomarkers for predicting tumorigenesis and metastasis in hepatocellular carcinoma. Oncotarget 2015; 6: 4505-4515.

[16] Dong L, Qi P, Xu MD, Ni SJ, Huang D, Xu QH, Weng WW, Tan C, Sheng WQ, Zhou XY and Du X. Circulating cudr, lsinct-5 and ptenp1 long noncoding rnas in sera distinguish patients with gastric cancer from healthy controls. Int J Cancer 2015; 137: 1128-1135.

[17] Arita T, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Shoda K, Kawaguchi T, Hirajima S, Nagata H, Kubota T, Fujiwara H, Okamoto K and Otsuji E. Circulating long non-coding rnas in plasma of patients with gastric cancer. Anticancer Res 2013; 33: 3185-3193.

[18] Crea F, Watahiki A, Quagliata L, Xue H, Pikor L, Parolia A, Wang Y, Lin D, Lam WL, Farrar WL, Isogai T, Morant R, Castori-Eppenberger S, Chi KN and Helgason CD. Identification of a long non-coding rna as a novel biomarker and potential therapeutic target for metastatic prostate cancer. Oncotarget 2014; 5: 764-774.

[19] Ren S, Wang F, Shen J, Sun Y, Xu W, Lu J, Wei M, Xu C, Wu C, Zhang Z, Gao X, Liu Z, Hou J and Huang J. Long non-coding rna metastasis associated in lung adenocarcinoma transcript 1 derived minirna as a novel plasma-based biomarker for diagnosing prostate cancer. Eur J Cancer 2013; 49: 2949-2959.

[20] Ma X, Yuan T, Yang C, Wang Z, Zang Y, Wu L and Zhuang L. X-inactive-specific transcript of peripheral blood cells is regulated by exosomal jpx and acts as a biomarker for female patients

with hepatocellular carcinoma. Ther Adv Med Oncol 2017; 9: 665-677.

[21] Ji J, Tang J, Deng L, Xie Y, Jiang R, Li G and Sun B. Linc00152 promotes proliferation in hepatocellular carcinoma by targeting epcam via the mtor signaling pathway. Oncotarget 2015; 6: 42813-42824.

[22] Wang Y, Wu P, Lin R, Rong L, Xue Y and Fang Y. Lncrna nalt interaction with notch1 promoted cell proliferation in pediatric t cell acute lymphoblastic leukemia. Sci Rep 2015; 5: 13749.