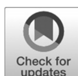
Research Article

Cancer Epidemiology, Biomarkers & Prevention



Serum Exosomal Long Noncoding RNAs *ENSG00000258332.1* and *LINC00635* for the Diagnosis and Prognosis of Hepatocellular Carcinoma

Hong Xu, Yueming Chen, Xueyan Dong, and Xianjun Wang

Abstract

Background: Increasing studies suggest that long noncoding RNAs (lncRNAs) are involved in carcinogenesis of human cancers and might be used as diagnostic biomarkers for cancers.

Methods: A total of 301 participants were recruited in the first part of the study, including a hepatocellular carcinoma (HCC) group ($n = 60$), liver cirrhosis (LC) group ($n = 85$), chronic hepatitis B (CHB) group ($n = 96$), and healthy subjects ($n = 60$). In the second part, we collected 55 HCC patients, 60 CHB patients, and 60 healthy subjects as an independent cohort to validate the ability of the experiential lncRNAs for identifying HCC from CHB. A commercial kit was used to isolate serum exosomes and total RNA. The relative levels of lnRNAs and *GAPDH* mRNA were measured with TaqMan PCR.

Results: The results showed that the levels of *ENSG00000258332.1* and *LINC00635* in the HCC group were significantly higher than those in the other groups (all $P < 0.05$). A high *ENSG00000258332.1* level in HCC was associated with portal vein tumor emboli, lymph node metastasis, TNM stage, and overall survival (OS; all $P < 0.05$), and a high *LINC00635* level was related to lymph node metastasis, TNM stage, and OS (all $P < 0.05$). *ENSG00000258332.1* discriminated HCC from CHB, gaining an area under the ROC curve (AUC) of 0.719 (cutoff value of 1.345); *LINC00635* gained an AUC of 0.750 (cutoff value of 1.690). Furthermore, the AUC for the combination of the 2 lncRNAs and serum AFP (cutoff value of 20 μg/L) was 0.894. The abilities of the 2 lncRNAs for identifying HCC from CHB were validated by an independent cohort.

Conclusions: The results suggested that the combination of serum exosomal *ENSG00000258332.1*, *LINC00635*, and AFP may be a valuable assay in diagnosis and prognosis of HCC.

Impact: Our data will shed light on exosomal lncRNAs as biomarkers for HCC. *Cancer Epidemiol Biomarkers Prev; 27(6); 710–6. ©2018 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadly cancers in the world. The morbidity rate of HCC ranks fifth, and the mortality rate in males is the second highest in frequency worldwide among all malignancies. The latest data estimated that there were approximately 782,000 new cases and 745,000 deaths due to liver cancer in 2012, especially in Asia and Africa (1). There are some major risk factors, including chronic hepatitis B and C virus (HBV, HCV) infection, aflatoxin B1 intake, smoking, excessive alcohol consumption, and diabetes mellitus, that contribute to the occurrence of HCC (2, 3). HBV infection is the leading pathogeny of HCC in 37 countries in the Western Pacific Region, including China, which is approaching 50% of the total incidence in the world (4).

At present, serum α-fetoprotein (AFP) acts as a common screening test for HCC, while the false-negative rate is almost 50% in HCC patients. Hence, AFP is not a precise marker because it provides low sensitivity and specificity (5). Fortunately, HCC has specific etiologic factors in comparison with the other malignant tumors. If the high-risk population could be supervised periodically with appropriate methods, we believe that the patients with HCC could have a definite diagnosis in time, and their life expectancy and quality could be greatly improved. Therefore, it is urgent to develop a potent diagnostic and prognostic indicator for HCC.

Over the past decade, the underlying molecular mechanisms of hepatocarcinogenesis have been elucidated partly. Increasingly evidences reveal that long noncoding RNAs (lncRNAs) play crucial roles in cancer biology, including HCC (6). Noncoding RNAs (ncRNAs) are divided into small RNAs (such as miRNA, siRNA, and piwi-interacting RNA) and lncRNAs based on nucleotide sequence length (7). LncRNAs are defined as transcripts of more than 200 nucleotides that structurally resemble mRNAs but have little or no potential for encoding proteins (8). Although lncRNAs exhibit opaque and complicated mechanisms in various biological processes, some literatures illustrated that lncRNAs participated in the pathogenesis and development of human cancers, such as *H19*, *HOTAIR*,

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), and maternally expressed gene 3 (9–12).

Exosomes are one type of membrane vesicles secreted into extracellular space, produced by almost all cells, executing the function of cell-to-cell communication. It has a diameter of 30 to 100 nm and is a diverse cell-derived vesicle containing a variety of proteins, ncRNA, DNA, mRNA, lipid, and bioactive peptides (13). In a former study, we pooled 4 pairs of sera from untreated HCC patients and healthy subjects into one pair of samples, extracted total exosomal RNA, and used RNA sequencing (RNA-seq) to screen differentially expressed transcripts, which was conducted by a commercial service (RiboBio). In the exosomal lncRNA data produced by RNA-seq, 252 differentially expressed transcripts were identified (Supplementary Table S1). *ENSG00000258332.1* and *LINC00635* were significantly upregulated in the transcripts. Specially, the medians of reads per million for the 2 lncRNAs in serum of HCC patients are higher than the other differential lncRNAs that benefit clinical detection, and the functions of the 2 lncRNAs have been partly explained in the recent studies (14–17). Therefore, it is necessary to assess whether serum exosomal *ENSG00000258332.1* and *LINC00635* are feasible and noninvasive biomarkers for HCC.

In this study, we measured the levels of *ENSG00000258332.1* and *LINC00635* in serum-derived exosomes from patients with HCC, liver cirrhosis (LC), and chronic hepatitis B (CHB) and healthy subjects using TaqMan real-time PCR. The abilities of *ENSG00000258332.1*, *LINC00635*, and AFP to identify HCC were analyzed using ROC curves, which were validated by an independent test. In addition, we evaluated the relationship between exosomal *ENSG00000258332.1* and *LINC00635* and clinicopathologic features in HCC patients, as well as overall survival (OS).

Materials and Methods

Patients

In the first part of the study, 241 patients were enrolled that visited Hangzhou First People's Hospital (Hangzhou, China) from January 2012 to June 2016. In detail, 60 HCC patients were diagnosed on the basis of serum AFP, liver ultrasound, CT, and MRI, and the diagnosis was confirmed by histopathologic examination; 85 LC patients were diagnosed using liver ultrasound, CT and MRI, and/or accompanying portal hypertension and hypersplenism; 96 CHB patients were diagnosed on the basis of HBV surface antigen (HBsAg)-positive and/or HBV DNA-positive serum for more than 6 months; 60 healthy subjects were collected from the physical examination center of Hangzhou First People's Hospital. In the second part of the study, we collected 55 HCC patients, 60 CHB patients, and 60 healthy subjects according to the inclusion criteria mentioned above from March 2017 to November 2017, which acted as an independent test to validate the abilities of the 2 lncRNAs for identifying HCC from CHB. The subjects with autoimmune hepatitis, alcoholic liver disease, Wilson disease, other types of viral hepatitis, and other major diseases were excluded. All participants were age and gender matched.

Institutional review board approval and informed consent

All subjects provided written informed consent, and this study was approved by the ethics committee of Hangzhou First People's Hospital. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Serum collection and exosomal RNA extraction

Four milliliters of fasting blood was drawn from each volunteer. Two milliliters of serum was isolated after centrifugation at 3,000 $\times$ g for 5 minutes, and approximately 2 mL of serum was subsequently acquired after centrifugation at 10,000 $\times$ g for 5 minutes. The prepared serum samples were stored at -70°C until further use.

One milliliter of sera was appended to a new tube, and 200 μL of total exosome isolation reagent (Thermo Fisher Scientific Co., Ltd.) was added. The mixture was mixed well by pipetting up and down until a homogenous solution was produced. The sample was incubated at 4°C for 30 minutes, followed by centrifugation at 10,000 $\times$ g for 10 minutes at 20°C. The obtained pellet was resuspended in 200 μL of PBS. Total exosomal RNA was extracted and purified using a commercial kit (Thermo Fisher Scientific Co., Ltd.), and 50 μL of total RNA was acquired eventually.

TaqMan real-time PCR

The primer and probe sequences for *ENSG00000258332.1*, *LINC00635* and *GAPDH* mRNA were designed using Primer Express v3.0 software (Applied Biosystems Co., Ltd.) and synthesized by Shanghai Huirui Biology Science & Technology Company. The primers for *ENSG00000258332.1* were (forward) 5′-TGCCATCAGGAAA ATGCACA-3′ and (reverse) 5′-CCATGTG-GAACTGTGAGCCT-3′; the probe for *ENSG00000258332.1* was 5′-FAM-TCACGCTGCTGATAAAGACATACCAGGGA-TAMRA- 3′. The primers for *LINC00635* were (forward) 5′-AGGCAAAG-GTTTGGATGGGAT-3′ and (reverse) 5′-AGGCATGTCTTTTCTC-CTGTTTT-3′; the probe for *LINC00635* was 5′-FAM-AATGT-GAGTTCGTGTCCTCTGCCAGACC-TAMRA-3′. The primers for *GAPDH* were (forward) 5′-GAGCTGAACGGGAAGCTCAC-3′ and (reverse) 5′-GCCTGCTTCACCACC TTCTT-3′; the probe for *GAPDH* was 5′-FAM-ACAGTCTC ACTTTATCGCCCCAGGCTGG -TAMRA-3′.

The PCR mixture contained 10 μL of 2 $\times$ PCR buffer, 0.4 μL of DNA polymerase, 0.4 μL of reverse transcriptase, 0.4 μL of each primer, 0.8 μL of each probe, 2 μL of RNA template, and nuclease-free water to a total volume of 20 μL (TaKaRa Inc.). PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems Co., Ltd.) under the following cycling conditions: 50°C for 15 minutes, 95°C for 5 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 40 seconds. *GAPDH* mRNA was used as an internal control; no-template control was included as negative control. For quantitative results, the relative expression level expression of each lncRNA was represented as a fold change using the $2^{-\Delta\Delta C_t}$ method (18). Each sample was assayed in duplicate, with an average of the two duplicates used for analysis.

Statistical analysis

Nonparametric variables are presented as the median and interquartile ranges, which were analyzed using the Mann-Whitney U test and Kruskal–Wallis H test. Categorical variables were compared using the χ^2 test. ROC curves were constructed, and the AUC were estimated to determine the feasibilities of using *ENSG00000258332.1*, *LINC00635*, and serum AFP levels as

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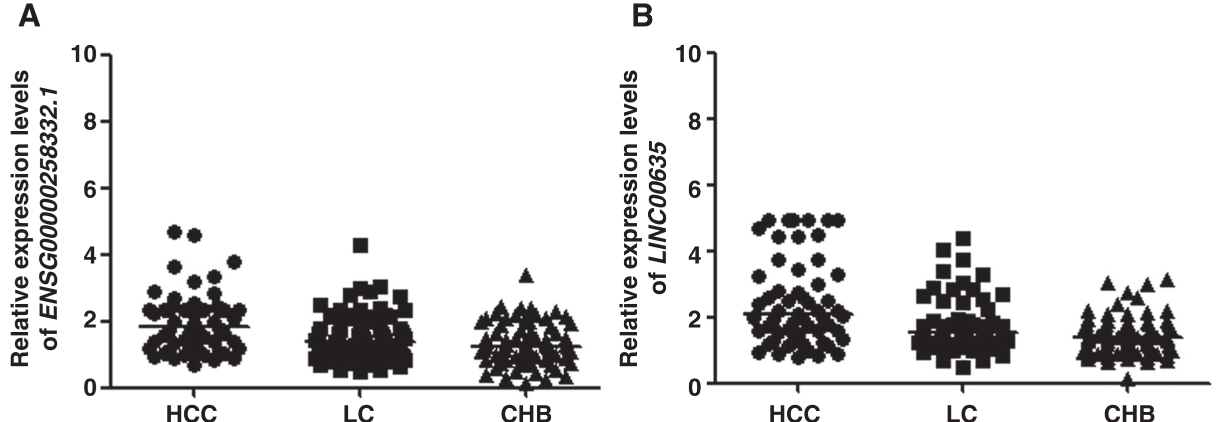

Figure 1.

A scatter plot for serum exosomal *ENSG00000258332.1* and *LINC00635* levels. **A,** There were significant differences in the levels of *ENSG00000258332.1* between 2 groups (HCC vs. LC, $P = 0.002$; HCC vs. CHB, $P = 0.001$), whereas there was no significant difference between LC patients and CHB patients ($P = 0.158$). **B,** The differences in the levels of *LINC00635* between 2 groups were similar to that of *ENSG00000258332.1* (HCC vs. LC, $P = 0.005$; HCC vs. CHB, $P = 0.002$; LC vs. CHB, $P = 0.042$, respectively).

biomarkers for HCC. OS curves were calculated from the day of confirmed HCC to death or the last follow-up observation using the Kaplan–Meier method; the differences between the high lncRNA level subset and low lncRNA level subset were assessed with a log-rank test. *P* values less than 0.05 were considered statistically significant. All data analyses were performed using SPSS software (version 16.0, SPSS Inc.).

Results

Quantification of exosomal *ENSG00000258332.1* and *LINC00635*

The relative levels of serum exosomal *ENSG00000258332.1* and *LINC00635* were detected in HCC ($n = 60$), LC ($n = 85$), CHB ($n = 96$), and healthy subjects ($n = 60$) from the first part of the study using TaqMan real-time PCR.

The levels of *ENSG00000258332.1* were 1.845 (1.175–2.330) in HCC patients, 1.360 (0.960–1.770) in LC patients, 1.240 (0.935–1.655) in CHB patients. There were significant differences in the levels of *ENSG00000258332.1* between 2 groups (HCC vs. LC, $P = 0.002$; HCC vs. CHB, $P = 0.001$), whereas there was no significant difference between LC patients and CHB patients ($P = 0.158$; Fig. 1A). The levels of *LINC00635* were 2.100 (1.460–3.430) in HCC patients, 1.510 (1.230–1.750) in LC patients, and 1.370 (1.018–1.770) in CHB patients. The difference in the levels of *LINC00635* between 2 groups were similar to that of ENSG00000258332.1 (HCC vs. LC, $P = 0.005$; HCC vs. CHB, $P = 0.002$; LC vs. CHB, $P = 0.042$, respectively; Fig. 1B).

To determine the reproducibility of *LINC00635* assay by TaqMan real-time PCR, the intra-assay variation was assessed by testing 3 mixed serum samples with different levels 10 times in a single run; meanwhile, the interassay variation was assessed by testing the same samples 10 times in 5 separate runs. The intra-assay coefficient of variation (CV) ranged from 0.91% to 1.89%, and the interassay CV ranged from 1.21% to 2.37% (Table 1).

The results indicated that the 2 lncRNAs showed overexpression between HCC group and CHB group, and the assay exhibited high reproducibility.

Expression of the markers in HCC correlation with clinical characteristics and operative status

Sixty HCC patients were selected from the first part of the study. We analyzed the relationship between the relative levels of exosomal *ENSG00000258332.1* and *LINC00635* and clinicopathologic variables, including gender, age, serum HBsAg status, histologic grade, portal vein tumor emboli, lymph node metastasis, tumor–node–metastasis (TNM) stage, and cirrhosis in HCC patients. No significant association was found between the lncRNAs and gender, age, serum HBsAg status, histologic grade, and cirrhosis (all $P > 0.05$), whereas *ENSG00000258332.1* level was associated with portal venous invasion, lymph node metastasis, and TNM stage ($P = 0.004$, $P = 0.028$, $P = 0.021$, respectively); *LINC00635* was linked to lymph node metastasis and TNM stage ($P = 0.043$, $P = 0.033$, respectively; Table 2). We also measured the levels of the 2 lncRNAs in 21 HCC patients, whose serum samples were collected preoperatively and postoperatively (visiting hospital day vs. 21 days after tumor resection). The levels of both lncRNAs were significantly reduced after surgery (all $P < 0.05$; Fig. 2A and B). The results showed that the 2 lncRNAs would be helpful to clinical staging and recurrence prediction for HCC patients.

Table 1. Reproducibility of TaqMan real-time PCR assay for *LINC00635*

	Intra-assay				Interassay			
Sample no.	Times of replication	Mean C_t	SD	CV (%)	Days of replication	Mean C_t	SD	CV (%)
1	10	25.39	0.23	0.91	5	25.27	0.37	1.46
2	10	27.10	0.45	1.67	5	27.18	0.33	1.21
3	10	30.18	0.57	1.89	5	30.35	0.72	2.37

Abbreviation: C_t, cycle threshold

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Table 2. Correlation between the relative levels of both exosomal lncRNAs and clinicopathologic variables in an independent set

Factors	*n*	*ENSG00000258332.1*	*P*	*LINC00635*	*P*
Gender					
Male	50	2.025 (1.493–2.435)	0.390	2.660 (1.650–4.115)	0.248
Female	10	1.850 (1.285–2.330)		3.290 (1.495–3.360)	
Age (y)					
≤50	11	2.330 (1.513–2.730)	0.411	2.370 (1.735–4.418)	0.943
>50	49	1.960 (1.420–2.330)		2.595 (1.543–3.730)	
HBsAg status					
Positive	44	2.150 (1.650–2.330)	0.158	2.790 (1.770–4.170)	0.251
Negative	16	1.650 (1.050–2.690)		1.950 (1.460–3.290)	
Tumor size (cm)					
<5	21	1.683 (1.096–2.024)	0.152	1.894 (1.270–2.926)	0.173
≥5	39	1.853 (1.206–2.247)		2.112 (1.194–3.283)	
Histologic grade					
I+II	29	1.773 (1.137–1.902)	0.252	1.922 (1.187–2.910)	0.375
III+IV	31	1.866 (1.184–2.338)		2.117 (1.192–3.095)	
Portal vein tumor emboli					
Yes	32	2.330 (1.815–2.570)	0.004	3.230 (2.008–3.730)	0.122
No	28	1.650 (1.295–2.060)		2.975 (1.460–4.540)	
Lymph node metastasis					
Yes	35	2.265 (1.760–2.530)	0.028	2.580 (1.941–3.722)	0.043
No	25	1.680 (1.586–2.150)		1.825 (1.390–3.155)	
TNM stage					
I+II	16	1.512 (1.243–2.091)	0.021	1.635 (1.245–2.741)	0.033
III+IV	44	2.226 (1.560–2.462)		2.247 (1.635–3.583)	
Cirrhosis					
Yes	42	1.925 (1.530–2.430)	0.401	2.980 (1.543–3.840)	0.744
No	18	1.836 (1.390–2.217)		2.460 (1.735–3.730)	

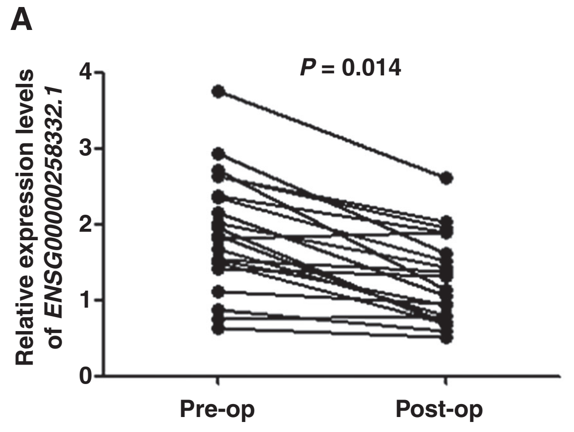
NOTE: Serum exosomal lncRNA levels are presented as the median and interquartile range. The histologic examination was conducted by referencing the standard Edmondson grade. Tumor stage was classified according to the TNM criteria of the International Union Against Cancer.

Powers of exosomal *ENSG00000258332.1* and *LINC00635* for discriminating HCC

We explored whether the relative levels of the 2 lncRNAs could discriminate HCC patients from CHB patients; ROC curve analyses were performed (the data from the first part of the study). The results showed that *ENSG00000258332.1* discriminated HCC patients from CHB patients, yielding an AUC of 0.719 [95% confidence interval (CI), 0.658–0.865; $P = 0.004$] at a cutoff value of 1.345 with 71.6% sensitivity and 83.4% specificity; *LINC00635* yielded an AUC of 0.750 (95% CI, 0.626–0.835; $P = 0.002$) at a cutoff value of 1.690 with 76.2% sensitivity and 77.7% specificity. Serum AFP at a cutoff value of 20 μg/L yielded an AUC of 0.666 (95% CI, 0.531–0.724; $P = 0.025$), with 54.7% sensitivity and 75.3% specificity. Furthermore, the AUC for the combination of the 3 markers was 0.894 (95% CI, 0.761–1.104; $P = 0.005$), with 83.6% sensitivity and 87.7% specificity (Fig. 3). The data suggest the combined assay could be a potential biomarker for HCC.

Kaplan–Meier analysis of OS

Sixty patients with HCC enrolled in the first part of the study were followed up with the median duration of 17.67 (2.31–33.73) months, and the OS rate was 38.3% (23/60). According to the median levels of *ENSG00000258332.1* (1.845) and



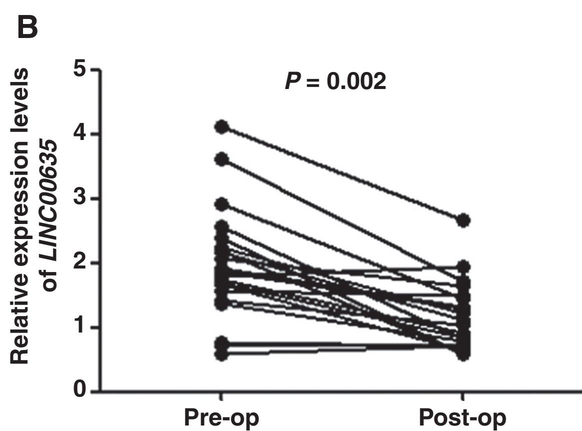


Figure 2.
Serum relative levels of exosomal lncRNAs in HCC patients varied in paired preoperative and postoperative samples. The relative levels of serum exosomal *ENSG00000258332.1* (**A**) and *LINC00635* (**B**) were significantly reduced 21 days after tumor resection in 21 HCC patients, as examined by TaqMan real-time PCR (all $P < 0.05$).

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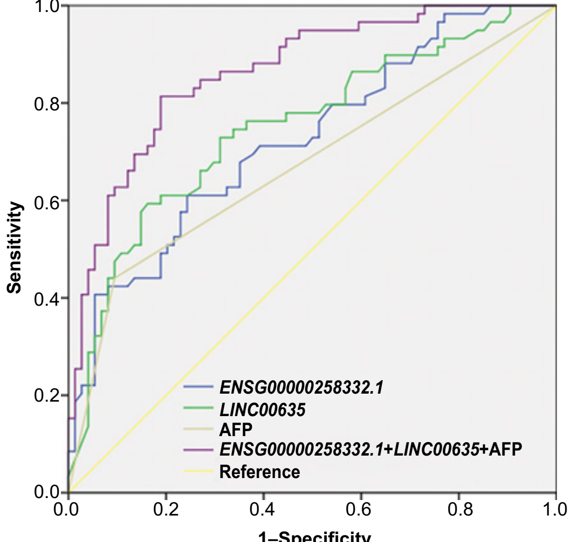

Figure 3.
ROC curve analyses for identifying HCC patients from CHB patients. Serum exosomal *ENSG00000258332.1* discriminated HCC patients from CHB patients obtaining an AUC of 0.719 at a cutoff value of 1.345; *LINC00635* yielded an AUC of 0.750 at a cutoff value of 1.690; serum AFP at a cutoff value of 20 μg/L yielded an AUC of 0.666; combined, the 3 markers yielded an AUC of 0.894 with 83.6% sensitivity and 87.7% specificity, respectively.

LINC00635 (2.100), 60 cases of HCC patients were divided into high-level and low-level subsets. Using a Kaplan–Meier analysis, we found that the high-level *ENSG00000258332.1* subset ($n = 29$) was associated with worse OS in HCC patients ($\chi^2 = 4.489$, $P = 0.034$; Fig. 4A); the high-level *LINC00635* subset ($n = 26$) was significantly related to worse OS ($\chi^2 = 4.348$, $P = 0.037$; Fig. 4B). The results revealed that the 2 lncRNAs could predict prognosis of HCC patients.

Validating the powers of the 2 lncRNAs for identifying HCC in an independent set

The following investigation was conducted by detecting serum exosomal levels of the 2 lncRNAs in an independent cohort, which consisted of 55 HCC patients, 60 CHB patients, and 60 healthy subjects. The relative levels of *ENSG00000258332.1* were 1.890 (1.240–2.410) in HCC patients and 1.190 (0.788–1.795) in CHB patients, and that of *LINC00635* were 2.085 (1.280–2.950) in HCC patients and 1.325 (1.045–1.688) in CHB patients. The differences in the levels of *ENSG00000258332.1* and *LINC00635* between 2 groups were obviously significant ($P = 0.004$, $P = 0.002$, respectively).

ENSG00000258332.1 discriminated HCC patients from CHB patients, yielding an AUC of 0.718 (95% CI, 0.646–0.822; $P = 0.009$) at a cutoff value of 1.366 with 73.5% sensitivity and 80.5% specificity; *LINC00635* yielded an AUC of 0.731 (95% CI, 0.651–0.817; $P = 0.004$) at a cutoff value of 1.532 with 79.6% sensitivity and 75.2% specificity. Serum AFP at a cutoff value of 20 μg/L yielded an AUC of 0.634 (95% CI, 0.531–0.717; $P = 0.021$), with 52.5% sensitivity and 74.1% specificity. Furthermore, the AUC for the combination of the 3 markers was 0.885 (95% CI, 0.749–1.025; $P = 0.012$), with 84.5% sensitivity and 85.3% specificity (Fig. 5). Besides, we also examined the relationship between the levels of the 2 lncRNAs and clinicopathologic features of HCC patients in the validation cohort (Supplementary Table S2). The results displayed the abilities of the 2 lncRNAs as biomarkers for HCC could be confirmed in the independent cohort.

Discussion

Typically, cancer cells secrete more exosomes than healthy cells, and the exosomes cargoes differ. Cancer cell–derived exosomes can provide a suitable microenvironment for cancer cell proliferation, drug resistance, angiogenesis, invasion, immune modulation, and premetastatic niche formation (19). In addition, exosomes are suitable for use as a "liquid biopsy" for malignancies, because they are very stable, easy to obtain, and present in almost all body liquids (20, 21).

The lncRNA *ZFAS1* can be transferred by exosomes to promote gastric cancer progression, which suggests that *ZFAS1* may be a diagnostic and prognostic biomarker for gastric cancer (22, 23). Selective enrichment of the lncRNAs *HOTAIR*, *HOX-AS-2*, *ANRIL*, and *linc-RoR* in urinary exosomes from urothelial cancer patients was reported to be associated with poor prognosis (24, 25). Exosomal *lncARSR* elevated in plasma in renal cell carcinoma could predict a poor response to sunitinib (26,

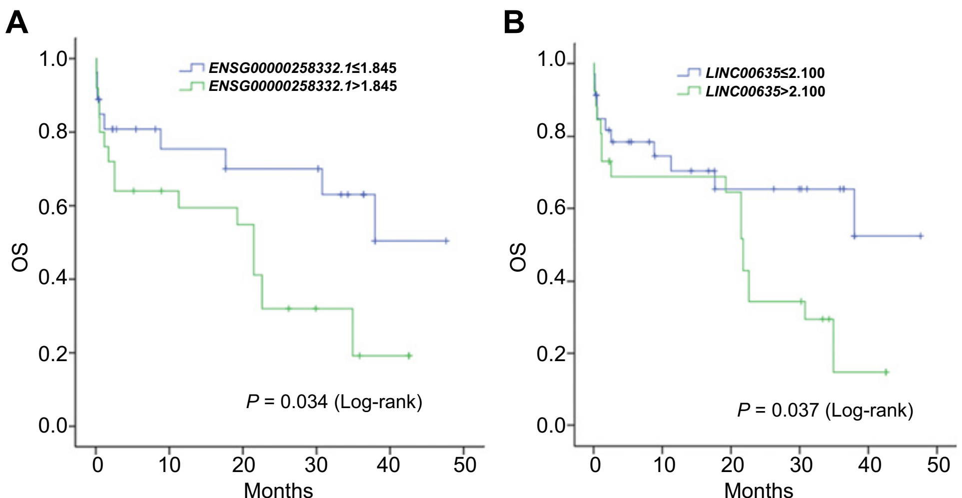

Figure 4.
Kaplan–Meier analysis of OS with log-rank test. Sixty patients with HCC enrolled in the first part of the study were followed up with the median duration of 17.67 (2.31–33.73) months, and the OS rate was 38.3% (23/60). **A,** According to the median level of *ENSG00000258332.1* (1.845), 29 cases of high-level subset and 31 cases of low-level subset were classified in 60 HCC patients, and high-level subset was associated with worse OS ($\chi^2 = 4.489$, $P = 0.034$). **B,** According to the median level of *LINC00635* (2.100), 26 cases of high-level subset and 34 cases of low-level subset were classified in 60 HCC patients, and high-level subset of *LINC00635* was correlated to worse OS ($\chi^2 = 4.348$, $P = 0.037$).

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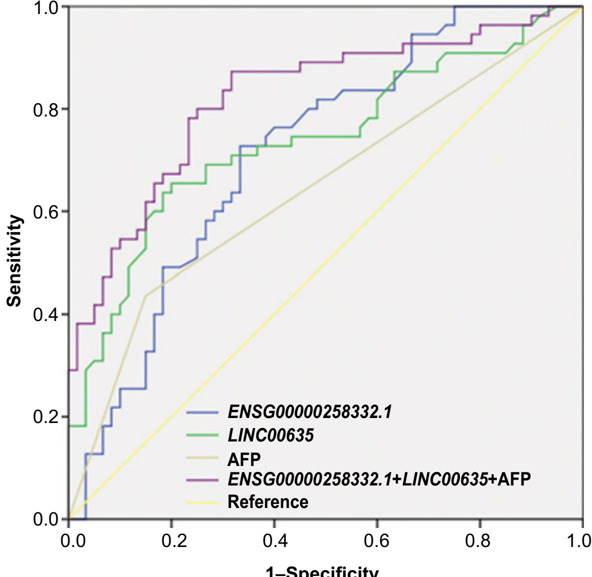

Figure 5.

ROC curve analyses for identifying HCC patients in an independent set. *ENSG00000258332.1* discriminated HCC patients from CHB patients, yielding an AUC of 0.718 (95% CI, 0.646–0.822; $P = 0.009$) at a cutoff value of 1.366; *LINC00635* yielded an AUC of 0.731 at a cutoff value of 1.532. Serum AFP at a cutoff value of 20 μg/L yielded an AUC of 0.634. Furthermore, the AUC for the combination of the 3 markers was 0.885 (95% CI, 0.749–1.025; $P = 0.012$), with 84.5% sensitivity and 85.3% specificity.

27). An intriguing study compared the efficacy of colorectal cancer prediction using total serum RNA and exosomal RNA, including 2 mRNAs and 1 lncRNA; the results showed that the AUC of total serum RNA (0.857) was inferior to exosomal RNA (0.936) in the same samples (28). Importantly, recent data indicated that lncRNAs exhibited tissue- and disease-specific expression. For example, *HULC*, which was identified as the first hepatocyte-specific lncRNA, was found to be highly upregulated in HCC (29–32). These studies highlight exosomal lncRNAs as a valuable source of various biomarkers for cancers. In fact, ExoDx Prostate (IntelliScore), a noninvasive urine test for clinical staging that assesses the expression of 3 exosomal RNAs associated with high-grade prostate cancer, was recently approved by the FDA (33, 34). However, the precise diagnostic and prognostic values of exosomal lncRNAs in HCC require elucidation.

In this study, we selected 2 serum exosomal lncRNAs as candidates to test the diagnostic value for HCC. Our results showed that the levels of both exosomal *ENSG00000258332.1* and *LINC00635* in the HCC group were significantly higher than that in CHB groups (all $P < 0.05$), and the assay exhibited high recovery. *ENSG00000258332.1* (cutoff value of 1.345) discriminated HCC patients from CHB patients with an AUC of 0.719 and with 71.6% sensitivity and 83.4% specificity, and the AUC for *LINC00635* (cutoff value of 1.690) was 0.750 with 76.2% sensitivity and 77.7% specificity. Furthermore, the AUC for the combination of *ENSG00000258332.1*, *LINC00635*, and AFP (cutoff value of 20 μg/L) was 0.894 with 83.6% sensitivity and 87.7% specificity. Thus, a combination of *ENSG00000258332.1*, *LINC00635*, and AFP could be a feasible diagnostic indicator for HCC.

When we analyzed the relationship between the relative levels of both lncRNAs and clinicopathologic characteristics of HCC patients, we found that a high *ENSG00000258332.1* level was associated with portal vein tumor emboli, lymph node metastasis, and TNM stage, and a high *LINC00635* level was related to lymph node metastasis and TNM stage (all $P < 0.05$). We also found that the levels of the 2 lncRNAs could help to predict life expectancy for HCC patients. In addition, the levels of both lncRNAs were significantly reduced after surgery. These results suggest the indicators would be advantageous to clinical staging and outcome prediction for HCC patients.

ENSG00000258332.1 maps to human chromosome 12: 74432866-74439608 forward strand, and *LINC00635* maps to human chromosome 3: 107841662-107878068 reverse strand. *ENSG00000258332.1* is also named as *LINC02394* (www.ensembl.org/Homo_sapiens/Gene). Vasconcelos and colleagues assessed thousands of lncRNAs encoded by *Schistosoma mansoni* genome at different parasite life-cycle stages (14). *SmLINC02394-IBu* were found to be upregulated in adults of schistosomula. This might indicate an importance of *SmLINC02394-IBu* on regulating some processes involved in the rapid adaptation of schistosomula from the free-living larvae to the early mammal parasitic stage, such as the worm body remodeling and defense against the host immune system. It hinted that exosomal *LINC02394* is involved in microenvironment remodeling and immune escape in human cancers. *LINC00635* as OncoLncs has been reported in cancer and is frequently overexpressed in lung adenocarcinoma (15, 16). On the other hand, it has been demonstrated that *LINC00635-001* silencing accompanied by gefitinib treatment suppressed Akt activation and sensitized HCC827-8-1 cells to gefitinib-induced cytotoxicity (17). Above all, the underlying carcinogenic mechanisms of the 2 lncRNAs have rarely been explained.

In conclusion, our study suggested that the combined analysis of serum exosomal *ENSG00000258332.1*, *LINC00635*, and serum AFP may be a novel useful biomarker for HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Chen
Development of methodology: X. Dong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Xu, X. Dong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Xu
Writing, review, and/or revision of the manuscript: H. Xu, Y. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Chen, X. Wang
Study supervision: Y. Chen, X. Wang

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