Circulating exosomal noncoding RNAs as prognostic biomarkers in human hepatocellular carcinoma

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Exosomal noncoding RNAs (ncRNAs) have unique expression profiles reflecting the characteristics of a tumor, and their role in tumor progression and metastasis is emerging. However, the significance of circulating exosomal ncRNAs in the prognosis of hepatocellular carcinoma (HCC) remains to be elucidated. We therefore determined the prognostic significance of circulating exosomal ncRNAs (miRNA-21 and lncRNA-ATB) for human HCC. This prospective study enrolled 79 HCC patients between October 2014 and September 2015. Exosomes were extracted from serum samples using the ExoQuick Exosome Precipitation Solution. To validate the isolation of the exosomes from serum, immunoblotting for exosome markers and characterization of nanoparticle using NanoSight were performed. NcRNAs were isolated from exosomes using the miRNeasy serum/plasma micro kit. Both circulating exosomal miRNA-21 and IncRNA-ATB were related to TNM stage and other prognostic factors, including the T stage and portal vein thrombosis. Multivariate analysis using the Cox regression test identified that both higher miRNA-21 and higher IncRNA-ATB were independent predictors of mortality and disease progression, along with larger tumor size and higher C-reactive protein (all p < 0.05). The overall survival and progression-free survival were significantly lower in patients with higher circulating levels of exosomal miRNA-21 (p<0.09) and IncRNA-ATB (p<0.0016) (log-rank test: p < 0.05). In conclusion, our study has provided strong evidence that circulating exosomal ncRNAs (miRNA-21 and IncRNA-ATB) are novel prognostic markers and therapeutic targets for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide and is a serious health problem in patients with chronic liver disease (CLD). 1 Being diagnosed at an advanced stage and a high recurrence rate are the main reasons for the poor prognosis of HCC. 2,3 Predicting the prognosis in HCC patients may affect the therapeutic approach and treatment outcomes, which indicates the importance of discovering prognostic markers in these patients. Although various biomarkers have been studied to predict the prognosis of HCC, reliable and simple prognostic factors have not been established yet. 4

Noncoding RNAs (ncRNAs) are classified according to their length into microRNAs (miRNAs) (19–25 nucleotides), messenger RNAs (mRNAs), and long noncoding RNAs (lncRNAs). These noncoding RNAs are of special interest because they have been found to be involved in tumor progression, invasion, and metastasis. 5,6 Their expression may be related to the clinical outcome of patients with cancer. 7

Key words: circulating biomarker, exosome, microRNA, lncRNA, hepatocellular carcinoma

Abbreviations: AFP: alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; CD: cluster of differentiation; cDNAs: complementary DNAs; CI: confidence interval; CLD: chronic liver disease; CRP: C-reactive protein; CT: computed tomography; EMT: epithelial-mesenchymal transition; HCC: hepatocellular carcinoma; HR: hazard ratio; LC: liver cirrhosis; lncRNAs: long noncoding RNA; miRNAs: microRNAs; MRI: magnetic resonance imaging; MVB: multivesicular body; ncRNAs: noncoding RNAs; TNM: tumor-node-metastasis.

Additional Supporting Information may be found in the online version of this article.

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Conflict of interest: The authors disclose no conflicts.

Grant sponsor: National Research Foundation of Korea; Grant numbers: 2017M3A9G8083382, NRF-2016R1C1B2011739, 2014R1A5A2009242
DOI: 10.1002/ijc.31931

History: Received 6 May 2018; Accepted 1 Oct 2018; Online 19 Oct 2018

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A growing body of evidence indicates that noncoding RNAs (ncRNAs), such as miRNAs and lncRNAs, play an important role in tumor progression and metastasis. Could particular ncRNAs also serve as serum biomarkers for cancer prognosis? In our study, the authors found that two specific ncRNAs in circulating exosomes were independent predictors of overall survival and disease progression in human hepatocellular carcinoma (HCC). These molecules might therefore provide valuable biomarkers to improve the clinical management of HCC, as well as potential therapeutic targets.

In the present study, we aimed to determine the prognostic relevance of two noninvasive circulating exosomal ncRNAs (miRNA-21 and lncRNA-ATB) for HCC.

Materials and Methods

Patients

HCC patients who visited Kyungpook National University Hospital, Daegu, South Korea between October 2014 and September 2015 were enrolled in this prospective study. Serum samples were collected from all of these patients before specific treatment for HCC. Exclusion criteria were age under 20 years, severe or uncontrolled medical illness, malignancy other than HCC, infection, and pregnancy.

The study was performed in accordance with the ethical guidelines of the Helsinki Declaration of 1975, as revised in 2008. All patients included in the study provided informed consent, and the study protocol was approved by the Institutional Review Board of Kyungpook National University Hospital.

Diagnosis of HCC and follow-up

HCC was diagnosed either pathologically or clinically using typical imaging modalities such as CT and/or MRI in accordance with the guideline of the European Association for the Study of the Liver. HCC was staged using the system of the Modified International Union Against Cancer and Barcelona Clinic Liver Cancer (BCLC) staging system. Liver cirrhosis (LC) was defined as a liver biopsy specimen showing evidence of cirrhosis (Metavir stage F4). If a histological assessment was not available, cirrhosis was diagnosed based on laboratory findings of a platelet count of less than 100,000/μL, radiologic findings including a surface nodularity, the presence of varices, clinical findings of complications of LC such as ascites and hepatic encephalopathy, and a fibroscan score of more than 12.5 kPa.

During the follow-up period, laboratory tests, including routine blood chemistry and alpha-fetoprotein (AFP) and imaging studies using computed tomography (CT) or magnetic resonance imaging (MRI) were performed every 3–6 months to monitor disease status. Disease progression was defined as recurrence after curative treatment, tumor growth according to mRECIST criteria in patients who could not be treated curatively, or death. The primary endpoint was overall survival and the secondary endpoint was disease progression.
Isolation and identification of exosomal RNAs from serum samples

Exosomes were extracted from serum samples using the ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA). Briefly, 500 μL of serum was mixed with 126 μL of this solution and then incubated for 30 min at 4 °C. The ExoQuick/serum mixture was then centrifuged at 1,500g for 30 min at room temperature, and the pellet obtained after removing supernatant was resuspended in 200 μL of phosphate-buffered saline. To validate the isolation of the exosomes from serum, immunoblotting was performed for the cluster of differentiation 9 (CD9), CD63, and tumor susceptibility gene 101 (TSG101), which are exosome markers, and for calnexin, which is an integral protein of the endoplasmic reticulum and is not expressed in the exosome. In addition, exosomes were investigated on the NanoSight LM10 instrument according to the manufacturer’s protocols (Malvern Instruments Inc.). The instrument was calibrated prior to each experimental run for nanoparticle size and quantity using standardized nanoparticle dilutions provided by the manufacturer. NTA 3.0 software was used to perform the particles tracking analysis.

NcRNAs were isolated from exosomes using the miRNeasy serum/plasma micro kit (QiaGen, Valencia, CA, USA). Sample-to-sample variations during RNA isolation were normalized by adding 25 fmol of synthetic C. elegans miRNA (cel-miR-39, Applied Biosystems, Foster City, CA, USA) to each serum sample, as described previously. For lncRNA expression analysis, complementary DNAs (cDNAs) were synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and the RT product was used for the subsequent qRT-PCR performed with the SYBR Green PCR Master Mix (Applied Biosystems) on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The after primer sequences were used for lncRNA-ATB: forward, 5'-TCTGGGCTGAGGCTGGTTGAC-3'; and reverse, 5'-ATCTCCTGGGTGCTGGAAGG-3'. The average expression levels of serum miRNA and lncRNA were normalized against that of cel-miR-39 using the 2−ΔΔCt method.

Statistical analysis

Data are presented as median (interquartile range) or n (%) values as appropriate. Categorical variables were compared using the chi-square test (or Fisher’s exact test), while continuous variables were compared using Mann–Whitney’s test (or the Kruskal–Wallis test). The optimal cutoff was calculated as the value that maximized the sum of sensitivity and specificity using time-dependent receiver operating characteristic curves and the areas under these curves. The factors associated with overall survival were identified by applying the multivariate Cox proportional-hazards regression test using variables that were significant in the univariate analysis. This method was also used to obtain the factors associated with HCC progression. The overall survival and cumulative disease progression rate were estimated using the Kaplan–Meier method. The hazard ratio (HR) and 95% confidence interval (CI) were also determined. A probability value of p < 0.05 was considered to be statistically significant. The statistical analyses were performed using SPSS (version 18.0, PASW Statistics Incorporated, Chicago, IL, USA).

Results

Patients

The baseline characteristics of the 79 patients (69 men and 10 women) enrolled in our study are summarized in Table 1. The median age of the study population was 59 years. The most common etiology of HCC was chronic hepatitis B (n = 62), followed by alcohol (n = 8) and chronic hepatitis C (n = 7). The tumors were smaller than 5 cm in 40 patients (50.6%), and 44 patients (55.7%) had multiple tumors. The median AFP level was 27.6 ng/mL.

Treatment outcomes of HCC patients

Patients were treated according to the BCLC guidelines. Ten patients underwent hepatic resection, 5 liver transplantation, 24 radiofrequency ablation, 9 chemoembolization, 17 systemic therapy (sorafenib), and 14 received best supportive care.

During the study period (median 14.0 months), disease progression was identified in 44 patients (55.7%) at a median of 3.4 months, and 34 patients (43.0%) died at a median of 3.4 months. The most common cause of death was progression of HCC (n = 29, 85.3%), followed by HCC rupture, bleeding, and hepatic failure (Supporting Information Table 1). The overall survival rates were 80.9%, 68.1%, and 61.7% at 3, 6, and 12 months, respectively. Patients who died had significantly larger tumor size, a higher proportion with multiple tumors; higher tumor stage, erythrocyte sedimentation rate, C-reactive protein (CRP) level, platelet count, aspartate aminotransferase level, total bilirubin level, and AFP level; and a significantly lower serum albumin level compared to patients who survived (p < 0.05) (Table 1).

Identification of serum exosomes and expression level of circulating exosomal ncRNAs

To confirm isolated exosome from serum samples, both immunoblotting analysis for exosome markers and characterization of nanoparticle using NanoSight were performed. We compared the expression of CD9, CD63, TSG101, and calnexin in isolated exosomal pellets from the sera of patients with those of the supernatant. The expression of exosome specific markers (CD9, CD63, and TSG101) were identified in the isolated exosomes, whereas calnexin, a negative marker of exosomes, was only expressed in the supernatant (Fig. 1a). In addition, isolated exosomes from normal healthy controls and HCC patients’ sera displayed mainly around 100–200 nm size and their concentrations were enough to analyses (Fig. 1b).
Notably, miR-21 was highly concentrated in exosome compared to remaining supernatant after isolation of the exosome. (Fig. 1c).

Since free circulating small RNAs could be included during isolation of exosomal RNAs from serum samples, we performed our own designed-experiments using two different nonhuman miRNAs which are not homologous with human sequence as spike-in-controls (Supporting Information Fig. 1). First, we directly added the spike-in-control A in human serum samples as a free circulating miRNA before ExoQuick treatment. Next, we performed isolation of exosome using our modified ExoQuick protocol. Subsequently, we included the spike-in-control B in exosome pellet, but not in the supernatant. In miRNAs analyses, we observed that the free circulating miRNA (spike-in-control A) was only detected in supernatant samples without exosome pellet.

Taken together, these data indicate that the exosomes were adequately purified, and circulating miR-21 is more highly expressed in exosome. Moreover, our experimental data suggest that free circulating small RNAs on the surface of exosome were very barely contaminated during our exosomal-RNA extraction step.

Both exosomal miRNA-21 and IncRNA-ATB were successfully detected in the HCC serum samples. The distribution of levels of circulating exosomal ncRNAs (miRNA-21 and IncRNA-ATB) are demonstrated in Supporting Information Figure 2. Compared to the TNM stage I and II, the levels of circulating exosomal ncRNAs (miRNA-21 and IncRNA-ATB) tended to increase in the TNM stage III and IV (p = 0.023, p = 0.070, respectively). When using the BCLC staging system, the levels of circulating exosomal ncRNAs (miRNA-21 and IncRNA-ATB) were also significantly increased in the advanced HCC group (BCLC stage C-D) compared to early HCC group (BCLC stage 0–B) (p = 0.018, p = 0.004, respectively).

Both serum exosomal miRNA-21 and IncRNA-ATB levels correlate with multiple prognostic factors of HCC

We evaluated correlations between the expression levels of these ncRNAs and clinical features. The patients were divided into two groups based on high and low expression levels of

| Table 1. Baseline characteristics of the study population (n = 79) |
|------------------|------------------|------------------|------------------|------------------|
| Demographic variables | All             | Death (n = 34, 43.0%) | Survival (n = 45, 57.0%) | p Value |
| Age, years | 59 (52–67) | 57.5 (51–64.5) | 60 (54.5–70.5) | 0.111 |
| Sex, male | 69 (87.3) | 29 (85.3) | 40 (88.9) | 0.738 |
| Etiology, HBV/HCV/alcohol/other | 62/7/8/2 (78.5/8.9/10.1/2.5) | 27/2/4/1 (79.4/5.9/10.1/2.5) | 35/5/4/1 (77.8/11.1/8.9/2.2) | 0.878 |
| Previous HCC treatment history | 20 (25.3) | 5 (14.7) | 15 (33.3) | 0.059 |

| Laboratory variables | | | | |
| ESR, mm/h | 14.5 (6.25–25.75) | 25 (14.5–37) | 9 (4–17) | 0.001 |
| CRP, mg/dl | 0.29 (0.29–1.07) | 1.17 (0.56–2.78) | 0.29 (0.18–0.29) | 0.001 |
| Platelet count, ×10^9/L | 148 (97–195) | 165.5 (117.75–234.25) | 128 (87.5–161) | 0.004 |
| Prothrombin time, INR | 1.13 (1.06–1.24) | 1.16 (1.07–1.26) | 1.11 (1.06–1.22) | 0.244 |
| Aspartate aminotransferase, IU/L | 41.00 (27–68) | 66 (30.75–108.75) | 38 (25.5–51) | 0.003 |
| Alanine aminotransferase, IU/L | 32.00 (24–49) | 37 (24.75–62.75) | 31 (23.5–43.5) | 0.231 |
| Total bilirubin, g/dl | 3.9 (3.3–4.1) | 3.6 (3.3–4.0) | 4.0 (3.6–4.2) | 0.002 |
| Creatinine, mg/dl | 0.88 (0.75–1.03) | 0.825 (0.72–1.07) | 0.92 (0.80–0.99) | 0.458 |
| Alpha-fetoprotein, ng/mL | 27.6 (6.3–1,512) | 210.45 (19.475–11,530.75) | 12.1 (4.6–150.15) | 0.001 |
| LC | 57 (72.2) | 28 (82.6) | 29 (64.4) | 0.079 |
| Child-Pugh class, A/B | 43/14 (75.4/24.6) | 19/9 (67.9/32.1) | 24/5 (82.8/17.2) | 0.191 |
| Presence of ascites | 17 (21.5) | 10 (29.4) | 7 (15.6) | 0.138 |

Abbreviations: HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; INR: international normalized ratio; LC: liver cirrhosis.

Data are expressed as median (interquartile range [IQR]) or n (%).
miRNA-21 (≥0.09 vs. <0.09) and lncRNA-ATB (≥0.0016 vs. <0.0016). Both miRNA-21 and lncRNA-ATB were related to the TNM stage, which correlates with the advanced disease state (both p < 0.05). These expression levels were also related to other prognostic factors including T stage and portal vein thrombosis. LncRNA-ATB was not associated with age, sex, the presence and severity of LC, or HCC etiology (Table 2).

**Both circulating exosomal miRNA-21 and lncRNA-ATB are independent predictors of overall survival in HCC**

In multivariate analysis, larger tumor size, higher CRP, higher miRNA-21 (HR = 2.869, 95% CI = 1.249–6.593), and higher lncRNA-ATB (HR = 2.169, 95% CI = 1.003–4.686) were identified as independent predictors of mortality (all p < 0.05) (Table 3). In subgroup analysis with 47 patients with stage III or IV, miRNA-21 was also found to be independently associated with overall survival (p = 0.015) (Supporting Information Table 2). The overall survival rates at 3, 6, and 12 months were significantly higher among HCC patients in the low miRNA-21 expression group (miRNA-21 < 0.09) (85.4%, 75.5%, and 69.0%, respectively) than among those in the high miRNA-21 expression group (miRNA-21 ≥ 0.09) (64.7%, 41.2%, and 35.3%) (log-rank test: p = 0.014). The overall survival rates at 3, 6, and 12 months were also significantly higher in HCC patients in the low lncRNA-ATB expression group (lncRNA-ATB <0.0016) (97.5%, 82.1%, and 79.5%, respectively) than in those in the high lncRNA-ATB expression group (lncRNA-ATB ≥0.0016) (64.1%, 53.8%, and 43.6%) (log-rank test: p = 0.005) (Figs. 2a and 2b).

**Both circulating exosomal miRNA-21 and lncRNA-ATB are independent predictors of disease progression in HCC**

Larger tumor size, higher CRP, presence of LC, higher miRNA-21 (HR = 2.530, 95% CI = 1.234–5.187), and higher lncRNA-ATB (HR = 2.550, 95% CI = 1.331–4.884) were identified as significant factors predicting the progression of HCC after adjusting for competing risks (all p < 0.005) (Table 4). In subgroup analysis with 47 patients with stage III or IV, miRNA-21 and lncRNA-ATB identified as an independent predictors of disease progression in multivariate analysis (p = 0.043, p = 0.012, respectively) (Supporting Information Table 3). The rates of disease progression at 3, 6, and 12 months were significantly higher in HCC patients in the high miRNA-21 expression group (miRNA-21 ≥ 0.09) (41.2%, 63.2%, and 77.9%, respectively) than in those in the low miRNA-21 expression group (miRNA-21 < 0.09) (21.4%, 35.0%, and 47.1%) (log-rank test: p = 0.012). The rates of disease progression at 3, 6, and 12 months were also significantly higher in the high lncRNA-ATB expression group (lncRNA-ATB ≥0.0016) (44.6%, 55.7%, and 66.8%, respectively) than in the low lncRNA-ATB expression group (lncRNA-ATB <0.0016) (7.7%, 26.7%, and 40.4%) (log-rank test: p = 0.004) (Figs. 2c and 2d).

**Discussion**

Circulating ncRNAs have recently emerged as novel biomarkers of cancer development and progression.39,40 Because exosomal ncRNAs can remain stable in serum and exhibit unique expression profiles reflecting the properties of cancer cells, they may serve as sensitive and noninvasive biomarkers...
for both diagnostic and prognostic purposes. In the present study we evaluated the prognostic significance of circulating exosomal miRNA-21 and lncRNA-ATB in HCC patients.

In our study, both the circulating exosomal miRNA-21 and lncRNA-ATB levels were found to be associated with an advanced disease state and other prognostic factors. After adjusting for other factors, miRNA-21 and lncRNA-ATB were independently related to the overall survival and disease progression in HCC patients. The survival rate was significantly lower in patients with higher circulating levels of

| Table 2. Correlations between expression levels of both serum exosomal miRNA-21 and IncRNA-ATB level and clinical characteristics (n = 79) |
| --- |
| **Serum exosomal miRNA-21 level** | **Serum exosomal lncRNA-ATB level** |
| Low group (miRNA-21 < 0.09) | High group (miRNA-21 ≥ 0.09) | p Value | Low group (lncRNA-ATB < 0.0016) | High group (lncRNA-ATB ≥ 0.0016) | p Value |
| Age, years (≤60/≥60) | 29/33 | 13/4 | 0.03 | 19/21 | 23/16 | 0.307 |
| Sex, male/female | 54/8 | 15/2 | 1 | 33/7 | 36/3 | 0.311 |
| Etiology, HBV/HCV/alcohol/others | 49/6/5/2 | 13/1/3/0 | 0.787 | 33/3/3/1 | 29/4/5/1 | 0.433 |
| Previous HCC treatment history, yes/no | 43/19 | 16/1 | 0.056 | 27/13 | 32/7 | 0.137 |
| Tumor variables | | | | | | |
| Size of tumor, <5 cm/≥5 cm | 35/27 | 5/12 | 0.048 | 23/17 | 17/22 | 0.216 |
| Number of tumor, single/multiple | 32/30 | 3/14 | 0.013 | 19/21 | 16/23 | 0.562 |
| PVT, no/yes | 46/16 | 7/10 | 0.01 | 34/6 | 19/20 | 0.001 |
| T stage, 1–2/3–4 | 29/33 | 3/14 | 0.03 | 21/19 | 11/28 | 0.028 |
| N stage, 0/1 | 59/2 | 17/0 | 1 | 40/0 | 36/3 | 0.116 |
| M stage, 0/1 | 59/3 | 15/2 | 0.292 | 39/1 | 35/4 | 0.201 |
| Modified UICC stage, I–II/III–IV | 29/33 | 3/14 | 0.03 | 21/19 | 11/28 | 0.028 |
| Laboratory variables | | | | | | |
| Aspartate aminotransferase, IU/L (<40/≥40) | 34/28 | 4/13 | 0.022 | 21/19 | 17/22 | 0.428 |
| Alanine aminotransferase, IU/L (<40/≥40) | 42/20 | 9/8 | 0.258 | 27/13 | 24/15 | 0.58 |
| Alpha-fetoprotein, ng/mL (<400/≥400) | 45/17 | 12/5 | 1 | 29/11 | 28/11 | 0.944 |
| LC, no/yes | 19/43 | 3/14 | 0.371 | 11/29 | 11/28 | 0.944 |
| Child-Pugh class, A/B | 37/6 | 6/8 | 0.004 | 23/6 | 20/8 | 0.955 |
| Presence of ascites, no/yes | 50/12 | 12/5 | 0.505 | 31/9 | 31/8 | 0.83 |

Abbreviations: HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; PVT: portal vein thrombosis; LC: liver cirrhosis.

| Table 3. Independent risk factors for overall survival in HCC patients (n = 79) |
| --- |
| **Variable** | **Univariate** | **Multivariate analysis using miRNA-21** | **Multivariate analysis using lncRNA-ATB** |
| | p Value | Hazard ratio (95% CI) | p Value | Hazard ratio (95% CI) |
| Number of tumor (multiple vs. single) | 0.004 | 5.961 (1.829–19.426) | 0.001 | 7.075 (2.158–23.199) |
| Size of tumor, cm (<5 cm vs. ≥5 cm) | <0.001 | 1.344 (1.170–1.543) | 0.005 | 1.204 (1.058–1.371) |
| N stage | 0.003 | 1.204 (1.058–1.371) |
| M stage | 0.001 | 2.869 (1.249–6.593) |
| CRP, mg/dL | <0.001 | 1.204 (1.058–1.371) |
| Platelet count, ×10^9/L | 0.003 | 2.869 (1.249–6.593) |
| Total bilirubin, mg/dL | <0.001 | 2.169 (1.003–4.686) |
| Serum albumin, g/dL | 0.003 | 2.169 (1.003–4.686) |
| Alpha-fetoprotein, ng/mL (>400 vs. ≤400) | 0.003 | 2.169 (1.003–4.686) |
| miRNA-21 (≥0.09 vs. <0.09) | 0.018 | 2.169 (1.003–4.686) |
| IncRNA-ATB (≥0.0016 vs. <0.0016) | 0.006 | 2.169 (1.003–4.686) |

Abbreviations: HCC: hepatocellular carcinoma; CI: confidence interval; CRP: C-reactive protein.
Figure 2. Kaplan–Meier plots of overall survival according to the miRNA-21 (miRNA-21 ≥ 0.09 (n = 17) vs. miRNA-21 < 0.09 (n = 62)) (a) and lncRNA-ATB (lncRNA-ATB ≥ 0.0016 (n = 39) vs. lncRNA-ATB < 0.0016 (n = 40)) (b) expression levels. (a, b) The survival rate was significantly lower in patients with higher circulating levels of exosomal miRNA-21 and lncRNA-ATB (log-rank test: both \( p < 0.05 \)). Kaplan–Meier plots of cumulative disease progression according to the miRNA-21 (miRNA-21 ≥ 0.09 (n = 17) vs. miRNA-21 < 0.09 (n = 62)) (c) and lncRNA-ATB (lncRNA-ATB ≥ 0.0016 (n = 39) vs. lncRNA-ATB < 0.0016 (n = 40)) (d) expression levels. (c, d) The progression rate was significantly higher in patients with higher circulating levels of exosomal miRNA-21 and lncRNA-ATB (log-rank test: both \( p < 0.05 \)). [Color figure can be viewed at wileyonlinelibrary.com]

Table 4. Independent risk factors for disease progression in HCC patients (n = 79)

| Variable                     | Univariate p Value | Multivariate analysis using miRNA-21 p Value | Hazard ratio (95% CI) | Multivariate analysis using lncRNA-ATB p Value | Hazard ratio (95% CI) |
|------------------------------|--------------------|----------------------------------------------|-----------------------|-----------------------------------------------|-----------------------|
| Number of tumor (multiple vs. single) | 0.001              |                                              |                       |                                              |                       |
| Size of tumor, cm (≥5 vs. <5)   | <0.001             | <0.001                                       | 4.568 (2.171–9.613)   | <0.001                                       | 5.907 (2.826–12.345)  |
| N stage                       | 0.002              |                                              |                       |                                              |                       |
| M stage                       | <0.001             |                                              |                       |                                              |                       |
| CRP, mg/dL                    | <0.001             | <0.001                                       | 1.239 (1.105–1.390)   | 0.034                                        | 1.127 (1.009–1.258)   |
| Platelet count, ×10^9/L       | 0.002              |                                              |                       |                                              |                       |
| Total bilirubin, mg/dL        | 0.004              |                                              |                       |                                              |                       |
| Serum albumin, g/dL           | 0.003              |                                              |                       |                                              |                       |
| Alpha-fetoprotein, ng/mL (≥400 vs. <400) | 0.024          |                                              |                       |                                              |                       |
| miRNA-21 (≥0.09 vs. <0.09)    | 0.015              | 0.011                                        | 2.530 (1.234–5.187)   | 0.005                                        | 2.550 (1.331–4.884)   |
| lncRNA-ATB (≥0.0016 vs. <0.0016) | 0.006             |                                              |                       |                                              |                       |
| LC                            | 0.041              |                                              |                       |                                              |                       |

Abbreviations: HCC: hepatocellular carcinoma; CI: confidence interval; CRP: C-reactive protein; LC: liver cirrhosis.
exosomal miRNA-21 and lncRNA-ATB. Our results suggest that circulating exosomal ncRNAs can serve as biomarkers for predicting the prognosis of HCC patients.

Our study has several strengths. First, the two analyzed circulating exosomal ncRNAs (miRNA-21 and lncRNA-ATB) remained meaningful even after adjusting for other factors. Especially, lncRNA-ATB can be used to predict the prognosis noninvasively regardless of age, sex, presence of LC, or etiology. These observations suggest that these serum exosomal ncRNAs are valuable prognostic markers of HCC. Second, recent studies also have revealed that exosomal ncRNAs are protected from endogenous RNase in the blood, whereas naked RNAs are rapidly degraded in blood. Therefore, the level of serum exosomal ncRNAs were more accurate and better for predicting the prognosis than those of serum nonexosomal ncRNAs. In our study, we determined the prognostic relevance using exosomal ncRNAs instead of naked ncRNAs, these exosomal ncRNAs may enable more accurate prediction of prognosis in real practice. Finally, we also showed that these circulating exosomal ncRNAs remained independent predictors of overall survival and disease progression in advanced stage with stage III–IV. Both of the ncRNAs in our study have also been reported to be associated with tumor proliferation and EMT in previous studies, thus they also can be considered as therapeutic targets.

Exosomes are small membranous vesicles derived from the membrane of MVB, which is late endosomal compartments. The presence of late endosome components like TSG101, Alix, and the tetraspanins CD9, CD63 identify the MVB origin of exosomes and characterize them as endosome-derived cellular vesicles. They are secreted into body fluids and selectively taken up by cells distal from their release, and cause alterations in the recipient cell and their microenvironment. Long range targeting, tissue specificity in uptake, and stability of exosomes in systemic circulation is promising as a target of novel therapy. Moreover, exosomes contain cell- and disease state-specific ncRNAs, miRNAs, and proteins that can aid tumor development, angiogenesis, proliferation, survival, and metastasis, and these exosomal profiles reflect the molecular signature of the cell, and provide a means for a liquid biopsy. Therefore, exosomal ncRNAs can be used as biomarkers for screening tool, diagnosis and prognosis.

According to previous studies, miRNA-21 targets PTEN and PDCD4, promoting proliferation, invasion, and migration as well as carcinogenesis, and inhibits negative regulations of the Ras/MEK/ERK pathway. LncRNA-ATB was reported to upregulate ZEB1 and ZEB2 and then induce EMT, tumor cell invasion, and metastasis in tissue. Although these studies have revealed that these ncRNAs are associated with a poor prognosis in HCC, the role of these ncRNAs were observed in tumor tissue. There have been only few studies on circulating exosomal ncRNAs in serum, especially lncRNA, as prognostic factors of HCC. In our study, we revealed the prognostic significance of two noninvasive circulating exosomal ncRNAs (miRNA-21 and lncRNA-ATB) in HCC patients. Moreover, this is the first study of circulating exosomal lncRNAs as a prognostic marker in human HCC.

While both miRNA-21 and lncRNA-ATB were independent predictors in HCC, there was no benefit of combinatorial assessment of both exosomal ncRNAs. The areas under the receiver operating characteristic curves of both exosomal ncRNAs for the prediction of overall survival and disease progression were not superior to those of exosomal miRNA-21 and lncRNA-ATB, respectively. In addition, miRNA-21 and lncRNA-ATB levels do not correlate across all patients (p = 0.781 by Pearson correlation analysis).

MiRNA-21 and lncRNA-ATB are also known to be associated with the diagnosis and prognosis of other malignancies, such as lymphoma, pancreatic cancer, glioblastoma, and colorectal cancer. Since these ncRNAs are not specific for HCC, high levels of these factors do not always mean diagnosis or poor prognosis of HCC. To overcome this limitation, these factors may need to be used with other tumor markers and imaging studies.

There are several limitations in the present study. First, we did not compare the level of circulating exosomal ncRNAs of HCC with those of normal controls and exosomal ncRNAs from HCC tissues. Because few data are available on the mechanisms regulating exosomal ncRNAs from tumor cells into the blood, the relationship between tissue and circulating levels of exosomal ncRNAs should be examined in future studies. Second, validation with an external cohort was not performed. In addition, chronic hepatitis B, the most common etiology of HCC in our study, accounts for 62 of 79 patients, thus we cannot assess the effect of underlying etiology on circulating exosomal ncRNAs. So further validation studies that include a large number of patients are needed, preferably in a multicenter study. Third, we did not observe the serial level change of circulating exosomal ncRNA before and after treatment in HCC patients. Finally, the effect of the degree of exosomal ncRNA expression on metastasis cannot be concluded from our results due to the short follow-up period and the small number of HCC patients with extrhepatic metastasis, further studies are required to resolve this issue.

In conclusion, our study provides strong evidence that circulating exosomal ncRNAs (miRNA-21 and lncRNA-ATB) can be novel prognostic biomarkers and therapeutic targets for HCC. Further validation studies that include a large number of patients are needed to confirm the potential prognostic value and clinical utility of both miRNA-21 and lncRNA-ATB in HCC patients.

Acknowledgements

Writing assistance: The English in this document has been checked by a professional editor who is native English speaker.
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