Bile Extracellular Vesicles from End-stage Liver Disease Patients Show Altered microRNA Content

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Abstract

**Background:** Extracellular vesicles (EVs) have recently attracted attention as novel diagnostic biomarkers and therapeutic tools. Several reports have correlated blood EVs with liver diseases. However, blood EVs do not reflect the liver state as it contains other systemically circulating EVs. Therefore, we focused on bile EVs, which are secreted directly from the liver, for identification of potential biomarkers of liver failure.

**Methods:** Bile samples were collected from liver transplant recipients (n = 21) diagnosed with end-stage liver disease (ESLD) and donors (normal liver, NL; n = 18) during transplantation. Bile EVs were extracted using ultracentrifugation.

**Results:** Nanoparticle tracking analysis showed that bile EV concentration was significantly higher in recipients than in donors. Among recipients, bile EV concentration was remarkably higher in those with hepatocellular carcinoma. Next-generation sequencing revealed 461 and 465 types of microRNAs (miRNAs) in donor and recipient bile EVs, respectively, with no significant difference in diversity between the groups. Among 43 high-expression miRNAs, the expression of 86.0% of the miRNAs was higher in the bile EVs of recipients than in those of donors. Quantitative PCR validation showed that the levels of miR-17, miR-92a, miR-25, miR-423, and miR-451a significantly increased in bile EVs of recipients. Levels of miR-17 were remarkably higher in recipients with alcoholic ESLD.

**Conclusions:** Secretion of EVs into the bile and their miRNA content increase in the ESLD state. Additionally, miRNA levels in bile EVs are not correlated with those in serum EVs. Bile EVs could be promising novel biomarkers for liver diseases.

**Introduction**

Liver cirrhosis (LC) is a terminal illness characterized by highly advanced liver fibrosis and caused by various chronic liver injuries. It can lead to hepatocellular carcinoma (HCC) and chronic liver failure, also called end-stage liver disease (ESLD) (1). ESLD is reported as the 13th most common cause of death worldwide, increasing from about 67,600 in 1980 (1.54% of global deaths) to more than 1 million (1.95%) in 2010 (2, 3). New effective treatment focusing on ameliorating liver fibrosis has been developed (4); however, liver transplantation is still the only curative treatment for ESLD patients in clinical settings. The development of novel treatment options for LC requires further elucidation of the molecular mechanism underlying LC.

 Recently, several studies have shown that extracellular vesicles (EVs) are secreted by various cells into bodily fluids, such as blood, urine, and ascites in normal and disease states (5). EVs are small membrane vesicles and can be classified into exosomes, microvesicles, and apoptotic bodies, depending on the mechanism through which they are produced and on their particle size. Exosomes are the smallest vesicles (30–100 nm in diameter), which are enveloped by a lipid bilayer membrane containing functional molecules derived from secretory cells, such as micro RNAs (miRNAs), messenger RNAs.
(mRNAs), and proteins, and they act as intercellular communication tools (5). The profile of encapsulated molecules in exosomes varies depending on the conditions of secretory cells (6, 7).

Several studies have reported on the relationship between EV characteristics and various disease states, including cases of liver disease (5, 8). In most liver disease studies, the analyses of EV characteristics were performed on those extracted from blood, since they are easy to collect. However, since EVs in circulating bodily fluids are composed of those secreted from different cells and organs, it is impossible to examine EVs originating from the liver alone in cases of liver disease. Therefore, in this study, we focused on EVs in the bile, which is secreted from liver epithelial cells. Both hepatocytes and cholangiocytes release EVs into the bile fluid (9, 10). EVs extracted from the bile are considered to reflect liver conditions more accurately than those found in the blood. Bile EVs have been reported to be useful in the diagnosis of pancreaticobiliary malignancies, including pancreatic adenocarcinoma and cholangiocarcinoma (11, 12). However, the relationship between bile EVs and liver diseases has not yet been unambiguously established.

The aim of this study was to elucidate the characteristics of bile EVs from individuals with ESLD compared to those from individuals with normal liver (NL). Our results could lead to the identification of specific EVs associated with liver disease that may serve as novel diagnostic and treatment options for liver disease.

Materials And Methods

The detailed experimental procedures are described in the Supplementary Material.

Patients and bile samples

From July 2015 to December 2016, 21 patients with ESLD who underwent living donor liver transplantation (LT) at Nagasaki University Hospital and 18 corresponding NL donors were enrolled in this study. This study was approved by the Ethics Committee of Nagasaki University (Approval Number: 15062234-2) and performed in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all patients. Bile samples were collected from the gallbladder resected during LT as samples for ESLD. Bile samples were also collected from the drainage tube inserted into the bile duct on the 7th and 14th day after LT. Bile samples were similarly collected from resected gallbladder of NL donors. In three NL donors, bile samples could not be collected because the bile had been leaking from the resected gallbladder. All bile samples were stored frozen at -80°C until analysis. Serum samples were also collected from the patients just before LT and stored in the same manner. The clinical characteristics and blood examination data of recipients and donors were obtained from medical records.

Extraction of EVs from bile
EVs were extracted from 5 mL of bile in all cases. Bile samples were centrifuged at 300 × g for 10 min at 4°C. Then, the supernatant was further centrifuged three times at 16,500 × g for 10 min at 4°C to remove cellular debris completely. Next, the supernatant was centrifuged twice at 150,000 × g for 70 min at 4°C to pellet EVs. The extracted EVs were resuspended in 200 µL phosphate buffered saline (PBS) and were stored in a low adsorption tube at 4°C until subsequent analysis. To confirm the quality and quantity of extracted EVs, the morphological characteristics were examined using a transmission electron microscope (TEM) (Kamakura Techno–Science, Inc., Tokyo, Japan). The concentration and particle size distribution were analyzed by nanoparticle tracking analysis (NTA) (Theoria Science, Tokyo, Japan), and the specific protein expression was analyzed by western blotting analysis for CD63 and the tumor susceptibility gene 101 (TSG101) protein.

Isolation of miRNA and next-generation sequencing (NGS)

After the addition of cel-miR-39 (0.01 ng/mL of bile sample) as spike-in for standardization, miRNA was extracted using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocols. The extracted miRNA was qualitatively and quantitatively evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). A barcoded cDNA library was prepared using SMARTer smRNA-Seq Kit for Illumina (Clontech Laboratories, Mountain View, CA) and sequenced with Illumina HiSeq 2500 (Illumina, San Diego, CA) for comprehensive evaluation.

Statistical analysis

Results are presented as median with minimum-maximum range. For comparison between two groups, Mann-Whitney U test and nonparametric Spearman correlation analysis were performed using StatFlex V6.0 (Artec, Osaka, Japan). Differences were considered to be statistically significant when the p-value was less than 0.05. Shannon index was analyzed by calculating diversity index H.

Results

Characteristics of the study participants

Bile samples were collected from 21 LT ESLD patients and 18 NL donors. The clinical characteristics of the ESLD patients and NL donors are shown in Table 1. All LT recipients had ESLD, and the background liver diseases were hepatitis C virus (HCV; n = 7), alcohol-related (n = 5), non-alcoholic steatohepatitis (NASH; n = 3), autoimmune hepatitis (AIH; n = 2), and the others (n = 4). HCC was complicated in 5 patients (23.8%), and tumor-node-metastasis (TNM) stages and incidences were: stage I: two cases (40%) and stage II: three cases (60%). The median Child-Pugh score and model for ESLD score were 11 and 19, respectively. Mild fatty liver was observed in 4 NL donors (22.2%). Bile samples obtained from the donors were used as healthy controls compared to ESLD.
Table 1
Baseline clinical characteristics of study participants

| Characteristic                                      | Recipients [n = 21] | Donors [n = 18] |
|----------------------------------------------------|---------------------|-----------------|
| Age [years], median [range]                        | 58 [17–68]          | 33 [22–64]      |
| Sex [male / female], n [%]                         | 10 [47.6] / 11 [52.4] | 12 [66.7] / 6 [33.3] |
| Background liver disease, n [%]                    | 7 [33.3]            |
| HCV                                                | 5 [23.8]            |
| Alcoholic                                          | 3 [14.3]            |
| NASH                                               | 2 [9.5]             |
| AIH                                                | 4 [19.0]            |
| Others†                                            |                     |
| Hepatocellular carcinoma [+ / -], n [%]            | 5 [23.8] / 16 [76.2] | 0 [0] / 18 [100] |
| TNM stage [I/ II], n [%]                           | 2 [40.0] / 3 [60.0] |
| Child-Pugh score, median [range]                   | 11 [8–14]           |
| MELD score, median [range]                         | 19 [8–40]           |
| ALT [IU/L], median [range]                         | 26 [16–71]          | 19.5 [9–40]     |
| Total bilirubin [mg/dl], median [range]            | 4.2 [0.7–61.3]      | 0.85 [0.5–1.3]  |
| Albumin [g/dl], median [range]                     | 2.7 [2.0-4.1]       | 4.7 [4.1–5.1]   |

Abbreviations: HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; AIH, autoimmune hepatitis; MELD, model for end-stage liver disease; ALT, alanine aminotransferase.

†Others indicates hepatitis B virus, primary biliary cholangitis, primary sclerosing cholangitis, and Wilson disease.

Characteristics of EVs extracted from bile

After extraction, the morphological characteristics of EVs were observed by TEM. Bile EVs were round and ranged from 30 to 100 nm in diameter, which was similar to what has been previously reported (13) (Fig. 1a). Subsequently, the concentration, size, and distribution of bile EVs were analyzed by NTA. The concentration of bile EVs was between $1.60 \times 10^{10}$ and $26.4 \times 10^{10}$ particles/mL, and the particle size peak was at about 100 nm (Fig. 1b). Furthermore, western blotting analysis showed the expression of CD63 and TSG101, which are key EV protein markers commonly expressed in exosome populations, in the extracted bile EVs (Fig. 1c). These results suggest that EVs extracted from bile were rich in exosomes.

Bile EV concentration increased in the liver disease state
NTA revealed that the concentration of bile EVs was significantly higher in ESLD patients than in the NL donors ($p = 0.008$). The median concentration of bile EVs was $14.14 \times 10^{10}$ particles/mL in the ESLD patients and $5.45 \times 10^{10}$ particles/mL in the NL donors (Fig. 2a). After LT, the concentration of bile EVs decreased to $4.66 \times 10^{10}$ particles/mL ($n = 17$) and to $2.78 \times 10^{10}$ particles/mL ($n = 15$) after seven and fourteen days, respectively; these values were significantly lower than those obtained before LT (before LT vs seven days after LT: $p < 0.001$, before LT vs fourteen days after LT: $p < 0.001$) (Fig. 2b). There was no significant difference in the concentrations of bile EVs between patient groups classified according to background disease that caused ESLD (Fig. 2c). On the other hand, the concentration of bile EVs in patients with HCC was $22.89 \times 10^{10}$ particles/mL, which was significantly higher than that in patients without HCC ($10.45 \times 10^{10}$ particles/mL) ($p = 0.026$) (Fig. 2d). The differences in EV concentrations based on the TNM stages of HCC were not statistically evaluated due to the small number of cases (data not shown). The particle size of bile EVs was also examined in the same manner. There were no significant differences in the particle size of bile EVs between ESLD patients and NL donors or between groups of patients classified according to clinical characteristics (Supplementary Fig. 1).

### Altered miRNA content in bile EVs extracted from patients with ESLD

Subsequently, NGS was performed to compare the miRNA content of bile EVs isolated from ESLD patients and NL donors. First, we comprehensively analyzed miRNA levels in 13 ESLD patients and 11 donors with NL. The total number of reads in the sequence was $9.41 \times 10^7$, and the median number of reads was $3.76 \times 10^6$ per sample ($1.48 \times 10^6$ to $6.54 \times 10^6$ reads per sample). The median number of miRNAs identified was 6,065 (465 types) for ESLD patients and 4,295 (461 types) for the NL donors (Supplementary Table 1). Comparison of miRNA diversity between both groups using Shannon index showed no significant difference between ESLD patients and NL donors ($p = 0.284$) (Supplementary Fig. 2). To identify miRNAs specifically contained in bile EVs of ESLD patients, we extracted 43 types of miRNAs that were detected at a median of $\geq 5$ reads per million (RPM) in bile EVs of either the ESLD patients or NL donors (Fig. 3). Of these, 38 types (88.4%) of miRNAs were found at higher levels in bile EVs of ESLD patients, 12 of which were significantly higher than those in bile EVs of NL donors (Supplementary Table 2). No miRNA was found at a significantly higher level in the NL donors than in the ESLD patients.

### Quantification of miRNA in bile EVs extracted from ESLD patients using real-time quantitative PCR (qPCR)

From the NGS analysis results, a total of six miRNAs, including five miRNAs (miR-17, miR-92a, miR-25, miR-423, and miR-451a) that were found at significantly high amounts in bile EVs of ESLD patients, and the liver-specific miR-122, were validated by qPCR. Similar to NGS results, there was no significant difference in the miR-122 levels in bile EVs between the two groups ($p = 0.949$), but the levels of miR-17,
miR-92a, miR-25, miR-423, and miR-451a were significantly higher in the ESLD patients than in the NL donors ($p = 0.002$, $p < 0.001$, $p < 0.001$, $p = 0.005$, and $p = 0.001$, respectively) (Fig. 4). Comparing patients who were grouped together based on the background liver disease, miR-17 expression was significantly higher in alcohol-induced ESLD than in other background diseases (HCV vs alcoholic: $p = 0.012$, alcoholic vs NASH: $p = 0.042$) (Fig. 5). On the other hand, HCC did not affect miRNA levels in bile EVs (Supplementary Fig. 3). To determine whether the changes in miRNA expression levels in bile EVs are similar to those in serum, levels of these six miRNAs were measured by qPCR using serum samples from each patient. However, no significant correlation between the change in expression levels of EVs in bile and in serum was observed in these miRNAs, indicating that the miRNA profile in bile EVs was not reflected in serum (Supplementary Fig. 4).

**Discussion**

Here, we compared bile EVs in ESLD patients who received LT with those of donors with normal liver for identification of potential biomarkers of ESLD. First, we found that the levels of EVs secreted into the bile increased in ESLD patients, particularly in patients with HCC. Second, there were no major changes in the overall diversity of miRNA in the bile EVs between the two study groups, but the total amount of miRNA was higher and the types of miRNA were changed dramatically in the bile EVs of ESLD patients. Third, we found that altered miRNA expression in bile EVs was not reflected in serum miRNA in ESLD patients. Furthermore, to our best knowledge, this study is the first analysis of bile EVs extracted from normal liver (healthy control).

EVs, which are secreted by a large variety of cells, vary in count and content depending on the type or health of the EV-producing cells. Thus, they have become an area of intense research as potential diagnostic biomarkers for various diseases (6, 7). A majority of these studies have focused on EVs extracted from blood samples. The relative ease of the extraction process has made EVs a promising clinically effective biomarker. However, EVs extracted from blood include EVs secreted from various organs, and therefore blood EVs are only an indirect reflection of changes in EV secreted from a certain type of cell or tissue. Importantly, they are not completely reflective of changes in the EV secretion profiles of hepatocytes in the state of liver disease (14). In contrast, bile EVs are secreted into the bile canaliculi, which do not interact with the sinusoids. Thus, the only cells that secrete EVs into bile canaliculi are hepatocytes and bile duct cells (10). Therefore, bile does not contain EVs secreted from other organs and from nonparenchymal liver cells, such as hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells. This means that changes in bile EVs more accurately reflect the changes in the EVs secreted from hepatocytes, which are exposed to the liver microenvironment, than do blood EVs. Indeed, many studies have reported the altered expression of various miRNAs in blood and liver tissue of LC patients (15–17). However, the miRNAs in bile EVs identified in this study differed greatly from their results, suggesting that bile EVs contain a unique miRNA profile independent of the blood and liver tissues samples.

Bile EV concentration was significantly higher in ESLD liver than in normal liver as revealed by NTA. Furthermore, bile EV concentration decreased promptly after transplantation with normal liver, reaching
levels similar to those in NL donors. This suggests that EV secretion by hepatocytes increased following exposure to the liver microenvironment during ESLD. We found no associations between bile EV concentration and underlying disease, alanine aminotransferase (ALT) levels, or hepatic reserve (data not shown). Several previous reports have stated that blood EV concentration increases with liver injury (18, 19). Changes in blood EV concentrations have been correlated with ALT, hepatic fibrosis, cell death, and pathological angiogenesis. In mouse models of nonalcoholic fatty liver disease, blood EV concentration increased early on after liver injury and subsequently displayed time-dependent changes (18). These studies showed that EV secretion increased several folds, but these likely reflect not only the EVs secreted by hepatocytes, but also systemic changes, including EV secretion by other organs that accompany fibrosis progression.

Patients with ESLD complicated with HCC had significantly higher EV concentrations than those without HCC. A recent study has reported the superiority of bile EVs for diagnosing malignant bile duct stenosis compared to tumor markers, such as serum Ca19-9 (11); thus, bile EV concentration may also be effective for diagnosing HCC. EV secretion is more elevated in cancer cells than in normal cells, and EVs from cancer cells are involved in angiogenesis, inflammation, cancer-related fibroblast differentiation, and epithelial-mesenchymal transition in the tumor microenvironment (20, 21). Increase in EV secretion by HCC itself may result in higher bile EV concentrations, but the pathway of EV secretion into the bile duct by HCC cells that have lost polarity and normal function is unknown, thus warranting further investigations.

Here, we found that the expression of five bile EV miRNAs, viz., miR-17, miR-92a, miR-25, miR-423, and miR-451a, significantly increased in the ESLD liver relative to the normal liver. Furthermore, a comparison of underlying disease or presence of HCC showed that miR-17 expression significantly increased in alcohol-induced ESLD, which is consistent with the report of markedly higher miR-17 and miR-18a levels in the liver tissue of a rodent model of alcohol-induced liver injury (22). miR-17 and miR-92a are components of the miR-17-92 cluster, and miR-25 composes the miR-106b-25 cluster; both clusters are inhibited by the transcription factor E2F and are known as oncogenic miRNAs (23, 24). It is possible that these EVs secreted by hepatocytes in the microenvironment of liver injury with fibrosis progression contribute to HCC progression and could be promising targets of new liver cancer treatments. In terms of the function of EVs released into the bile, an analysis of a rat model has shown that EV uptake occurs via cholangiocyte cilia to modulate bile duct cell growth (10), but how target cells are determined or how they functionally transmit their content is still unknown. The present study did not elucidate how EVs released by hepatocytes in the liver injury microenvironment act on bile duct cells, or whether they also act on the gastrointestinal tract after secretion. Future studies should investigate these aspects in more detail.

These results must be interpreted with caution, and some limitations should be borne in mind. First, since the study participants were recipients undergoing living donor LT, the sample size was small. Therefore, statistical analysis on the background factors of liver failure (presence of background liver disease and hepatocellular carcinoma) was insufficient. More cases are needed to clarify the effects of hepatocellular carcinoma progression and other background liver diseases on bile EVs. Second, in the analysis of
miRNA expression level, exogenous cel-miR-39 spiked-in was used for normalization. However, the deviation of miR-39 levels in bile EVs was large between individual cases (a few samples showed outliers in the scatter plot). Li et al. have similarly reported that the level of the same amount of miR-39 added as an internal control in bile varied upon qPCR quantification, suggesting that the efficiency of RNA extraction from bile was not constant among the samples (12). Therefore, further verification on the relationship between miRNA levels and disease state is needed. Additionally, the establishment of a reliable internal control for bile samples will help improve future studies. Furthermore, collecting bile samples from chronic liver disease patients is invasive and difficult; thus, it may have limited utility as a clinical biomarker. In contrast, bile samples can be obtained easily through the drainage tubes placed in patients after liver transplant. Future studies should uncover the benefits of bile EVs as a diagnostic tool of post-transplant complications, such as acute rejection, blood flow obstruction, and bile duct stricture.

In conclusion, EVs secreted from the liver into the bile change to reflect liver disease state. Furthermore, miRNA in bile EVs change as they do in blood, but the changes are independent of changes in serum miRNA. Various studies have already focused on EVs in blood samples, but we found that EVs in bile could also serve as new biomarkers that provide direct insights into the liver microenvironment in liver disease.

Declarations

Funding:
This study was not receiving any funding.

Conflicts of interest/Competing interests:
All authors declare that they have no conflict of interest.

Ethics approval:
This study was approved by the Ethics Committee of Nagasaki University (Approval Number: 15062234-2) and performed in accordance with the ethical principles of the Declaration of Helsinki.

Consent to participate:
Written informed consent was obtained from all patients.

Consent for publication:
All authors consent to the publication of the manuscript in Hepatology international.

**Availability of data and material:**

The data that support the findings of this study are available from the corresponding author, Satoshi Miuma, upon reasonable request.

**Code availability:**

Not applicable.

**Authors' contributions:**

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Suguru Nakashiki, Satoshi Miuma, Masaaki Hidaka, Akihiko Soyama, Yasuko Kanda, Masanori Fukushima, Masafumi Haraguchi, Ryu Sasaki, Hisamitsu Miyaaki, Tatsuki Ichikawa, Mitsuhisa Takatsuki, and Susumu Eguchi. Deep sequence analysis was performed by Suguru Nakashiki, Satoshi Miuma, Hiroyuki Mishima, Hiroshi Masumoto, and Koh-ichiro Yoshiura. The first draft of the manuscript was written by Suguru Nakashiki and Satoshi Miuma and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

**Fig. 1**

Figure 1

Characteristics of extracellular vesicles extracted from bile by ultracentrifugation. 

a. Morphological characteristics observed by transmission electron microscopy (TEM) showed that extracellular vesicles (EVs) had spherical structures with diameters of about 30–100 nm. Scale bar: 50 nm. 

b. The number and size distribution of EV particles were measured by nanoparticle tracking analysis (NTA). The size of EVs extracted from bile had a peak at 107 nm in mode value. 

c. Western blotting analysis showed expression of CD63 and tumor susceptibility gene 101 (TSG101), well-known exosome-specific protein markers.
Figure 2

Concentration of bile extracellular vesicles (EVs) in patients with end-stage liver disease (ESLD) before and after liver transplantation. Concentration of bile extracellular vesicles (EVs) was measured by nanoparticle tracking analysis. a. Compared with the normal liver group (donors, n = 18), the bile EV concentration is significantly higher in ESLD patients (recipients, n = 21) (p = 0.0081). Box-and-whiskers plots. Results are expressed as median, interquartile range, and minimum-maximum. b. EV
concentrations of individual cases were plotted from pre- to post-liver transplantation (postoperative, 1 and 2 weeks). The concentration of bile EVs decreased significantly over time after liver transplantation (1 week: \( p = 0.00061 \); 2 weeks: \( p = 0.14595 \)). Mann-Whitney U test and nonparametric Spearman correlation analysis were used to determine statistical significance. * \( p < 0.01 \).

c. Classification of ESLD patients based on background liver disease (hepatitis C virus (HCV): \( n = 7 \); alcohol-induced: \( n = 5 \), and non-alcoholic steatohepatitis (NASH): \( n = 3 \)), showed no significant difference in the concentration of bile EVs between these groups.

d. Concentration of bile EVs in ESLD patients with hepatocellular carcinoma (HCC, \( n = 5 \)) was significantly higher than that in healthy donors (\( n = 16 \)) (\( p = 0.02578 \)). Box-and-whiskers plots. Results are expressed as median, interquartile range, and minimum-maximum. Mann-Whitney U test and nonparametric Spearman correlation analysis were used to determine statistical significance. * \( p < 0.05 \).

**Figure 3**

Comparison of miRNA profiles in bile extracellular vesicles in patients with end-stage liver disease (ESLD) and those with normal liver (NL) by next-generation sequencing. Next-generation sequencing identified 43 miRNAs that had at least five reads per million in bile extracellular vesicles (EVs) extracted from patients with either normal liver or end-stage liver disease. Fold changes in the levels of these miRNAs in bile EVs from ESLD patients relative to the NL group are represented by volcano plots. The dot size indicates the level of miRNA contained in the EVs. The x-axis indicates the fold change, and the y-axis indicates the p value based on the Mann-Whitney U test. Above the dotted line are miRNAs that had \( p < 0.05 \).
Levels of miR-17, -92a, -25, -423, -451a, and -122 in bile extracellular vesicle from patients with end-stage liver disease (ESLD) and normal liver. Levels of miRNA in bile extracellular vesicles (EVs) were analyzed by real-time quantitative PCR. The miRNA levels were normalized against that of cel-miR-39 and were corrected by each EV particle concentration to examine the miRNA level per EV. Outliers were removed from the scatter plot. The levels of miR-17, miR-92a, miR-25, miR-423, and miR-451a significantly
increased in patients with ESLD (recipients, n = 21) compared to those with normal liver (donors, n = 16). On the other hand, the level of miR-122 was not significantly different between the two groups. Mann-Whitney U test and nonparametric Spearman correlation analysis were used to determine statistical significance. ** p < 0.01; *** p < 0.001.

**Figure 5**
Comparison of miR-17, -92a, -25, -423, -451a, and -122 levels in bile extracellular vesicles from patients with end-stage liver disease (ESLD) classified based on background liver disease. The miRNA levels in bile extracellular vesicles (EVs) were analyzed by real-time quantitative PCR. The miRNA levels were normalized against that of cel-miR-39 and were corrected by each EV particle concentration to determine miRNAs level per EV. The level of miR-17 was significantly higher in alcoholic end-stage liver disease (n = 5) than in end-stage liver disease due to hepatitis C virus (HCV; n = 7) and non-alcoholic steatohepatitis (NASH; n = 3). miR-92a was significantly higher in alcoholic end-stage liver disease than in end-stage liver disease due to HCV. There was no significant difference in other miRNAs levels between HCV-, alcohol-, and NASH-related end-stage liver disease. Mann-Whitney U test and nonparametric Spearman correlation analysis were used to determine statistical significance. * p < 0.05.

Supplementary Files

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