## Supplementary Information (SI)

**Article Title:** Bile extracellular vesicles from end-stage liver disease patients show altered microRNA content

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# SUPPLEMENTARY MATERIAL

## Experimental Procedures

### Diagnosis and staging of hepatocellular carcinoma

The diagnosis of hepatocellular carcinoma was comprehensively determined by various available diagnostic tools, including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and postoperative pathological examination. Tumor staging was defined according to the American Joint Committee on Cancer (AJCC)/International Union for Cancer Control (UICC) TNM system.

### *Western blot analysis*

The extracted EVs were lysed by the addition of radioimmunoprecipitation assay (RIPA) buffer and 0.5, 1, and 5 μg of protein from each lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA). The proteins were transferred onto 0.2-μm nitrocellulose membranes (Bio-Rad). After blocking by fat dried milk, the membranes were blotted with the primary antibodies against CD63 mouse antibody (1:200) (sc-5275; Santa Cruz Biotechnology, Dallas, TX) and TSG101 rabbit antibody (1:1000) (ab125011; Abcam, Cambridge, MA) for 1 h. The membranes were washed with PBS containing 0.05% of Tween 20 and incubated with the following secondary antibodies: for CD63: HRP-conjugated goat anti-mouse IgG (H+L) (1:2500) (G21040, Thermo Fisher Scientific,

Waltham, MA); for TSG101: HRP-conjugated goat anti-rabbit IgG (H+L) (1:2500) (G21234, Thermo Fisher Scientific) for 1 h. The Clarity Western ECL Substrate (Bio-Rad) was used for signal detection. All western blotting was performed at least in duplicate.

***Isolation of miRNA***

Extracted EVs (170 μL) were lysed in 1,000 μL of QIAzol Lysis reagent (QIAGEN, Tokyo, Japan) and incubated at room temperature for 30 min. Thereafter, cel-miR-39 (0.01 ng/mL of bile samples) was added as spike-in for standardization. miRNA was extracted using the miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocols for preparation of miRNA-enriched fractions. After purification using the RNeasy MinElute Cleanup Kit (QIAGEN), the miRNA was eluted with 30 μL of nuclease-free water. The extracted miRNA was qualitatively and quantitatively evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA).

***cDNA library construction and next-generation sequencing***

A barcoded cDNA library of the extracted miRNAs was prepared using SMARTer smRNA-Seq Kit for Illumina (Clontech Laboratories, Mountain View, CA) according to manufacturer's protocols. To comprehensively evaluate miRNAs, we analyzed the cDNA library with Illumina HiSeq 2500 using the HiSeq SR Rapid Cluster Kit v2, single-read flow cell and HiSeq Rapid SBS Kit v2, 50 Cycles

(Illumina, Clontech Laboratories).

***Sequencing data analysis***

FASTQ files obtained from sequencing were processed by the Cutadapt software package (25) version 1.13 for adapter trimming. A reference genome sequence was prepared using the following sequences: 1) canonical chromosomes for hg38, 2) the Epstein-Barr virus (EBV) decoy sequence, 3) the decoy JRGv2 sequence obtained from Japanese human genomes (26), 4) biallelic single-nucleotide variants (SNVs) in dbSNP147 for Novoalign calibration, and 5) sequences of *Arabidopsis thaliana* miR-163 and *Caenorhabditis elegans* miR-39-3p encompassed by 100-bp "N"s in both ends. Trimmed reads were mapped on the prepared reference sequence using Novoalign version 3.08 (Novocraft, Selangor, Malaysia) with the miRNA mode option followed by read sorting using Novosort (Novocraft). Mapped reads were annotated using an in-house script based on Ruby UCSC API (27, 28) accessing the University of California Santa Cruz (UCSC) genome browser tables TRNAs (as of November 4, 2015) and WgEncodeGencodeBasicV24 (as of December 9, 2015).

***Real-time quantitative RT-PCR of miRNAs***

We synthesized cDNA from the extracted miRNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). The quantitative RT-PCR was performed by the

LightCycler 480 Instrument II (Roche Diagnostics, Indianapolis, IN) using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific). The references for primers and hydrolysis probes are as follows: cel-miR-39 (TM: 000200), has-miR-122 (TM: 002245), has-miR-17 (TM: 002308), has-miR-92a (TM: 000431), has-miR-25 (TM: 000403), has-miR-423-5p (TM: 002340), mmu-miR-451 (TM: 001141) (Life Technologies, Tokyo, Japan).

***Reference***

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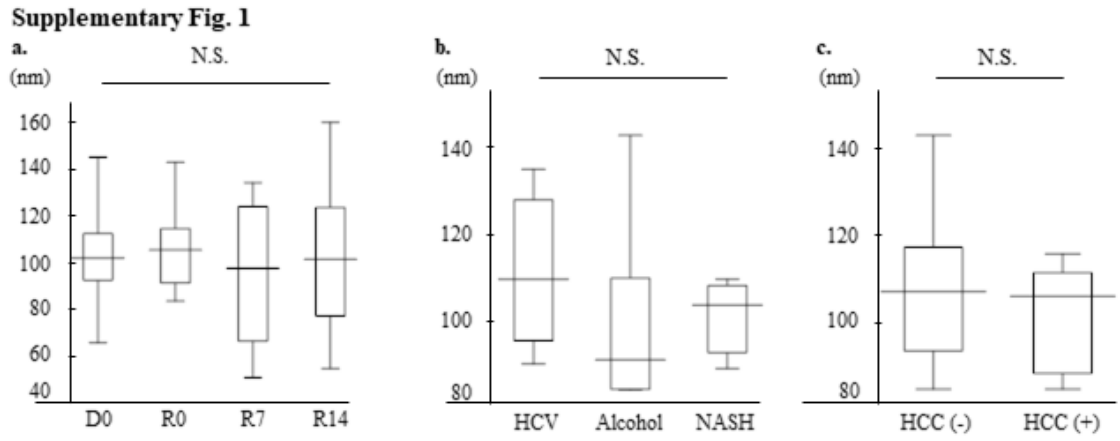
**Supplementary Table 1 Summary of deep sequence analysis data**

| | **Recipients (n = 13)** | **Donors (n = 11)** |
|---|---|---|
| Median number (range) of RNA reads/ sample | $3.82 \times 10^6$ ($1.48–5.64 \times 10^6$) | $3.93 \times 10^6$ ($2.19–6.54 \times 10^6$) |
| Median number (range) of miRNA reads | $6.07 \times 10^3$ ($3.79–15.6 \times 10^3$) | $4.30 \times 10^3$ ($1.88–23.0 \times 10^3$) |
| Median number (range) of miRNA variety | 465 (133–303) | 461 (109–244) |

**Supplementary Table 2 Reads per million of miRNA elevated in recipients as reported by deep sequence analysis**

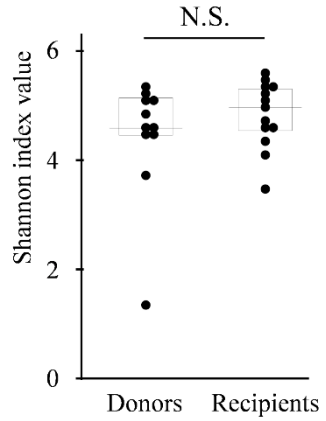
| | Recipients [n = 13] | Donors [n = 11] | Fold change [Recipient/Donor] | *P* value |
|---|---|---|---|---|
| miR-17 | 76.7 | 18.9 | 4.07 | 0.0162 |
| miR-25 | 61.8 | 8.76 | 7.06 | 0.0029 |
| miR-92A1 | 27.5 | 8.23 | 3.34 | 0.0162 |
| miR-23A | 15.9 | 5.14 | 3.09 | 0.0345 |
| miR-451 | 15.9 | 0.34 | 46.3 | 0.0251 |
| miR-423 | 10.1 | 3.06 | 3.30 | 0.0059 |
| miR-3184 | 10.1 | 3.06 | 3.30 | 0.0059 |
| miR-191 | 9.78 | 3.05 | 3.21 | 0.0257 |
| Let-7I | 9.19 | 2.37 | 3.87 | 0.0059 |
| miR-19B2 | 9.07 | 1.38 | 6.59 | 0.0117 |
| miR-16-2 | 9.03 | 0.76 | 11.8 | 0.0050 |
| miR-23B | 6.13 | 2.40 | 2.55 | 0.0162 |




**Supplementary Fig. 1 Size of bile extracellular vesicle in the patients with end-stage liver disease (ESLD)**

Size of bile extracellular vesicles (EVs) were measured by nanoparticle tracking analysis. **a.** There was no significant difference in EV particle size between the patients with ESLD and with normal liver, and pre- and post-liver transplantation (LT). D0, R0, R7, and R14 indicate bile EVs extracted from donor (normal liver) pre-LT, recipients (ESLD) pre-LT, recipients 7 days post-LT, and recipients 14 days post-LT, respectively. **b.** There was no significant difference in EV particle size between patients grouped according to background liver disease. **c.** There was no significant difference in EV particle size in the absence or presence of hepatocellular carcinoma. 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. N.S.: not significant.

**Supplementary Fig. 2**

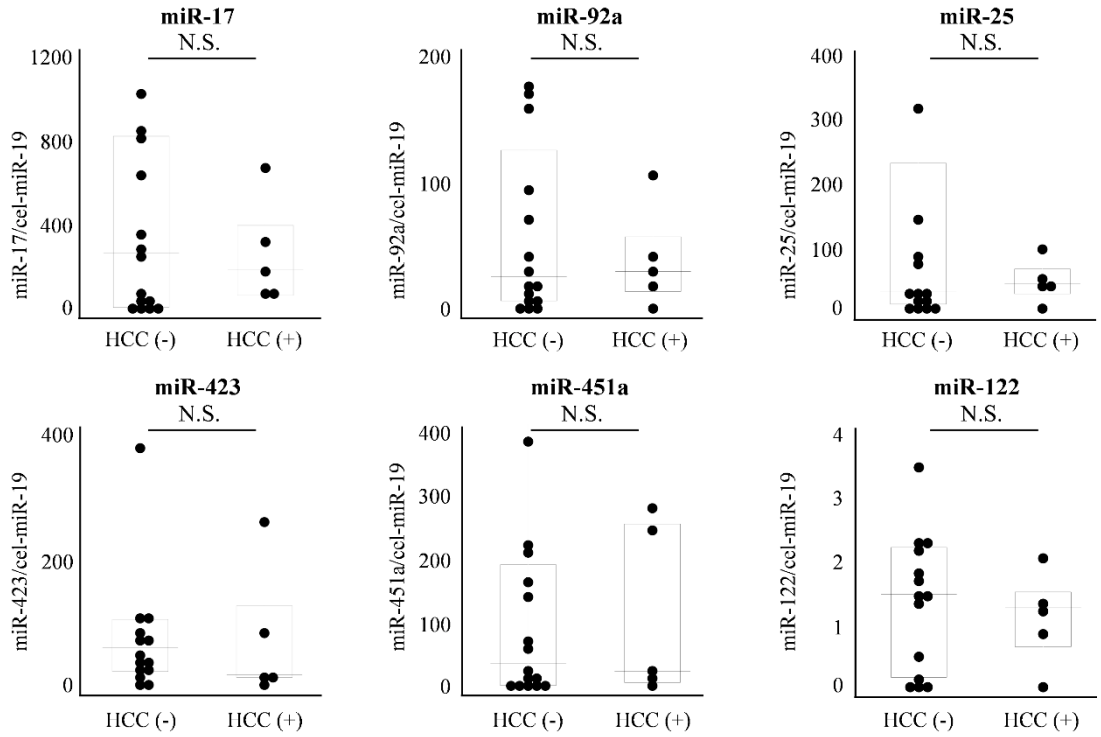



**Supplementary Fig. 2 Diversity of miRNA contained in bile extracellular vesicles in the patients with end-stage liver disease (ESLD)**

Diversity of miRNAs in bile extracellular vesicles (EVs) as identified by next-generation sequencing was analyzed. Shannon index indicated that the diversity of miRNAs in bile EVs was similar between donors (normal liver, n = 11) and recipients (ESLD, n = 13). 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.

N.S.: not significant

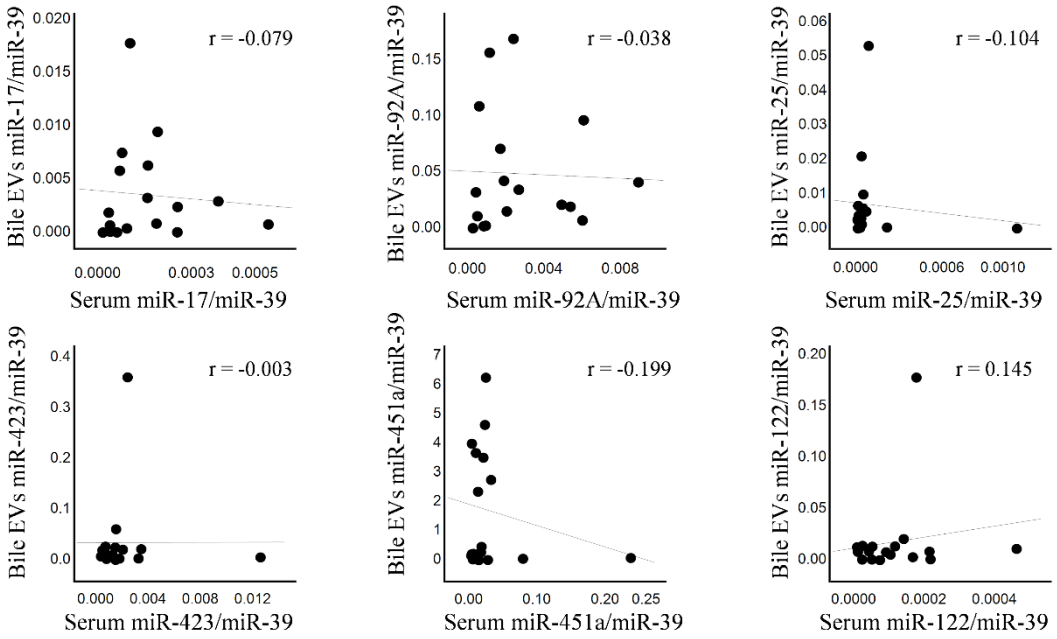
**Supplementary Fig. 3**




**Supplementary Fig. 3 Comparison of miR-17, -92a, -25, -423, -451a, and -122 levels in bile extracellular vesicles from patients with and without hepatocellular carcinoma (HCC)**

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 miRNA levels per EV. Outliers were removed from the scatter plot. There was no significant difference in miRNA levels in the presence or absence of hepatocellular carcinoma (HCC). 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. N.S.: not significant.

**Supplementary Fig. 4**




**Supplementary Fig. 4 Comparison of miR-17, -92a, -25, -423, -451a, and -122 levels between extracellular vesicles from bile and whole serum**

Levels of miRNA in extracellular vesicles (EVs) in bile and whole serum were analyzed by real-time quantitative PCR. The miRNA levels in bile EVs were normalized against that of cel-miR-39 and were corrected by the concentration of EV particles to determine miRNA content per EV. Serum miRNA levels were normalized against that of cel-miR-39. No significant correlation was observed between any of the miRNAs and the source of EVs. Mann-Whitney U test and nonparametric Spearman correlation analysis were used to determine statistical significance.