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Research article

Next-generation sequencing-based small RNA profiling of cerebrospinal fluid exosomes

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HIGHLIGHTS

- This study used NGS to analyse CSF and serum exosomal fractions.
- Exosomal miRNA profiles differed between CSF and serum.
- miR-1911-5p was specific for CSF exosomes.
- Our results have important implications for the origin of CSF exosomal miRNAs.

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ABSTRACT

MicroRNAs (miRNAs), particularly those found in human body fluids, have been suggested as potential biomarkers. Among various body fluids, the cerebrospinal fluid (CSF) shows promise as a profiling target for diagnosis and monitoring of neurological diseases. However, relevant genome-scale studies are limited and no studies have profiled exosomal miRNAs in CSF. Therefore, we conducted a next-generation sequencing-based genome-wide survey of small RNAs in the exosomal and non-exosomal (supernatant) fractions of healthy human CSF as well as serum in each donor. We observed miRNA enrichment in the exosomal fractions relative to the supernatant fractions of both CSF and serum. We also observed substantial differences in exosomal miRNA profiles between CSF and serum. Half of the reported brain miRNAs were found in CSF exosomal fractions. In particular, miR-1911-5p, specifically expressed in brain tissue, was detected in CSF but not in serum, as confirmed by digital PCR in three additional donors. Our data suggest that the brain is a major source of CSF exosomal miRNAs. Here we provide the important evidence that exosomal miRNAs in CSF may reflect brain pathophysiology.

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1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs regulating post-transcriptional gene expression. Evaluations of miRNA expression profiles in relatively easily available samples such as human body fluids have provided useful biomarkers of human diseases, particularly in the field of oncology [1]. Although serum and plasma miRNAs have been extensively investigated [2–4], cerebrospinal fluid (CSF) miRNAs are major targets in neurological

Abbreviations: CI, confidence interval; CPM, counts per million; CSF, cerebrospinal fluid; dPCR, digital PCR; HDL, high-density lipoprotein; NGS, next-generation sequencing.

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disease biomarker studies [5–8]. Accordingly, increasing numbers of CSF miRNA studies have been conducted, although most have evaluated a limited number of miRNA species using real-time PCR. There are only two recent comprehensive analyses on CSF miRNAs related to neurological diseases [9,10]; in those studies, miRNAs in total CSF, rather than fractions, were surveyed.

Body fluids contain lipid vesicles, or exosomes, secreted from cells [11,12]. These vesicles facilitate intercellular communication by transporting molecules, such as miRNAs, that could be used as biomarkers indicative of disease states [13–15]. Exosome-derived miRNAs have been investigated for cancer diagnostic and prognostic purposes [16]; however, a comprehensive analysis of CSF and serum exosomes has not yet been conducted. Given previous reports on exosome secretion by neurons and glial cells [17,18], an investigation on small RNA species in CSF exosomes would be of interest.

Therefore, we used next-generation sequencing (NGS) to conduct a comprehensive analysis of miRNA expression in the exosomal and supernatant fractions of both CSF and serum samples from healthy donors. A subsequent comparison of our results allowed us to provide an overview of the miRNA expression profile of CSF exosomes.

2. Materials and methods

2.1. Sample collection

The protocols for the collection and analysis of healthy donor samples were approved by the local ethics committee at Tokyo Medical and Dental University (No. 512) and the ethical committee at RIKEN Yokohama (H17-34).

The first batch of samples was derived from three donors and subjected to a genome-wide NGS-based survey. CSF samples were obtained via lumbar puncture, centrifuged to remove contaminating cells (500 × *g*, 10 min) and stored at −80 °C. Corresponding serum samples were simultaneously isolated from peripheral blood and stored at −80 °C. Samples were further divided into exosomal and supernatant fractions via ultracentrifugation (100,000 × *g*, 70 min, 4 °C), as previously described [19]. Seven millilitres of CSF and 3 ml of serum from each donor were subjected to ultracentrifugation. Total RNA was isolated from each individual fractionated sample (12 samples in total) using an miRNeasy Mini Kit and an RNeasy MinElute Cleanup Kit (both from Qiagen, Hilden, Germany).

The second batch of samples from three additional donors was subjected to focused validation via digital PCR (dPCR). CSF and serum exosomal fractions were isolated using the miRCURY Exosome Isolation Kit and subjected to RNA extraction using the miRCURY RNA Isolation Kit (both from Exiqon, Vedbæk, Denmark) according to the manufacturer's protocols (Fig. 1A).

Samples derived from other three donors were used in the comparison of the abovementioned two exosome isolation methods. The same samples were treated with ultracentrifugation and miRCURY Kits in the same manner as the first and second batch of samples, respectively. Extracted exosomal miRNA were analysed via dPCR.

2.2. Evaluation of isolated exosomes

Exosomes isolated from CSF via ultracentrifugation were processed for visualization by electron microscope (EM), as described in [19]. H-7100 EM (Hitachi, Tokyo, Japan) and Advantage-HS CCD camera (AMT, Woburn, USA) were used.

For western blot analysis of exosomes, primary antibodies were Mouse Anti-Human CD9 (COSMO BIO, Tokyo, Japan) and Mouse Anti-Calnexin (Santa Cruz Biotechnology, Dallas, USA). Secondary antibody was Peroxidase AffiniPure Goat Anti-Mouse IgG (Jackson Immuno Research, West Grove, USA).

Nanoparticle tracking analysis (NTA) [20] of exosomes were performed using NanoSight LM10 and NanoSight NTA 3.0 0068 software (Malvern Instruments, Worcestershire, UK). Exosome samples were diluted to optimal concentrations according to the manufacturer's protocols and recorded five times for 60 s. CSF exosome samples obtained with ultracentrifugation and miRCURY kit were diluted by 1:10 and 1:50, respectively. Serum exosome samples obtained with ultracentrifugation and miRCURY kit were diluted by 1:100 and 1:1000, respectively.

2.3. Small RNA sequencing

Small RNA libraries were prepared using a TruSeq Small RNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol; 10–30-base RNA molecules were targeted, and a 15-cycle PCR protocol was employed. Individual libraries were prepared with unique indexes, pooled and subjected to 50-base reads in single lanes of a HiSeq 2500 system (Illumina). The resulting reads were post-processed using decoding indexes to recover individual samples; adaptor sequences were trimmed, and sequences that either contained 'N' or were aligned with the ribosomal DNA repeat unit (GenBank accession HSU13369) were excluded.

2.4. Small RNA cluster construction and quantification

We aligned small RNA sequences to the human reference genome (hg19) using BWA (version 0.5.9) [21]. Alignments with high mapping quality (>20) were aggregated even when only one base pair overlapped to define the genomic regions (called small RNA clusters) that produced small RNAs. Clusters were annotated as miRNAs when their genomic coordinates overlapped with those of miRNAs registered in miRbase (version 20) [22]. Aligned reads in each cluster were counted to monitor the global small RNA expression landscape. The number of reads aligned within each individual cluster was normalized to counts per million (CPM) after applying a normalization factor based on the relative log expression method [23] via edgeR [24].

2.5. Sequencing data-based quantification of miRNAs

Rather than alignment with the human genome sequence, post-processed reads were analysed using the script 'quantifier.pl' in the miRdeep2 package (version 2.0.0.7; based on the miRbase database) for comparisons with data from previous sequence-based studies of total miRNA in CSF [9] or brain tissue [25]. Differences in methodologies led to differences in resulting data with respect to the quantification of small RNA clusters, including miRNAs; however, miRNA expression was represented in a manner consistent with the findings of a previous study [9].

2.6. dPCR

After purification, exosomal miRNAs were subjected to reverse transcription (RT) using TaqMan MultiScribe Reverse Transcriptase and TaqMan Probe (5×) (Applied BioSystems, Foster City, CA, USA). In order to equalise the starting amounts of biofluids, exosomal miRNA obtained from 25 μl of serum or CSF were subjected to each RT reaction. Fig. S1 shows the schematic flowchart of the validation experiments with dPCR. The thermal cycling conditions were as follows: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min, followed by a hold step at 4 °C. dPCR was performed using QuantStudio 3D Digital PCR Master Mix v2 and TaqMan Probe (20×) (Applied BioSystems) in accordance with the manufacturer's instructions.

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