Characterization of microRNAs expressed in the cystic legion of the liver of *Mus musculus* perorally infected with *Echinococcus multilocularis* Nemuro strain

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Abstract

Alveolar echinococcosis (AE) is a zoonosis caused by the metacestode of *Echinococcus multilocularis*. The published genome of *E. multilocularis* showed that approximately 86% of its genome is non-coding. Micro RNAs (miRNAs) are small non-coding regulatory RNAs, and recent studies on parasitic helminths expect miRNAs as a promising target for drug development and diagnostic markers. Prior to this study, only a few studies reported the *E. multilocularis* miRNA profiles in the intermediate host. The primary objective of this study was to characterize miRNA profiles via small RNA-seq in *E. multilocularis* Nemuro strain, a laboratory strain of Asian genotype, using mice perorally infected with the parasite eggs. The data were then compared with two
previously published small RNA-seq data. We identified 44 mature miRNAs as *E. multilocularis* origin out of the 68 mature miRNA sequences registered in the miRNA database miRbase. The highest quantities of miRNAs detected were miR-10-5p, followed by bantam-3p, let-7-5p, miR-61-3p, and miR-71-5p. The top two most abundant miRNAs (miR-10-5p and bantam-3p) accounted for approximately 80.9% of the total parasite miRNAs. The highly expressed miRNA repertoire is mostly comparable to that obtained from the previous experiment using secondary echinococcosis created by an intraperitoneal administration of metacestodes. A detailed characterization and functional annotations of these shared miRNAs will lead to a better understanding of parasitic dynamics, which could provide a basis for the development of novel diagnostic and treatment methods for AE.

**Keywords**

*Echinococcus multilocularis*, alveolar echinococcosis, microRNA, non-coding RNA, high-throughput sequencing
Main text

Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE), a neglected tropical disease acknowledged by the World Health Organization. Humans are infected by E. multilocularis as accidental intermediate hosts. After an asymptomatic incubation period of 5–15 years, patients start to show symptoms including cholestatic jaundice, abdominal pain, fatigue, and weight loss, which can ultimately lead to death once left untreated [10]. Diagnosis is performed by radiography or serological tests such as enzyme-linked immunosorbent assays (ELISA) [10], but these methods can only diagnose AE after a certain development of the metacestode stage. Difficulty in the detection of early stage E. multilocularis infection causes patients to be diagnosed at advanced stages, allowing fatal symptoms to occur.

Intermediate hosts are infected with the parasite by ingesting infective eggs containing oncospheres, which hatch and develop into metacestodes in the liver. The metacestode consists of an inner germinal layer that bud to form a brood capsule, which grows protoscolex inside [14]. Echinococcus multilocularis grows aggressively as it buds exogenously, infiltrating and colonizing surrounding tissues. It can also spread to adjacent locations or distant organs owing to its metastatic nature [9].
The *E. multilocularis* Europe genotype genome was sequenced [20], and preliminary analyses show that approximately 86% of the genome is non-coding, with further non-coding RNA analyses, including microRNA (miRNA) awaiting exploration. miRNAs have been identified in numerous organisms over diverse clades, including helminths, and their relevance as repressors of gene expression is now broadly recognized [1, 3]. The existence of the RNA induced silencing complex, which is needed for miRNA utilization, has been demonstrated in *E. multilocularis* by *in silico* analysis [8]. Subsequently, the presence of *E. multilocularis* miRNA has been confirmed experimentally using mice infected with the parasite [7]. However, this experiment was conducted using secondary echinococcosis created by an intraperitoneal administration of sieved metacestodes, which may result in atypical miRNA profiles compared to a natural *E. multilocularis* infection. Furthermore, *E. multilocularis* is divided into three genotypic clades: Asia, Europe, and North America [18] and previous studies on *E. multilocularis* miRNA solely used European genotypes [7, 8]. Hence experimental data using *E. multilocularis* with different genetic backgrounds, are necessary for a comprehensive understanding of miRNA profiles.

The primary objective of this study was to characterize miRNA expression profiles of *E. multilocularis* using Nemuro strain (Asian genotype), which has been
maintained in a cotton rat-dog cycle under laboratory conditions, in mice perorally infected with the parasite eggs. Furthermore, the obtained data were compared with two previously published *E. multilocularis* miRNA profiles using European strains.

*Echinococcus multilocularis* Nemuro strain was obtained from a male *Myodes rufocanus bedfordiae* caught in Nemuro city (43°16'57"N 145°30'57"E), Hokkaido, Japan in 1987. The parasite has been maintained in a specially designed safety facility (Biosafety Level 2 Enhanced) at the Hokkaido Institute of Public Health, Hokkaido, Japan. Six-week-old DBA/2 mice (Japan SLC, Japan) were infected with *E. multilocularis* Nemuro strain by perorally administrating parasite eggs (2,000 eggs/mouse). To characterize miRNAs expressed after cyst development, the samples were collected at 16 weeks post infection which allows protoscolex maturation in DBA/2 mice [16, 19]. The infected mice were euthanized by cervical dislocation under isoflurane anesthesia and their livers including *E. multilocularis* cysts were obtained and stored at −80°C until RNA extraction. All procedures performed in this study involving animals were in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, and the protocol for the animal experiments was approved by the ethics committee of the Hokkaido Institute of Public Health (study ID K26-3).
Total RNA was isolated from three *E. multilocularis*-infected mouse livers (30 g) using the Nucleospin® miRNA Kit (Machery-Nagel, Germany). RNA concentration was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). A Truseq® Small RNA Sample Preparation Kit (Illumina, USA) was used to prepare the Illumina sequencing library from the pooled total RNA (500 ng) of three mice. Two Illumina libraries, replicate nos. 1 and 2, were made as technical replicates. In brief, SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) was used to perform reverse transcription to adapter ligated small RNAs. The cDNAs obtained were then amplified and purified by extracting amplicons that accounted for RNA lengths between 20 and 33 bp. Agencourt AMPure XP (Beckman Coulter, USA) was used to further purify the amplicons. A High Sensitivity DNA Assay Kit (Agilent, USA) on an Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to determine the quality of the purified library. The library’s quantity was adjusted after being measured with a Kapa Library Quantification Kit (Kapa Biosystems, USA) and 7500 Fast real-time PCR system (Applied Biosystem, USA). After the measurement, the library was adjusted to 4 nM with a 1% spike-in of phiX control library (Illumina) and sequenced on the HiSeq 2000 sequencing system (Illumina). All procedures were carried out according to the manufacturers’ protocols. Raw sequence data in this study have been deposited in
DDBJ Sequence Read Archive with an accession number of DRA010502.

miRNA sequences of mouse (*Mus musculus*) and *E. multilocularis* were obtained from the public database miRBase version 22.1 (http://www.mir-base.org/).

All analyses were performed with CLC Genomics Workbench version 9.5.1 (Qiagen, Denmark). The reads were trimmed by removing index sequences, selected between 18 and 24 bp, and merged and sorted according to the same sequences using the “extract and count” command. The merged reads were annotated as *M. musculus* or *E. multilocularis* miRNAs using the “annotating and merging small RNA samples” command.

A total of 7,064,228 and 6,854,600 reads were initially obtained from replicate nos. 1 and 2, respectively. Of these reads, 3,313,066 and 3,629,090 reads were filtered as selected reads. An initial overview of sequencing results annotated 3,010,447 (90.9%) and 3,274,942 (90.2%) of the selected reads as pre-miRNA. Among them, 2,983,927 (99.1%) and 3,247,608 (99.2%) reads were of mouse origin, while 26,520 (0.9%) and 27,334 (0.8%) reads were of *E. multilocularis* origin. A list of the *E. multilocularis* mature miRNAs detected using the *E. multilocularis* miRbase database is shown in Supplementary Data 1. Further analysis was carried out using replicate no. 1 as this library detected more *E. multilocularis* miRNAs. Out of 36 precursors and 68 mature miRNAs
registered in miRbase for *E. multilocularis*, a total of 32 and 44 were detected from the Nemuro strain, respectively. The most abundant mature miRNA detected was miR-10-5p (n = 9,320), followed by bantam-3p (n = 6,213), let-7-5p (n = 1,859), miR-61-3p (n = 506), and miR-71-5p (n = 257). The top two most abundant miRNAs (miR-10-5p and bantam-3p) accounted for approximately 80.9% of the total parasite miRNAs (Figure 1).

A total of 555 mouse-derived mature miRNAs were detected (Supplementary Data 1). The most highly expressed mouse-derived mature miRNA was miR-22-3p (n = 609,309), followed by miR-192-5p (n = 140,335), miR-21a-5p (n = 104,063), miR-26a-5p (n = 73,797), and miR-148a-3p (n = 60,906) (Figure 1). miR-22-3p consisted 39.3% of all mouse-derived miRNAs and top 10 most abundant miRNAs accounted for 78.6% of the total mouse miRNAs (Figure 1).

Next, we compared our data with miRNA profiles obtained from two other studies. The details of each experiment (experimental animal strain, origin of *E. multilocularis*, infection route, and time of sampling) are summarized in Table 1. One study conducted by Cucher et al. [7] analyzed *E. multilocularis* miRNAs in mice with secondary echinococcosis, while the other study conducted by Boubaker et al. [2] reported miRNA profiles in the liver of the mice orally infected with *E. multilocularis* eggs. Since the latter study characterized only mouse miRNAs, we searched for parasite
miRNAs in the deposited sequence data (SRA nos. of SRX7826699-SRX7826701) using the pipeline mentioned above, leading to the detection of 6 *E. multilocularis* miRNAs (bantam-3p, let-7-5p, miR-1-3p, miR-9-5p, miR-61-3p, and miR-3479b-3p).

All 44 matured miRNAs obtained in the present study were found in the study of Cucher et al. [7], since the present miRbase database for *E. multilocularis* is structured using the result of Cucher et al. [7, 8]. A total of 24 *E. multilocularis* miRNAs registered in the database were not detected in the Nemuro strain (Figure 2). This could be attributed to the difference in the number of miRNA sequences obtained for each study; a total of 53,854 reads mapped to *E. multilocularis* miRNAs in this study and 1,497,226 and 2,169,374 reads mapped to *E. multilocularis* miRNAs in Cucher et al. [7]. In fact most of the miRNAs absent in this study showed only 0.042% or lower (1~623 reads) in proportion, implying that increased reads can detect the lowly expressed miRNAs. The only exception was miR-190-5p, which accounted for 0.508~0.558% of reads, and was ranked in 15th highest expressed *E. multilocularis* miRNA out of the 68 [8]. This may imply that miR-190-5p plays different roles in the cyst between genotypes or parasite infection routes. Since more than 99% of miRNA reads obtained in this study were mouse origin, further analysis using only cysts instead of liver tissue with cysts may lead to increased detection of lowly expressed parasite miRNAs.
Boubaker et al. [2] extracted RNA from the liver tissue of peri-parasitic area, precisely 3 to 4 mm adjacent to parasite lesions, in *E. multilocularis*-infected mice. Nonetheless, we could detect six *E. multilocularis* miRNAs from their sequencing data. The six miRNAs were also detected in the present study and 5 were highly expressed, suggesting that these miRNAs share necessary roles throughout the *E. multilocularis* cyst development between different strains. As excretory/secretory vesicles of *E. multilocularis* have been inferred to carry parasite miRNAs, these miRNAs could possibly have been transported to surrounding murine tissue.

*Echinococcus multilocularis* miRNA functions are beginning to be assessed [24], but most remain unknown. However, since some miRNAs are shared among different organisms, their functions can be speculated. In particular, miR-10-5p, the most abundantly expressed miRNA in *E. multilocularis* Nemuro strain, also exists in mice (mmu-miR-10a-5p) and humans (hsa-miR-10a-5p). This miRNA is upregulated during cancer in humans [15], and recent studies showed that miR-10a may have a causal role in metastases in gastric cancer [5] and glioma [23]. In the case of glioma, the overexpression of miR-10a causes epithelial–mesenchymal transition to occur, enabling glioma cells to be transferred to other parts of the body [23]. Because *E. multilocularis* infection causes tumor-like metastases in mice and humans, *E. multilocularis* miR-10 may play roles in
the expansion of metacestodes across host organs. As modulation of miRNA activities has been studied as a novel therapeutic approach in the field of cancer research [11, 17], parasite miRNAs including miR-10 could be potential drug targets. Nonetheless, since *E. multilocularis* miRNAs are expressed inside the cyst enclosed by the laminated layer, a proper delivery system should be developed in the drug design process.

microRNA can also be present in blood, and its usage as a tumor-specific marker is being considered in the field of oncology [21]. As parasite-derived miRNAs have recently been found in the host blood stream [4, 6, 12, 22], the use of miRNAs as biomarkers of parasite infection should be considered. In fact, a recent study revealed the presence of several *E. multilocularis* miRNAs in experimentally infected mice sera [12], indicating that certain miRNAs may serve as potential AE diagnostic biomarkers.

In conclusion, we detected 44 mature miRNAs of *E. multilocularis* Nemuro strain. The data obtained in this study will provide a reliable resource to supplement existing knowledge on *E. multilocularis* miRNAs. Further comparative experiments are required to reveal the effect of parasite strain, infected animal (species and strain), and mode of infection on the expression patterns of *E. multilocularis* miRNAs in the intermediate host. Nonetheless, this study showed that certain miRNAs are shared even among different infection conditions. Further characterization and functional annotation
of these miRNAs will lead to a better understanding of the parasite and host interactions which could enlighten new strategies to tackle AE.

Conflict of interest

The authors declare no conflict of interest.

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References


Table 1. Details of experimental design of *Echinococcus multilocularis* miRNA studies.

<table>
<thead>
<tr>
<th>Year</th>
<th>Mouse strain</th>
<th><em>E. multilocularis</em> origin</th>
<th>Infection route/Parasite material</th>
<th>Sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study$^1$</td>
<td>2020</td>
<td>DBA/2</td>
<td>Hokkaido, Japan</td>
<td>Oral/Egg</td>
</tr>
<tr>
<td>Cucher et al.$^2$</td>
<td>2015</td>
<td>CF1</td>
<td>Europe</td>
<td>Intraperitoneal/Cyst</td>
</tr>
<tr>
<td>Boubaker et al.$^3$</td>
<td>2020</td>
<td>C57BL/6</td>
<td>Switzerland</td>
<td>Oral/Egg</td>
</tr>
</tbody>
</table>

$^1$ Eggs obtained from experimental gerbil-dog cycle; $^2$ Infection route not clearly mentioned in paper; $^3$ Eggs obtained from hunted fox.
Figure legends

Figure 1. Percentage of most abundant miRNAs found in *Echinococcus multilocularis*-infected mice liver. Each graph shows the top 10 miRNAs that are expressed in *E. multilocularis* infected mouse. (A) is of *E. multilocularis* origin and (B) is of *M. musculus* origin. miR-10-3p and bantam-3p accounted for most (approximately 80%) expression in *E. multilocularis*. Data from replicate no. 1 are used.

Figure 2. Comparison of *Echinococcus multilocularis* miRNAs observed in two different studies. The numbers stand for the ranks when listed according to the miRNA abundance in each small RNA-seq. The list of miRNAs not detected in this study is shown on Supplementary Data 2. *miRNAs found in Boubanker et al., [2]. ND: Not detected.*