Experimental Parasitology 128 (2011) 116–120

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Angiostrongylus cantonensis: Identification and characterization of microRNAs in male and female adults

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ARTICLE INFO

Article history:
Received 2 January 2011
Received in revised form 17 February 2011
Accepted 21 February 2011
Available online 26 February 2011

Keywords:
Angiostrongylus cantonensis
Angiostrongyliasis
Eosinophilic meningitis
microRNAs (miRNAs)
Solexa deep sequencing

ABSTRACT

Angiostrongylus cantonensis causes eosinophilic meningitis and eosinophilic pleocytosis in humans and is of significant socio-economic importance globally. microRNAs (miRNAs) are endogenous small non-coding RNAs that play crucial roles in gene expression regulation, cellular function and defense, homeostasis and pathogenesis. They have been identified in a diverse range of organisms. The objective of this study was to determine and characterize miRNAs of female and male adults of *A. cantonensis* by Solexa deep sequencing. A total of 8861,260 and 10,957,957 high quality reads with 20 and 23 conserved miRNAs were obtained in females and males, respectively. No new miRNA sequence was found. Nucleotide bias analysis showed that uracil was the prominent nucleotide, particularly at positions of 1, 10, 14, 17 and 22, approximately at the beginning, middle and the end of the conserved miRNAs. To our knowledge, this is the first report of miRNA profiles in *A. cantonensis*, which may represent a new platform for studying regulation of genes and their networks in *A. cantonensis*.

1. Introduction

The zoonotic nematode *Angiostrongylus cantonensis* was first discovered in the pulmonary arteries and hearts of domestic rats in Guangzhou, China (Chen, 1935). Humans are non-permissive hosts and become infected by consuming raw or undercooked intermediate hosts (snails) or transport hosts (frogs and fish) infected with third-stage larvae (Ali et al., 2008). After ingestion, larvae migrate to the brain, spinal cord and nerve roots, leading to eosinophilic meningitis or meningoencephalitis (Noskin et al., 1992; Diao et al., 2009). Since the report of the first human case in Taiwan in 1945 (Rosen et al., 1961), the parasite has been found to infect humans and other mammals across a wide and ever-increasing distribution, such as in East Asia, Southeast Asia, the Pacific islands and the Caribbean (Wang et al., 2008).

In the last several years, several major outbreaks of the disease have been reported in endemic regions, especially in China (9 outbreaks in mainland China and 3 in Taiwan) (Slom et al., 2002; Bartschi et al., 2004; Lv et al., 2009). Additionally, an increasing number of travelers have been diagnosed with eosinophilic meningitis caused by *A. cantonensis* after returning from endemic regions (Lo and Gluckman, 2001; Slom et al., 2002; Bartschi et al., 2004; Podwall et al., 2004; Kumar et al., 2005; Leone et al., 2007; Ali et al., 2008; Lv et al., 2009). These recent outbreaks have caused great concern about *A. cantonensis* amongst the general public and the physicians.microRNAs (miRNAs) are regulatory non-coding small RNAs of 18–22 nucleotides in length with functions in post-transcriptional regulation, gene expression in eukaryotes by targeted RNA degradation and translational arrest (Bartel, 2004). Since the discovery of *lin-4* and *let-7* (Lau et al., 2001; Lee and Ambros, 2001; Ruby et al., 2006), miRNAs have been found in numerous organisms including viruses, plants and animals (Rhoades et al., 2002; Bentwich et al., 2005; Hussain et al., 2008), and there have been a number of studies of miRNAs in a variety

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doi:10.1016/j.exppara.2011.02.019

of helminthes, protozoans and arthropods (Xue et al., 2008; Chen et al., 2009; Devaney et al., 2010; Huang et al., 2009; Liu et al., 2010; Xu et al., 2010; Zeiner et al., 2010), but until now no miRNAs have been reported from *A. cantonensis*.

A thorough understanding of *A. cantonensis* physiology has important implications for finding effective control strategies and treatments for angiostrongyliasis which remains a substantial challenge in endemic areas. Elucidating the roles of miRNAs in parasite growth, development and its ability to infect mammalian hosts is important for the understanding of the complex life cycle of *A. cantonensis*. In this study, miRNA profiles of *A. cantonensis* female and male adults were investigated and compared using Solexa deep sequencing combined with bioinformatic analyses.

2. Material and methods

2.1. Preparation of parasite samples

A. cantonensis male and female adult worms were collected from the lung arteries of rats experimentally infected with *A. cantonensis*. Host-derived contaminants were removed by washing 5 times with sterile physiological saline. Adults were separated according to gender, placed RNase-free 1.5 ml-screw-top cryotubes containing RNAlater (Sigma) and stored at 4° C before transfer to −80° C until use. All animals were handled in strict accordance with good animal practice as defined by the Ethics and Management Committee for Laboratory Animals of National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, and all animal work was approved by this committee.

2.2. RNA preparation

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After preparation, RNA concentration and purity were evaluated spectrophotometrically at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Then RNA samples were stored at −80° C.

2.3. Construction of small RNA libraries and Solexa high-throughput sequencing

For small RNA library construction and deep sequencing, 18–30 nt RNAs were isolated and purified using 15% PAGE. Twenty mg of the purified small RNA from each sample was ligated with 3′and 5′ adaptors (Illumina, San Diego, CA. USA), and was reverse transcribed using standard RT-PCR. The cDNAs were then concatenated, cloned and sequenced with Solexa (Illumina, San Diego, CA).

After removal of low quality sequences, the reads were searched against GenBank and Rfam database to discover ncRNAs including tRNA, rRNA, sRNA, and snoRNA etc. Repeat Masker (http://www.repeatmasker.org) was used to eliminate the repetitive sequences for sequence analysis of candidate precursors. miRNAs of *Caenorhabditis elegans* in the public database miRBase of Sanger center were used for the analysis of conserved miRNA in *A. cantonensis*. To identify potential miRNA precursors, the clean reads were mapped onto the genome of *C. elegans*. The genomic regions containing candidate sequences with ~70 bp were used to predict secondary structures using Mfold http://www.bioinfo.rpi.edu/applications/mfold).

3. Results

3.1. Profile characteristics of short RNAs from A. cantonensis

In total, 11,314,665 and 13,095,827 reads were obtained with Solexa deep sequencing from female and male *A. cantonensis*, respectively. After filtering of low quality tags including 5′ and 3′ adaptors and contamination formed by adaptor-adaptor ligation, a total of 8861,260 and 10,957,957 reads of high quality were obtained (Table 1). Length distribution analysis showed that most reads were distributed among 21–23 nt in both genders. The highest percentage was 25.16% with reads of 22 nt long followed by 18.43% of 23 nt reads in females (Fig. 1A). In males, 31.56% of reads were 22 nt long followed by 27.93% of 21 nt reads in males (Fig. 1B).

Among the clean reads, 11.52% in female and 12.43% in males were identified as non-coding sRNAs, including tRNAs, rRNAs, siRNAs, snRNAs and snoRNAs. Allowing the maximum mismatch of 2-nt, 13,341 (1.99%) tRNAs, 42,416 (6.32%) rRNAs, 70 (0.01%) snoRNAs, 647 (0.10%) snRNAs, and 20,800 (3.10%) siRNAs were identified in female *A. cantonensis* (Table 1). 9957 (1.90%) tRNAs, 24,508 (4.68%) rRNAs, 82 (0.02%) snoRNAs, 592 (0.11%) snRNAs, and 29,962 (5.72%) siRNAs were identified in the male adults (Table 1). Except for the ncRNAs mentioned above, 5481,845 (61.68%) and 8232,986 (75.13%) of total reads had no match to the public database and were marked as un-annotated reads. Repeat-associated small RNAs coming from the high-repeat regions of the genome or transposon-region were found to belong to two types of repeats, LINE/Pao:0 and rRNA:1, with unique copy number of 278 (9571 in total) and 7861 (1104,010 in total) in females; 212 (6876 in total) and 7136 (193,672 in total) in males.

It was found that the total percentage of ncRNAs was at approximately the same level in each gender of *A. cantonensis* and the number of unique reads was much higher in male than in female. In females, 2001,020 (22.58%) sequences were perfectly mapped to the *C. elegans* genome, which included 12,962 (1.93%) unique sequences. In males, there were 2075,173 (18.94%) sRNAs mapped to the target genome, which contained 11,587 (2.21%) unique sequences.

3.2. Distribution of sRNAs between female and male A. cantonensis

Altogether, there were 1072,876 unique sRNAs (19,819,217 total reads) of male and female *A. cantonensis*, and 122,051 (11.38%) of these were found to be common to both genders of *A. cantonensis*, 548,720 (51.14%) reads were specific to female, and 402,105 (37.48%) reads were male-specific (Table 2 and Fig. 2A). While 92.25% (18,282,317) of the total reads were shared between the two genders, and only 4.19% (972,807 reads) and 2.85% (564,093 reads) were female and male specific, respectively (Table 2 and Fig. 2B).

3.3. Expression difference of known miRNAs

After removal of ncRNAs and repeat reads, 592, 899 un-annotated unique reads were left and blasted against known miRNA of *C. elegans* in the public database of miRBase which had 154 mature miRNA and 3 miRNA∗ sequences available. There were 20 (134 in total) and 23 (196 in total) conserved miRNAs found in females and males, respectively, while no miRNA∗ homolog of *C. elegans* was found in either gender. Compared with females, 11 miRNAs were expressed at a higher level in males and 7 miRNAs were expressed at approximately equal levels, while no miRNAs with reduced expression levels in males were found. The miRNAs