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ORIGINAL PAPER

Characterization of microRNAs in *Taenia saginata* of zoonotic significance by Solexa deep sequencing and bioinformatics analysis

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Abstract The beef tapeworm *Taenia saginata* infects human beings with symptoms ranging from nausea, abdominal discomfort to digestive disturbances and intestinal blockage. In the present study, microRNA (miRNA) expressing profile in adult *T. saginata* was analyzed using Solexa deep sequencing and bioinformatics analysis. A total of 15.8 million reads was obtained by Solexa sequencing, and 13.3 million clean reads (1.73 million unique sequences) was obtained after removing reads smaller than 18 nt. Ten conserved miRNAs corresponding to 607,382 reads were found when matching the reads against known miRNAs of *Schistosoma japonicum* in miRBase database. The *miR-71* had the most abundant expression in *T. saginata*, followed by *miR-219-5p*, but some other common miRNAs such as *let-7*, *miR-40*, and *miR-103* were not identified in *T. saginata.* Nucleotide bias analysis found that the known miRNAs showed high bias and the uracil was the dominant nucleotide, particularly at the first and 11th positions which were almost at the beginning and middle of conserved miRNAs. One novel miRNA (*Tsa-miR-001*) corresponding to ten precursors was identified and confirmed by stem-loop RT-PCR. To our knowledge, this is the first report of miRNA profiles in *T. saginata*, which will contribute to better understanding of the complex biology of this zoonotic trematode. The reported data of *T. saginata* miRNAs should provide valuable references for miRNA studies of closed related zoonotic *Taenia* cestodes such as *Taenia solium* and *Taenia asiatica*.

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Introduction

Taenia saginata is a food-borne zoonotic cestode which infects approximately 50 million humans worldwide, and approximately 50,000 people died from diseases related to this parasite annually (Wanzala et al. 2003). The adult *T. saginata* in the small intestines of humans can cause taeniasis with symptoms such as nausea, abdominal discomfort, epigastric pain, diarrhea, excessive appetite or loss of appetite, and intestinal blockage. Encysted larvae of *T. saginata* known as *Cysticercus bovis* reside in musculature of cattle, and cause bovine cysticercosis. Severe bovine cysticercosis may cause myocarditis or heart failure of the host (Matuchansky and Lenormand 1999; Lees et al. 2002). The

transmission of *T. saginata* between humans and cattle usually is related to primitive animal husbandry practices, inadequate meat inspection, poor sanitation conditions, and deficiency in management or control policies (Ogunremi and Benjamin 2010; Vicentini-Oliveira et al. 2010; McFadden et al. 2011).

MicroRNA (miRNA) is non-coding small RNA molecule of 18–24 nucleotides in length, playing essential roles in gene expressions of living organisms (Du and Zamore 2007; Liu et al. 2010). miRNAs have been identified in many organisms, such as viruses, plants, and mammals (Rhoades et al. 2002; Bentwich et al. 2005; Zhang et al. 2007; Hussain et al. 2008; Xue et al. 2009). Also, miRNAs have been identified in the free living nematode *Caenorhabditis elegans*, as well as some parasites of human health significance such as *Schistosoma japonicum*, *Clonorchis sinensis*, *Toxoplasma gondii*, *Trichinella spiralis*, and *Angiostrongylus cantonensis* (Rathjen et al. 2006; Kaufman and Miska 2010; Liu et al. 2010; Wang et al. 2010; Xu et al. 2010; Zeiner et al. 2010; Chen et al. 2011a, b). However, there have been no reports of miRNAs in *Taenia* cestodes.

Therefore, the objectives of the present study were to investigate the expression profile of miRNAs in adult *T. saginata*, and to identity potential novel miRNAs by a Solexa deep sequencing approach, combined with bioinformatics analysis and stem-loop real-time polymerase chain reaction (PCR).

Material and methods

Total RNA preparation and sequencing

The gravid proglottids of adult *T. saginata* were collected from feces of a naturally infected human from Guizhou province, China. After harvesting, the parasites were transferred immediately to sterile physiological saline (37°C) in a sterile beaker and washed five times to remove contaminants from the host. One gravid proglottid was cut into pieces and transferred to RNase-free 1.5-ml screw-top cryotube containing RNAlater (Sigma). The sample was kept at 4°C for overnight and then stored at −70°C until use. The taxonomic identity of the cestode was confirmed as *T. saginata* by PCR amplification and subsequent sequence analysis of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) (Jeon and Eom 2006).

Total RNA was prepared using Trizol (Invitrogen) according to the protocol provided by the manufacturer. Pure RNA was resuspended in distilled water and stored at −70°C. The small RNA isolation was performed as described previously (Lau et al. 2001; Xu et al. 2010). For Solexa sequencing, 10 μg of total RNAs were separated on Novex 15% denaturing acrylamide 8 M urea gel with 18–30 nt fragments being recovered. The fragments were added with 5′ and 3′ adaptors (Illumina, San Diego, CA, USA) to the both ends and reverse-transcribed with a RT-PCR kit. The cDNA was then sequenced with a Solexa sequencer at Huada Genomics Institute Co. Ltd, China. All the gels and kits for small RNA purification and amplification were bought from Invitrogen Co. Ltd.

Bioinformatics analysis

Initial Solexa data was computationally filtered to remove adaptors. Reads of 18–26 nt were mapped onto the genome of *S. japonicum* (http://lifecenter.sgst.cn/schistosoma/cn) using Short Oligo nucleotide Analysis Package software (SOAP, http://soap.genomics.org.cn). Further bioinformatics analysis including unique reads screening, candidate precursors, inverted repeats, family distribution of conserved miRNA and the nucleotide bias were performed as described by Xue et al. (2009).

Analysis of novel miRNA expression

Novel miRNA expression in adult *T. saginata* was analyzed using the stem-loop real-time reverse transcriptase polymerase chain reaction (RT-PCR) with SYBR Green. The PCR mix included cDNA (5 μl in 1:20 dilution), forward and reverse primers (5 μM each) and 10 μl 2× SYBR Green PCR Master Mix. The 18S rRNA gene of *T. saginata* (GenBank accession number DQ768166) was used as the endogenous control with forward primer (5′-GACTCAA CACGGGAAAACTCAC-3′) and reverse primer (5′-TCGGAATTAACCAGACAAA TCG -3′). The cycling conditions were as follows: 95°C for 10 min, followed by 28 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s. The threshold cycle (Ct) was defined as the cycle number at which the fluorescence intensity passes a predetermined threshold.

Results

Profile characteristics of short RNAs of *T. Saginata*

There were 15.8 million reads yielded with Solexa sequencing. After filtering low quality tags including 5′ and 3′ adaptors and adaptor-adaptor ligation, a total of 15.02 million reads with high quality were obtained. Length distribution analysis showed that most reads distributed among 20–23 nt. The highest percentage was 20.09% of 22 nt reads, followed by 16.09% of 21 nt reads (Fig. 1). After removing reads smaller than 18 nt (9.71%), a total of 13.3 million clean reads remained with 1.7 million unique sequences being obtained. Among the 13.3 million clean reads, 1.5 million ones (11.10%) were perfectly mapped onto the *S. japonicum* genome, which included 14,704 (0.85%) unique sequences.

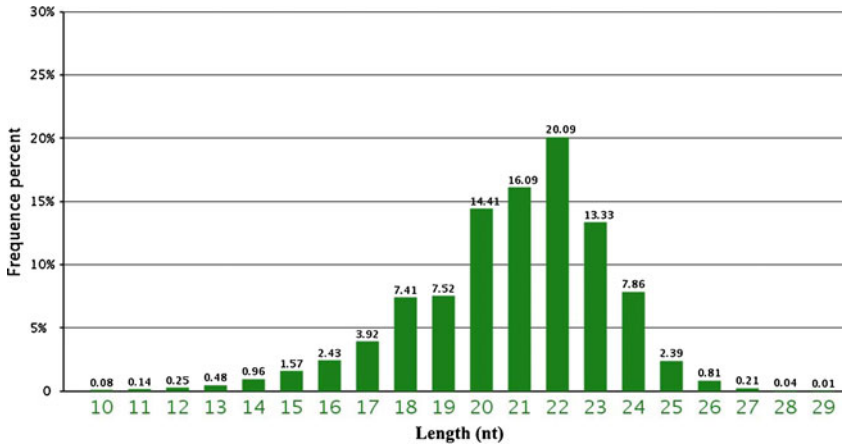

Fig. 1 Length distribution analysis of small RNAs of *Taenia saginata*. Analysis of the 15,022,805 reads with high qualities after filtering low quality tags, 5′ and 3′ adaptor and contamination formed by adaptor-adaptor ligation

Among the 13.3 million clean reads, 1,067,970 (8.03%) were found as rRNA, tRNA, snRNA, and snoRNA when searched against Rfam database. Repeat-associated small RNAs coming from high-repeat regions or transposon-region of genome were found as two types of repeats of LINE/RTE:0 and LINE/RTE:1 with 24,917 reads (188 unique ones). The percentage of known miRNA was 4.57% (607,382 total reads corresponding to 140 unique ones) focusing on ten conserved miRNAs. In addition to the known miRNAs, rRNA and repeats mentioned above, 11,265,223 (84.69%) reads had no match with public databases and marked as un-annotated RNAs. Among them, 1,679,098 were unique reads (Fig. 2).

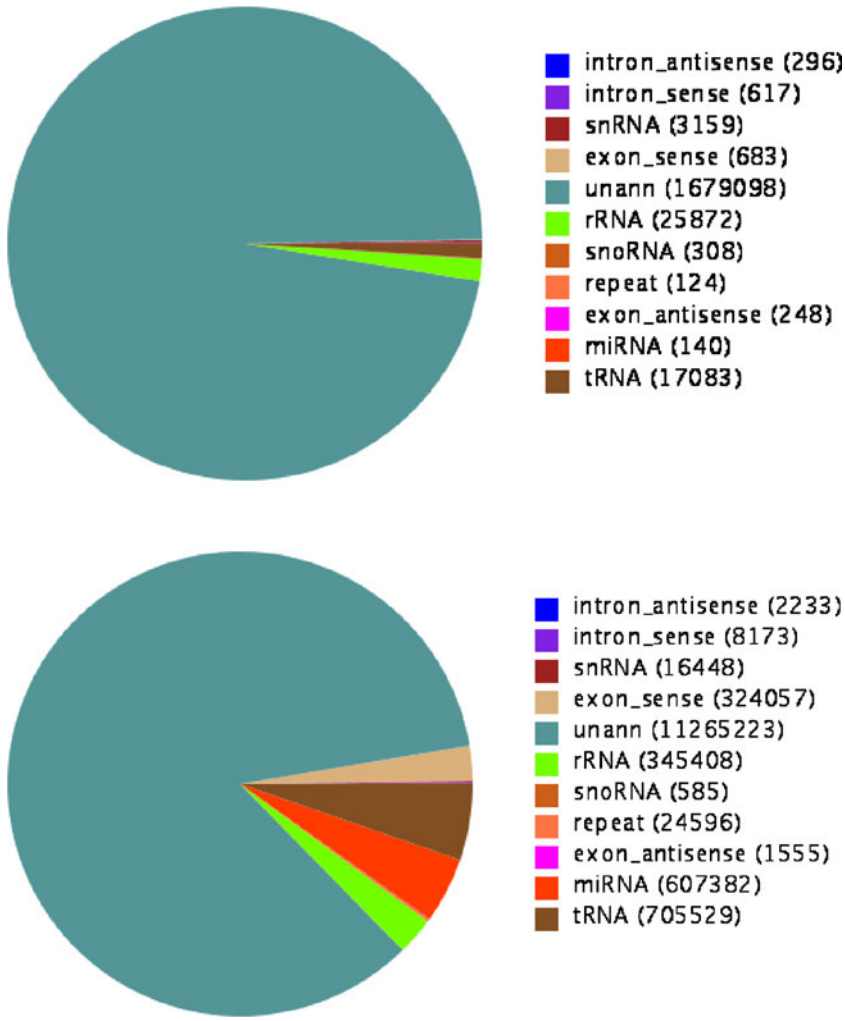

Fig. 2 Coverage of small RNAs in *Taenia saginata* by Solexa deep sequencing. *Top*: unique reads. *Bottom*: total reads

Identification of novel miRNA

The 1,679,098 un-annotated unique reads that matched *S. japonicum* genome were marked as novel miRNA candidates and used for novel miRNA predication. Among them, only one novel miRNA named *Tsa-miR-001* was found (Fig. 3). The *Tsa-miR-001* located on the 5′ end arm of the stem-loop structure with fold energy of −21.60 kcal/mol. It corresponded to ten precursors which had different locations on the *S. japonicum* genome. The most abundant precursor was located at the position of gi|158567012|gb|DS261745.1|:425:521:- 97(nt).

Expression predominance of known miRNAs in *T. Saginata*

Some kinds of miRNAs were expressed with high predominance. The *miR-71* had the most abundant reads with a percentage of 69.53% (422,285 reads). It was followed by *miR-219-5p* with a percentage of 15.80% with 95,989 reads (Table 1), whereas some other common miRNAs such as *let-7*, *miR-40*, and *miR-103,* were not found in *T. saginata. let-7* was the second miRNA found in *C. elegans*, and the *let-7* family was the biggest miRNA family among the conserved miRNAs in *C. elegans* and *C. sinensis*

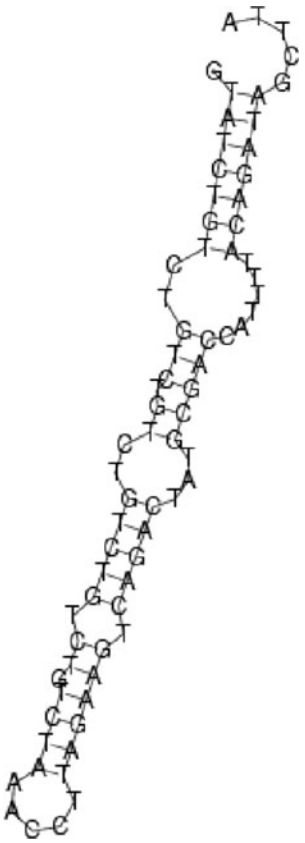

Fig. 3 Predicted structures of the novel miRNA precursors of *Taenia saginata* evaluated by RNA-fold software

Table 1 Known miRNAs and their copy numbers in *Taenia saginata*

Name	Copy numbers	Percentage (%)
miR-71	422,285	69.53
miR-219-5p	95,989	15.80
miR-7-5p	45,299	7.46
miR-277	40,152	6.61
miR-2b-3p	3,111	0.51
miR-124-3p	413	0.07
miR-10-5p	49	0.01
bantam	48	0.01
miR-307	31	0.01
miR-2a-3p	3	0

(Reinhart et al. 2000; Xu et al. 2010). However, the *let-7* or its family members were not identified in *T. saginata* in the present study.

Nucleotides bias of miRNAs in *T. Saginata*

Analysis of the 140 unique reads of known miRNAs showed that U was most often used as the first nucleotide in miRNAs of *T. saginata,* with an average percentage of 72.18%. (A + U) was found in most reads with a percentage of 88.25% in average, and it was 100% at 5th, 22th, and 24th nt, respectively. While G or C was seldom used as the first nucleotide with only 5.21% and 10.85%, respectively.

The nucleotide bias analysis at each position showed that A and U mainly located at the beginnings and the ends of reads, and U had high frequency in the 1st, 17th, and 11th positions with percentage of 99.98%, 99.43%, and 88.21%, respectively, while it did not appear at the 2nd, 10th and 24th positions. (A + U) distributed mainly in the front of reads, including the first and third to fifth nucleotides with percentages of 100%, 88.77%, 99.41%, and 100%. C and G had high percentage at the positions of sixth to ninth which belonged to the “seed region”. For example, G showed high percentages at the sixth nucleotide with percentage of 99.43%. The highest percentage of (G + C) was found at the sixth and 13th positions with a percentage of 100%. On the contrary, C and G were also found not to exist at some particular positions of miRNAs. For example, C did not exist at the 17th, 19th, and 23th positions, while G did not appear at the end positions, such as 22nd and 24th.

Quantification of *T. Saginata* miRNA expression

Using the modified stem-loop RT-PCR, the relative expression levels of the novel miRNA *Tsa-miR-001* relative to the 18S gene of *T. saginata* were calculated. Results showed that the relative expression level of *Tsa-miR-001* was very close to that of 18S gene with 7.44(E−06)±0.00 (Fig. 4).

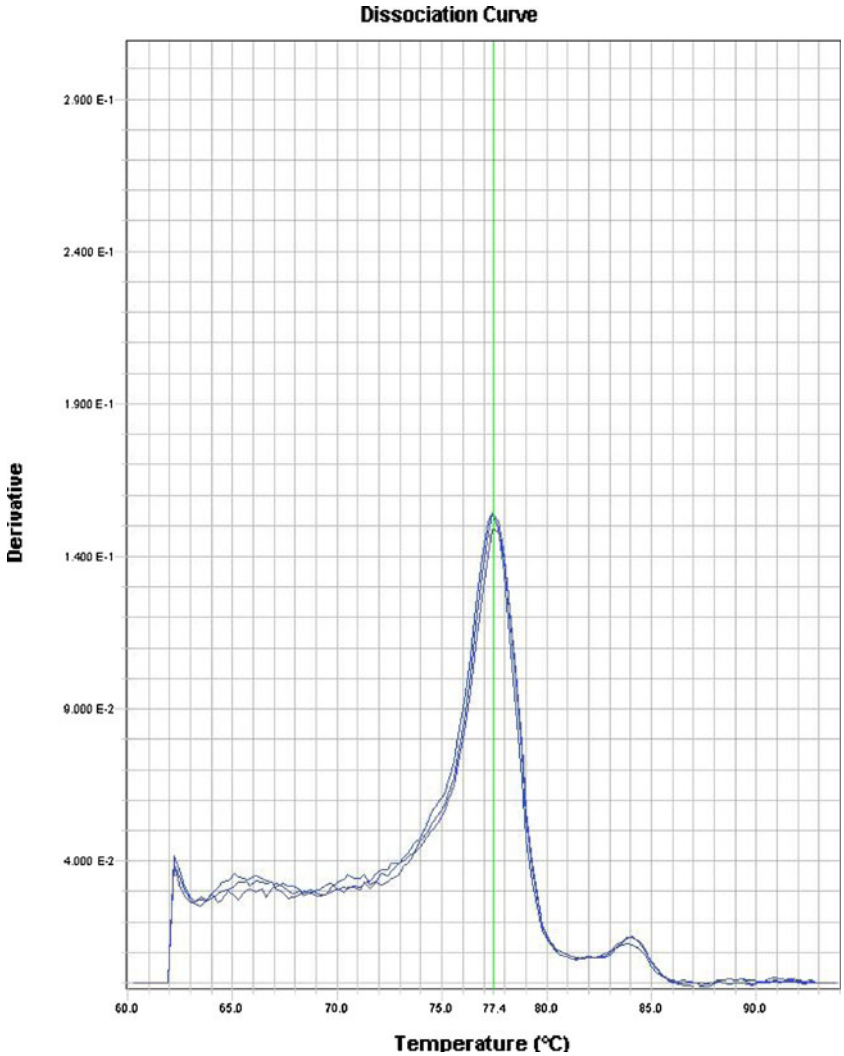

Fig. 4 Dissociation curve of the novel miRNA *Tsa-miR-001* by the modified stem-loop RT-PCR

Discussion

Given that miRNAs play significant roles in gene expression of living organisms, studies of miRNAs in human and animal parasites with complex life cycles are particular important for better understanding of the ability and the underlying mechanisms that the parasites respond to environmental and developmental signals as well as interaction between parasites and their hosts through miRNA-mediated gene expression (Liu et al. 2010; Xu et al. 2010; Al-Quraishy et al. 2011; Chen et al. 2011a, b; Delić et al. 2011). Therefore, the present study examined the expression profiles of miRNAs in the zoonotic cestode *T. saginata* by Solexa deep sequencing combined with bioinformatics analysis.

It was found that reads matched to the reference genome of *S. japonicum* was very low (11.10%), and only 14,704 unique ones out of 1,727,628 unique reads were perfectly matched, while it can reach as high as 70.5% or higher in some other species (Xue et al. 2009). The most possible reason for this might be that the reference genome used for matching analysis was the *S. japonicum* genome rather than *T. saginata*, because the *T. saginata* genome or other cestode species is not available at present publicly. For the same reason, we identified only one novel miRNA in adult *T. saginata*. When the genome of *T. saginata* or other closely related cestodes become available, more miRNAs will be found in *T. saginata*.

The conserved miRNAs were expressed with characteristics of high bias. The *miR-71* occupied most portions of the total reads, which indicated that the *miR-71* was essential for the survival of the cestode. In addition to the *miR-71*, some other miRNAs had particularly high copies in *T. saginata*, such as *miR-219*, *miR-7*, and *miR-277*. The *miR-71* and *miR-219* were also found in *C. sinensis* with high copies (Xu et al. 2010), indicating that these miRNAs might be involved in metabolism of these parasites.

U was the dominant nucleotide in mature miRNAs, especially in the first nucleotide. U showed a high frequency in the 1st and 11th positions which was almost at the

beginning and the middle of reads. Zhang et al. (2009) reported that the first, ninth and the terminal nucleotides were enriched with U, and the first and ninth positions were the edges of "seed region". However, in the present study, it was found that in the ninth and the end positions, the percentage of U was only 0% and 11.22%. At the sixth to ninth positions, G or C occupied an extremely high percentage. The 6th nucleotide was at the middle of "seed region" of a miRNA, and it was known that when miRNAs perform regulating functions, the "seed region" was the key sequence for base paring to the aimed gene, therefore high percentage of (G + C) at the sixth position will induce and enhance the function of a miRNA.

In conclusion, the present study represents the first characterization of *T. saginata* miRNAs, which will help to better understand the complex biology of this zoonotic parasite and to explore effective control methods for diseases caused by this important parasite. The reported data of *T. saginata* miRNAs should provide valuable references for miRNA studies of closed related zoonotic *Taenia* cestodes such as *Taenia solium* and *Taenia asiatica*.

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