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Verification of miRNAs in ginseng decoction by high-throughput sequencing and quantitative real-time PCR

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Abstract

Panax ginseng C. A. Meyer is a precious traditional Chinese medicine that has been clinically used for over thousands of years. In general, ginseng needs to be prepared to ginseng decoction before taking it. MicroRNAs are a class of small (18−24 nt), single-stranded molecules that regulate gene expression at the post-transcriptional level. Considering that ginseng miRNAs may be bioactive compounds, we used Illumina high-throughput sequencing and quantitative real-time PCR (qRT-PCR) to validate the existence of miRNAs in fresh ginseng decoction which have been boiled at high temperature. Our previous studies have demonstrated that there are several miRNAs in fresh ginseng. The roots of fresh *Panax ginseng* were prepared according to routine methods, from which miRNAs were extracted and sequenced. A total of 43 miRNAs were identified from water decoction by Illumina high-throughput sequencing, belonging to 71 miRNA families. The target genes of these miRNAs were predicted by sequencing, and were annotated by GO, KEGG and Nr databases. The functions of these target genes mainly included plant hormone signal transduction, transcription regulation, macromolecular metabolism and auxin signaling. Nine highly expressed miRNAs

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(miR159, miR167, miR396, miR166, miR168, miR156, miR165, miR162 and miR394) were verified by qRT-PCR, and the results of Illumina high-throughput sequencing and qRT-PCR were consistent. Results from this study indicate that miRNAs remained stable in *P. ginseng* after high-temperature boiling. Additionally, Illumina high-throughput sequencing was superior in the acquisition of higher amount of small RNAs.

Keywords: Bioinformatics, Molecular biology

1. Introduction

Panax ginseng C. A. Meyer which belongs to the *Araliaceae* family has been regarded as an important traditional Chinese medicine in north-eastern Asia. It has been used for human health for several millennia. During long-term empirical clinical use, ginseng is mostly prepared by boiling with water into decoctions (water extracts) for oral administration (S. S. Zhou et al., 2016). The presence of small molecules in the decoctions of *Glycyrrhiza uralensis*, *Lonicera japonica* (miR2911) and *Oryza sativa* (miR168a) has been well-proved (Shao et al., 2015; Zhang et al., 2012; Z. Zhou et al., 2015). Whether miRNAs exist in ginseng decoction is unknown, so studies on the molecular level are of great significance to the innovation of ginseng.

MiRNAs are endogenous non-protein coding RNAs with the lengths of approximately 22 nucleotides (nt), which negatively regulate gene expressions by complementarily binding the ORF or UTR regions of target messenger RNAs(Liang et al., 2010). They are widespread in animals and plants, being more stable *in vivo* (Ludwig et al., 2017). MiRNAs can be detected with a variety of technologies, including quantitative PCR, microarrays, solution-based hybridization, etc. (Roberts et al., 2015). Due to satisfactory technical performance, high-throughput sequencing is also a powerful method for the discovery and quantification of miRNAs(Alon et al., 2011). The aim of this study was to detect miRNAs in ginseng decoction by high-throughput sequencing, and to confirm the results by quantitative real-time PCR (qRT-PCR). The findings provide a theoretical basis for molecular pharmacology studies of ginseng.

2. Materials and methods

2.1. Preparation of plant materials

Six-year-old fresh ginseng plant originating from China Changbai Mountain (Jilin Province, China) was purchased from Qingping Chinese Herbal Medicine Market. The plant was washed by tap water to remove sticky soil, sterilized with 70% ethanol solution for 1 min, washed with sterile water, and dried on disinfected filter paper.

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Afterwards, fresh ginseng root (30 g) was chopped, added 8 times the amount of water, soaked for 30 min, boiled at 85 °C for 2 h, and filtered. Then the residue was added 8 times the amount of water and filtered. The two filtrates were combined, appropriately concentrated, and finally stored in a −80 °C refrigerator.

2.2. Small RNA library construction

MiRNAs were isolated from collected seedlings by miRNA kit (Bioteke, China) following the manufacturer's instructions. Polyacrylamide gel electrophoresis was used to enrich RNA molecules with the sizes of 18−30 nt. Then 3′ adapters were added and 36−44 nt RNAs were enriched, to which 5′ adapters were then ligated. The ligated products were reversely transcribed by PCR amplification, and 140-160bp PCR products were enriched to generate a cDNA library which was thereafter sequenced by using Illumina HiSeq^{TM} 2500 (Gene Denovo Biotechnology Co., Guangzhou, China).

2.3. Identification of known miRNAs in ginseng decoction

Reads obtained from sequencing included dirty reads containing adapters or low-quality bases. Dirty reads affected subsequent assembly and analysis. Thus, raw reads were further filtered according to the following rules:(i) removal of low-quality reads containing more than one low-quality (Q-value≤20) base or unknown nucleotides(N); (ii)removal of reads without 3′adapters or containing 5′adapters; (iii) removal of reads containing 3′ and 5′ adapters but no small RNA fragment between them; (iv)removal of reads containing ploy A in small RNA fragment; (v)removal of reads shorter than 18nt (not including adapters). All clean tags were aligned with small RNAs in the GenBank database (Release 209.0) to identify and to remove rRNA, scRNA, snoRNA, snRNA and tRNA. All the clean tags were also aligned with reference genome. These tags were removed because those mapped to exons or introns may be fragments from mRNA degradation. Moreover, the tags mapped to repeat sequences were also removed (Kozomara and Griffiths-Jones, 2014).

2.4. Identification of novel miRNAs in ginseng decoction

All clean tags were then searched against the miRBase database (Release 21.0; all plant species database) to identify known miRNAs (existing miRNAs). So far, the miRNA sequences of some species have not been included in the miRBase database. For these species, known miRNAs were identified through alignment with those of other species. All unannotated tags were aligned with reference genome. According to the genome positions and hairpin structures predicted by Mireap_v0.2 software, novel miRNA candidates were identified. After tags were annotated as mentioned previously, the annotation results were determined following a descending order

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of rRNA > existing miRNA > existing miRNA edit > known miRNA > repeat > exon > novel miRNA > intron. The tags that cannot be annotated as any of the above molecules were recorded as unann (Deng et al., 2017; Islam et al., 2015).

2.5. Analysis of miRNAs expression profiles in ginseng decoction

Total miRNA consists of existing miRNA, known miRNA and novel miRNA. The miRNA expression level in each sample was calculated and normalized to transcripts per million (TPM) using the equation below:

$$TPM = \text{Actual miRNA counts/total counts of clean tags} \times 10^6$$

In addition, the expression levels of existing miRNA, known miRNA and novel miRNA were also analyzed individually.

Meanwhile, miRNA families were analyzed to clarify whether the miRNAs existed in other species. The analysis result was marked as "+" or "-" which corresponded to existing or non-existing.

2.6. Prediction of miRNA target genes

Based on the sequences of existing, known and novel miRNAs, the candidate target genes were predicted as follows (Allen et al., 2005; Schwab et al., 2005):(i) no more than four mismatches between small RNA and target (G-Ubases: 0 to 5mismatches); (ii) no more than two adjacent mismatches between miRNA and target duplex; (iii) no adjacent mismatches between sites 2 to 12 of miRNA and target duplex (5′ of miRNA); (iv) no mismatches between sites 10−11 of miRNA and target duplex; (v) no more than 2 to 5 mismatches between sites 1 to 12 of miRNA and target duplex (5′ of miRNA); (vi) minimum free energy of miRNA and target duplex should be ≥74% of that of the miRNA bound to its perfect complement.

2.7. qRT-PCR

qRT-PCR was carried out to validate differentially expressed miRNAs and their corresponding target unigenes that were chosen randomly. Based on mature miRNA sequences, forward primers were designed, and reverse primers were frequently used ones. U6 was employed as endogenous control. By using M-MLV reverse transcriptase (Promega, M1701) first-strand cDNAswere synthesized, and amplifications were performed with SYBR Green qPCR Super Mix (Invitrogen, C11744500). For miRNA targets, primers were designed using Beacon Designer version7.7 (Promega, USA), with 18S rRNA as endogenous control gene. Amplifications were performed with SYBR Green Real-time PCR Master Mix (Toyobo, Japan) and ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, USA).

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There lative expression levels of miRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method and compared with those of their targets as described previously. Each sample was tested in triplicate.

3. Results

3.1. Analysis of small RNA library

After raw data were sequenced, low-quality reads were removed, leaving clean reads. Then the fragment types, numbers and percentages of small RNAs in ginseng decoction were analyzed by the Solexa system (Table 1). Additionally, the types and numbers of small RNAs were calculated and their distribution was analyzed statistically (Fig. 1).

The small RNAs of different samples can be compared based on the length distributions of clean reads. In general, the length distribution of plant sample has a peak at 21/25bp. In some cases, such as viral infection and drug therapy, samples are more prone to mRNA degradation, generating more fragments that render the peak abnormal. Probably, the samples *per se* underwent RNA degradation after being decocted at high temperature.

3.2. Identification of known miRNAs

To identify known miRNAs in ginseng decoction, we compared our database with the miRNA precursors in the plant miRNA database (Release 21.0) to construct an expression profile (Lang et al., 2011; Yin et al., 2008). We obtained 29 miRNAs from ginseng decoction, and identified the secondary structures of 5 of them (Table 2) which were expressed in most plant species. At the same time, we analyzed the family of these miRNAs to explore its species belonging to other species,

Table 1. Fragment types, numbers and percent ages of small RNAs in ginseng decoction.

Fragment type	Number	Percentage
Clean reads	11692788	100%
High quality	10902997	93.2455%
3′adapter_null	21900	0.2009%
Insert null	238881	2.1910%
5′adapter contaminants	100218	0.9192%
Smaller than 18nt	2660169	24.3985%
polyA	45	0.0004%
low cutoff	165172	1.5149%
clean_tags	7716612	70.7751%

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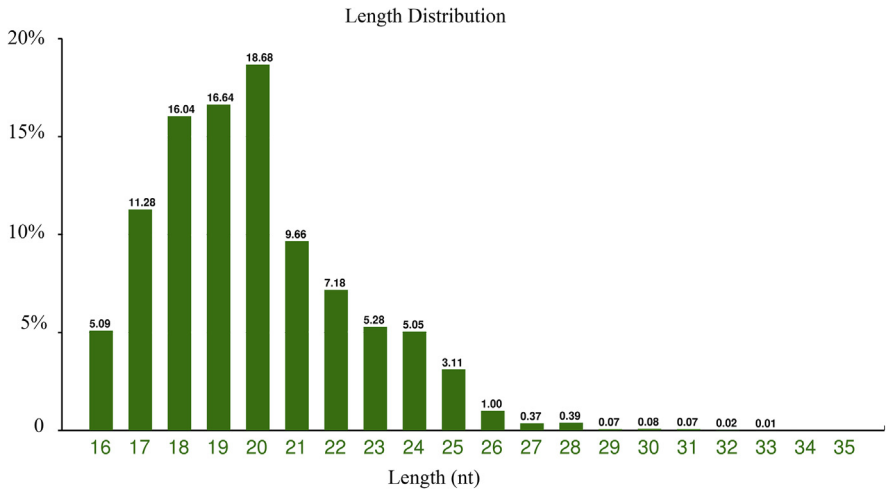

Fig. 1. Length distributions of small RNAs in ginseng decoction.

including *O. sativa*, *Arabidopsis thaliana*, *Populus tremula*, *Triticum aestivum*, *Solanum lycopersicum*, *Brassica oleracea*, *Saccharum officinarum*, *Vitis vinifera*, *Zea mays*, *Sorghum bicolor*, *Gossypium hirsutum*, *Glycine max* and other 23 species (Fig. 2). Comparing the miRNA sequences obtained from ginseng with conserved miRNAs in 23 plant species revealed that miR166, miR167 and miR159 existed in multiple plant families, indicating that they were widespread in plants.

3.3. Identification of novel miRNAs

The principle of prediction is that the upstream of hairpin structure is highly conserved and located on the two arms of such structure (Ge et al., 2013; Tsai et al., 2014). A conserved restriction site generally does not offset the lower folding free energy of the hairpin precursor. The precursor of landmark hairpin structure as well as highly conserved mature plant sequences were searched against a related nucleic acid sequence database using the homologous search alignment method. Intercepting a certain length of the genome sequence alignment revealed its secondary structure, enzyme site information, energy and other characteristics.

Table 2. Identification of known miRNAs from ginseng decoction.

Mature id	Hair pin id	Mature arm	Hair pin energe (kcal/mol)	Hair pin MFEI	Mature seq	Mature length (nt)
miR166-y	m0001	3p	−41.4	0.81	UCGGACCAGGCUUCAUUCCCC	21
miR167-x	m0002	5p	−64.9	1	UGAAGCUGCCAGCAUGAUCUA	21
miR319-y	m0003	3p	−82.6	0.94	UUGGACUGAAGGGAGCUCCCU	21
miR166-y	m0004	3p	−52	0.63	UCGGACCAGGCUUCAUUCCCC	21
miR159-y	m0005	3p	−79	0.95	UUUGGAUUGAAGGGAGCUCUA	21

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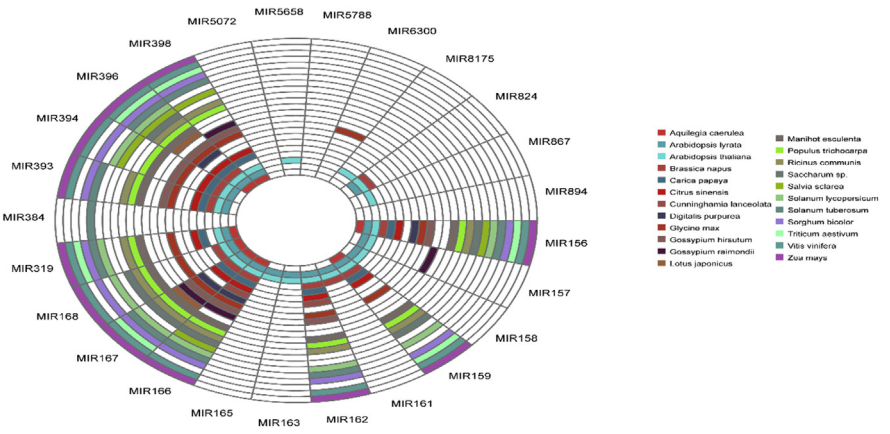

Fig. 2. Circular heat map of conserved miRNAs identified in ginseng among different model plants. Each color represents a plant species and white color represents the absence of miRNA. MiRNA that was found in at least 9 plants was considered as conserved.

To predict and to identify miRNAs newly discovered in ginseng decoction, we compared the genomes of plants with databases using Mireap_v0.2 software, and predicted the specific secondary structures of miRNAs to explore the possible existence of novel ones (He et al., 2015; Wan et al., 2012a; Wan et al., 2012b; Xu et al., 2015). We obtained a total of 84 sequences, 24 of which were aligned to the genome. Eventually, the specific structures of only 14 miRNAs were determined. As predicted by Mireap_v0.2 software, these 14 novel miRNAs were mostly 21 or 22 nt in length. The information of seven novel miRNAs is listed in Table 3.

3.4. Identified target genes of miRNAs

We performed complementary pairing of small RNAs and target genes by using PatMatch_v1.2 software, and then predicted the final results by programmed screening (Yan et al., 2005). Software prediction showed that 38 miRNAs in ginseng decoction corresponded to 2,238 target gene loci. Subsequently, all the predicted target genes

Table 3. Information of seven novel miRNAs.

Mature id	Hairpin id	Hairpin energe (kcal/mol)	Hairpin MFEI	Mature seq	Mature length (nt)
novel-m0001-3p	novel-m0001	−35.1	0.68	CCCGCCGGGGCGGGGGUGCCG	21
novel-m0003-5p	novel-m0003	−69.8	0.68	GAGGGAGAGGGAGAGGGAGAGA	22
novel-m0005-3p	novel-m0005	−61.6	0.5	GUGGUGGUGGUGGUGGUGGUGG	22
novel-m0010-3p	novel-m0010	−27.2	0.56	AACGGAUCGCCCACUUCCUAC	21
novel-m0010-5p	novel-m0010	−27.2	0.56	GGGAAUCGUGGGUGCCGUAGA	21
novel-m0011-3p	novel-m0011	−107.4	0.66	GUGGUGGUGGUGGUGGUGGUGG	22
novel-m0012-5p	novel-m0012	−37.8	0.65	GCGCGGUAGCGUUCGUGGUGG	21

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were enriched by GO analysis to study the biological functions of all miRNAs after boiling at high temperature (Fig. 3). These target genes mainly involved intracellular material formation and pathways (formation of intracellular membranes, formation of cytoplasmic proton transport channels, formation of the cell membrane system, etc.), signal transduction, macromolecular metabolism and auxin signaling pathways. These biological functions participated in the entire growth and development processes of plants, playing important roles in energy metabolism and regulation.

3.5. Validation of miRNAs and their target genes by qRT-PCR

Totally, we verified the expressions of 9 miRNAs retrieved from the database by qRT-PCR (Table 4, Fig. 4). The miRNA expressions of control group (fresh ginseng) and treatment group (ginseng decoction) were negatively correlated, which both decreased. Compared with the control group, the expression levels of target genes comp63622, comp59886, comp63996, comp63909 and comp47306 in the treatment group increased, whereas those of comp62398, comp64090, comp58543 and comp50760 decreased.

4. Discussion

Since the first report, miRNAs have been found to widely exist in the nature, and miRNAs also commonly in Chinese herbal plants. For example, Yang et al. found a large number of miRNAs in *Rehmannia glutinosa*, extracted them from roots, stems and leaves, and identified them by high-throughput sequencing (Yang et al., 2011). The small RNA database of ginseng root, stem, leaf and flower has been constructed using high-throughput sequencing, confirming the abundance of such RNAs(Arata et al., 2012; Wu et al., 2012).

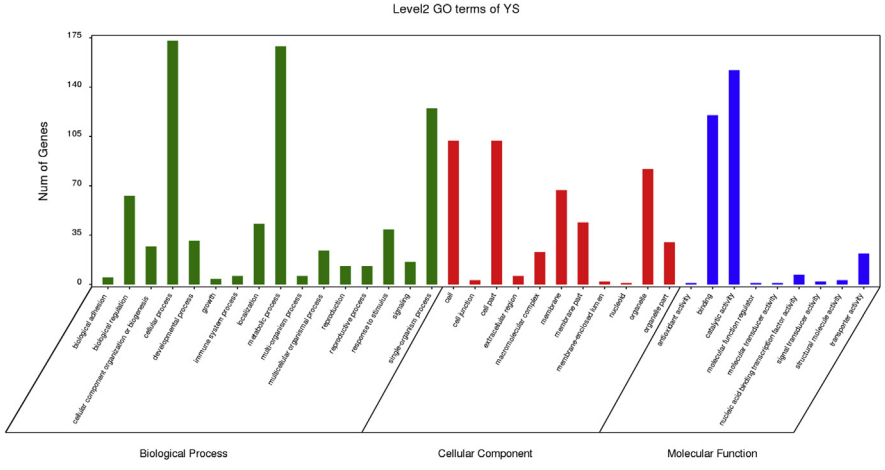

Fig. 3. GO enrichment analysis of all predicted target genes in ginseng.

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Table 4. Primers of miRNAs of ginseng decoction used in the study.

miRNA	Sequence
miR159-F	5′ ACACTCCAGCTGGGTTTGGATTGAAGGGAGCT
miR159-RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTAGAGCTC
miR167-F	5′ ACACTCCAGCTGGGTGAAGCTGCCAGCATGAT
miR167-RT	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTAGATCAT
miR396-F	5′ ACACTCCAGCTGGGTTCCACAGCTTTCTTGAA
miR396-RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAGTTCAA
miR166-F:	5′ ACACTCCAGCTGGGTCGGACCAGGCTTCATTC
miR166-RF:	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGGAATG
miR168-F:	5′ ACACTCCAGCTGGGTCGCTTGGTGCAGGTCGG
miR168-RT	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCCCGAC
miR156-F:	5′ ACACTCCAGCTGGGTTGACAGAAGAGAGTGAG
miR156-RT:	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGCTCAC
miR165-F:	5′ ACACTCCAGCTGGGTCGGACCAGGCTTCATCC
miR165-RT	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGGGATG
miR162-F:	5′ ACACTCCAGCTGGGTCGATAAACCTCTGCATC
miR162-RT	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTGGATGC
miR394-F:	5′ ACACTCCAGCTGGGTTGGCATTCTGTCCACC
miR394-RT:	5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGAGGTGG
miRNA-R	5′ CTCAACTGGTGTCGTGGA
U6-F:	5′ CTCGCTTCGGCAGCACA
U6-R:	5′ AACGCTTCACGAATTTGCGT
U6-RT:	5′ AACGCTTCACGAATTTGCGT

As a class of small, endogenous, single-stranded RNAs with the lengths of about 18−24 nt, miRNAs primarily regulate eukaryotic cells at the post-transcriptional level (Bartel, 2004). MiRNAs dominate in the growth and development of the body, metabolism and diseases.

Researchers have endeavored to identify miRNAs and then to study their functions. Bioinformatics analysis combined with high-throughput sequencing is one of the most effective methods for identifying miRNAs and predicting novel ones. Considerable miRNA sequences can be analyzed by high-throughput sequencing at once, and bioinformatics and software analyses allow large-scale prediction and identification of miRNAs(Xu et al., 2015).

MiRNAs in ginseng decoction were herein identified by high-throughput sequencing for the first time, and five known miRNAs and seven novel miRNAs were obtained by database comparison. Nine miRNAs and their target genes were selected for qRT-PCR, giving consistent results.

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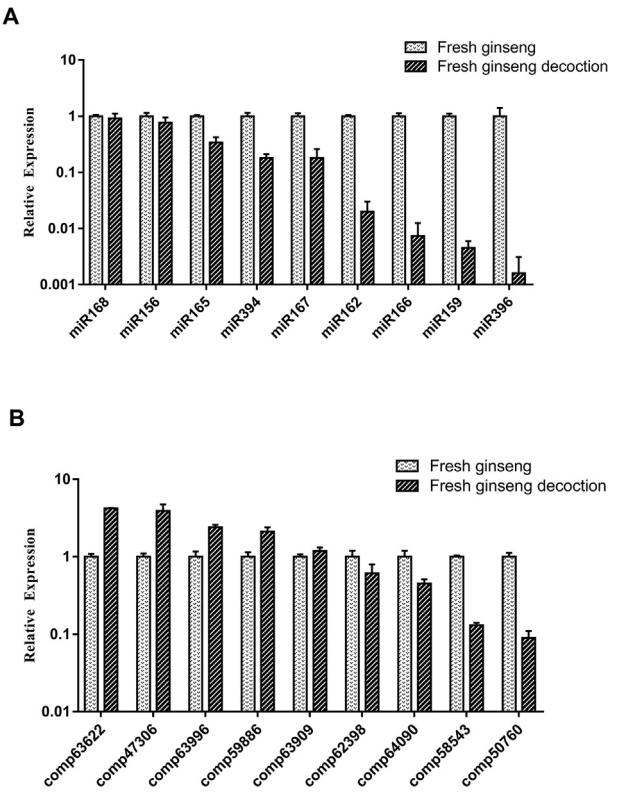

Fig. 4. Expressions of 9 miRNAs(A) and target genes(B).

Ginseng is a rare Chinese herbal medicine with a long history of clinical application. It was reported that *Panax ginseng* and its active constituents has been used for various diseases and proven its great efficacy in managing neurodegenerative diseases (Rajabian et al., 2019), cancer (Li and Qi, 2019), and immune-modulation (Riaz et al., 2019), ect. As many TCMs are hepatotoxic, ginseng, especially ginsenosides, is widely used in the adjunctive therapy of the liver diseases. The hepatoprotective mechanisms of ginsenosides, such as anti-oxidation (Ning et al., 2018), anti-inflammation and effect on drug metabolism (Sun et al., 2019) in liver, have been well studies.

The existing research on the effective material basis of ginseng mostly focuses on ginsenosides, ginseng polysaccharides and other components. However, it cannot fully reveal the complex clinical effects of ginseng. This paper studies ginseng, high-throughput sequencing and qRT-PCR from the perspective of miRNAs, which confirm the existence of miRNAs in ginseng decoction. What needs to be further discussed is whether miRNAs in ginseng decoction can enter the body and play a role. The content can be found in the master's thesis (Wang, 2018) partly. The study of honeysuckle gives inspiration (Zhou et al., 2015), which will provide reference for ginseng research.

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Declarations

Author contribution statement

Yingfang Wang: Conceived and designed the experiments, Wrote the paper.

Zemin Yang: Conceived and designed the experiments.

Mengyuan Peng, Wenjuan Wang: Analysed and interpreted the dara; Performed the experiments.

Zhihua He: performed the experiments.

Yanlin Chen, Jingjing Cao, Zhiyun Lin, Mengjuan Gong, Yongqin Yin: contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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