




Sequencing and analysis of micro RNAs in camel milk exosomes

RINILE WU^{1,2}, CAIFENG YUE^{1,2}, YINA XIE^{1,2},
YUERONG FU^{1,2}, XIN YU^{1,2}, ZHUOYUAN LI^{1,2} and
BIN YANG^{1,2*} 

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¹ College of Veterinary Medicine, Inner Mongolia Agricultural University, Hohhot, 010011, China

² Key Laboratory of Clinical Diagnosis and Treatment Technology in Animal Disease, Ministry of Agriculture, Hohhot, 010011, China

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SHORT COMMUNICATION



ABSTRACT

In order to study the species and functions of micro (mi)RNAs in the exosomes of camel milk, non-coding small (s)RNAs were sequenced and identified by Illumina sequencing technology, and the miRNA fraction was analysed by bioinformatics. After quality control, the average length of sRNA of camel milk exosomes was 18–24 nucleotides. A total of 2,659 miRNAs were identified, including 2,458 known, and 201 new miRNAs. Among the known miRNAs, miR-148a and let-7i had the highest expression levels. The results of gene ontology enrichment analysis indicated that the target genes of camel milk exosome miRNAs were involved in multicellular organismal, catabolic and other biological processes. They play role in the extracellular region, in the cytoskeleton and other cell components, in protein binding, but also have structural molecule activity and other molecular functions. According to the results of the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, the target genes of camel milk exosome miRNAs are involved in Alzheimer's disease, non-alcoholic fatty liver disease, *Staphylococcus aureus* infection and other pathological pathways. We speculate that the reported beneficial effect of camel milk in various pathologic conditions may be closely related to the regulatory function of the exosomal miRNAs exerted on target genes of the diseases.

KEYWORDS

camel milk, exosomes, miRNAs

Milk is one of the important food sources for human beings. Camel milk has attracted much attention because it contains high nutritional value and a variety of active functional ingredients (Shakeel et al., 2022). Many beneficial or even therapeutic effects have been attributed to camel milk, including antibacterial, antioxidant, anti-cancer, anti-inflammatory, immunomodulatory and hypoglycaemic effects (Behrouz et al., 2022).

Milk exosomes contain proteins, lipids, mRNAs, microRNAs (miRNAs), circular RNAs (circRNAs) and long non-coding (lnc)RNAs. Milk exosomes and their miRNAs can enter the systemic circulation and regulate gene expression in peripheral tissue cells (Melnik et al., 2021). MicroRNAs in human (Zhou et al., 2012), bovine (Yun et al., 2021) and porcine milk exosomes have been sequenced and identified (Gu et al., 2012). However, the types and functions of miRNAs in camel milk exosomes have not been reported. In this study, the sequence analysis of small (s)RNA in camel milk exosomes was helpful for the cognition of the miRNA types and functions of camel milk exosomes.

Fresh camel milk samples were collected individually from female Bactrian camels (*Camelus bactrianus*) at mid lactation period. The exosomes of camel milk were extracted by an exosome purification kit (Umibio, Science and Technology Group, Shanghai, China). Four sRNA libraries were constructed, and sequenced on a HiSeq 2,500 sequencing system (Illumina, San Diego, CA, USA) with 50-bp single-end reads.

Since there was no information regarding camel miRNA in the miRBase, a closely related species, the cattle was selected as the reference for the known miRNA information for camel

*Corresponding author.
Tel.: +8604714309196.
E-mail: yangbin@imau.edu.cn

milk exosomes. Mapped sRNA tags were used to search for known miRNA. The available software miREvo and miR-Deep2 were integrated to predict novel miRNA. Target genes of miRNAs were predicted by the miRanda algorithm. Gene Ontology (GO) enrichment analysis was used on the target gene candidates of differentially expressed miRNAs. To test the statistical enrichment of the target gene candidates in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, the KEGG Orthology-Based Annotation System (KOBAS) software was used.

In the four sequenced libraries, raw reads of 10332445, 10211088, 10283477 and 10203516, respectively, were identified in the exosomes of camel milk from the four samples. After quality control, the clean reads were 9731305 (94.18%), 9122333 (89.34%), 9422731 (91.63%) and 9139881 (89.58%), respectively (Table 1). The sequence length of sRNAs of camel milk exosomes was mainly distributed from 18 to 24 nucleotides (nt), with 22 nt accounting for the most of them.

The numbers of sRNA aligned to the reference sequence of the four samples were 7722899, 7102696, 6152393 and 5573984, respectively. The sRNA sequences contained miRNA (including known and novel miRNAs), ribosomal (r)RNA, transfer (t)RNA, small nuclear (sn)RNA, small nucleolar (sno)RNA, as well as repeat, exon, intron and other RNAs. Known miRNAs were the most abundant (Table 2).

The expression levels of miRNAs in the four samples were different, among which 6 miRNAs ranked among the top ten in each sample. These were miR-148a, let-7i, miR-200a, let-7b, miR-3596 and miR-26a (Table 3).

The results of the GO enrichment analysis of target genes of miRNAs showed that the largest number of enriched target genes are involved in multicellular organismal and catabolic processes. They also function in the extracellular region, in the cytoskeleton and other cell components with protein binding and structural molecule activity.

The target genes of miRNAs were enriched in 265 pathways. Among the top 20 pathways with Rich factor value, were the Alzheimer’s disease, non-alcoholic fatty liver disease (NAFLD) and *Staphylococcus aureus* infection pathways.

Among the most expressed miRNA species was miR-148a, followed by let-7i in all the four libraries. This study was consistent with the results of previous studies, in which also the highest expression of miR-148a has been found in human (Zhou et al., 2012), bovine (Chen et al., 2020) and porcine (Gu et al., 2012) milk exosomes. In this study, 9 let-7 family members were detected in camel milk exosomes, including let-7i, let-7b, let-7f, let-7g, let-7a-5p, let-7c, let-7d, let-7a-3p and let-7e, among them, the expression of let-7i was the highest. There are more let-7 family members in exosomes from camel milk than those reported from cow milk: let-7i and let-7e (Izumi et al., 2015).

Some highly expressed miRNAs in the exosomes of camel milk are closely related to neoplastic diseases. For example, miR-148a has been found to inhibit the proliferation and migration of glioblastoma (Xu et al., 2019), esophageal cancer (Wang et al., 2019) and colorectal cancer (Zhao et al., 2019). Members of the let-7 family have been described to be conserved in various host species (Su et al., 2012) and to be involved in anti-inflammatory and

Table 1. Summary of raw sequence data from camel milk exosomes

Sample	Reads	Bases	Error rate	Q20	Q30	GC content	Clean reads
Camel1	10332445	0.517G	0.01%	99.25%	97.29%	50.01%	9731305 (94.18%)
Camel2	10211088	0.511G	0.01%	98.64%	95.92%	50.63%	9122333 (89.34%)
Camel3	10283477	0.514G	0.01%	99.18%	97.29%	52.08%	9422731 (91.63%)
Camel4	10203516	0.510G	0.01%	99.07%	96.93%	52.81%	9139881 (89.58%)

Table 2. Classification and annotation of small RNAs from camel milk exosomes

Types	Camel1	Camel1 (percent)	Camel2	Camel2 (percent)	Camel3	Camel3 (percent)	Camel4	Camel4 (percent)
Total	7722899	100.00%	7102696	100.00%	6152393	100.00%	5573984	100.00%
Known miRNA	4800165	62.15%	4101531	57.75%	2784704	45.26%	2007719	36.02%
rRNA*	518326	6.71%	486126	6.84%	630940	10.26%	658844	11.82%
tRNA*	99240	1.29%	187249	2.64%	652689	10.61%	999479	17.93%
snRNA*	865	0.01%	989	0.01%	1270	0.02%	1209	0.02%
snoRNA*	17386	0.23%	17909	0.25%	17104	0.28%	17217	0.31%
Repeat	34350	0.44%	37604	0.53%	36607	0.60%	31204	0.56%
Novel miRNA	68397	0.89%	35198	0.50%	27499	0.45%	20716	0.37%
Exon	80768	1.05%	96942	1.36%	141919	2.31%	91439	1.64%
Intron	28700	0.37%	26871	0.38%	56292	0.91%	65083	1.17%
Other	2074702	26.86%	2112277	29.74%	1803369	29.31%	1681074	30.16%

*Abbreviations: r: ribosomal; t: transfer; sn: small nuclear; sno: small nucleolar



Table 3. Top 10 most highly expressed miRNAs in camel milk exosomes

sRNA readcount-1	Camel1 readcount	sRNA readcount-2	Camel2 readcount	sRNA readcount-3	Camel3 readcount	sRNA readcount-4	Camel4 readcount
miR-148a	2424865	miR-148a	2217849	miR-148a	1404625	miR-148a	797136
let-7i	488639	let-7i	326794	let-7i	302373	let-7i	199317
miR-30a-5p	263554	miR-200a	151570	miR-200c	131920	let-7b	173772
miR-200a	256635	let-7b	149404	miR-200a	118692	miR-3596	173772
miR-21-5p	188656	miR-3596	149404	let-7b	98650	miR-200c	101205
miR-148d	170446	miR-200c	142913	miR-3596	98650	miR-200a	92097
miR-99a-5p	129917	miR-99a-5p	125654	miR-26a	78672	miR-26a	64609
let-7b	113672	miR-21-5p	123288	miR-26c	78672	miR-26c	64600
miR-3596	113672	miR-26a	119071	miR-30d	72209	miR-30a-5p	63900
miR-26a	112311	miR-26c	119060	miR-99a-5p	67975	miR-30d	59500

anti-cancer activities (Bernstein et al., 2021). The miR-200a has been shown to inhibit epithelial-mesenchymal transition and tumor growth (Klicka et al., 2022). Several studies have reported that miR-26a has the effect of tumor suppression (Gao et al., 2020; Guo and Tian, 2020; Wei et al., 2020). Camel milk and its exosomes have been reported to play an anti-cancer role possibly by inducing apoptosis and inhibiting oxidative stress and inflammation (Badawy et al., 2018). We speculated that let-7i, let-7b, miR-200a and miR-26a in camel milk exosomes might have anti-inflammatory and anti-cancer effects.

The target genes of miRNAs in camel milk exosomes are enriched in a variety of diseases, such as Alzheimer’s disease, NAFLD, rheumatoid arthritis, type II diabetes mellitus, Huntington’s disease, Parkinson’s disease, inflammatory bowel disease and cancer. Previous reports have attributed a certain effect to camel milk in the treatment of some diseases, such as Alzheimer’s disease (Abdulkadir et al., 2021), NAFLD (Korish and Arafah, 2013), rheumatoid arthritis (Arab et al., 2017), diabetes (Hussain et al., 2021) and cancer (Al Nohair, 2021; Khan et al., 2021). We speculate that in these cases, the beneficial effect of camel milk may be closely related to the possible regulatory function of miRNAs in the exosomes on target genes of the diseases.

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