

Exosomal microRNAs in Human Breast Milk: Potential Effect on Neonatal Breast Milk Jaundice

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Abstract

Background: The pathogenesis of breast milk jaundice (BMJ) remains unrevealed. While UGT1A1 gene has been extensively investigated in neonatal BMJ cases, the reason for down-regulation of UGT1A1 gene in neonatal BMJ has not been completely elucidated. In this research, the authors attempt to speculate whether there was some gene regulatory substance exist in human milk and result in BMJ, such as miRNA. This research aims to demonstrate the association between the profile of exosomal miRNA in human milk and the occurrence of neonate breast milk jaundice.

Methods: A previous study conducted by Shanghai Children's Medical Center of the Shanghai Jiao Tong University School of Medicine and the UIB International Maternity Care Center regarding 12 mother-infant dyads were recruited from September 2016 to December 2016. The subjects were divided into two groups (BMJ and control) for exosomal miRNA screening. Four methods of transmission electron microscopy (TEM), a nanoparticle tracking analyzer (NTA), flow cytometry (FCM), and Western blotting were used to identify the exosome in human milk. Based on the previous study, this research determines the expression profile of miRNA in human milk exosomes by small RNA sequencing. Based on the biological information analysis, the authors not only screen the differentially expressed miRNA but also predict the target genes. Then, another 20 mother-infant dyads were recruited for realtime PCR assay to verify the difference of predicted microRNAs expression in breast milk exosomes and to explore the correlation between differentially expressed microRNAs in breast milk and neonatal breast milk jaundice.

Results: Human milk exosomes are rich in various types of microRNA, especially let-7g-5p, let-7b-5p, has-miR-21-5p, has-miR-375, has-miR-99a-5p, et al. The predicted target genes of miR-127-3p are statistically significantly overexpressed in BMJ group, including the gene of UGT1A1 which expresses key enzyme in process of bilirubin metabolism.

Conclusions: Exosomal miRNA-127-3p plays a potential effect on neonatal breast milk jaundice.

Background

Known as breast milk jaundice (BMJ), the prolonged unconjugated hyperbilirubinemia in infants that develops after 4–7 days of life associated with breast milk feeding is a common pediatric condition [1]. BMJ is distinguished from breastfeeding jaundice, which occurs in the first week after birth and is caused by insufficient intake [2]. Even though two conditions may overlap at the same patient, infants with BMJ are otherwise healthy, with normal weight gain, normal stool and urine output, as well as a normal physical examination [1]. In many cases, BMJ is a benign and self-limiting condition. Although exceedingly rare, bilirubin encephalopathy can occur in apparently healthy, full-term, breast-fed newborns diagnosed with BMJ, especially the infants with serum bilirubin level exceeds 342 mmol/L (20 mg/dL) [3, 4] which may cause irreversible neurological damage [5]. As of now, there is no reliable method for neither BMJ diagnosis nor early identification of bilirubin encephalopathy at initial stages of neonatal period [3].

Therefore, it is critically important and highly necessary to investigate the reason for higher bilirubin level in some neonates with BMJ.

At present, the etiology of BMJ still remains unknown. The cause of BMJ has been a subject of numerous investigations based on the assumption that either a factor in the breast milk itself or in the neonate could contribute to the observed clinical presentation. Nevertheless, some evidence suggests that BMJ is caused by combinations of genetic and environmental factors [4]. A polymorphic mutation (G71R) of bilirubin UDP-glucuronosyltransferase (UGT1A1) has been identified as a cause of BMJ, but the responsible components in breast milk remain unknown. OATP is involved in the absorption of free bilirubin by liver

cells and MRP is involved in the release of conjugated bilirubin [6]. Several breast milk components have been considered to be possibly associated with BMJ, such as pregnane-3 β , 20 α -diol [4], fatty acids [7, 8], β -glucuronidase [9], cytokines, and the epidermal growth factor (EGF). However, the effect of the exact role of the specific components in breast milk and their interaction with the genetic background on BMJ is currently unclear.

Human milk (HM) is optimal source of nutrition for infants as it contains various nutrients and bioactive factors that are necessary for infants health and development [10]. The bioactive components in human milk including secretory immunoglobulin A (sIgA), lactoferrin, oligosaccharide, the epidermal growth factor, and microRNAs, protect infants from infection and inflammation as well as make contribution to their immune maturation, organ development, and healthy microbial colonization [10–12].

MicroRNAs (miRNAs) are short endogenous non-coding RNAs that regulate gene expression at post-transcriptional level by binding to an mRNA target to either inhibit the translation of mRNA into protein and/or promote its degradation [13]. Studies have shown that miRNA derived from food could be absorbed in the gastrointestinal tract [14–16]. HM is rich in microRNA, and the exosomes in HM can protect microRNAs from digestion in the gastrointestinal tract [17]. Exosomes are micro-vesicles produced by almost all cell types that may serve as a critical inter-cell communication messenger [18].

Therefore, the objective of this research is to investigate the association between human milk exosomal microRNAs and neonatal BMJ.

Methods

Preparation of breast milk samples

6 mother-newborn pairs were classified into BMJ group if newborns were clinically diagnosed with BMJ and another 6 pairs of healthy newborns were allocated into control group. None of the recruited newborns had the *UGT1A1* mutation. A volume of 10 mL of human milk were collected from each mother. The milk samples were centrifuged at 2,000 \times g for 15 min at 4 $^{\circ}$ C to remove cells and fat globules. Then, the supernatants were centrifuged at 12,000 \times g for 30 min and further filtered through a

0.22-um PVDF filter to eliminate cells and cellular debris. Finally, the supernatants were stored at -80 °C until exosome enrichment and RNA extraction.

Preparation of exosome

Approximately 4 mL of the supernatant was next utilized for exosome preparation, using an exoRNeasy Serum/Plasma Maxi Kit (QIAGEN GmbH; Hilden, Germany)) as specified in the manufacturer's protocol.

Transmission electron microscopy (TEM)

The prepared exosome samples were placed in contact with a support membrane of copper mesh grids for 3–5 min. The grids were then air-dried for a few minutes. Further, the grids were stained with 3% phosphotungstic acid and dried for several minutes under a filament lamp. Finally, a transmission electron microscope JEM1230 (JEOL, Tokyo, Japan) was used to visualize the grids and take photos.

Nanoparticle tracking technology (NTA)

After dilution in PBS, the exosome size and concentration were measured by nanoparticle tracking analysis (NTA) using ZETASIZER Nano series-Nano-ZS (Malvern Instruments Ltd., Malvern, UK) based on the Stokes-Einstein principle.

Western blot analysis

Twenty micrograms of protein were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (GE Healthcare). The membranes were then blocked and incubated overnight with either a mouse anti-CD63 (1:1,000, Abcam) or rabbit anti-calnexin antibody (1:1,000, Novus). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG was used as a secondary antibody (diluted 1:2,000 in PBST). The bands obtained were scanned using ImageQuant LAS 4,000 mini densitometer (GE Healthcare Life Sciences).

Flow cytometry (FCM)

For the FCM analysis, 30 mg of exosomes (or 30 mg of FCS proteins as negative control) were incubated with 10 uL of 4-um diameter aldehyde/sulfate latex beads (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at room temperature in a final volume of 30–100 uL, followed by 2 h of gentle shaking in 1 mL PBS. The reaction was terminated by incubation for 30 min in 100 mM glycine. Exosome- or FCS-coated beads were next washed three times in FAM wash (3% FCS and 0.1% NaN₃ in PBS) and resuspended in 500 uL of FCM wash. A volume of 10 uL of coated beads was incubated for 1 h with PE-conjugated CD63 Ab and then washed and analyzed using a FAM Calibur.

Enrichment of exosomal RNA from the breast milk

The stored milk supernatants (3 mL) were filtered through a 0.22-um PVDF filter to eliminate cells and cellular debris. Approximately 2 mL of the supernatant was used for total RNA exosome preparation with

the exoRNeasy Serum/Plasma Maxi Kit (QIAGEN) following the manufacturer's instructions.

Small RNA sequencing

To isolate small RNA from the prepared total RNA, we separated RNA segments of different sizes by PAGE gel, selected the 18–30-nt size range, and recycled. Then, we linked a 5'-adenylated, 3'-blocked single-stranded DNA adapter to the 3' end of the selected small RNAs, and the RT primer was added and crossed to the RNA 3' and excessive free 3' adapter. 5' adaptor was then also linked to 5' end of the product. Finally, reverse extension of the RT primer was performed to synthesize strand cDNA. High-ping polymerase was utilized to amplify cDNA by PCR amplification. The PCR product with a size of 100–120 bp was separated by PAGE gel to eliminate the primer dimers and other byproducts. Further, the BGISEQ-500 sequencer (BGI-Shenzhen, Shenzhen, China) was employed to obtain raw data of small RNA. Raw sequencing data were subjected to data cleaning analysis first, which included elimination of the low-quality tags, 5' adaptor contaminants, from the 50-nt tags, and obtain credible clean tags. Then, the length distribution of the clean tags and common and specific sequences in the samples were established. The standard analysis will annotate the clean tags into different categories and those which cannot be annotated to any category are used to predict the novel miRNA. After obtaining the miRNA results, target prediction for miRNAs and GO enrichment and KEGG pathway for target genes will be analyzed.

Prediction of differential expressed miRNA target genes.

The computational prediction of miRNA targets is a critical initial step in the identification of miRNA:mRNA target interactions for experimental validation. Several software was used to find the possible targets in this research. The intersection targets were identified using appropriate filter conditions such as MFE, and the score was used for further analysis. In this study, the authors used miRanda and TargetScan to predict target.

Realtime PCR of miRNAs whose predicted target genes concluded *UGT1A1*

The milk of another 20 mother-newborn pairs, including BMJ group (n=10) and control group (n=10), was used for quantification of differentially expressed miRNAs of which the predicted target genes concluded *UGT1A1*. This study used the method of realtime PCR of pre-amplified miRNA (Table3-6 in supplement). Reverse transcription was performed using the miScript PCR Starter Kit (Qiagen Hilden, Germany), and then the reverse transcribed cDNA was diluted 5-fold. The pre-amplification system was prepared using a miScript PreAMP PCR Kit (Qiagen Hilden, Germany). The pre-amplified cDNA was diluted 20-fold with RNase-free water, gently mixed and placed on ice. After preparing a real-time PCR system, add 9 µl of the above mixture and 1 µl of cDNA to the quantitative tube, then cover the tube, mix gently, centrifuge briefly and prepared for realtime PCR.

Data analysis

The target miRNA and the external reference of each sample were simultaneously subjected to Realtime PCR reaction, and the data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Results

Identification of exosomes in human breast milk

Exosomes were isolated from human breast milk by exoRNeasy Serum/Plasma Maxi Kit (QIAGEN, GERMANY) and investigated using nanoparticle tracking technology (NTA) and transmission electron microscopy (TEM). The NTA results show that the diameter of more than 70% of the particles in the prepared exosome samples was within the range 20–200 nm, with a mean diameter of 88.98 nm and a peak value of 167.7 nm (Fig.1A). The ultrastructure of the exosomes in the human breast milk recorded by TEM is illustrated in Fig.1B. As can be seen in the figure, a classic morphological ultrastructure was visible, similar to that of the exosomes derived from human saliva, urine, and in vitro cells. The authors determine the expression of exosome membrane proteins by FACS. Both CD63 and CD81 were positive; 65.2% of the particles were CD63-positive and 87.2% of the particles CD81-positive (Fig. 1C). The results of the Western blotting analysis indicated that CD63 was positive, but calnexin was negative in the exosome samples (Fig.1D). The authors also observed that human milk exosomes contain a considerable number of RNA sequences that are shorter than 100 nt (Fig.2), confirming the abundance of miRNA-loaded exosomes in human breast milk.

Development- and immune-related miRNAs are abundant in human milk exosome

The size of the majority of small RNAs in the breast milk exosomes is between 19–24 nt. The size of 22 nt was predominant, which is basically in line with the length distribution range of the small RNAs in various species. Comparing the results obtained from the miRBase 21 database, this research found out that milk samples of both BMJ and control group include a variety of miRNAs. It is noteworthy that 420 and 417 unique miRNAs were detected in BMJ and control group, respectively. The research findings show that some miRNAs were highly expressed in all samples, such as let-7b-5p, let-7f-5p, hsa-miR-181a-5p, hsa-miR-30b-5p, hsa-miR-200a-3p, hsa-miR-148a-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-375, and has-miR-21-5p. Meanwhile, 322 and 124 unique novel miRNAs were predicted to be present in BMJ and control group, correspondingly.

Exosomal miRNAs in human milk possibly regulate the expression of genes associated with bilirubin metabolism

In Table 1, some significantly upregulated expressed miRNAs in BMJ group compared with those in the control group (Q-value close to 0) were listed. The target genes of miRNA were predicted by mirdbV5 and TargetScan7.1. Additionally, the target genes were analyzed by GO enrichment and KEGG pathway annotation. The GO entries of significant enrichment include molecular functions (MF), biological processes (BP), and cell components (CC). The GO analysis show that the target genes of miRNA were

significantly differentially expressed among two groups, which were mainly related to cellular process, metabolic process, immune system process. (Fig.4)

In this research, the authors found that hsa-miR-206, hsa-miR-224-5p, hsa-miR-127-3p, hsa-miR-409-3p, hsa-miR-574-3p, hsa-miR-431-5p and hsa-miR-138-2-3p were more upregulated in BMJ group compare to control group, and their target genes were predicted to include UGT1A1. In addition, Organic anion transport peptide (OATP) was also the target gene of hsa-miR-206, hsa-miR-431-5p, and hsa-miR-138-2-3p. In BMJ group, hsa-miR-128-3p and hsa-miR-4516 were also overexpressed. The prediction indicates that the target genes of hsa-miR-128-3p and hsa-miR-4516 include multidrug resistance are associated with protein 2 (MRP2).

Realtime PCR of five miRNAs whose predicted target genes concluded UGT1A1

In this research, among miRNAs with target genes including UGT1A1, five significantly differentially expressed miRNAs, included hsa-miR-206, hsa-miR-224-5p, hsa-miR-127-3p, hsa-miR-409-3p, and hsa-miR-574-3p, were selected for realtime PCR. The results found that the expression of hsa-miR-127-3p was statistically significantly overexpressed in BMJ group; while the expression of other four miRNAs between two groups had no statistically significant difference (Fig.3).

Discussions

Exosomal miRNAs in human milk are possibly associated with the development and immune regulation of neonates

The findings of the current study showed that certain miRNAs are relatively highly expressed in breast milk, including let-7b-5p, let-7f-5p, hsa-miR-181a-5p, hsa-miR-30b-5p, hsa-miR-200a-3p, hsa-miR-148a-3p, and hsa-miR-146a-5p, which are consistent with results of an earlier report that conducted in 2012 by Zhou [19]. Therefore, these highly expressed miRNAs are associated with the development and immunoregulation of neonates. miR-148a-3p, miR-30b-5p, and miR-200a-3p are considered as immune-related miRNAs [19]. For example, miR-30b-5p promotes cellular invasion and immunosuppression [20], and miR-200a-3p was associated with Hodgkin lymphoma [21]. Evidence in a previous publication shows that miR-148a-3p decreases NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling NF- κ B target gene expression [22]. However, in this research, the authors also found that hsa-miR-26a-5p, hsa-miR-375 and has-miR-21-5p were highly expressed in breast milk exosomes.

Exosomal miRNAs in human milk are possibly associated with the occurrence and development of BMJ in neonates

Neonatal breast milk jaundice often occurs in term infants and is due to the genetic background of neonates and breast milk components. Although the prognosis of most neonates with breast milk jaundice is benign, in some cases, this condition still requires hospitalization while some parents will also possibly stop breast-feeding. Bilirubin encephalopathy or kernicterus may occur in severe breast milk

jaundice infants, which possibly leads to hearing, visual, neurological damage, and even death of newborns [2]. In this research, the authors determine the effect of miRNA in breast milk exosomes on the pathogenesis of neonatal breast milk jaundice.

Uridine diphosphoglucuronyl transferase (UDPGT), OATP, and MRP participate in the complex process of bilirubin removal by liver cells. In addition, OATP1B1 and OATP1B3 are involved in the absorption of free bilirubin by liver cells, where it is later transformed into direct bilirubin (DBIL) through catalysis by UDPGT. Eventually, it is released into the bile drainage by the MRP. The UDPGT is a key enzyme that regulates the metabolism of bilirubin [6]. As can be observed in Table 2, in this investigation, three upregulated miRNAs were established in BMJ group (hsa-miR-206, hsa-miR-431-5p, and hsa-miR-138-2-3p), which were predicted to regulate the expression of the genes UGT1A1 and OATP. The target genes of hsa-miR-128-3p and hsa-miR-4516 include MRP2.

The UDPGT, a key enzyme that regulates the metabolism of bilirubin, is expressed by UGT1A1. UGT1A1 has been identified as a cause of BMJ by many scholars [6]. In this research, the authors found that there are some miRNAs with potential effect on expression of UGT1A1 in human milk exosome and hsa-miR-127-3p overexpressed in the BMJ group. Therefore, the effect of hsa-miR-127-3p on expression of UGT1A1 gene and its mechanism need to be further verified to reveal the pathogenesis of breast milk jaundice.

Conclusions

The present study is a comprehensive survey on levels of exosomal miRNA in human breast milk. Moreover, it was focused on the gene regulation network during the development of neonates. Immune- and development-related endogenous miRNAs were observed to be enriched in the exosomes of the two groups investigated. The authors also established the potential effect of exosomal hsa-miRNA-127-3p on the development of neonatal breast milk jaundice.

List Of Abbreviations

BMJ Breast Milk Jaundic

DBIL Direct Bilirubin

DHA Docosahexaenoic Acid

EVs Exosomes

Fads Fatty Acyl Desaturases

β -GD β -glucuronidase

G6PD Glucose-6-phosphate Dehydrogenase

ISEV Executive Committee of the International Society for Extracellular Vesicles

LA Linoleic Acid

LC-PUFA Long Chain Polyunsaturated Fatty Acid

MCS Multiple cloning site

miRNA microRNA

MRP2 Multidrug Resistance Protein 2

MVBs Multivesicular Bodies

OA Oleinic Acid

OATP Organic Anion Transporting Polypeptide

PPARs Peroxisome Proliferator-Activated Receptors

PUFA Polyunsaturated Fatty Acid

PXR Pregnane X Receptor

TEM Transmission Electron Microscopy

UCB Unconjugated Bilirubin

UDPGT Uridine Diphosphate Glucuronyl Transferase

3'UTR 3' Untranslated Regions 3'

Declarations

Declarations

Ethics approval and consent to participate The study was approved by the ethics committee of Shanghai Children's Medical Center (SCMCIRB-K2018097). Consent was obtained from the mothers and informed consent was signed before the research started.

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: LY collected data and samples, performed analysis, and drafted the initial version of the manuscript. NS carried out data interpretation and reviewed the manuscript. JL had primary responsibility for the study design, data analysis, and interpretation, and reviewed and revised this paper. WS was involved in samples and data collection. RH and YL were involved in the operation of the technology mentioned in this article. XM and LX supervised data collection and were involved in data interpretation. All authors have read and approved the manuscript, and ensured this is the case.

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Tables

Table 1 up-regulated miRNAs of BMJ group. Q value: The P value of each gene was corrected by multiple hypothesis tests, and the difference was twice or more, and the Q-value was less than or equal to 0.001, which was considered to be a significant differentially expressed gene.

miRNA	log2Ratio (BMJ/NC)	P value	Q value
hsa-miR-378f	7.176460312	0	0
hsa-miR-3656	5.834429699	0	0
hsa-miR-486-5p	5.713861414	0	0
hsa-miR-328-3p	5.313411717	0	0
hsa-miR-122-5p	4.834710776	0	0
hsa-miR-128-3p	3.483710778	0	0
hsa-let-7d-3p	3.420995915	0	0
hsa-miR-139-5p	3.360351398	0	0
hsa-miR-451a	2.970189727	0	0
hsa-miR-4532	2.566196373	0	0
hsa-miR-4516	2.447549406	0	0
hsa-miR-206	2.180318634	0	0
hsa-miR-3074-5p	2.12839887	0	0
hsa-miR-24-3p	1.531436553	0	0
hsa-miR-23a-3p	1.514647566	0	0
hsa-miR-27a-3p	1.493679286	0	0
hsa-miR-484	1.40228226	0	0
hsa-miR-4488	1.31999654	0	0

Table 2
miRNAs possibly associated with newborn breast milk jaundice

miRNA	log2Ratio (BMJ/NC)	P value	Q value
hsa-miR-378f	7.176460312	0	0
hsa-miR-3656	5.834429699	0	0
hsa-miR-486-5p	5.713861414	0	0
hsa-miR-328-3p	5.313411717	0	0
hsa-miR-122-5p	4.834710776	0	0
hsa-miR-128-3p	3.483710778	0	0
hsa-let-7d-3p	3.420995915	0	0
hsa-miR-139-5p	3.360351398	0	0
hsa-miR-451a	2.970189727	0	0
hsa-miR-4532	2.566196373	0	0
hsa-miR-4516	2.447549406	0	0
hsa-miR-206	2.180318634	0	0
hsa-miR-3074-5p	2.12839887	0	0
hsa-miR-24-3p	1.531436553	0	0
hsa-miR-23a-3p	1.514647566	0	0
hsa-miR-27a-3p	1.493679286	0	0
hsa-miR-484	1.40228226	0	0
hsa-miR-4488	1.31999654	0	0
MiRNA	log2Ratio (BMJ/NC)	P value	Target gene
hsa-miR-206	2.180318634	0	UGT1A1 OATP
hsa-miR-128-3p	3.483710778	0	MRP2
hsa-miR-4516	2.447549406	0	MRP2
hsa-miR-224-5p	1.014077245	1.27E-196	UGT1A1
hsa-miR-127-3p	2.25214727	1.99E-115	UGT1A1
hsa-miR-409-3p	5.274015621	6.46E-58	UGT1A1
hsa-miR-574-3p	1.577153131	4.83E-51	UGT1A1
hsa-miR-431-5p	7.039893908	4.68E-17	UGT1A1 OATP

miRNA	log2Ratio (BMJ/NC)	P value	Q value
hsa-miR-138-2-3p	4.998073732	2.87E-05	UGT1A1 OATP

Table 3

preamplification system. *: The miScript PreAMP Primer Mix was prepared by adding 10 μ l of the miRNA to be tested and the external reference cel-miR-19 primer miScript Primer Assay to RNase-free water to a total amount of 250 μ l.

Reagent	Volume(μ l)
5 \times miScript PreAMP Buffer	5
HotStarTaq DNA Polymerase	2
miScript PreAMP Primer Mix *	5
RNase-free water	7
miScript PreAMP Universal Primer	1
Diluted template cDNA	5
Total Volume	25

Table 4
reaction condition of
preamplification

Procedure	Time
95 °C	15 min
the following two steps cycle	
94 °C	30 sec
60°C	3 min
Cycles	12

Table 5
real-time PCR system

Reagent	Volume(μ l)
2 × QuantiTect SYBR Green PCR Master Mix	5
10 × miScript Universal Primer	1
10 × miScript Primer Assay	1
Template cDNA	1
RNase-free water	2

Table 6
reaction condition of Realtime PCR

Procedure	Time
95 °C	15 min
the following three steps cycle	
94 °C	15 sec
55°C	30 sec
70°C	30 sec (collect the fluorescent)
Cycles	40

Figures

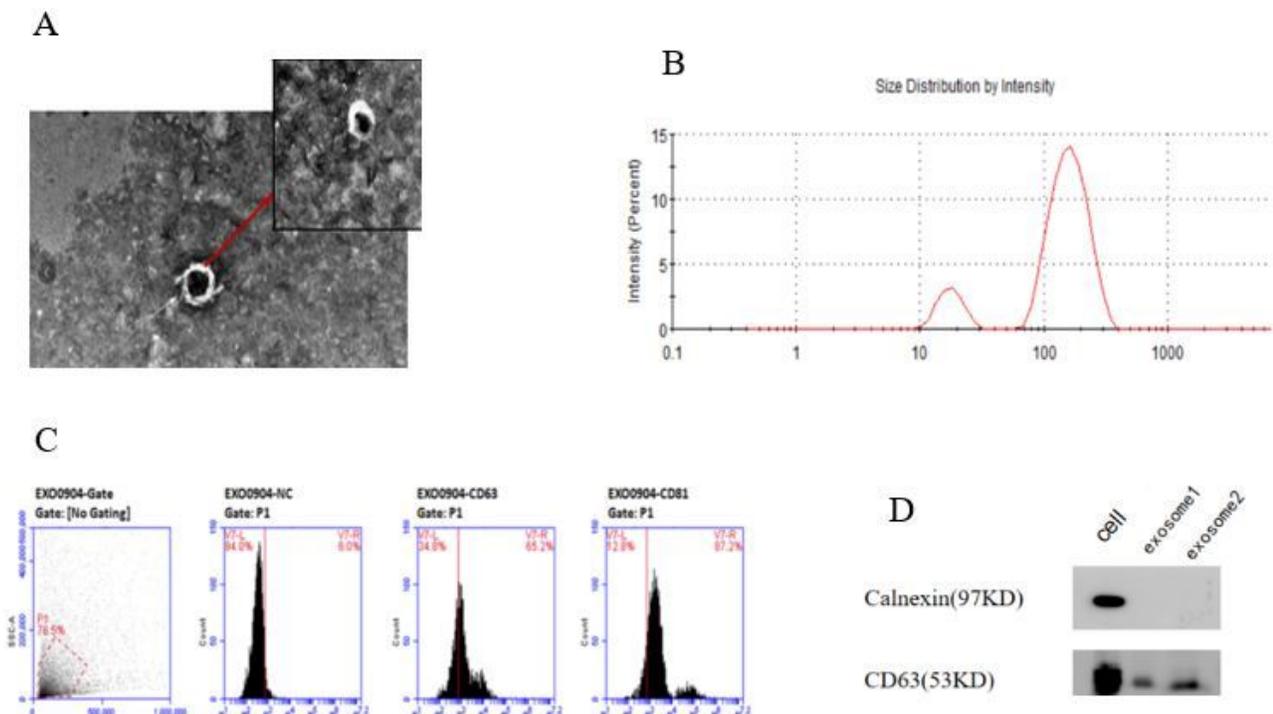


Figure 1

Identification of exosomes in human breast milk. (A) Morphological ultrastructure of the milk exosomes by TEM. (B). The particle size distribution of the milk exosomes detected using NTA (C) CD63 and CD81 expression of the milk exosomes by FACS. (D) Western blotting of milk exosomes.

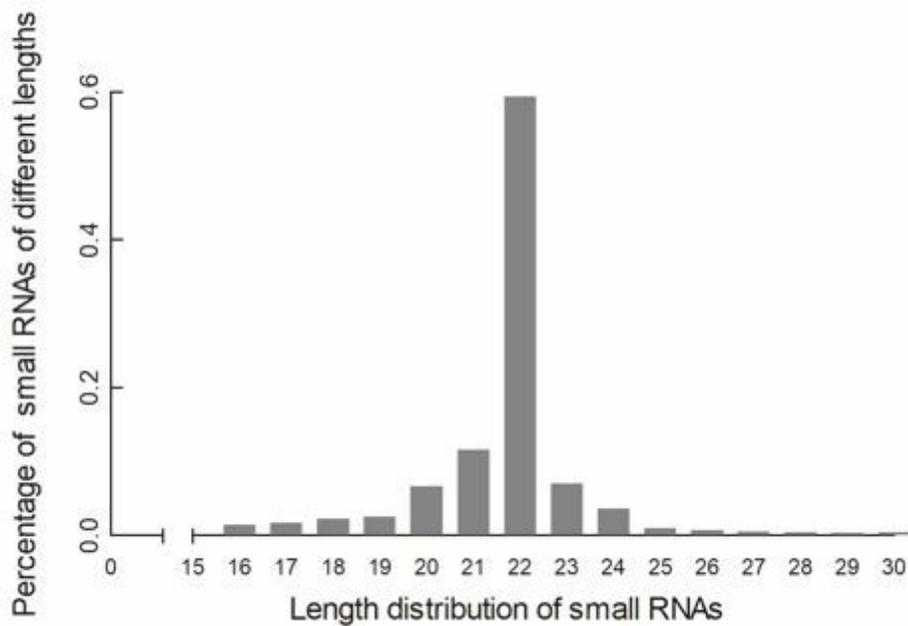


Figure 2

Length distribution of small RNAs

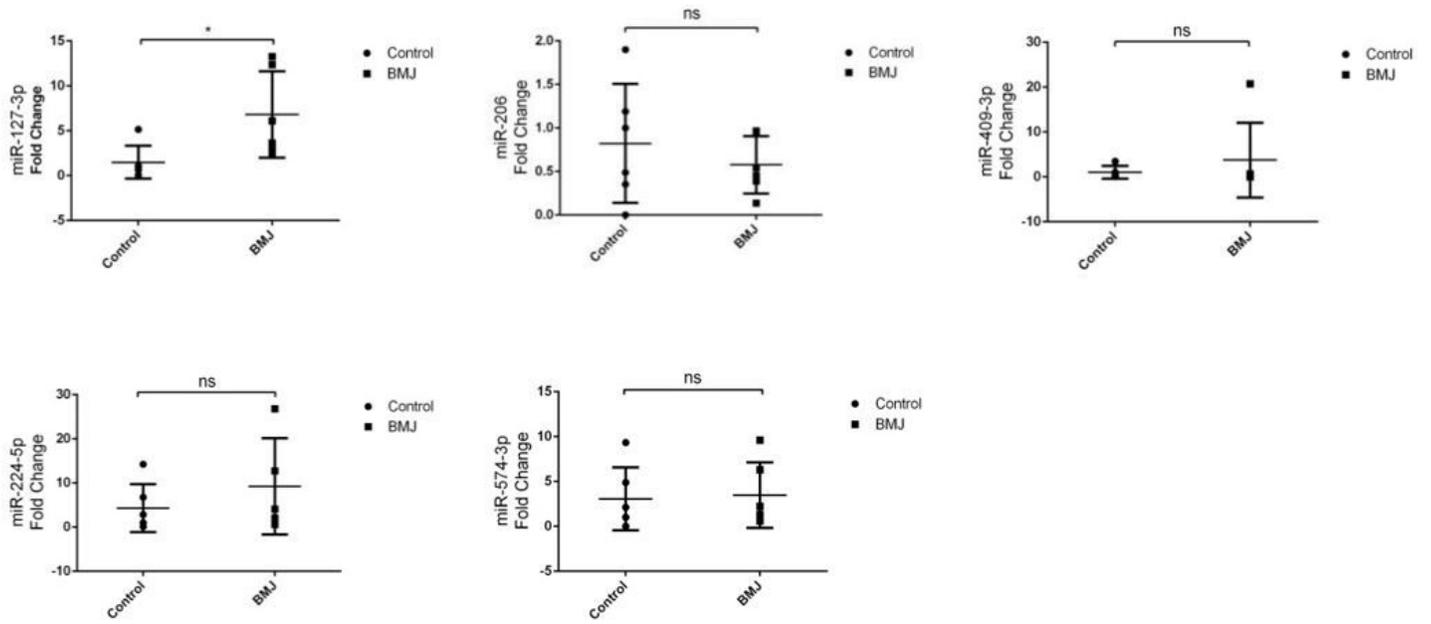


Figure 3

Pre-amplified miRNA real-time PCR.

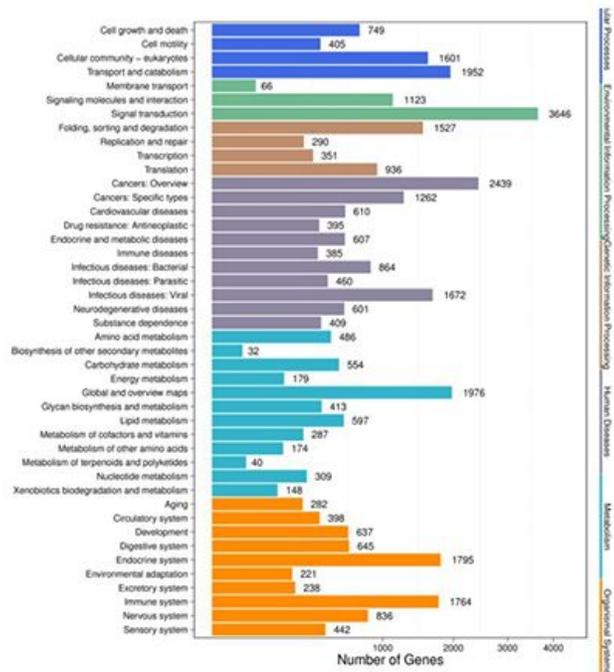
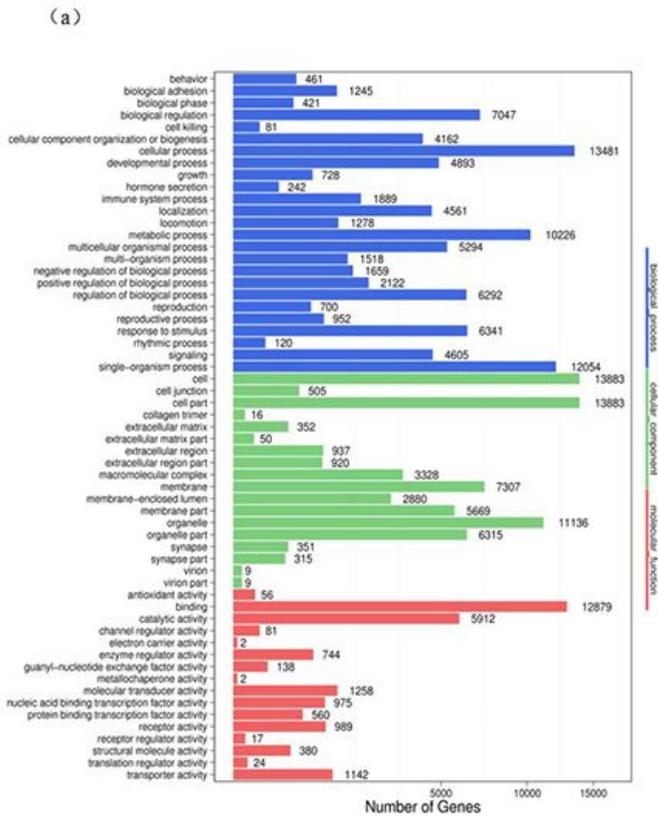


Figure 4

GO enrichment (a) and KEGG pathway annotation (b)