

Original Article



Relationship of MicroRNA according to Immune Components of Breast Milk in Korean Lactating Mothers

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ABSTRACT




Purpose: Human breast milk (HBM) contains immune components that produced and delivered from the mother along with nutrients necessary for the baby. MicroRNA (miRNA) is a small noncoding RNA molecule, that is used as an ideal biomarker for diagnosis and prognosis of various diseases and are more abundant in HBM. We analyzed and compared the immune components and miRNAs of HBM.

Methods: HBM were collected from 20 healthy breastfeeding mothers. We measured the amount of lactoferrin, lysozyme, and immunoglobulin A (IgA) and extracted the miRNAs from each breast milk samples. Next, the top 5 and bottom 5 expressed miRNAs were compared and analyzed based on the amounts of the 3 immune components.

Results: The mean levels and ranges of lactoferrin, lysozyme, and IgA were 6.33 (2.24–14.77)×10⁶ ng/mL, 9.90 (1.42–17.59)×10⁷ pg/mL, and 6.64 (0.48–20.01)×10⁵ ng/mL, respectively. The miRNAs concentration per 1 mL of skim milk was 40.54 (14.95–110.01) ng/μL. Comparing the bottom 5 and top 5 groups of each immune component, 19 miRNAs were significantly upregulated (6, 9, and 4 targeting lactoferrin, lysozyme, and IgA, respectively) and 21 were significantly downregulated (4, 9, and 8 targeting lactoferrin, lysozyme, and IgA, respectively). There were no miRNAs that were expressed significantly higher or lower in common to all 3 components. However, 2 and 3 miRNAs were commonly overexpressed and underexpressed, in the top 5 groups of lysozyme and IgA concentrations.

Conclusion: We identified the immune components and miRNAs in breast milk and found that each individual has different ingredients.

Keywords: Human milk; MicroRNAs; Lactoferrin; Lysozyme; Immunoglobulin A

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Conflict of Interest

The authors have no financial conflicts of interest.

INTRODUCTION

Milk is the main source of nutrition for various newborn mammals. Human breast milk (HBM) not only provides complete nutrition for the newborn but also provides bioactive components that guide the growth and development of the immune system and colonization of the intestinal microbiota, promoting both human health and protection against subsequent diseases during childhood, which can have lifelong effects [1,2]. Although most of the HBM is water (87%), it contains nutritional elements essential for infants, such as carbohydrates, proteins, lipids, and minerals, along with numerous bio-active components with immunocompetence and developmental functions [2]. The cumulative knowledge arising from myriad studies indicates that breastfeeding infants have a lower risk of diabetes, cancer, obesity, and infections than formula-fed infants [3]. Although the mechanisms are not yet fully understood, it has been postulated that they may arise from not only the microbiome but also from the biological macromolecules present in the breast milk, such as growth factors, antibodies, lactoferrin, lysozyme, and nucleic acids.

MicroRNAs (miRNAs) are small, noncoding RNAs of about 18–25 nucleotides in length. Present in eukaryotes, both plants and animals, miRNAs regulate genes at the post-transcriptional level by binding to target mRNAs or DNAs with complementary sequences and control a wide range of cellular functions, such as cell differentiation, proliferation, and cell death [4,5]. Downregulation of gene expression by miRNAs may play an important role in the development or progression of disease. As a result, miRNAs are postulated as possible biomarkers for the diagnosis or prognosis of various diseases, such as cancer, cardiovascular disease, and diabetes. Various tissue and cell miRNAs are found in extracellular fluids of animals (e.g., urine, milk, saliva, tears, plasma), and the abundance of miRNAs in the exosomes of breast milk is well-known [6-8]. However, it has only recently been assumed that miRNAs in breast milk may participate in the regulation of immunological and developmental processes in early human life [7,8].

In the past, research on breast milk has focused on its nutritional components. Since then, with the development of various analysis techniques, studies of the immunological components, bioactive components, and more recently, studies of the microbiome of HBM have come to the fore [9-11]. These various components and their interactions, directly and indirectly, affect the infant's health. However, while some of these components have been studied, the effects of each other and the clinical outcomes are unclear. Therefore, we conducted this study with the aim of analyzing, comparing, and confirming the association of miRNAs with lysozyme, lactoferrin, and immunoglobulin A (IgA), which are representative immune components of HBM.

MATERIALS AND METHODS

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Institutional Review Board (IRB) of Chung-Ang University Hospital, Seoul, Korea (IRB No.: 1921-008-399).

Collection of breast milk

HBM samples were collected by donation from healthy Korean breastfeeding mothers. The samples were collected from breastfeeding mothers aged 19 to 40 who have been

nursing for at least 2 weeks. We collected information on breastfeeding donor through questionnaires on age, fertility, past history, drug history, breastfeeding time, and gender, growth status, and disease history of infants. Samples of lactating mothers with special problems in pregnancy history and past history were excluded from the collection. In the case of premature or low birth weight in birth history, it was also excluded. HBM samples were collected in a refrigerated state or frozen at -20°C for one day, and they were frozen immediately after collection. The samples were analyzed immediately after thawing through pasteurization. The HBM samples were collected at least 25 mL, and 15 mL were used for immunologic components analysis, and the remaining 10 mL were used for miRNA analysis.

Analysis of immune components and extraction of exosomal RNA

We measured the amount of 3 common immune components in HBM through ELISA kits in each 5 mL of breast milk samples. Lactoferrin was measured using the human lactoferrin ELISA kit (HLF2 [ab108882], Abcam), lysozyme was measured through human lysozyme ELISA kit (ab267798, Abcam), and IgA was analyzed using human IgA ELISA kit (ab196263, Abcam) as per manufacturer's instructions.

Human breast milk fractionation and miRNA isolation

The remaining of 10 mL of HBM samples used for miRNAs analysis. To prepare nonfat skim milk fraction, HBM samples were centrifuged (3,000 g, 4°C , 10 minutes) twice, and fat, cells, and debris were removed. The skim milk samples were centrifuged (12,000 g, 4°C , 20 minutes) again, and sequentially filtered through 0.8 μm , 0.45 μm , and 0.22 μm syringe filters (Satorius AG) to remove residual fat and cell debris. MiRNAs of skim milk were extracted by filter column & phenol/guanidine method, using acid-phenol: chloroform and *mirVana* miRNA isolation kit (Invitrogen). The concentration and purity (260:280 ratio) of miRNAs were analyzed by NanoDropTM 1000 spectrophotometer (Thermo Scientific). The miRNA samples were used >20 ng RNA for small RNA-seq.

Library preparation and sequencing

The construction of library was performed using NEBNext Multiplex Small RNA Library Prep kit (New England BioLabs Inc.) according to the manufacturer's instructions. Briefly, for library construction, total RNA from each sample were used to ligate the adaptors and then cDNA was synthesized using reverse-transcriptase with adaptor-specific primers. PCR was performed for library amplification and libraries were carried out clean-up using QIAquick PCR Purification Kit (Quaigen Inc.) and polyacrylamide gel electrophoresis gel. The yield and size distribution of the small RNA libraries were assessed by the Agilent 2100 Bioanalyzer instrument for the High-sensitivity DNA Assay (Agilent Technologies Inc.). High-throughput sequences were produced by NextSeq500 system as way of single-end 75 sequencing (Illumina).

Data analysis

Sequence reads were mapped by bowtie2 software tool in order to obtain bam file. Mature miRNA sequence is used as a reference for mapping. Read counts mapped on mature miRNA sequence were extracted from the alignment file using bedtools v2.25.0 and Bioconductor that uses R statistical programming language [12,13]. Read counts were used to determine the expression level of miRNAs. The CPM-TMM normalization method was used for comparison between samples. MiRNA targets were predicted using miRWalk 2.0. Functional gene classification was performed by DIANA online software.

Comparison of miRNAs according to immune components

Because there were no specific problems with the donors of the collected samples or newborns breastfeeding through the corresponding breast milk, the 3 immune components to be analyzed were expected to be in the normal range in almost all samples. Therefore, the analysis of miRNA according to the immune component confirmed no differences according to the donor who provided the sample, but only differences according to the relatively high and low levels of the 3 immune components. The results of the top 5 and bottom 5 samples were confirmed after the analysis of each immune component, and miRNAs were investigated in the corresponding samples. In addition, the analysis of differentially expressed genes (DEGs) was used to identify miRNAs with significant expression, and the top 5 and bottom 5 were compared and analyzed based on the amount of the 3 immune components. We found miRNA with a normalized read count (log) value of 6 or higher, with the expression of miRNA increasing or decreasing by more than 2 times on a subgroup basis (t -test p -value < 0.05).

RESULTS

Participant information

A total of 20 breast milk donors were enrolled in the study, and their average age was 34.35 ± 3.69 years (range, 28–40 years). In delivery history, 11 (55%) were multipara and 9 (45%) were primipara, and their mean gestational age was 38.85 ± 1.09 weeks. The mean birth weight of newborn babies was 3.26 ± 0.35 kg, and the mean feeding duration from childbirth to breast milk sample collection was 20 ± 8.66 weeks (Table 1). There was no statistically significant difference between the variables of delivery history, including the mother's age, gestational age, and the birth weight of newborns.

Table 1. Baseline variables of breast milk donors

No	Age (yr)	Primipara	Delivery mode	Gestational age (wk)	Newborn birth weight (kg)	Feeding duration (wk)*
1	40	Yes	C/S	39	2.89	24
2	40	No	C/S	39	3.86	4
3	33	Yes	C/S	39	3.86	36
4	40	Yes	C/S	37	3.06	29
5	32	Yes	VD	38	2.82	26
6	34	No	VD	39	2.72	10
7	34	Yes	C/S	40	3.20	16
8	37	No	C/S	38	3.57	20
9	28	Yes	VD	39	3.41	23
10	30	Yes	VD	38	2.82	20
11	33	Yes	C/S	40	3.69	24
12	32	Yes	VD	40	3.52	21
13	38	No	C/S	38	3.18	17
14	38	No	C/S	38	3.62	6
15	32	No	C/S	38	3.20	12
16	32	No	C/S	39	3.28	15
17	28	Yes	C/S	40	3.05	28
18	35	Yes	C/S	40	3.35	6
19	35	No	VD	41	2.87	28
20	36	No	VD	37	3.31	25
Mean±SD	34.35±3.69			38.85±1.09	3.26±0.35	20.00±8.66

C/S: cesarian section, VD: vaginal delivery, SD: standard deviation.

*Duration of breastfeeding when sample collection.

Table 2. Immune components and microRNAs in 20 Korean female's breast milk

No	Lactoferrin (10^6 ng/mL)	IgA (10^5 ng/mL)	Lysozyme (10^7 pg/mL)	miRNA concentration (ng/ μ L)
1	4.35	7.45	12.53	63.02
2	3.76	4.33	3.89	61.80
3	4.50	11.03	14.14	14.95
4	2.47	6.71	12.79	29.25
5	9.63	7.79	12.44	50.58
6	2.24	5.11	2.66	26.03
7	3.21	0.62	12.50	34.16
8	3.66	10.99	12.49	28.03
9	2.73	7.09	7.62	23.34
10	13.20	4.41	12.66	98.08
11	14.15	13.21	5.64	110.01
12	3.84	4.78	10.83	18.30
13	7.50	7.08	17.48	47.75
14	4.89	0.79	4.57	34.14
15	10.98	8.04	8.32	51.36
16	4.92	3.06	1.42	36.60
17	3.53	0.48	7.53	20.00
18	7.13	0.65	4.01	21.39
19	5.20	9.14	16.96	24.10
20	14.77	20.01	17.59	17.98
Mean \pm SD	6.33 \pm 4.03	6.63 \pm 4.83	9.90 \pm 5.04	40.54 \pm 26.13

IgA: immunoglobulin A, miRNA: microRNA, SD: standard deviation.

Immune components in HBM

The concentrations of lactoferrin, IgA, lysozyme, and miRNAs in the collected breast milk samples were measured. The mean value of each immune component that was measured in breast milk were 6.33 \pm 4.03 ($\times 10^6$) ng/mL of lactoferrin, 6.63 \pm 4.83 ($\times 10^5$) ng/mL of IgA, and 9.90 \pm 5.04 ($\times 10^7$) pg/mL of lysozyme, and the mean concentration of extracted miRNAs was 40.54 \pm 26.13 ng/ μ L (**Table 2**). Although the mean concentrations of all 3 immune components in the breast milk of mothers who gave birth through vaginal delivery were higher than in the case of cesarean section, maternal age, delivery history, delivery method, and feeding duration of donors had no statistically significant effect on the concentrations of lactoferrin, IgA, and lysozyme in breast milk.

MicroRNAs in HBM and relation with immune components

A total of 2,588 miRNAs were detected in the donor's breast milk. In order to compare the expression of miRNAs present in breast milk, 5 samples with high concentrations and 5 with low concentrations were classified as subgroups for each immune component, and their composition was not significantly different. According to the results from the DEG analysis with a fold change (FC) threshold ($\log_2|FC|$) >2, several miRNAs were found to have statistically significantly increased or decreased expression in each subgroup. There were 10 (6 increased and 4 decreased), 12 (4 increased and 8 decreased), and 18 (9 increased and 9 decreased) miRNA species in the lactoferrin, IgA, and lysozyme subgroups, respectively. There were no miRNAs with significantly increased or decreased expression in common to all 3 subgroups (**Fig. 1**).

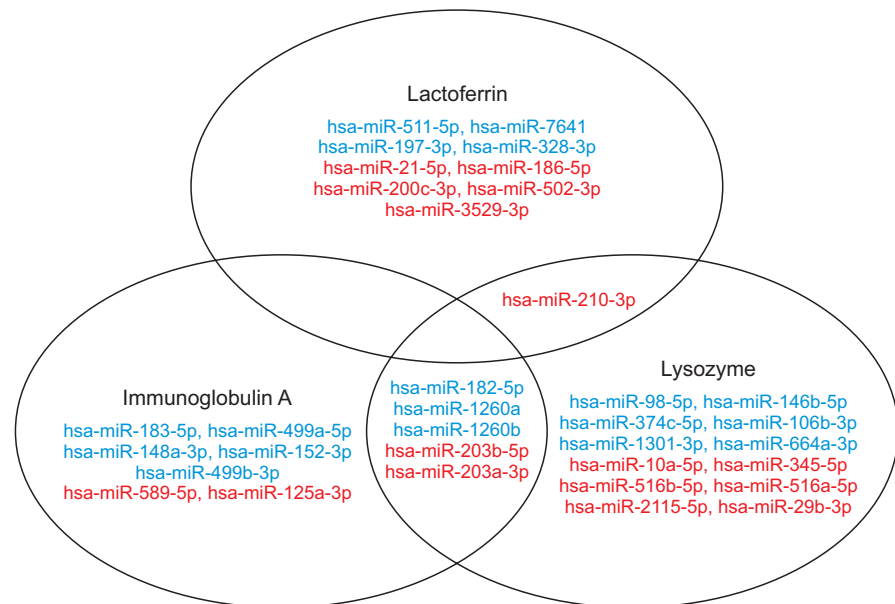


Fig. 1. MicroRNAs with increased or decreased expression in each immune component subgroup.

DISCUSSION

This study is the first to analyze miRNAs in HBM from Korean lactating mothers and also to decipher the association among important immune components in HBM. A total of 2,588 miRNAs were detected in the breast milk of 20 Korean breastfeeding mothers, and 34 miRNA species were shown with increased or decreased expression depending on the amounts of lactoferrin, IgA, and lysozyme.

In this study, the concentrations of lactoferrin, IgA, and lysozyme present in the breast milk of Korean breastfeeding mothers were $6.33 \pm 4.03 (\times 10^6)$ ng/mL, $6.63 \pm 4.83 (\times 10^5)$ ng/mL, and $9.90 \pm 5.04 (\times 10^7)$ pg/mL, respectively, and the results were similar to those in previous studies [14-16]. All breast milk samples were collected from mothers who gave birth between 38 and 41 weeks of gestation and continued breastfeeding for at least 1 month. There were no significant differences between the concentrations of each immune component and the mother's age, obstetric history, and delivery mode.

Since the first discovery of miRNAs in the early 1990s, approximately 2,600 species of human miRNAs have been identified [17]. MiRNAs regulate target genes to control their pathways, and many studies have been conducted on the role of miRNAs in the pathogenesis, detection, treatment, and prognosis of various diseases [18]. Among them, the most widely studied field is tumorigenesis and their detection and treatment, especially miRNAs associated with this have been referred to as oncomir [19,20]. MiRNAs are present in various human body fluids, such as serum, saliva, tears, seminal, amniotic, pleural, peritoneal, and cerebrospinal fluids, and the particular abundance of miRNAs in breast milk has already been highlighted [21,22].

HBM is a body fluid specifically designed for human babies, not only to supply nutrients to the baby but also to contribute to the formation of immunity and the growth and development of the infant. It is pertinent to study how miRNAs in breast milk will affect breastfed newborns and infants. Therefore, the analysis of miRNAs and immune

components in HBM is an ongoing area of research, but such studies have not yet been reported in Korea [8,23].

As already known, breast milk contains an abundance of immune components, such as various immunoglobulins and cytokines, and these components vary depending on the timing of breast milk from colostrum to transitional milk and mature milk, and delivery history, such as premature babies [14,24]. To reduce these differences, we measured the concentrations of lactoferrin, lysozyme, and IgA, which are most closely related to the formation of the immune system of breastfed infants among the myriad ingredients in breast milk.

In order to analyze the relationship between the expression of miRNAs and concentrations of lactoferrin, lysozyme, and IgA, we classified 5 breastfeeding mothers with high and low concentrations of each immune component as subgroups rather than simply comparing the expression of miRNAs according to each component of breast milk. Next, the miRNAs with significantly increased or decreased expression between these 2 subgroups were detected by DEG analysis. This approach allows analyzing the expression pattern according to specific conditions or processors by securing all genes expressed and finding miRNAs with higher expression compared to the control through multiples of the amount of miRNA expression, contrary to microarray, where only the secured genes can be analyzed.

In this study, we found that the expression of *hsa-miR-3529-3p* was most increased when the lactoferrin concentration in breast milk was relatively high. Although *hsa-miR-3529-3p* is known to be associated with more than 1,000 target genes, the clinical significance has not yet been revealed, except that it is known to be involved in the downregulation of the long noncoding RNA (lncRNA) prostate androgen-regulated transcript 1 (PART1), which has been implicated in the process of several cancer pathogenesises involving forkhead box protein C2 (FOXC2) [25]. In breast milk samples with a relatively high level of lysozyme, the expression of *hsa-miR-2115-5p* showed a marked positive correlation with the overall survival rate of patients with breast ductal and lobular cancers [26]. *Hsa-miR-125a-3p* is a miRNA highly expressed in breast milk with increased levels of IgA, and it is also believed to be an important tumor suppressor in the pathogenesis of several cancers, especially human epidermal growth factor receptor 2 (HER2)-amplified and HER2-overexpressing breast cancers by regulating breast cancer gene 1 (BRCA1) signaling and non-small cell lung cancer (NSCLC) by activating p53 signaling [27-29].

We found a total of 3 miRNAs (*hsa-miR-210-3p*, *hsa-miR-203a-3p*, and *hsa-miR-203b-5p*) with increased expression in HBM when 2 of 3 immune components were measured at relatively high concentrations, although there were no miRNAs with increased expression in common to all 3 subgroups. *Hsa-miR-210-3p*, whose expression was simultaneously increased in 2 subgroups with increased concentrations of lactoferrin and lysozyme, is one of the miRNAs under active study in myriad fields. It has been found significantly overexpressed in different human cancers and is suggested as a potential predictor of prognosis and treatment response in breast cancer, a biomarker of pancreatic ductal adenocarcinoma or chronic pancreatitis, and a new therapeutic target in NSCLC [17,30,31]. In addition, *hsa-miR-203a-3p*, which showed increased expression in both the lysozyme and IgA subgroups, is also implicated in several cancers, such as breast cancer, nasopharyngeal carcinoma, and cutaneous squamous cell carcinoma [32,33].

In this study, we found thousands of miRNAs in breast milk, of which dozens of miRNAs showed increased or decreased expression. However, it is not yet clear how they will affect

lactating mothers and breastfed newborns. Most of the previous studies on miRNAs have been related to cancer, and the analyzed samples were serum, not breast milk. However, numerous miRNAs and their target genes are being revealed daily, and studies have suggested that miRNAs could influence growth factors or regulators, activation of immune responses, and maturity of T cells and B cells [18]. Breast milk contains abundant antibodies and immune components derived from the mother, and together with the nutrients in breast milk, they play a major role in the growth and immunity of newborns [8]. Breastfeeding infants have lower risks of infection, obesity, and various metabolic diseases than formula-fed infants. It can be assumed that the miRNAs in breast milk also have direct or indirect effects.

This study, which attempted to find the relationship between miRNA and 3 immune components in breast milk from Korean lactating mothers, had several limitations. In the process of collecting breast milk samples, the timing of collection was varied, and the breastfeeding time was not controlled. In addition, the analysis could not be conducted by separating the breast milk immediately after breastfeeding. In our results, the concentrations of immune components in breast milk were not evenly raised or lowered, and various components showed inter-individual variability. Therefore, in order to minimize the difference in the composition of immune components in breast milk according to the timing of the postpartum period, only mature milk was analyzed, except for colostrum and transitional milk [8]. In addition, after pumping breast milk, its components may change depending on environmental factors during the storage of breast milk, which we prevented by thoroughly following the generally recommended procedure for the storage of breast milk. Second, secretory IgA is a representative immune component in breast milk, and it is difficult to represent all immune components in breast milk with only the 3 components we analyzed. Based on this study, we plan to collect more breast milk samples from further studies and analyze more diverse breast milk immune components or immune-related cytokines with miRNAs in the future. Many previous studies have found a relationship between the immune function of HBM and miRNAs, but many areas are still unclear, and more studies are needed.

CONCLUSION

This study was the first to analyze the expression of miRNAs in HBM from Korean lactating mothers in association with immune components, and although there was no statistical significance among their expressions, the concentrations of immune components, and the variables of subjects, we found that the expression of various miRNAs was increased or decreased according to the amount of 3 immune components in breast milk. In future follow-up studies, our results can be a basis for identifying the relationship between the miRNAs and immune components and their influence on the health, immunity, and growth of newborns and children.

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