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Detection of a single nucleotide polymorphism in the human α-lactalbumin gene: implications for human milk proteins☆

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Abstract

Variability in the protein composition of breast milk has been observed in many women and is believed to be due to natural variation of the human population. Single nucleotide polymorphisms (SNPs) are present throughout the entire human genome, but the impact of this variation on human milk composition and biological activity and infant nutrition and health is unclear. The goals of this study were to characterize a variant of human α-lactalbumin observed in milk from a Filipino population by determining the location of the polymorphism in the amino acid and genomic sequences of α-lactalbumin. Milk and blood samples were collected from 20 Filipino women, and milk samples were collected from an additional 450 women from nine different countries. α-Lactalbumin concentration was measured by high-performance liquid chromatography (HPLC), and milk samples containing the variant form of the protein were identified with both HPLC and mass spectrometry (MS). The molecular weight of the variant form was measured by MS, and the location of the polymorphism was narrowed down by protein reduction, alkylation and trypsin digestion. Genomic DNA was isolated from whole blood, and the polymorphism location and subject genotype were determined by amplifying the entire coding sequence of human α-lactalbumin by PCR, followed by DNA sequencing. A variant form of α-lactalbumin was observed in HPLC chromatograms, and the difference in molecular weight was determined by MS (wild type=14,070 Da, variant=14,056 Da). Protein reduction and digestion narrowed the polymorphism between the 33rd and 77th amino acid of the protein. The genetic polymorphism was identified as adenine to guanine, which translates to a substitution from isoleucine to valine at amino acid 46. The frequency of variation was higher in milk from China, Japan and Philippines, which suggests that this polymorphism is most prevalent in Asia. There are SNPs in the genome for human milk proteins and their implications for protein bioactivity and infant nutrition need to be considered.

Keywords: SNP; Infant nutrition; Lactation; Genetics; α-lactalbumin; Human milk

1. Introduction

Human milk contains a wide array of proteins that provide biological activities beneficial to the infant, including nutrient absorption, antimicrobial effects and immunostimulatory functions [1]. Many environmental factors have been shown to affect milk composition, including diet, exercise, stage of lactation and stress during labor [2–4]. However, normal variability also exists in milk composition between women. While the factors affecting normal variability between women are not well understood, it is possible that genetic factors may underlie some of the variability in milk composition.

Polymorphism in human milk proteins has received far less attention than that in bovine milk proteins, possibly because of a lack of commercial significance. There are spurious reports in the literature of genetic variants in the phosphorylation patterns of human β-casein and some mutations have been implied, often with limited documentation. We recently encountered prevalent polymorphism in the major human milk protein, α-lactalbumin, in milk samples from the Philippines and here describe its genetic and biochemical nature. Although this SNP is unlikely to

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affect the biological function of α-lactalbumin, it highlights the need to further study polymorphism among human milk proteins.

α-Lactalbumin plays a key role in lactose synthesis in the mammary gland and provides a major source of amino acids in human milk. Human α-lactalbumin contains 123 amino acids and has a molecular weight of about 14 kDa [5]. α-Lactalbumin functions as a regulatory subunit of the enzyme complex lactose synthase. It binds to the enzyme galactosyltransferase thereby ensuring synthesis of lactose from glucose and galactose [6,7]. It is a calcium metalloprotein [8] and has been suggested to have several biological functions in the infant in addition to its role in the mammary gland. These biological activities range from antibacterial and prebiotic activities to enhancement of trace element absorption [9–11]. In human milk, α-lactalbumin is a major milk protein and is present in concentrations of 1–2 g/L [11]. The α-lactalbumin gene consists of four exons spanning ~2.5 kb [12] and is located on chromosome 12 [13]. α-Lactalbumin-deficient mice produced by gene targeting show greatly thickened milk and containing little lactose, which supports the role of α-lactalbumin in both lactose synthesis and milk volume via osmotic influx of water [14,15].

The Human Genome project has revealed the extent of the genetic variability between individuals in the human population and the large number of single nucleotide polymorphisms (SNPs) in the human gene pool. The extent of variability is enormous, with the discovery of 1.42 million SNPs in the human genome [16]. However, the impact of SNPs in nutrition is just starting to be explored and the impact of SNPs on human milk proteins and infant nutrition is currently unknown. Proteomic methods, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS), and genomic tools, such as PCR and DNA sequencing, are currently available and widely accessible. In addition, collection of milk is a relatively noninvasive procedure. We have therefore started to characterize SNPs in human milk proteins to better understand their functional significance. In this study we identified a variant form of α-lactalbumin and revealed the location of the polymorphism in both the protein primary sequence and the genomic DNA sequence.

2. Material and methods

2.1. Sample collection and preparation

Breast milk and blood samples were collected from 20 women during mid-lactation, between 28 and 100 days postpartum, in the Philippines and stored at −20°C until α-lactalbumin protein analysis and SNP detection. Milk samples were diluted 2:5 with HPLC-grade water and centrifuged for 15,000 × *g* at 5°C for 30 min. The lipid layer was removed, and the aqueous phase was retained for HPLC and MS analyses. Additional milk samples from a previous study [17] were collected from women in Australia ($n=53$), Canada ($n=49$), Chile ($n=51$), China ($n=49$), Mexico ($n=49$), Japan ($n=50$), Philippines ($n=52$), United Kingdom ($n=50$) and the United States ($n=47$) and screened for variant α-lactalbumin. Genomic DNA was isolated from 200 μl of whole blood using the Qiamp DNA Blood Mini Kit (Qiagen, Valencia, CA).

2.2. High-performance liquid chromatography analysis

Milk proteins were separated by reverse-phase HPLC. Samples were loaded on a Jupiter C4 column (Phenomenex, Torrance, CA) using a Hewlett-Packard 1050 system (Agilent Technologies, Wilmington, DE). The column temperature was 30°C, and proteins were eluted with a 30-min mobile phase gradient from 40% acetonitrile, 0.1% trifluoroacetic acid to 55% acetonitrile, 0.1% trifluoroacetic acid and a 0.8 ml/min flow rate. Proteins were monitored at 210 and 280 nm by diode array. á-Lactalbumin concentrations for the 20 Filipino milk samples were determined by the total area under the curve of the chromatogram peak, and standards for the assay consisted of purified human α-lactalbumin (Sigma, St. Louis, MO).

2.3. Protein digestion and MS analysis

Trypsin digests were performed on reduced and either alkylated or non-alkylated protein fractions. The isolated proteins were reduced with 25 μl of 0.4 M ammonium bicarbonate and 5 μl of 45 mM dithiothreitol followed by incubation at 60°C for 5 min, then alkylated with 5 μl of 100 mM iodoacetamide and digested with 0.5 μg trypsin (Promega, TPCK modified) for 2 h at 37°C. Nonreduced samples were digested for 2.5 h. Digest mixtures were desalted (C18 Zip Tip, Waters, Milford, MA) and diluted into methanol/water/acetic acid (49:49:2), and analyzed by electrospray ionization mass spectrometry (LC-ESI-MS) on a Micromass QTOF II MS system (Waters). Mass spectra were deconvoluted using Micromass Masslynx software (Waters). Measurement of the intact protein molecular weights and calculated mass difference between the known and variant protein forms were accomplished by using data from isolated fractions.

2.4. Single nucleotide polymorphism detection

The four exons and three introns of human α-lactalbumin gene were amplified from 200 ng genomic DNA by PCR using Platinum Taq Hi Fidelity (Invitrogen, Carlsbad, CA) and the primers 5′-tcccaacatcccctccaaagat-3′ and 5′-tggctggattggttggacaagt-3′. Primers were designed using the Primer3 output program [18] and the DNA sequence of the human α-lactalbumin gene was obtained from the National Center for Biotechnology Information (NCBI, NT_029419). The PCR reaction consisted of an initial denaturation of 60 s at 94°C, followed by 30 cycles at 94°C for 30 s, 57°C for 45 s and 68°C for 5 min, and an additional extension of 68°C for 7 min. PCR products were cloned into a vector for sequencing using the TOPO XL PCR Cloning Kit

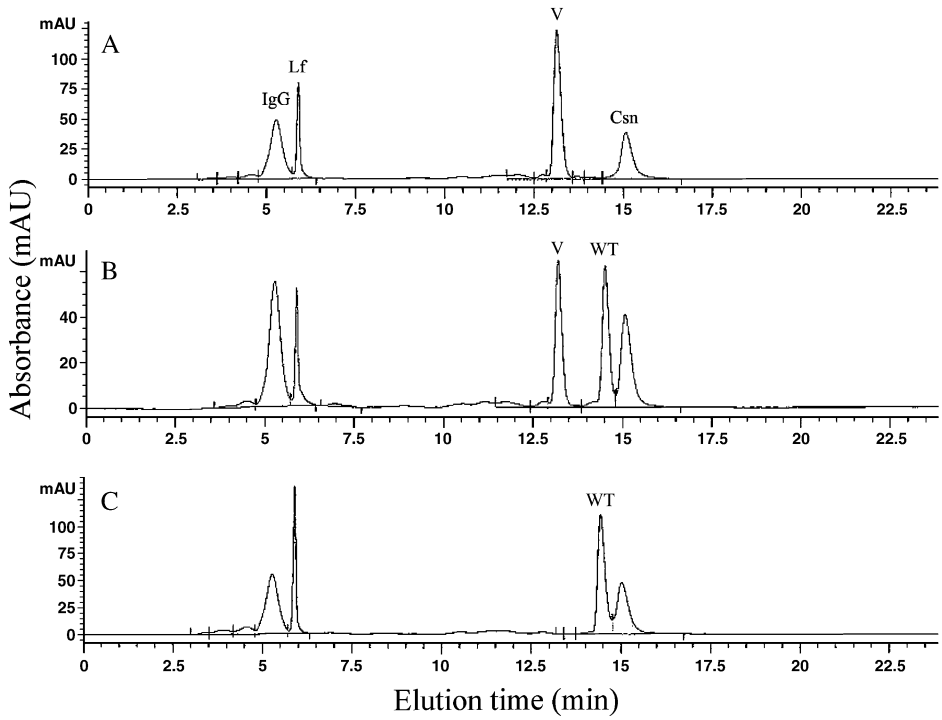

Fig. 1. Identification of a variant form of α-lactalbumin by HPLC. Sample chromatograms containing (A) only variant (homozygous variant), (B) both variant and wild type (heterozygous variant) and (C) only wild-type (homozygous wild type) forms of lactalbumin. The letters WT and V denote wild-type and variant forms, respectively. The other peaks are, from left to right, immunoglobulins (IgG), lactoferrin (Lf) and casein (Csn), respectively. Peak height corresponds to absorbance at 280 nm [mAU (absorbance units)], and *x*-axis corresponds to elution time (min).

(Invitrogen). The exons were sequenced by University of California, Davis, Division of Biological Sciences Automated DNA Sequencing Facility (Davis, CA) using the following four sequencing primers corresponding to á-lactalbumin intron sequences: 5′-aaggaagctggcaggctcag-3′, 5′-ggcaatgaagcctgaggtct-3′, 5′-aagctgatgattcctccagag-3′, 5′-gtccacgctcttccacttca-3′, with an ABI 3730 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems, Foster City, CA). Six samples, four heterozygotes and two wild types, were also sequenced in the opposite direction with the primer 5′-gagctttgccatctttggag-3′ to confirm the polymorphism. Sequence translations and analysis were performed using Bioedit software [19] and sequence chromatograms were visually analyzed for SNP detection. In addition, the sequences were compared with the reference mRNA sequence for α-lactalbumin provided by NCBI (NM_002289).

2.5. Statistics

Graphpad Prism [20] was used for data analysis and graphing α-lactalbumin concentration in milk. A two-tailed, two-sample equal variance *t* test was performed to compare concentrations of α-lactalbumin in milk from wild-type and heterozygous-variant women. A two-tailed, paired *t* test was used to compare concentrations of the wild-type and variant forms of α-lactalbumin in women with the heterozygous genotype. A chi-square test was used to compare the number of women who are wild type or possess at least one variant allele between women from Asian countries (China, Japan or Philippines) compared to all countries.

3. Results

The variant form of α-lactalbumin was detected in 7 of the 20 women by both HPLC and genomic DNA sequencing (Fig. 1). Variant α-lactalbumin was also detected in the milk of 77 women from different countries (Table 1).

Table 1
Number of women from different countries who contain either wild-type or variant forms of lactalbumin in milk

	Wild type	Variant	Gene frequency
China*	36	13	0.13
Japan*	43	7 (1)	0.08
Philippines*	39	33 (5)	0.28
Australia	50	3	0.028
Canada	45	4	0.041
Chile	47	4	0.039
Mexico	47	2	0.020
United Kingdom	42	8	0.08
United States	44	3	0.032
All countries	393	77 (6)	0.088

Wild type includes only women who are homozygous wild type.
Variant includes women who are either heterozygous or homozygous variant.
Number in parentheses denotes number of women who were homozygous for the variant.
* Denotes countries within Asia.

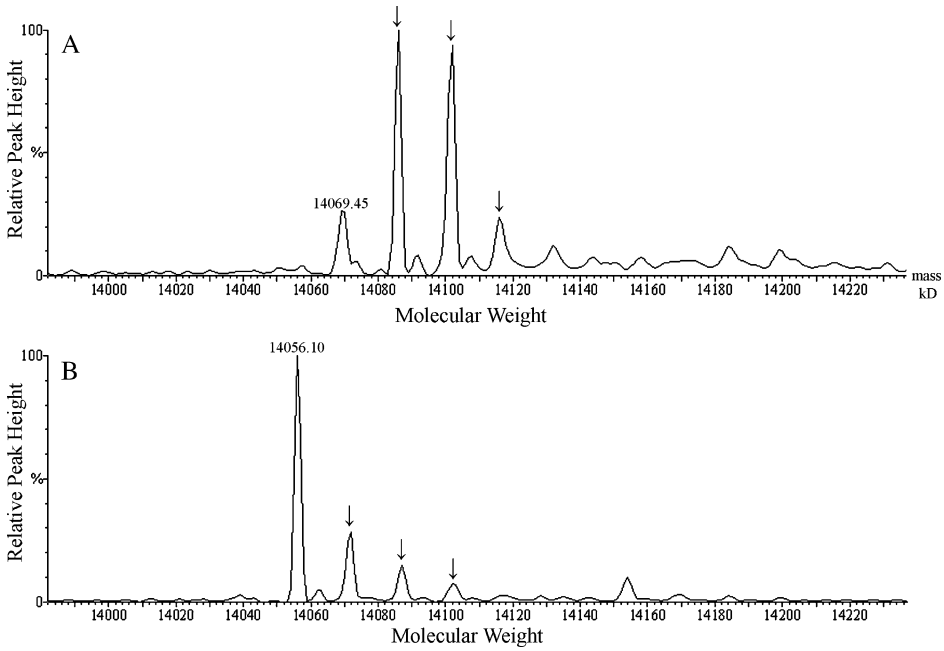

Fig. 2. Molecular weight determination of the variant form of α-lactalbumin by MS and deconvolution. Sample spectra containing (A) only wild-type (homozygous) and (B) only variant (homozygous) forms of α-lactalbumin. The numbers of the smallest molecular weight peaks correspond to the measured molecular weight of the protein (theoretical weight of lactalbumin = 14,070 Da [5]). Peak height corresponds to relative ion abundance, and the arrows denote peaks due to product oxidation.

Two peaks, roughly equal in height and half the amplitude compared to other samples (Fig. 1A and C), can be observed in the HPLC chromatograms of heterozygotes (Fig. 1B). Mass spectra (Fig. 2) showed that the wild-type and variant forms differ by 14 Da. After digestion and reduction, only the fractions containing the largest fragment, with amino acids 33–77 of α-lactalbumin, contained a variation in molecular weight, which localized the polymorphism to within this peptide, and the small difference in molecular weight suggested that the variation was due to a single amino acid substitution from glutamic acid to aspartic acid, leucine or isoleucine to valine, or threonine to serine. Further analysis of the peptide by manual comparison of the isotopic patterns narrowed down the mutation to either leucine or isoleucine, at amino acid 45 or 46, respectively.

DNA sequencing chromatograms of heterozygotes (Fig. 3A) detected both adenine and guanine at +136 on the coding strand, whereas only an adenine was detected in chromatograms of homozygotes (Fig. 3B). This polymorphism translates to an isoleucine to valine substitution at amino acid 46 (Fig. 4). There was no significant difference in α-lactalbumin concentration between homozygotes and heterozygotes (Fig. 5A) or between the wild-type and variant forms within heterozygotes (Fig. 5B), which suggests that the polymorphism does not influence the

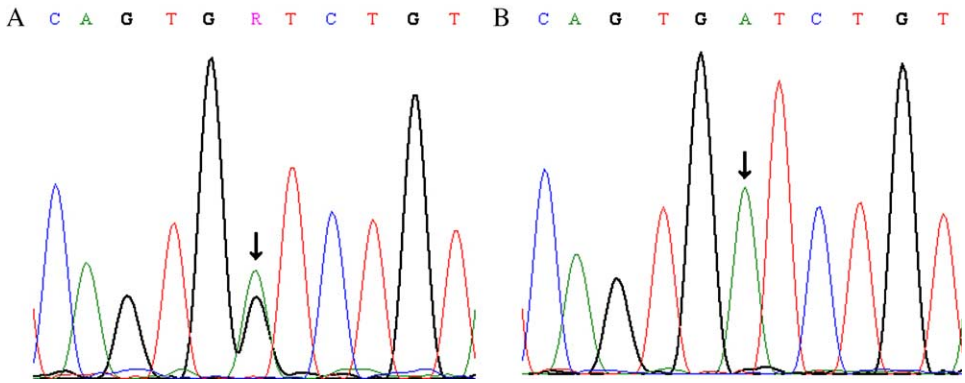

Fig. 3. Genotyping of women by analysis of DNA sequencing chromatograms. Sample chromatograms of (A) heterozygous, variant and (B) homozygous, wild-type α-lactalbumin genotypes are shown. The arrow denotes the location of the polymorphism.

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1    atgaggttctttgtccctctgttcctggtgggcatcctgttccctgccatcctggccaag  60
      M  R  F  F  V  P  L  F  L  V  G  I  L  F  P  A  I  L  A  K

61   caattcacaaaatgtgagctgtcccagctgctgaaagacatagatggttatggaggcatc 120
      Q  F  T  K  C  E  L  S  Q  L  L  K  D  I  D  G  Y  G  G  I

121  gctttgcctgaattgatctgtaccatgtttcacaccagtggttatgacacacaagccata 180
      A  L  P  E  L  g     C  T  M  F  H  T  S  G  Y  D  T  Q  A  I
                     I↔V

181  gttgaaaacaatgaaagcacggaatatggactcttccagatcagtaataagctttggtgc 240
      V  E  N  N  E  S  T  E  Y  G  L  F  Q  I  S  N  K  L  W  C

241  aagagcagccaggtccctcagtcaaggaacatctgtgacatctcctgtgacaagttcctg 300
      K  S  S  Q  V  P  Q  S  R  N  I  C  D  I  S  C  D  K  F  L

301  gatgatgacattactgatgacataatgtgtgccaagaagatcctggatattaaaggaatt 360
      D  D  D  I  T  D  D  I  M  C  A  K  K  I  L  D  I  K  G  I

361  gactactggttggcccataaagccctctgcactgagaagctggaacagtggctttgtgag 420
      D  Y  W  L  A  H  K  A  L  C  T  E  K  L  E  Q  W  L  C  E

421  aagttgtga
      K  L
```

Fig. 4. The coding sequence of human α-lactalbumin with location of polymorphism. Lower case letters represent the DNA sequence, and upper case letters represent the translated amino acid sequence. Numbering is relative to the start of the coding sequence. The location of the polymorphism is underlined within the sequence, and the variant nucleotide is listed underneath the wild-type nucleotide. A nucleotide change from adenine (A) to guanine (G) at +136 results in an amino acid change from isoleucine (I) to valine (V) at position 46.

expression or secretion of α-lactalbumin. The α-lactalbumin concentration of Filipino-variant homozygotes (2.76± 0.22 g/L, mean ± S.D., $n=5$) was not different from wild-type homozygotes or heterozygotes. There was no difference in milk lactose concentrations between homozygotes and heterozygotes (data not shown).

Out of the nine countries milk samples were collected from, the polymorphism was most prevalent in the Philippines and China, followed by the United Kingdom and Japan, and least common in Mexico and Australia (Table 1). The gene frequency of the variant allele is 0.18 in women from Asian countries and 0.042 in women outside Asia, and there were significantly more women who possess the variant gene (either heterozygous or homozygous variant) from Asian countries compared to other nations ($P<.0001$), which suggests that this polymorphism is concentrated within Asia. In addition, milk samples from 7 of 11 Filipino women living in Canada also possess the variation (data not shown).

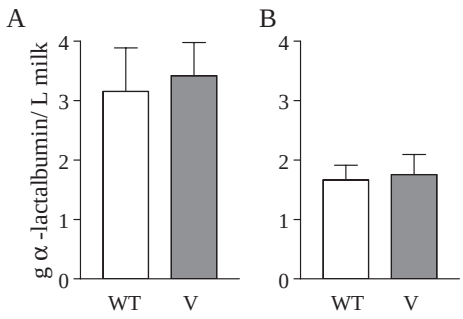

Fig. 5. α-Lactalbumin concentrations in milk of wild-type and heterozygous-variant women, as measure by HPLC. (Panel A) Concentrations of total α-lactalbumin in milk of homozygous wild-type (WT, $n=13$) and heterozygous variant (V, $n=7$) women. Values are means ± S.D. (Panel B) Concentrations of wild-type (WT) and variant (V) forms of α-lactalbumin in milk of heterozygous-variant women ($n=7$).

4. Discussion

Extensive research regarding polymorphisms and milk quality has been conducted in cows, where associations between genetic variation in milk composition and production have implications for the dairy industry. Variants that result in changes to the amino acid sequence have been detected in α_s-casein [21], β-casein [22], κ-casein [23], β-lactoglobulin [24] and α-lactalbumin [25]. For example, a polymorphism in α-lactalbumin is present in Droughtmaster cattle, resulting in an arginine to glutamine substitution [26]. In addition, variants that contain changes in noncoding regions have been detected in α_{s1}-casein [27], β-casein [28], β-lactoglobulin [29] and α-lactalbumin [30]. Variants in these regions have the potential to affect the concentration of the protein through alterations in the level of gene expression. For example, a mutation in the α_{s1}-casein gene results in a 371-bp insertion in the 3′-flanking region of the gene and results in lower concentrations of the variant form due to decreased mRNA stability of the gene [27]. Thus, polymorphisms can directly affect concentrations of individual milk proteins. A polymorphism in α-lactalbumin located 15 bp downstream of the transcription start site is associated with differences in milk production and composition in Holstein cows [30], although it is unclear if the polymorphism affects gene transcription.

Although many bovine milk polymorphisms have been characterized as “silent” because they have no known

effects on the functional properties of the proteins, there are some examples that modify milk protein properties and bioactivity. Polymorphism in bovine κ-casein has been shown to affect rennet clotting time, which is important in cheese production [31]. The enzymatic hydrolysis of the bond between phenylalanine 105 and methionine 106 is dependent upon the surrounding amino acid sequence and an arginine to histidine substitution at position 97 has been shown to affect the enzyme–substrate interaction and thus cleavage efficiency [32], probably due to an alteration in protein conformation. Since one of the larger fragments of human κ-casein formed by this hydrolysis, glycomacropeptide, has been shown to have bioactivity [33], the efficiency of release may affect its bioactivity and outcomes in the breast-fed infant. To date, no such polymorphism has been detected in human milk.

Genetic polymorphism may also affect posttranslational modification of milk proteins. Several milk proteins are phosphorylated or glycosylated and their bioactivity is dependent on the degree of phosphorylation/glycosylation [34,35]. If SNPs will transform serine or threonine residues to other amino acids that will not become phosphorylated, or if amino acids not involved in posttranslational modifications are substituted with such amino acids, changes in bioactivity may occur. Similarly, if asparagine residues needed for attachment of N-linked oligosaccharides are substituted, protein glycosylation may be altered. One example of the latter has been found in bovine β-lactoglobulin, where a substitution at amino acid 28 gives rise to a novel glycosylated variant [36].

SNPs that affect the amino acid sequence or posttranslational modification of milk proteins and their bioactivity have also been shown for lactoferrin. Velliyagounder et al. [37] expressed and purified two variants of human lactoferrin with either a lysine or arginine at position 29 in the N-terminus region of the protein. The variants showed no difference in iron-binding or bactericidal properties against a gram-negative strain of bacteria, but the lysine variant was shown to have greater bactericidal activity against two gram-positive strains of bacteria and more transcriptional activity of tracheal antimicrobial peptide mRNA. Although various forms of lactoferrin have not been reported in human milk, it is likely that this variant exists in the human population and may affect the bactericidal property of lactoferrin [38]. Lactoferrin has been shown to have several physiological activities, such as modulating immune function and antimicrobial activity, and enhancing cellular iron uptake and cell proliferation [39], and these are more or less dependent upon the remarkable resistance of lactoferrin against proteolytic degradation [40,41]. Recently, it was shown that bovine lactoferrin exists in two genetic variants, A and B, and that they are hydrolyzed by digestive enzymes to very different extent [42]. A glycan at asparagine 281 protects against tryptic attack at the subsequent lysine 282– serine 283 bond making it considerably more stable than the B form, which lacks the glycan at asparagine 281 and is relatively easily digested [43]. Thus, biological functions of lactoferrin in the gastrointestinal tract may be severely compromised. To date, the effect of polymorphism in human lactoferrin on its resistance against proteolysis has not been studied.

Our data indicate that there is variability in α-lactalbumin composition both between populations from different countries as well as within populations within a community. The polymorphism characterized in this study is unlikely to affect the biological function of α-lactalbumin. The impact of SNPs on human milk protein variability and infant nutrition represents a vast target area for further research.

Acknowledgments

The milk collection portion of the study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. The Committee on Research Implementation and Development, University of the Philippines Manila Institutional Review Board approved the study protocol and informed consent. Written informed consent was obtained from the women before study enrollment.

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