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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