Mouse mammary tumor like virus sequences in breast milk from healthy lactating women

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Abstract Mouse mammary tumor virus (MMTV) has been a long standing candidate as a potential cause of some human breast cancers. Forty years ago, electron microscopic images of MMTV-like particles were identified in milk from 5% of healthy lactating women. These observations, however, have not been confirmed by modern methods. The purpose of this study was to confirm the presence of MMTV-like DNA sequences in human milk from normal lactating women. Standard and in situ PCR analyses were conducted on DNA extracted from fresh breast milk samples collected from a group of 91 healthy lactating women volunteers. The MMTV-like viral positive PCR products were sequenced and a phylogenetic tree was constructed to compare these sequences. Immunohistochemistry analyses were performed on breast milk cells using polyclonal rabbit antibodies against affinity-purified MMTV envelope glycoproteins 52/36. MMTV-like envelope gene sequences were identified by PCR in 5% (4/91) of breast milk samples from healthy lactating women volunteers. These observations were confirmed by in situ PCR and immunohistochemistry using MMTV gp52/36 antibodies. These findings confirm the presence of MMTV-like gene sequences in human milk. As MMTV is transmitted via milk from mouse mothers to their newborn pups to cause mammary tumors when they become adults, this indicates a means of transmission of this virus in humans.

Keywords Mouse mammary tumor virus · MMTV · MMTV-like virus · Envelope protein · Hormonal influence · PCR

Introduction

Mouse mammary tumor virus-like virus gene sequences have a confirmed presence in some human breast tumors [1]. In mice, mouse mammary tumor virus (MMTV) is transmitted via milk to mouse pups, some of whom develop MMTV-associated mammary tumor as adults [2]. It has long been postulated that MMTV may be present in human milk and following ingestion in the gut during infancy may cause human breast cancers in adult life [3].

Dan Moore et al. [4] were able to identify images of putative MMTV particles in both mouse and human milk using electron microscopy in the 1970s. In addition, using the same electron microscopic techniques they demonstrated that the presence of these particles differed greatly in milk from American women with no family history of breast cancer (particles in 5% of subjects), American women with a family history of breast cancer (60%) and in 39% of Parsi women from Mumbai, India, who are known
to be at much higher risk of breast cancer than other Indian women [4]. The Moore group also demonstrated that human milk which contained putative MMTV particles had reverse transcriptase activity (an enzyme associated with oncogenic retroviruses) which was not found in particle-free milks [5]. A protein similar to the envelope protein of MMTV was also isolated and characterized from a pool of normal human milks [6]. These findings of MMTV in human milk, however, were controversial [7].

To our knowledge, there have been no published studies which used modern methods to identify MMTV in human milk. However our colleague, Caroline Ford, using polymerase chain reaction (PCR) techniques, was able to identify MMTV envelope gene sequences in the milk of 4 (6%) of 66 normal women (10 of whom reported a family history of breast cancer) [8].

Despite the identification of MMTV-like virus in human breast milk, there has been consistent evidence of a lack of association between breast feeding and breast cancer [9]. We have, however, shown previously that these past studies of breast feeding and breast cancer are potentially misleading as they have been based on questions relating to duration of breast feeding and not exposure to colostrum and breast milk during the first days of life [10]. We have demonstrated by direct observation, that 98% (n = 100) of newborn Australian infants were exposed to the breast with ingestion of colostrum [10]. This observation was in accord with a reported incidence of 82–99% exposure to the breast of 87,000 new born Australian infants. These observations are relevant because it is known that retroviruses (MMTV is a retrovirus) such as the human immunodeficiency virus (HIV), can be transmitted via human milk and that the concentration of HIV in milk is highest during the early post partum period [11].

The presence of human endogenous retroviruses (HERVs) in the human genome has many similar nucleotide sequences to MMTV. This has caused great difficulty with past investigations. The Pogo group in New York have addressed this problem by their identification and use of selected sequences of the MMTV env gene as primers in PCR analyses [12]. These selected primer sequences have very low homology to HERV-K10, the human endogenous retrovirus most closely related to MMTV [12–14]. These selected MMTV env primer sequences have been used in many subsequent studies [1]. For these reasons, in this current study we used these same sequences for both standard and in situ PCR analyses and for construction of a phylogenetic tree. In addition, we used immunohistochemical techniques to identify the presence of MMTV associated glycoprotein 52 in human milk cells. The identification of specific cell types in human milk was attempted. In this investigation, we have used modern methods to document the presence of MMTV-like envelope gene sequences in human milk samples from healthy lactating women. When used in isolation, none of these techniques offer conclusive outcomes, but when used in combination and give consistent outcomes this adds substantially to the validity of the data.

### Materials and methods

#### Breast milk samples

Fresh breast milk samples were collected from a group of 91 healthy lactating women volunteers (SESIAHS ethics approval 01/248). These volunteers completed a questionnaire prior to collection which included information on the age of the mother and baby, history of mastitis as well as any family history of breast cancer.

Breast milk samples (15 ml) were centrifuged at 3,000×g for 15 min and the upper lipid layer removed. The breast milk samples were then separated into 1.5 ml aliquots and stored at −80°C prior to nucleic acid extraction.

#### Nucleic acid extraction

Nucleic acid extraction and PCR reactions were performed in separate rooms to avoid contamination. The 1.5 ml breast milk aliquots were thawed and centrifuged at 13,000×g for 15 min to separate the supernatant from the cell pellet. The cell pellet was used for DNA extraction while the breast milk supernatant was used for genomic RNA extraction. In brief, RNA was extracted from the supernatant of the breast milk samples using Trizol LS Reagent (Invitrogen™, Carlsbad, CA, USA) according to manufacturer’s instructions. All extracted RNA samples were DNase treated using RQ1 RNase-Free DNase according to manufacturer’s instructions (Promega, Madison, USA). DNA was extracted from the cell pellet using methods previously published [16].

#### Standard PCR for MMTV-like virus and a cellular housekeeping gene (GAPDH)

PCR reactions were performed at 50 μl volumes using 300–500 ng of template DNA. Positive controls were template DNA extracted from NIH3T3 mouse fibroblast cell lines that carry endogenous MMTV sequences. The negative controls used were extraction controls and water controls. Extraction controls are controls where no sample material is added but the process of nucleic acid extraction is still performed. Extraction controls check for contamination during the process of extraction while water controls check for contamination during the process of amplification. PCR for human cellular glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) was performed to verify successful DNA extraction [16]. All GAPDH-positive samples were screened by nested PCR for a 356 bp env sequence of MMTV-like virus as described previously [16].

MMTV-like virus reverse transcriptase (RT-PCR)

All RT-PCR was performed using 1 μg of template RNA in 20 μl reaction volumes. The positive control used was RNA extracted from Australian wild mice mammary glands positive for MMTV [17]. Negative controls included in each run were reactions with reverse transcriptase omitted to detect possible traces of genomic DNA in the DNase-treated RNA preparations as well as water controls.

Reverse transcription was carried out at 42°C for 1 h in 5X AMV RT Reaction buffer (Promega, Madison, USA) [250 mM Tris–HCl (pH 8.3), 250 mM KCl, 50 mM MgCl2, 2.5 mM spermidine, and 50 mM DTT], MgCl2 (2.5 mmol/l), deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP 0.25 mmol/l each), dithiothreitol (2.5 mmol/l), 40 units RNaseOUT™ Ribonuclease Inhibitor (Invitrogen), 0.2 μl of primer 3L [12], 1.5 units of AMV reverse transcriptase (Promega, Madison, USA), and nuclease-free water to a volume of 20 μl.

Following reverse transcription, 1 μl of the cDNA was used in 20 μl standard reactions with primers 5L and 3L [12], followed by a nested PCR with primers 1× [12] and 2NR [12] as described previously [16].

Sequencing and phylogenetic analysis

MMTV-like viral positive PCR products from genomic RNA and DNA were sequenced using forward and reverse primers and analyzed with the ABI 3730 DNA capillary sequencer (AME Bioscience, USA) at the University of New South Wales DNA Analysis Facility. The nucleotide Blast online program was used to check for homology to other known sequences. Following the identification of homology to MMTV-like sequences, the amplified sequences were aligned against one another, against MMTV env sequence from Australian wild mice [17] and an HERV-K10 sequence using Clustal W [18]. A phylogenetic tree was constructed using MEGA [19] to compare these sequences.

In situ PCR for MMTV-like viral envelope (env) gene

A breast milk sample was spun at 3,000×g and the cell pellet was washed and resuspended in 1× PBS. Drops of the suspension were placed on microscope slides and air-dried. The slides were then fixed in cold methanol for 5 min. Prior to amplification, sections were digested with 0.1% Triton X-100 for 3 min followed by denaturation and washing in 0.1 M Tris (pH 8.0). In situ PCR was performed using a Hybaid Omnislide in situ PCR thermal cycler with 1× Buffer, 2.5 mM MgCl2, 0.25 mM of each dNTP, 10 pmol each of primer 5L and 3L [12], 0.75 nM of DIG-dUTP (Roche, Mannheim, Germany), and 1 unit of Hot Start Taq polymerase (Promega Corporation, Madison, USA) made up to a 75 μl reaction with nuclease-free water [20]. The reaction mix was placed within a Geneframe® (AB gene, United Kingdom) on the slide with a coverslip to maintain humidity and amplified using thermal cycling conditions of 95°C for 3 min followed by 30 rounds of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min with primers 1× and 2NR [12]. Slides were then washed and incubated with a 1:50 dilution of anti-DIG alkaline phosphatase Fab fragments (Roche, Germany) [20]. The signal was detected following incubation in a 1:50 dilution of NBT (0.33 g/ml)-BCIP (0.16 g/ml) substrate solution with 1 mM levamisole [20]. The slides were dehydrated in a series of alcohol baths and counterstained with eosin. They were then viewed under light microscopy and cells stained dark blue/purple were considered positive.

Immunohistochemistry for MMTV-like viral envelope (env) protein

Polyclonal rabbit antibodies against affinity-purified MMTV envelope gp52/36 were donated by Dr. Janet Butel (Department of Molecular Virology and Microbiology Baylor College of Medicine, Texas) and were prepared as described previously [21]. Breast milk cells that were methanol-fixed on glass slides were rehydrated in a series of ethanol baths and incubated with a 1:3,200 dilution of polyclonal gp52/36 antibodies followed by anti-rabbit horseradish-peroxidase labeled polylinker secondary antibodies (Dako, Glostrup, Denmark) and dianimobenzidine detection. Breast cancer tissues known to be positive for MMTV-like virus on PCR [22] were used as the positive control, while positive breast cancer tissue not incubated with the primary antibodies, and PCR-negative tissues as well as tissue sections incubated with normal rabbit serum (1:3,200 dilution) were used as negative controls. Microscopic examination was performed by two observers blinded to the tissue score and brown cytoplasmic staining was determined as positive staining.

Immunohistochemistry for cell markers on human breast milk cells

Slides from two representative MMTV-positive breast milk samples were stained for epithelial cells, lymphocytes, macrophages, and plasma cells. Anti-cytokeratin antibody (Antibodies-online, Aachen, Germany) was used to stain epithelial cells, anti-CD45 (human) (Antibodiesonline,
Aachen, Germany) for lymphocytes and anti-CD68 (Antibodies-online, Aachen, Germany) for macrophages. Anti-CD138, anti-VS38C, anti-kappa, and anti-lambda antibodies (all from Antibodies-online, Aachen, Germany) were used to stain plasma cells. All antibodies were used according to manufacturer's instructions.

Results

MMTV-like viral prevalence in human breast milk

MMTV-like viral DNA was detected in 5/91 (5%) of breast milk cell samples while MMTV-like viral RNA was detected in 2/91 (2%) of human breast milk supernatants (Table 1). The two MMTV-like viral RNA positive breast milk samples were not DNA positive and none of the DNA positive breast milks were RNA positive possibly due to variation in the level of MMTV-like viral replication and transcription in each sample.

There was a family history of breast cancer noted in the questionnaire from two of the five women that had MMTV-like viral DNA positive breast milk cells while 15 out of 17 women that were MMTV-like viral DNA negative, reported a positive family history. Both of the women with MMTV-like viral RNA positive breast milk supernatants did not note any family history of breast cancer.

Phylogenetic analysis of MMTV-like virus in human breast milk

Figures 1 and 2 show that the MMTV-like viral env sequences from human breast milk are interspersed in the phylogenetic tree among representative MMTV-like viral sequence from human breast cancer [22] and MMTV sequence from an Australian wild mouse [17], but separate from HERV-K10, the human endogenous retrovirus most closely related to MMTV based on pol gene alignments [13, 14]. The MMTV sequence from the mouse did not branch separately from MMTV-like virus in human breast milk or breast cancer. This suggests that the virus found in mice and humans is closely related.

Table 1 Prevalence of MMTV-like virus in human breast milk determined by DNA PCR, RT-PCR, and immunohistochemistry (IHC)

<table>
<thead>
<tr>
<th>Total No. tested by PCR</th>
<th>MMTV-like viral DNA positive</th>
<th>MMTV-like viral RNA positive</th>
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<tbody>
<tr>
<td>91</td>
<td>5/91 (5%)</td>
<td>2/91 (2%)</td>
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Table 1 Prevalence of MMTV-like virus in human breast milk determined by DNA PCR, RT-PCR, and immunohistochemistry (IHC)

Fig. 1 Sequence alignment of MMTV-positive env sequences from human breast milk. MMTV-like envelope gene DNA sequences from human breast milk (breast milk 1–7), human breast cancer (BC_AY161340), Australian wild mouse (AUS_wild mse) and HERV-K10 used in the phylogenetic tree (Fig. 1). The sequence homology between the human breast milk, human breast cancer, and wild mouse is very high. The sequence homology of HERV-K10 is very low compared to the other sequences. * indicates identical/consensus homology between sequences.

#breast milk 1: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 2: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 3: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 4: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 5: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 6: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 7: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#BC_AY161340: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#AUS_wild mse: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#HERV-K10: GGTAGAACCTTCTGCTGGAAATATCTCTTAAAGTCCCGAACAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---

#breast milk 1: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 2: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 3: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 4: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 5: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 6: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#breast milk 7: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#BC_AY161340: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#AUS_wild mse: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---
#HERV-K10: GCGCCTCAAGTGCAAGCTTTCTGCTGCTCCGAGCAAGATTTCTGCTCTAGGTCCCATACGAAAATGTTTTGCTTATGTT---

Fig. 2 Phylogenetic tree based on env and pol sequences from human breast milk samples (red boxes), human breast cancer (green boxes), Australian wild mouse (blue boxes), and HERV-K10 (dark green box) showing branch points and branching order.

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Staining for CD 45 (lymphocytes), CD 68 (macrophages), CD 138, and VS 38C (plasma cells) were all negative.

Discussion

In this study, we demonstrated the presence of MMTV-like viral RNA, DNA, and protein in cells in breast milk from healthy lactating women, with no evidence of mastitis or breast cancer.

Standard PCR is the best available technique for the identification of minute concentrations of viral sequences. However, as is well known, PCR techniques are subject to both false positive and false negative outcomes and are especially liable to contamination [15]. Therefore, corroboration of outcomes that are based on standard PCR is desirable. In situ PCR, while also subject to false positives and negatives, offers information about the cellular location of sequences and is also less liable to contamination than standard PCR. In addition, the use of immunohistochemistry with gp52/36 polyclonal antibodies allow the identification of the presence of MMTV envelope protein is useful but may not be specific [23]. Despite these reservations, the consistency of the outcomes using three different techniques, adds confidence to the findings. In addition, these current observations are consistent with those by Ford that MMTV env gene sequences were present in 6% of milk from normal women and by Moore et al. who identified putative MMTV particles also in approximately 5% of milk from normal women [4, 8].

Human endogenous retroviral (HERV)-K10 sequences are closely related but distinct from β retroviruses including MMTV-like virus, in the env/pol region of the viral genome [24]. Sequencing of the MMTV-positive amplicons demonstrated that MMTV-like viral env DNA was separate to HERV-K10, in agreement with previous studies by Mant et al. [24] (Fig. 1).

These current and past results indicate the presence of MMTV-like viral DNA, RNA, and protein in human breast milk. Accordingly, it is possible that MMTV-like virus is able to be transmitted via human breast milk as is

Fig. 2 Phylogenetic (rooted) tree of 252 bp of MMTV-like viral envelope (env) sequences. The tree shows the close relationship and lack of clustering of MMTV-like virus from human breast milk (breast milk 1, 2, 3, 4, 5, 6, 7) and breast cancer (Australian AY161340) compared to MMTV from an Australian wild mouse (AUS-wild mice) and a HERV-K10 sequence. The phylogenetic tree was constructed using the Neighbor Joining Method on MEGA and bootstrapped to replicate 1000 replications. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Fig. 3 Detection of MMTV-like viral envelope (env) DNA in putative epithelial cells from human breast milk. Panels a and b Positive MMTV env DNA stained blue in the nuclei of putative breast milk epithelial cells via in situ PCR. Panels c and d Negative in situ PCR no primer controls showing unstained nuclei indicating that the DNA in cells were not sheared and capable of self-priming. It is not possible to identify identical cells in both positive slides and negative control slides with in situ PCR studies based on centrifuged milk smears in contrast to fixed tissues. All images at 400× magnification.
MMTV in mice and other rodents. It is known that MMTV-like virus associated human breast cancer is more prevalent in gestational than other breast cancers, which demonstrates the influence of sex hormones [25]. Therefore, it is possible that the concentration of MMTV-like virus is high in colostrum and milk in the immediate post partum period when transmission of the virus is most likely to occur. This may explain the lack of association between breast feeding and breast cancer risk because the many past studies have not considered the exclusive exposure of new born infants to colostrum and breast milk during the newborn period [9]. There are crucial differences to the well being of newborn infants between those who have exclusive exposure to colostrum and breast milk and infants exposed to mixed feeding (colostrum, breast milk, and other liquids including water and foods) [26]. Exclusive breast feeding protects the integrity of the intestinal mucosa which offers a barrier to infections including HIV [26].

The cells typically found in human breast milk consist of macrophages, lymphocytes, and epithelial cells [27]. Although the exact nature of the cells containing MMTV-like viral DNA is unclear, our immunohistochemistry data indicate that these cells may represent exfoliated epithelial cells. In mice, lymphocytes are known to carry MMTV to the mammary gland during infection [28–30]. The nature of the cells infected with MMTV in breast milk from mice has not been investigated previously but our findings suggest that in humans, the virus could also be transported via plasma cells or macrophages to the breast where they gain entry into epithelial cells which are shed into breast milk during lactation.

The finding that MMTV-like virus is present in some human milk may also explain the 35% increased risk of breast cancer among women whose mothers had breast cancer [9]. The obvious alternate explanation for the increased risk is genetic susceptibility [31].

We are acutely aware of the adverse social implications of these findings as the benefits of breast feeding are overwhelming. However, it is our strong view, that it is essential to understand the biology of viruses and breast cancer so that rational responses can be developed. Knowledge about the transmission of HIV via human milk provides an excellent precedent [11]. This knowledge has stimulated research and has allowed the development of sound preventive and treatment strategies.

In 1942, John Bittner of the Jackson laboratories in Maine, USA, suggested that for mammary tumors to occur in mice three “agents” were required: (a) an active mammary tumor influence generally transferred by nursing, (b) hormonal stimulation of mammary tissue, and (c) an inherited susceptibility to the development of mammary tumors [3]. Some 70 years later, emerging evidence suggests these same three “agents” may have a role in some human breast cancers.

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Conflict of interest None.

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