Mouse Mammary Tumor Virus-like RNA Transcripts and DNA Are Found in Affected Cells of Human Breast Cancer

Caroline E. Ford,1,3 Margaret Faedo,1,2,3 and William D. Rawlinson1,2,3
1Virology Division, Department of Microbiology, South Eastern Sydney Area Laboratory Services, Prince of Wales Hospital, Randwick, Australia; and 2Faculty of Medical Sciences and 3Faculty of Biotechnology and Biomolecular Sciences, University of New South Wales, Kensington, Australia

ABSTRACT

Identifiable risk factors for the development of breast cancer include age, diet, family history, and lifetime estrogen exposure. An infectious agent (mouse mammary tumor virus; MMTV) is known to cause murine breast tumors and may be involved in the pathogenesis of human disease. Multiple studies have detected MMTV-like sequences in 30 to 60% of breast cancer samples and up to 1.8% of samples from normal breast. Using in situ PCR of MMTV-like sequences of formalin-fixed, paraffin-embedded breast tissue, viral sequences have been located in cancerous epithelial cells in breast acini of male and female breast tumors, but not in adjacent nonmalignant cells. MMTV-like sequences were also located in the epithelial cells of male gynecomastia samples. Using reverse transcriptase in situ PCR, RNA transcripts from the env gene were also detected within cancerous epithelial cells of 78% of DNA-positive tumors, 80% of gynecomastia samples, and 0% of normal tissues screened. This suggests the virus may be replicating in these cells. The epidemiologic and histopathological data are consistent with the association of an MMTV-like virus with breast cancers in men and women. The association with gynecomastia, a benign, possibly premalignant condition suggests hormonal influences, rather than cancer per se, may be the dominant factor in determining viral presence and replication.

INTRODUCTION

Breast cancer accounts for one fifth of all female cancers worldwide, yet the etiology of the disease remains unclear (1, 2). Many studies have identified risk factors for the development of breast cancer, such as age, diet, hormonal influences, and genetic predisposition (3–5), although a clear underlyng cause for the disease has not yet been established. Although it appears that most likely breast cancer is a multifactorial disease encompassing many different causes and factors, it has been suggested that an infectious agent may contribute to the development of human breast cancer (6–8). Other human cancers attributed to viral oncogenesis include cervical cancer and human papilloma virus, body cavity B-cell lymphoma and Epstein-Barr virus, Kaposi’s sarcoma and human herpesvirus 8, and hepatocellular carcinoma and hepatitis B and C viruses (9–12). Many animal models of cancer due to viruses exist, including breast cancer in mice due to the oncogenic retrovirus, mouse mammary tumor virus (MMTV; refs. 13, 14). MMTV has been shown to infect other tissues of the mouse, it is generally only the mammary gland that is malignantly transformed, with replication occurring within the epithelial cells of the mammary gland (14).

Because the advent of molecular technologies allowing the discrimination between MMTV and human endogenous retroviruses, MMTV-like sequences have been detected in numerous studies in up to 60% of breast biopsies from women with breast cancer (15–18), including the population studied in this article (19). Further studies have shown that these same sequences are rarely found in normal breast tissue from women with benign breast conditions, and adjacent normal tissue from women with breast cancer specimens positive for MMTV-like sequences (17, 19, 20). These studies support the link between an MMTV-like virus and human breast cancer, although this link has never been shown to be causal. These MMTV-like sequences will be hereafter referred to as MMTV, although this does not infer that these viral env sequences are zoonotic infection with MMTV.

One of the first steps in establishing causality between a virus and a disease is to show that the virus is consistently associated with the disease and is not associated with the disease-free state (21). This has previously been shown in at least three populations (15, 17, 18). We have additionally addressed this in previous studies of premalignant breast conditions and through a longitudinal cohort study (19). Another step is to show exclusive co-localization of virus with cancer cells (21). In this article, we now describe the development of an in situ PCR and reverse transcriptase-PCR method for the detection of MMTV in human breast cancer, to determine where in the breast MMTV RNA transcripts and DNA are located.

MATERIALS AND METHODS

Specimens and Samples. Breast cancer tissue specimens were formalin-fixed, paraffin-embedded, archival material from men and women who had undergone breast surgery at hospitals in Sydney, Australia. Archival breast tissue was also obtained from men with gynecomastia. Histologic analysis was carried out on all of the specimens with H&E staining (under South Eastern Area Health Service ethics approval 00/189).

DNA Extraction and PCR. DNA was extracted from formalin-fixed, paraffin-embedded tissues and nested PCR for a 356-bp env region of MMTV done as described previously (17).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene PCR was used to test the integrity of DNA extracted from archival material. Cycling conditions for GAPDH PCR with primers (GAPF, 5’ AAACCCCATCACCTCTCCCA 3; and GAPR, 5’GGCAGTATGGCATG-GACTG 3) consisted of an initial 5-minute denaturation at 95°C followed by 30 rounds of 94°C 30 seconds, 57°C 30 seconds, 72°C 30 seconds, and a final extension at 72°C for 3 minutes.

**In situ PCR Detection of MMTV.** In situ PCR targeted the 356-bp env region of MMTV, amplified previously from breast tissue (17), as this region has low homology to endogenous retroviruses (15). Four-micron sections of breast tissue were cut onto silanized slides for in situ PCR and reverse transcriptase-PCR. Paraffin was removed by heating slides at 70°C for 10 minutes followed by a 10-minute wash in xylene. Slides were washed sequentially in 100, 95, and 80% EtOH solutions and 0.1 mol/L Tris (pH 8.0) buffer for 10 minutes in each. Before amplification, sections were digested with 0.01% Triton X-100 followed by denaturation and washing in 0.1 mol/L Tris (pH 8.0) buffer. In situ PCR was done with a Hybaid Omnislide in situ PCR thermal reactor. Reaction mix containing deionized water, MgCl2 (2.5 mmol/L), Taq buffer [50 mmol/L KCl and 10 mmol/L Tris (pH 8.3)], deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP 0.25 mmol/L), primer 1 × (10 pmol), and primer 2NR (10 pmol; refs. 15, 17), Taq polymerase (1U, Promega, Madison, WI), and DIG-11-dUTP (0.01 mmol/L; Roche, Mannheim, Germany) was placed within a Geneframe (Abgene, Surrey, United Kingdom) on the slide, with a coverslip on top to maintain humidity. Cycling conditions consisted of an initial denaturation of 94°C for 5 minutes followed by 20 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 40 seconds, and a final extension of 72°C for 3 minutes.

A positive control (MMTV-positive breast tissue), negative control (MMTV-negative breast tissue), and controls omitting DIG-11-dUTP (to confirm incorporation of DIG label), omitting primers (to confirm signal was the result of specific amplification), and controls omitting reverse transcriptase were also included in experiments to confirm the specificity of the PCR for MMTV. After amplification, the Geneframe and coverslip were removed, and slides were washed in 1× SSC buffer at room temperature. Slides were then washed in Buffer 1 [Tris 100 mmol/L and NaCl 150 mmol/L (pH 7.5)] for 10 minutes, followed by immersion in blocking buffer (Tris 100 mmol/L and NaCl 150 mmol/L), 0.6% Triton X-100, and 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes. A 1:500 dilution of anti-DIG-alkaline phosphatase Fab fragments (Roche) prepared in blocking buffer was added, and slides were incubated for one hour at room temperature. Slides were washed in Buffer 1 and subsequently Buffer 3 [Tris 100 mmol/L, NaCl 150 mmol/L, and MgCl2 50 mmol/L (pH 9.0)], then a 1:50 dilution of nitroblue tetrazolium (0.33g/mL) to 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 0.16g/mL) substrate solution in Buffer 3 containing levamisole (1 mmol/L) was prepared, and color development was monitored. Once dark blue/purple staining was observed within cells, usually after 30 to 40 minutes, the reaction was stopped by removing excess substrate and washing in 1× SSC buffer and deionized water. Slides were immersed in a 70% EtOH solution, a 95% EtOH solution, and counterstained with eosin (6% w/v in 90% EtOH). Coverslips were placed on the slides, and sections were viewed under a light microscope. Positive results were defined as those slides containing cells with a specific, dark blue/purple stain, and negative results were defined as those exhibiting no or nonspecific background staining in cells.

**In situ Reverse Transcription-PCR Detection of MMTV.** The same 356-bp MMTV env region amplified with in situ PCR was targeted with reverse transcriptase in situ PCR. Specimens for reverse transcriptase in situ PCR were processed and dewaxed as for standard in situ PCR. Specimens were subjected to a 16-hour overnight DNase digestion before amplification. Reaction mix consisted of diethyl pyrocarbonate-treated water, MgCl2 (2.5 mmol/L), Taq Buffer [50 mmol/L KCl, and 10 mmol/L Tris (pH 8.3)], deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP 0.25 mmol/L), primer 1 × (10 pmol), primer 2NR (10 pmol; ref. 15), Taq polymerase (1 unit, Promega), DIG-11-dUTP (0.01 mmol/L; Roche), and reverse transcriptase (1 unit, Promega). Samples were incubated at 42°C for one hour preceding amplification to produce cDNA template. Separate equipment was used than for in situ PCR to avoid contamination, and controls omitting reverse transcriptase were included to detect any false positives from contaminating or residual DNA. The DNase digestion step was omitted as another control to confirm the specificity of the reverse transcriptase-PCR reaction.

**GAPDH In situ PCR.** In situ PCR was also done on tissues for the presence of GAPDH, a housekeeping gene. Methods were identical as for MMTV in situ PCR with the substitution of GAPDH primers (GAPF, GAPR) for MMTV primers. Cycling conditions and all of the other steps were identical. Controls as outlined for MMTV in situ PCR were also undertaken for GAPDH in situ PCR.

**In situ Hybridization Detection of MMTV.** Paraffin wax was removed as described previously for in situ PCR. After Tris-HCl wash, endogenous biotin in the tissue was blocked with avidin/biotin (Vector Laboratories, Inc.). Slides were then washed in 1× PBS for 10 minutes. Before hybridization, sections were digested with 0.01% Triton X-100, followed by denaturation, and washing in 0.1 mol/L Tris (pH 8.0) buffer. A hybridization cocktail was added to each slide, consisting of 100 μL hybridization buffer (2× SSC, 10% dextran, and 50% deionized formamide), 5 μL BSA (10 mg/mL), and ~5 ng of biotin-labeled MMTV probe (5′B TACATCGCCTGTGTTAC 3′). A glass coverslip was then added to each slide, and slides placed in the Hybaid in situ PCR machine and incubated for 3 hours at 55°C. After incubation slides were washed in Tris-HCl for 10 minutes, transferred to ice-cold 100% EtOH for 10 minutes, then rehydrated in 80% EtOH for 5 minutes. Slides were washed once more in Tris-HCl for 10 minutes, then 100 μL of alkaline phosphatase conjugated avidin [diluted 1:100 in buffer containing 100 mmol/L Tris (pH 9) and 150 mmol/L NaCl] added to each slide and incubated at 37°C for 30 minutes. Slides were washed in 1× PBS for 10 minutes, then 100 μL of nitroblue...
tetrazolium/BCIP [1:50 dilution of nitroblue tetrazolium (0.33g/mL) to BCIP (0.16g/mL) substrate solution in 0.1 mol/L Tris to 0.05 mol/L MgCl2 to 0.1 mol/L NaCl; 1 mmol/L levamisole] was added to each slide and incubated in the dark for ~1 hour until color development. Slides were counterstained and coverslipped, as for in situ PCR, and viewed under light microscopy.

RESULTS

MMTV sequences were detected with in situ PCR in cancerous epithelial cells of the acini of breast cancer specimens from women and men (Fig. 1; Table 1). The staining appeared to be generally nuclear. MMTV was detected in all grades of infiltrating ductal carcinoma, as well as ductal carcinoma in situ in female patients. A total of 33 female breast cancer samples were positive on in situ PCR, all of which were positive on solution PCR for MMTV. Staining was consistently noted in all of the cancerous epithelial cells of the breast tissue sections, but not in connective tissues or areas of normal histology on all 33 female breast cancer sections examined. Multiple (at least 2) sections were examined for each specimen, at 100× then 400× on a light microscope.

One case of an MMTV positive, histologically normal...
female breast tissue was also tested with in situ PCR, and an area of epithelial cells shown to stain positive for MMTV (Fig. 1E). The specimen was diagnosed as fibrocystic disease, and the patient from which the tissue was excised developed breast cancer 2 years later in the ipsilateral breast (also positive for MMTV), as discussed previously in more detail (19, 23).

MMTV sequences were also detected in the cancerous epithelial cells of six breast cancer samples and in the normal epithelial cells surrounding breast ducts in 10 gynecomastia samples from male patients (Table 1; Fig. 1). Staining in male breast cancer samples was detected in all areas of cancerous cells, in gynecomastia samples staining was found only in particular breast ducts. Approximately 50 to 80% of all cancerous cells, in gynecomastia samples staining was found positively, and the involved ducts were reproducible in consecutive slides on repeat testing.

Control sections were consistently negative for MMTV. The control reactions omitting Taq, DIG-11-dUTP, primers, or substituting nonsense primers confirmed the veracity of the results. As well as the controls outlined above, >40 breast cancer samples negative for MMTV on solution PCR (17) were also negative with in situ PCR. An additional 20 normal breast tissue samples that were negative for MMTV on solution PCR (17) were again negative on in situ PCR (Fig. 1F).

Viral transcripts from the env gene were detected with reverse transcriptase in situ PCR in all forms of breast tissue described above. The transcripts were found in the tissues in the same locations as MMTV detected with in situ PCR (epithelial cells). When slides were superimposed, the same cancerous epithelial cells stained positive for both MMTV DNA and RNA (Fig. 1, F1–F3). Controls with no reverse transcriptase included in the reaction mix were always negative for MMTV, indicating that the sequences detected via reverse transcriptase in situ PCR were because of amplification of RNA, and not contaminating DNA. Expression of the env region was detected with reverse transcriptase in situ PCR in 78% (40 of 51 samples) of samples positive for MMTV and in 0% of samples negative for MMTV on solution PCR (Table 1). The nine samples positive for MMTV on in situ PCR, but not reverse transcriptase in situ PCR (Table 1), showed absolutely no staining for MMTV RNA in any of the cells that were positive for MMTV DNA. Representative results of duplicate experiments are shown in Fig. 1.

### DISCUSSION

MMTV sequences were detected in the cancerous epithelial cells of female and male breast cancer patients and were not detected in the surrounding normal breast tissue or in normal breast tissue removed for cosmetic reasons from unaffected women. Previous studies with solution PCR to test breast cancer and normal breast tissue from the same individuals are consistent with this (20). This study reported >30% of breast cancer sections positive for MMTV and <1% of normal breast tissue positive for MMTV from the same women (20). We have also reported similar findings of extremely low positivity in both unrelated (normal breast tissue from cosmetic surgery) and related normal breast tissue (non-malignant breast tissue excised from the same breast containing MMTV sequences in the cancerous tissue; ref. 19).

One of the first tenets to be satisfied in establishing causality between an infectious agent and a disease is to show that the suspected etiological agent is consistently associated with the disease and is not associated with the disease-free state (21). MMTV sequences have been shown to be strongly associated with breast cancer and not with normal breast tissue in a number of populations with multiple methods, including the new technique described here of MMTV in situ PCR (15–20, 23–26). However, although not detected in normal breast tissue, we have detected MMTV in male gynecomastia for the first time. Gynecomastia is considered a benign breast disorder, although there is controversy as to the impact on the risk of subsequent breast cancer in men (27). Gynecomastia results from a disruption in normal hormone levels, which are most commonly elevated estrogen levels as a result of exogenous (drugs and alcohol) or endogenous factors (27). Elevated estrogen levels have also been associated with an increased risk of breast cancer in women, as well as in mice infected with MMTV (2, 28–30).

The finding of these sequences in epithelial cells of gynecomastia may have several explanations: (a) gynecomastia may be viewed as a precursor to breast cancer, and the detection of MMTV in a (possible) premalignant breast condition may support the hypothesis that MMTV sequences are associated with the development of breast cancer; or (b) gynecomastia may be viewed as a tumor-free state, and the detection of MMTV may not support the causal association between these sequences and breast cancer. If these gynecomastia results are taken alone, then these scenarios are equally likely. However, because of the consistent finding of the absence of MMTV in normal breast tissue related or unrelated to breast cancer, the first hypothesis appears more plausible, although unproven, at this time. These results also suggest that the hormonal milieu of the breast may be an important factor in additional investigations of the role of these sequences in breast cancer.

Seventy-eight percent of samples positive for MMTV were also shown to be positive with reverse transcriptase in situ PCR, whereas none of >60 samples negative for MMTV were positive via reverse transcriptase in situ PCR. These data are similar to the 66% (12 of 18) MMTV RNA expression in MMTV-positive, fresh breast cancer tissue samples described previously.

### Table 1  Detection of MMTV-like sequences with in-situ PCR and RT in-situ PCR of breast tissues previously positive for MMTV on solution PCR

<table>
<thead>
<tr>
<th>Gender</th>
<th>Pathology</th>
<th>No. tested</th>
<th>No. positive IS-PCR</th>
<th>No. positive RT IS-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>DCIS</td>
<td>2</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IDC I</td>
<td>8</td>
<td>8 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IDC II</td>
<td>13</td>
<td>13 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IDC III</td>
<td>10</td>
<td>10 (100%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>20</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>IDC II</td>
<td>5</td>
<td>5 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IDC III</td>
<td>1</td>
<td>1 (100%)</td>
<td></td>
</tr>
<tr>
<td>Gynecomastia</td>
<td>10</td>
<td>10</td>
<td>10 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DCIS, ductal carcinoma in-situ; IDC, infiltrating ductal carcinoma; IS-PCR, in-situ PCR; RT IS-PCR, reverse transcription in-situ PCR.
Retroviral Infection in Breast Cancer

(24). The detection of RNA transcripts with reverse transcriptase in situ PCR indicates that the MMTV sequences are being expressed, that the virus is active at a molecular level in the breast, and that viral DNA and RNA are physically coincident. Whether viral replication is occurring is unknown, because virus transcription and protein expression can occur at different levels than virus replication and DNA production (31).

There remains no evidence to date that MMTV is the direct cause of breast cancer in humans. It is possible these sequences represent an epiphenomenon; a virus that takes advantage of the proliferative nature of cancer cells as an ideal environment for growth and development (32), or a virus known to have hormone responsive elements responding to the hormonal milieu of human breast tissue (33). However, the detection of both viral DNA and RNA, and the detection only in cancerous epithelial cells and not normal tissue, suggests that MMTV may be a contributing factor in breast carcinogenesis.

Positive signals resulting from in situ PCR and reverse transcriptase in situ PCR appeared generally nuclear rather than cytoplasmic. If MMTV DNA detected via in situ PCR represents an inserted provirus, it would be expected that the positive signal would be located in the nucleus of the cancerous cells. Retroviruses act by entering the cytoplasm of a cell, where reverse transcriptase transcribes viral RNA to cDNA. This cDNA is then replicated and inserted into the host nucleic acid, in the nucleus. Cellular polymerases transcribe RNA, which is packaged into a new virion in the cytoplasm, and released to continue infecting other cells. Following on from this model, MMTV RNA would also be expected to be located in the nucleus if the gene was being transcribed.

The origin of MMTV sequences detected in human breast cancer is unknown. Some studies have suggested that these sequences represent a human form of MMTV (termed the human homologue of mouse mammary tumor virus; ref. 7), whereas others have suggested that these sequences are not, in fact, a separate virus, but rather MMTV acting as a zoonotic virus (34). An exogenous source of MMTV is supported by the geographical differences in breast cancer and MMTV sequence positivity rates in different countries around the world (16, 17, 34, 35).

MMTV sequences have now been detected in up to 40% of breast cancer samples from women in Western countries and in <2% of normal breast tissue by three separate research groups in three different populations (15, 17, 18). The morphologic data presented here, showing MMTV sequences and transcripts in cancerous epithelial cells of the breast, but not in surrounding normal breast tissue, add additional support to the hypothesis that MMTV is involved in human breast cancer (7). Given these results and the complexity of carcinogenesis, additional molecular pathogenetic studies are needed to distinguish causality from association.

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