Progression from Normal Breast Pathology to Breast Cancer Is Associated with Increasing Prevalence of Mouse Mammary Tumor Virus-Like Sequences in Men and Women

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ABSTRACT

Mouse mammary tumor virus (MMTV)-like sequences have been found in up to 40% of breast cancer samples but in <2% of normal breast tissue samples from Australian women studied by our group. Screening of a larger and more diverse cohort of female breast cancer samples has now shown a correlation of MMTV-like sequences with the severity (grade) of breast cancer. Thirty-two percent (43 of 136) of female breast cancer samples were positive for MMTV-like sequences when screened using PCR. A significant gradient of MMTV positivity was observed with increasing severity of cancer from 25% of infiltrating ductal carcinoma (IDC) grade I tumors to 34% of IDC grade II tumors (P = 0.00034) and 38% of IDC grade III tumors (P = 0.00002). We also report for the first time the detection of MMTV-like sequences in 62% (8 of 13) of male breast cancer samples and 19% (10 of 52) of male gynecomastia samples screened. MMTV-like sequences were demonstrated in various premalignant breast lesions of females, including fibroadenoma (20%) and fibrocystic disease (28%) samples, at a significantly higher prevalence than that seen in normal breast tissue (1.8%; P = 0.00001). Study of a longitudinal cohort of female breast cancer patients indicated that MMTV was coincident with tumor but was not present when tumor was absent on histology. These results support the association of MMTV-like sequences with development of breast tumors in men and women and suggest association of MMTV with increasing severity of cancer.

INTRODUCTION

The possible role of mouse mammary tumor virus (MMTV)-like sequences in human breast cancer remains controversial. The majority of recent reports linking MMTV and human breast cancer have come from one research group (1–4), including sequences of two complete proviral structures from human breast cancer tissue (5). A second group has confirmed these results and also reported the presence of MMTV-like sequences in lymphomas (6). We have independently reported the presence of MMTV-like sequences in 42% of Australian breast cancer samples and 1.8% of normal breast tissue samples (7), similar to the results in studies of American, Argentinian, and Italian women (1, 2). However, there have recently been two small studies reporting the absence of MMTV-like sequences in breast tumors from Italian (8) and Austrian women (9). Although all other studies to date demonstrate strong associations between the presence of MMTV-like sequences and human breast cancer, no causal link has yet been shown.

Investigations into the role of MMTV-like sequences in the progression from normal breast pathology to breast cancer were undertaken by examining a longitudinal cohort of breast cancer patients. We sought to determine whether MMTV-like sequences would be present in nonmalignant (NM) tissues and whether cases of MMTV-like sequences would be detected before positive breast cancer to further support the causal role of MMTV in tumorigenesis.

The prevalence of MMTV-like sequences has been shown to increase with severity of breast cancer from 1.8% in normal breast tissue to 26% in cases of ductal carcinoma in situ to 54% in infiltrating ductal carcinoma (IDC) cases in previous studies of Australian women (7). We have subsequently followed up the two cases of MMTV sequence-positive normal breast tissue (2 of 111, 1.8%) and learned that breast cancer was present in one patient before testing for MMTV and in one patient after testing for MMTV, in the contralateral and ipsilateral breasts, respectively (10). We have tested a larger and more diverse cohort of female breast cancer samples in this study to confirm our previous results showing a high prevalence of MMTV-like sequences in the Australian population. We also sought to correlate virus positivity with the specific grade of cancer in cases of IDC.

An area of controversy in the etiology of breast cancer is the effect of previous benign breast conditions (often referred to as premalignant breast lesions), such as fibrocystic disease, hyperplasia, and fibroadenoma, on the risk of subsequent breast cancer. These are relatively common breast lesions in women, although their contribution to the overall risk of breast cancer remains unclear (11, 12). Although not all women with a premalignant breast lesion develop breast cancer, it appears that a large number of patients do, and the reasons for this association remain unclear (13–17). Explanations include the involvement of an oncogenic virus (18) and the coexistence of hormonal factors in benign and malignant conditions.

Gynecomastia is a benign breast disorder of males, characterized by enlargement of the breasts, that often occurs during puberty and other periods of hormonal imbalance in the breast (19). The role of gynecomastia as a risk factor for breast cancer is contentious and is further complicated by the fact that many males choose to undergo surgery after the diagnosis of gynecomastia, thereby reducing the incidence of subsequent breast cancer (20).

The vast majority of reports linking MMTV-like sequences and breast cancer have studied tissue from women only. Male breast cancer is a rare yet severe cancer of men (21). Subsequently, due to the rarity of this disease in comparison with female breast cancer, much of what we know about the disease has been extrapolated from women (22). No clear cause of male breast cancer has yet been determined, and the risk factors for the development of the disease in men are still unclear (23). There has been one published study of the association between MMTV and male breast cancer (24). This study has potential confounders because human endogenous retroviruses with close sequence homology to MMTV may also have been detected by the methods used (1, 24). We have therefore examined for the first time using molecular techniques the prevalence of these gene sequences in male breast cancer samples.

In summary, this study uses a new approach to examine the possibility of involvement of a MMTV-like virus in the progression from
normal breast pathology to breast cancer. The prevalence of MMTV-like sequences was studied in female premalignant breast lesions, a graded cohort of female breast cancer samples, a longitudinal cohort of women with breast cancer, males with gynecomastia, and males with breast cancer. Results demonstrate the increasing prevalence of MMTV-like sequences in more severe tumors, association with tumors during overt cancer, and an apparent gradation of prevalence with the progression from normal to malignant pathology. These data support association of virus with tumorigenesis but also indicate wider prevalence of the virus in premalignant conditions.

**MATERIALS AND METHODS**

**Cell Lines and Tissues.** The NIH3T3 (MMTV-positive murine cell line) and MCF-7 (MMTV-like env-positive human breast tumor cell line) cell lines were cultured according to conditions recommended by the manufacturer (American Type Culture Collection, Manassas, VA) and used as positive controls for PCR.

Tissue culture was conducted in a separate laboratory in a separate area of the hospital to the main laboratory. All breast tissue samples were from tissue banked, paraffin-embedded tissue samples selected from the archives of the Department of Anatomical Pathology, Prince of Wales Hospital (Sydney, Australia) between the years 1995 and 2003. This study was approved by the South Eastern Sydney Area Health Services Ethics Committee (00/189). Samples in the longitudinal cohort were selected on the basis of the availability of multiple biopsies taken between 1995 and 2003. Breast tissue samples were graded histopathologically as either high- or low-grade ductal carcinoma in situ; grade I, II, or III IDC; infiltrating lobular carcinoma; or NM/normal tissue by an experienced histopathologist, and grading was confirmed by a second histopathologist (R. C.) (25, 26). DNA was extracted and quantitated from the cell line, original breast cancer samples, subsequent episodes, and premalignant breast lesions as described previously (7).

**Detection of MMTV-Like Sequences using PCR.** Samples were tested for housekeeping genes and MMTV-like sequences using standard PCR precautions and procedures to avoid contamination, as described previously (7, 27). Due to problems with fragmentation of DNA from formalin fixation (28, 29), a new internal primer (MMTV 5F, GTATGAAGCAGGATGGGTAGA) was specifically designed to amplify a smaller (190-bp) template of MMTV envelope gene, thereby allowing improved amplification of MMTV-like sequences. The first round of amplification using primers 1X and 2NR (1) was performed as described previously (7), followed by a semi-nested PCR step (primers MMTV 5F and 2NR), with cycling conditions of 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 72°C for 3 min. Samples positive using PCR were retested and sequenced to confirm the presence of MMTV-like sequences in the sample and rule out amplification of nonspecific or endogenous retroviral sequences (7). Sequences were analyzed using BLAST and EclustalW programs from the Australian National Genomic Information Service to compare sequences between positive longitudinal samples and previously reported MMTV-like sequences amplified from human breast tissue.

**Statistical Analysis.** Statistical analysis (Fisher’s exact test) was performed using the computer program SPSS Version 6.1.

**RESULTS**

**Longitudinal Cohort.** Thirty-six cases of breast cancer were tested using PCR, and 10 (28%) of these were positive for MMTV-like sequences (Fig. 1; Table 1). Four of the 10 MMTV-positive patients (patients A-D) had NM breast tissue excised adjacent to the MMTV-positive breast cancer tissue. All four cases consisted of an original local excision to remove tumor, followed by a full mastectomy to remove additional tissue and clear tumor margins. All of the NM tissues were negative for MMTV-like sequences. An additional four patients (patients D-G) with recurrences of breast cancer had both breast cancer samples positive for MMTV-like sequences. These four cases consisted of local excisions to remove tumor, followed by the removal of additional tissue and/or mastectomy. Sequencing of the samples from initial and recurrent tumors showed 100% nucleotide identity and between 96.5% and 97.4% identity to the MMTV C3H strain (GenBank accession numbers AY496179-AY496184). There were two cases in which the second biopsy specimen (following original mastectomy to remove breast tumor) was located elsewhere than the breast (patients H and I). In patient H, in whom the second tumor biopsy was located in the cervix, MMTV-like sequences were detected, whereas in patient I, in whom the second tumor biopsy was located in the neck, no MMTV-like sequences were detected. There was one case of a lymph node excised (axillary dissection) following MMTV-like gene sequence-positive breast cancer that was also positive for MMTV-like sequences (patient J). The lymph node was histopathologically graded as NM but was positive on PCR for...
MMTV-like sequences. This was not due to contamination because appropriate controls and procedures were undertaken as described above, and the result were reproducible. All secondary tumor biopsies were negative from the 26 breast cancer samples negative on initial PCR for MMTV-like sequences. Twenty-two of the 26 negative tumor samples were positive (32%). Of these 43 positive samples, a gradient of positivity was observed with increasing severity of breast cancer. The percentage of MMTV-positive tumors was significantly higher in IDC grade II (34%; \( P = 0.00034 \)) and IDC grade III tumors (38%; \( P = 0.000002 \)) when compared with IDC grade I tumors (23%; Table 2).

### Female Samples

An additional 136 female breast cancer samples were tested for the presence of MMTV-like sequences, and 43 samples were positive (32%). Of these 43 positive samples, a gradient of positivity was observed with increasing severity of breast cancer. The percentage of MMTV-positive tumors was significantly higher in IDC grade II (34%; \( P = 0.00034 \))) and IDC grade III tumors (38%; \( P = 0.000002 \)) when compared with IDC grade I tumors (23%; Table 2). Common breast lesions of females, identified as premalignant by some authors (11–17), were also tested for MMTV-like sequences because these conditions have been suspected to be risk factors for subsequent breast cancer. Five of 25 (20%) fibroadenomas, 7 of 25 (28%) fibrocystic disease, and 1 of 4 (25%) hyperplasia samples tested were positive for MMTV-like sequences. Sequences amplified from female premalignant tissues were between 95.8% and 97.6% similar to the MMTV C3H strain (GenBank accession numbers AY600608–AY600615). The sequence differences to MMTV C3H noted in female premalignant breast conditions were not different from those noted previously in female breast cancer samples (7). The difference in prevalence of MMTV-like sequences between all premalignant breast lesions and normal breast tissues was found to be highly significant (\( P = 0.000001 \); Table 2).

### Male Samples

Due to the extreme rarity of male breast cancer in the Australian and other populations, establishment of a large cohort was difficult (30). Eight of 13 (62%) male breast cancer samples were positive for MMTV-like sequences. Sequences amplified from male breast cancer tissue were between 96.6% and 98.6% similar to the MMTV C3H strain (GenBank accession numbers AY600596–AY600601). These 13 samples consisted of 8 IDC grade II tumors, 2 IDC grade III tumors, 2 ductal carcinoma in situ tumors, and 1 adenocarcinoma. Testing of male gynecomastia samples for the presence of MMTV-like sequences showed that 10 of 52 (19%) were positive, and these sequences were between 96.5% and 97.4% similar to the MMTV C3H strain (GenBank accession numbers AY600602–AY600607; Table 2). Whereas there were significant sequence differences between isolates from male breast cancer and gynecomastia and the MMTV C3H strain, there were no significant differences between sequences amplified from males and those amplified from females.

### DISCUSSION

These data contribute to the growing number of studies supporting the involvement (causal or epiphenomenon) of MMTV-like sequences in breast cancer. The presence of MMTV-like sequences in malignant breast tissue was highly significant (\( P = 0.000001 \); Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Tissue</th>
<th>Pathology</th>
<th>Surgery details</th>
<th>MMTV PCR</th>
<th>Time period (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>55</td>
<td>Breast</td>
<td>IDC II</td>
<td>Local excision</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>Breast</td>
<td>NM</td>
<td>Mastectomy</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>37</td>
<td>Breast</td>
<td>IDC I</td>
<td>Local excision</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>Breast</td>
<td>NM</td>
<td>Removal of additional tissue</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>64</td>
<td>Breast</td>
<td>IDC II</td>
<td>Local excision</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>65</td>
<td>Breast</td>
<td>IDC III</td>
<td>Local excision</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>50</td>
<td>Breast</td>
<td>IDC II</td>
<td>Removal of additional tissue</td>
<td>+</td>
<td>0.5</td>
</tr>
<tr>
<td>H</td>
<td>78</td>
<td>Breast</td>
<td>IDC II</td>
<td>Mastectomy</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>66</td>
<td>Breast</td>
<td>IDC II</td>
<td>Mastectomy</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>47</td>
<td>Breast</td>
<td>IDC I</td>
<td>Local excision</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymph Node</td>
<td>NM</td>
<td>Axillary dissection</td>
<td>+</td>
<td>0.75</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Total no. studied</th>
<th>MMTV PCR positive [no. (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>111</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>25</td>
<td>5 (20)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>4</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Fibrocystic</td>
<td>25</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Female breast cancer (total)</td>
<td>136</td>
<td>43 (32)</td>
</tr>
<tr>
<td>IDC ≤ grade I</td>
<td>8</td>
<td>2 (25)</td>
</tr>
<tr>
<td>IDC ≥ grade I</td>
<td>40</td>
<td>9 (23)</td>
</tr>
<tr>
<td>IDC grade II</td>
<td>56</td>
<td>19 (34)</td>
</tr>
<tr>
<td>IDC grade III</td>
<td>40</td>
<td>15 (38)</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gynecomastia</td>
<td>52</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Male breast cancer (total)</td>
<td>13</td>
<td>8 (62)</td>
</tr>
<tr>
<td>IDCIS</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>IDC grade II</td>
<td>8</td>
<td>5 (63)</td>
</tr>
<tr>
<td>IDC grade III</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>

**Table 2**: Summary of archival breast tissue samples tested using seminested PCR, showing the percentage of samples positive for MMTV-like sequences.

### Notes

`a` MMTV, mouse mammary tumor virus; IDCIS, ductal carcinoma in situ; IDC, infiltrating ductal carcinoma.

`b` Normal breast tissue was from reduction mammoplasties, as described previously (7).

One positive was shown to have had prior breast cancer; the other positive had subsequent breast cancer (10).

`d` Ductal carcinoma in situ and infiltrating ductal carcinoma were classified according to Refs. 25 and 26.

}\[P = 0.00034\] compared with IDC grade II; \( P = 0.000002 \) compared with IDC grade III (Fisher’s exact test).
in human breast cancer. There are several postulates that need to be fulfilled to establish a causal link between an agent and tumor development (31, 32). Firstly, it must be conclusively shown that the putative causative agent is consistently associated with the disease in question. Three independent groups have now demonstrated the presence of MMTV-like sequences in a large proportion of breast cancer samples, as opposed to normal breast samples (1, 6, 7). Additionally, MMTV-like sequences have also consistently been shown to be present in a very low percentage of normal breast tissues (0–1.8%), including those from within the same breast (1, 7, 33). Furthermore, the longitudinal study reported here shows that all NM breast tissues excised up to 24 months after breast cancer surgery are negative for MMTV-like sequences. The specificity of detecting MMTV-like sequences in virus-associated tumors is also supported by the 26 cases of breast cancer that were initially negative for MMTV and remained negative in their secondary tumor biopsy samples, which were located in the breast or elsewhere. The one other cancer positive for MMTV-like sequences was a case of papillary serous adenocarcinoma of the endocervix, a gynecological carcinoma (34, 35). This carcinoma occurred 4 years after a MMTV-like sequence-positive breast cancer was treated in the same woman.

The second postulate to be fulfilled in proving causality is to show that the suspected causal agent precedes the disease (31, 32). This has not yet been demonstrated in relation to MMTV-like sequences and human breast cancer. We have attempted to address this postulate by studying longitudinal breast cancer samples from a cohort of women, to follow the progression from normal breast pathology to breast cancer. The second approach taken to address this postulate was to test benign and possibly precancerous breast disease samples from both males and females for MMTV-like sequences.

For the first time, a clear gradient of samples positive for MMTV-like sequences with increasing severity of breast cancer has been demonstrated. The percentage of female breast cancer samples positive for MMTV-like sequences increased from 23% of IDC grade I tumors to 34% of IDC grade II tumors to 38% of IDC grade III tumors. Furthermore, the prevalence of MMTV-like sequences in premalignant breast lesions (20–28%) was lower than that of cancerous tissue yet significantly higher (P = 0.00001) than that recorded in normal breast tissue (1.8%; Ref. 7). The recent description that the two cases of MMTV-positive normal breast tissue both had episodes of breast cancer adds further support to the association of a MMTV-like virus with breast cancer (10). The gradient of MMTV prevalence with severity was also found in tumors taken from the male cohort, with 19% of gynecomastia samples positive for MMTV-like sequences compared with 62% of male breast cancer samples. The presence of MMTV-like sequences in fibroadenoma, fibrocystic disease, and gynecomastia samples is intriguing. There is evidence that these conditions increase a patient’s risk for subsequent breast cancer. If these are premalignant conditions, the presence of sequences from a possible oncogenic virus is of great interest. This raises the possibility that the presence of these sequences may act as a marker for future carcinogenesis. However, the possibility that these sequences do not contribute to the etiology of breast cancer and are instead an epiphenomenon cannot be ruled out at this stage. The absence of MMTV-like sequences from normal breast tissues anatomically related (NM tissue adjacent to MMTV-positive cancer tissue) and anatomically unrelated (normal breast tissue from reduction mammoplasties) to cancerous tissues suggests that these MMTV-like sequences do play a role in development of breast cell oncogenesis.

The connection between MMTV-like sequences and hormones is not yet clear; however, the results from this study suggest that some link exists. Breast cancer is a hormonally related tumor, as is endometrial carcinoma, both of which have been shown to be positive for MMTV-like sequences. The female premalignant breast lesions and male gynecomastia samples with significant rates of MMTV positivity (20–28% and 19%, Table 2) have strong links to hormonal imbalances of the breast (36, 37). Both MMTV and the putative human homolog of the virus have been shown to have hormone-responsive elements in the long terminal repeat regions of their genomes (5, 38). We have previously proposed a multifactorial model for breast carcinogenesis involving viral infection, hormones, and genetic elements (18), and these additional data are consistent with such a model.

Overall, these new data support the association of a MMTV-like virus with human breast cancer. This study was conducted independently of earlier studies reporting a high prevalence of MMTV-like sequences in human breast cancer (1, 6) and is in direct contrast to two recent negative reports questioning the association of MMTV-like sequences with breast cancer (8, 9). We have carefully refined the detection of MMTV-like sequences in formalin-fixed, paraffin-embedded breast tissues and used a different approach to the question of causality or epiphenomenon than previously undertaken. This is the first report of MMTV-like sequences in male gynecomastia samples and the first report to use molecular techniques to describe the prevalence of these sequences in male breast cancer and female premalignant breast lesions. These data, taken together, add to the growing number of studies implicating a MMTV-like virus in human breast cancer, although a clear causal relationship of MMTV to breast cancer remains to be established.

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