Featured Article

Clonal Isolation of Different Strains of Mouse Mammary Tumor Virus-Like DNA Sequences from Both the Breast Tumors and Non-Hodgkin’s Lymphomas of Individual Patients Diagnosed with Both Malignancies

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ABSTRACT

Purpose: In a previous study, we had detected the presence of mouse mammary tumor virus (MMTV)-like envelope (ENV) gene sequences in both the breast tumors and non-Hodgkin’s lymphoma tissue of two of our breast tumor patients who had been diagnosed simultaneously with both malignancies. The aim of this study was to determine if MMTV-like DNA sequences are present in the breast tumors and non-Hodgkin’s lymphomas of additional patients suffering from both malignancies and if so to characterize these sequences in detail.

Experimental Design: DNA was extracted from formalin-fixed, paraffin-embedded tissue sample blocks of breast tumors and non-Hodgkin’s lymphomas from patients suffering from both malignancies. A 250-bp region of the MMTV ENV gene and a 630-bp region of the MMTV long terminal repeat (LTR) open reading frame (ORF) that encodes the MMTV superantigen (sag) gene were amplified by PCR from the isolated DNA. Amplified products were analyzed by Southern blotting, cloned, and sequenced.

Results: MMTV-like ENV and LTR sequences were detected in both the breast tumors and non-Hodgkin’s lymphomas of 6 of 12 patients suffering from both malignancies. A novel mutant of the MMTV ENV gene was identified in these patients. Characterization of the MMTV-like LTR highly variable sag sequences revealed total or nearly total identity to three distinct MMTV proviruses from two different branches of the MMTV phylogenetic tree.

Conclusions: The presence of MMTV-like ENV and LTR sequences in both the breast tumors and non-Hodgkin’s lymphomas of 6 additional patients suggests a possible involvement of these sequences in these two malignancies. MMTV-like LTR sequence homology to different MMTV proviruses revealed the presence of more than one strain of MMTV-like sequences in each individual suggesting the possibility of multiple infections in these patients.

INTRODUCTION

Non-Hodgkin’s lymphoma is significantly more prevalent in women with breast cancer than in the general population. We have shown that in 87 patients with both malignancies 78 patients (89.7%) had breast cancer diagnosed first or concurrently with non-Hodgkin’s lymphoma (1). The percentage of patients expected to have this temporal relationship between the two diagnoses was between 34% and 45% and, thus, in our study breast cancer was diagnosed before or simultaneously with lymphoma more frequently than expected (P < 0.001). This statistically significant increased incidence of non-Hodgkin’s lymphoma in breast cancer patients suggests a relationship between the development of these two malignancies that is not related to therapy for either neoplasm. Numerous additional studies have also reported the occurrence of breast cancer and lymphoma in the same patient and have suggested a common link between breast cancer and non-Hodgkin’s lymphoma (2–6).

In the inbred mouse system the betaretrovirus mouse mammary tumor virus (MMTV) supplies a common link between the development of breast cancer and lymphoma. MMTV is the etiological agent for the development of breast tumors (7–9) as well as certain B- and T-cell lymphomas in the mouse (10–12). Published data from three separate laboratories including our own have strongly suggested the involvement of MMTV-like sequences in a certain percentage of human breast tumors (13–16). The possibility that MMTV-like sequences may also be involved in human lymphoma development was suggested by the finding that two of the breast tumor patients in our earlier study that had been diagnosed simultaneously with non-Hodgkin’s lymphoma were also positive for the MMTV ENV gene in their malignant lymphoid tissue (15). Additional laboratory observations suggesting a relationship between human breast cancer and lymphoma include the increased incidence of lymphoma seen in mice inoculated with human mammary cancer extracts (17) and the frequent development of mouse T-cell
lymphomas after implantation of human inflammatory breast cancer cells in nude mice (18).

In this report we present data for the presence of MMTV-like envelope (ENV) and long terminal repeat (LTR) sequences in both the breast tumors and non-Hodgkin’s lymphomas of 6 patients suffering from both malignancies. A novel mutant of the MMTV ENV gene was identified in these patients. Phylogenetic characterization of the MMTV-like LTR sequences revealed that more than one strain of MMTV-related sequences could be isolated from individual patients.

MATERIALS AND METHODS

Human Tissue. Formalin-fixed, paraffin-embedded tissue sample blocks of breast tumors and non-Hodgkin’s lymphomas from patients suffering from both malignancies were obtained under a protocol approved by the Institutional Review Board of Our Lady of Mercy Medical Center. Hematoxylin and eosin stained slides of each paraffin block were made and read to determine the location of malignancy in each sample block. Blocks were shaved into 5-µm thick serial sections, and two sections from each specimen were used for DNA extraction. DNA was isolated using a microwave technique (19). DNA from normal lymphocytes of normal individuals was isolated using the QIAamp mini kit (Qiagen, Valencia, CA). DNA from lymphocytes of patients suffering from acute lymphocytic leukemia was kindly provided by Dr. Janis Racevskis (Our Lady of Mercy Medical Center, Comprehensive Cancer Center, Bronx, NY). To determine the quality of the isolated DNA, either p53 or globin primers was used in PCR, and the resulting amplified products were electrophoresed in 1% agarose gels.

PCR. Conditions for MMTV ENV gene amplification were the same as described previously with the exceptions that MgCl$_2$ concentration was increased to 3 mM and 3.5 mM, each 50 µL reaction contained 0.5 µL (0.5 units) of Perfect Match PCR Enhancer (Stratagene, La Jolla, CA), and reactions using primers designated ENV 2 and ENV 3 (13, 15) were not nested. PCR conditions for amplification of MMTV LTR sequences were the same as for envelope with the exception that MgCl$_2$ concentration was at 3.5 mmol/L. Primers (LTR 3 and LTR 5) and cycling conditions were those of Wang et al. (20). To detect possible contamination of master mix components, a reaction without template DNA was routinely tested. P53 or globin primers were also used as control for master mix and polymerase activity. The PCR product was analyzed by electrophoresis in 1% agarose gels. dX 174 RF DNA cut with the restriction enzyme HaeIII was used as a marker to identify the size of the PCR products.

Hybridization. PCR products were hybridized on Southern blots (21) under stringent hybridization conditions to either a 23-bp probe specific for DNA sequences present in exogenous MMTV ENV sequences but not present in endogenous human endogenous retroviral sequence (HERV-K) DNA (13, 15, 22, 23) or to a 20-bp probe specific for the MMTV LTR and also not present in endogenous human endogenous retroviral sequence (HERV-K) DNA (20, 22, 23). The 23-bp ENV probe, which extended from position 7822–7845 in the MMTV genome, and the 20-bp LTR probe, which extended from position 972–991 or 9545–9564 in the MMTV genome (24), were 5’ end-labeled with [32P]ATP using the RTS T4 kinase labeling kit (Life Technologies, Inc., Rockville, MD). Stringent hybridization conditions were as described previously (15, 23, 25).

Cloning and Sequencing of PCR Products. In most cases amplified DNA products were cloned directly from the PCR tube using the TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies, Inc., Carlsbad, CA). In some instances PCR products were eluted from agarose gels using the Qiagen QIAquick Gel Extraction kit and then cloned as described above. DNA sequencing was performed by Genemed Synthesis (South San Francisco, CA). The resulting sequences were compared to known published sequences and to sequences in the GenBank. Genbank accession nos. for ENV sequences are AY659980-AY659985 and for LTR sequences the nos. are AY652964-AY652978.

Phylogenetic Analysis. The LTR sequences obtained from cloned fragments after PCR amplification were compared to known exogenous and endogenous MMTV sequences in the GenBank database. LTR sequences were aligned using DNAAssist 2.0 software and analyzed using the Phylogenetic Analysis Using Parsimony (PAUP 4.0b10) program (26). A heuristic search using a branch-swapping algorithm of tree-bisection-reconnection was used for 100 bootstrap replicates examining a matrix of 29 taxa with 668 nucleotides of aligned sequence to generate a bootstrap 50% majority-rule consensus tree. All of the nucleotides were given equal weight; 391 were constant, 137 were parsimony-uninformative, and 140 were parsimony-informative. The JYG (27), FM (28), and SW21 (29) sequences from Asian mice were used to root the tree.

RESULTS

Presence of MMTV-Like Sequences in Both Breast Tumors and Non-Hodgkin’s Lymphomas. DNA from breast tumors and lymphomas of 12 patients suffering from both malignancies was screened using PCR with primers specific for a 250-bp region of the MMTV ENV gene and primers specific for a 630-bp region within the MMTV LTR open reading frame (ORF) that codes for the MMTV superantigen (sag) gene (30–32). Primer sequences used for these amplifications are specific for the MMTV ENV and LTR genes and are not found in any human endogenous retroviral sequences such as the human endogenous retroviral sequence-Ks (HERV-Ks) (13, 15, 20, 22, 23). Table 1 describes the malignancies of each of the 12 patients whose tissues were analyzed for MMTV-like ENV and LTR sequences and the ages at which their two malignancies were diagnosed. Multiple histologic types of both breast cancer and non-Hodgkin’s lymphoma were present in the 12 patients. Six patients (Table 1, A–F) contained both breast tumor and lymphoma DNA that was amplified by the MMTV primers into the expected size DNA fragments. Amplification of DNA of lymphocytes from 4 normal individuals and from lymphocytes of 10 patients suffering from acute lymphocytic leukemia was negative for the presence of MMTV-like ENV or LTR sequences.

MMTV-Like ENV Gene Sequences in Both Breast Tumors and Non-Hodgkin’s Lymphomas. Fig. 1A, top panel, represents the ethidium bromide-stained agarose gel electrophoresis of an MMTV ENV primed PCR from 3 different
patients suffering from both diseases who are MMTV-like ENV gene positive, and Fig. 1B, top panel, is the hybridized Southern blot. Lanes 2, 4, and 5 contain breast tumor DNA whereas lanes 3, 6, and 7, respectively, represent lymphoma DNA from the corresponding patient. Lane 8 is DNA amplified from an MMTV-like ENV gene-positive human breast tumor of a breast cancer patient who does not have lymphoma. Fig. 1A, bottom panel, represents the ethidium bromide-stained agarose gel electrophoresis of an MMTV ENV primed PCR from 2 breast tumor lymphoma patients (1 positive and 1 negative for the MMTV-like ENV gene sequences), normal lymphocyte DNA, and lymphocytes from leukemic patients, and Fig. 1B, bottom panel, is the hybridized Southern blot. Lanes 2 and 3 contain breast tumor and lymphoma tissue DNA, respectively, from 1 of 6 MMTV-like ENV gene-positive patients, lanes 4 and 5 contain breast tumor and lymphoma tissue DNA, respectively, from 1 of 6 MMTV-like ENV gene-negative patients, lanes 6 and 7 contain lymphocyte DNA from 2 of 4 normal individuals, and lanes 8 and 9 contain DNA from lymphocytes of 2 of 10 leukemic patients analyzed. Lane 1 in both panels represents our negative control that contains no template DNA. Southern blot hybridization was done under stringent hybridization conditions to avoid any endogenous sequences that might interact with the probe. Positive hybridization results with the radiolabeled 23-mer oligonucleotide probe that contained MMTV ENV gene sequences indicated that this MMTV-specific sequence was present in the amplified 250-bp fragment and that the bands in the agarose gel were not artifactual. Negative hybridization results reinforced the ethidium bromide staining data.

The amplified MMTV ENV gene-specific 250-bp sequences present in the breast tumors and lymphomas of each of the 6 positive patients were cloned using the Invitrogen TOPO TA Cloning kit for sequencing. A total of 21 clones was sequenced from the amplified lymphoma DNA and 17 from the amplified breast DNA. The sequences of the amplified fragments were shown to be 98–100% homologous to MMTV in this 250-bp region of the ENV gene. Fig. 2 contains the cloned MMTV-like ENV gene sequences from the lymphoma tissue (Fig. 2A) and the breast tissue (Fig. 2B) of the 6 breast cancer/non-Hodgkin’s lymphoma patients compared with GR, C3H, and BR6 strains of MMTV. This 250-bp domain of the MMTV ENV gene in the GR strain is 100% homologous to the C3H strain and 98% homologous to the BR6 strain (24, 33, 34). Except for the mutation described below, 18 lymphoma clones (3 per patient) were identical to the GR/C3H sequence in this 250-bp region, whereas 3 lymphoma clones (2 from patient B and 1 from

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/BCA</th>
<th>BCA/histology</th>
<th>Age/NHL</th>
<th>NHL/histology</th>
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<tr>
<td>A</td>
<td>69</td>
<td>Infiltrating lobular carcinoma</td>
<td>83</td>
<td>B-cell diffuse, intermediate grade</td>
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<td>B</td>
<td>70</td>
<td>Infiltrating ductal carcinoma</td>
<td>70</td>
<td>Follicular in one axillary lymph node, mixed small cleaved cell and large cell type</td>
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<tr>
<td>C</td>
<td>63</td>
<td>Carcinoma in situ, high grade comedonecrosis</td>
<td>63</td>
<td>T-cell diffuse, mixed, in laryngeal nodule and supraclavicular node</td>
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<tr>
<td>D</td>
<td>78</td>
<td>Infiltrating ductal carcinoma</td>
<td>79</td>
<td>Nodular, poorly differentiated, lymphocytic, mesenteric lymph nodes</td>
</tr>
<tr>
<td>E</td>
<td>68</td>
<td>Infiltrating ductal carcinoma</td>
<td>86</td>
<td>T-cell, left inguinal node and left adjacent skin nodule</td>
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<tr>
<td>F</td>
<td>61</td>
<td>Infiltrating poorly differentiated ductal carcinoma, intraductal comedo carcinoma</td>
<td>61</td>
<td>Nodular poorly differentiated lymphocytic in 3 proximal axillary, 1 midaxillary, and the paratracheal lymph nodes</td>
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<td>G</td>
<td>56</td>
<td>Infiltrating ductal carcinoma</td>
<td>70</td>
<td>Diffuse, large cell</td>
</tr>
<tr>
<td>H</td>
<td>72</td>
<td>Infiltrating ductal carcinoma</td>
<td>72</td>
<td>Diffuse mixed small cell in multiple axillary lymph nodes</td>
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<tr>
<td>I</td>
<td>59</td>
<td>Infiltrating ductal carcinoma</td>
<td>47</td>
<td>Diffuse, large cell involving left inguinal node</td>
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<tr>
<td>J</td>
<td>61</td>
<td>Intraductal carcinoma</td>
<td>64</td>
<td>T-cell, small cell and intermediate size</td>
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<tr>
<td>K</td>
<td>82</td>
<td>Infiltrating ductal carcinoma</td>
<td>83</td>
<td>Diffuse, large cell</td>
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<tr>
<td>L</td>
<td>67</td>
<td>Intraductal carcinoma</td>
<td>78</td>
<td>Large cell</td>
</tr>
</tbody>
</table>

Abbreviations: BCA, breast cancer; NHL, non-Hodgkin’s lymphoma.

Table 1 Patients diagnosed with both BCA and NHL

Fig. 1 Amplification of 250 bp of MMTV-like ENV gene by PCR. A, ethidium bromide stained 1% agarose gel electrophoresis; B, Southern blot hybridization using 5’ P32 end-labeled 23-mer probe. Top, lanes 2, 4, and 5, breast tumor DNA from MMTV-like ENV gene-positive breast cancer/non-Hodgkin’s lymphoma patients; lanes 3, 6, and 7, represent respectively lymphoma DNA from the corresponding patient; lane 8, breast tumor DNA from MMTV-like ENV gene-positive breast cancer patient not diagnosed with lymphoma; bottom, lanes 2 and 3, breast tumor and lymphoma tissue DNA, respectively, from MMTV-like ENV gene-positive patient; lanes 4 and 5, breast tumor and lymphoma tissue DNA, respectively, from MMTV-like ENV gene-negative patient; lanes 6 and 7, normal lymphocyte DNA; lanes 8 and 9, DNA from lymphocytes of leukemic patients. Lane 1 in both panels, negative control contains no template DNA; M, molecular weight marker. Arrow, 250-bp band.
Fig. 2  Sequences of the 250-bp PCR ENV gene product amplified from the DNA of breast cancer tissue and non-Hodgkin’s lymphoma tissue from 6 patients suffering from both malignancies compared with the GR, C3H, and BR6 strains of MMTV. *, same nucleotide. The numbers 1, 2, and 3, indicate the three locations where the BR6 strain differs from the C3H and GR strains in this region of the MMTV ENV gene. The A and ↓ indicate the location at which a mutation was found in the amplified ENV gene of the breast and lymphoma patients. The numbers 7656 and 7905 indicate the location of the MMTV 250-bp ENV gene sequence within the MMTV genome (24). A, 18 LYM represents the sequences of 18 cloned ENV gene products from lymphoma tissue of 6 patients (3 clones per patient), which contained the mutation and were totally homologous to the GR and C3H strains of MMTV in this region of the ENV gene. 3 LYM represent the 3 additional lymphoma clones (2 from patient B and 1 from patient D), which also contained the mutation but were similar to BR6 at position 3. B, 8 Br represents sequences of 8 cloned ENV gene products from the breast tumors of 6 patients (1 each from patients A-D) that were 100% homologous to C3H/GR. 3 Br represents 3 clones (2 from patient E and 1 from patient F) that were similar to BR6 at position 2, and 2 Br represents 2 clones (patient A) that had homology to BR6 at positions 2 and 3.
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A mutation fragment present in all 21 of the clones and, thus, in every patient differed at position noted 3 from GR/C3H but were identical with BR/6 at this site (Fig. 2A). The mutation fragment present in all 21 of the clones and, thus, in every patient D) differed at position noted 3 from GR/C3H but were the same as BR/6 at this site (Fig. 2A). Within the 250-bp PCR fragment present in all 21 of the clones and, thus, in every lymphoma sample, we detected the presence of a single base change mutation denoted by the arrow in Fig. 2A. The mutation of a G to A resulted in the replacement of an alanine (GCA) with a threonine (ACA) in these samples.

Breast tumors differed from the lymphomas in that in addition to having sequences containing the mutation described above, every breast tumor also contained at least one cloned ENV sequence without the mutation. Thus, although both normal and mutated forms of the ENV gene were found in the breast tumors, only the mutated form of the ENV gene has been seen in the lymphomas. All of the 8 cloned MMTV-like breast sequences (1 each from patients A-D and 2 each from patients E and F) containing the mutation had 100% sequence homology to the GR and C3H strains of MMTV (Fig. 2B). Of the 9 breast clones containing no mutation, 4 clones (1 each from patients A-D) were 100% homologous to C3H/GR, 3 clones (2 from patient E and 1 from patient F) were similar to BR6 at position 2, and 2 clones (patient A) had homology to BR6 at position 2 and 3.

MMTV-Like LTR Sequences in Both Breast Tumors and Non-Hodgkin’s Lymphomas. Fig. 3A, top panel, represents the ethidium bromide-stained agarose gel electrophoresis of an MMTV LTR-primed PCR from 4 different MMTV-like LTR-positive patients suffering from both diseases, and Fig. 3B, top panel, is the hybridized Southern blot. Lanes 2–5 contain breast tumor DNA, whereas lanes 6–9, respectively, represent lymphoma DNA from the corresponding patient. Fig. 3A, bottom panel, represents the ethidium bromide-stained agarose gel electrophoresis of an MMTV LTR-primed PCR from 2 breast tumor/lymphoma patients (1 positive and 1 negative for the MMTV-like LTR gene), 2 normal individuals, and 2 leukemic patients, and Fig. 1B. bottom panel, is the hybridized Southern blot. Lanes 2 and 3 contain breast tumor and lymphoma tissue DNA, respectively, from 1 of 6 MMTV-like ENV-gene positive patients, lanes 4 and 5 contain breast tumor and lymphoma tissue DNA, respectively, from 1 of 6 MMTV-like ENV gene-negative patients, lanes 6 and 7 contain lymphocyte DNA from 2 of 4 normal individuals, and lanes 8 and 9 contain DNA from lymphocytes of 2 of 10 leukemic patients tested. Lane 1 in both panels represents our negative control that contains no template DNA. The amplified 630-bp LTR DNA products from 4 breast tumors (Fig. 3, top panel, lanes 2–5) and from 3 lymphomas (Fig. 3, top panel, lanes 6, 7, and 9) were successfully cloned and sequenced. Multiple clones were analyzed from each patient. On occasion we have seen PCR products as doublets in the MMTV-like LTR region. Because for the most part cloning was done directly from the PCR reaction using the Invitrogen TOPO-TA cloning kit individual doublet bands were not isolated before cloning.

The U3 region of the LTR of MMTV harbors an ORF that encodes a glycoprotein sag that is present in all of the exogenous and endogenous MMTV proviruses (30–32). The sag protein interacts with the immune system of mice to cause either deletion or expansion of specific subsets of T cells that express a particular T-cell receptor V-beta(B) chain corresponding to the hypervariable region of the sag protein. Although highly conserved, the MMTV sag sequences are not identical with ~35% of the total variation clustered at the hypervariable COOH terminus. This COOH-terminal variation is responsible for the specificity of the deletion and/or expansion of Vβ-carrying T lymphocytes in mice. This variation present in the COOH terminus is specific to each provirus and, thus, allows for identification of the presence of different viral strains. To analyze relationships between viral strains, phylogenetic trees have been constructed on the basis of alignments of LTR ORF sequences (35).

In the conserved regions of the LTR ORF all 15 of the clones (7 from breast and 8 from lymphomas) shared 96–98% homology with numerous strains of MMTV and with each other. Thus, no one clone was 100% homologous to another clone or to any other published MMTV LTR sequence. Within the highly variable COOH terminus of the sag gene multiple clones isolated from individual patients showed sequence differences from known strains of MMTV and from each other. However, all of the isolated clones were either identical or nearly identical to either the MMTV endogenous proviral sequences Mtv-8 (36, 37) or Mtv-6 (38) and Mtv-1 (39, 40) as shown in Fig. 4. Thus, comparison of the highly variable regions of the MMTV sag with these regions in our PCR-amplified products resulted in either total or nearly total homology of each of our clones to one of three different MMTV-like proviruses. Each of the 4 patients for which cloned and sequenced MMTV-like LTR fragments were available contained COOH-terminal sag sequences that were homologous to more than one MMTV provirus. Table 2 lists the MMTV-like LTR clones present in each of 4 patients (Table 1, A–D) and the MMTV provirus to which the COOH-terminal sag sequences in these clones are homologous.

The results of phylogenetic analysis of the entire 630-bp LTR ORF sequences isolated from patients suffering from both

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**Fig. 3** Amplification of 630 bp of MMTV-like LTR gene by PCR. A, ethidium bromide stained 1% agarose gel electrophoresis; B, Southern blot hybridization using 5’ P32 end-labeled 20-mer probe. Top, lanes 2–5 represent breast tumor DNA from MMTV-like LTR gene positive breast cancer/non-Hodgkin’s lymphoma patients; lanes 6–9 represent, respectively, lymphoma DNA from corresponding patient; bottom, lanes 2 and 3, breast tumor and lymphoma tissue DNA, respectively, from MMTV-like LTR gene-positive patient, lanes 4 and 5, breast tumor and lymphoma tissue DNA, respectively, from MMTV-like LTR negative patient; lanes 6 and 7, normal lymphocyte DNA; lanes 8 and 9, lymphocyte DNA from leukemic patients. Lane 1 in both panels, negative control contains no template DNA; M, molecular weight marker. Arrow, 630-bp band.
breast cancer and non-Hodgkin’s lymphoma are shown in Fig. 5. Both breast tumor-derived clones and lymphoma-derived clones segregate to two separate branches of the MMTV phylogenetic tree (35). Moreover, clones isolated from each individual patient segregate to these two separate branches. Patient A has two lymphoma-derived clones segregating to the upper branch and one breast tumor derived clone and another lymphoma derived clone segregating to the lower branch. Patient B has two lymphoma-derived clones and one breast tumor-derived clone segregating to the upper branch and one breast tumor-derived clone and one lymphoma-derived clone on the lower branch. Patient C has one breast-derived clone on one branch and one breast-derived clone on the other branch, and no lymphoma-derived clones were available for patient C. Patient D has one lymphoma clone on the upper branch and two breast tumor-derived clones and one lymphoma-derived clone on the lower branch. Previously identified MMTV-like LTR sequences isolated from human patients also segregate on these two branches of the MMTV tree. MMTV-like LTR sequences AF346815 and AF243039 isolated from human breast tumors (14, 20) and an MMTV-like LTR isolate from gestational breast cancer AY325271 (41) segregate with the upper branch of the tree, whereas the MMTV-like LTR isolates from primary biliary cirrhosis patients, AF513913 and AF512923 (42), segregate to the lower branch of the tree.

**DISCUSSION**

Numerous reports have suggested a relationship between human breast cancer and non-Hodgkin’s lymphoma (1–6). Our results that indicate the presence of MMTV-like ENV and LTR sequences in both the breast tumors and non-Hodgkin’s lymphomas of 6 patients suffering from both malignancies suggest that a viral etiology may exist in certain cases of patients suffering from both malignancies. In addition, we have previously detected MMTV-like ENV gene sequences in the breast tumors and non-Hodgkin’s lymphomas of two additional women who had been diagnosed simultaneously with both malignancies and in the non-Hodgkin’s lymphomas of 3 of 19 patients who had been diagnosed with lymphoma alone (15). The 250-bp PCR-derived ENV gene sequences found in these malignancies were 98–100% homologous to the MMTV ENV gene. A number of our ENV gene clones contained a single base change that resulted in the replacement of an alanine with a threonine. The location of this amino acid change at position 317 in the MMTV gp 52 ENV sequence is not near the MMTV receptor binding site that is located at amino acid positions 40–44 (43). Sequences from multiple clones isolated from each of the 6 individual patients indicated the presence of both mutated and nonmutated ENV gene sequences in breast tumors but only the mutated form in lymphomas. It is possible that the
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Table 2  Cloned MMTV-like LTR sag gene sequences from the same breast cancer/non-Hodgkin’s lymphoma patient that are homologous to more than one MMTV provirus

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<th>Patient</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Breast LTR clone A-B1 homologous to Mtv-6</td>
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<tr>
<td></td>
<td>Breast LTR clones A-L1 and A-L2 homologous to Mtv-8; A-L3 homologous to Mtv-1</td>
</tr>
<tr>
<td>B</td>
<td>Breast LTR clones B-B2 homologous to Mtv-8, B-B3 homologous to Mtv-1</td>
</tr>
<tr>
<td></td>
<td>Breast LTR clones B-L4, B-L5 homologous to Mtv-8; B-L6 homologous to Mtv-1</td>
</tr>
<tr>
<td>C</td>
<td>Breast LTR clones C-B4 homologous to Mtv-6; C-B5 homologous to Mtv-8</td>
</tr>
<tr>
<td>D</td>
<td>Breast LTR clones D-B6, D-B7 homologous to Mtv-1</td>
</tr>
<tr>
<td></td>
<td>Breast LTR clones D-L7 homologous to Mtv-1; D-L8 homologous to Mtv-8</td>
</tr>
</tbody>
</table>

“wild-type” virus mutates in the breast cancer and that the mutant is the only virus that is able to survive in the lymphocyte precursor giving rise to lymphomas. However, we had seen this mutation for the first time a few years ago in a male patient who was diagnosed with only non-Hodgkin’s lymphoma and whose malignant tissue was positive for the MMTV-like ENV gene.³ This mutation has not been detected in MMTV-like ENV DNA sequences from >30 breast tumors from patients suffering from only breast cancer (13, 15, 44).³

The MMTV-like LTR sequences present in both the breast tumors and lymphomas of our breast tumor/lymphoma patients contain the ORF that codes for the MMTV sag gene. Although highly homologous to MMTV, none of our cloned LTR sequences is totally identical throughout the amplified 630 bp to each other or to any previously published MMTV LTR sequence, including those isolated from human patients (14, 20, 41, 42). We believe this difference in individual clones derived from our breast tumor/lymphoma patients suggests that our PCR products were not amplified from a point source mutation. In the mouse system, the MMTV variant known as Type B leukemogenic virus that is associated with lymphoma development contains a deletion in the LTR ORF that results in a truncated sag (45, 46). We have not detected such a deletion in the LTR-cloned sequences from our lymphoma patients. Recent work has shown, however, that the truncated Type B leukemogenic virus sag protein is dispensable for virally induced T-cell lymphomas (47).

Different laboratory mouse strains contain a characteristic subset of the known MMTV proviruses, and because this variation present in the COOH terminus of the LTR ORF sag gene is specific to each MMTV provirus, it thus allows for identification of the presence of different viral strains (48). Comparing the differences found at the COOH termini of different MMTV proviral DNAs, Brandt-Carlson et al. (35) established an MMTV phylogenetic tree that grouped the known proviruses into three to four families: two separate families of genetically transmitted endogenous proviruses, which they labeled A (containing MMTV proviruses Mtv-1 and Mtv-6) and C1 (containing MMTV provirus Mtv-8), a family containing both endogenous and exogenous proviruses designated B, and an exogenous virus-producing family called C2. Phylogenetic analysis of the 630-bp sequences of our cloned LTR ORF sag gene clones to known MMTV LTR sequences showed that our clones fell into the two families A and C1 containing genetically transmitted MMTV endogenous proviruses. The finding that our clones segregate to two branches of the MMTV phylogenetic tree and do not form a distinct branch of their own argues for their being mouse derived and not human homologues of the mouse sequences from the 4 patients are listed (U40459) are endogenous proviral sequences of MMTV. Sequences used to root the tree. Mtv-8 (J0222), Mtv-1 (X63024), and Mtv-6 (27), FM (28), and SW21 (29) sequences are from Asian mice and were used to root the tree. Mtv-8 (J0222), Mtv-1 (X63024), and Mtv-6 (U40459) are endogenous proviral sequences of MMTV. Sequences from the 4 patients are listed A to D and the individual clones are labeled B or L to designate their isolation from breast tumor or lymphoma. Previously published human isolates include AF346815, AF243039, and AF3513923 from primary biliary cirrhosis patients (42).

![Phylogenetic analysis of human breast tumor and non-Hodgkin’s lymphoma MMTV-like LTR sequences. A matrix composed of 29 taxa was generated with 668 nucleotides of aligned LTR sequence. All nucleotides were given equal weight; 391 were constant, 137 were parsimony-uninformative, and 140 were parsimony-informative. The tree was generated with a heuristic search and is the result of 1000-iteration bootstrap procedure using the program PAUP (26). Percentages are the frequencies of assortment in an individual branch after the bootstrap procedure and indicate the robustness of branch assignments. Branch lengths are indicative of the number of nucleotide changes to individual branch points (small numbers and see scale bar). The JYG (27), FM (28), and SW21 (29) sequences are from Asian mice and were used to root the tree. Mtv-8 (J0222), Mtv-1 (X63024), and Mtv-6 (U40459) are endogenous proviral sequences of MMTV. Sequences from the 4 patients are listed A to D and the individual clones are labeled B or L to designate their isolation from breast tumor or lymphoma. Previously published human isolates include AF346815, AF243039, and AF3513923 from primary biliary cirrhosis patients (42).](image-url)

³ Unpublished observations.
4 B. G-T. Pogo, personal communication.
sequence. Interestingly, all of the prior human LTR isolates with homology to MMTV, AF346815, AF243039, AY325271, AF513913, and AF513923 (14, 20, 41, 42) have also been associated with these two branches of the phylogenetic tree; however, no one patient has been reported to show association with more than one strain of MMTV.

Our data are the first to suggest that more than one strain of MMTV-like sequences may be present in individual patients suffering from both breast cancer and non-Hodgkin’s lymphoma. These data also provide evidence for possible multiple infections in the same patient. Although there are arguments for the rarity of multiple infections to occur (49, 50), the possibility that these infections can occur sequentially has been demonstrated in mice (51). Recent data from Sarkar et al. (52) showing that RIII/Sa mice with a high incidence of mammary tumors produce two strains of exogenous MMTV and one potential endogenous strain of MMTV suggests the possibility of infection by multiple strains of MMTV from a single strain of mouse.

Although the MMTV proviruses that have the greatest homology to our cloned sequences are endogenous to the inbred mouse, they have not been detected as endogenous sequences in the human genome (42). The suggestion that MMTV exogenous infection can occur in humans is highly controversial, yet it is consistent with our phylogenetic data and with numerous previous studies that suggest such an infection may be occurring (13–16, 20, 41, 44, 53). Most recently, exogenous MMTV infection has been implicated in another human disease, primary biliary cirrhosis (42). Viral particles were identified in biliary epithelium by electron microscopy, and retroviral MMTV-like nucleotide sequences were cloned from patients with primary biliary cirrhosis. Normal biliary epithelial cells were shown to develop phenotypic manifestation of primary biliary cirrhosis when cocultivated in serial passage with supernatants containing the MMTV-like betaretrovirus from primary biliary cirrhosis patients or with MMTV.

In the mouse, exogenous MMTV is passed to offspring through the milk of the mother and replicates in gut-associated lymphoid tissue before traveling to the breast and salivary epithelium. The primary targets for exogenous MMTV infection in the mouse are the T and B cells located in Peyer's patches of the gastrointestinal tracts of neonatally infected pups (54). The MMTV-infected B cells express the MMTV gag on their surface that, in turn, recognizes and stimulates T cells with a Vβ sequence corresponding to the hypervariable region of the gag protein. Different strains of MMTV will stimulate different T-cell receptors, and increased T-cell expansion allows, in turn, for increased B-cell expansion supplying more cells for MMTV infection (55). The MMTV-like beta retrovirus in primary biliary cirrhosis patients appears to be preferentially located in lymphoid tissues with a limited viral abundance in liver where the end organ damage occurs in this disease (42). Similarly, human breast tumors appear to have a limited abundance of MMTV-like sequences, whereas the presence of MMTV-like sequences has been reported in human lymphocytes of breast cancer patients (56, 57). These findings argue for the possible ability of MMTV to somehow infect human lymphoid tissue. If indeed such infection of human B and T cells can occur this may allow not only for the passage of MMTV to the breast as in the mouse but also to function as an etiological agent of disease in the lymphoid tissue itself. Spontaneous lymphomas arise in >90% of SJL mice by the age of 12 months and are of B-cell origin (58). The cells of these lymphomas stimulate T cells via expression of endogenous MMTV gag, and the cytokines produced by these activated T cells promote growth of the lymphomas. In addition, it has been shown that MMTV gag can stimulate human T cells (59).

Certainly the presence of viral-like sequences in any disease can only suggest that such sequences may be related to the etiology of the disease. However, the presence of different strains of MMTV-like sequences in the same two human malignancies that are also caused by this virus in the mouse and the presence of MMTV-like sequences in an additional human disease in which in vitro infection by the mouse virus itself mimics characteristics of the disease certainly strengthens the argument for additional research into the possibility of a viral etiology in these diseases.

ACKNOWLEDGMENTS

We thank Dr. Bashir Alobed and Dr. Stanley Oiseth of the Department of Pathology at Our Lady of Mercy Medical Center (OLM) for their expertise in evaluating our paraffin blocks for study. We also thank Marie Alice Lilazois and Julio Aviles in OLM Pathology for their excellent skill in preparing shavings of paraffin blocks for DNA isolation. We very much appreciate the efforts of Patricia Marino and Kelly Brown in our department for their work in locating breast cancer/non-Hodgkin’s lymphoma patients. We thank Elizabeth Montgomery of the Johns Hopkins Medical Institute for her contribution of paraffin blocks for our study.

REFERENCES


Clonal Isolation of Different Strains of Mouse Mammary Tumor Virus-Like DNA Sequences from Both the Breast Tumors and Non-Hodgkin's Lymphomas of Individual Patients Diagnosed with Both Malignancies
