# Sequences Homologous to the Mouse Mammary Tumor Virus *env* Gene in Human Breast Carcinoma Correlate with Overexpression of Laminin Receptor[1]

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## ABSTRACT

**We previously reported that a 660-bp sequence that is homologous to the *env* gene of the mouse mammary tumor virus (MMTV) but not to endogenous retroviruses or to other known genes was present in 38% of human breast cancers and in some breast cancer cell lines studied (Y. Wang *et al.*, Cancer Res., *55:* 5173–5179, 1995). Here, we have investigated whether the MMTV-like sequences were associated with the clinical, pathological, and molecular parameters that have been reported to define two subsets of human breast cancers.**

**Archival breast carcinoma samples were analyzed for four clinical parameters, obtained from patients' records, and for six pathological characteristics. Expression of c-erbB-2, p53, bcl-2, progesterone receptor, laminin receptor, and cathepsin D was detected by immunochemistry using monoclonal antibodies. PCRs were used to amplify 250 bp of the MMTV *env* gene-like sequence. The $\chi^2$, log-rank, and generalized Wilcoxon tests were used to analyze the data.**

**The MMTV *env* gene-like sequence was detected in 37.7% of the samples. The presence of this sequence was not significantly associated with any of the pathological clinical or biological parameters studied. It did correlate, however, with expression of the laminin receptor, a marker for invasiveness and poor prognosis. This is the first phenotypic characterization of human breast cancers containing retroviral sequences.**

Received 12/7/98; revised 5/4/99; accepted 5/10/99.

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[1] Supported by the T. J. Martell Foundation for Leukemia, Cancer and AIDS Research; the Chemotherapy Foundation; the Kash Family Foundation; and the Ellen Block Research Fund.

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## INTRODUCTION

Evidence that an agent similar to the MMTV[3] may be involved in the development of human breast cancer has been controversial, as obtained from older investigative techniques (1). We have recently found that a 660-bp sequence that is homologous to the *env* gene of MMTV but not to endogenous retroviruses or to other known genes is present in 38% of human breast cancers and in several breast cancer cell lines (2). This sequence is expressed in 66% of the human breast cancers that contain it and in all of the breast cancer cell lines that have the sequence (3). The sequence is not present in other tumors, and only rarely has it been found in normal tissues of patients or controls (2).

In our previous publications (2, 3), 90% of the samples studied were invasive ductal carcinomas with or without positive nodes, and because of this, it was not possible to correlate the presence of MMTV-like sequences with pathological types (2). Moreover, other clinical characteristics did not indicate the type of tumor with which the retroviral sequence was associated. We have, however, found that the MMTV *env* gene-like sequence is present more frequently in tumors from women with a family history of breast cancer, tumors discovered during pregnancy or lactation, and breast cancers from certain geographical locations (4).

Examination of clinical, pathological, and molecular parameters by multicorrespondence analysis has led to the definition of two distinct subsets of breast carcinomas with different outcomes, suggesting that different events lead to transformation (5). Here, we tested the hypothesis that MMTV-like sequences were associated with one of the two subsets. A panel of 74 archival breast carcinoma coded samples previously distributed between the two types was then analyzed for the presence of MMTV *env* gene-like sequences.

The results indicated that the presence of the *env* sequence is not associated with any of the pathological or clinical parameters that determine the two categories or with other molecular markers analyzed, with one exception. A positive correlation was found between the presence of the *env* sequence and increased expression of the monomeric laminin receptor of $M_r$ 67,000.

## MATERIALS AND METHODS

Paraffin-embedded sections of breast carcinomas were obtained from patients operated upon at the Istituto Nazionale per lo Studio e la Cura dei Tumori (Milan, Italy). Primary tumor

[3] The abbreviations used are: MMTV, mouse mammary tumor virus; MAb, monoclonal antibody; PgR, progesterone receptor; OS, overall survival; DFS, disease-free survival.

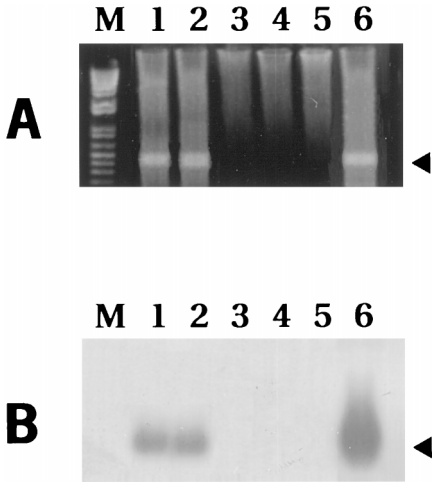


*Fig. 1* Amplification of 250 bp of MMTV-like *env* gene. DNA was extracted from paraffin-embedded breast carcinomas. PCR was performed using primers 2N and 3N. *A*, 1% agarose gel electrophoresis. *B*, Southern blot hybridization using 5′ $^{32}$P-labeled 2aN as probe. *Lanes M*, 1-kb plus ladder; *Lanes 1* and *2*, *env*-positive samples; *Lanes 3* and *4*, *env*-negative samples; *Lanes 5*, negative control (without DNA); *Lanes 6*, positive control (EK2 cell line).

size and axillary nodal status were obtained from pathological records. H&E-stained slides were used for assessment of the histological type, Bloom-Richardson grading, necrosis, leukocyte infiltration, and mitotic index.

Immunohistochemistry was performed as reported previously (5), using MAbs against c-erbB-2 (MAb cB11, 1:60; Ylem, Avezzazno, AQ, Italy), p53 (MAb DO7, 1:500; Novocastra, Newcastle-upon Tyne, United Kingdom), bcl-2 (MAb 100, 1:20; a gift from Dr. David Mason, Royal Marsden National Health Service Trust, Sutton, United Kingdom), PgR (MAb 1A6, 1:20; DBA, Segrate, MI, Italy), laminin receptor (MLuC5), and cathepsin D (1:300; Novocastra). Immunostaining was performed by a peroxidase-streptavidin method on paraffin-embedded material.

DNA extraction and amplification of MMTV *env* gene-like sequences and of the estrogen receptor gene were performed as described previously (2), with some modifications. Primers 2N and 3N were used to amplify a 250-bp segment of the *env* gene. The product of the reaction was run on a 1% agarose gel along with a 1-kb plus DNA ladder (Life Technologies, Inc.) as a molecular weight marker. DNA from EK2 cells (2) was amplified as a positive control. Hybridization was performed using the $^{32}$P-labeled oligomer 2aN as a probe and conditions as described (2) Sequences for primers were as follows: 2N, 5′-CCT ACA TCT GCC TGT GTT AC (positions 1386–1405); 3N, 5′-ATC TGT GGC ATA CCT AAA GG (positions 1640–1621); and 2aN, 5′-CCG TAC GTG CTG CTA CCT GTA (positions 1557–1577). The amplification reaction was carried out with 100 pmol of primers using PCR BEADS from Amersham Pharmacia Biotech, UK, Ltd. Each reaction contained, in 25 μl, the following components: 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each nucleotide, and stabilizers. Thermocycling was performed in a DNA cycler (Perkin-Elmer Corp.) by denaturation at 94°C for 1–5 min, annealing at 55°C for 1 min, and elongation at 72°C for 1–5 min for 35 cycles.

*Table 1* Correlation between MMTV *env* sequences and clinical parameters

| | *env*-positive tumors | | *env*-negative tumors | |
|---|---|---|---|---|
| Parameter | No. cases/ total | % | No. cases/ total | % |
| Tumor diameter of <2 cm | 16/26 | 62 | 26/43 | 60 |
| Nodal positivity | 11/23[a] | 48 | 26/41[a] | 63 |
| Age of <50 yr | 10/26 | 38 | 16/43 | 37 |
| Premenopause | 12/26 | 46 | 15/41[a] | 37 |

[a] For some cases the data are missing.

**Statistical Methods.** Relationships between the presence or absence of MMTV *env* gene-like sequences and the individual characteristics that determined the patient categorization (5) were assessed by the $\chi^2$ test. The $\chi^2$ test was also used to compare the proportions of *env*-positive cases in the three laminin receptor groups (0, <50, and ≥50% laminin receptor-positive tumor cells). Survival comparisons between MMTV *env* gene-positive and negative patients were made by the log-rank and generalized Wilcoxon tests for censored survival data (6).

## RESULTS

**MMTV *env* Gene-like Sequences.** The presence of the 250-bp sequence was studied in breast carcinoma DNAs that had first been tested for DNA quality control, by amplification of a 150-bp sequence of the estrogen receptor gene (2). Seventy-four samples were processed, but only 69 amplified the estrogen receptor. These 69 were then used for amplification of the 250-bp *env* gene sequence. By Southern blot hybridization with an internal probe, 26 (37.7%) of the breast carcinomas showed the MMTV *env* gene-like sequence. A typical gel/hybridization result is shown in Fig. 1.

**Correlation of MMTV *env* Sequences with Clinical Parameters.** The results from the analysis of four clinical parameters (tumor size, node metastasis, age, and menopausal status) were compared with the presence or absence of the *env* sequence. No significant difference was found in the four parameters studied with respect to presence or absence of MMTV *env* gene-like sequences (Table 1).

In addition, median OS and median DFS were also analyzed. Median DFS was 7.8 years for *env*-positive patients *versus* 14.9 years for negative patients. Median OS was 13.1 years for *env*-positive tumor patients *versus* 15.1 years for *env*-negative tumor patients. With the sample size studied, these differences were not statistically significant.

**Correlation between MMTV *env* Sequences and Pathological Parameters.** Six pathological characteristics were correlated with the presence of *env* gene sequences: ductal histotype, associated ductal carcinoma *in situ*, grade III, lymphoid infiltration, presence of necrosis, and a high mitotic index. None was found to correlate with the presence of *env* gene sequences. The results are shown in Table 2.

*Table 2* Correlation between MMTV *env* sequences and pathological parameters

| Parameter | *env*-positive tumors | | *env*-negative tumors | |
|---|---|---|---|---|
| | No. cases/ total | % | No. cases/ total | % |
| Ductal histotype | 19/26 | 73 | 34/43 | 79 |
| Associated DCIS[a] | 5/26 | 19 | 12/43 | 28 |
| Grade III | 13/26 | 50 | 21/43 | 49 |
| Lymphoid infiltration | 10/26 | 38 | 19/43 | 44 |
| Presence of necrosis | 13/26 | 50 | 21/43 | 49 |
| High mitotic index | 13/26 | 50 | 21/43 | 49 |

[a] DCIS, ductal carcinoma *in situ*.

*Table 3* Correlation between MMTV *env* sequences and molecular parameters

| Parameter | *env*-positive tumors | | *env*-negative tumors | |
|---|---|---|---|---|
| | No. cases/ total | % | No. cases/ total | % |
| c-erbB-2 overexpression | 10/26 | 38 | 14/43 | 33 |
| p53 alterations | 12/26 | 46 | 13/43 | 30 |
| PgR expression | 13/26 | 50 | 22/43 | 51 |
| Bcl-2 expression | 15/26 | 58 | 22/43 | 51 |
| Cathepsin D expression | 9/26 | 35 | 23/43 | 53 |
| Laminin receptor expression | 22/26[a] | 85 | 25/43 | 58 |

[a] $P = 0.02$.

**Correlation between MMTV *env* Sequences and Biological Markers.** Six molecular markers were also analyzed in *env*-positive and -negative tumors. There was no statistically significant correlation between overexpression of c-erbB2, p53 mutation, expression of bcl-2, PgR, or cathepsin D, with presence of *env* sequences (Table 3). When the relationship between *env* gene sequences and the laminin receptor expression was examined, however, a statistically significant correlation was found ($P = 0.02$; Table 3). A comparison between the number of cells expressing the laminin receptor and *env* gene sequences indicated that the frequency of MMTV *env*-positive tumors increased with the number of cells expressing the receptor ($P = 0.04$), as shown in Table 4.

As expected by the lack of association between the presence of MMTV sequences and any of the eight parameters included to evaluate the tumor phenotype, *i.e.*, lymphoid infiltration, necrosis, mitosis, c-erbB-2, p53, PgR, and bcl-2 (5), the phenotype classification did not correlate with MMTV sequence because 13 of 35 phenotype A tumors *versus* 13 of 34 phenotype B tumors were MMTV positive.

## DISCUSSION

These results obtained in tumors from Italian women demonstrate that 37.7% of the breast carcinomas analyzed contain MMTV *env* gene-like sequences, extending our previous findings of 38% in American women (2). These carcinomas are equally represented in the two phenotypes described by Ménard *et al.* (5). Furthermore, the component parts of Ménard's index exhibit no correlation with the presence of *env* gene sequences. Individual analysis of four clinical, six pathological, and five molecular parameters showed no correlation with the presence of retroviral sequences, indicating that the presence of the MMTV-like sequences are not confined to any tumor subset.

*Table 4* Correlation between MMTV *env* sequences and laminin receptor levels

| % laminin receptor-positive tumor cells | *env*-positive tumors | | *P* |
|---|---|---|---|
| | No. cases/total | % | |
| 0 | 4/22 | 18 | |
| <50 | 10/25 | 40 | 0.04 |
| ≥50 | 12/22 | 55 | |

The survival data indicate a shorter DFS for MMTV-positive tumor patients but a similar OS. The difference in DFS, although quite impressive, does not reach statistical significance because the analyzed series is too small. The different behaviors of DFS and OS suggest differences in the sites of relapse or tumor behavior after relapse between the MMTV-positive and -negative tumors. These observations require testing a large series of patients that are similarly treated to address this issue appropriately.

From the different molecular characteristics that are not part of the Ménard's index, a positive correlation was found between the presence of MMTV-like *env* gene detection and laminin receptor expression. How are these two parameters associated? The $M_r$ 67,000 protein, characterized as laminin receptor, has been found to be significantly increased in a variety of cancer cells (7). Its overexpression, observed as increase in frequency of tumor cells scoring as strongly positive, has been recorded in 44- 55% of the breast cancers, and it has been correlated with invasive phenotype and poor prognosis (5, 8, 9). Interestingly, the laminin receptor has been found to bind several different molecules, and it has been shown to be a receptor for Sindbis virus in mammalian cells (10) and also for the prion responsible for the spongiform encephalopathies (11). This indicates the intrinsic capability of this receptor to react with proteins. It has been also found to be part of the translation machinery in a variety of tumor cells, including mouse leukemia cells infected with the Friend virus (12). Covalently bound fatty acids have recently been detected in the laminin receptor, thus providing a mechanism for membrane association (13). It is provocative to speculate that the laminin receptor can also act as an MMTV-like virus receptor. Recently, a potential receptor for MMTV has been isolated from mouse cells. A human homologue to this receptor was also found to be expressed in several human tissues (14). Because there is no sequence homology between the MMTV receptor and the laminin receptor or between laminin and gp52 (the envelope glycoprotein of MMTV), the role of the laminin receptor as a possible receptor for a virus similar to MMTV cannot be sustained on this evidence. Virus penetration is a multistep process, however, that may have more than one membrane associated protein or receptor. Furthermore, viruses may use the intrinsic cellular mechanisms for recognition and adhesion (15).

The origin of the MMTV *env* gene-like sequences in humans, whether exogenous or endogenous, is still unknown. We have detected other MMTV-like genes in human breast cancer

that are highly homologous to those of MMTV but not to any other known endogenous retroviral sequence (16). These results support the presence in 38% of American and Italian women's breast cancers of a human virus similar to MMTV. The possibility that unknown endogenous retroviral sequences are amplified in breast carcinomas, although highly unlikely, cannot be completely ruled out. It is significant that MMTV variants that can infect human cells have been described (17).

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