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Tumorigenesis and Neoplastic Progression

A Mouse Mammary Tumor Virus *env*-Like Exogenous Sequence Is Strictly Related to Progression of Human Sporadic Breast Carcinoma

Chiara Maria Mazzanti,* Mohammad Al Hamad,* Giovanni Fanelli,* Cristian Scatena,* Francesca Zammarchi,* Katia Zavaglia,* Francesca Lessi,* Mauro Pistello,† Antonio Giuseppe Naccarato,* and Generoso Bevilacqua*

From the Division of Surgical, Molecular, and Ultrastructural Pathology and the Department of Experimental Pathology,† University of Pisa and Pisa University Hospital, Pisa, Italy*

A viral etiology of human breast cancer (HBC) has been postulated for decades since the identification of mouse mammary tumor virus (MMTV). The detection of MMTV *env*-like exogenous sequences (MMTVels) in 30% to 40% of invasive HBCs increased attention to this hypothesis. Looking for MMTVels during cancer progression may contribute to a better understanding of their role in HBC. Herein, we analyzed HBC preinvasive lesions for the presence of MMTVels. Samples were obtained by laser microdissection of FFPE tissues: 20 usual-type ductal hyperplasias, 22 atypical ductal hyperplasias (ADHs), 49 ductal carcinomas *in situ* (DCISs), 20 infiltrating ductal carcinomas (IDCs), and 26 normal epithelial cells collateral to a DCIS or an IDC. Controls included reductive mammoplastic tissue, thyroid and colon carcinoma, and blood samples from healthy donors. MMTVels were detected by fluorescence-nested PCR. DNA samples from the tissues of nine patients were analyzed by real-time quantitative PCR, revealing a different viral load correlated with stage of progression. Furthermore, as never previously described, the presence of MMTVels was investigated by chromogenic *in situ* hybridization. MMTVels were found in 19% of normal epithelial cells collateral to a DCIS or an IDC, 27% of ADHs, 82% of DCISs, and 35% of IDCs. No MMTVels were found in the control samples. Quantitative PCR and chromogenic *in situ* hybridization confirmed these results. These data could contribute to our understanding of the role of MMTVels in HBC. *(Am J Pathol 2011, 179:2083–2090; DOI: 10.1016/j.ajpath.2011.06.046)*

Despite the fact that breast carcinoma represents the most frequent cancer in women and that it has been largely studied worldwide for many decades, its etiology is largely unknown. The hereditary transmission of pathogenetic mutations of some predisposing genes, such as *BRCA1* and *BRCA2*, is the only factor recognized as causative in this disease.[1] Although the pathogenetic role of estrogens is well demonstrated, sound evidence of their etiological effect is still lacking.[2] A minimal percentage of cases are caused by radiation.[3]

On the other hand, in mice, the etiological role of mouse mammary tumor virus (MMTV) in the development of tumors of the mammary gland has been demonstrated for a long time.[4] Notably, much of what is known about the pathogenesis of human breast carcinoma (HBC) was learned by the experimental model of the MMTV-induced mouse mammary tumors.[5,6] In particular, the concept of cancer progression and the recognition of the so-called preinvasive lesions as morphological steps of its development are based on the MMTV model.[7] Moreover, the promotional role of estrogens was built on the observations conducted in mice.[8,9] The strong similarities between the human and the murine disease led to a half-century quest for a possible viral etiology of breast carcinoma in women. MMTV viral antigens were found in human breast tumors,[10] MMTV particles were described in human cells and milk,[11,12] and MMTV sequences were

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C.M.M. and M.A.H. contributed equally to this work.

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Current address of F.Z: Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, New York, New York.

Address reprint requests to Generoso Bevilacqua, M.D., Division of Surgical, Molecular, and Ultrastructural Pathology, University of Pisa and Pisa University Hospital, via Roma 57, 56126 Pisa, Italy. E-mail: generoso.bevilacqua@med.unipi.it.

found in humans.[13] Unfortunately, these data were never considered conclusive mainly because of their scarce reproducibility. Moreover, until a few years ago, the involvement of viruses in the etiology of human solid tumors did not receive much attention and the researchers were not stimulated in this direction. Finally, the identification of endogenous MMTV sequences in humans was a discouraging element.

In 1995, Wang et al[14] selected a 660-bp region of MMTV with a nucleotide sequence identity of only 16% to human endogenous retroviral element K10, which is highly similar to MMTV, and 90% to 98% identical to the MMTV envelope (*env*) gene. By using MMTV-specific primers located in the 660-bp region, Wang et al[14] identified an MMTV *env* gene–like exogenous sequence (MMTVels) in 38% of a series of human infiltrating breast carcinomas. On the other hand, MMTVels was found in only 2% of normal human breast samples. Several other groups[15–17] were able to confirm these data; however, negative results were also published.[18,19] These discrepant findings could be the consequence of differences in technical procedures, tissue heterogeneity, and the facts that there are only a few copies of *env* sequences present and earlier techniques were not highly sensitive. In 2006, Zammarchi et al,[20] from this laboratory, designed a rigorous methodological approach able to overcome these difficulties, based on the application of a laser microdissection procedure and a highly sensitive fluorescence-nested PCR. The MMTVels was detected in 33% of a series of HBCs, whereas normal breast tissues and other types of human tumors were negative for it.

Today, the idea that a virus could be involved in the etiology of HBC is receiving much more attention that in the past. In general, most research[21,22] has been restricted to the demonstration of viral particle proteins in tumor cells. However, the ability of MMTV to infect human cells was demonstrated.[23]

MMTV, if really involved in breast human carcinogenesis, could act as a promoting agent or as an etiological/pathogenic cofactor linked to some steps of cancer progression, such as infiltration or metastasis. The determination of where MMTV exogenous sequences appear during cancer progression could help unveil their role in HBC. This study analyzes the presence of MMTVels in a series of preinvasive breast lesions [ie, usual ductal epithelial hyperplasia (UDH), atypical ductal epithelial hyperplasia (ADH), ductal carcinoma *in situ* (DCIS), and infiltrating ductal carcinoma (IDC)]. Moreover, the contemporary presence of the exogenous viral sequence in normal epithelium, preinvasive lesions, and infiltrating cancer, all from the same patient, was also investigated. Results demonstrate an early appearance of MMTV *env*-like sequences in HBC progression. Real-time quantitative PCR (qPCR) fully confirms the PCR data. For the first time, to our knowledge, we report the presence of the MMTV *env*-like viral sequence by chromogenic *in situ* hybridization (CISH) experiments, which detected viral sequence hybridization signals in the nuclei of DCIS and IDC cells.

Materials and Methods

All tissues, formalin fixed and paraffin embedded, were collected and archived (from January 1, 2005 to December 31, 2009) at the Division of Surgical, Molecular, and Ultrastructural Pathology, University of Pisa, Pisa, Italy. Epithelial cells (normal, hyperplastic, dysplastic, and neoplastic) were obtained by laser microdissection. DNA was extracted from microdissected tissues, and MMTV *env*-like sequences were detected by fluorescence-nested PCR and sequenced.[20] In nine cases, real-time qPCR was performed.

Histological Criteria

The adjective collateral is used to indicate normal epithelial cells (NECs) present in the same histological section, hosting a preinvasive lesion or an infiltrating carcinoma, and preinvasive lesions present in the same histological section, hosting an infiltrating carcinoma. UDH indicates a benign ductal epithelial proliferative lesion characterized by secondary lumens and streaming of the central proliferative cells. ADH has cytological and architectural features of low-grade DCIS present in one or more ducts or in an aggregate not exceeding 0.2 cm. DCIS can be of high, intermediate, or low grade: i) pleomorphic large cells with abundant mitoses and with variable architecture, often solid and with central necrotic debris; ii) uniform cells with small nuclei and frequently with a cribriform or micropapillary configuration; and iii) neoplastic nuclei, showing pleomorphism of a degree between high- and low-grade DCIS.[24–26]

Specimens

The specimens included 26 NECs collateral to DCIS or IDC; 20 UDHs noncollateral to DCIS or IDC; 22 ADHs noncollateral to DCIS or IDC; 49 DCISs, 20 of which were collateral to IDC; and 20 IDCs. Of the DCISs, 15 were poorly differentiated, 33 were moderately differentiated, and one was well differentiated. The negative control included 20 NECs from reductive mammoplastic tissues, six papillary thyroid carcinomas, six colon adenocarcinomas, and DNA extracted from blood samples from six healthy blood donors. The DNA of a mouse infected by MMTV was the positive control.

Laser Microdissection

A Leica ASLMD automatic laser microdissector (Leica Microsystems, Wetzlar, Germany) was used to select the epithelial cell population to be studied. Sections (6-μm thick) were cut from each case using a new microtome blade for each slide, obtaining a total of 10,000 to 15,000 cells. Stromal and inflammatory cells were carefully excluded. Because of the long experience of the laboratory with this method, there was no difficulty in selecting areas of interest.[20]

DNA Extraction

Microdissected samples were kept overnight in lysis buffer containing proteinase K. Samples were processed for specific PCR amplification the next day. To avoid cross contamination, blank DNA samples (water) were processed in parallel with the tissue samples. The quality and amount of extracted DNA were evaluated by agarose gel electrophoresis and UV spectrophotometry (NanoDrop 2000, Thermoscientific, Wilmington, DE).

DNA Amplification Suitability

DNA was checked for the absence of PCR inhibitors by amplifying *HERV-K10* provirus target templates as positive controls. The cycle conditions were as follows: one cycle at 94°C for 10 minutes; 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 7 minutes in 30-μL reaction mixture containing 1× PCR Buffer [500 mmol/L KCl, 150 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L $MgCl_2$, 200 μmol/L each 2′-deoxyribonucleoside 5′-triphosphate, 0.5 μmol/L of forward and reverse primer, and 2.5 U AmpliTaq Gold; Applied Biosystems]. DNA samples were considered free of PCR inhibitors if *HERV-K10* amplicons were clearly visible on a 1.8% agarose gel.

MMTV Sequence Detection

Fluorescence-nested PCR was used to detect the presence of the MMTV *env*-like sequence. Generated fluorescent amplicons were sized on an automatic DNA sequencer. The pairs of primer were designed on the basis of the sequence available in GenBank (accession no. AF243039). Slight changes were introduced to our previous procedure[20] to adapt it to paraffin-embedded tissues. They mainly consisted of decreasing the length of the amplicons to a maximum of 250 bp. The outer primers yield a 248-bp fragment from nucleotide positions 231 to 480 of MMTV-like *env*, and the inner primers yield a 202-bp fragment (nucleotide positions 231 and 431). Sequences of the outer primers for the first PCR were as follows: forward, 5′-GATGGTATGAAGCAGGATGG-3′; and reverse, 5′-CCTCTTTTCTCTATATCTATTAGCTGAGGTAATC-3′. For the nested PCR, the forward primer sequence was the same as the one previously listed, whereas the reverse sequence was reverse nested (5′-AAGGGTAAGTAACACAGGCAGATGTA-3′). Both PCRs were performed in 50 μL containing 1× standard PCR buffer [1.5 mm $MgCl_2$, 200 μm each 2′-deoxyribonucleoside 5′-triphosphate, 0.5 μmol/L unlabeled reverse primer (MWG Biotech, Ebersberg, Germany), 0.5 μmol/L 6-FAM–labeled forward primer (Applied Biosystems, Milan, Italy), and 2.5 U AmpliTaq Gold]. Input target template was 500-ng genomic DNA in the first-round PCR and 2 μL of first-round PCR product in the second round. The amplification profile was as follows: one cycle at 94°C for 10 minutes; 40 (first-round) and 30 (second-round) cycles at 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 7 minutes. To exclude PCR contamination, water controls and negative DNA samples were included every five samples in each run. Fluorescent amplicons were analyzed by capillary electrophoresis and appeared as peaks in an electropherogram. The amplicon size was extrapolated from a molecular size ladder resuspended in PCR buffer and run in parallel. Briefly, 3 μL of PCR products from both amplification rounds were mixed with 0.5 μL of ROX-labeled size standard (Gene Scan 400 HD ROX; Applied Biosystems) and 11.5 μL of formamide (Hi-Di Formamide; Applied Biosystems). After denaturation at 95°C for 3 minutes, samples were loaded onto an ABI PRISM 3100 automatic genetic analyzer and analyzed using GENESCAN software, version 3.1 (Applied Biosystems, Foster City, CA).

Sequencing of MMTV env*-Like PCR Fragments and CISH Probe*

MMTVels PCR fragments and MMTV CISH probe were purified on agarose gels using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and sequenced on an ABI PRISM 3130XL instrument (Applied Biosystems). Each sequence was aligned by using the BLAST search function (Basic Local Alignment Search Tool, *http://blast.ncbi.nlm.nih.gov*, last accessed March 8, 2010) to MMTV sequences deposited in GenBank.

MMTV Real-Time PCR Assay

Extracted DNA from nine patients was analyzed by qPCR. In two patients, NEC, DCIS, and IDC were available, whereas in the other seven patients, only DCIS and IDC were available. Briefly, a 25-μL PCR mixture was prepared using 2× Master Mix (MesaGreeen qPCR; Eurogentec, San Diego, CA) and 0.5 μL of MMTV primer (0.3 μmol/L) for the *env* region (nested PCR, 202 bp). The viral load was determined as the mean of triplicate sample values. The amplification of DNA from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a single-copy gene, was used as an internal reference control. Four 10-fold dilutions (1×10^1 to 1×10^4 copies/mL) of genomic human DNA (GAPDH) and of MMTV *env* amplicon were included as standard curves on each plate. MMTV *env*-like and corresponding GAPDH PCR measurements were performed in the same plate to minimize experimental errors. An MMTV-positive control DNA extracted from a mouse known to be infected with the virus and a template-negative control (PCR mix only) were also included in each PCR run. The PCRs were performed on the Rotor-Gene Q (Qiagen). The standard curves were plotted by the Rotor-Gene software detector system printing the C_T values against each known concentration of standards (MMTV or GAPDH sequence), and MMTV copy number per DNA sample was calculated by the detector.

CISH Analyses

The MMTV CISH probe was prepared in-house by amplification of a 2.7-kb PCR product using primers previously described.[27] The PCR was conducted in a 50-μL volume

reaction containing 3 U of DNA polymerase enzyme (Takara Bio Inc., Shiga, Japan), 5 μL of 10× PCR buffer, 2 μL of 50 mmol/L $MgCl_2$, 8 μL of 2.5 mmol/L 2′-deoxyribonucleoside 5′-triphosphate, 0.2 μL of each primer (5L and LTR3),[27] and 1 μg of mouse genomic DNA positive for the MMTV virus. The PCR conditions were as follows: 94°C for 2 minutes, followed by 40 cycles of 94°C for 5 seconds and 68°C for 5 minutes. The amplified probe was labeled using the DIG-NICK translation mix kit (Roche, Basel, Switzerland). The CISH experiments were performed as follows: paraffin-embedded sections were cut (4-μm thick), mounted onto positively charged microscope slides, and left to dry overnight at 37°C. Sections were then deparaffinized and rehydrated. Antigen retrieval was achieved by heat retrieval using a water bath (Dako, Glostrup, Denmark). The slides were placed in Coplin jars (Sigma-Aldrich, St. Louis, MO) containing enough 0.01 mol/L sodium citrate solution (pH 6.0) to cover the sections, then incubated in a water bath at 80°C for 30 minutes. To expose the nuclear components, proteinase K was applied on the sections for 10 minutes at 37°C. The denaturation and hybridization procedures were achieved by applying 500 ng of MMTV CISH probe on the sections for 5 minutes at 80°C and for 16 hours at 37°C, respectively, using a ThermoBrite hybridizer (ABBOTT, Abbott Park, IL). Finally, the results were detected by applying anti-digoxigenin-AP conjugate for 30 minutes at 37°C, which produced a characteristic nuclear black stain using NBT-BCIP substrate.

For each run of staining, positive and negative control slides were also prepared. The positive control slides were prepared from a case known to be positive for MMTVels in the PCR study. The negative control slides were prepared from the same tissue block but incubated with hybridization buffer instead of the MMTV CISH probe, from breast tissue negative for the presence of viral sequences, and from colon cancer tissue.

Statistical Analyses

A one-way analysis of variance test was used to statistically evaluate the differences in viral copy numbers between the early and later stages of tumor progression in the same patient.

Results

Our use of laser microdissection shows that only neoplastic cells were selected for molecular studies, when comparing representative DCIS before laser microdissection (Figure 1A) with DCIS after laser microdissection (Figure 1B).

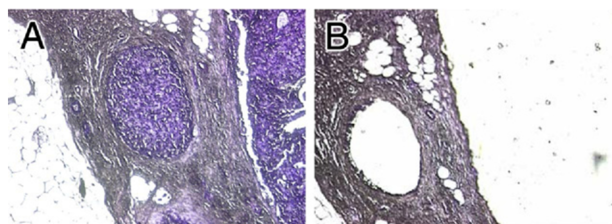

Figure 1. Accuracy of laser microdissection. The comparison between the two pictures demonstrates that only neoplastic cells were selected for molecular studies. DCIS before (**A**) and after (**B**) laser microdissection.

Table 1. Presence and Frequency of MMTVels in 49 DCISs and in Concomitant c-NECs and IDCs

Sample no.	c-NECs	DCISs	IDCs	Sample no.	c-NECs	DCISs	IDCs
1	+	+	+	26	NA	+	−
2	+	+	−	27	NA	+	−
3	+	+	−	28	NA	+	NA
4	+	+	−	29	NA	+	NA
5	+	+	NA	30	NA	+	NA
6	−	+	+	31	NA	+	NA
7	−	+	+	32	NA	+	NA
8	−	+	+	33	NA	+	NA
9	−	+	+	34	NA	+	NA
10	NA	+	+	35	NA	+	NA
11	NA	+	+	36	NA	+	NA
12	−	+	−	37	NA	+	NA
13	−	+	−	38	NA	+	NA
14	−	+	−	39	NA	+	NA
15	NA	+	−	40	NA	+	NA
16	NA	+	−	41	−	−	−
17	−	+	−	42	−	−	NA
18	−	+	NA	43	−	−	NA
19	−	+	NA	44	−	−	NA
20	−	+	NA	45	−	−	NA
21	−	+	NA	46	NA	−	−
22	−	+	NA	47	NA	−	NA
23	−	+	NA	48	NA	−	NA
24	−	+	NA	49	NA	−	NA
25	−	+	NA				

Positive results were as follows: c-NECs, 5 (19.0%) of 26; DCISs, 40 (82.0%) of 49; IDCs, 7 (35.0%) of 20; DCISs + IDCs, 7 (35.0%) of 20; DCISs + IDCs + c-NECs, 1 (8.0%) of 13; and c-NECs + DCISs, 5 (24%) of 21.

NA, not assessed.

Presence and Frequency of MMTV *env*-Like Sequences

The presence and the frequency of MMTVels in normal breast and in all phases of breast cancer progression are described later and reported in Table 1 and in Figure 2. For NECs, exogenous MMTVels were found in 19% (5 of

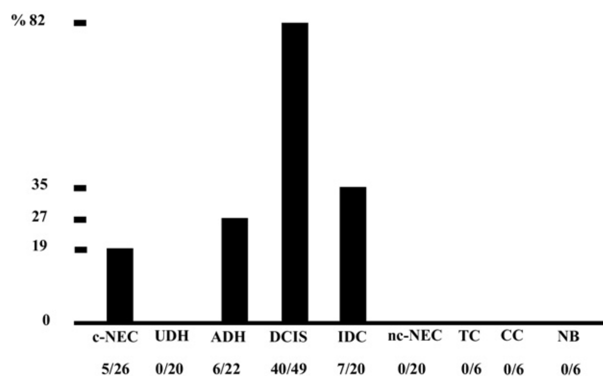

Figure 2. Percentage of MMTVels-positive cases in different histological stages of breast cancer progression and in control tissues. CC, colon carcinoma; c-NEC, NECs collateral to DCIS or IDC; NB, normal blood samples; nc-NEC, NECs noncollateral to DCIS or IDC (reductive mammoplasty); TC, thyroid carcinoma.

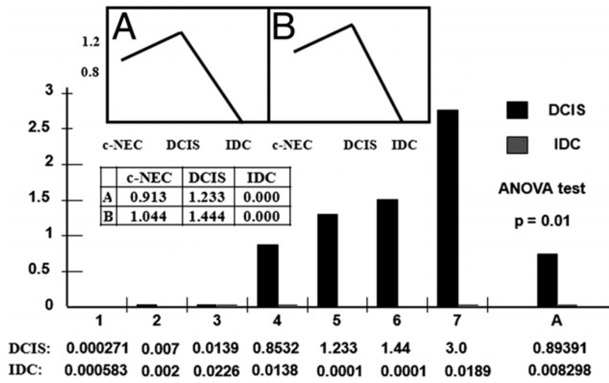

Figure 3. MMTVels qPCR conducted on seven samples for which both DCIS and IDC were available. The viral load was determined as the mean of triplicate sample values represented by the virus/GAPDH copy number gene ratio. A statistical difference was found in MMTV copy number between the two stages, showing a dramatic decrease moving from DCIS to IDC (P = 0.01). In two patients, NECs collateral to DCIS or IDC, DCIS, and IDC were available (**insets: A** and **B**). In each of them, there is an increase in MMTV DNA copy number moving from normal epithelium to DCIS, whereas there is a complete loss of the virus in IDC. ANOVA, analysis of variance.

26) NECs collateral to DCIS or IDC. The 20 cases of normal breast samples obtained by reductive mammoplasty were all negative. For ductal epithelial hyperplasia, the 20 cases of UDH were all negative for MMTVels, but exogenous MMTV *env*-like sequences were found in 27% (6 of 22) samples of ADH. For breast carcinoma, MMTVels were found in 82% (40 of 49) of DCIS samples; 35% (7 of 20) of IDC samples were positive. As for controls, the six cases of infiltrating papillary thyroid carcinoma, the six cases of colon adenocarcinoma, and the six DNA samples of healthy donors were negative. Regarding different histological structures obtained from the same patient, normal breast and IDC were also available from 13 patients with DCIS positive for MMTVels. In one case (case 1), all three samples were positive; in four cases (cases 6 to 9), both DCIS and IDC were positive, whereas the normal structures were negative; in three cases (cases 2 to 4), normal structures and DCIS were positive and IDC was negative; and in six cases (cases 12 to 17), only DCISs were positive. In the six patients (cases 41 to 46) with DCISs negative for MMTVels, in whom normal structures and/or IDC were available, the normal structures and IDCs were negative. Of four cases of positive DCISs in which IDCs were available, the latter was positive in two cases and negative in two cases (cases 10 to 11 and 26 to 27, respectively). In nine cases of positive DCISs in which only normal gland structures were also available, eight (cases 18 to 25) of the latter were negative and one (case 5) was positive. In the 16 cases with only DCISs available, 13 (cases 28 to 40) were positive and three (cases 47 to 49) were negative.

Sequence Detection

To determine whether the amplified fragments were homologous to MMTV, the 248-bp amplicons were sequenced and aligned to the corresponding region of the prototype MMTV sequence and MMTV (GenBank accession no. AY152 721 and no. AF243039, respectively). Multiple nucleotide alignment showed 97% homology to both MMTV and human mammary tumor virus *env* sequences. Finally, no significant hits were found when the two sequences were compared with the human genome sequences available in GenBank, indicating that these amplicons were not of human genomic or endogenous retrovirus origin.

MMTV Viral Load with qPCR

qPCR was conducted on DNA extracted from DCIS and IDC from seven different patients. GAPDH was used as the internal gene reference control; therefore, each bar represents the MMTV/GAPDH DNA copy number ratio (Figure 3). The average of the MMTV/GAPDH DNA copy number ratio (Figure 3A) for DCIS and IDC of seven samples is also shown. There was a statistically significant dramatic decrease in MMTV copy number between the two stages (P = 0.01). qPCR was also conducted on DNA extracted from NEC, DCIS, and IDC from two different patients (Figure 3). In each patient, the PCR result was positive in NECs and DCISs but negative in IDCs. In both patients, there was an increase in MMTV DNA copy number after the progression from normal epithelium to DCIS but a complete loss of the virus in the invasive stage.

MMTV CISH Detection

As shown in Figure 4, MMTV-like sequences were successfully detected by CISH in DCIS samples. None of the negative cases revealed any CISH signal. The CISH signals were present inside the nuclei (Figure 4B), whereas Figure 4A shows the complete absence of nuclear signal. The multiple number of signals coming from each nucleus varies among cells, with an average of 3.5; in addition, there are much fewer cells with hybridization signals in the IDC than in the DCIS cases (data not shown).

Discussion

The etiology of the sporadic breast carcinoma is unknown, with the exception of rare radiation-induced neoplasms.[3] The promoting role of estrogens has been well established for many years, but sound evidence of their possible causative action is still lacking.[2]

The molecular approach to breast cancer pathogenesis clearly demonstrates that the classic distinction between ductal and lobular carcinoma is giving place to a

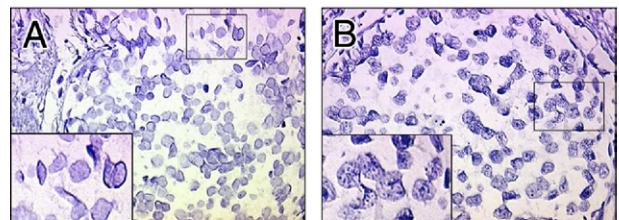

Figure 4. CISH on DCIS using a 2.7-kb MMTV probe. Negative (**A**) and positive (**B**) cases, in which numerous hybridization signals are evident in the nuclei of cancer cells. **Insets** are a magnification of the area in the small square.

new terminology based on biomolecular characteristics.[28–30] The provisory molecular classification in use is clarifying that the group of ductal carcinoma, accounting for approximately 80% of all types of breast carcinoma, contains an unpredictable number of different molecular subtypes. This molecular heterogeneity could be the consequence of different causative agents or of different transforming pathways caused by the same agent. In summary, no etiological factor has been identified for sporadic breast cancer; it is possible that different factors exist; and it is also possible that one or a few factors can give origin to the many molecular pathways of breast carcinoma by using different transforming mechanisms.

A possible viral etiology is receiving a certain consideration, mainly because the demonstration of the causative role of human papilloma virus for carcinoma of the cervix[31] has opened this new frontier. In addition, several articles[20–22] have demonstrated the presence of exogenous MMTV sequences in infiltrating human tumors. The amount of information about the possible existence of a human MTV that has accumulated during the past several decades is considerable: i) viral particles in milk and tumor tissues,[11,12,21,32] ii) reverse transcriptase in milk,[33] iii) MMTV antigens in serum and tumors,[34] iv) the epidemiological observation that HBC incidence is higher in geographic areas where *Mus domesticus* is the most prevalent mouse species,[35] v) the association between breast cancer and lymphoma,[36] vi) the occurrence of breast cancer in husbands and wives,[37,38] and finally, vii) the presence of exogenous MMTV sequences in 30% to 40% of HBCs.[14,20] Interestingly, a recent article[39] shows a higher expression of Wnt1 protein in human DCISs and IDCs positive for MMTVels. Moreover, another recent article[40] describes the presence of MMTV-like sequences in milk from healthy lactating women. Unfortunately, none of these data, alone or combined, is sufficient to demonstrate that the most common cancer in women is caused by MMTV. On the other hand, there is no element clearly against this hypothesis, whereas the quantity and quality of data justify continuing to explore this possibility.

Concerning the MMTV *env*-like sequence, it seems possible that some negative results were the consequence of the low quantity of *env* sequences and/or technical and methodological problems. A recent article[20] from this laboratory demonstrates that a correct approach can avoid these limitations (in particular, a laser microdissection procedure to obtain a pure and enriched population of tumor cells and a highly sensitive fluorescence-nested PCR for DNA analysis). On the basis of all of the data in the literature, it is reasonable to conclude that the presence of exogenous sequences of MMTV in infiltrating HBC is real and that the percentage of positive cases is between 30% and 40%. A recent article[41] on human RNA rumor viruses acknowledges that our previous PCR/laser microdissection approach,[20] together with *in situ* PCR studies,[42] supports the specific association of MMTV-like DNA and RNA with tumor cells and strengthens the argument that MMTV-like sequences are present in some HBCs.

If MMTVels is involved in human breast carcinogenesis, it could play an early, intermediate, or late role. In other words, it could behave as a transforming agent, an etiological cofactor, and/or a pathogenetic agent. The establishment of when exogenous viral sequences appear during the natural history of the disease would represent an interesting contribution to answer the previous question. This article investigates, for the first time to our knowledge, the presence of MMTVels in preinvasive lesions, infiltrating carcinomas, and normal tissues. MMTV exogenous viral sequences are absent in UDHs but are present in 27% of ADHs, 82% of DCISs, and 35% of IDCs. Normal epithelium collateral to DCIS is positive in 19% of cases, whereas in noncollateral NEC, it is always negative (eg, when obtained by reductive mammoplasty) in healthy women.

These data seem well in line with the model of progression usually accepted for breast cancer: normal epithelium, UDH, ADH, DCIS, and IDC.[43–48] ADH and DCIS are considered obligatory precursors of IDC; ADH and low-grade DCIS are included in the same category of DIN1, ductal intraepithelial neoplasia. Usual-type epithelial hyperplasia represents a common finding in human breast, as the result of a hormonal derearrangement. In fact, it is considered to be not related to carcinogenesis, as shown by its substantial polyclonality, the absence of chromosomal alterations, and the absence of dysregulation in adhesion and extracellular matrix pathways. However, there are a few cases (3%) that are monoclonal, with the occurrence of loss of heterozygosity in up to 15% of cases, with few chromosomal aberrations. Normal epithelium can show molecular alterations similar to those occurring in cancer, mainly in areas close to an invasive carcinoma, as expected by the hypothesis of field carcinogenesis occurring in breast and other organs. In summary, the few epithelial cells transformed by the unknown carcinogenic agent in a normal-looking breast glandular structure, under the proliferative stimulus of estrogens, can give origin to small neoplastic clones in a few UDHs, which is a frequent benign alteration. The subsequent expansion of the neoplastic clones, with the accumulation of genomic aberrations, gives origin to ADHs, in which nuclear atypia is easily evident.

Exogenous MMTVels are present in normal epithelium close to IDCs in a small percentage of cases and have not been found in normal epithelium of normal breast tissue obtained by reductive mammoplasty or in UDHs not associated with IDCs. MMTVels frequency is already high in ADHs, then dramatically increases in IDCs, and finally in IDCs goes back to a value similar to that of ADHs.

The presence of MMTVels in IDCs, and in normal breast, ADH, and DCIS, demonstrates that these exogenous sequences represent an event bound to early steps of carcinogenesis. The 35% positivity in IDCs is consistent with previous data. The absence of positivity in normal breast not associated with cancer, the few positive cases in normal glandular structures close to IDCs, and the absence of positivity in UDHs are in line with the field carcinogenesis model and the biological characteristics of UDHs. The only apparently controversial result is the high frequency of MMTV *env*-like sequences in DCIS (>80%), with a signifi-

cantly lower frequency in the two lesions immediately upstream and downstream, ADH and IDC.

However, the high level of technological accuracy and the robust controls used in this study strongly support the reliability of the results. A possible explanation for the strong decrease in positive cases moving from DCIS to IDC can be that MMTVels are relevant for transformation, but less important for transition, of DCISs to IDCs. Moreover, MMTVels could be lost as a consequence of the high level of chromosomal rearrangement characterizing IDCs. A selective depletion of tumor cells expressing MMTV antigens can also be assumed, as a consequence of their identification and elimination by the immune system. These data are supported by results obtained by qPCR, demonstrating that the MMTV *env*-like sequence copy number is small and that there is a dramatic reduction moving from DCIS to IDC. In addition, the copy number increases from normal cells to DCIS.

The progressive increase in the number of positive cases moving from normal cells to DCISs was previously demonstrated by Ford et al[16] during the dedifferentiation of IDCs, moving from low- to high-grade cases. On the other side, the benign lesions they studied (ie, fibroadenoma and fibrocystic disease) are not comparable with the preinvasive lesions analyzed herein.

Furthermore, and most important in this study, we report for the first time to our knowledge, the presence of the MMTV viral sequence by performing CISH experiments in which we use a homemade probe of 2.7 kb amplified from genomic DNA of a mouse infected with the MMTV virus. We clearly demonstrate, as shown in Figure 4, the presence of the viral hybridization signal in the nuclei of DCIS and IDC cells. The multiple signals coming from each nucleus vary among cells and reflect the average number of signals (3.5) found by Liu et al[27] in 2001, when they conducted fluorescence in situ hybridization experiments on breast cancer cell lines in metaphase. In addition, the number of cells that present viral hybridization signals is much fewer in the IDC cases than in the DCISs, supporting the concept of a loss of viral sequence load with the progression of the disease and, therefore, enforcing the hypothesis that the viral sequences are likely involved only in the initial transformation process.

Finally, a secondary, but not negligible, result of this study is the demonstration that MMTV *env* gene-like sequences can be easily detected in formalin-fixed, paraffin-embedded tissues.

In conclusion, exogenous MMTV sequences are easily identifiable in the early steps of breast cancer progression, suggesting their possible role in the transformation process, at least as an etiological cofactor. On the other hand, the strong reduction of positive cases moving from DCIS to IDC suggests that they are not relevant for the progression of the disease. Our data encourage us to pursue further research to definitively determine whether there is a viral etiology of HBC. The isolation of the MMTV virus from patients and the detection of its integration site would represent relevant contributions.

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