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Short communication

# Detection of human mammary tumor virus proteins in human breast cancer cells

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A B S T R A C T

Mouse mammary tumor virus (MMTV) has been proven to induce mammary cancer in mice. MMTV-like *env* gene sequences have been detected in one-third of the human breast tumors studied. The whole proviral structure with 95% homology to MMTV was found in two human breast tumors and was designated as human mammary tumor virus (HMTV). HMTV viral particles with betaretroviral features have been isolated. In addition, a retrovirus called human betaretrovirus (HBRV), homologous to the mentioned retroviruses, has been isolated from tissues of patients with primary biliary cirrhosis.

In this report, the expression of HMTV envelope (Env) and capsid (Ca) was detected in 10 primary cultures of human breast cancer containing HMTV sequences (MSSM) by Western blot and fluorescence activated cell sorting (FACS), using a panel of antibodies against HMTV Env, HBRV Env and Ca and the MMTV Env Gp36 and Ca P27 proteins. By contrast, HMTV proteins did not react with antibody against the MMTV Env Gp52 protein. All the antibodies detected MMTV proteins with exception of two out of four monoclonal antibodies against HMTV Env. Approximately 13% of the MSSM cells showed HMTV protein expression by FACS analysis.

This report shows the expression of HMTV proteins for the first time in human breast cancer cells using a panel of antibodies against HMTV, HBRV and MMTV proteins. This should be taken into consideration when MMTV antibodies are used to detect HMTV proteins in human tissues.

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In 1936, Bittner discovered the presence of an agent in the milk able to cause mammary tumors in mice (Bittner, 1936). Years later, it was identified as a betaretrovirus, mouse mammary tumor virus (MMTV). The search for a similar retrovirus in human breast cancer had been hampered by the presence of Human Endogenous Retrovirus (HERV) until the association of a retrovirus homologous to MMTV with human breast cancer had been shown (Wang et al., 1995, 2001; Etkind et al., 2000; Ford et al., 2003; Levine et al., 2004; Zammarchi et al., 2006; Luo et al., 2006; Zapata-Benavides et al., 2007; Hachana et al., 2008). A 660-bp sequence of the MMTV envelope (*env*) gene, with no significant homology to any human sequences reported in the GenBank, was present in 38% of breast cancers studied in the USA. Its mRNA has been detected in most of them (Wang et al., 1995, 1998). The sequence was detected in the tumor but not in the normal breast tissue of the same patient indicating its exogenous origin (Melana et al., 2001). A complete provirus structure with 95% homology to MMTV has been described and designated as human mammary tumor virus (HMTV) (Liu et al., 2001). This proviral structure seems to be replicative competent and its Long Terminal Repeat (LTR) contains several hormone responsive elements and the Open Reading Frame (ORF) for the superantigen (Sag) (Wang et al., 2004). In addition, the *sag* gene has been cloned and shown to be functional (Wang et al., 2004). Recently, betaretroviral particles from primary cultures of human breast cancer containing HMTV sequence cells (MSSM cells) have been isolated and characterized (Melana et al., 2007). The virion RNA was more than 90% homologous to MMTV RNA and to the HMTV proviral DNA identified previously (Melana et al., 2007).

Human betaretrovirus (HBRV) sequences have been isolated from biliary epithelium of patients with primary biliary cirrhosis (Xu et al., 2003, 2004). The HBRV *env* and capsid (*ca*) sequences were cloned and polyclonal antibodies were produced in rabbit against Env and Ca. Recently, MMTV-like virus (MMTV-LV) *env* gene sequences were found in tissue from patients with liver disorders but not in normal livers (Johal et al., 2009).

Previous studies have demonstrated the expression of MMTV Env proteins in breast cancer tissues and its absence in normal

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mammary tissues (Mesa Tejada et al., 1978; Yang et al., 1978; Litvinov and Golovkina, 1989; Lloyd et al., 1983). These results were challenged when Env has also been shown in a pool of milk from 300 healthy women (Dion et al., 1980), from whom 6% are expected to develop mammary tumors. Furthermore, another study indicated that an anti-MMTV Gp52 polyclonal antibody reacted with an unknown protein isolated from a human cDNA expression library (Hareuveni and Lathe, 1990).

The association of an MMTV-like agent with human pathologies is still controversial since MMTV is considered to be exclusively a mouse virus unable to infect human cells. However, infection of a number of human cells lines, including mammary cells by MMTV has been shown (Indik et al., 2005, 2007).

In this communication, the expression of HMTV proteins was investigated in MSSM cells using a panel of antibodies against HMTV, HBRV and MMTV proteins in an attempt to identify antibodies that can be used to detect viral proteins in tissues. The detection of HMTV proteins provided another tool to search its presence in human tissues.

*Cells*: MSSM cells were obtained following the Institutional Review Board recommendations from discarded ascitic fluids or pleural effusions obtained from patients with metastatic breast cancer (Fernandez-Cobo et al., 2006a). Cells were grown in Mammary Epithelial Growth Media (MEGM) (Cambrex Bio Science Corp, Charles City, IA). Culture media and fluids were tested for mycoplasma by a specific polymerase chain reaction (PCR). A normal human breast derived cell line (MCF10F) was obtained from American Tissue Culture Collection (ATCC, Manassas, VA) grown in MEGM with 5% horse serum (Soule et al., 1990). MM5MT (ATCC), a mouse mammary cell line infected with MMTV (Owens and Hackett, 1972), was grown and processed in another laboratory to prevent possible cross-contamination.

*Anti-P1, P2, P3 and P4 monoclonal antibodies*: Amino acid sequences from the 660-bp *env* gene sequence (GenBank accession: AF239172) were examined according the Jamison–Wolf antigenicity index (PeptideStructure) (Jamison and Wolf, 1988). The sequence designated P1 (H-LKRPGFQEHEMI-OH), P2 (H-LLGLPHLIDIEKRGSTFHIS-OH), P3 (H-CRLTNCLDSSAYDYA-OH) and P4 (H-DIGDEPWFDDSA-OH) were predicted to be the most immunogenic and were synthesized at the Microchemistry Laboratory of the New York Blood Center, NY, NY. Murine monoclonal antibodies, designated mAbP1, mAbP2, mAbP3 and mAbP4, were prepared by Viro/Dynamics, Hawthorne, NY.

*Anti-HBRV-Su and -Ca polyclonal antibodies*: Polyclonal antibodies against HBRV Env protein (pAbSu) and against HBRV-Ca protein (pAbCa) raised in rabbits. Both antibodies and the recombinant proteins (HBRV-Su) and (HBRV-Ca) used as controls were gifts from Dr. Sakalian (Melana et al., 2007).

*Anti-Gp52, Gp36 and P27 monoclonal antibodies*: Monoclonal antibodies against the MMTV Gp52, Gp36 and P27 proteins called mAbGp52, mAbGp36 and mAbP27 were gifts from Dr. Tatyana Golovkina (University of Chicago) (Purdy et al., 2003).

*α-Tubulin monoclonal antibody*: A mouse monoclonal antibody against α-tubulin, designated pAbTu was employed (Sigma–Aldrich Corp, St. Louis, MO) to compare protein loading.

*Western blot assays*: Protein lysates were prepared from approximately $1 \times 10^7$ cells. Confluent cell monolayers were washed with phosphate buffered saline (PBS), treated with RIPA lysis buffer (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 15 min at 4 °C, and then scrapped from the plates. Lysates were clarified by centrifugation at 12,000 rpm for 10 min. Protein concentration was determined by Bradford method (Bio-Rad Labs Corp, Hercules, CA). Equal amounts of protein from each sample were loaded onto an SDS-PAGE–10% polyacrylamide gel, followed by transfer onto nylon membranes. After blocking with blotto (Santa Cruz Biotechnology Inc), Western blot analysis was performed using the described primary antibodies. Proteins were visualized using the respective horseradish peroxidase-labeled sheep IgG (GE Healthcare Bio-Sciences, Piscataway, NJ) as secondary antibodies, followed by enhanced chemiluminescence (GE Healthcare Bio-Sciences).

*Flow cytometry analyses*: Cells were detached from the flasks using a non-enzymatic cell dissociation buffer (Sigma–Aldrich Corp). After FcR block, $5 \times 10^6$ cells were stained in a two-step protocol with primary antibody followed by anti-mouse IgG FITC

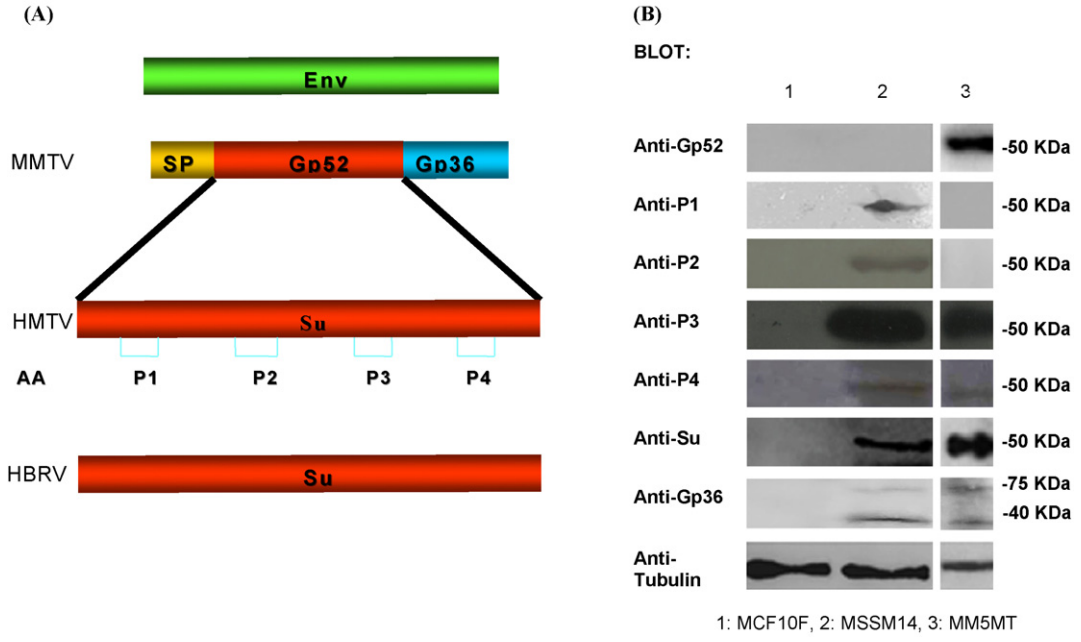


**Fig. 1.** Western blot analysis of HMTV Env protein in MCF10F, MSSM14 and MM5MT cells. (A) Schema of MMTV gPr73 Env protein precursor, Gp52 and Gp36 proteins from MMTV, HMTV Env and HBRV-Su protein. The localization of the synthetic peptides that interact with the antibodies is indicated. (B) Detection of HMTV Env protein using mAbGp52, mAbP1, mAbP2, mAbP3 and mAbP4, pAbSu and mAbGp36. Equal amount of proteins were loaded. The Western blots conditions and the origin of the antibodies are described in the text.

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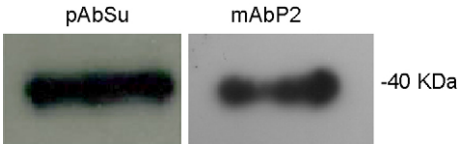


**Fig. 2.** Western Blot analysis of recombinant HBRV-Su protein with pAbpSu and mAbP2. Equal amounts of HBRV-Su protein was loaded and Western blots were carried out as described in the text using the indicated antibodies.

coupled antibody (Invitrogen Inc, Carlsbad, CA) and then subjected to fluorescence activated cell sorting (FACS) analysis. Cell counts were obtained using a FACScan cytometer (Becton Dickinson Corp, Franklin Lake, NJ). Approximately 20,000 cells were analyzed per sample with dead cells excluded using FSC/SCC profiles. Surface marker analysis was undertaken with Cell Quest software.

*Detection of HMTV env sequences*: DNA extraction and detection of HMTV *env* sequences were carried out under the conditions recommend by Wang et al. (1995).

*Expression of viral proteins in breast cancer cells*: Ten MSSM cells used in these experiments were obtained from discarded ascites fluid or pleural effusions of patients with breast cancer (Fernandez-Cobo et al., 2006a; Melana et al., 2007). The presence of HMTV *env* gene sequences was confirmed by PCR (Wang et al., 1995). Retroviral particles were observed by electronic microscopy, and their cDNA sequences corresponded to HMTV (Melana et al., 2007). The expression of HMTV Env was investigated in MSSM cells by Western Blot using mAbGp52, mAbP1, mAbP2, mAbP3, mAbP4, pAbSu and mAbGp36 antibodies (Fig. 1A). The specificity of the mAbP1, mAbP2, mAbP3 and mAbP4 antibodies was confirmed by competition experiments (data not shown). Fig. 1 depicts the results of Western blot analyses using MCF10F, MSSM14 and MM5MT protein extracts with different antibodies. MCF10F cells did not react with any of the antibodies while MSSM cells reacted with mAbP1, mAbP2, mAbP3, mAbP4, pAbSu and mAbGp36 but not with mAbGp52, which only showed some activity against MSSM cells when it was incubated without dilution at 37 °C, for 3 h. Similar results were obtained with the other MSSM cells. Western blot of the recombinant HBRV-Su protein, whose molecular weight was of 38 kDa, was used as a control. This protein reacted with the HMTV antibodies as well as against pAbSu. The reactivity of pAbSu and mAbp2 to HBRV-Su is shown in Fig. 2. To rule out that the non-reacting antibodies were inactivated, they were used in Western blots of MM5MT cells, a MMTV infected murine cell line. As seen in Fig. 1B, the MM5MT cells showed reactivity to mAbGp52, mAbP3 and mAbP4 but not to mAbP1 and mAbP2. In addition, reactivity to mAbP27 and to pAbCa (Fig. 3) was similar in MSSM cells and in MM5MT cells but it was absent in MCF10F cells. Both antibodies reacted with the recombinant HBRV-Ca protein (data not shown). The expression of α-tubulin in the same protein extracts is shown as a control for protein loading (Figs. 1B and 3B). To determine how many MSSM cells expressed the HMTV Env protein, FACS analyses were performed using mAbP2 and mAbGp52 in MSSM and MM5MT cells. Around 13% of MSSM6 cells reacted with mAbP2 but only 1.5% with mAbGp52, while only 7.44% of the MM5MT cells reacted with mAbP2 and most of them (95%) reacted with mAbGp52 (Fig. 4).

The results reported above have demonstrated clearly expression of HMTV Env and Ca proteins in MSSM cells and their absence in normal human breast cells. The results have also indicated that the HTMV Env protein did not react as well with mAbGp52 as it did with antibodies obtained against HMTV peptides and HBRV proteins. This could explain some of the contradictory results reported on the detection of MMTV-like Env protein in human tissues using anti-MMTV Env antibodies (Selmi et al., 2004). Furthermore, it is important to note that MMTV Env Gp52 protein is recognized by mAbP3 and mAbP4 antibodies as well as by mAbGp52 but not by mAbP1 and mAbP2 antibodies. No difference was found in the detection of HMTV-Ca protein by mAbP27 and pAbCa antibodies by Western blot.

Why is mAbGp52 not reactive with HMTV Env protein? Several explanations can be considered:

MAbGp52 only reacted with HMTV Env protein when it was incubated without dilution at 37 °C for 3 h while antibodies against HMTV Env were only incubated for 1 h at room temperature. These results suggest low affinity between mAbGp52 and HMTV Env protein.

The antigenic site(s) recognized by mAbGp52 are different from those, which are recognized by the anti-HMTV Env and pAbSu antibodies. There are no major differences between the MMTV and HMTV *env* gene sequences (Wang et al., 1998) that could result in an altered protein structure. It is not known what one amino acid change, as it was described in certain human isolates (Etkind et al., 2004, 2008), could affect the antigenic site(s).

It is important to emphasize that the HBRV antibodies against HBRV proteins, which were cloned in bacteria, whereas the MMTV antibodies were obtained against the entire glycoprotein (Purdy et al., 2003). By contrast, HMTV antibodies were raised against peptides derived from HMTV *env* gene sequences that were 100% homologous to the MMTV *env* gene. This difference in immunoreactivity is only seen in the Env protein, which is involved in receptor binding and in starting a signaling cascade (Katz et al., 2005).

FACS analysis indicated that only 13% of MSSM express HMTV Env protein. Previously, it was shown by immunofluorescence that 15% of the MSSM7 cells reacted with mAbp2, and 12% by FACS (Melana et al., 2007). Similarly, some MCF7 cell sublines were found to react with mAbP2 (Fernandez-Cobo et al., 2006b).

In conclusion, expression of HMTV proteins was detected in MSSM cells. The HMTV Env protein was detected by mAbP1, mAbP2, mAbP3, mAbP4 and pAbSu as well as by mAbGp36 but not by mAbGp52. In contrast, MMTV Env protein reacted with

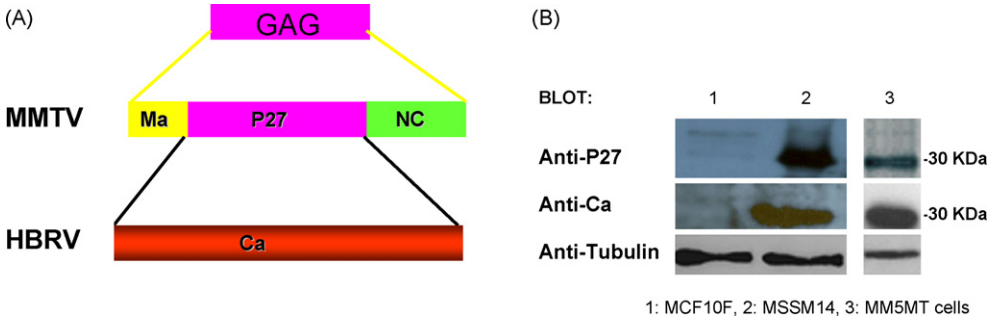


**Fig. 3.** Western blot analysis of HMTV-Ca protein in MCF10F, MSSM14 and MM5MT cells with mAbP27 and pAbCa. (A) Schema of MMTV-P27 proteins from MMTV and HBRV-Ca protein. (B) Detection of HMTV-Ca protein using mAbP27 and pAbCa. Equal amount of proteins were loaded. The Western blots were carried out as described in the text using the indicated antibodies.

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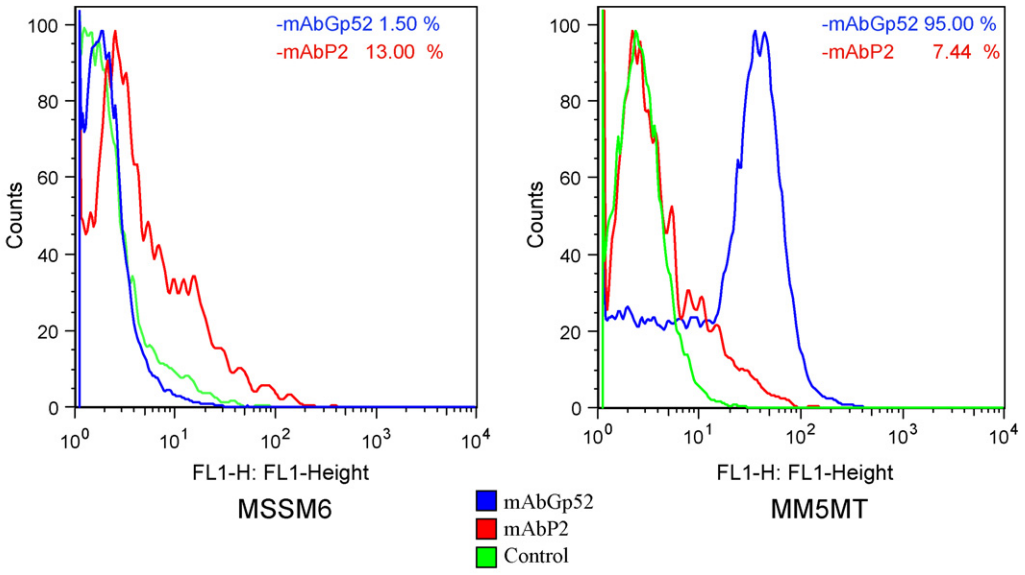


**Fig. 4.** Comparison of surface expression of HMTV and MMTV Env in MSSM6 and MM5MT cells. MSSM6 and MM5MT cells were stained with mAbP2 and mAbGp52, followed by FITC-labeled anti-mouse IgG antibody and analyzed by FACS. The percentage of HMTV Env and MMTV Env expressing cells is shown. Green line represents cells not exposed to primary antibody, red line represents cells stained with mAbP2 and blue line represents cells stained with mAbGp52.

mAbP3 and mAbP4 as well as by mAbGp52 and mAbGp36 but not by mAbP1 and mAbP2 antibodies. There is no difference in the reactivity against MMTV and HMTV-Ca proteins to mAbP27 and pAbCa antibodies. These results should be taken into consideration for the detection of HMTV proteins in human tissues, since this can serve as another tool for investigating the presence of HMTV in human tissue. This study identified HMTV proteins for the first time in breast tumor cells and it paves the way for their detection in human specimens.

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