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Short Communication

Differential Expression of the MT-1E Gene in Estrogen-Receptor-Positive and -Negative Human Breast Cancer Cell Lines

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The goal of this study was to determine which of the 10 functional metallothionein (MT) genes are expressed in four human breast cancer cell lines and whether expression varies among the cell lines. Using reverse transcription polymerase chain reaction (RT-PCR) technology, it was shown that there was no expression of mRNA for the MT-1A, MT-1B, MT-1F, MT-1G, MT-1H, MT-3, and MT-4 genes in any of the four cell lines. All four cell lines were shown to express mRNA for the MT-2A and MT-1X genes. The expression level of mRNA for the MT-2A gene demonstrated modest differences among the cell lines, whereas expression of the MT-1X gene was consistent. In contrast, mRNA for the MT-1E gene was expressed in only two of the four cell lines and expression correlated to the estrogen receptor status of the cell lines. The two estrogen-receptor-positive cell lines showed no mRNA expression for the MT-1E gene. In the two estrogen-receptor-negative cell lines, mRNA expression for the MT-1E gene was elevated with expression levels similar to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. The cellular content of MT protein was also shown to be elevated in the estrogen-receptor-negative cell lines that express MT-1E mRNA. These results suggest a possible relationship between estrogen receptor status and MT-1E gene expression in human breast cancer. ***(Am J Pathol 1998, 152:23–27)***

Since 1993, at least seven studies have suggested that the immunohistochemically determined expression of metallothionein (MT) has prognostic significance in breast cancer.[1–7] The first of these, by Schmid and coworkers,[1] investigated the pattern of MT immunohistochemical reactivity in 86 cases of routinely fixed and paraffin-embedded primary breast carcinomas. MT overexpression was found in the invasive components of 7 of 32 pT1 and 17 of 28 pT2 invasive ductal carcinomas, whereas all 26 invasive lobular carcinomas yielded weak or negative results. Fourteen of seventeen pT2 and two of seven pT1 invasive ductal carcinomas with MT overexpression developed metastases during follow-up with poor prognostic outcome. In contrast, only 3 of 11 pT2 and none of the 25 pT1 cases without MT overexpression had a poor clinical course. The difference was shown to have a high level of significance. This initial study concluded that MT overexpression was associated with a poor prognosis, particularly in pT2 invasive ductal breast carcinomas. These findings regarding MT overexpression in ductal breast cancer have been confirmed and extended by subsequent investigations.[2–7]

Although these studies demonstrate the potential use of MT as a prognostic marker, further extension to the level of gene expression is limited by the fact that the MT antibody reveals the overall expression of a family of genes rather than a specific single gene product. In humans, the MTs are encoded by a family of genes located at 16q13 consisting of 10 functional MT isoforms (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3, and MT-4) and 7 nonfunctional MT isoforms (MT-1C, MT-1D, MT-1I, MT-1J, MT-1K, MT-1L, and MT-2B).[8–12] This raises the possibility that the prognostic significance of MT expression in human ductal breast cancer might be further enhanced if specific MT gene expression patterns were known. However, the nucleotide bases comprising the amino acid coding regions of all of the MT genes are highly conserved, and it is unlikely that specific antibodies can be made to the individual isoforms. An alternative approach, using reverse transcription polymerase chain reaction technology (RT-

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PCR), has been developed to detect mRNA for each of the 10 active MT genes.[12] This approach is based on the fact that each active MT gene has 5′ and 3′ untranslated regions that do exhibit sequence divergence, allowing the generation of specific primers for the RT-PCR analysis of each active MT gene. As an initial step in determining the possible significance of MT isoform gene expression in human ductal breast cancer, MT isoform-specific gene expression was determined in two estrogen-receptor-positive and two estrogen-receptor-negative cell lines.

Materials and Methods

Cell Culture

The cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) with corresponding designations: HTB 22 (MCF7), HTB 133 (T-47D), HTB 126 (Hs 578T), and HTB 26 (MDA-MB-231). The HTB 22 and HTB 133 cell lines are estrogen receptor positive; the HTB 126 and HTB 26 cell lines are estrogen receptor negative. The cells were grown in Dulbecco's modified Eagles' medium supplemented with 10% (v/v) fetal calf serum. The cells were passaged at a 1:4 ratio on attaining confluence.

RNA Isolation and RT-PCR

The human breast cancer cell lines were grown to confluence in 75-cm^2 T flasks. Total RNA was isolated from the cultures according to the protocol supplied with TRI REAGENT (Molecular Research Center, Cincinnati, OH) as described previously.[12] The concentration and purity of the RNA samples were determined using spectrophotometer scans in the ultraviolet region and ethidium bromide (EtBr) visualization of intact 18 S and 28 S RNA bands after agarose gel electrophoresis.

Total RNA (0.5 μg) was reverse transcribed using murine leukemia virus reverse transcriptase (50 U) in 1X PCR buffer (50 mmol/L KCl and 10 mmol/L Tris/HCl, pH 8.3), 5 mmol/L $MgCl_2$ solution, 20 U of RNase inhibitor, 1 mmol/L each of the dNTPs and 2.5 μmol/L random hexanucleotide primers. The samples were reverse transcribed for 20 minutes at 42°C, followed by a 5-minute denaturation step at 99°C using a DNA thermocycler (Perkin-Elmer-Cetus 9600, Norwalk, CT). The reverse transcribed product was then used for PCR amplification using the AmpliTaq DNA polymerase enzyme (2.5 U) and the specific upstream and downstream primers at a concentration of 0.075 μmol/L each. The primers developed for the analysis of each of the active MT genes have been previously described along with the sources of positive control RNA for each isoform.[12] Primers for the determination of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were obtained commercially (Clontech, Palo Alto, CA). The thermocycler was programmed to cycle at 95°C for a 2-minute initial step and then 95°C for 15 seconds and 68°C for 30 seconds with a final elongation step at 68°C for 7 minutes. Controls for each PCR reaction included a no-template control where water was added instead of the RNA and a no-reverse-transcriptase control where water was added instead of the enzyme. Samples were removed at 30, 35, and 40 PCR cycles to assure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers.

Immunolocalization

Cell cultures grown on SuperCell culture slides (Curtin Matheson Scientific, Houston, Texas) were rinsed with phosphate-buffered saline (PBS) and fixed for 10 minutes in calcium-acetate-buffered formalin. After fixation, the chamber slide with attached cells was rinsed for a minimum of 30 minutes in PBS. Immunostaining was done by the indirect avidin-biotin-peroxidase procedure with the Vectastain kit (Vector Laboratories, Burlingame, CA).

MT was localized using a monoclonal mouse anti-horse antibody (DAKO-MT, E9, Dako, Carpinteria, CA) as the primary antibody. Controls consisted of omission of the primary antibody from the staining sequence.

MT Protein Determination

Proteins were isolated from cells grown in 75-cm^2 T-flasks. Cells were washed twice with PBS and lysed directly in the flask by addition of 400 μl of hot (85°C) 1X SDS buffer (2% SDS, 100 mmol/L dithiothreitol, and 50 mmol/L Tris/HCl, pH 6.8). The cell lysate was heated in a boiling water bath for 10 minutes. DNA was sheared by repeated passage through a 23-gauge needle. The samples were centrifuged at 10,000 × *g* for 10 minutes at room temperature, and the supernatant was transferred to a new tube. The concentration of protein in the samples was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). MT protein was determined by immunoblotting with the antibody used in immunolocalization studies.[13]

Results and Discussion

The goal of this study was to determine the expression profiles of the 10 active MT genes for four human breast cancer cell lines. Total RNA was isolated from each cell line, and 500 ng was subjected to RT-PCR analysis using primers specific for each of the 10 human MT genes. At 40 PCR cycles, this analysis disclosed that there was no expression of mRNA representing the MT-1A, -1B, -1F, -1G, or -1H or MT-3 or -4 genes in any of the four cell lines. The remaining three MT genes, MT-2A, MT-1X, and MT-1E, were expressed and demonstrated distinct patterns of expression among the four cell lines (Figures 1 and 2). The RT-PCR reaction demonstrated the expression of MT-2A mRNA at 40 cycles in each of the four breast cancer cell lines (Figure 1, top panel). Although RT-PCR as performed is not quantitative, a qualitative assessment of the levels of MT-2A mRNA expression could be determined for the four cell lines. The PCR was

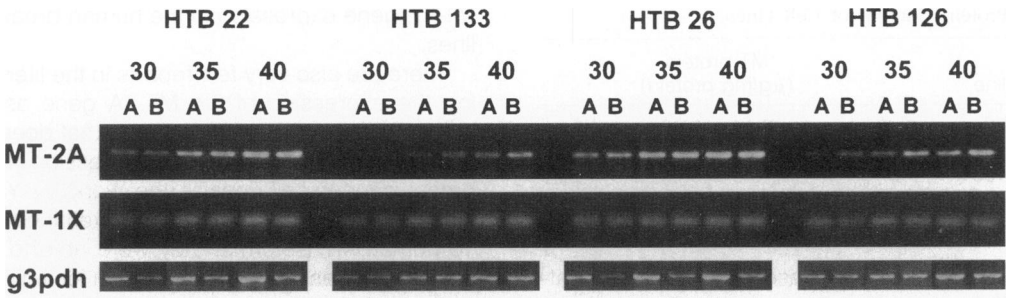

Figure 1. MT-2A, MT-1X, and G3PDH expression at 30, 35, and 40 PCR cycles. RT-PCR was used to analyze total RNA samples derived from four breast cancer cell lines (HTB 22, HTB 133, HTB 26, and HTB 126). Aliquots were removed at 30, 35, and 40 PCR cycles, electrophoresed on agarose gels, and stained with EtBr. mRNA expression is shown for MT-2A (**top**), MT-1X (**middle**), and G3PDH (**bottom**). Duplicate samples represented by the letters A and B are shown in each case.

halted at 30, 35, and 40 cycles to ensure a comparison at a linear portion of the reaction cycles. An examination of the PCR products at 30 reaction cycles demonstrated that mRNA for the MT-2A gene was detected for the HTB 22, HTB 26, and HTB 126 cell lines but not for the HTB 133 cell line. A visual assessment of reaction product intensity at 30 cycles also showed that the HTB 26 cell line had the highest expression of MT-2A mRNA, followed by the HTB 22 cell line. It was not until 35 reaction cycles that a convincing reaction product for MT-2A mRNA was detected in the HTB 133 cell line. Due to the nature of the PCR it is speculative to calculate specific percent differences in expression among the four cell lines; however, based on the cycle number required for detection of the reaction product, the fluorescent intensity of the EtBr-stained reaction product, and the fact that expression of the housekeeping gene (G3PDH) mRNA was relatively constant, a qualitative estimate of MT-2A expression among the cell lines can be made. The qualitative ranking of MT-2A expression among the four cell lines is as follows: HTB 26 > HTB 22 > HTB 126 > HTB 133. A reaction product correlating to the expression of MT-1X mRNA was also detected in all four breast cancer cell lines after 40 cycles of amplification (Figure 1, middle panel). Examination at earlier PCR cycles also demonstrated comparable intensity of expression of MT-1X for each cell line, suggesting little difference in the level of expression of MT-1X mRNA among the cell lines. An analysis of G3PDH mRNA expression (Figure 1, bottom panel) was included to control for possible effects (ie, inhibitors) of the individual RNA preparations on the PCR reaction.

The expression pattern of the MT-1E gene among the four breast cancer cell lines proved to be the most interesting. Two of the breast cancer cell lines (HTB 22 and HTB 133) demonstrated no expression of MT-1E mRNA at 40 reaction cycles, whereas the other two cell lines (HTB 126 and HTB 26) demonstrated MT-1E reaction products (Figure 2). This difference in expression correlates with the estrogen receptor status of the cell lines, the estrogen-receptor-positive cell lines showing no expression of MT-1E mRNA. Control reactions using identical total RNA samples demonstrated similar expression of G3PDH mRNA among the four cell lines, as shown in Figure 1.

Additional significance of this finding is suggested by the levels of expression in the two cell lines expressing MT-1E, where at 30 PCR cycles an easily identified fluorescent band was detected. This level of expression is similar to that found in identical reactions for the housekeeping gene G3PDH, suggesting that MT-1E mRNA has a high level of expression and is not a minor mRNA transcript in these two cell lines.

To determine whether these differences in mRNA expression of the three MT genes were productive, the total MT protein content of each cell line was determined (Table 1). This demonstrated that the HTB 126 and HTB 26 cell lines had significantly higher MT protein content than the HTB 22 and HTB 133 cell lines, correlating with the expression pattern of mRNA for the MT-1E gene. Localization of MT protein was also determined by immunohistochemical techniques using the identical antibody for the four cell lines grown on glass slides. Each cell line demonstrated a variable pattern of localization of MT

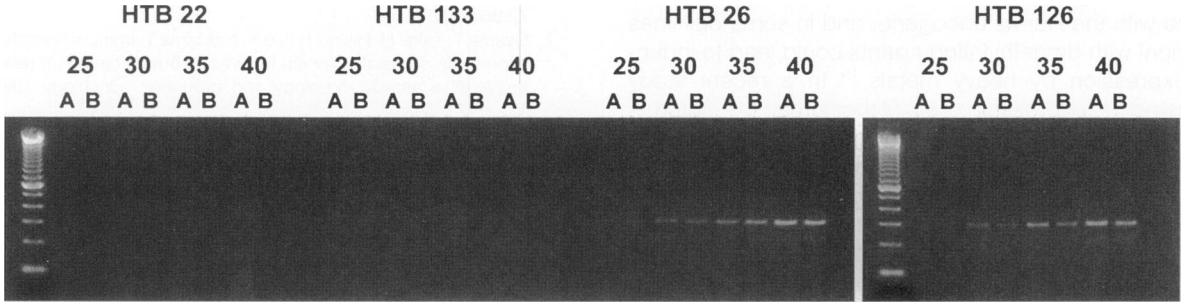

Figure 2. MT-1E expression at 25, 30, 35, and 40 PCR cycles. RT-PCR was used to analyze total RNA samples derived from four breast cancer cell lines (HTB 22, HTB 133, HTB 26, and HTB 126). Aliquots were removed at 25, 30, 35, and 40 PCR cycles, electrophoresed on agarose gels, and stained with EtBr. Duplicate samples represented by the letters A and B are shown in each case.

Table 1. MT Protein Content of Cell Lines

Cell line	MT protein (μg/mg protein)
HTB 22	3.14 ± 0.16
HTB 133	3.30 ± 0.12
HTB 126	4.48 ± 0.46
HTB 26	5.66 ± 0.18

between the nucleus and cytoplasm. Overall, these patterns did not show a convincing correlation to the expression of MT protein, to MT-1E mRNA, or to the estrogen receptor status of the cells (data not shown).

A significant finding in the present study is the correlation between the expression of MT-1E mRNA and expression of the estrogen receptor in these cell lines. The two cell lines known to have the estrogen receptor express no mRNA for the MT-1E gene, whereas the two cell lines known not to have the estrogen receptor express very high levels of MT-1E mRNA. The finding that estrogen-receptor-negative breast cancer cell lines express the MT-1E gene and produce more MT protein corresponds with clinical studies that assign a prognostic significance to MT overexpression in ductal breast cancer. The study by Fresno and co-workers[2] demonstrated a statistically significant association of MT overexpression with histological and nuclear grade and an inverse relationship with estrogen receptor status. It was also demonstrated that MT immunostaining predicted a worse prognosis in the subgroup of lymph-node-negative and estrogen-receptor-negative patients. Haerslev and co-workers[3] extended the prognostic relationship for MT staining and survival to lymph-node-positive patients. The current study is the first to suggest that the MT-1E gene may mediate the overexpression of MT protein in breast cancer, be related to estrogen receptor status, and influence patient prognosis. However, these preliminary observations will need to be confirmed in patient tissue and additional cell lines.

Unfortunately, very little information is available regarding the MT-1E gene and its regulation. Most of what is known has been obtained from cultured cells. The MT-1E gene was isolated and the genomic sequence obtained in 1985.[14] In this report, the MT-1E gene was shown to be expressed in human hepatoblastoma cells and to confer heavy metal resistance when transfected into cell lines. Subsequently, it was demonstrated that basal expression of the MT-1E gene was elevated in cultured cells transformed with the Ha-*ras* oncogene, and in some cell lines treatment with demethylating agents could lead to inducible expression by heavy metals.[15] In a recent study assessing the expression of six of the human MT isoforms in three sets of cisplatin-sensitive and -resistant cell lines, the expression of MT-1E was noted only in the SSC25 head and neck squamous cell carcinoma cell line.[16] As expression of MT-1E occurred in only one of the three cisplatin-resistant cell lines, it was concluded that MT-1E does not play a role in cellular resistance to cisplatin. Based on the limited studies available, there is not sufficient information to allow speculation on the mechanism for a relationship between estrogen receptor status and MT-1E gene expression in the human breast cancer cell lines.

There are also very few reports in the literature regarding the expression of the MT-2A gene as it relates to human cancer. A significant study that does relate to the current finding of MT-2A gene expression in all four of the breast cancer cell lines is the work by Yang and co-workers.[16] Although the authors examined only 6 of the 10 human MT isoforms, they were able to demonstrate that MT-2A was the only MT isoform for which expression correlated with resistance to cisplatin in all three pairs of cisplatin-resistant and -sensitive cell lines. The fact that MT-2A was expressed in all four breast cancer cell lines and that the level of expression varied among the cell lines suggests that MT-2A expression could also play a role in the development of chemotherapeutic resistance to some agents in breast cancer.

There are only two published reports regarding the expression of the MT-1X gene. One of these is the initial isolation and sequence of the 1X gene,[9] and the other is the documentation of expression in the human kidney.[12] Until more is known about the regulation of the MT-1X gene, the significance of its expression in the breast cancer cell lines will remain unknown.

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