Expression of the Tumor-associated Gene MN: A Potential Biomarker for Human Renal Cell Carcinoma

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Abstract

MN is a novel cell surface antigen originally detected in human HeLa cells. Although it is also expressed in normal gastric mucosa, this antigen was previously found to be expressed in cells with a malignant phenotype in certain tissues of the female genital tract (cervix and ovary). Using an oligonucleotide primer set specific for MN-complimentary DNA, we performed reverse transcription-PCR assays on RNAs extracted from human cell lines and tissues to evaluate whether this marker might be expressed at other sites. RNA libraries extracted from normal human heart, lung, kidney, prostate, peripheral blood, brain, placenta, and muscle were negative for MN expression. RNAs extracted from liver and pancreatic tissue were positive for MN expression. Three of six renal cancer cell lines tested revealed MN expression. In addition, 12 of 17 samples of human renal cell carcinoma tissue tested positive for MN, all 12 of which were clear cell adenocarcinomas. This survey identified a unique association of MN expression with renal cell cancers, especially those of the clear cell variety, suggesting that MN is a potential marker for the diagnosis, staging, and therapeutic monitoring of renal cell carcinoma in humans.

Introduction

For several forms of solid tumors, the process of tumor detection and staging has been greatly improved by the development of assays that detect and measure tumor-specific markers in specimens of patient tissues or body fluids. As exemplified by the use of prostate-specific antigen screening for prostate cancer (1), such assays have the potential of revolutionizing the clinical approach to the diagnosis, staging, and monitoring of the therapeutic response of human malignancies. Many of these tumor marker assays are based on immunological detection of the tumor marker protein, but increasingly, there are tumor detection methods that involve DNA- and RNA-based surveys of patient specimens (2–4). With the use of PCR technology to amplify distinct genetic sequences that are markers of the malignant cell (5–7), new methods allow the detection of small numbers of cancer cells in patient blood specimens.

With regard to kidney cancer, it is estimated that 28,800 new cases will be diagnosed in 1997, resulting in approximately 11,700 deaths (8). The majority of these cases will be caused by renal cell carcinoma. If these tumors are detected while still confined to the kidney, radical nephrectomy results in excellent long-term survival. Unfortunately, symptoms of this disease rarely occur before metastatic spread, and once renal cell carcinoma has metastasized, survival rates are less than 10% at 5 years (9). At the present time, no suitable diagnostic tumor marker exists for renal cell carcinoma detection, staging, or response to therapy.

Recently, we used a PCR-based technique to perform a survey of benign and malignant human cell lines and tissues for the expression of a putative malignant marker cell previously referred to as MN. The MN protein was first detected on the cell surface of the highly malignant cervical cancer cell line HeLa (10). MN expression was also detected in human cervical and ovarian tumor specimens but not in normal cervical or ovarian tissue, and these results suggest that MN might be a useful marker for screening for certain gynecological malignancies (11). In these earlier studies, MN expression was also detected in normal epithelial cells of the gastric mucosa (11, 12), but its restriction to this normal tissue was not felt to interfere with its value as a tumor-specific marker in tissues outside of the gastrointestinal tract.

The availability of the complete cDNA sequence for the MN gene product (10) allowed us to design specific PCR primers that only amplify a portion of the MN cDNA in a RT-PCR assay. In our survey of human cells and tissues, we have focused on whether MN expression can be identified in human urogenital tract tissues. Using this RT-PCR assay, RNAs from a series of normal human tissues were tested for MN expression. The distribution of MN expression in benign and malignant tissues of the human genitourinary tract was subsequently characterized. The results of our preliminary survey, presented here, suggest that the MN gene product might be a useful marker in the diagnosis and molecular staging of renal cell carcinoma.

Materials and Methods

Cell Culture. Six human renal carcinoma cell lines (SKRC-01, SKRC-08, SKRC-09, SKRC-17, SKRC-39, and SKRC-42; Ref. 13) and the human prostate cancer cell line LnCaP were maintained in RPMI media supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. HeLa cells were obtained as a kind gift from Dr. S. Silverstein (Columbia University, New York, NY).

Human Tissues. All primary human tissues used in this study were obtained through a protocol approved by the institutional review board at Columbia Presbyterian Medical Center. All patients involved in this study granted informed consent to participate. Approximately 1 g of fresh normal renal parenchymal tissue and 1 g of renal cancer were obtained at the time of radical nephrectomy. In total, 17 specimens containing renal cancer were available, and 10 of these specimens had matched tissues obtained from the normal area of the resected kidney. In addition, one specimen of normal human kidney was obtained from a patient undergoing radical nephroureterectomy for transitional cell cancer of the renal pelvis. A human prostate was obtained at the time of transurethral resection for bladder outlet obstruction. Tissues were snap-frozen in liquid nitrogen and then stored at —80°C. Frozen sections of each specimen were cut and analyzed to confirm the appropriate presence/absence of renal cell cancer.

Human cDNA libraries for liver, placenta, pancreas, heart, skeletal muscle,
brain, kidney, and lung packaged in Agt11 phage were obtained from Clontech (Palo Alto, CA).

Peripheral blood (8 ml) was obtained from five normal healthy male and female volunteers. Nucleated cells were separated in Ficoll gradient cell separation tube (Vacutainer #362761; Becton-Dickson, Piscataway, NJ) as described previously (6). The nucleated cells were then removed from the buffy coat fraction, washed once in PBS, and pelleted. Cell pellets were stored at -80°C before RNA extraction.

RNA Extraction. RNA extraction was performed using a guanidinium thiocyanate-phenol chloroform technique (14) using the RNazol B reagent of Tel-Test (Friendswood, TX). Cells grown in culture were scraped into medium, centrifuged, washed, and then directly homogenized (using a Brinkman tissue disrupter) in 2 μl of RNazol B/20 mm² of cells growing in a monolayer. Frozen tissues were pulverized under liquid nitrogen, and the frozen powder was likewise extracted with RNazol B. The RNA pellet obtained after isopropanol and ethanol precipitation was dried under a vacuum drier and resuspended in 50 μl of RNase-free water. RNA yield was quantified using UV spectrophotometry at 260 nm. RNA specimens were stored in aliquots at -80°C before RT.

RT of RNA. One μg of RNA was added to 0.5 μg of oligo(deoxythymidylic acid primer (Life Technologies, Inc.) and brought to a final volume of 20 μl. The samples were placed at 65°C for 5 min and then cooled on ice. The primer RNA mixture was then combined with 200 units of Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, Inc.) and 10 units of cloned RNase inhibitor. The master mixture was completed by adding 1 mm deoxynucleotide triphosphate, 50 mm Tris-HCl, 75 mM potassium chloride, 3 mM magnesium chloride, and 10 mM DTT. The RT reaction was then carried out at 37°C for 90 min. Samples were stored at -20°C.

PCR Assay for MN. Oligonucleotide primers specific for MN cDNA were designed using the Oligo Software application system. The two primers (5'-GGGACAGAACAGGGATGAC-3' and 5'-AAAGGCCGCTAGGTTGAAA-3') were chosen so that they would amplify a particular 386-bp region of MN cDNA (from position 433 to 819 of the cDNA sequence). The 5' primer was designed to span the splice junction between the first and second exon within the MN genomic sequence. This design should prevent the amplification of any product from genomic MN DNA that might contaminate RNA specimens. PCR was then performed on the cDNAs with a denaturing temperature of 94°C (1 min), an annealing temperature of 57°C (1 min), and an extension temperature of 72°C (1 min) for 35 cycles (Perkin-Elmer Corp. thermocycler 7000).

As a control for RNA integrity, a separate portion of cDNA was amplified using primers specific for human glyceraldehyde-3-phosphate dehydrogenase primers. Positive and negative PCR controls were provided by HeLa cell RNA (strongly positive for MN), which was reverse-transcribed to cDNA (to provide a positive control) or not reverse-transcribed (to provide a negative control or a control for contamination).

PCR products were then separated by electrophoresis on a 1% agarose gel. DNA fragments were visualized and photographed under UV light subsequent to ethidium bromide staining. The expected band for MN is 386 bp, and this was identified by comigration of a DNA marker ladder electrophoresed in an adjacent lane.

Cloning and Sequencing of the MN PCR Product. After amplification using the MN primers, the positive 386-bp DNA fragment was excised from the agarose gel (subsequent to RT-PCR assay of HeLa Cell RNA) and directly cloned into the TA cloning vector (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. The cloned 386-bp fragment was then sequenced using standard dideoxynucleotide techniques to confirm appropriate homology with the MN transcript.

Results

Detection, Cloning, and Sequencing of the MN RT-PCR Product. Using oligonucleotide primers that were designed to amplify a 386-bp portion of MN cDNA, we performed a RT-PCR assay on HeLa cell RNA, the first reported source of MN protein. The PCR reaction products were electrophoresed on a 1% agarose gel, and ethidium bromide staining of this gel identified the 386-bp band characteristic of amplification of MN cDNA. The identity of the 386-bp PCR product as a fragment of MN cDNA was confirmed by cloning and subsequent sequencing of the amplified DNA fragment.

The sequence of this product was identical to the region of MN cDNA from position 433 to position 819 of the cDNA sequence (12), the region that the primers were designed to amplify. A negative control tested with this RT-PCR assay (using non-reverse-transcribed HeLa cell RNA) did not show any amplified products, and likewise, a direct PCR amplification of purified human genomic DNA did not show any amplified products. Based on primer design, the genomic DNA-based amplification product would be expected to exceed the optimum length for most PCR-based detection systems.

MN Expression in Benign Human Tissue. Representative cDNA libraries were obtained from nine different normal human tissues. These cDNA libraries were directly amplified using the MN primers in a standard PCR reaction. The MN PCR product was not detected in the reaction products from human nucleated blood cells, heart, lung, brain, skeletal muscle, placenta, or kidney. A distinct 386-bp band characteristic of MN cDNA was detected, however, in the reaction products of the human liver and pancreatic cDNA libraries (Fig. 1).

MN Expression in Cultured Human Cells. Total RNA was extracted from human prostate and kidney cancer cell lines grown in culture. Although the RNA extracted from the LnCaP cell line (malignant human prostate) showed no expression of the MN transcript, three of six human renal cell carcinoma lines revealed strong expression of MN (SKRC-01, SKRC-08, and SKRC-09; Fig. 2). The cell surface marker G250 has been detected on the surface of certain renal cell carcinoma lines (15). These three cell lines have previously been shown by immunohistochemistry to express the renal cancer cell surface marker G250, whereas the cell lines that did not express MN are known to be G250 negative.4

MN Expression in Human Renal Tissue. The finding of MN expression in renal carcinoma cell lines prompted further investigation into MN expression in clinical samples of benign and malignant renal tissue. Normal human renal tissue and primary renal carcinomas were obtained after radical nephrectomy of renal cancer patients. The RT-PCR analysis of RNAs from these specimens revealed positive MN expression in 12 of 17 renal tumors tested. Histologically, all of the MN-positive specimens were pure clear cell carcinomas (Table 1). For 10 of these 17 renal cancer patients, samples of normal renal tissue were obtained from sites within the ipsilateral kidney, and no evidence of MN expression was detected in any of these specimens (Fig. 3).

Fig. 1. RT-PCR assay identifies the expression of the 386-bp MN cDNA message in the HeLa cell line. No expression is seen in cDNA libraries from human heart, kidney, lung, muscle, placenta, or brain. MN expression is seen in the human pancreas and liver. Human glyceraldehyde-3-phosphate dehydrogenase primers were used in a parallel reaction to confirm RNA integrity.

4 N. H. Bander, unpublished data.
Discussion

Based on ultrastructural and immunohistochemical evidence, renal cell carcinoma is thought to arise from cells of the proximal tubule (16). Although renal cancer cells share many surface markers with renal tubular cells, no single protein has proven effective as a serum marker in renal cancer. Serum levels of proteins such as erythropoietin and ferritin have been used to follow patients after nephrectomy to detect recurrent disease (17—20). However, these markers have shown limited clinical usefulness in the diagnosis and staging of renal cancer. Based on the results obtained in our survey of human cell lines, cDNA libraries, and tissues, we believe that the gene product referred to as MN might have potential value as a marker of certain types of renal cell cancer in humans.

The novel tumor-associated protein MN was first isolated from the human cervical cancer cell line HeLa by Der and Stanbridge (10). MN has subsequently been characterized and found to be a 51-kDa cell surface protein. The complimentary DNA sequence and intron exon map of the MN coding region have also recently been elucidated (12, 21). The MN gene seems to be a member of the carbonic anhydrase family, and its role in oncogenesis remains unclear. However, this protein is able to induce a malignant phenotype in cell culture experiments. When MN cDNA was transfected into NIH3T3 cells, the resulting cells demonstrated loss of cell-cell growth inhibition, failed to grow in monolayers, and acquired a spindle-shaped morphology. In addition, transfection of MN resulted in a loss of cellular dependence on growth factors, shorter doubling times, and an increase in DNA synthesis (12). Based on these experiments, Pastorek et al. proposed that the MN protein may play a role in cellular growth regulation (12).

This survey reports on the distribution of expression of the MN gene in several benign and malignant human tissues. The most important results of this survey indicate that MN mRNA was never detected in RNAs extracted from normal renal parenchyma (RT-PCR negative from all 10 normal specimens examined). In contrast, in our RT-PCR analysis of 17 human renal tumors, 12 were strongly positive for MN expression. All of these tumors were found to have a pure clear cell adenocarcinoma histology. Interestingly, MN expression was not observed in granular or papillary tumors of the kidney. The small number of specimens in this category limits any statistical significance; however, we are currently expanding our series of patients to evaluate whether MN expression is a distinct marker of clear cell adenocarcinoma of the kidney, the most common form of renal cancer, and not of other forms.

The expression of the MN mRNA in the tissue from human liver and pancreas has not been described before. However, MN is expressed in gastric mucosa of normal subjects and is perhaps localized to tissues of the upper gastrointestinal tract.

The expression of MN in the three human renal tumor cell lines (SKRC-01, SKRC-08, and SKRC-09) is relevant, because these cells have previously been shown to express the renal cell carcinoma-specific cell surface marker G250. The absence of MN expression in SKRC-17, SKRC-39, and SKRC-42 is consistent with this association, because these three cell lines have been shown to be G250 negative. The G250 gene has been cloned and sequenced, and preliminary analysis indicates homology to the MN gene (22). The precise relationship between MN gene expression and G250 expression, however, will require further study.

Curative treatment of renal cell carcinoma is available for organ-confined disease, and investigation into the immunotherapy of metastatic disease continues. Unfortunately, there is no well-established renal carcinoma-specific marker available at this time. An effective marker could be used to detect early recurrences, monitor response to immunotherapy regimens, detect extra renal spread at presentation, or even diagnose renal cancer in cases of complex renal masses. With

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**Table 1: MN expression in 17 human renal tumors and normal renal tissue**

<table>
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**a** Pt: patient.  
**b** Pos: positive; neg, negative; NA, not available.
techniques such as the RT-PCR assay, the clinician in the future may rely on highly sensitive techniques to detect microscopic evidence of disease progression. Our results here suggest that MN might be a useful marker to develop for this purpose. The potential role of MN in the molecular staging of renal cell carcinoma is currently being evaluated at our institution using this RT-PCR-based assay on peripheral blood specimens.

References

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