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Quantitation of Membrane Type Serine Protease 1 (MT-SP1) in Transformed and Normal Cells

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Membrane type serine protease 1 (MT-SP1) is a representative member of a large family of related enzymes known as type II transmembrane serine proteases or membrane type serine proteases. MT-SP1 has been implicated in the selective proteolysis of key extracellular substrates but its physiological role is still not fully understood. MT-SP1 expression at the protein and RNA level has been previously examined by non-quantitative methods such as *in situ* hybridization, Northern blotting and immunohistochemistry. To establish an introductory understanding of the quantitative mRNA expression of MT-SP1 and to correlate these levels with urokinase-type plasminogen activator receptor (uPAR), a key component of extracellular proteolysis, quantitative RT-PCR was carried out. RNA expression was analyzed in 34 human cancer cell lines, 26 human tissues and 18 primary human breast cancer tissue samples. MT-SP1 mRNA is highly expressed in many breast, ovarian, prostate and colon cancer cell lines and normal human tissues of endodermal origin. At the transcript level, MT-SP1 shows a highly statistically significant correlation (Pearson's product moment correlation r = 0.784, $p < 0.001$) with uPAR in human breast cancer tissue. The exact role of MT-SP1 in concert with proteins such as uPAR and other members of the plasminogen activator cascade has yet to be ascertained. However, the significant correlation between MT-SP1 and uPAR transcript levels in this initial study suggests further work to establish the role of MT-SP1 as a possible prognostic, diagnostic or therapeutic target for breast cancer.

Key words: Breast / Cancer / MT-SP1 / Protease / Serine.

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Introduction

Central to the pathogenic potential of cancer is the ability of a cancer cell to invade through basement membrane and metastasize. The process of metastasis involves a sequence of interrelated steps that includes invasion of local host tissue, intravasation, extravasation and permeation through the basement membrane (Hanahan and Weinberg, 2000). A number of natural tissue barriers, including interstitial connective tissue and basement membranes, must be degraded by proteases during the process of cancer invasion and metastasis (Tryggvason *et al.*, 1987). The role of proteases, however, ranges beyond simple degradation of the proteinacious extracellular matrix (ECM) to regulation of cell signaling and metabolism (Sternlicht and Werb, 2001). Proteolytic activities, such as ECM degradation and regulation of cell signaling, are absolutely required for the complex orchestration of cancer cell invasion and metastasis. This makes cancer-related proteases a particularly interesting subclass of regulatory enzymes to study.

Several classes of proteases are upregulated in cancer including the serine proteases of the plasminogen activating cascade and the matrix metalloproteinases (MMP). Recently, a new family of membrane-anchored proteases, known as the type II transmembrane serine proteases (TTSP), was identified (Hooper *et al.*, 2001). As with both the plasminogen activating cascade and the MMPs, a majority of the TTSPs that have been characterized are upregulated in cancer (Tanimoto *et al.*, 1997; Underwood *et al.*, 2000; Wallrapp *et al.*, 2000; Dhanasekaran *et al.*, 2001; Oberst *et al.*, 2001). Members of the TTSP family of proteases have been described independently by several groups (Figure 1) (Paoloni-Giacobino *et al.*, 1997; Lin *et al.*, 1999b; Takeuchi *et al.*, 1999; Hooper *et al.*, 2001). Membrane type serine protease 1 (MT-SP1), a member of this family, is closely related to the best-known and first member of this family of trypsin-like serine proteases, enteropeptidase. Expressed in many tissue types, including both breast and prostate tissue, MT-SP1 [also independently cloned by Lin *et al.* (1999b) and termed matriptase, and by Kim *et al.* (1999) from mouse and termed epithin] has been implicated in

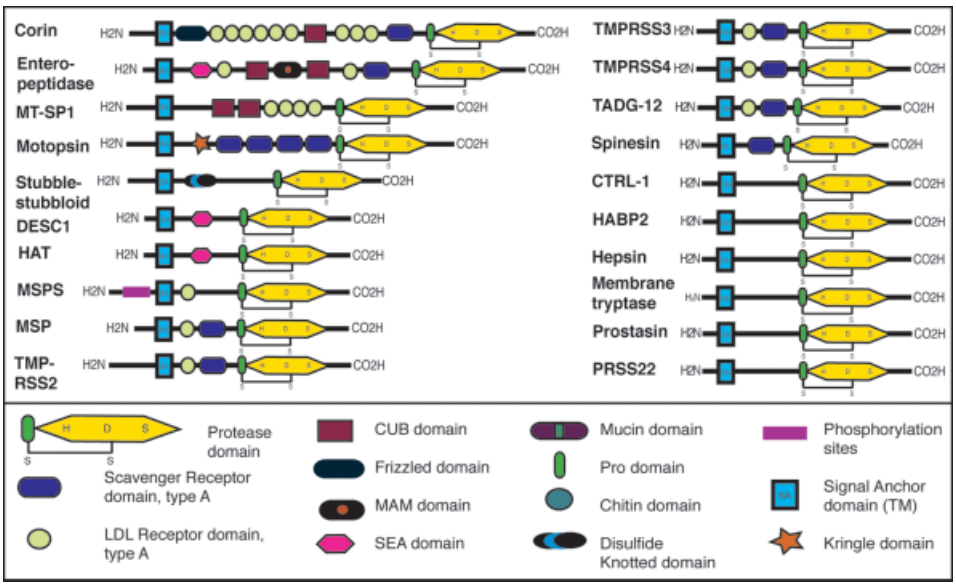

Fig. 1 Domain Diagram of Membrane Type Serine Protease Family Members.
The membrane type serine protease family, also known as the TTSP family, is represented figuratively by domain. Members of the TTSP family are multi-domain proteins that exhibit a characteristic structure: an N-terminal cytoplasmic tail, a transmembrane domain, protein-protein interaction domains and a C-terminal protease domain.

prostate, breast and ovarian cancer progression and metastasis (Takeuchi *et al.*, 1999, 2000; Oberst *et al.*, 2001). The role of this protease in breast cancer is, however, not well established. Expression at the mRNA level was confirmed in breast cancer cell lines, but the mRNA expression pattern of MT-SP1 in human breast cancer tissue samples has not yet been studied (Lin *et al.*, 1999a).

The first evidence that supports the differential activity of MT-SP1 in normal *versus* cancerous tissue was recently described in the case of ovarian cancer. This report suggests that while MT-SP1 protein levels (measured by immunohistochemistry) are not substantially different in ovarian tumors *versus* normal ovarian tissue, the expression of a cognate inhibitor, hepatocyte growth factor activator inhibitor 1 (HAI-1), does decrease with increasing cancer grade (Oberst *et al.*, 2002). This argues that perhaps MT-SP1 activity, and not overall expression, is altered in cancer *versus* normal tissues. Both immunohistochemistry and *in situ* hybridization can readily identify cell types that express a given protein or mRNA, but these methods are of limited use in precise protein or mRNA quantification. While densitometric analysis can be applied to methods such as immunohistochemistry and *in situ* hybridization, this may not be the most accurate means by which to determine differential gene expression in multiple tissue samples. In order to more rigorously study gene expression at the mRNA level other methods, such as quantitative RT-PCR, must be employed.

A number of methods have been applied to understand possible functions of MT-SP1. These include identifying expression patterns, putative substrates and signaling pathways involving this protease. A cadre of combinatorial chemical, biochemical and biological methods has already been used to identify possible substrates of MT-SP1. Tools such as tetrapeptide positional scanning synthetic combinatorial library screening and substrate bacteriophage display have been used to profile the catalytic domain of MT-SP1. The extended substrate specificity was shown to be P4-K/R, P3-Q/R, P2-S/A, P1-R. A candidate approach was then used to identify possible substrates. Currently the repertoire of macromolecules that MT-SP1 cleaves includes hepatocyte growth factor (HGF), single chain urokinase-type plasminogen activator (sc-uPA) and protease activated receptor-2 (PAR-2) (Lee *et al.*, 2000; Takeuchi *et al.*, 2000). These are all in very good agreement with predicted substrate specificity from the aforementioned combinatorial methods. While identification of putative substrates is a valuable approach, genetic removal (knockout) and inhibition of MT-SP1 are two other distinct, but related, approaches to creating a comprehensive understanding of the biological role of MT-SP1. Phenotypic characterization of the mouse that has the MT-SP1 homolog, epithin, knocked out has provided useful information (List *et al.*, 2002). The MT-SP1 null mouse, however, is short-lived dying within 48 hours of birth. This complicates experimental approaches to address the role of MT-SP1 in late stage cancers. Additionally, epithin is only 81% identical to MT-SP1 raising concern about the functional identity between the two enzymes. Furthermore, there always exists the potential for compensation of the lack of epithin activity from the other TTSP family members. Chemical genetic methods using both small molecule and macromolecular inhibitors

of MT-SP1 provides a complementary approach to elucidate possible roles of MT-SP1 *in vivo*. Indeed, a bis-benzamidine (K_i=208 nM) was recently identified as an inhibitor of MT-SP1 with a 13 fold higher K_i against thrombin in comparison to MT-SP1 (Enyedy *et al.*, 2001). Further inhibitor development will play an instrumental role in both *in vivo* and *in vitro* assays of MT-SP1 function.

Underlying the utility of all of these methods to characterize and describe MT-SP1 function is a clear understanding of the expression profile of the enzyme. Once established, the profile can be compared to known and putative cancer markers. One protease cascade that is well known to be involved with the extracellular matrix remodeling associated with neoplasia is the plasminogen cascade (Duffy, 1990; Bell, 1996). Given the evidence that MT-SP1 is able to efficiently activate uPA, a potent activator of the plasminogen cascade, exploration of the interrelationships between MT-SP1 and members of the uPA system is warranted (Lee *et al.*, 2000; Takeuchi *et al.*, 2000). Such studies may lead to identification of possible roles of MT-SP1 with respect to both the uPA system and neoplasia progression. Elements of the uPA system such as uPA itself, urokinase type plasminogen activator receptor (uPAR), and a potent serpin inhibitor of uPA known as plasminogen activator inhibitor 1 (PAI-1) have been studied by others for their role(s) in cancer. Furthermore, uPA, uPAR and PAI-1 have also been examined for their use as possible breast cancer markers (Broet *et al.*, 1999; Foekens *et al.*, 2000; Kotzsch *et al.*, 2000; de Witte *et al.*, 2001; Look *et al.*, 2002). While uPA, PAI-1 and uPAR are all possible breast cancer markers whose expression can be studied at the protein level by serum ELISAs or immunohistochemistry, uPAR is unique in that it is a membrane protein. Due to its localization at the cell surface, protein level studies are complicated by the difficulty in extracting uPAR from tissue samples. An extraction method has been developed for the isolation of the uPAR-containing fraction from tissues but uPAR expression may be more easily quantified by RNA based methods (Bouchet-Bernet *et al.*, 1996). RNA-based methods also allow for comparison of transcript levels without the added problems associated with correlating protein expression levels in samples obtained by different extraction methods. The tissue distribution of MT-SP1, which has been described by others in a non-quantitative manner (by *in situ* hybridization, Northern blotting and immunohistochemistry), is known to be broad. Both uPAR and MT-SP1 are postulated to play a role in breast cancer due to their high level expression in a variety of breast cancer cell lines, but examination of the expression of MT-SP1 at the protein or mRNA level in breast cancer tissues has, to date, not been reported.

Results

RNA samples were obtained from a variety of sources. When available, commercial suppliers were used, providing two independent sources of 26 normal human tissues. In addition, mRNA was isolated from 34 cancer cell lines as well as from 18 primary tumors of cancer patients with varying stages of breast cancer. Relevant clinical and pathological details of the primary breast cancer tissue samples are provided (Table 1).

Table 1 Patient Characteristics for Primary Breast Cancer Tissue Samples Used in TaqMan® Determination of MT-SP1 and uPAR Transcript Levels.

Parameter[a]	n	%
Age (yr)		
≤39	3	17
40–49	3	17
50–59	2	11
60–69	1	6
70–79	5	28
≥80	4	22
Menopausal status		
Premenopausal	6	33
Postmenopausal	12	67
Tumor size (cm)		
≤2	2	11
2.1–5	8	44
>5	6	33
Histological type		
Ductal carcinoma	16	89
Lobular carcinoma	2	11
Tumor Grade		
I	2	11
II	8	44
III	4	22
IV	2	11
Receptor status		
Receptor positive	15	83
Receptor negative	3	17
Positive lymph nodes		
0	3	17
1–3	4	22
>3	10	56

[a] Parametric data is presented in as complete a manner as allowed by patient clinical history. In some cases, such data was not fully available.

MT-SP1 Is Expressed in a Variety of Epithelial Cancer Cell Lines

Quantitative RT-PCR (Sybr Green detection) was used to measure MT-SP1 transcript levels in 34 cancer cell lines (Figure 2). Initial experiments were performed on cell lines of various origins (two vascular endothelial, seven breast, ten colon, eight prostate, two fibroblast and one each of ovary, lung, fibrosarcoma, and teratocarcinoma; Figure 2). Relative values of expression are reported and all samples are normalized to levels of β-actin and hypoxanthine phosphoribosyl transferase (HPRT). In order to quantitatively assess the difference in transcript levels between the cell lines studied, the MT-SP1 mRNA level is reported as a percentage of the MT-SP1 level in HT29,

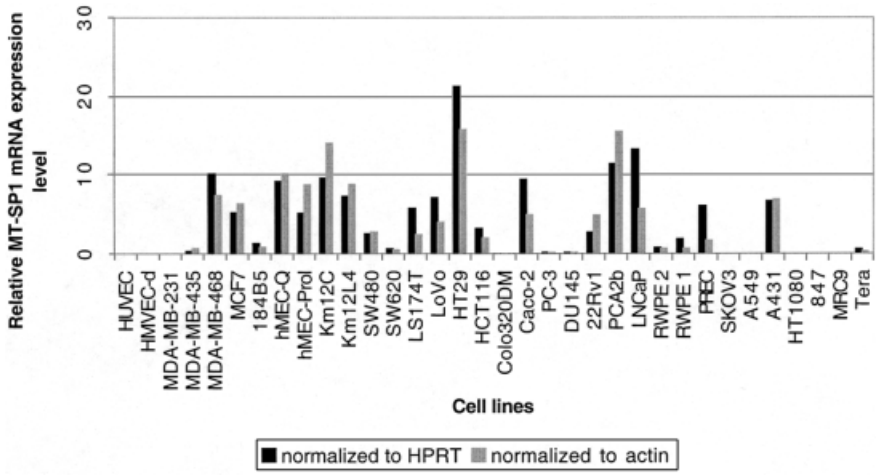

Fig. 2 Expression of MT-SP1 in Cancer Cell Lines.
Quantitative RT-PCR analysis of several human cancer cell lines shows high transcript levels of MT-SP1 in cancer cell lines of breast, prostate, colon and ovary. Expression levels of MT-SP1 are normalized to those of actin, a cytoskeletal gene (light shading) and HPRT (dark shading). HUVEC and HMVEC are vascular endothelial cell lines. MDA-MB-231 to hMEC-Prol are breast cell lines (tumorigenic lines followed by normal lines). Km12C to Caco-2 are colon cancer cell lines. PC-3 to PREC are prostate cell lines (tumorigenic lines followed by normal lines). The remaining cell lines are SKOV3 (ovarian cancer), A549 (lung carcinoma), A431 (epidermal cancer), HT1080 (fibrosarcoma), 847 (fibroblast), MRC9 (skin fibroblast) and Tera (teratocarcinoma).

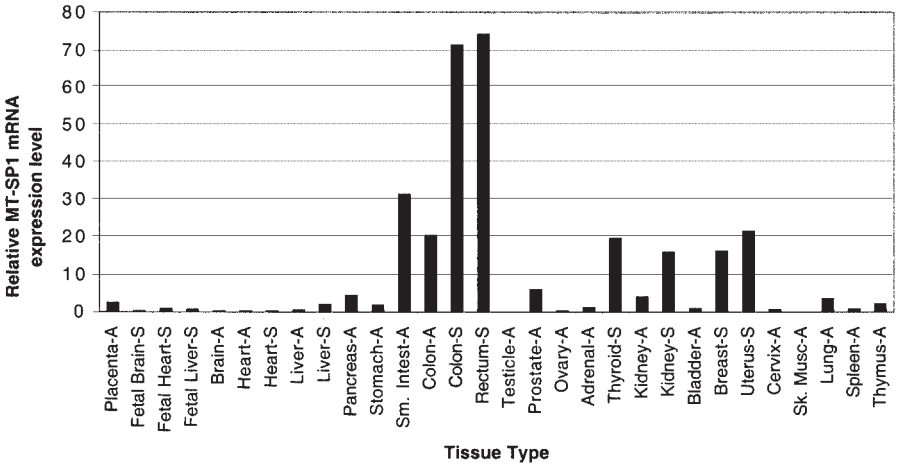

Fig. 3 Expression of MT-SP1 in Normal Human Tissue Measured by Quantitative RT-PCR Analysis.
Transcript levels of MT-SP1 are detected in rectum > colon > small intestine > uterus, thyroid, breast, kidney > prostate > pancreas > lung, placenta, liver, stomach > adrenal gland, bladder, spleen, cervix, fetal heart and fetal liver. The dark shaded bar indicates MT-SP1 mRNA expression level relative to the control gene HPRT. Total RNA for this assay was acquired from two sources: Stratagene (S) and Ambion (A). The mRNA expression of MT-SP1 in tissues of the same type from the two different sources is not perfectly correlated. Transcript levels of MT-SP1 are routinely many-fold higher in the samples purchased from Stratagene in comparison to those purchased from Ambion (liver, colon, kidney). This may reflect the small pools from which these tissues are generally derived, comprising one to three patient samples.

the cell line with the highest transcript levels of MT-SP1. MT-SP1 mRNA expression is seen in the primary breast cell line 184B5 (5% of HT29 MT-SP1 transcript levels normalized to actin, 6% of HT29 MT-SP1 transcript levels normalized to HPRT), but is four- to nine-fold less than in some tumorigenic breast cell lines such as MCF-7 (40%, 24%) and MDA-MB-468 (46%, 47%). Based on this trend, one would expect the other human mammary epithelial cell lines studied (hMEC-Q or quiescent and hMEC-Prol or proliferating) to have low levels of MT-SP1 transcript. In contrast, both these cell lines express MT-SP1 mRNA at high levels (hMEC-Q: 64%, 43% and hMEC-Prol: 55%, 24%) similar to those observed in MDA-MB-468 (46%, 47%) and MCF-7 (40%, 24%). The tumorigenic prostate cell lines 22Rv1 (31%, 12%), PCA-2b (99%, 54%), and LNCaP (36%, 62%) showed a trend of higher MT-SP1 transcript levels than the non-tumorigenic prostate cell lines RWPE 1 (3%, 8%) and

PREC (10%, 28%). However, certain prostate cancer cell lines such as PC-3 (0%, 0%) and DU 145 (0%, 0%), exhibited undetectable levels of MT-SP1 by this method. Strikingly, in the cancers studied, MT-SP1 is not universally expressed in all tumorigenic lines. For example, SKOV3 (0%, 0%), an ovarian cancer cell line, shows no expression of MT-SP1. The colon cancer cell lines studied, particularly HT29 (100%, 100%), Km12C (89%, 45%) and Km12L4 (56%, 34%), exhibit remarkably high transcript levels of MT-SP1. In fact, HT29 has the highest level of MT-SP1 mRNA expression in the panel of cell lines examined. Certain colon cancer cell lines such as SW620 (3%, 3%) and Colo320DM (0%, 0%) exhibit negligible MT-SP1 transcript levels. No MT-SP1 transcript is detected in the endothelial cell lines HUVEC (0%, 0%) and HMVEC-d (0%, 0%) as well as the lung cancer cell line A549 (0%, 0%), the fibrosarcoma cell line HT1080 (0%, 0%), the fibroblast line 847 (0%, 0%), and the skin fibroblast cell line MRC9 (0%, 0%). The teratocarcinoma cell line Tera exhibits very low levels of MT-SP1 transcript (1%, 2%). Somewhat surprisingly, MT-SP1 transcript levels are significantly higher in the epidermal cancer cell line A431 (44%, 31%) than in the other cell lines of non-endodermal origin. Overall, expression is seen in some, but not all, colon, breast and prostate cancer cell lines and generally not in the control cell lines of non-endodermal origin (vascular endothelial, lung, fibroblast).

MT-SP1 Is Expressed in a Range of Normal Human Tissue Types

In order to understand the pattern of MT-SP1 expression in normal human tissues quantitative RT-PCR (Sybr Green detection) was utilized to measure MT-SP1 transcript levels in a variety of tissues (Figure 3). Relative values of mRNA expression are reported and all samples are normalized to HPRT levels. There is fairly good agreement in the transcript levels measured in tissue samples from two commercial sources. Transcript levels of MT-SP1 are found to be highest in the rectum. MT-SP1 transcript levels in order from highest magnitude to lowest magnitude are rectum > colon > small intestine > uterus, thyroid, breast, kidney > prostate > pancreas > lung, placenta, liver, stomach > adrenal gland, bladder, spleen, cervix, fetal heart and fetal liver. The mRNA expression of MT-SP1 in tissues of the same type from the two different sources is not perfectly correlated and was found to be many-fold higher in samples from one commercial supplier over the other (Figure 3).

MT-SP1 Is Expressed in Human Breast Cancer Tissue Samples

TaqMan® analysis was performed on 18 primary breast cancer tissue samples, HMEC (a transformed epithelial cell line) and five common breast cancer cell lines (600MPE, MDA-MB-436, MCF7, T47D, BT474) (Figure 4). MT-SP1 levels were normalized to those of β-glucuronidase (GUSB). Transcript levels that clustered into a similar range were observed in all 18 breast cancer tissue samples (Figure 4A). This includes five samples from patients who experienced tumor recurrence and 11 patients who did not experience recurrence. The 600MPE, MCF-7, T47D and BT474 breast cancer cell lines also express MT-SP1 at levels similar to those observed in breast tissue specimens. In contrast, transcript levels of MT-SP1 are approximately three orders of magnitude lower in MDA-MB-436, one of the five breast cancer cell lines studied (Figure 4B). MT-SP1 transcript is detected in primary tumors in both tissue samples from five patients who did experience tumor recurrence and 11 patient who did not experience recurrence without a significant difference between the two groups. No significant correlation was found between MT-SP1 transcript levels and clinical follow-up data, which included age, menopausal status, nodal status, tumor size or grade. A significant fraction of the patients in this small cohort tended to risk factors for

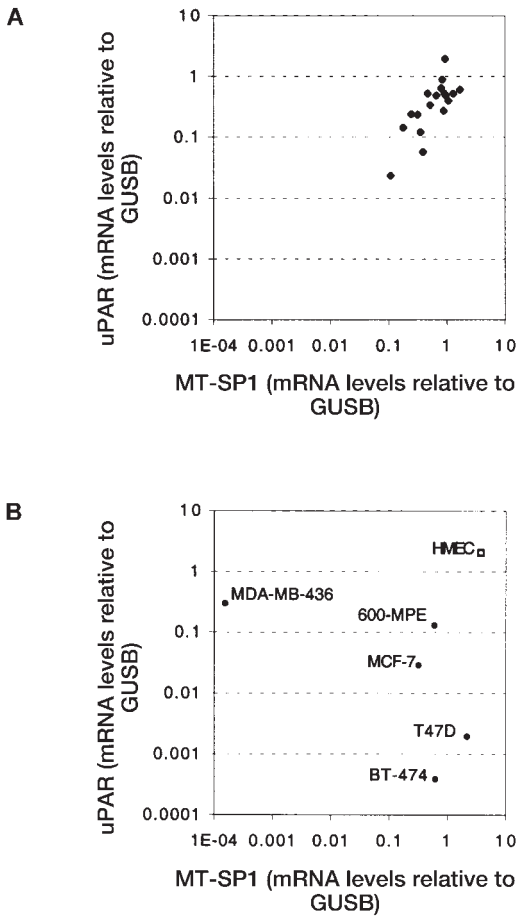

Fig. 4 Expression of MT-SP1 and uPAR in Breast Cancer Tissue Samples and Breast Cancer Cell Lines Measured by TaqMan®.
The x- and y-axes show the relative expression of MT-SP1 and uPAR to the control gene GUSB, respectively. (A) MT-SP1 expression shows a nearly 1:1 correlation with that of uPAR, a key component of extracellular proteolysis in 18 breast cancer tissue samples. (B) Breast cancer cell lines exhibit less tightly correlated expression levels of MT-SP1 and uPAR. Cancer cell lines are labeled by name.

recurrence of breast cancer and increased mortality (Table 1): most had large tumors, 33% larger than 5 cm, and 44% between 2.1 and 5 cm. In addition, 1/3 were either Stage III or IV. Over 50% of the patients had >3 lymph nodes with metastatic cancer.

MT-SP1 and uPAR Expression Are Closely Correlated in Breast Cancer Tissue Samples But Not All Breast Cancer Cell Lines

TaqMan® analysis of the breast cancer tissue samples shows a highly statistically significant correlation (defined as $p < 0.001$) of MT-SP1 and uPAR (Pearson's r=0.784, $p < 0.001$). The Pearson's product moment correlation coefficient calculation is based on an analysis of the log values of MT-SP1 and uPAR transcript levels. HMEC has higher overall transcript levels of both MT-SP1 and uPAR compared to the cancer cell lines and the breast cancer tissue specimens. As is the case for the breast cancer tissue samples the levels of MT-SP1 and uPAR do, however, correlate within the same order of magnitude in HMEC. In contrast, three of the five cancer cell lines show MT-SP1 and uPAR transcript levels that differ by two to four orders of magnitude. These cancer cell lines are MDA-MB-436, BT474 and T47D. One cell line, MDA-MB-436, has a much lower level of MT-SP1 transcripts than the other samples while retaining high levels of uPAR transcripts. In contrast, BT474 and T47D have very high MT-SP1 transcript levels but very low uPAR transcript levels.

Discussion

Our initial studies of proteases involved in prostate cancer led to the discovery of MT-SP1 (Takeuchi *et al.*, 1999). While it is possible that MT-SP1 plays a role specifically in prostate cancer, we have opted to take an unbiased approach to studying the roles of novel proteins, such as MT-SP1, in human biology and disease. To provide a more systematic analysis of the mRNA expression pattern of MT-SP1 we have quantified MT-SP1 transcript in an array of normal tissues and cancer cell lines. Future *in vitro* experiments can be guided by cell line mRNA expression data and *in vivo* applications such as the design of tissue specific transgenic models can be directed by tissue level mRNA expression data.

Transformed Cell Lines

Previous reports have demonstrated mRNA and protein expression of MT-SP1 in normal and cancerous cells from a wide variety of human tissue sources and human prostate, breast and ovarian cancer cell lines (Lin *et al.*, 1999a; Takeuchi *et al.*, 1999; Oberst *et al.*, 2001). MT-SP1 transcript levels were previously detected by Northern blotting in two immortalized breast epithelial cell lines, 13 breast cancer cell lines and two ovarian cancer cell lines (Oberst *et al.*, 2001). To expand the understanding of MT-SP1 expression in a larger set of normal and cancer cell lines, quantitative RT-PCR (Sybr Green detection) of 34 cancer cell lines was used to measure MT-SP1 transcript levels in a variety of cancer cell lines. Interestingly, MT-SP1 expression was highest in the colon cancer cell line HT29. Most of the studies of MT-SP1 in human cancer and tissues, to date, have been reported in the context of breast, ovarian and prostate cancer. There are initial suggestions of the importance of MT-SP1 (also termed SNC19) in colon cancer, but biochemical experimentation to confirm this suggestion has not yet been reported (Zhang *et al.*, 1998; Cao *et al.*, 2001). The data presented here in conjunction with the previous reports of MT-SP1 in colorectal cancer cell lines suggest that MT-SP1 may be a key player in normal colon biology and colon cancer. Four of the seven breast cell lines (two tumorigenic and two normal), two of the eight prostate cell lines (both tumorigenic) and four of the ten colon cancer cell lines expressed MT-SP1 mRNA at high levels (defined as greater than 30% of the highest level of expression of MT-SP1 when normalized to actin). The mRNA level expression information provided here should help to direct future cell-based experimentation oriented towards studying the role of MT-SP1 in normal and cancer biology.

Normal Human Tissue

While cell line expression information is certainly valuable in establishing model systems for biological study, understanding MT-SP1 expression in normal tissue samples is more biologically relevant. In the quantitative RT-PCR analysis presented here, the mRNA expression pattern of MT-SP1 was rectum > colon > small intestine > uterus, thyroid, breast, kidney > prostate > pancreas > lung, placenta, liver, stomach > adrenal gland, bladder, spleen, cervix, fetal heart and fetal liver. This is in good general agreement with previously published Northern data (Takeuchi *et al.*, 1999; Cao *et al.*, 2001). MT-SP1 transcripts are found primarily in cells of endodermal origin and are prevalent in glandular, exocrine cells such as colon, salivary gland, pancreas, small intestine, breast and prostate. Of note, MT-SP1 mRNA expression in stomach, small intestine and spleen is seen to a greater extent in published Northern blotting data than in the data presented here. Northern blots, however, are reliable only insofar as gleaning a qualitative view of expression and may not be effective in quantitating protein levels in both tissue and cell line samples. With the quantification of transcript levels in both normal tissues and a variety of cancer cell lines, it is clear that MT-SP1 mRNA is expressed in both normal tissue and cancer cell lines of a broad range of tissue types. While a definitive statement on overall MT-SP1 expression in cancer *versus* normal states cannot be made, MT-SP1 appears to be involved in both normal and cancerous tissue biology. The data presented here support a general trend of higher levels of MT-SP1 transcript in breast and prostate carcinoma cell lines versus normal breast and prostate cell lines.

Human Tissue Samples

In order to assess the role of MT-SP1 (and other family members) in human cancer, studying expression of MT-SP1 in human cancer tissue samples is of paramount importance. Immunohistochemistry of ovarian tumors showed MT-SP1 staining at both early and advanced stages. While MT-SP1 protein levels were fairly constant at different stages of ovarian cancer, advanced stage tumors were found to express MT-SP1 in the absence of the proposed inhibitor of MT-SP1, HAI-1 (Oberst *et al.*, 2002). Expression in breast cancer tissue samples, on the other hand, has not yet been reported. Understanding breast cancer expression of MT-SP1 was deemed salient because of the very high level of expression of the protease in both human breast milk (a product of the ductal epithelium) (Lin *et al.*, 1999a,b) and a variety of breast cancer cell lines as previously addressed (Oberst *et al.*, 2001). To this end, the transcript levels of MT-SP1 in human breast cancer tissue were determined by quantitative RT-PCR. MT-SP1 mRNA levels clustered within one order of magnitude for all the cancer tissue samples, HMEC and four of the five cancer cell lines studied. HMEC, a cell line that is thought to represent normal breast tissue, exhibited transcript levels of MT-SP1 that were notably higher than in the breast cancer tissue samples. It is possible that MT-SP1 is slightly down-regulated in cancer versus normal breast tissue but this will require further study.

While MT-SP1 has not yet been established as a prognostic marker in breast cancer, there is support for uPA, uPAR and PAI-1 as possible prognostic indicators in breast cancer (Del Vecchio *et al.*, 1993; Jankun *et al.*, 1993; Broet *et al.*, 1999; Look and Foekens, 1999; Foekens *et al.*, 2000; Guyton *et al.*, 2000; Pedersen *et al.*, 2000; de Witte *et al.*, 2001; Mazar, 2001; Harbeck *et al.*, 2002; Look *et al.*, 2002; Riisbro *et al.*, 2002). Once activated by proteolytic cleavage, the mature uPA bound to uPAR is thought to activate plasminogen and the associated downstream signaling cascade. Previous reports indicate conflicting results regarding the role of uPAR as a possible prognostic; whereas some groups have found uPAR to be an indicator of breast cancer progression, uPA and PAI-1 are thought to be stronger markers of prognosis (Foekens *et al.*, 2000; Harbeck *et al.*, 2002; Look *et al.*, 2002). Hypothesizing that the difficulty in measuring uPAR protein levels accurately by ELISA may be the result of incomplete extraction methods, mRNA-based studies were carried out to quantify uPAR expression in a variety of primary breast cancer tissue samples. Because MT-SP1 has been shown to activate sc-uPA we hypothesized that the expression levels of two membrane bound members of this cascade (MT-SP1 and uPAR) would be well correlated. TaqMan® analysis performed on 18 breast cancer tissue samples, HMEC (a transformed epithelial cell line) and five commonly used breast cancer cell lines supported this hypothesis by showing a tight correlation between MT-SP1 and uPAR mRNA expression. The correlation between MT-SP1 and a putative tumor marker suggests that MT-SP1 may be a valuable tumor prognostic marker, if not a target for diagnostics and therapeutics, in the future. Furthermore, correlation of the two transcript levels with each other suggests the intriguing possibility that regulation of their expression is transcriptionally coordinated at the biochemical level. This would not be surprising given their potential physiological interactions. It will be interesting to determine whether other components of this cascade, such as uPA, have similar transcript levels. While the primary breast cancer tissue samples showed a highly statistically significant correlation between MT-SP1 and uPAR, three of the five cancer cell lines exhibited MT-SP1 and uPAR transcript levels that differed by two to four orders of magnitude. Accordingly, these are inappropriate cell lines to explore the possible biological relationships between uPAR and MT-SP1.

Proteases have long been associated with the basic ability of cancerous cells to invade through basement membrane and thus metastasize. With the advent of current research and a paradigm shift in the study of proteolysis, proteolytic enzymes are now implicated in all stages of cancer progression including growth at both primary and metastatic sites, angiogenesis, migration as well as invasion and the physical process of cancer metastasis (Monsky and Chen, 1993; Koblinski *et al.*, 2000). Known to exhibit alterations in expression, localization and activity in tumors (both cancerous cells and surrounding, tumor-associated stroma) *versus* normal cells (Koblinski *et al.*, 2000), proteases may prove to be a prime avenue of research from both biological and therapeutic standpoints. MT-SP1 or other TTSP family members may hold promise as a future prognostic tools, diagnostics or therapeutic targets. Even if MT-SP1 expression remains unchanged, changes in enzyme activity, membrane localization or cleavage and secretion of the protease into ductal lumen may be altered in cancer versus normal tissues. To this end, a clear understanding of protease expression in human tissues will be central in the effort to delineate the role(s) of MT-SP1 in normal and tumor biology.

The previous identification of sc-uPA as a putative substrate of MT-SP1 provides good evidence for the involvement of MT-SP1 in the plasminogen activation cascade; the relationship between MT-SP1 and uPAR provides additional support to this hypothesis. Such observations suggest the importance of MT-SP1 and related proteases in cancer and with the correlation of MT-SP1 to uPAR in a variety of breast cancer tissue specimens, studies of a clear biochemical link between the two proteins should be forthcoming.

Materials and Methods

Quantitative RT-PCR to Determine MT-SP1 Transcript Levels in Human Cancer Cell Lines

A collection of 34 human cell lines was used including HUVEC (umbilical cord-derived vascular endothelium) and HMVEC (der-

mal-derived normal microvascular endothelium); Km12C, Km12L4, SW480, SW620, LS174T, LoVo, HT29, HCT116, and Colo320DM (colon cancer); 184B5, hMEC-Q, and hMEC-Prol (non-tumorigenic breast); MDA-MB-231 (Estrogen Receptor (ER) negative), MDA-MB-435 (ER negative), MDA-MB-468 (ER negative), MCF-7 (ER positive); PC-3, DU 145, 22Rv1, Pca 2b, LnCaP, RWPE 2 (prostate cancer); RWPE 1 and PREC (normal prostate); SKOV3 (ovarian cancer), A549 (lung cancer), A431 (epidermal cancer) HT1080 (fibrosarcoma), 847 (fibroblast), MRC9 (skin fibroblast), and Tera (teratocarcinoma). These cells were grown as recommended by the ATCC. Total RNA was isolated from these cell lines using the High Pure RNA Isolation Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). RNA was reverse transcribed using MMLV reverse transcriptase and RNasin (Ambion, Austin, TX, USA) and oligo-d$(T)_{18}$ synthesized at Chiron Corporation. Two µl of each 20µl RT reaction was then quantified for MT-SP1 by real-time quantitative PCR using a Gene Amp 5700 and Sybr Green PCR Master Mix from Applied Biosystems (Foster City, CA, USA) (Schneeberger *et al.*, 1995). The resulting quantities for MT-SP1 message level were normalized to actin and HPRT message levels from the same sample to normalize for variations in reverse transcription. Primers used for quantitative PCR were synthesized at Chiron Corporation with the following sequences:

MT-SP1-forward 5'-TTCCTGGGCTTGCACGACCA-3' and MT-SP1-reverse 5'-TGCCGGTTTCTCC-AGCTCCA-3'; β-actin forward 5'-GGGAAATCGTGCGTGACATTAAG-3' and β-actin reverse 5'-TGATCTCCTTCTGCATCCTGTCGG-3'. HPRT forward 5'-AGGCAGTATAATCCAAAGATG-3' and HPRT reverse 5'-GTCAAGGGCATATCCTACAAC-3'. Primers were used at a final concentration of 180 nM. PCR was conducted using the following cycle parameters: 95°C for 10 min for 1 cycle, (95°C for 15 s, 60°C for 1 min) for 45 cycles.

Quantitative RT-PCR to Determine MT-SP1 Transcript Levels in Human Tissue Samples

Total RNA from 26 human tissues was purchased from two commercial sources [Stratagene (S), La Jolla, CA, USA, and Ambion (A), Austin, TX, USA]. These samples included placenta, fetal brain, fetal heart, fetal liver, brain, heart, liver, pancreas, stomach, small intestine, colon, rectum, testicle, prostate, ovary, adrenal gland, thyroid, kidney, bladder, breast, uterus, cervix, skeletal muscle, lung, spleen and thymus. RNA was reverse transcribed and analyzed by quantitative RT-PCR as described in the methods section above. MT-SP1 transcript levels were normalized to those of HPRT. Primers used for quantitative PCR were as described in the methods section for quantitative PCR of MT-SP1 in cancer cell lines.

TaqMan® Measurement of mRNA Expression of MT-SP1 and Urokinase Type Plasminogen Activator Receptor (uPAR) in Breast Cancer Tissue Samples

Eighteen primary breast cancer tissue samples, five breast cancer cell lines (600MPE, MDA-MB-436, MCF-7, T47D, BT474) and HMEC (human mammary epithelial cell) were analyzed by TaqMan® to determine transcript level of MT-SP1 and uPAR. The 18 primary breast cancer tissues were obtained from patient samples with paired clinical follow up data, which included age, menopausal status, nodal status, tumor size and grade (Table 1). Snap-frozen tumors were dissected and tissue with >70% tumor was used for RNA isolations using Trizol Reagent (Gibco-BRL Life Technologies, Carlsbad, CA, USA). RT reactions were performed as described (Raja *et al.*, 2000). Expression levels of MT-SP1 and uPAR are normalized to that of GUSB. Primers used for MT-SP1 were: forward Primer 5'-CCTCCTCTTGGTCTTGCTGG-3'; Reverse Primer 5'-CATTTGTGATCCTCAT-GTAGCCA-3'. The TaqMan® probe used for MT-SP1 was 5'-FAM-CATTTGCAGTACCGGGACGTGCG-3'TAMRA. Primers used for uPAR were: forward primer 5'-GATCACCAGCCTTACCGAGGT-3'; reverse primer 5'-GCCCCTCTCACAGCTCATGT-3'). The TaqMan® probe used for uPAR was: 5'-FAM-CACCTCTGGCCGGGCTGTCACCTAT-3'TAMRA.

For each gene, PCR was conducted in triplicate with 50 µl reaction volumes of 1× PCR buffer A (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 0.4 µM each primer, 200 µM each dNTP, 100 nM probe and 0.025 u/µl *Taq* Gold (Applied Biosystems). PCR was conducted using the following cycle parameters: 95°C for 12 min, one cycle; (95°C for 20 sec, 60°C for 1 min), 40 cycles.

Analysis was carried out using the sequence detection software supplied with the ABI 7700 (Applied Biosystems) as described (Ginzinger *et al.*, 2000).

The Pearson's product moment correlation between log values of the transcript levels of MT-SP1 and uPAR was calculated to be 0.784. For 18 patient samples and a correlation coefficient of 0.7, the probability of the null hypothesis being true (no correlation between the transcript levels of MT-SP1 and uPAR) is 0.1%. Given the correlation coefficient (r) value of 0.784, the *p* value is calculated to be $p < 0.001$. The correlation can therefore deemed 'highly significant' as the *p* value is less than 0.01 (Taylor, 1982).

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