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TSP50, A Possible Protease in Human Testes, Is Activated in Breast Cancer Epithelial Cells[1]

Jidong Shan, Liming Yuan, Qianxun Xiao, Nicholas Chiorazzi, Daniel Budman, Saul Teichberg, and Hao-peng Xu[2]

Departments of Molecular Oncology [J. S., L. Y., H-p. X.], Pathology [Q. X.], Rheumatology/Clinical Immunology [N. C.], and Electron Microscopy [S. T.] and Hematology/Oncology Medicine [D. B.], North Shore-Long Island Jewish Research Institute, New York University School of Medicine, Manhasset, New York 11030

ABSTRACT

Initial studies have identified *TSP50* as a human testes-specific gene that is demethylated in breast cancer. In this study, we will present new data related to the *TSP50* gene. We have found that the *TSP50* gene product shares a similar enzymatic structure with many serine proteases. However, the most critical catalytic site, serine, has been replaced by threonine. Western analysis revealed that in human testes, the TSP50 antibody detected two closely positioned protein bands whose estimated molecular masses were 37 kDa, whereas in a large portion of breast cancer tissues, but not normal control tissues, only one band was present. Immunohistochemistry assays found TSP50 proteins located in the spermatocytes of human testes, whereas *in situ* hybridization and immunohistochemistry confirmed that gene activation in breast tumors took place in malignant mammary epithelial cells. These results suggested that the normal function of the *TSP50* gene was involved in spermatogenesis, whereas the up-regulation of *TSP50* in many breast cancer patients not only indicated that it might be a novel biomarker for this disease but also encouraged us to further explore the possibility of whether it was an oncogene.

INTRODUCTION

Previously, we reported that a novel gene, *TSP50*, was discovered by a hypomethylated DNA fragment isolated from human breast cancer cells by the methylation-sensitive representational difference analysis technique (1). It was homologous to many serine proteases (2–5) and specifically expressed in testes. However, its transcripts were detected in some breast cancer tissues (1). These preliminary observations encouraged us to search for more information about *TSP50*, and as a result, new data have been generated. First, using an aa[3] alignment with seven serine proteases, we found that if TSP50 possesses enzymatic activity, then it could be classified as a novel protease because its catalytic triad is different from that of traditional serine proteases (2–8). To understand the biological function of TSP50, its polyclonal antibodies were generated. The antibody was used to detect TSP50 in human testes by Western blot assay. We found that two closely positioned protein bands in the testes lysates were recognized by the antibody. To determine the cellular location of TSP50 in human testes, an IHC assay was performed. The results showed antibody staining located in the spermatocytes, but not in the mature sperms. This finding suggested that one function of the *TSP50* gene was its involvement in spermatogenesis. In addition, the possible expression of *TSP50* at a translation level in 53 breast cancer samples was analyzed by Western blot. It was found that in 92% of those samples, the antibody detected the TSP50 protein, whereas the protein was not detected in all of the normal matched tissues. The surprisingly high positive detection rate of the TSP50 protein in breast cancer could make it an attractive molecular marker for this disease. To determine the cellular locations of *TSP50* transcripts and proteins in breast cancer tissues, ISH and IHC assays were carried out. It was discovered that the gene transcripts and products were located in malignant cells, but not in normal epithelial cells. These results demonstrated that abnormal expression of the *TSP50* gene correlated with human breast cancer.

MATERIALS AND METHODS

aa Sequence Analysis. The sequence of TSP50 was compared with that of seven serine proteases by the NIH Blast 2 Sequence program. Multiple sequence alignment was established by Vector Nti Suite software (InforMax).

Plasmid Construction and Fusion Protein Purification. A major portion of the *TSP50* gene, which encodes 325 aa from position 35 to 360, was generated by PCR amplification using sense primer GTAGGATCCGCGAGGGGAAGCCCCGG and reverse primer CCGAATTCTTATCACTGCCCGTTGAGGCAGTCC. The sense or reverse primer contained *Bam*HI or *Eco*RI restriction enzyme recognition sites, respectively, for cloning purposes. The PCR product was sequenced to verify that no mutations were created during PCR amplification. This product, which encoded a protein with about 320 aa (TSP50/320), was cleaved with *Bam*HI and *Eco*RI and subcloned into GST fusion vector pGEX-4T-3 (Amersham Pharmacia Biotech), which was also digested with *Bam*HI and *Eco*RI. Expression of GST (26 kDa) or GST/TSP50/320 (60 kDa) fusion proteins was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C in *Escherichia coli* for 3.5 h. GST and GST/TSP50/320 fusion proteins were purified from bacterial lysate by affinity chromatography using glutathione-Sepharose 4B. The TSP50/320 protein was cleaved from the fusion protein with thrombin following the manufacturer's procedure (Amersham Pharmacia Biotech).

Preparation of Affinity-purified Polyclonal Antibody, AT-50. Rabbit polyclonal antibodies were generated against the TSP50-specific peptide pep-50, corresponding to the 156–206 aa position, which was a nonhomologous hydrophilic region within the catalytic domain. The sequence of pep-50 was Ile-Trp-Arg-Asp-Val-Ile-Tyr-Ser-Val-Arg-Val-Gly–Ser-Pro-Trp-Ile-Asp-Gln-Met-Thr-Gln-Thr-Ala-Ser-Asp-Val-Pro-Val-Leu-Gln-Val-Ile-Met-His-Ser-Arg-Tyr-Arg-Ala-Gln-Arg-Phe-Trp-Ser-Phe-Val-Gly-Gln-Ala-Asn, and it was synthesized at the Facility of Molecular Genetics, North Shore-Long Island Jewish Research Institute. pep-50 (100 μg) was emulsified with an equal volume of complete Freund's adjuvant and injected into New Zealand rabbits. Booster injections of 50 μg of pep-50 were performed every 3 weeks until a high-titer antiserum (1:1500 dilution) was detected by Western analysis in which the *E coli* cell lysate containing induced GST/TSP50/320 fusion protein served as antigen. The preimmune serum was used as a negative control. The affinity-purified TSP50 antibody, AT-50, was obtained by pep-50 affinity chromatography. The attached AT-50 was released from the column by elution buffer with a low pH value. The AT-50 solution was neutralized immediately and brought to its original volume so that it would be comparable with both AT-50 and the preimmune serum, which were diluted to the same degree. The quality of AT-50 was confirmed by Western blot in which affinity-purified GST/TSP50/320, TSP50/320, and GST proteins were used as antigens. The antibody for GST (Invitrogen) was also used to detect those antigens, which served as positive and negative controls.

Preparation of Tissue Lysates and Western Analysis. Dissected human breast tissues were frozen immediately in liquid nitrogen and stored at −70°C. Tissues were homogenized in liquid nitrogen, and proteins were released in the lysate buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide,

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[2] To whom requests for reprints should be addressed, at R132 Research Building, 350 Community Drive, Manhasset, NY 11030. Phone: (516) 562-1638; Fax: (516) 562-1605; E-mail: hduffy@nshs.edu.

[3] The abbreviations used are: aa, amino acid(s); IHC, immunohistochemistry; ISH, *in situ* hybridization; ITDC, intraductal carcinoma; IFDC, infiltrating ductal carcinoma; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; DIG, digoxigenin; RT-PCR, reverse transcription-PCR.

0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% NP40, and 0.5% sodium deoxycholate]. Protein concentration in each lysate was quantified using the bicinchoninic acid protein assay reagent kit (Micro BCA kit; Pierce). Protein (50 or 100 μg) was separated on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membranes were blocked with PBS containing 0.15 M NaCl, 0.1% Tween 20, and 5% dry milk and then incubated with AT-50 (1:1000), followed by goat antirabbit IgG HRP conjugates. The color assays were performed according to the manufacturer's instructions (Clontech Inc.). The membranes were restained with Coomassie Blue, which served as the quantitative control for protein loading.

IHC Assay. The tissue sections (5-μm thick) were fixed in formalin, embedded in paraffin, and then deparaffinized and rehydrated by standard procedures. To quench endogenous peroxidase activity, the sections were incubated in 0.3% hydrogen peroxide for 15 min. To perform IHC analysis, the Vectastain Elite ABC Kit (Vector Laboratories) was used. After three treatments with washing buffer [0.05 M Tris-HCl (pH 7.6)], sections were incubated in the following solutions until the desired stain intensity developed: (*a*) normal goat serum (1.5% in PBS) for 20 min; (*b*) AT-50 (1:300 dilution) for 30 min; (*c*) wash buffer for 5 min; (*d*) HRP-conjugated goat antirabbit antibodies (1:200 dilution) for 30 min; (*e*) wash buffer for 5 min; (*f*) Vectastain Elite ABC reagent for 30 min; (*g*) wash buffer for 5 min; and (*h*) peroxidase substrate solution. The sections were then dehydrated and mounted with Aqua-Mount (Lierner Laboratories). Negative control sections were incubated with preimmune serum (1:300 dilution).

ISH Assay. A partial *TSP50* sequence (672 bp), which encodes aa 98–322, was released from the full-length *TSP50* cDNA in the Bluescript plasmid by *Sal*I and *Pml* I digestion. Both ends of the truncated cDNA were filled in and subcloned into a Bluescript plasmid. The 5′ end was close to the T7 promoter, whereas the 3′ end was close to the T3 promoter. Antisense RNA (riboprobe) was synthesized by using T3 RNA polymerase. During the synthesis of RNAs, DIG-labeled UTP was incorporated according to the Amersham Pharmacia protocol. The sense probe was synthesized by using the T7 promoter and served as the negative control. Both probes were used to detect mRNAs of *TSP50* in sections of human breast tissue, which were prepared as described previously. Sections were hybridized with either the riboprobe or sense probe at 50°C overnight. An anti-DIG antibody conjugated with HRP was used to recognize DIG-labeled RNA. After an enzyme-catalyzed color reaction, the sections were dehydrated and mounted with Aqua-Mount.

RESULTS

TSP50 Could Encode a Novel Type of Protease. The aa sequence alignment of TSP50 with seven serine proteases demonstrated that it shares 26–36% identity and similar enzymatic structures with those proteases (Fig. 1). TSP50 contains the first two sites of the catalytic triad, His and Asp, at position 159 and 206, respectively. However, the third site, Ser, at position 310, which the serine protease nomenclature is based on, was replaced by Thr. Although Thr is structurally similar to Ser, to our knowledge this was the first time such a substitution has been reported. Therefore, if TSP50 is a protease, then it could represent a novel classification because of its Thr^{310} residue substitution, which could play an important catalytic role. In addition, its substrate could be different from those of serine proteases. These speculations are currently under investigation.

Generation of the Affinity-purified Antibody for TSP50. The affinity-purified antibody (AT-50) was obtained as described (see "Materials and Methods"). The quality of AT-50 was confirmed by a Western blot in which the antibody for GST was also used as the control. Western analysis showed that both the GST antibody and AT-50 recognized the GST/TSP50/320 fusion protein, whereas AT-50, but not the GST antibody, detected TSP50/320 (Fig. 2*A*). To detect the TSP50 protein in a Western blot assay, the optimal dilution of AT-50 was found to be 1:1000.

Western Analysis Revealed that TSP50 was Expressed in Human Testes and Breast Cancer Tissues. Previous Northern analysis of *TSP50* expression in 16 different types of human tissues found that it was specifically and highly expressed in human testes (1). This result suggested that the TSP50 protein existed in human testes. To test this hypothesis, two testes lysates were separated by a SDS-PAGE gel. The proteins were then transformed onto a nylon membrane,

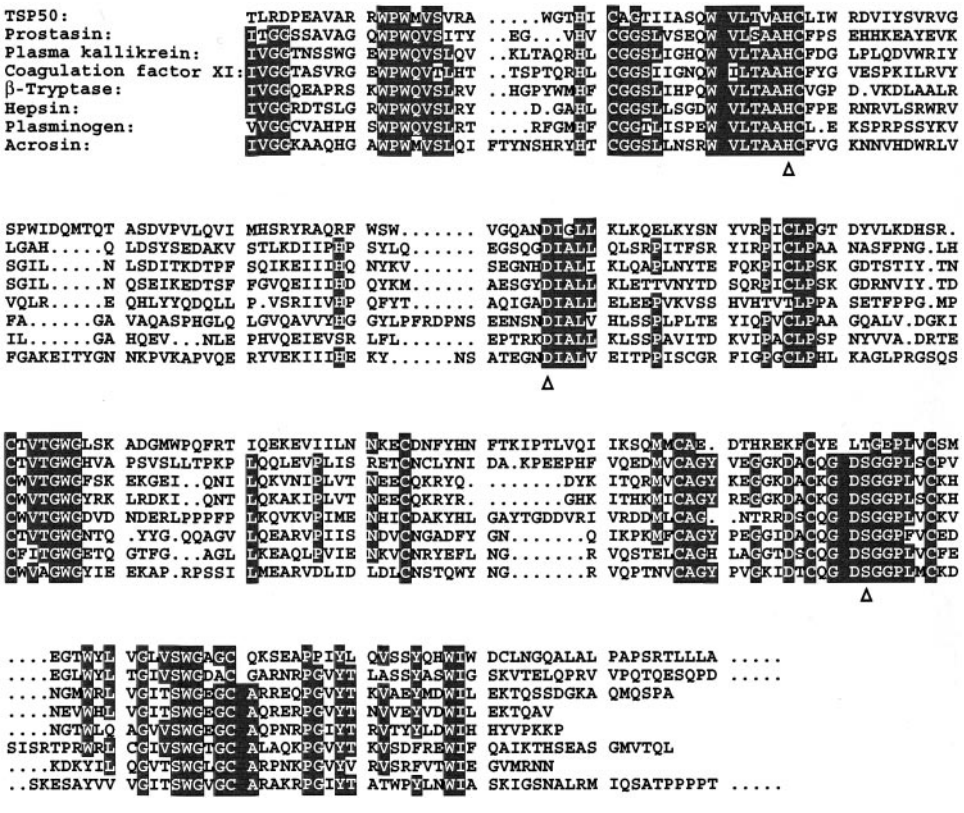

Fig. 1. Comparison of the deduced TSP50 aa sequence with other serine proteases. The aa sequences of these proteases correspond to the mature form of β-trypsin or the catalytic chains of acrosin, prostasin, plasma kallikrein, coagulation factor XI, hepsin, and plasminogen. The aa residues that are highly conserved are *shaded*, and the catalytic triad of histidine, aspartic acid, and serine is indicated by *triangles*. *Dots* represent gaps to bring the sequence into better alignment.

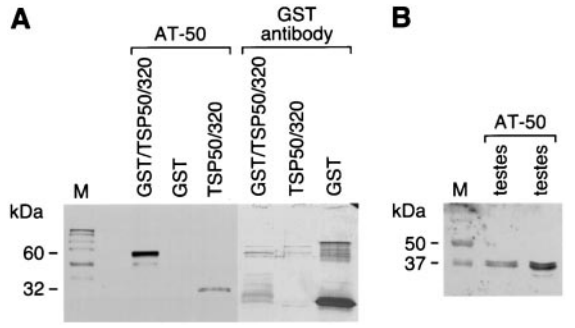

Fig. 2. *A*, Western analysis for the affinity-purified TSP50 antibody, AT-50. *Lane M* contains protein molecular mass markers in kilodaltons. The antibody used is indicated *above the bracket*, whereas the antigens used are listed *above each lane*. *Lanes GST/TSP50/320* and *TSP50/320* contain 50 ng of protein, whereas the *Lane GST* contains 100 ng of protein. *B*, results of the Western blot for TSP50 expression in two human testes tissue lysates. *Lane M* contains protein molecular mass markers in kilodaltons. Each sample line contains 50 μg of protein prepared from each tissue. For working conditions, AT-50 and GST antibody were diluted 1:1000 and 1:2000, respectively. This experiment was repeated three times.

where they interacted with AT-50. The protein bands recognized by the antibody were signaled by HRP color assay. We found that the antibody detected two bands in both testes lysates. These two protein bands were very close to each other, and their molecular masses were estimated to be 37 kDa (Fig. 2*B*). These molecular masses were lower than the predicted molecular mass (43 kDa), a difference that was most likely caused by posttranslational proteolytic cleavage. Although previous RT-PCR results demonstrated that the *TSP50* gene was abnormally expressed in some human breast cancer biopsies, we did not know whether this abnormality was also reflected at a translation level. With the availability of the AT-50 antibody, we examined TSP50 protein levels in 53 breast cancer biopsies by Western blot. Among these samples, 29 were paired with their matched normal tissues, and 24 were nonpaired tumor biopsies. The positive control tissue used was human testes. Among the 53 samples, 49 (27 of 29 paired samples, and 22 of 24 nonpaired breast cancer tissues) expressed the TSP50 protein, whereas all 29 normal controls did not (Fig. 3). Clearly, the *TSP50* gene was selectively activated in the breast cancer samples.

IHC Demonstrated That TSP50 Was Expressed in the Spermatocytes and Malignant Mammary Epithelial Cells. To understand the biological function of the *TSP50* gene, the subcellular locations in human testes and breast tumors were explored by performing IHC assays using the AT-50 antibody and preimmune serum (negative control). The TSP50 protein was signaled by goat antirabbit antibodies conjugated with HRP. No signals were generated for the human testes by the negative control. However, the AT-50 antibody indicated that TSP50 existed in the cytoplasm and nuclei of early, middle, and late stage spermatocytes, but not in mature spermatozoa (Fig. 4, *A* and *B*). These findings suggested that the function of TSP50 could be involved in the initialization and progression of spermatogenesis, a developmental process involved in mitosis and meiosis. To understand where *TSP50* was activated in breast tumors, tissue sections prepared from two types of tumors, ITDC and IFDC, were incubated with AT-50 or with the preimmune serum. The results obtained from the ITDC section, in which both normal ductal structures and tumor cells coexisted, showed that TSP50 was expressed in malignant cells, but not in normal epithelial cells (Fig. 4*C*). In addition, TSP50 was also found in cancer epithelial cells, but not in the coexisting lymphocytes in the IFDC section (Fig. 4, *D* and *E*). In summary, the *TSP50* gene was normally expressed in spermatocytes but abnormally expressed in breast cancer epithelial cells.

ISH Analysis Also Demonstrated That TSP50 Was Activated in Malignant Epithelial Cells in Breast Cancer. To further confirm the IHC results, ISH experiments, in which *TSP50* transcripts are precisely hybridized to their antisense probes under stringent conditions, were performed. In the ISH assays, the endogenous RNAs in tumor and normal breast tissue sections were incubated with DIG-labeled sense (negative control) or antisense *TSP50* riboprobes. The *in situ*-hybridized probes were then recognized by anti-DIG antibodies conjugated with HRP and signaled by color staining. The results showed that the riboprobes hybridized to the target sequences marked by *brown staining* in malignant epithelial cells but did not cause staining in normal epithelial cells (Fig. 4, *F* and *G*). This data suggested that cancer cells, but not normal epithelial cells, contained the *TSP50* transcript. In addition, the sense probe did not cause staining in cancer epithelial cells (Fig. 4*H*). This information was

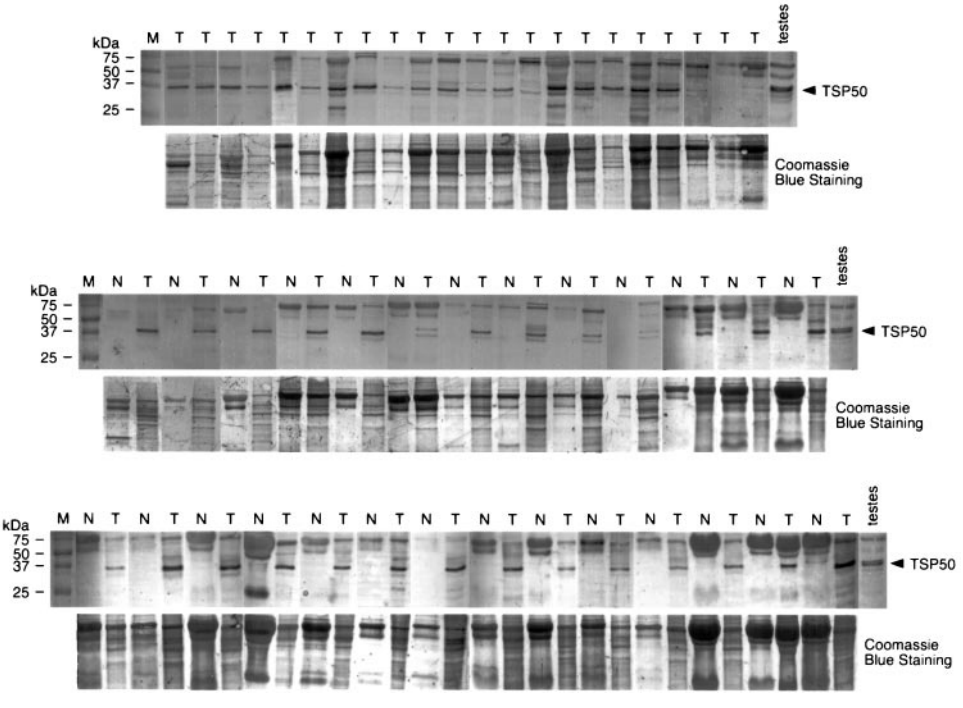

Fig. 3. Results of the Western blot for TSP50 expression in normal and breast cancer tissues. Normal and tumor tissues are labeled with *N* and *T*, respectively. For easy comparison, each paired normal tissue and tumor tissue obtained from the same patient are arranged next to each other. *Lane M* contains protein molecular mass markers in kilodaltons. Western blot membranes that were restained with Coomassie Blue are displayed in the *bottom panels* of each segment. For most samples, 100 μg of protein were loaded in each lane. For some of the normal controls, excessive amounts of protein were loaded. The AT-50 antibody was diluted 1:1000. Human testes served as positive controls (*solid arrows*). The experiment was repeated three times.

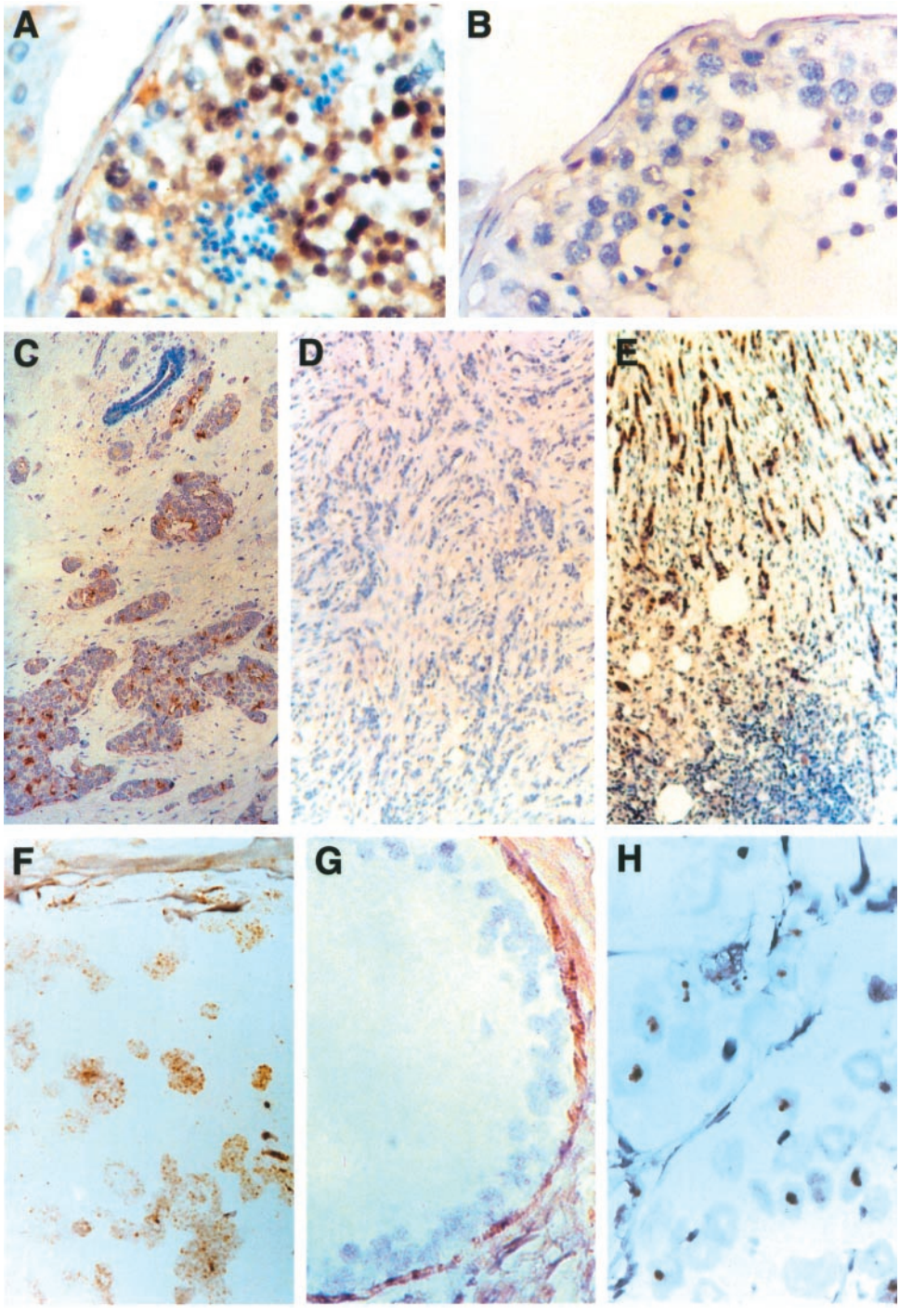

Fig. 4. *A* and *B*, results of IHC assays for *TSP50* expression in the spermatocytes of human testes. In *A*, the tissue section was incubated with AT-50. In *B*, the tissue section was incubated with preimmune serum. Positive and negative staining are signaled by *brown* and *blue staining*, respectively. The sperms, which are much smaller than the spermatocytes, are stained *blue*. *C*−*E*, results of IHC for the TSP50 protein in human breast cancer tissue sections (*C*, ITDC; *D* and *E*, IFDC). In *C*, the section was incubated with AT-50. Cancer and normal duct epithelial cells were stained *brown* and *blue*, respectively. In *D*, the section was incubated with preimmune serum, which served as a negative control. In *E*, the section was interacted with AT-50. Cancer epithelial cells were stained *brown*. The surrounding lymphocytes, internal negative controls, were stained *blue*. *F*−*H*, the ISH results for detecting the *TSP50* transcript in cancer and normal breast tissue sections. In *F* and *G*, cancer and normal (negative control) sections were incubated with riboprobe. Cancer and normal epithelial cells are indicated by *brown* and *blue*. In *H*, the tumor section was incubated with a sense probe, which also served as negative control. *Blue staining* represents the counterstaining by hematoxylin. For working conditions, both AT-50 and preimmune serum were diluted 1:300. Magnification, ×150. Both experiments were repeated three times.

consistent with that observed in the IHC assays. We also noticed brown staining in the extracellular matrix, which could be caused by endogenous HRP or nonspecific binding of the riboprobe.

DISCUSSION

Recently, the study of DNA methylation patterns in human breast cancer cells by the methylation-sensitive representational difference analysis technique has led to the discovery of a novel gene, *TSP50* (1). Preliminary results suggested that it was a serine protease homologue and that its transcripts were found predominantly in human testes but were not visible in 15 other tissues examined. Most importantly, RT-PCR results indicated that *TSP50* was expressed in some breast cancer biopsies. These findings have led us to further characterize this new gene and its possible relationship with human breast cancer.

First, the aa sequence of TSP50 was aligned with that of seven serine proteases that belong to the chymotrypsin clan SA group (9, 10). In this group, a common order for the catalytic triad, His (H), Asp (D), and Ser (S), is usually found. The alignment comparison showed that TSP50 shared similar enzymatic structure with the seven proteases. For example, the linear positions of the two catalytic sites (H and D) in TSP50 are comparable with the conserved patterns (Fig. 1). However, the third one, S, which is considered the enzymatic active site, has been replaced by a Thr residue in TSP50. To our knowledge, such a substitution has not been reported previously. However, it has been documented that the NH_2-terminal Thr of proteasomes, which

belong to the amidase group, is the catalytic nucleophile (11, 12). The Thr^{310} in TSP50 was not a sequencing error nor was it caused by point mutation because two DNA fragments containing the corresponding Thr codon were independently isolated twice in our laboratory. First it was obtained from a human placenta genomic library, and then it was obtained from a human testes cDNA library. In addition, an expressed sequence tag fragment (GenBank accession number AW139922), which was isolated from the NCI-CGAP-Sub 3 library by Dr. R. Strausberg, encoded 95 aa that were 100% identical to the 3′ region of TSP50, including the Thr^{310} residue. Furthermore, the 4 aa surrounding the Thr residue in TSP50 were also different from the conserved residues observed in many serine proteases. Because previous studies suggest that the linear order of catalytic site residues and the clusters of conserved aa around the catalytic residues are the most important factors used to classify a protease (9–13), we speculate that TSP50 could be a novel type of protease. This hypothesis needs to be proven experimentally, and we will undertake its verification in the near future. In addition, the unique structure of TSP50 indicates that it may have a specialized substrate. We are interested in identifying this substrate because it will allow us to design an enzymatic assay to evaluate the protein expression levels in different formations. Sequence analysis also found that TSP50 contains two hydrophobic regions: one located at the NH_2-terminal, which is assumed to be a secretory signal peptide (14); and another located at the COOH-terminal, which is predicted to be a membrane attachment signal. Recently, several human membrane-anchored serine proteases have been identified, and they are believed to be released from the cell membrane by enzyme cleavage (6, 15, 16).

Although the transcripts of *TSP50* were previously detected in human testes and some breast cancer tissues, this does not necessarily carry over to the translational level. Therefore, to confirm that *TSP50* is indeed expressed in those tissues, Western blots were performed. The results disclosed that the gene products were detected in testes and breast cancer specimens, but not in normal breast tissues. These findings indicated that the normal function of TSP50 could participate in human regeneration, whereas its activation correlated with human breast cancer. We also noticed that the TSP50 detection rate in breast cancer patients by Western blot was much higher (~90%) than that obtained from RT-PCR assays (~30%; Ref. 1). This disparity could be due to RNA degradation in the specimens that resulted in a low positive scoring by the RT-PCR method. Another possibility is that the TSP50 transcripts in some tumors were quite low and, as a result, could not survive during RNA purification and cDNA preparation. However, this small amount of RNA might have a very low turnover rate, which could generate enough protein to be detected by Western blot. We believe that the data generated from Western blot analysis are more reliable than data obtained from RT-PCR because proteins are much more stable than RNA, and their quantities should reflect with greater accuracy the levels of gene expression. Western blot analysis also found that the antibody recognized two bands in close proximity with molecular masses of approximately 37 kDa in testes, whereas in most breast cancer samples, only the smaller band was detected. This could be due to different microenvironments in breast tissue as compared with testes. It has been reported that several genes were expressed in spermatogenetic and somatic cells when their products were in different forms (17, 18). To determine the cellular location of TSP50 in human testes, the IHC technique was used. The results showed that the TSP50 protein resides specifically in the nucleus and cytoplasm of spermatogenetic cells at different stages, but not in the spermatozoa. These findings suggest that the normal function of TSP50 is involved in spermatogenesis, a developmental process, with stem cells (spermatogonia) giving rise to more specialized cells (spermatocytes). This, in turn, yields highly specialized cells (spermatids) that undergo a remarkable morphogenetic transformation to become spermatozoa (19, 20). In addition, IHC analysis revealed that the malignant epithelial cells expressed TSP50, whereas the normal mammary epithelial cells and surrounding lymphocytes did not. Because IHC results demonstrated that the normal epithelial cells were stained blue, the nonspecific protein bands observed in the Western blots of both normal and tumor tissues (Fig. 3) should not be contributed to by normal epithelial cells. Furthermore, the absence or presence of *TSP50* transcripts in normal or malignant epithelial cells was also verified by ISH analysis, a technique that is more specific than IHC in determining the cellular location of a gene product.

Taken as a whole, our studies have discovered that the native function of the *TSP50* gene was involved in spermatogenesis and that the *TSP50* gene was expressed in a vast majority of breast cancer patients tested. Based on these data, one might make a connection between the influence of *TSP50* on spermatogenesis and tumor growth, because in both cases cells are vigorously proliferated. Whether the *TSP50* gene is tumorigenic and whether its product possesses enzymatic activity remain to be determined. If this is so, designation of inhibitors to block its biological function could be an invaluable strategy for breast cancer treatment.

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