The Hepatocyte Growth Factor Regulatory Factors in Human Breast Cancer

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ABSTRACT

Purpose: Hepatocyte growth factor (HGF) stimulates tumor cell-cell interactions, matrix adhesion, migration, invasion, and angiogenesis. This factor is produced as an inactive precursor called pro-HGF, which requires proteolytic conversion, by HGF activator (HGFA) and matriptase, to evoke a biological response. Two new HGF inhibitors, HAI-1 and HAI-2, inhibit the generation of biologically active HGF, through their interaction with HGFA. This study determined the expression of this HGF regulatory system in breast cancer. We examined HGF, the HGF receptor (c-Met), HGFA, matriptase, and the activation inhibitors (HAI-1 and HAI-2), tissues from patients with breast cancer.

Experimental Design: Breast cancer tissue (n = 100) and normal background tissue (n = 20) was obtained immediately after surgery. The median follow-up for the patients was 72 months. HGF, c-Met, HGFA, matriptase-1, HAI-1, and HAI-2 expression was quantified using real-time quantitative PCR. The distribution of these factors in mammary tissues was also examined through immunohistochemistry.

Results: The breast cancer specimens expressed a significantly higher level of HGF, c-Met, HGFA, HAI-1, and HAI-2, but not matriptase, compared with the normal background tissues. Tumor tissues from node-positive patients expressed a higher level of HGFA than from the patients without nodal involvement. Interestingly, HAI-2 was expressed to a lower degree in positive nodes than that of the node-negative breast cancer tissues. HAI-1 and HAI-2 were both significantly reduced in grade 3 tumors compared with the well-differentiated tumors. In addition, on comparison of Tumor-Node-Metastasis (TNM) classification groups, HAI-2 was also found to be statistically lower in the TNM 3 breast cancer group when compared with TNM groups 1 and 2, thus associated with a poor prognosis.

Conclusions: This study shows that there are aberrant levels of HGF, c-Met, HGFA, HAI-1, and HAI-2 expressed in breast cancer tissues compared with background breast tissue. HAI-1 and HAI-2 are expressed to a significantly lower level in poorly differentiated breast tumors, and HAI-2 is also inversely correlated with nodal involvement and tumor spread. Overall a low level of HAI-2 in the breast cancer tissues was associated with an overall poor outlook. Therefore, the HGF regulatory system may have an important role in the progression of breast cancer.

INTRODUCTION

Hepatocyte growth factor (HGF)/scatter factor plays a well-recognized role in the process of tumor invasion and metastases. HGF stimulates proliferation, dissociation, migration, and invasion in a wide variety of tumor cells, and is a known potent angiogenic factor (1–3). Tumor-stromal interactions are known to facilitate the metastatic spread of cancer. Stromal fibroblasts are the main source of HGF, as HGF is synthesized and secreted as an inactive precursor called pro-HGF from these cells. However, some tumor cells themselves have been shown to produce HGF, as HGF transcripts and the protein itself have been detected in breast cancer cells (4). Elevated serum HGF levels have also been reported in patients with breast cancer (5–7), and the removal of malignant breast tumors has resulted in a decrease in serum HGF levels (8), therefore suggesting that the primary source of HGF in these patients may have been the tumor cells themselves or stromal cells within the tumor tissue. HGF levels have also been shown to correlate with disease progression, with levels rising in cases of recurrence (9, 10). However, another breast cancer study revealed that the immunoreactive HGF level was a stronger independent predictor of recurrence and survival than lymph node involvement (11).

The biological functions of HGF are stimulated through the binding of HGF to its specific receptor, c-Met. The binding of HGF to the c-Met receptor induces the activation of the c-met tyrosine kinase, which, in turn, results in the phosphorylation of COOH-terminal tyrosine residues and subsequent recruitment of intracellular signaling molecules. c-Met receptor status in various tumor tissues has been evaluated, and its presence has been reported in tumors of the thyroid, ovary, pancreas, breast, prostate, and gastrointestinal tract (12–18). The degree of c-Met expression has been correlated with tumor progression and poor outcome in breast cancer patients (19, 20). In addition, c-Met has also been reported to be an independent prognostic factor in breast cancer (21).
For HGF to influence metastatic spread it must first be transformed from the inactive single-chain form, pro-HGF, to the biologically active form of HGF. A number of factors possess HGF-converting ability, but the major converter of HGF is a serine protease known as HGF activator (HGFA; 22). The liver is the main source of HGFA, and secretes HGFA in a precursor form, which is converted to the active form of HGFA by thrombin (23). In addition, matriptase has also been shown to be able to activate pro-HGF (24). Recent studies have described two new serine protease inhibitors with the ability to bind to HGFA, and, thus, block its HGF-activating properties. These inhibitors are called HGFA inhibitor type-1 and type-2 (HAI-1 and HAI-2; Refs. 25, 26). HAI-1 also possesses the ability to inhibit matriptase action (27).

The role of HGF and c-Met in human cancer metastasis is well established (28); however, less is understood of the generation of biologically active HGF by HGFA, matriptase, and the role of the HGFA inhibitors. We reported previously that the MRC5 fibroblast cell line synthesized a high level of the HGF, c-Met, and HGFA transcripts. However, these fibroblasts revealed little or no HAI presence with which to inhibit HGF activity (29). Very little is known about this HGF regulatory system in breast cancer. For this reason, we quantified mRNA expression of the factors of the HGF regulatory system in 120 breast tissue specimens (20 normal background samples and 100 cancer samples) from breast cancer patients.

Our study is the first to quantitate the expression level of the components of the HGF regulatory system. We examined how the HGF system functioned in breast cancer, because the degree to which these factors are expressed may contribute to clinical parameters and prognosis of patients with breast cancer.

**MATERIALS AND METHODS**

**Specimens.** Breast samples (120) were obtained from breast cancer patients (20 were background normal breast tissue and 100 were breast cancer tissue). These tissues were collected immediately after mastectomies and snap-frozen in liquid nitrogen. Background normal mammary tissues were removed from the same patients. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumor deposits.

**Total Cellular RNA Preparation.** Total cellular RNA was isolated from the homogenized breast samples using the AB Gene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Epsom, Surrey, United Kingdom). The concentration of RNA was determined through spectrophotometric measurement (WPA UV 1101; Biotech Photometer, Cambridge, United Kingdom).

**Reverse Transcription-PCR.** cDNA was prepared using 1 µg of the RNA sample and a reverse transcription kit (Advanced Biotechnologies Ltd.). The quality of DNA was verified using β-actin. PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer, Norwalk, CT). β-Actin was used to confirm the quality of the cDNA; the conditions for PCR were 40 s at 94°C for denaturation, 60 s at 58°C for annealing, and 90 s at 72°C for elongation (26 cycles). β-Actin forward and reverse primers were 5'-ATGATATCGC-CGGCTGCCTGTC-3' and 5'-GCCTCGGTGAGGATCTTCA-3', respectively, and gave products ~0.58 kb. PCR products were then loaded onto a 1.0% agarose gel and ethidium bromide stained.

**Cloning of the HGF Regulatory System Factors into Plasmid Vectors.** HGF/scatter factor, c-Met, HGFA, HAI-1, and HAI-2 were amplified from the breast sample cDNA using the primer pairs in Table 1. Appropriate PCR products were then cloned using the pCR 2.1-TOPO vector (Invitrogen, Groningen, the Netherlands). Bacterial colonies were grown on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside coated Luria-Bertani agar plate, and white colonies were examined via PCR, using the primer pairs from Table 1, to confirm the presence of the appropriate factor. Plasmid purification was performed using the using a plasmid mini purification kit (Qiagen, Crawley, United Kingdom). Plasmids were then sequenced to confirm that they possessed the appropriate DNA sequence (BigDye; PE Applied Biosystems, Warrington, United Kingdom). The concentration of each plasmid was determined using a spectrophotometer. The number of copies of the specific sequence (copy number) was calculated based on the size and concentration of the plasmid, and serial logarithmic dilutions were prepared. These calculated plasmid dilutions were used as internal standards.

**Real-time Quantitative PCR.** The iCycler IQ system (Bio-Rad, Camberley, United Kingdom), was used to quantify the level (shown as copies/µl from internal standard) of HGF,

**Table 1** Primers used for PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte growth factor</td>
<td>Forward</td>
<td>ATGGATCCAGCACCTGAGATAAAAACC</td>
</tr>
<tr>
<td>c-Met</td>
<td>Reverse</td>
<td>GACATTTGTAGGGTGGTATC</td>
</tr>
<tr>
<td>Matriptase</td>
<td>Forward</td>
<td>ATATGAGGCCCCGCCGTCGTTT</td>
</tr>
<tr>
<td>Hepatocyte growth factor activator</td>
<td>Reverse</td>
<td>ATTCATACGGGGCGCTTCACA</td>
</tr>
<tr>
<td>HAI-1</td>
<td>Forward</td>
<td>AGGACACAAAGTGCCAGATTG</td>
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<td>HAI-2</td>
<td>Forward</td>
<td>TCGGACTTGCAGTGAAGGTC</td>
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<tr>
<td>HAI-1</td>
<td>Reverse</td>
<td>TCAGAGGGGCGGCTGGTGT</td>
</tr>
<tr>
<td>HAI-2</td>
<td>Reverse</td>
<td>ATGGCCGAGCTGTGCGGCG</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

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**Real-time Quantitative PCR.** The iCycler IQ system (Bio-Rad, Camberley, United Kingdom), was used to quantify the level (shown as copies/µl from internal standard) of HGF,
HGF Regulators in Breast Cancer

Table 2  Primers and probes for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>Hepatocyte growth factor</td>
<td>Forward</td>
<td>GCACTGAAGATAAAAAACCAA (ACTGAACCTGACGTACA/GTTTCTCCTTATCAAA)</td>
</tr>
<tr>
<td></td>
<td>Reverse (z-sequence)</td>
<td>CTTITATCAGAA (ACTGAACCTGACGTACA/GAGCCAAA GTCCCTTCTAT)</td>
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<tr>
<td>c-Met</td>
<td>Forward (z-sequence)</td>
<td>ATCGAATGCAATTGATGAT (ACTGAACCTGACGTACA/CCTTGACCTTTCTT)</td>
</tr>
<tr>
<td>Matriptase</td>
<td>Reverse</td>
<td>CTTTGAGGCCACCTTCTTT (ACTGAACCTGACGTACA/GGTTAGTGAGTGTGTA)</td>
</tr>
<tr>
<td>Hepatocyte growth factor activator</td>
<td>Forward (z-sequence)</td>
<td>GACGGCTGTCAACATC (ACTGAACCTGACGTACA/CAGGCTG AGGTGCTG)</td>
</tr>
<tr>
<td></td>
<td>Reverse (z-sequence)</td>
<td>CCACCGTCTGACTATGAC (ACTGAACCTGACGTACA/GCTAGAAT GCACCTTCTC)</td>
</tr>
<tr>
<td>HAI-1</td>
<td>Forward (z-sequence)</td>
<td>GACCATCCACGACTTCTG (ACTGAACCTGACGTACA/AGTGACATT GTACACCAC)</td>
</tr>
<tr>
<td>HAI-2</td>
<td>Forward (z-sequence)</td>
<td>CTTTGAGGCCACCTTCTTT (ACTGAACCTGACGTACA/GGTTAGTGAGTGTGTA)</td>
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</tbody>
</table>

Table 2 Primers and probes for real-time quantitative PCR

- c-Met, HGFA, HAI-1, HAI-2, matriptase-1, and matriptase-2 in the breast specimens. Breast cDNA samples were then examined for each of these factors, along with the appropriate set of plasmid standards and negative controls. Primer sets and probes used in this technique are described in Table 2. These molecules of the HGF regulatory system all used the Amplifluor system (Intergen Inc., New York) and quantitative PCR master mix (ABgene, Surrey, England), which was used in conjunction with a universal probe (UniPrimer; Refs. 30, 31). This probe recognized a specific sequence (z sequence), which had been incorporated into the primers. Real-time quantitative PCR conditions for this Amplifluor system were 95°C for 15 s, 55°C for 60 s, and 72°C for 20 s. The results of the test molecules were normalized against levels of β-actin, using a β-actin quantitation kit from Perkin-Elmers (Perkin-Elmers, Surrey, England; Ref. 30).

HAI-1 and HAI-2 Antibody Production, Purification, and Analysis of Specificity. The HAI-1 peptide was generated from the COOH-terminal region (sequence: DTEHLVNTHTTPL), whereas the HAI-2 peptide was generated to correspond to a region in NH2-terminal of the protein (sequence: DRERSIHDFFCFLVSKV) and synthesized by MWG GmbH (Milton Keynes, England). These peptides were attached to the KLH and injected into rabbit with complete Freund’s solution, following a standard procedure. The antibodies we obtained after a series of purification steps. Antisera were obtained after series injection and booster injections. Immunoglobulin was purified using a protein-A Sepharose affinity column (Sigma, Poole, Dorset, England). Briefly, the rabbit serum underwent delipidation by mixing 1,1,2-trichloro-trifluoroethane with the rabbit serum in a 3:2 mixture and underwent agitation for 30 min. Then the mix was spun down at 5000 rpm for 10 min and the top layer kept for purification. Delipidated antisera was diluted with PBS buffer and loaded onto the Sepharose column (equilibrated with 10 column volumes of 10 mM PBS; pH to 7.4). The column was then washed with 7 column volumes of PBS. Elution of the bound antibodies was achieved with 5 column volumes of elution buffer (11.1g Glycine-HCl brought to volume of 1 liter with dH2O then pH to 2.7). One/ml fractions were collected into tubes containing neutralization buffer (12.1 g Tris, 4.2 ml HCl, brought to 100 ml and pH to 8.0), and mixed immediately. Purified HAI-1 and HAI-2 antibodies were then stored in 50% glycerol at −20°C.

The specificity of the antibodies was verified using the synthetic peptides and cell lysates. The breast cancer cell lines used were ZR-751, MCF-7, MDA-MB 231, MDA-MB 435, and MDA-MB 453; the prostate cancer cell lines were DU-145, PC-3, and CA-HPV; also human endothelial cells (HECV) and a human fibroblast cell line (MRC5) were examined. HAI-1 and HAI-2 peptides were blotted onto a nitrocellulose membrane, at four different concentrations, to confirm that the purified antibodies specifically recognized the peptide that they were raised against. A variety of human normal and cancer cell line lysates were also examined with Western blot analysis to confirm the presence of HAI-1 and HAI-2 proteins.

Immunohistochemical Staining of Breast Specimens. Frozen sections of breast tumor and background tissue were cut at a thickness of 6 μm using a cryostat. The sections were mounted on super frost plus microscope slides, air-dried, and then fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in “Optimax” wash buffer for 5–10 min to rehydrate. Sections were incubated for 20 min in a 0.6% BSA blocking solution and probed with the primary antibodies (anti-HGF, anti-MET, and anti-HGFA antibodies were from Santa-Cruz Biotechnologies, and anti-HAI-1 and anti-HAI-2 as purified above). After extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multiplex Swine anti- goat/mouse/rabbit immunoglobulin; Dako Inc.). After washings, the avidin-biotin complex (Vector Laboratories) was then applied to the sections, followed by extensive washing steps. Diaminobenzidine chromogen (Vector Laboratories) was then added to the sections and incubated in the dark for 5 min. Sections were then counterstained in Gill’s Hematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a coverslip.
Results of the HGF Regulatory System in Breast Specimens. We assessed the results obtained using nonpaired Student’s t test (one-sided).

Results
Expression of HGF, c-Met, HGFA, HAI-1, HAI-2, and Matriptase in the Breast Tissues. We first studied the mRNA expression of HGF, c-Met, HGFA, matriptase, HAI-1, and HAI-2 in the breast specimens (tumor n = 100, background n = 20). Fig. 1 shows the reverse transcription-PCR expression pattern of these molecules in paired breast tissues (T = tumor; n = background). Breast tissues, both normal and tumor, were found to express these factors of the HGF regulatory system.

Quantitative-PCR revealed that the expression levels were elevated in the cancer specimens for all of the factors assessed, except matriptase, which exhibited a lower, but not significant, level in tumor tissues (0.67 ± 0.29 in tumor versus 4.3 ± 3.5 in normal background; P = 0.31). HGF was expressed at low levels in the normal breast samples (5.2 ± 12.1 copies/μl), but was elevated significantly (P < 0.001) in the breast cancer samples (48.8 ± 107 copies/μl; see Fig. 2A). HGF receptor was found at high levels in the normal breast tissues (9,339.4 ± 16,210 copies/μl); however, the level was significantly increased (P < 0.05) in the cancer tissues (22,155 ± 58,037 copies/μl; see Fig. 2B). An additional observation made in the study was the wide variance between different samples. This is probably a reflection of the suggestion that mRNA is more volatile and subject to active regulations in the cells (32).

We have additionally demonstrated that the transcript levels of HGF, c-Met, and HAI-2 were all significantly elevated in the cancer samples compared with the normal breast tissues. HGFA was observed at low levels in the normal background samples (12.9 ± 22.8 copies/μl), but the HGFA levels in the cancer samples were elevated (46.3 ± 95.8 copies/μl), and this increase was statistically significant (P < 0.05; see Fig. 2C). We also report that the HAI-1 and HAI-2 transcripts were expressed in the breast specimens. HAI-1 was found to be expressed at a significantly higher level (P < 0.01) in the breast cancer tissues (1141.6 ± 1983 copies/μl) compared with the background tissues (524.7 ± 541.7 copies/μl; see Fig. 2D). Again, HAI-2 continued this trend showing a lower level in the normal breast tissue (126.7 ± 169.7 copies/μl), compared with a highly significant increase (P < 0.0001) of HAI-2 expression in the breast cancer tissues (448.2 ± 527.6 copies/μl; see Fig. 2E). Matriptase did not display any significant differences.

An Increased Expression of HGF Was Associated with Poor Patient Prognosis. We assessed the survival status of the breast cancer patients over a 6-year follow-up period. Patients were divided into two groups, the patients who were alive and well with no recurrence or metastasis were assigned to the good prognosis group (n = 69). The poor prognosis group contained the patients who had local recurrence, had metastasis to a distant site, or had died as a result of breast cancer (n = 24; patients who died of other unrelated causes were excluded, n = 7).

The amount of c-Met, HGFA, matriptase, and the HGFA inhibitors in the breast cancer patients did not significantly vary between good prognosis and bad prognosis. However, the level of HGF was found to be elevated in the patients with a poor prognosis. Studies have suggested previously that HGF itself may act as a prognostic factor in breast cancer due to this cytokine ability to facilitate metastatic spread (33). The patients with a good prognosis expressed 43.7 ± 104.7 copies/μl of HGF, whereas patients with a bad prognosis showed an increase in HGF expression with 62.7 ± 119.8 copies/μl. However, with this sample number the results are yet to reach statistical significance.

The Involvement of the HGF Regulatory System in Nodal Status. We examined the components in the HGF regulatory system with regard to the nodal status of these breast cancer patients. The results showed that for HGF, matriptase, c-Met, and HAI-1 there was no difference between the quantity of these transcripts in patients with (n = 40) or without (n = 60) nodal involvement.

HGF was found at a lower level (38 ± 71.9 copies/μl) in the node-negative patients, compared with the node-positive patient samples (59.4 ± 124.1 copies/μl), although these findings have yet to reach statistically significant levels. Also, patients without nodal involvement had a higher level of HAI-2 (440.8 ± 568.2 copies/μl) compared with patients with nodal involvement (341.3 ± 368.1 copies/μl), although this did not reach significance. This lower level of HAI-2 in breast cancer patients with nodal involvement would mean less suppression of HGFA, which itself appeared to be increased, and would, therefore, increase the level of active HGF present in the surrounding environment.

The HGFA Inhibitors (HAI-1 and HAI-2) Were Significantly Reduced in Grade 3 Tumors. We went on to analyze the levels of the molecules in relation to tumor grade (grade 1, n = 20; grade 2, n = 33; and grade, 3 n = 47). HAI-1 and HAI-2 were both decreased in the poorly differentiated grade 3 tumors of the breast cancer specimens. HAI-1 was found at a statistically lower level (P < 0.05) in grade 3 tumors (821.7 ± 1203.3 copies/μl) when examined against patients with grade 1 tumors (1897.7 ± 3799.1 copies/μl; see Table 3). HAI-2 ex-
expression in grade 3 tumors was found to be greatly reduced (236 ± 365.2 copies/µl), compared against both grade 2 tumors (545 ± 542.8 copies/µl) and patients with grade 1 tumors (478.7 ± 522 copies/µl). Importantly, these grade 3 HAI-2 levels were significantly reduced in comparison with both grade 1 and grade 2 tumors (P < 0.001; see Table 3).

The relationships between the levels of the transcripts and histological types were also analyzed. There was a significantly higher level of HGFA in ductal tumors (52.5 ± 11.1; n = 83) compared with lobular tumors (31.5 ± 27.8; n = 11; P < 0.05). Although the other tumors showed lower, but nonsignificant, levels of the transcript, the sample number of other tumor types was too small for a convincing analysis (mucinous, n = 2; medullary, n = 2; tubular, n = 2). Similarly, significantly higher levels of HAI-1 were seen in ductal tumors (1082 ± 241) when compared with lobular tumors (442 ± 278; P = 0.027). There were no significant differences in other molecules, namely HGFA, c-Met, HAI-1, and matriptase.

**Tumor Node Metastasis (TNM) Classification.** This study also analyzed the HGF system in relation to the TNM staging (TNM-1, n = 55; TNM-2, n = 35; and TNM-3, n = 7). Values for HGF, c-Met, HGFA, matriptase, and HAI-1 did not reveal any significant differences. However, the quantity of HAI-2 mRNA expressed in TNM group 3 (58.5 ± 61 copies/µl) was reduced dramatically (P < 0.001) compared with TNM groups 1 and 2 (475.2 ± 497.8 and 521.6 ± 622 copies/µl, respectively). TNM group 3 identified the breast cancer patients with an overall poor outlook; therefore, HAI-2 was inversely correlated with tumor spread and would allow a more potent influence from HGF (see Fig. 3). TNM group 4 was excluded from the analysis due to low sample number in the group (n = 3).

**HGF Regulatory System Staining of Breast Specimens.** For the current study, we first raised polyclonal antibodies to human HAI-1 and HAI-2. Purified IgG from antisera specifically recognized the respective peptides used to generate antibody. We show that these antibodies are specific against HAI-1 and HAI-2, as the HAI-1 antibody identified the HAI-1 peptide, and HAI-2 only recognized the HAI-2 peptide (Fig. 4).

In addition, these antibodies identified HAI-1 and HAI-2 in a variety of normal and cancer cell lines. Specific bands at M_66,000 and M_34,000 were identified, which corresponded to

<table>
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<tr>
<th>Table 3</th>
<th>Mean copy number/µl for grade of breast cancer tumors</th>
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<tr>
<td>Factor</td>
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<tr>
<td>Hepatocyte growth factor</td>
<td>50.3 ± 100.9</td>
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<tr>
<td>c-Met</td>
<td>15342 ± 19676</td>
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<td>Hepatocyte growth factor activator</td>
<td>37.82 ± 59</td>
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<td>Matriptase</td>
<td>0.385 ± 0.196</td>
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<td>HAI-1</td>
<td>1897.7 ± 3799.1</td>
</tr>
<tr>
<td>HAI-2</td>
<td>478.7 ± 522</td>
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* NS, not significant.
the mature HAI-1 and HAI-2 proteins, respectively. HAI-1 was weakly expressed in the endothelial and fibroblast cells, and in the more invasive cell lines (PC-3 and MDA-MB-231), whereas HAI-1 was expressed to a higher degree in the cells with low invasive ability (DU-145). HAI-2 was expressed in the majority of cell lines. In addition, the human fibroblast cell line (MRC5) responsible for synthesizing bioactive hepatocyte growth factor had little or no inhibitory presence.

These purified antibodies, together with those antibodies, obtained commercially, to human HGF, c-Met, and HGFA, were used for immunohistochemical staining using frozen sectioned tissues (Fig. 6). HGF shows staining in the background breast tissue samples, and is to be expected, as the stromal tissue is a major source of HGF (Fig. 6A, arrow). However, the degree of staining for HGF in the breast cancer tissue is greatly increased (Fig. 6B), c-Met also shows some staining in background tissue (mainly in epithelial cells, Fig. 6C, arrow), although again the results show a higher level of c-Met expressed by the breast cancer tissues (Fig. 6D). HGFA was weakly expressed in background tissues, but was found to be elevated in breast cancer, mainly in breast cancer cells (Fig. 6F, arrow).

Both HAI-1 and HAI-2 showed a high degree of staining in the background tissues. The staining in normal tissues was largely confined to epithelial cells (Fig. 6, G and I, indicated by arrows). In contrast to the other factors of the HGF system, these inhibitors revealed a lower level of staining in the cancer cells in the breast tissues (Fig. 6, H and J).

**DISCUSSION**

The importance of the HGF regulatory system in cancer metastasis is still not fully understood. Our study examined the mRNA expression levels as well as distribution pattern of the proteins of the HGF regulatory system. HGF is synthesized and secreted as an inactive precursor called pro-HGF. Pro-HGF requires proteolytic processing, by HGFA, to generate the active form of HGF. This active cytokine is capable of evoking a variety of prometastatic responses in tumor cells, mediated through the binding and phosphorylation of the c-Met receptor. Inhibitors to HGFA action have been discovered recently (HAI-1 and HAI-2), which possess the ability to attach to HGFA and prevent it from converting pro-HGF to HGF. Therefore, these new inhibitors act as a means of suppressing the influence of HGF on cancer cells. The degree to which HGF, c-Met, HGFA, matriptase, HAI-1, and HAI-2 are expressed determines the biological activity of HGF. Therefore, these factors have direct bearing on the metastatic spread of cancer cells as mediated by HGF.
The significance of HGF and c-Met in cancer metastasis has been well documented (3, 28). Our study confirms that the breast cancer tissues have an increased level of the HGF and c-Met transcripts compared with the normal background tissues. These breast tissues in general synthesized a low level of the HGF transcript, although the HGF receptor, c-Met, was produced at very high levels in these tissues. A recent study shows that HGF levels are elevated in the sera of recurrent breast cancer patients (6). Previous studies have described HGF and c-Met as potential prognostic indicators, because both are associated with invasive breast cancer and may be causally linked to metastasis (4, 7, 19, 20, 34, 35). We also observed that the breast cancer patients with a poor prognosis expressed a higher level of HGF than the patients who remained disease free.

At present, there are no studies on the roles or expression of HGFA, HAI-1, and HAI-2 in the clinical outcomes of human cancers, including breast cancer. Given the pivotal role of HGF in migration, invasion, and angiogenesis of cancer cells, proteases such as HGFA may play a vital role in the progression of tumors. Therefore, in this context the serine proteinase inhibitors, such as HAI-1 and HAI-2, could be considered to suppress the tumor-progressive influence. This study was the first to examine and quantitate HGFA, matriptase, HAI-1, and HAI-2 mRNA expression in breast samples. During the course of preparation of the article, a similar report has shown increased staining of matriptase, HAI-1, and MET in human breast tumors, using a tissue array (36).

We report that the mRNA levels of HGFA are elevated significantly in breast cancer tissues. An increase in HGFA expression would allow for a more potent influence from HGF, because a higher level of pro-HGF would be processed. Surprisingly, we also report the dramatic increase in HAI-1 and HAI-2 expression, which would limit the action of HGFA. These results were unexpected, because it was thought that HAI expression would be lessened in cancer states, thus explaining the greater influence of HGF in cancer metastasis. HAI-1 was suggested recently to play a role in the hepatocellular carcinoma progression, because 35% of hepatocellular carcinoma tissues were stained, whereas HAI-1 staining was absent in normal liver tissues (37). One study reports that HAI-1 immunoreactivity in colorectal tissues is decreased in cancer cells compared with the adjacent nonneoplastic tissue (38), but surprisingly, the same study reports HAI-1 to be up-regulated in carcinoma cells showing interactions with the host cells, especially at the invasion front. HAI-1 was shown previously to be up-regulated in cancer cells showing cell injury (39). Our results revealed that HAI-1 expression was significantly lower in poorly differentiated tumors compared with the grade 1 tumors, suggesting that HAI-1 may have other functions independent of its HGFA inhibitory activity. It does regulate HGF activity but also plays roles in tissue injury and a possible role at the invasion front in cancer (40). Although this study shows the levels of HAI-1 and HAI-2 transcripts to be higher in breast cancer tissues (Fig. 2), the immunohistochemical study revealed that the staining of both inhibitors was lower in cancer cells, compared with normal mammary epithelial cells (Fig. 6, G–J). This discrepancy may reflect the cellularity difference between normal and tumor tissues.

Expression of HAI-2 has been found to be both increased and conserved in cancer cells compared with their normal counterpart (41, 42). HAI-2 has been separately identified as KOP, a Kunitz-type serine protease inhibitor overexpressed in pancreatic cancer (41), and also as bikunin, a factor reported to inhibit metastasis and tumor invasion through the possible suppression of cell-associated plasmin activity, and expression of urokinase-type plasminogen activator receptor mRNA and protein (42). The exact role of HAI-2 in the body and in tumor progression is unknown at present. This study has shown that although HAI-2 is up-regulated in breast cancer tissues, the degree of HAI-2 expression is inversely correlated with tumor spread. HAI-2 was found to be lower in node-negative patients and was significantly decreased in grade 3 tumors compared with both grade 1
and grade 2 tumors. We have shown that poorly differentiated grade 3 tumors possess a significantly lower level of both HAI-1 and HAI-2; therefore, suppression of HGFA is lessened, resulting in increased levels of active HGF. Thus, a decrease in HAI expression was associated with poor tumor differentiation. Also, we reveal that TNM group 3 tumors have dramatically lower levels of HAI-2 compared with both TNM groups 1 and 2. In these situations, the low levels of HAI-2 are associated with an overall poor outlook for the breast cancer patient. This decrease in HAI-2 would allow for a more potent influence from HGF on the breast cancer cells due to the lessening of HGFA suppression.

Some studies are beginning to show that serine protease inhibitors expressed in cancer cells are related to a worse prognosis in cancer patients (40, 43–46). The serine protease inhibitors of plasminogen activator (PAI-1 and PAI-2), a protease involved in tumor cell invasion and metastasis, are an example. It is reported that a high protein level of PAI-1 in tumor tissue is a prognostic indicator associated with a bad prognosis in various tumors (44, 47, 48). However, to date it is unclear whether such serine protease inhibitors have a direct role to tumor progression or may be due to the increased proliferation and differentiation of the tumor cells.

HAI-1 and HAI-2 are a unique class of serine protease inhibitors, as they are synthesized as transmembrane glycoproteins rather than secreted forms. They are type 1 transmembrane proteins and have two Kunitz-type serine protease domains, the first of which is thought to be responsible for the HGFA-inhibitory action (49), although the target protease(s) for the second Kunitz domain is unclear. The protein is secreted through ectodomain shedding through proteolytic cleavage at the juxtamembrane part of the protein; this release of the inhibitors could decide the function of HAI-1 and HAI-2. Whereas in the transmembrane form, only HAI-1 can bind to HGFA, this is a reversible reaction and may act as a means of pooling the available HGFA on the cell surface at a desired site to ensure a concentrated pericellular HGFA activity, whereupon it may be released to activate HGF (50). It appears to be the released forms of HAI-1 and HAI-2 that inhibit HGFA action most effectively.

HAI-1 in the mature form is Mr 66,000 and when shed is Mr 58,000 or Mr 40,000 (2 forms). The Mr 58,000 form contains both Kunitz domains, whereas the Mr 40,000 form only contains the first Kunitz domain (51). This group also reports that the Mr 58,000 form displays less potent binding to HGFA than the membrane-associated form and the Mr 40,000-secreted form. So proteolytic processing to generate HAI-1 with only one Kunitz domain may be the variable that regulates the main HGFA-inhibiting activity of HAI-1 (52), although this is still unclear. Therefore it appears that HAI-1 and HAI-2 do not appear to be just negative regulators of HGFA activity. Although HAI-1 and HAI-2 possess similar structure, immunohistochemical staining and other reports suggest they have a variety of different functions in the body, which may be unrelated to their action against the HGFA and may be due to other events happening at that time in the body. Their physiological roles in the body may be governed by whether they are in their transmembrane form or their secreted form, if they are composed of one Kunitz-domain or two, and what their target protease is in a particular situation.

These results suggest that these HGFA inhibitors require more attention for their roles in tissue regeneration, tumor invasion, and metastasis.

In summary, this study reports that the components of the HGFA regulatory system are aberrantly expressed in breast cancer. The HGFA, c-Met, HGFA, HAI-1, and HAI-2 transcripts were all expressed at significantly higher levels in the breast cancer samples compared with the normal background samples. This was the first study to quantitate HGFA and HAI expression in breast cancer. Importantly, the HAI-1 and HAI-2 serine protease inhibitors are also significantly decreased in poorly differentiated grade 3 tumors, compared with both grade 1 and 2 tumors. HAI-2 was also inversely correlated with nodal involvement and tumor spread. A low level of HAI-2 was associated with overall poor outlook for the breast cancer patient. The current study, together with that of Kang et al. (36), indicates the important role of the HGFA regulators in the development and progression of breast cancer. It also reveals that these molecules may be potential targets in cancer intervention.

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