Detection of hepatocyte growth factor/scatter factor receptor (c-Met) in axillary clearance after mastectomy for breast cancer using reverse transcriptase-polymerase chain reaction

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ABSTRACT

The diverse biological effects of hepatocyte growth factor/scatter factor (HGF/SF) are mediated by c-Met which is preferentially expressed on epithelial cells. Met signaling has a role in normal cellular activities, and may be associated with development and progression of malignant processes.

In this study presence of Met in the axillary drainage from patients who underwent conservative operations for breast cancer, and its prognostic significance was examined. Sixty-two consecutive patients with invasive ductal carcinoma of the breast which were suitable for breast-conserving treatment participated in the study. The output of the drain that had been placed in the axilla during the operation was collected, and the presence of Met and β-actin were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) assays. The data were compared with the pathological features of the tumor and the axillary lymph nodes, and with the estrogen and progesterone receptors status.

RT-PCR of the axillary lymphatic drainage was positive for Met in 46 (74.2%) of the patients and positive assays were correlated with increase in tumor size and grade of capillary and lymphatic invasion, as well as with lymph node metastasis ($P < 0.02$, for all comparisons). All 24 patients with axillary lymph node metastases in comparison with those without lymph node (57.9%) metastases had positive assays for Met. While all ten patients with tumor involvement in the margins of the resection had positive assays for Met in their lymphatic fluid, only 36 out of 52 patients (69.2%) were positive for met assay. Finally, Met showed negative correlations with positive estrogen and progesterone receptor assays ($P<0.02$).

From the results of this study it may concluded that Met can be detected in the axillary fluids of patients with breast cancer and its expression in the axillary drainage may be a potential prognostic factor. This finding might be useful in therapeutic considerations since a positive assay for Met in histologically node-negative patients might indicates the need to search for node microinvasion or involvement of the excision margins with tumor

Keywords: Conservative Breast Ductal Carcinoma Management

INTRODUCTION

As many as 30% of patients with breast cancer who have undergone curative surgery and show no evidence of locoregional or distant disease still have recurrent disease within 5-10 years (1,2). Some of these treatment failures may be attributed to residual disease in the breast or axillary lymph nodes (3). The limitation of routine histopathologic examination of the tumor margins and the dissected lymph node specimen is well known (4). Contemporary methods of detection, including CT scan, magnetic resonance imaging, bone scintigraphy and flow cytometry, have limited sensitivity and specificity (5,6). Micrometastases can be found by immuno-histochemistry or polymerase chain reaction in 10-30% of the patients who have shown free of disease by the conventional histological methods (7,8). The prognostic importance of micro-metastases found by these sensitive methods are now being evaluated (9-11).

Hepatocyte growth factor/scatter factor (HGF/SF) is a paracrine factor produced primarily by mesenchymal cells. HGF/SF induces mitogenic and morphogenic changes, including rapid membrane ruffling, formation of microspikes, and increased cellular motility (12,13). The diverse biological effects of HGF/SF are all mediated by Met, which is preferentially expressed on epithelial cells (14). This receptor-ligand pair is

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essential for normal embryonic development (15,16). Whereas Met signaling clearly has a role in normal cellular processes, this signaling pathway has also been implicated in tumor development and progression. Met signaling can increases tumorigenicity, induces cell motility, and enhances in vitro invasiveness and in vivo metastasis (14, 17-20). In addition, Met signaling can increases the production of protease and urokinase, which are associated with extracellular matrix/basal membrane degradation and are important in metastasis (14, 19).

Operations for breast cancer include either mastectomy or breast-conserving surgery, and consist of wide local excision of the tumor with margins of intact breast tissue ('lumpectomy') and axillary lymph node dissection. Drains are inserted in the dissected axilla in most of these operations, to avoid accumulation of lymphatic fluid.

The aim of this study was to examine whether Met can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in the axillary drainage from patients who have undergone conservative operations for breast cancer, and to assess correlations between the mRNA expression of Met in the collected fluid and prognostic factors of breast cancer

MATERIAL AND METHODS

Patients and Operations

Sixty-two consecutive women with invasive ductal carcinoma of the breast, who were suitable for breast-conserving treatment, participated in the study. Only patients who had underwent conserving breast surgery and in them correlations of Met-HGF/SF expression with both the tumor margins and the status of the axillary lymph nodes could be evaluated were included in the study. The diagnosis of cancer was established by needle core biopsy ('Trucut'), which was performed 2 weeks before operation. All patients underwent wide local excision and axillary lymph node dissection. Non-palpable tumors were localized by mammography before surgery. The axillary dissection was performed with a separate incision, and level I and II axillary lymph nodes were removed. The breast incisions were closed without drainage, and all axillae were drained postoperatively by closed vacuum drains. The output of the drain when it was less than 25 ml per 24 hrs collected and measured every 24 hours. The presence of Met-HGF/SF and β-actin were assessed in the fluid, which was collected during the second postoperative day because during the first 24 hours it might contains many erythrocytes and debris.

Pathological Examinations

The resected specimen was divided into 5 mm slices and, each of them was evaluated macroscopically for the presence of tumor and its distance from the margins of the specimen. All slices from tumor were embedded with paraffin, sliced again into 4 μm pieces, and stained with hematoxylin-eosin. Tumor type, size, grade, capillary or lymphatic invasion, and the distance from the margins were evaluated microscopically for margin involvent. All axillary lymph nodes were paraffin embedded, divided into 4 μm slides and assessed for the presence of micrometastases.

Receptor Assays

Estrogen (ER) and progesterone receptors (PR) were assessed in the tumor by immuno-histochemical assays with mouse monoclonal antibodies in accordance with the manufacturer's instruction (Novocastra Laboratories). The 'quick score', a simple combination of the proportion of the stained cells and a measure of intensity of staining (21) was employed and a cut-off value of 2 or more was taken as negative criteria for ER or PR.

RT-PCR Assays

Total RNA was extracted from axillary lymphatic fluid by the Tri Reagent procedure, in accordance with the manufacturer's instruction (Sigma). Reverse transcription was performed with 1-2 μg of total RNA. The first strand of cDNA was generated with 0.5 μg of$(dT)_{15}$ primer (Gibco-BRL) using 200 units of subscripts II RNAse-H-reverse transcriptase (Gibco-BRL). This was incubated for 50 min at 42°C, followed by inactivation for 15 min at 70°C. To detect Met transcript, PCR was performed on 3 μl of cDNA with MP1 primer (5'-GGAATCGAGCTGC-GAGA-3') and MP2 primer (5'-TCCAAC-ATGCAGTTTCTTGC-3'). To detect β-actin transcript, PCR was performed on 3 μl of cDNA with MP1 primer (5'-CTCTTCCAGCCTT-CCTTCCT-3') and MP2 primer (5'-AGCAC-TGTGTTGGCG-TACAG-3'). Cycling conditions consisted of 35 cycles with denaturation steps at 94 °C for 30 s, hybridization steps at 55°C for 30 s and an extension step at 72°C for 1 min. The β-actin and c-Met RT-PCRs were performed simultaneously, under the same conditions. The limit of sensitivity of the RT-PCR system for Met was 1 pg of total RNA.

Immunohistochemical Analyses

Staining was performed with an antibody against hepatocyte growth factor-α receptor (HGFα-R; H-145; Santa Cruz Biotechnology, California, USA). Five μm sections mounted on Super

Frost/plus glass were processed by a labelled streptavidin-biotin method with a Histostain Plus kit (Zymed, California, USA). Heat-induced antigen retrieval was performed by temperature-controlled microwave treatment with a H2800 model processor for 12 min in 10 mM citrate buffer, pH 6.0, at 97°C. The sections were treated for 5 min with 3% H_2O_2, followed by 10 min incubation with the universal blocker, Cas-block (Zymed). The sections were incubated for 32 min with 1:20 diluted HGFα-R antibody. A biotinylated second antibody was applied for 10 min, followed by incubation with horse radish peroxidase-conjugated streptavidin for 10 min. The slides were washed with Optimax wash buffer (BioGenex, California, USA) after each incubation. The immunoreaction was revealed by a horse radish peroxidase-based chromo-gen. The sections were then counterstained and cleared in xylene. Controlled staining was performed with human liver tissue. The brown colure (diaminobenzidine) of the membranes of tumor cells was interpreted as positive reactivity.

Control Group

To verify that in normal persons there is no Met in the axilla the axillary drainage of 20 patients with malignant melanoma had negative axillary sentinel lymph node were examined. The lesions were located in the upper limbs, and the lymph node basins were found to be in the axilla. Sentinel lymph nodes were found in all of patients and in none of them melanoma was involved in frozen sections or in paraffin-embedded and stained slices.

Statistical Evaluation

Correlations between various clinicopatho-logical parameters and RT-PCR assays in lymphatic fluid were analyzed by the χ^2 test ($P < 0.05$).

RESULTS

Sixty-two consecutive female patients who underwent breast-conserving surgery for operable invasive duct carcinoma of the breast between 1 May 2004 and 31 October 2006 were included in this investigation. Patients with tumors that had invaded the chest wall or skin, or with inflammatory carcinoma, were excluded. The mean age of the patients was 58 ± 16 years. Forty-six patients (74.2%) had undergone lumpectomy for palpable masses, and in sixteen women (25.8%) wire-guided excision of non-palpable tumors had been performed. The tumor size was 0-1 cm in 20 women (32.2%), 1-2 cm in 18 (29.0%), 2-5 cm in 22 (35.4%), and larger than 5 cm in 2 (3.2%) of women. Eight patients (12.9%) had grade I tumors, 38 (61.3%) had grade II lesions, and grade III tumors were found in 16 (25.8%). Lymphatic and capillary invasion were noted in 20 (32.2%) and 22 (35.4%) patients, respectively. In attempt to achieve free margins, ten patients (16.1%) had to undergo re-excision owing to incomplete resections. Four of these patients had wire-guided excisions.

Table 1. Correlation between Met status and tumor size

Tumor size (cm)	Met-positive patients (%)
0-1	10/20 (50)
1-2	12/18 (66)
2-5	22/22 (100)
> 5	2/2 (100)

Table 2. Correlation between Met status and axillary lymph node involvement

Lymph node involvement	Met-positive patients (%)
None	22/38 (57.9)
1-3	16/16 (100)
4-10	8/8 (100)

The collected axillary fluids were assessed by RT-PCR for Met and β-actin. The β-actin RT-PCR served as positive control and gave strong signals in all cases, indicating that both RNA preparation and cDNA synthesis were successful. The RT-PCR assays were positive for Met in 46 (74.2%) of the breast cancer patients. In all patients of the control group, RT-PCR gave positive results for β-actin but was negative for Met.

The correlations between tumor size and the presence of Met in the axillary fluid are shown in Table 1. Positive RT-PCR assays for Met in the lymphatic fluids were associated with increase in tumor size. Twenty-two of 38 patients (57.9%) with T1 tumors (tumor size 0-2 cm) had positive Met in the axillary fluid (50% and 66% for tumor sizes of 0-1 and 1-2 cm in respectively), compared with 24 of 24 patients (100%) with T2 and T3 tumors ($P < 0.01$).

Table 3. Correlation between Met status, capillary and lymphatic invasion and tumor grade

Measure	Status	Met-positive patients (%)
Capillary invasion	Present	22/22 (100)
	Absent	24/40 (60)
Lymphatic invasion	Present	20/20 (100)
	Absent	26/42 (61.9)
Grade	I	4/8 (50)
	II	28/38 (73.6)
	III	14/16 (87.5)

Table 4. Correlation between Met status, estrogen receptor and progesterone receptor

Receptor status	Met-positive patients (%)
ER-positive	14 /28 (50)
ER-negative	32/34 (94.1)
PR-positive	6/20 (30)
PR-negative	40/42 (95.2)

ER, estrogen receptor; PR, progesterone receptor.

All ten patients with tumor involvement in the margins of the resection had positive RT-PCR assays for Met in their lymphatic fluid, compared with 36 of 52 positive assays (69.2%) for patients without involved margins ($P < 0.04$). The associations between tumor grades, capillary and lymphatic invasion, and the presence of Met in the axillary fluid are presented in Table 3. Patients with lymphatic invasion of the tumor were all Met-positive (20 of 20), and patients without lymphatic invasion had 61.9% (26 of 42) positive assays for Met ($P < 0.02$). Capillary invasion of the tumor was also found to be in correlation with Met; although all 22 patients with capillary invasion had positive RT-PCR assays for Met in their axillary fluid, the assays were positive in only 60% (24 of 40) patients without capillary invasion ($P < 0.02$). The presence of Met in the axillary drainage was also correlated with higher tumor grade: of which 50% were of grade I tumors, and 87.5% were of grade III lesions ($P < 0.05$).

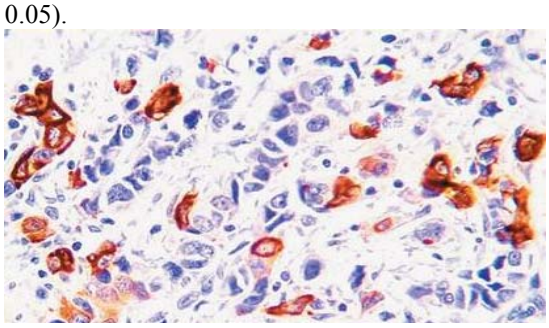

Figure 1. Invasive Duct Carcinoma with moderately positive anti-estrogen receptor staining**.** (×400)

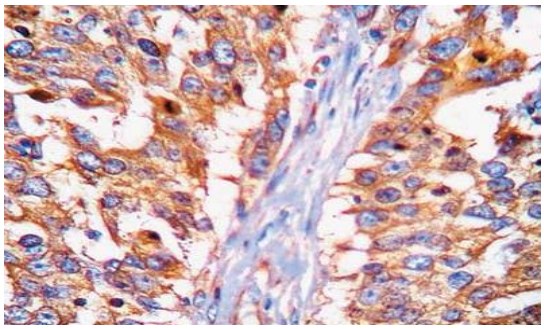

Figure 2. Invasive Duct Carcinoma with strongly Positive anti-Progesterone receptor staining**.** (×400)

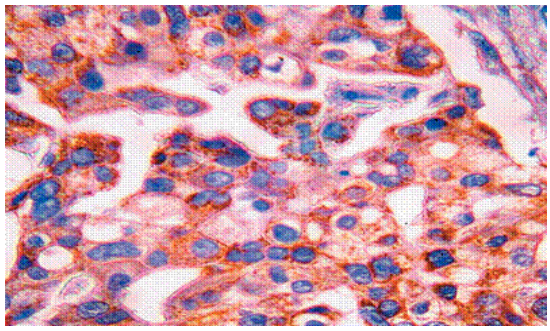

Figure 3. Invasive Duct Carcinoma with Positive staining for anti-hepatocyte growth factor-α receptor [c-Met] (×400)

The correlations between the ER and PR status and RT-PCR assays for Met in the axillary fluid are shown in Table 4. Thirty-four patients had ER-negative tumors (Figure 1), of which 32 (94.1%) had Met-positive assays in the axillary fluid. In fourteen (87.5%) out of the sixteen patients with Met-negative assays the ER were positive. Similarly, 42 patients had PR-negative tumors, of which 40 (95.2%) of these were positive for Met. PR-positive tumors (Figure 2) were found in fourteen out of sixteen patients (87.5%) where assays for Met in the axillary drainage were negative.

All the paraffin-embedded sections of the resected primary breast cancers were assessed by immunohistochemistry staining for Met (Figure 3). In 56 patients (90.3%) the primary tumor stained positively for Met and none of the tumors that had negative staining had a positive Met RT-PCR assay in the axillary drainage. In addition, the patients with negative staining had no involvement of lymph nodes.

DISCUSSION

HGF/SF is synthesized as a pro-HGF, and once activated by the HGF activator (HGFA), the complex HGF/SF-Met affects numerous normal cellular processes (22). HGF/SF-Met is also involved in malignant transformation, presumably as a mediator in interaction between tumor and stroma, which enhances tumor progression, and also angiogenesis.(23, 34). In invasive ductal carcinoma, stronger expression of HGF/SF seems to be associated with tubule formation (25). HGF/SF is over-expressed in breast carcinoma in situ and invasive ductal carcinoma in comparison with normal breast tissue. Normal mammary ducts within infiltrating cancer showed intermediate levels of HGF/SF. This finding suggests that the expression of these proteins in breast cancer are regulated by soluble factors produced by the tumor cells (26, 27). High levels of expression of HGF and Met are associated with invasive breast

cancer, and might be causally linked to early recurrences, metastatic disease and shortening survival of breast cancer patients (28, 29). High levels of HGF/SF detected within breast tumor extracts are correlated with larger tumor size and shorter relapse-free and overall survival compared with tumors with low HGF/SF concentration (30).
The activation of HGF/SF by HGFA might be modified by two HGFA inhibitors, HAI-1 and HAI-2. Highly invasive breast cancer cells express large amounts of HGF and Met, and no HAI-1, whereas breast cancer cells with low invasive potential have low levels of HGF and Met, and high levels of HAI-1 (22). In a mouse model system HGF antagonists suppressed in situ conversion of pancreatic tumors into invasive cancer (23). It seems that regulation of the HGF/SF stimulation and inhibition is associated with the metastatic potential of tumor cells, and knowledge of the status of HGFA, HAI-1 and HAI-2, in addition to Met, might provide useful information.
HGF/SF and Met have been found in a variety of tumors, (31-33) and in lymph nodes of patients with no tumor,(34) but never in the fluid drained from the tumor bed or the lymph node basin. In this study we evaluated whether Met can be detected in the axillary drainage of breast cancer patients, and the significance of its expression in the lymphatic fluid. Studying the expression of Met in the axillary fluid is a simple, non-invasive procedure because drains are routinely inserted during axillary lymph node dissections. The collected fluid is readily available, and RT-PCR is a short assay with minimum artifacts.
The axillary fluid after breast and axillary lymph node operations includes erythrocytes, lymphocytes, epithelial and tumor cells. One of the goals of this study was to examine whether tumor cells can be detected in the axillary drainage by RT-PCR assays for Met. To determine the source of Met in the axillary fluid in breast cancer patients and to exclude the possibility that the source was related to surgical trauma, we evaluated a control group of melanoma patients with negative axillary sentinel lymph nodes. In none of the control patients the axillary drainage Met-was positive. In 56 patients the primary tumor stained positively for Met and none of the tumors that had negative immunostaining showed a positive Met RT-PCR assay in the axillary drainage. Thus, it seems likely that the source of Met in the axillary fluid of node-negative breast cancer patients is the tumor in the breast.
The results show that Met can be detected in the axillary drainage, and Met is associated with unfavorable prognostic factors. Positive assays for Met are correlated with tumor size, grade, lymphatic invasion, tumor involvement of the margins of the resected specimen, the existence of metastases in the lymph nodes, and the numbers of lymph nodes with tumor. Met was associated with larger tumors, and in none of the patients with tumors larger than 2 cm the assay was negative. Moreover, Met was never negative in patients with metastatic carcinoma in the lymph node. Negative staining for ER and PR is associated with unfavorable prognosis, and the assays for Met in the axillary fluid were positive in most receptor-negative tumors.
One could postulate that because Met was highly correlated with tumor size, its expression implies aggressive behavior of the malignant processes. The significance of HGF/SF as a marker of poor prognosis might also be associated with its effects on acquired resistance to anti-cancer drugs (35, 36). HGF/SF protects DNA Damaging of cancer cells by chemotherapeutic agents through pathways involving signaling from Met to phosphoinositide 3-kinase and c-Akt (29). Human breast cancer cells, pre-incubated with HGF/SF and then exposed to Adriamycin, exhibit an altered pattern of gene expression compared with cells treated with Adriamycin alone. Cells treated with HGF/SF and Adriamycin also had modified cell line regulation and signal transduction might suggests mechanisms by which HGF/SF exerts its protective activity (31).

CONCLUSION

From the results of this study it might be concluded that the expression of Met in the axillary drainage might have prognostic significances. More importantly, its expression in histologically node-negative patients necessitates the search for node micrometastasis or involvement of the excision margins by tumor. Therefore, RT-PCR for Met in the axillary fluid of patients who undergo breast-conserving surgery for breast cancer might have influences on therapy. The results of this study justify prospective investigation on a larger scale, and the clinical significance of the presence of Met positivity in the axillary drainage fluid in breast cancer patients should be defined by an extended follow-up study.

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