Detection of Hepatocyte Growth Factor/Scatter Factor Receptor (c-Met) and NUC1 from the Axillary Fluid Drainage in Patients after Breast Cancer Surgery

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Abstract

Background: Drains are inserted in the dissected axilla of most patients during surgery for breast cancer.

Objective: To evaluate the presence and prognostic value of MUC1 and Met-hepatocyte growth factor/scatter factor in the axillary drainage of these patients.

Methods: The study group included 40 consecutive patients with invasive ductal carcinoma of the breast who were suitable for breast-conserving treatment; 20 malignant melanoma patients found to have negative axillary sentinel lymph node served as the control group. The output of the drains, which had been placed in the axilla during operation, was collected, and the presence of MUC1, Met-HGF/SF and β-actin were assessed in the lymphatic fluid by reverse transcription-polymerase chain reaction assays. The data were compared to the pathologic features of the tumor and the axillary lymph nodes, and to the estrogen and progesterone receptors status.

Results: RT-PCR assays of the axillary lymphatic drainage were positive for MUC1 and Met-HGF/SF in 15 (37.5%) and 26 (65%) of the patients, respectively. Patients in whom MUC1 and Met-HGF/SF were not found in the axillary fluid had smaller tumors and less capillary and lymphatic invasion, compared to patients with positive assays ($P < 0.0$ for all these comparisons). The lymph nodes were negative for metastases in all patients with negative assays ($P < 0.001$). The presence of MUC1 and Met-HGF/SF showed negative correlations with the estrogen and progesterone receptors ($P < 0.05$).

Conclusion: MUC1 and Met-HGF/SF can be detected in the axillary fluids of patients with breast cancer. The expression of both tumor markers in the axillary drainage is strongly associated with unfavorable tumor features and can be used as a prognostic factor.

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Patients with breast cancer who have undergone curative surgery and show no evidence of loco regional or distant disease still have recurrent disease at a rate as high as 30% over 5–10 years [1]. Some of these treatment failures may be attributed to residual disease in the breast or axillary lymph nodes [2]. The limitation of routine histopathologic examination of the tumor margins and the dissected lymph node specimen is well known [3]. Surgery for breast cancer involves either mastectomy or breast-conserving surgery, consisting 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 in order to prevent the accumulation of lymphatic fluid. We hypothesized that this fluid contains products of cancer cells that may be used as prognostic factors.

Mucins, including MUC1, are produced by epithelial tissues and neoplasms. MUC1 was found in solid tumors, and it has been reported that the expression of the gene coding for it is suitable for the detection of disseminated breast cancer cells in lymph nodes [4]. Hepatocyte growth factor/scatter factor is a paracrine factor produced primarily by mesenchymal cells. HGF/SF induces mitogenic and morphogenic changes, including rapid membrane ruffling, formation of micro-spikes, and increased cellular motility. The diverse biological effects of HGF/SF are all mediated by Met – the HFG/SF receptor that is preferentially expressed on epithelial cell [5]. Although Met-HGF/SF signaling clearly plays a role in numerous normal cellular processes, this signaling pathway has also been implicated in tumor development and progression. Met-HGF/SF signaling can increase tumorigenicity, induce cell motility, and enhance *in vitro* invasiveness and *in vivo* metastasis [5–7]. While MUC1 and Met-HGF/SF were found in malignant tissues [8–12], their expressions in the lymphatic drainage of the tumor bed have not yet been assessed.

Using RT-PCR we assessed the presence of MUC1 and Met-HGF/SF in the axillary drainage of patients who underwent operations for breast cancer and evaluated the mRNA expressions with prognostic factors of breast cancer.

Patients and Methods

Patients and operations

We studied 40 consecutive women with invasive ductal carcinoma of the breast who were suitable for breast-conserving treatment. In order to evaluate the correlations of MUC1 and Met-HGF/SF expression with both the tumor margins and the status of the axillary lymph nodes we included only patients who underwent breast-conserving surgery. The diagnosis of cancer was established by wide bore needle biopsies (Trucut) performed 2 weeks prior to surgery. All the patients underwent wide local excision and axillary lymph node dissection by the same team. Non-palpable tumors were localized by mammography prior to surgery by wire insertion. During surgery a wide resection around the wire was performed, followed by specimen mammography for verification of complete resection of the tumors. The axillary dissection was performed using a separate incision, and level I and II axillary lymph nodes were removed. The breast incisions were closed without drainage, and all axillaries were drained postoperatively by closed vacuum drains

HGF/SF = hepatocyte growth factor/scatter factor
RT-PCR = reverse transcription-polymerase chain reaction

(ch-14, Biometrix, USA). The output of the drain was collected and measured every 24 hours, and the drains were removed when the output was less than 25 ml/24 hr. The presence of MUC1 and Met-HGF/SF and β-actin was assessed in the fluid collected on the second postoperative day since fluid may contain many erythrocytes and debris during the first 24 hours.

Pathologic examinations

The resected specimen was covered circumferentially by black Indian ink and Bouin's solution and then sliced into 5 mm slices. Each slice was macroscopically evaluated for the presence of tumor and its distance from the margins of the specimen. All slices with tumor were paraffin-embedded, sliced again into 4 μ slices, and stained with hematoxylin and eosin. Microscopic evaluations for the presence of tumor and margin involvement were performed by one pathologist using x 40 magnification. Additional information such as tumor type, size and grade, and capillary or lymphatic invasion and the distance form the margins was also evaluated. All axillary lymph node were paraffin-embedded and sliced into 4 μ slices and assessed for the presence of micrometastases.

Receptor assays

The estrogen receptors and progesterone receptors were studied in the tumor by immunohistochemical assay using mouse monoclonal antibody (NCL-ER-6F11, NCL-PGR-312) according to the manufacturer's instruction (Novocastra Laboratories, UK). We use the "quick score," a simple combination of the proportion of cells staining plus a measure of intensity of staining [13]. A cutoff value of 2 was referred to as ER or PR-negative.

RT-PCR assays

Total RNA was extracted from axillary lymphatic fluid using the TRI REAGENT procedure according to the manufacturer's instructions (Sigma, USA). Reverse transcription was performed using 1–2 μg of total RNA. The first strand of cDNA was generated with 0.5 μg oligo-$(dT)_{15}$ primer (Gibco BRL, USA) using 200 units of subscripts II RNAse-H-Reverse transcriptase (Gibco BRL). This was incubated for 50 minutes at 42°C, followed by inactivation at 70°C for 15 min. Cycling conditions included 35 cycles with denaturation steps at 94°C for 30 seconds, hybridization steps at 55°C for 30 sec and extension steps. at 72°C for 1 min. The products were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide. The primer sequences used for RT-PCR for MUC1, Met and β-actin are summarized in Table 1. The limit of sensitivity of the RT-PCR system for Met was 1 pg of total RNA.

ER = estrogen receptors
PR = progesterone receptors

Table 1. Primer sequence used for RT-PCR

Primer sequence		
MUC-1	5'-CGTCGTGGACATTGATGGTACC-3'	5'-GGTACCTCCTCTCACCTCCTCCAA–3'
Met	5'-GGAATCGAGCTGCGAGA-3'	5'-TCCAACATGCAGTTTCTTGC-3'
β-actin	5'-CTCTTCCAGCCTTCC TTCCT-3'	5'-AGCACTGTGTTGGCGTACAG-3'

Control group

In order to verify the absence of either MUC1 or Met-HGF/SF in patients without breast cancer we examined the axillary drainage in 20 patients with malignant melanoma who were found to have a negative axillary sentinel lymph node. The lesions were located in the upper limbs and the lymph node basins were located in the axillas. Sentinel lymph nodes were found in all these patients and none was involved with melanoma in frozen sections or in paraffin-embedded and stained slices. Immunohistochemical staining was performed on the primary melanoma with an antibody for HGFα-receptor (H-145 Santa Cruz Biotechnology, CA, USA).

Statistical evaluation

Statistical analysis for correlation between the various clinico-pathologic parameters and RT-PCR assays in lymphatic fluid was performed using the chi-square test at a significance level of 5%. All statistical analyses were performed with the Statistical Package for the Social Sciences Software (SPSS, Chicago, IL, USA).

Results

The study group comprised 40 consecutive women who underwent breast-conserving surgery for operable invasive duct carcinoma of the breast. Patients with tumors that invaded the chest wall or skin, or with inflammatory carcinoma, were not included in the study. The mean age was 58 ± 16 years. Thirty-one patients (77.5%) underwent lumpectomies for palpable masses, and in 9 women (22.5%) wire-guided excisions for non-palpable tumors were performed. The tumor size was 0–1 cm in 12 patients (30%), 1–2 cm in 13 (32.5%), 2–5 cm in 14 (35.%), and larger than 5 cm in one patient (2.52%). Eight patients (20%) had grade I tumors, 23 (57.5%) had grade II lesions, and 9 (22.5%) had grade III tumors. Lymphatic and capillary invasion was noted in 11 (27.5%) and 14 (35%) patients, respectively. While attempting to achieve free margins, five patients (12.5%) had to undergo re-excision due to incomplete resections. Two of these patients had wire-guided excisions.

The collected axillary fluids were assessed by RT-PCR for MUC1, Met-HGF/SF and β-actin. The β-actin RT-PCR served as a 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 MUC1 and Met-HGF/SF in 15 (37.5%) and 26 (65%) of the breast cancer patients, respectively. The patients were divided into three groups: a) 15 patients (37.5%) with positive assays for both MUC1 and Met-HGF/SF, b) 11 patients (27.5%) with positive assays for Met-HGF/SF but negative assays for MUC1 (there were no Met-HGF/SF-negative MUC1-positive patients), and c) 14 patients (35%) in whom both assays were negative. In all patients in the control group RT-PCR gave positive results for β-actin but was negative for MUC1 as well as for Met-HGF/SF. All the paraffin-embedded sections of the resected primary melanoma were assessed by immunohistochemical staining for Met and were found negative.

The correlations between tumor size and the presence of MUC1 and Met-HGF/SF in the axillary fluid are shown in Table 2. Positive RT-PCR assays for MUC1 or Met-HGF/SF in the

lymphatic fluids were associated with increasing tumor size. This finding was more pronounced when both assays were positive. Among the group of 25 patients with a tumor size of 0–2 cm, 14 (56%) were both MUC1 and Met-HGF/SF negative compared with 5 patients (20%) in whom the assay revealed positive MUC1 and Met-HGF/SF ($P < 0.05$). It is noteworthy that there were no T2 tumors when assays for both MUC1 and Met-HGF/SF were negative.

The average number of axillary lymph nodes was 17 ($\pm$ 4), and all dissected nodes were examined for the presence of metastases. Sixteen patients (40%) had axillary lymph node metastases by hematoxylin and eosin staining. Axillary lymph node involvement was strongly associated with the presence of MUC1 and Met-HGF/SF in the lymphatic fluid [Table 2]. All patients with axillary lymph node metastases had positive RT-PCR assays for Met-HGF/SF, and in 14 of the patients in whom both assays were positive (93.3%) the lymph nodes were involved with tumor. In all patients without MUC1 and Met-HGF/SF in the axillary fluid there were no metastases in the lymph nodes ($P < 0.01$).

The associations between tumor grade, capillary and lymphatic invasion, and the presence of MUC1 and Met-HGF/SF in the axillary fluid are presented in Table 2. In patients in whom both assays were negative there was neither capillary nor lymphatic invasion, and 13 of 14 patients (92.8%) had grade I and grade II tumors. The ratios of the capillary and lymphatic invasions in patients in whom only the Met-HGF/SF assays were positive were still low (27.2% and 9%, respectively), however they increased to 73.3% when both assays were positive ($P < 0.02$).

Table 2. Correlation between MUC1/Met presence and tumor size, lymphatic involvement, capillary and lymphatic invasion and receptor status

	Met +	Muc +	Met +	Muc –	Met –	Muc –	
	Patients	%	Patients	%	Patients	%	*P* value
Tumor size (cm)							
a. – 1	1	6.6	3	27.2	8	57.1	
b. – 2	4	26.6	3	27.2	6	42.9	
c. – 5	9	60	5	45.6	–		
> 5	1	6.6	–		–		
Total	15		11		14		P < 0.05
Lymph node involvement							
Absent	1	6.6	9	81.2	14	100	
Present	14	93.3	2	18.8	–		
Total	15		11		14		< 0.01
Capillary invasion							
Present	11	73.3	3	27.2	–		
Absent	4	26.7	8	72.8	14	100	< 0.05
Lymphatic invasion							
Present	10	66.6	1	9	–		
Absent	5	33.4	10	91	14	100	< 0.05
Grade							
I	1	6.6	1	9	6	42.8	
II	9	53.3	7	63.6	7	50	
III	5	33.3	3	27.2	1	7.2	< 0.05
Receptor status							
ER +	3	20	5	45	12	85.7	
ER –	12	80	6	55	2	14.3	< 0.02
PR +	–		5	45	13	92.8	
PR –	15	100	6	55	1	7.2	< 0.02

The correlations between the estrogen and progesterone receptor status and RT-PCR assays for MUC1 and Met-HGF/SF in the axillary fluid are shown in Table 2. In most of the patients with positive stains for either estrogen or progesterone (85.7% and 92.8% respectively) the assays for both MUC1 and Met-HGF/SF were negative. The receptor status of the patients with one positive assay was almost equally divided, but when both assays were positive the ER were negative in 80% of the patients and the PR were negative in all of them ($P < 0.02$).

Discussion

In all patients in this series with T1N0 breast cancer the RT-PCR assays for both MUC1 and Met-HGF/SF were negative. Similarly, the assays were never positive in patients without lymphatic or capillary invasion and had negative correlation with the hormonal receptor status. On the other hand, the presence of mRNA of MUC1 and Met-HF/SF was associated with unfavorable prognostic features. We did not evaluate the specificity of the assays, but they were negative in all control patients with negative sentinel axillary lymph nodes. Sentinel lymph node biopsies are highly accurate and their ability to predict the long-term nodal status was reported in over 90% of patients [14–16]. This is also our personal experience with melanoma and breast cancer patients who underwent axillary sentinel lymph node biopsies

MUC1 is a transmembrane glycoprotein that is over-expressed and aberrantly glycosylated in more than 90% of breast cancer cells and in various other malignant cells [4,8–12]. The status of the axillary lymph nodes is a most important prognostic parameter in breast cancer, and it was postulated that traditional histopathologic methods might miss micro-metastases within [3]. The efficacy of MUC1 assays in detecting axillary lymph node micro-metastases was investigated in several studies, and the significance of MUC1 expression and its role as a diagnostic and/or prognostic factor have been extensively evaluated in tumor tissues, lymph nodes, hematopoietic cells and in the serum [4,17–19]. MUC1 may also be used in cancer therapy and its potential in antitumor immunotherapy has recently attracted much interest [20]. We found that MUC1 can be detected in the axillary drainage and that its presence may have a prognostic significance. However, we could not determine whether MUC1 and Met-HGF/SF in the axillary fluid originated from the tumor, lymphatic channels, or from metastases in the lymph nodes.

HGF/SF is synthesized as a pro-HGF and, once activated, the complex Met-HGF/SF affects numerous normal cellular processes [21]. Met-HGF/SF is also involved in malignant transformation, presumably as a mediator in tumor-stromal interaction, which enhances tumor progression and invasiveness, and angiogenesis [22]. HGF/SF is over-expressed in breast carcinoma *in situ* and invasive ductal carcinoma, as compared with normal breast tissue. Normal mammary ducts within infiltrating cancer showed intermediate levels of HGF/SF. This finding may suggest that the expression of these proteins in breast cancer is regulated by soluble factors produced by the tumor cells. High levels of HGF and Met expression are associated with invasive breast cancer and may be causally linked to early recurrences, metastatic disease and

shortened survival of breast cancer patients. Moreover, high levels of HGF/SF detected within breast tumor extracts are correlated with larger tumor size and short relapse-free and less overall survival compared to tumors with low HGF/SF concentration [23].

HGF/SF and Met are abundant in highly invasive breast cancer cells, while low levels of their expression were found in less invasive breast cancer cells [21]. The significance of HGF/SF as a marker of poor prognosis may be associated with its effects on acquired resistance to anti-cancer drugs [24]. The axillary fluid of most patients in this series (65%) contained Met-HGF/SF, and Met-HGF/SF assays were never negative in patients with MUC1-positive assays.

Our results indicate that the associations between Met-HGF/SF and tumor size and grade were more predominant than their relations with MUC1, while the latter showed a stronger linkage with lymph node involvement and with capillary and lymphatic invasion. It appears that both MUC1 and Met-HGF/SF had a negative correlation with the hormonal receptors status. It is noteworthy that when both assays were positive, all tumors were negative for progesterone receptors and 80% were negative for estrogen receptors.

During our work on this manuscript a new study, which focused on the number of lymph nodes examined in patients with T1/T2 N0 breast cancer, found a significantly higher risk of death from breast cancer in patients with 0–10 nodes examined compared to 20 or more nodes examined [25]. Studying the expression of mRNA in the axillary fluid is a simple non-invasive procedure, since drains are routinely inserted into the axilla during axillary lymph node dissection and minimally invasive procedures such as sentinel lymph node biopsy and the collected fluid are readily available. The RT-PCT methods that were used in this study are routine short assays with minimum artifacts. The results suggest that the presence of both MUC1 and Met-HGF/SF in the axillary drainage has a significant prognostic value. Their expression in histologically node-negative patients point to the need to search for nodal microinvasion and may have a prognostic role.

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