Altered Expression of Metastasis-Associated and Regulatory Molecules in Effusions from Breast Cancer Patients: A Novel Model for Tumor Progression

Ben Davidson,1 Sophia Konstantinovsky,2 Søren Nielsen,3 Hiem Phuc Dong,3 Aasmund Berner,1 Mogens Vyberg,3 and Reuven Reich2,4

1Department of Pathology, The Norwegian Radium Hospital, Montebello, University of Oslo, Oslo, Norway; 2Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel; and 3Department of Pathology, Aalborg University, Aalborg, Denmark. 2David R. Bloom Center for Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel.

ABSTRACT

Purpose: The aim of this study was to characterize phenotypic alterations along the progression of breast carcinoma from primary tumor to pleural effusion through analysis of the expression of proteases, laminin receptors (LRSs), and transcription factors involved in invasion and metastasis.

Experimental Design: The material studied consisted of 60 malignant pleural effusions from breast cancer patients and 60 corresponding solid tumors (37 primary and 31 metastatic tumors). Expression of matrix metalloproteinases (MMPs (MMP-1, MMP-2, MMP-9, and MMP-14)), the MMP inhibitor tissue inhibitor of metalloproteinase-2, the MMP inducer EMMPRIN, the 67-kDa LRs, the α6 integrin subunit, and the transcription factors AP-2, Ets-1, and PEA3 was studied using immunohistochemistry, mRNA in situ hybridization, reverse transcription-polymerase chain reaction, zymography, and flow cytometry. Hormone receptor (estrogen receptor and progesterone receptor) status and c-erbB-2 status were also studied.

Results: Significantly reduced estrogen receptor (P < 0.001) and progesterone receptor (P = 0.001) expression was seen in effusions compared with primary tumors, with opposite findings for c-erbB-2 (P = 0.003). Tumor cell MMP-2 protein expression in effusions was higher than that in primary tumors (P < 0.001) and lymph node metastases (P = 0.01). In situ hybridization demonstrated higher MMP-2 (P = 0.007), PEA3 (P = 0.038), and EMMPRIN (P = 0.026) mRNA expression in effusions. The time to progression from primary tumor to effusion was significantly shorter for patients whose primary tumors expressed MMP-1 (P = 0.016) and who expressed the 67-kDa LR protein in primary tumor (P = 0.007) and effusion (P = 0.015).

Conclusions: Our data provide documented evidence of molecular events that occur during the progression of breast carcinoma from primary tumor to effusion. The coordinated up-regulation of MMP-2 and Ets transcription factors in carcinoma cells in effusions is in full agreement with our previous reports linking these factors to poor prognosis in ovarian cancer. The rapid progression to effusion in cases showing MMP-1 and 67-kDa LR expression in primary tumor cells links aggressive clinical behavior with expression of metastasis-associated molecules in this setting.

INTRODUCTION

Breast cancer is the most common malignancy in women, second in mortality only to lung cancer (1). Breast cancer metastasizes most frequently to axillary lymph nodes, but any organ may be involved. Metastatic spread to serosal surfaces involves primarily the pleural cavity, where breast cancer is the etiology of approximately 25% of malignant effusions (2, 3). However, breast carcinoma metastasis may be infrequently found in the pericardial and peritoneal cavity as well (4). Involvement of the pleural cavity by breast carcinoma may occur at any point of time of the clinical course and may be the sole manifestation of metastatic disease (5). It portends an extremely poor prognosis, with a median survival of 5 (5) and 11 (6) months in two series.

Despite the magnitude of the clinical problem, studies of the biology of breast cancer have focused exclusively on primary tumors and solid metastases. Consequently, the biological characteristics of breast carcinoma cells in effusions have been poorly characterized at both the phenotypic and genotypic level, and their potential differences from primary and metastatic solid tumors are largely unknown. Elucidation of these potential differences may aid in explaining why the appearance of pleural effusion in breast cancer is associated with a rapidly fatal disease.

Tumor progression is a multistep process. Epithelial cells acquire the malignant phenotype during an initial growth phase in an in situ lesion, which is contained by a basement membrane (BM). The BM, a thin layer of extracellular matrix that physiologically supports epithelial, endothelial, and mesenchymal cells, is composed of several proteins, including laminins, the major noncollagenous protein, type IV collagen, and proteoglycans (7). Breaching and degradation of the BM is a key event in...
these processes and signifies the transition from a contained in situ lesion to an invasive and potentially disseminated tumor in epithelial malignancies.

Invasion and metastasis involve a large number of molecules, including angiogenic factors, growth factors and their receptors, adhesion molecules, proteases, intracellular signaling molecules, and transcription factors (8–15). These molecules are related in terms of expression and function and cross-talk through defective signaling pathways and impaired regulation of vital cellular mechanisms, including cell death, proliferation, and response to growth-stimulatory and -inhibitory signals (15).

Attachment to laminin is a key event in the process of local and vascular invasion and therefore plays an important role in tumor progression. Laminin receptors fall into two categories, integrin and nonintegrin receptors. The integrins involved in attachment to laminin include the α5β1, α5β3, α6β1, and α6β1 integrins, as well as two family members that contain the αo integrin subunit, the αo1β1 and αo1β3 integrins (12–14). The latter two are specific for laminin. The non-integrin 67-kDa laminin receptor [LR (laminin-binding protein)] was isolated from the membrane of cancer cells in 1983 (16, 17). The LR precursor presumably becomes the mature 67-kDa LR molecule through homodimerization of two molecules and/or heterodimerization with another unknown protein (18). Expression of the LR precursor or the 67-kDa LR has been shown to be up-regulated by cytokines, inflammatory agents, and extracellular matrix proteins such as laminin and fibronectin. The receptor has been postulated to be coregulated and coexpressed with α6 integrin in vitro models. Its expression has been found in a wide range of malignancies, and in many of these, it correlated with poor differentiation, disease progression, and poor survival (18, 19).

Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent enzymes, are central mediators of the biology of tumor metastasis because of their ability to degrade BM and extracellular matrix components (20). Two members of the family, MMP-2 and MMP-9, are the proteases involved in BM degradation. MMP-2 synthesis is in turn induced by laminin (21). EMMPRIN (extracellular matrix metalloproteinase inductor), a 58-kDa glycoprotein adhesion molecule belonging to the immunoglobulin superfamily (22), is one of the molecules involved in the regulation of MMP expression. The enzymes whose synthesis is known to be up-regulated as a consequence of stimulation by EMMPRIN are MMP-1, MMP-2, and MMP-3 (23). In addition, EMMPRIN is able to bind to MMP-1 on the surface of tumor cells (24). EMMPRIN has been shown to colocalize with the α5β1 and αo5β1 integrins at the cell membrane (25).

Ets transcription factors contain a DNA-binding domain (the Ets domain) that confers the ability to bind to DNA sequences having the core motif 5'-GGAA/T-3' (26). They are further divided into subfamilies based on the sequence of the Ets domain and the presence of additional conserved domains (26, 27). Ets members are involved in a variety of physiologic and pathological processes, including embryogenesis, wound healing, and tumor progression (28, 29). This is largely through their ability to activate the transcription of proteases, including urokinase-type plasminogen activator (30) and MMP (31), as well as that of tissue inhibitors of MMP (TIMP; 31) and the β3 integrin subunit (32). The activation of proteolytic enzyme transcription is central to the metastatic process, owing to proteolytic enzymes role in both angiogenesis and tumor invasion.

AP-2 transcription factors are a family of three highly homologous genes that regulate a large number of molecules, including the c-Kit receptor, MUC-18, estrogen receptor (ER), MMP-2 and MMP-9, and HER-2/neu (33). As with Ets transcription factors, AP-2 expression is central for the regulation of developmental and cancer-related events (33).

The objective of the present study was to analyze the expression of metastasis-associated molecules in breast cancer cells in effusions and compare it with that of tumor cells in solid tumors. The studied molecules consisted of proteases (MMP-1, MMP-2, MMP-9, and MMP-14) and molecules that regulate their expression (the MMP inhibitor TIMP-2, EMMPRIN, and the transcription factors AP-2, Ets-1, and PEA3). In view of the central role of LRs in tumor progression of ovarian carcinoma to effusions (34), as well as the role of LRs in MMP regulation in experimental models (35), we additionally analyzed the expression of 67-kDa LR and the α6 integrin subunit. Hormone receptor [ER and progesterone receptor (PgR)] status and c-erbB-2 status were studied due to the central role of these molecules in the biology of breast carcinoma. Our results document for the first time extensive molecular changes undergone by breast cancer cells in the progression from primary tumor to pleural effusion and show data supporting coexpression of regulatory and effector molecules at the latter site. We additionally report on the predictive role of some of these molecules with respect to the time to progression (TTP) from primary tumor to effusion.

**MATERIALS AND METHODS**

**Patients and Clinicopathologic Data.** The study cohort consisted of 55 female patients with histologically verified breast cancer. Patient age ranged from 35 to 85 years at diagnosis. Forty-eight women were diagnosed with infiltrating duct carcinoma, six were diagnosed with lobular carcinoma, and one was diagnosed with a tumor combining both histologic types. Tumor grade was available for 44 ductal carcinomas and was as follows: 2 grade 1 tumors; 33 grade 2 tumors; and 9 grade 3 tumors.

The period of time from primary diagnosis to the sampling of pleural effusion ranged from 0 (simultaneous) to 19 years. For the purpose of this study, this period was defined as TTP.

**Effusion Specimens.** The material consisted of a total of 60 pleural effusions from the above-mentioned patients, all submitted for routine diagnostic purposes to the Section of Cytology, Department of Pathology, Norwegian Radium Hospital and the Section of Cytology, Department of Pathology, Aalborg Hospital during the period of January 1998 to September 2002. Submitted specimens arrived within minutes after tapping and were processed immediately. Material was sufficient for the preparation of paraffin-embedded cell blocks from 49 effusions. Additional material was suspended and frozen in equal volumes of RPMI 1640 supplemented with 20% fetal calf serum and 20% dimethyl sulfoxide. Smears and cell block sections from all specimens underwent morphologic evaluation by experienced cytopathologists and were further characterized using immunocytochemistry with broad antibody panels against cancer and mesothelial epitopes, as detailed previously (36, 37).
Patient consent was obtained according to institutional and national guidelines.

Solid Tumors. Thirty-seven consenting primary tumors, 18 lymph node metastases, and 13 locoregional recurrence specimens from 37 patients were available for comparative analyses. Diagnoses, histologic typing, and tumor grade were reviewed for all cases.

Immunocytochemistry. All specimens for whom a paraffin-embedded block was available (49 effusions and 68 solid lesions) were studied using the antibodies listed below, with the exception of 67-kDa LR and MMP-1, which were studied in 38 effusions and 39 solid lesions due to limited material. The antibodies used were as follows: ER (1:50; DakoCytomation, Glostrup, Denmark); PgR (1:50; DakoCytomation); c-erbB-2 (1:1,500; DakoCytomation); MMP-1 (1:50; Becton Dickinson, San Jose, CA); MMP-2 (1:50; Becton Dickinson); MMP-9 (1:50; Becton Dickinson); EMMPRIN (1:300; Santa Cruz Biotechnology, Santa Cruz, CA); and 67-kDa LR (1:160; NeoMarkers, Fremont, CA). Pretreatment consisted of microwaving with Tris-EDTA buffer (pH 9.0) for all antibodies except for EMMPRIN and 67-kDa LR, which were pretreated by microwaving using citrate buffer (pH 6.0) as buffer. Slides were immunostained at the Departments of Pathology at Aalborg Hospital and the Norwegian Radium Hospital. Staining was performed using the EnVision+ peroxidase system (DakoCytomation). Positive controls consisted of arrays of different tissues or specimens found to express the studied protein in previous studies.

In situ Hybridization. Specific antisense oligonucleotide DNA probes for the mRNA transcripts of MMP-2, MMP-9, TIMP-2, EMMPRIN, 67-kDa LR, the α5 integrin subunit, Ets-1, and PEA3 were obtained from Research Genetics (Huntsville, AL). Probe sequences (5’ to 3’) were as follows (38–43): MMP-2, 5’-TGGGCTACGGCCCGGCGGCGTGCG-3’; MMP-9, 5’-CCGGTCCACCTCGCTGGCGCTCCGGU-3’; TIMP-2, 5’-CCAGGGAGGTATGCAGGACCG-3’; EMMPRIN, 5’-CAAGGACCGAGCAGAGAATCG-3’; and 67-kDa LR, which were pretreated by microwaving using citrate buffer (pH 6.0) as buffer. Slides were immunostained at the Departments of Pathology at Aalborg Hospital and the Norwegian Radium Hospital. Staining was performed using the EnVision+ peroxidase system (DakoCytomation). Positive controls consisted of arrays of different tissues or specimens found to express the studied protein in previous studies.

Flow Cytometry. Six pleural effusions were studied for protein expression of the α5 integrin subunit using the FACSCalibur flow cytometer (Becton Dickinson), as described previously (45); the sole modification was the exclusive use of primary conjugated antibodies. Phycocyanin-conjugated antibodies directed against the α5 integrin subunit were purchased from Santa Cruz Biotechnology. The Ber-EP4 and anti-epithelial membrane antigen antibodies, both established markers directed against epithelial epitopes, were obtained from DakoCytomation. Antibodies against the macrophage protein CD14 (Diaotec, Oslo, Norway) and the pan-leukocyte marker CD45 (Becton Dickinson) were also used. Controls consisted of a mixture of the Ber-EP4–positive breast carcinoma cell line (T47-D) and human mononuclear leukocytes.

Evaluation of flow cytometry immunophenotyping was performed as described previously (45). Any number of positive cells was considered sufficient for analysis.

Reverse Transcription-Polymerase Chain Reaction. Twenty-two pleural effusions were studied for mRNA expression of MMP-2, MMP-9, TIMP-2, MMP-14 [membrane type-1 MMP (MT1-MMP)], 67-kDa LR, the α5 integrin subunit, and AP-2 using reverse transcription-polymerase chain reaction (RT-PCR). Specimens analyzed in this test consisted of effusions with a large number of tumor cells. Morphology and immunohistochemistry (IHC) using diagnostic markers for tumor cells, mesothelial cells, and leukocytes showed that 12 effusions contained >50% (up to 95%) carcinoma cells, whereas the remaining 10 specimens approached this value. RNA was isolated using the Tri Reagent kit (Sigma, St. Louis, MO) and reverse transcribed using the Moloney murine leukemia virus reverse transcription kit (Promega, Madison, WI). Primer sequences were as follows: (a) 28S, 5’-gtcaccaccaattacaaggtc (sense) and 5’-gagttccttgagttgacgcc (antisense; 212 bp); (b) MMP-2, 5’-ccctcctccctcctccttg (sense) and 5’-aaccatcactctcactctctc (antisense; 327 bp); (c) MMP-9, 5’-cctctacggcctact (sense) and 5’-gacctcgagttgctc (antisense; 611 bp); (d) MT1-MMP, 5’-ccctcctccctcctcctcttg (sense) and 5’-ctcctcctccctcctcctcctctcctctcct (antisense; 532 bp); (e) TIMP-2, 5’-cacacccactctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctcta
Cycle parameters were as follows: (a) 28S, heating at 94°C for 5 minutes, denaturation at 94°C for 15 seconds, annealing at 63°C for 20 seconds, and extension at 72°C for 10 seconds, for 17 cycles; (b) MMP-2, heating at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes, for 33 cycles; (c) MMP-9, heating at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute, and extension at 72°C for 1.5 minutes, for 33 cycles; (d) MT1-MMP, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, for 34 cycles; (e) TIMP-2, heating at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1.5 minutes, for 35 cycles; (f) α6 integrin, heating at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes, for 31 cycles; (g) 67-kDa LR, heating at 94°C for 5 minutes, denaturation at 94°C for 1.5 minutes, annealing at 50°C for 1.5 minutes, and extension at 72°C for 2 minutes, for 31 cycles; (h) AP-2α, heating at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute, for 30 cycles; (i) AP-2γ, heating at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, for 30 cycles.

Products were separated on 1.5% agarose gels, isolated using the Invisorb Spin DNA extraction kit (Invitek GmbH, Berlin, Germany), and sequenced. The HT-1080 fibrosarcoma cell line was used as control in all reactions. Gels were photographed by the Kodak EDAS 290 system. Densitometer analysis of films was performed using the National Institutes of Health Image program. The A375SM melanoma cell line was used as control. Activity was scored as low, moderate, or high, corresponding to densitometry scores of 1 to 100, 101 to 1,000, and >1,000, respectively.

**Statistical Analysis.** ISH and IHC results in tumor cells were evaluated statistically, applying the SPSS–PC package (version 10.1; SPSS, Chicago, IL). P < 0.05 was considered statistically significant. Comparative analyses evaluating protein expression of the studied molecules in tumor cells in effusions, primary tumors, and lymph node metastases were executed using the Wilcoxon signed ranks test, as were comparative analyses of mRNA expression in carcinoma cells in effusions versus primary tumors. Lymph node expression results were not included in the mRNA expression analysis due to the small number of cases analyzed. Local recurrences were not statistically analyzed for comparative protein or mRNA expression for the same reason. Studies of the association between staining results and TTP and analyses of coexpression of the studied molecules were undertaken using the χ² test. Univariate survival analyses for effusion specimens were executed using the Kaplan-Meier method and log-rank test. All statistical analyses were two-sided.

**RESULTS**

**Hormone Receptor Status and c-erbB-2 Expression Are Significantly Altered in Breast Carcinoma Cells in Effusions.** Our first goal was to compare the expression of molecules that have an established role in the biology and prognosis of breast carcinoma at the different anatomic sites. ER, PgR, and c-erbB-2 staining results (percentage of stained cells) are shown in Table 1. Wilcoxon test showed reduced expression of both ER (P < 0.001) and PgR (P = 0.001) in effusions compared with primary tumors (Fig. 1A and B). PgR expression was also reduced in lymph nodes compared with primary tumors (P = 0.046). A case to case analysis of matched primary tumors and effusions (37 paired lesions) showed reduced ER in effusions in 18 specimens, expression that was similar to the corresponding
primary tumor in 17 cases (of which 5 were negative at both sites), and increased expression in 2 cases. The data for PgR were as follows: reduced expression in 20 cases, similar expression in 14 cases (of which 8 were negative at both sites), and increased expression in 2 cases. The data for the comparative analysis of 37 paired primary tumors and effusions were as follows: (a) staining extent, reduced expression in 9 effusions, similar expression in 15 cases, and increased expression in 13 cases; and (b) staining intensity, reduced expression in 3 effusions, similar expression in 18 cases (all showing intensity = 3 in both primary tumor and effusion), and increased expression in 16 cases.

MMP-2, EMMPRIN, and PEA3 Are Up-Regulated in Breast Carcinoma Cells in Effusions. The next analysis dealt with site-related expression of metastasis-associated molecules in the studied material. IHC with antibodies directed against MMP-1, MMP-2, and MMP-9 showed that MMP-2 protein expression clearly predominated over that of MMP-1 and MMP-9 in carcinoma cells. Expression of the two other enzymes (most often limited to <10% of cells) was as follows: (a) MMP-1, 19 of 49 effusions, 8 of 37 primary tumors, 7 of 18 lymph nodes, and 4 of 13 local recurrences; and (b) MMP-9, 14 of 49 effusions, 10 of 37 primary tumors, 5 of 18 lymph nodes, and 5 of 13 local recurrences. MMP-2 was also frequently expressed in stromal (34 tumors) and endothelial (all 37 tumors) cells. MMP-9 protein was found in stromal cells in 11 cases. Wilcoxon test showed significantly elevated expression of MMP-2 in effusions compared with both primary tumors ($P < 0.001$) and lymph nodes ($P = 0.01$; Fig. 1E and F). ISH results for MMP-2, MMP-9, and TIMP-2 showed significant up-regulation of MMP-2 mRNA expression in carcinoma cells in effusions compared with primary tumors, in agreement with IHC results ($P = 0.007$). MMP-9 mRNA was expressed in 30 of 33 effusions, 16 of 20 primary tumors, 6 of 10 lymph node metastases, and 8 of 10 local recurrences ($P > 0.05$ in Wilcoxon test). TIMP-2 mRNA was detected in 27 of 33 effusions, 5 of 10 primary tumors, 0 of 6 lymph node metastases (4 failed tests), and 3 of 10 local recurrences ($P = 0.046$ for the comparison of effusions and lymph node metastases). An example of the d(T) control for mRNA integrity is shown in Fig. 2A. Positive and negative examples of MMP-2, MMP-9, and TIMP-2 ISH are shown in Fig. 2B–G. Zymography documented gelatinolytic activity of MMP-2 and MMP-9 in the majority of effusions, with significant correlation between MMP-2 protein expression and activity in 15 effusions for which both tests were available ($P = 0.02$; Fig. 3). Statistical analysis showed no differences in MMP activity between specimens containing >50% tumor cells and those containing ≤50% tumor cells.

EMMPRIN mRNA expression was significantly higher in effusions compared with primary tumors ($P = 0.026$). EMMPRIN protein expression was higher in effusions compared with all solid lesion sites (total = 29 of 49 versus 27 of 68

Fig. 1 Breast cancer cells in effusions show altered expression of established prognostic markers and metastasis-associated molecules compared with primary tumors and lymph node metastases. A–H show a comparative view of a lymph node metastasis and pleural effusion from a single patient diagnosed with ductal breast carcinoma. Down-regulation of ER (A and B) is accompanied by up-regulation of c-erbB-2 (C and D), MMP-2 (E and F), and EMMPRIN (G and H) in tumor cells.
positive lesions; Fig. 1G and H), but this difference failed to reach significance.

The 67-kDa LR and the α6 integrin subunit mRNA were found in the majority of effusions and approximately 50% of solid lesions (25 of 33 effusions and 20 of 40 solid lesions for 67-kDa LR; 26 of 33 effusions and 19 of 40 solid lesions for the α6 integrin subunit). No significant site-related differences were seen (P > 0.05). Expression of the 67-kDa LR protein was seen in 15 of 38 effusions, 14 of 21 primary tumors, 10 of 14 lymph nodes, and 0 of 4 locoregional recurrences. A positive example and a negative example of α6 integrin subunit ISH are shown in Fig. 2H and I. Protein expression of the α6 integrin subunit in cancer cells was confirmed in six of six effusions using flow cytometry (Fig. 4).

Messenger RNA of the Ets transcription factors PEA3 and Ets-1 was studied using ISH. PEA3 mRNA was significantly up-regulated in cancer cells in effusions compared with primary tumors (P = 0.038). Ets-1 mRNA was found in the majority of effusions (22 of 33) and in approximately 50% of solid lesions (11 of 20 primary tumors, 4 of 10 lymph node metastases, and 5 of 10 local recurrences; P > 0.05 in Wilcoxon test). A positive example and a negative example of PEA3 ISH are shown in Fig. 2J and K.
higher expression of the low molecular weight isoform of TIMP-2 molecules between specimens containing LR (shown). Association with that of P (mRNA colocalized with MMP-2 P/H11005 mRNA with EMMPRIN in carcinoma cells in (46). In the present study, analysis of IHC results showed coexpression of MMP-9 with EMMPRIN in carcinoma cells in effusions (P = 0.002). ISH showed coexpression of MMP-2 (P = 0.046) and MMP-9 (P = 0.034) mRNA with EMMPRIN. MMP-2 mRNA colocalized with 67-kDa LR (P = 0.001), Ets-1 (P = 0.004), and PEA3 (P = 0.002) mRNA, as did MMP-9 with PEA3 (P = 0.004). EMMPRIN mRNA expression showed association with that of Ets-1 (P = 0.023), as did α6 integrin (P < 0.001) and 67-kDa LR (P = 0.003) mRNA with PEA3 (data not shown).

RT-PCR results supported and expanded the ISH data. Higher expression of MMP-2 correlated with higher expression of TIMP-2 (P = 0.001), MT1-MMP (P = 0.04), the low molecular weight isoform of α6 integrin (P = 0.001), and 67-kDa LR (P = 0.04). Higher expression of MMP-9 correlated with higher expression of the low molecular weight isoform of α6 integrin (P = 0.009) and 67-kDa LR (P = 0.011). Higher expression of TIMP-2 correlated with higher expression of 67-kDa LR (P = 0.018), whereas higher expression of MT1-MMP correlated with higher expression of the high molecular weight isoform of α6 integrin (P = 0.043) and 67-kDa LR (P = 0.003). Higher expression of the high molecular weight isoform of α6 integrin correlated with higher expression of AP-2α (P = 0.011), whereas higher expression of 67-kDa LR correlated with higher expression of AP-2γ (P = 0.008). Statistical analysis of RT-PCR results showed no differences in expression of these molecules between specimens containing >50% tumor cells and those containing ≤50% tumor cells (data not shown).

MMP-1 and 67-kDa LR Are Novel Predictors of Disease Progression to Effusions in Breast Carcinoma. In the last analysis, we wished to analyze the potential value of the studied molecules in predicting a more rapid progression in our cohort. In Kaplan-Meier analysis, protein expression of MMP-1 in primary tumors (P = 0.016; Fig. 6A) and protein expression of 67-kDa LR in effusions (P = 0.015; Fig. 6B) and primary tumors (P = 0.007; Fig. 6C) predicted shorter TTP.

DISCUSSION

Despite an increase in incidence, death from breast cancer has been on the decline in the last decade, reflecting both the use of mammography-based screening and improved treatment combining multiagent chemotherapy and tamoxifen (47). Unfortunately, as with other cancers, these encouraging trends have little effect on the clinical course once the disease is at its final phase. Metastatic spread in the form of pleural effusion signifies end-stage disease in breast cancer, but this clinical observation has not been supported by any scientific evidence to date.

We reported recently (48) on altered expression of the nerve growth factor receptors TrkA and p75 in breast carcinoma cells in pleural effusion compared with primary tumors and lymph node metastases, thereby providing molecular data regarding tumor progression to effusion in this disease. In the present study, we analyzed site-related expression of metastasis-associated molecules that share known biological pathways and have a previously documented role in breast and other carcinomas.

Hormone receptors and c-erbB-2 are established molecular prognostic markers in breast cancer (49, 50) and are often targeted with therapeutic intention in both localized and metastatic breast cancer, including in the presence of malignant effusion. Obviously, use of these agents is of benefit to the patient only when their molecular target is present on cancer cells, an issue that has never been investigated in effusions from
breast cancer patients. We report significant reduction in the expression of hormone receptors compared with primary tumors and (for PgR) lymph node metastases. Notably, hormone receptor expression was also considerably lower in effusions compared with locoregional recurrences, despite the temporal proximity of disease recurrence at these sites in most patients. Staining intensity for c-erbB-2 was similarly highest in effusions, with intermediate levels in locoregional recurrences and significantly lower expression in primary tumors and lymph node metastases. These data suggest that the presence of breast carcinoma cells in effusion represents biologically a more advanced phase in tumor progression compared with metastatic spread to both lymph nodes and locoregional sites and that these specimens need to be routinely evaluated before the selection of therapy.

MMPs are the protease family with the most widely documented role in cancer (9, 11, 20). Numerous studies have dealt with MMP expression in solid breast cancer and cannot be mentioned here due to space limitations (reviewed in ref. 51). We analyzed the expression of MMP-2, an enzyme with a documented prognostic role in breast carcinoma (51), as well as two additional members of this family, MMP-1 and MMP-9, all

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Effusions</th>
<th>Primary tumors</th>
<th>Lymph nodes</th>
<th>Locoregional recurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 protein</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>MMP-2 mRNA</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>EMMPRIN mRNA</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PEA3 mRNA</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE. Values represent the percentage of stained cells (0 = 0%, 1 = 1–10%, 2 = 11–50%, and 3 = 51–100%).
of which were previously shown to be expressed in effusions from ovarian cancer patients (52). Our results show that MMP-2 is the predominant MMP among the studied proteases at all tumor sites, with further up-regulation in effusions at both the mRNA and protein levels. Surprisingly, MMP-1 protein expression, although limited to <5% of cells in most specimens, was the finding associated with more rapid progression to effusion. This may reflect the addition of an enzyme with different substrate specificity to the gelatinases (MMP-2 and MMP-9) and the generally higher expression of MMP-2 at all sites. Thus, MMP-2 may have a central biological role in disease progression (as also evidenced by the up-regulation in effusions), but it does not aid in predicting TTP within a group of patients with uniformly poor outcome.

The expression of EMMPRIN and TIMP-2, two molecules involved in the activation of MMP-2, was additionally up-regulated in effusions, compared with primary tumors and lymph nodes, respectively. These findings are in agreement with the central role of MMP-2 in disease progression in our cohort and with previous data documenting the role of TIMP-2 as a predictor of poor outcome in breast (53) and ovarian (39) carcinoma.

Altered expression of receptors for laminin, a major component of the BM, has a central role in mediating cellular events related to adhesion and dissemination of cancer cells and has been the subject of intensive research in solid breast cancer. In the largest study published to date, 67-kDa LR protein expression independently predicted poor prognosis in 1,160 breast carcinomas (54). Four additional studies of have found correlation with relapse-free survival but not overall survival (55), no correlation (56, 57), and correlation with better outcome (58). Higher expression of the $\alpha_6$ integrin subunit correlated with enhanced metastatic ability in vitro (59) and with poor outcome in two clinical studies of breast carcinoma (60, 61). We found comparable expression of 67-kDa LR and $\alpha_6$ integrin subunit mRNA at all anatomic sites, with higher 67-kDa LR protein expression in solid tumors. We reported recently (34) that loss of mRNA expression of the $\alpha_6$ integrin subunit predicts poor survival in ovarian carcinoma. In the present study, mRNA expression of both LRs did not predict TTP. However, in agreement with the study of Martignone et al. (54), protein expression of 67-kDa LR in both primary tumors and effusions predicted shorter TTP, suggesting that in this cohort, the retained ability to bind laminin facilitates disease progression. Despite the small number of effusions analyzed for $\alpha_6$ integrin subunit protein expression, its presence in cancer cells in 6 of 6 specimens may support this hypothesis and is in agreement with the reports linking $\alpha_6$ integrin subunit expression with aggressive disease (60, 61).

The role of PEA3 or its human homologue, E1AF, has previously been studied in breast cancer, but data are inconclusive. PEA3 has been shown to be a downstream target of Her2/neu (62), and PEA3 mRNA expression showed an association with HER2/neu expression (63) and poor survival (64) in clinical specimens of breast carcinoma. Conversely, PEA3 expression suppressed HER2/neu expression in human breast and ovarian carcinoma (41) and mediated apoptosis in the SKBr-3 breast carcinoma cell line (65), whereas E1AF expression showed no correlation to HER2/neu or overall survival in a recent study (66). The latter group found Ets-1 to be an independent predictor of relapse-free survival using real-time RT-PCR (67). We found mRNA copies of Ets-1 and PEA3 in a roughly similar number of cases, but only PEA3 was up-regulated in effusions compared with primary carcinomas. Ets-1 and PEA3 mRNA expression did not correlate with c-ErbB-2 expression (data not shown) or TTP. These results suggest, as observed for MMP-2, that PEA3 is involved in disease progression to effusion but does not aid in segregating patients into subgroups within this patient group. The concomitant increase in PEA3 and c-ErbB-2 expression in effusions is of interest, but in the absence of correlation between these findings, there is little data to support the existence of a regulatory pathway between the two molecules in our material.
Coexpression of Ets transcription factors and MMP was reported previously (68) in the stroma surrounding breast carcinomas and was recently documented (69) in tumor material using real-time RT-PCR. In the present study, we found a significant correlation between mRNA expression of MMP, TIMP-2, EMMPRIN, LRs, and Ets transcription factors using both ISH and RT-PCR. These data follow our recent observations in clinical ovarian carcinoma (46) and suggest the existence of regulatory pathways that follow in vitro models in clinical breast cancer. In addition, expression of the AP-2α and AP-2γ isoforms was seen in the majority of specimens and correlated with expression of LRs. AP-2γ has recently been shown to be regulated by estrogens (70) and to regulate ErbB-3 (71), whereas the AP-2α isoform negatively regulates mammary gland growth and differentiation (72), making clinical studies of the expression of these factors relevant.

The above-detailed altered expression of cancer-related and metastasis-associated molecules may well reflect true tumor progression, a hypothesis that is the more plausible one, at least in the case of hormone receptors and c-erbB-2. As for the remaining molecules analyzed in this study, the alternative hypothesis, i.e., that of changes in growth conditions (fluid versus tissue) and/or the microenvironment (stromal cells and vessels versus mesothelial cells), needs to be considered. Growth in fluid affects cross-talk between cell populations and may result in different architecture and spatial growth pattern of tumor cell clusters, thereby affecting adhesion and signaling pathways. In support of the latter hypothesis, we have previously shown that carcinoma cells in effusions differ from those in both primary tumors and solid metastases and that growth factor and growth factor receptor expression are among the frequently altered molecules in this setting (48, 73). Elucidation of this issue requires further research.

In conclusion, we present a new cohort consisting of breast carcinoma patients with tumor spread to the pleural space. Breast carcinoma cells in pleural effusion show up-regulation of several cancer- and metastasis-associated molecules and down-regulate hormone receptor expression. Coexpression of these molecules suggests the presence of biological pathways regulating prometastatic signaling. MMP-1 and the 67-kDa LR are novel predictors of rapid progression in this cohort. These data may aid in the choice of therapeutic targets for this patient population.

ACKNOWLEDGMENTS

We gratefully acknowledge the competent technical help of Inger-Liv Nordli, Mai Nguyen, Erika Thorbjørnsen, and Ann Larsen (IHC) at the Department of Pathology, The Norwegian Radium Hospital.

REFERENCES


Altered Expression of Metastasis-Associated and Regulatory Molecules in Effusions from Breast Cancer Patients: A Novel Model for Tumor Progression

Ben Davidson, Sophya Konstantinovsky, Søren Nielsen, et al.