Changes in Cytoskeletal Protein Composition Indicative of an Epithelial-Mesenchymal Transition in Human Micrometastatic and Primary Breast Carcinoma Cells

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Abstract

Purpose: The bone marrow is a frequent and clinically important homing site for early disseminated breast cancer cells. Here, we aimed to profile the protein expression of these cells using unique cell line models and to evaluate the prognostic relevance of candidate gene expression for breast cancer patients.

Experimental Design: To identify expression patterns characteristic for micrometastatic cells, three different cell lines (BC-K1, BC-P1, and BC-S1) established by SV40 immortalization of cancer cells isolated from the bone marrow of patients with breast cancer were compared with MCF-7 breast cancer and SV40 immortalized normal breast ductal cells (MTSV-1.7) using two-dimensional gel electrophoresis followed by MALDI-ToF analysis. The prognostic significance and clinicopathologic associations of selected differentially expressed proteins were evaluated using high-density breast cancer tissue microarrays.

Results: In contrast to MCF-7 and MTSV1-7 reference cell lines, all micrometastatic cancer cell lines displayed loss of epithelial cytokeratins (CK8, CK18, and CK19) and ectopic expression of vimentin commonly present in mesenchymal cells. Immunohistochemical analysis of 2,517 samples of breast cancer further showed that loss of cytokeratin and ectopic vimentin expression were significantly associated with a higher tumor grade, high mitotic index, and negative estrogen/progesterone-receptor status. Although in univariate analyses significantly related to clinical outcome, none of the cytokeratins analyzed were independently associated with either overall or cancer-specific survival.

Conclusions: Micrometastatic cancer cells exhibit marked changes in the expression pattern of cytoskeletal proteins indicative of an epithelial-mesenchymal transition. This phenotypical change could already be detected in primary tumors and is associated with the aggressive behavior of breast cancer cells *in vivo*.

Breast cancer, the most common malignancy in females, kills ~300,000 women worldwide every year and is the main reason for cancer-related death in the postoperative development of distant metastasis in secondary organs (1). Many of the patients who are primarily diagnosed as having apparently localized or regional breast cancer eventually develop distant metastases. Clinical experience showed that in early clinical stages, cells could already dissociate from the tumor's parenchyma via the hematogenous route and colonize the bone marrow, the clinically most relevant site of metastatic disease in patients with solid epithelial tumors (2). Often, many years after resection of the primary tumor and treatment of cancer patients, micrometastatic cancer cells can grow out to form overt metastases. Indeed, evidence is accumulating that the presence of occult carcinoma cells of epithelial origin in the bone marrow is an independent risk factor in breast cancer (3–5). Understanding the biology of these dormant tumor cells in the bone marrow is therefore pivotal for the development of novel therapeutic approaches for the treatment of breast cancer patients.

The identification, enrichment, and molecular characterization of micrometastatic cancer cells in the bone marrow are hampered by the fact that these disseminated tumor cells have an extremely low frequency of 10^{-6} nucleated bone marrow cells. Recently, however, micrometastatic tumor cell lines generated from the bone marrow of patients with breast cancer were established by immortalization with the SV40 large T antigen (6). These cells show the phenotypic and genotypic features of epithelial tumor cells as well as of metastases and therefore might represent valuable model systems of micrometastasis (7, 8).

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Received 3/21/05; revised 7/27/05; accepted 8/16/05.

Grant support: Deutsche Forschungsgemeinschaft, Bonn, Germany (K. Pantel).

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doi:10.1158/1078-0432.CCR-05-0632

In this study, we were aiming to identify molecular determinants characteristic for micrometastatic cancer cells using a proteomics-based approach. Comparison of the proteome of three micrometastatic cancer cell lines generated by SV40 immortalization of cancer cells isolated from the bone marrow of breast cancer patients with MCF-7 and MTSV1.7 breast carcinoma cell lines revealed a significant down-regulation of cytokeratins (CK8, CK18, and CK19) and a concurrent up-regulation of vimentin expression as compared with controls, suggesting that micrometastatic cancer cells may have undergone an epithelial-mesenchymal transition. An immunohistochemical analysis of 2,517 tumor specimens using high-density breast cancer prognosis tissue microarrays (TMA) further showed that in univariate analyses, down-regulation of CK8, CK18, and CK19 is significantly linked with reduced overall or cancer-related survival in subgroups of patients with breast cancer. Taken together, these findings provide further support that the epithelial-mesenchymal transition is relevant in breast cancer progression.

Materials and Methods

Cell culture. Micrometastatic cancer cell lines BC-K1, BC-P1, and BC-S1 established by SV40 large T antigen immortalization of micrometastatic cells isolated from bone marrow of patients with breast cancer (6, 7), and the human mammary carcinoma cell lines MTSV-1.7 and MCF-7 were cultured as described elsewhere (9).

Two-dimensional gel electrophoresis. Monolayers were washed twice with PBS and cells were harvested using a cell scraper followed by centrifugation at 600 × *g* at 4°C. Cells were lysed in extraction buffer consisting of 8 mol/L urea, 2% (w/v) CHAPS, 20 mmol/L DTT, 250 units of endonuclease (all from Sigma, Steinheim, Germany) by incubation for 30 minutes at room temperature. After lysis, cell extracts were cleared by centrifugation for 10 minutes at 9,300 × *g* and the protein concentration was determined using a Bradford assay (Bio-Rad, Munich, Germany).

Whole cell homogenates were separated by isoelectric focusing using in-gel rehydration buffer consisting of 7 mol/L urea, 2 mol/L thiorurea, 2% CHAPS, 0.5% IPG Buffer, and 10 mmol/L DTT (pH 4-7) immobilized IPG-Strip (Amersham Biosciences, Freiburg, Germany) essentially as described by Görg et al. (10, 11), followed by 12% SDS-PAGE using a Bio-Rad Protean II XL chamber. To visualize protein spots and to identify differentially expressed polypeptides by visual inspection, gels obtained from triplicate experiments were silver-stained as described before (12) and scanned using an Epson expression 1680 pro scanner.

In-gel digestion of proteins and mass spectrometry. To analyze spots by mass spectrometry, gels were stained with G-colloidal Coomassie brilliant blue (Sigma) according to the manufacturer's instructions. For the identification of proteins, in-gel digestion and mass spectrometry was done as described in Rohaly et al. (13).

Western blot analysis. Fifty-microgram aliquots of cell lysates were separated on denaturing 12% polyacrylamide gels, electroblotted onto nitrocellulose membranes (Amersham Biosciences), and blocked with 4% skim milk powder in PBS. Subsequently, the membranes were incubated at room temperature with monoclonal antibodies specific for CK8, CK18, and CK19 (all from Progen, Heidelberg, Germany; 1:500 dilution), anti pan-cytokeratin (A45-B/B3; Micromet, Munich, Germany 1:500), vimentin (Sigma; clone V9, 1:1,000 dilution), or HSC 70 (Santa Cruz Biotechnology, Heidelberg, Germany, 1:2,000 dilution) followed by horseradish peroxidase–conjugated rabbit anti-mouse IgG (DAKO Diagnostika GmbH, Hamburg, Germany) for 2 hours each. After each individual antibody incubation, the membranes were washed with PBS containing 0.05% Tween 20 (Sigma). Signals were developed using the enhanced chemiluminescence system (Amersham Biosciences).

Immunohistochemical analysis using breast cancer tissue microarrays. To analyze the prognostic relevance of CK8, CK19, and vimentin expression, a breast cancer prognosis TMA containing a total of 2,219 formalin-fixed, paraffin-embedded primary breast tumor specimens with available clinical follow-up (median follow-up period was 51 months ranging from 1 to 150 months) and histopathologic data was used (14). This study was approved by the University of Basel Ethics Committee. Institutional Review Board approval was obtained before samples were collected.

Automatic immunostaining was done in the DAKO autostainer using mouse monoclonal antibodies specific for human CK19 (DAKO; clone BA17 at 4 μg/mL), CK8 (DAKO; clone 35βH11; at 22 μg/mL), and CK18 (DAKO; clone DC10 at 1:100 dilution) and the DAKO ChemMate detection kit. The staining procedure was done as described in Woelfle et al. (15).

For vimentin immunohistochemistry, microwave pretreatment was followed by inactivation of endogenous peroxidase activity in 0.3% H_2O_2/methanol for 30 minutes and blocking of nonspecific binding sites by incubation with 10% horse serum in PBS for 30 minutes. After incubation with the primary antibody (anti-vimentin; Boehringer Mannheim, Mannheim, Germany, 1:160) for 16 hours at 4°C, with biotinylated secondary horse anti-mouse antibody, and with avidin/biotin/peroxidase complex reagent (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes each, vimentin expression was visualized using 3,3′-diaminobenzidine. Finally, slides were counterstained with hematoxylin. The number of immunostained cells in each tumor sample of the TMA was estimated by light microscopy.

As we concentrated on the most predominant protein expression changes (i.e., absence or presence) in our proteome analysis of micrometastatic cancer cells, CK8, CK18, and CK19 staining results were also grouped into absence (i.e., <10% stained tumor cells, "cytokeratin-negative") or presence (i.e., ≥10% stained tumor cells, "cytokeratin-positive") of expression. Similarly, vimentin expression was judged positive when >10% of tumor cells stained positive. Besides standardization of the immunostaining procedure (all samples were analyzed under absolutely identical conditions for 1 day with one set of reagents) the small sample size minimizes interobserver variability. As shown previously, intraspecimen variation was also small in the TMA studies (16, 17). In addition, the staining results were related to clinical outcome, which also argues against "chance findings" or "technical errors."

Statistical analysis. The χ^2 test was used to evaluate the relationship between CK8, CK18, and CK19 or vimentin expression and other known risk factors or histologic variables. Kaplan-Meier life-table curves were used to visualize for tumor-specific or overall survival. The log-rank test was used to compare significance of differences between the curves. $P < 0.05$ was considered statistically significant. All tests were two-tailed. The joint effects with already recognized prognostically relevant variables were examined via Cox proportional hazard analysis. pT status, pN status, tumor histology, and tumor grading were entered stepwise forward into the model to test these covariables for possible prognostic joint effects with cytokeratin and vimentin expression. For statistical analyses, we used SPSS software for PC (version 11 for Windows).

To analyze whether cytokeratin and/or vimentin expression are involved in characteristic pattern associated with overall or cancer-related survival, a multivariate classification and regression tree analysis that included classical breast cancer prognosis factors was done (DTREG, V 4.0).

Results

Proteomics-based analysis of micrometastatic cell lines. Using two-dimensional gel electrophoresis in conjunction with MALDI-ToF mass spectrometry, we compared three distinct micrometastatic breast cancer cell lines (BC-K1, BC-P1, BC-S1) established by SV40 large T antigen immortalization of micrometastatic cells isolated from bone marrow of patients with

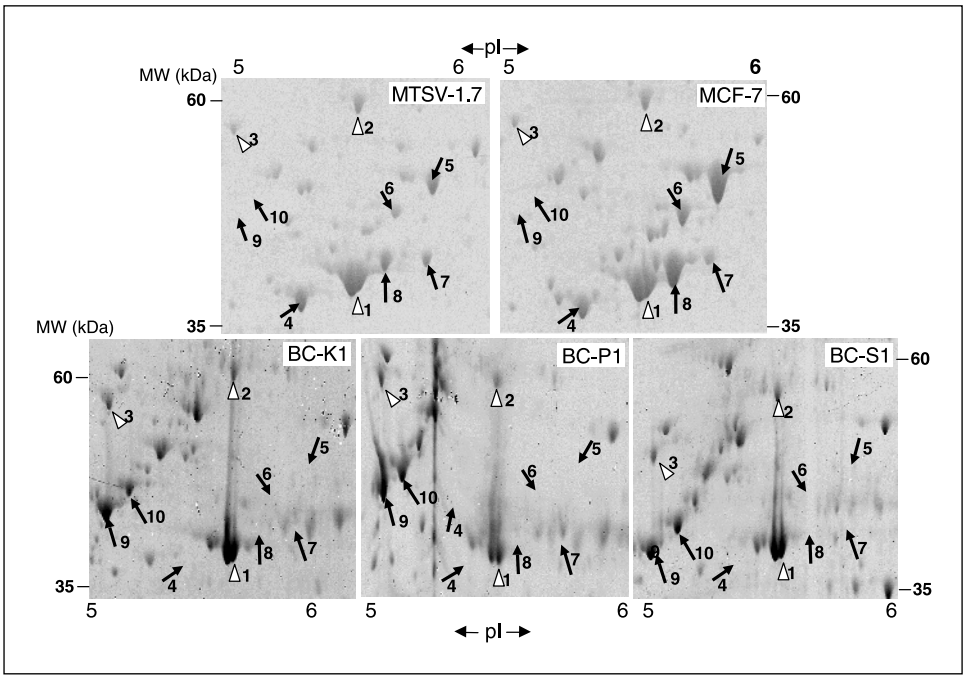

Fig. 1. Representative two-dimensional PAGE protein patterns of micrometastatic cancer cell lines BC-K1, BC-P1, BC-S1 and reference cell lines MTSV-1.7 and MCF-7. Five hundred – microgram aliquots of cell extracts of the cell lines indicated were subjected to two-dimensional gel electrophoresis as described in Materials and Methods. Individual protein spots were visualized by G-colloidal Coomassie brilliant blue staining of gels. Commonly (△) or differentially (➡) expressed polypeptides selected for MALDI-ToF analysis.

breast cancer (7) with (*a*) SV40 immortalized MTSV-1.7 cells representing nonmalignant mammary epithelial cells (18) and (*b*) MCF-7 breast cancer cells.

Following two-dimensional gel electrophoresis of cell extracts prepared from micrometastatic and reference cell lines, protein spots were visualized by colloidal Coomassie-blue staining. A close-up of representative two-dimensional PAGE protein patterns of all five cell lines examined in this study is shown in Fig. 1. Differentially expressed polypeptides identified by visual inspection were excised from gels and subsequently analyzed by MALDI-ToF mass spectrometry as detailed in Materials and Methods. As we were mainly interested in the identification of differentially expressed polypeptides which potentially could serve as molecular markers for micrometastatic

Table 1. Proteins identified by two-dimensional electrophoresis and MALDI-ToF analysis

Protein	Spot*	Accession no.†	Protein variables				Control cells		Micrometastatic cells		
			Calculated†		Observed‡		MCF-7	MTSV-1.7	BC-K1	BC-P1	BC-S1
			Molecular weight (kDa)	Isoelectric point	Molecular weight (kDa)	Isoelectric point					
β-Actin	1	Q96334	40.9	5.7	50.0	5.5	+§	+	+	+	+
HSP60	2	P10809	61.0	5.5	60.0	5.5	+	+	+	+	+
β-Tubulin	3	P07437	48.8	5.5	50.0	5.2	+	+	+	+	+
CK19	4	P08727	44.0	5.0	40.0	5.0	+	+	–§	–	–
CK8	5	P05787	53.6	5.5	55.0	5.6	+	+	–	–	–
CK8 (279 amino acids)	6	P05787	30.8	5.0	50.0	5.5	+	+	–	–	–
CK18	7	P05783	47.3	5.2	45.0	5.6	+	+	–	–	–
CK18 (424 amino acids)	8	P05783	47.3	5.3	45.0	5.6	+	+	–	–	–
Vimentin	9	A25074	53.6	5.0	50.0	4.9	–	–	+	+	+
Vimentin variant	10	P08670	53.5	5.0	50.0	5.0	–	–	+	+	+

*Spots correspond to those marked in Fig. 1.
†Accession numbers and calculated molecular weights derived from SwissProt/TrEMBL database.
‡Calculation based on migration of standard and known proteins.
§Presence (+) or absence (–) of corresponding proteins in cell lines listed.

disease, we concentrated on the most predominant protein expression changes (absence or presence). A total of 10 protein spots were analyzed by MALDI-ToF mass spectrometry, 3 of which were common to all cell lines, and 7 exhibited a differential expression pattern among the cell lines investigated in this study (see Fig. 1). The data obtained are listed in Table 1.

Commonly expressed polypeptides identified as β-actin (spot #1), HSP 60 (spot #2,) and β-tubulin (spot #3) served as reference spots to facilitate identification of differentially expressed proteins. Two proteins (spots #9 and #10) exclusively expressed in all three micrometastatic cell lines (BC-K1, BC-P1, and BC-S1), but not in MTSV-1.7 and MCF-7 reference cell lines, represented the cytoskeletal protein vimentin. In contrast, expression of proteins corresponding to spots #4, #5, #6, #7, and #8 only were found in MTSV-1.7 and MCF-7 cells, but not in any of the three micrometastatic cell lines. Analysis of these spots by MALDI-ToF mass spectrometry revealed identity with CK19 (spot #4), CK8 (spots #5 and #6), and CK18 (spots #7 and #8), respectively. The characteristics of identified proteins are summarized in Table 1.

As the identification of proteins by mass spectrometry (MALDI-ToF) is based on the mass to charge ratio of individual peptide fragments which also could be derived from closely related members of cytokeratin family of proteins, we additionally confirmed our results by 2D Western blot analysis using a panel of monoclonal antibodies specific for the suspected proteins (data not shown).

To further show differential expression of vimentin and CK8, CK18, and CK19 in micrometastatic cell lines (BC-K1, BC-P1, and BC-S1) as compared with MTSV-1.7 and MCF-7 reference cell lines, one-dimensional immunoblots were done. The results shown in Fig. 2 confirmed the expression of vimentin and the absence of CK8, CK18, and CK19 expression in micrometastatic cancer cell lines, indicating that these cell lines indeed have undergone dramatic changes in their cytoskeletal scaffolds.

Taken that these cells initially were identified using the pan-cytokeratin monoclonal antibody A45-B/B3 (6), loss of cytokeratin expression in micrometastatic cancer cell lines was surprising. To investigate this discrepancy in greater detail, an additional immunoblot experiment was done using cell extracts from all five cell lines used in this study and the pan-cytokeratin monoclonal antibody A45-B/B3. As shown in Fig. 2, in MCF-7 and MTSV-1.7 cells, a wide range of bands were detectable using the A45-B/B3 antibody. In striking contrast, only a faint band of similar size was detectable in micrometastatic cancer cells (BC-K1, BC-P1, and BC-S1). Accordingly, these cells also stained positive in immunofluorescence analysis using the A45-B/B3 antibody (data not shown). Further experiments using antibodies specific for CK5/6, CK7, and CK14 showed no reactivity with proteins extracted from micrometastatic cell lines by immunoblot analysis, suggesting that the reactivity of the A45-B/B3 antibody with micrometastatic cancer cell lines was due to the expression of a yet unidentified member of the cytokeratin family of proteins.

Immunohistochemical analysis of breast cancer tissue microarrays for expression of cytokeratins and vimentin. The proteomic approach revealed differences in the cytoskeletal architecture of micrometastatic cancer cells, particularly in CK8, CK18, and CK19 as well as vimentin expression when compared with reference cells. To examine whether changes in CK8, CK18, CK19 and vimentin expression correlate with clinicopathologic variables *in vivo*, an immunohistochemical approach using a high-density TMA yielding 2,517 interpretable tumor sample stainings was employed.

Because we focused on loss of expression of cytoskeleton proteins in breast tumors (<10% stained tumor cells), we were not concerned with the certain variation in the staining intensities of normal tissues. Moreover, we were interested in the role of these molecules in tumor progression/metastasis (and not tumor initiation), and therefore the comparison of tumor tissue with normal tissue from the same patient was not within the scope of our present investigation. Mammary carcinoma tissue specimens exhibited high variability of cytokeratin expression (Table 2). Complete loss of CK8, CK18, and CK19 expression and ectopic vimentin expression was observed in 21.1%, 14.1%, 13.4% and 13.9%, respectively, of all tumors analyzed with no significant differences between younger and older patients (Table 2). Furthermore, a small percentage of tumor samples (5.0%) was found to be completely negative for all three cytokeratins (data not shown). Our data also show a significant correlation between ectopic vimentin expression and loss of one or more cytokeratins in tumor cells ($P < 0.001$).

We also analyzed the putative association between intermediate filament protein expression and histologic tumor type and found that CK8, CK18, and CK19 expression was completely lost in 19.8%, 13.8%, and 13.8% of ductal as well as 14.9%, 8.5%, and 10.3% of lobular carcinomas, respectively. On the other hand, in 13.2% of ductal and 8.4% of lobular carcinomas, an ectopic expression of vimentin in tumor cells was observed. A group of special-type tumors ($n = 355$; Table 2) exhibited a higher incidence of CK8-negative (32.4%) and CK18-negative (19.3%) or vimentin-expressing (22.0%) tumors than ductal or lobular carcinomas.

As shown in Table 2, loss of CK8, CK18, or CK19 expression was significantly correlated with risk factors of unfavorable

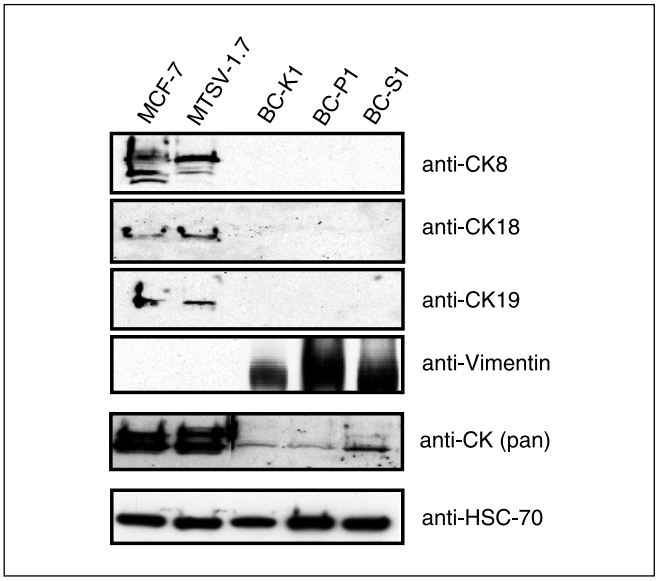

Fig. 2. Differential expression of cytokeratins and vimentin in micrometastatic cancer cell lines BC-K1, BC-P1, BC-S1 and reference cell lines MTSV-1.7 and MCF-7. Fifty-microgram aliquots of whole cell extracts were electrophoresed through 12% SDS polyacrylamide gels, and following transfer onto nitrocellulose membranes, were subjected to Western blot analysis using monoclonal antibodies specific for CK8, CK18, CK19, and vimentin. Monoclonal antibody A45-B/B3 recognizes an epitope common to various cytokeratins. Detection of HSC-70 was used to show equal loading.

Table 2. CK8, CK18, and CK19 and vimentin expression in primary breast tumors in relation to various clinicopathologic factors

	Number of cases*	CK8		CK18		CK19		Vimentin	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Total		78.9%	21.1%	85.9%	14.1%	86.6%	13.4%	13.9%	86.1%
Age (y)									
≤50	414	75.0%	25.0%	85.4%	14.6%	88.8%	11.2%	17.5%	82.5%
≥51	1,468	77.9%	22.1%	85.4%	14.6%	85.5%	14.9%	13.8%	86.2%
P		0.205		0.900		0.120		0.080	
Histologic type									
Ductal carcinoma	1,552	80.2%	19.8%	86.2%	13.8%	86.2%	13.8%	13.2%	86.8%
Lobular carcinoma	312	85.1%	32.4%	91.5%	8.5%	89.7%	10.3%	8.4%	91.6%
Other	355	67.6%	32.4%	80.7%	19.3%	85.3%	14.7%	22.0%	88.0%
P		≤0.001		≤0.001		0.190		≤0.001	
Tumor size									
pT_1	823	82.3%	17.7%	90.7%	9.3%	87.8%	12.2%	13.5%	86.5%
pT_2	1,023	75.8%	24.2%	82.8%	17.2%	85.2%	14.8%	15.6%	84.4%
pT_3	124	73.3%	26.7%	80.4%	19.6%	86.1%	13.9%	14.2%	85.8%
pT_4	242	83.3%	16.7%	87.2%	12.8%	88.3%	11.7%	8.1%	91.9%
P		0.540		0.048		0.990		0.110	
Lymph node status									
pN_0	952	76.5%	23.5%	84.7%	15.3%	85.3%	14.7%	16.0%	84.0%
$pN_{1/2}$	915	79.3%	20.7%	87.2%	12.8%	87.2%	12.8%	12.9%	87.1%
P		0.170		0.190		0.330		0.070	
Histologic grade									
G_1	546	86.2%	13.8%	94.5%	5.5%	89.7%	10.3%	8.4%	91.6%
G_2	844	84.7%	15.3%	91.0%	9.0%	88.7%	11.3%	9.5%	90.5%
G_3	656	66.8%	33.2%	73.8%	26.2%	82.4%	17.6%	24.3%	75.7%
P		≤0.001		≤0.001		≤0.001		≤0.001	
Mitotic index†									
M_1	1,111	85.2%	14.8%	93.6%	6.4%	90.0%	10.0%	8.8%	91.2%
M_2	356	81.4%	18.6%	86.9%	13.1%	86.9%	13.1%	11.2%	88.8%
M_3	574	66.5%	33.5%	72.4%	27.6%	81.5%	18.5%	25.8%	74.2%
P		≤0.001		≤0.001		≤0.001		≤0.001	
Estrogen receptor									
Positive	1,522	87.6%	12.4%	91.4%	8.6%	88.3%	11.7%	7.0%	93.0%
Negative	420	50.2%	49.8%	67.5%	32.5%	80.6%	19.4%	35.3%	64.7%
P		≤0.001		≤0.001		≤0.001		≤0.001	
Progesterone receptor									
Positive	650	92.3%	7.7%	94.5%	5.5%	91.1%	8.9%	8.0%	92.0%
Negative	1,259	73.0%	27.0%	82.0%	18.0%	84.6%	15.4%	17.4%	82.6%
P		≤0.001		≤0.001		≤0.001		≤0.001	

*Differences in the number of cases were due to the absence of tissue or the presence of necrotic/damaged tissues in the samples and availability of clinicopathologic information.

† M_1, <10 mitoses per high-power field; M_2, 10 to 20 mitoses per high-power field and M_3, >20 mitoses per high-power field.

prognosis, such as high tumor grade ($P < 0.001$, respectively) and high mitotic index ($P < 0.001$, respectively). In addition, down-regulation of CK18 also was associated with increasing tumor size (pT stage; $P = 0.048$). In contrast, no significant correlation was observed between loss of CK8, CK18, or CK19 expression and lymph node status (Table 2). Likewise, ectopic vimentin expression in tumor cells correlated significantly with high tumor grade ($P < 0.001$) and high mitotic index ($P < 0.001$) but not with lymph node status ($P = 0.07$; Table 2). Moreover, both loss of CK8, CK18, or CK19 expression and ectopic vimentin expression were significantly related to the absence of estrogen/progesterone receptor expression in breast cancer samples ($P < 0.001$, respectively).

Prognostic significance of intermediate filament protein expression. The prognostic significance of intermediate filament proteins CK8, CK18, and CK19 as well as vimentin for patients where clinical follow-up data and interpretable stainings were available was evaluated using high-density TMAs. Kaplan-Meier analysis showed a significant association between loss of CK18 (Fig. 3A), but not CK8, CK19, or ectopic vimentin expression

and reduced overall survival (P = 0.02). Furthermore, a significant correlation between loss of CK8 expression and cancer-related survival was found (P = 0.019; data not shown).

When subsequently evaluating the putative association between cytokeratin and vimentin expression and prognosis of distinct subgroups of breast cancer patients, an association between loss of CK19 expression and overall survival for patients <50 years old (a cutoff that roughly distinguishes between pre- and postmenopausal women) was found (P < 0.001; Fig. 3B). In addition, for patients <50 years old, loss of CK19 expression was also significantly associated with cancer-related survival (P = 0.036; data not shown).

We also did separate Kaplan-Meier analyses in subgroups of patients with and without lymph node metastases as well as in subgroups representing different tumor stages. No significant correlation between intermediate filament protein expression and survival of patients with nodal-negative breast cancer (pN_0) was observed (data not shown). In node-positive patients (pN_1 and pN_2), loss of CK8 and CK18 expression was a significant prognosticator for overall survival (P = 0.004 and P = 0.034, respectively; Fig. 3C and D), whereas loss of CK8 expression was a significant prognosticator for cancer-related survival (P = 0.008; data not shown). Kaplan-Meier analyses on subgroups with different tumor stages confirmed the prognostic effect of CK8 and CK19 expression for overall (CK19, P = 0.02) or cancer-specific survival (CK8, P = 0.002) of patients with tumors staged as pT2 (data not shown).

However, multivariate analyses including the histopathologic variables tumor stage, lymph node status, and grade of differentiation showed that the expression of CK8, CK18, CK19, or vimentin were not independent prognostic variables with regard to overall or cancer-related survival in the whole patient group or in the subgroups. Because therapeutic regimens may also have an influence on clinical outcome, we included information regarding therapy (available for 812 patients) into multivariate analyses. This analysis did not reveal an independent association of therapy with clinical outcome. However, in the subgroup of patients receiving hormonal

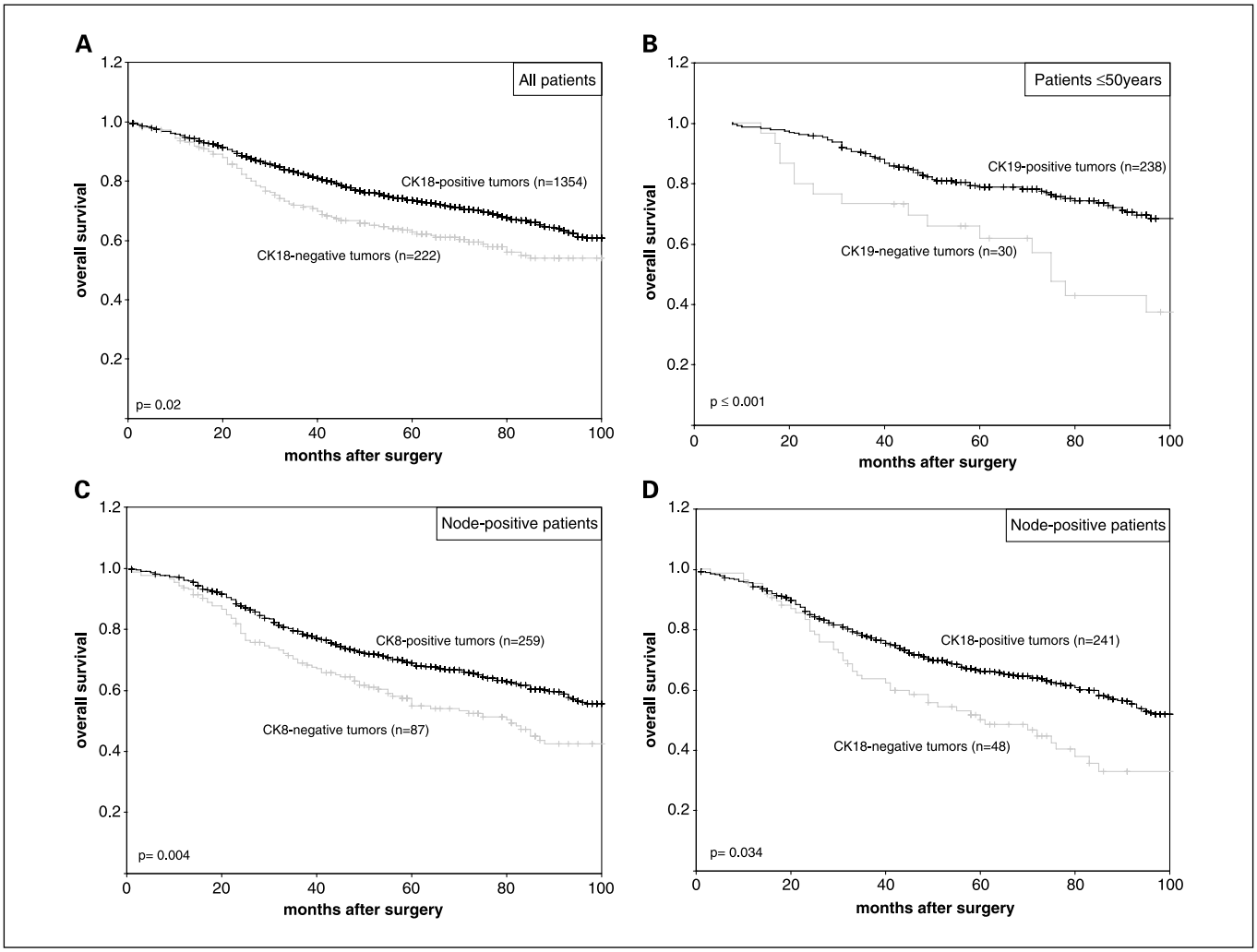

Fig. 3. Survival of patients with breast cancer in relation to CK8, CK18, and CK19 expression. Kaplan-Meier analyses were done by evaluating samples wherein CK8, CK18, and CK19 immunohistochemical stainings yielded interpretable results and clinical follow-up information on overall survival was available. When breast cancer patients of all ages were analyzed, a significant correlation between loss of CK18 expression with overall survival was observed (*A*). For patients ≤50 years old, a significant correlation between loss of CK19 expression and shortened overall survival was observed (*B*). Separate Kaplan-Meier analyses in subgroups of patients with and without lymph node metastases revealed that loss of CK8 (*C*) or CK18 (*D*) expression was a significant prognosticator for overall survival.

therapy (alone or in combination with chemotherapy), both overall and cancer-related survival were significantly decreased in CK8-negative compared with CK8-positive tumors (data not shown). Nevertheless, this relationship was not independent of lymph node status, tumor stage, and differentiation grade of the primary tumor.

To possibly identify patterns of molecular or clinical characteristics that are associated with clinical outcomes, a regression tree analysis was done. However, none of the molecular markers analyzed was identified by the regression tree analysis to be associated with the median survival time. In contrast, the classical prognostic factors (e.g., nodal status, tumor size, and grading) were clearly identified by this analysis.

Discussion

Early dissemination of tumor cells to the bone marrow frequently occurs in patients with breast cancer. Although the prognostic relevance of disseminated tumor cells in this organ is widely accepted, the molecular characteristics of micrometastatic cancer cells in bone marrow still remain to be elucidated (2). In this study, we therefore aimed to further characterize micrometastatic cancer cells by analyzing the protein expression patterns of three distinct micrometastatic breast cancer cell lines. These cells represent valuable and unique model systems of micrometastasis in that they have been reported to share several phenotypic and genotypic features of epithelial tumor cells (7, 8). By comparing protein expression patterns of these cell lines with (*a*) SV40 immortalized normal breast ductal cells (MTSV-1.7; ref. 18) and (*b*) MCF-7 breast cancer cells using two-dimensional gel electrophoresis in conjunction with MALDI-ToF analysis, a significant down-regulation of CK8, CK18, and CK19 and a concurrent up-regulation of vimentin expression was found.

In normal mammary epithelium, luminal duct cells usually express CK8, CK18, and CK19 (19). The majority of mammary carcinomas which exhibit the luminal phenotype express one or more of the cytokeratins (20) and monoclonal antibodies directed against CK8, CK18, and CK19 have therefore been used to identify primary and metastatic breast cancer cells (19, 21). Interestingly, a shift from cytokeratin intermediate filaments to vimentin, which was found in all three micrometastatic cancer cell lines analyzed in this study, suggests that these cells have undergone an epithelial-mesenchymal transition (22). Although epithelial-mesenchymal transition is not only defined by changes in the organization of the cytoskeleton but also by other criteria, including loss of epithelial cell polarity, disassembly of tight junctions, adherens junctions and desmosomes, as well as increased cell motility (23), results obtained for micrometastatic cell lines indicate that these cells have acquired a rather mesenchymal phenotype, possibly reflecting the increased motility and invasiveness of disseminating tumor cells. Accordingly, we previously showed that a reduced expression of CK8, CK18, and CK19 in primary breast carcinomas was associated with the presence of micrometastatic tumor cells in bone marrow (24).

For decades, intermediate filament proteins have been used as molecular markers in diagnostic histopathology (19, 25, 26). An increasing body of evidence suggests, however, that aberrant expression of individual cytokeratins or vimentin results in abnormal cell behavior. For example, CK8 knockout mice developed colorectal hyperplasia (27) and exhibited disturbances in cell-cycle regulation mechanisms driving cells into the G_2 phase (28). Immunohistochemical analysis of breast cancer tissues showed that reduced CK18 expression was correlated with poor clinical outcome (15). Also, down-regulation of CK19 expression and overexpression of vimentin were found in highly aggressive breast cancer cell lines exhibiting strong migratory and invasive abilities (29). A direct functional contribution of vimentin to epithelial cell migration and invasion was shown by antisense-mediated down-regulation of vimentin expression in highly invasive MDA-MB-231 breast cancer cells (30), further substantiating the functional relevance of intermediate filament proteins in breast cancer progression.

To investigate whether CK8, CK18, CK19 or ectopic vimentin expression in primary mammary carcinomas is correlated with clinicopathologic variables, a comprehensive immunohistochemical study was conducted. We have applied commercially available monoclonal antibodies with published specificities and used the well-established TMA technology (13). Based on 2,517 interpretable tumor samples using a high-density breast cancer TMA, we found loss of CK8, CK18, or CK19 expression and ectopic vimentin expression in a significant number of breast cancer specimens, which correlated with risk factors indicating an unfavorable prognosis, such as high tumor grade, high mitotic index, and absence of estrogen receptor/progesterone receptor expression. Among several other characteristics such as a high proliferative potential and expression of p53 and epithelial growth factor receptor, loss of expression of luminal cytokeratins in conjunction with lack of estrogen receptor/progesterone receptor expression, similar to that found in this study, is indicative of the more aggressive basal-like phenotype of breast carcinomas (31, 32).

In this study, we further showed that loss of cytokeratin expression was associated with the clinical outcome of patients with breast cancer. In particular, suppression of CK18 expression was significantly associated with overall survival of breast cancer patients ($P = 0.02$), whereas loss of CK8 expression was related to cancer-specific survival ($P = 0.019$). Although CK19 expression was not of prognostic relevance when breast cancer patients of all ages were analyzed, however, a subgroup analysis of patients <50 years old showed a relation between loss of CK19 expression and both overall and cancer-specific survival ($P < 0.001$ and $P = 0.036$, respectively). In general, tumors from breast cancer patients <50 years old tend to be more aggressive (33, 34) and down-regulation of CK19 accompanies a subset of carcinomas with a particularly poor clinical outcome.

Although axillary lymph node status, as determined by histologic examination, is still considered the most accepted prognostic indicator in invasive breast cancer (35, 36), negative lymph nodes do not preclude aggressive disease and subsequent distant disease. Accordingly, nodal-positive patients might still exhibit a variable clinical outcome. The identification of additional molecular markers predictive for the behavior of individual tumors is a challenge in breast cancer research. Therefore, we also did separate Kaplan-Meier analyses in subgroups of patients with and without lymph

node metastases. In univariate analyses, loss of CK8 and CK18 expression was significantly linked with clinical outcome in node-positive patients, suggesting that suppression of cytokeratin expression might be a feature of more advanced stages of primary breast cancer. The strong relationship between loss of cytokeratin expression and clinical outcome in this group of patients indicates that epithelial-mesenchymal transition seemed to be more relevant for breast tumor cells in $pN_{1/2}$ patients. Multivariate analyses including well-known prognostic variables for breast cancer, however, showed that cytokeratin expression was not independently associated with overall and cancer-related survival. Furthermore, we cannot exclude the influence of adjuvant therapy on the observed associations with clinical outcome but this influence did not seem to be very strong.

The finding that the expression of some cytokeratins was associated with clinical outcome in univariate analysis, but not in traditional multivariate analysis, suggests that interactions between cytokeratins and clinical outcome and/or clinicopathologic variables exist. To identify patterns of characteristics that include the analyzed cytokeratins/vimentin and that are independently associated with either overall or cancer-related survival, a classification and regression tree analysis was done. However, this analysis revealed that neither CK8, CK18, CK19 nor vimentin expression are part of the characteristic patterns independently associated with clinical outcome. Conflicting results regarding the prognostic importance of vimentin expression for patients with breast cancer have been reported in the literature. Although several studies indicate that the presence of vimentin is correlated with poor prognosis for patients with breast cancer (37–39), our results are more in line with data recently published by Heatley and coworkers (40) who could not find an association between vimentin expression and the poor survival of patients with ductal carcinomas of the breast. Such discrepant results may be due to the low number of patients included in many other studies as well as to inherent problems of immunohistochemistry such as different antibodies, staining protocols, or variations of tissue processing variables in different studies.

In summary, our study contributed to a better understanding of the molecular characteristics of micrometastatic breast cancer cells through the identification of major changes in the cytoskeletal architecture of these cells. Loss of CK8, CK18, and CK19 expression and a concurrent up-regulation of vimentin suggests that disseminated tumor cells have acquired a mesenchymal-like, aggressive phenotype. Evidence is provided that loss of cytokeratin expression already occurs in the primary tumor and is associated with factors predictive for an unfavorable prognosis for patients with breast cancer. Looking at cytokeratin expression in lymph node micrometastases or even single disseminated cancer cells in distant organs (e.g., bone marrow as one of the prominent sites of hematogenous metastasis in breast cancer) will be an interesting future project. A better understanding of the mechanisms underlying this profound phenotypical change may open new avenues for therapeutic interventions aimed to prevent cancer metastases.

Acknowledgments

We thank Sonja Santjer for excellent technical assistance.

References

1. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999;83:18–29.
2. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. Nat Rev Cancer 2004;4:448–56.
3. Braun S, Pantel K, Muller P, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med 2000;342:525–33.
4. Janni W, Hepp F, Rjosk D, et al. The fate and prognostic value of occult metastatic cells in the bone marrow of patients with breast carcinoma between primary treatment and recurrence. Cancer 2001;92:46–53.
5. Heiss MM, Allgayer H, Gruetzner KU, et al. Individual development and uPA-receptor expression of disseminated tumour cells in bone marrow: a reference to early systemic disease in solid cancer. Nat Med 1995;1:1035–9.
6. Pantel K, Dickmanns A, Zippelius A, et al. Establishment of micrometastatic carcinoma cell lines: a novel source of tumor cell vaccines. J Natl Cancer Inst 1995;87:1162–8.
7. Putz E, Witter K, Offner S, et al. Phenotypic characteristics of cell lines derived from disseminated cancer cells in bone marrow of patients with solid epithelial tumors: establishment of working models for human micrometastases. Cancer Res 1999;59:241–8.
8. Kraus J, Pantel K, Pinkel D, Albertson DG, Speicher MR. High-resolution genomic profiling of occult micrometastatic tumor cells. Genes Chromosomes Cancer 2003;36:159–66.
9. Assmann V, Marshall JF, Fieber C, Hofmann M, Hart IR. The human hyaluronan receptor RHAMM is expressed as an intracellular protein in breast cancer. J Cell Sci 1998;111:1685–94.
10. Görg A, Postel W, Gunther S. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 1988;9:531–46.
11. Görg A, Postel W, Baumer M, Weiss W. Two-dimensional polyacrylamide gel electrophoresis, with immobilized pH gradients in the first dimension, of barley seed proteins: discrimination of cultivars with different malting grades. Electrophoresis 1992;13:192–203.
12. Heukeshoven J, Dernick R. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 1985;6:103–12.
13. Rohaly G, Chemnitz J, Dehde S, et al. A novel human p53 isoform is an essential element of the ATR-intra-S phase checkpoint. Cell 2005;122:21–32.
14. Simon R, Sauter G. Tissue microarrays for miniaturized high-throughput molecular profiling of tumors. Exp Hematol 2002;30:1365–672.
15. Woelfle U, Sauter G, Santjer S, Braakenhoff RH, Pantel K. Downregulated expression of cytokeratin 18 promotes progression of human breast cancer. Clin Cancer Res 2004;10:2670–4.
16. Nocito A, Bubendorf L, Maria Tinner E, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Pathol 2001;194:349–57.
17. Torhorst J, Bucher C, Kononen J, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 2001;159:2249–56.
18. Bartek J, Bartekova J, Kyprianou N, et al. Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proc Natl Acad Sci U S A 1991;88:3520–4.
19. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11–24.
20. Abd El-Rehim DM, Pinder SE, Paish CE, et al. Expression of luminal and basal cytokeratins in human breast carcinoma. J Pathol 2004;203:661–71.
21. Malzahn K, Mitze M, Thoenes M, Moll R. Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. Virchows Arch 1998;433:119–29.
22. Vincent-Salomon A, Thiery JP. Epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res 2003;5:101–6.
23. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–54.
24. Woelfle U, Cloos J, Sauter G, et al. Molecular signature associated with bone marrow micrometastasis in human breast cancer. Cancer Res 2003;63:5679–84.
25. Pantel K, Cote RJ, Fodstad O. Detection and clinical importance of micrometastatic disease. J Natl Cancer Inst 1999;91:1113–24.
26. Coulombe PA, Omary MB. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. Curr Opin Cell Biol 2002;14:110–22.
27. Baribault H, Price J, Miyai K, Oshima RG. Mid-gestational lethality in mice lacking keratin 8. Genes Dev 1993;7:1191–202.
28. Toivola DM, Nieminen MI, Hesse M, et al. Disturbances in hepatic cell-cycle regulation in mice with assembly-deficient keratins 8/18. Hepatology 2001;34:1174–83.
29. Zajchowski DA, Bartholdi MF, Gong Y, et al. Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res 2001;61:5168–78.
30. Hendrix MJ, Seftor A, Seftor RE, Trevor KT.

Experimental coexpression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. Am J Pathol 1997;150:483–95.

31. Nielsen T, Hsu FD, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 2004;10:5367–74.

32. Korsching EP, Agelopoulos J, Eisenacher K, et al. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest 2002;82:1525–33.

33. Weber-Mangal S, Sinn HP, Popp S, et al. Breast cancer in young women (<35 years):genomic aberrations detected by comparative genomic hybridization. Int J Cancer 2003;107:583–92.

34. Chung M CHR, Bland KI, Wanebo HJ. Younger women with breast carcinoma have a poorer prognosis than older woman. Cancer 1996;77:97–103.

35. Weidner N, Cady B, Goodson WH III. Pathologic prognostic factors for patients with breast carcinoma. Which factors are important. Surg Oncol Clin N Am 1997;6:415–62.

36. Ellis P, Smith J, Ashley S, et al. Clinical prognostic and predictive factors for primary chemotherapy in operable breast cancer. J Clin Oncol 1998;16:107–14.

37. Domagala W, Lasota J, Dukowicz A, et al. Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas. Am J Pathol 1990;137:1299–304.

38. Domagala W, Striker G, Szadowska A, Dukowicz A, Harezga B, Osborn M. p53 protein and vimentin in invasive ductal NOS breast carcinoma—relationship with survival and sites of metastases. Eur J Cancer 1994;30A:1527–34.

39. Domagala W, Markiewski M, Harezga B, Dukowicz A, Osborn M. Prognostic significance of tumor cell proliferation rate as determined by the MIB-1 antibody in breast carcinoma: its relationship with vimentin and p53 protein. Clin Cancer Res 1996;2:147–54.

40. Heatley MK, Ewings P, Odling Smee W, Maxwell P, Toner PG. Vimentin expression does not assist in predicting survival in ductal carcinoma of the breast. Pathology 2002;34:230–2.

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Clin Cancer Res 2005;11:8006-8014.