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Rapid Communication

Low nm23 Protein Expression in Infiltrating Ductal Breast Carcinomas Correlates with Reduced Patient Survival

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Protein levels corresponding to nm23 were determined in normal and neoplastic breast tissues by immunoperoxidase staining. Nm23 protein levels were highest in normal breast epithelium, and lower in intraductal carcinomas. Based on nm23 staining, 39 infiltrating ductal carcinomas were separated into two groups: tumors with homogeneously high nm23 protein content, and tumors with low staining in either a homogeneous or heterogeneous pattern. Patients with low nm23 staining tumors, determined by three pathologists independently, had reduced survival times ($\alpha = 0.034$, $\alpha = 0.012$, $\alpha = 0.052$ by the log rank test). Nm23 expression approached significance as an independent predictor of survival in Cox's proportional hazards model. The data provide the first correlation of low nm23 protein expression and reduced breast carcinoma patient survival. (Am J Pathol 1991, 139:245–250)

Carcinoma of the breast remains a significant cause of morbidity and mortality among women. A major research goal is to identify the genetic events that underlie tumorigenesis and metastatic progression in breast cells. These findings, in turn, may enable the development of prognostic and therapeutic strategies.

Nm23 was identified on the basis of its reduced steady-state RNA levels in five highly metastatic K-1735 melanoma cell lines, as compared with two related, low metastatic potential K-1735 melanoma cell lines.[1] In murine K-1735 melanoma cells, transfection of murine nm23-1 cDNA resulted in a reduced incidence of primary tumor formation, and significant reductions in tumor metastatic potential.[2] In human breast carcinomas, low nm23 RNA levels were correlated with the presence of lymph node metastases at surgery,[3] and significantly reduced diseasefree and overall survival.[4] Although the RNA data indicate the potential prognostic significance of nm23 expression, the technical requirements of preparing tumor RNA and performing and quantitating Northern or *in situ* hybridizations will likely preclude its widespread clinical use. We therefore prepared affinity purified rabbit antibodies to synthetic nm23 peptides, which identified 17 and 18.5 kDa bands on Western blots containing lysates of a human breast carcinoma cell line.[5] In the present study, we report the first attempt at quantitation of nm23 protein content in normal and neoplastic breast tissues, determined by immunoperoxidase staining using anti-nm23 peptide 11 antibody, and its correlation with patient survival.

Materials and Methods

Cohort Selection

Fifty-seven cases from the files of the University of Jacksonville were assessed for nm23 staining patterns. Thirteen of these were excluded, as they were subclassifiable as histological types with survivals different from

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routine infiltrating ductal carcinomas. These included medullary, mucinous, and papillary carcinomas. Five more cases were excluded from analysis because they were lost to followup. All 57 cases were used in evaluation of staining patterns and variability; 39 cases were correlated to survival.

Immunohistochemical Staining

Five micrometer sections from formalin-fixed, paraffin embedded breast tumors were mounted on poly-lysine coated slides, air dried, and heated at 60°C for 20 minutes. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 30 minutes. Nonspecific antibody binding was blocked with PBS, 2% goat serum for 20 minutes. Tissue sections were incubated with affinity purified anti-nm23 peptide 11 antibody (5), and 1 ng/ml, in 0.1% (w/v) BSA for 60 minutes at 20°C. After rinses in 10 mM PBS, the primary antibody was detected with an avidin-biotin system (Vector laboratories, Burlingame), using 0.5 mg/ml diaminobenzidene (Sigma, St. Louis) for 6 minutes as the chromogen. Sections were lightly counterstained in Mayer's hematoxylin. There was no difference in staining intensity between peripheral and central portions of tumor, except for the "edge effect" common to immunohistochemistry.

Evaluation

Slides were examined under low power (4× objective) to identify regions containing low-staining invasive tumor cells. In cases of multiple areas of low intensity, all of these areas were scored; in sections in which all of the staining appeared intense, a random field was selected. The proportion of high- and low-staining tumor cells in each selected field was determined by counting individual tumor cells at high magnification. At least 100 tumor cells were scored per 40× field. Two scoring systems were utilized; Pathologist A noted both intensity (0, equivalent to background staining of the acellular stroma; +1, light brown stain slightly darker than background; +2, moderate brown stain; +3, intense brown stain equivalent to normal breast epithelium) and relative abundance (1 = less than 5% of the cells, 2 = 5–25%. 3 = 26–50%, 4 = 51–75%, 5 = 76–100%). Tumors were placed in the low-staining category if the combined abundance scores for staining intensity categories 0 and 1 was 5 or greater. Pathologists B and C used a simplified scoring system, which recorded the percentage of 0–1+ intensity versus 2–3+ intensity tumor cells in the 40× field(s). The cutoff points for Pathologists B and C was selected by cut-point analysis, at 30 and 40% weak staining tumor cells in the 40× field, respectively.

Histopathological, patient and clinical course data. Data regarding age, race, tumor size, lymph node status, and clinical outcome were obtained from the tumor registry (University of Florida Health Science Center), and coded in a dichotomized form for statistical analysis of results: Age, <50 years vs. ≥50; race, black or white; tumor size, <2 cm vs. ≥2 cm; lymph node status, 0 vs. any positive axillary lymph nodes or evidence of distant metastases; clinical outcome, dead of disease vs. all other possibilities.

Statistical Evaluation. The end point for analysis was death due to disease with all other observations being censored. Differences among the positively and negatively staining groups were assessed with the log rank statistic for censored data (6). The predictive ability of the prognostic variables was determined using Cox's proportional hazards linear regression model (7). All analyses were performed using SAS software (SAS Institute, Cary, NC).

Results

Affinity purified rabbit antibody to a synthetic internal, hydrophilic fragment of nm23 (peptide 11) was previously demonstrated to specifically identify nm23 protein on Western blots.[5] Immunoperoxidase staining of formalin fixed, paraffin embedded breast tissue sections was inhibitable by preincubation of the nm23 antibody with a molar excess of peptide 11, and was not demonstrable with a control affinity purified antibody to peptide 65, the predicted amino acid sequence 5′ of the initiating methionine,[5] (data not shown). Staining of normal human breast epithelium using anti-peptide 11 antibody is shown in Figure 1A. Staining intensity was greater than that of the neoplastic tissues and was predominately cytoplasmic and perinuclear. Occasional negative staining cells were observed. Intraductal carcinomas generally exhibited significant nm23 protein content, although less than normal epithelium, and often a greater amount of nuclear staining. In Figure 1B, tumor cells in the intraductal portion of the carcinoma exhibited significant nm23 staining, whereas cells invading into the stroma were lower in nm23 protein content. Thus, in a single lesion a quantitative reduction in nm23 expression was observed in association with histological evidence of invasion.

Nm23 staining of 39 infiltrating ductal carcinomas was predominantely cytoplasmic and perinuclear, and heterogeneous in intensity. Many tumors contained areas of low-staining tumor cells, either as clusters of tumor cells or single infiltrating cells, in otherwise high-staining sections. An example of this pattern of expression is shown on Figure 1C,D. Since low expression of nm23 RNA has

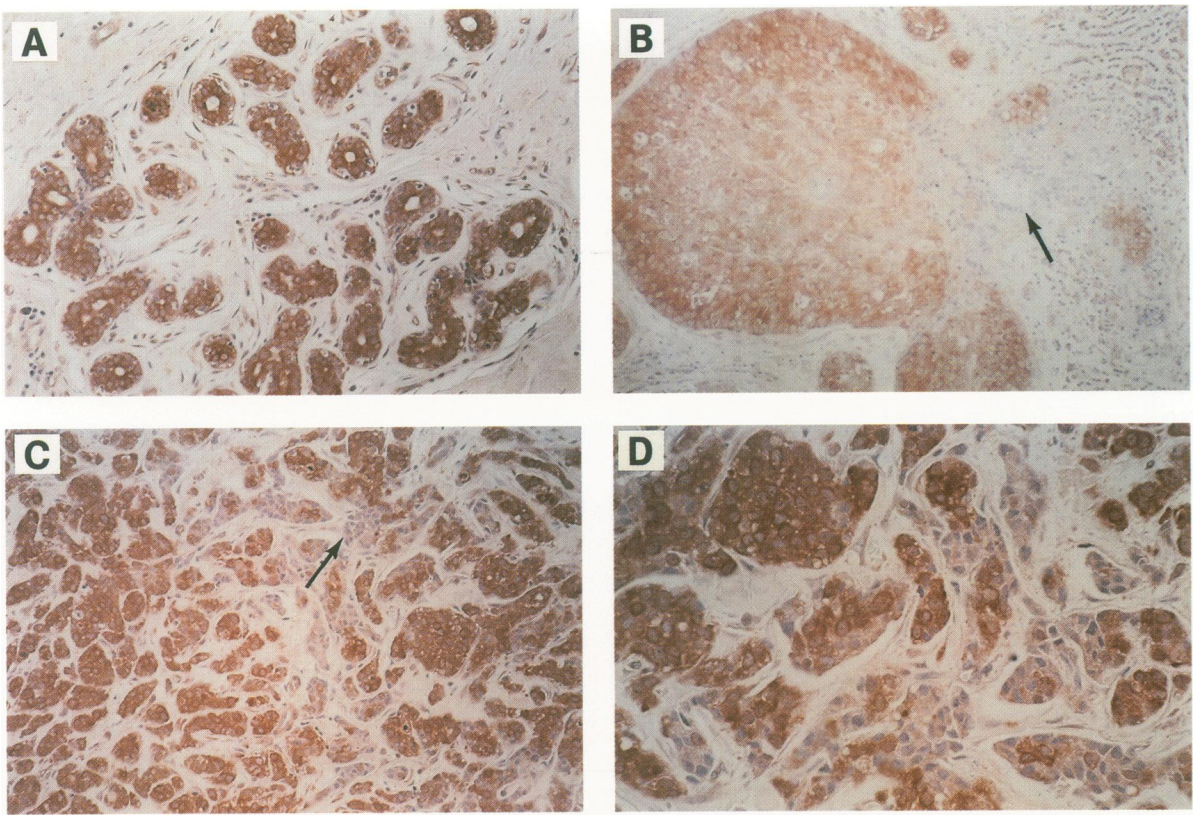

Figure 1. *Photomicrographs of immunoperoxidase staining of normal and neoplastic breast tissues using anti-nm23 antibody. Sections from formalin fixed, paraffin embedded tissues were stained with affinity purified anti-nm23 peptide 11 antibody, which was detected with an avidin–biotin system using diaminobenzidene as the chromogen. Slides were lightly counterstained with hematoxylin.* **A**: *Normal breast epithelium (×200);* **B**: *Intraductal and infiltrating ductal carcinoma (×80). Arrow indicates the infiltrating tumor cells with decreased nm23 protein content;* **C**: *Infiltrating ductal carcinoma (×200), showing nests of low staining tumor cells (arrow) among otherwise high-staining cells;* **D**: *High-power magnification (×800) of the low-staining area from (***C***).*

been associated with high metastatic potential in murine melanomas and human breast carcinomas,[1,3–4] a scoring system was designed for the infiltrating ductal cohort to quantitate the presence of low-staining tumor cells, as opposed to the mean staining intensity throughout the section. The cohort was evaluated independently by three pathologists (RB, KPJ, EW), and the data are summarized on Figure 2 and Table 1. The three pathologists assigned an average of 59–64% of the tumors to the low-staining intensity category. Using the log rank test, two of the three pathologists identified a subgroup of patients with significantly reduced overall survival. The survival of the low-staining subset of patients identified by the third pathologist approached statistical significance ($\alpha = 0.052$). The graph of survival percentages in this case (Figure 2) indicates that the lack of statistical significance stems from the equivalent survival percentages of the low- and high-staining subgroups within the first 4 years of followup; thereafter, the survival percentages of the two subgroups diverged.

Analysis of the infiltrating ductal carcinoma cohort using Cox's proportional hazards linear regression indicated that lymph node involvement was an independent predictor in this model, in agreement with numerous other prognostic studies. Other factors such as patient age or race, and tumor size were not independent predictors and did not change the predictive value of lymph node involvement. Nm23 staining was significant as an independent predictor separate from lymph node status when scored by two pathologists, and approached significance ($\alpha = 0.066$) by the third.

Three variables that could contribute to scoring variations of the slides have been assessed: (1) staining variability between different affinity purifications of the antibody; (2) intraobserver variability in obtaining a numerical score; (3) interobserver variability in scoring slides. In the experiment shown in Table 2, consecutive sections of eight tumors were stained with two different affinity purifications of anti-nm23 peptide 11 antibody, and were evaluated in a blinded manner by a single pathologist. The percent of tumor cells scored by one pathologist in identical fields in two separate staining runs varied by less than 9%. However, in case S84-8884, two different low-staining foci in the same tumor showed markedly different proportions of high- versus low-staining tumor cells. It is likely in this tumor, and throughout the cohort,

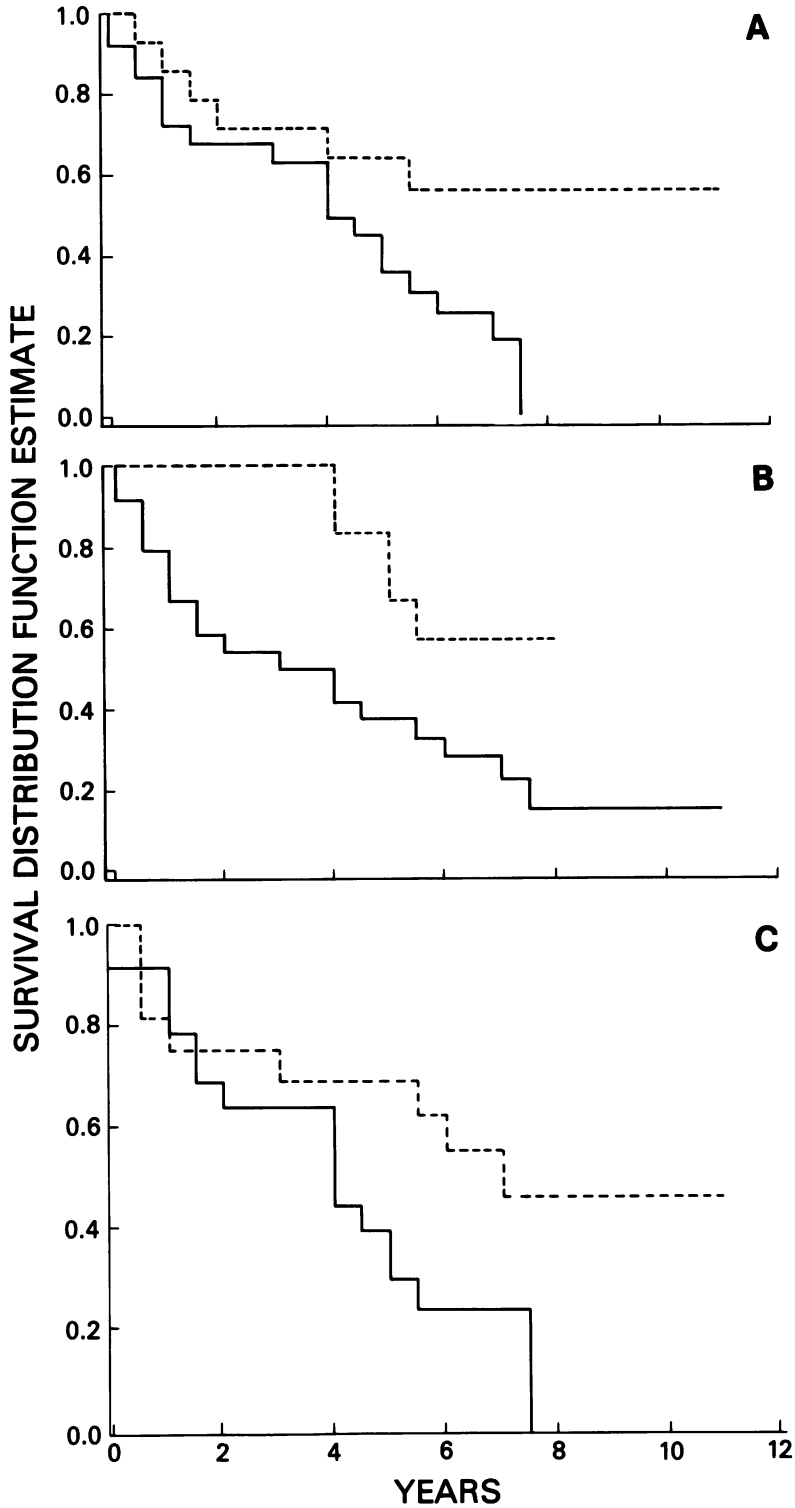

Figure 2. *Survival of patients with low and high nm23 protein content tumors. Thirty-nine infiltrating ductal carcinomas were scored for low or high nm23 protein content by three pathologists independently, blind to clinical course data. The survival of 39 patients with low and high nm23 protein content as determined by each pathologist is shown on the upper, middle and lower graphs. --------, patients with high nm23 content tumors; ——, patients with low nm23 content tumors.*

that the pathologist's identification of the low-staining area to score under higher magnification is a more significant source of variability than either lot of affinity purified antibody or intraobserver accuracy. When the survival percentages were recalculated for the cases in which all three pathologists agreed, significant differences in the survival of patients with low- and high-staining tumors were observed using the log-rank test ($\alpha = 0.03$).

Table 1. *Quantitative Analysis of nm23 Protein Expression in Human Infiltrating Ductal Breast Carcinomas**

	Pathologist A	Pathologist B	Pathologist C
Tumors with high nm23 staining	14	14	16
Tumors with low nm23 staining	25	25	23
Log-rank test for differences in patient survival	$P = 0.034$	$P = 0.012$	$P = 0.052$
Cox's proportional hazards linear regression—nm23 as an independent predictor	$P = 0.046$	$P = 0.021$	$P = 0.066$
Other variables in Cox's proportional hazards linear regression			
Age $P = 0.41$			
Race $P = 0.10$			
Primary tumor size $P = 0.32$			
Lymph node involvement $P = 0.05$			

* Thirty-nine primary infiltrating ductal carcinomas were stained with anti-nm23 peptide 11 antibody, and the tumors scored as low or high nm23 protein content as described in the "Methods" section by three pathologists independently. Variables were compared with patient survival.

Discussion

Low steady state nm23 RNA levels in infiltrating ductal breast carcinoma was previously associated with the presence of lymph node metastases,[3] and significantly reduced disease free and overall survival.[4] This report presents the first quantitation of nm23 protein levels in normal and neoplastic breast tissues. In a limited series of infiltrating ductal carcinomas, two pathologists independently identified a subgroup of patients with low-staining tumors that had significantly shorter survival times, and in which nm23 was an independent predictor on Cox's model. Both overall patient survival and independence in Cox's model approached significance when determined by a third pathologist.

The scoring system that was utilized was unique, based on the quantitation of low- and high-staining tumor cells in a selected low-staining field. Analysis of consecutive sections suggested that the lot of affinity-purified antibody and intra-observer variability did not contribute to variability in scoring. Data from Table 2, however, indicate that the pathologist's selection of the lowest staining area to score under high magnification is a possible source of variability in this protocol.

The development of specific monoclonal antibodies to nm23 proteins may improve nm23 protein quantitation in breast carcinoma. Two human nm23 genes have been discovered, nm23-H1[5] and nm23-H2.[8] Both encode 17 kDa proteins that are approximately 88% identical in amino acid sequence. Using probes to their 3′ untranslated regions, we determined the specific expression of nm23-H1 and nm23-H2 RNA levels in breast carcinoma lines and tumors.[8] nm23-H1 RNA levels were decreased in tumor cells of high metastatic potential to a greater extent than nm23-H2, but nm23-H2 was the more abundant message. Based on their predicted amino acid sequences, anti-peptide 11 antibody may detect both of these proteins. Strong homologies have also been reported between nm23 and nucleoside diphosphate (NDP) kinases.[9] The total number of NDP kinase genes and their homology to nm23 are not known. Therefore, the antibody used may detect additional non-nm23 NDP kinases. Monoclonal antibodies that detect nm23-H1 specifically, for instance, may enable the pathologist to more easily and reliably detect low-staining tumor sections.

The data presented are consistent with the hypothesis that reduced nm23 expression is associated with high

Table 2. *Effect of Antibody Purification Lot, Intraobserver Variability and Selection of Lowest Staining Area on nm23 Scoring**

Accession number	Scoring in lowest staining region under 40× magnification			
	Antibody purification #1		Antibody purification #2	
	Weak cells (%)	Strong cells (%)	Weak cells (%)	Strong cells (%)
S84-4912-7	213 (71)	87 (29)	222 (76)	69 (24)
S84-4130-7	83 (28)	217 (72)	85 (28)	217 (72)
S79-1695	299 (93)	22 (7)	305 (94)	19 (6)
S84-3068	128 (40)	192 (60)	122 (41)	178 (59)
S86-4338	50 (16)	270 (84)	57 (16)	246 (84)
S84-6381	264 (82)	59 (18)	269 (87)	41 (13)
S84-8147	221 (66)	115 (34)	235 (66)	119 (34)
S84-8884 (Reg. A)	143 (44)	185 (56)	121 (35)	221 (65)
S84-8884 (Reg. B)	214 (60)	147 (40)	229 (65)	121 (35)

* Two consecutive sections of eight tumors were stained with different affinity purifications of anti-nm23 peptide 11 antibody. The area or areas of lowest staining tumor cells were scored under 40× magnification by one pathologist, blinded to the slide accession number.

metastatic potential in primary breast carcinoma. The data also indicate the need for retrospective studies of tumors from lymph node-negative patients and premalignant lesions, in which prognostic data are critical to therapeutic decisions.

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