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13. ABSTRACT *(Maximum 200 words)*

This is the final report for this grant. There were two major papers and several abstracts published from this work. The main goals were to determine the mechanism of action of nm23 and to determine if the expression of this anti-oncogene can predict the metastatic spread of disease. The over-expression of this gene has been reported to prevent tumors from spreading from the primary site to distant sites in a nude mouse model and has been shown to be prognostic indicator for a good prognosis.

We wanted to relate its expression in 150 primary breast cancer tumors to other markers that have been shown to be of prognostic use. These markers included protease-related factors, steroid receptors, proliferative activity and ploidy status. Our results indicated that there was no relationship to these markers. We did find that over-expression of nm23 in a breast cancer cell line resulted in a significant decrease in growth-factor-stimulated motility.

Using two different cell lines, we were not able to demonstrate that nm23 over-expression resulted in a significant decrease in metastatic potential in a nude mouse model. This was after a 4 to 8 fold expression over the parent cell line. A survey of cell lines and xenographs did indicate that over expression of nm23 was related to a lower metastatic potential. We also demonstrated that over-expression of nm23 in breast cancer tissue as determined by either western blot analysis or by immunohistochemistry was not related to prognosis, to stage, to grade or to nodal status. The role of nm23 in breast cancer is still unknown.

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Timothy E Kute

PI - Signature Date

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ATTACHMENTS

PAPER #1:

Russell RL, et al nm23- relationship to the metastatic potential of br4ast carcinoma cell lines, primary human xenografts, and lymph node negative breast carcinoma patients Cancer 79: p 1158-1165,1997

PAPER #2:

Russell RL, et al Relationship of nm23 to proteolytic factors, proliferation and motility in breast cancer tissues and cell lines Brit J. Cancer 78: Sept 15,1998

PAPER #3

Dauphinee M et al Effect of nm23 expression levels in GI-101A and its effect on metastatic potential in athymic nude mice. Abstract presented at the 25th annual meeting of the International Breast Cancer Meeting in San Antonio, Tex Nov, 1997 (Abstract # 366)

PAPER #4

Kute TE, et al Low cathepsin d and low plasminogen activator type I inhibitor in tumor cytosols defines a group of node negative breast cancer patients with low risk of recurrence Br. Can. Res. Treat 47: 9-16,1998
(note not directly related to project but could be of interest to reviewer)

INTRODUCTION

This grant started in July of 1994 and was concluded in July of 1998. It was proposed by the army review for 2 years of funding for 2 aims (1 and 2). The PI requested 3 years with three aims at the 2 year funding rate. This was approved and in July of 1997, it was extended for 1 year at no additional cost. This project has produced 2 direct papers on this subject and 5 different abstracts.

The goal of this project was to study the mechanism of action of an anti-oncogene, nm23, and to demonstrate that its action was to reduce metastatic disease. The aims of this project are listed below:

1. TO CORRELATE NM23 EXPRESSION WITH FACTORS INVOLVED IN PROTEOLYTIC DEGRADATION

2. TO LOCALIZE NM23 EXPRESSION IN RELATIONSHIP TO PROTEOLYTIC FACTORS USING IMMUNOHISTOCHEMISTRY

3. TO DECREASE METASTATIC POTENTIAL BY TRANSFECTION OF NM23 INTO BREAST CANCER CELL LINES CONTAINING A lacZ MARKER GENE.

The studies on each of these aims will be discussed in the body of this report. I will use the published papers and abstracts to present the data since it would be more concise. The methods used are presented in these papers.

We have also done additional studies to address the function of nm23 in breast cancer. These will be discussed after the aims are presented. These addition studies include:

1. Role of nm23 in motility of breast cancer cells
2. Role of nm23 in clinical outcome of node negative breast cancer (retrospective study)
3. Role of nm23 expression to nodal status, grade, stage, and clinical outcome (prospective study)
4. Role of nm23 in predicting response to high dose chemotherapy and bone marrow transplant treatment
5. Relationship of nm23 to steroid receptors, ploidy and proliferation

It is our opinion that from the limited funds a good deal of information has been obtained on breast cancer from this grant.

BODY OF THE REPORT

Aim#1

In aim #1, we wanted to correlate the expression of nm23 to other proteolytic factors. This was done on 150 samples where nm23 expression was determined by western blot analysis (see methods and Figure 1, paper #1). It should be noted that nm23 has two forms, H-1 and H-2 and both of these factors were analyzed in these studies. At the same time, the expression of various proteolytic factors was also determined on the same tumor extract (see methods paper #2). Our hypothesis was that nm23 was able to down regulate metastatic disease by down regulation of factors that caused tumor cells to spread out from the primary site. The factors used in these studies were picked based on other clinical trials that suggested that they were involved in a poor prognosis of the disease (1-7).

Our results demonstrated NO inverse correlation between nm23 expression and cathepsin D, urokinase plasminogen activator, urokinase plasminogen activator receptor, or urokinase plasminogen activator inhibitor. The correlations are given in table 3-bottom 4 items in paper #2. We were able to confirm these results by transfecting tumor cells with nm23 and measuring the expression of these factors. The results demonstrate that there is no correlation of nm23 expression to these factors in these transfected cells (figure 1 paper 2).

Aim #2

In aim#2, we wanted to demonstrate that nm23 expression down-regulated the protease activity at unique cellular locations. This involved the measurement of nm23 and the above mentioned proteolytic factors using immunohistochemistry. This was a more difficult process than was anticipated. As reported in the last annual review, the expression of nm23 is very difficult to measure. We found in 87 cases that most of the cells were positive. We also found that the levels of positively did not agree with the expression of nm23 as defined by western blot analysis. The other antibodies to the proteolytic factors demonstrated low expression or were not able to be determined due to high background. In conclusion, we could not show any relationship of nm23 expression to proteolytic factors expression using immuhohistochemistry.

We have recently tried antibodies specific for the H-1 and H-2 isoforms (8). These studies gave similar results compared to the polyclonal antibodies. We found that in 28 cases almost all stained greater than 80%. There are some changes in intensity and this has also been evaluated. We have therefore asked the question if this nm23 staining by either the polyclonal or the monoclonal antibodies has any relationship to prognosis or response to treatment. Node negative breast cancer trials and chemo/BMT treatment studies will be presented later in this report to address this question.

Aim#3

In aim#3, we wanted to demonstrate that transfection of nm23 into two different cell lines would decrease the metastatic potential. We used MDA-MB-231, which had previously been transfected with lacZ to better define the metastatic site in the lung tissue.

The details of this procedure are discussed in paper #2. Our results indicate that there is a decrease in the metastatic potential but that it is not significant (see Table 4 paper #2). In order to confirm these results, we collaborated with the Goodwin Institute of Cancer Research and transfected a second cell line, GI-101, with nm23. The results of this work were presented in an abstract at the 20th annual San Antonio Breast Cancer Symposium (see enclosed abstract). After transfection of the nm23 into GI-101A, we observed increased nm23 levels but no decrease in lung metastasis using the GI-101A cell line. It could be that the cell line GI-101A is not as metastatic as the primary tumor, GI-101 (see abstract), but it is clear that the nm23 expression did not alter the metastatic potential. This data does not agree with previous published results (9,10) but it confirms the previous results that nm23 does not provide a significant reduction in metastatic potential from our studies.

We also looked at known metastatic potentials from various cell lines and xenografts in relationship to their nm23 expression. Our results are published on table 1 paper #1. There was an inverse relationship between nm23-H1 expression and metastatic potential. The p value was 0.19 and the R-value was -0.51 for this relationship.

OTHER STUDIES NOT INCLUDED IN AIMS

1. Role of nm23 in motility

A second mechanism for reduction in metastatic potential is the reduction in motility. Previous work by Kantor (11) and others (12) have demonstrated that expression of high levels of nm23 result in a reduction in the motility of the cell. We, therefore, contacted Dr. Kantor and sent him our parent and two nm23 transfected MDA-MB-231 cell lines. He then performed the motility studies on these cell lines using a Boyden chamber assay procedure. The methods and results are discussed in paper #2 (see methods and Figure 3). Clearly, there is a significant reduction in growth factor stimulated motility as a function of nm23 expression. Our results demonstrated that clone 40 which had a 4 fold increase in nm23 over the parent cell line was intermediate in motility compared to parent and the clone 47 which had an 8 fold increase in nm23 expression. Preliminary work suggested that the motility was not due to changes in integrin expression (data not presented).

2. Role of nm23 in clinical outcome of node negative study

In this study, we performed a case control study of 40 patients for expression of nm23 by immunohistochemistry (figure 2 paper #1). There were 19 non-recurrent cases and 21 recurrent patients. They were matched for various characteristics and length of follow-up. (See table 2 of paper #1) We used a polyclonal antibody that detects both H-1 and H-2 forms of nm23. The results demonstrate that nm23 expression does not relate to recurrence of disease in these patients (figure 3 paper #1). There was no optimal cut-off value that would separate recurrent from non-recurrent patients.

3. **Role of nm23 expression to nodal status, grade, stage and clinical outcome (prospective study)**

In this study, we related the nm23 expression in the patients from aim#1 to nodal status, grade, stage, and clinical outcome. There were 144 patients for this study with a mean follow-up time of 2.5 years. The mean nm23 expression for either H-1 or H-2 was then compared between node negative and node positive patients.

		Mean Value +/- StdErr*		
		Node Negative	Node Positive	Significance
	# Of patients	61	63	
NM 23-H1		0.70 (0.067)	0.63 (0.069)	p = 0.43
NM23-H2		0.55 (0.05)	0.49 (0.05)	p = 0.38

* Units of ng/μg of protein extract

As is evidenced in the above table, in this sample there was no mean difference of nm23 expression to nodal status. This result is different from some of the literature results (13). We also attempted to relate the number of positive nodes to expression of nm23. Our results indicated a direct correlation between number of nodes positive and an increase in the expression of nm23-H1 and nm23 -H-2 (p= 0.02 and 0.03 respectively). This is not the expected result.

It was also suspected that the grade of the tumor might be correlated to the mean nm23 expression of the tumor. Our results do indicate that the mean nm23 expression is higher in grade 1 tumors compared to grade 2 and 3 tumors which is what would have been predictive. However, there were only 7 patients with grade 1 tumors and the overall hypothesis of 'no difference in mean values of H1 (or H2)' between the three groups is not rejected as is evidenced by the following results.

	Mean value +/- StdErr *			
	Grade 1	Grade 2	Grade 3	SIGNIFICANCE
NM23-H1	1.08 (0.2)	0.65 (0.08)	0.67 (0.06)	p = 0.13
Nm23-H2	0.75 (0.15)	0.47 (0.04)	0.55 (0.040	p = 0.18

* Units of ng/μg of protein extract

It was also hypothesized that the overall stage of the tumor might be inversely related to the mean nm23 expression. Our data on these patients do not demonstrate that this is a true observation as demonstrated by the following table:

	Mean value +/- StdErr *					
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Significance
Nm23 H-1	0.71(0.11)	0.63 (0.07)	0.66(0.09)	0.73(0.13)	1.11(0.2)	p = 0.24
NM23 H-2	0.54(0.08)	0.51(0.05)	0.51(0.07)	0.58(0.09)	0.82(0.15)	p = 0.36

* units of ng/μg of protein extract

In this group, there were 34 deaths and 21 recurrences of 134 evaluable patients. The expression of nm23 was borderline predictive of poor prognosis for either death or recurrence rate in this sample as shown in the following table:

	Overall Survival		Disease Free Survival	
	Rel. Risk	P value	Rel. Risk	P value
NM23 H-1	1.28	0.08	1.32	0.11
NM23 H-2	1.31	0.19	1.66	0.05

These data would suggest that nm23 is *not* acting as a 'risk protector' as described in the literature (14,15) but rather as a possible risk factor for poor prognosis. This is tentative since all of these patients underwent varying treatment regimens and are of varying stage, grade and nodal status. It is suggestive that since there is little or no relationship of nm23 expression with markers such as nodal status, grade or stage, then there would be no relationship to prognosis. Multivariate analysis which was adjusted for stage, grade, nodal status, PAI-1, UPA, UPAR, and Cathepsin-D, did not indicate that expression of nm23 of either isotype was predictive of prognosis (all $p > 0.17$) although the trend for poorer prognosis was still evident (relative risks ranging from 1.28 to 2.42).

4. Role of nm23 in predicting response to high dose chemotherapy and bone marrow transplant treatment.

In this study, we attempted to use the expression of either H-1 or H-2 by immunohistochemistry for predicting the response to high dose chemotherapy and bone marrow transplant. There were 28 patients with node positive disease but no observable distant metastatic disease. We predicted that patients whose tumors had high levels of nm23 would be less metastatic and therefore would have a longer disease survival. To adequately test this hypothesis, we analyzed nm23 H-1 and nm23 H-2 for percent of tumor cells staining and intensity of this stain using specific antibodies (8). There were 7 treatment failures in the 28 patients with a median follow-up of 17 months. The overall disease free survival after treatment was 85.1% and 65.5% for years one and two respectively. Statistical analysis was performed but due to the low number of events and the short follow-up, the results can not be evaluated at this time. Further follow-up and more patients added to this study will better address of role of nm23 expression in the

prediction of the patient's response to high dose chemotherapy and bone marrow transplant treatment. We believe that such analysis would help in determining which patients are good candidates for this expensive and toxic treatment.

5. Role of nm23 to steroid receptors, ploidy status and proliferation

Since my laboratory is a cancer marker laboratory, we were able to compare the levels of nm23 expression to other markers that have been related to prognosis. These markers include estrogen receptors, progesterone receptors, ploidy status, and S% activity. The methods for assays are discussed in paper #2. Our results indicate that neither nm23 H-1 or nm23 H-2 expression is related to any of these markers. These data are presented in table 2 and the first half of table 3. We did observe an inverse relationship of nm23 H-1 expression with % S phase and a direct relationship with G-1 phase (table #3) which were of borderline significance. In order to further evaluate this observation, we performed "in vitro" and "in vivo" growth curves on cells lines with different nm23 expressions. These results are given in figure 2 paper #2. They demonstrate that 4 fold or 8-fold differences in nm23 expressions are not sufficient to change the growth potential of these cells. It was therefore concluded that nm23 in not a regulator of cell cycle. This is in contrast to the literature (16).

In conclusion, these data would lead one to believe that nm23 might regulate metastatic potential by decreasing growth factor stimulated motility. It would not effect the proteolytic expression or growth rates of tumor cells. Its role in predicting prognosis is still an unanswered question. There was a recent paper, which demonstrated that high nm23 H-1 expression predicted a lower recurrence rate in node negative patients (15). This is very exciting but the definition of high and low expression is not clear. They state that high expression of nm23 means "all tumor cells must be positive" while tumors that have "cold spots" where there is no expression would be considered negative. This might be difficult to standardize in the "light of current immunohistochemistry."

To the reviewer of this grant, I would like to thank you for your comments on this work over the years. I hope that you have enjoyed reading this work.

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nm23—Relationship to the Metastatic Potential of Breast Carcinoma Cell Lines, Primary Human Xenografts, and Lymph Node Negative Breast Carcinoma Patients

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BACKGROUND. Since the discovery of nm23 (nonmetastatic) by Steeg et al. in 1988, a number of tumor cohort studies have shown an inverse relationship between the levels of expression of the nm23-H1 protein and disease aggressiveness and tumor metastatic potential.

METHODS. The relationship between the expression of nm23 protein and the metastatic potential of human breast carcinoma was analyzed in cell lines, xenografts, and in a retrospective lymph node negative breast carcinoma population. The lymph node negative breast carcinoma study was comprised of 40 patients: 19 with nonrecurrent and 21 with recurrent disease. The 40 patients were matched according to age, cathepsin D, tumor size, percent S-phase, DNA ploidy, steroid receptor status, and tumor grade. Nm23-H1 protein levels in cell lines and xenografts were analyzed quantitatively using Western blot analyses and semiquantitatively in tissue sections using immunocytochemistry. Immunocytochemical analysis of lymph node negative breast tumors was graded as the percent of tumor staining positive for nm23 and the intensity of staining. The metastatic potentials of the cell lines and xenografts were assessed as the ability to form metastatic lesions in nude mice. In the lymph node negative breast carcinoma patients, the metastatic potential was characterized as the incidence of breast carcinoma recurrence.

RESULTS. The MCF-7 cell line expressed four- and tenfold higher levels of nm23-H1 than the highly metastatic MDA-MB-435 and MDA-MB-231 cells, respectively. Among the xenografts and cell lines, there was an inverse correlation between nm23-H1 expression and metastatic potential in athymic nude mice (correlation coefficient [R] = −0.51). The differences between the levels of nm23-H1 among the metastatic and nonmetastatic cell lines and xenografts were not statistically significant. Statistical analyses indicated that neither the intensity nor the percent of tumor staining positive for nm23 expression was correlated to the recurrence of breast carcinoma in the lymph node negative patient population that had been matched for other clinical prognostic markers.

CONCLUSIONS. There was an inverse correlation (R = −0.51) between the levels of nm23-H1 expression in cell lines and xenografts and the metastatic potential in nude mice. In the retrospective lymph node negative breast carcinoma population, no clear association was demonstrated between the expression of nm23 and breast carcinoma recurrence. This observation suggests the nm23 expression does not predict outcome in lymph node negative breast carcinoma patients. ***Cancer* 1997;79:1158–65.** © *1997 American Cancer Society.*

KEYWORDS: Breast carcinoma, clinical, metastasis, lymph node-negative, nm23, immunohistochemistry.

Although the mortality associated with breast carcinoma is most certainly a result of metastatic disease, a clear understanding of the mechanisms involved in the progression of a primary tumor toward metastasis have not been established. The discovery of the nm23 gene,[1] and its subsequent identification as a putative suppressor of metastases,[2,3] has elicited a great deal of interest in the mechanisms by which the nm23-H1 protein may suppress the formation of metastatic lesions.

The nm23 gene was first identified in a murine melanoma cell line using differential hybridization techniques to characterize clones having high and low metastatic potential.[1] Studies demonstrated that transfection of the nm23-H1 gene into highly metastatic cell lines resulted in significantly fewer metastatic lesions in athymic nude mice.[4,5] Several human breast tumor cohort studies have shown that reduced nm23-H1 protein or RNA expression was associated with more aggressive disease.[2,6–11] Other human tumor cohort studies have demonstrated a similar inverse correlation between the levels of nm23 and overall survival/stage or metastatic potential of hepatocellular, melanoma, gastric, prostatic, and ovarian carcinomas.[12] However, in both neuroblastoma and pancreatic tumor studies, elevated levels of nm23 were associated with more aggressive disease.[12]

Two human nm23 genes have been identified, nm23-H1[13] and nm23-H2,[14] that encode for distinct proteins that are 88% homologous. Nm23-H1 and nm23-H2 are identical to human nucleoside-diphosphate kinase (NDPK) A and B, respectively.[15] Although this observation attributed a biologic activity to nm23, no correlation between NDPK enzymatic activity and the suppression of metastasis has been shown.[5,16]

This study was undertaken to analyze breast carcinoma expression of nm23 protein levels in a variety of different sources including breast carcinoma cell lines, primary breast carcinoma xenografts, and a retrospective lymph node negative breast carcinoma population matched for other significant prognostic markers. The levels of nm23-H1 expression in these sources were correlated to metastatic potential based on athymic nude mouse studies or clinical follow-up of breast carcinoma patients.

METHODS

Lymph Node Negative Breast Carcinoma Characterization

A lymph node negative population was selected because this is the patient population in which it would be most useful to predict the risk for recurrence. The original patient population from which this sample was drawn has been described previously in detail.[17] At the time of selection of the pilot study group, 26 patients from the original group had suffered a recurrence. The recurrent patients were selected and matched with a nonrecurrent group of lymph node negative patients from the original population. Criteria that were used to match the recurrent and nonrecurrent groups included age, cathepsin D concentration, tumor size, percent S-phase, percent aneuploidy, steroid receptor status, and tumor grade.

Human Mammary Tumor Xenografts and Cell Lines

The human mammary cell lines MCF-7, MDA-MB-231, and MDA-MB-435 were grown according to recommended procedures and maintained in log growth phase by passaging 1–2 times per week. Human xenografts were established by repeated passages of human breast tumors into nude mice as has been previously described.[18–20] The mammary tumor xenograft GI-101 was developed by J. Hurst at the Goodwin Institute for Cancer Research, Inc., Plantation, Florida.[18] Xenografts UISO-BCA-1 and UISO-NMT-BCA-4 were established by Dr. R. R. Mehta.[19] Human breast carcinoma xenografts MAXI-401, MAXF-713, MAXF-713 with matrigel, and MAXF-583 were originally established by Dr. H. H. Fiebig, Frieburg University, Frieburg, Germany.[20] The xenograft OHSTMAM-4 was established by D. J. Dykes, Southern Research Institute, Birmingham, Alabama.[19] Tumor extracts were prepared from xenografts and cell pellets using methods as previously described.[21]

Metastatic Potential Analysis

Athymic nude mice 5 to 6 weeks old were treated with either human tumor homogenates, or tumor cell suspensions as described previously.[19] In the case of the MCF-7 tumors, cells were injected into animals treated with 60-day release estrogen pellets (0.5 mg) to ensure tumor development. The growth of each tumor was monitored and the animals were sacrificed at the end of the experiment (6–8 weeks). The animals were then dissected to determine whether any metastatic disease was present. Metastatic disease was determined by hematoxylin and eosin staining of the lungs and other suspected organs of the animal. Any positive tumor site outside the primary tumor was considered a metastasis.

In the case of the lymph node negative breast carcinoma population, metastatic potential was defined as breast carcinoma recurrence.

Western Blot Procedure

Tumor extracts (30–50 μg/lane) from xenografts or cell line pellets were boiled in sodium dodecyl sulfate (SDS)-lysis buffer and analyzed using standard SDS-polyacrylamide gel electrophoresis and Western blot

procedures. Protein loading was evaluated by staining the nitrocellulose membrane with Coomassie blue dye after protein transfer. Each gel contained molecular weight markers and purified nm23-H1 protein standards ranging from 2–160 ng. Nitrocellulose membranes (Hybond-C extra; Amersham Life Sciences, Arlington Heights, IL) containing nm23-H1 standards and test samples were blocked using a 5% casein-Tris buffer. The blots were incubated 2 hours at room temperature with 0.193 μg/mL polyclonal anti-nm23 antibody. The partially purified polyclonal antibody was a generous gift from Oncologix™ (Gaithersburg, MD) and recognizes nm23-H1 and nm23-H2 proteins in human breast tumor specimens. The polyclonal antibody to nm23 was evaluated and compared with the peptide 11 antibody[4] and both antibodies were shown to have comparable immune-reactivity (data not shown). The blots were then washed and the primary antibody was labeled with 1:2000 dilution of biotinylated donkey antirabbit antibody (Amersham Life Sciences). Further washing was followed by the labeling of the biotinylated antibody with 1:2000 dilution of streptavidin-horseradish-peroxidase enzyme (Amersham Life Sciences). Finally, the blots were immersed in ECL™ chemiluminescent reagent (Amersham Life Sciences) and the nm23 proteins labeled with the enzyme were detected on X-ray film.

Quantification of the levels of nm23-H1 (17 kilodaltons) in the tumor extracts was performed using the standard curve of the known quantities of nm23-H1 and scanning laser densitometry. Known quantities of nm23-H1 were plotted against optical density X mm and a standard curve was plotted for each experiment.

Immunohistochemistry for nm23

Frequently in the clinical environment, the availability of fresh tumor tissue is limited. Therefore the current analyses have been extended to include immunohistochemical staining techniques performed on formalin fixed, paraffin embedded tissues. The polyclonal antibody to nm23 recognizes both nm23-H1 and nm23-H2 by Western and immunohistochemical analyses and has reactivity comparable to peptide 11 antibody as described by Leone et al.[5] (data not shown).

The immunohistochemical analyses of nm23 in breast carcinoma patients were performed according to standard procedures. Briefly, human breast tumors were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4–5 μ) were cut from each breast tumor specimen, deparaffinized in xylene, and rehydrated in a graded series of ethyl alcohol. The tissues were then washed in distilled water for 2–5 minutes.

The polyclonal antibody concentration was established at 19.3 μg/mL. Tissue specimens were analyzed for nm23-H1 using an automated staining system (Ventana 320 ES™; Ventana Biotek System, Inc., Tucson, AZ) and manufacturer recommended reagents necessary for the detection of primary antibody using AEC (3-amino ethylcarbazole) as the chromogen. Pretreatment of the tissues with alkaline protease (protease 2; Ventana Medical Systems, Inc.) for 8 minutes enhanced the sensitivity of the detection of nm23.

One pathologist (K. R. G.) performed all immunohistochemical analyses without knowledge of patient outcome. Extensive analysis of the literature has shown considerable variability with respect to the methods of grading nm23 expression by immunohistochemical techniques. Nm23 has been shown by others to be related to metastatic potential,[12] therefore, the authors analyzed the invasive and intraductal components of each breast carcinoma specimen independently. The criteria for the analysis of each specimen, included the cytoplasmic staining intensity of the breast tumor (0–3+), the percentage of tumor that stained at the given intensity, and the percentage of tumor in the section. Independent evaluations were performed for intraductal and invasive tumors in each specimen.

Statistical Analyses

Twenty-one recurrent patients were chosen retrospectively followed by 19 matched controls. Matching was attempted according to age, cathepsin D, tumor size, percent S-phase, DNA ploidy, steroid receptor status, and tumor grade. The two groups were homogeneous with respect to most of the matched factors with the exception of steroid receptor status, in which the recurrent group had more steroid receptor positive patients. The two-sample Student's t test was used to compare continuous variables (age, cathepsin D, and tumor size), and either the Fisher's exact test or Pearsons chi-square test was used to compare ordinal variables.

The association between nm23 status (>40% vs. $\leq$40%) and recurrence and overall survival was assessed by Kaplan–Meier analysis,[22] in which the log rank test was used to determine level of significance. Similar statistical analyses were performed using 20%, 50%, 60% and 80% cutoff values for nm23 positivity and the relationship with disease free and overall survival.

The relationship between nm23-H1 expression and metastatic potential was assessed using Pearson's product moment correlation coefficient. Because the analyses for cell line and xenografts were different, partial correlation coefficients were calculated.

The Cox proportional hazards model[23] was used to assess differences in recurrence and overall survival between the two nm23 groups after adjustment for other covariants.

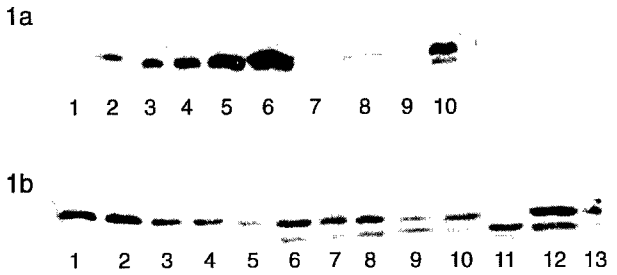

FIGURE 1. Western blot analysis of human breast carcinoma cell lines and xenografts. (a) The Western blot of the breast carcinoma cell lines MDA-MB-435 and MCF-7 is shown. The standard curve contained 10, 20, 40, 80, and 160 ng of purified nm23-H1 protein in Lanes 2-6, respectively. Lanes 8 and 10 contain cell pellet extracts from MDA-MB-435 and MCF-7 cell lines, respectively. Lanes 1, 7, and 9 are blank. (b) Xenografts from human breast tumors grown in nude mice were analyzed by Western blot analysis for nm23 concentrations. Lanes 1–5 represent known nm23-H1 standards and contain 100, 75, 50, 30, and 20 ng of purified protein, respectively. Lanes 6–13 represent nm23 levels in xenografts UISO-BCA-1, UISO-BCA-4, MAXF-401, MAXF-583, MAXF-713 (with matrigel), MAXF-713, OHSTMAM-4, and GI101 as described. Lanes 6 and 7 are nonmetastatic xenografts in athymic nude mice and Lanes 8–13 are metastatic xenografts. The upper bands represent nm23-H1 and the lower bands in the tumor extracts are nm23-H2. All nm23-H1 values for each tumor were calculated from the standard curve shown.

TABLE 1
Expression of nm23-H1 in Breast Carcinoma Cell Lines, and Xenografts Derived from Primary Human Tumors

Cell type	Expression of nm23-H1 (ng/μg of protein) No.	Mean (± SE)	Metastatic potential Mets/animal (%)
MCF-7	6	1.642 (0.137)	2/6 (33)
MDA-MB-231	7	0.173 (0.047)	5/7 (71)
MDA-MB-435	5	0.369 (0.118)	5/10 (50)
Xenograft[a]	8		
UISO-BCA-1	8	0.542 (0.076)	0/50 (0)
UISO-BCA-4	8	0.429 (0.172)	0/50 (0)
OSHTMAM-4	7	0.605 (0.086)	1/5 (20)
MAX-F-401	6	0.388 (0.047)	3/8 (38)
MAX-583	8	0.291 (0.074)	1/5 (20)
MAX-713	7	0.182 (0.069)	1/5 (20)
MAX-713 (mtg)[b]	8	0.137 (0.039)	1/5 (20)
GI-101	9	0.258 (0.038)	3/5 (60)

SE: standard error; Mets: metastases; mtg: matrigel.
[a] Human breast carcinoma specimens were transferred to nude mice. The resulting tumor grown in nude mice was analyzed for nm23-H1 by Western blot.
[b] Xenograft was injected in matrigel (mtg).

RESULTS

nm23 Expression in Human Breast Carcinoma Cell Lines and Xenografts and the Correlation of nm23-H1 Concentration with Metastatic Potential

The authors evaluated nm23-H1 protein levels in breast carcinoma cell lines and nude mouse xenografts of primary human tumors using Western blot analyses. Figure 1 shows typical Western blots containing unknown samples and standards having known concentrations of nm23. The nm23 values of the unknowns were calculated using a standard curve of the standards contained in each gel. In Figure 1a Lanes 2–6 contain nm23-H1 standards (10–160 ng), and Lanes 7 and 9 contain tumor extracts from MDA-MB-435 and MCF-7 cells, respectively. Lanes 1, 7, and 9 are blank. The levels of nm23-H1 in the three cell lines vary considerably. MCF-7 cells expressed the highest nm23-H1 levels and contained four to ten times higher levels than either MDA-MB-435 and MDA-MB-231, respectively (Table 1). A broad range of values for nm23-H1 was observed among the eight primary human tumor xenografts (Fig. 1b). Lanes 1–5 contain purified nm23-H1 protein and Lanes 6–13 represent eight different breast cancer xenografts. Lanes 6 and 7 represent xenografts that are nonmetastatic in nude mice and Lanes 8–13 represent xenografts that are metastatic in nude mice. The authors utilized the ability to quantify the levels of nm23 in tumor extracts and cell lines to evaluate the variability of nm23 H1 levels in these tissues. The levels of nm23 ± standard error were compared with the metastatic potential of cell lines (MCF-7, MDA-MB-231, and MDA-MB-435), and eight human xenografts (Table 1).

TABLE 2
Characterization of Lymph Node Negative Breast Carcinoma Patients as a Function of Recurrence

Factor	Nonrecurrent N = 19 Mean ± SE	(%)	Recurrent N = 21 Mean ± SE	(%)
Age (yrs)	58.7 ± 15.8		62.9 ± 14.7	
Cathepsin D	54.1 ± 18.5		60.8 ± 29.4	
Tumor size	1.9 ± 0.9		2.0 ± 1.0	
% S-phase[a]	7.3 ± 5.6		6.9 ± 3.7	
% Tumor[b]	41.0 ± 25.2		53.2 ± 25.9	
% Aneuploid		67		56
% ER positive		33		67
% PR positive		35		65
Tumor grade[c] (no.)				
1	0		1	
2	7		6	
3	8		11	
Follow-up time (years)				
Range	3.3–13.8		0.7–9.2	
Median	5.1		2.7	
Mean	6.3		2.9	

SE: standard error; ER: estrogen receptor; PR: progesterone receptor.
[a] The nonrecurrent group contained 12 patients and the recurrent group contained 15 patients for percent S-phase analysis by flow cytometry.
[b] All slides used in this study contained sufficient tumor for the analysis. The mean percent tumor ± the standard error of the tumors are represented here.
[c] Tumor grade was available for 15 nonrecurrent and 18 recurrent patients.

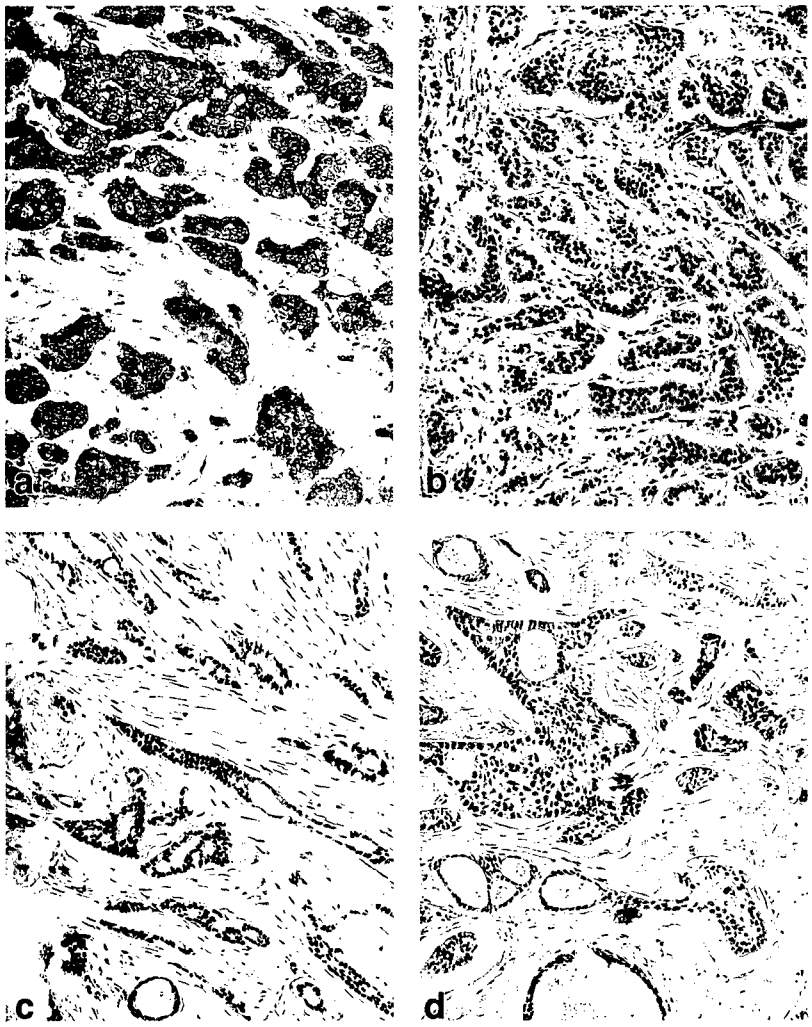

FIGURE 2. Immunohistochemical analyses of nm23 in human breast tumors. Human breast carcinoma tissues were stained for nm23 using polyclonal anti-nm23 antibody as described. (a) An invasive breast carcinoma staining positive for nm23 (100% of the tumor expresses nm23). (b) The negative control for this tissue. (c) An invasive breast tissue that was graded as nm23 negative because <40% of the tumor stained for nm23. (d) The negative control for this tissue.

The levels of expression of nm23 were correlated to the incidence of metastatic lesions in nude mice (described as the metastatic potential). The differences between the metastatic and nonmetastatic tissues and cell lines were not significant ($P = 0.19$) although a negative correlation (correlation coefficient [R] = −0.51) was observed when there appeared to be a trend toward the nonmetastatic tissues expressing higher levels of nm23.

Immunohistochemical Analyses of nm23 and Prognosis of Lymph Node Negative Breast Carcinoma Patients

A lymph node negative breast carcinoma pilot study was selected as a means of evaluating the effect of nm23 expression on the metastatic potential of a clinical breast carcinoma population. The recurrent patients were selected and matched with a nonrecurrent group of lymph node negative patients from the original population. Criteria that were used to match the recurrent and nonrecurrent groups are shown in Table 2. The mean time of follow-up was significantly longer in the nonrecurrent group to reduce selection bias as a result of short follow-up times. Although there was a trend toward nonrecurrent patients expressing lower steroid receptor positivity, this difference was not statistically significant using the Fisher's exact test ($P = 0.06$ and $P = 0.18$ for estrogen and progesterone receptors, respectively). Because steroid receptors have not

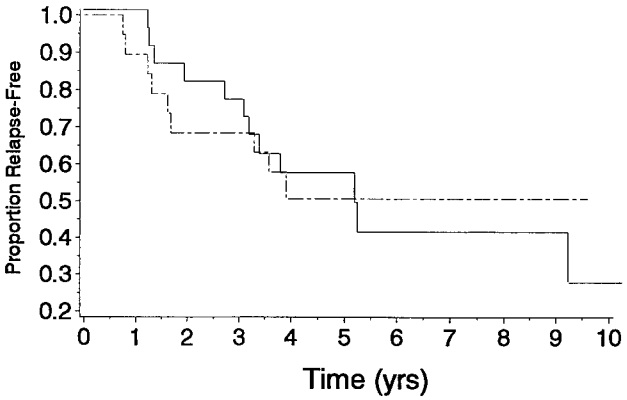

FIGURE 3. Relationship between nm23 positivity and disease free survival in lymph node negative breast carcinoma patients. The nm23 analysis by immunohistochemistry in the lymph node negative breast carcinoma study was plotted as a function of relapse free survival using the method of Kaplan and Meier.[22] The patients with negative nm23 staining intensity are identified with a solid line (N = 21 with 12 relapse events). Those patients that were nm23 positive were identified with a dashed line (N = 19 with 9 relapse events). Positivity for nm23 was defined as ≥40% of the tumor staining. No prognostic advantage was observed in patients who had nm23 positive tumors.

been proven to be prognostic in the complete clinical trial,[17] it was believed that the differences in receptor status compatibility would not be critical. In addition, because the nonrecurrent group had a lower percentage of steroid receptor positivity than the recurrent group, the selective advantage for survival would, if anything, give a benefit to the recurrent group.

Because the staining intensity is a variable that is highly subjective and can have considerable variability within and between experiments, nm23 positivity was also graded according to the percent of invasive or intraductal breast tissue that expressed nm23. In the patient population studied, only 16 of the 40 specimens analyzed contained an intraductal component, whereas 40 of the patients analyzed contained an invasive component. Thus, these analyses only focused on the invasive component. Tissue with ≤40% positivity was considered negative for nm23. The 40% cutoff value was derived from the literature[24] and the patient distribution, which was roughly divided in half. Figure 2 shows two different breast carcinoma tissues and their respective negative controls. Figure 2a is an example of a breast tissue that was graded as positive (100%) for nm23 expression and Figure 2c was graded as nm23 negative because <40% of the invasive tissue was stained for nm23. Figures 2b and 2d are the negative controls for Figures 2a and 2c, respectively. In the intraductal carcinoma tissues evaluated, 14 of the 16 (88%) specimens were positive for nm23 staining. In the two intraductal tissues negative for nm23 staining, only one was in the recurrent patient group. Among the invasive tumors evaluated, 21 of the 40 (53%) were negative for nm23 staining; of these, 12 were from recurrent and 9 were from nonrecurrent patients. The nm23 staining in all breast samples analyzed to date has been cytoplasmic.

The Kaplan–Meier disease free survival curve as a function of nm23 positivity is shown in Figure 3. The degree of nm23 positivity (<40% vs. ≥40%) did not offer a selective advantage against recurrence or survival, (Table 3) as would be predicted by the hypothesis that nm23 has antimetastatic properties. In addition, there was no statistically significant difference between nm23 positive (≥40% positive) and negative (<40% positive) populations with respect to the prognostic markers analyzed (Table 3). Additional statistical analyses were performed using 20%, 50%, 60%, and 80% cutpoints for nm23 positivity. These analyses showed that none of the cutpoints predicted either disease free or overall survival (data not shown).

DISCUSSION

The authors' experience in the analysis of breast carcinoma patient specimens, tissue culture cell lines, and cell lysates from primary human breast carcinoma xenografts, by both immunohistochemical and Western blot analysis, has shown that in some cases one procedure may be superior to the other. With human tumor cell lines grown in athymic nude mice models, the Western blot technique provides superior information compared with immunohistochemistry procedures because there is a high level of nonspecific tissue reactivity of mouse-derived tumors with certain detection antibodies. These tissues are easily analyzed for nm23-H1 and nm23-H2 proteins using Western blot techniques (Table 1). Immunohistochemistry may be more valuable in the analysis of human breast carcinoma, particularly in patients with very small tumor size, in whom extracts may not be available.

The authors have shown that nm23-H1 levels vary considerably between cell lines, xenografts, and breast carcinoma patients. The current data indicated that MCF-7 cells can metastasize to the lung; however, careful histologic analysis must be performed for detection of micrometastatic lesions. This observation is in agreement with Shafie et al.[25] The authors' data suggested a trend ($R = -0.51$) for lower nm23-H1 levels in both the xenografts and the cell lines that had high metastatic potential in athymic nude mice, compared with their nonmetastatic counterparts. More cell lines and xenografts need to be analyzed to prove whether this is a significant observation.

The analyses of the relationship between disease

TABLE 3
Comparison of Prognostic Factors as a Function of nm23 Positivity

Factor	nm23 NEG N = 21 Mean ± SE	(No.)	nm23 POS N = 19 Mean ± SE	(No.)	*P* value significance
Age (yrs)	61.0 ± 13.20		61.0 ± 17.50		0.860
Cathepsin D	51.9 ± 22.39		63.9 ± 26.26		0.098
Tumor size	1.9 ± 0.88		2.0 ± 1.03		0.684
% S-phase[a]	7.0 ± 5.20		7.2 ± 3.88		0.806
% Tumor[b]	48.6 ± 24.91		47.8 ± 26.26		0.684
% Aneuploid		63		57	1.000
% ER Positive		52		53	1.000
% PR Positive		62		47	0.750
Tumor Grade[c]					
1	0		1		0.424
2	8		5		
3	9		10		
Outcome	**(No.)**	**(%)**	**No.**	**(%)**	
No. of recurrences	12	(57)	9	(47)	0.752
No. of deaths	5	(24)	7	(37)	0.494

NEG: negative; POS: positive; SE: standard error; ER: estrogen receptor; PR: progesterone receptor.

[a] The nonrecurrent group contained 12 patients and the recurrent group contained 15 patients for percent S-phase analysis by flow cytometry.

[b] All slides analyzed contained a sufficient tumor component. The percent of tumor per slide analyzed is represented here.

[c] Tumor grade was available for 15 nonrecurrent and 18 recurrent patients.

recurrence, overall survival, and the levels of nm23 as measured by immunohistochemistry in the lymph node negative breast carcinoma population were not significant. The limitations of a retrospective study and the small number of recurrent and nonrecurrent patients may be responsible for the lack of correlation between nm23 levels and the risk of disease recurrence. However, these two groups were evenly matched for various clinical factors that might relate to disease recurrence. The purpose of matching these patients was to determine whether nm23 was an independent prognostic indicator. Based on the observations in this study, nm23 may not be an independent indicator of disease progression or aggressiveness.

Although several human breast carcinoma studies have shown an association between nm23 levels and patient survival and/or prognosis,[6–8] to the authors' knowledge this is the first attempt to control for all other prognostic factors that may obscure the true role of nm23 expression in patient survival. In particular, the association of low nm23 expression and lymph node involvement has proven significant in different studies.[2,6–7,9] Because this study selected only lymph node negative breast carcinoma patients, perhaps this is a reason for the lack of correlation of disease recurrence with nm23 positivity. Because lymph node involvement at the time of diagnosis is known to be associated with a poor prognosis, the next stage of analysis must be the predictive ability of nm23 levels for disease recurrence and survival in lymph node negative patients. From the purely clinical perspective, the usefulness of nm23 as a prognostic marker must be proven in the unknown risk group (lymph node negative at diagnosis). In this matched retrospective lymph node negative breast carcinoma population, nm23 expression was not predictive of either disease recurrence or overall survival. It is possible that there are potentially recurrent patients still undetected in the nonrecurrent data set. The probability of this was reduced by extending the follow-up time in the nonrecurrent patients; however, the range for the time to recurrence was between 0.7 and 9.2 years; therefore, there may be more undetected recurrences in the nonrecurrent group (Table 3).

The xenograft and cell line data support the possibility that nm23 levels may be related to metastatic potential but only more extensive prospective clinical studies will clarify the potential for nm23 as a predictor of clinical outcome in lymph node negative breast carcinoma.

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Relationship of nm23 to proteolytic factors, proliferation and motility in breast cancer tissues and cell lines

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Summary Low expression of the antimetastatic gene *nm23* has been associated with shorter overall survival in breast cancer. To better understand the mechanism(s) of action of this protein, we compared the levels of the nm23 protein in 152 breast cancer samples with other factors known to be involved in metastasis or related to prognosis. There was no significant relationship between either of the nm23 isoforms and cathepsin D (Cat-D), urokinase plasminogen activator (uPA), its inhibitor (PAI-1), steroid hormone receptors or ploidy status. A marginal inverse correlation was observed between per cent S-phase and *nm23-H1* expression ($r = -0.193$, $P = 0.047$) and a positive correlation was observed between uPA receptor (uPAR) and both *nm23-H1* ($r = 0.263$, $P = 0.0018$) and *nm23-H2* ($r = 0.230$, $P = 0.0064$). The *nm23-H1* gene was transfected into MDA-MB-231 human breast cancer cells and 12 clones were selected, of which two were characterized extensively. We found no significant differences in Cat-D, uPA, PAI-1 or uPAR, as a function of *nm23* expression in either the MDA-MB-231 cells or the transfected clones. Compared with the parent cell line, we did observe a dose-dependent decrease in growth factor-stimulated motility and a decrease in metastatic potential in two clones with four- and eightfold elevated *nm23-H1* expression, whereas the proliferative activities were similar. We conclude that the decreased metastatic potential might be related to down-regulation of growth factor-stimulated motility.

Keywords: nm23; protease; tumour suppressor; human breast cancer; transfection; motility

The genotypic alterations that accompany and/or determine the metastatic phenotype of cancer cells are not well characterized, but metastatic progression is thought to involve the accumulation of functionally additive genetic defects (Liotta and Steeg, 1991; Stracke and Liotta, 1992).

A specific gene family related to non-metastatic invasiveness of cancer was first characterized in 1988 and called *nm23*. This gene was identified using differential hybridization techniques in K1735 murine melanoma clones having different metastatic potentials (Steeg et al, 1988). The first member of the family, *nm23-H1*, has demonstrated antimetastatic properties in animal models (Leone et al 1991, 1993), and levels of nm23-H1 protein or RNA have shown an inverse correlation with lymph node status and patient survival in a number of human breast cancer studies (Bevilacqua et al, 1989; Barnes et al, 1991; Hennessy et al, 1991; Hirayama et al, 1991; Royds et al, 1993). A second member of this family, *nm23-H2* (Stahl et al, 1991), encodes for a protein that has 88% homology to nm23-H1. Finally, a third member of this family, *Dr-nm23*, has been isolated and shows a 65% homology to the other members and contains several of the key domains. This protein is found to be increased in leukaemia blast crisis and inhibits differentiation and induces apoptosis (Venturelli et al, 1995). Whereas the preponderance of evidence suggests that nm23-H1 protein expression is related to lymph node metastasis and patient survival, there is still some controversial data that dispute the role of *nm23* expression levels as predictive of nodal involvement and breast cancer patient survival (Goodall et al, 1994; Sawan et al, 1994; Russell et al, 1997). Correlation between nm23-H1 levels and prognosis of a number of other tumour types has also been demonstrated (De La Rosa et al, 1995).

No clear molecular mechanism of action that explains the antimetastatic role of nm23 has been demonstrated so far. Transfection of the *nm23-H1* gene in MDA-MB-435 breast cancer cells has been associated with reduced motility in response to growth factors (Kantor et al, 1993) and the development of ducts in vitro (Howlett et al, 1994). These observations suggest a role for nm23-H1 in motility responsiveness and tissue development. The nm23-H1 and nm23-H2 proteins are identical to the nucleoside diphosphate kinase (NDPK) A and B respectively (Gills et al, 1991). The kinase activity, however, has been dissociated from the antimetastatic role of nm23 (Leone et al, 1993; Sastre-Garau et al, 1992). The nm23-H2 protein has been identified as the human PuF factor (Ji et al, 1994; Postel, 1996), which is a transcriptional activator of the *c-myc* proto-oncogene. Although there has not been a positive correlation between the levels of expression of *nm23-H2* and metastatic potential, very few studies have been performed to address this issue.

The urokinase pathway of plasminogen activation and other proteolytic enzyme systems are thought to be involved in extracellular matrix degradation, facilitating tumour invasion and metastasis (Rochefort, 1990; Ossowski, 1992; Christensen et al, 1996). Additionally, the production of plasmin through the uPA cascade

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has been associated with the activation of latent metalloproteases and the regulation of certain growth factors (Sato and Rifkin, 1989; Lyons et al, 1990; Campbell et al, 1992). A number of studies have shown that patient survival is independently associated with the levels of proteolytic enzymes, their receptors and inhibitors (Duffy et al, 1990; Rochefort 1990; Kute et al 1992, 1997; Grøndahl-Hansen et al., 1993; Jänicke et al., 1993; Foekens et al., 1994; 1997).

This study was undertaken to evaluate the relationship between the levels of nm23 (H1 and H2) and uPA, uPAR, PAI-1 and Cat-D as a means of defining a biological mechanism for the antimetastatic effect of these nm23 isoforms. The levels of these isoforms were also compared with other cancer markers such as steroid receptors, %S activity and ploidy status. Additionally, nm23-H1-transfected breast cancer cells were used to evaluate any direct effect of nm23-H1 expression on invasion-related factors, proliferation, growth factor-stimulated motility and metastatic potential.

METHODS

Breast cancer tissue accrual and extraction

Human breast cancer tissue was acquired in a prospective manner from 152 patients diagnosed with breast cancer. The evaluation of various prognostic markers including nm23 was performed as part of a routine breast cancer panel. Fresh tissue was acquired directly from the operating room when surgery occurred in the hospital. When the sample was transported from an external site, the fresh tissue was transported on dry ice and immediately transferred to – 70°C until the tests could be performed. The only restrictive basis for selection of potential breast cancer tissues for inclusion in this study was the presence of an adequate tissue sample following routine breast cancer sample analyses (steroid hormone receptor status, DNA index and cell cycle kinetics). As a quality control, adjacent sections to all samples were analysed by standard haemoloxylin and eosin (H&E) histochemistry in order to verify tumour tissue content. The fresh breast cancer tissues were processed for biochemical steroid receptor analyses using standard tissue homogenization and high-speed centrifugation techniques (Kute et al, 1992). The resulting extract was analysed for total protein (Bio-Rad assay) and the various markers as described below.

nm23 measurement

nm23-H1 and nm23-H2 proteins were measured by a Western blot analysis using a standard curve containing known quantities of nm23-H1 and as described in detail previously (Russell et al, 1997). The detection limit was 250 pg μl^{-1}. Breast tumour extracts were analysed using SDS-PAGE electrophoresis and the levels of nm23 were measured by densitometry from the standard curve run concurrently with patient samples. A polyclonal antibody to nm23 was used. This antibody has been shown (Russell et al, 1997) to have similar reactivity in Western blot analysis to the peptide 11 antibody described by Leone *et al* (1993). The polyclonal antibody was a generous gift from Oncologix and recognizes both nm23-H1 and nm23-H2 by Western blot and immunohistochemical analyses (Russell et al, 1997).

Cat-D, uPA, uPAR and PAI-1 measurement

The analysis of Cat-D was performed using a commercially available radioimmunometric (RIA) kit (CIS Bio International, Bedford, MA, USA) with triplicate measurements for each sample (Kute et al, 1992). The detection limit was 31 pg ml^{-1}. The urokinase plasminogen activator (uPA), its receptor uPAR and its inhibitor (PAI-1) were measured using previously described ELISA techniques (Grøndahl-Hansen et al, 1993; Rosenquist et al, 1993; Rønne et al 1995) with detection limits of 25 pg ml^{-1}, 16 pg ml^{-1}, and 25 pg ml^{-1} respectively. In order to eliminate sampling artefacts, uPA, PAI-1 and uPAR analyses were performed using the same tissue extracts that had been previously analysed for nm23 and Cat-D at our institution.

Additional breast cancer prognostic markers

The evaluation of oestrogen and progesterone receptors, DNA ploidy and per cent S-phase was performed prospectively to determine the relationship between nm23 and these prognostic markers. Steroid receptor status was performed using either standard biochemical (Kute et al, 1992) or immunohistochemical methods (Barnes et al, 1996). Tumours were considered steroid receptor positive if they contained more than 10 fmol of receptor per mg of protein or if the steroid receptor was present in more than 10% of the tumour nuclei as seen in the immunohistochemistry procedure. DNA ploidy status and per cent S-phase were evaluated using flow cytometry (Kute et al, 1992) on fresh tissue. The percentage of tumour cells in S-phase was determined using the Modfit™ software analysis program (Kute et al, 1992).

Characterization and transfection of MDA-MB-231-BAG cells

The human breast cancer cell line, MDA-MB-231-BAG, (containing a *lacZ* gene) was previously described and stains positive for X-gal when fixed in glutaraldehyde and incubated overnight in X-gal staining reagent (Brünner et al, 1992). The BAG vector facilitated the detection of micrometastatic lesions in various tissues of the animals. Both H&E and X-gal staining were performed on all lungs and suspicious growths when examining the mice for metastatic disease.

The MDA-MB-231-BAG cells were transfected with the cDNA for nm23-H1 (kindly provided by Dr PS Steeg), which was inserted into the multiple cloning site of the delta pCEP4 vector using *Bam*HI and *Xho*I restriction enzymes. This vector contains the hygromycin selectable marker and uses the CMV early promoter to drive transcription of the inserted sequence. This vector was modified by the deletion of the EBNA and ori-O sequence to prevent episomal transcription and thus force integration of the vector into chromosomal DNA (Bunting and Townsend, 1996). The transfection was performed using standard calcium phosphate precipitation techniques and the successfully transfected clones were selected in 0.67 mg ml^{-1} hygromycin-containing medium. The dose of hygromycin chosen for selection was 99.9% cytotoxic to the parental cell line using clonogenic assays. Twelve clones were selected, two of which contained four (clone 40) and eight (clone 47)-fold elevated levels of nm23 when compared with the parental cells that contained 0.173 ± 0.047 ng μg^{-1} nm23-H1 protein as determined by Western blot analyses.

The cell pellets of the MDA-MB-231-BAG cells and nm23-H1 transfected clones were homogenized manually using 20 strokes in a ground glass mortar and pestle apparatus. All subsequent analyses for Cat-D, uPA, uPAR and PAI-1 expression were performed as described for the breast tumour samples.

Table 1 Description of parameters used in the study

Parameter	*n*	Median	Mean ± s d	Range
% S-phase	110	12.0	14.04 ± 9.37	0–36.00
% G_1 phase	110	81.0	79.5 ± 11.23	51–97
nm23-H1[a]	147	0.44	0.57 ± 0.48	0–2.77
nm23-H2[a]	147	0.30	0.42 ± 0.34	0–2.00
Cathepsin D[b]	150	45.3	51.40 ± 2.41	5.06–173.10
PAI-1[c]	144	0.96	2.20 ± 0.26	0–22.37
uPA-R[c]	144	0.92	1.40 ± 0.19	0–22.37
uPA[c]	144	0.75	1.38 ± 0.20	0–19.48

[a]ng μg^{-1} protein. [b]pmol mg^{-1} protein. [c]ng mg^{-1} protein.

The measurement of in vitro proliferation of MDA-MB-231-BAG-and MDA-MB-231-BAG-nm23-H1-transfected clones was performed by adding 0.4 million cells per flask and measuring the cell number in triplicate over the course of 7 days (see Figure 2B).

Motility studies

The evaluation of the motility of MDA-MB-231-BAG cells and the transfectants was performed without prior knowledge of nm23-H1 levels by Dr Kantor. Motility was assessed using a modified Boyden chamber assay as previously described (Kantor et al, 1993). The growth factors used in this study were 0.5% serum, lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF). The concentrations of growth factors used are listed in the legend to Figure 3.

Animal studies

Two sites on the hindquarters of the 6-week-old female athymic nude mice (Balb/c purchased from Goodwin Institute for Cancer Research, Plantation, FL, USA) were inoculated with 10^6 cells (MDA-MB-231-BAG, clones 40 or 47) each. Ten animals were included in each group. The tumours were allowed to grow for 32 days, at which time the surviving animals were sacrificed. The in vivo growth of the MDA-MB-231-BAG- and MDA-MB-231-BAG-nm23-H1-transfected cells was assessed over the course of a 5-week period of time using two-dimensional analysis of each of two tumour sites on each of ten animals. The mean tumour size was evaluated for the parent and each of the two nm23-H1-transfected clones.

The lungs were removed from each animal and, during the autopsy, any suspicious area was also collected. These tissues were fixed with glutaraldehyde and then stained for β-galactosidase activity as previously described (Brünner et al, 1992). Subsequently, the tissues were formalin fixed and paraffin embedded. The analysis of the mouse lungs and other suspicious growths was performed by a pathologist (K Geisinger) using both H&E and X-gal-stained tissues. Each lung or suspected tissue was analysed using a multilevel technique with a minimum of 3–4 levels (50 μm apart) to enhance the detection of micrometastatic disease. Any animal tissue that contained tumour cells was defined as positive for metastasis.

Table 2 Relationship of nm23-H1 and nm23-H2 to steroid receptor status and ploidy

		nm23-H1 (ng μg^{-1} protein)			nm23-H2 (ng μg^{-1} protein)		
	n	Median	Mean (s.d.)	*P*-value	Median	Mean (s.d.)	*P*-value
ER⁻	77	0.456	0.540 (0.483)	0.48	0.290	0.404 (0.342)	0.48
ER⁺	70	0.490	0.579 (0.485)		0.317	0.425 (0.343)	
PR⁻	84	0.510	0.579 (0.486)	0.60	0.299	0.420 (0.348)	0.65
PR⁺	62	0.442	0.564 (0.488)		0.306	0.409 (0.346)	
Diploid	46	0.506	0.551 (0.345)	0.66	0.366	0.404 (0.244)	0.52
Aneuploid	94	0.445	0.618 (0.698)		0.296	0.441 (0.494)	

Table 3 Relationship of nm23-H1 and nm23-H2 to each other and to other prognostic markers

Factor		nm23-H1		nm23-H2	
	n	*r*-value	*P*-value	*r*-value	*P*-value
nm-23-H1 (ng μg^{-1} protein)	147			0.751	0.0001
% S-phase	106	–0.193	0.047	–0.122	0.21
% G_1 phase	106	0.172	0.078	0.107	0.27
Cathepsin D (pmol mg^{-1})	145	0.112	0.18	0.048	0.57
uPA (ng mg^{-1} protein)	139	0.096	0.26	0.046	0.59
uPAR (ng mg^{-1} protein)	139	0.263	0.0018	0.230	0.0064
PAI-1 (ng mg^{-1} protein)	139	0.139	0.10	0.125	0.14

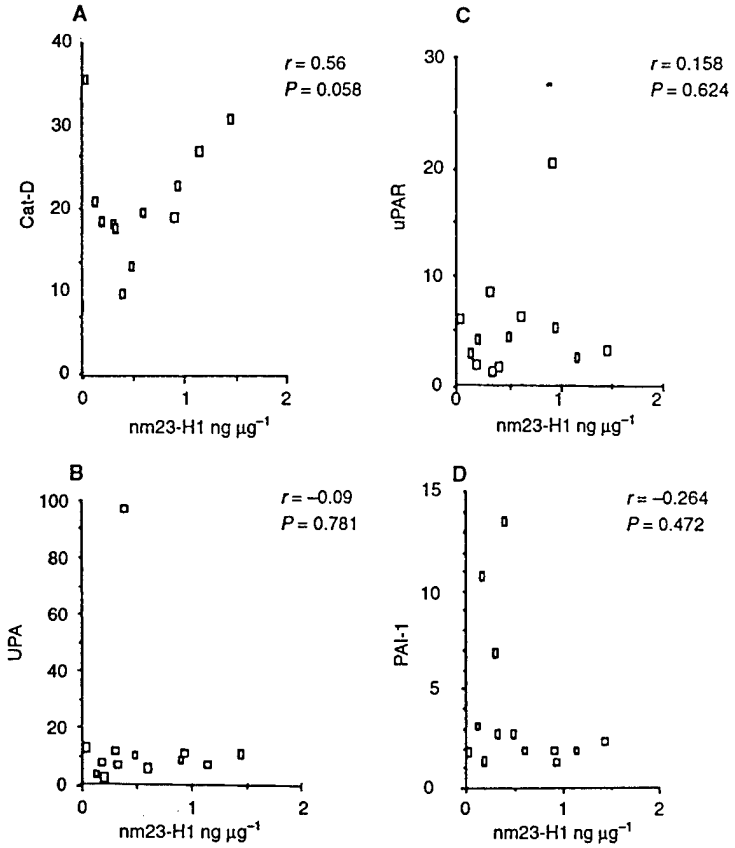

Figure 1 Cell pellets were obtained from MDA-MB-231-BAG cells and each of 12 clones transfected with nm23-H1. The lysates were used to measure nm23-H1 in relation to Cat-D (A), uPAR (B), uPA (C) and PAI-1 (D). The comparison between the levels of nm23-H1 expression and the expression of each of these proteases and protease-related factors demonstrated no significant correlation

Statistical analyses

Relationships between nm23 isoforms and Cat-D, uPA, uPAR, PAI-1, per cent S-phase, were evaluated using Spearman correlation coefficients. The relationships involving nm23-H1 and nm23-H2 with steroid receptor and DNA ploidy status were analysed using the Wilcoxon ranked-sum statistics. Fisher's exact test was used to assess difference in per cent metastatic potential in Table 3. Analyses of the effect of nm23-H1 expression on motility were performed using analyses of variance.

RESULTS

Assessment of the distribution of nm23-H1 and nm23-H2 protein levels revealed a great deviation from normality. Both log and square root transforms of the data did not improve the situation, prompting use of non-parametric techniques in the analysis. Descriptive statistics including the median, mean with standard deviation and ranges for nm23-H1 and H2, per cent S-phase, proteases (Cat-D, uPA), PAI-1 and uPAR for this group of patients are given in Table 1. The patient population exhibited a wide variability with respect to the levels of the markers analysed as would be expected from a random, prospective analysis. The levels of expression of the proteolytic factors are consistent with our previous findings (Kute et al, 1997). Of the tumours analysed, 68% were aneuploid, 47% were oestrogen receptor positive and 42% were progesterone receptor positive (Table 2). The DNA ploidy and steroid receptor status fall within the normal limits for a breast cancer population.

Analyses of the correlation between the levels of nm23 isoforms and per cent S-phase, Cat-D, uPA, uPAR and PAI-1 are shown in Table 3. There was a strong correlation between the levels of *nm23-H1* and *H2* expression within a given tissue ($r = 0.75$, $P = 0.0001$). This observation is not surprising considering the co-ordinate regulation of these two genes. There was no relationship between the levels of nm23-H1 or -H2 and the proteases Cat-D and uPA, or the uPA inhibitor, PAI-1. Whereas there was a positive direct correlation between both nm23-H1 and -H2 with uPAR expression ($r = 0.26$, $P = 0.0018$, and $r = 0.23$, $P = 0.0064$, respectively), the relatively low correlation coefficient values call the biological relevance of these observations into question. As elevated levels of uPAR have been shown to have a poor prognosis in breast cancer (Grøndahl-Hansen et al, 1995), the positive correlation between uPAR and the antimetastatic gene *nm23* was therefore unexpected. Although the magnitude was low, there was a significant inverse relationship between nm23-H1 and per cent S-phase ($r = -0.19$, $P = 0.047$). Using G_1 as an inverse of proliferation, a direct correlation was observed as one would expect (Table 2B). Further evaluation of the relationship between nm23-H1 and per cent S- and G_1 phases of aneuploid and diploid populations was performed independently and did not improve the correlation coefficient (data not shown).

The statistical analysis of the relationship between nm23-H1 and -H2 levels with ER, PR and DNA ploidy is shown in Table 2.

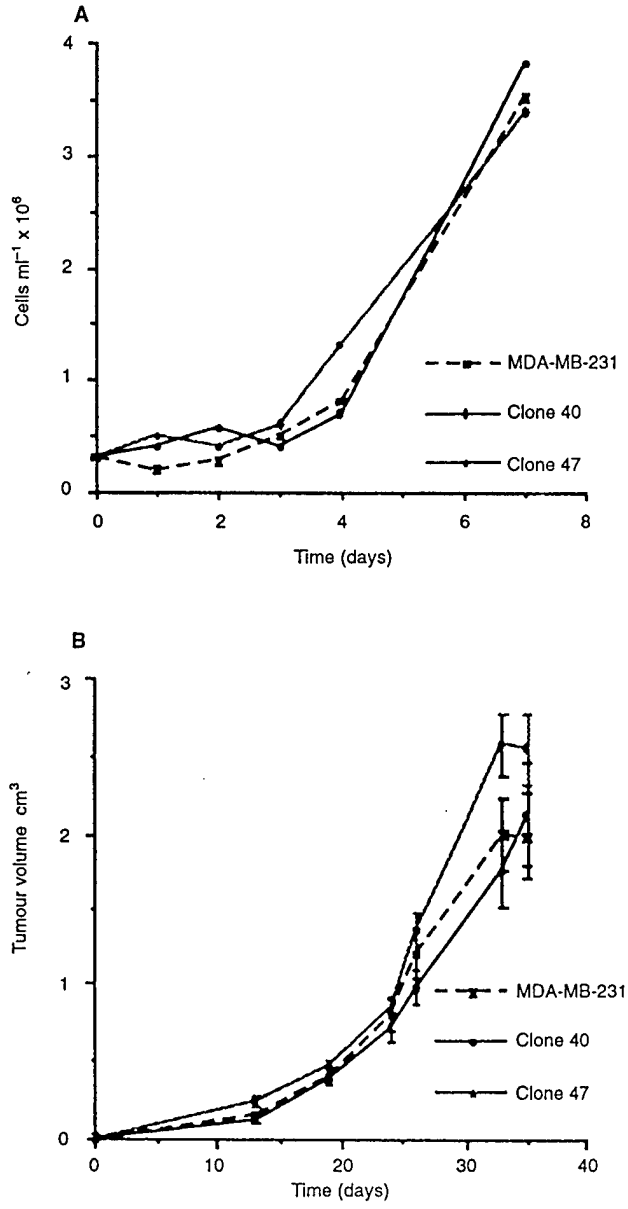

Figure 2 A MDA-MB-231-BAG cells and clones 40 and 47 (4×10^5) were added to 25-cm^2 flasks containing DMEM, 10% FCS, P/S, L-glutamine and non-essential amino acids. Cells were harvested with 0.25% trypsin–versine daily for 7 days and cells counted. B MDA-MB-231-BAG cells and clones 40 and 47 were injected into the hind quarters of athymic nude mice and the resulting tumours were measured every 7–12 days for 32 days

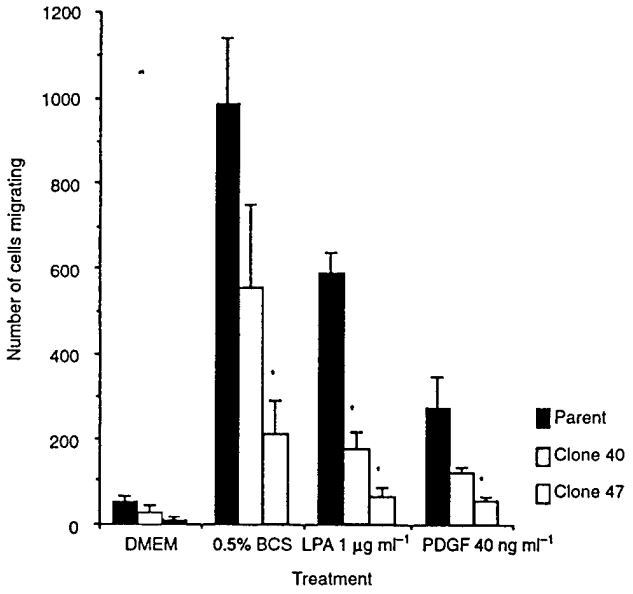

Figure 3 MDA-MB-231-BAG parent and clones 40 and 47 were analysed for random (DMEM) and growth factor-stimulated motility using a modified Boyden chamber assay. Results represent the mean of six measurements from two independent experiments. *Results that were statistically different from untransfected cells using analysis of variance

Table 4 Metastatic potential of MDA-MB-231-BAG and two nm23-H1 transfected clones

Group	No.	Lung mets	Other mets	Overall mets	(%)
MDA-MB-231 parent	7	3/7	3/7	5/7	(71)
Clone 40	8	1/8	3/8	3/8	(38)
Clone 47	10	4/10	1/10	4/10	(40)

These data demonstrate that there is no statistically significant relationship between these parameters

To address further the relationship between the expression of the Cat-D, uPA, uPAR and PAI-1, and nm23-H1, the human breast cancer cell line (MDA-MB-231-BAG) was transfected with the modified cDNA for nm23-H1 (Leone et al, 1991).

Cell pellets of the 12 nm23-H1-transfected clones were obtained from log growth cells and cell extracts were analysed for the levels of Cat-D, PAI-1, uPA and uPAR using the same methods as for the breast tissue extracts. There was no significant relationship between the level of expression of nm23-H1 and any of these factors (Figure 1), although there was a trend towards high nm23-H1 expression in association with high Cat-D levels ($r = 0.56$, $P = 0.058$). These data thus support the observations described above concerning the prospective breast cancer study, which suggests that nm23-H1 levels are not highly related to the expression of Cat-D, uPA, PAI-1 or uPAR. As this comparison is using a pure population of cancer cells, one would speculate that it would correct for sampling artefacts that are always present when using solid tumour tissues.

Of the 12 transfectants, two clones were selected (clone 40 and 47) that contained four- and eightfold elevated levels of nm23-H1, respectively compared with the untransfected parental cell line, which contained 0.173 ± 0.047 ng µg^{-1} protein. These clones were evaluated for proliferative activity with both in vitro and in vivo systems and compared with the growth characteristics of the parent cell line. The data are shown in Figure 2A and B. There was no significant difference between the proliferation of clones 40 and 47 (high nm23-H1) and the proliferative rate of the untransfected MDA-MB-231 BAG (parent) cells in tissue culture when analysed daily for 7 days (Figure 2A). The injection of 10^6 cells into the hindquarters of nude mice resulted in similar rates of growth of MDA-MB-231-BAG parent and clones 40 and 47 as determined by weekly two-dimensional measurements of tumour size for 32 days (Figure 2B).

We also performed studies in nude mice to determine whether the MDA-MB-231-BAG cells transfected with nm23-H1 showed a reduced metastatic potential when compared with the parental cell line, which expresses very low levels of nm23-H1. The advantage of using the MDA-MB-231-BAG cells to detect the metastatic lesions in nude mice is evident in that micrometastatic lesions are easily detected when the excised mouse lungs have been treated with the β-galactosidase substrate, X-gal. Whereas the MDA-MB-435-BAG cells tend to produce large metastatic foci in the lungs of nude mice (Brünner et al, 1992), the MDA-MB-231-BAG cells produce very small foci that are not readily detected by the eye.

Each nude mouse was injected with 10^6 MDA-MB-231-BAG cells or MDA-MB-231-BAG-nm23-H1 clones into the hind quarter. The tumours were allowed to grow (Figure 2B) for 32 days, at which time the animals were sacrificed. The lungs and any visually suspicious growths were analysed for the presence of metastatic cells. The data depicting the metastatic potential of the nm23-transfected cells are shown in Table 4. These data demonstrate that injection of the nude mice with the MDA-MB-231-BAG-nm23-H1 clones resulted in between 40% and 44% fewer animals developing metastases, compared with mice injected with the untransfected cells. These preliminary data thus show a trend that high expression of nm23 results in a lowering of the metastatic potential of the cells. However, analysis of the data using Fisher's exact test demonstrated that the groups were not significantly different. During the period of tumour growth in these animals, the control group lost three of ten animals, whereas the transfected groups lost only 3 out of 20 animals. It is not known why the control animals seemed to die prematurely as all the animals were given the same amount of tumour burden at the start. One of the animals in the transfected group could be used in the metastatic potential analysis (Table 4).

The in vitro motility of the MDA-MB-231-BAG cells and nm23-H1-transfected clones was investigated using a modified Boyden chamber assay (Kantor et al, 1993). The results of theses studies are summarized in Figure 3. Whereas there was no difference in random (unstimulated) motility between untransfected MDA-MB-231-BAG cells and nm23-H1-transfected clones, there was a significant reduction in growth factor [0.5% bovine calf serum (BCS), 1 μg ml^{-1} lysophosphatidic acid (LPA) and 40 ng ml^{-1} platelet derived growth factor (PDGF)]-induced motility in the clones. The differences in growth factor-stimulated motility are represented as the mean of six replicates performed in two independent experiments. Figure 3 shows that the number of cells migrating in response to LPA, PGDF and 0.5% serum decreased in a dose-dependent manner as a function of nm23-H1 concentration. These data suggest a possible role for nm23-H1 in the regulation of motility responsiveness to growth factor stimulation. This effect on motility has been previously demonstrated in the MDA-MB-435 breast cancer cell line (Kantor et al, 1993). Our results confirm this important observation in a different breast cancer cell line, indicating that down-regulation of motility by nm23 in breast cancer tissues may be a necessary feature of the biological mechanism of action of this protein.

DISCUSSION

The biological mechanism(s) by which nm23 attenuates metastatic disease has not been clearly defined. The hypothesis that nm23-H1 may serve a role in the regulation of motility, proliferation, proteases (uPA, and Cat-D), protease inhibitor (PAI-1) or protease receptor (uPAR) expression was investigated in solid tumors and in cells transfected with nm23-H1 to establish a role for nm23 in metastasis suppression.

In 152 human breast tumour extracts, there was no relationship between the levels of nm23-H1 or -H2 and DNA ploidy, steroid receptor status, proteases (Cat-D, and uPA) or PAI-1. Analyses of the relationship between the levels of nm23 isoforms and the expression of uPAR showed a significant correlation. This observation was unexpected given that elevated levels of uPAR have been associated with poor prognosis in breast cancer patients (Grøndahl-Hansen et al, 1995), and elevated levels of nm23-H1 have been associated with a good prognosis. Although this statistical observation cannot be overlooked, it is difficult to explain what the relationship between uPAR and nm23-H1 may be. Expression of components of the uPA system have been localized to different types of cells in breast cancer tissues and the expression level of these components seems to vary with tumour differentiation (Christensen et al, 1996) Would this be a factor in how these markers predict prognosis? In a recent retrospective study of a small number of patients, nm23 and erbB-2 expression by immunohistochemistry predicted disease-free survival in a univariate analysis. Other factors such as cathepsin D and p53 were of borderline utility (Han et al, 1997). This is encouraging but more studies need to be done. It is also very important to determine what method of analysis for these markers would yield the best results. Prospective studies that investigate patient prognosis would be especially useful in determining the clinical significance of these markers. Our present studies are ongoing with this patient population but the mean follow-up time at present is less than 2 years and would therefore not yield valid information at this time.

Although there was a statistically significant inverse relationship between the levels of nm23-H1 and per cent S-phase in the patient tumour population, the correlation was low and the in vitro and in vivo MDA-MB-231-BAG clonal data suggest that nm23 expression is not related to proliferation. Yet a positive correlation between per cent S-phase and nm23 RNA expression has been shown in both breast cancer cell lines and solid tumours, using RNA extraction, [^{3}H]-thymidine labelling and flow cytometry (Caligo et al, 1995). It is, therefore, possible that the relationship between nm23 and per cent S-phase is relevant and the analysis of clonal populations for similar correlations is not representative of the multiple factors that modulate proliferation in vivo. If this is the case, only large prospective clinical studies can adequately address this issue.

The role of nm23 as a metastasis-suppressor gene is suggested in the animal experiments where 40–50% fewer animals developed metastases with the MDA-MB-231-BAG clones expressing elevated levels of nm23-H1. Although not statistically significant, these data are in general agreement with the observation by Leone et al (1993) using the MDA-MB-435 human breast cancer. They demonstrated a 78% reduction in metastatic lesions in animals injected with the nm23-H1-transfected cell line compared with the mock-transfected cells. The reasons as to why our results are less pronounced are unknown except that we used a different cell line and our protocols were different. Although it is hard to quantify the amount of metastatic disease in the lungs using standard histological analysis, we did find that the amount of cancer cells in the lung tissue was higher in the animals with the parental tumours than in the animals with the nm23-transfected cells. Further studies need to be performed to quantify the tumour load in these animals.

The role of nm23-H1 in suppressing growth factor-stimulated motility is in line with the suggested antimetastatic mechanism of action of nm23. The MDA-MB-435 breast cancer cell line exhibited reduced response to motility-stimulating factors following nm23-H1 transfection (Kantor et al, 1993). Recently, site-directed mutagenesis studies have identified critical amino acids required for this motility responsiveness (MacDonald et al, 1996). This study showed that mutation of either proline-96 to serine or serine-120 to glycine, caused MDA-MB-435-wtH1-transfected cells to revert to parental levels of motility responsiveness to 0.5% serum or autotaxin. Recent studies also using site-directed mutagenesis of serine-120 and proline-96 have attributed a biological mechanism for the abrogation of motility suppression by these mutations (Freije et al, 1997). The analyses of purified nm23 mutants of proline-96 and serine-120 demonstrated alterations in autophosphorylation and histidine kinase activity (Freije et al, 1997). The combined observations of MacDonald et al and Freije et al suggest a biological link between motility responsiveness and structure and function of nm23-H1. Freije et al propose that metastatic potential may be related to protein histidine kinase activity where increased activity favours the non-metastatic state.

Three key events in the metastatic cascade (proteolysis of extracellular matrix, motility and proliferation/colonization) have been addressed in this study. The data suggest that only changes in growth factor-stimulated motility is related to nm23-H1 expression. We are currently investigating the possibility of a common signal transduction pathway that is affected by the levels of nm23-H1 expression.

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366 **EFFECT OF NM23 EXPRESSION LEVELS IN GI-101A AND ITS EFFECT ON METASTATIC POTENTIAL IN ATHYMIC NUDE MICE.** Dauphinée M*, Raney S, Russell R, Gaisinger K, Kute T. The Goodwin Institute for Cancer Research, Plantation, Florida 33313, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27157-1072.

The nm23 gene, a putative metastasis suppressor gene, was originally identified by its reduced expression in highly metastatic K-1735 murine melanoma cell lines, as compared to related, low metastatic melanoma cell lines. GI-101A, a human mammary tumor cell line, was transfected with a hygromycin selected nm23 vector and compared with the mock transfected original cell line for differences in tumorigenicity and lung metastasis following injection and growth in athymic nude mice. The original tumor GI-101 was an aggressive, infiltrating ductal adenocarcinoma (stage IIIa, T3N2MX). The cell line GI-101A is an adherent, non-fastidious tumor with a doubling time of 48 hours which is also metastatic to the lungs.

Cell Line/ Tumor	nm23 Expression (ng/μg ± SE)[1]	# Assays	# Mets/# Animals[2]
GI-101	0.258 (0.038)	9	46/50 (92%)
GI-101A	0.380 (0.026)	3	5/13 (39%)
Clone 10	1.990 (0.615)	3	8/15 (53%)
Clone 15	2.350 (0.388)	3	5/13 (39%)

[1]Nm23 expression was done by western analysis as described in Cancer 1997, 79: 1158-1165.

[2]The lungs were sectioned at 6 different levels. GI-101 data was pooled over a two year period.

The cell line GI-101A, compared to tumor GI-101, has similar levels of nm23, but less potential for metastasis. After transfection of nm23 into GI-101A, we observed increased nm23 levels but no increase in lung metastasis.

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368 METASTATIC POTENTIAL AND IMPAIRED MACROPHAGE MEDIATED CYTOTOXICITY ARE DIRECTLY CORRELATED WITH ANNEXIN I EXPRESSION IN RAT MAMMARY ADENOCARCINOMA. Scot D. Pencil University of Texas Medical Branch, Galveston TX 77555

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Report

Low cathepsin D and low plasminogen activator type 1 inhibitor in tumor cytosols defines a group of node negative breast cancer patients with low risk of recurrence

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Key words: prognostic markers, proteases, protease inhibitors, node negative

Summary

Prognostic factors are highly needed to divide node negative breast cancer patients into groups of low versus high risk of recurrence and death. In order to invade and spread, cancer cells must degrade extracellular matrix proteins. Accordingly, tumor levels of molecules involved in this degradation might be associated with patient outcome. Previous work has demonstrated that high levels of the aspartyl protease cathepsin D in breast cancer are associated with a poor prognosis and similar findings have been reported for molecules involved in the urokinase pathway of plasminogen activation. Interactions between different protease systems have been described and data suggest that several proteolytic enzymes may be operable at the same time in a tumor.

In the present study we measured cathepsin D (n = 162), uPA (n = 116), uPAR (n = 109) and PAI-1 (n = 135) in tumor cytosols obtained from a population of node negative breast cancer patients. A significant correlation was found between levels of uPA, uPAR, and PAI-1. Levels of cathepsin D were directly related to levels of uPA and uPAR. With a median observation time of 4.81 years, univariate survival analyses showed that high levels of uPA and cathepsin D significantly predicted a shorter disease free survival, while only high levels of cathepsin D were able to significantly predict a shorter overall survival. Tumor levels of uPAR and PAI-1 gave mixed results depending on the cut-off point choosen. Interestingly, multivariate analysis demonstrated that PAI-1 and cathepsin D were independent significant prognostic indicators for disease-free survival while only cathepsin D was helpful in overall survival. The five year relapse rate of patients with low PAI-1 and low cathepsin D was 13% while patients who had greater than the median value for both of these molecules had a 5 year relapse rate of 40%. These data would indicate that at least two different protease systems are active in spread of node negative breast cancer and that measurement of these molecules may aid in the decisions to be made when offering adjuvant treatment to these patients.

Introduction

The invasive nature of malignant tumors is due to the coordinated expression of several families of proteolytic enzymes [1]. This proteolytic activity is regulated by a dynamic equilibrium between the proteases and their inhibitors, as well by their binding to specific cell surface receptors. While invasion

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and metastasis are the primary reasons for the mortality associated with cancer progression, several investigators have reported an association between patient outcome and tumor content of proteolytic enzymes, their receptors, and their inhibitors [2–7].

The lysosomal protease cathepsin D was the first protease to be associated with prognosis in breast cancer, high levels of this enzyme being a marker of short recurrence free survival and short overall survival [6, 7]. Later, components of the serine protease system, urokinase plasminogen activator (uPA), its receptor uPAR, and the type one inhibitor (PAI-1) have all been shown to be significant predictors of patient outcome in node negative and node positive breast cancer [2–5, 8], with high levels predicting a poor prognosis. The above studies were all performed by either ELISA or RIA procedures. Lastly, high levels of the cysteine proteases cathepsin L and B were shown to be related to prognosis in breast cancer [9], while data on collagenases and stromelysins as prognostic markers in breast cancer are still scarce.

Although they are significant independent prognostic markers in breast cancer, none of these molecules provide a definite prognostic separation of node negative patients. Interactions between the different enzymatic systems have been described [10, 11], e.g. cathepsin D can activate cathepsin B, which in turn regulates extracellular collagenases. Cathepsin B can activate pro-uPA, resulting in the formation of plasmin which in turn can activate some pro-collagenases [10, 11]. These data suggest that several proteolytic enzymes may be operable at the same time in a tumor and work in a cascade mechanism. Also, a certain degree of functional overlap regarding substrate specificity may exist between the different proteolytic pathways, which is supported by the apparent lack of major changes in phenotype in mice gene-disrupted for various proteases [12]. Thus, establishment of a proteolytic profile, e.g. measurement of several different proteolytic enzyme systems in breast tumors, might more exclusively relate to patient outcome than the level of a single protease, or its receptor or inhibitor.

By measuring components of the serine protease system and the lysosomal protease cathepsin D in tumor cytosols obtained from node negative breast cancer patients, we here show that when combining cathepsin D and PAI-1 values, a group of patients characterized by having low cathepsin D and low PAI-1 content in their tumors can be identified. The five year recurrence rate of this group is only 13% compared with 35% in the group of patients whose tumors contain high levels of both cathepsin D and PAI-1.

Materials and methods

Patients and tumor tissue

A total of 162 patients were included in the study. The median follow-up time was 4.81 years with a range of 0.3 to 14 yrs. All patients included were defined as having node negative breast cancer based on the histological inspection of at least 4 ipsilateral axillary lymph nodes. Information on tumor size, tumor grade, and patient age at the time of diagnosis was obtained from the pathology reports. Data on clinical follow-up was initially obtained by direct correspondence to the physician and then was followed by annual letters or by use of local tumor registries. There were 142 patients with clinical follow-up, with 27 deaths and 29 relapses. None of the patients had any adjuvant therapy except for local radiation treatment. Disease-free survival (DFS) was defined as the time from initial diagnosis to first recurrence, and overall survival (OS) was defined as the time from initial diagnosis until death. The cause of death was breast cancer in 80% of the cases.

Remaining cytosols from tumor tissue sent to the steroid hormone receptor laboratory for estrogen and progesterone receptor measurements were stored at – 75° C until used in the present study. Cytosols were prepared as previously described [13]. All of the extracted tumors were confirmed by histology to contain cancer tissue.

Steroid receptors

These methods have previously been published [13]. The receptors were determined by the biochemical method with 10 or more femtomoles/mg of protein being considered positive. The protein

content of the cytosolic extracts was determined by the Waddell procedure [13].

Cathepsin D measurements

Previous results have been reported and were used in this paper in order to better define the full extent of the analysis [7]. All samples were normalized to pmoles per mg of protein. Only the data from the RIA procedure were used here. Similar results were obtained using a biochemical assay for cathepsin D [7].

ELISA measurements

Remaining cytosolic tumor extracts from the steroid hormone receptor assays were used to determine the content of uPA, uPAR, and PAI-1. The various ELISAs used have previously been described [3, 8, 14]. It should be noted that the extraction procedure in these studies used low salt and non-detergent buffers and will therefore give lower values. uPA and PAI-1 were measured as international and interim units [3], respectively, and uPAR as ng [8]. All values were calculated as per mg protein in the cytosols. The number of patients with defined variables was different due to lack of cytosol for the later assays or failure of the results to be interpretable.

Statistical methods

Univariate, descriptive statistics, and frequencies were generated for the continuous and categorical variables. Spearman's correlation was used to assess the relationship between the various prognostic factors. The Kaplan-Meier method [15] was utilized to estimate DFS and OS times. Log-rank tests were used to assess the univariate effects of variables on DFS and OS times. Cox's proportional hazards model [16] was used to examine the differences in DFS and OS after adjustment for other covariates.

Results

Characteristics of patients

Table 1 shows a summary of the characteristics of the 162 patients included in the study. Based on the clinical data presented, this group of patients is considered representative of a node negative breast cancer population with a mean age of 60.5 years, and a mean tumor size of approximately 2 cm, with most of the tumors being grade II (42%) or grade III (37%). Also the results of the estrogen and progesterone receptor assays were similar in distribu-

Table 1. Characterization of patients

Characteristics		Number	Mean	Median	Range
Age		162	60.5	62	32 to 95
Tumor size (cm)		159	2.03	2.00	0.15 to 6.0
Tumor grade	I	27 (20%)			
(N = 132)	II	56 (42%)			
	III	49 (37%)			
Steroid receptors (fmoles/mg of protein)					
Estrogen receptor (% positive = 59%)		162	72.7	18.5	0 to 811
Progesterone receptor (% positive = 44%)		161	71.7	0.0	0 to 580
Cathepsin D analysis (pmoles/mg of protein)					
RIA assay		159	52.3	51.8	0 to 160
uPA system analysis (ELISA procedure)					
uPA (IU/mg protein)		116	0.064	0.040	0 to 0.406
PAI-1 (Interim units/mg protein)		135	1.14	0.72	0 to 9.16
uPAR (ng/mg protein)		109	0.70	0.51	0.11 to 3.45

Table 2. Significant correlations of the various markers

Markers analyzed	Number	P value	R value
Estrogen receptor to progesterone receptor	161	0.0010	0.65
Cathepsin D to uPA	116	0.0019	0.28
uPA to tumor grade	89	0.0109	0.27
uPA to uPAR	103	0.0039	0.28
uPA to PAI-1	116	0.0228	0.21
PAI-1 to uPAR	109	0.0026	0.28
Cathepsin D to uPAR	109	0.0322	0.21

tion to what has been reported in the literature [17]. The median values of uPA, uPAR, PAI-1, and cathepsin D found in the present study are all in the ranges of those reported previously [3, 7, 8].

Correlations between uPA, uPAR, PAI-1, and cathepsin D

Table 2 shows the significant correlations between the investigated molecules. It should be noted that all measurements were performed on the same cytosolic fraction prepared in the same manner. uPA, uPAR, and PAI-1 were weakly but significantly correlated to each other. By including cathepsin D, it was found that cathepsin D was significantly correlated with uPA and uPAR (Table 2), while no correlation was found between cathepsin D and PAI-1 ($p = 0.82$ and $r = 0.02$). We also observed a strong correlation between estrogen and progesterone receptors. There was no significant correlation of cathepsin D or PAI-1 with either ploidy or %S as defined by flow cytometry (data not shown).

Prognostic significance

When treating the data as continuous variables (procedure 1 Table 3), the only clinical parameter which seemed to predict overall survival was tumor grade. For the biochemical parameters using continuous variable analysis, high levels of PAI-1 and cathepsin D predicted a shorter DFS and high levels of cathepsin D predicted a shorter OS. In order to define a better cut-off value, univariate analyses were performed using a 30% positive to 70% negative cut point (procedure 2 Table 3). This cut point was chosen from a clinical viewpoint since it is known that 30% of the patients with node negative disease will have a recurrence over 10 years. Table 3 shows the results of these analyses. It appears that

Table 3. Univariate analysis of factors included in this study

Factors analyzed	Calculated values*					
	Disease free survival			Overall survival		
	# 1	# 2	# 3	# 1	# 2	# 3
Clinical factors						
Age	NS*	NS	NS	NS	NS	NS
Tumor size	NS	NS	NS	NS	NS	NS
Tumor grade	NS	–	–	0.04	–	–
Biochemical factors						
Cathepsin D	0.04	0.0002	0.0005	0.003	0.007	0.01
uPA	NS	0.005	0.05	0.08	NS	NS
PAI-1	0.04	0.07	NS	NS	NS	NS
uPAR	0.06	0.07	0.02	NS	NS	NS
Estrogen receptor	NS	NS	NS	NS	NS	NS
Progesterone receptor	NS	NS	NS	NS	NS	NS

* NS equals any value greater than 0.10.

1 analysis – data from analysis using the actual numbers with no cut point analysis.

2 analysis – data from analysis using 30% of high values compared to a 70% of the lower values. This is more representative of a clinical recurrence rate as seen in patients.

3 analysis – data from analysis using the median value as the cut point.

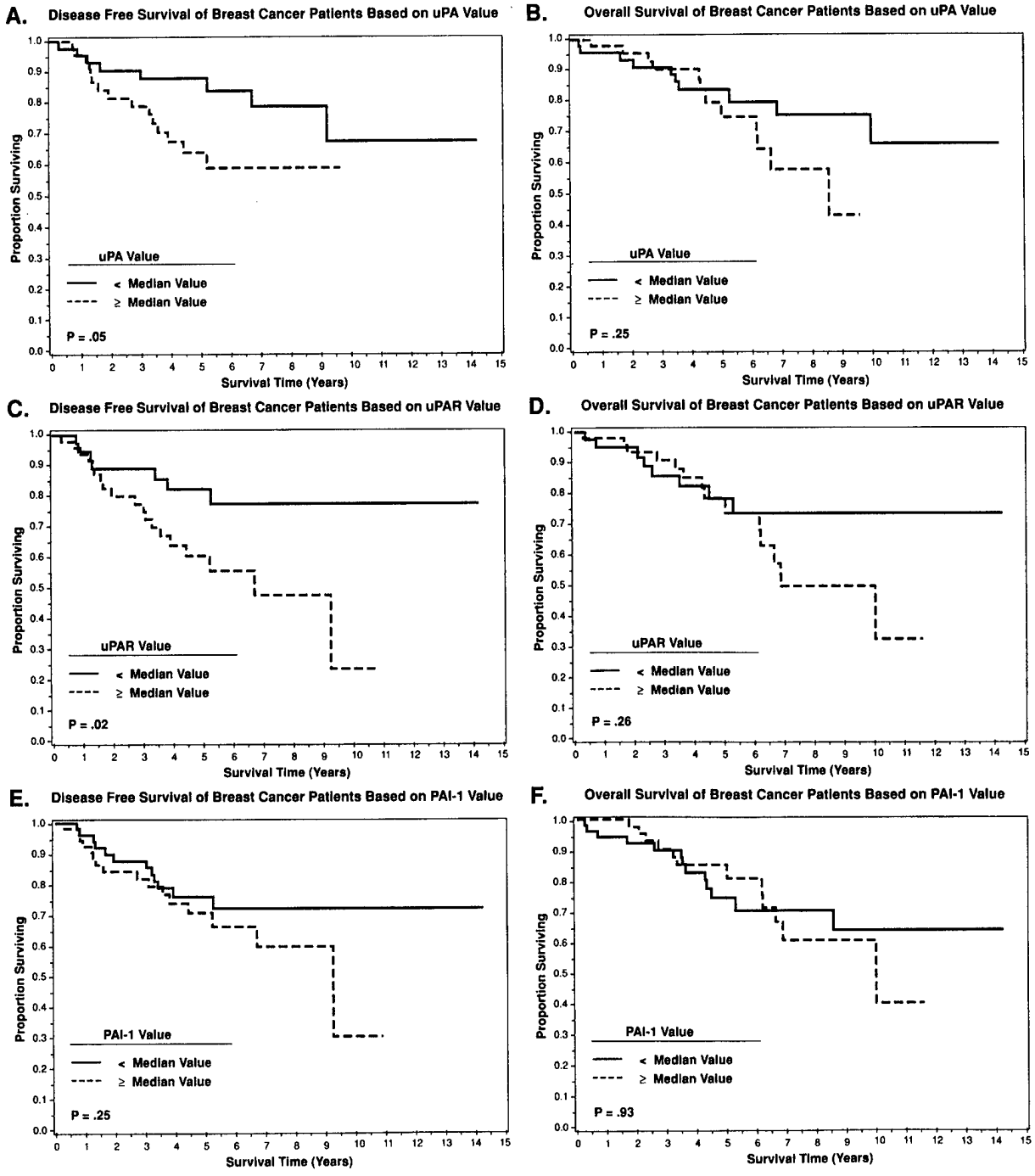

Figure 1. The disease free survival and overall survival for patients expressing uPA, uPAR, and PAI-1. Patients were divided into below the median value (——) or equal to or above (----) the median value for the various prognostic markers. Panels A, C, and E represent disease free survival for uPA, uPAR, and PAI-1, respectively. Panels B, D, and F represent overall survival for uPA, uPAR, and PAI-1, respectively. The median values for uPA, uPAR, and PAI-1 are 0.04 IU/mg protein, 0.51 ng/mg protein, and 0.73 interim units/mg protein respectively. The p values are given in the figures.

high level of cathepsin D predict a significantly shorter DFS and a significantly shorter OS. High tumor level of uPA significantly predicts a shorter DFS with this cut point procedure, while no significant relation was found between uPA level and OS. With respect to PAI-1 and uPAR, there was a trend toward an association of low values predicting a longer DFS. Finally, the cut point set at the median value (procedure 3 Table 3). Kaplan-Meier curves for DFS and OS for each of the measured components of the uPA system using the median value as cut-off point demonstrate that only uPA and uPAR predict DFS and none of the uPA system components predict OS (Figure 1). Previously, data have already been published which indicates that high cathepsin D levels were able to predict shorter DFS and OS using the median value, 52 pmoles/mg of protein [7].

Multivariate analysis

In order to compare the prognostic impact of tumor cytosolic levels of uPA, uPAR, PAI-1, and cathepsin D with that of other parameters, multivariate analyses were performed where variables were eliminated from the model singly in a backwards fashion and retained only if the P value was less than 0.05 (Table 4). The analysis was performed only for those patients for whom full data were available using the continuous variable results. There were 90 patients analyzed with 23 relapses and 21 deaths. PAI-1 was found to be significant for DFS with a p value of 0.0001. The relative risk for a 0.1 unit change was 8% with a confidence interval of 4% to 12%. Cytosolic cathepsin D was found to be a statistically significant independent variable for DFS and OS with respective p values of 0.01 and 0.007. The relative risk for a 10 unit increase of cathepsin D was 17% and 18% for DFS and OS respectively, with corresponding confidence intervals of 6% to 32% and 5% to 31%.

Table 4. Multivariate analysis of factors used in this study

Factors analyzed	Reported p values for group	
	disease free survival	overall survival
Tumor size	0.67	0.65
Age	0.57	0.51
Estrogen receptor	0.40	0.92
Progesterone receptor	0.99	0.58
uPAR	0.62	0.45
uPA	0.35	0.34
PAI-1	0.0001	0.82
Cathepsin D	0.01	0.007

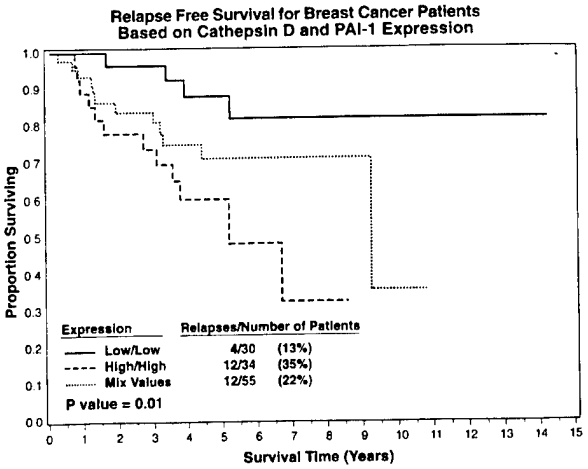

Figure 2. The disease free survival for patients expressing cathepsin D and PAI-1. The patients were divided into below the median or equal or above the median values for cathepsin D and PAI-1. They were then grouped in three subgroups: both factors below the median (——), both factors above the median (----), or patients with one above and one below the median (.....). The number of patients in each of the groups with clinical follow-up was 30, 34, and 55 respectively. Separation into these groups could significantly divide the patients into different prognostic groups (p = 0.01).

Prognostic significance of PAI-1 and cathepsin D

Since cathepsin D and PAI-1 appeared to be the only statistically significant independent parameters for DFS in the multivariate analysis (Table 4), a univariate analysis was performed comparing DFS between patients with cathepsin D and PAI-1 levels above the median value with those with levels below. As seen in Figure 2, DFS was significantly better when both the cathepsin D and PAI-1 levels were below rather than above the median values. Patients whose tumors had one value below and one above the median presented with an intermedi-

ate prognosis. This was highly significant ($p = 0.01$). A comparison of relative risk between the low/low group and the high/high group was 0.2 with 95% confidence limits of 0.06 to 0.74. The primary driving force for this interaction was the cathepsin D value.

Discussion

In view of a 30% 10-year recurrence rate in node negative breast cancer, it becomes most urgently needed to establish prognostic factors which will define subgroups of patients with low versus high risk of recurrence. Patients at low risk would not need further treatment while high risk node negative breast cancer patients should be offered systemic therapy.

It is now well established that proteolytic enzymes, their receptors, and their inhibitors are actively involved in cancer cell invasion and metastasis [1]. Accordingly, several studies have shown that high tumor levels of these molecules are significantly related to a shorter disease free survival and shorter overall survival [2–9]. Recently, experimental data have been presented suggesting strong interaction and cooperativity between different families of proteases [10, 11]. We have in the present study measured components of two different enzyme systems, namely the serine protease uPA, its receptor, and its inhibitor type 1, as well as the aspartyl protease cathepsin D, in breast tumor cytosols obtained from 162 node negative breast cancer patients. The important new finding is that the lysosomal protease cathepsin D and the plasminogen activator inhibitor PAI-1 are independent prognostic markers which when combined can be used to divide node negative breast cancer patients into highly significantly different prognostic groups. It is interesting that PAI-1 was of borderline utility for predicting prognosis from a univariate analysis (Table 3 and Figure 1) but is important in the multivariate analysis (Table 4, Figure 2). The reason for this is not clear but must involve a selection process of which certain markers become redundant while others appear to become important.

A number of prior studies have demonstrated that uPA is a prognostic marker in node negative breast cancer [4]. When using either the 30% vs. 70% cut off selection or the median value, we also found in univariate analysis uPA to be of prognostic significance for DFS in this group of node negative patients, while multivariate analysis revealed that uPA was of no independent prognostic value when the other factors were included in the Cox model. In accordance with our previous study [8], uPAR failed in univariate and multivariate analyses to be a significant predictor of prognosis in node negative patients.

One other study has measured both cathepsin D and PAI-1 in the same tumor extracts [5]. They also found in univariate analysis cathepsin D and PAI-1 to be prognostic markers, but cathepsin D had no prognostic impact in the multivariate analysis.

Garcia et al. have demonstrated that transfection of non-metastatic cells with cathepsin D results in an increase in spread of the disease to the liver in an animal model [18]. This would suggest that cathepsin D is involved in the spread of the disease, but it is not clear how this enzyme might work since its pH optimum activity is 3.5. However, Briozzo et al. have demonstrated that cathepsin D is active at pH 5.5 when the substrates are extracellular matrix components [19]. An extensive review by Dr. Ravdin of the literature on cathepsin D would indicate that cathepsin D is still controversial and needs better defined studies [20], and in a recent editorial by Rochefort, it is suggested that it is not valid to compare cathepsin D studies which use different methods of analysis since immunohistochemistry and RIA or ELISA give different results [21]. It is not clear if cathepsin D could be done on only node negative breast cancer tissues or should be done on all breast cancer tissues. Since cathepsin D is still being requested by many oncologists and surgeons, it would be very important to better define its role in the treatment of breast cancer. Our study attempted to define cathepsin D in combination with other proteases or protease factors that have been shown to be independent prognostic markers.

In summary, there are many papers on prognostic markers in node negative breast cancer. Dr. McGuire published a paper attempting to give a consensus of this area [22]. He demonstrated that

multiple markers provide a better understanding than any single marker. Our data here would suggest that in the protease area cathepsin D and PAI-1 should be considered as markers that should be included in the prognostic panel. Our study is a retrospective study, which provides some error, but none of the patients were treated with adjuvant therapy which could also affect the results. Future studies need to be done on patients in clinical trials in a prospective clinical study. Furthermore, the assays performed here were all done on tumor extracted material and it might be useful to look at immunohistochemistry as a second method of analysis. The question on who to treat and how to treat patients with node negative breast cancer is still an open question that needs to be answered in both the clinical and basic science environment.

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