Immunoenzymatically Determined Pepsinogen C Concentration in Breast Tumor Cytosols: An Independent Favorable Prognostic Factor in Node-positive Patients[1]

Andreas Scorilas, Eleftherios P. Diamandis,[2] Michael A. Levesque, Anastasia Papanastasiou-Diamandi, M. Javad Khosravi, Maurizia Giai, Riccardo Ponzone, Riccardo Roagna, Piero Sismondi, and Carlos López-Otin

Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5G 1X5 and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M5G 1L5 [A. S., E. P. D., M. A. L.]; Diagnostic Systems Laboratories Canada Inc., Toronto, Ontario, Canada M5G 1X5 [A. P-D., M. J. K.]; Department of Gynecologic Oncology, University of Turin, Turin, Italy 10121 [M. G., R. P., R. R., P. S.]; and Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Oviedo, Oviedo, Spain 33006 [C. L-O.]

ABSTRACT

The aim of this study was to determine the concentration and to evaluate the prognostic value of pepsinogen C (PepC) in breast cancer patients. PepC is an aspartic proteinase that is involved in the digestion of proteins in the stomach and is also synthesized by a subset of human breast tumors. PepC concentrations were measured with a highly sensitive immunofluorometric assay, which uses two monoclonal antibodies that are specific for PepC and has a detection limit of 0.1 ng/ml. Breast tumor cytosols from 151 patients (median follow-up, 67 months), stratified according to nodal status, were evaluated. An optimal cutoff value, equal to 1.75 ng/mg of extracted protein, was first defined by statistical analysis. PepC status was then compared with other established prognostic factors, in terms of disease-free survival (DFS) and overall survival (OS). High PepC concentrations were found in small ($P = 0.003$) and well-differentiated tumors ($P = 0.042$) as well as in stage I ($P = 0.003$) and node-negative patients ($P = 0.040$). Statistically significant associations of PepC concentration with patient age and estrogen receptor and progesterone receptor status were not observed. In univariate Cox regression analysis of the entire cohort of patients, negative PepC proved to be a significant predictor of reduced DFS ($P = 0.0086$) and OS ($P = 0.025$). Multivariate analysis in subgroups of patients defined by nodal status indicated that PepC status was a strong predictor of DFS ($P = 0.0039$) and the strongest factor for predicting OS ($P = 0.0046$) in node-positive but not in node-negative patients. Our results suggest that PepC may be used as an independent favorable prognostic factor in node-positive breast cancer patients because there were no significant associations between PepC and the other prognostic factors evaluated in this group of patients.

INTRODUCTION

A variety of proteinases are overproduced, either by epithelial cells or by surrounding stromal cells of the host tissue (1). These enzymes include matrix metalloproteinases as well as serine, cysteine, and aspartic proteinases. Several clinical studies have shown that overexpression of these enzymes in breast tumors may be associated with poor clinical outcomes (2–4).

PepC[3] is the precursor of pepsin C, an aspartic proteinase that is synthesized primarily in the gastric mucosa and secreted into the gastric lumen, where it is converted to the corresponding active enzyme under acidic conditions (5, 6). PepC, also known as progastricsin, is widely distributed in the gastrointestinal tract and, in some species, such as rodents, constitutes the major proteolytic enzyme present in the gastric juice (7). Isolation and characterization of cDNA and genomic clones for human PepC has shown that this protein is composed of a single polypeptide chain of 488 residues, with significant sequence similarity to other aspartic proteinases, such as pepsinogen A, procathepsin D, procathepsin E, and prorenin (8).

The association of PepC with human breast pathology, including breast cancer, was suggested after it was found that PepC is a major proteolytic enzyme in the cyst fluid from women with gross cystic disease of the breast (9, 10). PepC accumulation in cyst fluid is a pathological entity that is thought to be linked to androgen dysfunction (11, 12). Several groups have also demonstrated that normal prostate and prostatic carcinomas are able to produce PepC (13, 14). Of particular interest have been findings that PepC expression in breast carcinomas was associated with pathological and biochemical features of less aggressive disease and with favorable prognostic outcome (15). These findings parallel closely those demonstrating the favorable prognostic value in breast cancer of PSA (16). Because both *PSA* and *PepC* are androgen-regulated genes, we

Received 12/22/98; revised 3/25/99; accepted 4/9/99.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This research was supported by the Canadian Breast Cancer Research Initiative and by Nordion International, Kanata, Ontario, Canada.
[2] To whom requests for reprints should be addressed, at Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5. Phone: (416) 586-8443; Fax: (416) 586-8628; E-mail: ediamandis@mtsinai.on.ca.

[3] The abbreviations used are: PepC, pepsinogen C; PSA, prostate-specific antigen; DFS, disease-free survival; OS, overall survival; ER, estrogen receptor; PR, progesterone receptor; RR, relative risk.

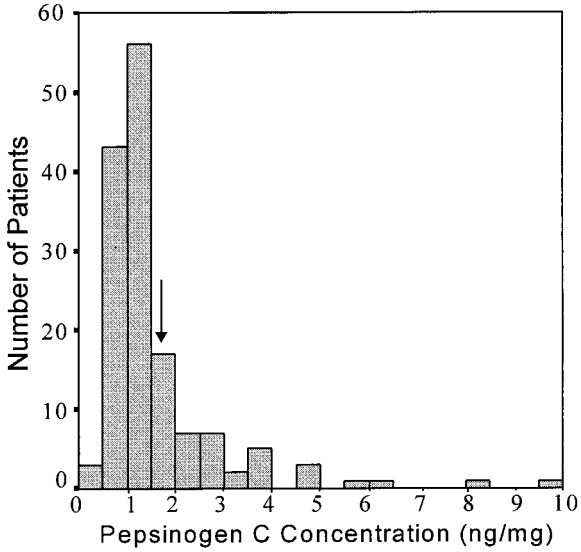

Fig. 1 Distribution of PepC concentrations in 151 human breast carcinomas. The median concentration was 1.16 ng/mg protein. *Arrow*, selected cutoff level, 1.75 ng/mg protein (75th percentile).

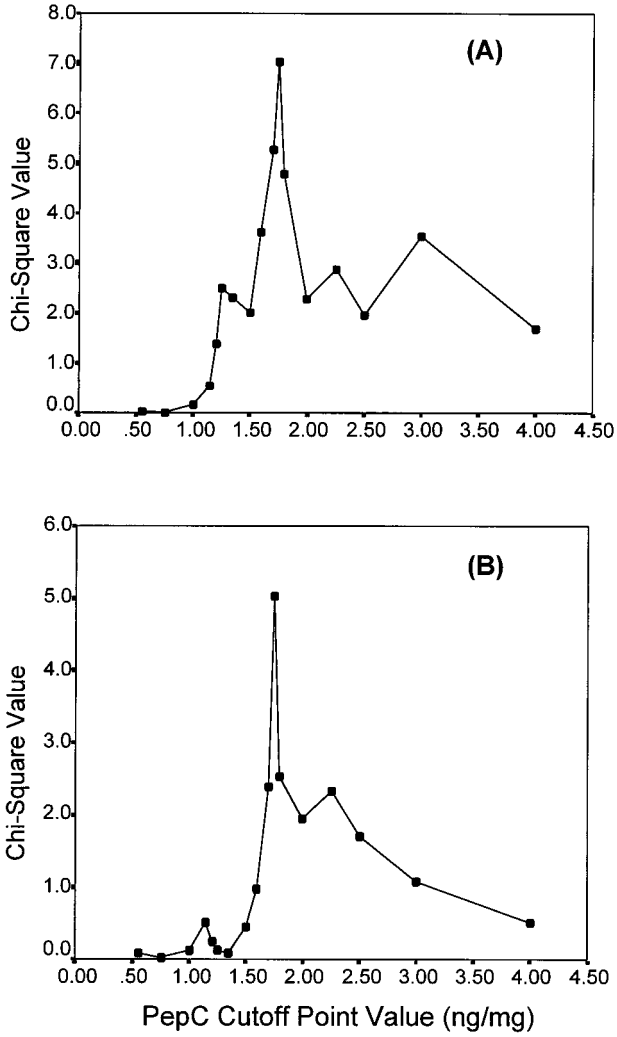

Fig. 2 Determination of optimal cutoff value for PepC status for prediction of relapse-free survival (*A*) and OS (*B*) of breast cancer patients. The χ^2 values obtained at each cutoff value are plotted against the value itself.

hypothesized that they may have similar or complementary prognostic values in breast cancer patients. In this study, we determined the concentration of PepC with a highly sensitive immunofluorometric assay in breast tumor cytosols from 151 patients and evaluated the prognostic value of this measurement. PepC values were compared with other established prognostic factors in terms of DFS and OS using univariate and multivariate analyses.

MATERIALS AND METHODS

Study Population. Included in this study were tumor specimens from 151 patients undergoing surgical treatment for primary breast carcinoma at the Department of Gynecological Oncology at the University of Turin, Turin, Italy, during the period from January 1988 to December 1992. Tumor tissue had been frozen in liquid nitrogen immediately after surgery. The selection criteria for the specimens included the availability of sufficient tissue mass for extraction and assay; the patients represented 60% of new cases of breast cancer diagnosed and treated at the above institution during the accrual period. This study had been approved by the Ethics and Research Committee at the University of Toronto and by the Institutional Review Board of the University of Turin.

The ages of the patients ranged from 25 to 93 years; the median age was 54 years. Twenty-five % of the patients were under the age of 45 years, 25% were between 45 and 55 years, and 50% were aged 56 years or older. All patients had a histologically confirmed diagnosis of primary breast cancer and received no treatment before surgery. Modified radical mastectomy with axillary lymph node dissection was performed on 95% of the patients. For the patients who had axillary node dissection, the positivity rate for cancer involvement of lymph nodes was 61.5%. The sizes of the tumors resected during surgery ranged from 0.8 to 7.0 cm and the mean and median sizes were 2.7 and 2.5 cm, respectively. Clinical staging was performed according to the Postsurgical International Union Against Cancer tumor-node-metastasis classification system (17). Of 150 patients for whom the stage was known, 45 (30%), 87 (58%), 7 (4.6%), and 11 (7.4%) were stage I, II, III, and IV, respectively. Histological grade of the tumors was determined according to criteria reported by Bloom and Richardson (18) and was known for 107 patients: 6 patients (5.6%) had grade I, 55 (51.4%) had grade II, and 44 patients (41.1%) had grade III tumors. Most of the tumors (70%) were of invasive ductal histological type, whereas the remaining tumor were invasive lobular (12.6%), ductal *in situ* (2%), medullary (2.7%), papillary (2.7%), tubular (2%), inflammatory (2.7%), tubulo-lobular (2.7%), cribriform (1.3%), and muciparous (1.3%). Postoperative treatment was known for all patients. Whereas 30% received no further treatment after tumor resection, 24% were given adjuvant chemotherapy only, 41% were treated with en-

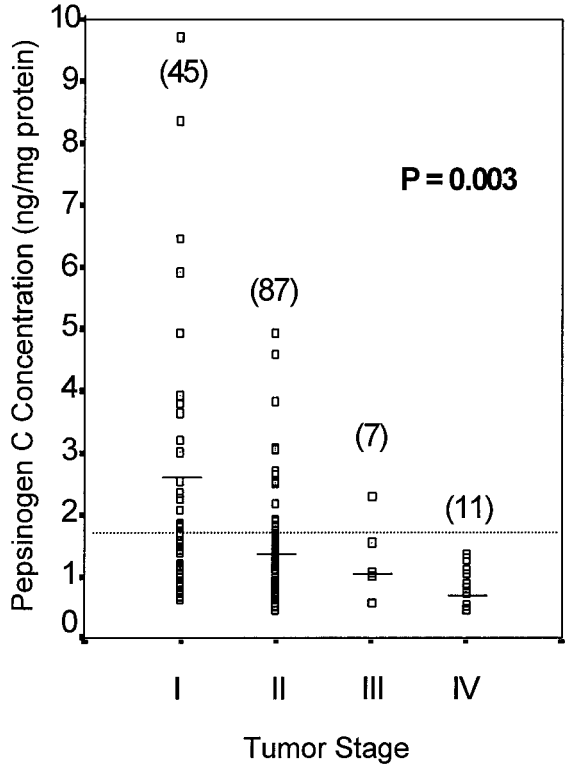

Fig. 3 Relationship between PepC concentration and tumor-node-metastasis stage. The PepC concentrations in 150 primary breast cancer cytosols are plotted according to stage (I, II, III, or IV). *P* was determined by the Kruskal-Wallis test. The *numbers in parentheses* indicate the number of patients in each group. The *broken line* indicates the cutoff level of 1.75 ng/mg protein that was used in survival analysis. *Horizontal lines*, mean PepC concentrations for each stage of cancer.

Table 1 Relationships between PepC status[a] and other variables

Variable	Total no. of patients	No. of patients (%) PepC negative	No. of patients (%) PepC positive	*P*[b]
Age (yr)				
<45	38	32 (84.2)	6 (15.8)	
45–55	38	27 (73.0)	10 (27.0)	NS[c]
>55	75	52 (71.2)	21 (28.8)	
Tumor size (cm)				
<2	43	26 (60.5)	17 (39.5)	0.008[d]
≥2	105	83 (81.4)	19 (18.6)	
Nodal status				
Negative	55	36 (67.9)	17 (32.1)	NS[d]
Positive	88	69 (79.3)	18 (20.7)	
Grade[e]				
I–II	63	41 (68.3)	19 (31.7)	0.019[d]
III	44	39 (88.6)	5 (11.4)	
Histology				
Ductal	106	79 (76.0)	25 (24.0)	
Lobular	19	15 (78.9)	4 (21.1)	NS[c]
Other	26	17 (68.0)	8 (32.0)	
Stage[f]				
I	45	27 (60.0)	18 (40.0)	
II	87	66 (78.6)	18 (21.4)	0.008[c]
III–IV	18	17 (94.4)	1 (5.6)	
ER status[g]				
Negative	48	35 (74.5)	12 (25.5)	NS[d]
Positive	99	73 (75.3)	24 (24.7)	
PR status[g]				
Negative	53	36 (72.0)	14 (28.0)	NS[d]
Positive	93	71 (76.3)	22 (23.7)	
Adjuvant treatment				
None	44	32 (72.7)	12 (27.3)	NS[c]
Tamoxifen	68	47 (69.1)	21 (30.9)	
Chemotherapy ± tamoxifen	36	32 (88.9)	4 (11.1)	

[a] Cutoff point, 1.75 ng/mg.
[b] NS, not significant ($P > 0.05$).
[c] χ^2 test.
[d] Fisher's exact test.
[e] Bloom-Richardson grading system.
[f] Tumor-node-metastasis system.
[g] Cutoff point, 10 fmol/mg.

docrine therapy only, and 5% were given both chemotherapy and endocrine therapy. Disease relapse was defined as the first documented evidence of local or regional axillary recurrence or distant metastasis.

Follow-up information was available for 148 patients and included survival status (alive or deceased) and disease status (disease free or recurrence/metastasis), along with the dates of the events and cause of death, if applicable. The relapse-free survival time in each case was the time interval between the date of surgical removal of the primary cancer and the date of the first documented evidence of relapse. The OS time was the time interval between the date of surgery and the date of death or the date of last follow-up for those who were alive at the end of the study.

Preparation of Cytosolic Extracts. Tumor tissues were stored at −80°C until their pulverization and cytosolic extraction. The extraction procedure consisted of treatment of the tissue powders (10–50 mg) with a cell lysis buffer (500 μl) containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 g/liter NP40 surfactant, and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice and subsequent separation of cell debris from the cytosols by centrifugation at 15,000 × *g* for 30 min at 4°C. Supernatants were assayed for PepC and total protein immediately after centrifugation.

Steroid Hormone Receptor Analyses. Tumor specimens ($n = 151$) were pulverized in liquid nitrogen and homogenized in buffer, and the cytosolic fractions were obtained by ultracentrifugation and quantified for steroid hormone receptors, as described elsewhere (19). The results of the dual ligand-binding assay, in which dextran-coated charcoal was used to separate bound from free ligand, were interpreted by Scatchard analysis (20). Protein concentrations of the cytosols were determined by the method of Lowry *et al*. (21). Tumors with ER and PR concentrations below or equal to 10 fmol/mg protein were considered as receptor negative, whereas tumors with receptor concentrations above such values were considered positive, as followed previously (22, 23). On the basis of these cutoffs, 99 (67.3%) and 93 (63.7%) of 147 and 146 breast carcinomas were ER and PR positive, respectively.

PepC Immunoassay. We have used a quantitative immunofluorometric assay to determine the PepC concentrations in the tumor extracts (10), which were assayed without dilution and in duplicate. The assay, which has been described previ-

ously and is now commercially available from Diagnostic Systems Laboratories (Webster, TX), uses two monoclonal antibodies specific for PepC and has a detection limit of 0.1 ng/ml. All PepC concentrations in ng/ml were converted to ng of PepC per mg of total protein to compensate for the amount of tissue extracted from each tumor.

Statistical Analysis. For analysis of data, patients were subdivided into groups based on different clinical or pathological parameters. Because the distribution of PepC concentrations was not Gaussian, the analysis of differences in PepC values between two groups was performed with the nonparametric Mann-Whitney *U* test. Similarly, relationships between more than two groups were determined by the Kruskal-Wallis test. In this analysis, PepC was used as a continuous variable. PepC values were also classified into two categories (PepC-positive and -negative groups), and associations between PepC status and other qualitative variables were analyzed using the χ^2 and Fisher's exact tests, where appropriate. An optimal cutoff point, equal to 1.75 ng/mg, was found by χ^2 analysis. ER and PR values were categorized into positive and negative status, as described above. The cutoff value for tumor size was 2 cm. Lymph node status was either positive (any positive number of nodes) or negative. Age was categorized into three groups: <45 years, 45–55 years, and >55 years. Survival analyses were performed by constructing Kaplan-Meier DFS and OS curves (24), where differences between curves were evaluated by the log-rank test as well as by estimating the RRs for relapse and death using the Cox proportional hazards regression model (25). Only patients for whom the status of all variables was known were included in the multivariate regression models, which incorporated PepC and all other variables for which the patients were characterized. Selection of prognostic variables with the highest significant effect in relapse-free survival and OS was performed in the Cox's model using the stepwise regression option from SPSS software (SPSS Inc., Richmond, CA). Only variables for which P was <0.05 were retained in the final model.

RESULTS

Distribution of PepC Concentration and Relationship to Other Prognostic Variables

The PepC concentration of the 151 cytosolic samples varied widely from 0 to 9.71 ng/mg; the median was 1.16 ng/mg, and the mean was 1.59 ng/mg. Fig. 1 shows the distribution of these concentrations, which was slightly positively skewed. An optimal cutoff value was defined by χ^2 analysis, based on the ability of PepC values to predict the DFS and OS of the study population. As shown in Fig. 2, a value of 1.75 ng/mg protein was shown to be the optimal cutoff ($\chi^2 = 7.0$, $P = 0.008$, and $\chi^2 = 5.0$, $P = 0.026$, for DFS and OS, respectively). This cutoff (75th percentile) identifies 25% of patients as being PepC positive. PepC positivity was found more frequently in small ($P = 0.008$), well-differentiated tumors ($P = 0.019$), as well as in patients with stage I disease ($P = 0.003$; Fig. 3). No significant associations between PepC status and patient age, steroid hormone receptors, and histological type were observed (Table 1). A weak association was found between PepC concentration and lymph node status ($P = 0.042$; Fig. 4).

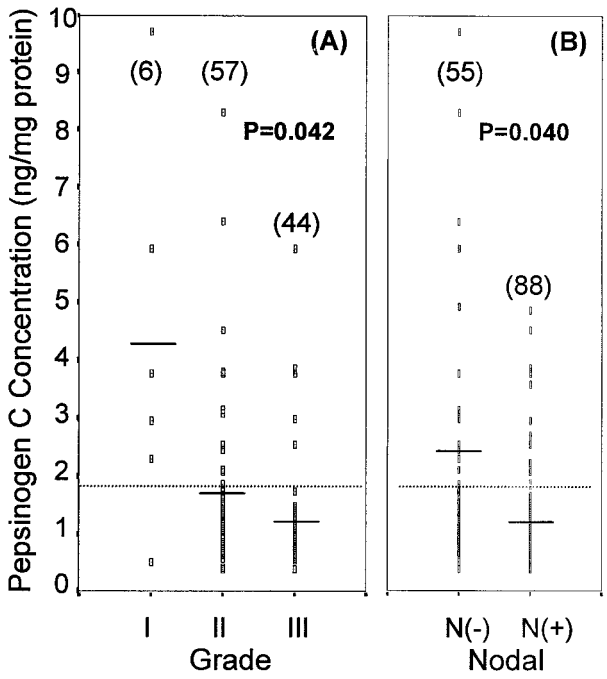

Fig. 4 Relationship between PepC concentration and tumor grade (*A*) as well as the nodal status (*B*). The PepC concentrations are plotted according to grade (I, II, or III) and axillary lymph node status [*N(+)* or *N(−)*, lymph node-positive or -negative, respectively]. *P* was determined from Kruskal-Wallis (*A*) and Mann-Whitney (*B*) tests. *Numbers in parentheses* indicate the number of patients in each group. The *broken line* indicates the cutoff level of 1.75 ng/mg protein that was used in survival analysis. *Horizontal lines*, mean PepC concentrations.

PepC Protein as a Predictor of Breast Cancer Patient Survival

Univariate and Multivariate Analysis. Follow-up information was available for 148 of the 151 patients included in the study. During their respective follow-up periods, 56 patients (37.1%) developed cancer relapse, and 39 (25.8%) died. In Cox univariate survival analysis, the risks of relapse and death were not significantly related to PepC, considered as a continuous variable. However, significantly reduced risks for both relapse and death were shown to be associated with PepC positivity using the PepC cutoff of 1.75 ng/mg (Table 2). These regression models showed that there was an extensive reduction in risk of relapse and death in patients with PepC-positive cancer compared to those with PepC-negative disease. The Kaplan-Meier survival curves (Fig. 5) also show that PepC-positive patients had more favorable DFS and OS rates than did PepC-negative patients. The difference in survival rates between the two groups was greater for DFS than for OS. In the multivariate analysis of PepC, the Cox regression models were adjusted for age, nodal status, tumor size, and ER and PR status, all of which were used as categorical variables, except tumor size, which was considered a continuous variable, as described above. Tumor grade was not included in the multivariate analysis because of the relatively large number of patients for which this variable was unknown. Patient age, tumor size, and nodal status were, thus, shown to be independent factors for predicting both DFS and

Table 2 Association between PepC and breast cancer survival

	DFS			OS		
Variable	Univariate *P*	Multivariate P^a	RR (95% CI)[b]	Univariate *P*	Multivariate P^a	RR (95% CI)[b]
Patient's age[c]						
A	0.16	0.79	1.06 (0.69–1.59)	0.43	0.047	1.61 (1.01–2.59)
B	<0.001	0.0023	0.52 (0.34–0.79)	0.012	0.0041	0.44 (0.25–0.77)
Tumor size[d]	0.0032	0.012	1.34 (1.07–1.68)	0.022	0.024	1.38 (1.04–1.83)
Nodal status	0.018	0.022	2.12 (1.11–4.07)	0.017	0.021	2.53 (1.08–5.88)
Grade[e]	0.18			0.28		
ER[f]	0.091			0.033		
PR[f]	0.33			0.64		
PepC status[g]	0.0086	0.032	0.39 (0.16–0.92)	0.025		
Node-positive patients ($n = 88$)						
Patient's age[c]						
A	0.024	0.76	0.92 (0.57–1.49)	0.86		
B	<0.001	0.0073	0.49 (0.30–0.82)	0.022		
Tumor size[d]	0.054			0.13		
Grade[e]	0.39			0.40		
ER[f]	0.22			0.061	0.047	0.45 (0.20–0.98)
PR[f]	0.76			0.41		
PepC status[g]	0.0045	0.0039	0.19 (0.04–0.80)	0.019	0.0046	0.12 (0.02–0.83)
Node-negative patients ($n = 55$)						
Patient's age[c]						
A	0.62			0.021	0.023	3.33 (2.06–5.34)
B	0.039			0.23	0.083	0.28 (0.07–1.17)
Tumor size[d]	0.10			0.41		
Grade[e]	0.16			0.25		
ER[f]	0.089			0.31		
PR[f]	0.45			0.91		
PepC status[g]	0.89			0.51		

[a] *P*s in multivariate analyses are from the final models in which only variables with $P < 0.05$ were retained.
[b] RRs with 95% confidence intervals are presented only for the retained variables that were significant in the multivariate analysis. CI, confidence interval.
[c] A, 45–55 years of age *versus* <45 years old. B, >55 years old *versus* <45 years old.
[d] Test for trend.
[e] Grade was not included in multivariate analysis because of a large number of missing values.
[f] Positive compared with negative (cutoff point, 10 fmol/mg protein).
[g] Positive compared with negative (cutoff point, 1.75 ng/mg protein).

OS. PepC significantly added to the prognostic power in the multivariate model in analysis for DFS (RR = 0.39; $P = 0.032$) but not for OS.

Univariate and Multivariate Analysis in Patients Classified by Nodal Status. Because node-positive patients are substantially different from node-negative patients in terms of their prognosis and treatment administered after the surgery, univariate and multivariate Cox regression models were developed to evaluate the effect of PepC on DFS and OS for each of the two groups of patients. The results are shown in Table 2 and Fig. 6. Breast cancer patients with tumors that were positive for PepC tended to have a 30–45% reduction in risk for relapse or death. PepC was an independent factor for predicting DFS (RR = 0.19; $P = 0.0039$) and OS (RR = 0.12; $P = 0.0046$) in node-positive patients. Age and ER significantly added to the prognostic power in the multivariate model in analysis for DFS and OS, respectively. When the relationship between PepC and survival was examined in node-negative patients, none of the differences were statistically significant.

DISCUSSION

This study was designed to investigate whether PepC concentrations determined by an immunofluorometric assay have prognostic value in primary breast carcinoma. Our findings have demonstrated the clinical relevance of PepC as an independent favorable prognostic indicator of lymph node-positive but not of node-negative breast cancer. To our knowledge, only one previous study, by Vizoso *et al*. (15), has addressed the relationship between PepC expression in breast tumor tissue and survival outcome. These authors similarly reported evidence for favorable prognosis conferred by PepC expression in their entire cohort of breast cancer patients but did not provide data, suggesting differences between node-positive and node-negative

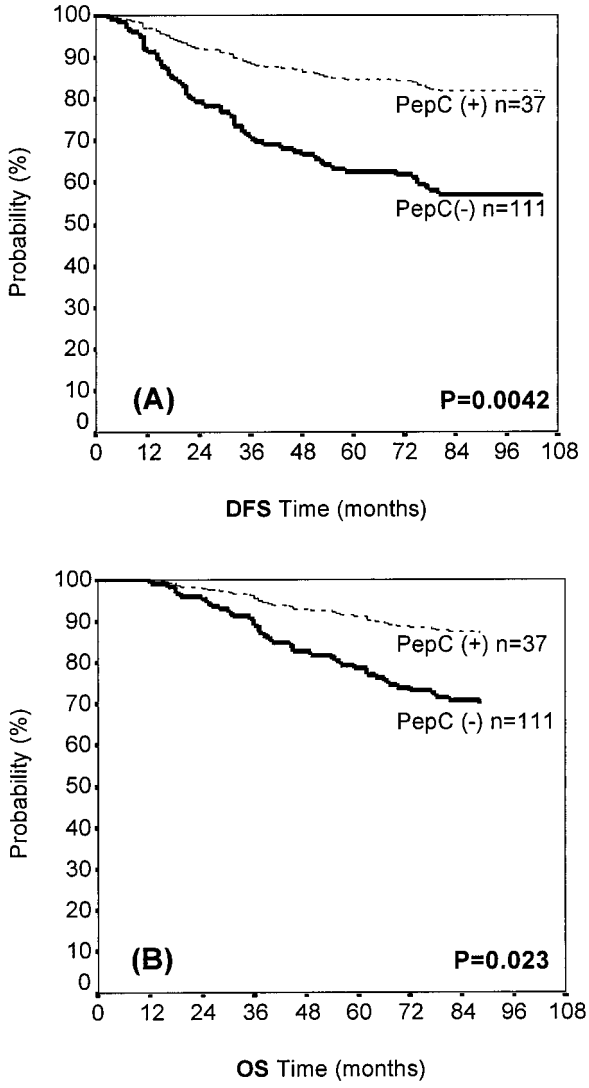

Fig. 5 DFS (*A*) and OS (*B*) curves in patients with PepC-positive and -negative breast tumor cytosols, followed for a median of 67 months. The cutoff value for PepC positivity was 1.75 ng/mg protein.

patients with respect to the effect of PepC on outcome. Whereas the detection of PepC expression in this previous study was performed by immunoperoxidase staining using a polyclonal antibody, whereby 33% of 243 cases were defined as PepC positive, in the work described here, 25% of 151 breast cancer patients were classified as PepC positive on the basis of having PepC concentrations, measured by a quantitative ELISA technique using two monoclonal antibodies, which exceeded a statistically determined optimal cutoff level. The availability of several other clinicopathological features for our sample of breast cancer patients permitted the multivariate examination of each variable for its independent contribution to DFS and OS. Thus shown to be independent markers of prognosis were nodal status, tumor size, patient age, and PepC status, among which only PepC and older age (>55 years) indicated favorable outcome. Two other proteins expressed in breast tumor tissues, PSA and pS2, have also been previously shown to be favorable prognostic indicators (16, 26, 27). In other studies, the prognostic impact of some biochemical markers have been shown to be dependent on lymph node status. For instance, cathepsin D, c-myc, and pS2 protein were found to have independent prognostic value in node-negative breast cancer patients (3, 27–29), whereas c-erbB2 oncoprotein was shown to be the strongest predictive factor of poor short-term prognosis followed by p53 protein in lymph node-positive breast cancer (30, 31).

Because very little is known about the physiological role of PepC in breast tissue, a hypothesis explaining the mechanism by which PepC expression may confer a favorable breast cancer prognosis, especially in node-positive patients, is, at present, difficult to formulate. In contrast to its function in the gastric lumen or to those of matrix metalloproteinases and aspartic proteases such as cathepsin D in the interstitium, PepC may not become functionally active as a proteolytic enzyme in breast cancer tissue, given that it is secreted as a precursor of high molecular weight that requires exposure to pH conditions lower than those found in the extracellular matrix (9). However, because large acidic vesicles within breast cancer cells have been demonstrated (32), local activation of secreted proPepC cannot be excluded. Whether PepC acts upon substrates such as matrix structural components, sequestered growth factors, cytokines, their binding proteins, or other extracellular constituents remains to be determined experimentally. In light of the fact that PepC is not synthesized by mammary epithelium under normal conditions and is expressed only in a subset of breast carcinomas (33), its function may not be required either for the maintenance of breast tissue function or for breast tumorigenesis but may simply reflect hormonal alterations involved in the breast cancer development.

Studies on the regulation of the PepC gene have revealed that it is up-regulated by androgens, glucocorticoids, and progesterone but not by estradiol (34), in contrast to the estrogen responsiveness of another gene, *pS2*, which displays the same pattern of tissue specificity as PepC (35). Furthermore, high levels of both PepC and pS2 are associated with favorable breast cancer prognosis (27). The relationship between steroid hormone responsiveness and PepC expression in breast tumor tissue may be reflected by our finding that PepC concentrations were higher in well-differentiated, low-grade lesions, which typically express steroid hormone receptors. In light of these considerations and the fact that poorly differentiated, high-grade tumors are frequently independent of steroid hormone regulation, it is possible that PepC may serve as a better indicator of a functional pathway than the presence of the steroid hormone receptors themselves. Because not all breast cancers respond to endocrine manipulation (36), it has been speculated that the physical existence of the receptors may not necessarily constitute proof of their functionality. Defective receptors have been shown to exist which do not have the ability to form complexes with their ligands or to bind to the hormone response elements in target genes (37). The results of our study may further indicate the ability of PepC to predict response of breast cancer patients to hormonal manipulation, given that only 13% of node-positive patients who received postoperative tamoxifen treatment and whose tumors expressed high PepC relapsed and died, compared to a 35% relapse rate and a 25% death rate of

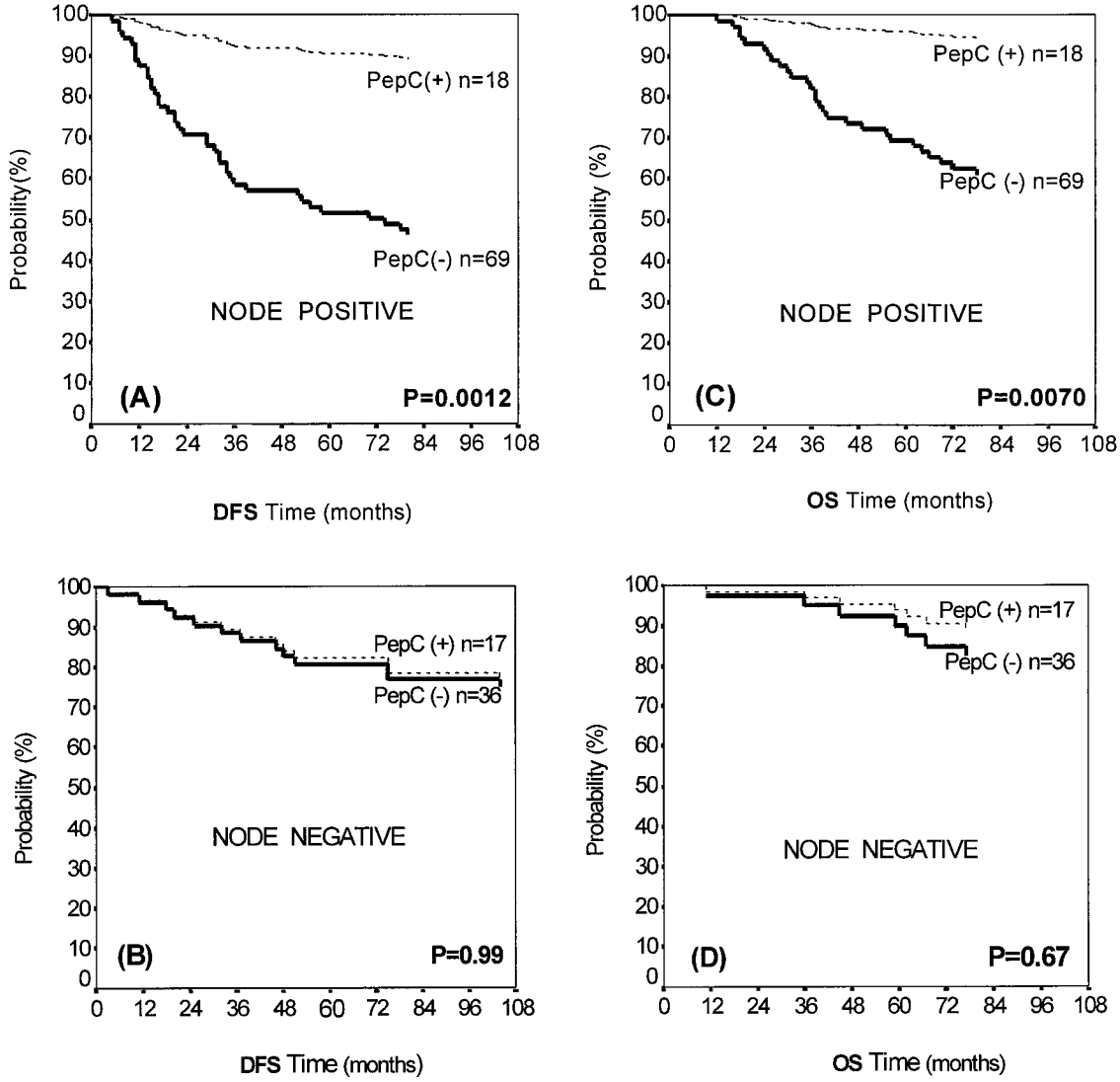

Fig. 6 DFS (*A* and *B*) and OS (*C* and *D*) curves of patients with PepC-positive and -negative breast cancer, stratified by their nodal status: node positive (*A* and *C*) or node negative (*B* and *D*). The cutoff value for PepC positivity was 1.75 ng/mg protein.

similarly treated node-positive patients whose tumors were PepC-negative (data not shown). Additional studies of hormonally treated patients for whom response criteria are clearly defined are needed to confirm these preliminary observations regarding PepC and tamoxifen responsiveness.

In summary, we found that PepC was present in 25% of breast cancer tissues at concentrations of >1.75 ng/mg protein. PepC was more frequently present in small tumors and in tumors of lower grade as well as in early-stage disease. The difference between the means of PepC concentrations in node-positive and -negative patients was of borderline significance. No significant association between PepC status and steroid hormone receptor status was observed. Node-positive breast cancer patients with tumors positive for PepC tended to have a marked reduction in the risk for relapse or death. This difference in survival remained significant after clinical and pathological features, also related to survival, were taken into consideration. Therefore, the measurement of PepC concentrations in tumor extracts may provide additional information related to breast cancer prognosis, particularly in node-positive patients.

REFERENCES

1. López-Otin, C., and Diamandis, E. P. Breast and prostate cancer: an analysis of common epidemiological, genetic, and biochemical features. Endocr. Rev., *19:* 365–396, 1998.

2. Basset, P., Bellocq, J. P., Wolf, C., Stoll, I., Hutin, P., Limacher, J. M., Podhajcer, O. L., Chenard, M. P., Rio, M. C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature (Lond.), *348:* 699–704, 1990.

3. Scorilas, A., Yotis, I., Gouriotis, D., Keramopoulos, A., Ampela, K., Trangas, T., and Talieri, M. Cathepsin-D and c-erbB2 have an additive prognostic value for breast cancer patients. Anticancer Res., *13:* 1895–1900, 1993.

4. Ardavanis, A., Scorilas, A., Amanatidou, A., Gerakini, F., Missitzis, I., Garoufali, A., Pissakas, G., Pateras, C., Apostolikas, N., Rigatos, N.,

and Yotis, I. Cathepsin-D concentration in tumour cytosols improves the accuracy of prognostic evaluation of primary breast cancer. Anticancer Res., *17:* 1405–1410, 1997.

5. Foltmann, B. Gastric proteinases: structure, function, evolution and mechanism of action. Essays Biochem., *17:* 52–84, 1981.

6. Moore, S. A., Sielecki, A. R., Chernaia, M. M., Tarasova, N. I., and James, M. N. G. Crystal and molecular structures of human progastricsin at 1.62 Å resolution. J. Mol. Biol., *247:* 466–485, 1995.

7. Samloff, I. M. Peptic ulcer: the many proteinases of aggression. Gastroenterology, *96:* 586–595, 1989.

8. Hayano, T., Sogawa, K., Ichihara, Y., Fujii-Kuriyama, Y., and Takahashi, K. Primary structure of human pepsinogen C. J. Biol. Chem., *263:* 1382–1385, 1988.

9. Sanchez, L. M., Freije, J. P., Merino, A. M., Vizoso, F., Foltmann, B., and López-Otin, C. Isolation and characterization of a pepsin C zymogen produced by human breast tissues. J. Biol. Chem., *267:* 24725–24731, 1992.

10. Diamandis, E. P., Nadkarni, S., Bhaumik, B., Abdelrahman, A., Melegos, D. N., Borchert, G., Black, M. H., Alonso, M., Salas, A., de los Toyos, J. R., Sampedro, A., and López-Otin, C. Immunofluorometric assay of pepsinogen C and preliminary clinical applications. Clin. Chem., *43:* 1365–1371, 1997.

11. Haagensen, C. D. The relationship of cystic disease to carcinoma of the breast. *In:* C. D Haagensen (ed.), Diseases of the Breast, pp. 168–172. Philadelphia: Saunders, 1971.

12. Mazoujian, G., and Haagensen, D. E. The immunopathology of gross cystic disease fluid proteins. Ann. N. Y. Acad. Sci., *586:* 188–197, 1990.

13. Reid, W. A., Liddle, C. N., Svasti, J., and Kay, J. Gastricsin in the benign and malignant prostate. J. Clin. Pathol., *38:* 639–643, 1985.

14. Szecsi, P. B., Halgreen, H., Wong, R. N. S., Kjaer, T., and Tang, J. The cellular origin, cDNA and N-terminal sequences of human seminal progastricsin. Biol. Reprod., *53:* 227–233, 1995.

15. Vizoso, F., Sanchez, L. M., Diez-Itza, I., Merino, A. M., and López-Otin, C. Pepsinogen C is a new prognostic marker in primary breast cancer. J. Clin. Oncol., *13:* 54–61, 1995.

16. Yu, H., Levesque, M., Clark, G., and Diamandis, E. Prognostic value of prostate specific antigen for women with breast cancer: a large U. S. cohort study. Clin. Cancer Res., *4:* 1489–1497, 1998.

17. Spiessl, B., Beahrs, O. H., Hermanek, P., Hutter, R. V. P., Scheibe, O., Sobin, L. H., and Wagner, G. Illustrated Guide to the TNM/pTNM Classification of Malignant Tumours: TNM Atlas, Ed. 3. New York: Springer-Verlag, 1989.

18. Bloom, H. J. G., and Richardson, W. W. Histological grading and prognosis in breast cancer. Br. J. Cancer, *11:* 359–377, 1957.

19. Dressler, L. G., Seamer, L. C., Owens, M. A., Clark, G. M., and McGuire, W. L. DNA flow cytometry and prognostic factors in 1131 frozen breast cancer specimens. Cancer (Phila.), *61:* 420–427, 1988.

20. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci., *51:* 660–672, 1949.

21. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with folin-phenol reagent. J. Biol. Chem., *193*: 265–275, 1951.

22. Alexiera-Figusch, J., van Putten, W. L. S, Blankestein M. A, Blonk-Van Der wijst, J., and Klijn, J. G. M. The prognostic value and relationships of patient characteristics, estrogen and progestin receptors and site of relapse in primary breast cancer. Cancer (Phila.), *61:* 758–768, 1988.

23. Reiher, A., Kolb, R., Reiner, G., Jakesz, R., Schemper, M., and Spona, J. Prognostic significance of steroid hormone receptor and histopathological characterization of human breast cancer. J. Cancer Res. Clin. Oncol., *113:* 285–290, 1987.

24. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.

25. Cox., D. R. Regression models and life tables. R. Stat. Soc. B, *34:* 187–202, 1972.

26. Diamandis, E. P., Yu, H., and Sutherland, D. J. A. Detection of prostate specific antigen immunoreactivity in breast tumors. Breast Cancer Res. Treat., *32:* 291–300, 1994.

27. Foekens, J. A., van Putten, W. L. J., and Portegen, H. Prognostic value of pS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J. Clin. Oncol., *11:* 899–908, 1993.

28. Berns, E. M., Klijn, G. M., van Putten, W. L., Staveren, I. L., Portegen, H., and Foekens, J. A. c-myc amplification is a better prognostic factor than HER2/new amplification in primary breast cancer. Cancer Res., *52:* 1107–1113, 1992.

29. Ardavanis, A., Scorilas, A., Gerakini, F., Loukeri, A., Pateras, C., Apostolikas, N., Stravolemos, K., and Yiotis, I. Cathepsin D may help in discriminating node-negative breast cancer patients at risk for local-regional recurrence. Anticancer Res., *18:* 2885–2890, 1998.

30. Scorilas, A., Yotis, I., Stravolemos, K., Gouriotis, D., Keramopoulos, A., Ampela, K., Talieri, M., and Trangas, T. c-erbB2 overexpression may be used as an independent prognostic factor for breast cancer patients. Anticancer Res., *15:* 1543–1548, 1995.

31. Eissa, S., Khalifa, A., el-Gharib, A., Salah, N., and Mohamed, M. K. Multivariate analysis of DNA ploidy, p53, c-erbB-2 proteins, EGFR, and steroid hormone receptors for short-term prognosis in breast cancer. Anticancer Res., *17:* 3091–3097, 1997.

32. Briozzo, P., Morisset, M., Capony, F., Rougeot, C., and Rochefort, H. *In vitro* degradation of extracellular matrix with M_r 52,000 cathepsin D secreted by breast cancer cells. Cancer Res., *48:* 3688–3692, 1988.

33. Diez-Itza. I., Merino, A. M., Tolivia, J., Vizoso, F., Sanchez, L. M., and López-Otin, C. Expression of pepsinogen C in human breast tumors and correlation with clinicopathologic parameters. Br. J. Cancer, *68:* 637–640, 1993.

34. Balbin, M., and López-Otin, C. Hormonal regulation of the human pepsinogen C gene in breast cancer cells. Identification of a *cis*-acting element mediating its induction by androgens, glucocorticoids, and progesterone. J. Biol. Chem., *271:* 15175–15181, 1996.

35. Rio, M. C., Bellocq, J. P., Daniel, J. Y., Tomasetto, C., Lathe, R., Chenard, M. P., Batzenschlager, A., and Chambon, P. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. Science (Washington DC), *241:* 705–708, 1988.

36. Muss, H. B. Endocrine therapy for advanced breast cancer: a review. Breast Cancer Res. Treat., *21:* 15–26, 1992.

37. Leygue, E. R., Watson, P. H., and Murphy, L. C. Estrogen receptor variants in normal human mammary tissue. J. Natl. Cancer Inst. (Bethesda), *88:* 284–290, 1996.