

Prognostic Significance of the Metastasis-associated Protein Osteopontin in Human Breast Cancer¹

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ABSTRACT

The adhesive glycoprophosphoprotein (OPN) is capable of inducing metastasis in rodent models of breast cancer. We now show that a monoclonal antibody to rat OPN recognizes specifically human OPN using Western blotting techniques and used it to assess the prognostic significance of OPN in primary tumors of a group of 333 patients treated between 1976 and 1982 for operable stage I and stage II breast cancer. The antibody stains immunocytochemically normal breast tissue weakly but pregnant/lactating tissue and 66% of the carcinomas strongly, leaving the remaining 34% as negatively stained. In addition to the carcinoma cells, some host reactive stromal cells, macrophages, lymphocytes, and blood vessels are also stained, but these have been excluded in the following analyses. There is a significant association of staining of carcinomas for OPN with some tumor variables reported previously to be associated with patient outcome: high histological grade ($P = 0.024$), staining for *c-erbB-3* ($P < 0.001$), p53 ($P = 0.014$), pS2 ($P = 0.025$), and borderline significance for progesterone receptor ($P = 0.089$). The association of staining for OPN with survival times of the patients has been evaluated using life tables over 14–20 years of follow-up (mean 16 years) and analyzed using generalized Wilcoxon statistics. Of the patients who have been classified as OPN-negative, 94% are alive, but only 26% of those classified as OPN-positive are alive after 19 years of follow-up. This association is highly significant ($P < 0.0001$); the former have a median survival of >228 months and the latter 68 months. When the patients are divided into separate classes based on the percentage of carcinoma cells staining for OPN, the five classes show a progressive decrease in survival with increasing percentage of stained carcinoma cells, and this association is also highly significant ($P < 0.0001$). Other tumor variables that show a significant association with patient survival times in this group of patients include nodal status, tumor size, histological grade, staining for *c-erbB-2*, estrogen receptor α , or p53. Analysis of the association of patients with carcinomas staining for OPN and their survival in subgroups defined by these tumor variables shows that positive staining for OPN in each subgroup is associated with poorer survival. There is little difference in patient survival times in the OPN-negative group of patients with or without any of the other tumor variables examined. Multivariate regression analysis for 202 patients shows that staining for OPN is most highly correlated with patients' deaths ($P < 0.0001$), but involved lymph nodes ($P = 0.0007$), fixed tumors ($P = 0.0008$), and staining for estrogen receptor α ($P = 0.008$) are also significant independent prognostic variables with that for *c-erbB-2* being of borderline significance ($P = 0.060$). These results suggest that in this group of patients, the presence of the metastasis-associated protein OPN is tightly correlated with patient demise.

INTRODUCTION

Most deaths of women with breast cancer arise not as a result of the growth of the primary tumor but from its metastatic spread to distant sites in the body (1, 2). Therefore, prognostic factors are required to identify those patients who are at most risk of dying from metastatic spread so that they can be treated more aggressively than those with indolent, nonmetastatic breast carcinomas. Extensive research throughout the last century has emphasized the prognostic significance of a number of pathological factors, which include the size of the primary tumor, the histological grade (3), and the appearance of tumor deposits in the draining lymph nodes of the primary breast carcinoma (4). With the advent of molecular biology over the last 20 years, gene products have been discovered largely in model systems that relate to the development of breast cancer. These gene products are involved, *inter alia*, in controlling cell proliferation [*e.g.*, *c-erbB-2* (5, 6) and *c-erbB-3* (7, 8)], cell differentiation [*e.g.*, ER α (9–11),⁵ pS2 (12), and PgR (13, 14)], cell death [*e.g.*, p53 (15, 16)], and cell invasion [*e.g.*, cathepsin D (14, 17)] in tissue cultured systems. However, these molecular changes have been of more limited use to date than the pathological factors in predicting patient death from metastatic disease (18), because they presumably relate more to the growth of the primary tumor and not necessarily to the development of distant metastases.

One molecule that has been strongly implicated in progression and metastasis of rodent models of cancer is the secreted adhesive glycoprophosphoprotein OPN, because its enhanced expression is often associated with transformation of stromal and epithelial cell lines (19–21). For example transfection of mouse NIH3T3 cells with the *H-ras* oncogene causes enhanced production of OPN and an increased metastatic potential in nude mice (22), whereas specific suppression of OPN mRNA in the *ras* transfectants results in a reduction of metastatic ability (23). Moreover, direct transfection of an expression vector for rat OPN into a Rama cell line, Rama 37, which yields only benign, nonmetastatic tumors in the mammary fat pads of syngeneic rats (24), causes these cells to metastasize predominantly to the draining lymph nodes and lungs when reinserted into similar animals (25, 26). In humans, OPN is expressed by a number of different cell types including osteoblasts, arterial smooth muscle cells, leukocytes, both activated macrophages and T cells, and various types of epithelial cells (27). In the breast, OPN is normally expressed by the secretory cells, but occasionally by nonsecretory epithelial cells (28). In the clinical setting, high circulating levels of OPN have been reported in patients with metastatic breast cancer (29) to be associated with decreased times of survival (30), whereas OPN has also been detected in the primary tumors of patients with breast cancer, particularly in the infiltrating macrophages (28, 31, 32). Pilot studies have also identified OPN mRNA and protein in invading carcinoma cells in about a quarter of lymph node-negative breast cancer patients, and

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⁵The abbreviations used are: ER α , estrogen receptor α ; CI, confidence interval; d.f., degrees of freedom; Ig, immunoglobulin; MAb, monoclonal antibody; OPN, osteopontin; PgR, progesterone receptor; Rama, rat mammary; RR, relative risk.

tumor cell OPN positivity above an optimized cutoff has been reported to be significantly associated with decreased disease-free and decreased overall survival of the patients (33). We now investigate, using immunocytochemical techniques, the presence of OPN in specimens of primary breast carcinomas from a comparatively large group of patients with 14–20 years follow-up to assess its relationship with other potential prognostic factors and their association with patient death from metastatic disease.

MATERIALS AND METHODS

Patients and Specimens. The 333 unselected patients presented with operable (stage I and stage II) breast cancer between the years 1976 and 1982 to general surgery clinics in the Merseyside Region of the North West of England, as reported previously (6, 11, 17). The vast majority (99.4%) of the patients were Caucasian and were treated by either modified radical mastectomy in 83% of patients or simple mastectomy with sampling of axillary lymph nodes in 17% of patients. The lymph nodes were recorded as containing or not containing carcinoma on histological examinations, with no additional breakdown of the numbers of nodes involved. The range of patient ages was 31–89 (mean age 57 years) at the time of presentation. All of the patients had invasive carcinomas, and the distribution of tumor sizes (T_1 , <2 cm; T_2 , 2–5 cm; T_3 , >5 cm in diameter; and T_4 fixed to the chest wall; 321 patients), nodal status (239 patients), menopausal status (302 patients), and some histological grades (I–III) have been recorded previously (11); others were assessed more recently (294 patients in total). This represents 96%, 72%, and 88% of the patients with known tumor size, nodal status, and histological grading, respectively. The patients were staged clinically according to the international Tumor-Node-Metastasis system. The absence of metastatic disease at the time of presentation was confirmed by skeletal survey or bone scan and in some patients by urinary hydroxyproline estimations. Only patients with operable cancer (T_{1-4} , N_{0-1} , M_0) were included in the study. No patients in this group received any adjuvant systemic therapy. Follow-up information was obtained from the Merseyside Cancer Registry for patients in this study and was updated for patient survival to August 31, 1995. The period of follow-up ranged from 14 to 20 years with a mean of 16 years. The accuracy of this data was subsequently checked by inspection of General Practitioner records to confirm whether patients were alive, dead of cancer, or dead of other causes. Local Ethics Committee approval was obtained and the patient data anonymized. Archival formalin-fixed, paraffin-embedded specimens were obtained from the primary tumors, and they were predominantly invasive ductal carcinoma of no special type (NOS), with only 1.5% of special type (colloid and medullary).

Serology. Mouse MAb MBIII B10₁ of the isotype IgG1 raised against rat OPN was purchased from the Developmental Studies Hybridoma Bank (34), developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA). It recognized rat, human, rabbit, and mouse OPN in Western blots, and in immunocytochemistry; several batches were purchased giving the same results. Rabbit antiserum to human OPN (LF-123) was a gift of Dr. Larry Fisher (National Institute of Dental and Craniofacial Research/NIH, Bethesda, MD; Ref. 35). It was raised against the recombinant carboxyl half of human OPN, starting at the thrombin site, and did not contain the RGD domain (36). Mouse MAbs to human ER α (ID5), PgR (NCL-PGR), pS2, and *c-erbB-3*, respectively, were purchased from Dako (Ely, United Kingdom), Novocastra (Newcastle-upon-Tyne, United Kingdom), CIS Bio (Gif-sur-Yvette, France) and Novocastra. The rabbit polyclonal antibody to the mutant form of p53 (CM1) was a gift from Professor David Lane (Dundee, United Kingdom). All of these antibodies recognized the correct sized antigens on Western blots from SDS-polyacrylamide gels of extracts from human breast tumor tissue (37).

Immunocytochemistry. Histological sections on 3-aminopropyltriethoxysilane-coated slides were cut at 4 μ m from the formalin-fixed, paraffin-embedded specimens, and endogenous peroxidase activity was removed with H₂O₂ (38). Antigen retrieval was undertaken routinely for ER α , PgR, pS2, and p53 (37). Indirect immunocytochemistry was carried out using a commercially available antibody biotin complex containing horseradish peroxidase as described previously (38). The MAb to OPN was diluted to 1:30, whereas the rabbit antiserum was diluted to 1:300, the sections were incubated at room

temperature overnight for 16 h for the MAb or for 3 h for the polyclonal antibody, and bound antibody was detected with 1:200 diluted biotinylated sheep antimouse Ig (Amersham Int., Bucks, United Kingdom) or 1:200 biotinylated donkey antirabbit Ig (Amersham Int.) followed by antibody biotin complex (Dako Ltd). The bound complex was visualized with 3,3'-diaminobenzidine (Sigma, Poole, United Kingdom) and 0.003% H₂O₂. The cellular nuclei were counterstained blue with Mayers' hemalum and the sections mounted in DPX (Merck Ltd., Poole, United Kingdom). Blocked antibody was prepared as above with 50 μ g/ml full-length human recombinant OPN obtained from Dr. Larry Fisher (39) or with 10 μ g/ml commercial human recombinant OPN from Chemicon International (see below). Immunocytochemical staining of carcinoma specimens for ER α (323 patients), PgR (315 patients), pS2 (327 patients), and *c-erbB-3* (321 patients) with MAbs, and for p53 (330 patients) with polyclonal antibodies was conducted as above using the relevant secondary antibody except that incubations were for 90 min (37), similar to that for *c-erbB-2* (327 patients) and cathepsin D (256 patients) described previously (6, 17).

Slides from all 333 of the specimens stained by the MAb to rat OPN were analyzed independently by two observers using light microscopy. The percentage of carcinoma cells with cytoplasmic/membranous staining was recorded from two sections of each specimen, 10 fields/section at \times 200 magnification. Staining for OPN was evaluated initially in six classes: negative (-), <1%; borderline (\pm), 1–5%; intermediate (+), 5–25%; moderate (++), 25–50%; strong (+++), 50–75%; and very strong (++++), 75–100% of the carcinoma cells stained. Because the strong and very strong groups were small, they were combined into one class (++++). For two-way analyses the negative and borderline classes were usually combined into one group (-/ \pm) and all of the genuinely positive classes in another group (+/+/+/+/+/++++). Increasing the concentration of antibody to OPN 10-fold or substituting the mouse MAb to rat OPN with the rabbit polyclonal antibody to human recombinant OPN gave identical results for 16 specimens examined at random. The cutoff values for the other immunocytochemically stained markers between those groups of patients designated negatively or positively stained also included the borderline staining group with the unstained group, unless otherwise specified (6, 17, 38). Photographs were recorded on a Reichert Polyvar microscope fitted with a Wratten 44 blue green filter (38).

Protein Samples and Western Blotting. Pure full-length recombinant human OPN (39) and bovine OPN (40) were gifts of Dr. Larry Fisher and Professor Esben Sorensen (Aarhus University, Aarhus, Denmark), respectively. Additionally, full-length human recombinant OPN (CC 1074) was purchased from Chemicon International (Harrow, United Kingdom). Samples of human breast cancer specimens were converted to a powder while still frozen using a brass pestle and mortar at -70°C, and the resulting frozen and powdered tissue was rapidly transferred into either 16 or 8 ml of a guanidinium isothiocyanate buffer system (41) containing an elevated concentration of 2 mercaptoethanol (42). The tissue powder was solubilized immediately in a Polytron homogenizer at 16,000 rpm, centrifuged at 7,700 \times g for 10 min, and the supernatant centrifuged on a cushion of 5.7 M CsCl for 18 h at 120,000 \times g as described previously (42). The resultant supernatant above the CsCl cushion was dialyzed (cutoff M_r 3,500) against 10 mM (NH₄)₂CO₃, lyophilized, redissolved in sample buffer containing 2% (w/v) SDS together with glycerol, bromophenol blue, and 2-mercaptoethanol (43). An additional SDS extract of a human osteosarcoma cell line (SC2235) was purchased from Autogen Bioclear (Colne, United Kingdom). The samples containing equal amounts of protein were boiled, sonicated, and electrophoresed on 0.1% (w/v) SDS-10% (w/v) polyacrylamide gels (43) together with molecular weight markers and OPN standards. Proteins were transferred to Immobilon P membranes (Millipore, Watford, United Kingdom), which were incubated with "blocking" buffer containing 3% (w/v) dried, defatted milk, for 45 min at room temperature and then with anti-OPN Ig, diluted as in the figure legends. In some experiments, 10 μ g/ml commercial human recombinant OPN or 1 mg/ml bovine OPN was present to provide a blocked antibody control. Membranes were then incubated with horseradish peroxidase-conjugated rabbit antimouse (for MAb) or swine antirabbit (for the polyclonal antibody) Ig, and bound antibodies were detected with the Super Signal West Pico Chemiluminescence System (Pierce and Warriner, Chester, United Kingdom) and exposure to Fuji RX film (44). Images of the film were scanned using a Shimadzu C9000 flying spot densitometer, and the integrated densities of area under the peaks were recorded as described previously (42, 44).

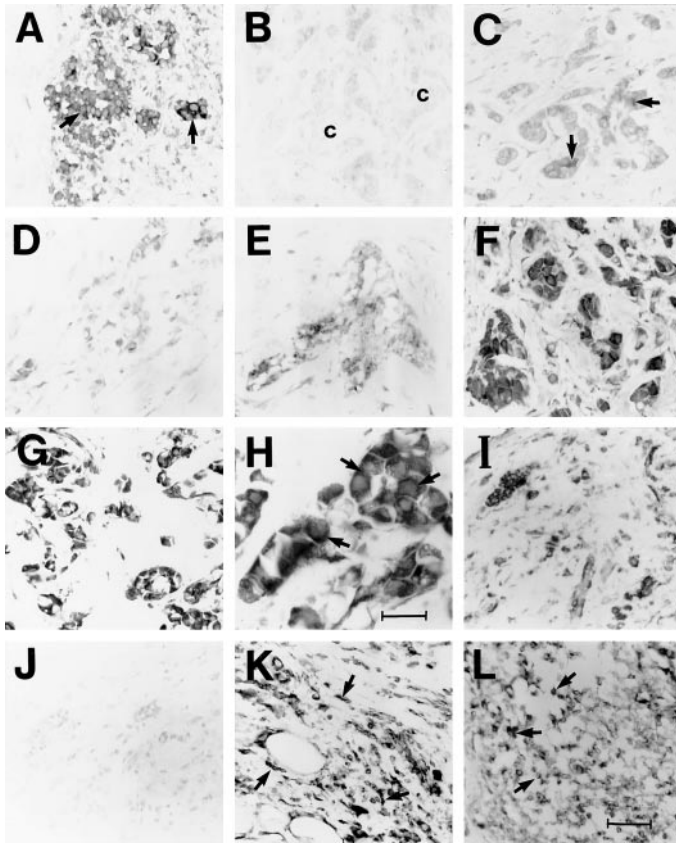


Fig. 1. Immunocytochemical staining with MAb to OPN. A, normal breast from a pregnant female showing staining of epithelial cells in lobules (arrows). B, invasive carcinoma (c) showing no immunocytochemical staining. C, invasive carcinoma showing borderline staining (\pm) of the occasional malignant cell (arrows). D and E, invasive carcinoma showing intermediate staining of the malignant cells for (D) class + and moderate staining for (E) class ++. F and G, invasive carcinoma showing strong staining of the malignant cells for (F) class +++ and very strong staining for (G) class +++++. H, higher magnification of G showing strong cytoplasmic staining of malignant cells (arrows). I and J, adjacent sections of the same invasive carcinoma incubated with either (I) MAb to OPN showing strong staining or (J) the same MAb preincubated with human recombinant OPN showing no staining. K and L, reactive stromal cells (arrows, K) and lymphocytic cells/macrophages (arrows, L) in an invasive but unstained carcinoma, both showing strong staining. Magnification, A–G and I–L, $\times 230$; H, $\times 580$. Bars, A–G and I–L, 50 μm ; H, 20 μm .

Statistical Methods. The association of immunocytochemical staining for OPN with other tumor variables was assessed using Fisher's exact test; two-sided values of *P* were given (45). These variables on the same group of patients included tumor size, histological grade, nodal status, menopausal status, patient age (11), and presence of *c-erbB-2* (6), *c-erbB-3*, ER α , pS2, PgR, p53 (37), and cathepsin D (17). The cutoff values between those groups of patients designated negatively or positively immunocytochemically stained for the marker proteins were set at 5% and, therefore, included the borderline staining group with the unstained group, unless otherwise specified (6, 17, 38). The degree of agreement between observers was assessed using the Kappa statistic; a value of >0.61 was taken to be a satisfactory level of agreement (45).

The association of the staining for OPN in breast cancers with patient survival was evaluated using life tables constructed from survival data with Kaplan-Meier plots and analyzed using generalized Wilcoxon (Gehan) statistics (45). Those patients who died of causes other than cancer were treated as censored observations (6). To assess unadjusted RR for survival and 95% CI, a Cox univariate analysis was performed with $\text{RR} = \text{antilog}_e \beta$, and 95% CI obtained from the SE of β , β being the constant in the exponential decay term used for modeling (45). To determine whether the association of patient survival with OPN was independent of other prognostic factors shown to approach significance in univariate analysis, a multivariate analysis was performed using the Cox proportional hazards model (46). Other potential prognostic factors measured on the same group of patients included tumor size,

histological grade, nodal status (11), the presence of *c-erbB-2* (6), cathepsin D (17), ER α , PgR, pS2, p53, and *c-erbB-3* (38). Data processing and statistical analyses were performed using Excel version 97 (Microsoft Corp., Redmond, WA) and Statistical Package for the Social Sciences, version 10.0 (SPSS Inc., Chicago, IL).

RESULTS

Immunocytochemical Staining for OPN. When histological sections from 2 normal breasts or 10 samples from uninvolved breast tissue from cancerous breasts were incubated with MAb to rat OPN, the majority of the parenchymal tissue was relatively unstained (not shown), although tissue from pregnant/lactating women showed extensive staining of alveolar cells and luminal secretions (Fig. 1A). When 333 breast carcinomas were examined, 47 (14%) were unstained (Fig. 1B), 65 (20%) possessed borderline staining (Fig. 1C), and the remaining 221 (66%) were stained to various degrees by MAb to OPN. These positively stained carcinomas were subdivided into 59 (18%) intermediate (Fig. 1D), 95 (28%) moderate (Fig. 1E), and 67 (20%) strong (Fig. 1F) or very strong (Fig. 1G) staining carcinomas, depending on the average percentage of stained carcinoma cells present in the specimen (Table 1). The strong staining and very strong staining groups were combined to equalize, somewhat the numbers in each quartile staining class. The staining was confined mainly to the cytoplasm (Fig. 1H) and could be abolished by prior incubation of the MAb with human recombinant OPN (Figs. 1, I and J). The assessment of staining class was made only on the malignant cells; however positive staining was also present on normal blood vessels, certain reactive stromal cells (Fig. 1K), and lymphocytic cells/macrophages (Fig. 1L). The same results were obtained with 16 specimens selected at random and stained with a rabbit polyclonal antibody to human OPN, although backgrounds were higher than with the MAb to rat OPN.

The borderline class was defined as carcinomas possessing 1–5% of the malignant cells stained for OPN (Table 1). For the purpose of most analyses the borderline staining carcinomas were combined with the unstained carcinomas into one group of negatively staining carcinomas, leaving the clearly positive staining carcinomas as the other group. There was some variability in the assessment of staining by the two observers on the same histological section for these two broad classes of negatively and positively stained carcinomas. However, there was agreement in 96% of the slides, corresponding to a κ value of 0.93, which represents a good degree of consistency between observers. In addition, intratumor heterogeneity was determined by comparing the category of staining allocated independently to two well-separated sections from the same tumor. In 10% of all of the histological sections analyzed, heterogeneity of staining was sufficiently high to influence whether a section was classified as negatively or positively stained. In those cases two additional sections were immunocytochemically stained and analyzed to obtain a consensus result.

Table 1 Frequency distribution of immunocytochemical staining of carcinomas for OPN

| Percentage of carcinoma cells staining ^a | Classification of staining | Number of carcinomas ^b | Percentage of carcinomas |
|---|----------------------------|-----------------------------------|--------------------------|
| <1 | – | 47 | 14 |
| 1–5 | \pm | 65 | 20 |
| 5–25 | + | 59 | 18 |
| 25–50 | ++ | 95 | 28 |
| 50–100 | +++ / +++++ | 167 | 20 |
| Total | | 333 | 100 |

^a Two sections, 10 fields per section, read by two independent observers for sections stained by MAb to OPN.

^b Number of carcinomas in each staining class, only the carcinoma cells were scored.

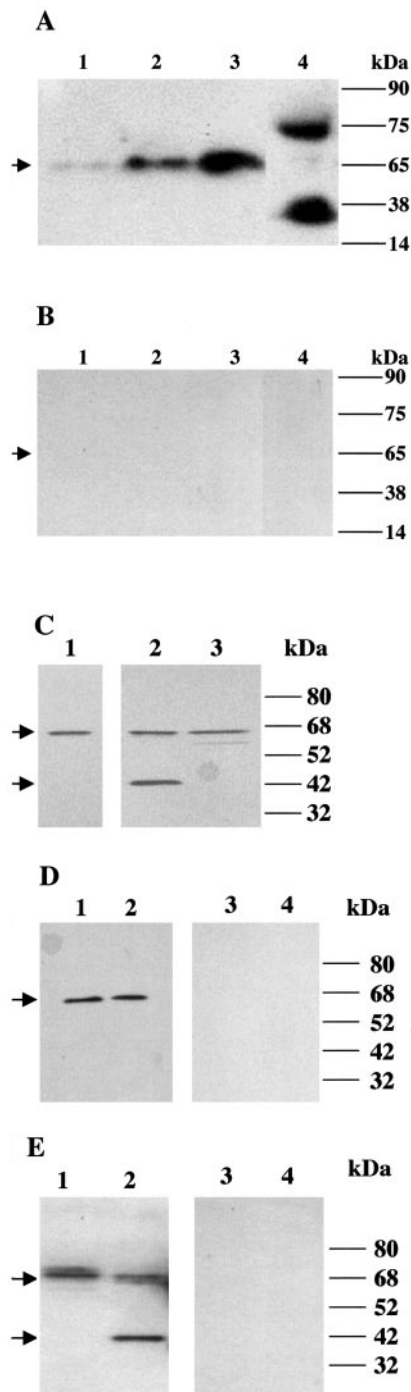


Fig. 2. Detection of OPN by Western blotting. A and B, protein samples (16 μ g) of SDS extracts of invasive carcinomas of the following classes of immunocytochemical staining for OPN: class \pm (Lane 1), class ++ (Lane 2), class ++++ (Lane 3), and a 16- μ g sample of pure bovine OPN (Lane 4) were subject to electrophoresis on 10% (w/v) polyacrylamide SDS gels, and the proteins were transferred to the Immobilon P membrane (see "Materials and Methods"). The filter was incubated with A, a 1:500 dilution of MAb to rat OPN for 2 h or B, the same antibody preincubated for 2 h with 1 mg/ml bovine OPN for the same time period all at 4°C. The filters were additionally incubated with a 1:5000 dilution of peroxidase-conjugated swine antimouse IgG for 1 h. Bound antibody was detected by chemiluminescence and exposure to Fuji RX film. The position of human OPN is shown on the left side of the panels by the arrows and that of molecular weight markers is shown on the right side. Scanning densitometry yielded the following values for the relative proportions of immunoreactive OPN: 5.43, 35.14, and 78.83 for samples with (mean \pm SD, 10 points) 3 ± 2 , 37.5 ± 5 , and $75 \pm 10\%$ carcinoma cells stained for OPN, respectively. Least squares regression analysis fitting of these points to a straight line through the origin yielded $r^2 = 0.994$, $P < 0.001$, where $r^2 = 1$ is a perfect fit. C-E, protein samples of 2 μ g of pure human recombinant OPN (Lane 1), 16 μ g of SDS extract of class ++++ invasive breast carcinoma above (Lane 2), and additionally in C 16 μ g of SDS extract of a human osteosarcoma cell line (Lane 3) were subjected to SDS-PAGE and transferred to Immobilon P membranes. The filters were incubated in C (Lane 1) and D

Western Blot Analysis for OPN. When tested in Western blots of extracts of selected carcinoma specimens, the MAb to OPN recognized a protein of M_r 65,000 (Fig. 2A, Lanes 1–3), consistent with the size of OPN from human tumors (47, 48), as well as cross-reacting with pure bovine OPN of M_r 75,000 (Fig. 2A, Lane 4; Ref. 40). The level of staining of the Western blots increased from very little for the borderline class, through intermediate for the moderate class, to strong for the strong staining immunocytochemical class (Fig. 2A, Lanes 1–3). The binding of the MAb to the M_r 65,000 protein in the sample extracts and to bovine OPN was inhibited completely when bovine OPN at a concentration of 1 mg/ml was preincubated with MAb to OPN before the blotting procedure (Fig. 2B, Lanes 1–4). The relative proportions of immunoreactive OPN determined by scanning densitometry of Western blots of 3 selected samples showed a good correlation with the percentage of immunocytochemically stained carcinoma cells from histological sections of the same specimens (correlation coefficient $r^2 = 0.99$, $P < 0.001$; Fig. 2A). In addition to the protein at M_r 75,000, Western blots of pure bovine OPN produced a smaller molecular weight protein of $\sim M_r$ 38,000 of which the appearance was also inhibited by prior incubation of the MAb with pure bovine OPN (Fig. 2A, Lane 4). This is probably similar to the cleavage product reported previously (40).

When the two antibodies were compared, the MAb to rat OPN reacted with a single M_r 65,000 polypeptide, whereas the polyclonal antibody to human OPN reacted with an additional M_r 45,000 polypeptide from breast cancer extracts that were immunocytochemically positive for OPN (Fig. 2C, Lanes 1 and 2). The polyclonal antibody failed to recognize this smaller polypeptide in extracts of an osteosarcoma cell line (Fig. 2C, Lane 3). Both the MAb to rat OPN (Fig. 2D, Lanes 1 and 2) and the polyclonal antibody to human OPN (Fig. 2E, Lanes 1 and 2) reacted with human recombinant OPN to a similar extent, and this protein coelectrophoresed with the M_r 65,000 polypeptide detected in extracts from OPN-positive breast cancers. Prior incubation of both antibodies with pure recombinant human OPN blocked their ability to recognize recombinant human OPN (Fig. 2, D and E, Lane 3) and the M_r 65,000 polypeptide in the breast cancer extracts (Fig. 2, D and E, Lane 4), as well as the M_r 45,000 polypeptide detected by only the rabbit antiserum to human OPN (Fig. 2E, Lane 4). Because the MAb to rat OPN recognized one discrete polypeptide corresponding closely to the molecular weight of human recombinant OPN, the MAb was the antibody of choice for this study.

Association of OPN with Other Tumor Variables. The presence of definitely positive immunocytochemical staining for OPN in the carcinoma cells was cross-tabulated with other tumor variables reported to be predictive of patient outcome, including tumor size, histological grade, nodal status, menopausal status, and the presence of *c-erbB-2*, cathepsin D, ER α , PgR, pS2, p53, and *c-erbB-3*, and assessed using Fisher's exact test (Table 2). Of the pathological factors, only the presence of high histological grade showed a statistically significant association with immunocytochemical staining for OPN in the primary tumor; 30% of OPN-positive tumors were classified as grade III compared with 17% of those that were classified as OPN-negative (Fisher's exact test, $P = 0.024$). There was a statistically significant association of carcinomas staining for OPN with

(Lanes 1 and 2) with 1:500 dilution of MAb to rat OPN; in D (Lanes 3 and 4) with the same MAb preincubated for 2 h with 10 μ g/ml human recombinant OPN; in C (Lanes 2 and 3) and E (Lanes 1 and 2) with a 1:5000 dilution of rabbit antiserum to human OPN; or in E (Lanes 3 and 4) with the same rabbit antiserum preincubated for 2 h with 10 μ g/ml human recombinant OPN. Bound antibodies were detected as above, except that peroxidase-conjugated swine antirabbit Ig was used for those filters incubated with the rabbit antisera. The position of recombinant human OPN and an additional M_r 45,000 band is shown on the left side of the panels by arrows and that of molecular weight markers on the right side.

Table 2 Association of immunocytochemical staining for OPN with other tumor variables

| Tumor variable ^a | OPN-negative no ^b (%) | OPN-positive no ^b (%) | Statistical significance ^c |
|-----------------------------|----------------------------------|----------------------------------|---------------------------------------|
| Grade 1, 2 | 68 (83) | 129 (70) | 0.024 |
| Grade 3 | 14 (17) | 56 (30) | |
| LN- | 49 (61) | 82 (52) | 0.17 |
| LN+ | 31 (39) | 77 (48) | |
| C-erbB-3- | 68 (59) | 66 (31) | <0.001 |
| C-erbB-3+ | 47 (41) | 146 (69) | |
| p53- | 82 (71) | 125 (57) | 0.014 |
| p53+ | 34 (29) | 95 (43) | |
| PgR- | 74 (68) | 124 (58) | 0.089 |
| PgR+ | 34 (32) | 89 (42) | |
| pS2- | 81 (70) | 123 (57) | 0.025 |
| pS2+ | 35 (30) | 94 (43) | |

^a Grade, histological grade 3 vs. histological grades 1, 2; LN, lymph node with tumor (+) or without tumor deposits (-); c-erbB-3, the presence (+) or absence (-) of immunocytochemical staining for the c-erbB-3 receptor (38); p53, the presence (+) or absence (-) of staining for p53 (38); PgR, the presence (+) or absence (-) of staining for the progesterone receptor (38); pS2, the presence (+) or absence (-) of staining for pS2 (38).

^b Number of patients with carcinomas either classified as staining (positive) or not staining (negative) for OPN. Parentheses show the percentage of patients.

^c Probability, P between paired samples from Fisher's exact test (two-sided value).

positive staining for the potential molecular markers c-erbB-3 ($P < 0.001$), p53 ($P = 0.014$), and pS2 ($P = 0.025$; Table 2). There was also a tendency for more PgR-positive carcinomas to be positive for OPN when compared with the PgR-negative carcinomas, but this association did not achieve statistical significance ($P = 0.089$; Table 2). The remaining tumor variables measured, tumor size, lymph node status, menopausal status, and staining for c-erbB-2, cathepsin D, and ER α , showed no significant association with positive staining for OPN in this group of patients, and there was no obvious age or other demographic difference between patients with OPN-negative and OPN-positive carcinomas (not shown). The very slight trend in association between lymph node status and positive staining for OPN was not significant ($P = 0.17$; Table 2).

Association of OPN with Patient Survival. The association of immunocytochemical staining and the cumulative proportion of patients surviving at yearly intervals after the time of presentation is shown in Fig. 3A. Of the 112 patients who were classified as OPN-negative, ~94% were alive at the census date, in comparison with ~26% of the 221 patients classified as OPN-positive. The median survival of patients with tumors classified as OPN-negative was >228 months in comparison with that of patients with a tumor classified as positive of 68 months. The data show that over the time period of 19 years, the survival of patients with OPN-positive carcinomas was highly significantly worse than those patients with carcinomas classified as OPN-negative (Wilcoxon test, $P < 0.0001$). This difference in survival of patients between the two groups was statistically significant after 1 year of follow-up (Wilcoxon test, $\chi^2 = 8.0$; 1 d.f.; $P = 0.005$). Thereafter increasing follow-up times increased the significance of the difference (e.g., for 2 years $\chi^2 = 17.4$, 1 d.f., $P < 0.0001$; for 3 years $\chi^2 = 31.5$; for 4 years $\chi^2 = 42.2$; for 5 years $\chi^2 = 55.4$; and for 19 years $\chi^2 = 95.4$). When the results are expressed in terms of RR of a patient surviving, women with OPN-negative and borderline carcinomas had an unadjusted RR for survival of 21 (95% CI, 9–49) compared with the OPN-positive group.

When the patients were divided into their separate classes based on the percentage of carcinoma cells staining immunocytochemically for OPN and analyzed separately for their outcome, then the five curves showed a progressive decrease in survival and were highly significantly different (Wilcoxon test, $P < 0.0001$; Fig. 3B). These overall differences were significant after 1 year (Wilcoxon test $\chi^2 = 10.5$;

4 d.f.; $P = 0.034$). The completely negative stained group of 47 patients (-) showed all 100% survived with a median survival of >228 months. The borderline group of 65 patients (\pm) showed 90% surviving with a median survival of >216 months, the intermediate group of 59 patients (+) showed 45% surviving with median survival of 177 months, the moderate group of 95 patients (++) showed 22% surviving with median survival of 56.8 months, and the strong staining group of 67 patients (+++/++++) showed only 16% surviving with median survival of 55.7 months (Table 3). The differences between the first four consecutively paired classes of patients were significant (Wilcoxon test, $P \leq 0.034$), but that between the moderate (++) and strong staining (+++/++++) classes was not ($P = 0.54$). These differences between the first four consecutively paired classes of patients became statistically significant after 10, 3, and 5 years, respectively. This progressive decrease in patient survival for the different staining classes was also reflected in a progressive decrease in unadjusted RR for survival varying from 6.9 (95% CI, 2.8–17), through 13.8 (95% CI, 6.0–32), to 16 (95% CI, 6.8–37) for the borderline versus intermediate (\pm versus +), intermediate versus moderate (+ versus ++), and moderate versus strong (++ versus +++/++++) staining classes, respectively (Table 3). However, the comparisons with the group of patients with unstained carcinomas may be open to question because there were no deaths, and, therefore, all 47 of the observations were censored in this group (Fig. 3B). Hence the RR between the unstained versus the borderline staining class (- versus \pm) was not determinable (Table 3).

Association of OPN and Other Tumor Variables with Patient Survival. In addition to staining for OPN, the other tumor variables, which showed a significant association with survival time for this group of 333 patients at the same census date were: nodal status (Wilcoxon test, $P < 0.0001$; RR = 2.1), tumor size ($P = 0.0014$; RR = 1.5), histological grade ($P = 0.0049$; RR = 1.52), staining for c-erbB-2 ($P = 0.0024$; RR = 1.7), ER α ($P = 0.025$; RR = 0.73), and p53 ($P = 0.020$; RR = 1.4). The association of staining for PgR, pS2, c-erbB-3, and cathepsin D with survival time failed to reach statistical significance in this group of patients at the 5% cutoff level (Table 4). Most of the tumor variables were associated in a negative manner with survival times of the patients, but ER α showed a positive association with survival times (Table 4). However, all of these tumor variables had smaller values of the Wilcoxon statistic χ^2 and RR than those for OPN. Therefore, these tumor variables were less significantly associated with patient survival time and were associated with less of a risk to the patient than OPN (Table 4).

The association of patients with carcinomas either staining or not staining for OPN and their survival in subgroups defined by the different tumor variables described above were analyzed. The borderline cases of carcinoma cell staining for OPN were included, once again, in the unstained carcinoma cell group of patients to ensure sufficient numbers. In all of these subgroups of patients, staining for OPN was associated with poorer survival time, including that for nodal status (Table 5). Three of the tumor variables studied were associated with a statistically significant reduction in patient survival time over that obtained with OPN alone: involved lymph nodes, fixed tumors (T₄), and staining for c-erbB-2 (Table 5). The results for histological grade III, p53, and cathepsin D showed a similar trend to the other three tumor variables above but were not significant (Table 5). Three of the tumor variables studied were associated with a statistically significant increase in patient survival times over that obtained with OPN alone: staining for ER α ($P = 0.0001$), PgR ($P = 0.006$), and c-erbB-3 ($P = 0.004$; Table 5). The results for OPN patients without or with pS2 showed a similar trend, but this was not statistically significant (Table 5). Univariate analyses for survival of the patients with OPN-positive carcinomas showed that RRs for not

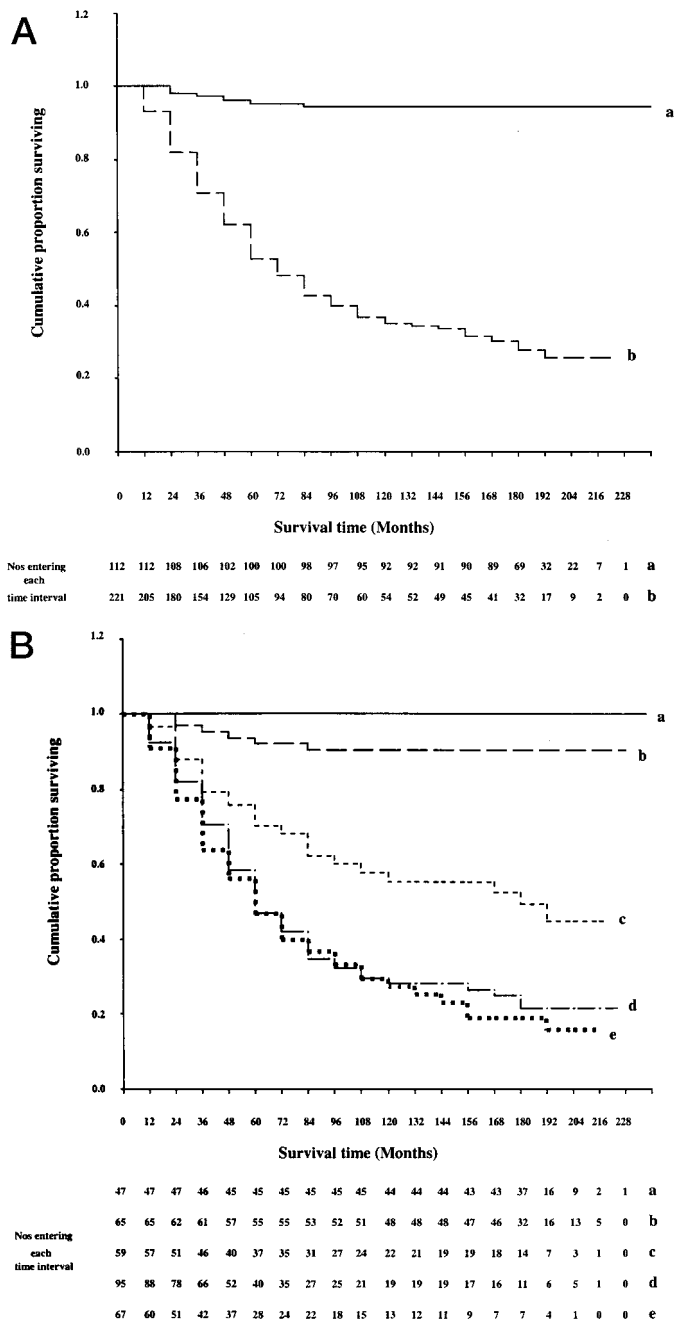


Fig. 3. Association of immunocytochemical staining for OPN with overall survival of patients for (A) a cutoff of 5% between the two staining classes and for (B) different classes of immunocytochemical staining. In A, the cumulative proportion of surviving patients as a percentage of the total for each year after presentation for either (a) patients with carcinomas classified as negatively staining (—) or (b) positively staining (---) for OPN is shown. For OPN-negative carcinomas, 100% corresponds to 112 patients, and 100% for OPN-positive carcinomas corresponds to 221 patients. There were 106 censored observations in a (17 dead of other causes) and 75 in b (41 dead of other causes). The cumulative proportions surviving \pm 1.96 SD to give 95% CIs were (a) 0.95 ± 0.02 and (b) 0.48 ± 0.03 at 5 years; (a) 0.94 ± 0.02 and (b) 0.34 ± 0.03 at 10 years; (a) 0.94 ± 0.02 and (b) 0.26 ± 0.03 at 15 years; and (a) 0.94 ± 0.02 and (b) 0.26 ± 0.03 at 20 years. The two curves are highly significantly different (Wilcoxon statistic $\chi^2 = 95.4$, 1 d.f., $P < 0.0001$). In B, the cumulative proportion of surviving patients as a percentage of the total for each year after presentation of (a) patients with carcinoma cells classified as completely negative staining (—; 100% = 47 patients); (b) \pm borderline staining (---; 100% = 65 patients); (c) + intermediate staining (- - -; 100% = 59 patients); (d) ++ moderate staining (- · - · -; 100% = 95 patients); and (e) ++++/++++ strong staining (●●●; 100% = 67 patients) for OPN is shown. There were 47 censored observations in a (4 dead of other causes); 59 in b (13 dead of other causes); 32 in c (17 dead of other causes); 27 in d (13 dead of other causes); and 16 in e (11 dead of other causes). The cumulative proportions surviving \pm 1.96 SD to give 95% CIs were (a) 1.00 ± 0.00 , (b) 0.92 ± 0.03 , (c) 0.68 ± 0.06 , (d) 0.42 ± 0.05 , and (e) 0.40 ± 0.06 at 5 years; (a) 1.00 ± 0.00 , (b) 0.90 ± 0.04 , (c) 0.55 ± 0.07 , (d) 0.28 ± 0.05 , and (e) 0.25 ± 0.06 at 10 years; (a) 1.00 ± 0.00 , (b) 0.90 ± 0.04 , (c)

having one of the other tumor variables (Table 5) were similar in most cases, except for *c-erbB-3*, to those in the unselected patients (Table 4); the node-negative, T_4 -negative, *c-erbB-2*-negative, and ER-positive patients had the highest RRs of ~ 2 -fold (Table 5). The survival times for patients with OPN-negative tumors with or without any one of the other tumor variables were, with the exception of ER α , not significantly different (not shown). The median survival times and cumulative proportion of patients surviving for patients with OPN-negative tumors with either tumor variable-negative or tumor variable-positive tumors was >216 months and $>87\%$, respectively, in every case (not shown). However, it is possible that the statistical validity may have been influenced by the numbers of patients in the subgroups analyzed.

To determine whether the seven tumor variables that were significant in the univariate analyses were independent of one another, they were all included in a multivariate regression analysis for the 202 patients available with full data sets (see "Materials and Methods"). After analysis using this model, the first variable to emerge and the most significant of all was staining for OPN, followed by involved lymph nodes, fixed tumors T_4 , staining for ER α , and borderline significance for *c-erbB-2* (Table 6). On controlling the data for OPN, nodal status, fixed tumors T_4 , ER α , and *c-erbB-2*, there was no significant association between tumor sizes T_1 - T_3 , grades I-III, p53, and patient survival (Cox analysis, residual $P = 0.77$). Consistent with this result, the univariate analysis for tumor size gave the most significant and shortest survival for patients when groups with fixed tumors (T_4) were compared with the rest (T_1 - T_3 ; Wilcoxon test, $\chi^2 = 9.19$; 1 d.f.; $P = 0.0024$). None of the possible pairwise or higher order interaction terms in the multivariate analysis was statistically significant, implying that the effect of staining for OPN on patient survival is similar over the various prognostic groups defined by nodal status, tumor fixation, ER α , and *c-erbB-2*. When the results were expressed in terms of the RR of a patient surviving, and adjusted for nodal status, tumor size T_4 , and staining for ER α and for *c-erbB-2* in multivariate analysis, the RR for survival of women with OPN-negative carcinomas was 13 (95% CI, 5.6-30), an effect that was still much higher than that of all of the other factors (Table 6). In the same multivariate analysis the individual contributions that grade and p53 staining made to the RR for survival of the patients were lost (Table 6).

DISCUSSION

The purpose of this present investigation has been to establish using immunocytochemical techniques, the level of expression of the glycoposphoprotein OPN in carcinomas of patients presenting with primary breast cancer and its clinical significance. We have found that 66% of the carcinomas are stained positively by the mouse MAb to rat OPN and that 20% possessed borderline staining of between 1 and 5% of the carcinoma cells stained for OPN. This percentage is largely in agreement with the overall staining levels of 70%-80% reported previously (32, 33, 49, 50), except the very high level of 87% reported by Kim *et al.* (Ref. 51; see later). In much of the present work the borderline cases have been included with the negatively stained carcinomas unless otherwise stated. In agreement with previous reports (28, 31, 33), the staining in specimens is not restricted to only

0.45 ± 0.08 , (d) 0.22 ± 0.05 , and (e) 0.16 ± 0.05 at 15 years; and (a) 1.0 ± 0.00 , (b) 0.90 ± 0.04 , (c) 0.45 ± 0.08 , (d) 0.22 ± 0.05 , and (e) 0.16 ± 0.05 at 20 years. The five curves are highly significantly different (Wilcoxon statistic $\chi^2 = 109.3$, 4 d.f., $P < 0.0001$) and significantly different in the pairwise combinations for a with b ($\chi^2 = 4.50$, 1 d.f., $P = 0.034$), b with c ($\chi^2 = 18.75$, 1 d.f., $P < 0.0001$), c with d ($\chi^2 = 7.92$, 1 d.f., $P = 0.005$), but not for d with e ($\chi^2 = 0.38$, 1 d.f., $P = 0.53$).

Table 3 Summary of association of different staining classes with patient survival times

| Class ^a | Patient number ^b | Median survival ^c (months) | Cumulative survival ^c (%) | χ^2 ^d | P ^d | RR ^e | 95% CI ^e |
|--------------------|-----------------------------|---------------------------------------|--------------------------------------|-----------------------|----------------|-----------------|---------------------|
| - | 47 | >228 | 100 | } | 4.5 | 0.034 | n.d. ^f |
| ± | 65 | >216 | 90 | | | | |
| + | 59 | 177 | 45 | | | | |
| ++ | 95 | 56.8 | 22 | | | | |
| +++ /++++ | 67 | 55.7 | 16 | | | | |

^a Immunocytochemical staining class for OPN defined in Table 1.

^b Total number assessed for the group.

^c Median survival in months and cumulative proportion of patients surviving evaluated using life tables constructed from survival data ("Materials and Methods").

^d χ^2 and probability (P) were determined by the generalised Wilcoxon (Gehan) test using 1 d.f. ("Materials and Methods").

^e RR and 95% CI were determined using a Cox's univariate analysis with 1 d.f. ("Materials and Methods").

^f n.d., not determinable since no events in the negative (-) group of patients.

carcinoma cells, because variable, often reduced levels are detected in normal parenchymal breast tissue and in some reactive stromal fibroblast-like cells, macrophages, lymphocytes, and blood vessels. Although the overall proportion of breast carcinomas reported capable of being stained with antibodies to OPN is relatively consistent, the relative proportion, because of stained host cells, particularly macrophages/lymphocytes, and because of the stained carcinoma cells, has varied considerably (28, 31, 33). This variation in relative proportion of host to carcinoma cells producing OPN is also reflected in the level of its mRNA using *in situ* hybridization techniques with nucleic acid probes (33, 49, 50). Because an early study reported that carcinoma cells contained immunodetectable OPN but not hybridizable mRNA, whereas host macrophages contained both, it was suggested that OPN secreted by macrophages may bind to the carcinoma cells rather than be synthesized *de novo* (28). More recent studies have shown that some of the OPN-containing cells also contain OPN mRNA (33, 49, 50), but this finding does not exclude the possibility that OPN could be transferred from host to a variable proportion of carcinoma cells in some way. However, our present assessment has been undertaken on only the carcinoma cells in the tumor specimens and their ability to stain for immunoreactive OPN, irrespective of how the OPN is generated.

The rather heterogeneous cellular staining pattern for OPN observed here is not an artifact of tissue preservation attributable, for instance, to lack of accessibility of the antigen for its specific antibody for the following reasons: (a) the same results have been obtained in pilot experiments using frozen sections and with carcinomas preserved in Methacarn (38) or formalin as paraffin-embedded sections; (b) increasing the concentration of the MAb 10-fold or incubating for periods >16 h fails to increase the assessment of staining, although incubating for short 1–3 h periods reduces the levels of staining, and hence assessment, appreciably; and (c) attempts at antigen retrieval by prior microwaving (52) for osseous deposits (47) or Pronase digestion (38) failed to increase the assessment. The immunocytochemical staining for OPN is also specific for this molecule for the following reasons: (a) incubation of human recombinant OPN with the mouse MAb to OPN before its use completely inhibits the immunocytochemical staining of all of the carcinoma cells as well as of lactating breast parenchymal cells and reactive stromal cells in the invasive carcinomas; (b) the same staining patterns have been achieved with different batches of commercial mouse MAb to rat OPN and in a small sample with a rabbit polyclonal antibody to human recombinant OPN; and (c) the mouse MAb to OPN reacted with pure bovine OPN from milk and produced a single band of the same molecular weight as human tumor OPN (47, 48) in extracts of selected positively staining carcinomas when tested by Western blotting techniques. The second smaller band of ~M_r 38,000 in the bovine milk OPN probably corresponds to a cleavage product (47, 53), as reported previously (40). In addition,

interobserver and intratumor variability in immunocytochemical staining for OPN is sufficiently small (4% and 10%, respectively) not to affect appreciably the reported results. Finally, the different classes of immunocytochemical staining of the carcinoma specimens based on the proportion of OPN immunoreactive carcinoma cells may also reflect the levels of expressed OPN protein, because the levels of OPN immunoreactive protein, as determined by Western blotting, are linearly correlated with the percentage of stained carcinoma cells by immunocytochemistry, albeit for only a very few tumor specimens tested.

In this and previous studies with this group of patients (6, 11, 38) the tumor variables, which show a significant association with survival time of the patients, are nodal status (P < 0.0001), tumor size (P = 0.014), histological grade (P = 0.0049), and staining for *c-erbB-2* (P = 0.0024), ER α (P = 0.025), and p53 (P = 0.020), for the full follow-up period of 19 years. Immunocytochemical staining for PgR, pS2, *c-erbB-3*, and cathepsin D show only trends in association with survival times in this group of patients at the 5% cutoff level and are not statistically significant, despite their significant association in other groups of patients (7, 8, 12–14). The association of OPN and another metastasis-inducing protein, S100A4, assessed in this group of patients (38) is the subject of a future communication.

When the above tumor variables, which represent potential prognostic markers for patient outcome, are tested for association with immunocytochemical staining for OPN in the primary carcinomas, only high histological grade, and staining for *c-erbB-3*, pS2, and for p53 show statistical significance, and a trend is also established for

Table 4 Association of tumor variables with patient survival times

| Tumor variable | Patient | χ^2 ^a | P ^a | RR ^b | 95% CI ^b |
|---------------------------------|---------|-----------------------|----------------|-------------------|------------------------|
| Tumor size ^c | 321 | 10.59 | 0.014 | 1.49 ^d | 1.13–1.95 ^d |
| Histological grade ^e | 294 | 10.66 | 0.0049 | 1.52 ^f | 1.19–1.93 ^f |
| Nodal status ^g | 239 | 17.29 | <0.0001 | 2.06 | 1.42–3.00 |
| OPN ^h | 333 | 95.4 | <0.0001 | 21.5 | 9.5–48.9 |
| <i>C-erbB-2</i> ^h | 327 | 9.22 | 0.0024 | 1.66 | 1.16–2.39 |
| <i>C-erbB-3</i> ^h | 321 | 0.72 | 0.40 | 1.31 | 0.93–1.84 |
| ER α ^h | 323 | 5.00 | 0.025 | 0.73 | 0.53–1.02 |
| PgR ^h | 315 | 2.21 | 0.14 | 0.84 | 0.60–1.18 |
| pS2 ^h | 327 | 0.71 | 0.40 | 1.25 | 0.90–1.73 |
| p53 ^h | 330 | 5.37 | 0.020 | 1.37 | 0.99–1.89 |
| Cathepsin D ^h | 256 | 0.24 | 0.62 | 1.12 | 0.77–1.62 |

^a χ^2 and probability (P) were determined by the generalised Wilcoxon (Gehan) test ("Materials and Methods").

^b RR and 95% CI were determined using a Cox univariate analysis with 1 d.f. ("Materials and Methods").

^c T₁ vs. T₂ vs. T₃ vs. T₄, P for 3 d.f.

^d RR mean for 1 step; T₁ vs. T₂ = 1.35 (95% CI, 0.72–2.53), T₁ vs. T₃ = 1.59 (0.79–3.18); T₁ vs. T₄ = 3.30 (1.46–7.45), all 1 d.f.

^e Histological grade: I vs. II vs. III; P for 2 d.f.

^f RR mean for 1 step; I vs. II = 1.68 (95% CI, 1.08–2.61), I vs. III = 2.30 (1.42–3.71) all 1 d.f.

^g No nodes vs. 1 or more nodes with tumor; P for 1 d.f.

^h Negative vs. positive staining, 5% cutoff.

Table 5 Summary of the survival of OPN-positive patients divided into groups by the other tumor variables

| Tumor variable (TV) | Median survival ^a (months) | | Cumulative survival ^d (%) | | χ^2 ^b | P ^b | RR ^c | 95% CI ^c |
|--|---------------------------------------|-------|--------------------------------------|------|-----------------------|----------------|-----------------|---------------------|
| | TV- | TV+ | TV- | TV+ | | | | |
| Tumor size T ₄ ^d | 74.3 | 31.0 | 26.6 | 10.0 | 7.1 | 0.008 | 2.43 | 1.36-4.33 |
| Histol. grade III ^e | 78.9 | 54.7 | 28.2 | 20.9 | 2.0 | 0.16 | 1.35 | 0.94-1.95 |
| Nodal status ^f | 108.8 | 49.7 | 33.0 | 16.3 | 14.3 | 0.0002 | 1.99 | 1.35-2.93 |
| C-erbB-2 ^g | 82.5 | 40.1 | 29.0 | 13.3 | 12.6 | 0.0004 | 2.04 | 1.41-2.95 |
| C-erbB-3 ^g | 45.8 | 79.5 | 18.9 | 30.1 | 8.5 | 0.004 | 0.63 | 0.44-0.89 |
| ER _α ^g | 51.6 | 107.7 | 15.9 | 35.7 | 16.4 | 0.0001 | 0.50 | 0.36-0.71 |
| PgR ^g | 57.5 | 99.4 | 23.1 | 30.6 | 7.6 | 0.006 | 0.66 | 0.47-0.94 |
| pS2 ^g | 63.4 | 74.0 | 26.0 | 26.4 | 1.31 | 0.25 | 0.90 | 0.64-1.26 |
| p53 ^g | 78.7 | 55.7 | 20.9 | 32.3 | 1.12 | 0.29 | 1.05 | 0.75-1.46 |
| Cathepsin D ^g | 72.6 | 63.4 | 26.2 | 26.2 | 0.04 | 0.83 | 0.94 | 0.64-1.37 |

^a Median survival in months and cumulative survival (%) of patients with OPN-positive tumors and either not expressing (TV-) or expressing (TV+) one of the other tumor variables.

^b Wilcoxon statistic (χ^2) and probability for 1 d.f. (P) for the significance of the difference of the other tumor variable for the OPN-positive patients.

^c RR for survival and 95% CI were determined using a Cox univariate analysis for 1 d.f.

^d Tumor size, not fixed (T₁, T₂, T₃) vs. fixed tumors (T₄).

^e Histological grade (Histol. grade) of tumor, low (I, II) vs. high (III) grade.

^f Nodal status, no nodes vs. 1 or more nodes involved with tumor.

^g Negative vs. positive staining.

staining for PgR. Coexpression of OPN and p53 has been reported previously in a primary tumor of a single patient who later developed OPN and p53 coexpressing metastases before death (54). These results may suggest that the same underlying change(s) is responsible for the altered expression of those tumor variables, histological grade, c-erbB-3, pS2, p53, and possibly PgR, which show some correlation with OPN. However, the fact that there is a significant correlation between the presence of OPN and histological grade but not with other major pathological tumor variables associated with poor patient prognosis, that of involved lymph nodes and tumor size, may reflect the characteristics of the tumors in this group of patients and the size of the sample. In this study, a minority (18% and 6%, respectively) of the tumors are classified as T₃ and T₄; these are small fractions in comparison with other groups of patients (5). Moreover, nodal status has been undertaken on only 77% of the tumors in this study. These smaller numbers may make tests for associations less meaningful.

In this paper the overall survival for patients with carcinomas containing immunocytochemically detectable levels of OPN is shown to be significantly worse than for those patients with carcinomas considered negative for OPN. The degree of association at $P < 0.0001$ using Wilcoxon statistics is more significant than with most other tumor variables in this group of patients and is similar to the most significant association shown thus far for involved lymph nodes ($P < 0.0001$; Table 4). When the Kaplan-Meier plots are analyzed using log-rank sums, a similar level of significance is achieved (log-rank $\chi^2 = 108$; 1 d.f.; $P < 0.0001$). The median survival times of >231 months for the negatively stained and 65 (95% CI, 49-80) months for the positively stained group obtained using log-rank sums (not shown) compare favorably with >228 months for the negatively stained and 68 months for the positively stained group obtained using Wilcoxon statistics (Fig. 3A). This relationship for OPN achieved statistical significance after 1 year and remained statistically significant for the full 19 years of follow-up of the patients. Moreover, when

the patients are grouped in classes according to the percentage of carcinoma cells staining for OPN: $<1\%$, 1-5%, 5-25%, 25-50%, and 50-100%, they are also associated with increasing levels of deaths in the same order of ranking. Moreover, all of the classes except the last two are significantly different by 10 years of follow-up (Fig. 3B). Once again Kaplan-Meier plots followed by analysis of log-rank sums gave very similar results to those in Fig. 3B and Table 3 [$\chi^2 = 130.7$, 4 d.f., $P < 0.0001$; median survival times were >228 , >225 , 172 (95% CI, 80-263), 55.4 (95% CI, 42-69), and 51.3 (95% CI, 36-66) for patients with carcinomas of class -, ±, +, ++, and +++/++++, respectively (not shown)]. This result shows that not only the presence but also the proportion of carcinoma cells staining for OPN are correlated with the time of demise of the patients (Table 3). This result also suggests that the levels of immunoreactive OPN may be correlated with their time of demise, because the percentage of cells stained for OPN is probably directly related to the levels of OPN in the tumor specimens (Fig. 2). However, it should be noted that there were no deaths in 47 cases in the unstained class (-) and only 6 deaths in 65 cases in the borderline staining class (±) of patients, and these very low percentages may cast some doubt on the overall statistical test for significance. In addition, the fact that in this study many patients are required to obtain a statistically meaningful result may mean that small fluctuations in the data can alter considerably the level of significance of the results. This is exemplified by the fact that there is no statistically significant difference between the time of survival of the moderate staining (++) and the strong staining classes (+++/++++), whereas there are significant differences between the other pairs of consecutive staining classes (Table 3). It may be that there are too few patients with strong staining carcinomas to verify this effect statistically using 5% confidence limits. However, the fact that the presence of immunoreactive OPN is so tightly correlated with early demise of this group of patients may suggest that this change is closely associated with their cause of death. Because OPN has been

Table 6 Regression coefficients for the Cox proportional hazards model for cancer-related deaths

| Tumor variable ^a | Coefficient β^b | SE ^c | χ^2 ^d | R value ^e | P ^f | RR ^g | 95% CI ^g |
|-----------------------------|-----------------------|-----------------|-----------------------|----------------------|----------------|-----------------|---------------------|
| OPN | 2.56 | 0.43 | 35.7 | 0.19 | <0.0001 | 12.9 | 5.6-30 |
| Nodal status | 0.72 | 0.21 | 11.4 | 0.098 | 0.0007 | 2.1 | 1.4-3.1 |
| Tumor size T ₄ | 1.39 | 0.42 | 11.1 | 0.097 | 0.0009 | 4.0 | 1.8-9.1 |
| ER _α | -0.57 | 0.22 | 7.05 | -0.072 | 0.008 | 0.56 | 0.37-0.86 |
| c-erbB-2 | 0.46 | 0.24 | 3.5 | 0.040 | 0.060 | 1.58 | 0.98-2.50 |

^a Tumor variable that showed a statistically significant association with patient survival times in the univariate analysis. There were 202 patient cases available with full data sets. Only comparison between patients with involved lymph nodes, all tumor sizes (T₁-T₄), all histological grades (I-III), staining for OPN, c-erbB-2, ER_α and p53 were made. ^b Value of β parameter in the Cox's multiple regression analysis ("Materials and Methods"). ^c SE of β . ^d Cox's statistic χ^2 . ^e Regression coefficient for the variable. ^f Probability from Cox's statistic χ^2 , 1 d.f. in each case. Overall $\chi^2 = 84.1$, 5 d.f., $P < 0.0001$; residual $\chi^2 = 2.52$, 5 d.f., $P = 0.77$. ^g RR for survival and 95% CI from multivariate analysis.

strongly associated with tumor progression (22, 23) and can cause metastasis in rodent models (25, 26), it is possible that OPN, among other metastasis-inducing proteins (e.g., S100A4; Ref. 38), is causing early deaths by its ability to induce metastasis in humans as well.

When smaller subgroups of patients are analyzed for their survival times, small fluctuations in data may have an even more pronounced effect on the level of significance of the results than when analyzed overall. Moreover, patient numbers may be too small to observe a significant effect, particularly when interobserver error and intratumor heterogeneity are taken into consideration (14). Nevertheless, when subgroups of patients with carcinomas classified as positive or negative for OPN and for another tumor variable are examined, there is little difference in patient survival in the OPN-negative group of patients with or without any one of the other tumor variables. The median survival is >216 months in all of the cases.⁶ These results suggest that the presence of OPN in the tumor is the more dominant factor examined at predicting patient outcome. Moreover, these results are consistent with those obtained in Cox's multivariate regression analysis model, where the presence of OPN is also found to have the most significant association with patient death. There is also reasonable agreement between the results obtained in the univariate and multivariate regression analyses where OPN positivity, involved lymph nodes, fixed tumors T₄, and ER α positivity are all significant independent prognostic indicators, with *c-erbB-2* positivity being of borderline significance (Tables 4 and 6). The fact that histological grade and p53 are rejected as independent prognostic factors in the Cox's multivariate regression analysis may suggest that they are confounded with one or more of the independent prognostic variables in the proportional hazards model. Although the presence of any of the other tumor variables in OPN-negative carcinomas does not appear to be associated with an alteration in the survival times of the patients, their presence in OPN-positive carcinomas is often associated with a significant change in the patient median times in either a negative (lymph nodes, T₄, and *c-erbB-2*) or positive (ER α , PgR, and *c-erbB-3*) manner (Table 5). Whether this synergy with OPN is associated with an increase or reduction in the patient median survival times is largely consistent with their effects in univariate analyses (Table 4). However, although patients with tumors with high histological grade (III) or the presence of p53 show significant association with decreased survival times in univariate analyses (Table 4), patients with OPN-positive, grade III/p53-positive tumors show no significant decrease in survival times over patients with only OPN-positive tumors (Table 5). This result, coupled with the mutual association in the primary carcinomas of these three tumor variables (Table 2), may suggest that their alterations in the primary tumor are interrelated. The other two pathological tumor variables, lymph node status and tumor size, on the other hand, probably operate at least in part, through OPN-independent events, consistent with the results obtained in the Cox's proportional hazards model (Table 6).

In contrast to this report and that of Tuck *et al.* (33), Kim *et al.* (51) failed to find an association of immunocytochemical staining for OPN and any clinicopathological parameters in 253 cases of breast cancer or with patient survival for 215 patients over a 5-year follow-up period (51). However, their proportion of 87% of patients with positive carcinomas for OPN was considerably higher than other reports (32, 33, 49, 50), including the present study of 66% of patients with positive carcinomas. Most of the staining in that report which was recorded as positive fell into a diffuse and weak or a diffuse and strong category (51). The former category of diffuse and weak staining was not considered positive in our present study, because it was

largely seen only with the polyclonal antibody to human OPN, the same antibody as used by Kim *et al.* (51) and even then very variably. Therefore, the main difference between the two studies was that in the present study the cutoff between what was considered negatively and positively stained carcinoma cells was more stringently applied. Whether this diffuse and weak staining is largely because of the extra M_r 45,000 polypeptide recognized by the polyclonal antiserum to human OPN in extracts of breast cancers remains to be determined.

How OPN is overexpressed and its role in human breast cancer is not clear. Although it is generally accepted that OPN occurs in the carcinoma cells themselves, the relative proportion sequestered from host cells such as activated macrophages and/or lymphocytes, and that produced *in situ* has varied considerably (28, 31, 32), but at least a sizeable proportion is now believed to arise from overexpression of OPN mRNA in the carcinoma cells themselves (33, 49, 50). In the breast OPN is expressed normally at a discrete time during lactation (55), presumably controlled by the levels of hormones and other morphogenetic signals (53, 56). Permanent perturbation of the balance of these signals may lead to its overproduction. For example, the receptors for two important hormones, which activate OPN transcription, vitamin D₃ (57), and ER α (9), are overexpressed, a region 4q25–26 in close proximity to the gene for OPN 4q21–25 is frequently deleted (58), and regulatory DNA sequences that bind a potential inhibitory factor for the OPN promoter related to the Wnt-signaling pathway, Tcf-4 (44), are amplified in some human breast cancers (26). However, the dominant mechanism(s) remains to be determined. Although the precise functions of the secreted OPN molecule are unknown, its unique GRGDS sequence, which can interact with integrin receptors, particularly of the $\alpha_v\beta$ type (59, 60), suggest that it can mediate cell attachment, cell migration, chemotaxis, and intracellular signaling (53), including protection against cytotoxic attack by the host (61, 62). The different signals that OPN can elicit in the same and different cells may be explained by the existence of multiple heterodimeric combinations of integrin chains that can ligate OPN and by variant forms of OPN (53). Moreover, OPN is also an extracellular ligand for CD44 (63), which is the main cell-surface receptor for hyaluronate. The CD44 family of receptors mediate cellular responses similar to those of integrins, and variants may interact with OPN in the development of certain gastric cancers, particularly their lymphogenous metastases (64). Recently, immunocytochemical studies have revealed an intracellular, perimembranous location for OPN where it colocalizes with CD44 and ezrin-radixin-moesin proteins in migrating embryonic fibroblastic cells, activated macrophages, and metastatic breast cancer cells (65). It is suggested that the CD44-ezrin-radixin-moesin-OPN complex found at the leading edge of migrating cells (65) represents a novel adhesion complex, which is formed in rapidly migrating cells. Moreover, the extracellular OPN may provide temporary (CD44) or more substantial ($\alpha_v\beta_3$) attachment complexes required for motility as well as for chemotaxis of the migratory cells (53).

It is unlikely that variation in cellular properties caused by OPN (27) is the sole property required to establish the metastatic cascade in rodent models, let alone in human breast cancer (66, 67). Even in a rodent model, transfection of an expression vector for OPN alone into a normal, nontumorigenic Rama cell line, Rama 704, fails to induce any pathological change when the transformants are reintroduced into syngeneic rats.⁶ It is only when an expression vector for OPN is transfected into a neoplastic, probably Ha-*ras*-transformed Rama cell line, Rama 37 (24), that the metastatic phenotype is demonstrable in animals (25). Therefore, it may be anticipated that interactions with growth-promoting oncogenes and other metastasis-inducing molecules with complementary properties to those of OPN (38) are required for the overall process of metastasis. It also remains to be

⁶ Unpublished observations.

determined how widespread the association between OPN and patient survival will prove to be, not only in breast but in other metastatic carcinomas. Thus far pilot studies on a small group of lymph node-negative breast cancer (33) and gastric cancer (64) patients have shown a positive association with patient demise and disease progression, respectively, whereas in ovarian cancers a surprising association with low malignant tumors has been reported (48). Our results show that in one large group of breast cancer patients with up to 19 years follow-up, the presence of the adhesive glycoprophosphoprotein OPN, which can cause metastasis in rodent models, is associated with a poor prognosis, almost certainly caused by metastatic spread from the primary tumor.

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