Microvessel density, p53 overexpression, and apoptosis in invasive breast carcinoma

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Abstract

Aims—To investigate the possibility of a correlation among microvessel density, p53 overexpression, and apoptosis in invasive breast carcinoma.

Methods—Microvessel density was analysed in 105 cases of invasive breast carcinoma by immunohistology using anti-factor VIII related antibody. The results were correlated with the immunohistochemical expression of p53 and the apoptotic index, detected using the *in situ* end labelling of fragmented DNA method (TUNEL). Assessment was made with a CAS 200 image analyser. All these studies were performed on formalin fixed, paraffin wax embedded tissue sections of tumour samples.

Results—The mean (SD) microvessel count was 47.2 (51.1), with a range from 7 to 250. Thirty five (33%) carcinomas showed overexpression of p53 protein. The apoptotic index of tumours ranged from 0.0 to 28.0, with a mean (SD) of 1.7 (3.2). The results showed that there was a significant inverse correlation between microvessel density and p53 expression ($p = 0.04$; odds ratio, 0.37). In contrast, no correlation was identified between the microvessel density and apoptotic index.

Conclusions—These results suggest that in invasive breast carcinoma the p53 overexpression phenotype downregulates tumour neoangiogenesis, as does the wild-type of p53 protein. In addition, they suggest that apoptosis and neoangiogenesis in these tumours are independent processes.

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Keywords: breast cancer; microvessel density; apoptosis; p53

Neoangiogenesis represents a critical step in the metastatic cascade and the morphological evaluation of newly formed vessels may provide further information on the biological pattern and behaviour of cancers.^{1,2} The formation of new blood vessels might permit the expansion of primary tumours and their migration to secondary sites. Endothelial cells can stimulate growth of tumour cells by production of growth factors. This paracrine effect of neovascularisation, together with the perfusion of nutrients and oxygen and removal of catabolites, permits expansion of a tumour or its metastases.³ Hence, the microvessel count, which gives a measure of tumour angiogenesis,

may provide useful information on the biological pathway of tumours. In humans, the first evidence showing the predictive role of microvessel quantitation in metastatic relapse was reported in cutaneous melanoma.⁴ These initial findings have been confirmed by several studies in breast,^{5–12} prostatic,^{13,14} head and neck,¹⁵ lung,¹⁶ colorectal,¹⁷ brain,¹⁸ and renal cancers.¹⁹

The vessels in tumour samples may be identified by monoclonal antibodies that recognise factor VIII related antigens and, thus, highlight vascular endothelium. Angiogenesis is controlled by the local balance between factors that stimulate new vessel growth and factors that inhibit it.²⁰ In most normal tissues, inhibitory influences predominate, and usually cells derived from these tissues do not stimulate angiogenesis. In contrast, tumour cells, which must attract new vessels in order to grow and metastasise efficiently,²¹ are potently angiogenic as a result of the decreased production of inhibitors and the increased secretion of inducers.²² As normal cells progress towards malignancy, they must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth. To examine the effect of p53 on angiogenesis, Dameron *et al*²³ used fibroblasts cultured from Li Fraumeni patients. These patients had inherited one wild-type (WT) and one mutant allele of the p53 gene and as a result had an elevated risk of developing sarcomas and other tumours in which the remaining WT allele is inactivated.²⁴ They reported that the switch to an angiogenic phenotype was found to coincide with loss of the WT allele of the p53 tumour suppressor gene and to be the result of reduced expression of thombospondin-1 (TSP-1), a potent inhibitor of angiogenesis. Transfection assays revealed that p53 can stimulate the endogenous TSP-1 gene and regulate TSP-1 promoter sequences positively. Their data indicated that, in fibroblasts, WT p53 inhibits angiogenesis through regulation of TSP-1 synthesis.

Abnormalities of p53 are probably the most common genetic abnormalities in human cancer.²⁵ p53 gene alterations result in various mutant p53 proteins that have in common a change in conformation and greater stability. The resulting longer half life permits their accumulation and demonstration by immunohistochemistry.²⁶ The mutant proteins not only lose tumour suppressor function, but can act as dominant oncogenes.²⁷

The exact role of apoptosis in mammary epithelial homeostasis and the development of

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breast cancer remains to be elucidated. Although the regulation of apoptosis and angiogenesis is elusive, defective (that is, oncogenic) regulation may exert an important effect in breast cancer.²⁸ Holmgren *et al*²⁹ have found experimental data studying dormant lung metastases under angiogenesis suppression in mice. They show that inhibition of angiogenesis limits tumour growth by elevating the incidence of apoptosis. Their studies demonstrate that apoptosis in micrometastases is reduced significantly after induction of angiogenesis, although the high proliferation rate remains unchanged. Growth of the metastases can occur because of increased survival of the tumour cells.

In this report we analysed microvessel density in 105 cases of invasive breast carcinoma using immunohistochemical methods and correlated these results with the immunohistochemical expression of p53 and the apoptotic index determined by the terminal deoxynucleotidyl transferase (TdT) mediated biotinylated deoxyuridine-triphosphate nick end labelling (TUNEL) method to investigate the possibility of a correlation among microvessel density, p53 expression, and apoptotic index.

Methods

MATERIAL

Data for this study were collected from 105 consecutive, unselected, invasive breast cancer women patients who had undergone breast cancer surgery at the Ramón y Cajal Hospital of Madrid from January to June 1995. Local excision or modified radical mastectomy and axillary lymph node excision were performed on all patients.

HISTOLOGICAL EXAMINATION

Histological diagnosis was performed on haematoxylin and eosin stained, paraffin wax sections. Cases were classified according to the WHO criteria. The differentiation grading of invasive breast carcinoma was defined according to the Bloom-Richardson system modified by Elston *et al*³⁰ as: well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III).

IMMUNOHISTOCHEMISTRY

Paraffin wax sections of all cases were immunostained with monoclonal antibodies against p53 (Novocastra, Newcastle upon Tyne, UK; clone DO7, which recognises both WT and mutant p53 proteins; 1/50 dilution), MIB1; (Immunotech, Marseille, France; which is equivalent to Ki67 for paraffin embedded, microwave processed sections; 1/200 dilution), antioestrogen receptor (Novocastra; clone NCL-ER-LH2; 1/200 dilution), and factor VIII related antibody (Dako, Glostrup, Denmark; 1/200 dilution), using microwave mediated antigen retrieval and the streptavidin-biotin complex labelled with alkaline phosphatase procedure. Levamisole was used to inhibit endogenous alkaline phosphatase. Simultaneous staining of known positive cases was used as a positive control for p53 and oes-

trogen receptor antibodies and a hyperplastic tonsil was used as a positive control for MIB1 antibody. Incubation of parallel slides omitting the specific antibody was performed as a negative control.

IN SITU END LABELLING METHOD

Apoptotic cells were identified by TUNEL staining as described by Gavrieli *et al*.³¹ After deparaffinisation and rehydration, paraffin embedded sections adhered to poly L lysine coated slides were incubated with 20 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 15 minutes at 37°C and then washed with distilled water. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxidase in methanol for 10 minutes. The sections were rinsed with distilled water and immersed in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), TdT (0.3 eu/µl; Boehringer Mannheim), and biotinylated deoxyuridine-triphosphate (dUTP) (Boehringer Mannheim; 0.01 nmol/µl). TdT buffer was added to cover the sections and they were then incubated at 37°C for 60 minutes. The sections were rinsed in distilled water, immersed in phosphate buffered saline (PBS), and covered with 10% normal rabbit serum for 20 minutes. Subsequently, they were incubated with streptavidin-peroxidase complex for 30 minutes, and immersed in PBS. Finally, they were developed with diaminobenzidine solution, lightly counter stained with haematoxylin, and mounted. Positive controls were obtained by DNase (Boehringer Mannheim) treatment of the sections after peroxidase inactivation. The high contrast obtained between staining of apoptotic cells and the background is amenable to automated quantitation of apoptotic cells.

QUANTITATIVE STUDIES

Quantitative immunohistochemistry investigation with the quantitative nuclear antigen application of the Computerized Analyzer System (CAS 200; Becton Dickinson, San José, California, USA) was used to score individual nuclei for the presence of both the p53 protein and TUNEL label. This program has the capacity to measure the percentage of cell nuclei in tissue sections having the p53 protein and TUNEL signal and the density (or concentration) of protein in these nuclei. The positive stain value was measured, which represents the percentage of the sum total optical density for the positive TUNEL or p53 nuclear areas divided by the sum total optical density of all of the nuclei, expressed as a percentage. This represents the protein density of the nuclear material measured. Nuclear boundary optical density and antibody threshold were adjusted for each case examined, to allow the results to be compared. Representative fields down to a minimum of 30 000 µm² (approximately five fields with a 45× objective and a 10× ocular lens). Section analysis was carried out at random focusing in tumoral areas.

In the statistical analysis, p53 overexpression was established when more than 20% of

tumorous cells were p53 positive. p53 positive cases always showed a diffuse pattern of p53 expression, however, in negative cases the positive cells were scattered. The apoptotic index was graded using the same application and proceedings, as: 1 (TUNEL positive cells fewer than 1% of the total cells), 2 (1–3% TUNEL positive cells), and 3 (more than 3% TUNEL positive cells). Great care was taken to exclude areas of necrosis because in necrotic cells sufficient DNA strand breaks may occur to allow detection.³² The growth fraction was quantified with the Quantitative Proliferation Index application of the CAS 200. This program measures the percentage of proliferating cells (MIB1 immunostaining) in a tissue section or single cell cytology preparation. In the tissue section mode proliferation index count was measured: immunostained nuclear area is first divided by the individual cell nuclear antibody size estimate, and the nuclear area is divided by the nuclear size estimate. Nuclear boundary optical density and antibody threshold were adjusted for each case examined, in order to allow the results to be compared. Representative fields down to a minimum of 30 000 μm^2 (approximately five fields with a 45 $\times$ objective and 10 $\times$ ocular lens). Section analysis was carried out at random focusing in tumoral areas. The quantification of the growth fraction was graded as: low growth fraction (positive cells fewer than 15% of the total cells), medium growth fraction (15–25% positive cells) and high growth fraction (more than 25% positive cells). In the statistical analysis the quantification of the growth fraction was graded as: low growth fraction (positive cells fewer than 15% of the total cells) and medium/high growth fraction (more than 15% positive cells).

The quantitative oestrogen/progesterone application for the CAS 200 Analyzer is used to measure the percentage of cell nuclei that have oestrogen receptors and the density of receptors in those nuclei. The antibody threshold is determined first on the control specimen. This is an adjacent tissue section to the one being analysed, processed by the same staining, but without the antibody application. This slide is analysed in the control mode, and the threshold is determined automatically. Expression was considered positive when more than 5% of tumoral cells were oestrogen receptor positive.

VESSEL GRADING AND COUNTING

Vessel grading and counting were performed using the retrieved tumour blocks. Immunohistochemical detection of factor VIII related antibody was used for identifying microvessels and these were scored using the microvessel counting protocol and the criteria developed by Weidner *et al.*³³ Microvessels were counted simultaneously by two observers, without any knowledge of the clinical circumstances or the histological appearance of the tumours. The stained sections were screened at low power microscopic magnification (2.5 $\times$ or 10 $\times$) for selecting the densest vascular regions of the tumours. Usually, these neovascular “hot spots” were found at the tumour margins, whereas the lowest vascular areas appeared in

the sclerotic tumour areas. Any single red-brown stained cell or cluster of cells, clearly distinguishable from the background, was counted as a vessel. Branching structures were counted as a single vessel unless there was a break in the continuity of the structure. The presence of either a lumen or erythrocytes in the lumen (although often seen) was not required to classify a structure as a vessel. These criteria for counting could be applied reliably to all 105 cases included in the study. Individual microvessel counts were made on a 200 $\times$ field (20 $\times$ objective and 10 $\times$ ocular, equivalent to 0.6648 mm^2 per 200 $\times$ field) within the invasive tumour neovascular hot spot. Vessels were counted in four areas and the highest count was taken for further analysis. Depending on this number, tumours were graded as: group 1 (fewer than 25 vessels/field), group 2 (25–100 vessels/field) and group 3 (101 or more vessels/field). Microvessels were also categorised using the mean microvessel density of all patients to classify patients into high and low microvessel density groups and, moreover, into fewer than 25 vessels/field and 25 or more vessels/field.

STATISTICAL ANALYSIS

The crude association between studied parameters was evaluated by the χ^2 test and multivariate association by logistic regression. Statistical analysis was performed with a PRESTA package; p values of 0.05 or less were considered to be statistically significant. The maximum model analysed, with backward elimination, the probability of the dependent variable and each of the independent variables occurring simultaneously. The dependent variable included in the logistic model was p53 protein overexpression and independent variables were microvessel density, histological grading and typing, growth fraction, tumour diameter, oestrogen receptor, and node status.

Results

HISTOLOGICAL, IMMUNOHISTOCHEMICAL, AND MOLECULAR ANALYSIS

The size of tumours ranged from 0.5 to 9.0 cm, with a mean (SD) of 2.9 (1.6) cm, and 48 tumours were less than 2 cm in diameter (46.7%). The results of the histological classification of the cases were as follows: 78 cases (74.2%) were infiltrating ductal carcinomas, 12 (11.4%) cases were infiltrating lobular carcinomas, five (4.7%) cases were tubular carcinomas, two (1.9%) cases were medullary carcinomas, two (1.9%) cases were mucinous carcinomas, and six (5.8%) cases were combined carcinomas.

The analysis of the histological grading of the 105 cases of invasive carcinoma revealed that 37 (35.2%) tumours were classified as grade I, 36 (34.2%) as grade II, and 32 (30.4%) as grade III.

The study of the lymph node status did not identify axillary metastases in 47 (44.7%) cases, one to three axillary metastases were identified in 27 (25.7%) cases, and 31 (29.5%) cases had more than three axillary metastases.

Table 1 Correlation between microvessel density and pathological parameters in infiltrating breast carcinoma

	Microvessel density			p value*
	< 25	25–100	> 100	
p53 (%)				< 0.007
Negative (n = 70)	30 (42.8)	34 (48.5)	6 (8.5)	NS
Positive (n = 35)	24 (68.5)	6 (17.1)	5 (14.2)	
Histological grade (%)				
I (n = 37)	18 (48.6)	17 (45.9)	2 (5.4)	NS
II (n = 36)	18 (50)	12 (33.3)	6 (16.6)	
III (n = 32)	18 (56.2)	11 (34.3)	3 (9.3)	
Tumour diameter (%)				NS
< 2 cm (n = 48)	26 (54.1)	18 (37.5)	4 (8.3)	NS
≥ 2 cm (n = 57)	28 (49.1)	22 (38.5)	7 (12.2)	
Node status (%)				NS
Negative (n = 47)	28 (59.5)	14 (29.7)	5 (10.6)	NS
Positive (n = 58)	26 (44.8)	26 (44.8)	6 (10.3)	
Oestrogen receptor (%)				NS
Negative (n = 34)	21 (61.7)	13 (38.2)	0 (0)	NS
Positive (n = 71)	33 (46.4)	27 (38)	10 (14)	
Growth fraction (%)				NS
Low (n = 49)	20 (40.8)	23 (46.9)	6 (12.2)	NS
Medium (n = 15)	10 (66.6)	3 (20)	2 (13.3)	
High (n = 41)	24 (58.5)	14 (34.1)	3 (7.3)	
Apoptotic index (%)				NS
< 1 (n = 53)	26 (49)	22 (41.5)	5 (9.4)	NS
1–3 (n = 37)	21 (56.7)	11 (29.7)	5 (13.5)	
> 3 (n = 7)	7 (46.6)	7 (46.6)	1 (6.6)	

Values are mean (SD).
* χ^2 test.

Table 2 Multivariate analyses by logistic regression between microvessel density and p53

	OR	95% CI OR	χ^2	p value
Microvessel density	0.37805	0.14349–0.99601	3.87339	0.0463
Nodes	3.76791	1.22732–11.56757	5.37276	0.01944
Tumour size	2.55980	0.93310–7.02236	3.33252	0.06443
Histological grade	1.55600	0.45175–5.35944	0.49094	0.50935
Oestrogen receptor	0.59521	0.22821–1.55238	1.12532	0.28893

Association controlled by nodes (node negative; node positive); tumour size (< 2 cm; ≥ 2 cm); histological grade (I, II, III); oestrogen receptor (negative; positive).
Model $\chi^2 = 26.79/p = 0.00008$.
OR, odds ratio; CI, confidence interval.

The immunohistochemical analysis demonstrated that 71 (67%) carcinomas were positive for the oestrogen receptor. The results of the growth fraction measurements revealed that the growth fraction was low in 49 (46%) tumours, medium in 15 (14%) tumours, and high in 41 (39%) tumours. Overexpression of p53 protein was shown in 35 (33%) carcinomas.

The microvessel count ranged between 7 and 250, with a mean (SD) of 47.2 (51.1). Fifty four tumours (51.4%) had fewer than 25 microvessels/field, 40 (38%) tumours had 25–100 microvessels/field and 11 (10.4%) tumours had more than 100 microvessels/field.

The analysis of the apoptotic index showed that 53 (50%) cases had an index of less than 1. In 37 cases (35%) the apoptotic index was between 1 and 3, and in 15 (14%) tumours the apoptotic index was more than 3; the apoptotic index of tumours ranged from 0.0 to 28.0, with a mean (SD) of 1.7 (3.2).

UNIVARIATE ANALYSIS

p53 overexpression was significantly associated with microvessel density ($p = 0.0076$; $\chi^2 = 9.78$). The correlation was persistent with other categories of microvessel counts and these variables were associated inversely. Thirty (43%) p53 negative tumours were microvessel density group 1, and 40 (69%) p53 positive tumours were microvessel density

group 1. We did not find a correlation between microvessel density and apoptotic index ($p = 0.76$; $\chi^2 = 0.09$), or with any other variables, yet, a trend with tumour size was observed ($p = 0.09$) (table 1).

MULTIVARIATE ANALYSIS

The maximum likelihood logarithm test indicated that the proposed model is statistically significant ($p = 0.00008$). The odds ratio to association of p53 overexpression and microvessel density was less than 1 (0.37805), confirming the inverse relation between these variables. The variable with the highest confusion effect was the lymph node status (table 2).

Discussion

The predictive role of microvessel quantitation on the biological behaviour of breast carcinoma has been confirmed by different studies. However, the results published recently by several authors question the validity of using microvessel densities as a prognostic indicator in lymph node negative breast cancer.^{34–42}

In the present study, the number of microvessels was determined using the anti-factor VIII antibody. The median number of microvessels per 200× field was 47, which is near to that reported in previous studies or slightly lower,^{1 33 41} because our field for counting (0.66 mm²) was rather different from the area of the field recommended for optimum results (0.74 mm²).¹ The endothelial signal obtained was clear and intense, although other authors have reported better results using the JC70 monoclonal antibody (CD34) directly against platelet endothelium cell adhesion molecules.^{7 15} Horak *et al*⁷ reported that CD31 was the most sensitive immunostain for determining microvessel counts in invasive breast carcinoma. Although apparently more sensitive, CD31 is less specific than factor VIII related antigen. CD31 crossreacts mildly with fibroblasts and even with some tumour cells (as does CD34),⁴³ and CD31 crossreacts strongly with plasma cells. In fact, the crossreactivity of CD31 with plasma cells can obscure the microvessels in those tumours with a prominent inflammatory background containing plasma cells, particularly when using low power microscopic examination to select the most densely vascular regions of the tumour.⁴⁴

Probably, abnormalities of p53 are the most common genetic abnormalities in human cancer.²⁵ It has been estimated that p53 gene mutations and protein accumulation occur in 14–58% of invasive breast carcinoma.^{45–51} To our knowledge, there are few reports about the relation between p53 expression and microvessel density in human cancers.^{7 10 40 52} In all these studies, p53 expression was not associated with microvessel density. Microvessel density has been found to be associated significantly with p53 expression by Gasparini *et al*¹⁵ in head and neck squamous cell carcinoma, Vermeulen *et al*⁸ in colorectal adenocarcinoma, and Bochner *et al*⁹ in invasive transitional cell carcinoma of the bladder. We have found that the χ^2 test and multivariate analysis showed significant and inverse association between these two variables

in invasive breast cancer. To our knowledge, no other reports have found this association between microvessel density and p53 expression. Although immunohistochemical detection of p53 protein is not entirely specific for mutation in this tumour suppressor gene, our results disagree with the *in vitro* results of Dameron *et al*²³ on the association of mutation or loss of WT p53 with loss of thrombospondin production and a shift in the balance towards stimulation of angiogenesis. These discrepancies could be a technical problem. However, we have used the original technique described by Weidner *et al*,³³ which is usually accepted to assess angiogenic activity. In addition, the cut off point for p53 overexpression positive tumours guaranteed that p53 expression was present in more than occasional cells. Charpin *et al*² reported that a cut off point of 20% for the immunostained surface is most appropriate for assessing the prognostic significance of p53 immunodetection. Moreover, in the statistical study we used a different cut off point for microvessel density and also showed a significant inverse relation between both variables.

Considerable functional differences are known to exist between WT and mutant p53 proteins.³³ The biological effects of p53 protein accumulation in tumours may, in part, depend on where they can still mediate protective mechanisms, such as angiogenesis inhibition. However, it is often difficult to assess the incidence of p53 overexpression, p53 mutation, and the level of concordance between these alterations. Soong *et al*¹⁵ provide evidence that immunohistochemical detection of p53 protein accumulation does not always indicate the presence of a gene mutation. These and other studies⁵⁴⁻⁵⁵ indicate that the accumulation of p53 might confer "tumour suppressor-like" angiogenic protective effects.

Our results suggest that in human invasive breast cancer angiogenic activity determined by microvessel density does not correlate with the apoptotic cell count, measured by means of enzymatic detection of DNA fragmentation.³¹ To our knowledge, these results have not been reported previously. The essential interaction between the endothelial cell compartment and the tumour cell compartment in cancer is very complex and the specific understanding of those interactions may well play a key role in breast cancer prognosis.

In summary, we report a significant inverse correlation between microvessel density and immunohistochemical detection of p53, suggesting that in invasive breast carcinoma the accumulation of p53 could produce a down-regulation of neoangiogenesis, also implying that neoangiogenesis and apoptosis are not related in these tumours. Future efforts should be directed towards elucidating the relation between the presence of p53 gene mutations detected by sequential analysis and neoangiogenesis in breast carcinoma.

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