Virchows Arch (1998) 432:113–117

ORIGINAL ARTICLE

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Vascular density as a prognostic indicator for invasive ductal breast carcinoma

Received: 28 April 1997 / Accepted: 16 August 1997

Abstract Estimated vascular density obtained with the aid of antibodies against endothelial cells has been claimed to be an independent prognostic indicator for invasive ductal breast carcinoma. Since 1991 most studies have counted the number of vessels with the optic microscope. We have performed immunohistochemical staining for Factor VIII on formaldehyde-fixed, paraffin-embedded primary invasive ductal carcinomas from 112 patients, with a minimal follow-up time of 60 months, who had received postoperative chemoradiation therapy. We have performed a manual count with a 20× objective of the vessels in the vascular hot-spot identified in a 4× field. We analysed the association of this factor with epidemiological risk factors, histopathological features, hormonal receptor status and p53 and *c-erbB-2* expression and the influence on prognosis. In univariate analysis vascular density is a significant prognostic indicator in both node-negative and node-positive patients, together with staging, Baak's morphometric multiparametric index, tumour size and histological grade. However, in multivariate analysis only tumour staging and vascular density are independent prognostic factors in breast carcinoma.

Key words Invasive ductal carcinoma · Vascular density · Angiogenesis · Prognostic factors · Oncogenes

Introduction

Breast carcinoma is the most frequent tumour in European women and girls (18% of all tumours [11]). Today the incidence seems to be on the increase, although this is not accompanied by a parallel increase in mortality, owing to earlier diagnosis [13].

Great efforts have been made to identify prognostic factors in breast carcinoma. It is well known that tumours with the same TNM stage or histological type show fairly heterogeneous biological behaviour, and in recent years there have therefore been many studies on the contribution of oncogenes, mainly *p53* [15] and *c-erbB-2* [2, 19] to the origin and prognosis of various tumours, including breast carcinoma. In 1991 Weidner et al. [18] performed the first study on the prognostic influence of neovascularization on 49 patients with ductal carcinoma of the breast. The promising results reported by these authors led several groups to the analysis of this possible prognostic factor [1, 5, 12], which might open up new therapeutic approaches [14] in some patients. However, the results have not been homogeneous and several reports have not confirmed this prognostic influence [7, 17]. The aim of this study is to estimate the possible influence of vascular density on the prognosis of patients affected by ductal carcinoma in our hospital and to examine its relationship to other prognostic factors.

Materials and methods

The study group was based on 112 patients treated at the Hospital Clínico San Carlos (Madrid, Spain) between 1984 and 1990 by radical mastectomy for invasive ductal breast carcinoma. All of them received postoperative chemoradiation therapy following the treatment protocol employed by the oncologists at this hospital.

The clinical records were obtained on admission and follow-up information from the hospital records or directly from the patients. Only patients with a follow-up time of at least 60 months (5 years) were included in our study. Patients who died from intercurrent disease or were lost to follow-up in this period were also excluded.

Histological sections of formaldehyde-fixed, paraffin-embedded material stained with haematoxylin-eosin were reviewed at the time of the study. The following variables were recorded: histological grade (according to the Elston and Ellis score reported in 1991 [3]) and the morphometric prognostic index (reported by Baak in 1985 [16]).

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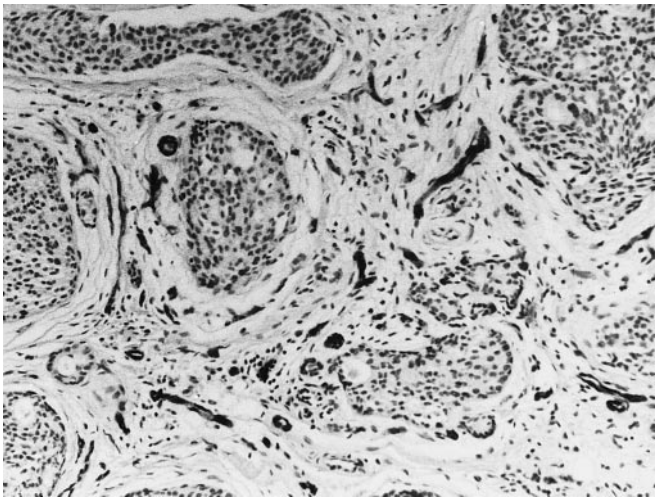

Fig. 1 Vascular hot-spot with a 4× objective. This vascular hot-spot was located at the periphery of the tumour in most cases. Immunohistochemistry for Factor VIII, ×40

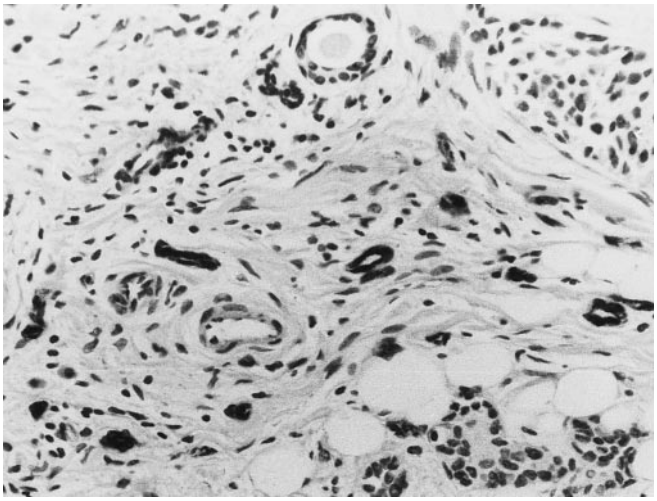

Fig. 2 Example of the fields in which we performed the vascular counts. Immunohistochemistry for Factor VIII, ×200

Immunostaining was performed on paraffin-embedded archival tissue, following the avidin–biotin peroxidase technique. In brief, sections 4–6 mm thick were cut, air dried for 15 min, heat fixed at 42ºC and then air dried overnight at room temperature. The slides were stored at room temperature until use. After deparaffinization with xylene, endogenous peroxidase activity was eliminated treating the slides with $EtOH/H_2O_2$ for 30 min at room temperature. The sections were either trypsinized for 30 min at 60ºC (factor VIII) or microwaved in a 700-W microwave oven in sodium citrate buffer for two 7-min cycles with replenishment of the buffer between cycles, and allowed to cool at room temperature. Then the slides were incubated with the diluted primary antibody at 4ºC overnight in a humidified chamber. Biotinylated anti-mouse IgG and avidin–biotin peroxidase complex were added in sequence. The sections were then incubated with 3,3′ diaminobenzidine tetrachloride (DAB) for 7–10 min for visualization of the peroxidase reaction. After washing in water for some min, the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol, cleared in xylene and mounted. The antibodies employed in the study were: p53 (clone DO7 diluted at 1/50, Novocastra), c-erbB-2 (clone CB11 diluted at 1/40, Medac Diagnostika), oestrogen receptor (clone ER1D5, prediluted, Immunotech), progesterone receptor (clone 1AG, diluted at 1/10, Novocastra) and Factor VIII (prediluted, Enzo diagnostics).

p53 protein was considered positive when at least 10% of the tumour cells showed nuclear staining. Only cases showing distinct membrane staining in more than 50% of the tumour cells were considered positive for c-erbB-2 protein. Oestrogen and progesterone receptors (ER, PR) were considered positive when more than 10% of the cells showed nuclear staining.

For the vascular counts, we followed the criteria reported by Gasparini et al. [5]. First, we identified the vascular hot-spot with a 4× objective (Fig. 1) and then measured the number of vessels in this area (0.74 mm^2) with a 20× objective (Fig. 2). Following the first experimental design reported by Weidner [18], we obtained two values: the number of vessels in the most vascular field and the median value of three fields in the most highly vascular area. Both values were expressed as vascular density (vessels/mm^2).

Statistical tests were performed using the BMDP (Biomedical Data) Statistical Software Package run on a Convex C3210 computer. Frequency tables were tested for association using the Chi-square test. We also analysed the possible influence of the different factors on survival (estimated with the relapse free survival, RFS). The pattern of RFS was estimated by means of the product-limit (Kaplan-Meier) method on the basis of a 5-year median follow-up period. The role of each of the prognostic factors (univariate analysis) was evaluated using the log-rank test (comparison of two Kaplan-Meier curves), and their joint effect (multivariate analysis) with Cox's proportional risk model.

Results

The results of the study are summarized in Table 1.

The vascular density in our series ranged between 10.83 and 151.6 vessels/mm^2 (median 54.329) for the most highly vascular field and 9.03 and 146.22 vessels/mm^2 (median 51.376) for the median of the three most vascular fields. First, we compared these two values, obtaining a correlation factor of 0.99 (value of r, significant $P<0.05$). This almost perfect correlation between these two variables allows us to discard one of the measures and employ only the vascular density in the most highly vascular field.

As Table 1 shows, the vascular density measured with the optical microscope has no relationship with any of the variables estimated in our study.

To analyse the prognostic influence of the vascular density in ductal carcinoma, we divided the patients into two groups, testing this prognostic factor with the log-rank test, starting with the median vessel count and trying at 10-microvessel intervals. With this technique, the first value at which microvessel density behaved as a prognostic indicator for breast cancer patients was 48 vessels/mm^2 (Fig. 3). The remaining factors were analysed with the same test, and only tumour staging, tumour size, histological grade, Baak's morphometric multiparametric index, and vascular density behaved as significant prognostic factors in both node-negative and node-positive patients, and *c-erbB-2* expression in node-negative patients.

Only tumour staging and vascular density behaved as independent prognostic indicators in invasive ductal carcinoma of the breast in this series.

Table 1 Correlation between vascular density in the manual count and biological, histological and clinical parameters

	Low (< = 48/mm²) No. of patients (%)		High (>48/mm²) No. of patients (%)
ER+	11 (10%)		18 (16%)
ER–	46 (41%)		37 (33%)
		NS (P = 0.15)	
PgR+	19 (16.9%)		23 (20.6%)
PgR–	38 (33.9%)		32 (28.6%)
		NS (P = 0.464)	
p53+	20 (18%)		22 (19.6%)
p53–	37 (33%)		33 (29.4%)
		NS (P = 0.732)	
c-erbB-2+	10 (9%)		9 (8%)
c-erbB-2–	47 (42%)		46 (41%)
		NS (P = 0.931)	
Menopausal status			
Premenopausal	22 (19.6%)		19 (16.9%)
Postmenopausal	35 (31.2%)		36 (32.1%)
		NS (P = 0.803)	
Grade			
G1	8 (7.2%)		9 (8.1%)
G2	24 (21.6%)		26 (23.4%)
G3	25 (22.5%)		19 (17.1%)
		NS (P = 0.64)	
Morphometric prognostic index (Baak)			
<0.6	27 (24.1%)		23 (20.5%)
= >0.6	30 (26.8%)		32 (28.6%)
		NS (P = 0.68)	
Tumor size			
<2 cm	21 (18.7%)		20 (17.8%)
2–4 cm	28 (25%)		25 (22.3%)
>4 cm	8 (7.1%)		10 (8.9%)
		NS (P = 0.826)	
Nodes			
N+	30 (26.8%)		37 (33%)
N–	27 (24.1%)		18 (16.1%)
		NS (P = 0.16)	
Stage			
I	10 (8.9%)		11 (9.8%)
II	27 (24.1%)		26 (23.2%)
III	20 (17.8%)		18 (16.1%)
		NS (P = 0.934)	

NS: Non-significant

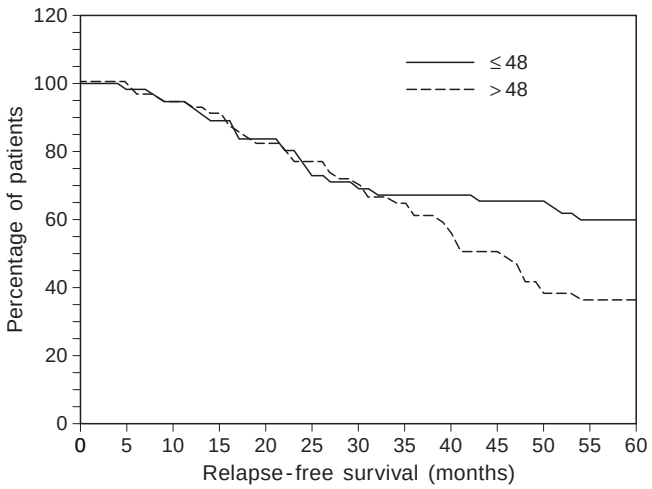

Fig. 3 Survival plotted against manual vessel count

Discussion

Analysis of the prognostic influence of neovascularization in breast cancer patients involves several difficulties. First, which endothelial marker should one employ? To date, several studies have analysed the possible prognostic influence of vascular density in breast cancer and also in other solid tumours [9, 10]. The first studies all employed Factor VIII, but since Horak's report [8] of a study with CD31 (platelet/endothelial cell adhesion molecule antibodies), many workers [5, 6] have chosen this endothelial marker, as it does not stain the lymphatic endothelium and has better sensitivity (detecting 33% vessels more than Factor VIII). However, Gasparini et al. reported a disadvantage of CD31 – it stains plasma cells nonspecifically and its expression can be altered by inflammatory reactions. This can cause diagnostic problems, mainly in cases with an intense lymphoplasmacytoid infiltrate and when image analysis systems are used. As the intention of our work was to complement the

Table 2 Summary of the results of some of the first studies about the prognostic influence of vascularization

References	No. of patients	Node+	Prognostic value	Marker	Unit (vessels)
[18]	49	30	Yes	Factor VIII	100 in a 200 field
[8]	103	39	Yes	CD31	100/mm^2
[1]	120	32	Yes	Factor VIII	84 in a 200 field
[6]	254	0	Yes	CD31	70 in a 200 field

manual count with an automized one carried out with an image analyser, we preferred Factor VIII to CD31.

The second problem is the lack of homogeneity between different studies on the number of vessels, which divides the patients into high- and low-risk groups, and selection of the unit to use for the estimation of neovascularization (Table 2). Authors such as Weidner employed an absolute number of vessels, while some others have employed vascular density. These two systems cause great difficulty in attempts to compare the results obtained by different groups. In our series we chose to use vascular density and calculated the first value that divided the patients into two groups with a different prognosis (see "Materials and methods").

In our series we confirmed the prognostic influence of this factor reported by several authors [1, 5, 12] in both the univariate and the multivariate analysis. We feel our series is interesting in two senses. First, all our patients even early-stage patients had received chemoradiation therapy after surgery, as this was the treatment protocol followed in our hospital at that time. Our results seem to support recent findings by other authors, such as Gasparini et al. [6] and Toi et al. [14], suggesting that vascular density might be important in predicting resistance to chemoradiation. The prognosis of patients with high vascular density has remained poor despite aggressive therapy, and it is possible that in this group of patients other kinds of therapy, including some of the antiangiogenic drugs currently under study, might be more effective. Secondly, we have noted that in most cases the prognostic effect of neovascularization appears after 30 months, behaving as a medium-term prognostic indicator. This has not been reported by any other authors. In reviewing the literature, we have noticed that in the report of Gasparini et al. [6] the log-rank curves show a similar tendency in T2 patients. We find this fact of special interest, as it might be the reason for the lack of prognostic significance of vascularization reported by such authors as Hall et al. [7]: series that do not reach a long enough follow-up time might fail to show the prognostic influence of this factor.

As for the relationship between vascular density and other prognostic variables, our study has failed to show any significant relation. Weidner et al. [18] showed that every 10-microvessel increase in a 200× field was associated with a 1.59-fold increase in the risk of node metastases. Most studies have confirmed the trend of node-positive patients to have more vessels, although studies like that of Miliaras et al. [12] have shown an inverse, although nonsignificant trend. In our patients the vascular density has been higher in node-positive patients (56.8 vs 50.6 vessels/mm^2), but the difference did not reach statistical significance. Several authors have shown a higher vascular density in premenopausal and younger patients [6, 12], in larger tumours [8] or with higher histological grades [6, 8], but none of these relations have been confirmed in our patients.

Several reports have analysed the possible association between oncogenes and neovascularization [6, 8], but none has shown any significant association between them. In Gasparini's report stated that both p53 and neovascularization were significant prognostic indicators for breast cancer patients, but according to Horak's report p53 showed no prognostic influence and only neovascularization was a significant prognostic indicator in univariate and multivariate analysis.

An interesting suggestion, which cannot be demonstrated in our series, is a possible relation between histological type and vascular density. Some reports have shown that the number of vessels is smaller in lobular carcinomas than in ductal carcinomas and that vascular density does not behave as a significant prognostic indicator in this histological type of breast tumour (Morphopopoulos et al.). Although we cannot estimate this association, as our study was restricted to ductal carcinomas, the most frequent histological type, we feel this finding supports the heterogeneity of the disease we call breast carcinoma. When studying prognostic factors in this disease we should clearly state which histological kind of breast carcinoma we are dealing with.

Although vascular density in our series has behaved as a significant prognostic indicator for both node-negative and node-positive patients, we feel that there is a great need for homogeneity in the measurement system before this factor can be accepted as a useful prognostic tool for ductal cancer patients that may even open up alternative therapeutic approaches. We regard subjectivity and lack of homogeneity in the measurement of the vessels as potential sources of error, which could be avoided by applying the measurement criteria reported by Gasparini et al. and by applying image analysers, which could reduce this dangerous subjectivity.

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