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Assessment of Tumour Vascularity as a Prognostic Factor in Lymph Node Negative Invasive Breast Cancer

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The association between tumour vascularity and relapse was examined in 93 patients with lymph node negative (LNN) invasive breast cancer. Factor VIII-related antibody was used to stain the microvessels. Vascularity was defined by the number of vessels per field counted in the area of highest vascular density at 100 × magnification. These vascular counts were divided into three groups of vascular density (group 1: <67, group 2: 68–100, group 3: >101 vessels/field). Cross-tabulation analysis revealed a significant relationship between vascular density and tumour grade ($P = 0.027$). No association was found between vascularity and tumour size, tumour type, age or menopausal status. Survival analysis showed no association between vascularity and relapse- free ($P = 0.92$) or overall survival ($P = 0.99$). Significant associations between tumour grade and relapse-free ($P = 0.0048$) and overall survival ($P = 0.0064$) and between tumour size at the cut off of 15 mm diameter and relapse-free ($P = 0.0097$) and overall survival ($P = 0.0271$) were found. When grade was taken into account the effect of tumour size became non-significant ($P = 0.059$). Our results suggest that assessment of vascularity is not an independent prognostic factor in LNN invasive breast cancer.
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INTRODUCTION

THE EXPERIMENTAL evidence suggesting that tumour growth beyond a certain diameter depends upon neovascularisation [1] has recently been investigated in clinical studies. A relationship between vascular density and metastatic disease was reported in cutaneous melanoma [2] and non-small cell lung cancer [3]. In invasive breast cancer various studies have reported an association between high vascular density and the presence of metastases. For example, Weidner *et al.* [4] compared histological specimens of 30 patients with lymph node positive or distant metastatic disease with 19 patients without any metastases and found a significant correlation between microvascular density and metastatic disease. Harris *et al.* [5] reported a similar relationship for 73 lymph node negative and 42 lymph node positive cases. Gasparini *et al.* [6] found a significant correlation between microvascular density and metastatic disease, relapse-free and overall survival in 161 early breast cancer patients, independent of their lymph node status at a median of 42 months follow-up. By contrast Hall *et al.* [7] found no association between vascularity and metastatic disease in 87 breast tumours. Among patients with lymph node negative (LNN) breast cancer Bosari *et al.* [8] found that those with high vessel counts were more likely to relapse than those with low vessel counts and Sahin *et al.* [9] found higher vascular counts in 9 LNN patients who developed metastatic disease compared with 63 patients who did not develop metastases.

Associations between vascularity and other variables were not uniformly found. Harris *et al.* [5] reported an association with poor differentiation and tumour size, Gasparini *et al.* [6] an association of vascularity with tumour grade and Bosari *et al.* [8] reported an association between vascularity and vascular invasion. Hall *et al.* [7] found no association with any of these variables.

Most of the above studies suggest that vascularity might be a valuable prognostic factor for metastatic breast cancer. Whether vascularity can be used to predict relapse in LNN breast cancer has not been fully investigated. Because of the need for new prognostic factors in LNN breast cancer, we have examined the relationship between vascularity and relapse in a cohort of LNN patients at a median of more than 10 years follow-up, in which period a relapse rate of 30% can be anticipated.

PATIENTS AND METHODS

Patients and material

We examined 93 patients in whom a radical mastectomy and axillary lymph node clearance had consecutively been performed between 1976 and 1981. The patients were selected on the basis that there was no axillary lymph node involvement or evidence of distant metastases at presentation. A median of 17 lymph nodes (range 5–47) were examined. 47 patients were premenopausal and 46 postmenopausal and their median age was 51 years (range 26–72 years). Median follow-up was 12 years 11 months (range 3 months–15 years 9 months). At the time of analysis 26 patients (28%) were known to have relapsed. This number represents a probability of 0.695 at both median and longest follow-up. The first site of relapse was local in 10 cases, local and opposite breast in 2 cases, distant in 10 cases and local and distant in 4 cases.

One or two paraffin-embedded blocks and haematoxylin–eosin-stained slides of the original primary tumour were retrieved from files. All original slides of these blocks were reviewed by a pathologist. The tumour type was classified

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invasive ductal in 79 of the cases, invasive lobular in 4, medullary in 4, tubular in 4 and invasive cribiform in 2 of the cases. Tumours other than invasive ductal were analysed as one group.

The overall tumour grade was assessed according to a modification of the Bloom and Richardson grading method [10] based on the three following subgrades: tubular differentiation, nuclear grade and mitoses. Except for the four lobular cases all 93 tumours were graded. The tumour grades in 79 ductal carcinomas were grade I in 28 tumours, grade II in 20 tumours and grade III in 31 tumours. All four medullary carcinomas were grade III and the two cribiform and four tubular carcinomas were grade I. The mean tumour size recorded from the original pathology records was 18.7 ± 8.2 mm (median 20, range 5–45 mm) for 89 cases and unknown in 4 cases. Tumour size was analysed both as a continuous variable and, in groups which were divided using three different cut off points, namely 10 mm, 15 mm and 20 mm diameter. The histological parameters vascular invasion, necrosis, lymphocytic and plasma cell infiltration were also recorded and classified into three groups for each parameter. Group 1 represented the absence and group 3 the highest expression of each variable.

Vessel staining, grading and counting

Sections (3–4 μm thick) were cut from the retrieved tumour blocks and stained with factor VIII-related antibody (Dakopatts, Denmark) using the avidin–biotin peroxidase method (Vectastain, Vector Laboratories) [11]. As mercury was used at fixation, the sections were first dewaxed and then washed with 3% Lugol's iodine and 5% sodium thiosulphate in order to remove the mercury. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol. Predigestion with 0.1% pronase (Boehringer Mannheim) was used to unmask hidden epitopes. The stained sections were screened at 50 × magnification to identify the areas of highest microvascular density. Those areas were found at the tumour margins, whereas the lowest vascularity appeared to be in sclerotic tumour areas. A dense rim of neovascularisation was noticed around areas of carcinoma *in situ*. Those areas were not included in the counts. Some background staining was often noticed but did not interfere with vessel counting in the investigated cases. Any single brown stained cell or cluster of cells, which was 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 present, were not required to classify a structure as a vessel. These criteria for counting could be reliably applied to all 93 cases included in the study.

A square grid was used to count the vessels. This grid defined an area of 0.4761 mm^2 per field at 100 × magnification and 0.1225 mm^2 per field at 200 × magnification. Vessels within the grid and those touching the outline of the grid were included in the count. Vessels were counted in four areas of highest vascular density at both magnifications. The highest count was taken for further analysis. Vascularity or vascular density was defined first of all by the maximum vascular count at 100 × magnification. Depending on this number tumours were classified in one of three vascular density groups. Group 1 included tumours with less than 68 vessels/field, group 2 included from 68–100 vessels/field and group 3, 101 or more vessels/field.

To define tumour microvascular heterogenity two methods were used. Firstly, in 2 cases, 16 serial sections were cut from one block, the sections were stained and the microvessel counts recorded in the areas of highest vascularity. Secondly, in 41 cases, two blocks of the same tumours were retrieved, sections were cut, stained and the vessels were counted in the four areas of highest vascular density for each block. In these cases the highest count for each block was used to compare the two blocks, whereas the highest count out of the two blocks was taken for further analysis.

All sections were counted by one investigator. Ten sections were counted twice by the same investigator with at least 3 days interval between the first and second counting and also by a second investigator, who used the same counting criteria. The results (intra- and inter-observer) agreed within 10 vessels at 100 × magnification and within five vessels at 200 × magnification.

Statistical analysis

The vessel counts were analysed as continuous variable and in three statistically logical levels for each magnification. Level I represented 1–60, level II 61–90 and level III 91 + vessels/field at 100 × magnification. At 200 × magnification these figures were 1–25 for level I, 26–35 for level II and 36+ for level III.

The relationship between vessel counts and tumour parameters was examined with non-parametric tests and correlations and the relationship between vascularity and those parameters was examined using contingency tables (SPSS-X software V3.12, Chicago, Illinois, U.S.A.).

Survival analyses were done using the Pearson χ^2 test and log rank test (in-house program) and the Cox regression model (BMDP, Los Angeles, U.S.A.). The proportional hazards model was used to identify which variables together accounted for a statistically significant amount of variation in disease-free survival.

RESULTS

Vascularity

Table 1 shows the number of vessels counted in the area of highest vascular density and the groups of vascular density. These results reveal no difference in vascular counts or density for relapsed compared to non-relapsed patients or patients with local compared to distant relapses.

Table 1. Vascularity in tumour specimens of breast carcinoma patients

Patients (*n*)	Vascular counts* at magnification		Vascular density (group)†
	100 ×	200 ×	
All (93)	79.7 ± 29.8	29.6 ± 10.6	1.83 ± 0.76
	72 (32–156)	28 (11–66)	2 (1–3)
Non-relapsed (67)	80.5 ± 30.0	30.2 ± 10.4	1.84 ± 0.75
	73 (34–156)	29 (14–60)	2 (1–3)
All relapsed (26)	77.5 ± 29.7	28.2 ± 11.2	1.80 ± 0.78
	72 (32–154)	25 (11–16)	2 (1–3)
Local relapsed (12)	76.7 ± 28.2	27.8 ± 9.8	1.83 ± 0.72
	75 (32–124)	27.5 (11–45)	2 (1–3)
Distant relapsed (14)	78.21 ± 31.97	28.5 ± 12.6	1.79 ± 0.80
	70.5 (39–154)	25 (18–66)	2 (1–3)

*†figures represent mean + S.D., median (range); *actual counts are given. †groups of vascular density are based on the highest vascular counts at 100 × magnification as follows: group 1: 1–67 vessels/field, group 2: 68–100 vessels/field, group 3: 101+ vessels/field.

No relationship between the relapse rate and vascular density group was found. In group 1, 28% of the patients relapsed, in group 2, 31% and in group 3, 25% of the patients.

In Table 2 variables are compared for relapsed and non-relapsed patients. No apparent difference was found regarding vascular invasion, necrosis, lymphocytic and plasma-cell infiltration between relapsed and non-relapsed cases. The tumour grade, components of grade and size, however, were evidently higher in relapsed compared to non-relapsed patients. Statistical analysis of these differences was not carried out as this was statistically not justifiable (see discussion).

Cross-tabulation by Pearson χ^2 test (Table 3) showed an association between vascular density and tumour grade ($P = 0.027$) and the subgrade tubular differentiation ($P = 0.036$) only. When only ductal carcinomas were considered the associations between vascular density and grade ($P = 0.079$) and tubular differentiation ($P = 0.068$) were not significant. Vascular density was not associated with tumour type and tumour size (see Table 3). There was no relationship between vascular density and the first site of relapse ($P = 0.47$), number of lymph nodes removed ($P = 0.22$), menopausal status ($P = 0.32$) and age ($P = 0.055$), although there was a trend towards a higher vascular density at younger age.

Heterogeneity

Tumour heterogeneity regarding vascular density was investigated in order to determine whether the density in one slide per tumour is representative for the whole tumour.

Table 2. Mean, standard deviation, median and range for several characteristics in relapsed and non-relapsed patients

Characteristic	Relapsed patients ($n = 26$)	Non-relapsed patients ($n = 67$)
Vascular invasion	1.27 ± 0.45	1.15 ± 0.36
(group)	1 (1–3)	1 (1–3)
Necrosis	1.42 ± 0.50	1.20 ± 0.40
(group)	1 (1–3)	1 (1–3)
Lymphocytic infiltration	2.65 ± 0.69	2.74 ± 0.59
(group)	3 (1–3)	3 (1–3)
Plasma cell infiltration	1.65 ± 0.49	1.68 ± 0.47
(group)	2 (1–3)	2 (1–3)
Tumour grade	2.52 ± 0.59	1.94 ± 0.84
	3 (1–3)	2 (1–3)
Tubular differentiation	2.69 ± 0.47	2.12 ± 0.76
(subgrade)	3 (2–3)	2 (1–3)
Nuclear grade	2.38 ± 0.50	2.17 ± 0.69
(subgrade)	2 (2–3)	2 (1–3)
Mitotic grade	2.12 ± 0.86	1.76 ± 0.86
(subgrade)	2 (1–3)	1 (1–3)
Size (mm)	21.7 ± 8.2	17.6 ± 7.8
	20 (5–45)	15 (5–40)
Lymph nodes removed	16.3 ± 6.8	18.3 ± 8.0
(no.)	14.5 (7–30)	17 (5–47)
Age (years)	53.1 ± 9.4	51.3 ± 10.4
	52.5 (33–72)	50 (26–69)
Disease-free follow-up months, median (range)	49.6 (2.9–125.5)	142.5+ (3.0–189.3+)

Values are given as mean ± S.D., median (range); groups for vascular invasion: 1 = absent, 2 = possible, 3 = present; necrosis: 1 = absent, 2 = +, 3 = ++, lymphocytic and plasma cell infiltration each: 1 = absent, 2 = ±/+, 3 = ++/+++.

Table 3. Cross tabulation of vascular density with other tumour variables

Variable	Vascular density group* 1 (n)	2 (n)	3 (n)	Total (n)	Pearson χ^2 P-value
Tumour type					
Ductal	32	32	15	79	
Others	4	5	5	14	0.36
Vascular invasion					
−ve	28	31	16	75	
+ve	8	5	4	17	0.65
Tumour necrosis					
−ve	30	26	12	68	
+ve	6	10	8	24	0.16
Lymphocytic infiltration					
−ve	5	3	0	8	
+ve	2	5	3	10	0.34
++ve	29	28	17	74	
Plasma cell infiltration					
−ve	14	11	5	30	
+ve	22	25	15	62	0.54
All tumours except lobular					
Tumour grade					
1	11	10	5	26	
2	15	11	2	28	0.027†
3	8	14	13	35	
Tubular differentiation					
1	7	5	3	15	
2	19	14	3	36	0.036†
3	9	15	14	38	
Nuclear grade					
1	5	4	2	11	
2	20	16	10	46	0.86
3	10	14	8	32	
Mitotic grade					
1	18	14	7	39	
2	9	8	4	21	0.52
3	8	12	9	29	
Tumour size					
0–10	8	8	2	18	
11+	26	27	18	71	0.43
0–15	16	18	4	38	
16+	18	17	16	51	0.062
0–20	29	24	12	65	
21+	5	11	8	24	0.097

(n) number of patients. *For classification of vascular density groups see methods and legend to Table 1. †Significant.

The results of the 2 cases in which 16 serial sections were counted suggest that the microvessel counts within a given block of tumour are quite constant. The number of vessels per field present in the first and the last sections of two blocks are shown in Table 4. In 41 cases, two blocks from the same tumour were examined. The vessels were counted at 100 × and 200 × magnification and were classified according to the vascular density groups described in the methods. Two vessel counts that fell into the same group were classified as concordant. Vessel counts that fell into adjacent vascular groups were still classified as concordant if at least one of the counts was within 5% of the borderline value. The concordance in terms of density groups occurred in 71% of the cases (Table 5). Table 5 also shows that

Table 4. Heterogeneity within one tumour block

	Section 1 magnification		Section 16 magnification	
	100 × (*n*)	200 × (*n*)	100 × (*n*)	200 × (*n*)
Case 1	77	43	81	43
Case 2	53	17	55	21

(*n*) = no. of vessels/field.

Table 5. Heterogeneity in vascularity between blocks of the same tumour

Variable	Difference None *n* (%)	1 level *n* (%)	2 levels *n* (%)
Vascular density	29 (71)	10 (24)	2 (5)
100 × magnification	32 (78)	6 (15)	3 (7)
200 × magnification	31 (76)	9 (22)	1 (2)

n = no. of cases. Values which are not concordant may belong to adjacent groups (i.e. 1 difference level) or to non-adjacent groups (i.e. 2 difference levels). Levels at 100 × magnification are: I:1–60, II:61–90, III:91+ vessels/field. Levels at 200 × magnification are: I:1–25, II:26–35, III:36+ vessels/field.
Vascular density groups are: 1:1–67, 2:68–100, 3:101+ vessels/field.

the percentage of concordant values varied between 71 and 78% depending on the classification of vascular levels or groups used.

Survival

Survival analysis by the log rank test showed no significant correlations between disease-free or overall survival and vessel counts at 100 × magnification ($P = 0.86$ and $P = 0.53$, respectively), at 200 × magnification ($P = 0.46$ and $P = 0.42$, respectively) or with vascular density groups ($P = 0.92$ and $P = 0.99$, respectively). For all cases tumour grade correlated significantly with disease-free survival ($P = 0.0048$) and overall survival ($P = 0.0064$) (Fig. 1a and b). In addition, the proportional hazards model showed that patients with grade I tumours were much less likely to relapse than patients with grade III tumours (hazard ratio 0.071) whereas this correlation was not found between grade II and III tumours (hazard ratio 0.69). Univariate analysis revealed that the subgrade tubular

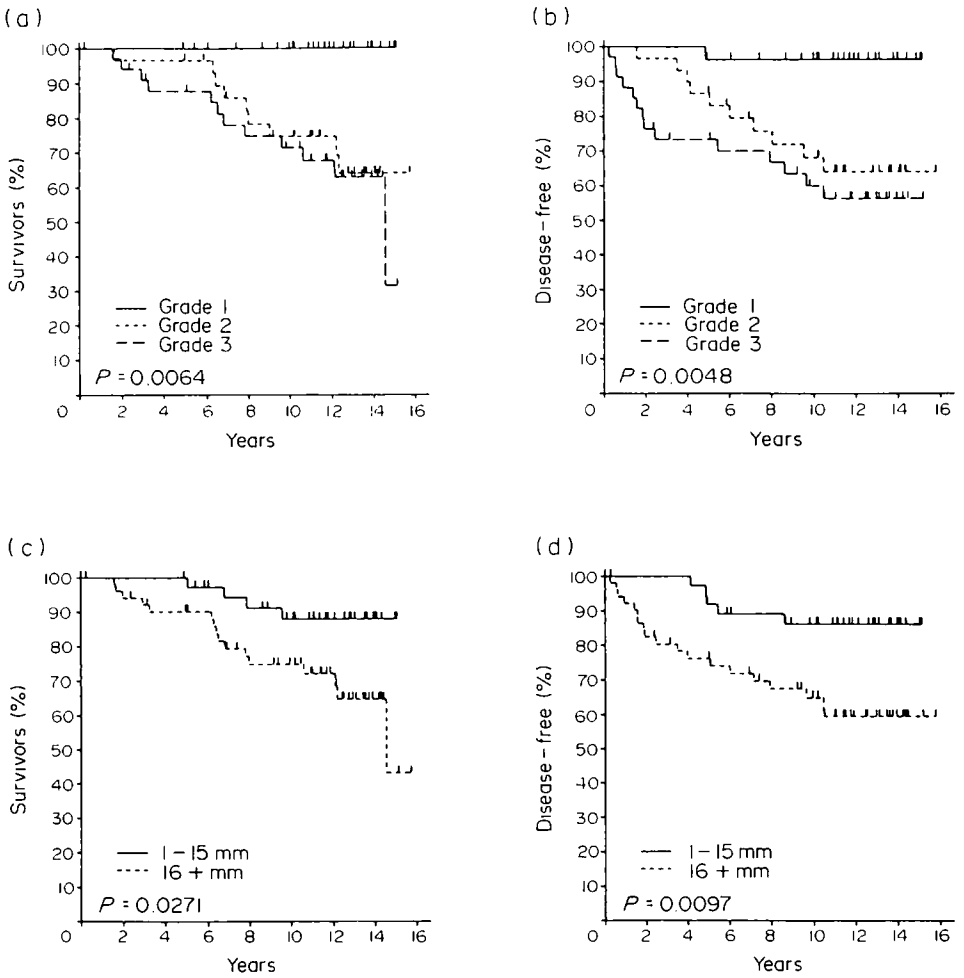

Fig. 1. Relationship between survival, tumour grade and tumour size. Relationship between tumour grade and overall (a) and disease-free survival (b). Relationship between tumour size and overall (c) and disease-free survival (d). Tumour size was assessed at a cut-off point of 15 mm diameter. Dashes represent the time at which individual cases were censored.

differentiation also correlated significantly with disease-free ($P = 0.008$) and overall survival ($P = 0.008$). Of the other histological parameters, necrosis showed significant association ($P = 0.029$) with disease-free survival only. Univariate analysis also revealed a significant correlation between tumour size and disease-free survival ($P = 0.033$). Multivariate analysis by Cox regression showed that when grade was taken into account necrosis ($P = 0.40$) and tumour size ($P = 0.059$) became non-significant.

Further analysis of tumour size revealed a significant difference in disease-free ($P = 0.0097$) and overall survival ($P = 0.027$) for patients with tumours up to 15 mm compared with tumours of 16 mm or more (Fig. 1 c and d). When tumour size was divided in less than or over 20 mm there was a significant difference in disease-free survival only ($P = 0.038$).

DISCUSSION

Various studies have examined the role of vascularity as a prognostic factor by comparing lymph node positive and LNN breast cancer patients. The results were not consistent although the general consensus was that increased vascular density occurred when metastatic disease was present [4–8]. Our study addresses the question of whether vascularity could be used as a prognostic factor in invasive breast cancer patients with no evidence of metastatic disease at presentation.

Our results indicate that vascularity in LNN invasive breast cancer correlates with tumour grade and in particular with the subgrade tubular differentiation but does not predict relapse or survival. Multivariate analysis revealed that tumour grade was the only significant variable associated with disease-free survival, although tumour size was only just non-significant at the 5% level. In the non-relapsed group of patients, 10 (10.7%) were lost from follow-up after 5 years and 15 (16.1%) were lost after 10 years. As information about a possible late relapse of these patients is missing, in our study it would have been statistically not justifiable to analyse the results in Tables 1 and 2 by χ^2 test. Other studies have used this test to analyse similar data [3, 4, 6]. In these studies the number of patients lost from follow-up was not stated.

The heterogeneity study reveals only a slight difference in vessel counts between serial sections of one block, but points out that tumour heterogeneity between blocks may introduce a considerable error in the assessment of vascular density.

Factor VIII-related antibody has routinely been used for staining endothelial cells in paraffin-embedded sections [3, 4, 6, 7, 9]. In one study [8] factor VIII was compared with blood group isoantigens and found to be superior for staining purposes. In another study [5] CD31 was used to stain the vessels.

With regard to the interpretation of the staining results it has to be taken into account that factor VIII-related antibody stains both blood and lymphatic vessels [12] and that the intensity and selectivity of the staining results may also depend on the type of the antibody used. Enzymatic predigestion is often required to enhance the signal and loss of antigenicity may occur attributable to technical aspects of fixation and temperatures reached during routine paraffin-embedding [13–15]. Therefore, it would be of value to perform comparative studies with a panel of antibodies to define whether the variability of staining results could be attributed to the choice of the antibody.

In conclusion, with the caveat that heterogeneity in the vascularity of tumours may account for different results, our study shows that vascularity is not valuable as independent prognostic factor in LNN invasive breast cancer.

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