

ORIGINAL ARTICLE

Thymidine phosphorylase expression and stromal vascularity in ductal carcinoma in situ of the breast

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Aims: Periductal angiogenesis in ductal carcinoma in situ is associated with an increased risk of subsequently developing a recurrence. This study aimed to (1) identify the relation between periductal and stromal vascularity and recurrence and (2) determine whether thymidine phosphorylase (TP) is associated with angiogenesis or recurrence in ductal carcinoma in situ (DCIS).

Methods: Twenty cases of DCIS that did not subsequently recur, 20 that developed a subsequent in situ recurrence, and 12 that developed a subsequent invasive recurrence were investigated. Periductal and stromal (hotspot) microvessel density were determined quantitatively using antibodies to CD34 and von Willebrandt factor (vWF). TP expression by DCIS was assessed semiquantitatively using the H score method.

Results: Stromal and periductal microvessel density assessed by anti-vWF gave similar mean values, and showed a strong positive correlation. When angiogenesis was assessed with anti-CD34 this association was lost. Not only were the mean values for both types of microvessel density higher than those obtained with anti-vWF, but the periductal microvessel density was significantly greater than the stromal microvessel density. TP expression was associated with stromal microvessel density assessed with anti-vWF, but not with anti-CD34. TP expression was not related to recurrence. No significant difference was identified in TP expression or stromal vascularity in DCIS between cases that recurred as DCIS and those that recurred as invasive carcinoma.

Conclusions: Recurrent in situ or invasive disease after excision of DCIS does not appear to be related to stromal microvessel density or to TP expression by DCIS cells.

Since the introduction of the UK National Breast Screening Programme the frequency with which ductal carcinoma in situ (DCIS) is detected has increased from less than 5% to approximately 20% of all carcinomas.¹ DCIS is a non-fatal disease and is potentially curable. However, if following initial treatment recurrent disease develops, then approximately 50% of the recurrences are as invasive carcinoma.^{2–3} Although standard histological parameters such as DCIS nuclear grade, size, and the status of the excision margins predict disease recurrence to some extent,^{2–3} no factors are known to predict the development of recurrent invasive disease.

There is increasing evidence that angiogenesis has a role as a prognostic factor for invasive breast cancer.^{4–9} In DCIS two different vascular patterns have been identified, increased stromal vascular density and an increase in periductal vessels.^{10–11} We have previously shown that an increase in microvessel density (MVD) associated with this last pattern is associated with the development of an invasive recurrence.¹²

“There is increasing evidence that angiogenesis has a role as a prognostic factor for invasive breast cancer”

Thymidine phosphorylase (TP) is thought to play a role in endothelial cell migration and differentiation,¹³ and has been shown to be present within DCIS.¹⁴ The aim of our present study was to see whether TP is associated with the increased periductal vascularity previously identified, or with disease recurrence, and to see whether stromal vascularity is as important as periductal vascularity in determining disease recurrence.

MATERIALS AND METHODS

Patients and tumours (table 1)

Records from 355 patients with DCIS in the Merseyside region were examined. Thirty two patients were identified who were known to have subsequently developed recurrent disease, 20 as recurrent DCIS and 12 as recurrent invasive carcinoma. In addition, 20 cases of DCIS with no history of recurrence after initial treatment were retrieved. These 20 cases had similar clinical and histological features, in addition to initial treatment. Histological features included nuclear grade, pathological grade, tumour size, and excision margin (table 1). We used the same cases in our previous study.¹² The original haematoxylin and eosin stained sections were reviewed by two pathologists (BSS and JPS) for classification following the guidelines of the European Commission and the UK National Breast Screening Programme.^{15–16} A representative block for each patient was selected for subsequent immunostaining.

Immunohistochemistry

Sections were stained using monoclonal anti-CD34 (Qbend/10; Dako, Glostrup, Denmark) and polyclonal antihuman von Willebrandt factor (anti-vWF; Dako). These two antibodies were chosen because in a previous study they had been shown to detect different subpopulations of blood vessels.¹²

Abbreviations: BSA, bovine serum albumin; DCIS, ductal carcinoma in situ; IMS, industrial methylated spirits; MVD, microvessel density; TBS, Tris buffered saline; TP, thymidine phosphorylase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrandt factor

Table 1 Clinical and pathological data from the three groups of patients with ductal carcinoma in situ

	Non-recurrence	In situ recurrence	Invasive recurrence
Number of patients	20	20	12
Age (years)			
Range	49–87	45–78	43–82
Mean	66.2	65.6	63.3
Follow up (months)			
Range	29–138	9–98	7–52
Mean	94	43	48
Death	2 (both not related to breast cancer)	0	1 (died of breast cancer)
Surgery			
Wide local excision	18	20	10
Mastectomy	2	0	2
Adjuvant treatment			
None	9	9	5
Hormone	11	11	7
Radiotherapy	0	0	0
Others	0	0	0
Nuclear grade			
Low	4	3	4
Intermediate	4	6	2
High	12	11	6
Size (mm)			
Range	1–65	5–20	1–22
Mean	17	13	12
No of cases not assessable	0	9	3
Excision margin (mm)			
<1	7	5	3
1 < to 10	4	4	0
>10	5	4	3
No of cases not assessable	4	8	6

An antibody to TP was also used (Clone P-GF.44C; NeoMarkers, Fremont, California, USA). Sections were dewaxed through two changes of xylene and industrial methylated spirits (IMS). Endogenous peroxidase activity was blocked with a mixture of H₂O₂/methanol (12 ml H₂O₂ in 400 ml methanol) for 12 minutes. Antigen retrieval was performed for the endothelial markers by treating the sections with 0.2 g of trypsin and 0.4 g of calcium chloride in 440 ml Tris buffered saline (TBS; 50mM Tris/HCL, 150mM NaCl, pH 7.4) at 37°C for 20 minutes. A pretreatment was not required for the TP antibody. Before staining with the polyclonal antiserum, sections were treated with a mixture of 5% bovine serum albumin (BSA)/TBS for 10 minutes.

The antibodies were diluted 1/20 for anti-CD34, 1/100 for anti-TP, and 1/1000 for anti-vWF in 5% BSA/TBS. The sections were incubated with the primary antibodies at room temperature for 40 minutes. Secondary antibodies were incubated for 40 minutes using EnVision labelled polymer (mouse or rabbit as appropriate). Sections were washed with TBS between incubation steps. 3,3'-Diaminobenzidine was used as a chromogen. The last two steps were carried out using a commercial kit (EnVisionTM + System; Dako, Carpinteria, California, USA).

The nuclei were counterstained with haematoxylin solution. The sections were dehydrated through four changes of IMS and three changes of xylene before being mounted in resinous mountant (DPX; BDH Laboratory Supplies, Poole, Dorset, UK). Omission of the primary antibody was used as a negative control and a case of DCIS with known endothelial and TP staining was used as a positive control for each batch of immunohistochemistry.

Assessment of tumour periductal vascularity

Periductal vascular density evaluation required the assessment of completely transected ducts involved by DCIS. Counting started from the upper right of all stained sections, moving downwards and to the left. All or the first 50 foci

encountered were assessed on each section. The area of each individual focus of DCIS was measured at ×200 magnification using an image analysis system (Zeis Axiohome with software version 3.0; Germany). In addition, the area with a circumference 100 μm from the edge of an individual focus of DCIS was measured. The area required for MVD assessment was thus calculated by subtracting the first measurement from the second. The microvessels within the appropriate area around each DCIS focus were then counted at high magnification (×400). Eligible microvessels included any immunostained endothelial cell or cluster of cells around a visible lumen clearly separated from adjacent microvessels, tumour cells, and other connective tissue components. The presence of red blood cells was not required. It was not possible to distinguish blood and lymphatic vessels. Where vessels were in clusters, each was counted as separate if it met the above criteria. The highest periductal MVD around a single duct and the mean periductal MVD for the section were then calculated.

Assessment of tumour stromal vascularity

Microvessel counts were determined as described by Weidner *et al.*⁴ Areas with high vascularisation were identified by scanning the sections at low power (×40 magnification). Only those stromal areas at least 1 mm from the edge of the nearest tumour were assessed. Up to 30 high power fields (magnification, ×200; field size, 0.32 mm²) were examined. The five highest counts were recorded and the hotspot (highest) MVD, the mean of the three highest counts, and the mean of the five highest counts were calculated. However, the results obtained with the three different counts were similar.

Assessment of TP expression

Sections were examined at ×40 and ×100 magnification. TP expression in DCIS cells was both cytoplasmic and nuclear, with the cytoplasmic form predominating. Nuclear and

cytoplasmic staining were both counted as evidence of positive staining. An H score for each section was then calculated. This was produced by multiplying the percentage of cells staining with each individual intensity score (0, negative; 1, weak; 2, intermediate; and 3, strong) and then adding the numbers together to produce a score ranging from 0 to 300.

The data were analysed by means of the non-parametric Mann Whitney and Kruskal Wallis tests and Spearman's and Pearson's correlation coefficients using SPSS Version 10.0 software for Windows 97/NT.

RESULTS

Table 1 summarises the clinical and histological details of the 52 cases of DCIS studied. All the cases studied are retrospective cases and thus the size or excision status could not be evaluated in all the cases.

Comparison of periductal and stromal MVD vWF

The mean stromal MVD and the mean periductal MVD were both 160 vessels/mm². A strong positive correlation was identified between stromal and periductal density, regardless of how measured (for example, hotspot, mean of three highest stromal counts, mean of five highest stromal counts, highest periductal density, or mean periductal density). The weakest correlation was between hotspot and mean periductal MVD (Pearson: lowest $r = 0.464$; highest $p < 0.001$).

There was no significant correlation between nuclear grade and stromal or periductal MVD in the entire group of DCIS cases (Spearman: lowest $p = 0.3$). No significant difference was identified in stromal vascularity between DCIS cases that did and did not subsequently recur, either as DCIS or as invasive carcinoma (Mann-Whitney: lowest $p = 0.9$).

CD34 (figs 1 and 2)

The mean stromal MVD was 182 vessels/mm² and was higher than that seen for vWF (Mann-Whitney: $p = 0.037$). The mean periductal density was 210 vessels/mm² and was again higher than that seen for vWF (Mann-Whitney: $p < 0.001$). Furthermore, in contrast to that seen for vWF, the periductal MVD was significantly higher than the stromal MVD, regardless of how measured (Mann-Whitney: highest $p = 0.037$). Unlike staining with the anti-vWF antibody, there was no significant correlation noted between stromal and periductal vascularity when the anti-CD34 antibody was used (Pearson: lowest $p = 0.1$).

There was also no significant correlation between nuclear grade and periductal or stromal vascularity (Spearman:

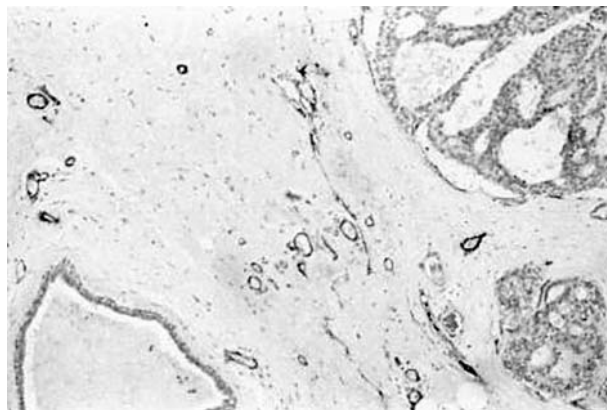


Figure 2 Stromal microvessels stained with the anti-CD34 antibody (low power field).

lowest $p = 0.4$). This finding was again similar to that seen with the anti-vWF antibody. In addition, there was no significant difference in stromal vascularity between DCIS cases that did and did not recur, either as DCIS or invasive carcinoma, after initial treatment (Mann-Whitney: lowest $p = 0.5$).

TP staining in DCIS (figs 3 4, and 5)

When the H scores for TP staining were compared between cases of DCIS that subsequently recurred or did not recur, either as DCIS or invasive carcinoma, no difference was identified (Mann-Whitney: $p = 0.181$). A negative correlation was seen between the TP H score and DCIS nuclear grade (Spearman: $r = 0.292$; $p = 0.049$). The TP H score correlated with stromal (Pearson: highest $p = 0.035$) but not periductal (Pearson: lowest $p = 0.091$) vWF MVD. In contrast, for CD34, no significant correlation was identified between the TP H score and stromal or periductal vascularity (Pearson: lowest $p = 0.598$).

DISCUSSION

Angiogenesis is thought to play an important role in the progression of invasive cancer.⁴⁻⁹ It also appears to play a role in the development of hyperplastic and other precancerous lesions.^{10-11 17} Two vascular patterns have been described in DCIS,^{10 11} a diffuse stromal increase and an increase in periductal blood vessels. An increase in periductal MVD is seen in 23–62% of DCIS cases. Our previous study showed that an increase in periductal MVD around DCIS predicts the development of a recurrence, particularly an invasive

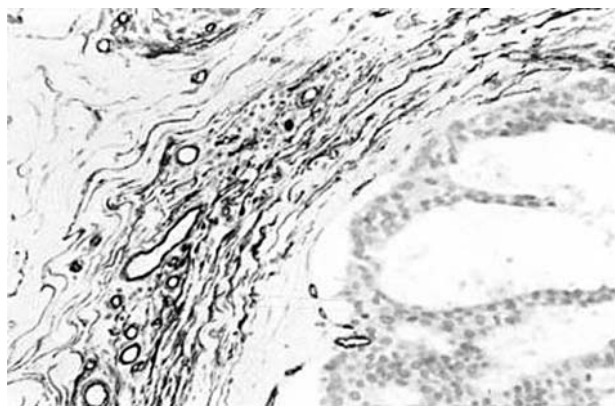


Figure 1 Periductal microvessels stained with the anti-CD34 antibody (high power field).

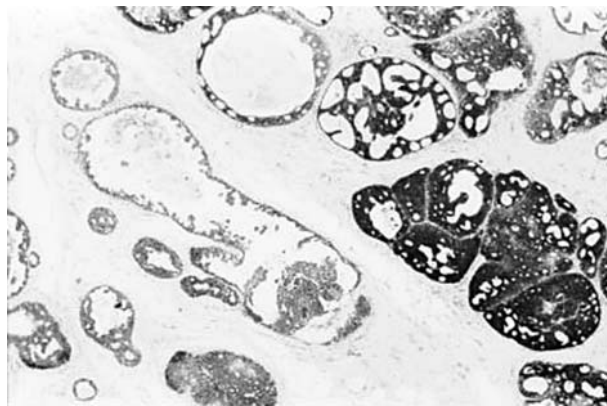


Figure 3 Thymidine phosphorylase expression in ductal carcinoma in situ parenchymal cells (low power field).

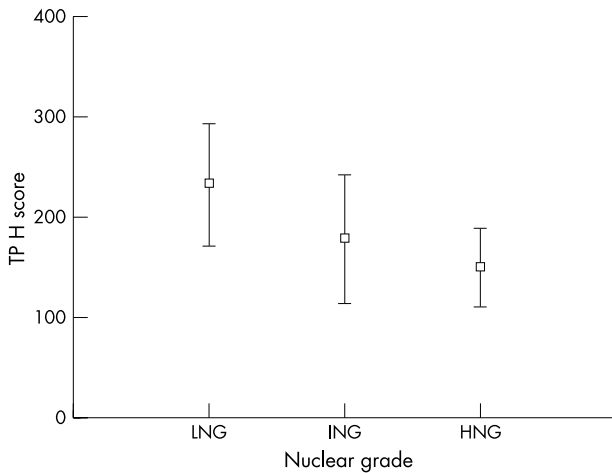


Figure 4 Relation between thymidine phosphorylase (TP) expression and nuclear grade in all cases of ductal carcinoma in situ. HNG, high nuclear grade; ING, intermediate nuclear grade.

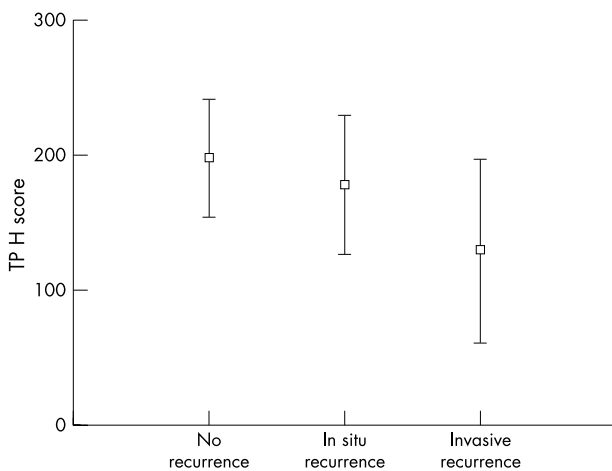


Figure 5 Relation between thymidine phosphorylase (TP) expression and clinical outcome.

recurrence.¹² The highest MVD for CD34 was seen in cases of DCIS that subsequently developed an invasive recurrence and the lowest in cases of DCIS that were not known to have developed recurrent disease.¹²

It has also been proposed that the increased periductal MVD may result from angiogenic factors secreted by tumour cells.¹¹ TP is thought to have a role in endothelial cell migration and differentiation,¹³ and has been shown to be associated with increased periductal MVD in DCIS.¹⁴ Therefore, we proposed that the increased periductal MVD in those cases of DCIS that subsequently recurred was a result of TP expression. However, we found no significant correlation between TP expression and disease recurrence or with periductal MVD. TP expression did show a correlation with stromal vWF stained vessels but not those stained for CD34. Thus TP does not appear to be related to disease recurrence and possibly only influences certain subtypes of vessel. Other factors associated with angiogenesis, such as vascular endothelial growth factor (VEGF), may have a more important role. Valtola *et al* examined the role of VEGF receptor 3 (VEGFR-3) and its ligand VEGF-C in DCIS.¹⁸ They noted that VEGFR-3 was prominent in vessels adjacent to the basal lamina of affected ducts and its ligand was localised in the cytoplasm of the tumour cells. These results suggest that

the role of VEGF-C involves modifying the permeabilities of both blood and lymphatic vessels. High levels of expression of VEGF mRNA and its receptor mRNA have been found in both tumour cells and microvessel endothelial cells.¹⁹ Guidi *et al* showed that there was a significant correlation between a high Ki-61 proliferation index and high microvessel counts,²⁰ in addition to VEGF expression.²¹

Previous studies^{10 11 14 22} have used qualitative or semi-quantitative assessments of periductal vascularity and have defined periductal vascularity as the rim or necklace of vessels that are adjacent to the basement membrane, or vessels that are within a defined distance from the basement membrane. We have used a precise quantitative method, which includes those stromal vessels that are within 100 µm of the tumour, in addition to the rim or necklace of vessels. To identify a separate population of blood vessels within the tumour we counted stromal MVD. The vessels counted were situated at least 1 mm from the nearest DCIS involved duct to minimise the effects of tumour proximity on the blood vessels. However, this would not exclude the effect of factors released by the tumour cells into the blood and lymphatic system. This difference in assessment of MVD does not allow direct comparisons between our current study and previous studies.^{10 14}

“Although thymidine phosphorylase expression may play a role in the development and progression of invasive breast cancer, it does not appear to be important in the development of disease recurrence following treatment for ductal carcinoma in situ.”

There is some evidence to suggest that TP expression in invasive breast cancer correlates with MVD and may have prognostic value,^{23 24} although other studies have not found this to be the case.^{22 25 26} TP has also been shown to correlate with the relapse rate in DCIS, although the same study did not show a significant correlation between relapse free survival and TP expression.¹⁵ In our present study, when the H scores for TP expression were compared between patients who did or did not subsequently develop a recurrence, no difference was seen. Although TP expression may play a role in the development and progression of invasive breast cancer, it does not appear to be important in the development of disease recurrence following treatment for DCIS.

TP can also be induced by hypoxia and low pH.²⁷ Therefore, high grade DCIS with comedo necrosis would be expected to be more likely to express TP. Surprisingly, in our study we found that the TP H score showed a negative correlation with grade, even though most of our high grade DCIS cases showed at least focal comedo necrosis. Interestingly Lee *et al* found no relation between TP expression and necrosis.²² Possibly the hypoxia present in DCIS is not a strong enough stimulus to induce TP expression, or perhaps the cells in high grade DCIS are so genetically deranged that they are unable to respond to the hypoxia by upregulation of TP.

Although two patterns of vascularity have been described in DCIS, the relation between these two patterns is unclear.^{10 11 26} Guidi *et al* found increased stromal vascularity in 25% of cases and the presence of a periductal vascular cuff in 35%.¹⁰ However, they could not show an association between stromal and perivascular density. In contrast, when measuring vWF expression, we found a strong correlation between stromal and periductal vascularity, with similar values for each. When measuring vascular density by means of CD34 expression, the values were higher than those seen for vWF. There was also a difference between stromal and periductal vascularity, with the highest values being seen for

Take home messages

- An increase in microvessel density around ductal carcinoma in situ (DCIS) did not predict the development of a recurrence, whether in situ or invasive, in contrast to our earlier study
- In addition, thymidine phosphorylase expression by DCIS cells did not appear to be related to recurrent in situ or invasive disease after excision of DCIS

periductal vascularity. The difference between the stromal and periductal counts probably results from an increase in CD34+ α WF blood vessels, and it may be that this phenotype of blood vessel in most important in angiogenesis and disease progression.

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