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Angiogenesis in invasive breast carcinoma—a prospective study of tumour heterogeneity

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Abstract

Assessment of angiogenesis has been reported to be an independent prognostic factor in breast cancer, while other studies have been negative. This study prospectively investigates the degree of intratumoral microvessel heterogeneity and the possible influence on the results. From 21 invasive breast cancers six 4μ sections were cut. Sections ($n=126$) were stained immunohistochemically with a CD31 monoclonal antibody (JC70). In each section, three areas with the most intense neovascularisation (hot spots) were identified and the microvessel density (MVD) was obtained by counting vessels at 200× magnification. The variation between sections contributed more to the total variance than variation between different tumours: 45.0 and 37.3%, respectively, according to a nested ANOVA analysis. Paired comparisons of two sections at a time from the same tumour showed a concordance in 59.0% (95% Confidence Interval (CI): (55.3–62.8)) with reference to a tentative cut-off level. Our study demonstrates that assessment of MVD in hot spots is questionable to measure angiogenesis due to the considerable intratumoral heterogeneity.

Keywords: Breast cancer; Angiogenesis; Immunohistochemistry; Heterogeneity

1. Introduction

Angiogenesis, i.e. the ability of the tumour to induce formation of new blood vessels in the tumour stroma, is a prerequisite for tumour growth [1]. Tumour blood vessels can be visualised with immunohistochemical staining of tumour sections, using monoclonal antibodies to endothelial cell antigens. Most investigators have used an antibody to the factor VIII related antigen (FVIIIRag) expressed by endothelial cells or an antibody (JC70) to the platelet/endothelial cell adhesion molecule (PECAM) or CD31 [2]. In the comparison between those two markers, CD31 has been claimed to have a higher sensitivity [3–5].

Several studies have demonstrated that microvessel density (MVD) can independently predict poor prognosis in operable breast cancer [3–8] including lymph-node negative patients [8–12]. However, some authors could not find any prognostic value for angiogenesis assays [13–18].

One important reason for the contradictory results may well be methodological problems. Whereas inter-observer variability seems manageable [19], some reports claim intratumoral heterogeneity to be a more problematic aspect [15].

The observer's ability to identify the areas of highest vascular density, which is an important step in the methodology [2,20], is most likely partly dependent on intratumoral heterogeneity.

Due to conflicting results in the literature, the aim of this study was to investigate if the intratumoral angiogenic pattern demonstrates a significant variation which may influence the results. Therefore, we meticulously analysed 21 consecutive primary breast cancers.

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2. Patients and methods

Twenty-one invasive breast carcinomas were received as fresh specimens at the Department of Pathology of Örebro during May to October 1994. No preoperative treatment of breast cancer was allowed. The whole tumour was sectioned in 5-mm slices. Fixation was run overnight in 4% buffered formaldehyde solution. After dehydration, all tumour material was completely embedded in paraffin. Haematoxylin and eosin (H&E) staining of all tumours was done for routine assessment of histological type and grade [21].

Two to 15 blocks were obtained from each tumour. Six 4μ sections were cut per tumour, i.e. the same number of sections per tumour regardless of the tumour size (Fig. 1). Sections from different levels within one or more blocks were cut from tumours with less than six blocks. In all cases, sections were separated as widely and evenly as possible within the tumours with a minimal distance of 1.5 mm. The position of all sections within the tumour was registered and labelled A–F.

The decision to analyse six sections per tumour was due to the fact that an increase in the number of sections would substantially increase the extent of the time-consuming vessel counting procedure. We also arbitrarily found it reasonable to believe that six sections per tumour would be sufficient to detect a significant degree of intratumoral variation.

2.1. Assessment of vascularisation

Six 4μ sections from each tumour were stained with a monoclonal antibody to CD31 (JC70 Dako AS Glostrup, Denmark) labelled with the ABC method (Dako Duet kit, DAKO AS Glostrup, Denmark). The sections were predigested with trypsin (Chymotrypsin Sigma, Stockholm Sweden) for 30 min at 37 °C. After washing in Tris-buffered saline (TBS), the sections were pre-incubated with normal goat serum for 10 min to block unspecific staining before the JC70 antibody was applied for 30 min. After washing in TBS, the biotinylated goat anti-mouse/rabbit antibody was applied for 30 min, TBS washing was repeated before the peroxidase-conjugated strept–avidin–biotin complex was added to the sections for 30 min. The colour was induced by the addition of the electron donator diaminobenzidine (DAB) for 10 min. The sections were counterstained with Mayer's haematoxylin.

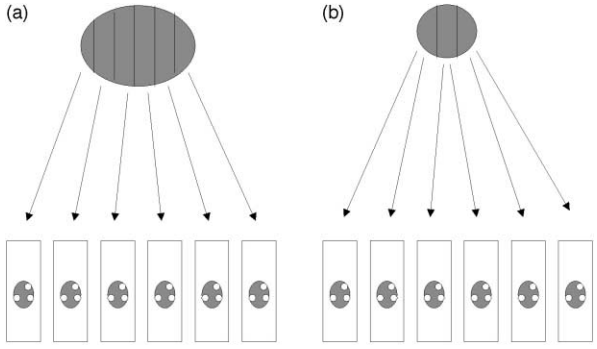

Fig. 1. Example on how the tumours were handled. The tumours were sectioned in 5-mm slices. All tumour material was paraffin-embedded so that 2–15 blocks were obtained from each tumour. Six 4μ sections were cut per tumour. From the tumour with six blocks (a), one section from each block was cut. From tumours with more than six blocks, the six sections were cut from six different blocks that were chosen to ensure that the sections were separated as widely and evenly as possible. From tumours with less than six blocks (b), sections were cut from different areas within one or more blocks. The sections were separated as widely and evenly as possible with a minimal distance of 1.5 mm.

2.2. Counting of vessels

All sections were coded and shuffled to ensure a blind assessment. The MVD assessment followed the recommended procedure in a recent overview [2]. The quality of the intratumoral staining was judged using blood vessels in adjacent benign breast tissue as internal positive controls. The most vascularised areas of the tumour tissue were located at low magnification (10× oculars with 4× and 10× objectives). Vessels were counted in three 200× fields (0.72 mm^2). Microvessels in sclerotic areas within the tumour and immediately adjacent areas of normal breast tissue were disregarded. Any brown staining endothelial cell or cluster of endothelial cells that was separate from the adjacent microvessels was considered to be a single countable microvessel. All slides were assessed simultaneously by two observers using a conference microscope.

2.3. Statistical analysis

We used the Statistica software (Statsoft, OK, USA) for calculation of the standard deviation, confidence intervals (CI), and the independent two-sided *t*-test for comparisons between groups. Variability of MVD between different sections from the same tumour was expressed by the coefficient of variation (CV) which is defined as 100× the standard deviation divided by the mean. A nested ANOVA (Statistica software) was used to analyse the proportion of the total variance to which each sampling level contributed. For this analysis we considered cases, intersection and intrasection as three levels in a hierarchic model; the cases constituted the highest level in which the intersection level was nested. The lowest level, intrasection, was then nested in the intersection level. If a method is to detect meaningful differences between the different tumours, the relative variance of the case level must be clearly larger than the relative variance of the methodological levels, i.e. intersection and intrasection.

3. Results

Median age of the patients was 69 years (range 35–88 years). The median size of the 21 tumours was 20 mm (range 10–40 mm). Lymph-node status, histological type and tumour grade according to the World Health Organization (WHO) are given in Table 1.

In 5 patients, the quality of immunostaining was not judged as satisfactory in a proportion of the six sections (Table 2). All eight sections with questionable staining were stained a second time with a highly vascularised tumour section as a positive control; none of them turned out to be assessable by this procedure.

3.1. Measures of variation

The mean of all MVDs ($n=345$) was 82.5/200XHPF (median 75, range 21–196). The mean of the highest scores from each section ($n=115$) was 93.3 (median 86.5, range 40–196) The mean of the highest score from each tumour ($n=21$) was 128.4 (median 120, range 87–196) (Table 2). A nested ANOVA of variance components was performed in order to assess the contribution of the three sampling levels to the total variance. The highest level of the hierarchic model, the different tumours, contributed with 37.3% of the total variance. The corresponding figures for the methodological levels, the intersection and intrasection levels, were 45.0 and 17.7%, respectively. Thus, variation between different sections of the tumours contributed more to the total variance than did variation between different tumours. The highest MVD from each section was plotted in order to visualise the intratumoral heterogeneity (Fig. 2a). The CV of all maximum scores per section within each tumour was 23.7% (10.9–45.9). If the means of the three readings on every slide were used, the results of the analyses were similar to those obtained with maximum MVD (Fig. 2b).

The intersection variability can be further exemplified by the fact that the mean of all the highest scores from each tumour was exceeded with $>20\%$ by the highest count in 19/21 cases. The CV was analysed in different subgroups with reference to tumour size ($\leqslant 20$ mm versus >21 mm) and the number of blocks taken (2–5 blocks versus >5). The independent two-sided t-test was used in this analysis, but no differences could be found.

3.2. Application of potential cut-offs

To become clinically useful a factor such as MVD is usually dichotomised into a high MVD and a low MVD category. In this analysis, we chose the median of all the highest scores (86.5/200XHPF) from each section ($n=115$) as a tentative cut-off level, which was applied to the scores from each sectioning level (A–F). In this way, we had six sets of dichotomised scores, each representing a different part of the tumours. We then compared the results from one set of sections with a second set of sections (A–B; A–C; A–D; A–E; A–F; B–C, etc.) and calculated the proportion of tumours for

Table 1
Patient and tumour characteristics

Age, median (years)	69 (range 35–88)
Tumour size, median (mm)	20 (range 10–40)
Type of surgery (%)	
Mastectomy	9 (43)
Breast conserving	12 (57)
Nodal status, numbers (%)	
Negative	11 (52)
Positive	8 (38)
Not assessed	2 (10)
Histological type	
Ductal	18 (86)
Lobular	1 (5)
Medullary	1 (5)
Mucinous	1 (5)
Histological grade according to WHO of ductal carcinomas	
Grade 1	3 (17)
Grade 2	8 (44)
Grade 3	7 (39)

WHO, World Health Organization.

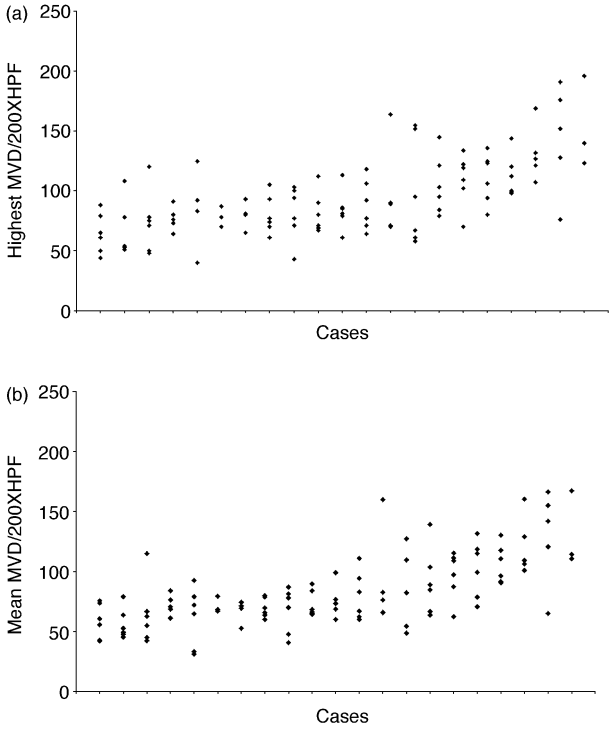

Fig. 2. (a) The highest MVDs from all assessable sections ($n=115$) are plotted. Each mark on the X-axis represents one tumour. The tumours are ordered by increasing mean microvessel density (MVD). (b) The means of three hot spot microvessel densities (MVDs) within each assessable section ($n=115$) are plotted. Each mark on the X-axis represents one tumour. The tumours are ordered by increasing mean MVD.

Table 2
The highest MVD in all 115 assessable sections: the tumours are ordered from lowest mean MVD (top) to highest (bottom)

T-size (mm)	Blocks no.	Sections, highest of three counts						Mean (A–F)	CV%
		A	B	C	D	E	F		
15	4	44	50	88	65	79	61	64.5	26.0
10	2	51	53	54	54	78	108	66.3	34.3
22	5	48	71	75	78	120	50	73.7	35.4
19	5	91	76	73	64	80	64	74.7	13.8
15	3	83	40	83	92	125	40	77.2	42.3
10	3	–[b]	–[b]	70	87	–[a]	78	78.3	10.9
22	7	–[a]	–[a]	65	81	93	80	79.8	14.4
28	6	61	77	105	93	70	74	80.0	20.2
20	3	100	43	71	94	103	77	81.3	27.9
16	5	69	67	71	80	90	112	81.5	21.1
11	2	79	113	61	86	81	85	84.2	19.9
17	7	64	77	106	92	118	71	88.0	24.0
29	7	70	89	71	90	–[a]	164	96.8	40.0
35	7	152	95	155	61	58	67	98.0	45.9
30	4	84	145	121	95	79	103	104.5	23.7
11	5	134	122	70	102	109	119	109.3	20.3
22	4	94	80	106	125	123	136	110.7	19.1
21	5	120	98	100	112	144	99	112.2	15.9
15	3	169	–[b]	127	132	121	107	131.2	17.6
30	15	76	176	191	128	152	–[a]	144.6	31.3
40	10	–[a]	–[a]	123	–[a]	140	196	153.0	25.0

MVD, microvessel density; CV%, % coefficient of variation.
Mean (A–F) is mean value of highest scores.
a Section not adequately stained.
b No invasive cancer within section.

which both results of a pair were concordant with reference to the cut-off level.

Fifteen comparisons were thus made for each of the 21 tumours. In these paired comparisons the mean proportion of concordant results was 59.0% (95% CI (55.3:62.8)). The result was similar if the upper tertile (101.3/200XHPF) was chosen as a cut-off level, 64.7% (95% CI (60.0:69.5)). This example shows that more than one third of the dichotomised estimates will change from high to low or from low to high if the analysis is made on a second section from another part of the tumour.

The results of this analysis did not change if the two most peripheral sections (A and F) were omitted.

4. Discussion

This study demonstrates that there is a marked intratumoral variation of neovascularisation. In the nested ANOVA analysis, the variation between different sections contributed more to the total variance than the variation between different tumours. This means that there is a risk that a result of a hot spot MVD assessment is more influenced by the sampling procedure than the true angiogenic capacity of the tumour.

The influence of intratumoral heterogeneity on the results has been addressed in previous studies but, as noted below, there has been no standardised way of designing or reporting studies on heterogeneity. In a study using a single section from 220 breast tumours, Axelsson and co-writers [15] could not find any prognostic value of MVD. They drew the conclusion that this was mainly due to variability from field to field within the same section. The difference between average score and maximum score exceeded 20% in 49% of the cases. The corresponding figure in our study was a difference of more than 20% in 19% of the sections ($n = 115$). In contrast to the study by Axelsson and colleagues our study investigated the variability between multiple sections from each tumour which was shown to be even more pronounced than the variation within each section. A finding that is in agreement with a Dutch study [22], in which all of the available blocks (2–4) from 10 breast cancers were used. Their data showed that the variation between blocks contributed more to the total variation than the variation between tumours which is identical to our results. The CVs for different blocks from the same tumour in the Dutch study ranged from 5.7 to 54.9%. Although the sampling technique was not identical to our study, the corresponding range of CVs in our material, based on twice as many cases, is quite similar (10.9–45.9%).

Bosari and co-workers [7] examined all available blocks (on average three) from 120 tumours. The three highest scores from all slides were recorded. They noted that heterogeneity is a potential pitfall, in 14% of the cases the difference between the average and the maximum score was more than 20%. However, it is unclear if this moderate variation was predominantly the result of intra- or intersection heterogeneity.

Another important methodological study on 40 primary breast cancers [19] concentrated on the reproducibility of different counting procedures. Hansen and co-workers found that 78% of the total variance was due to biological variation between different tumours, whereas 22% was due to methodological and intratumoral variation. Since intertumoural variation was the major contributor to the total variance, regardless of the counting method, Hansen and co-workers supported the continued use of MVD in future prognostic studies. However, the relevance of these findings is unclear since the Danish group selected 40 archival tumours in order to obtain varying degrees of MVD, which could lead to an overestimation of the intertumoral variation. Moreover, their estimation of a moderate degree of intratumoral heterogeneity is based on one section per case. The majority of studies utilising sections from more than one block [14,22], including the present study, have demonstrated a high degree of intratumoral variation. This supports the view that intratumoral heterogeneity is the most reasonable

explanation to account for the conflicting data on the usefulness of MVD as a prognostic factor.

It has been argued that the probability of finding the hot spots can be increased by counting the 10 apparently highest fields [23], this suggestion seems to be supported by our findings. The problem with this approach is that if one counts a large number of apparent hot spots it is likely that one will end up with a high score in most tumours. In our material, all tumours had at least one score above the median MVD of all 115 sections.

In our study, more than one-third of the tumours had a discordant classification with reference to the cut-off levels if two sections from different parts of the tumours were compared. The reproducibility of assignment to high and low MVD groups when sampling is done from different blocks has been addressed before [14,23]. Van Hoef and co-workers [14] retrospectively analysed MVD in 93 breast cancers, without finding any independent prognostic information. In 41 of the tumours, MVD was assessed in two sections from two different blocks. This comparison showed a concordance with reference to MVD categories of 71–78%. Although their figures are slightly higher than ours, the authors concluded that this moderate degree of concordance could introduce a substantial error in the method. Another English study [23] included an analysis of three different blocks from each tumour. Since 85% of the patients were correctly assigned to the high MVD and low MVD groups, regardless of whether one or three sections were taken into account, they drew the conclusion that the MVD measured in one section is representative of whole tumour vascularity. Even if the figure presented by Martin and co-workers is somewhat better than those presented by Van Hoef and co-authors and by ourselves, it is still obvious that intratumoral differences between different parts of the tumours constitutes a major source of error when tumours are grouped according to a cut-off level. Moreover, the introduction of a cut-off level and thereby dichotomising a continuous variable is associated with a loss of information. In addition, the selection of an unbiased cut-off level can be problematic [24]. Nevertheless, most published studies on angiogenesis as a prognostic factor in breast cancer present dichotomised data and this is the main reason why we did an analysis of the concordance with reference to high and low MVD classification of different sections from the same tumour in this study.

One problem in our study was the failure to stain all sections satisfactorily. Altogether there were eight sections (6.3%) that were judged as inadequate (Table 2). Up to 13% of inadequate stainings using antibodies against CD31 have been reported [5]. Factor VIIIRag is probably somewhat more reliable with inadequate stainings in the range of 1–4% [5,7]. One article also reported the CD34 antibody to be more reliable compared with CD31 [5] whereas another group preferred the use of CD31 [3]. Thus, which antibody to use is still under debate.

Although this study deals with some of the problems associated with assessment of tumour vascularisation, there is clear evidence that angiogenesis is a necessary step in the progression of solid tumours [25]. It has also been demonstrated that specific angiogenesis inhibitors can cause tumour regression [26] and potentiate other cancer therapies [27]. These facts suggest that assessment of neovascularisation in primary tumours should have the potential to become a useful prognostic factor. Other approaches such as analyses of angiogenic peptides [28–30] deserves further investigation.

In conclusion, the results of this study demonstrate that the most widespread method of assessment, MVD in hot spots, is questionable due to considerable intratumoral heterogeneity. The methods of assessing angiogenesis in tumours must be improved before they can be implemented in routine pathology.

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