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Breast carcinoma vascularity: A comparison of manual microvessel count and Chalkley count

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Summary. Manual counting of microvessels as intratumoral microvessel density (MVD) and Chalkley counting have been used in several studies to assess the prognostic impact of vascularity in invasive breast carcinomas. In our present study, the aim was to evaluate the prognostic value of angiogenesis in invasive breast carcinoma assessed by MVD and Chalkley techniques in the same series of patients. A total of 498 breast carcinoma patients with median follow up time 85 months were evaluated. The tumour vascularity was quantified by both manual microvessel count (MVD) and Chalkley count in CD34 stained breast carcinoma slides by a single investigator blinded to clinical information. Other relevant clinicopathological parameters were noted, including breast cancer related death and both loco-regional and systemic relapse. The patients were stratified by converting MVD and Chalkley counts to categorical variables to assess prognostic impact, and results were compared. High vascular grades using MVD count did not demonstrate any prognostic significance for breast cancer specific survival (BCSS) or distant disease free survival (DDFS) either in whole patient group (BCSS, p=0.517, DDFS, p=0.301) or in non-treated node negative patients (p>0.05). Chalkley count showed prognostic significance for both DDFS and BCSS in whole patient group (p<0.001) and also in untreated node negative patient group (p<0.05). In multivariate analysis, Chalkley count, but not MVD, retained the prognostic value for BCSS (p=0.007) and DDFS (p=0.014). The Chalkley count for assessing angiogenesis in invasive breast carcinomas demonstrated prognostic value. The Chalkley method appears to be the better method in estimating the prognostic impact of vascularity in invasive breast carcinomas.

Key words: MVD, Chalkley, Breast, Carcinoma, Vascularity

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Introduction

Angiogenesis is important for tumour growth and progression. Beyond a certain size, tumours need formation of new vessels to continue growth (Folkman, 1990). Tumour vascularity in invasive breast carcinoma has been extensively investigated in relation to its prognostic significance. Different methods, such as visual vascular grading, manual counting of microvessels in defined microscopic field areas known as microvessel density (MVD), and the Chalkley counting method, have been applied to quantify tumour vascularity after immunostaining for different types of endothelial markers, like FVIII, CD31 and CD34 (Fox and Harris, 2004; Uzzan et al., 2004).

Manual counting of microvessels in the most vascularized areas of the tumour (hot spots) as MVD reported by Weidner et al. (1991, 1992) has been widely used in evaluation of angiogenesis in invasive breast carcinomas (Uzzan et al., 2004). In several reports, MVD has been shown to be of prognostic significance (Weidner et al., 1991, 1992; Bosari et al., 1992; Gasparini et al., 1994, 1998; Toi et al., 1995; Martin et al., 1997; Heimann et al., 1998; de Jong et al., 2000). In other publications, it did not have any impact on the prognosis (Axelsson et al., 1995; Goulding et al., 1995; Clahsen et al., 1998; Fridman et al., 2000; Guidi et al., 2000; Vincent-Salomon et al., 2001; Goffin et al., 2003). The MVD method is, to a certain degree, observer

dependent and subjective (Hansen et al., 1998) and various field sizes and adaptations of the method have been applied (Uzzan et al., 2004). The Chalkley counting method uses an eyepiece graticule for the assessment of vascularity and estimates a relative area of microvessels (Fox et al., 1995a). The Chalkley eyepiece graticule is a circle containing randomly placed black dots. The circle is applied onto a hotspot (a highly vascularized area under low power scanning) and rotated to allow a maximum number of dots to be on or within the vascular profiles (Fox et al., 1994). The prognostic significance of Chalkley estimates of breast cancer vascularity has been reported (Fox et al., 1994, 1995a,b; Hansen et al., 2000a, 2004). This method is reported to be quick, feasible and less subjective compared to other techniques (Fox et al., 1995a; Hansen et al., 2004).

Most of the studies in breast cancer angiogenesis have used one of these methods of quantification and evaluated the prognostic value of vascularity in breast carcinoma (Uzzan et al., 2004). Variability and reproducibility of MVD and Chalkley methods have been extensively studied (Hansen et al., 1998). However, only few reports have compared the prognostic value of MVD and Chalkley counting methods in the same series of breast carcinoma patients (Fox et al., 2000; Offersen et al., 2003; Hansen et al., 2004). Thus, the aim of this study was to further elucidate the relation between MVD and Chalkley methods by evaluating their clinical impact, examining a series of breast carcinomas.

Materials and methods

Patients and tumours

We have examined 498 primary breast carcinomas for which paraffin blocks with adequate tumour tissue were available from the 920 patients enrolled in the Oslo Breast Cancer Micrometastasis Project from 1995 to 1998. The relationship between disseminated tumour cells in bone marrow, clinico-pathological parameters and its prognostic significance has been reported previously (Naume et al., 2001, 2004; Wiedswang et al., 2003). Relevant clinico- pathological data were obtained from the database of the Oslo Micrometastasis Study of this series of patients. Chalkley estimates of vascularity and its relationship with disseminated tumour cells in bone marrow, clinical outcome and other parameters have been reported earlier (Dhakal et al., 2008). Clinico-pathological characteristics of patients are given in Table 1 (Modified from Table 1 in earlier report) (Dhakal et al., 2008).

During the follow-up period, ranging from 1 to 125 months (median 85 months), 112 of 491 (22.8%) patients with available information for systemic relapse experienced distant metastases and 50 (10%) patients had local recurrences. Four hundred and seventy six patients had available relapse follow-up time for distant disease free survival (DDFS) calculations. Eighty six of the 498 patients (17.3%) died of breast cancer disease during the same period. In 457 cases, where we had available information about surgical treatment, 326 (71%) had breast conservation surgery and 131 (29%) had modified radical mastectomy. Of the 478 patients with information about non-surgical treatment, 224 (47%) had received radiation therapy and 254 (53%)

Table 1. Clinico-pathological characteristics of patients (n=498).

Characteristics	Number (n=498)	Percentage
Necrosis		
Presence	40	8.5
Absence	458	91.5
Histologic types		
IDC	356	71.5
ILC	93	18.7
Others*	49	9.8
Histologic grade		
I	108	21.7
II	251	50.4
III	139	27.9
ER		
Positive	381	76.5
Negative	117	23.5
PgR		
Positive	288	57.8
Negative	210	42.2
LN status		
N0	308	63.0
N+‡	180	37.0
Inflammation		
Minimal/mild	401	80.5
Moderate/marked	97	19.5
Tumour stroma relation		
Tumour>stroma	130	26.1
Tumour<stroma	368	73.9
p53 expression		
Positive	114	22.9
Negative	383	77.1
Tumour status		
T1	271	54.4
T2	190	38.2
T3-4†	25	5.0
TX	12	2.4
Vascular invasion		
Presence	108	21.7
Absence	390	78.3
HR status§		
Positive	404	81.1
Negative	94	18.9
c-erbB- 2 status		
Positive	32	6.5
Negative	462	93.5

IDC, infiltrating ductal carcinoma; ILC, invasive lobular carcinoma; BM, bone marrow; HR, hormone receptor; ER, estrogen receptor; PgR, progesterone receptor; LN, lymph node status.

§ HR status positive: ER and/or PgR positive; HR status negative: ER and PgR negative. *Other subtypes include mucinous, neuroendocrine, medullary and mixed carcinomas. ‡: Number of pN1=114, pN2=47 and pN3=19. †: T3=23 and T4=2.

post operative systemic adjuvant therapy (chemotherapy and/or tamoxifen) according to the Norwegian guidelines. The standard adjuvant chemotherapy regimen consisted of six cycles, every three weeks, of intravenous cyclophosphamide 600 mg/m^2, methotrexate 40mg/m^2, and fluorouracil 600 mg/ m^2. The patients who had received preoperative chemotherapy or who had metastases at the time of diagnosis or within 1 month after operation were not included in the present study.

Morphology

The primary tumours were classified according to the WHO recommendations (Ellis et al., 2003) and graded according to Elston and Ellis (1991). The presence of vascular invasion was recorded from the H&E slides. Tumour size was extracted from the original pathology report. The presence of necrosis in the tumour was noted. Inflammatory cell infiltrates were subjectively graded into two categories as minimal/mild and moderate /marked. The relationship between tumour cell mass and tumour stroma (tumour/stroma ratio) was subjectively classified into two categories; tumour cells more than tumour stroma, and tumour cells less than tumour stroma.

Immunohistochemistry

Four micrometer thick sections with adequate tumour tissue were cut from the formalin-fixed paraffin-embedded blocks and immunostained as follows (Dhakal et al., 2008). Deparaffinized sections were microwaved in Tris/EDTA pH 9.0 to unmask the epitopes, followed by treatment with 0.03% hydrogen peroxide (H_2O_2) for 5 minutes to block endogeneous peroxidase. The sections were incubated with the monoclonal murine antibody (IgG1) QBEND-10 (Monosan, Netherlands) against CD34 in 1:200 dilution for 30 minutes at room temperature, then with peroxidase labelled polymer conjugated to goat antimouse antibody for 30 minutes, and finally with 3-3'- diaminobenzidine tetrahydrochloride for 10 minutes (Dako EnVision™ + System Peroxidase (DAB) (K4007, DakoCytomation, CA, USA)). The sections were counterstained with haematoxylin. The immunostaining was performed by Dako Autostainer. Appropriate positive and negative controls were included in the series.

Vascular quantification

MVD method

MVD was estimated using a light microscope (Nikon, Eclipse E400) by counting the microvessels at 200 magnification as reported by Weidner et al. (1991, 1992). The immunostained sections for CD34 were carefully scanned at low power (at 40 and 100 magnifications), and then the three areas, considered to have the highest vascularity in the section (hotspots) were selected. Microvessels were counted manually in a single microscopic field in each hotspot at 200 magnification (high power field) as MVD score. In the present study, one high power microscopic field used for MVD count had an area of 0.916 mm^2. The highest MVD score among the three hotspot counts was used for further analyses. As in previous studies (Weidner et al., 1991, 1992), any CD34 stained endothelial cells or clusters of endothelial cells with or without lumen lying separately from adjacent vessel profiles were counted as a vessel.

Chalkley method

Chalkley counting procedure was done according to the proposal from a recent international consensus meeting (Vermeulen et al., 2002). The counting procedure, the reproducibility and the prognostic value of this method have been reported (Fox et al., 1994, 1995a,b; Hansen et al., 1998, 2000a; Dhakal et al., 2008). A selection of hotspots was made in the same way as described for manual MVD counting. Then, a 25 point Chalkley eyepiece graticule was applied to each hotspot area at 200 magnification with a Chalkley grid area of 0.1886 mm^2 (Nikon microscope, Eclipse E400). The dots in the graticule hitting any stained vessel or endothelial cell were counted in each hotspot selected. The highest score of the three hotspot counts was used for further analyses.
Counts were performed in areas of invasive carcinoma, including tumour margin. Sclerotic and necrotic areas were avoided. Both the MVD and Chalkley counts were performed as separate procedures on the same occasion. All Chalkley and MVD counts were performed by the same observer without knowledge of clinical data, bone marrow status or patient's prognostic outcome.

Statistical analysis

We used a predetermined cut-off (≥ 7) for Chalkley counts to categorize the patients into high and low vascular groups as reported earlier from this series (Dhakal et al., 2008). The predetermined cut-off was the 67th percentile (high tertile) value, for both Chalkley counts and MVD counts to analyse their prognostic values and relationship with other tumour characteristics. This cut-off was based on an earlier report (Fox et al., 1995a) which showed the high tertile or 67th percentile of Chalkley counts is of prognostic significance in breast carcinoma, compared to another two tertiles. For comparison, we preselected the 67th percentile (high tertile) also for MVD, represented by the cut-off value ≥ 87 vessels per high power field.

We also used tertiles and binary variable with median value as cut-offs for MVD and Chalkley counts for comparison of the prognostic significance. Chalkley count had discrete absolute values with a narrow range. The Chalkley tertile cut offs used were <5, 5-7 and ≥ 7.

The number of patients in Chalkley tertiles is unequal with around 40% in high tertile, 34% in the middle and 26% in lower tertile groups. MVD had a wider range of absolute values. Despite the discrete nature of the MVD counts, the number of patients in each tertile group is almost the same.

Correlation between MVD and Chalkley count was tested by Pearson's r and scatter plotting as continuous variables. Univariate survival estimation was performed by Kaplan-Meier analysis. P values were computed by log rank test. The primary end point for the survival analysis was breast cancer specific survival (BCSS) measured from the date of surgery to breast cancer related death, or otherwise censured at the time of the last follow up visit or at non cancer related death. Secondary end points were time to loco -regional and systemic relapse and were measured in the same way. Metastases in the skeleton, liver, lungs, or CNS were recorded as systemic relapse. Kaplan-Meier survival curves for time to distant recurrences, and breast cancer specific deaths were constructed. Cox proportional hazard regression was used for multivariate (stepwise backward elimination) analyses of prognostic impact of relevant variables. In the multivariate analyses, the number of parameters was restricted to approximately 10% of the number of events (systemic relapse/breast cancer related death) in population analyzed. The Pearson's Chi squared test was used to compare MVD, Chalkley count and clinico-pathological variables. For statistical analyses, the SPSS software was used.

Results

Patient and tumour characteristics

The patient and tumour characteristics are presented in Table-1 and have also previously been reported (Dhakal et al., 2008).

MVD, Chalkley count and relation with different parameters

The MVD ranged from 14 to 319 vessels/0.916 mm^2 (at 200 magnification; median 73, mean 81.1, SD 38.9). The tertile cut-offs were at 60 and 87 vessels. Of 498 cases, 331 (66.5%) were with low vascularity, and 167 (33.5%)) with high vascularity, when dichotomized by ≥ 87 vessels. The Chalkley counts ranged from 2 to 13 (median 6, mean 6.02, SD 1.97). Two hundred and one (40%) were with high vascularity and 297 (60%) with low vascularity when dichotomized by predetermined value ≥ 7. Table 2 shows the comparison between MVD and Chalkley count in relation to patient- and tumour characteristics, as also previously reported for Chalkley count (Dhakal et al., 2008). The MVD was associated with vascular invasion (p=0.043, Chi squared), and tumour cell mass>tumour stroma (p<0.001). The high Chalkley count was associated with hormone receptor status, p53, vascular invasion, presence of necrosis, presence of moderate to marked chronic inflammatory cell infiltrate, histologic grade, histologic type (IDC vs. non-IDC), pT status and p53 expression (p<0.001 to p≤ 0.006, Chi square). A correlation between MVD and Chalkley count was detected (Pearson's r=0.40, p<0.001). However, the results also show variability between the two methods in the estimation of vascularity, as shown in the scatter plot in Figure 1.

Some cases showed different results when the MVD and Chalkley methods were applied. In Figure 2, immunohistochemical (IHC) patterns of the MVD and Chalkley categorisation are presented. Figure 2C is an

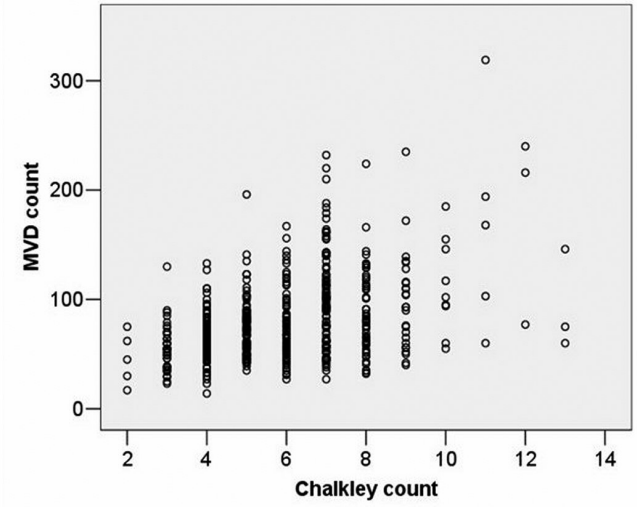

Fig. 1. Scatter plot of MVD and Chalkley counts showing fair correlation (r=0.40, p<0.001).

Table 2. Association of MVD and Chalkley count with other clinico-pathological variables (n=498).

Variables	MVD	Chalkley count
Histological type ‡	0.936	<0.001
LN status §	0.886	0.281
Histologic grade	0.262	<0.001
pTumour status	0.524	<0.001
Necrosis	0.366	<0.001
Inflammation	0.91	0.003
Tumour cells/tumour stroma ratio	<0.001	<0.001
HR status	0.72	<0.001
P53 status	0.543	0.005
c-erbB-2	0.752	0.257
PgR status	0.492	<0.001
ER status	0.617	<0.001
Vascular invasion	0.043	0.006
BM status	0.492	0.127

BM, bone marrow; HR, hormone receptor; ER, estrogen receptor; PgR, progesterone receptor; LN, lymph node status; MVD, microvessel density. The association between tumour vascularity and other clinicopathological variables were tested by chi square tests. ‡: *P* calculated as ductal carcinoma versus lobular and other histologic types §: *P* calculated as node negative versus node positive cases.

example of low MVD/high Chalkley group. Here, microvessels are in close proximity, branching and interconnected. This gives relatively low MVD count and high Chalkley count. In this group, apart from above example, vascular pattern that could give high Chalkley count and low MVD ranged from relatively larger sized vessels to focal proliferation with close proximity of microvessels. Both MVD and Chalkley counts are low in Figure 2A whereas both are high in Figure 2D. An example of low Chalkley count and high MVD is presented in Figure 2B.

We also observed that high MVD/low Chalkley group was seen in 16% of invasive lobular carcinomas (ILC) compared to 10% of infiltrating ductal carcinomas (IDC) whereas low MVD/ high Chalkley and high MVD/high Chalkley groups were seen in higher percentage of IDC (22.5% and 23.6%) compared to ILC (5.3% and 14%). Low MVD/ low Chalkley group was in higher percentage among ILC (64.5%) compared to IDC (44.1%).

Survival analyses

Kaplan-Meier survival probabilities of Chalkley count (Fig. 3C,D) showed significantly reduced breast cancer specific survival and distant disease free survival (DDFS, $p<0.001$; BCSS, $p<0.001$; log-rank) for dichotomized high, as compared to low vascular groups. Node negative patients not receiving systemic adjuvant therapy were separately analysed, and showed reduced BCSS ($p=0.037$, log-rank) and DDFS ($p=0.004$, log-rank) (Dhakal et al., 2008). On the other hand, MVD, as dichotomized variable by 67th percentile cut-off, did not demonstrate any prognostic significance for all patients

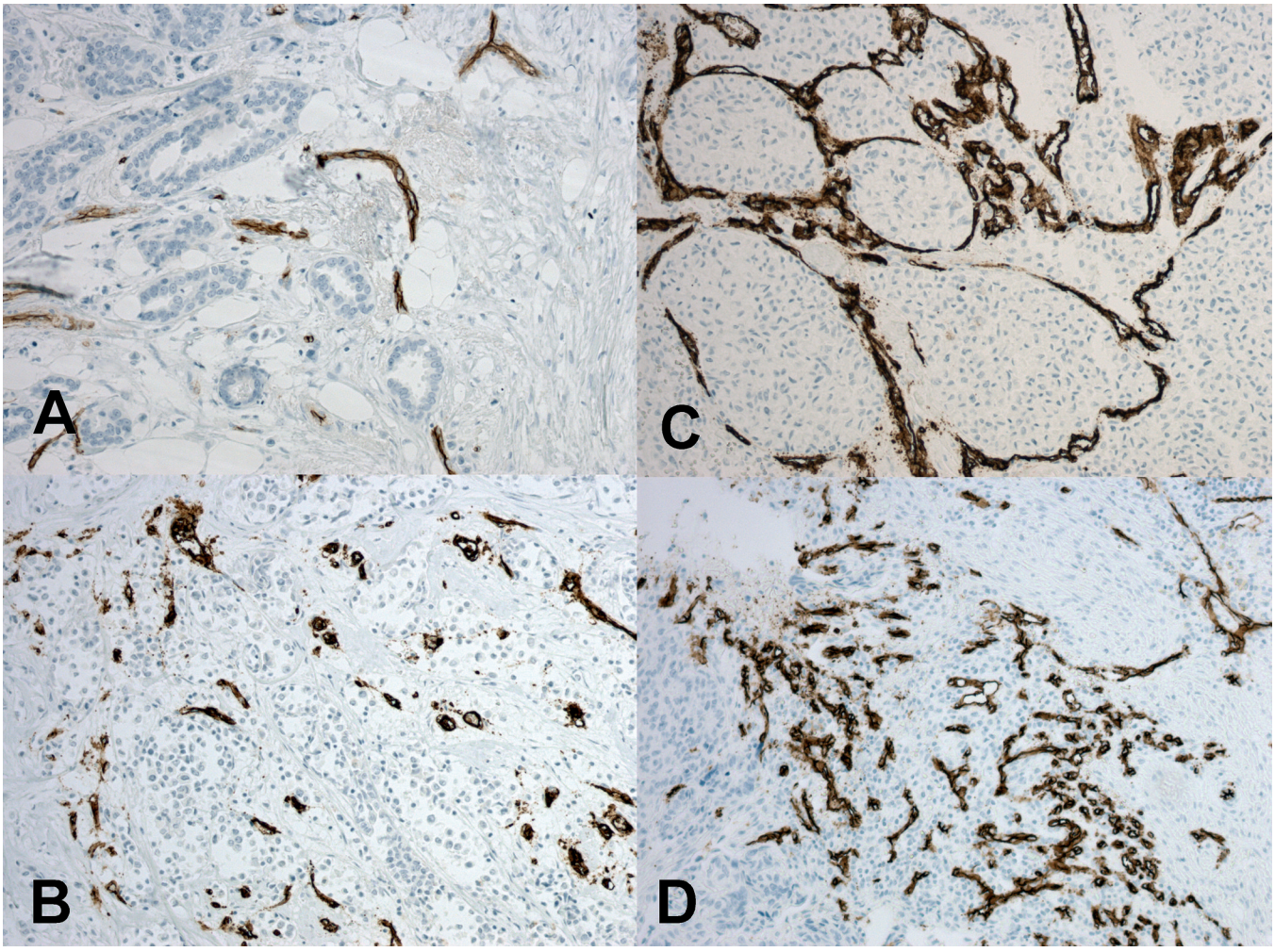

Fig. 2. Microphotographs of breast carcinomas stained for CD34 and measured using both MVD and Chalkley methods with low MVD/ low Chalkley **(A)**, high MVD/low Chalkley **(B)**, low MVD/high Chalkley **(C)** and high MVD/ high Chalkley **(D)** categorizations. All images were taken by a Leica DFC-320 digital camera at 10 magnification with a Plan-neofluar 10x/0.30 objective lens in Axiophot microscope (Zeiss Germany).

(DDFS, p=0.499; BCSS, p=0.435 log-rank) (Fig. 3 and Tables 3, 4), nor for the untreated node negative cases (BCSS, p=0.894; DDFS, p=0.744). We examined prognostic significance of MVD by dichotomizing by median value. This also did not show prognostic significance [except a trend for shorter DDFS (p=0.058, log-rank) in non treated pN0 group]. We further examined the prognostic significance of Chalkley count and MVD by categorizing into tertiles. Chalkley count tertiles showed prognostic significance in univariate survival analysis whereas MVD tertiles did not (Tables 3, 4).

Other variables that showed significant prognostic value for BCSS in univariate analysis, included in multivariate analysis, were nodal status, vascular invasion, pT status, histologic grade, BM status, HR status (all with p<0.001; log rank), tumour necrosis, and inflammatory infiltrates (p<0.05; log-rank). P53 and c-erbB2 were not included in multivariate analysis due to statistical restriction (Dhakal et al., 2008). However, p53 and c-erbB2 did not reach statistical significance when tested in multivariate analysis in the entire cohort of patients (Wiedswang et al., 2003). MVD and Chalkley count were introduced separately into the multivariate models. Systemic therapy status was also included.

In multivariate analysis (Tables 3, 4), the Chalkley

Table 3. Distant disease free survival (DDFS) in relation to MVD and Chalkley count.

	MVD				Chalkley count			
	Univariate analysis (log-rank)	Multivariate analysis (n=432) (Cox regression)			Univariate analysis (log-rank)	Multivariate analysis (n=432) (Cox regression)		
	P value	*P* value	RR	CI	*P* value	*P* value	RR	CI
Binary variable: preselected cut off ‡	0.499	0.708	1.086	0.706-1.669	<0.001	0.014	1.715,	1.113-2.64
Binary variable: Median cut off	0.301	0.869	1.034	0.697-1.534	0.006	0.939	0.982	0.616-1.566
Tertiles	0.339	0.919			<0.001	0.03		
Low			1				1	
Middle		0.864	0.956	0.570-1.603		0.226	0.673	0.354-1.277
High		0.822	1.060	0.637-1.763		0.309	1.344	0.760-2.376

P value ≤ 0.05 is taken as significant and >0.05 non-significant. ‡: Chalkley count results as reported previously (Dhakal et al., 2008). RR, relative risk; CI, confidence interval; MVD, microvessel density; DDFS, distant disease free survival. Note: Multivariate analysis by Cox regression performed for DDFS included histologic grades, vascular invasion, inflammation, bone marrow status(BM), necrosis, pT status, pN status, hormone receptor status, systemic adjuvant therapy and MVD or Chalkley count in the multivariate models. All three types of categorizations of MVD and Chalkley count as shown in the table were tested separately in both univariate and multivariate analyses. Only findings of vascular categorizations are presented in the table for the comparison of prognostic impact of MVD and Chalkley count. Other variables retaining the prognostic significance in the multivariate analysis are histologic grades, pN status, vascular invasion, BM status and hormone receptor status. Also, high tertile of Chalkley count showed prognostic significance compared to middle tertile (data not shown).

Table 4. Breast cancer specific survival (BCSS) in relation to MVD and Chalkley count.

	MVD				Chalkley count			
	Univariate analysis (log-rank)	Multivariate analysis (n=451) (Cox regression)			Univariate analysis (log-rank)	Multivariate analysis (n=451) (Cox regression)		
	P	*P*	RR	CI	*P* value	*P* value	RR	CI
Binary variable: preselected cut off ‡	0.435	0.885	1.036	0.639-1.661	<0.001	0.007	2.064	1.218-3.496
Binary variable: Median cut off	0.517	0.931	0.980	0.627-1.533	<0.001	0.513	1.214	0.680-2.167
Tertiles	0.438	0.977			<0.001	0.023		
Low			1				1	
Middle		0.875	0.954	0.533-1.708		0.481	0.745	0.329-1.689
High		0.968	1.012	0.573-1.786		0.148	1.709	0.877-3.533

P value ≤ 0.05 is taken as significant and >0.05 non-significant. ‡: Chalkley count results as reported previously (Dhakal et al., 2008). RR, relative risk; CI, confidence interval; BCSS, breast cancer specific survival; MVD, microvessel density. Note: Multivariate analysis for BCSS by Cox regression was performed that included histologic grades, vascular invasion, bone marrow status (BM status), inflammation, necrosis, pT status, pN status, hormone receptor status, systemic therapy and MVD or Chalkley counts in the models. All three types of categorizations of MVD and Chalkley count as shown in the table were tested separately in both univariate and multivariate analyses. Only the findings of vascular categorizations have been presented in the table for the comparison of prognostic impact of MVD and Chalkley count. Other variables in the models retaining prognostic significance in the multivariate analysis for BCSS were vascular invasion, necrosis, pN status, pT status, vascular invasion, BM status and hormone receptor status. Also, high tertile of Chalkley count showed prognostic significance compared to middle tertile (data not shown).

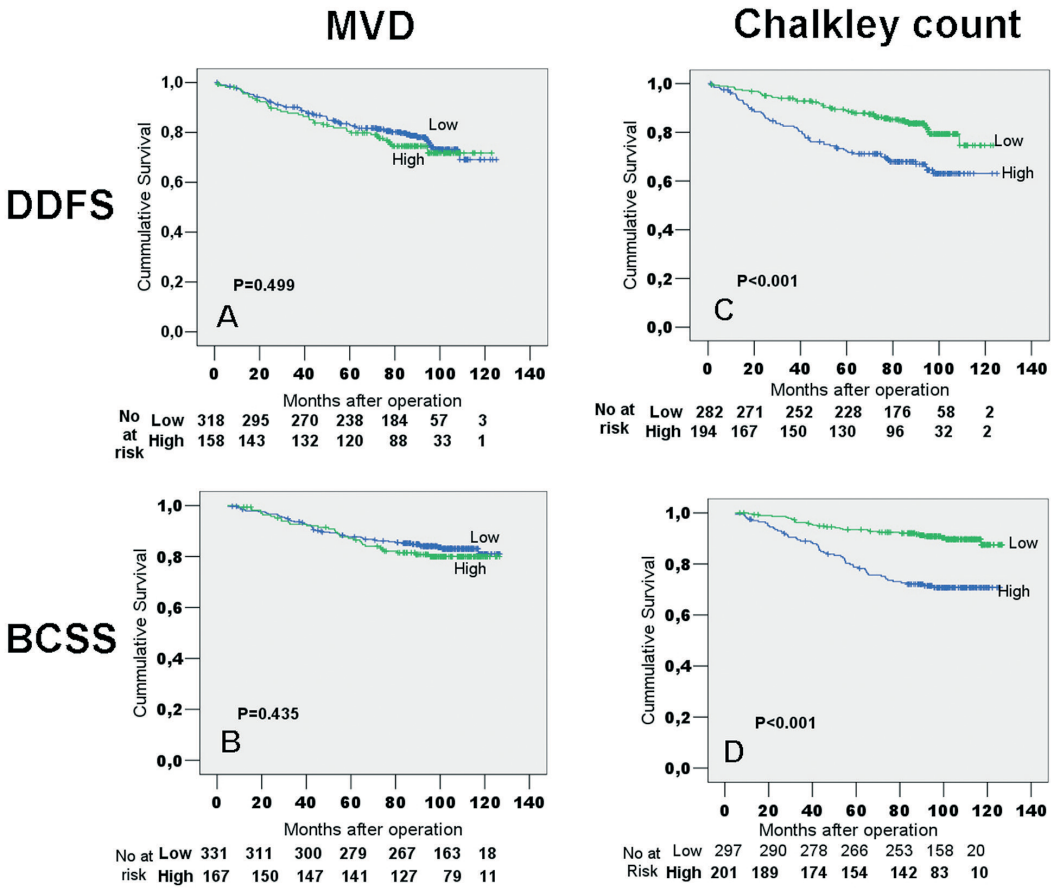

Fig. 3. Kaplan-Meier survival curves for both MVD and Chalkley counts as binary variables stratified in high and low vascular groups with cut - off 67th percentile (high tertile). **A.** Distant disease free survival (DDFS) for MVD. **B.** Breast cancer specific survival (BCSS) for MVD. **C.** DDFS for Chalkley count. **D**, BCSS for Chalkley count. Fig 3C and Fig 3D are modified from Fig 1A and 1E in an earlier report (Dhakal et al., 2008).

count retained the prognostic significance for breast cancer specific survival (BCSS) and DDFS when markers showing prognostic significance in univariate analysis were included. MVD did not attain statistical significance either for DDFS or BCSS (Tables 3, 4).

We further examined the prognostic significance by combining MVD and Chalkley count into four categories as low MVD/low Chalkley, high MVD/low Chalkley, low MVD/high Chalkley and high MVD /high Chalkley to see their prognostic relationship with each other (Fig. 4). The prognostic significance of these combinations was tested by Kaplan-Meier survival analysis and showed prognostic significance for both BCSS and DDFS (BCSS, p<0.001, DDFS, p<0.001) (Fig. 4A,B). On pair-wise comparisons by log rank test, both high MVD/high Chalkley and low MVD/ high Chalkley groups demonstrated significantly reduced survivals compared to low MVD/low Chalkley and high MVD/low Chalkley combinations [BCSS: high MVD/high Chalkley & low MVD/ high Chalkley versus low MVD/low Chalkley (p<0.001) & high MVD/low Chalkley (p=0.029 and 0.002) and DDFS: high MVD/high Chalkley (p=0.007), low MVD/high Chalkley (p<0.001) compared to low MVD/low Chalkley, and low MVD/high Chalkley versus high MVD/low Chalkley (p=0.014)]. However, the difference between low MVD/ high Chalkley and high MVD/high Chalkley did not reach statistical significance (BCSS, p=0.15 & DDFS, p=0.273).

Discussion

The prognostic significance of angiogenesis in invasive breast carcinoma has been demonstrated in several studies (Weidner et al., 1991, 1992; Gasparini et al., 1994; Fox et al., 1995a; Hansen et al., 2000a). Different methods for the estimation of tumour vascularity have been applied. MVD, Chalkley method and subjective visual grading of the tumour vascularity have been used to stratify invasive breast carcinoma patients in different prognostic groups (Weidner et al., 1991; Fox et al., 1995a; Hansen et al., 2000b; Fox and Harris, 2004).

A subjective selection of hotspots (areas with relatively intense vascularity in the tumour section under low power) followed by manual counting of microvessels per high power field as MVD estimate (Weidner et al., 1992) or counting of graticule spots

hitting the stained microvessels under higher power as Chalkley count (Fox et al., 1995a,b) has been most widely used. Shortcomings and limitations of these methods have been reported (Hansen et al., 1998; Vermeulen et al., 2002; Fox and Harris, 2004).

MVD count has shown prognostic significance in invasive breast cancer patients in various studies (Weidner et al., 1991, 1992; Gasparini et al., 1994; Lipponen et al., 1994; Toi et al., 1995; Vermeulen et al., 1997; Martin et al., 1997; Gasparini et al., 1998; de Jong et al., 2000; Koukourakis et al., 2003; Tsutsui et al., 2005) while others did not find this relation (Van Hoef et al., 1993; Axelsson et al., 1995; Costello et al., 1995; Goulding et al., 1995; Fridman et al., 2000; Guidi et al., 2000; Vincent-Salomon et al., 2001; Ludovini et al., 2003; Goffin et al., 2003). In these studies, different magnifications, field sizes, and cut-off values were used. Although the hotspot selection was done in the same way in most of these studies, some methodological variations in MVD technique did exist.

Also, the Chalkley method has been used to assess the prognostic importance of angiogenesis in invasive breast carcinoma. In most of these studies, prognostic significance was demonstrated (Fox et al., 1994, 1995a,b; Hansen et al., 2000a, 2004 Offersen et al., 2002), but few reports showed negative results (Fox et al., 2000, 2001). Some of the studies using the Chalkley method for vascularity assessment applied the same cut-off value to stratify breast carcinoma patients into different prognostic groups. Despite various graticule sizes, it was reported to have prognostic significance (Fox et al., 1995a,b; Hansen et al., 2000a, 2004). Hansen et al reported methodological differences for both MVD and Chalkley methods, but with less observer variation with the Chalkley method. The Chalkley counting method is considered to be simple, quick and less subjective (Hansen et al., 1998).

In the present study, we applied both the MVD and the Chalkley counting methods in evaluating breast carcinoma angiogenesis in 498 patient samples. The MVD did not show association with most of the clinical and pathological variables and had no prognostic significance in our patient cohort (Tables 2-4). We also examined MVD using the median value to dichotomize and the tertiles. This did not demonstrate prognostic significance. On the other hand, Chalkley count showed a strong association with most of the clinico-pathological variables in our series, and turned out to be a strong prognostic indicator for both BCSS and DDFS in univariate analyses. All these results are in accordance with two previously published studies which have analysed for both MVD and Chalkley counts (Offersen et al., 2003; Hansen et al., 2004). The prognostic significance of Chalkley count was also retained in multivariate analysis, in line with the study of 330 breast carcinomas by Hansen et al. (2004). In the same study, MVD and Chalkley count were significantly associated with each other ($r=0.46$, $p<0.001$) similar to what has been found in our study (Pearson's $r=0.40$; $p<0.001$). Similar results were also reported by Offerson et al ($p<0.0001$, Pearson's $r=0.70$) (Offersen et al., 2003). No prognostic significance was demonstrated either by MVD or Chalkley count in another series of invasive breast carcinomas (Fox et al., 2000). The Chalkley counting and the manual microvessel counting were performed by different observers (Axelsson et al., 1995;

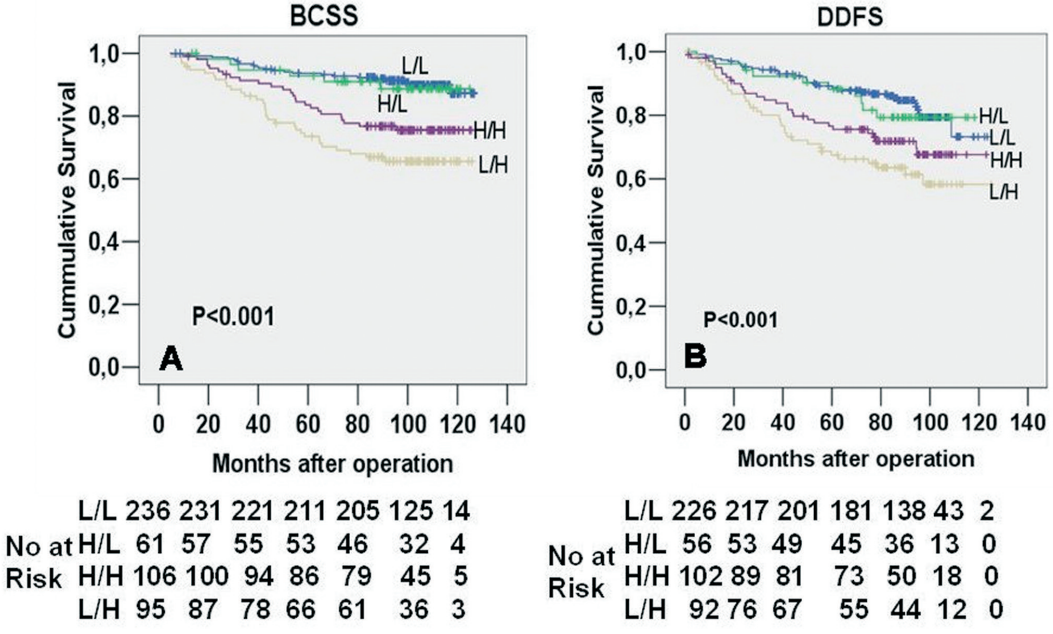

Fig. 4. Kaplan-Meier survival curves for both breast cancer specific survival (BCSS) and distant disease free survival (DDFS) plotted as combinations of MVD and Chalkley count into 4 groups as low MVD/low Chalkley count (L/L), high MVD/low Chalkley count (H/L), low MVD/high Chalkley count (L/H) and high MVD/high Chalkley count (H/H) for binary variables stratified by preselected cut-offs. **A.** Breast cancer specific survival (BCSS). **B.** Distant disease free survival (DDFS).

Fox et al., 2000), in contrast to this study.

By combining both the MVD and the Chalkley count, we demonstrated significantly reduced survivals for low MVD/high Chalkley and high MVD/high Chalkley groups of patients compared to the other two combinations (Fig. 4A,B). The high MVD/high Chalkley group similar to low MVD/high Chalkley group showed significantly reduced survivals compared to low MVD/low Chalkley and high MVD/low Chalkley groups. However, the difference between low MVD/high Chalkley and high MVD/high Chalkley did not reach statistical significance. Though high MVD alone did not show prognostic significance, high MVD in combination with high Chalkley count demonstrated prognostic significance. On the other hand, high Chalkley count with or without high MVD demonstrated prognostic significance. This indicates the role of microvessel size and vascular area on the prognostic impact of tumour vascularity in invasive breast carcinomas.

The reason for such different observations between MVD and Chalkley count in terms of prognostic value in breast carcinoma, despite the significant correlation between the counts by the two methods, is not clear. However, the variations inherent to methods may be an explanation for such discrepancies (Hansen et al., 1998; Offersen et al., 2003). MVD estimating vessel number per high power field has greater observer variability compared to Chalkley count (Hansen et al., 1998). We observed in the present study that larger vessel size could give higher Chalkley count, despite a comparatively lower number of the vessels counted, clearly shown in the scatter plot (Fig. 1). A similar observation was made in breast carcinoma by Hansen et al. (2004). MVD and Chalkley methods give counts on tumour vascularity in two different ways (Fox et al., 1995a; Offersen et al., 2003; Hansen et al., 2004). The Chalkley method estimates a relative vascular area, irrespective of number of vessels and is also easy to use and count (Fox et al., 1994, 1995a). Independent of the size, single stained endothelial cells or clusters of endothelial cells with or without lumen lying separately from each other are counted as a vessel in MVD method (Weidner et al., 1991, 1992). This might give rise to a higher counts. But possibly, microvessels or vascular area might have to reach a certain size or level and get connected to the general vascular system to take part in the process of tumour cell dissemination (Figs. 2, 4) as also reported by Hansen et al. (2004). This might explain why the prognostic value of the two methods differed, despite a significant correlation between the counts by the two methods. Furthermore, branching and closely connected microvessels (as illustrated in Fig. 2C), and relatively larger vessels as well as focal proliferation with closely lying vessels might have given high Chalkley count in combination with low MVD. Our findings are supported by Goffin et al, reporting that focal glomeruloid proliferation of microvessels has prognostic significance in breast carcinoma, while MVD count does not (Goffin et al., 2003).

In the present study, the field area for the manual microvessel count was about five times larger than the Chalkley graticule field area. Though investigators have found that the MVD count in smaller field area gives stronger prognostic impact compared to the count in a larger area (Vermeulen et al., 1997; de Jong et al., 2000), MVD count in field size as large as 1.56 mm^2 has been reported to be of prognostic significance (de Jong et al., 2000). Furthermore, MVD count in field areas of 0.63 mm^2 (Fridman et al., 2000) and 0.754 mm^2 (Hansen et al., 2004) did not demonstrate prognostic significance either. Microvessel counts (MVD) even in a smaller field area (0.25 mm^2), closer to the field size for Chalkley count, has not demonstrated prognostic significance compared to the Chalkley count (Offersen et al., 2003).

Our present observations support previous reports (Fox et al., 1995a,b; Hansen et al., 2000a, 2004) that the same and near the same Chalkley cut-off values could demonstrate prognostic significance despite the use of various magnifications, graticule field sizes, and score selection. For MVD, it is difficult to pre-select a cut-off in a similar way for routine use. This, combined with the observation that the Chalkley count provides better prognostic information than the MVD count, supports Chalkley count as the preferred method for estimating angiogenesis in breast carcinoma patients.

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