

Microvessel Density at Presentation Predicts Subsequent Muscle Invasion in Superficial Bladder Cancer

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ABSTRACT

Purpose: The purpose of this study was to determine whether angiogenesis, as measured by microvessel density (MVD), at presentation is related to subsequent progression of superficial bladder cancer (SBC).

Experimental Design: Archived primary bladder tumors from 180 patients were stained with a monoclonal antibody against cluster determinant 34 to label vessels. Image analysis was used to count MVD in 30 randomly selected areas in each case.

Results: Of the 170 patients evaluated, 37 progressed to muscle invasive disease. A strong association was found between the intensity of angiogenesis and clinical stage, pT1 tumors having a higher MVD than pTa disease. The median MVD was significantly higher at presentation in those patients that subsequently developed progressive SBC than in those that did not progress ($P < 0.0001$). pT1 ($P = 0.001$), grade 3 disease ($P = 0.002$), and MVD ($P = 0.008$) were found to predict subsequent disease progression on univariable analysis. Both MVD ($P = 0.007$) and pT1 disease ($P = 0.044$) remained significant predictive factors for subsequent disease progression on multivariable analysis.

Conclusion: MVD in SBC at presentation is significantly higher in those cases that subsequently progress to muscle invasive disease.

INTRODUCTION

Bladder cancer presents as superficial disease in 70–90% of cases (1). Although SBC² is confined to the mucosa (pTa disease) or subepithelial layer (pT1), between 15 and 30% of cases will progress to muscle invasive disease (2). It is not

possible at presentation to accurately predict subsequent invasion. However, the presence of pT1 disease, grade 3 morphology, and concomitant carcinoma *in situ* are all associated with a greater risk of progression (3).

As the majority of bladder malignancies present as superficial disease and only a small percentage of these progress, any method of determining which cases are at risk of subsequent progression would be valuable, particularly if it represented a potential target for therapy. In other solid tumors, such as those of the prostate (4), breast (5), colon (6), and lung (7), a proangiogenic phenotype, described by a high MVD, has been associated with a poor outcome. Angiogenesis, the formation of a new blood supply from an existing vasculature, is essential for tumor growth > 1–2 mm in diameter. Hence, a high MVD in SBC might reasonably be expected to be associated with an increased risk of progression. Although a high MVD has been associated with a poor outcome in invasive bladder cancer (8, 9) for some time, this association has never been satisfactorily shown in superficial disease.

It has been suggested that the methodology of MVD measurement is the reason for this apparent failure and that the commonly used techniques are inadequate either because of observer bias or incompatibility with SBC's distinct morphology (10). There have been many methods described to measure MVD in tissue sections. As the counting of vessels is time consuming and tedious, these have been directed at minimizing the amount of counting required to obtain an MVD value for a specimen. Sampling methods have therefore been devised, most famously, the "hot spot" method. These methods, however, require that a tissue has some homogeneity. The pathological specimens of SBC are particularly varied in histological architecture. This is attributable not only to the fronded, papillary type but also to the piecemeal specimens that are retrieved by transurethral resection of bladder tumors (11).

In this study, we applied not only a random counting technique but counted in multiple areas to reduce sampling error. To allow clinical feasibility, an image analysis system was used to determine whether angiogenesis in early disease is associated with later progression in SBC.

MATERIALS AND METHODS

A total of 180 cases of SBC was identified from computerized pathological records. Only cases where the tissue from the first resection was available and >12 months follow-up was recorded were included. Cases where stage progression was recorded at the second cystoscopic examination were not included, because histological under-staging at initial cystoscopy of these cases was assumed. First review cystoscopies were carried out at a median of 4 months after first resection (range 2–18 months). The median age of the patients in the study was 70 years (range 24–95). The group consisted of 140 males and

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² The abbreviations used are: SBC, superficial bladder cancer; BCG, *Bacillus Calmette-Guérin*; MVD, microvessel density.

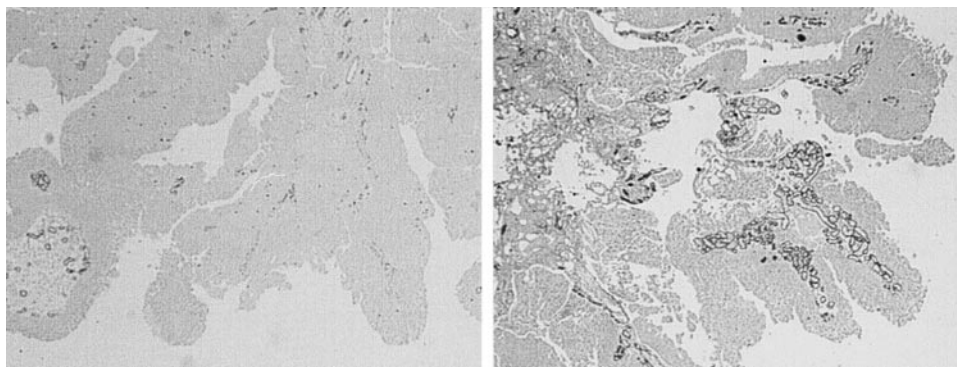


Fig. 1 SBC. Vessels stained with cluster determinant 34 and a light counter stain of hematoxylin. Magnification: $\times 20$. A, area of low MVD; B, area of high MVD.

40 females (ratio m:f = 3.5:1). The median follow-up was 57 months (range 12–280).

Validation of the method of vessel staining and computer image analysis has been described in detail previously (12). In summary, monoclonal antibody against cluster determinant 34, a surface molecule on endothelial cells, was used to identify microvessels using the avidin-biotin complex streptavidin biotin immunoperoxidase development method (DAKO). Sections (5 μ m) were cut from routinely fixed archived paraffin-embedded tissue and mounted on silane slides. The sections were deparaffinized and rehydrated, exposed to 6% hydrogen peroxide solution for 10 min to block endogenous peroxidase, and then washed in Tris-buffered saline followed by normal goat serum. Primary antibody was applied and incubated overnight at 4°C. After further washing in PBS, secondary linking antibody (reagent C) and then the streptavidin peroxidase complex (reagents A+B) were applied, each for 30 min, followed by washing in phosphate buffer. The chromogen 3,3-diaminobenzidinetetrahydrochloride was enhanced with nickel sulfate in 1 M acetate buffer. This was applied to the sections for 10 min and washed thoroughly with water. A very light counterstain (not >1-s exposure) of Mayer's hematoxylin was then applied.

Vessels were counted using an image analysis system specifically calibrated for this study. Images were captured using a JVC KYF50 3-chip color video camera connected to a Nikon microscope with a Scion cg-7 frame-grabber attached to an Apple Macintosh G-3 computer. A computer macro written within NIH Image (an image analysis software package) converted color images to grayscale and counted the number of vessels. The system was programmed to follow the same rules as manual counting. Any separate stained area was counted as a single vessel regardless of the presence of a lumen. Vessels touching the right or lower border were not counted.

Images were captured at a magnification of $\times 200$ (Fig. 1). As the nature of SBC specimens is fragmentary, the area of tissue in the field was measured, and the vessel count was expressed per square millimeter. To determine the number of areas to be counted in each section, the method of cumulative mean was used.

Statistical analysis was performed using SPSS version 10.

RESULTS

Of the 180 patients, 42 (23%) progressed to muscle invasive disease, and 138 (77%) did not progress. These two study

Table 1 Distribution of histological grade within stage type at presentation for all 180 patients

		Grade			Total
		1	2	3	
Stage	pTa	50	46	10	106
	pT1	7	37	30	74
Total		57	83	40	180

groups were comparable; the median age of those patients that progressed (71.5 years) did not significantly differ from those that did not progress (70 years; $P = 0.122$; Mann-Whitney), and neither did the gender distribution ($P = 0.158$; χ^2). The length of follow-up for the two groups was also similar, $P = 0.08$ (Mann-Whitney).

At presentation, 106 (59%) cases were pTa, and 74 (41%) cases were pT1. Fifty-seven (32%) were grade 1, 83 (46%) were grade 2, and 40 (22%) were grade 3. The distribution of histological grade within stage type is displayed in Table 1.

Of the 170 cases, 50 had intravesical chemotherapy, 23 had intravesical BCG immunotherapy, and 9 had both BCG and chemotherapy. The median time to recurrence was 15 months (range 2–90). The median time to progression was 34 months (range 7–144).

The number of random areas required to be counted to achieve <10% variation in cumulative mean ranged from 6 to 28 (median 16). Therefore, to ensure a representative sample was taken, a minimum of 30 random areas was counted in each case. However, in 10 cases, the tissue available was insufficient to count 30 random areas. The total number of areas available for counting in these cases ranged from 5 to 27 (median 17). The final sample available for analysis, counting 30 random areas in each case, included 37 (22%) cases that subsequently progressed to muscle invasive disease and 133 (78%) cases that did not, 170 cases in total.

The area of capture on the computer screen was 0.122 mm². The mean area of tissue measured per case ranged between 0.100 and 0.141 mm². The median MVD of all cases was 73.2 vessels/mm² (SD 42.71). The median MVD of those cases that progressed was 94.36 vessels/mm² (SD 41.24). The median MVD in those cases that did not progress was 68.78 vessels/mm² (SD 41.06). There was a statistically significant difference in MVD between the two groups ($P < 0.0001$; Mann-Whitney);

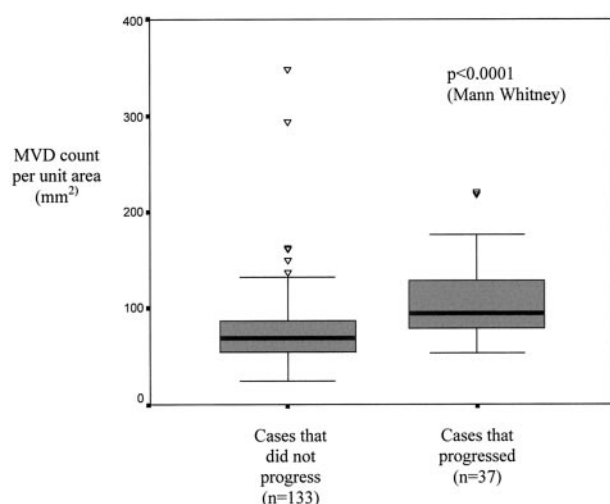


Fig. 2 Graph of MVD at presentation in SBC in cases that subsequently progressed and cases that did not progress.

Table 2 Univariable analysis using binary logistic regression

Factors at presentation predicting subsequent progression of superficial bladder cancer.

	Odds ratio	95% Confidence interval		Significance
Stage	3.940	1.814	8.557	$P = 0.001$
Grade	2.300	1.346	3.929	$P = 0.002$
MVD per mm^2	0.747	0.603	0.927	$P = 0.008$

Fig. 2). The highest vessel count per captured area measured in each case ranged from 9 to 88 (median 27). The highest vessel count in cases that progressed ranged from 17 to 75 (median 33). The highest vessel count in cases that did not progress ranged from 9 to 88 (median 26). There was a statistically significant difference between the maximum vessel count of the two groups ($P = 0.00014$; Mann-Whitney). Including the mean MVD from the 10 cases in which insufficient material was available to count 30 random areas did not reduce the significance of the result ($P < 0.0001$).

The median MVD in pT_a tumors at presentation was 65.61 vessels/ mm^2 and 85.45 vessels/ mm^2 in pT₁ tumors. There was a significant difference between these two groups ($P = 0.005$; Mann-Whitney). The median MVD in grade 1 tumors at presentation was 68.78 vessels/ mm^2 , 75.77 vessels/ mm^2 in grade 2, and 81.32 vessels/ mm^2 in grade 3. There was no significant difference between grades 1 and 2 ($P = 0.429$), 2 and 3 ($P = 0.415$), or 1 and 3 ($P = 0.19$).

For the 170 patient group, the following factors were found to significantly predict progression when analyzed using a univariate binary logistic regression model: (a) stage pT₁ disease ($P = 0.001$); (b) high grade ($P = 0.002$); and (c) MVD ($P = 0.008$; Table 2). However, in a multivariable analysis, only MVD and stage remained significant predictors of progression ($P = 0.007$ and 0.044 , respectively; Table 3). Treatment with intravesical chemotherapy or BCG was not a significant factor with regards to outcome.

Table 3 Multivariable analysis using binary logistic regression

Factors at presentation predicting subsequent progression of superficial bladder cancer.

	Odds ratio	95% Confidence interval		Significance
Stage	2.497	1.026	6.073	$P = 0.044$
Grade	1.689	0.909	3.135	$P = 0.097$
MVD per mm^2	1.013	1.004	1.023	$P = 0.007$

DISCUSSION

Angiogenesis, the formation of new vessels from preexisting venules, is central to tumor biology. Quantification of angiogenesis by measurement of MVD has shown that in many tumor types, a high level of angiogenic activity is associated with aggressive tumor behavior. In urological malignancies, MVD has been extensively investigated as a prognostic tool. However, as we have shown previously, the measurement of angiogenesis by this method is subject to a multitude of differing techniques (10). A high MVD in invasive bladder cancer has been associated with a poor outcome (13). This has not been the case in SBC where results have been (8, 9) differing and contrary (11, 14, 15). This may be attributable to insufficient sampling of the sections for MVD counting. Dinney *et al.* looked at between six and eight areas within the tissue section. Ozer *et al.* and Sagol *et al.* both looked at “representative” areas, but neither specified the number. Different techniques have been used to measure MVD. The commonest used is the “hot spot” technique (5). This involves the subjective selection of the most vascular area in the section seen at low magnification and was used by Dinney *et al.* (14) in pT₁ bladder cancer, but it was not found to be clinically useful. A stereotactic grid to estimate vessel density has also been used in SBC in an attempt to compensate for the fragmentary nature of the tumor. Using this method, however, Sagol *et al.* and Ogura *et al.* (11, 16) found no correlation between MVD and outcome.

To eliminate the subjectivity of choosing areas (*i.e.*, hot spots) we counted in random areas within each section. The number of areas required to be sampled to give a MVD representative of the whole section was calculated using the method of cumulative mean. This ensured adequate count sampling to establish a true mean for that tissue section. This technique was also used by Philp *et al.* (17). They examined MVD in a series of bladder cancers that included superficial cases, but these were not analyzed separately. They found that counting in 12 areas was sufficient. In our pure SBC case mix, we found that sampling of between 6 and 28 areas was required and, therefore, chose 30 areas as our sample number. This higher requirement supports the hypothesis that SBC tissue is more heterogeneous and, therefore, requires greater sampling for accurate assessment of MVD.

The fragmentary nature of SBC specimens is caused by their papillary architecture and the piecemeal resection of the tumors by the transurethral technique. To allow for the variation in the amount of tissue (0.1–0.141 mm^2) in the randomly selected areas, vessel counts were expressed per millimeter squared of tissue.

Subsequent analysis of the maximum vessel count in each

case of SBC was also performed. A significant difference in the maximum vessel count was demonstrated between the disease progression and nonprogression groups. The measurement still relies on the analysis of 30 areas in each case, which were chosen randomly with no guarantee of including the most vascular spot. Therefore, although interesting, this result should not be directly compared with Weidner's hot spot counting method.

In summary, this study shows that MVD can be measured in SBC and demonstrates a significantly higher MVD in those cases that subsequently progress. Binary logistic regression found MVD to be a predictive factor in the subsequent progression of SBC. Although this study examines a large sample of SBC cases, it is retrospective. A prospective trial of this method would better determine its clinical use. Furthermore, despite the highly significant difference shown, there is considerable overlap in MVD between the two groups (Fig. 2). This would also benefit from further examination by a prospective study. Use of the Image Analysis System described to measure MVD in SBC allows multiple areas to be counted with relative ease and would facilitate a large prospective study. Many areas ($n = 30$) were sampled in each case to achieve representation of that section as a whole. The use of random areas prevents the assessors' bias found in manual counting methods by removing the need to select specific areas of tumor. Angiogenesis is a complex multistep process involving many tumor and host cytokines. Measurement of a single angiogenic indicator, even a downstream denominator such as MVD, is unlikely to ever be powerful enough to use alone as a prognostic factor. It is more likely that a combination of markers would lead to a biological angiogenic model predictive of future risk.

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