Microvascular Density In The Evaluation Of Neoplastic Cervical Lesions

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Keywords: Angiogenesis, CD31, Cervix, Microvascular density, Neoplasm

Posted Date: September 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-75444/v1

Abstract

Aims:

To evaluate the diagnostic value of CD31 immunohistochemical (IHC) investigation in neoplastic lesions of the uterine cervix among patients of Hospital Tuanku Jaafar (HTJ), Seremban, Malaysia.

Methods:

This is a retrospective correlation study constituting 105 cases in total, selected over a 10-year period from March 2005 to March 2015.

It was conducted in the research laboratories of the International Medical University, Bukit Jalil, and sample collection was performed at the Department of Pathology of HTJ. Patients diagnosed with “cervicitis”, “cervical intraepithelial neoplasia (CIN) grades I through III” and “squamous cell carcinoma” were included in the study. Non-epithelial cervical lesions were excluded.

Demographic data and representative paraffin blocks with H&E slides of each case were reviewed. Sectioned samples were stained immunohistochemically with CD31 to mark endothelial cells. Staining density of the microvessels was then observed under high power. The average of three hotspots per sample was obtained and recorded as the microvascular density (MVD) score. Means for each diagnostic group were tabulated and results were statistically analysed using SPSS version 18.

Results:

Patients’ age ranged from 23 to 85 years with mean of 49.2 years. Cases of carcinoma were most prevalent among 60-69 year-olds. Mean MVD score increases with increasing dysplasia from cervicitis (8.84), CIN (12.57) to carcinoma cervix (29.6).

Conclusion:

MVD CD31 immunohistochemistry investigation can aid in differentiating cervical lesions; benign, intraepithelial neoplasia and invasive malignancy, and could play an adjuvant role in diagnosing neoplastic cervical lesions.

Introduction

Cervical cancer is the 3rd leading cause of death among women of child-bearing age worldwide especially in developing and under-developed countries. Over half a million new cervical cancer cases accounted for 7.5% of total female cancer cases and over 275,000 mortalities among females in year 2012.[1] The single most influential factor affecting quality of life and indeed survival rate is early detection and intervention.

The current gold standard for cervical cancer screening is the Pap smear test developed by Papanicolou in the 1960s. However, over the past 50 years, there has been a substantial amount of cervical cancers still occurring worldwide and also amongst adequately screened women,[2] proving diagnostic limitation to this well-known screening method. Due to subjective criteria and requirement of sophisticated technology, interpretation of Pap test is subject to marked inter- and intra- observer variability and possesses relatively low sensitivity and specificity for cervical neoplasia.

Histology assessment plays an important role in diagnosing precancerous lesions and also invasive squamous cell carcinoma of the cervix (SCC).[3] The Bethesda system is the current standard for interpretation of cervical tissue samples from the Pap screening test. A notable feature of this grading system is differentiation between LSIL and HSIL which corresponds to CIN 1 and CIN 2, respectively, which marks the line between high likelihood of regression versus higher likelihood of progression to carcinoma. What it lacks is a more refined method of marking the transition of benign intraepithelial lesions to high grade dysplasia. This leads to test repetition, consideration of unnecessary and more invasive diagnostic procedures for further work-up and a possibility of over treatment. [4, 5]

Over the past 50 years, there has been a growing body of evidence supporting antiangiogenetic therapy in cancer treatment; proposing that angiogenesis studies could aid in early diagnosis of cervical cancer, in addition to established histocytological methods. The relationship between the degree of neovascularization (development of new blood vessels to supply nutrients to the growing tumour) and the aggressiveness of the tumour is well-established. Tumours with high microvascular density seem to have a worse prognosis than those with low microvascular density.[6, 7] It is therefore plausible to assume that inclusion of an assessment of the degree of neovascularization could facilitate improvements of accuracy of cervical lesion assessment under the microscope.

This study was conducted to establish a direct correlation between MVD and aggressiveness of cervical lesions using CD31 as the endothelial marker. Weidner et al. developed a method to calculate microvascular density (MVD) within tumours in 1991. Under light microscopy, areas with the highest densities of vessel were identified and named hotspots. By then counting the number of stained vessels and EC with collapsed lumen under higher power, and using the highest number calculated for each hotspot, a MVD score is obtained.

CD31 and CD34 are known to be expressed on endothelial cells and are thus excellent candidates for use as biomarkers for identifying blood vessels, proving useful for quantitative analysis of angiogenesis. Cluster differentiation 31 (CD31); also known as platelet endothelial cell adhesion molecule (PECAM-1) is a glycoprotein that is encoded by the PECAM1 gene on chromosome 17 in humans.[1, 6, 7, 8] This protein is a member of the immunoglobulin superfamily and plays a role in angiogenesis, leukocyte migration, integrin activation and removal of terminal neutrophils. It is found on endothelial cells, platelets, megakaryocytes, granulocytes, neutrophils, macrophages and Kupffer cells, monocytes, osteoclasts and some T-cells strains (i.e. T/NK cells, lymphocytes), and also makes up a significant portion of endothelial

intercellular junctions.[1, 6, 7, 8] Malignant endothelial cells also commonly retain this antigen, so CD31 immunohistochemistry can also be used to demonstrate angiomas and angiosarcomas.[9]

Methods

The study is a retrospective correlation analysis to test the degree of CD31 expression among three (3) groups of diagnosed cervical lesions; cervicitis, CIN and SCC cervix. Inclusion criterion is confirmed cases with the afore mentioned three diagnoses. The exclusion criterion is any diagnosis other than the three.

Selected cases were reviewed with histomorphological exam to verify degree of dysplasia or neoplasia and paraffin blocks were examined for integrity prior to inclusion into sample population. These blocks were then sectioned and stained for IHC examination to assess CD31 expression and MVD. Results obtained were then subjected to statistical analysis.

A series of 150 patients with cervical lesions ranging from cervicitis (45), CIN (65) to carcinoma (40) were retrospectively studied. Specimens were obtained either, via routine Pap smear prior to surgical treatment or from hysterectomy specimens. Age of the patients ranged from 23 to 86 years (mean 46.5 years). All cases were selected from the archives of the Department of Pathology of HTJ Seremban, derived from the period from March 2007 to March 2015, based on availability of representative paraffin blocks and H&E slides.

An experienced pathologist verified all histological diagnoses. All lesions were classified using the histopathological criteria of the World Health Organization (WHO) classification, and staging was made according to the American Joint Committee on Cancer system. Normal tonsil samples were used as a control group for IHC staining.

The consent was obtained from the Director of HTJ, Seremban to access and utilize the cervical tissue samples in HTJ. The study was approved by the Ethics Committee of IMU, BMS-I1-2015(06), and is registered under the National Medical Research Registry (NMRR) and is Medical Research & Ethics Committee approved, NMRR-15-1253-25215.

Paraffin-embedded blocks were cut serially into 4-micron sections using a microtome (Leica 1205, USA) and mounted onto silanized glass slides. All IHC methods done in this study followed a standard protocol by the manufacturers with minor optimisation to suit local laboratory conditions.

Monoclonal CD31 antibody (clone: mouse, DAKO, USA) was used at a dilution of 1:50 for 30 min at room temperature. Tonsil tissue was used as a positive control. The CD31 positivity was indicated by the presence of cytoplasmic or membranous brown staining. Microvessel counting was performed in areas with maximal neovascularization within the tumors, outside any areas of artifact, necrosis, or inflammation, and without prior knowledge of the patient outcome. It was performed in a blind fashion with patient's diagnosis and outcome withheld.

Sections were scanned at low and medium magnifications to identify the areas of greatest density of stained microvessels in the cervical stroma “hot spots”. These were found to be below the basement membrane in cervicitis (Fig. 1), adjacent to the greatest depth of invasion in CIN (Fig. 2) and anywhere in the tumor masses of SCC (Fig. 3).

Within these areas of high vascularization, the MVD was determined in three randomly chosen fields, at a magnification of 400. Any brown-staining endothelial cell or a separate cell cluster was considered one single countable microvessel. Vessel lumens and red blood cells were not required for counting as a microvessel. Unstained lumens were considered artifacts even if they contained blood or tumor cells. At 100x magnification, counts were made of all distinct brown staining endothelial cells over three fields in each slide. The microvessel density (MVD) was defined as the average value of the three readings.

The data were analysed with SPSS-PC package (version 11.0, SPSS, Chicago, IL). Statistical analysis included the analysis of variance (ANOVA) and Tukey post-hoc tests for evaluation of MVD counts between cervicitis, CIN (CIN1–3), and SCC groups.

The means of MVD counts from various groups were evaluated by independent sample t-test. IHC scoring was made based on the Allred and methods used by other IHC studies on breast, urinary bladder and prostate cancer. Scanning at 40x, 3 brightest spots with the densest staining were identified for each slide, and the number of visibly identifiable microvessels was recorded. The mean of these three readings was then obtained to be used as the MVD score for that slide. This was to ensure a representative evaluation of the entire section.

Results

The study population constituted patients ranging from 23 to 85 years old with a unimodal distribution and a median age of 46.5 years. The median age group in cervicitis is in the 40–49 age group, like the CIN group. The median age for the SCC group, however, is higher at 57.22 putting the 50–59 age group as the most prevalent followed by 60–69 year-olds.

The racial prevalence showed highest occurrence among Malays which is consistent with the general population makeup of the country. However, there appeared to be a higher prevalence of cervical lesions among Indians considering they make up only 11% of the population on the national census.

IHC showed positivity for CD31 in microvessels of tissue samples of all 3 groups as shown in Figs. 1 through 3. Mean MVD score, calculated as the average MVD count among samples within the group showed an increasing pattern as the lesion advances from benign to malignant. (Table 1)

Table 1: Mean MVD score across all groups

Groups	MVD Min	MVD Max	MVD Mean
Cervicitis	1.3	22	8.84
CIN	4.6	24	12.57
Carcinoma Cervix	12	48	29.6

The data was analysed using GraphPad Software version 2015, to obtain descriptive statistics and confidence interval of means. Results were then run through Student t-test to compare the means and analysed for statistical significance. A p-value of less than 0.05 is considered significant. Data was also processed with SPSS version 18 to undergo one-way ANOVA with post-hoc Tukey HSD test to verify significance of differences of means.

Table 2 records differences in mean scores between groups. The difference of means between the groups was verified as statistically significant. The difference of means within the cervicitis and CIN groups was found to be statistically insignificant. This is likely due to small sample size.

Table 2
Multiple comparison between means of study groups using Tukey post-hoc test. P < 0.05 to be considered significant. Generated using SPSS.

(I) Diagnoses	(J) Diagnoses	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
scc	cervicitis	20.774*	1.631	.000	16.24	25.31
dimensi on3	CIN 1	20.921*	2.079	.000	15.14	26.70
cervicitis	CIN 2	18.723*	2.034	.000	13.07	24.38
dimension3	CIN 3	11.468*	1.994	.000	5.93	17.01
CIN 1	scc	-20.774*	1.631	.000	-25.31	-16.24
dimension3 dimension2	CIN 1	.147	1.970	1.000	-5.32	5.62
CIN 2	CIN 2	-2.050	1.922	.823	-7.39	3.29
dimension3	CIN 3	-9.306*	1.880	.000	-14.53	-4.08
CIN 3	scc cervicitis CIN 2	-20.921*	2.079	.000	-26.70	-15.14
dimension3	CIN 3	– .147	1.970	1.000	-5.62	5.32
		-2.198	2.315	.877	-8.63	4.23
		-9.453*	2.279	.001	-15.79	-3.12
	scc cervicitis CIN 1	-18.723*	2.034	.000	-24.38	-13.07
	CIN 3	2.050	1.922	.823	-3.29	7.39
		2.198	2.315	.877	-4.23	8.63
		-7.255*	2.239	.014	-13.47	-1.04
	scc cervicitis CIN 1	-11.468*	1.994	.000	-17.01	-5.93
	CIN 2	9.306*	1.880	.000	4.08	14.53
		9.453*	2.279	.001	3.12	15.79
		7.255*	2.239	.014	1.04	13.47

1. IHC stained cervicitis sample to illustrate microvascular density

2. IHC stained CIN sample to illustrate microvascular density

3. IHC stained SCC sample to illustrate microvascular density

Discussion

In the study, the youngest patient age was 23 years old and the oldest was 85 years old with a mean of 49.2 years. This reflects the selection of cases for cervical cancer screening in Malaysia which offers sexually active women between ages of 20 and 65 years Pap testing. The cervical cancer group was older, and cervicitis was most prevalent in the younger age groups under 50 years of age. This is in line with the natural history of cervical cancer, where most low-grade dysplasia and HPV infection regresses. The older population may not have been benefited from early screening prior to presentation to medical attention and thus are often caught at a later stage of disease. It takes approximately 10–20 years for a HPV infection to progress to invasive carcinoma in which most of them will resolve in the HPV mild dysplasia stage.[10]

The variability of MVD count within the same diagnosis group reflects the technical difficulty in obtaining and sampling unique variable tumours in individuals but also illustrates the upward trend of increasing MVD with lesion maturity. This variability also proved the utility and need of obtaining a mean measurement from a few hotspots to improve data reliability.

Irregular blood vessels found on colposcopic examinations are well-documented as being consistent with invasive cervical cancer. Although insightful, these observations cannot characterize the biological relationship between angiogenesis and severity of tumour lesions. There is a need to study the direct correlation between MVD and stage of the lesions and in this study, CD31, a known, well-studied angiogenesis marker was used as a means to quantify MVD for that purpose. There have been controversies in previous studies evaluating IHC-measured MVD and its prognostic significance using other vascular markers (Factor VIII, CD34). High MVD has been associated with a worse outcome in stage IB patients, but has also been shown to be associated with improved survival in other studies.[7] The incongruences have been attributed to variability of the population studies, antibody detection and quantification methods used and treatment modalities employed.

In a study by Dellas et al. which evaluated the relationship between MVD, histopathologic parameters and clinical outcomes of invasive cervical carcinoma using CD31, a similar pattern of direct correlation was found. The compelling results in our studies indicate that CD31 MVD is correlated with the degree of neoplasia in cervical lesions. We hypothesize that elevated CD31 MVD could be a surrogate for cervical tumours complexity and be used as an adjunct in addition to conventional histology examinations.

A portion of slides did not stain satisfactorily despite positive control within the same batch. Considering same techniques were applied to those which stained positively within the same batch the cause was likely due to poor tissue condition. Damaged tissue such as those with haemorrhage, air and etc. are likely to degrade faster even under formalin-fixed paraffin embedded condition.

Conclusion

109 cases of cervical lesions were collected from Hospital Tuanku Ja’afar Seremban Malaysia dating from March 2007 to March 2015. The carcinoma group were considerably older with a mean age of 57.2

years, compared to 43.9 and 41.4 for the cervicitis and CIN groups respectively. The mean MVD count for the carcinoma group was 29.6 and showed a linear ascending pattern from cervicitis (8.84) and CIN (12.57). The mean MVD score from CIN 1 to CIN 3 also showed a linear ascending correlation with 8.69, 10.89 and 18.15 respectively. The statistical analysis of mean MVD between cervical lesions indicate that the observed data showed p-value < 0.05 indicating the results were significant. Therefore, null hypothesis is rejected and the alternate hypothesis that CD31 immunohistochemistry investigation can aid to differentiate cervical lesions into benign, intraepithelial neoplasia and invasive malignancy, and could play an adjuvant role in diagnosing neoplastic cervical lesions is accepted. It is noted that as the disease progresses, the CD31 expression increases from 0–100%.

This study was limited by the relatively small sample size which only represented a small cohort of the Malaysian population. Also, we had limited ourselves to the study of CD31 as an angiogenesis marker as it is currently the best profiled for microvascular density studies in cancers. Recommendations for further studies encompass inclusion of a larger study population with inclusion of at least 2 other angiogenesis markers, i.e. VEGF and CD34, for comparison.

Abbreviations

CD
Cluster of differentiation.
CIN
Cervical intraepithelial neoplasia
IHC
Immunohistochemistry
MVD
Mean vascular density

Declarations

Ethics approval: The study was approved by International Medical University Joint committee for ethics. The study was approved by the Ethics Committee of IMU, BMS-I1-2015(06), and is registered under the National Medical Research Registry (NMRR) and is Medical Research & Ethics Committee approved, NMRR-15-1253-25215.

Consent for publication: The consent was obtained from the Director of Hospital Tuanku Ja'afar, Seremban and Head of pathology department to access and utilize the cervical tissue samples in HTJ.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: International Medical University Malaysia

Authors' contribution:

All authors had access to the data and an important role in writing the paper. All authors read and approved the final manuscript.

Acknowledgement: We sincerely acknowledge the technical staff of the department of pathology for their assistance in obtaining the tissue samples.

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Figures

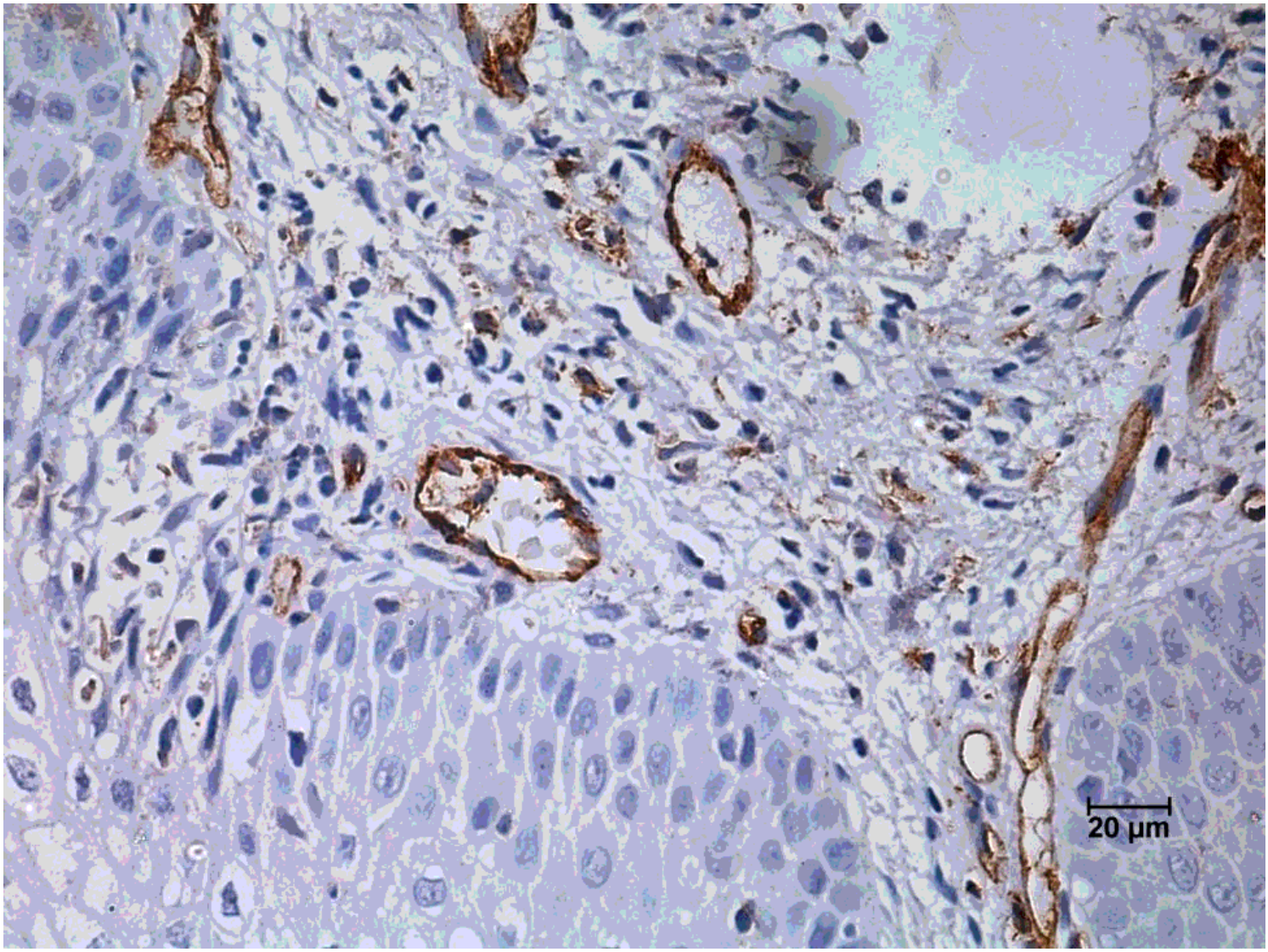

Figure 1

IHC stained cervicitis sample to illustrate microvascular density

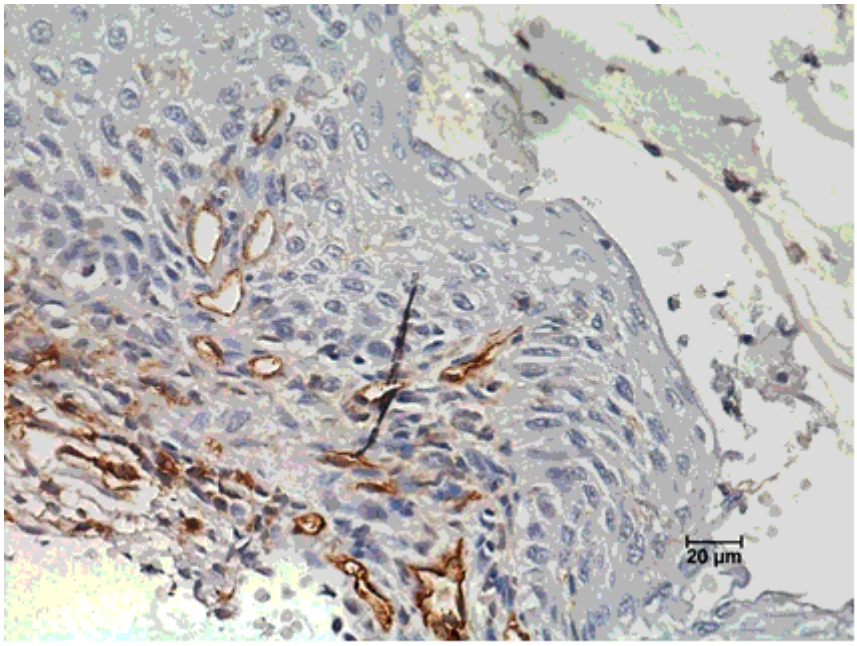

Figure 2

IHC stained CIN sample to illustrate microvascular density

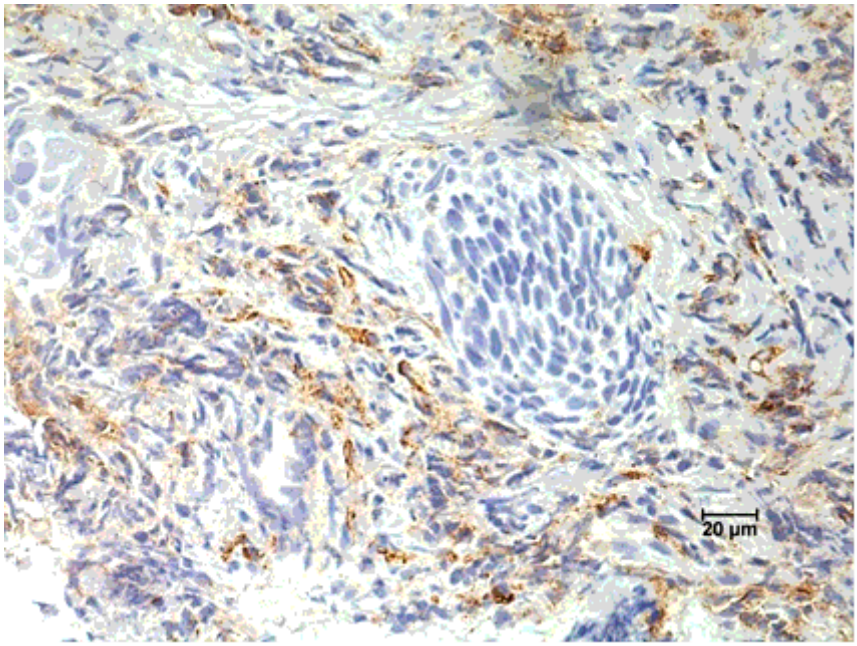

Figure 3

IHC stained SCC sample to illustrate microvascular density