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Significance of assessment of microvessel density (MVD) and mast cell density (MCD) by combined immunohistochemical and histochemical methods in cutaneous malignancies

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Abstract
Introduction: Angiogenesis is a complex event mediated by several angiogenic factors released from cancer cells and immune cells. There is compelling evidence that mast cell accumulation among the peritumoral inflammatory infiltrates contributes to a favourable microenvironment for tumor development and metastasis. The present study aimed to evaluate the relationship between mast cells and microvessels by applying the double staining CD34/toluidine blue in cutaneous malignancies.
Materials and Methods: The study consisted of 50 cases of cutaneous malignancies. Combined Immunohistochemical and Histochemical methods used to study the microvessels and mast cells.
Results: A significant correlation between the mast cell count and microvessel density was observed in all 3 cutaneous malignancies. Statistical analysis of the linear correlation between MCD and MVD was also performed ($P<0.05$ was statistically significant).
Conclusion: Mast cell density may have a role in angiogenesis of these tumors and might be responsible for their aggressive behavior.

Keywords: Cutaneous malignancies, mast cell, microvessels, CD34, Toluidine blue

Introduction
Angiogenesis is the process of formation of new blood vessels from preexisting ones. This process is an absolute requirement for growth, maintenance and metastasis of most solid tumors [1]. The tumor angiogenesis is also essential for the progression and metastasis [1]. Mast cells are present in the perivascular space normally and several studies have shown that these cells may play an important role in tumor progression by stimulating angiogenesis by releasing several angiogenic factors [2]. Studies on experimentally induced tumors in mice have shown mast cell accumulation close to the tumor cells before the onset of angiogenesis [3], and those induced in mast cell-deficient mice showed both reduced angiogenesis and metastatic potential [4]. However there is limited data available regarding angiogenesis in cutaneous malignancies. The role of mast cells in tumor stroma is also a controversial topic which needs further clarification. Hence this was undertaken to study the correlation between microvessel density (MVD) and mast cell density (MCD) in cutaneous malignancies and to perform a double Immunohistochemical/Histochemical technique, i.e.CD34 and toluidine blue.

Materials and Methods
The study included 50 cases (26 squamous cell carcinoma, 14 basal cell carcinoma and 10 malignant melanoma) of cutaneous malignancies. Morphologic staining of the 5um sections was performed using the standand Hematoxylin and eosin stain. Immunohistochemistry was performed on sections which showed tumor surrounded by numerous blood vessels using a monoclonal antibody against cluster determinant 34 (CD34) followed by toluidine blue stain for detecting mast cells in the same vicinity.
To count the microvessels, we followed the method described by William *et al.* [5] The three most vascularized areas ('hot spots') within tumor and the peritumoral areas were chosen at low magnification (40) and vessels were counted in a representative high magnification (× 400; 0.152 mm^2; 0.44 mm diameter) field in each of these three areas and averaged. Single

immunoreactive endothelial cells, or endothelial cell clusters separate from other microvessels, were counted as individual microvessels. Endothelial staining in large vessels with tunica media, and nonspecific staining of nonendothelial structures, were disregarded in microvessel counts. Subsequently in the same fields the mast cells highlighted with toluidine blue stain were counted and then averaged. The assessment was done by 2 observers and by third observer whenever the counts differed by more than 10%.

The pattern of blood vessels ie single, immature or mature were identified in peritumoral and intratumoral areas of squamous cell carcinomas and compared with the grade of tumor.

Statistical analyses were performed using the SPSS-PC package (version 10.0 SPSS, Chicago, 2003). p value < 0.05 was considered as statistically significant.

Results

Our sample size was 50 cases which included 26 cases of squamous cell carcinoma, 14 cases of basal cell carcinoma and 10 cases of malignant melanoma. Histopathologic diagnosis was established on Hematoxylin and Eosin stained sections. Our study included 30 (60%) males and 20 (40%) females with the age ranging from 35-87yrs and a median age of 55.

The microvessel density counted by vascular hot spot method [Figure 1a] was higher for squamous cell carcinomas. We identified all types of tumor blood vessels (single endothelial cells, immature, and mature types) [Figure 1b] but most of the blood vessels had lumen and thin wall probably without perivascular cells around them.

Immature vessels were more in number in poorly differentiated carcinomas. Microvessel density (MVD) and mast cell density (MCD) was more in the peritumoral region [Figure 1c] when compared to the intratumoral region. The microvessels counts ranged from 20-50 (mean 31.60) and mast cell counts ranged from 18-40 (mean 25.10). [Figure 1d] The density of mast cells within the tumors were less ranging from 2-10 (mean 5.30).

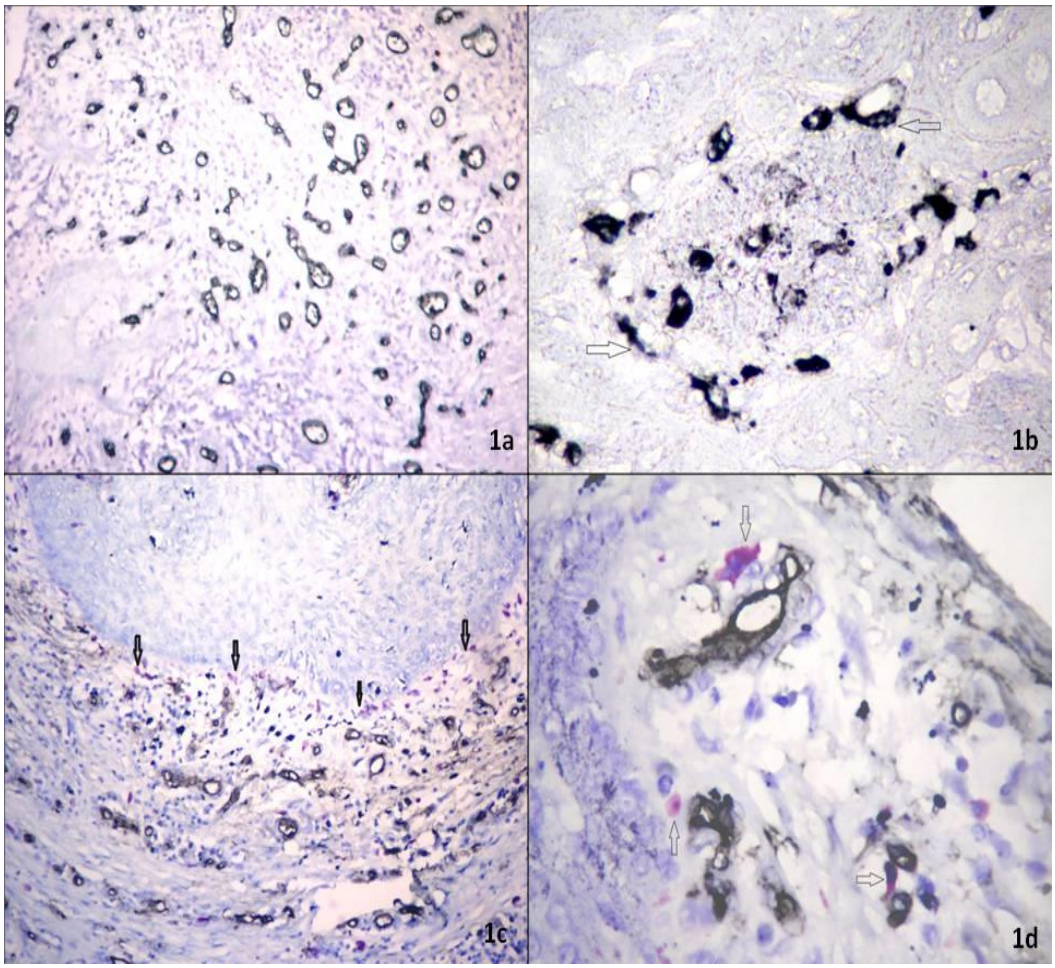


Fig 1: Microphotograph showing a) vascular hot spots (CD34/toluidine blue, X100) b) single, immature and mature blood vessels (CD34/toluidine blue, X100) c) peritumoral mast cells and micro vessels (CD34/toluidine blue, X100) d) mast cells in the vicinity of the blood vessel (CD34/toluidine blue, X400).

We employed paired 't' test to compare the mean difference of MVD and MCD in both peritumoral and intratumoral areas. In the peritumoral areas the mean difference of MVD and MCD was 4.36 with a T value of 11.577 and p value <0.001 which is statistically significant, whereas in the intratumoral area it was not significant. Peritumoral areas showed more blood vessels with high number of degranulated mast cells around them. [Figure 2a] Inside the tumor the blood vessels positive for CD34 was small and most of them without a lumen. [Figure 2b] The density of mast cells within the tumors were less ranging from 0-10 cells/HPF.

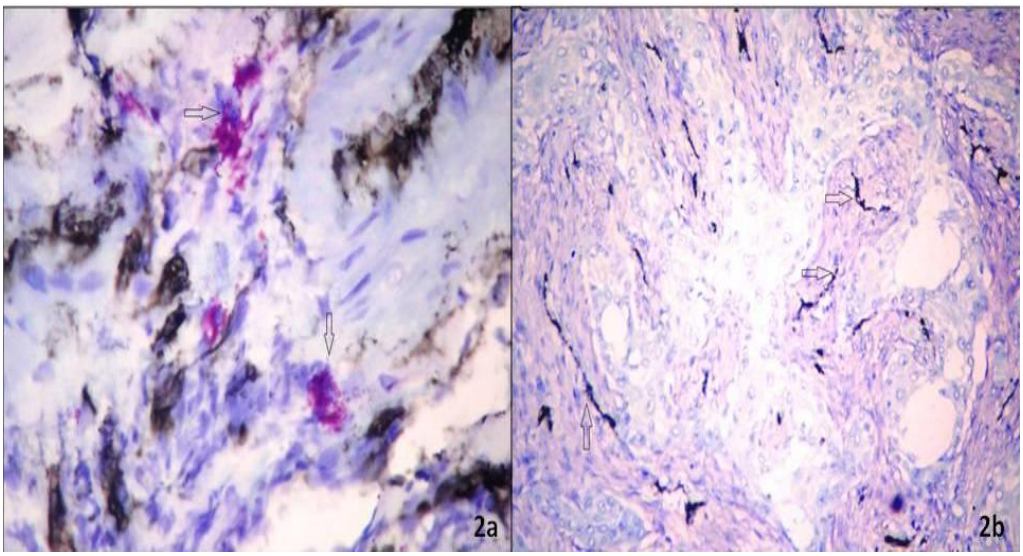
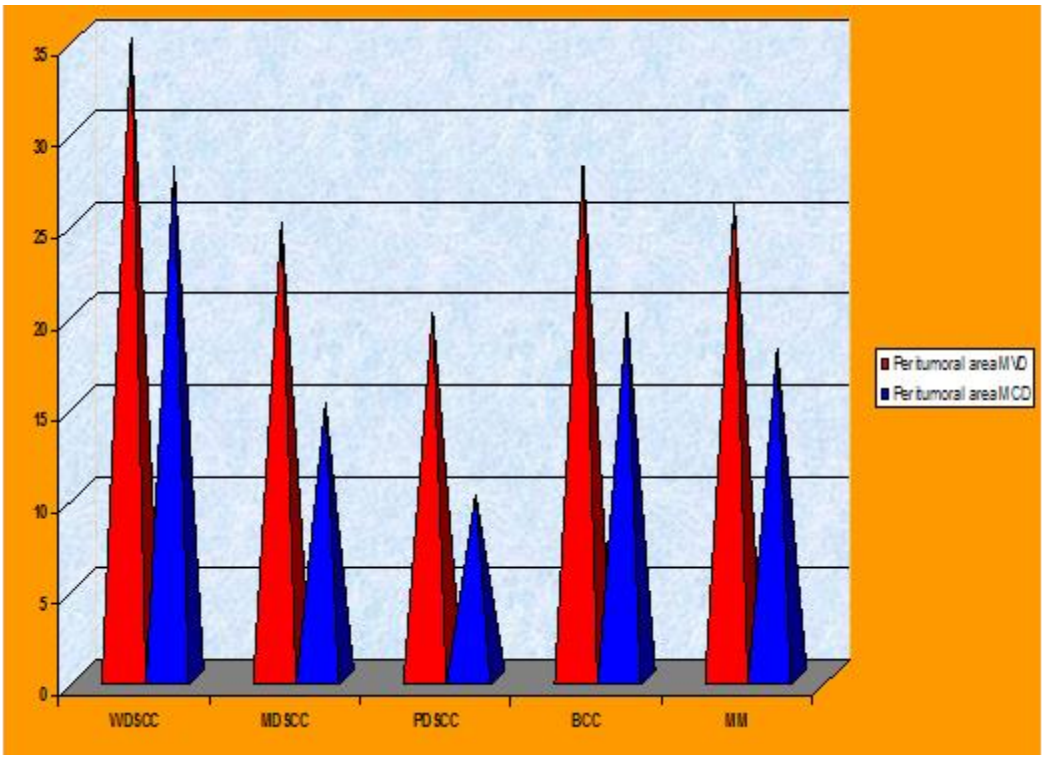


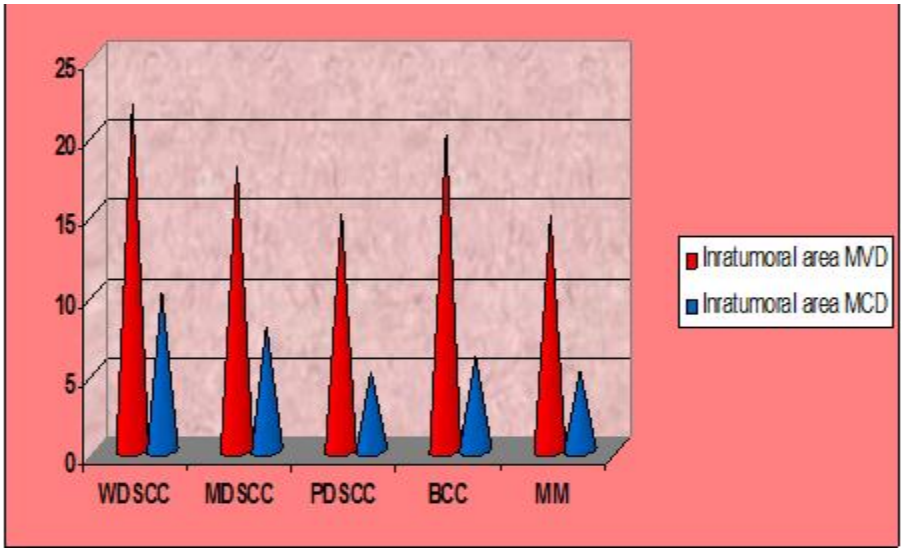
Fig 2: Microphotograph showing a) blood vessels with degranulated mast cells (CD34/toluidine blue, X400) b) intratumoral blood vessels without lumen (CD34/toluidine blue, X100)

To correlate the MVD and MCD we employed Pearson correlation co-efficient. In the peritumoral area it is positive and significant at 0.01 level (r=0.940) p value <0.001 (Graph 1).



Graph 1: Distribution of microvessels and mast cells in peritumoral area.
X-axis - WDSCC – Well differentiated squamous cell carcinoma, MDSCC –Moderately differentiated SCC, PDSCC – Poorly differentiated SCC, BCC – Basal cell carcinoma, MM – Malignant melanoma
Y –axis – microvessel and mast cell counts

In the intratumoral areas there is weak positive correlation and hence it is not statistically significant. Pearson's (r) coefficient=0.203, p value =0.157 (Graph 2).



Graph 2: Distribution of microvessels and mast cells in intratumoral area
X-axis - WDSCC – Well differentiated squamous cell carcinoma, MDSCC –Moderately differentiated SCC, PDSCC – Poorly differentiated SCC, BCC – Basal cell carcinoma, MM – Malignant melanoma
Y –axis – microvessel and mast cell counts

Discussion

Angiogenesis, the process of formation and growth of new blood vessels, plays a tremendous role in tumor development and its progression. Angiogenesis is a crucial step needed for solid tumor progression which involves the growth, invasion and metastasis [6]. Many experimental studies have shown that angiogenesis facilitates the spread of primary tumor by increasing its proliferative rate. Microvessel proliferation increases the vascularity of the tumor which is targeted by the invasive cancer cells thus aiding in the metastatic process. The newly formed blood vessels are leaky and immature, thus make the vascular invasion much easier [7].

Malignant transformation usually evokes an immune response, which morphologically manifests as peritumoral and intratumoral inflammatory cell infiltrates. The activated tumor cells recruit inflammatory cells such as mast cells, macrophages and fibroblasts [8]. Studies have shown that angiogenesis in malignant tumors may be induced by tumor cells via secretion of various types of angiogenic factors and inflammatory cells, including mast cells, via secretion of their angiogenic factors [9]. Chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, and tumors such as hemangiomas, carcinomas, lymphomas and multiple myelomas may show mast cells in close association with angiogenesis [9, 10]. The importance of mast cells lies in the fact that mast cells produce factors such as histamine, tryptase and a variety of multifunctional cytokines and growth factors, such as Interleukin (IL-6 and IL-8), Tumor necrosis factor (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), Transforming growth factor (TGF-β), Fibroblast growth factor (FGF-2) and Vascular endothelial growth factor (VEGF-A) [11, 12], release heparin from secretory granules which stimulate angiogenesis by promoting endothelial cell proliferation and migration [13].

In our study we employed histochemical stain toluidine blue and demonstrated mast cells at the periphery of the tumor. Mast cells have been observed at the edge of the tumor in case of cutaneous malignancies [14]. Histopathological studies on the human basal cell and squamous cell carcinomas have shown that the mast cell density is increased in the aggressive forms [14].

Our results have shown a significant positive correlation between the number of microvessels and mast cells, proven with correlation index of $P<0.001$.

Coussens *et al* [15] showed that mast cells contributed to neovascularization in squamous cell carcinoma. Our results obtained with combined immunohistochemical and histochemical stains CD34/toluidine showed the existence of degranulated mast cells close to a high number of blood vessels intensely stained with a different morphology than normal ones.

So the question arises about the exact role of mast cells in stimulating angiogenesis and initiating tumor progression.

There are many studies explaining the possible role of mast cells in tumorigenesis of cutaneous malignancies. Several pathways or mechanisms are evident from recent studies.

a) **Immunosuppression:** On exposure to Ultraviolet-B radiation, the mast cells are activated, which in turn stimulates the release of neuropeptides from neural c-fibers. These neuropeptides in turn trigger histamine secretion from mast cells, leading to suppression of the cellular immune system [16].
b) **Angiogenesis:** The major source of potent angiogenic factor ie vascular endothelial growth factor (VEGF) in basal cell carcinoma and malignant melanoma is the mast cell. VEGF also induces leakage of other angiogenic factors across the endothelial cell wall into the matrix [17]. Endothelial cell migration is facilitated by mast cell protease and heparin secreted from the mast cell granules assists in blood-borne metastasis [18].
c) **Degradation of extracellular matrix:** This process can be directly through its own proteases or indirectly via interaction with other cells [19] and initiated by tryptase and other mast cell mediators such as FGF-2, TGF-β, IL-3 and IL-4 that stimulate collagenase [18]. This step is essential for spread of tumors.
d) **Mitogenesis:** Recent evidence supports that some of the mast cell mediators such as FGF-2 and IL-8 are directly mitogenic to melanocytes [20].

Several studies have shown association between angiogenesis, tumor dissemination and their outcome. Correlations have been reported between microvessel counts and prognosis of melanomas and carcinomas of the breast, lung, prostate, head and neck, as well as other tumors [21]

Blair *et al* proved that mast cells around basocellular carcinoma and melanoma are a major source of VEGF [22]. High vascular density associated with mast cell density suggests the cooperation of both elements in tumor angiogenesis, possibly through the secretion of VEGF [22]. Our results correlated to the data from the literature with respect to high number of mast cells in cutaneous malignancies.

A study by Kwon *et al* showed a positive correlation between mast cell density and microvessel density in invasive breast carcinomas [23]. Elpek *et al* in their study showed a significant association between MVD and MCD and proposed their prognostic significance in squamous cell carcinoma of the oesophagus [24].

Our study showed significant correlation between mast cell density and microvessel density suggesting a possible role of mast cells in angiogenesis of cutaneous malignancies.

In our study, we also employed combined immunohistochemical/histochemical technique for simultaneous demonstration of microvessels and mast cells using anti-CD34 and toluidine blue stain. This technique is cost effective as well as time saving.

There are new modalities of treatment of solid tumors which includes mast cell anti- degranulation and anti-angiogenic therapy [25]. So assessment of MVD and MCD in cutaneous malignancies may aid clinicians in instituting new therapeutic modalities to halt the further development and progression of tumors.

Conclusion

The CD34/toluidine blue method is a simple and rapid allowing an optimal correlation between the number of vessels and mast cells on the same section. Mast cells may have a role in angiogenesis of cutaneous malignancies and might be responsible for their aggressive behaviour. Further studies are recommended to study the role of chemical mediators and growth factors released by mast cells and their role in angiogenesis and affect on tumor progression. This may aid the clinician in employing targeted therapy for treatment of cancers.

Further studies are recommended using large sample size and IHC markers for mast cells.

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