

Comparison of Vascular Hot Spots Method with Randomly Chosen Microscopic Fields Method in the Morphometric Assessment of Neovascularization in OSCC.

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ABSTRACT

Background: Despite all efforts made to develop predictive biomarkers for antiangiogenic therapies, no unambiguous markers have been identified so far. The most widely used method of quantifying neovascularity is immunohistochemistry, by which microvessel density is determined using endothelial markers, such as CD34. There are several methods to morphometrically assess neovascularization in OSCC. Aims and Objectives: To compare vascular hot spots method with randomly chosen microscopic fields method in the morphometric assessment of neovascularization in OSCC. **Methods:** Our study included 30 paraffin embedded tissue blocks of diagnosed cases of OSCC and 10 controls. Tissue sections of 4μ thickness were taken and immunostaining by anti CD34 monoclonal antibody was performed to demonstrate endothelial cells. Endothelial areas and Microvascular density was assessed by hot spot method and randomly chosen microscopic fields method using computerized image morphometric analysis and. The obtained results were tabulated and compared. **Results:** A statistically significant difference was found between normal mucosa and carcinoma (P<0.001). Endothelial areas values measured by the hot spot method were superior than the values obtained by the random field method. **Conclusion:** Among both the methods, hot spots method was found to be superior than random chosen microscopic fields method.

Keywords: Angiogenesis, CD 31, Endothelial area, Immunohistochemistry, Neovascularization.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck, and its incidence has increased in recent years. Despite great progress in chemotherapy, radiotherapy, and targeted therapy in the last three decades, the prognosis of OSCC is poor due to aggressive local invasion and metastasis, leading to recurrence. Thus, OSCC is still a challenging disease to treat in the field of head and neck cancer.^[1]

The major cause of malignancy-related deaths is metastatic spread of tumor cells. Tumor cells can metastasize from the primary site via the vessels which are already present in the tumor.

the growth of new vessels which provide a new escape route for tumor cells. Microvascular density (MVD) and Endothelial Areas (EA) are by far the most commonly used and reliable predictors for metastasis.^[2]

Quantification of angiogenesis is done through the staining of blood vessels with different endothelial markers including CD31, CD34, CD105, and factor VIII. Cells showing CD34 is an intercellular adhesion protein and cell surface glycoprotein with molecular weight of 105 to 120 CDS. CD34 expression are normally found in the umbilical cord and bone marrow as hematopoietic cells, as well as endothelial cells. Antibody of Anti-CD34 is connected to membrane protein of Sialomucin. Then, it would enable to detect of endothelial cells whether in precursor form or mature cells.^[3,4]

Morphometric quantification of tumoral microvessels is the most frequently used method of estimating tumor angiogenesis. Various methods have been applied to evaluate vascularity; each has its own shortcomings.^[5]

Since the distribution of vessels within tumors is heterogeneous, different methods have been proposed to adequately evaluate representative tumor areas. They are based on the estimation of

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Alternatively, neoangiogenesis or lymphangiogenesis into the tumor could promote

microvasculature in areas of the tumor showing the greatest vascular density - the so-called 'hot spot'. Thus, first at low magnification, tumor areas containing the maximum number of microvessels are identified. The most widely used method of assessing vascular areas involves the determination, at high magnification, of the number of microvessels or the number of randomly positioned dots (vessel cross sections) located within the hot spot. It has been suggested that endothelial cell proliferation is particularly active in these highly vascularized regions and that hot-spot identification may be critical to the accurate assessment of angiogenic potential and thus tumor progression. Unfortunately, all methods of angiogenesis assessment involving hot-spot determination have serious shortcomings. The most striking limitation is high intra- and inter observer variability. Hot-spot determination is highly subjective, and results are difficult to reproduce. It is of note that reproducibility is not necessarily optimized by choosing the same hot spot at a low magnification, in that variation in the selection of a higher power microscopic field often yields different, quite variable counts.^[3-5]

We carried out a study to compare vascular hot spots method with randomly chosen microscopic fields method in the morphometric assessment of neovascularization in OSCC.

MATERIALS AND METHODS

After obtaining the institutional ethical committee approval, the present study was undertaken by retrieving previous records and paraffin embedded tissue blocks of diagnosed cases of OSCC (30 cases) and normal mucosa (10). As control, normal oral mucosa specimens from the patient's buccal flap raised during surgical removal of impacted mandibular third molars were taken. 30 cases of OSCC included 10 cases of well differentiated carcinoma, 10 cases of moderately differentiated carcinoma and 10 cases of poorly differentiated carcinoma.

Immunohistochemistry Procedure:

Tissue sections of 4 μ thickness were taken and immunostaining by anti CD34 monoclonal antibody was performed to demonstrate endothelial cells. The deparaffinised sections were placed in a coupling jar containing 0.01 M citrate buffer (pH – 6.0) and given three cycles of 5 minutes boiling in a microwave oven at 450 $^{\circ}$ c and allowed to cool to room temperature. The sections were then placed in a humid chamber and rinsed twice with distilled water and phosphate-buffered saline (PBS) and covered with Peroxide block, incubated for 5-10 minutes and then drained and gently blotted. Then, the sections were covered with Power block and incubated for 10 minutes and drained and gently blotted. The slide was covered with primary

antibody (Bio Genex ready to use super sensitive antibodies) and incubated for 60 minutes, then rinsed well with buffer and blotted around the sections. Then sections were covered with poly HRP reagent and incubated for 30 minutes then rinsed thoroughly with buffer. After wiping off excess of buffer, the sections were incubated with DAB (Di amino Benzidine tetra hydrochloride) substrate solution for 10 minutes and then gently rinsed with distilled water. The sections were immersed in a Harris haematoxylin for 30 seconds, washed gently under running tap water, dedifferentiated by dipping in 1% acid alcohol, then dehydrated by dipping in xylene and mounted in DPX, a non aqueous permanent mounting medium. The anti CD34 antibody highlighted the micro vessels by staining endothelial cell membrane [Figure 1-3]. In each case, the slide demonstrating the vessels better was taken for morphometric measurements in image analysis. Calibration was done with "Image Pro" "calibration wizard" to measure with millimeter scale.

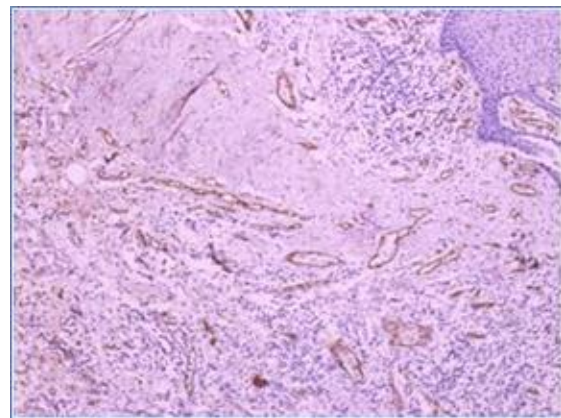


Figure 1: CD-34 immunostaining for angiogenesis in WDOSCC (10x).

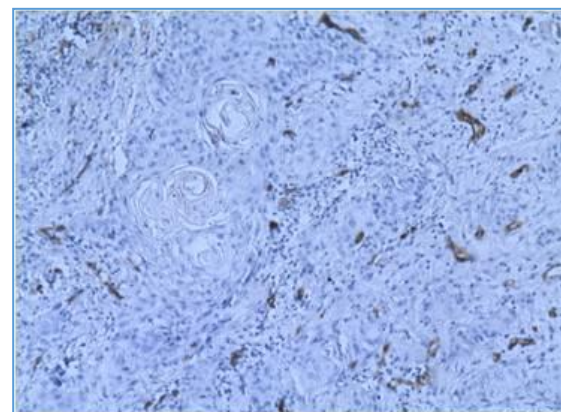


Figure 2: CD-34 immunostaining for angiogenesis in MDOSCC (10x).

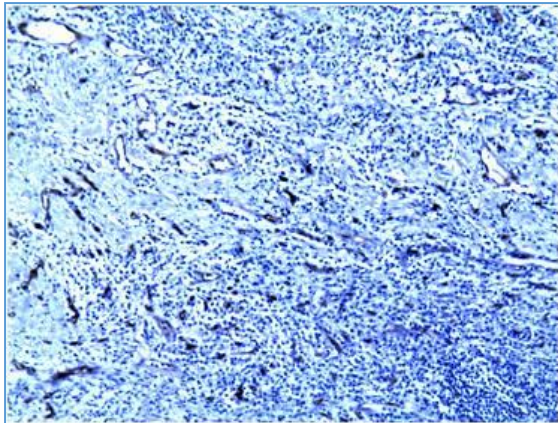


Figure 3: CD-34 immunostaining for angiogenesis in PDOSCC (10x).

MVD and EA Assessment and Comparison of methods:

MVD and EA were assessed by hot spot method using computerized image morphometric analysis and also by randomly chosen microscopic fields method as described in previous studies. The obtained results were tabulated and compared.^[6]

Areas representative of the invasive component of the cancer were selected from sections stained with hematoxylin and eosin. Microvessels were counted and assessed for morphometric parameters (area and perimeter) using “Image Pro” software. Individual microvessels were marked along the endothelial cells as “areas of interest” and the software generated results for each image in millimeters. Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Vessel lumens, although usually present, were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Image analysis was performed without knowledge of the subject’s outcome, the presence or absence of metastases, or any other subject variable. Four images were captured and assessed corresponding to the four hotspots for each slide so that heterogeneity of the tumor tissue was taken into consideration and enough areas were evaluated to represent the tumor vessel conditions and the collected data truly represent the staining conditions in the sections. We used the hot spot method (Weidner et al. 1991) and the random fields method (Oh et al. 2001).^[7,8]

Statistical analysis

The statistical software SPSS 11.0 and Systat 8.0 were used for the analysis of the data. For each method, a two-way random effect analysis of variance was used to estimate three sources of variability: due to different tissues, due to different observers, and unexplained variability (which was confounded with the tissue x observer interaction effect). The mean sums of squares were used to

estimate the variance components. Ratios of the standard deviation and the coefficient of variation (standard deviation divided by the average of all observations for the method and multiplied by 100) were used to summarize the variability associated with each method.

RESULTS

A statistically significant difference was found between normal mucosa and carcinoma ($P < 0.001$). Endothelial Areas (EA) values measured by the hot spot method were higher than the values obtained by the random field method [Table 1 & Figure 1].

Table 1: Summary of variability of the two methods.

Variable	Hot Spot Method	Random field Method
Average level of endothelial area (% of tumor area)	14.16	4.20
Unexplained variability (experimental error) sd1	2.75	0.84
Tumor-to-tumor variability sd2	4.02	1.05
Interobserver variability sd3	0.84	0.18
Coefficient of variation	18.43	19.75
Ratio of tumor-to-tumor variability to unexplained variability sd2/sd1	1.46	1.25
Percent disagreement	40.2	36.0

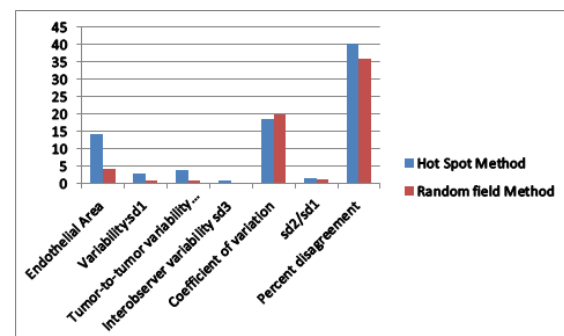


Figure 1: Summary of variability of the two methods.

DISCUSSION

Oral cancer is the sixth most common cancer worldwide. More than 90% of all oral cancers are squamous cell carcinoma (SCC).^[9] Blood vessels form during embryogenesis via two processes; vasculogenesis, whereby endothelial cells are born from progenitor cell types; and angiogenesis, in which new capillaries sprout from existing vessels. New vessels in an adult are formed only by angiogenesis and may be activated by an appropriate stimulus such as wound healing and tumor growth.^[10]

Folkman et al recognized that quantitation of the tumor vasculature might play an important role in predicting tumor behavior and patient management. They therefore developed a microscopic angiogenesis grading system, designated the “MAGS” score, calculated by measuring vessel

number, endothelial cell hyperplasia, and cytology in tinctorially stained tissue sections. Interest in grading tumor angiogenesis was rekindled in the 1980s with the advent of nonspecific endothelial markers, but only in the last five to ten years, with the advent of more specific endothelial markers, have quantitation studies on tissues have been performed. Most studies have employed a method based on that developed by Weidner et al.^[7] in which blood vessels are immunohistochemically highlighted and the number of microvessels quantified in the most vascular areas (so called “hot spots”) of the tumor. Few studies have shown that microvessel density is a powerful prognostic tool in many human tumor types.^[10-12]

The selection of vascular hot spots is subjective and depends on the experience and training of the observer (Vermeulen et al., 1997). There is little agreement as to the optimal number of hot spots to assess, which currently ranges from 1 to 5. In many studies the mean value of the vessel count in four fields was retained as the final value of MVD, others counted at least two fields for each tumor. This number also has an important bearing on the efficacy of the method, since tumors have a limited number of identifiable hot spots. Vascularization quantification on randomly chosen microscopic fields is dependent on the arbitrary selection of a limited number of fields in a restricted area of a tumor section and does not take into consideration the heterogeneous distribution of microvessels in tumor tissue (Vermeulen et al., 1996). A higher magnification gives an increased resolution, which enables more microvessels to be identified, but to the detriment that all fields at too high a magnification become an angiogenic hot spot. Conversely, low magnification, with its lower resolution, will identify a smaller number of vessels, and will dilute out the hot spot.^[5,13]

CD34 is an acceptable and most reproducible marker in many laboratories, but it will highlight perivascular stromal cells and has been noted to stain a wide variety of stromal neoplasms.^[5,6]

In this study we used the EA to quantify the tumor vascularization. Measurement of this parameter does not require the distinction of individual microvessels and may better reflect the interaction between tumor cells and peripheral blood, as previously suggested (Fox et al. 1997; Simpson et al. 1996).^[12,14] However, we showed that other parameters of vascularization, such as the MVD, can also be quantified by the same method using Metamorph software. Whether EA measurement is superior to MVD measurement to assess tumor vascularization is controversial in the literature (Simpson et al. 1996).^[14] Consistent with previous reports, we found no significant correlation between the EA and the MVD.

On comparison, no significant association was seen between MVD and patient's age, gender, site of

tumor and various risk factors in our study. Similar observations were also made in various other studies.^[15]

We found that EA values measured by the hot spot method were higher than the values obtained by the random field method. Our findings are in agreement with that of Christophe et al.^[16]

A limitation to our study is the relatively small sample size and therefore a follow-up study with more samples is advised. For a reliable and reproducible assessment of angiogenesis for all of the assays, validation procedures and quality control protocols are mandatory. Hence, studies on a much larger sample and using antibodies to identify the angiogenesis and its role in tumor progression.

CONCLUSION

It is likely that neoangiogenesis may become an integral part of a more consistent tumor staging system and routine prognostic evaluation. Among both the methods, hot spots method was found to be superior than chosen microscopic fields method, hence hot spots method constitutes a time efficient and reproducible method for quantification of tumor vascularization.

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