Lack of Correlation Between Microvascular Density and Pathological Features and Outcomes in Sinonasal and Oral Mucosal Melanomas

Elizabeth H. Kerr · Dezhi Wang · James S. Lewis Jr. ·
Nasser Said-Al-Naief · Omar Hameed

Received: 6 December 2010 / Accepted: 3 March 2011 / Published online: 19 March 2011
© Springer Science+Business Media, LLC 2011

Abstract Unlike its cutaneous counterpart, prognostic markers for primary mucosal malignant melanoma have not been well elucidated. It has been recently demonstrated that microvascular density (MVD) in cutaneous malignant melanoma has a significant negative correlation with survival; however, this has not been well-studied in mucosal malignant melanoma of the head and neck. This study explores the potential association between MVD, various histological parameters, and the outcome of a series of sinonasal and oral mucosal melanomas. Nineteen such cases were immunostained with CD31 and the MVD was calculated by using Bioquant Image Analysis Software (R and M Biometrics, Nashville, TN). These cases included

16 sinonasal and 3 oral cavity tumors. The 1, 2, 3, 4 and 5 years overall survival rates were 75, 57, 61, 46 and 46%, respectively. The MVD of the tumors ranged from 25.7 to 732 vessels/mm² (mean 142.8 vessels/mm²; median 84.7 vessels/mm²). There was no significant correlation between the MVD and the different clinicopathological features seen within the tumors. There was also no correlation between the MVD and relapse free and overall survival. The results of this study suggest that MVD does not correlate with outcome in mucosal melanoma of the head and neck as seen in cutaneous melanomas. Further larger studies are needed to identify predictive and prognostic markers in such melanomas.

E. H. Kerr · D. Wang · O. Hameed
Department of Pathology, University of Alabama
at Birmingham, Birmingham, AL, USA

J. S. Lewis Jr.
Department of Pathology and Immunology, Washington
University School of Medicine, St. Louis, MO, USA

N. Said-Al-Naief
Department of Pathology, University of the Pacific,
San Francisco, CA, USA

O. Hameed
Department of Surgery, University of Alabama at Birmingham,
Birmingham, AL, USA

O. Hameed
Comprehensive Cancer Center, University of Alabama
at Birmingham, Birmingham, AL, USA

O. Hameed (✉)
Division of Anatomic Pathology, University of Alabama at
Birmingham, 619 19th Street South, NP 3550, Birmingham,
AL 35294-6823, USA
e-mail: ohameed@uab.edu

Keywords Melanoma · Mucosal malignant melanoma ·
Sinonasal tract · Nasal · Oral · Microvascular density ·
CD31

Introduction

Primary mucosal malignant melanoma of the head and neck is rare, accounting for only 0.8–3.7% of all melanomas [1]. Despite the fact that the majority of patients present with localized disease, they have a very poor prognosis that is significantly worse than that of their cutaneous counterparts [2]. This poor outcome is demonstrated by 5-year disease-free survival rates that are usually less than 10% [1]. Accordingly, there is certainly a need for identification of biological markers that might provide useful prognostic information and, more importantly, help predict response to novel adjuvant therapies.

It is well accepted that tumor growth and metastasis are dependent upon tumor angiogenesis, a finding that has spurred significant progress in using anti-angiogenic agents

for cancer therapy. In addition, a positive correlation exists between tumor microvascular density (MVD) and clinicopathological features of certain neoplasms such as in breast carcinoma where the MVD correlates with the rate of nodal metastasis and survival [3]. Similarly, a correlation has been reported between MVD and invasive behavior in a variety of other solid tumors, including cutaneous melanoma [4].

Given that MVD has been found to correlate with outcomes in cutaneous melanoma, the primary aim of this study was to determine whether MVD is also a reliable prognostic marker in mucosal malignant melanoma of the head and neck. The secondary aim was to determine whether there is any correlation between the MVD and various histological features in these neoplasms.

Materials and Methods

Patient Selection and Clinicopathological Features

Following respective institutional review board approval, a retrospective review of surgical pathology database of 2 institutions (University of Alabama at Birmingham, AL and Washington University School of Medicine in St. Louis, MO) was performed to identify cases of primary mucosal malignant melanoma of the head and neck diagnosed over a consecutive 15 years (1993–2008) period. Clinical information was obtained from medical records while pathological features were determined from an unpublished detailed histological review of the archival pathological material performed by two of the authors (E. K and N. S-A). This review determined the predominant histological pattern (spindle vs. other), mitotic rate, and the presence or absence of melanin pigmentation, necrosis, ulceration, and vascular invasion.

Immunohistochemistry

Immunohistochemical staining to determine MVD was performed on a semi-automated immunostainer (Benchmark XT, Ventana Medical Systems, Tuscon, AZ) utilizing a biotin-free, multimer-based technology detection system. Briefly, 5 μ m sections were obtained from a representative formalin-fixed, paraffin-embedded block for each case. After deparaffinization, rehydration, and heat induced epitope retrieval, immunohistochemistry with anti-CD31 antibody (clone JC70; 1:16 dilution, Cell Marque, Rocklin, CA) was applied followed by the secondary antibody. The chromogen diaminobenzidine tetrachloride (DAB) was used to visualize the antibody-antigen complex and hematoxylin was used as the counterstain. Appropriate

positive and negative control slides were prepared in tandem with the cases.

Analysis of Microvascular Density

Except for 3 cases where the tumor area was too small and its entirety was used for calculation, the MVD of each tumor was calculated based on Weidner's method [5] whereby ten 0.25 mm² areas within the tumor were randomly selected at 40 $\times$ magnification for evaluation of MVD. Bioquant Image Analysis software (R&M Biometrics, Nashville, TN) was then used to "lock" on these preselected areas (Fig. 1b). The degree of CD31 staining in each area was then evaluated at 200 $\times$ magnification whereby each immunoreactive (brown-staining) endothelial cell or endothelial cell cluster, clearly separate from immediately adjacent microvessels in contact with the selected field was considered a single, countable microvessel (Fig. 1c, d). Assessment of microvessel density was performed without knowledge of the clinicopathological information including patients' outcome.

Statistical Analysis

The relationship between MVD and relapse-free survival (RFS), overall survival (OS), and other clinical and pathological features of the tumors was determined by the student *t* test, Pearson's correlation coefficient utilizing the SPSS for Windows statistical software (Ver. 16, SPSS Inc., Chicago). Kaplan–Meier curves and Cox regression analysis were also used to study the relationship between MVD and survival. A *P* value of 0.05 was considered statistically significant.

Results

Clinical and Pathological Features

Nineteen patients with invasive primary mucosal malignant melanoma of the head and neck were identified, including 13 men and 6 women, 52–88 years of age (mean 68 years). Sixteen (84%) of the lesions arose in the sinonasal tract and 3 (16%) of the lesions arose in the oral cavity and all underwent surgical excision. One patient died in the immediate postoperative period and 2 patients were lost to follow up. After a median follow up of 23 months (range 4–117 months), all remaining patients developed recurrences and/or metastasis. Local recurrences developed in 14 (74%) patients after a median of 12 months (range 1–105 months) and distant metastasis developed in 11 (58%) patients after a median of 13 months (range

Fig. 1 A sinonasal malignant melanoma (a) immunostained with CD31 (b–d) for microvascular density analysis using Bioquant Image Analysis software. An example of a randomly selected 0.25 mm² area (b, red box) where single or grouped immunoreactive cells

(representing individual microvessels) are identified (c). Multiple randomly selected such areas (d) are then used to calculate the microvascular density

1–96 months). Twelve (70%) patients died with disease after a median of 20 months (range 4–117 months).

Histologically, 8 (42%) of the cases had a predominantly spindle cell pattern and 9 (47%) were pigmented (Fig. 2a, b). Seventeen (89%) of the melanomas showed at least focal necrosis (Fig. 2c), 14 (74%) showed ulceration (Fig. 2d), and 13 (68%) had areas of vascular invasion. The mitotic rate ranged from 2 to 60 per 10 high power fields (mean 22; median 19).

Microvascular Density and Relationship to Clinicopathological Features including Relapse-Free Survival and Overall Survival

The MVD of the tumors was variable, ranging from 25.7 to 732 vessels/mm² (mean 142.8 vessels/mm²; median, 84.7 vessels/mm²). The mean MVD of the 3 oral cavity lesions (250.5; range 181.5–304.8 vessels/mm²) was higher than that of the sinonasal lesions (119.3; range 25.7–732 vessels/mm²) ($P > 0.05$; Fig. 3). There were also no statistically significant differences in the MVD when stratified by the age and gender of the patients, the tumor histological pattern, the mitotic rate, as well as the presence or absence of melanin pigmentation, necrosis, ulceration and vascular invasion (Table 1).

Patients who were alive with disease ($n = 4$) at the end of the study had a mean MVD count (59.3; range 30.7–93 vessels/mm²) that was less than the remaining patients who died of disease (159; range 25.7–732 vessels/mm²) ($P > 0.05$). Although patients were more likely to be alive if they had a MVD less than 32 vessels/mm² ($P = 0.052$), there was no correlation between the MVD and RFS ($r = -0.024$; $P = 0.9$) or OS ($r = -0.024$; $P = 0.9$). Moreover, Kaplan–Meier survival curves (*not shown*) overlapped significantly and Cox regression analysis failed to show statistically significant differences in outcomes.

Discussion

In this study, we have shown that MVD did not correlate with outcome in primary sinonasal and oral malignant melanomas. Moreover, there was no association between MVD and particular clinical or pathological features in these tumors.

Primary mucosal malignant melanoma of the head and neck is an uncommon but aggressive tumor. The literature reports 5-year disease-free survival rates that are usually <10% [1]. The poor prognosis of these tumors was confirmed in our study. Even though all the patients at the time

Fig. 2 Different histological features encountered in head and neck malignant melanomas included a spindle cell pattern (a), prominent melanin pigmentation (b), necrosis (c) and surface ulceration (d)

Fig. 3 Distribution of microvascular density (vessels/mm²) in sinonasal and oral mucosal melanomas

of diagnosis had localized disease, all of the patients with follow up information went on to develop recurrences and/or metastasis within a median follow up of 23 months (range 4–117 months). Local recurrences developed in 14 (74%) patients and distant metastasis developed in 11 (58%) patients. Twelve (63%) patients died with disease after a median of 20 months (range 4–117 months). The factors behind such universally bad outcomes in mucosal melanomas of the head and neck are mostly unknown, as are potential markers that might help predict response to novel adjuvant therapeutic regimens.

Table 1 Mean microvascular density (MVD) (*all differences were not statistically significant*) of head and neck malignant melanoma stratified by clinical and pathological features

Variable	Breakdown	Mean MVD (range)
Location	Oral (3)	250.5 (181.5–304.8)
	Sinonasal (16)	119.3 (25.7–732)
Gender	Females (6)	214.7 (49.7–732)
	Males (13)	112.4 (25.7–304.8)
Predominant histology	Spindle (8)	124.4 (37.6–304.8)
	Other (11)	159.5 (25.7–732)
Melanin pigmentation	Present (9)	112.9 (37.6–256)
	Absent (10)	173.3 (25.7–732)
Necrosis	Present (17)	146.2 (25.7–732)
	Absent (2)	131.9 (82.3–181.5)
Vascular invasion	Present (13)	121.4 (25.7–304.8)
	Absent (6)	195.3 (31.2–732)
Local recurrence	Yes (14)	144.4 (25.7–732)
	No (5)	145.6 (37.6–304.8)
Distant metastasis	Yes (11)	163 (25.7–732)
	No (8)	119.6 (31.2–304.8)

There are numerous studies in the literature showing that MVD correlates with aggressive tumor behavior, as measured by decreased patient survival, increase incidence of metastasis, and higher stage at presentation. Weidner and

colleagues first showed this relationship in patients with breast carcinoma [3], with MVD further shown to be a predictor of aggressive tumor behavior in patients with head-and-neck squamous cell carcinoma, non-small-cell lung carcinoma, cholangiocarcinoma, transitional cell carcinoma of the bladder, renal cell carcinoma, prostate carcinoma, ovarian carcinoma, and cervical squamous cell carcinoma [6–14].

MVD has also been previously explored in cutaneous malignant melanoma with some studies showing them to have significantly higher MVD counts than benign nevi [15, 16]. Other investigators have shown an association between increased vascularity and progression of cutaneous malignant melanoma from the radial growth phase to the vertical growth phase as well as with tumor thickness [17–19]. Finally, both Neitzel et al. [20] and Demirkesen et al. [4] reported that metastatic melanomas had significantly higher mean MVD counts than non-metastatic melanomas. These studies certainly suggest that MVD is associated with tumor progression and could mark for more aggressive disease, potentially also in mucosal melanomas of the head and neck, for which, to the best of our knowledge, MVD studies are lacking.

One of the findings in our study was a higher overall average MVD in sinonasal and oral mucosal melanomas compared to cutaneous melanomas. It is unclear whether different MVD calculation methods might account, at least in part, for such differences, but other possible explanations include topographic variation in the MVD related to the site of tumor origin (mucosa vs. skin) as well as inherent biological differences within the tumors at these two locations. Additional studies, evaluating angiogenic factor and receptor expression levels for example, would be useful to further investigate this.

The main finding in our study was that, in contrast to cutaneous melanomas and the above mentioned solid tumors, MVD did not correlate with a worse outcome in primary head and neck mucosal malignant melanomas. Other studies that did not show an association between MVD and outcome include those by Axelsson et al. and several others in which MVD did not predict metastasis-free survival or overall survival in patients with invasive breast carcinoma [21–23]. Leedy et al. and Rutgers et al. also found that MVD was not useful in determining outcome in patients with squamous cell carcinoma of the tongue or cervix, respectively [24, 25]. Accordingly, one has to consider that the association between MVD and tumor outcome is not uniform among different neoplastic lesions.

Other potential explanations for the lack of association between MVD and outcome in this study (and potentially others) include small sample size (which is somewhat similar to many case series of sinonasal and oral melanomas), as well as the universally bad outcome of these tumors. Again one

has to consider that heterogeneous methodologies used to calculate MVD among different studies might also play a role. In our study, we chose to highlight the tumor vessels with an antibody to CD31 because it has been shown that MVD is better assessed by CD31 (or CD34) than by factor VIII-related antigen [26, 27], often used in other studies. In addition, we selected random areas of the tumor [5] with the help of computer software to minimize potential inter and intraobserver variability in selecting particular areas at the growing edge of the tumor or the most vascularized “hot spot” areas. Finally, some studies calculate the highest number of microvessels in any single 200× field whereas we did an average of multiple fields to correct for potential heterogeneity. Nevertheless, we were also unable to find a correlation between MVD and outcome when we used the highest MVD in any field for calculation (*data not shown*). One has to conclude that, until there is a standardized method for evaluating MVD, it will remain difficult to compare different studies and conflicting reports will occur.

In summary, the results of this study suggest that, in contrast to cutaneous melanoma, MVD does not correlate with outcome in sinonasal and oral mucosal melanomas. Moreover, there was no association between MVD and particular clinical or pathological features of these tumors. Although this might be related to different MVD evaluation methods, one cannot exclude underlying biological differences and further study is warranted.

References

1. Bachar G, Loh KS, O'Sullivan B, et al. Mucosal melanomas of the head and neck: experience of the Princess Margaret hospital. *Head Neck*. 2008;30(10):1325–31.
2. Manolidis S, Donald PJ. Malignant mucosal melanoma of the head and neck: review of the literature and report of 14 patients. *Cancer*. 1997;80(8):1373–86.
3. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. *N Engl J Med*. 1991;324(1):1–8.
4. Demirkesen C, Buyukpinarbasili N, Ramazanoglu R, et al. The correlation of angiogenesis with metastasis in primary cutaneous melanoma: a comparative analysis of microvessel density, expression of vascular endothelial growth factor and basic fibroblastic growth factor. *Pathology*. 2006;38(2):132–7.
5. Weidner N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. *Breast Cancer Res Treat*. 1995;36(2):169–80.
6. Alcalde RE, Shintani S, Yoshihama Y, et al. Cell proliferation and tumor angiogenesis in oral squamous cell carcinoma. *Anti-cancer Res*. 1995;15(4):1417–22.
7. Fontanini G, Bigini D, Vignati S, et al. Microvessel count predicts metastatic disease and survival in non-small cell lung cancer. *J Pathol*. 1995;177(1):57–63.
8. Mobius C, Demuth C, Aigner T, et al. Evaluation of VEGF-A expression and microvascular density as prognostic factors in extrahepatic cholangiocarcinoma. *Eur J Surg Oncol*. 2007;33(8):1025–9.

9. Jaeger TM, Weidner N, Chew K, et al. Tumor angiogenesis correlates with lymph node metastases in invasive bladder cancer. *J Urol*. 1995;154(1):69–71.
10. Yoshino S, Kato M, Okada K. Prognostic significance of microvessel count in low stage renal cell carcinoma. *Int J Urol*. 1995;2(3):156–60.
11. Weidner N, Carroll PR, Flax J, et al. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol*. 1993;143(2):401–9.
12. Brawer MK, Deering RE, Brown M, et al. Predictors of pathologic stage in prostatic carcinoma. The role of neovascularity. *Cancer*. 1994;73(3):678–87.
13. Palmer JE, Sant Cassia LJ, Irwin CJ, et al. Prognostic value of measurements of angiogenesis in serous carcinoma of the ovary. *Int J Gynecol Pathol*. 2007;26(4):395–403.
14. Bremer GL, Tiebosch AT, van der Putten HW, et al. Tumor angiogenesis: an independent prognostic parameter in cervical cancer. *Am J Obstet Gynecol*. 1996;174(1 Pt 1):126–31.
15. Tu YT, Tao J, Liu YQ, et al. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in human malignant melanoma and their relation to angiogenesis. *Clin Exp Dermatol*. 2006;31(3):413–8.
16. Einspahr JG, Thomas TL, Saboda K, et al. Expression of vascular endothelial growth factor in early cutaneous melanocytic lesion progression. *Cancer*. 2007;110(11):2519–27.
17. Erhard H, Rietveld FJ, van Altena MC, et al. Transition of horizontal to vertical growth phase melanoma is accompanied by induction of vascular endothelial growth factor expression and angiogenesis. *Melanoma Res*. 1997;7(Suppl 2):S19–26.
18. Marcoval J, Moreno A, Graells J, et al. Angiogenesis and malignant melanoma. Angiogenesis is related to the development of vertical (tumorigenic) growth phase. *J Cutan Pathol*. 1997;24(4):212–8.
19. Rongioletti F, Miracco C, Gambini C, et al. Tumor vascularity as a prognostic indicator in intermediate-thickness (0.76–4 mm) cutaneous melanoma. A quantitative assay. *Am J Dermatopathol*. 1996;18(5):474–7.
20. Neitzel LT, Neitzel CD, Magee KL, et al. Angiogenesis correlates with metastasis in melanoma. *Ann Surg Oncol*. 1999;6(1):70–4.
21. Axelsson K, Ljung BM, Moore DH, et al. Tumor angiogenesis as a prognostic assay for invasive ductal breast carcinoma. *J Natl Cancer Inst*. 1995;87(13):997–1008.
22. Costello P, McCann A, Carney DN, et al. Prognostic significance of microvessel density in lymph node negative breast carcinoma. *Hum Pathol*. 1995;26(11):1181–4.
23. Goulding H, Abdul Rashid NF, Robertson JF, et al. Assessment of angiogenesis in breast carcinoma: an important factor in prognosis? *Hum Pathol*. 1995;26(11):1196–200.
24. Leedy DA, Trune DR, Kronz JD, et al. Tumor angiogenesis, the p53 antigen, and cervical metastasis in squamous carcinoma of the tongue. *Otolaryngol Head Neck Surg*. 1994;111(4):417–22.
25. Rutgers JL, Mattox TF, Vargas MP. Angiogenesis in uterine cervical squamous cell carcinoma. *Int J Gynecol Pathol*. 1995;14(2):114–8.
26. Weidner N. Chapter 14. Measuring intratumoral microvessel density. *Methods Enzymol*. 2008;444:305–23.
27. Wang D, Stockard CR, Harkins L, et al. Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. *Biotech Histochem*. 2008;83:179–89.