ORIGINAL ARTICLE

The role of VEGF in melanoma progression

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Background: Melanoma is the most serious skin cancer. There is an established correlation between thickness and aggressiveness of the tumor. Nevertheless, the potential value of vascular endothelial growth factor (VEGF) in correlation with tumor progression remains unresolved. **Materials and Methods**: Thirty seven paraffin blocks of cutaneous melanoma were obtained from Pathology department of Al-zahra hospital between 2005 and 2010. The sections were stained with monoclonal mouse antibodies (mAbs) against vascular endothelial growth factor A and evaluated by distribution of expression of VEGF in tumor cells as 0, 0%; 1, 1%--25%; 2, 25%--50%; 3, >50% and the staining intensity from 0 (negative) to 3 (strong). The sum of intensity score and distribution score was then calculated as the VEGF index. The relationship between VEGF expression (distribution, intensity, and index) and tumor progression (vertical and radial growth, Clark's level, and Breslow's depth) was studied. SPSS software was used to analyze the data by ANOVA, and chi-square tests. **Results**: 51.4% of the patients showed vertical growth pattern. Mean Breslow's depth was 1.84 ± 1.79 mm. There was a significant association between growth pattern and VEGF distribution, intensity and index ($P = 0.006$, $P = 0.005$, and $P = 0.001$ respectively). VEGF distribution, intensity, and index all had correlation with Breslow's depth as well (ANOVA test: $P = 0.003$, $P < 0.001$, and $P < 0.001$ respectively) VEGF index had also correlation with Clark's level, but this was not seen for VEGF distribution and intensity. **Conclusion**: VEGF expression (both VEGF distribution and intensity) is associated with progression of malignant melanoma. VEGF index can explain this association better.

Key words: : Melanoma, vascular endothelial growth factor, Breslow's depth

INTRODUCTION

Melanoma is the most serious form of skin malignancies. It's the sixth most common cancers in the United States and is one of the most fatal malignancies that affect young adults.[1] The incidence of melanoma has increased recently, but it is not clear whether this increase is due to environmental factors or early detection.[2] Previous studies have shown that tumor thickness in millimeters (Breslow's depth), depth related to skin structures (Clark level), type of melanoma, presence of ulceration, presence of lymphatic/perineural invasion affect the prognosis.[3,4] Breslow's depth is one of the most important determinants of the current AJCC TNM staging system for malignant melanoma which acts as a valuable prognostic factor.[5]

It is well known that the prediction of biological behavior of malignant melanomas is difficult on the basis of histological criteria. Thin melanomas may develop metastases and thick melanomas may remain localized for many years[6] Interaction between the tumor and stroma is considered critical in carcinogenesis, tumor invasion, and metastasis.[7] The induction of new blood vessel growth formation from a pre-existing vascular bed has been reported as a parameter of potential prognostic value in solid tumors, which may facilitate tumor growth and metastasis.[8] Tumor angiogenesis is controlled by a variety of angiogenic factors. The dominant growth factor controlling angiogenesis is vascular endothelial growth factor (VEGF).[9]

VEGF produced by a variety of cell types, comprises of six different proteins, including: placental growth factor, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and orf virus VEGF (VEGF-E). It appears to play an active role in the induction, maintenance, and growth of vascular endothelial cells. VEGF-C and VEGF-D have been shown to regulate lymphatic angiogenesis.[10,11] VEGF expression has been found to be absent in normal melanocytes but upregulated in malignant melanoma cells.[12]

Nevertheless, the potential prognostic value of VEGF in human cutaneous melanomas as well as its correlation with tumor progression is still unresolved and some studies have not shown any significant prognostic value for this marker.[13-15] Some of these contradictory results might be explained by the non-standardized

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assessments of VEGF. In the present study, we have investigated the relationship between VEGF expression with cutaneous melanoma progression. The presence of a significant relationship between VEGF expression and tumor progression in cutaneous melanomas will make VEGF a good target for antiangiogenic treatments in melanomas of the skin.

MATERIALS AND METHODS

Patients and specimens

This is a retrospective cross sectional study. Paraffin embedded tissue blocks of 37 patients with cutaneous melanoma from the pathology archive of Al-Zahra Hospital (Isfahan, Iran) between years 2005 and 2010 entered the study using the simple sampling method. The inclusion criteria were untreated cutaneous melanomas removed by excisional biopsy, fixed in formalin and embedded in paraffin with a confirmed diagnosis of melanoma in hematoxylin and eosin stained sections. Cases were excluded if the tumor was incompletely excised or was the recurrent lesion.

The clinical and histopathological characteristics of the specimens were retrieved from the pathology reports and verified by a pathologist. These included age, gender, histologic classification, Breslow's depth, Clark's level, anatomical location of the tumor, and ulceration. Radial growth phase (RGP) melanoma was defined as melanoma less than 0.76 mm depth by Breslow score. Melanomas with more than 0.76 mm Breslow's depth were considered as vertical growth phase (VGP) melanoma.[16]

Immunohistochemical analysis

4 μm sections prepared from the paraffin embedded tissue specimens were immunohistochemically stained using the immunoperoxidase-streptavidin-biotin complex method. We used a commercial anti-VEGF antibody (monoclonal mouse anti-human antibody, Clone VG1, Dako Co, code no.: M7273) to investigate the expression of isoforms of VEGF-A including VEGF-121, VEGF-165, and VEGF-189. The antibody was VEGF-A specific without any cross-reaction with VEGF-B, VEGF-C or placental growth factor (PIGF). Sections were first dewaxed for 15 min before rehydration in graded alcohols. Antigen retrieval was performed with microwave treatment in phosphate-buffered saline (PBS) (pH 9.0) for 10 min at 120W. Sections were immersed in 3% hydrogen peroxide (H_2O_2) for 5 min to block the endogenous peroxidase. After rinsing in PBS, the sections were incubated with the primary antibody (anti-VEGF antibody, used at a dilution of 1:50) for 30 min at room temperature. After washing with PBS, appropriate biotinylated secondary antibody was applied for 1 h at room temperature. Thereafter, the slides were washed with phosphate--buffered saline and treated with streptavidin--peroxidase conjugate (1:500; Amersham Pharmacia Biotech, Bucks, UK) for 25 min at room temperature. The sections were exposed to 3,3¢-diaminobenzidine tetrahydrochloride solution (DBT) as chromogen and 0.1% H_2O_2 for 5 min and counterstained with hematoxylin. For negative controls, primary antibody was replaced by buffer. Since epidermal keratinocytes normally express various forms of VEGF,[17] these cells were used as the internal positive control. Using diaminobenzidine as the chromogen results in a color closely resembling melanin color. To avoid false positive results, each stained specimen was compared with its negative control (which used buffer and stained with hematoxylin only).

Assessment of immunostaining

To assess the immunoreactivity, the specimens were viewed at ×40 and ×200 magnifications in the same lighting condition. Negative control of each specimen was simultaneously examined to avoid false positive results. The stained sections were scored by two independent observers.

The first parameter was the intensity of VEGF reactivity. The intensity was scored from 0 to 3 by comparing staining of melanoma cells with normal keratinocytes as follow: 0, no difference between malignant melanocytes and keratinocytes; 1, staining of melanoma cells slightly stronger than keratinocytes; 2, staining of melanoma cells moderately stronger than keratinocytes, and 3, staining of melanoma cells greatly stronger than keratinocytes.

Since, in cases with VEGF reactivity, the reaction was not necessarily seen in all melanoma cells, a second score named distribution score was given to each specimen which reflected the proportion of tumor cells that were positive for VEGF. To do this, 1000 cells were studied in each case and the percentage of positive cells was calculated. The percentage was then translated into a semiquantitative score as follow: score 0–0% of VEGF-positive tumor cells; score 1, 1% -25% of VEGF-positive tumor cells; score 2, 25%-50% of VEGF-positive tumor cells; and score 3, >50% of VEGF-positive tumor cells.

Finally, the sum of intensity score and distribution score in each case was calculated and considered as the VEGF index. This index was interpreted as follow: negative, 0--2, intermediate, 3--4, and strong, 5--6 .

Statistical analysis

The data were analyzed by SPSS software using one-way analysis of variance (ANOVA) and chi-square tests. Results were considered as statistically significant if the *P*-value was < 0.05.

RESULTS

The mean age of the patients was 51.16 ± 7.89 years (min: 37, max: 67 years). Other clinicopathologic characteristics of the specimens have been summarized in Table 1. The specimens showed more frequently lower degrees of VEGF expression regarding both intensity and distribution of the marker. [Figure 1] These data have been summarized in Table 2.

Eighteen (48.6%) samples showed radial growth pattern and the remainder showed vertical growth pattern. We observed that in the groups with VEGF distribution of more than 50% or between 25% and 50%, more samples showed vertical growth pattern. Chi-square test showed a significant statistical difference in VEGF distribution between the two groups of radial and vertical growth patterns. (Pearson chi-square $P = 0.006$)

Table 1: Clinicopathologic characteristics of patients

Mean age ± SD (years)	51.16 ± 7.89
Male (%)	20 (54.1)
Histopathological classification (%)	
Superficial spreading	16 (43.2)
Nodular	9 (24.3)
Lentigo maligna	7 (18.9)
Acral lentiginous	5 (13.5)
Anatomical Location (%)	
Head and neck	15 (40.5)
Upper extremity	7 (18.9)
Lower extremity	10 (27)
Trunk	5 (13.5)
With ulcer (%)	13 (35.1)
Clark's level (%)	
Melanoma in situ	15 (40.5)
Invasion to the basal layer epidermis	3 (8.1)
Invasion to the papillary dermis	7 (18.9)
Invasion to the reticular dermis	6 (16.2)
Invasion to the subcutaneous fat	6 (16.2)
Growth Pattern (%)	
Radial	18 (48.6)
Vertical	19 (51.4)
Mean Breslow's depth ± SD (mm)	1.84 ± 1.79

Table 2: Vascular endothelial growth factor expression in specimens

VEGF distribution (%)	
1-25	17 (45.9)
25–50	13 (35.1)
>50	7 (18.9)
VEGF intensity (%)	
0	19 (51.4)
+1	3 (8.1)
+2	10 (27)
+3	5 (13.5)
VEGF index (%)	
Negative	20 (54.1)
Intermediate	9 (24.3)
Strong	8 (21.6)

The difference between VEGF intensity was also statistically significant between the two groups with radial and vertical growth patterns of melanoma. (Pearson chi-square $P = 0.005$). Proportion between radial growth pattern/vertical growth pattern, in subgroups with VEGF intensity 0 and +1, was nearly the same (2.8 and 2, respectively). It was the same in subgroups with VEGF intensity +2 and +3 (0.11 and 0.25, respectively).

Finally, when we studied the relationship between the growth pattern and VEGF index, we observed an excellent statistically significant relationship. (Pearson chi-square $P = 0.001$) [Figure 2] Proportion between radial growth pattern/vertical growth pattern in negative, intermediate, and strong VEGF index were 3, 0.5, and 0, respectively.

Comparison between VEGF distribution with depth of invasion by Clark's level showed that in patients with high VEGF distribution the tumor invaded deeply to the dermis, but this association was not statistically significant (Pearson chi-square $P = 0.059$) This comparison with VEGF intensity showed a statistically association between them, (Pearson chi-square $P = 0.002$) so that all patients with invasion to reticular dermis and subcutaneous fat (Clark's level 4 and 5) had VEGF intensity +2 and +3. Finally, comparison of VEGF index with Clark's level invasion also showed a significant association between them. [Figure 3] (Pearson chi-square $P = 0.002$)

Although VEGF distribution was shown to increase with increased Breslow's depth (ANOVA $P = 0.003$), LSD Post Hoc analysis showed that this was not the case when comparing Breslow's depth between the VEGF distribution subgroups of 25%--50% and more than 50%. VEGF intensity was also observed to increase with increased Breslow's depth (ANOVA P value < 0.001). However, LSD Post Hoc analysis showed that this was not the case between subgroups (0 and +1) and (+2 and +3).

Finally, we studied the relationship between VEGF index and Breslow's depth and observed a significant relationship between the two parameters. ($P < 0.001$) [Figure 4] Interestingly, post-hoc analysis showed this significant relationship in all subgroups of VEGF index.

DISCUSSION

Although the direct role of VEGF in angiogenesis is not clear yet, it seems that VEGF causes proliferation of endothelial cells and prevents the death of these cells by inducing anti-apoptotic proteins.[18-21] Studies have shown that VEGF plays

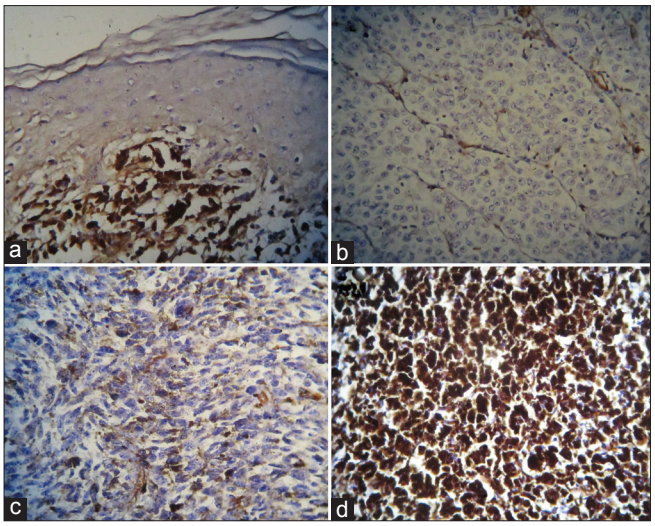

Figure 1: IHC staining of melanoma with VEGF marker. keratinocytes normally express VEGF mildly, these cells were used as the internal positive control. (a) Intensity in cases of (b, c and d) are 0, +1 and +3, respectively

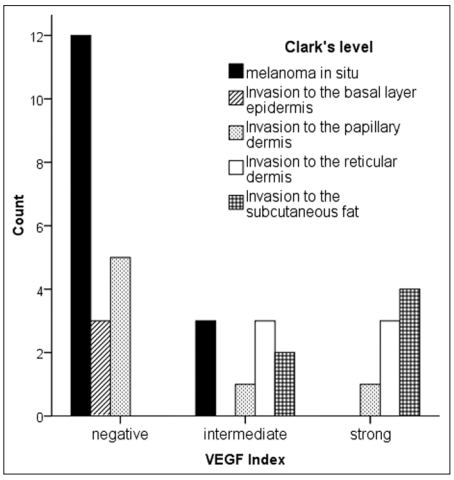

Figure 3: Association between Clark's level and VEGF index. Chi-square $P = 0.002$

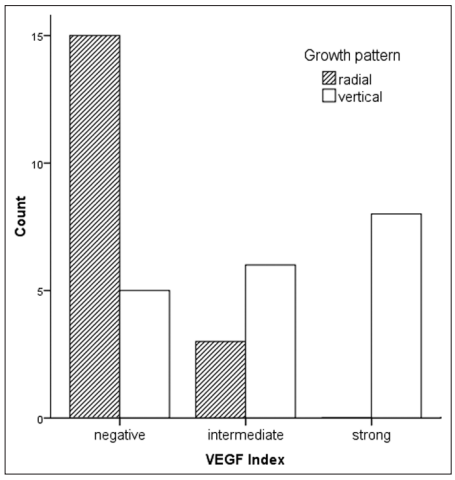

Figure 2: Comparison between VEGF index with growth pattern of malignant melanoma, Pearson chi-square $P = 0.001$

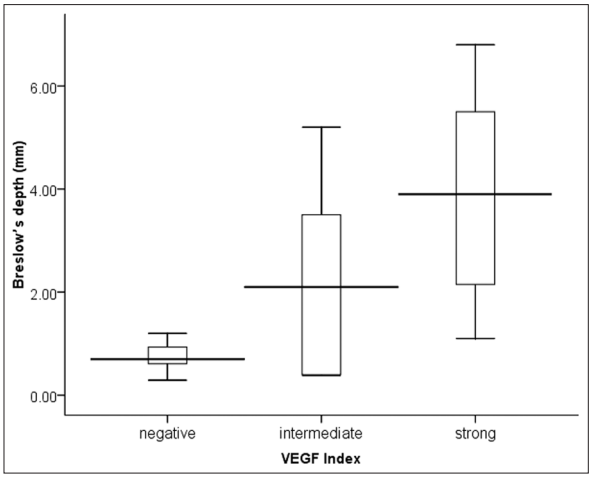

Figure 4: Association between Breslow's depth (mm) and VEGF index. ANOVA $P < 0.001$

these roles through binding to the high affinity receptors of Flt-1 and KDR/Flk-1.[22,23] Interestingly, these receptors are also found on the melanocytic cells[24] and thus VEGF exerts an autocrine effect in growth of melanoma. However, Herold-Mende *et al*.[25] showed that the simultaneous expression of VEGF and its receptors in tumor cells may inhibit tumor proliferation by decreasing the amount of oxygen and nutrients. Folberg *et al*.[26] findings may explain why VEGF expression is higher in advanced tumors. They showed that highly invasive and metastatic melanomas can create vascular channels without endothelial coverage. In addition, VEGF triggers metalloproteinase production in endothelial and melanocytic cells. These compounds with extracellular matrix degradation shall be tumor spread and metastasis and the other hand they induce angiogenesis.[21,27,28]

Although the mechanisms that cause progression of dysplastic nevus to malignant melanoma is not clear yet, neoangiogenesis undoubtedly plays an important role in this process.[29] Recent studies have shown that elevated levels of VEGF in malignant melanoma is more related to its increased production by transformed melanocytes rather than production resulted from tumor growth induced hypoxia.[30,31] Tas *et al*.[21] showed that VEGF serum levels were higher in patients with melanoma compared to the healthy individuals. Moreover in melanoma group, the serum levels were higher in greater tumor thicknesses. This relationship has also been observed in some other studies.[32-34] However, in some of the studies, this difference in the serum levels of VEGF was only seen between the melanoma patients and healthy individuals.[35,36]

Einspahr *et al*.[37] showed that intensity and distribution of VEGF expression are greater in dysplastic nevi compared to benign nevi. Several studies have shown that VEGF expression increases during transition from horizontal to vertical phase of melanoma growth.[38-42] The expression

of VEGF in some of these studies has been evaluated by PCR technique.[41,42] But in some studies based on immunohistochemistry method, the results were completely reverse.[43,44]

In the present study, we showed a relationship between VEGF expression in melanoma cells and progression of the tumor from horizontal to vertical growth phase. This association was seen with both the intensity and distribution of VEGF expression. Most importantly, when the combination of intensity and percentage of VEGF expression was applied, this relationship was even more significant. Our study similar to the previous studies did not show any association between VEGF expression and age and gender of the melanoma patients.[21,33]

Furthermore, we also found a significant correlation between expression of VEGF and Breslow's depth of the tumor. Here again, the relationship was even stronger when intensity and percentage of expression were considered together as VEGF index. Although, Breslow's depth is an important prognostic indicator in melanoma, some previous studies have not shown any association between VEGF expression and prognosis of melanoma.[13,14,35] The relationship between VEGF serum levels and prognosis of melanoma has also shown conflicting results.[33,34] In a recent study, a significant relationship was observed between the prognosis and expression of VEGF on melanoma samples.[45] The relationship between VEGF expression and Breslow index has also been reported in a recent study.[46] These results suggest that VEGF expression is a potential indicator of melanoma progression. According to our results, it is better to use a combination of intensity and percentage of the stained cells. Some of the discrepancies between the findings of various studies in this field could be attributable to the sensitivity of the staining techniques, the method of antigen-retrieval and type of the used antibodies. As it can be seen the recent results are all in one hand and are consistent with serum results. Obviously, we cannot correctly judge influence of VEGF expression on the prognosis of melanoma, in this study.

Finally, we can say that VEGF expression (both distribution and intensity) is associated with progression of malignant melanoma and VEGF index can explain this association better. However, since the data concerning patients' survival were not available in this study, it is obvious that we cannot exactly judge the influence of VEGF expression on the prognosis of melanoma.

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AUTHORS' CONTRIBUTIONS

PR and MM participated in designing this study and supervising the research project. All the experiments were carried out by AN as a part of his thesis. MAR had sent specimens and data to lab. ME and PT collected the data and wrote the manuscript.

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