

## Clinical and Prognostic Significance of Survivin, AKT and VEGF in Primary Mucosal Oral Melanoma

ORIANA SIMONETTI<sup>1\*</sup>, GUENDALINA LUCARINI<sup>2\*</sup>, CORRADO RUBINI<sup>3</sup>,  
RAFFAELLA LAZZARINI<sup>2</sup>, ROBERTO DI PRIMIO<sup>2</sup> and ANNAMARIA OFFIDANI<sup>1</sup>

Departments of <sup>1</sup>Clinic and Molecular Sciences-Dermatology, <sup>2</sup>Clinic and Molecular Sciences-Histology and  
<sup>3</sup>Biomedical Sciences and Public Health-Pathological Anatomy and Histopathology,  
Polytechnic University of Marche, Ancona, Italy

**Abstract.** Aim: To investigate survivin, AKT and VEGF expression in primary mucosal oral melanoma and explore their correlation with clinicopathological features and prognosis. Patients and Methods: Twenty malignant primary oral melanomas were immunostained with antibodies against survivin, AKT, VEGF, CD34. Histological parameters and disease-specific survival were related to marker expressions. Results: Survivin localization was both nuclear and cytoplasmic with a higher expression of nuclear survivin. High melanocyte survivin expression significantly correlated with higher thickness of primary melanoma. A significant positive correlation was found between melanocyte survivin and phospho Akt and VEGF expression. Survivin was significantly associated with the presence of metastasis. High melanocyte survivin and high endothelial VEGF expression were inversely correlated to both overall and disease-specific 5-year survival. Conclusion: Survivin, via AKT and VEGF, seems to play an important role in oral melanoma and could represent an important prognostic marker of melanoma progression.

Oral melanoma is an aggressive tumour because of the absence of symptoms in the early stage of the disease, which delays diagnosis; thus, it is diagnosed at an advanced stage (1, 2). The rich vascular and lymphatic supply of mucosal sites leads to a greater inclination of local and distal recurrence, as well as regional and distant metastases, making it one of the most biologically-unpredictable human neoplasms (2). An acquired

apoptosis resistance has been observed in advanced melanoma tumor cells (3) and, although multiple mechanisms have been identified (3), survivin over-expression has been suggested to play an important role in the pathogenesis and progression of various types of cancer (4). Survivin belongs to the inhibitor of apoptosis protein (IAP) family. It has a dual cellular function both as an inhibitor of apoptosis, related to its direct or indirect inhibition of caspase activity, and a regulator of mitosis (4, 5). In normal melanocytes, survivin is either not commonly detected or its expression is significantly lower than in melanomas (6). Conversely, it is highly expressed in many embryonic tissues, as well as in a number of cancers, including breast, lung, melanoma, leukemia, lymphoma, colon and pancreas (7). In the last decade, growing attention has been paid on the Akt, a serine/threonine kinase that regulates cell growth, cell cycle progression, survival and anti-apoptosis. Dysregulation of Akt has been observed in various cancers (8-9). Akt activation has been found in 70% of melanomas (10) and mediates melanoma cell migration, invasion and metastasis in various model systems (11-12). In particular, activation of Akt has also been shown to convert a melanoma cell line from radial to vertical growth phenotype (13). Recently, AKT activation has been linked to over-expression of survivin (14). Angiogenesis is a prerequisite for tumor growth and the microvessel density (MVD) has been shown to correlate with a higher incidence of metastases and a worse prognosis in malignant melanoma (15-16). The primary effector of angiogenesis in tumors is vascular endothelial growth factor (VEGF). It increases the permeability of microvessels to circulating macromolecules (17) and also acts as a selective endothelial cell mitogen (18). Previous studies have shown that VEGF expression is related to the expression of survivin in many cancers (7, 19).

Survivin, AKT and VEGF are both important regulators in tumor growth but there are no reports investigating the clinical value of these factors in oral melanoma.

The present study was designed to investigate the expression of survivin, AKT and VEGF in primary oral

\*These Authors contributed equally to this study.

Correspondence to: Oriana Simonetti, MD, Department of Dermatology, Polytechnic University of Marche-United Hospitals, Via Conca 71, Ancona, 60020, Italy. Tel: +39 0715963494, Fax: +39 0715963446, e-mail: o.simonetti@univpm.it

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melanoma and to explore their correlation with clinicopathological features and prognosis. Moreover, the interrelationship between these factors was evaluated.

## Materials and Methods

**Mucous tumor samples.** Twenty primary oral malignant melanomas were used for our present study. All specimens were obtained in fifteen years from the Archives of the Section of Anatomic Pathology of the Polytechnic University of the Marche Region, Italy. The use of human mucosal tissues in this study was approved by the medical ethical committee of the Polytechnic University of the Marche Region and the experimental work was performed in accordance with the Declaration of Helsinki guidelines. All recruited patients gave their informed consent. The clinical parameters assessed were sex, age, site and size of tumor, as well as outcome.

Histological diagnosis was based on the usual criteria (20). The infiltration levels were measured by the Prasad's method (21). Main tumour cell type was defined as epithelioid, spindle, mixed and undifferentiated. Tumour thickness was measured using an ocular micrometer from the deepest invasive melanoma component to the most superficial layer of the mucosa (when intact surface was present).

**Immunohistochemical staining and scoring.** Immunohistochemical analysis was performed on 6- $\mu$ m-thick paraffin-embedded histological sections. After deparaffinization and rehydration, sections were treated with microwave for heat-induced epitope retrieval in 10 mmol/l sodium citrate buffer (pH 6.0). Then, the sections were incubated with the following monoclonal antibodies: anti-CD34 (clone My10) (dilution 1:20; BD Biosciences, San Jose, CA, USA), anti-VEGF-165 (clone C-1, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-survivin (clone D-8, dilution 1:100; Santa Cruz Biotechnology) and with the polyclonal antiphospho-Ser-473 of Akt antibody (1:50 dilution; Cell Signaling Technology, Beverly, MA, USA). The immunohistochemical reaction and antibody visualization were performed as previously described (16).

All counts were performed separately by two investigators, blinded to the patient outcome (C.R. and G.L.). The density of microvessels (MVD) was defined by manually counting the number of CD34-positive small vessels in a 400 $\times$  microscopic field (0.16 mm<sup>2</sup> per field) (22). Any stained cell or cluster that was clearly separated from adjacent microvessels was considered as a single countable microvessel. The average of the microvessels in five fields was calculated and MVD was expressed as mean CD34-positive vessel count per mm<sup>2</sup>.

The endothelial cell and melanocyte reactivity for VEGF and survivin and the melanocyte reactivity for antiphospho-Ser-473 of Akt were evaluated in at least 10 fields/sample at  $\times$ 400 magnification and quantified as a percentage of the total counted cells. Images were captured with a Nikon DS-Vi1 digital camera (Nikon Instruments, EuropeBV, Kingston, Surrey, England) connected to a computer. The fields evaluating the most positive, moderately and less positive areas were randomly selected. The area of each field (0.07 mm<sup>2</sup>) was standardized using the NIS Elements BR 3.22 imaging software (Nikon Instruments). A percentage of positive cells <40 and a percentage of positive cells >40 were considered as low and high expression, respectively.

To evaluate intra and inter-observer variability, counting was performed three times by each examiner on each slide. Only the mean value was calculated for each case and used for statistical

analysis. The k value was >0.80 showing a substantial agreement between the two observers and among different observations of the same observer.

**Statistical analysis.** All data are represented as the means $\pm$ SD (standard deviation). The Pearson's correlation was used to test the relationship between marker expressions and between marker expression and clinicopathological features. Survival parameters were compared using Kaplan-Meier curves and the log-rank test. *p*-Values of <0.05 were considered statistically significant.

## Results

**Clinical features and pathological characteristics.** We observed that more male were affected by this tumor than females (2:1) with an age range of 38-89 years (mean age=65.5 years). Common tumour site was the palate (n=16, 65%); four cases involved the vestibular mucosa. Eighteen cases were strongly pigmented and two were amelanotic. We found two patients with in situ melanoma, while the tumor was invasive in 18 cases; 8 patients showed a tumour limited to the lamina propria (44.4%). In the rest of the patients, the tumour affected the muscle and the bone (Level III) (55.5%). The thickness of the tumor was between 1.3 mm to 10.00 mm (mean=4.8 $\pm$ 1.9). Tumours were prevalently epithelioid (9 cases), 7 patients showed a spindle cell-like appearance and 4 exhibited a combination of the two. The average size of the tumor was 0.75 cm (range from 0.4 to 1.7 cm). Eight patients had no evidence of metastasis, while twelve patients (60%) developed lymph node (4 patients) and/or distant metastases in the follow-up (8 patients).

Out of the 18 live cases presenting invasive melanoma, 3 presented the disease and 5 did not resulting in a 5-year survival rate of 35.7%. Level III patients had lower mean survival time (24 months), while level II had a survival time of 40 months (*p*=0.01 by the log-rank test). Different cell type tumours presented no variation in survival rate.

Immunostaining patterns of survivin, phospho-Akt, VEGF and CD34. Immunohistochemical staining revealed oral primary melanomas with an especially variable expression of surviving, which was particularly heterogenic compared to its intracellular localization. Survivin expression was found in tumoral melanocytes and endothelial cells. We observed that its localization was both nuclear (survivin-N) and cytoplasmic (survivin-C) (Figure 1A, E). Although survivin was detected in all the samples in both cytosolic and nuclear compartment, it was more abundant in the latter. Most melanomas showed simultaneous cytoplasmic and nuclear survivin staining (Table I).

Melanocyte survivin-N expression was low (<40% of positive cells) in 7 patients out of 20 (35%) and low survivin-C in 10 of 20 (50%), while high expression of survivin-N (>40% of positive cells) was observed in 13 out of 20 (65%) and cytoplasmic in 8 of 20 (40%) (Table I).



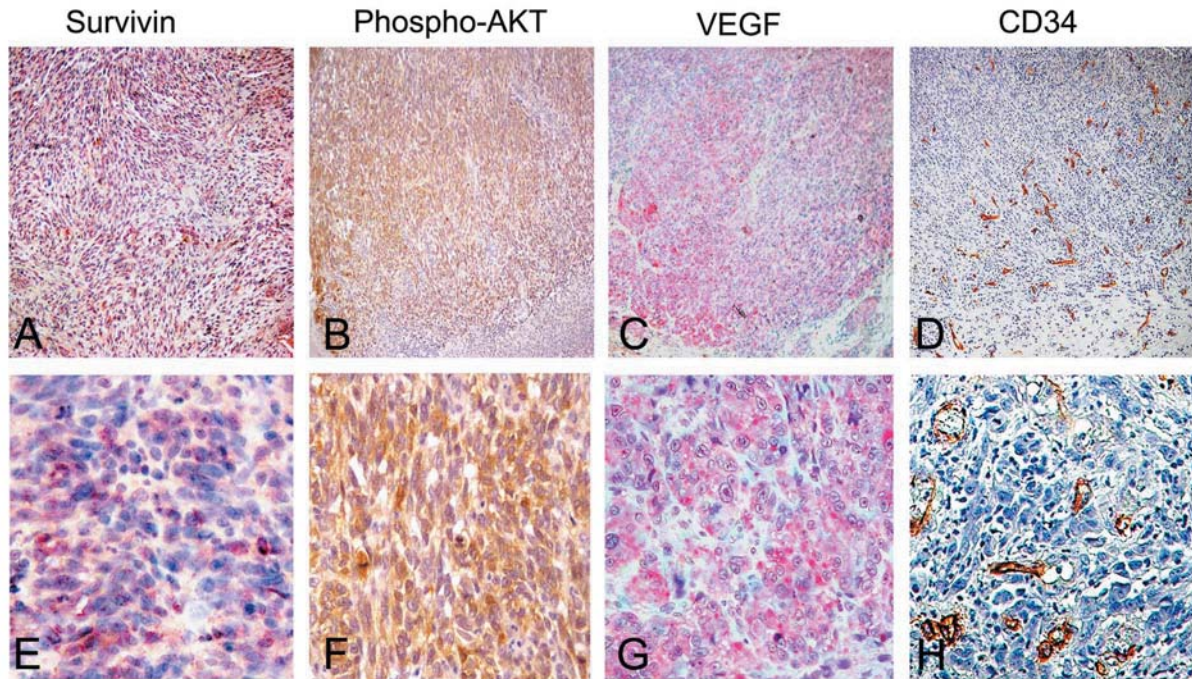


Figure1. Immunohistochemical staining of survivin, phospho-AKT, VEGF and CD34 in primary oral melanoma. (A and E) Simultaneous cytoplasmic and nuclear survivin staining; (B and F) Phospho- Akt is located in the melanocyte cell membrane, cytoplasm and, to a lesser degree, nucleus; (C and G) High VEGF expression in tumour melanocytes; (D and H) CD34-positive microvessels. Magnification x200 for A, B, C and D;  $\times 400$  for E, F, G and H.

Endothelial survivin-N expression was low in 5 patients out of 20 (25%) and low survivin-C in 3 of 20 (15%), while high expression of survivin-N was found in 14 out of 20 (70%) and survivin-C in 11 of 20 (55%) (Table II).

Phospho-Akt was located in the cell membrane, cytoplasm and, to a lesser degree, nucleus of malignant melanocytes (Figure 1B, F). Various levels of phospho-Akt expression were observed: low expression was observed in 30% of patients, while high expression in 70% of patients (Table I).

VEGF expression was found in the cytoplasm of melanocytes and endothelial cells (Figure 1C, G). Thirty percent of patients showed low melanocyte VEGF expression and 25% of patients showed low endothelial VEGF expression, while high expression was seen in melanocytes of 70% of patients and in endothelial cells (75%), respectively (Tables I and II).

CD34-positive vessels were counted and microvessel density (MVD) was defined (Figure 1D, H). The mean MVD was  $100.33 \pm 31.83$ . Thirty percent of the patients showed low MVD, while the remaining patients showed high MVD (Table II).

**Correlation between markers' expression.** A significant positive correlation was found between melanocyte survivin and phospho Akt expression ( $p=0.000$ ;  $r=0.868$ ) (Figure 2A).

Significant positive correlations were detected when comparing melanocyte survivin expression to melanocyte VEGF expression ( $p=0.000$ ;  $r=0.899$ ) (Figure 2B) and between endothelial cell survivin and endothelial VEGF expression ( $p=0.017$ ;  $r=0.669$ ) (Figure 2C). Endothelial VEGF expression also correlated with MVD ( $p=0.034$ ;  $r=0.671$ ).

**Marker staining and correlation with clinicopathological features.** To assess whether marker staining correlated with clinicopathological parameters of the patients, we analyzed their expression level in melanomas with different thicknesses since tumor thickness is a well-known prognostic marker for patients with primary melanoma. We found that high melanocyte survivin expression significantly correlated with higher thickness of primary melanoma. ( $p=0.004$ ,  $r=0.792$ ). Melanoma thickness was also positively correlated with AKT and melanocyte VEGF expression ( $p=0.004$ ,  $r=0.758$  and  $p=0.021$ ,  $r=0.653$ , respectively) and with MVD ( $p=0.028$ ,  $r=0.656$ ).

Moreover, survivin expression was significantly associated with the presence of metastasis ( $p=0.015$ ,  $r=0.709$ ). We also observed a positive correlation between melanocyte VEGF expression, MVD, AKT expression, melanoma thickness and presence of metastasis ( $p=0.041$ ,  $r=0.623$ ;  $p=0.003$ ,  $r=0.777$ ;  $p=0.004$ ,  $r=0.756$  and  $p=0.033$ ,  $r=0.615$ , respectively).

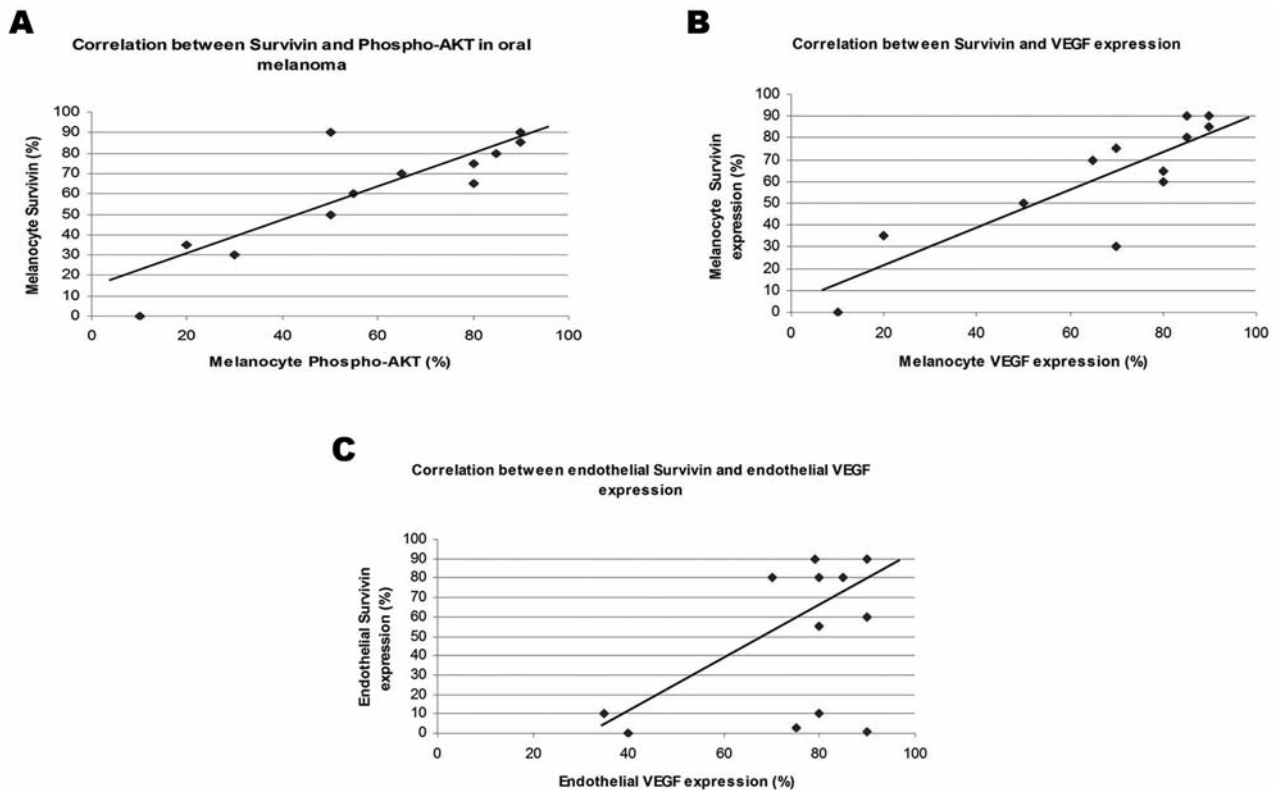


Figure 2. Correlation between individual markers: significant positive correlations are observed between (A) melanocyte survivin and phospho-AKT expression ( $p=0.000$ ;  $r=0.868$ ), (B) melanocyte survivin and VEGF expression ( $p=0.000$ ;  $r=0.899$ ) and (C) endothelial survivin and VEGF expression ( $p=0.017$ ;  $r=0.669$ ).

Table I. Immunostaining of melanocyte survivin, AKT and VEGF in oral melanoma patients.

Melanoma thickness mm (n. patients)	Metastasis n. patients (%)	Melanocytes							
		Low Survivin-N(+) n. patients (%)	High Survivin-N(+) n. patients (%)	Low Survivin-C(+) n. patients (%)	High Survivin-C(+) n. patients (%)	Low VEGF n. patients (%)	High VEGF n. patients (%)	Low AKT n. patients (%)	High AKT n. patients (%)
<1.5 (3)	-	3 (100)	-	2 (66)	-	3 (100)	-	3 (100)	-
1.5-4.00 (9)	5 (55)	4 (44.5)	5 (55.5)	4 (44)	4 (44)	3 (33)	6 (66)	3 (33)	6 (66)
>4.00 (8)	7(87)	-	8 (100)	4 (50)	4 (50)	-	8 (100)	-	8 (100)

Low, Positive cells <40% of the total counted cells; High, positive cells >40% of the total counted cells; Survivin-N, nuclear survivin; Survivin-C, cytoplasmic survivin.

*Survivin, phospho AKT, VEGF, MVD and disease specific survival.* To evaluate whether survivin, phospho AKT, VEGF expression and MVD correlates with a worse prognosis in oral primary melanoma, Kaplan-Meier survival curves were constructed using overall or disease-specific 5-year survival. We

considered a percentage of positive cells <40 as low expression and a percentage of positive cells >40 as high expression.

Our data revealed that high MVD (Figure 3), high melanocyte survivin (Figure 4) and high endothelial VEGF expression (Figure 5) were inversely correlated with both

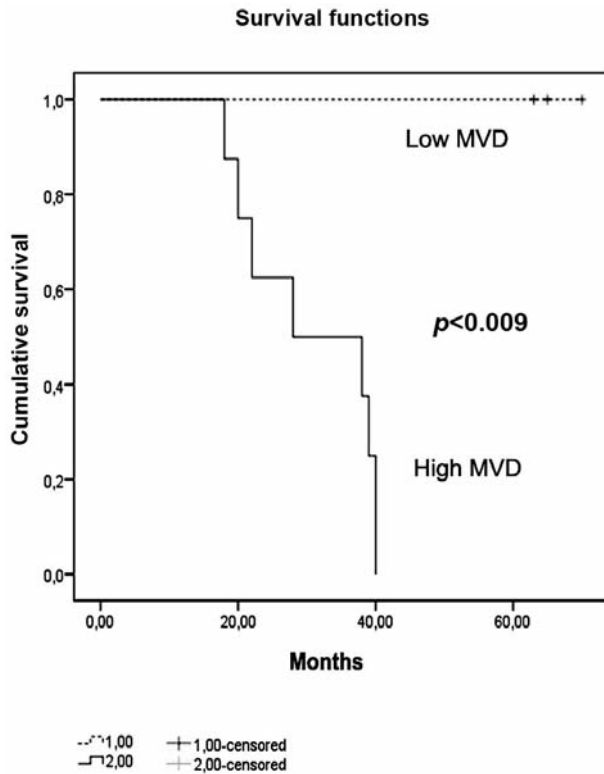


Figure 3. Kaplan-Meier curve for disease-free survival in relation to MVD divided into groups of high and low expression. A percentage of positive cells <40 and a percentage of positive cells >40 were considered as low and high expression, respectively. The log-rank test was used to calculate significance. Cum survival: cumulative survival.

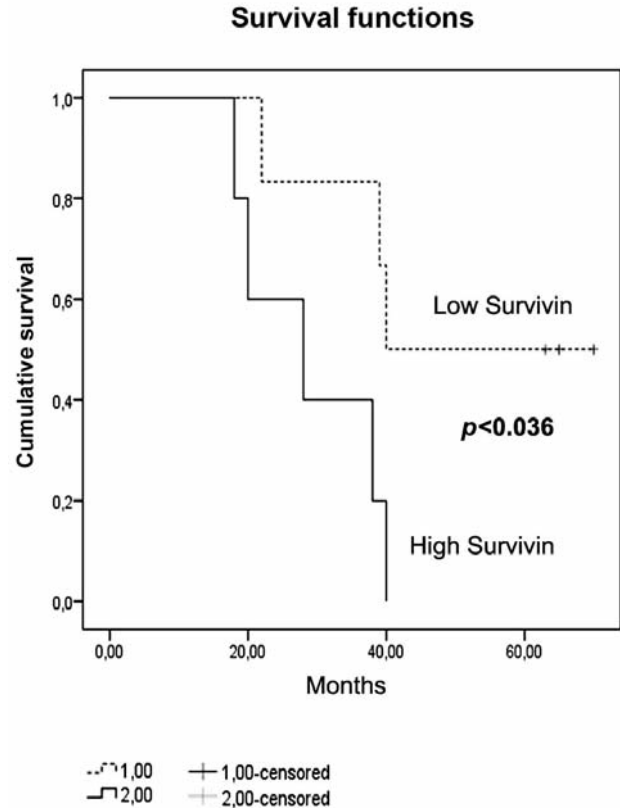


Figure 4. Kaplan-Meier curve for disease-free survival in relation to survivin expression divided into groups of high and low expression. A percentage of positive cells <40 and a percentage of positive cells >40 were considered as low and high expression, respectively. The log-rank test was used to calculate significance. Cum survival: cumulative survival.

overall and disease-specific 5-year survival (log-rank test;  $p=0.009$ ,  $p=0.036$ ,  $p=0.037$ , respectively).

## Discussion

Survivin localizes in the nucleus and cytoplasm of cells (23), whereas nuclear expression was found to be more closely related to malignant potential (24, 25). In primary cutaneous melanoma, survivin was mostly observed in the nuclear compartment, whereas it was equally distributed in both the nuclear and cytoplasmic compartment in metastases (26). Chen *et al.* (27) detected both cytoplasmic and nuclear survivin in primary mucosal melanoma, although only the nuclear pool was significantly correlated with poor outcome of the patients (28). Our results confirmed these data since we observed that survivin localization was both nuclear and cytoplasmic with a higher expression of survivin-N in 65% of all cases. We also observed, in accordance with other studies (29), that a higher melanocyte survivin positivity significantly correlated with a higher thickness of primary melanomas.

Survivin is essential for mitosis but has also been implicated in protecting cells from apoptosis. Moreover, the nuclear pool has been suggested to be involved in promoting cell proliferation, whereas the cytoplasmic pool may be related to control of cell survival. Therefore, strong nuclear staining of survivin may reflect an increase in mitotic events (30). The suppression of apoptosis and the increase of cell proliferation by survivin could be a mechanism responsible for high oral melanoma thickness and invasion.

In addition, survivin seems to play an important role in the formation of new blood vessels as well. Apoptosis inhibition is closely correlated with angiogenesis in the occurrence, development, invasion, recurrence and metastasis of tumours (31).

The process of apoptosis is regulated not only by survivin but also by VEGF (32). Furthermore, the positive expression of VEGF in tumour angiogenesis promotes and induces high expression of survivin, which then up-regulates the expression of angiopoietin-1, a crucial factor for maintaining



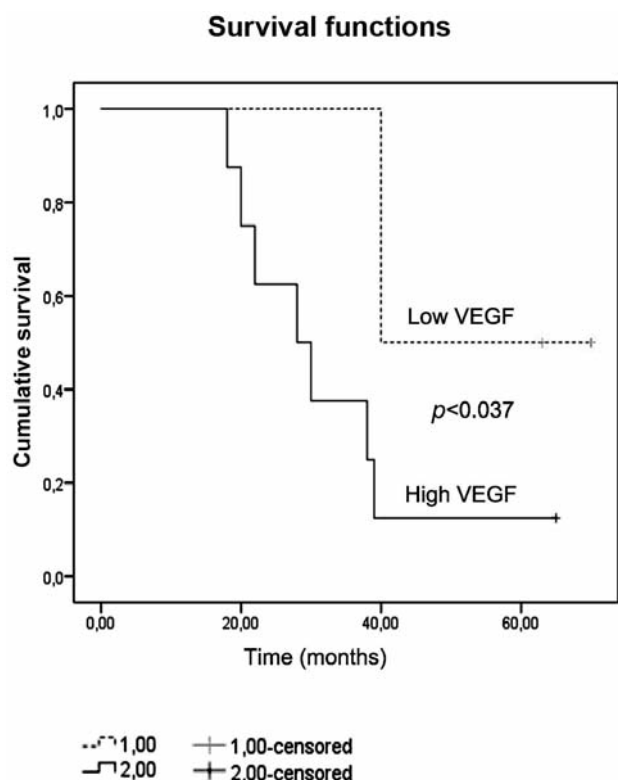


Figure 5. Kaplan-Meier curve for disease-free survival in relation to endothelial VEGF expression divided into groups of high and low expression. A percentage of positive cells  $<40$  and a percentage of positive cells  $>40$  were considered as low and high expression, respectively. The log-rank test was used to calculate significance.

vascular stability and lumen formation during the course of angiogenesis (33).

In our study, endothelial survivin-N expression was high in 14 out of 20 specimens (70%) and survivin-C in 11 out of 20 (55%); interestingly, in all cases of melanoma with a thickness  $>4$  mm. In addition, VEGF showed a high expression in melanocytes in 70% of melanoma specimens and in endothelial cells in 75% of specimens; moreover, endothelial VEGF expression correlated with MVD. Significant positive correlations were observed between melanocyte survivin expression and melanocyte VEGF expression and between endothelial cell survivin and endothelial VEGF expression. These positive correlations provide a cue that survivin may be strongly related with angiogenesis factors. A previous study on glioma demonstrated that tumor cells, which highly express survivin, not only drive their own proliferation but also provide adjacent tumor cells with growth factors, recruit angiogenesis-related cells into the region and promote tumour angiogenesis and, consequently, tumour progression (32).

Connor *et al.* (34) observed that survivin is a novel growth factor-inducible protective gene expressed by endothelial cells during angiogenesis. It has also been reported that survivin is induced by VEGF *via* a PI3 K/AKT pathway (35). Other researchers have suggested that the inhibition of apoptosis by survivin and the stimulation of tumour angiogenesis by VEGF may be interlinked and that they can influence each other through signal transduction networks collaboratively regulating the biological behavior of cancer (7). Many authors have reported that survivin expression was significantly associated with the presence of metastasis and, interestingly, correlated to an unfavourable prognosis in several tumours (27, 36). Our data highlighted that, in oral melanoma, a higher expression of melanocyte survivin and VEGF, together with a higher MVD, were inversely correlated with both overall and disease-specific 5-year survival in accordance with a study on primary cutaneous melanoma (28) and metastatic (stage IV) melanoma (37). We also observed that melanoma thickness was positively correlated with melanocyte VEGF expression and MVD. VEGF is known for sustaining tumour growth *via* its angiogenic properties but it can also elicit an inhibitory effect on dendritic cell differentiation and maturation, thus enhancing tumour survival (38-40).

In our study, oral melanoma thickness was also positively correlated with AKT; a significant positive correlation was found between melanocyte survivin and phospho AKT. In addition, AKT and melanocyte survivin-N expression correlated with the presence of metastasis. AKT activation has been reported to be linked to over-expression of survivin (14). Dhawan *et al.* (41) suggested that constitutive Akt activation plays an important role in human melanoma. Although it may not be essential for the initiation of melanoma, Akt activation facilitates melanoma progression, possibly by enhancing cell survival through up-regulation of the nuclear factor- $\kappa$ B and escape from apoptosis (14). Akt activation is required for both survivin-enhanced melanoma cell migration and invasion. McKenzie *et al.* (42) suggested a pathway whereby survivin promotes melanoma cell migration *via* Akt-dependent expression of the  $\alpha$ -5 integrin. The authors showed that survivin expression in melanocytes and its over-expression in melanoma cells enhanced cell migration and invasion (43).

In summary, the results of this report suggest that survivin, *via* AKT and VEGF, plays an important role in oral melanoma. We, thus, provide compelling evidence for the use of survivin as an important prognostic marker of melanoma progression. In addition, since resistance to conventional chemotherapy is the main obstacle in treating melanoma and the low therapeutic efficacy in this disease is likely due to a relative inability to induce apoptosis (44), we suggest that survivin, by activating a number of diverse downstream proliferative and antiapoptotic pathways, could be a promising target for future molecular based therapy.

Table II. Immunostaining of endothelial cell survivin, VEGF and MVD in oral melanoma patients.

Melanoma thickness mm (n. patients)	Metastasis n. patients (%)	Endothelial cells							
		Low Survivin-N(+) n. patients (%)	High Survivin-N(+) n. patients (%)	Low Survivin-C(+) n. patients (%)	High Survivin-C(+) n. patients (%)	Low VEGF n. patients (%)	High VEGF n. patients (%)	Low MVD n. patients (%)	High MVD n. patients (%)
<1.5 (3)	-	1 (33.3)	2 (66)	1 (33.3)	1 (33.3)	2 (66)	1 (33.3)	2 (66)	-
1.5-4.00 (9)	5 (55)	4 (44.5)	4 (44)	2 (22)	4 (44)	3 (33)	6 (66)	4 (44)	5 (55.5)
>4.00 (8)	7(87)	-	8 (100)	-	6 (66)	-	8 (100)	-	8 (100)

Low, Positive cells <40% of the total counted cells; High, positive cells >40% of the total counted cells; MVD, microvessel density; Survivin-N, nuclear survivin; Survivin-C, cytoplasmic survivin

## Conflicts of Interest

None declared

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