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CD31 and CD34 expression as immunohistochemical markers of endothelial transdifferentiation in human cutaneous melanoma

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Abstract. *Introduction*: Vasculogenic mimicry, as previously described in aggressive melanoma, is characterized by the *de novo* generation of intratumoral patterned vascular channels, composed of PAS-positive basement membrane in the absence of endothelial cells, providing additional microcirculation, in support to the classic tumoral angiogenesis. *Methods*: We investigated the immunohistochemical expression of two endothelial markers, CD31 and CD34, in tumoral cells of 60 melanomas (45 primary cutaneous and 15 metastatic) as possible evidence of vasculogenic mimicry. In addition we investigated the relationship between CD31 and CD34 expression and three pathological markers such as Clark's level, and skin ulceration, predictive of melanoma's aggressive behaviour, and mitotic index. *Results*: No cases of common melanocytic nevi immunoreacted with CD31 or CD34. Random CD31 immunoreactivity was present in 6% of Clark's level I/II, 50% of Clark's level III and 80% Clark's level IV/V. CD34 was negative in Clark's level I/II but randomly stained the 20% and 55% of level III and IV/V respectively. 66% (10/15) of metastatic melanomas were CD31 positive showing a canalicular immunostaining pattern, conversely CD34 expression was never found. 7/8 cutaneous ulcerated melanomas immunostained for CD31 and 4/8 for CD34. CD31 immunostained 88% high/intermediate MI, and 53% of low MI melanomas. CD34 decorated the 29% of high/intermediate and 38% of low MI melanomas. *Conclusions*: CD31 and CD34 immunoreactivity closely parallel both the different morphologic steps of melanocytic tumor progression and the presence of histological parameters related to the aggressive behaviour. Their expression could be related to endothelial transdifferentiation of melanoma cells although a consequent functional role has not been demonstrated yet.

Keywords: Malignant melanoma, vasculogenic mimicry, tumor-cell plasticity

Abbreviations: PAS, periodic acid-Schiff; MI, mitotic index.

1. Introduction

It is well known that in aggressive primary and metastatic melanomas, tumor cells generate, and subsequently line, acellular, microcirculatory patterned channels delimited by PAS-positive extracellular matrix. It has also been demonstrated that the presence of such PAS-positive *loops* and *network* patterned channels are associated to a worst clinical prognosis. On the whole, this *de novo* generation of non-endothelial cell-lined channels delimited by extracellular matrix, mimicking but not strictly representing a vasculogenic event, has been termed "vasculogenic mimicry" [13,20].

It is now assumed that vasculogenic mimicry is a complex process characterized by (i) the ability of aggressive melanoma cells to form extracellular matrix (ECM)-rich vasculogenic-like networks surrounding clusters of tumoral cells, when seeded on three-dimensional matrix of collagen I; (ii) the upregulation, as revealed by the gene-expression profiling in aggressive melanoma-cells, of genes involved in angiogenesis and vasculogenesis, such as the genes en-

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coding vascular endothelial (VE)-cadherin (CDH5), erythropoietin-producing hepatocellular carcinoma-A2 (EPHA2), and laminin 5 gamma2-chain (LAMC2); (iii) the activation of the vasculogenic-mimicry signalling cascade [17,27]. In the last five years, the vasculogenic mimicry has been described *in vivo* in a large series of malignant human tumours, and the association with aggressive tumour cells and advanced-stage disease has become evident both *in vivo* than *in vitro* [15,28,30]. The particular ability of aggressive tumor cells to generate patterned vascular-like networks could be a further attempt to provide an intratumoral paracirculation that form independently and/or simultaneously with angiogenesis and/or vessel co-option [2,8]. It is becoming evident that this intratumoral, tumor-cell-lined, ECM-rich, patterned network, can provide an extravascular fluid pathway, now known as the fluid-conducting meshwork [3,21]. Although a close association of tumor-cell-lined networks with angiogenic vessels has been revealed in the experimental models, in the human it is not actually clear whether a direct morphological anastomosis exists between the endothelium-lined vasculature and the tumor-cell-lined, fluid-conducting meshwork. Certainly this progressive tumor-host interaction is the result of the destructive tumor growth and remodelling, probably representing a form of survival mechanism that provides for the nutrient exchange.

Related to the vasculogenic mimicry, and to the gain of an endothelial phenotype, is the CD31 and CD34 expression by tumor cells. CD31 (PECAM-1; platelet/endothelial cell adhesion molecule-1) is a single chain type-1 transmembrane protein that plays a role in adhesive interactions between adjacent endothelial cells as well as between leukocytes and endothelial cells [25]. CD34 is a single-chain transmembrane glycoprotein expressed on haematopoietic precursor cells and on capillary endothelial cells [10]. The immunohistochemical detection of these two molecules has been largely used in microvessel density assessment in primary melanoma, providing reliable prognostic information on the risk of recurrence [6,7]. In 1999 Maniotis et al. in their pivotal article [20] described the CD31 immunohistochemical staining of uveal melanoma cells proximal to the patterned matrix-associated intratumoral channels whereas CD34 immunostaining was limited to the lumen contents around red blood cells. Subsequently, both the CD31 and CD34 over expression has been detected by molecular methods in aggressive breast, ovarian and prostatic tumor cells [15,28,30] associated with deregulation of the genes involved in vasculogenic mimicry. The present work was aimed at evaluating the immunohistochemical expression of CD31 and CD34 in common melanocytic nevi and malignant primary and metastatic melanoma cells. We assumed that these two molecules could be related to the acquisition of an endothelial phenotype in tumor cells, occurring during the vasculogenic-mimicry as expression of tumor cell plasticity. To date this is the first attempt to correlate the immunodetection of CD31 and CD34 in melanoma cells during tumor progression. Our final goal was to investigate the possible relationship between CD31 and/or CD34 expression and the presence of three histopathological markers related to aggressive behaviour in cutaneous melanomas such as Clark's level, skin ulceration and mitotic index [1,4,9].

2. Material and methods

2.1. Tissue specimens

Seventy cases of nevo-melanocytic tumors surgically resected between 2004 and 2005 were retrieved from the files of the Pathology Unit, I.R.C.C, Candiolo. The spectrum of selected lesions was so ranged: common melanocytic nevi (CMN; $n = 10$); *in situ*/microinvasive cutaneous malignant melanomas (Clark's level I-II; $n = 15$); invasive cutaneous malignant melanomas (Clark's level III-V; $n = 30$); malignant melanomas, metastatic ($n = 15$).

2.2. Histology and immunohistochemistry

Five micrometer tissue sections were cut from formalin-fixed, paraffin-embedded tissue blocks and stained with hematoxylin and eosin. Clark's level and presence/absence of skin ulceration were re-evaluated in 45 cutaneous malignant melanomas. Mitotic Index (number of mitotic figures / 10 High Power Field; three HPF $= 1\ mm^2$) was calculated in level III to V and metastatic melanomas and scored as follows: Low: $<$10 mitotic figures / 10 HPF; Intermediate: 10–20 mitotic figures / 10 HPF; High: $>$20 mitotic figures / 10 HPF. Serial tissue sections prepared for immunohistochemical analysis were deparaffinized and hydrated through a series of xylenes and alcohols. All sections underwent microwave antigen retrieval in 0.01 M citrate buffer pH 6.0. for 10 min. The sections were incubated with monoclonal mouse anti CD31 antibody

(clone JC70A; Dako, Carpinteria, Ca), dilution 4:100, or with monoclonal mouse anti CD34 antibody (Ylem, Rome, Italy), dilution 20:100, and then automatically immunostained on the Dako TechMateInstruments, using the ChemMate Detection Kit, Peroxidase/DAB, Rabbit/Mouse (Dako). The slides were finally washed for 5 min. in running tap water and then counterstained with Harris' hematoxylin.

2.3. CD31 and CD34 immunohistochemical evaluation and scoring:

- Negative: $<5\%$ immunostained tumoral cells $(-)$
- Positive: $\geqslant 5\%$ immunostained tumoral cells: $(+)$ 5–10%; $(++)$ $>10\%$

One of us (AMP) quantitatively rated the immunohistochemically stained slides. Particular care was taken to discriminate between tumor immunoreactive cells and CD31/CD34-positive endothelium.

2.4. Statistical analysis

The Chi-square test and Fisher's exact test (when appropriate) were used to infer proportions when assessing the degree of correlation among immunohistochemistry, histotypes and histological features. A probability value <0.05 was accepted as statistically significant.

3. Results

3.1. Histologic features

All the Clark's levels indicated in the Surgical Pathology Reports were confirmed. Skin ulceration was present in 8/45 cutaneous melanomas (all Clark's level IV and V). M I in cutaneous melanomas was ranked Low in 8/10 (80%) level III and 5/20 (25%) level IV/V, Intermediate in 1/10 (10%) level III and 8/20 (40%) level IV/V, High in 1/10 (10%) level III and 7/20 (35%) level IV/V. Eight out of 15 metastatic melanomas showed High M I; Intermediate (5/15) and Low (2/15) MI were calculated in the remaining two cases.

3.2. CD31-CD34 immunohistochemistry (Fig. 1)

3.2.1. Common melanocytic nevi

Both junctional and dermal melanocytes in 10 CMN were negative for CD31 and CD34. Both antibodies always decorated dermal endothelial cells, considered as positive control.

3.2.2. In situ/microinvasive malignant melanomas (Clark's level I/II)

One level II melanoma (1/15, 6.6%) immunoreacted with CD31 (p: n.s. vs. CMN). The antibody clearly decorated the cytoplasmic membrane in 5% of scattered tumoral cells located in papillary dermis. CD34 was negative in all cases. Dermal endothelium was always found immunoreactive.

3.2.3. Invasive malignant melanomas (Clark's level III)

CD31 and CD34 immunoreactivity was detected in 6/10 (60%; $p = 0.007$ vs level I/II) and 2/10 (20%; $p =$ n.s. vs level I/II) of Clark's level III invasive melanomas, respectively. CD31 decorated the cytoplasmic membrane of 5% of neoplastic cells in 5/6 cases and 15% in 1/6. CD34 decorated 5% and 10% of neoplastic cells in the two positive cases. Dermal endothelium was considered as positive control.

3.2.4. Invasive malignant melanomas (Clark's level IV/V)

CD31 and CD34 immunostained 16/20 (80%; $p =$ n.s. vs level III; $p = 0.003$ vs level I/II) and 8/20 (40%; $p =$ ns vs level III; $p = 0.006$ vs. level I/II) Clark's level IV/V malignant melanomas respectively. CD31 decorated the cytoplasmic membrane in 9.4% of tumoral cells randomly scattered or less frequently clustered in small groups throughout the lesion (Fig. 2A). CD34 was positive in 5.5% of tumoral cells, showing the same intratumor distribution (Fig. 2B).

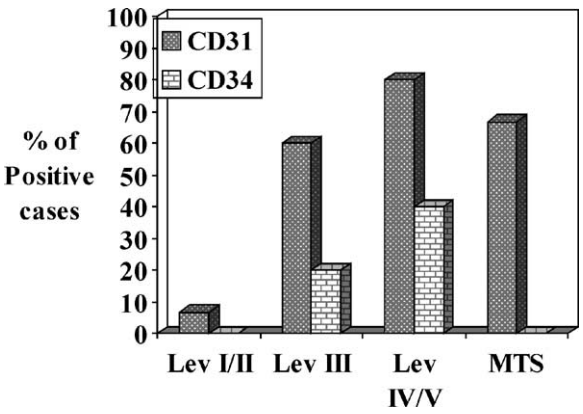

Fig. 1. CD31 and CD34 immunoreactivity in Clark's level I–V, and metastatic melanomas (MTS): graphic representation showing the percentages of immunolabeled cases.

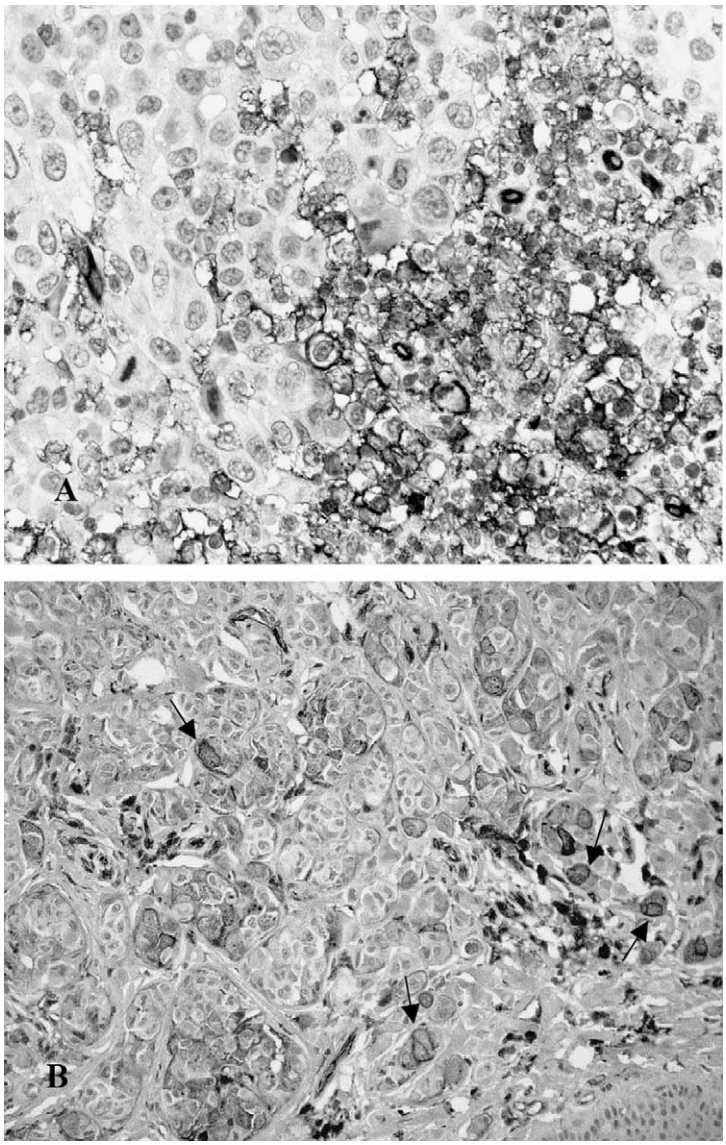

Fig. 2. CD31 (A) and CD34 (B) immunoreactivity in Clark's level IV melanoma: strong and complete immunostaining of cytoplasmic membranes in tumoral cells (*arrows*) (Original magnification ×400).

3.2.5. Metastatic melanomas

Ten out of 15 (66.6%)metastatic melanomas immunostained with CD31 (8/10 score + and 2/10 score ++). The antibody stained 6% tumoral cells mostly nested in scattered small groups (6–10 elements). The immunoreactivity selectively decorated only small segments of cytoplasmic membrane delineating a multifocal canalicular-like pattern (Fig. 3). Conversely, CD34 failed to stain tumor cells in all the 6 cases ($p = 0.006$ vs level IV/V).

3.3. CD31-CD34 immunohistochemistry: correlations with Mitotic Index and skin ulceration (Figs 4 and 5)

Eight of nine (88%) Intermediate and 7/8 (87.5%) High Mitotic Index-melanomas were CD31 positive. The average number of positive melanoma-cells ranged between 5–10% (score +) in the 87% of Intermediate MI group and in 71% of High MI-melanomas. CD31 also decorated 7 out of 13 (53.8%) Low MI-melanomas, four of which scored (+) and three scored

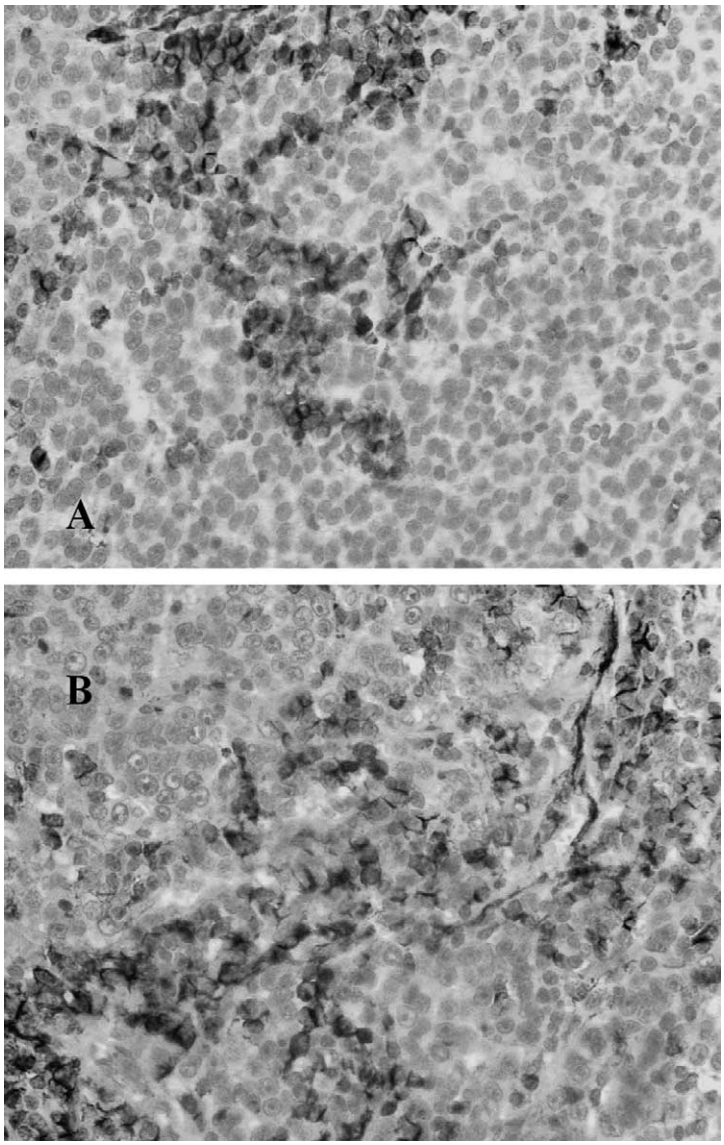

Fig. 3. CD31 immunoreactivity in metastatic melanoma: canalicular pattern (Original magnification ×400).

(++) (p: n.s. vs intermediate MI and High MI). CD34 immunostained 5/13 (38.4%) Low MI melanomas (score +), 3/9 (33.3%) Intermediate MI (2/9 score + ; 1/9 score ++) and 2/8 (25%) High MI melanomas. Finally, 7/8 (87.5%) of cutaneous ulcerated melanomas were CD31-positive (score +: 4 cases; ++: 3 cases) and 4/8 (50%) were CD34-positive (score +: 3 cases; ++: 1 case). Conversely, 15 out of 22 (68%), and 6 out of 22 (27%) nonulcerated melanomas turned out to be CD31+ and CD34+, respectively ($p = 0.03$ vs ulcerated melanomas).

4. Discussion

The induction of an adequate tumor vasculature in response to the increasing request of oxygen and metabolites is of paramount importance for tumor growth and progression. Access to the host vascular system and the generation of a tumor blood supply are rate-limiting steps in these processes. Most of the tumors, when entering the "vascular phase", provide their blood supply by activating angiogenesis ("angiogenic switch") [2]. Recently, a novel process of tu-

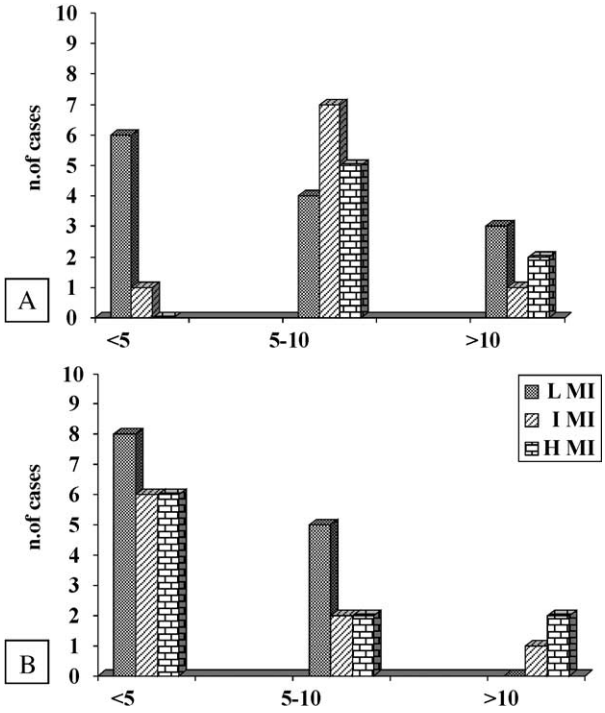

Fig. 4. Distribution of CD31 (A) and CD34 (B) in Clark's level III–V melanoma cells in relation to Mitotic Index (L = Low, I = Intermediate, H = High MI; <5, 5–10, >10: CD31 and CD34 immunohistochemical scoring.)

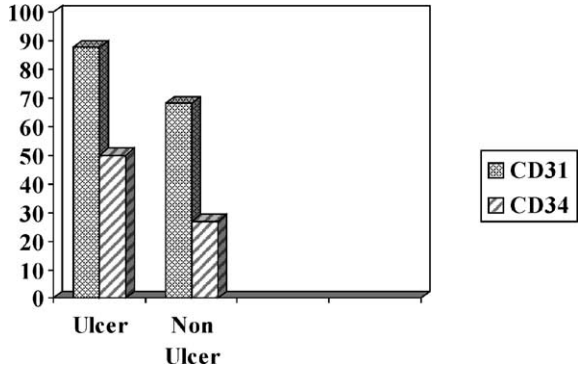

Fig. 5. CD31 and CD34 expression in ulcerated vs non ulcerated Clark's level III–V melanomas: graphic representation showing the percentages of immunolabeled cases.

mor perfusion has been described and termed "vasculogenic mimicry". Originally observed in aggressive melanoma cells, the vasculogenic mimicry refers to the tumor cells ability to express endothelium-associated genes and form a characteristic PAS-positive vasculogenic-like patterned network in three-dimensional culture. CD31 and CD34 are two endothelium-related molecules whose immunohistochemical expression has been described in human aggressive malignant melanoma in relation to the vasculogenic mimicry or associated to the ability of malignant melanoma to simulate a variety of soft tissue tumors [18]. In particular, CD31 immunoreactivity was demonstrated in primary uveal melanoma cells adjacent to the lumen of intratumoral vascular channels, in the absence of an endothelial lining. However the existence of a functional network providing a unique intratumoral paracirculation effectively anastomized with the endothelium-lined vasculature is still controversial [1,18,24], and its biological significance and prognostic role remain unclear [12, 19,22,23]. In this work the immunohistochemical expression of CD31 and CD34, never found in normal melanocytes, has been demonstrated in 22/30 (73%) and 10/30 (33.3%) invasive cutaneous melanoma cells, respectively. CD31, but not CD34, also stained 1/15 microinvasive melanoma and 10/15 melanoma metastasis. CD31 and CD34 expression closely parallel the morphologic phases of melanocytic tumor progression corresponding to Clark's levels of dermal infiltration and their associated metastatic potential. CD31 and CD34 immunoreactivity, in fact, was absent in the vast majority (93%) of *in situ* and microinvasive melanomas, and peaked the highest percentages of positive cells in invasive, Clark level IV and V melanomas. Furthermore, the expression of CD31 and CD34 in ulcerated melanomas (but not their distribution within the Intermediate and High M.I. groups) strongly support the existence of a direct correlation between the two vascular markers and the degree of aggressive behaviour in tumorigenic, Clark's Level III to V melanomas.

A patternless distribution of CD31 and CD34-positive tumor cells was found in all the invasive cutaneous melanomas. Immunohistochemical findings, therefore, do not seem to correspond the regular organization of PAS-positive *loops* and *networks* characterizing the vasculogenic mimicry [11,13,20]. The random immunoreactivity found in our cases could be related to the genotypical deregulation and to the ability of the few stained tumor cells to express multiple molecular phenotypes, rather than being associated to a specifically committed functional acquisition. In particular the appearance of markers associated with endothelial cells and their precursors could only represent a rough attempt of transdifferentiation, in accordance with the emerging concept of tumor cell plasticity [14,16]. The patternless and casual distribution, and

the relative low percentage of positive tumoral cells are consistent with an epigenetic event within a scenario in which the progressive and constant tumor growth dramatically increases the request of adequate blood supply that only the more structured angiogenic switch, blood vessel co-option and probably the classical vasculogenic mimicry can provide [26]. CD31 and CD34 immunohistochemical detection in human cutaneous melanomas could be therefore interpreted as an endothelial transdifferentiation attempt without a corresponding functional acquisition (i.e. ineffective contribute to tumor angiogenesis and/or absence of connection to the host vascular system). Alternatively, CD31 and/or CD34 immunoreactivity could be related to the increased expression of genes involved in vasculogenic mimicry, occurring in areas of tumor characterized by low oxygen tension, as described in experimental tumoral models under hypoxia [31]. In favour of this interpretation is the coexistence of skin ulceration and high MI in two invasive, level V melanomas characterized by the highest percentage of CD31 and CD34 positive cells. Conversely, in all the CD31-positive melanoma metastases, the peculiar, multifocal, canalicular-like immunoreactivity, although limited to a low percentage of tumoral cells, could represent a later phenomenon, probably related to the vascular transdifferentiation pathways besides vasculogenic mimicry, appearing in the most advanced step of neoplastic invasive growth [5] when the main effort is to implant and to expand an effective, blood sustained, metastatic growth, and to create an appropriate metastatic microenvironment.

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