Microinvasive Radial Growth Phase of Cutaneous Melanoma: A Histopathological and Immunohistochemical Study with Diagnostic Implications

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ABSTRACT Cutaneous melanoma (M) can develop through two progression phases: the radial growth phase of M (RGPM) and the vertical one. This distinction has a practical relevance in defining lesions with potential for a metastatic course. We analyzed the morphological attributes (intraepidermal proliferation type, inflammatory infiltrate, mitogenicity, Breslow thickness, Clark level, ulceration) and the immunohistochemical profile (S100, Melan A, HMB45, p16INK4a, CD117, Ki67, Cyclin D1, E Cadherin, Podoplanin) of 12 microinvasive RGPMs in absence of regression, with almost 10 years of follow-up. Immunohistochemistry (IHC) revealed that S100, Melan A, and HMB45 maintain a high expression in M cells in both epidermal and dermal compartments. Interestingly, an overexpression of p16INK4a in the nests of dermal microinvasion has been ascertained in all our cases. On the other hand, we found an attenuation of expression for CD117, Ki67, Cyclin D1, and E Cadherin in the migration phase from the epidermis to dermis. Each phase in M progression appears characterized by a specific immunohistochemical profile, as a result of molecular alterations. The long-term follow-up of our case series showed that microinvasive RGPM without regression is not tumorigenic and is devoid of metastatic potential; therefore, its accurate categorization is important. Conversely, microinvasive RGPM with regression should be classified as melanocytic tumor with uncertain biological potential. IHC for p16INK4a can be helpful in the diagnosis of microinvasive M on challenging cutaneous biopsies. Moreover, it can be applied as an immunohistochemical discriminator to distinguish microinvasive RGPM from *in situ* RGPM and microinvasive RGPM from dysplastic nevi.

KEY WORDS: melanoma, radial growth phase (RGP), p16INK4a (cdkn2a), CD117 (c-Kit), histology, immunohistochemistry

INTRODUCTION

Primary cutaneous melanoma (M) can develop through two well recognizable phases: the radial growth phase M (RGPM) and the vertical growth phase M (VGPM). This distinction derives from the concept of tumor progression and has a practical relevance in defining lesions with metastatic potential. The first complete and accurate description of RGPM was reported by Clark *et al*. in 1975, who described it

as a "lesion composed of melanoma cells within the epidermis and papillary dermis with a lack of biologic potential for metastasis in spite of invasion of the papillary dermis for some years" (1). The lesion is in fact characterized by a proliferation of malignant melanocytes confined to the epidermis (*in situ* M), or the malignant melanocytes invade the papillary dermis (microinvasive M), but, by definition, there is no evidence of tumor lump formation (2). RGPM is considered to lack metastatic competence (2,3). In microinvasive RGPM, the migration of neoplastic melanocytes into papillary dermis is often termed "invasion", but it is not necessarily accompanied by the acquisition of the capacity to survive, proliferate, or invade vessels and stroma (4,5). On the other hand, VGPM is characterized by the presence of true tumor nodules in the dermis. The two most important histologic features that help define the dermal invasion in VGPM are: dermal nests larger than any epidermal/junctional nest (expansive growth) and the presence of mitotic activity in the dermal component (6). All Ms at the third Clark level or deeper can be classified as VGPMs. For lesions at the second Clark level, the distinction between RGPM and VGPM can be difficult to make, and while inter-observer agreement among experienced dermatopathologists is good, concordance among non-specialists or less experienced observers is poor. In the present paper, we analyze the histopathological attributes and the immunohistochemical profile of 12 microinvasive RGPMs without regression, emphasizing that an accurate categorization of this kind of lesion is extremely important because it leads to excellent patient survival.

PATIENTS AND METHODS

The present study was a retrospective single-cohort study that included 12 cases of microinvasive RGPM, in absence of regression, diagnosed between 2003 and 2005 at the Institute of Pathology in Rovereto (Italy), with almost 10 years of follow-up and paraffin blocks available for immunohistochemistry (IHC). We adopted reproducible inclusion criteria for microinvasive RGPM.

Firstly, we defined microinvasive RGPM based on the presence of a few isolated melanoma cells or clusters in the papillary dermis, numbering up to 15 cells in individual sections with dermal nests smaller than those seen in the overlying epidermal component. Each epidermal component was categorized as lentiginous or pagetoid (nested). An increased number of atypical melanocytes along the dermal-epidermal junction were considered "lentiginous proliferation". In this case, the melanocytes were contiguous to each other and the proliferation was continuous;

the granules of pigment were often abundant and coarse, while the borders of the lesion were usually impalpable. Conversely, the pagetoid proliferation corresponded to an increased number of atypical epithelioid melanocytes, arranged singly and in nests near to the dermal-epidermal junction, with upward extension into the stratum corneum. In this instance, the nuclear atypia was moderate to severe; the borders of the lesion were often palpable.

Secondly, we defined regression as an area of increased vascularity and delicate fibroplasia in the papillary dermis, presenting a dermal infiltrate of lymphocytes and melanophages in absence of melanoma cells.

In our study, we excluded 25 cases of microinvasive RGPM with regression, 15 cases in which stained tissue sections were technically poor (8 cases with no residual invasion on IHC stained slides and 7 cases in which no consensus could be reached) and 15 cases in which the follow-up was not possible. Moreover, we considered only complete excisional biopsies and, therefore, a further 13 cases of microinvasive RGPM were excluded because the shave biopsies did not include complete tumors. All the slides were originally reviewed for routine histological attributes by authors without knowing patient outcomes. The reviewed attributes included: proliferation type of the intraepidermal component of microinvasive RGPM, band-like inflammatory infiltrate, dermal mitotic figures/mm^2 (mitogenicity), Breslow thickness, Clark level and ulceration. Eighteen sections were cut for each paraffin block (sub-seriation). After deparaffinization, hydration, endogenous peroxidase blocking, and heat-induced antigen retrieval, the tissue sections were incubated for 30 minutes at room temperature with anti-S100 protein (clone S1/61/69, prediluted; Leica Biosystems, Wetzlar, Germany), anti-Melan A (clone A103, prediluted; Leica), anti-Melanosome (clone HMB45, prediluted; Leica), anti-p16INK4a (clone E6H4, prediluted; Leica), anti-CD117 (clone T595, prediluted; Leica), anti-Ki67 antigen (clone MM1, prediluted; Leica), anti-Cyclin D1 (clone P2D11F11, prediluted; Leica), anti-E Cadherin (clone 36B5, prediluted; Leica), and anti-Podoplanin (clone D2-40 prediluted; Leica). A biotinylated secondary antibody was applied, and the staining product was detected with avidin-biotin complex (ABC) against a hematoxylin counterstain. Detection of the staining reaction was achieved by an enzyme-conjugated polymer complex adapted for automatic stainers from Leica Biosystems, with 3-3' diaminobenzidine tetrahydrochloride (DAB) or new fuchsine as chromogens. Each immunoreaction was evaluated by all authors and scored using a semi-quantitative method as follows: negative staining (-),
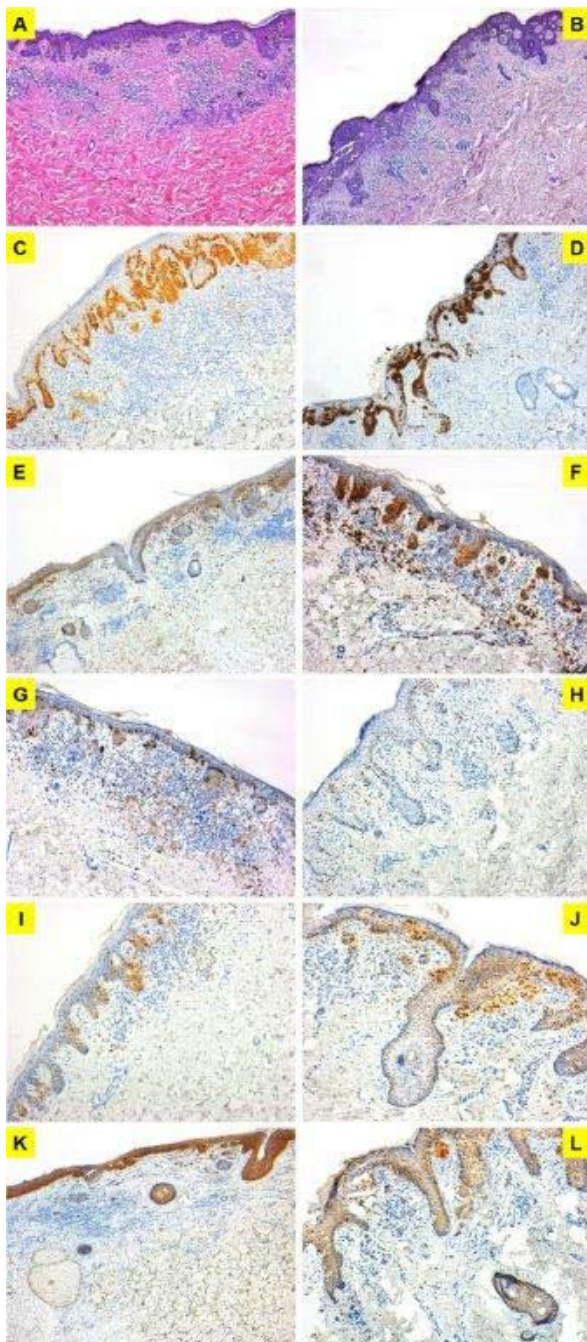
Figure 1. Imaging sequence of lentiginous (left row) versus nested (right row) type microinvasive radial growth phase melanoma (RGPM), in absence of regression, with related immunohistochemical profiles. (A) lentiginous type, hematoxylin and eosin (H&E) ×4; (B) nested type, H&E ×4; (C) anti-S100 protein, clone S1/61/69, prediluted, Leica Biosystems, Wetzlar, Germany, ×10; (D) anti-Melan A, clone A103, prediluted, Leica, ×4; (E) and (F) anti-CD117 clone T595, prediluted, Leica, ×4 and ×10; (G) and (H) anti-Ki67, clone MM1, prediluted, Leica, ×10 and ×10; (I) and (J) anti-Cyclin D1, clone P2D11F11, prediluted, Leica, ×10 and ×10; (K) and (L) anti-E Cadherin, clone 36B5, prediluted, Leica, ×4 and ×10).

low positive staining (+), moderate positive staining (++), and high positive staining (+++). The macroscopic features of the lesions were also considered, according to the ABCDE rule (asymmetry, border irregularity, color variegation, diameter, and evolution in time).

RESULTS

From the analysis of our case series, all microinvasive RGPMs were asymptomatic with no history of change, not all lesions were symmetric and flat or slightly raised, and they could be identified only by an alert physician. The age at the time of diagnosis ranged from 42 to 72 years with a mean of 59 years. The mean Breslow thickness was 0.4 mm (range 0.2-1.1 mm); all the lesions reached the second level of Clark. The epidermal component was pagetoid in 8 cases, while it was lentiginous in the remaining cases (Figure 1). The band-like inflammatory infiltrate was absent in 5 cases, not brisk in 2 cases, and brisk in the remaining cases. In 3 cases, 1 mitosis/mm^2 was observed, and in 1 case 2 mitotic figures/mm^2 were observed (no mitoses in the remaining cases). The length varied from 0.3 cm to 2 cm (mean 0.9 cm), while the width measured from 0.2 cm to 1.2 cm (mean 0.7 cm). Ulceration, nodules, and regression were absent (Figure 2). The immunohistochemical findings, reported in Table 1, revealed that S100 protein, Melan A, and Melanosome (HMB45) maintained a high expression in M cells in both epidermal and dermal compartments (Figure 1). Interestingly, an overexpression of p16INK4a in the nests of dermal microinvasion was found in all our cases (Figure 3). Conversely, an attenuation of expression in the migration phase from the epidermis to dermis was observed for CD117 (c-Kit), Ki67 antigen, Cyclin D1, and E Cadherin (Figure 1). The immunohistochemical assay for podoplanin,
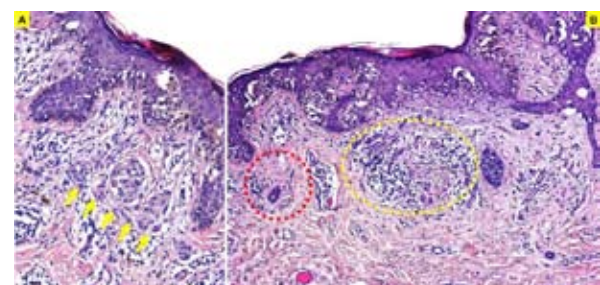

Figure 2. Microinvasive radial growth phase melanoma (RGPM) with regression: the yellow arrows point to nests of papillary dermal microinvasion: (A), hematoxylin and eosin (H&E) ×10. Areas of complete (red circle) or partial (yellow oval) regression can be observed: (B), H&E ×4. This histopathological variant has with unknown biological potential and for this reason it has been excluded by our series.

performed on every case, did not reveal any lymphatic invasion.

DISCUSSION

In microinvasive RGPM the recognition of papillary dermal microinvasion is facilitated by observing tumor cells separated from the junctional component through the interposition of dermal collagen, generally beneath the plane of the epidermal rete ridges or basal layer of the epidermis. Problems in the classification of dermal migration arise on histologically indeterminate cases, or when, as result of cross sections, large nests of melanocytes connected to the epidermis or adnexal structures appear free in the dermis. According to our findings, the IHC for p16INK4a protein and Melan A (or HMB45 or S100), together with serial sectioning, can resolve these disputes. In the analysis of our case series of microinvasive RGPM at the second Clark level, a precursor of tumorigenic VGPM, we constantly detected an immunohistochemical overexpression of p16INK4a in the nests of
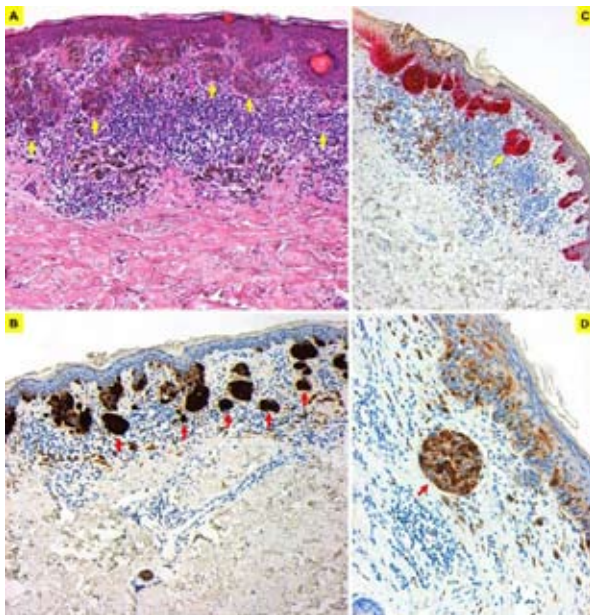

Figure 3. Microinvasive radial growth phase melanoma (RGPM) without regression: nests of papillary dermal microinvasion, which evoke a superficial band-like lymphocytic reaction, are indicated with yellow arrows: (A), hematoxylin and eosin (H&E) ×10. The intense brown-stained neoplastic nests overexpress p16INK4a protein: (B), red arrows: as revealed by immunohistochemical (IHC) assay: (B), clone E6H4, prediluted; chromogen 3-3′ diaminobenzidine tetrahydrochloride, ×10. A nest of dermal microinvasion (yellow arrow) is well red-stained by IHC for anti-Melan A: (C), clone A103, prediluted; chromogen new fuchsine, ×10. At higher magnification, the same nest (red arrow) shows an overexpression of p16INK4a protein if compared to the above epidermal compartment: (D), clone E6H4, prediluted, ×20.

dermal microinvasion. This finding appears to be a valuable aid in the diagnosis of microinvasive RGPM and agrees with the results reported by Strickler *et al.* (7). The authors have in fact examined the association of p16INK4a, WT1, and Fli-1 in 18 cases of RGPM and in 15 cases of VGPM: p16INK4a staining was strong in 15 RGPM (83%), WT1 in 17 RGPM (100%), and Fli-1 at least focally in 6 of 18 RGPM (33%). The infiltrative component of VGPM stained positively for Fli-1 in 9 of 14 cases (64%), intensely for WT1 in 10 of 14 cases (71%), and strongly for p16INK4a in only 2 of 15 cases (13%). RGPM tends to evoke a striking host response in the papillary dermis, typically a dense cellular infiltrate of lymphocytes and monocytes/macrophages in a perivascular or band-like fashion. In our series, the band-like inflammatory infiltrate was brisk in 5 cases, not brisk in 2 cases, and absent in the remaining cases. When a brisk infiltrate is encountered, the IHC for melanocytic cells, such as Melan A, HMB45, or S100, allows detection of single melanoma cells scattered among the inflammation in the papillary dermis, facilitating the achievement of a conclusive diagnosis.

In revising our cases, we noted that regression in RGPM can be complete or partial and diffuse or focal. Complete regression can be defined as an area within the melanoma where there is fibroplasia and usually lymphocytic infiltrate, often with melanophages and prominent vessels, but without melanoma cells in the dermis. Adjacent to this area of regressive fibroplasia, melanoma cells may be found in the epidermis, dermis, or in both. Partial regression is the disappearance of parts of the neoplasia without complete replacement of the tumor by inflammatory cells, melanophages, and fibrosis, and with focal persistence of melanoma cells. Theoretically, diffuse regression occurs when the entire tumor is affected by regression, while in focal regression the regression can only be found in a segment of the tumor. According to Guerry *et al.* (4), RGPM in absence of regression is biologically indolent and lacks metastatic competence. Some authors agree that RGPM regression is an adverse prognostic factor (8-9). Yun *et al.* (10) demonstrated that the adverse effect of RGPM regression is mediated through the increased lymphatic vessel density and/or lymphatic invasion in the area of complete regression, whereas a partial regression does not appear to be associated with worse prognosis. On the other hand, Kaur *et al.* (11) proposed a histopathological staging system for regression, concluding that regression is associated with a favorable prognosis in thin Ms. An Australian study on 1716 patients with RGPM showed metastases in 67 patients (3.9%), in all of whom M showed regression in their primary tu-

Table 1. Semi-quantitative immunohistochemical scoring for the epidermal (green) and dermal (red) components of the 12 studied cases of microinvasive radial growth phase melanoma (RGPM) without regression. The relative ratios for each immuno-marker are shown in parenthesis

IMMUNOHISTOCHEMICAL MARKER	EPIDERMAL COMPARTMENT (ratio)				DERMAL COMPARTMENT (ratio)			
S100 protein				+++ (12/12)				+++ (12/12)
Melan A				+++ (12/12)				+++ (12/12)
Melanosome (HMB45)				+++ (12/12)				+++ (12/12)
p16INK4a		+ (2/12)	++ (10/12)					+++ (12/12)
CD117 (c-Kit)			++ (12/12)		- (6/12)	+ (6/12)		
Ki67 antigen			++ (12/12)		- (2/12)	+ (10/12)		
Cyclin D1			++ (12/12)		- (9/12)	+ (3/12)		
E Cadherin			++ (12/12)		- (10/12)	+ (2/12)		

mors (12). In our opinion, the variable results in these reported studies may be explained by the different criteria used to define regression (complete vs partial; diffuse vs focal). In the present study, we have excluded 25 cases of microinvasive RGPM with regression because we believe that microinvasive RGPM with regression should be not considered non-tumorigenic, but rather a variant of RGPM with uncertain biological potential (13). For this reason, in these cases the use of the term "non-tumorigenic" is to be avoided and sentinel node biopsy should be considered, independently from the Breslow thickness.

Starting from the assumption that the main genes implicated in the pathogenesis of melanoma are CD-KN2A, encoding for p16INK4a, and several transcript variants such as p14ARF, C-KIT, MITF (microphthalmia-associated transcription factor), N-RAS, and B-RAF (14), we investigated the first two genic pathways immunohistochemically, being the two most suitable to date from an immunohistochemical point of view, together with the cell cycle (Ki67, Cyclin D1) and intercellular adhesion (E Cadherin).

Our immunohistochemical results have demonstrated an overexpression of p16INK4a in the dermal compartment for microinvasive RGPM, but also an attenuation of expression in the M cells for CD117, Ki67 antigen, Cyclin D1, and E Cadherin during the migration phase from the epidermis to the dermis. These findings support the concept that each phase in M progression is characterized by a specific immunohistochemical profile as a result of molecular alterations.

Firstly, the expression profile of CD117, the mast/stem cell growth factor receptor, was decreased in the dermal compartment with respect to the epidermal one in all microinvasive RGPMs of our series. Similarly, Montone *et al.* (15) found that CD117 is expressed in normal melanocytes, benign nevi, dysplastic nevi, and non-tumorigenic RGPM but not in tumorigenic M and metastases. In the model of M progression (RGPM followed by VGPM), Alexeev *et al.* (16) hypothesized that melanoma cells lose CD117 expression to acquire proliferative advantage and escape from epidermal boundaries, and our results support this hypothesis.

Secondly, the proliferative activity in RGPM is practically confined to the lesional cells in the epidermal compartment. The dermal microinvasive cells

show low reactivity for Ki67 antigen because the melanoma cells are not tumorigenic in RGPM without regression. The immunohistochemical assay for Ki67 antigen can thus be so considered a useful test to exclude potentially tumorigenic M. Similar findings have been reported by others authors (17-18). For example, Gimotty *et al.* (19) demonstrated that dermal Ki67 expression was lower than epidermal Ki67 expression in RGPM and that dermal Ki67 expression and dermal mitotic rates were higher in VGPM in comparison with RGPM. These data support that only tumorigenic M exhibits significant proliferative activity in the dermis.

Thirdly, Cyclin D1 maintained the same expression profile of CD117, Ki67, and E Cadherin in our immunohistochemical investigation. Alonso *et al.* (20) examined 165 M samples from 88 patients corresponding to RGPM, VGPM, and metastases. The authors found that cyclin D1 was expressed in 48% of RGPMs (12 of 25), with significant loss of expression in VGPM. Alonso *et al.* showed that melanoma cells progress through the deregulation of the molecular mechanisms, controlling proliferation and programmed death, in accord with the article by Molumbres and Barbacid (21).

Fourthly, the adhesiveness loss of melanoma cells to both basal laminae and adjacent keratinocytes, consequent to a downregulation of the cell adhesion protein E Cadherin (22), promoted tumor microinvasion in RGPMs in our case series.

CONCLUSION

Long-term follow-up in our case series has shown that microinvasive RGPM without regression is not tumorigenic and is devoid of metastatic potential. Conversely, microinvasive RGPM with regression should be classified as melanocytic tumors with uncertain biological potential. The IHC for p16INK4a protein allows confirmation of dermal microinvasion and can be helpful in the diagnosis of microinvasive M in cutaneous biopsies of difficult interpretation. In particular, it can be applied as an immunohistochemical discriminator to distinguish microinvasive RGPM from *in situ* RGPM and microinvasive RGPM from dysplastic nevi.

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