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Loss of dipeptidyl peptidase IV immunostaining discriminates malignant melanomas from deep penetrating nevi

Alexander Roesch, Sina Wittschier, Bernd Becker, Michael Landthaler and Thomas Vogt

Department of Dermatology, University of Regensburg, Regensburg, Germany

The deep penetrating nevus is a rare variant of benign melanocytic nevus with histologic features mimicking vertical growth phase, nodular malignant melanoma. In this study, we expand on the search for new complementary discriminating markers by analyzing a selection of both cell cycle-related factors, such as retinoblastoma protein and phospho-retinoblastoma protein Ser795 as indicators for retinoblastoma protein activation/inactivation status, and invasion-related factors, such as matrix metalloproteinase-1, matrix metalloproteinase-2, membrane-type matrix metalloproteinase-1 and integrin β3. MIB-1/Ki-67 was analyzed as an example for a common proliferation marker. Dipeptidyl peptidase IV/CD26 was analyzed as a marker affecting both proliferation and invasion of malignant melanocytic tumors. Semiquantitative assessment of both immunolocalization and immunoreactivity of retinoblastoma protein and phospho-retinoblastoma protein Ser795, MIB-1/Ki-67, matrix metalloproteinase-1, matrix metalloproteinase-2, membrane-type matrix metalloproteinase-1 and integrin β3 revealed no consistent differences between deep penetrating nevi ($n=14$) and matched cases of nodular malignant melanomas ($n=10$). Matrix metalloproteinase-1 and matrix metalloproteinase-2 immunostaining of some deep penetrating nevi even exceeded that of nodular malignant melanomas. Membrane-type matrix metalloproteinase-1 expression scores of nodular malignant melanomas were higher than those of deep penetrating nevi, which was, however, not significantly discriminative. In contrast, immunostaining of dipeptidyl peptidase IV was significantly discriminative due to a consistent lack of dipeptidyl peptidase IV-expression in nodular malignant melanomas. These results add evidence that among the selected markers supposed to be relevant for melanoma progression the presence of dipeptidyl peptidase IV can be used to support diagnosis of deep penetrating nevi in doubtful cases. As loss of dipeptidyl peptidase IV may also be causally linked to the transition of invasive to metastatic phenotypes, the molecular mechanisms downstream of dipeptidyl peptidase IV deserve to be studied in more detail in future investigations.
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In 1989, Seab *et al*[1] reported a series of benign invasive pigmented melanocytic tumors and coined the term deep penetrating nevus. Barnhill *et al*[2,3] reported a histomorphologically similar melanocytic lesion using the term plexiform spindle cell nevus. However, the plexiform spindle cell nevus is more plaque-like and surrounds the superficial neurovascular plexus. Deep penetrating nevi mostly appear as darkly pigmented papules or nodules with no or mild epidermal changes. They are most commonly found in the face, on the upper trunk or proximal extremities of patients at the age of 10–30 years.[1,4] Histopathologic growth patterns are often worrisome showing a wedge-shaped invasive growth extending from the upper dermis into the subcutaneous fat tissue not rarely following preformed structures, for example hair follicles or sweat glands.[1,5]

Cytological pleomorphism defined as a variation of cell size and shape together with hyperchromasia, one hallmark of malignant melanomas, is reported to occur also in the deeper, invasive portions of deep penetrating nevi.[5] Further deep penetrating nevus features that complicate the distinction from malignant melanoma are the presence of inflammatory stroma reactions observed in 75% of deep penetrating nevi, lack of melanocytic maturation in the deep portion and even some possible degree of lesional asymmetry.[4–6] In some cases, only histological features together with clinical follow-up information can confirm that a lesion was truly benign.

Correspondence: Dr A Roesch, MD, Department of Dermatology, University of Regensburg, Franz-Josef Strauss-Allee 11, D-93053 Regensburg, Germany.
E-mail: alexander.roesch@klinik.uni-regensburg.de
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Thus, as several studies have emphasized, deep penetrating nevi are often mistaken for vertical growth phase nodular malignant melanomas, both clinically and histologically.[1,2,5,7–10] Estimates exist that, depending on the criteria used for classification, misdiagnoses as melanoma occur in 29–40% of the cases.[1,4,5] The opposite, that is taking advanced melanomas as deep penetrating nevi may also occur.

Unfortunately, to this end immunohistochemical analyses also failed to differentiate deep penetrating nevus from nodular malignant melanoma.[1,11] To the best of our knowledge, to date, only one study exists suggesting PCNA (proliferating cell nuclear antigen) as a possibly discriminating marker.[12] PCNA represents an accessory protein of DNA δ-polymerase which is increased during the late G1 growth phase and peaks in the S phase of the cellular cycle.

Beyond the clinical demand for precise diagnosis, the deep penetrating nevus may also represent a valuable natural model for melanocytic invasion without metastatic potential, thereby, querying the role of MMPs in melanomas. Here, we expand on the search for complementary discriminating markers analyzing either pRb/phospho-pRb and MIB-1/Ki-67 as a direct read-out of cell cycle progression and proliferation or invasion-related proteins such as MMPs or, finally, a marker related to invasion as well as proliferation, DPPIV.

Materials and methods

Tissue Samples and Immunohistochemistry

Tumor material and clinical follow-up information of 14 deep penetrating nevi and a set of matched nodular malignant melanomas ($n=12$) was collected at the Department of Dermatology, University of Regensburg, Germany. Written consent of the patients was obtained prior to surgery. Conventional paraffin embedded tissue sections were deparaffinized and rehydrated according to standard protocols. After inhibition of endogenous peroxidase activity with hydrogen peroxide, three different protocols for antigen retrieval were performed: (1) For MIB-1/Ki-67, integrin β3-, phospho-pRb Ser795- and total pRb-staining, the sections were incubated at 100°C for 30 min with citrate buffer (pH 6.0). (2) For DPPIV-detection, EDTA buffer (pH 8.0) was used. (3) MMP-1-, MMP-2 and MT1-MMP-staining required pretreatment with 150 μl pepsin for 25 min at 37°C. After washing with PBS, samples were blocked with SuperBlock™ (Zytomed, Berlin, Germany) and subsequently incubated with a 1:100 dilution of the primary antibody for 35 min at 37°C. For detection of total pRb and phosphorylated pRb, *Rb (4H1) Monoclonal antibody* and *Phospho-Rb (Ser795) Monoclonal antibody*, respectively, were used from Cell Signaling Technology/New England Biolabs GmbH, Frankfurt, Germany. Ki-67 was detected by the *MIB-1 antibody*, DakoCytomation, Glostrup, Denmark. Integrin β3-, MMP-1-, MMP-2- and MT1-MMP-detection was performed with *anti-human-CD61 (CBL479)-*, *anti-human-MMP-1 (MAB3307)-*, *anti-human-MMP-2 (MAB13405)-* and *anti-human-MT1-MMP (MAB3317)*-antibodies from Chemicon, Hampshire, UK. *Anti-human-CD26 (D068-1)* antibody from MBL, Woburn, USA was used for DPPIV-staining. For detection of all primary antibodies, the avidin–biotin complex method was performed using a biotinylated secondary antibody (ZytoChemPlus Anti-Broad Spectrum™) together with avidin-conjugated horseradish peroxidase (ZytoChemPlus HRP™ Zytomed, Berlin, Germany) and AEC Substrate Chromogen™ (DakoCytomation, Hamburg, Germany) according to the manufacturers' recommendations.

Immunohistochemical Evaluation

To reduce bias, immunostaining was assessed by two independent investigators (AR, SW) in a blinded fashion. Inter- and intra-examiner reproducibility was 82 and 88%, respectively. Immunoreactivity was scored using uniform criteria to maintain the reproducibility of the method. The relative quantity of immunostaining was recorded considering the expression in tumor cells (nuclear, cytoplasmatic or membranous staining) as well as in the surrounding extracellular matrix (in case of MMPs).

Considering nuclear staining of total pRb, pRb Ser795 and MIB-1/Ki-67, for each sample, three representative fields of vision were evaluated at $\times 400$ magnification in a tumor region with maximum and a region with minimum staining as described previously.[13,14] Briefly, the number of positive stained nuclei was estimated as percentage of all nuclei (p) per field of vision. In addition, the staining intensities (i) of positive cells were scaled from grade 1, when the cells showed a slight granular staining pattern, to grade 4, when the cells had completely filled nuclei. Grades 2 and 3 were assigned to intermediate staining intensities. Subsequently, a maximum and minimum expression score (ES_{max}, ES_{min}) were calculated for each tumor sample according to following formula shown below. To facilitate comparability among different markers, in this study, the expression information of the whole tumor (ES_{total}) was calculated as average value from ES_{max} and ES_{min}.

$$ES_{max} = \frac{(p_1 + p_2 + p_3)}{3} \times \frac{(i_1 + i_2 + i_3)}{3}$$

$$ES_{min} = \frac{(p_1 + p_2 + p_3)}{3} \times \frac{(i_1 + i_2 + i_3)}{3}$$

$$ES_{total} = \frac{ES_{max} + ES_{min}}{2}$$

p_x is the percentage of stained cells per field of vision X, i_x the staining intensity of positive

cells per fields of vision X, ES the expression score.

Integrin β3- and DPPIV exhibit a more complex staining pattern in both entities the deep penetrating nevus and nodular malignant melanoma, respectively. Consequently, the mean staining percentage (*p*) and intensity (*i*) were semiquantitatively assessed considering the whole tumor section and not selected fields of vision. Total expression scores (ES_{total}) were calculated in accordance with the algorithm shown above.

Concerning MMP-1, MMP-2 und MT1-MMP also the whole tumor section including stromal surroundings was considered. However, the staining intensity (*i*) was not taken into account because intensities were invariable if staining was possible.

$$ES_{total} = \frac{(p_1 + p_2 + p_3)}{3}$$

Statistical Analysis

The SPSS 13.0 (SPSS Inc, Erkrath, Germany) software package was used to perform statistical analyses. For assessment of relative protein expression levels the nonparametric Mann–Whitney *U*-test was applied.

Results

Immunohistochemical Staining of Cellular Proliferation Markers: Consistency with Previous Studies, but Inapplicability as Discriminating Markers

Since a previous study has suggested differential expression of PCNA[12] in deep penetrating nevi *vs* melanomas, we were interested if this is also true for the common proliferation marker MIB-1/Ki-67 which is expressed from the mid-G1 phase of the cell cycle through S, G2 and M-phases and the major central cell cycle regulator, pRb. As reported recently by our group, a paradox progressive overexpression of the cell cycle controlling pRb and its increased inactivation due to phosphorylation are hallmarks of malignant melanomas.[13]

According to our former observations,[13] there was an intratumoral heterogeneity of total pRb and phospho-pRb Ser795 expression in nodular malignant melanomas. Particularly at the subepidermal lateral and dermo-invasive portions, both markers revealed high expression scores in most samples ($ES_{max} > 100$). In contrast to our previous findings on common benign nevi,[13] 33% of the deep penetrating nevus specimens showed a melanoma-like high deep dermal total pRb expression with $ES_{max} > 100$. Furthermore, even 50% of the deep penetrating nevi evinced a dermal infiltration of phospho-pRb Ser795 positive cells indicating inactive pRb, that is a cell cycle permissive status, very similar to nodular malignant melanomas. In addition to this accumulation of immunoreactivity in the tumor periphery, the majority of deep penetrating nevi showed staining signals for both markers in a more diffusely distributed pattern. In case of pRb, 67% and, in case of phospho-pRb Ser795, 80% of the deep penetrating nevi evinced such diffuse immunolocalization.

The determination of total expression scores (ES_{total}, see Materials and methods) revealed a higher expression of pRb and phospho-pRb Ser795 in nodular malignant melanomas (mean $ES_{total-pRb}$ 143, mean $ES_{total-p-pRb\ Ser795}$ 63) than in deep penetrating nevi (mean $ES_{total-pRb}$ 70, mean $ES_{total-p-pRb\ Ser795}$ 53), a finding consistent with our latest data on common benign nevi *vs* nodular malignant melanomas[13] and with previous data on PCNA[12] (Figure 1a and b). Since this differential regulation was only significant for total pRb ($P_{pRb} < 0.005$, $P_{p-pRb\ Ser795} < 0.1$), applicability of the pRb/phospho-pRb ratio as discriminative marker for deep penetrating nevi remains doubtful.

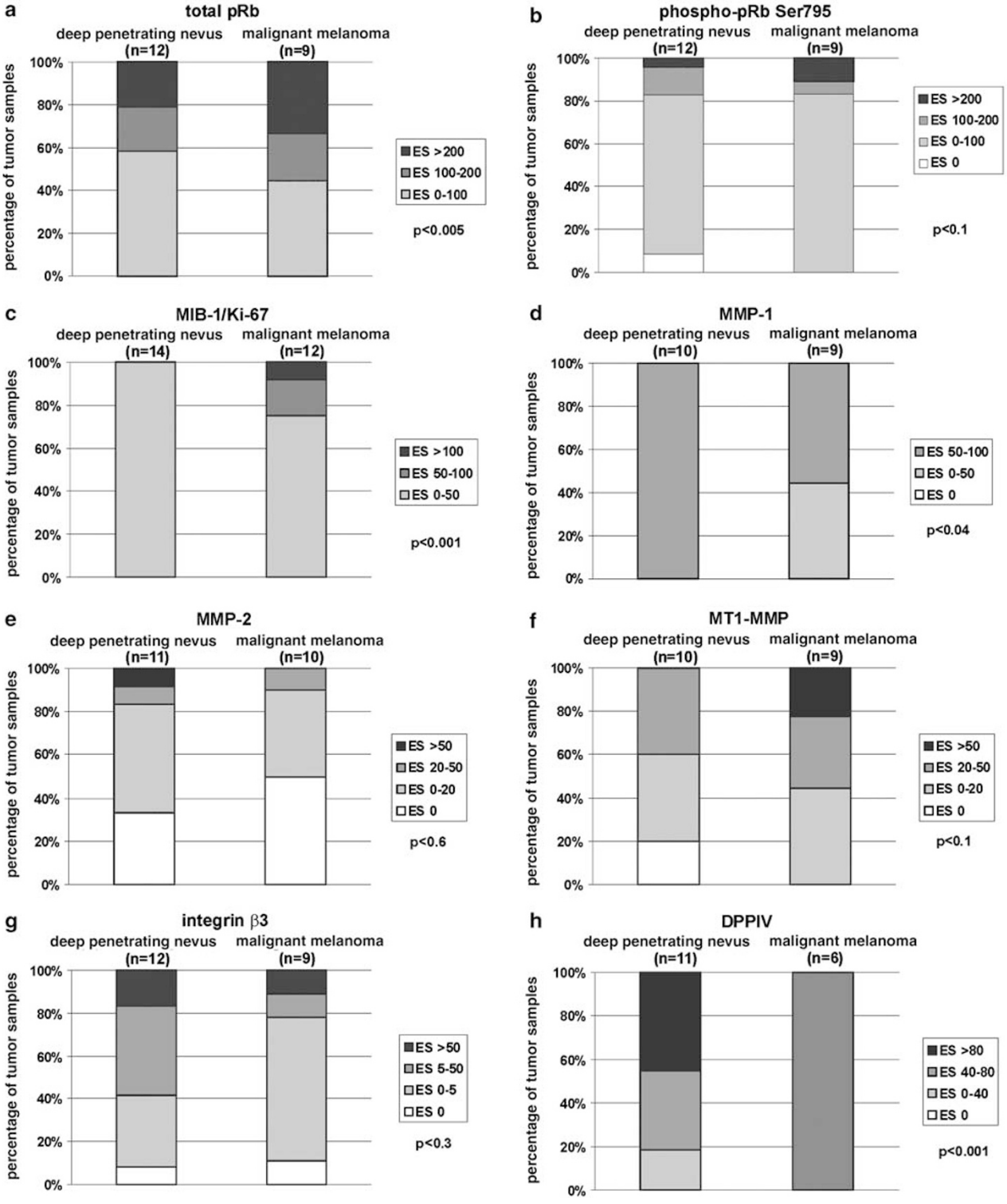
Also the evaluation of MIB-1/Ki-67 staining revealed a statistically significant trend to higher

Figure 1 Comparative immunohistochemical staining of cell proliferation-related factors, such as retinoblastoma protein (pRb), phospho-pRb Ser795 and MIB-1/Ki-67, as well as invasion-related factors, such as matrix metalloproteinase-1 (MMP-1), MMP-2, MT1-MMP and integrin β3. Dipeptidyl peptidase IV/CD26 (DPPIV) was analyzed as a marker affecting both proliferation and invasion of malignant melanocytic tumors. (**a**) Semiquantitative evaluation of total pRb staining of 12 deep penetrating nevi and 9 nodular malignant melanomas shown as percentage of samples within three categories of expression scores (for definition see Materials and methods). There is a statistically significant trend to higher scores in nodular malignant melanomas (*U*-test $P < 0.005$), but practically this marker might not be applicable for differentiation due to its broad intra- and intertumoral heterogeneity in immunoreactivity. (**b**) Evaluation of phospho pRb Ser795 staining within four categories of expression scores. There is a trend to higher scores in nodular malignant melanomas, but also not applicable for differentiation (*U*-test: $P < 0.1$). (**c**) Evaluation of MIB-1/Ki-67 staining shows a statistically significant trend to higher scores in nodular malignant melanomas (*U*-test $P < 0.001$). However, practically this marker might not be applicable for differentiation due to its high intertumoral expression variance. (**d**) MMP-1 expression scores showing a statistically significant trend to increased expression in deep penetrating nevi compared to nodular malignant melanomas (*U*-test $P < 0.04$). (**e**) MMP-2 expression scores suggesting an increased expression in deep penetrating nevi, however, also not applicable due to lacking statistical significance (*U*-test $P < 0.6$). (**f**) Increase of the overall MT1-MMP expression in nodular malignant melanomas compared to deep penetrating nevi, statistically not significant (*U*-test $P < 0.1$), probably due to broad inter- and intratumoral variance. (**g**) 'Melanoma-mimicking' integrin β3 expression in deep penetrating nevi, that is not significant based on the ES values (*U*-test $P < 0.3$). (**h**) DPPIV truly discriminates between deep penetrating nevi and nodular malignant melanomas (*U*-test $P < 0.001$). In contrast to all other markers analyzed, only DPPIV shows both a consistent immunoreactivity, that is low intertumoral heterogeneity, as well as a high statistical significance.

scores in melanomas (mean $ES_{total-MIB-1/Ki-67}$ was 31 for nodular malignant melanomas *vs* three for deep penetrating nevi, *U*-test $P<0.001$). However, practically this marker might not be applicable for differentiation due to its high intertumoral expression variance in melanomas (SD for nodular malignant melanomas was 51).

Immunohistochemical Staining of Markers Representing Invasive Potential: New Aspects of Differential Expression, but Unclear Applicability as Discriminating Markers

Degradation and remodeling of the tumor surrounding extracellular matrix is considered as an essential step in melanoma progression. As repeatedly described,



the increased coexpression of a subset of matrix metalloproteinases (MMP-2, MT1-MMP) and integrin $\beta 3$ is correlated with early melanoma invasion.[15–18] The inductio n of MMP-1 is reported to be a late event marker during cell invasion of advanced melanomas.[19]

The signal distribution of MMP-1, MMP-2, MT1-MMP and integrin $\beta 3$ revealed a complex expression pattern, notably in both analyzed entities. Beside high expression signals at the dermo-invasive front, most tumor samples additionally showed a focal staining pattern in association with cutaneous adnexes and sometimes a more diffuse staining pattern was observed. Concerning the discriminative power, none of the staining patterns, that is focal, diffuse or dermo-invasive, could validly separate deep penetrating nevi from nodular malignant melanomas. Interestingly, the invasive front staining of MMP-2 and integrin $\beta 3$, seen in 46 and 36% of the deep penetrating nevi, even exceeded the fraction of positive nodular malignant melanomas (20 and 11%). Subepidermal MT1-MMP signals were found in 78% of all nodular malignant melanomas *vs* 10% of deep penetrating nevi.

Concerning the relative distribution of total expression scores, integrin $\beta 3$ (Figure 1f) and MMP-1 (Figure 1c) appeared approximately equal within both entities. According to this, the mean $ES_{total-integrin\ \beta 3}$ of both entities did not differ significantly ($P<0.3$). The comparative analysis of $ES_{total-MMP-1}$ of deep penetrating nevi and nodular malignant melanomas only revealed a weak upregulation in deep penetrating nevi with $P<0.04$. Semiquantitative expression of MMP-2 in deep penetrating nevi (mean $ES_{total-MMP-2}$ 14) even exceeded that of nodular malignant melanomas twice as much (mean $ES_{total-MMP-2}$ 8, Figure 1d). However, since this difference could not reach statistical significance ($P<0.6$), this parameter seems also not applicable for discrimination. The relative distribution of MT1-MMP expression scores showed an inverse staining result with higher values in melanoma samples (mean $ES_{total-MT1-MMP}$ 36) than in deep penetrating nevi (mean $ES_{total\ MT1-MMP}$ 19, Figure 1e), but this regulation also failed to reach statistical significance ($P<0.1$).

Immunohistochemical Staining of DPPIV: a Possible Marker for Discrimination Between Deep Penetrating Nevi and Nodular Malignant Melanomas

Previous studies have shown that DPPIV is almost invariably lost during melanoma progression, which may affect both melanoma proliferation and invasion.[20–22]

In contrast to the other markers in this study, DPPIV staining clearly discriminates the two entities (Figure 1g). The mean $ES_{total-DPPIV}$ for deep penetrating nevi (87) significantly exceeded the mean $ES_{total-DPPIV}$ of nodular malignant melanomas (9) with $P<0.001$. All deep penetrating nevi stained positive. 46% ($n=5$) of all deep penetrating nevi showed an $ES_{total-DPPIV}>80$ and 36% ($n=4$) revealed an ES_{total} between 80 and 40.18% ($n=2$) had an ES_{total} lower than 40. In contrast, the highest ES_{total} reached in a nodular malignant melanoma was 38. All other melanoma samples showed expression scores lower than 10. The intratumoral immunolocalization of DPPIV in both entities showed a diffuse expression pattern with focal accumulation close to cutaneous adnexes or at the tumor periphery (Figure 2).

Discussion

Distinction of primary melanoma and deep penetrating nevus was always the major topic when the deep penetrating nevus was investigated in the past. In the first study on deep penetrating nevi, Seab[1] tried to make a distinction from melanoma using the proliferative capacity estimated by simply counting the number of mitoses. Lack of mitoses in deep penetrating nevi and the absence of a rapid clinical growth were suggested as critical features of the deep penetrating nevus. Also results from Mehregan[12] demonstrated subtle differences of PCNA expression in deep penetrating nevi seemingly corroborating Seab's data.

Consistent with our previous findings on the progressive increase of the major cell cycle switch pRb during melanoma development,[13] semiquantitative immunoreactivity of pRb significantly outweighed in nodular malignant melanomas compared to deep penetrating nevi. However, in contrast to these former observations also reporting a progressive increase of phosphorylated, inactive pRb, here, the differential expression of phospho-pRb Ser795 lacked statistical significance, particularly due to broad intertumoral staining variations. Strikingly, compared to the results on common benign nevi,[13] both pRb and phospho-pRb Ser795 showed a high deep dermal immunolocalization in a considerable subset of the deep penetrating nevus specimens indicating inactive pRb, that is a cell cycle permissive status, very similar to nodular malignant melanoma.

Over the last years, MIB-1/Ki-67 expression has been repeatedly discussed to be correlated with disease progression and prognosis in large cohorts of melanoma patients.[23,24] However, for differentiation of single indistinct cases of deep penetrating nevus from nodular malignant melanoma, this marker might practically not be applicable due to its high intertumoral expression variability, even in thick melanomas.[24]

Next to cellular proliferation, remodeling of the tumor-surrounding stroma by matrix metalloproteinases is considered as a most essential step for tumor progression.[25] In accordance with previous

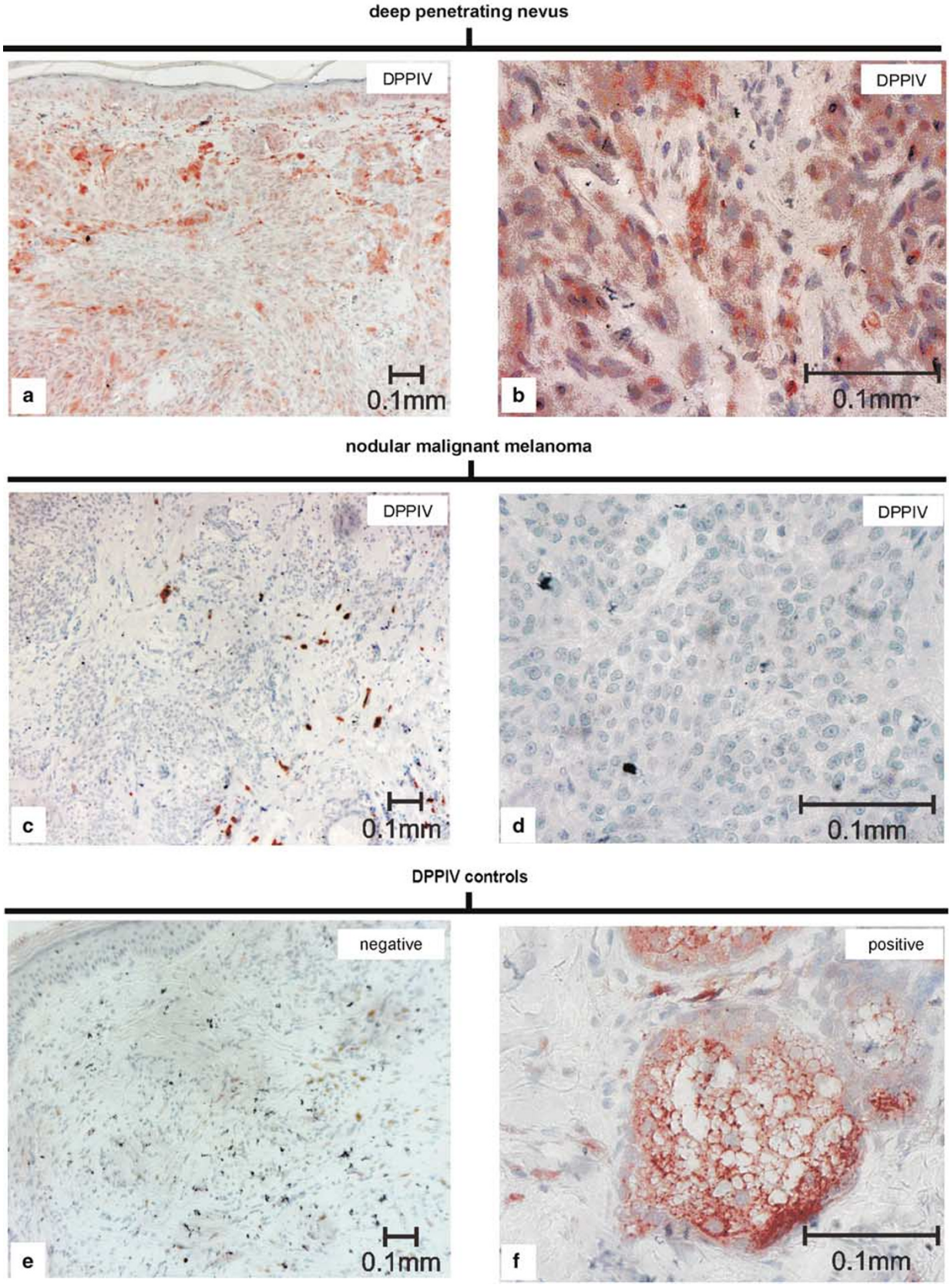


Figure 2 Immunoreactivity and immunolocalization of DPPIV in deep penetrating nevi *vs* nodular malignant melanomas. (**a**) DPPIV expression in a deep penetrating nevus specimen with its typical diffuse and, in part, focally accumulated staining pattern (× 100 overview). (**b**) × 400 magnification. (**c**) Example of a nodular malignant melanoma showing only single positive cells (× 100), (**d**) × 400 magnification. (**e**) Negative control (× 100 overview), (**f**) Positive control (sebaceous gland, × 400 magnification).

studies on MMP expression in melanomas,[16,17,26] MMP-1, MMP-2, integrin β3 and MT1-MMP were specifically overexpressed at the dermo-invasive front of nodular malignant melanomas also in this series. But, a more important result of this study is that most of these 'major suspects' considered as integral parts of melanoma progression leading to metastatic disease were not much different in deep penetrating nevi. After statistical evaluation of immunoreactivity, only MT1-MMP showed a trend to upregulation in nodular malignant melanomas. However, similar to most other invasion markers analyzed, it failed to show a differential immunoreactivity with statistical significance. Especially in the case of MT1-MMP, the lack of significance and, therefore, the inapplicability as a consistent discriminating marker is probably due to the broad variance of staining signals among the different tumor samples of both entities.

Most interestingly, only DPPIV kept its promise to be a protein that possibly discriminates true melanocytic malignancy and deep penetrating nevi. DPPIV is a cell surface peptidase expressed by normal melanocytes and common nevi, but primary and advanced malignant melanomas almost invariably lose or alter their DPPIV expression.[20–22] These previous observations made DPPIV a prime candidate for this study. Functionally, DPPIV re-expression leads to a loss of tumorigenicity, anchorage-independent growth, redifferentiation and an acquired dependence on exogenous growth factors.[22] Thus, high DPPIV expression was found correlated with less metastatic potential *in vivo*.[27] The effect of DPPIV appears to be either mediated through the upregulation of other factors such as E-cadherin and tissue inhibitors of matrix metalloproteinases[28] or by its ability to bind components of the extracellular matrix such as collagen or fibronectin.[27,29,30]

Beyond those practical implications of this study, our observations add to the evidence that the deep penetrating nevus could serve as a valuable, natural model of melanocytic progression and to dissect invasion mechanisms from metastatic potential. In this regard, the expression of MMP-1, MMP-2 was exceptional, since their immunoreactivity in deep penetrating nevi was unexpectedly high. In case of MMP-1, this upregulation in deep penetrating nevi even reached statistical significance. This is surprising since, the induction of MMP-1 has been suggested to be a pivotal late event in the progression of advanced melanomas not compatible with any benign condition[16,26,31] and even dysplastic nevi as well as early melanomas usually lack a significant MMP-1 and MMP-2 expression.[17,32,33]

We conclude that among the 'major suspects' reflecting melanocytic tumor progress, DPPIV bears the highest potential to be further exploited as a marker in doubtful cases. Moreover, due to its emerging functional role in invasion/proliferation of various cancers, the molecular mechanisms downstream of DPPIV deserve to be studied in more detail.

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