

Another Point of View on p16 and Ki67 Expression in Melanocytic and Non-Melanocytic Cutaneous Lesions

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SUMMARY The new analysis of the expression of cell cycle regulators (used in various neoplasms) and the nominal immunohistologic assays still represent valid and feasible diagnostic methods in most pathology practice. We examined 114 paraffin-embedded histologic specimens of melanocytic cutaneous lesions. The primary objective of this study was to explore diagnostic potential of the two important cell cycle regulators, p16 and Ki67, also evaluating the variations of expression by use of a semi quantitative graded scale. Another aim was to study the hypothetical correlation between p16 expression (in melanocytic and non-melanocytic lesions) and two independent variables, i.e. patient age and anatomical sites (sun exposed or non-sun exposed) of the lesions. Cell population was considered positive for antibody-specific p16 and Ki67 when at least 33% of the cells showed well-defined nuclear and/or cytoplasmic staining. A special p16 and Ki67 trend was found only in Spitz nevus, atypical Spitz nevus and invasive malignant melanoma (MM). No discriminative values were found regarding other lesions (junctional melanocytic nevus, MM in situ, superficially spreading MM, non-melanoma skin cancers). p16 was over-expressed on sun exposed sites and hypo-expressed on non-sun exposed areas, yielding a statistically significant correlation ($p < 0.03$). According to patient age, p16 was over-expressed in patients aged ≥ 61 and hypo-expressed in patients aged ≤ 60 ($p = 0.09$).

KEY WORDS: melanocytic lesions, p16 protein, Ki67 protein, malignant melanoma, cell cycle regulators, non-melanocytic skin cancers, predictive factors

INTRODUCTION

The cell cycle (controlled by several checkpoints) plays an important role in the pathogenesis of many cancers. Melanoma and non-melanoma skin cancers are an important global health problem, for this reason early detection and surgical excision of the tumors represent the principal goals. The immunohistochemistry progress can play an important role to improve histologic diagnosis. In this regard, the new analysis of the expression of cell cycle regulators (used in various neoplasms) and the nominal immunohistologic assays still represent valid and feasible diagnostic methods in the pathology practice (1).

Starting from previous studies, we decided to offer another point of view on the use of two important cell cycle regulators. The primary objective of this study was to explore diagnostic potential of the two important cell cycle regulators, p16 and Ki67, also evaluating the variations of their expression, using a semi quantitative graded scale. Another aim was to study the hypothetical correlation between p16 expression (in melanocytic and non-melanocytic lesions) and two independent variables, i.e. patient age and anatomical area (sun exposed or non-sun exposed) of the lesions analyzed.

MATERIALS AND METHODS

We examined 114 paraffin-embedded histologic specimens of melanocytic and non-melanocytic cutaneous lesions, which were stored at the Dermatopathology Unit of our Institute. Melanocytic lesions included melanocytic nevus (MN), Spitz nevus (SN), atypical Spitz nevus (ASN) and malignant melanoma (MM), while non-melanocytic lesions (NMC) included basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Five groups were analyzed.

Specimens were fixed in 10% buffered formalin. The sections were routinely processed and embedded in paraffin. Where necessary, in addition to hematoxylin-eosin staining, conventional immunohistologic stains (S100, HMB45, Melan-A) were performed.

In all histologic samples, double staining with p16 and Ki67 proteins was performed using a commercial immunohistochemical kit, normally used for simultaneous qualitative detection of p16 and Ki67 proteins in cervical cytology preparations. The distinct reaction products analyzed were brown and red. The brown staining of cells (cytoplasm and/or nuclei) indicates p16 expression, while red staining of cells (nuclei) indicates Ki67 expression (Fig. 1a). Cells stained for both antigens exhibit brown cytoplasmic staining with typically pronounced red nuclei.

Cell population was considered positive for antibody-specific p16 and Ki67 when at least 33% of the cells showed well-defined nuclear and/or cytoplasmic staining; for this reason, we arbitrarily used an accurate semi-quantitative graded scale, as follows: 0%; 0%-11%; 12%-22%; 23%-33%; 34%-66%; and 67%-100%.

Then, we evaluated if two independent variables could be predictive factors for p16 expression (either p16 over- or hypo-expression). To establish predictors, we analyzed age (≤ 60 or ≥ 61 years) and if the primary cutaneous lesions were present on sun exposed or non-sun exposed areas. Sun exposed sites were the face, neck, scalp (if absence of hairs) and back of the hands.

Assuming that the effects of the predictor variables were constant over time, we used Spearman's coefficient between p16 expression rate and the predictors (age and presence or absence of lesions on sun exposed areas).

A *p* value < 0.05 was considered statistically significant.

RESULTS

Epidemiological data

Out of 114 study patients, 63 (55.3%) were males and 51 (44.7%) females; median age of the entire co-

hort was 42 years. Melanocytic nevus was diagnosed in 29 males and 25 females (median age 42.81 years), MM in seven males and six females (median age 44.3 years), ASN in six males and five females (median age 35.6 years), and SN in six males and three females (median age 36.5 years). Regarding NMC, there were 23 (85.2%) BCC and four (14.8%) SCC. Eleven patients were female and 16 male (median age 60.5 years).

Fifty-four (47.4%) histologic samples corresponded to MN, 13 (11.4%) to MM, 11 (9.6%) to ASN, 9 (7.9%) to SN and 27 (23.7%) to NMC. Among MN, histopathologic analysis showed 17 (31.48%) junctional MN, 30 (55.55%) compound MN, 3 (5.55%) dermal MN, 3 (5.55%) dysplastic MN and 1 (1.84%) desmoplastic MN. Regarding MM, 10 (76.92%) were superficially spreading and 3 (23.08%) were invasive MM. Breslow thickness ranged between 0.4 mm and 1.4 mm.

P16 and Ki67 analysis

In 37 of 54 MN, the percentage of p16 positive cells was medium strong, between 34% and 100%; in all cases, the intensity was the same throughout the lesion thickness. The remaining 17 MN showed a medium low staining intensity (12%-33%). Regarding Ki67, 42 lesions showed low intensity (ranging between 0% and 22%), while the remaining 12 lesions showed a wide range of positivity (between 34% and 100%), with poor discriminative results (Fig. 1a, b).

All 9 cases of SN were positive ($\geq 33\%$) on staining for p16 and the cellular immune reaction was very strong (ranging between 67% and 100%) in 7 cases. Concerning Ki67, cellular reactivity was always medium (between 23% and 34%), but slightly higher than the majority of MN (Fig. 1c).

All 11 cases of ASN were positive for p16 and Ki67 staining and the immune reaction ranged between 34% and 66%, while Ki67 also showed cellular positivity between 34% and 66% (Fig. 1d). In these cases, p16 immune reaction was lower and Ki67 higher as compared with SN.

Regarding MM, we found double p16 cellular positivity: between 0% and 33% (in 6 cases) and between 34% and 100% (in 7 cases). In the first group, we recorded very low values of p16 (0%-11%) only in invasive MM; in these cases, p16 decreased progressively in the dermal melanocytic population. Regarding Ki67, histologic specimens showed high positivity (67%-100%) only in invasive MM (Fig. 2a). Finally, in NMC lesions, we found a high prevalence (69.6% of all NMC lesions) of lesions with p16 expression between 34% and 66% (Fig. 2b), while the remaining specimens showed p16 expression $\leq 34\%$. Regarding Ki67, 71.4% of the lesions showed 23%-33% positiv-

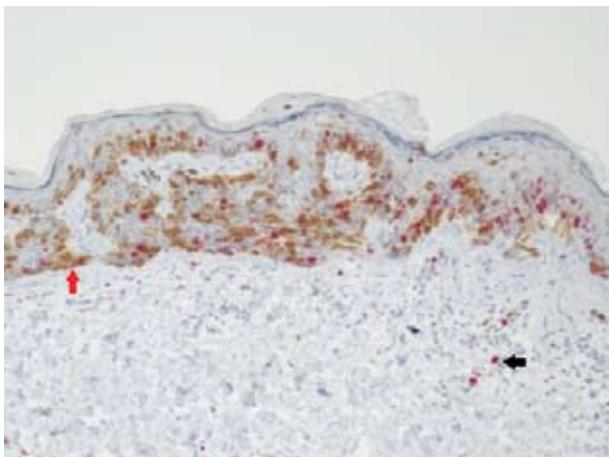


Figure 1a

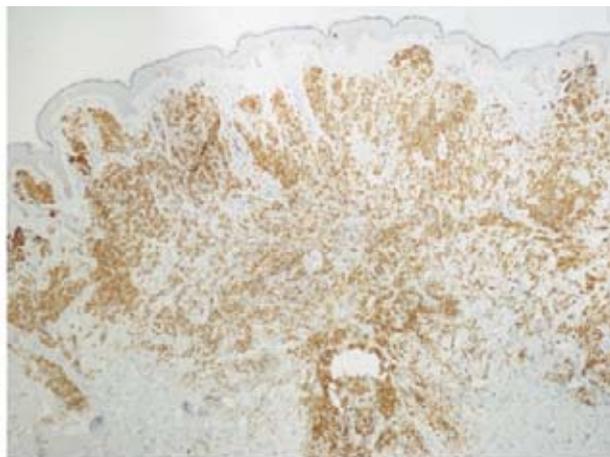


Figure 1b

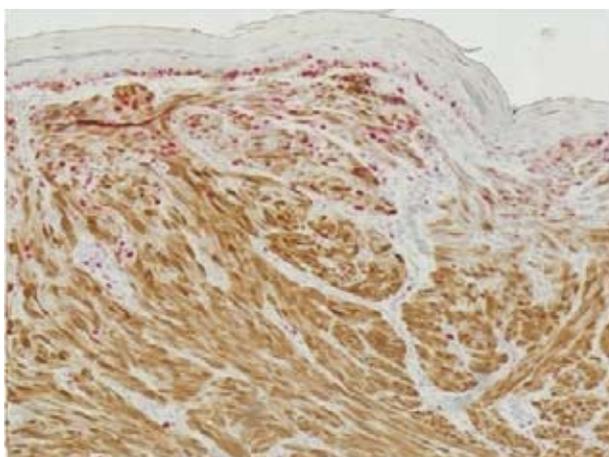


Figure 1c

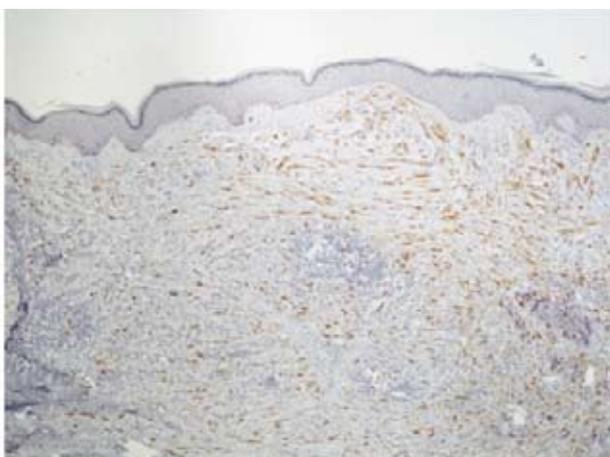


Figure 1d

Figure 1. (a) Junctional melanocytic nevus: medium p16 and Ki67 expression. Black arrow indicates Ki67 expression; red arrow indicates p16 expression (p16-Ki67; X20); (b) dermal nevus: p16 medium high expression and Ki67 very low expression (p16-Ki67; X10); (c) Spitz nevus: p16 overexpression and Ki67 medium expression (p16-Ki67; X20); (d) atypical Spitz nevus: reduction of p16 expression (compared to dermal and Spitz nevus) and medium low Ki67 expression (p16-Ki67; X10).

ity, while the remaining 28.6% of the lesions showed expression of 0%-11%.

Accordingly, a special p16 and Ki67 trend was found only in SN, ASN and invasive MM, whereas in other lesions (junctional MN, in situ MM, superficially spreading MM, NMC), no discriminative values were found.

p16 expression according to age and sun exposed or non-sun exposed areas

According to age, 28 patients were aged ≥ 61 and 87 patients ≤ 60 ; the patients aged ≤ 60 showed a medium p16 expression of 12%-22%, while those aged ≥ 61 showed a p16 positivity of 34-66% ($p = 0.09$;

Spearman's coefficient - 0.158). Regarding anatomical sites, 45 (39.5%) patients had cutaneous lesions removed on sun exposed areas and 69 (60.5%) patients on non-sun exposed areas. On nonparametric Spearman's coefficient test, we found that lesions removed on sun exposed sites showed p16 expression of 34%-66% (Fig. 2b) and those removed on non-sun-exposed areas showed p16 expression of 12%-22% ($p < 0.03$; Spearman's coefficient - 0.185).

Briefly, p16 was hyper-expressed on sun exposed sites and hypo-expressed on non-sun exposed areas, yielding a statistically significant correlation ($p < 0.03$); p16 was over-expressed in patients aged ≥ 61 and hypo-expressed in those aged ≤ 60 ($p = 0.09$).

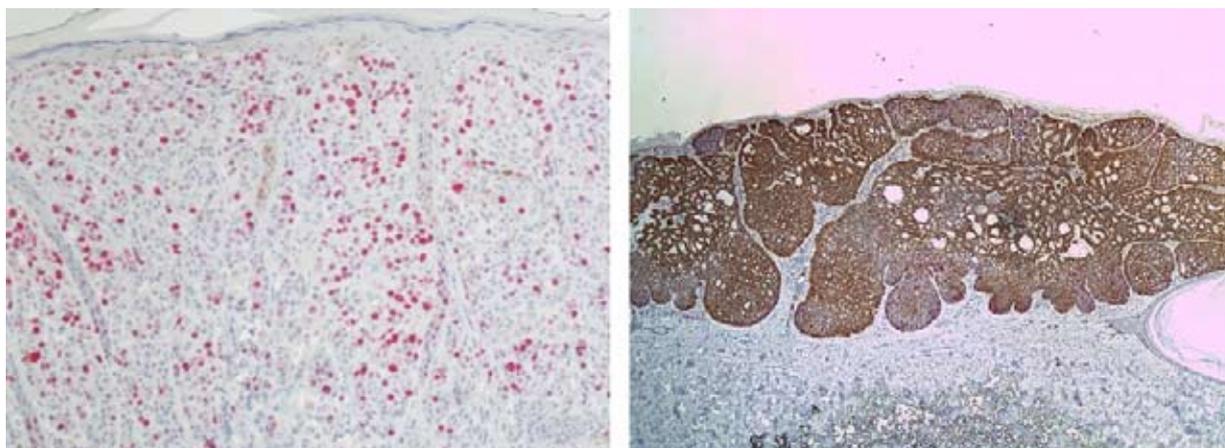


Figure 2. (a) Invasive melanoma: loss of p16 expression and high Ki-67 expression of cell population (p16-Ki67; X20); (b) basal cell carcinoma of sun exposed site (age ≥ 61) showing p16 hyper-expression (p16-Ki67; X10).

DISCUSSION

p16 is a protein product of the CDKN2A familial melanoma gene. p16 inhibits the G₁ cyclin-dependent kinases (CDK_s) 4 and 6. In the absence of functional p16, these CDK_s phosphorylate and inactivate the retinoblastoma tumor suppressor gene product, resulting in cell cycle regression and, ultimately, increased cellular proliferation (2,3). In 1995, Pollock *et al.* reported partial and homozygous deletions of CDKN2A gene in melanoma cell lines (4). At the same time, mutations in other melanoma (MM) cell lines were described, occurring most commonly in the exon 1 or 2 of the CDKN2A gene located near dipyrimidine sites, commonly associated with damage with ultraviolet rays (5). Reed *et al.* and Talve *et al.* were the first to report that the p16 loss was related to the depth of invasion (6,7). These results were also confirmed in our invasive MM samples.

In current analysis, most MN, in situ MM and superficially spreading MM showed a medium high p16 cellular positivity, without a discriminative value. However, a recent study speculates that p16 inactivation is not strictly timed event in MM tumor genesis and that it is not always inactivated in the MM cancer sequence (8). In this regard, it is also important to emphasize that p16 positivity in superficially spreading MM could also reassure the pathologist on the possible superficial localization of the malignant cells and, therefore, on a lower rate of recurrence and metastases.

We speculate that this can be valid, especially for questionable lesions of about 1.00 mm of Breslow thickness and/or with an important inflammatory infiltrate. In a previous analysis, no correlation of p16 decreasing score was found with Breslow thickness,

but only with increasing stage (9). In the current study, however, we found the loss of p16 to increase with increasing Breslow thickness and depth of melanocytic lesions.

Ki67 is a nuclear protein present in two isoforms: 345 kDa and 395 kDa, and it is expressed by proliferating cells in late G₁, S, G₂ and M phases, but not in G₀ phase (10). In our cohort, we found a proportional increase of Ki67 staining from benign to malignant melanocytic lesions. An exceptional trend was highlighted. In fact, 77.7% of MN and SN showed a very low Ki67 cellular positivity, while ASN and invasive MM showed strong cellular positivity (these values were not maintained in superficial MM).

Distinction between ASN and MM remains one of the most important areas in pathology. Although ASN have generally been reported to have a good prognosis, well documented cases of metastases and death exist. For the malignant potential of these lesions, clinicians often make decisions to treat them as MM, also performing sentinel lymph node (11). In this regard, p16 and Ki67, used in conjunction, can give further help in the discrimination of these lesions, especially when evaluated in the same tissue samples.

Regarding NMC, the analysis detected p16 protein in 69.6% of the lesions, ranging between 34% and 66%, while Ki67 expression was medium low (22%-33%) in 71.4% of the lesions. All these results were similar to those reported by other authors (12-14). In our study, no case of invasive NMC was found. According to previous studies (12-15) and our report, we think that currently there are too few and discordant results on p16 expression in NMC that cannot ascertain correlation between p16 expression and the pos-

sible malignant progression and/or tumor invasion. Further studies with larger samples are required.

Another two important aims of our study were to evaluate the correlation between p16 expression and age (≤ 60 and ≥ 61), and between p16 expression and sun exposed or non-sun-exposed areas. Sun exposed lesions showed a medium high p16 expression, ranging between 33% and 66%, while no exposed sites had lower values (11%-22%); Spearman's nonparametric test yielded a statistically significant result ($p < 0.03$), which was consistent with previous studies (15-18). In this regard, we suppose that the overexpression in the lesions on exposed sites can be correlated with the response of human melanocytes and keratinocytes to UVB irradiation and that the relative p16 pathway activation is associated with better response to a possible invasive malignant process.

Unlike previous studies, we also decided to explore the possible correlation between p16 expression and the patient relative age. Among 114 lesions, patients aged ≤ 60 showed a medium p16 expression (12%-22%), while it was 33%-66% in patients aged ≥ 61 ($p = 0.09$). In a previous work, Nielsen *et al.* studied p16 expression in adult and young tissues, and found that p16 expression was present in some tissues (such as Langerhans islets in the pancreas, thyroid, gland of stomach, and melanocytes in the skin), while in younger and infant patients, p16 expression was present only in the thymic Hassall's corpuscles, which is the only organ programmed for an early senescence progress (19). Although there are no statistically significant results ($p = 0.09$), according to our study, in elderly skin, both the increased susceptibility to UVB and p16 response to more frequent carcinogenic process can play important roles; this can explain the higher p16 expression in older than in younger tissues, also found in current analysis.

CONCLUSION

A special p16 and Ki67 trend was found only in SN, ASN and invasive MM. Sun exposed area showed a statistically significant correlation with p16 hyperexpression; this value was not maintained for the age of the patients.

References

1. Evan G, Polisar NL, Wick M. Immunohistochemical evaluation of p16^{INK4A}, E-cadherin and cyclin D1 expression in melanoma and Spitz tumors. *Am J Clin Pathol* 2010;133:370-9.
2. Hillard NJ, Krahl D, Sellheyer K. p16 Expression differentiates between desmoplastic Spitz nevus and desmoplastic melanoma. *J Cutan Pathol* 2009;36:753-9.
3. Bosserhoff AK. Novel biomarkers in malignant melanoma. *Clin Chim Acta* 2006;367:28-35.
4. Pollock PM, Yu F, Qiu L, Parsons PG, Hayward NK. Evidence of u.v. induction of CDKN2 mutations in melanoma cell lines. *Oncogene* 1995;11:663-8.
5. Liu Q, Neuhausen S, McClure M, Frye C, Weaver-Feldhaus J, Gruis NA, *et al.* CDKN2A (MTS1) tumor suppressor gene mutations in human tumor cells lines. *Oncogene* 1995;11:2455.
6. Reed JA, Loganzo F Jr, Shea CR, Walker GJ, Flores JF, Glendening JM, *et al.* Loss of expression of the p16/cyclin-dependent kinases inhibitor 2 tumor suppressor gene in melanocyte lesions correlates with invasive stage of tumor progression. *Cancer Res* 1995;55:2173.
7. Talve L, Sauroja I, Collan Y, Punnonen K, Ekfors T. Loss of expression of the p16INK4/CDKN A gene in cutaneous malignant melanoma correlates with tumor cell proliferation and invasive stage. *Int J Cancer* 1997;74:255.
8. Sanki A, Li W, Colman M, Karim RZ, Thompson JF, Scolyer RA. Reduced expression of p16 and p27 is correlated with tumor progression in cutaneous melanoma. *Pathology* 2007;39:551-7.
9. AlDhaybi R, Agoumi M, Gagné I, McCuaig C, Powell J, Kokta V. p16 Expression differentiates between childhood malignant melanomas and Spitz nevi. *J Am Acad Dermatol* 2011;65:357-63.
10. Lindboe CF, Torp SH. Comparison of Ki67 equivalent antibodies. *J Clin Pathol* 2002;55:467-71.
11. Miteva M, Lazova R. Spitz nevus and atypical spitzoid neoplasm. *Semin Cutan Med Surg* 2010;29:165-73.
12. Paolini F, Carbone A, Benevolo M, Silipo V, Rollo F, Covelto R, *et al.* Human papillomaviruses, p16INK4a and Akt expression in basal cell carcinoma. *J Exp Clin Cancer Res* 2011;30:108.
13. Conscience I, Jovenin N, Coissard C, Lorenzato M, Durlach A, Grange F, *et al.* p16 Is overexpressed in cutaneous carcinomas located on sun-exposed areas. *Eur J Dermatol* 2006;16:518-22.
14. Eshkooor SA, Ismail P, Rahman SA, Oskour SA. p16 Gene expression in basal cell carcinoma. *Arch Med Res* 2008;39:668-73.
15. Svensson S, Nilsson K, Ringberg A, Landberg G. Invade or proliferate? Two contrasting events in malignant behavior governed by p16INK4a and an intact Rb pathway illustrated by a model system of basal cell carcinoma. *Cancer Res* 2003;63:1737-42.

16. Pavey S, Conroy S, Russell T, Gabrielli B. Ultraviolet radiation induces p16 expression in human skin. *Cancer Res* 1999;59:4185-9.
17. Chazal M, Marionnet C, Michel L, Mollier K, Dazard JE, Della Valle V, *et al.* p16(INK4A) is implicated in both the immediate and adaptive response of human keratinocytes to UVB irradiation. *Oncogene* 2002;21:2652-61.
18. Abd Elmageed ZY, Gaur RL, Williams M, Abdraboh ME, Rao PN, Raj MH, *et al.* Characterization of coordinated immediate responses by p16INK4A and p53 pathways in UVB-irradiated human skin cells. *J Invest Dermatol* 2009;129:175-83.
19. Nielsen GP, Stemmer-Rachamimov AO, Shaw J, Shaw J, Roy JE, Koh J, *et al.* Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues. *Lab Invest* 1999;79:1137-43.