Virchows Archiv A Pathol Anat (1993) 423:359–364

Virchows Archiv A
Pathological Anatomy and Histopathology

Proliferative activity of primary cutaneous melanocytic tumours

Takeshi Kuwata, Masanobu Kitagawa, Tsutomu Kasuga

The Department of Pathology, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan

Received May 27, 1993 / Received after revision August 9, 1993 / Accepted August 10, 1993

Abstract. The expression of proliferating cell nuclear antigen (PCNA) was examined in formalin-fixed paraffin-embedded tissue sections from 41 lesions (27 melanocytic nevi, 3 atypical nevi and 11 malignant melanomas) to determine the proliferative activity of primary cutaneous melanocytic tumours. Most of the malignant melanomas had more than 7% PCNA-positive cells ($9.2 \pm 0.5\%$), while the melanocytic nevi manifested less than 1% PCNA-positive cells ($0.4 \pm 0.1\%$). Atypical nevi exhibited an intermediate, but still significantly lower, labelling ratio when compared with malignant melanomas ($0.8 \pm 0.2\%$). The proliferative activity of the lesions was compared between portions at different depths in the skin (epidermal, upper dermal and lower dermal location). In cases of malignant melanoma, the proliferative activity was higher in the deeper portion of dermis whereas PCNA-positive cells in melanocytic nevi were located in the upper dermis predominantly. Thus the PCNA labelling ratio of malignant melanoma and/or melanocytic nevus cells located in the epidermodermal junction was not necessarily higher than that of malignant melanoma and/or melanocytic nevus cells in the dermis. These results indicate that staining with PCNA would be very useful in the differentiation of malignant melanoma from melanocytic nevi manifesting cellular and/or structural atypia by virtue of a significant difference in the proportion of PCNA-positive cells. Although malignant melanomas have higher proliferative activity than melanocytic nevi in the deeper dermis, junctional activity in melanocytic tumours does not indicate cell proliferation.

Key words: Malignant melanoma – Melanocytic nevus – Proliferative activity – Proliferating cell nuclear antigen – Immunohistochemistry

Correspondence to: T. Kuwata

Introduction

Proliferative activity in tumour cells correlates with the degree of malignancy in various neoplasms (Ellison et al. 1987; Hall et al. 1990; McGurrin et al. 1987). In primary cutaneous melanocytic tumours, many studies have been conducted on the mitotic index, labelling index with tritiated thymidine or immunohistochemical determination of Ki-67 antigen in malignant melanoma cells (Kaudewitz et al. 1989; Kopf et al. 1987; Moretti et al. 1990; Schmoeckel and Braun-Falco 1978; Shirakawa et al. 1970; Smolle et al. 1989; Takahashi et al. 1991; Urso et al. 1992; Worth et al. 1989). Most of these studies have shown that the proliferative activity of melanoma cells correlates well with the prognosis. However, the cell kinetics of melanocytic nevi have not been clarified; mitotic figures in melanocytic nevi are extremely rare (Bentley-Phillips and Marks 1976; Smolle et al. 1989; Urso et al. 1992).

Proliferating cell nuclear antigen (PCNA) was originally described as an antigen recognized by an antibody reacting with antigens in proliferating cell nuclei from patients with systemic lupus erythematosus (Hall and Levinson 1990; Miyachi et al. 1978; Quinn and Wright 1990). Further studies on PCNA have revealed that this antigen is identical to the auxiliary protein of DNA polymerase-δ and is concentrated in proliferating cells with a peak in S phase in normal tissue and in neoplasms (Almendri et al. 1987; Bravo et al. 1987; Morris and Mathews 1989; Prelich et al. 1987). The results of immunohistochemical techniques using PCNA correlate well with the results of assays using tritiated thymidine (Ogata et al. 1987). Recently, several monoclonal antibodies against PCNA have been found to react on formalin-fixed paraffin-embedded tissues and proliferative activity of specific cells and the localization of proliferating cells has been evaluated in various tissues (Garcia et al. 1989; Hall et al. 1990; Takahashi et al. 1991). In this study, proliferative activity of benign and malignant cutaneous melanocytic tumours was evaluated by an immunohistochemical technique using monoclonal anti-

body against PCNA. Several cases of borderline lesions, melanocytic nevi with cellular and/or structure atypia (atypical nevi), were also examined. In addition to the ratio of proliferating cells in the whole tumour, the numbers of proliferating cells were compared at different depths in the tumours. Areas were selected from the epidermal (junctional), upper dermal and lower dermal level of each lesion. The efficacy of this staining in informing the discussion on diagnosis and histogenesis of melanocytic tumours was assessed.

Materials and methods

Forty-one surgically removed melanocytic tumours of the skin were examined. These included 27 melanocytic nevi and 11 primary cutaneous malignant melanomas. Three atypical nevi, which showed cellular and/or structural atypia, were also examined. The atypical nevi were defined by several features of melanocytic lesions including: cellular atypia of melanocytic cells, abnormal pigmentation in the keratinizing layer of the epidermis, abnormal foci of melanocytic cells in the upper layer of the epidermis, fusion of the rete ridges, and an inflammatory response. According to the World Health Organization Melanoma Program (Clemente et al. 1991), these atypical nevi have satisfied more than two minor criteria for the diagnosis of dysplastic nevi; however, the lesions have not necessarily satisfied the two major criteria. Thus we have preferred the term atypical nevus rather than dysplastic nevus. The age and sex of patients, and size and localization of tumours, are summarized in Table 1. Melanocytic nevi were subdivided into junctional nevi (10 cases), compound nevi (CN; 8 cases) and intradermal nevi (IDN; 9 cases), and malignant melanoma into lentigo maligna melanoma (3 cases), superficial spreading melanoma (SSM; 5 cases) and nodular melanoma (NM; 3 cases). Malignant melanomas were also classified by the level of tumour invasion and thickness according to Clark's classification and Breslow's measurement, respectively (Breslow 1975; Clark et al. 1969). All material was routinely fixed with 10% phosphate-buffered formalin and embedded in paraffin.

Sections, 3 μm thick, mounted on slides, were immunostained by labelled avidin-biotin methods as described previously (Giorno 1984). Anti-PCNA antibody (PC10, Novocastra), biotinylated rabbit anti-mouse immunoglobulin (Dako) and peroxidase-conjugated streptavidin (Dako) were employed as first, second and third incubation, respectively. The first incubation was performed overnight at 4° C. Finally, slides were developed by 3,3′-diaminobenzidine and nuclear counterstained by methylgreen. Additional depigmentation was employed by the treatment with 0.03 M disodium-hydrogen phosphate and 3% hydrogen peroxide for 2 h in the cases containing numerous melanin pigments.

Because many factors in preparation of the sections have been known to influence the intensity of immunostaining with anti-PCNA antibody (Hall et al. 1990), we confirmed in all cases that some of the keratinocytes of the epidermal basal layer and hair follicles were regularly stained positively as an integral control. The PCNA-positive tumour cells were counted on the sections and expressed as a percentage of total nucleated cells counted. In principle, more than 500 cells were observed from randomly selected areas of tumours and the PCNA-positive cells were counted. In addition, in order to evaluate the PCNA-positive cell ratio at different levels, positive cells were examined and compared in epidermal, upper dermal and lower dermal areas of each lesion. When the lesion was too small to count more than 500 cells, all the tumour cells in the section were counted. Lesions containing less than 100 tumour cells were excluded from the study.

Results

To compare the proliferative activity of benign, atypical and malignant tumours of melanocytic origin, the PCNA labelling ratio was examined in melanocytic nevi, atypical nevi and malignant melanoma. Figure 1 a–d shows the histological features of melanocytic nevi and the immunohistochemical localization of PCNA-positive cells. As shown in Table 2, the percentage of PCNA-positive cells in melanocytic nevi was $0.4 \pm 0.1\%$ (range 0–1.7%) of total tumour cells. In cases of atypical nevi, $0.8 \pm 0.2\%$ (range 0.5–1.8%) of total tumour cells were labelled with the anti-PCNA antibody (Fig. 1 e, f). The difference in the proportion of PCNA-positive cells was not significant between melanocytic nevi and atypical nevi. In contrast, PCNA-positive cells were frequently observed in malignant melanoma (Fig. 1 g, h), in which the percentage of positive cells was $9.2 \pm 0.5\%$ (range 7.5–11.7%) of total tumour cells. The differences in the proportion of PCNA-positive cells were significant between melanocytic nevi and malignant melanoma ($P < 0.01$), and atypical nevi and malignant melanoma ($P < 0.01$). However, the ratios were not significantly different between different subtypes of malignant melanomas (lentigo maligna, superficial spreading and nodular melanoma).

In order to clarify what portion of the tumour shows the most frequent labelling with PCNA, the PCNA-positive cell ratios were evaluated at three different levels of the tumours: epidermal, upper dermal and lower dermal areas. In most cases of CN, the PCNA-positive cells were most frequent in the upper dermal region (0.6 ± 0.2). In all cases of IDN, the PCNA-positive cell ratio

Table 1. Clinical data of cases in the study

Histological type	Age (years) mean (range)	Sex (female/male)	Size (cm)	Site (*n*)				Total
				head	trunk	extremity	acrum	
Junctional nevi	27.3 (6–70)	9/1	2–4				10	10
Compound nevi	34.9 (16–65)	6/2	5–8	3	3	1	1	8
Intradermal nevi	33.6 (17–45)	7/2	3–5		7	1	1	9
Atypical nevi	26.7 (15–33)	3/0	5–6		2	1		3
Lentigo maligna melanoma	39.3 (21–45)	2/1	3–6			2	1	3
Superficial spreading melanoma	25.0 (8–40)	5/0	8–20			3	2	5
Nodular melanoma	35.3 (15–63)	2/1	4–16	1	1	1		3

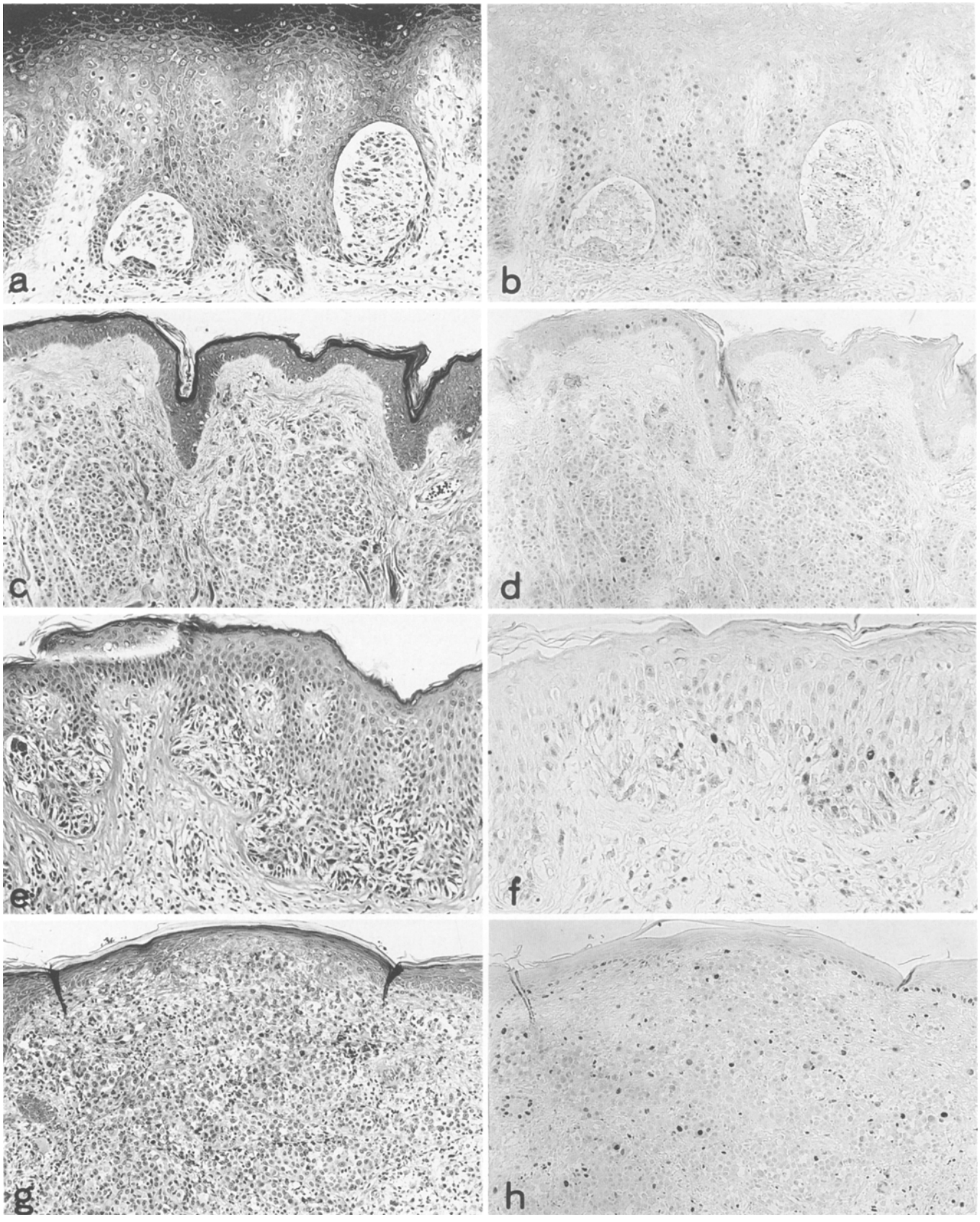

Fig. 1. **a** Junctional nevus. Focal accumulation of melanocytic cells in the epidermodermal junction. H&E, ×130. **b** Junctional nevus showing rare positive staining for proliferating cell nuclear antigen (PCNA); labelled avidin biotin (LAB), ×130. **c** Intradermal nevus. H&E, ×110. **d** Rare PCNA-positive cells in intradermal nevus. LAB, ×110. **e** Atypical nevus showing fusion of the rete ridges, lymphoid cell infiltration and lamellar fibrosis. H&E, ×130. **f** Several PCNA-positive cells in the atypical nevus. LAB, ×210. **g** Malignant melanoma (nodular melanoma). H&E, ×110. **h** PCNA-positive cells scattered in malignant melanoma. LAB, ×110

Table 2. Histological type of cutaneous melanocytic tumours and proliferative activity

Histological type	Proliferative activity (%) (mean ± SE)
Melanocytic nevi (total)	*0.4 ± 0.1**
Junctional nevi	0.3 ± 0.2
Compound nevi	0.6 ± 0.1
Intradermal nevi	0.4 ± 0.1
Atypical nevi	*0.8 ± 0.2***
Malignant melanoma (total)	*9.2 ± 0.5****
Lentigo maligna melanoma	8.6 ± 1.1
Superficial spreading melanoma	9.1 ± 0.8
Nodular melanoma	9.7 ± 0.4

The differences were significant between proliferating activity of melanocytic nevi* and malignant melanoma*** ($P < 0.001$), and atypical nevi** and malignant melanoma*** ($P < 0.001$) by Student's *t*-test

was higher in upper dermal areas than in the lower dermal area. Thus the PCNA-positive cells of melanocytic nevi with intradermal lesions (CN and IDN) were located in the upper dermis predominantly. In contrast with melanocytic nevi, PCNA-positive cells in malignant melanomas were mainly distributed in deeper portions (Table 3). SSM revealed a higher PCNA-positive cell ratio in the upper dermal area than in the epidermal area. Furthermore, in all cases of NM, PCNA-positive cell ratios in the lower dermal area (10.3 ± 0.6) were higher than in the upper dermal area (8.4 ± 0.8).

For a comparison of tumour progression and the proliferative activity of tumour cells pathological prognostic factors including tumour size, mitotic index, depth of invasion (Clark's level) and tumour thickness (Breslow's measurement) were determined in malignant melanomas. Tumour size, mitotic index of tumour cells and tumour thickness did not correlate well with the proliferative activity of tumour cells (data not shown). However, the depth of tumour invasion correlated with labelling with PCNA, i.e. tumours invading beneath the reticular dermis (levels IV or V according to Clark's classification), had higher proliferative activity (9.9 ± 0.5) than those in levels I, II and III (7.9 ± 0.3; Table 4).

Table 4. Clark's level and proliferative activity of primary cutaneous malignant melanoma

Clark's level	Proliferative activity [range (mean ± SE)]
I–III	7.5– 8.7 (7.9 ± 0.3)
IV–V	7.5–11.7 (9.9 ± 0.5)

The proliferative activity of malignant melanoma in level I–III was lower than that in level IV–V by Student's *t*-test ($P < 0.05$)

Discussion

Several studies have shown that the proliferative activity of malignant melanoma is higher than that of melanocytic nevi. Our present study also showed that malignant melanoma possessed higher proliferative activity than melanocytic nevi, although the mean values of the PCNA labelling ratio of malignant melanoma and melanocytic nevi were higher than in previous studies using tritiated thymidine or Ki-67 (Smolle et al. 1989; Urso et al. 1992). This difference may result, in part, from the difference in the period in the cell cycle that the assay could detect and the difference in half-life of the products detected (Hall et al. 1990). In a previous study using immunohistochemistry with anti-PCNA antibody, quite a few melanocytic nevi contained PCNA-positive cells (Takahashi et al. 1991), although most cases of melanocytic nevi revealed PCNA-positive cells in our study. This discrepancy may be caused by the fact that the antigenicity of PCNA is weak and is influenced by the interval and the manner of fixation (Hall et al. 1990). In the present study all materials were fixed for no more than 48 h and, further, cases were selected by the appropriate reactivity in normal epidermal cells in the same section.

The distribution of the proliferating cell in melanocytic nevi has not yet been evaluated. Previously, the cells in the epidermodermal junctional lesions were assumed to possess high proliferative activity, determined as "junctional activity". However, recent studies on cell kinetics of melanocytic nevi do not support excess activity at this site (Bentley-Phillips and Marks 1976; Smolle et al. 1989). Smolle et al. and Urso et al. have shown

Table 3. Proliferative activity and depth of melanocytic cells

Histological typing	Proliferating activity (mean ± SE)		
	epidermis	upper dermis	lower dermis
Melanocytic nevi	*0.2 ± 0.1*	*0.6 ± 0.1*	*0.1 ± 0.1*
Junctional nevi	0.3 ± 0.2	–	–
Compound nevi	0.1 ± 0.1	0.6 ± 0.2	0.1 ± 0.1
Intradermal nevi	–	0.6 ± 0.1	0.1 ± 0.1
Atypical nevi	*1.1 ± 0.4*	*0.5*[a]	–
Malignant melanoma	*9.0 ± 0.7*	*9.1 ± 0.5*	*10.3 ± 0.6*
Lentigo maligna melanoma	8.6 ± 1.1	10.6	–
Superficial spreading melanoma	9.2 ± 0.9	9.3 ± 0.9	–
Nodular melanoma	–	8.4 ± 0.8	10.3 ± 0.6

[a] Only one case was available for evaluation

that the proliferating cells were distributed throughout the whole lesion of melanocytic nevi, although superficial melanocytes would be metabolically more active than the cells in deeper lesions (Bentley-Phillips and Marks 1976; Smolle et al. 1989). Our study showed that PCNA-positive cells were mainly located in the upper dermal parts of the lesion and thus the proliferative activity of the upper dermal region was higher than that of the epidermal part of the lesion. "Junctional activity" does not mean high rate of cellular proliferation. In contrast with melanocytic nevi, malignant melanomas showed a greater proliferative activity in deeper lesions.

Correlation of histological features of malignant melanoma with prognosis of the patients has been well studied (Breslow 1975; Giorno 1984; Kopf et al. 1987; Schmoeckel and Braun-Falco 1978; Shirakawa et al. 1970). Mitotic index, tumour thickness and proliferative activity, including mitotic index, revealed a good correlation with prognosis. In the present study, the mitotic index of malignant melanoma did not correlate well with PCNA-positive cell ratio, and we have assumed that because the values of mitotic index were very small and possessed high deviations, proliferative activity, detected by PCNA, was more accurate than the mitotic index. In this context proliferative activity assessed by anti-PCNA antibody is useful in predicting prognosis.

Concerning the depth of invasion (Clark's level), proliferative activity of melanoma cells was significantly higher in cases with deeper invasion when compared with cases with superficial invasion, although the differences were small. It has been reported that proliferative activity of malignant melanoma increases as the tumour exhibits deeper invasion into the skin (Moretti et al. 1990; Smolle et al. 1989; Takahashi et al. 1991). Because malignant melanoma remains in the epidermis for a long period before vertical growth begins, proliferative activity of invasive malignant melanoma has been thought to be higher than that of non-invasive lesions (Clark et al. 1969, 1984; McGovern et al. 1973). However, the results of our study suggest that malignant melanoma acquires a high proliferative activity at an early stage of tumour development. A number of factors, including various host responses, might contribute in preventing malignant melanoma from vertical growth initially (Ackerman 1980). The fact that the malignant melanoma with deeper invasion has a poor prognosis is probably related to the fact that these more advanced cases are more likely to metastasize. Our study revealed little relationship between proliferative activity and histological subtypes of malignant melanoma, which supports the unifying concept of malignant melanoma. Some studies have suggested that histological subtypes are significantly related to their prognosis (Ackerman and David 1986; Shirakawa et al. 1970).

In conclusion, we emphasize the availability of anti-PCNA antibody for the differential diagnosis of malignant melanoma from atypical nevi. A PCNA-positive cell ratio in malignant melanoma is easily distinguished from that of a melanocytic nevus even when the lesions show cellular and/or structural atypia. The results support the concept that dysplastic nevi have little relationship to malignant melanoma in terms of proliferative activity (Ackerman and Mihara 1985).

References

Ackerman AB (1980) Malignant melanoma: a unifying concept. Hum Pathol 11:591–595

Ackerman AB, David KM (1986) A unifying concept of malignant melanoma: biologic aspects. Hum Pathol 17:438–440

Ackerman AB, Mihara I (1985) Dysplasia, dysplastic melanocytes, dysplastic nevi, dysplastic nevus syndrome, and the malignant melanomas. Hum Pathol 16:87–91

Almendri MJ, Huebsch D, Blundell AP, Macdonald-Bravo H, Bravo R (1987) Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc Natl Acad Sci USA 84:1575–1579

Bentley-Phillips C, Marks R (1976) Cell division and metabolic activity of naevus cells. Br J Dermatopathol 94:557–563

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature 326:515–517

Breslow A (1975) Tumor thickness, level of invasion and node dissection in stage I cutaneous melanoma. Ann Surg 182:572–575

Clark WH Jr, From L, Bernardino EA, Mihm MC (1969) The histogenesis and biologic behavior of primary human malignant melanoma of the skin. Cancer Res 29:705–726

Clark WH Jr, Elder DE, Guerry D, Epstein MN, Green MH, Horn MV (1984) A study of tumor progression. Hum Pathol 15:1147–1165

Clemente C, Cochran AJ, Elder DE, Levene A, Mackie RM, Mihm MC Jr, Rilke F, Cascinelli N, Fitzpatrick TB, Sober AJ (1991) Histopathologic diagnosis of dysplastic nevi: concordance among pathologists convened by the world health organization melanoma programme. Hum Pathol 22:313–319

Ellison DJ, Nathwani BN, Metter GE, Turner RR, Burke JS, Miller TP, Mann RB, Barcos M, Kjeldsberg CR, Winberg CD, Whitcomb CC (1987) Mitotic counts in follicular lymphomas. Hum Pathol 18:502–505

Garcia RL, Coltrera MD, Grown AM (1989) Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. Am J Pathol 134:733–739

Giorno R (1984) A comparison of two immunoperoxidase staining methods based on the avidin-biotin interaction. Diagn Immunol 2:161–166

Hall PA, Levinson DA (1990) Assessment of cell proliferation in histological material. J Clin Pathol 43:184–192

Hall PA, Levison DA, Woods AL, Yu CCW, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections. J Pathol 162:285–294

Kaudewitz P, Braun-Falco O, Ernst M, Landthaler M, Stoltz W, Gerdes J (1989) Tumor cell growth fractions in human malignant melanomas and correlation to histopathologic tumor grading. Am J Pathol 134:1063–1068

Kopf AW, Gross DF, Rogers GS, Rigel DS, Hellman LJ, Levenstein M, Welkovich B, Friedman RJ, Roses DF, Bart RS, Mintzis MM, Gumport SL (1987) Prognostic index for malignant melanoma. Cancer 59:1236–1241

McGovern VJ, Mihm MC, Bailly C, Booth JC, Clark WH, Cochran AJ, Hardy EG, Hicks JD, Levene A, Lewis MG, Little JH, Milton GW (1973) The classification of malignant melanoma and its histologic reporting. Cancer 32:1446–1457

McGurrin JF, Doria MI, Dawson PJ, Karrison T, Stein HO, Franklin WA (1987) Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of breast. Cancer 59:1744–1750

Miyachi K, Fritzler MJ, Tan EM (1978) Autoantibody to a nuclear antigen in proliferating cells. J Immunol 121:2228–2234

Moretti S, Massobrio R, Brogelli L, Novelli M, Giannotti B, Bernengo MG (1990) Ki67 antigen expression correlates with tumor progression and HLA-DR antigen expression in melanocytic lesions. J Invest Dermatol 95:320–324

Morris GF, Mathews MB (1989) Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 264:13856–13864

Ogata K, Kurki P, Celis JE, Nakamura RM, Tan EM (1987) Monoclonal antibodies to a nuclear protein (PCNA/Cyclin) associated with DNA replication. Exp Cell Res 168:475–486

Prelich G, Tan C, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxilliary protein. Nature 326:517–518

Quinn CM, Wright NA (1990) The clinical assessment of proliferation and growth in human tumours. J Pathol 160:93–102

Schmoeckel C, Braun-Falco O (1978) Prognostic index in malignant melanoma. Arch Dermatol 114:871–873

Shirakawa S, Luce JK, Tannock I, Feri E III (1970) Cell proliferation in human melanoma. J Clin Invest 49:1188–1199

Smolle J, Soyer H-P, Kerl H (1989) Proliferative activity of cutaneous melanocytic tumors defined by Ki-67 monoclonal antibody. Am J Dermatopathol 11:301–307

Takahashi H, Strutton GM, Parsons PG (1991) Determination of proliferating fractions in malignant melanomas by anti-PCNA/cyclin monoclonal antibody. Histopathology 18:221–227

Urso C, Bondi R, Balzi M, Sculba E, Mauri P, Becciolini A, Tarocchi S, Vallecchi C (1992) Cell kinetics of melanocytes in common and dysplastic nevi and in primary and metastatic cutaneous melanoma. Pathol Res Pract 188:323–329

Worth AJ, Gallagher RP, Elwood JM, Yang PC, Lamb C, Spinelli JJ, Wood WS, Threlfall WJ, Hill GB (1989) Pathologic prognostic factors for cutaneous malignant melanoma. Int J Cancer 43:370–375