FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 50, No. 2, 2012
pp. 263–269

ORIGINAL STUDY

VIA MEDICA

Expression of cyclins A and E in melanocytic skin lesions and its correlation with some clinicopathologic features

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Abstract: Cyclins play a fundamental role in the cell cycle. Recent studies have focused on their role in the development of various malignancies. The objective of this study was to evaluate and compare the expression of cyclins A and E in common nevi, dysplastic nevi and malignant melanomas, and to investigate the relationship between cyclin expression and some pathological parameters such as tumor thickness, ulceration, regression, and mitotic rate, as well as several clinical and phenotypic parameters such as skin phototype, hair and eye color, number of nevi, personal or family melanoma history, and personal history of nonmelanoma skin cancer (NMSC). A total of 102 melanocytic skin lesions, including 30 common nevi, 38 dysplastic nevi and 34 melanomas, were examined. Expression of cyclins was detected by immunohistochemistry and quantified as a percentage of immunostained cell nuclei in each sample. Significant differences in expression of both cyclins were found between all lesion types: the median percentage of cyclin A-positive nuclei was 8.2% in melanomas, 3.4% in dysplastic nevi, and 0.95% in common nevi ($p < 0.001$). The corresponding percentages for cyclin E were 9.5%, 4.25% and 1.44% ($p < 0.001$). Expression of both cyclins was significantly higher among patients with a personal history of NMSC. Cyclin A was also significantly overexpressed in patients with a high total nevus count (TNC) compared to moderate and low TNC. Expression of cyclins did not significantly correlate with the other clinicopathologic features investigated. These findings indicate the possible involvement of cyclins A and E in the pathogenesis of malignant melanoma. Our results also show a potential diagnostic significance of these cyclins as markers allowing discrimination between dysplastic nevi and melanoma. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 263–269)*

Key words: cell cycle, cyclin A, cyclin E, melanoma, nevus

Introduction

Progression through the cell cycle is controlled by cyclins, cyclin-dependent kinases (CDK), various transcription factors, suppressor gene products, and inhibitory proteins. Cyclin E, in conjunction with CDK2, regulates essential processes at the G1/S boundary of the cell cycle. Cyclin A, co-operating first with CDK2 and then with CDK1, plays a crucial role in DNA replication in the S phase and induces the cell to enter the M phase [1].

Altered expression of cyclins A and E has been demonstrated in different malignancies. Cyclin A overexpression was detected in several myeloid leukemia cell lines [2], and its level was also found to correlate with astrocytoma malignancy [3]. In colorectal cancer, high levels of cyclin A were associated with

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a poor prognosis [4]. A similar relationship was demonstrated in non-Hodgkin's lymphoma, non-small-cell lung carcinoma, esophageal squamous cell carcinoma, prostatic cancer, carcinomas of the renal pelvis and ureter, osteosarcoma and other soft tissue and smooth muscle tumors [5–13]. The clinical significance of cyclin E overexpression has been proved in laryngeal squamous cell carcinoma, non-small cell lung cancer, endometrial adenocarcinoma, human papilloma virus-related cervical neoplasia, testicular germ cell tumors, gastric and pancreatic cancer and hepatocellular carcinoma [14–21]. The association of cyclin E overexpression with tumorigenesis has also been observed in lymphomas, acute myelogenous leukemia, chronic lymphocytic leukemias and osteosarcomas [10, 22–24]. The role of cyclin E has been extensively investigated in breast cancer; the results showed that it was not only a factor involved in carcinogenesis, but also could be a valuable prognostic marker and biochemical marker of tamoxifen treatment efficacy [25].

In dermatological research, most clinical and immunohistochemical studies have concerned the expression of cyclin D1 and D3 in a variety of benign and malignant tumors including melanocytic lesions. Our previous study on expression of cyclins D1 and D3 in melanocytic lesions demonstrated that G1/S abnormalities were crucial for the progression of malignant melanoma, and that enhanced cyclin D1 and D3 expression was associated with increased melanocyte proliferation in both melanoma and dysplastic nevi [26]. On the other hand, the expression of cyclins A and E in melanocytic skin lesions has been the subject of only a few studies published to date, and some results are discordant. In the study by Georgieva et al. [27], significant overexpression of cyclin E was observed in primary and metastatic melanomas compared to nevi. Increased expression of cyclin E in melanomas was also reported by Bales et al. [28] and Tang et al. [29]. Bales et al. [30] provided evidence that low molecular isoforms of cyclin E forms are involved in the regulation of human melanoma progression and invasion. There is, however, some controversy about the role of cyclin A in melanomas. Most authors have reported its overexpression [29, 31, 32], and some of them have suggested a strong correlation of that cyclin with melanoma cell proliferation [33, 34] and indicated its potential as a prognostic factor for patients with superficial spreading melanomas [35]. On the other hand, Georgieva et al. [27] found very weak expression of cyclin A in melanomas, not different to that in nevi.

In the present study, we investigated by immunohistochemistry the expression of cyclins A and E in common nevi, dysplastic nevi, and malignant melanomas in correlation with (1) pathological parameters, such as tumor thickness (Breslow scale), ulceration, regression, and mitotic rate, as well as (2) several clinical and phenotypic parameters known to correlate with the incidence of melanoma, such as skin phototype, hair and eye color, nevus count, personal or family melanoma history, and personal history of nonmelanoma skin cancer.

Material and methods

Patients. Melanocytic skin lesions (n = 102) were collected from 52 male and 50 female patients at the Department of Dermatology, Jagiellonian University Medical College, Krakow, Poland. The mean age of the whole group was 44.1 years (SEM ± 1.91). Melanomas were diagnosed in 17 males and 17 females (mean age 62.1 ± 2.39 years), dysplastic nevi in 20 males and 18 females (mean age 34.8 ± 2.88 years), and common nevi in 15 patients of each sex (mean age 36.5 ± ± 2.88 years). The age differences between sexes were statistically insignificant. None of the patients with melanoma had metastases. The study was approved by the Ethics Committee of the Jagiellonian University in May 2008. Written informed consent was obtained from each patient following the guidelines of the Helsinki Declaration.

Dermoscopy and clinical examination. The lesions were selected for removal by dermoscopy using an Eris Medical videodermatoscope. Surgical excision of melanocytic skin lesions for histopathological evaluation was recommended when the lesion showed one of the following clinical or dermoscopic features: (1) benign nevus located in highly traumatic area; (2) the presence of two out of three criteria of the three-point checklist as a screening method [36]; or (3) the presence of one or two melanoma-specific local criteria. The samples collected included 30 common nevi (compound nevi — 16, intradermal nevi — 10, junctional nevi — 2, combined nevi — 2), 38 dysplastic nevi and 34 melanomas including nodular melanoma — 14, superficial spreading melanoma — 11, lentigo maligna melanoma — 6, acral lentiginous melanoma — 1, and undefined melanoma — 2.

During routine dermatological examination, data concerning phenotypic features such as skin phototype according to Fitzpatrick [37], eye and hair color and total nevus count were collected.

Skin phototype II was classified as 'fair' and III, IV — as 'dark'. There were no patients with I, V or VI phototypes. Hair color was classified as fair hair (blond(e), red, light brown) or dark hair (dark brown, black). Eye color was also divided into two groups: fair eyes (blue, green) and dark eyes (brown, black).

The total nevus count was divided into three groups: < 20 nevi (low nevus count); 21–100 nevi (moderate nevus count); and > 100 nevi (high nevus count).

Patients also filled out a questionnaire [38] which contained questions concerning their personal and/or family melanoma history, and personal history of nonmelanoma skin cancer.

Histopathology and immunohistochemistry. The excised samples were fixed in 10% buffered formalin and embedded in paraffin. All lesions were evaluated using the standard histopathological criteria (hematoxylin and eosin-stained 6 μm sections). The histological identification of dysplastic nevi was based on the presence of junctional nests of melanocytes often exhibiting large pleomorphic nuclei and prominent nucleoli, uniformly elongated rete ridges and lymphocytic infiltrations [39].

For each melanoma sample, tumor thickness, ulceration, regression and mitotic rate were evaluated. Tumor thickness was classified as thin (< 1 mm) or thick (≥ 1 mm). Ulceration (area devoid of epidermis) was coded as 0 — absent, or 1 — present. The histological features of regression included reduction in melanocyte density, as well as fibrosis often accompanied by prominent small vessels and lymphocytic infiltration [40]. Regression was coded as 0 — absent, or 1 — present. The mitotic rate (MR) was estimated in a 1-mm ˛area, by beginning in the field with the highest numbers of mitoses and then by counting in subsequent non-overlapping fields, as recommended by the 1982 Pathology Workshop [41, 42]. Due to the heterogeneity of our melanoma group (regarding differences in the number of mitotic figures per 1 mm˛ between cases) we decided to code MR as 0 — no mitoses or 1 — mitoses present [43].

For immunohistochemistry, deparaffinized 6 μm sections were subjected to antigen retrieval in 10 mM citrate buffer, pH 6.0 (5 min preincubation followed by 9 min in microwave oven, 160 W, at 100°C, and 30 minutes in a vapor-bath at 80°C). Next, the sections were incubated overnight at 4°C with primary mouse antibodies against cyclin A or E (NCL Cyclin A, NCL cyclin E, Novocastra Laboratories Ltd., Newcastle, UK, dilution 1:50 and 1:40, respectively), followed by secondary goat anti-mouse Cy3-conjugated antibody (Jackson IR, West Grove, PA, USA, code no. 115-165-146, dilution 1:400). Cell nuclei were counterstained with DAPI (Sigma, St Louis, MO, USA). Only nuclear immunostaining was scored as positive. In each sample, cyclin expression was assessed as percentage of cyclin-positive nuclei in the examined lesion. Microscopic examination, digital imaging of sections and image analysis were carried out using an Olympus BX-50 bright field/epifluorescence microscope equipped with DP-71 camera (Olympus, Japan) and PC-based image analysis software (AnalySIS-FIVE®, Soft Imaging System GmbH, Münster, Germany).

Statistical analysis. The relationship between expression of cyclins, the type of melanocytic skin lesion (common nevi, dysplastic nevi, melanoma) and other features was evaluated by nonparametric ANOVA rank Kruskal–Wallis plus *post-hoc* and Mann–Whitney tests, with $p < 0.05$ as the condition for statistical significance. For analysis of qualitative parameters, contingency tables with Pearson's chi-square test for independence were used.

Results

All investigated lesion samples showed expression of both cyclins; immunostaining was mostly observed in cell nuclei (Figure 1), with some cells also exhibiting weak cytoplasmic staining. In all types of melanocytic lesions (common nevi, dysplastic nevi and melanoma), the distribution of cyclin-positive cells was uniform. In normal skin (healthy margins of the excised lesions), the expression of cyclins A and E was not detected.

Statistically significant differences in the expression of both cyclins were observed between all skin lesion types ($p < 0.001$, Figure 2). The median percentage of cyclin A-positive nuclei was 8.2% in melanomas, 3.4% in dysplastic nevi and 0.95% in common nevi, with differences between melanomas and both dysplastic and common nevi statistically significant at $p < 0.001$, and between dysplastic and common nevi at $p = 0.045$. The corresponding percentages for cyclin E were 9.5%, 4.25% and 1.44%, with differences between melanomas and dysplastic nevi significant at $p = 0.02$, between melanomas and common nevi at $p < 0.001$, and between dysplastic and common nevi at $p = 0.008$.

In the melanoma group, there were 11 cases (32.35%) of thin melanoma (< 1 mm) and 21 cases (61.76%) of thick melanoma (≥ 1 mm including 3 cases > 4 mm); in two cases (LM, SSM *in situ*) the Breslow scale was not counted. However, no significant correlations were observed between cyclin expression and tumor thickness. No significant differences in cyclin A and E scores were found either in melanoma group patients with ulceration or regression presented in histopathological examination, or in relation to mitotic rate.

Statistically significant differences ($p < 0.001$) were observed between all groups (melanoma, common nevi and dysplastic nevi) in total nevus count. The largest high TNC proportion (65%) and the smallest low TNC proportion (0%) were demonstrated in the melanoma group. Differences in cyclin A expression were statistically significant between patients with high TNC compared to moderate and low TNC (8.15% vs. 5.76% vs. 3.98%, $p=0.0056$). Differences in cyclin E expression did not show significance (Figure 3).

No significant correlations were found between cyclin A and E expression and such phenotypic features as hair color, eye color or skin phototype, neither in the whole group, nor in any separately investi-

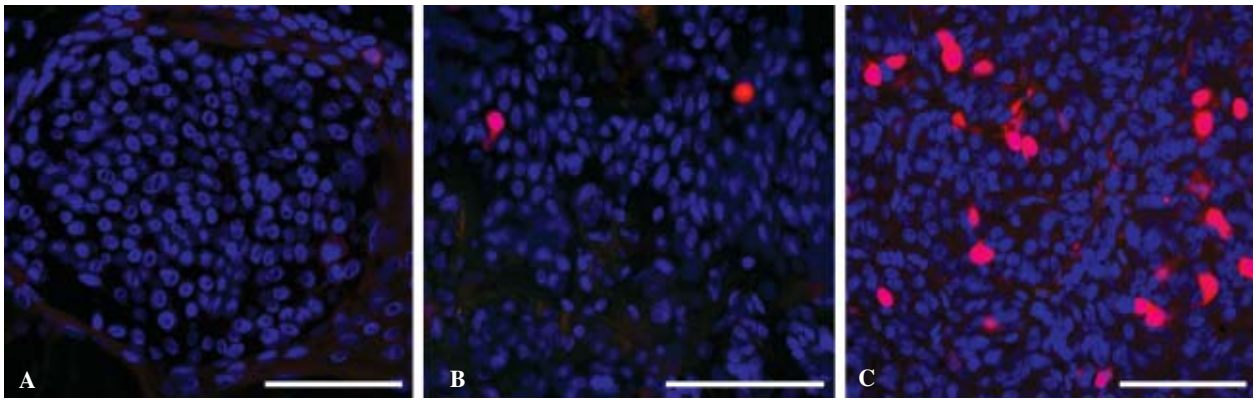

Figure 1. Representative micrographs showing exemplary cyclin immunostaining in melanocytic skin lesions: cyclin A-positive nuclei (red) in common nevus (**A**), dysplastic nevus (**B**) and melanoma (**C**). Nuclei counterstained by DAPI (blue). Scale bar = 100 μm

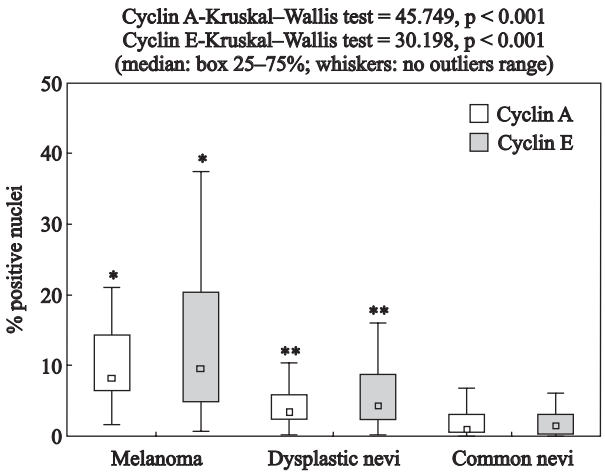

Figure 2. Cyclin A and E expression in three types of melanocytic skin lesion. Asterisk: significantly different ($p < 0.05$) from dysplastic nevi and common nevi; double asterisk: significantly different from melanoma and common nevi

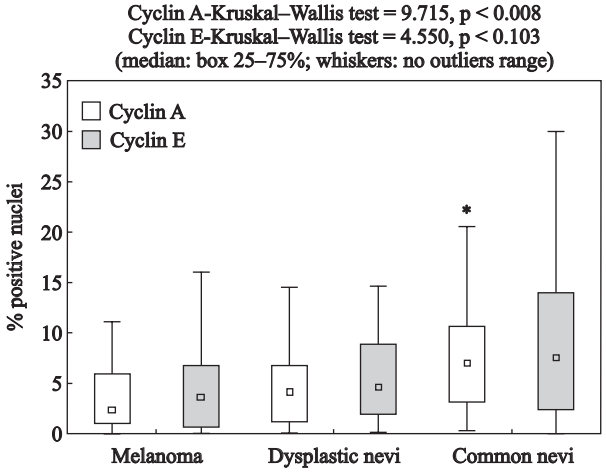

Figure 3. Correlation between cyclin A and E expression and total nevus count (low: < 20 nevi; moderate: 21–100 nevi; high: > 100 nevi). Asterisk: significantly different ($p < 0.05$) from moderate and low total nevus count

gated group with the particular type of melanocytic skin lesion.

For the whole investigated population of patients, we checked a possible relationship between cyclin A and E expression and personal or family melanoma history, as well as personal history of NMSC. Expression of both cyclins was significantly higher only among patients with a personal history of NMSC (Figure 4).

Discussion

In our study, all investigated lesion samples showed expression of both cyclins, with statistically significant differences between melanomas and dysplastic nevi, between melanomas and common nevi, and between dysplastic and common nevi. Both cyclins revealed significantly lower expression in dysplastic nevi than in melanomas, but higher than in common nevi.

A similar pattern was observed by us in the case of cyclin D3 [26] and we suggest that it could serve as a suitable diagnostic marker for differentiation of these lesions in histologically uncertain cases. Whether the same can be postulated for cyclins A and E separately is rather doubtful, since the level of significance is lower, although the study was carried out on a larger number of lesion samples. However, a joint examination of the three cyclins could provide a useful and more reliable diagnostic approach in such cases.

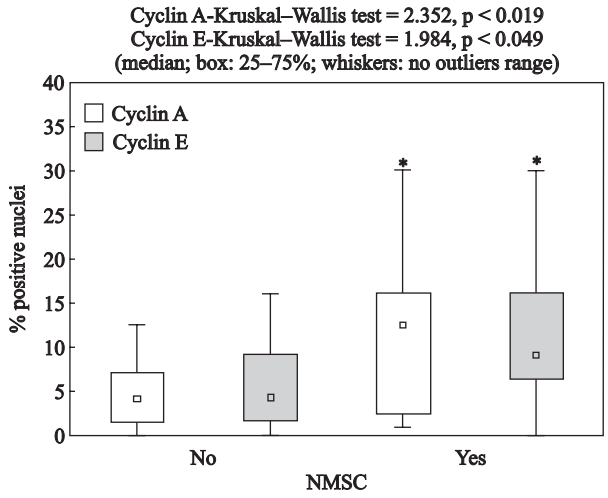

Figure 4. Correlation between cyclin A and E expression and personal history of nonmelanoma skin cancer (NMSC). Asterisk: significantly different ($p < 0.05$) from the group with no NMSC history

There is no available data about cyclin A and cyclin E expression in dysplastic nevi in comparison with melanoma. In our study, melanomas showed significantly higher expression of cyclins A and E compared to dysplastic nevi. That latter group of melanocytic lesions still remains a potentially challenging diagnostic problem for the pathologist because of its histological similarities with some melanomas. In the studies comparing expression of cyclins in melanocytic nevi and melanomas, dysplastic nevi were usually not included in the analysis as a separate group. In ambiguous cases, an additional marker is needed to improve diagnostic accuracy [44]. Cyclins examined in this study seem to be potential candidates.

'Fair' hair, eyes and skin (Fitzpatrick phototype I, II) are the characteristic phenotypic features of a 'typical' patient with melanoma [45]. In patients examined in this study, a significant predominance of fair hair was observed in all groups, and of fair skin in melanoma patients, while no significance was found for the occurrence of fair/dark eyes. There were, however, no significant correlations between cyclin A and E expression and these phenotypic features.

It has been demonstrated that total nevus count and the count of dysplastic nevi positively correlates with melanoma risk [45, 46]. The expression of cyclin A (but not E) was significantly higher in patients with high total nevus count compared to those with moderate and low total nevus count, suggesting that cyclin A might be involved in the formation of multiple nevi.

Several clinical and histological parameters influence melanoma prognosis. Of these, the most important for primary tumors are tumor thickness (Breslow scale), mitotic rate and the presence of ulceration or regression. In problematic cases, these parameters are not always sufficient and additional data is needed to clarify the prognosis [43, 44, 47].

In the melanoma group, there was no correlation between cyclin A and E expression and tumor thickness, irrespective of tumor type. Other authors, however, have reported such a correlation. Flørenes et al. [35] who investigated cyclin A expression in 172 primary, 73 metastatic melanomas and ten benign nevi found tumor thickness to be the strongest prognostic marker and cyclin A to be an independent prognostic predictor of relapse-free survival. Similar results were demonstrated by Tran et al. [33], who found a statistically significant correlation between expression of cyclin A and tumor thickness and mitotic index.

In this study, there was no statistically significant correlation of cyclin A and E expression with mitotic rate. This is quite surprising, and might be due to a relative scarcity of mitotic figures in our material.

As we did not show statistically significant differences in cyclin A and cyclin E expression among melanoma group patients with or without ulceration or regression presented in histopathological examination, the studied cyclins do not seem to have any prognostic significance in this context. Accordingly, neither ulceration nor the location of primary tumors had an impact as a prognostic factor in the study of Florenes et al. [35].

A patient with a personal history of melanoma is clearly at greater risk for subsequent melanoma. For melanoma patients with a positive family history, the risk for a second primary melanoma increases to 19% [45]. Among our patients, only five had a personal, and six a family, melanoma history. So, because of the low number of such cases, the absence of significant differences in cyclin A and E expression in relation to melanoma history should not be regarded as proven, especially since expression of both cyclins was significantly higher among patients with a personal history of NMSC.

In summary, the present study has shown overexpression of cyclins A and E in melanoma compared to dysplastic and common nevi. A positive correlation between those cyclins' immunoreactivity and some clinical parameters of melanoma implicates their role in the pathogenesis of this neoplasm. Our findings also indicate a potential diagnostic significance of cyclins A and E as proliferation markers in melanocytic lesions.

Acknowledgements

This study was supported by research grant 501/NKL/ /145/L from the Jagiellonian University Medical College.

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Submitted:16 September, 2011
Accepted after reviews: 23 November, 2011