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Source / Izvornik: **Collegium antropologicum, 2007, 31 - Supplement 1, 17 - 22**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:263269

Coll. Antropol. **31** (2007) Suppl. 1: 17–22
Original scientific paper

Expression of Cell Cycle and Apoptosis Regulatory Proteins and Telomerase in Melanocitic Lesions

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A B S T R A C T

To gain insight into the role and association of cell cycle and apoptosis regulatory proteins and telomerase activity in the course of progression of melanocitic lesions we have examined immunohistochemicaly, expression and the distribution of p53, bcl-2, Ki-67 and telomerase in 25 samples of common and dysplastic nevi, and 45 samples of primary invasive melanomas. Protein p53 expression was significantly increased in dysplastic as compared with common nevi and melanomas ($p<0.001$). Bcl-2 protein expression was significantly increased in melanomas as compared with common aquired and dysplastic nevi ($p=0.001$). Nevi and melanomas exhibited clear-cut differences in terms of Ki-67 expression. Telomerase expression was significantly increased in melanomas as compared with common acquired ($p=0.014$) and dysplastic nevi ($p<0.001$). Enhanced telomerase activity in association with increased bcl-2 expression in the course of melanoma progression could contribute to development and progression of melanoma.

***Key words**: bcl-2, melanoma, nevi, telomerase*

Introduction

Melanoma is the most devastating form of skin cancer. It is becoming clear that solar ultraviolet radiation is a main culprit in the etiology of melanoma the same as in basal and squamous cell carcinomas. Although sun exposure probably plays a primary or supporting role in melanoma, etiology of these tumors is probably variable and multifactorial. It has become clear that molecular events regulating cell survival, growth arrest, apoptosis and cell differentiation, are important contributors to the overall kinetics of benign and malignant cell growth[1–3]. p53 protein is well-described tumor suppressor that has a central role in the initiation of apoptosis and cell cycle control whereas Bcl-2 and its homologous proteins have emerged as one of the most important regulators of programmed cell death, playing a crucial role in the balance between cell survival and cell death[3–6]. The pro-survival proteins such as bcl-2/bcl-x block apoptosis, whereas bax-like proteins bax and bak induce apoptosis[5–7].

On the other hand, cell proliferation capacity, tumorigenesis and immortalization have been controlled by telomerase. Telomerase is a ribonucleoprotein DNA polymerase that is responsible for maintaining the length of telomeres on the end of chromosomes[8]. The role of telomeres is to protect chromosomes from degradation and aberrant recombination during replication prolonging the life span of the cell. This enzyme is repressed in human somatic cells, with a few exceptions, resulting in progressive loss of telomeres and shortening of the chromosome with successive cell divisions[9]. Finally, chromosomes reach a critical length at which cell division ceases, senescence begins, and the cell ultimately undergoes apoptosis or cell death. Contrary, telomerase is highly expressed in >85% of cancer cells including melanoma[10,11].

Little is known about correlation of apoptosis regulatory proteins, proliferation and other biomarkers of ma-

Received for publication November 2, 2006

lignancy, such as telomerase in melanocytic lesions. Activation of telomerase and deregulation of apoptosis regulatory proteins contribute to pathogenesis of a significant number of human malignances. To gain insight into the role of cell cycle and apoptosis regulatory proteins and telomerase activity in the course of progression of melanocitic lesions as well as their association we have examined expression and distribution of p53, bcl-2, Ki-67 and telomerase in common nevi, dysplastic nevi and melanomas.

Materials and Methods

Patients and skin specimens

Formalin fixed, paraffin-embedded archival tissue blocks from 50 benign melanocitic lesions (25 common acquired nevi and 25 dysplastic nevi) and 45 melanomas (primary invasive melanomas) were retrieved from Department of Pathology, Rijeka University School of Medicine, Croatia. 4 m-thick sections were stained with hematoxylin-eosin and two pathologists examined each slide independently.

Tissues were obtained from 50 (52.63%) male and 45 (47.37%) female patients. Median age of the patients was 52.10 years (range 48–69 years), 60.87 years (range 49–71 years) and 62.50 years (range 46–76 years) for common acquired nevi, dysplastic nevi and melanoma, respectively.

Immunohistochemical staining

The following antibodies and dilutions were used: mouse monoclonal antihuman p53 antibody, DO7 (DAKO A/S, Glostrup, DK), at a dilution of 1:50, rabbit monoclonal antihuman Ki-67 antibody, MIB-1 (DAKO A/S, Glostrup, DK), at a dilution of 1:50, mouse monoclonal antihuman bcl-2 antibody, Clone 124 (DAKO A/S, Glostrup, DK), mouse monoclonal antihuman TELOMERASE (Catalitic unit-hTERT) antibody (NOVOCASTRA, Newcastle upon Tyne, UK). Specimen of gastric cancer, oral mucosa, lymph node and tonsil served as positive control samples for p53, Ki-67, bcl-2 and telomerase, respectively. Additional sections were run in parallel with omission of the primary antibodies and served as negative control.

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated by washing in absolute and diluted ethyl alcohol and distilled water. Staining of p53, Ki-67 and bcl-2 proteins was carried out after sections were treated with antigen retrieval solution (DAKO Chem Mate, Glostrup, DK) at a dilution of 1:9 and placed in microwave for 20 minutes for antigen retrieval. This was followed by the standard ABC (avidin-biotin complex) procedure for 130 minutes in DAKO Techmate Immunostainer (Techmate Horizon, serial No. 30097, LJL Biosystems Inc., USA).

Immunostaining results of p53, Ki-67 and bcl-2 were quantified and expressed as percentage of positive keratinocytes out of 1000 cells counted in each section. For telomerase the extent of staining was independently evaluated by two observers and recorded semi-quantitatively as determined by the percentage of lesional cells (0%/–, 1–25%/1+, 26–75%/2+, and >75%/3+) as described previously[10]. The intensity of telomerase staining was graded as follows: 0 (negative), 1+ (weakly positive), 2+ (moderately positive), and 3+ (intensively positive) as described previously[10]. Counting fields were randomly selected to minimize possible bias.

Statistics

The normality of data was tested using Kolmogorov-Smirnov test. Since the distributions were normal, the results were presented as mean ± standard deviation and 95% confidence intervals. We used the parametric test one-way ANOVA to compare differences in p53, bcl-2 and Ki-67 expression between tested groups, and Tukey-HSD test for post-hoc analysis. We computed the correlation matrix using Pearson's product-moment correlation for analysis of correlations between positively stained of p53, Ki-67, bcl-2 proteins and telomerase in all three groups.

All statistical analyses were performed using Statistica for Windows (release 6.1., StaSoft. Inc., Tulsa, OK, USA). The level of statistical significance was set at 0.05.

Results

Nuclear staining for p53 protein was present in 35% of common nevi, 48% of dysplastic nevi and 47% of melanomas. Positive p53 cells were distrubuted in a diffuse pattern throughout melanocytic lesions. Expression values of p53, Ki-67, bcl-2 and telomerase are shown in Figure 1. Protein p53 expression was significantly increased in dysplastic nevi as compared with common nevi and

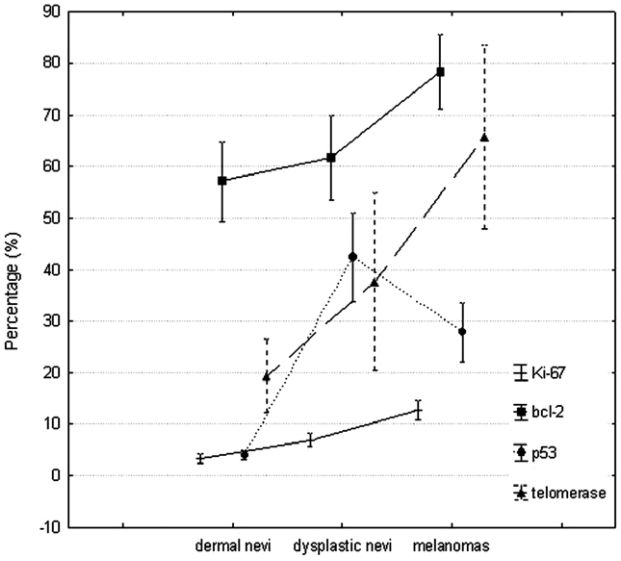

*Fig. 1. Expression of p53, Ki-67, bcl-2 and telomerase in melanocitic lesions.*for p53, Ki-67, bcl-2 and telomerase statistical significance is p<0.0001.*

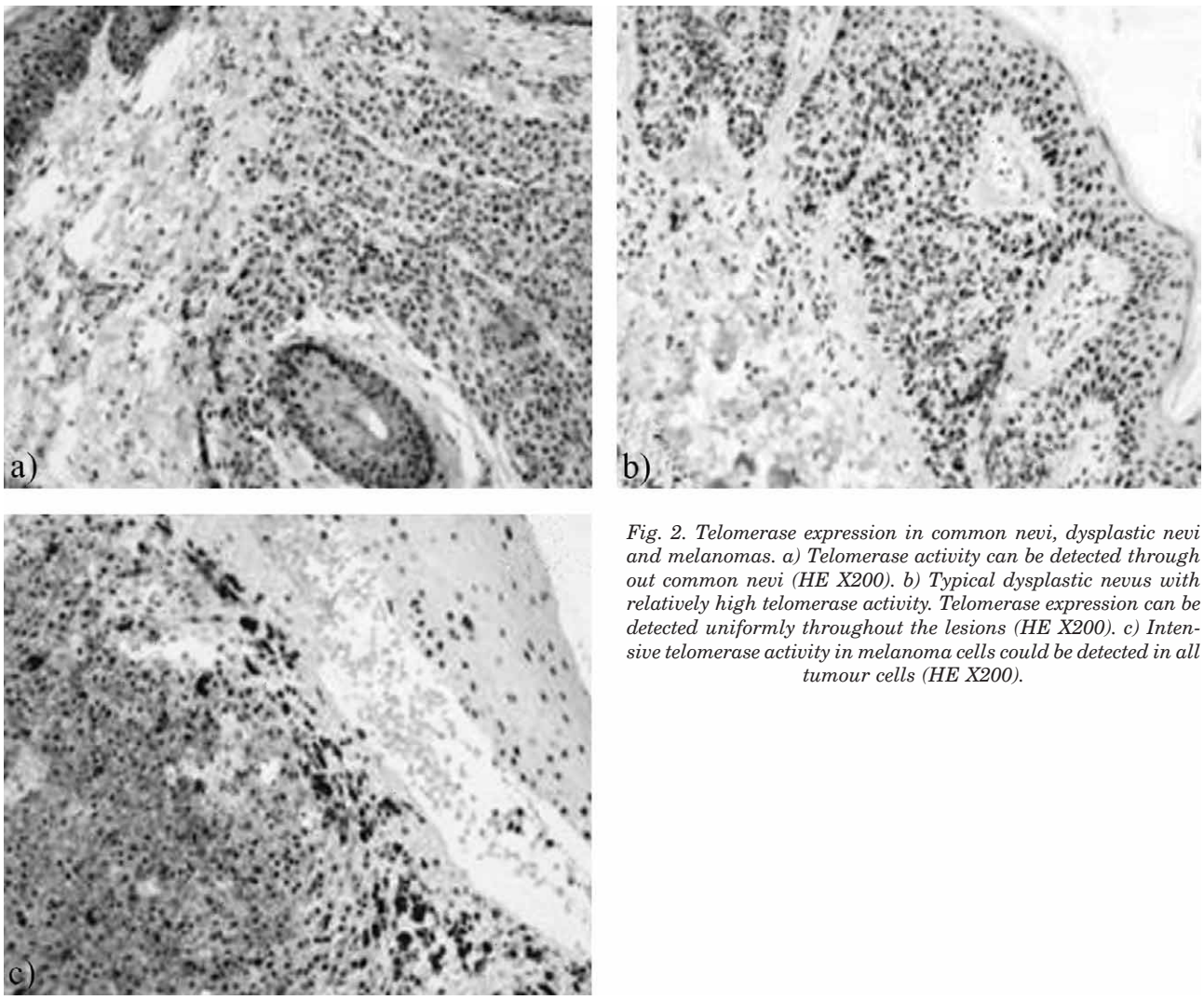

Fig. 2. Telomerase expression in common nevi, dysplastic nevi and melanomas. a) Telomerase activity can be detected through out common nevi (HE X200). b) Typical dysplastic nevus with relatively high telomerase activity. Telomerase expression can be detected uniformly throughout the lesions (HE X200). c) Intensive telomerase activity in melanoma cells could be detected in all tumour cells (HE X200).

melanomas ($p<0.001$). The lowest values were detected in common nevi and were significantly lower as compared with melanomas ($p<0.001$).

Nuclear expression of Ki-67 protein was detected in all cases examined. Ki-67 positivity showed same distribution pattern as p53-positivity in common and dysplastic nevi and melanomas. We also found some positivity in dysplastic and morphologically normal skin surrounding dysplastic nevi and especially melanomas. Nevi and melanomas exhibited clear-cut differences in terms of Ki-67 expression. The most intense cell proliferation was detected in melanomas and was significantly increased as compared to both nevi groups ($p<0.0001$). Also, Ki-67 protein expression was significantly lower in common nevi as compared with dysplastic nevi ($p<0.0001$).

Strong expression of Bcl-2 was detected in 41% of common nevi, 46% of dysplastic nevi and 54% of melanoma samples, similarly to previous study[6]. The Bcl-2 positive cells were distributed diffusely in all melanocytic lesions. Bcl-2 protein expression was significantly increased in melanomas as compared with common acquired and dysplastic nevi ($p=0.001$), while difference between two nevi groups did not reach statistical significance ($p=0.680$).

Human telomerase reverse transcriptase (hTERT) staining was detected in all melanocytic lesions examined as previously shown by other studies[10]. Telomerase expression was significantly increased in melanomas as compared with common acquired ($p=0.014$) and dysplastic nevi ($p<0.001$), while the difference between the two nevi groups did not reach statistical significance ($p= 0.138$). The pattern of hTERT expression was uniform throughout the lesions, with the same degree of staining in the lower half as the upper half of the dermal component in benign melanocytic nevi and melanomas (Figure 2.). Staining intensity was significantly increased in melanomas as compared to both nevi groups ($p<0.05$) and in dysplastic nevi as compared to common nevi ($p<0.05$). The nucleus, especially nucleolus, showed greater staining intensity as compared with the cytoplasm of the melanocytes.

We found positive, statistically significant correlation between p53 and bcl-2 expression in dysplastic nevi ($p < 0.001$, and $r=0.531$) but correlation did not reach statistical significance in dermal nevi ($p=0.618$; $r=0.077$) and in melanomas ($p=0.716$; $r=–0.056$).

In common nevi, there was a positive significant correlation between Ki-67 and telomerase expression ($p=0.006$; $r=0.790$) while correlation between bcl-2 and telomerase expression ($p=0.890$; $r=0.050$) and between p53 and telomerase ($p=0.980$; $r=–0.008$) did not reach statistical significance.

In dysplastic nevi, correlations between telomerase and Ki-67 expression ($p=0.362$; $r=0.323$), p53 and telomerase ($p=0.859$; $r=0.064$) as well as between telomerase and bcl-2 ($p=0.627$; $r=–0.176$) were not statistically significant.

In all melanocytic lesions examined there was a positive correlation between p53 and Ki-67 protein expression or a tendency toward higher p53 protein expression with increasing cell proliferation ($p=0.318$; $p<0.05$; $p < 0.05$; with $r=0.790$, $r=0.531$, $r=0.187$; for common nevi, dysplastic nevi and melanomas, respectively).

Similarly, in melanomas correlations between telomerase and Ki-67 expression ($p=0.318$; $r=0.352$), p53 and telomerase ($p=0.605$; $r=0.187$) as well as between telomerase and bcl-2 ($p=0.418$; $r=–0.289$) were not statistically significant.

Discussion

Telomerase activity has been evaluated in many tumors for diagnostic purposes, and an increase has been linked with tumor progression. Previous studies mainly used polymerase chain reaction (PCR)-based TRAP (telomeric repeat amplification protocol) method or *in situ* hybridization assays to quantify the level of telomerase activity [8,11,12].

We have detected increasing trend of hTERT expression, going from common nevi to dysplastic nevi and primary melanoma which suggests a role of telomerase in tumor progression. Our data also show some overlapping results for hTERT expression and staining intensity in different melanocytic lesions (melanomas and dysplastic nevi). Thus, hTERT expression is not a reliable for discrimination between benign and malignant melanocytic lesions in paraffin-embedded tissues. All of these findings are in agreement with previously published studies[8,10,11].

Some studies suggested increase in bcl-2 protein expression with advancing melanoma as well as associated increase of other anti-apoptotic proteins such as bcl-x. These results could reflect increased malignant potential caused by inhibition of apoptosis and growth advantage of metastatic melanoma cells[6]. However, other results suggested a decrease of anti-apoptotic proteins[13] or a detectable level of bcl-2 in all tissues of melanocytic origin[14]. Although the widespread expression of bcl-2 in both nevi groups and melanomas implies that altered expression of this oncoprotein is not the only factor involved in melanoma progression, significant increase of bcl-2 detected in melanomas suggests its contributory role in the process of malignant alteration and progression. Alterations in apoptotic proteins expression in association with increased telomerase activity probably play a synergic role in malignant transformation and melanoma progression.

Significantly higher p53 protein expression in dysplastic nevi as compared with melanomas suggests the necessity of other contributory factors in the course of progression to melanoma. Bcl-2 down regulation has been shown to occur preferentially in melanoma cells with mutated p53[15]. Our results are in accordance with previous studies and suggest that the p53 gene is not frequently mutated in melanoma[16,17]. Since most genotoxic agents act primarily via p53 to induce apoptosis, one would expect for melanoma cells to be sensitive to DNA damaging. However, exact cause of treatment failure in metastatic melanoma remains unsolved. This lack of p53 mutations may be explained in part by CDKN2A aberrations affecting p14ARF and also by inactivation of other downstream apoptotic effectors[16].

In vitro and *in vivo* studies suggested that hTERT is downregulated by wild-type p53 and telomerase activity upregulated by bcl-2 expression[18] but we have detected no significant correlation between p53 protein and telomerase expression in examined melanocytic lesions. These results suggest that telomerase activity is not always dependent on the presence of cell cycle regulating as well as pro-apoptotic and anti-apoptotic proteins.

It has been suggested that telomerase reactivation is associated with increased immunohistochemical expression of bcl-2 protein, and is thought to prevent apoptosis in normal cells[19]. In examined melanocytic lesions we have found no correlation between telomerase and bcl-2 expression confirming that telomerase reactivation is independent of bcl-2 protein expression in the course of progression from common and dysplastic nevi to melanoma. On the other hand, telomerase activity has been found to correlate with bcl-2 and bcl-xL expression as well as with reduced apoptosis in head and neck cancer[20].

A strong correlation between telomerase activity and proliferation markers in skin melanocytic lesions suggested that telomerase activity could be used as a marker for cell proliferation as various other methods, including Ki-67 protein expression[8,11]. Differences in correlation between telomerase and proliferation markers could be explained by recent results showing that the overexpression of hTERT subunit promoted stem-cell proliferation without changes in length of telomeres[21,22], but again not all proliferating cells express telomerase[23].

We have detected a significant increase of Ki-67 protein expression and telomerase activity in progression from common to dysplastic nevi and melanoma, but significant positive correlation was detected only in common nevi, while in dysplastic lesions and melanomas there was only a tendency of higher telomerase activity in the faster proliferating lesions. These findings suggest

that Ki-67 proliferation index and telomerase activity do not always correlate significantly in all tumors, as it has been shown previously for B-cell lymphoma[24]. The activation of telomerase in the absence of proportionally increased proliferative activity has been suggested to reflect a step toward immortalization, although transformation to full-blown malignancy may require additional molecular events[11]. A relation between telomerase activity and cell proliferation does exist since cell terminal differentiation and cell-cycle exit are accompanied by telomerase activity down-regulation, whereas telomerase is re-expressed following the re-entry of quiescent cells into the cell cycle[8,9]. Given the fact that cancers are heterogeneous and not all of their cells are telomerase-competent, telomerase activity might therefore also vary according to the percentage of proliferative cells vs. quiescent/differentiated and/or normal cells that are present in a tumor sample[9]. It has been proposed that the regulatory mechanisms controlling telomerase activity in melanoma rely on the transcription and alternative splicing of human telomerase[25]. There is evidence that telomerase has an active anti-apoptotic role[26]. These findings could explain unclear correlation between telomerase and Ki-67 protein expression detected in dysplastic nevi and melanomas.

Cases of cutaneous melanoma with an intermediate Ki-67 index and high telomerase activity have been described to follow a fatal course, whereas no progression was observed in a case of melanoma with higher Ki-67 index and low telomerase activity. These findings suggest that telomerase activity correlates poorly with the Ki-67 index while being closely related with the clinical outcome[11].

Telomerase is activated and upregulated in melanomas as compared to its precursor lesions dysplastic and common nevi. In majority of melanocytic lesions telomerase activity does not correlate with bcl-2, Ki-67 and p53 protein expression. Mechanisms that promote both cell proliferation and cell survival appear to be activated in melanoma but telomerase activation and bcl-2 expression are independent events. In addition to enhanced telomerase activity and its possible anti-apoptotic roll[26], increased bcl-2 expression in the course of melanoma progression could contribute to development and progression of melanoma.

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EKSPRESIJA PROTEINA STANIČNOG CIKLUSA, REGULATORA APOPTOZE I TELOMERAZE U MELANOCITNIM LEZIJAMA

S A Ž E T A K

Da bi dobili uvid u povezanost i ulogu proteina staničnog ciklusa, proteina regulatora apoptoze i telomerazne aktivnosti tijekom procesa napredovanja melanocitnih lezija imunohistokemijski smo ispitali razinu izražaja i distribucije p53, blc-2, Ki-67 i telomeraze kod 25 uzoraka običnih i displastičnih nevusa i 45 uzoraka primarnih invazivnih melanoma. Ekspresija p53 proteina je bila statistički značajno povećana kod displastičnih nevusa u usporedbi sa običnim nevusima i melanomima ($p<0.001$). Razina izražaja Bcl-2 proteina je bila statistički značajno povećana kod melanoma u usporedbi sa običnim stečenim i displastičnim nevusima ($p=0.001$). Nevusi i melanomi su pokazali jasne razlike u smislu Ki-67 ekspresije. Telomerazna ekspresija je bila statistički značajno povećana kod melanoma u usporedbi sa običnim stečenim ($p=0.014$) i displastičkim nevusima ($p<0.001$). Povećana telomerazna aktivnost zajedno sa povećanom bcl-2 ekspresijom u procesu progresije melanoma mogla bi doprinijeti nastanku i razvoju melanoma.