Correlation of cell cycle regulatory proteins (p53 and p16^{ink4a}) and bcl-2 oncoprotein with mitotic index and thickness of primary cutaneous malignant melanoma

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Abstract

The purpose of the study was to determine the frequency of expression p53 and p16INK4a proteins and bcl-2 oncoprotein in malignant skin melanoma and to determine their correlation with the proliferative index and tumor thickness. The study involved 53 patients: 27 (51%) male and 26 (49%) female. Mitotic index showed a correlation with p53 protein expression, a negative correlation with p16INK4a protein expression. Statistically significant correlations were determined between the Breslow tumor thickness, Clark invasion level and p53 protein expression, as well as Breslow tumor thickness and bcl-2 oncoprotein expression (p<0.05), whereas there was no correlation between the p16INK4a protein expression and melanoma thicknes and Clark invasion level. Overexpression p53 protein and bcl-2 oncoprotein, with the loss p16INK4a protein of expression in the nodular melanoma, confirms a frequent loss of function of these tumor suppressor gene and oncogene, and indicates a vertical tumor growth phase. The loss of tumor suppression function the p53 protein and bcl-2 oncoprotein overexpression in cutaneous melanoma correlates with larger tumor thickness, whereas the overexpression of mutated p53 protein and loss p16INK4a protein of expression indicate a higher proliferative tumour potential. Therefore, these evaluated proteins may be the aggressive biological tumour activity markers.

Introduction

Malignant melanoma of the skin (MMS) is a melanocyte system tumor which is produced by the malignant melanocyte transformation generated by the neural crest [1, 2, 3]. MMS is characterized by high malignant potential and high mortality rate, as well as a rapid occurrence rate in the late years, especially in women [4]. Multistep tumorigenesis is an evolving process that involves the inactivation of tumor suppressor genes, activation of oncogenes and defects of the reparatory genes which can induce inadequate gene reparation [5]. The most common oncogene defect occurs at the control site G1/S which mediates the cell S phase onset [6-13]. Melanoma is not an exception in the tumor biology and its occurrence is predominantly the result of the gene mutations accumulation whose key role is the regulation of cellular proliferation, differentiation, apoptosis or some other pathways of cellular death [9, 10]. Many studies have shown a certain amount of damage

and loss of tumor suppressor genes p16 and p53 with MMS patients, which is why they are considered to be responsible for the tumor carcinogenesis process [12-19]. The tumor suppressor gene p53 has a central role in the cell cycle and apoptosis regulation. The mutation of the p53 gene, one of the most common genetic defects in human tumors, generates defects in the control site of the cell cycle and genetic instability of some cancerous cells [6, 7, 12, 20-24]. The protein p16, the product of cyclin-dependent kinase N2a gene, is also known as p16^{INK4a}, p16-MTS1 or cyclin-dependent kinase 4/CDK4 inhibitor [6, 12, 13, 14]. The frequent mutations in melanoma are on the CDKN2A locus (typical target is p16^{INK4}) which suggests a key role for this protein in cell cycle control management in melanocytes [7-13]. It has been observed that the p16^{INK4a} protein expression in melanocytic lesions decreases from benign to malignant and metastatic lesions [25]. A bcl-2 oncoprotein overexpression can stop the apoptosis process which induces cell proliferation. Bcl-2 oncoprotein expression can be noticed in both benign and malignant melanocyte tumors, suggesting that it is not a key role in the malignant melanocyte transformation [26, 27]. The purpose of this study was to determine the status of the tumor suppression proteins expression (p53 and p16^{ink4a}) and bcl-2 oncoprotein in the primary ma-

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lignant melanoma of the skin and to analyse their correlation with the mitotic index and tumor thickness.

Materials and Methods

The study involved 53 samples of the primary malignant melanoma of the skin. The tumors were surgically removed using the elliptic skin incision (excisional biopsy). The histopathological parameters were determined for each tumor, including the following: size (maximum diameter in cm), thickness in mm (Breslow), histological cellular tumor type, tumor invasion level (Clark), ulceration, mitotic number/mitotic index and the pathological stage of the disease. The pathological stage /pT/ was determined according to the *American Joint Committee on Cancer, 2009*. We analyzed the p53 protein mutation expression level, tumor suppressor protein p16^INK4a and bcl-2 oncoprotein expression. The samples were stored in a neutral, buffered 4% formaline solution, for 18-24 hours, dehydrated in ethanol of progressive concentration and embedded in paraffin. From the paraffin blocks, the tissue was cut into 4-5 μm thin samples, stained with the standard hematoxylin-eosine (HE) method and microscopically analysed using Leica DM1000 (Wetzlar, Germany), with digital microscopic camera Leica EC3 (Wetzlar, Germany) with Leica LAS EZ imaging software V 1.8.0.

Immunohistochemical analysis
The detection of the target markers - antigen, was performed using commercially available monoclonal anti-human antibodies (Table 1.) and highly sensitive and specifically marked streptavidin-biotin complex immunoassays (DAKO LSAB⁺-HRP kit). The chromogen was 3.3'-diaminobenzidin (DAB), and the slides were lightly counterstained with Mayer hematoxylin (Merck, Germany). All the reactants were produced by the DAKO Company (Glostrup, Denmark). During the tissue staining, the positive and negative control samples were simultaneously stained in order to confirm the specificity and quality of the immunohistochemical analysis. The mutated p53 and p16 and bcl-2 protein expression evaluation was performed based on the intensity of the immunohistochemical nuclear (p53 and p16^INK4a) staining and cytoplasmic staining (p16^INK4a i Bcl-2) and immunoreactive tumor cell percentage. The positive immunohistochemical

reaction was observed as a light and dark brown colouring of the tumor nuclei. The p16 tumor cells were rarely visible - cytoplasmic colouring of light brown. The semiquantitative analysis is calculated as the percentage of the positive cells in relation to the total tumor cells in the field. Immunohistochemical staining intensity determine as (1+ = mild; 2+ = moderate; 3+ = intensive) and the tumor positive cell percentage (<5% no immunoreactive cells; 6-25% some positive cells; 26-75% many positive cells; >75% most positive cells).

Morphometric analysis
Tumor thickness measurements
The tumor thickness was determined according to Breslow using an ocular micrometer and suitable computer software on a microscope with a digital camera. The thickness was determined at the widest tumor area starting from the upper line of the granulated epidermal layer or the bottom line of ulceration, vertically into the depth, to the last tumor coast or individual tumor cell. The thickness was measured in millimeters
Mitotic activity
The number of mitoses (mitotic index, MI) was determined in the tumor areas with the largest number of the mitotic figures at 10 fields of the highest microscopic amplification (x400 for each sample), (the total mitoses number/10 HighPowerField - HPF). The MI was classified in the following manner: low index 1-6 mitotic figures/10 HPF; high index > 6 mitotic figures/10HPF.

Statistical analysis
The data obtained during the research was filed in a specially created data base. The results were statistically processed using the descriptive and analytical statistic methods based on the: mean value (x), standard deviation (SD), standard error (SE), Student's t-test in some groups in order to evaluate the statistical importance of mean value differences, MANOVA and Pearson's correlation.

Results

The mean age of patients was 59.2±12.7 years; the youngest patient was 27 years old, the oldest 85 years. There were 27 (50.9%) male and 26 (49.1%) female patients. There were two types of the tumor based on the cellu-

TABLE 1. Primary antibodies review used in the immunoassays

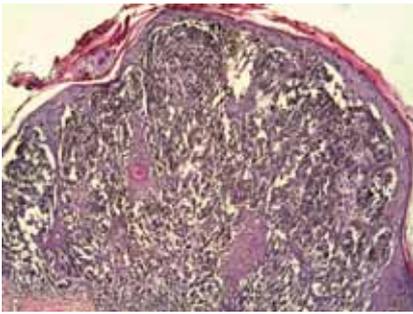| Antibody	Catalogue no.	Clone	Source	Company	Epitop Retrieval	Dilution	Hromogen
CINtec™ p16^INK4a Histology kit	K5334	E6H4	mouse	DAKO	Target retrieval Solution, pH 9.0 S2367 MW	KitVisualisation system EnVision TM+	DAB
p 53 protein	M7001	DO-7	mouse	DAKO	0.05M citric buffer pH 6.0 MW	1:50	DAB
Bcl-2 oncoprotein	M0887	124	mouse	DAKO	0.05M citric buffer pH 6.0 MW	1:50	DAB

FIGURE 1. Malignant melanoma of the skin, nodular type (H&E, x20).
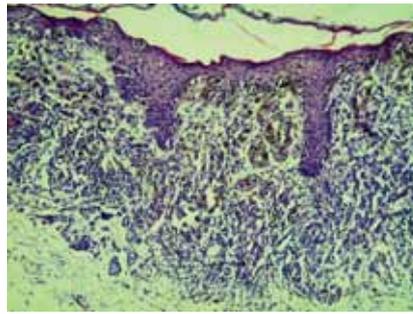

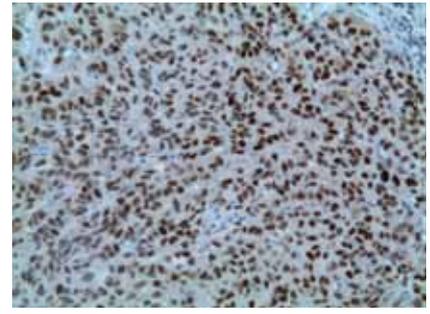**FIGURE 2.** Malignant melanoma of the skin, superficially spreading type (H&E, x20).

FIGURE 3. Diffuse nuclear immunoreactivity moderate intensity for p53 mutated protein of malignant melanoma skin, (IH, x20).

lar type: in thirty-three patients (62.3%) melanomas were nodular type (nodular melanoma, NM), (Figure 1), and 20 (37.7%) melanomas were of superficially spreading type (superficially spreading melanoma, SSM), (Figure 2).

According to the patient's sex, the most common MMS anatomical localizations in women were the lower extremities, lower legs - 10 patients (38.5%); the most common anatomical localization in men was the back - 16 cases (59%). Statistically significant differences between NM and SSM were found for the mean age value (p<0.05), Breslow tumor thickness, invasion level by Clark, WHO tumor stage, ulceration, mitosis number (p<0.01), histological cellular tumor type (p<0.05) and maximum tumor diameter (p<0.01), whereas the sex variable was not statistically significant.

The p53 protein mutated form expression was found in 46 (87%) MMS. All 33 (100%) NM showed a positive reaction to this protein, while in the SSM it was found in 13 (65%) cases. A positive nuclear reaction was found in over 75% tumor cells in 23 (70%) NM, moderate staining intensity (Figure 3), while SSM showed a positive reaction mild intensity of nuclear staining (percentage was 25-75% of tumor cells). Statistically relevant differences between NM and SSM were found for the percentage of p53 positive cells and p53 intensity of the immuno reaction (p<0.01).

The loss of p16^INK4a protein expression was found in 28 (53%) MMS. A negative immuno reaction to p16^INK4a protein was found in 23 (70%) NM cases and in 5 (25%) SSM cases. On the other hand, 17 (75%) SSM showed a positive p16^INK4a protein expression, 100% of tumour cells were immunoreactive, and the staining intensity was moderate and intensive. The P16^INK4a immunoreactive tumour cells MMS also showed the cytoplasmatic light

brown staining (Figure 4). The immunoreaction intensity for the p16^INK4a protein varied due to the different tumor segments; a moderate reaction was found in the upper parts of the tumor. Statistically relevant differences between the NM and SSM were found in the percentage of the p16^INK4a positive cells (p<0.01) and p16^INK4a reaction intensity (p<0.05). The bcl-2 oncoprotein expression was found in 50 (94%) MMS. A positive immunoreactivity to this protein was found in 32 (97%) NM and 18 (90%) SSM. With 27 (82%) NM, an immunoreaction was noticed in over 90% of the tumor cells, with significant staining intensity (Figure 5). A positive immunoreaction was observed in 14 (70%) SSM with over 75% of the tumor cells of the moderate cytoplasmic staining level. There were no significant statistical differences between the NM and SSM for the percentage of the bcl-2 positive cells and bcl-2 immunoreaction intensity. However, there were statistically significant correlations between the mitotic number and p53 positive cell percentage as well as the mitotic number and p53 reaction intensity. Moreover, there was a statistically significant negative correlation between the mitotic number and p16^INK4a positive cells percentage and between the mitotic number and p16^INK4a reaction intensity (p < 0.05); whereas there was no significant difference between the mitotic number and bcl-2 positive cells percentage, and between the mitotic number and bcl-2 reaction intensity (Table 2).
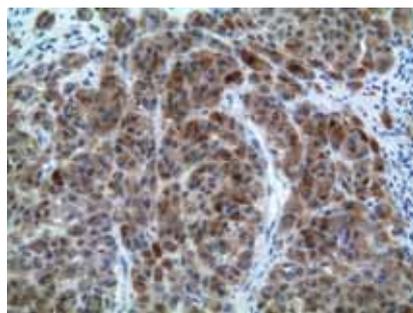

FIGURE 4. Diffuse nuclear and cytoplasmic immunoreactivity for p16 protein of the tumor cells malignant melanoma of the skin, (IH, x20).
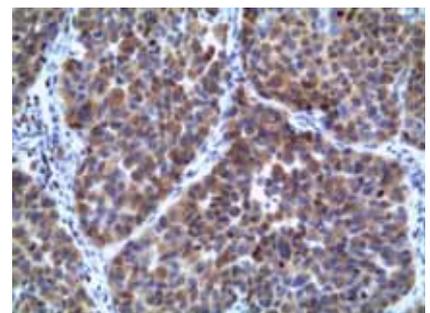

FIGURE 5. Diffuse cytoplasmic immunoreactivity for Bcl-2 oncoprotein of the tumor cells MMS, (IH, x20).

TABLE 2. Correlation of cell cycle regulation proteins (p53 and p16INK4a) and bcl-2 oncoprotein with mitotic index and Breslow thickness and Clark level MMS

Variable	p53 %	p53 Int	p16 %	p16 Int	Bcl-2 %	Bcl-2 Int
Mitotic index	0.28*	0.30*	-0.34*	-0.32*	0.11	0.05
Breslow, thickness	0.47*	0.20	-0.02	0.01	0.34*	0.20
Clark, level	0.64*	0.40*	-0.15	-0.07	0.21	0.07

* statistically significant correlation, (p < 0.05)

Statistically significant correlations were observed between the Breslow tumor thickness and p53 positive cell percentage and between the Breslow tumor thickness and bcl-2 positive cells percentage. Moreover, there were some significant correlations between the Clark tumour invasion levels and p53 positive cells percentage and between Clark tumour invasion level and p53 reaction intensity (p < 0.05). Other correlations were not significantly relevant (Table 2).

Discussion

MMS is one of the most studied tumors due to the vagueness of the tumor's etiological and biological aspects [4, 5, 7, 9, 10]. In our research, the largest amount of the NM (50%) consisted of epithelioid cells only, and then histologically combined tumors, epithelioid and spindle cells, whereas the smallest amount of the nodular melanoma consisted of spindle type cells. However, the largest amount of the SSM (75%) consisted of epithelium cells exclusively. In general, most types of melanoma exhibit a combined histological image, with a particular dominant cell type, which was the case with our subjects - the dominant form being the epithelioid cell type [7]. The most affected anatomical area in women was the lower extremites area, lower leg, almost 40% of the cases, whereas in men it was the trunk 60%, the back 52% of the cases. Generally speaking, the most common melanoma occurrence in men is on the trunk, in women on the lower extremites, which is confirmed by the data from our research [2, 7]. Tumor ulceration as an independent prognosis factor may also characterise cutaneous melanomas with a vertical growth phase in the late disease stage. The ulceration was a common characteristics of the NM in our group, and was observed in 28 (85%) of the cases, while in the SSM group it was present in only 2 cases (10%). Our results confirm a significant difference between the NM and SSM compared to the present ulceration which other studies also confirm [7, 28, 29]. In our group, the NM patients had a higher pathological stage (pT/WHO) compared to the SSM. This histopathological parameter also showed a statistically significant difference [7]. Although it is known that the cutaneous melanoma is a cellular growth and development deficiency, some genetic mechanisms responsible for its initiation and progression remain unclear [8, 9, 10]. Loss of function of p16 tumor sup-

pressor gene and mutation p53 tumour suppressor gene in malignant melanoma suggest the importance of their roles in the tumor pathogenesis [7, 13, 4, 21]. The results of our study show a high level of nuclear expression of the p53 protein mutated form with thick skin melanoma, which is also confirmed by the data from other studies [15, 30]. The correlation between p53 protein overexpression and MI suggests a high proliferative tumor potential. Our data is confirmed by a research by Soares de Sa et al. [28] who believe that an overexpression of the p53 protein in correlation with a high mitotic rate probably points to the diminishing of the S-G2 control site in high proliferative melanoma. We have encountered a frequent p16INK4a protein expression loss in patients with NM, as opposed to the SSM patients who have an overexpression of this protein. The permanent loss of the protein expression in NM suggests the lack of its function, late stage, and a possible role in the tumor progression, which is confirmed by the results from other authors [14, 16, 17, 18, 25]. The p16INK4a protein expression in melanocyte lesions decreases from benign to malignant and metastatic lesions [25]. We have observed a statistically relevant negative correlation between MI and p16INK4a protein expression, while Talve et al. [17] do not show a correlation between p16 protein and MI in a group of 79 malignant melanoma. There is, however, a correlation between the number of Ki-67 positive cells and p16INK4a protein expression loss. Mihic-Probst et al. [19] have also found a significantly higher proliferative activity with the melanoma of low p16 protein expression. The difference in these correlations may be due to a different p16INK4a protein expression which can lead to the existence of various p16INK4a protein mutations and their impact on the primary MMS proliferative activity. Our study shows a high level of bcl-2 oncoprotein expression in MMS, which can also be confirmed by the other research data [26, 27, 31, 32]. This protein is expressed with both benign and malignant lesions, has no diagnostic significance and is not a key aspect in the malignant melanocyte transformation [27]. Leiter et al. [32] have found an increased bcl-2 and bcl-2 related genes bcl-xLm bcl-xS and Bax expression in malignant melanoma during the disease progression as the result of the apoptosis inhibition and higher tumor malignant potential. We didn't find a correlation between bcl-2 oncoprotein expression and MI which suggests that this protein has no proliferative role in malignant melanoma of the skin. After the tumor thickness, MI is the second statistically relevant prognostic parameter for MMS. The melanoma prognosis is good if the mitotic index is low (< 6 mitoses/mm²), and bad if the MI is high (> 6 mitoses/mm²). Many multivariate analyses have shown a relation of MI with tumor thickness in a way that mitosis remains a significant independent variable for the primary cutaneous melanoma

[7, 28, 29]. Most of the published studies show a correlation of the tumor thickness with other prognostic factors such as ulceration, anatomical localisation, sex and age of patients, inflammatory response, mitotic index, vascular invasion and microscopic satellitosis. Their results show that the tumor thickness is the most powerful prognostic parameter for localised cutaneous melanoma [29, 33]. Both benign melanocyte tumors and malignant melanoma exprimate the p53 protein. Although the immunohistochemical reaction for this protein has no diagnostic relevance, numerous data have shown that the p53 protein expression increases in relation to the MMS thickness suggesting the presence of aggressive melanoma forms [21]. Our study, as well as Yamamoto et al. [21], also shows a high mutated p53 form expression in MMS and a positive correlation with tumor thickness. Compared to our results, other researches show no correlation between the p53 mutation overexpression and tumor size [22]. Although our study shows a high level of mutated p53 protein expression in MMS, the possible diagnostic significance of this marker may need to be assessed with a larger subject group. Our results show that there is no correlation between the Breslow tumor thickness and Clark invasion level degree compared to p16 protein expression, which is confirmed by other authors [14, 16, 18]. Moreover, Straume et al. [14] and Reed et al. [18] in their studies have found a significant connection of the p16 protein nuclear expression loss and vascular tumor invasion, but not so with Breslow tumor thickness or Clark invasion level. In contrast to our results and the studies mentioned above, some other authors suggest that the level of melanoma invasion after Clark is greatly increased with p16 negative melanoma [17]. We found a high level of bcl-2 oncoprotein expression with MMS and a positive correlation with Breslow tumor thickness, which may occur as a result of apoptosis inhibition which favorizes tumor cells survival and enables disease progression [20, 27, 30]. In addition, we found a combined p53 and bcl-2 protein overexpression with cutaneous melanoma patients, which also causes a higher malignant tumor potential [31].

Conclusion

Overexpression mutated p53 protein and bcl-2 oncoprotein, with the loss of expression p16 protein in the nodular melanoma, as shown by our study, confirms a common loss of function these tumor suppressor genes and oncogenes, and indicates a vertical tumor growth phase. Our results show that the loss of tumor suppression function and the p53 protein and bcl-2 oncoprotein overexpression in malignant of melanoma of the skin correlate with larger tumor thickness value, whereas the overexpression mutated p53 protein and the

loss of expression p16^INK4a protein indicate a higher proliferative tumour potential. Therefore, the evaluated proteins may serve as the aggressive biological tumour activity markers.

Declaration of interest

Authors do not have any commercial affiliations, or potential conflicts of interest associated with this work submitted for publication.

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