

BETA-CATENIN EXPRESSION IN MALIGNANT MELANOMA

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ABSTRACT – Beta-catenin is bound to E-cadherin in adherens junction formation, but also functions as a signaling molecule in the WNT pathway. We investigated beta-catenin expression in 41 superficial spreading melanomas. Our melanoma sample was analysed by immunohistochemistry and evaluated by image analysis as staining density, i.e. light permeability (LP).

In normal skin beta-catenin showed homogenous membranous staining of the epidermis. Comparison of relative LP for beta-catenin in tumor tissue and adjacent skin did not show any differences (157.8 compared to 156.6; $t = -1.087$, $P = 0.283$). However, the cellular location of beta-catenin changed considerably in melanoma. The protein was observed in the cytoplasm in 32% of patients, in 29% in the cell membrane, in 24% in both the cytoplasm and membrane, in 5% in the cytoplasm and nucleus, while in 9.8% of patients beta-catenin could not be observed.

There was a marked difference in beta-catenin distribution correlated to the Clark stages of melanoma progression. Patients with Clark 4 and 5 had significantly less beta-catenin than patients with Clark 2 and 3 ($\chi^2 = 11.3$; $P=0.01$).

Our results suggest that changes of beta-catenin levels have roles in melanoma development mechanisms and could be used as markers of disease progression.

Key words: *malignant melanoma, beta-catenin, image analysis*

Introduction

Although several molecular studies have examined alterations in oncogenesis and tumor suppressor genes in malignant melanoma^{1,2}, the etiology and pathogenesis of this neoplasm is still not clear and needs to be elucidated. Main candidates responsible for melanoma development and progression are CDKN2A³ and CDK4⁴ genes residing on chromosomes 9p21 and 12q14, as well as MC1R gene located at 16q24^{5,6}. These genes together with signal transduction pathways in which they are implied are primarily changed in hereditary melanoma.

Sporadic forms of melanoma are characterized by changes of: BRAF⁷, ras⁸, c-met⁹ and PTEN¹⁰ genes, but findings on their involvement are inconsistent and inconclusive.

In our study we tried to investigate expression of CTNNB1 gene by analyzing its product - beta-catenin in a series of superficial spreading malignant melanomas.

Beta-catenin is a 92-kd protein first identified as a molecule associated with the cytoplasmic tail of E-cadherin^{11,12}, transmembrane glycoprotein generally localized on the surfaces of epithelial cells in a region of cell-cell contact that is known as the adherens junction^{13,14}. Beta-catenin shows great homology to Armadillo of *Drosophila melanogaster*¹⁵ and plays a role linking E-cadherin to the cytoskeleton. In the cytoplasm, beta-catenin forms a complex together with adenomatous polyposis coli (APC) tumor suppressor protein, axin and

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GSK3 β . In the absence of a WNT signal, beta-catenin is phosphorylated and targeted for degradation by the ubiquitin/proteasome machinery¹⁶. Besides its role in normal cells, this gene can play a major role in malignant cell transformation acting as an oncogene. In response to WNT signalling, but also as a result of oncogenic mutation, cytoplasmic beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus, where it finds a partner, a member of the DNA binding protein family LEF/TCF (T cell factor-lymphoid enhancer factor)¹⁷. Together they activate new gene expression programs. One of the target genes for beta-catenin/TCF encodes c-MYC protein¹⁸, explaining why constitutive activation of the WNT pathway can lead to cancer.

There are several reasons why we propose studying beta-catenin protein expression in malignant melanoma. It has been well documented that WNT genes, together with other components of WNT signaling pathway, are implicated in cancer^{19,20}, and especially in neoplasms of epithelial origin. Pigment producing cells of the epidermis, melanocytes, undergo malignant transformation in melanoma. Melanocytes are of neuroectodermal origin and during embryonal development they migrate to the epidermis. In normal human epidermis melanocytes reside at the basement membrane and through their dendrite processes contact multiple keratinocytes, the predominant cell type in the epidermis. Keratinocytes play essential role in regulating melanocyte proliferation and phenotype²¹. The regulation is mediated by a major adhesion molecule between these two cell types – E-cadherin. In vitro loss of contact with keratinocytes causes melanocytes to dedifferentiate and to express melanoma-associated adhesion molecules²².

And finally, melanoma shows a very high tendency for metastasis, a process preceded by adherens junction disassembly, while beta-catenin acts as an oncogene and is indicative of invasiveness.

In order to identify novel molecular targets involved in malignant melanoma development, the aim of our investigation was to detect and quantify expression changes of beta-catenin. We also, aimed to correlate the level of immunohistochemical staining to clinicopathological parameters.

Materials and Methods

Melanoma samples

A total of 41 primary superficial spreading melanomas were collected from the Department of Pathology University Hospital Centre *Rebro, Zagreb*. The patients had no family history of melanoma. Tumor tissue specimens and adjacent skin were obtained after surgery during time period 1998-2004. Thirty normal skin tissue specimens were collected from the Department of Plastic Surgery Clinical Hospital Centre *Rebro, Zagreb*. The age of patients varied from 28 to 82 (mean age=56.8; median 62 years), and there were 25 females and 16 males. The mean ages at diagnosis for two sexes were (F=55.1; M=59.5).

The samples were formalin fixed, paraffin embedded and 4 mm thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). A pathologist classified primary tumors as superficial spreading melanomas. The histopathological classification was assessed according to Clark²³. The distribution of different Clark stages in our sample showed that prevailing stages were 4 and 3, 36.6% and 29.3% respectively. Clark 2 represented 17.1%, Clark 5 -17% of the total sample, while Clark 1 was not present.

The local Ethical committee approved our study and the patients gave informed consent.

Immunohistochemistry

Sections were immunostained using the biotin-avidin-horseradish peroxidase method. Deparaffinized and rehydrated sections were microwaved in Dako Target Retrieval Solution (Dako Corporation, U.S.A.) three times for 5 minutes at 800 W to unmask epitopes. To block endogenous peroxidase activity the cells were fixed in methanol containing 3% H₂O₂. Non-specific binding was blocked by the application of normal rabbit serum for 30 minutes in a humid chamber. Slides were blotted and the primary antibodies, at optimized dilutions of 1 : 200 (beta-catenin), were applied for 30 minutes at room temperature. The antibodies used were: Monoclonal Mouse anti-human Beta-catenin – 1, (Dako).

After incubation slides were washed 3 times in PBS/goat serum. Secondary link antibody was applied for 25 minutes. The washing was repeated and the slides were incubated with streptavidin horseradish peroxidase for another 25 minutes. All chemicals were from DakoCytomation.

Negative control in each experiment was the sample that underwent same staining procedure with the exclusion of the primary antibody. Normal skin sections and normal renal tissue sections served as positive controls. The analysis of the labeling was performed by two independent observers, *i.e.* blinded pathologists, experts in the field.

Image analysis

Protein expression was then quantified with the aid of Olympus BH-2 microscope and Image Analyzer. For each sample, the intensity of staining in a well defined area was evaluated. Region of interest was chosen as one representative field under x 100 magnification, in the centre of either the malignant tissue or apparently normal tissue with well preserved tissue components. All density measurements were calibrated against its slide characteristics *i.e.* transparency.

Staining intensity was assessed using image analyzing software manufactured by Vamstec (Zagreb, Croatia) by measuring densities on different areas of tissue section. Density was depicted as the intensity of light retained by tissue or tissue transparency, and expressed in grey scale pixels ranging 0-255. Zero representing no transmission of light, and 255 total transparency. Density in the area of tumor location was compared to density of the most distant normal tissue in the same section.

Statistical Analysis

Following statistical methods were used: A nonparametric Kruskal-Wallis test was used to correlate expression of beta-catenin in tumor tissues to Clark stages. The correlation between Clark stages and patients' sex and age at diagnosis was also evaluated using Kruskal-Wallis. T-test was used to compare levels of beta-catenin expression in tumor tissues to the levels of its expression in normal adjacent skin. Mann-Whitney test was used to evaluate the relationship of expression intensity of beta-catenin to its cellular location. All statistical evaluations were performed according to the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Beta-catenin expression

Forty one superficial spreading melanomas as well as 30 normal skin sections were analyzed by immunohistochemistry for protein expression of beta-catenin. In normal skin, as well as in morphologically normal skin adjacent to melanoma, beta-catenin showed homogenous membranous staining of the epidermis.

Contrary to the situation in the normal skin, the cellular location of beta-catenin in melanoma has changed. The protein could visually be determined in the cytoplasm in 32% of analyzed patients, in 29% in the cell

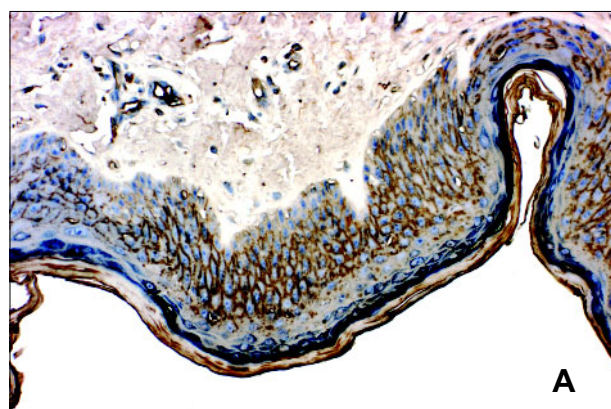
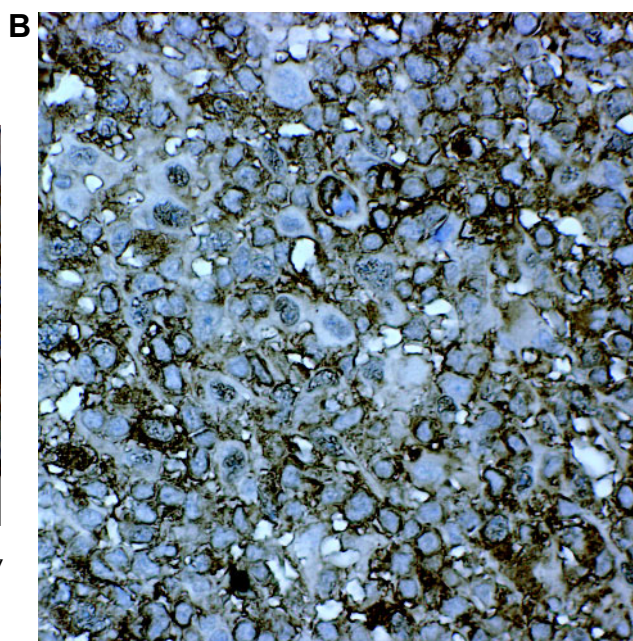
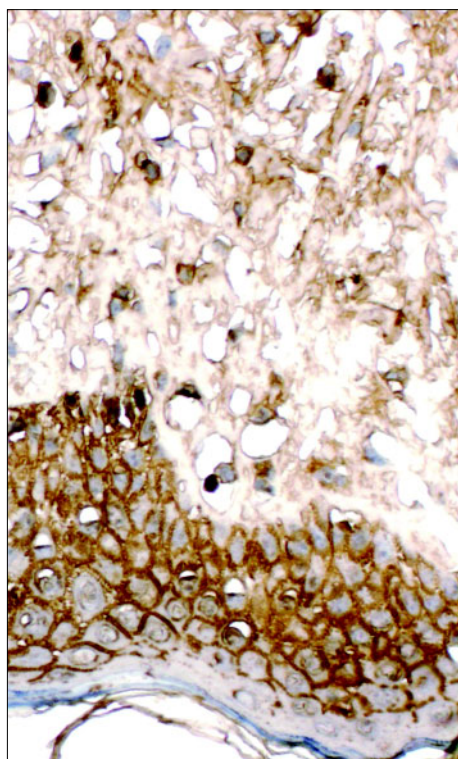


Figure 1. Shows beta-catenin staining in normal skin (A), and different locations of the protein in melanoma (B-cytoplasmic, C- membranous and cytoplasmic).





membrane, in 24% in both the cytoplasm and membrane, in 5% in the cytoplasm and nucleus, while in 9.8% of patients beta-catenin could not be observed (Figure 1).

Image analysis and its correlation to the histopathological stages

The obtained results were evaluated by Image analysis as staining density, i.e. light permeability (LP). To further specify the role that beta-catenin plays in melanoma rise and progression we correlated all data collected. Before interpretation of our results we had to standardize the values that Image analysis gave us. The analysis of 30 normal skin samples unrelated to melanoma showed that beta-catenin levels were lower in nor-

Table 1. The distribution of beta-catenin protein in melanoma cells for the total sample analyzed.

Location	Distribution percentage	
Cytoplasm	32%	13/41
Membrane	29%	12/41
Membrane and cytoplasm	24%	10/41
Cytoplasm and nucleus	5%	2/41
Could not be visualized	10%	4/41

mal skin of non-melanoma patients than beta-catenin levels in the skin adjacent to melanoma. Mean value of light permeabilities for beta-catenin was 147. This number enabled us to calculate relative LP increase or decrease of beta-catenin protein in tumor tissue and adjacent skin. We introduced variables of relative increase of protein expression in tumor tissue and adjacent skin defined as:

$$\text{Btmrel} = \text{btm}/\text{bxnorms} \times 100 \text{ and } \text{Bskinrel} = \text{bskin}/\text{bxnorms} \times 100.$$

Where Btmrel represents relative increase or decrease of LP in tumor tissue for beta-catenin; btm denotes measured LP in tumor tissue; bxnorms values of LP in normal skin, Bskinrel – relative increase or decrease of LP in the adjacent skin; and bskin measured values of LP in the adjacent skin.

Comparison of mean values of relative LP for beta-catenin in tumor tissue and the adjacent skin did not show any relevant differences (157.8 compared to 156.6; $t = -1.087$, $P = 0.283$).

On the other hand, as we have pointed out earlier, the location of beta-catenin protein has changed in melanoma cells. We divided our sample in two groups based on the presence of the protein in the cytoplasm. Comparison of mean values of the LP of samples in which beta-catenin was visually determined in the cytoplasm to that of cases where beta-catenin was not visually present in the cytoplasm was not statistically significant.

The histopathological Clark stages (stages 2–5 were present in our sample) of melanoma progression were correlated with the expression of the beta-catenin pro-

Table 2. A nonparametric Kruskal-Wallis test was used to correlate expression of beta-catenin in melanoma to Clark stages.

	Clark	N	Rank ¹
<u>Btmrel</u> ⁵	2	7	13.36
	3	12	14.79
	4	15	26.57
	5	7	27.36
	Total	41	
χ^2			11.300
³ D. fr.			3
⁴ St. sign			0,010

¹ Mean value of the rank

³ Degrees of freedom

⁴ Statistical significance

⁵ Relative light permeability increase in tumor for beta-catenin protein

tein. A significant difference in beta-catenin expression was noted in the different Clark stages ($\chi^2 = 11.3$; $P=0.01$). Patients with Clark 4 and 5 had significantly less beta-catenin in melanoma cells than patients with Clark 2 and 3 (table 2).

Kruskal-Wallis tested correlation of the severity of Clark stages to the sex and age of patients, but demonstrated no difference in distribution of Clark stages between men and women and younger and older group of patients. The analysis of beta-catenin levels according to the sex of the patient did not show significant differences between men and women ($P=0.261$).

Discussion

The genetics of melanoma is very diverse and does not include changes in a single gene or signaling pathway. It is now apparent that tumor malignancy can in certain aspects be explained by alterations in the adhesive properties of neoplastic cells. With this in mind, we offered a potential candidate, beta-catenin, whose expression patterns were quantified by Image analysis in a set of 41 superficial spreading malignant melanomas.

It is known that during melanoma progression normal epidermal homeostasis is impaired, and melanocytes escape the control of neighboring keratinocytes. Later on, loss of E-cadherin is accompanied by emerging of functional N-cadherin expression. The switch from E-cadherin expression in melanocyte to N-cadherin expression in melanoma cells enables homotypic interaction between melanoma cells, facilitates the formation of gap junctions with fibroblasts and endothelial cells, which all enables migration and survival of melanoma cells²².

Our first finding on beta-catenin's expression in melanoma showed a shift from exclusively membranous staining in normal skin to different locations in the transformed cells. Although quantification of beta-catenin protein expression showed that image analysis values in our melanoma sample and adjacent skin are almost identical, the location of the protein was quite different in melanoma. In the biggest percentage of our sample (32%) the protein was localized exclusively in the cytoplasm which could indicate that beta-catenin acquired its signaling role. The disruption or no formation of adherens junctions generates free cytoplasmic beta-catenin and thus promote the positive WNT signal. The question whether downregulation of main adherens junction molecule— E-cadherin is quicker in

melanocyte transformation, precedes expression of beta-catenin and, therefore, disables binding or whether the quantities of the two proteins are expressed independently remains unsolved.

Twenty-nine percent of our melanoma sample had beta-catenin localized in the membrane. Other authors²⁴ have found membranous staining in a bigger part of their melanoma sample (83%). This finding could be explained by the fact that transformed melanocytes express N-cadherin and other types of cadherin molecules on their surfaces and that beta-catenin can bind to the cytoplasmic domain of N-cadherin, same as it binds to E-cadherin in normal skin. We can speculate that membranous localization of beta-catenin in melanoma is the result of its binding to N-cadherin.

Many investigators have reported on cytoplasmic localization of beta-catenin in melanoma cells. Mælandsmo and coworkers²⁵ on a sample of 106 superficial spreading melanomas detected cytoplasmic beta-catenin in 71% of their sample (and in more than 50% of cells of each sample). On the other hand Kageshita *et al.*²⁶ showed that 42% of melanocytic nevi they investigated had exclusively cytoplasmic staining, 10% mixed cytoplasmic and membranous, and 44% cytoplasmic and nuclear. However, melanomas that same authors investigated showed cytoplasmic staining in 70% of cases, and cytoplasmic and membranous staining in 20% which is in consent to our 24%. Mælandsmo and coworkers²⁵ also found that beta-catenin is totally absent in 5% of their cases, a bit lower number than our finding of 10%. Different numbers that we and other authors obtained could be explained by different sample sizes, different evaluation of the staining and the use of monoclonal antibodies that may have different sensitivities.

Rubinfeld *et al.*²⁷ have indicated the possibility of beta-catenin's role as an oncogene in melanoma initiation and progression. They observed changes in WNT signaling in 28% of analyzed melanoma cell lines. The accumulation of cytoplasmic beta-catenin and mutations on 2 spots responsible for GSK-3-beta-kynase phosphorylation was detected in 4 out of 25 of these cell lines. The results that other authors obtained working on primary melanomas²⁴ and combining immunohistochemistry with sequencing of exon 3 of beta-catenin (most mutated exon) demonstrated a different picture. Although nuclear and/or cytoplasmic localization of beta-catenin was found in less than third of melanomas investigated, a somatic mutation was detected in only one of the 50 tumors sequenced. On a set of 62 melanoma

cell lines Pollock and Hayward²⁸ confirmed that the mutations of beta-catenin's exon 3 are a rare event in melanoma cell lines. Those findings, contradictory to Rubinfeld's, suggested that high frequency of beta-catenin mutations could be the consequence of *in vitro* cultivation, or that the cells that harbor beta-catenin mutations are preferentially established as cell lines.

The incidence of malignant melanomas is increasing, and once metastasized the prognosis is poor. Thus, early identification of patients with a high risk of progression is of major importance. Besides thickness of the primary tumor, few prognostic markers predicting patient outcome are available.

The analysis and correlations of the Image analysis values of protein expression to clinical parameters, histopathological diagnosis, age at diagnosis and sex of the patients, have demonstrated interesting results. The most interesting is definitely the finding that beta-catenin expression significantly changes depending on Clark stage. Patients with Clark 4 and 5 have significantly less beta-catenin protein ($P=0.01$) than patients with Clark 2 and 3. This finding is in accordance to other authors. Mælandsmo *et al.*²⁵ and Kageshita *et al.*²⁶ point out that beta-catenin is lost or decreasingly expressed in advanced lesions, while Krengel and coworkers²⁹ noticed significant downregulation of beta-catenin expression from primary to metastizing lesions. The significant correlation between loss of beta-catenin and the disease progression is a potential marker of melanoma progression.

The results of our study show that beta-catenin may have an important role in melanoma development and progression. Changes in beta-catenin protein levels were correlated with the malignancy stage which, after additional studies, could be used as a molecular marker of disease evolution and progression.

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Sažetak

IZRAŽENOST BETA-KATENINA U MALIGNOM MELANOMU

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Beta-katenin vezan je za molekulu E-kadherina u zonulama adherens, ali također funkcionira i kao signalna molekula u putu prijenosa signala WNT. U ovom radu istraživali smo ekspresiju ovoga proteina u uzorku od 41 superficijelno širećeg malignog melanoma. Metoda imunohistokemije korištena je za analizu beta-kateninske izraženosti, a razina ekspresije kvantificirana je image analizom kao gustoća obojenja, odnosno, propusnost svjetla. U zdravoj koži beta-katenin pokazivao je homogeno membransko obojenje u epidermisu. Usporedba relativne propusnosti svjetla za izraženost beta-katenina u tumorskom tkivu i pripadajućoj koži nije pokazala razlike u propusnosti svjetla (157.8 prema 156.6; $t = -1.087$, $P = 0.283$). Međutim, stanični smještaj ispitivanog proteina uvelike se promijenio. Opazili smo citoplazmatski smještaj beta-katenina u 32% bolesnika, membranski smještaj u 29%, citoplazmatski i membranski u 24%, a u samo 5% citoplazmatski i smještaj u jezgri tumorskih stanica. U 10% ispitanih bolesnika nismo detektirali beta-kateninsku izraženost. Znakovita razlika primijećena je u razdiobi beta-katenina po stupnjevima melanomske progresije (Clark). Bolesnici s Clarkom 4 i 5 imali su znakovito manje beta-katenina od bolesnika s Clarkom 2 i 3 ($\chi^2 = 11.3$; $P=0.01$).

Naši rezultati pokazuju da promjene u izraženosti beta-katenina imaju ulogu u mehanizmima nastanka melanoma i da postoji mogućnost korištenja beta-katenina kao biljega progresije bolesti.

Ključne riječi: *melanom, beta-katenin*