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## **E-cadherin and beta-catenin expression patterns in malignant melanoma assessed by image analysis**

**Nives Pećina-Šlaus<sup>1</sup>, Martina Žigmund<sup>1</sup>, Vesna Kušec<sup>2</sup>, Tamara Nikuševa Martić<sup>1</sup>, Mirjana Čačić<sup>3</sup>, Mario Šlaus<sup>4</sup>**

<sup>1</sup>Department of Biology, Croatian Institute for brain research, School of Medicine, University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia, e-mail: nina@mef.hr, tel. 385 01 4566952

<sup>2</sup>Clinical Institute of Laboratory Diagnosis, Clinical Hospital Centre Zagreb, Kišpatićeva 12, 10 000 Zagreb, Croatia

<sup>3</sup>Department of pathology, Clinical Hospital Centre Zagreb, Kišpatićeva 12, 10 000 Zagreb, Croatia

<sup>4</sup>Department of Forensic Medicine, School of Medicine University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia

Corresponding author: Nives Pećina-Šlaus<sup>1</sup>

Department of Biology, School of Medicine,

University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia,

e-mail: nina@mef.hr, tel. +385 1 46 21 140, fax:+385 1 45 50 744; +385 1 45 96

942; +385 1 49 20 050

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

**Running title:** E-cadherin and beta-catenin in melanoma.

**Abstract**

**Background.** We investigated the expression of E-cadherin and beta-catenin in melanoma. Both proteins are components of adherens junctions, but also play signaling roles in the wnt signal transduction pathway.

**Materials and Methods.** Seventy malignant melanomas were analysed by immunohistochemistry and evaluated by image analysis as staining density, i.e. light permeability (LP).

**Results.** Comparison of mean values of relative LP for E-cadherin and beta-catenin in tumor tissue shows that levels of E-cadherin protein are significantly lower (259.67 to 116.23;  $t=22.7$ ;  $P=0.000$ ). The comparison of mean values of the relative LP of E-cadherin in melanoma to the LP in the adjacent normal skin also shows that the expression of E-cadherin in tumor is significantly lower (256.06 to 169.87;  $t=11.55$ ,  $P=0.000$ ). Beta-catenin was observed in the cytoplasm in 30.6% of patients, in 24.2% in the cell membrane, in 21% in both the cytoplasm and membrane, in 1.6% in the membrane and nucleus, in 4.8% in the cytoplasm and nucleus, while in 17.7% of patients' beta-catenin could not be observed. Patients with Clark 4 and 5 had significantly less beta-catenin than patients with Clark 2 and 3 ( $\chi^2 = 12.854$ ;  $P=0.005$ ).

**Conclusions.** Changes in E-cadherin and beta-catenin levels have important roles in melanoma and could be used as molecular markers of disease progression.

## Introduction

In our study we tried to investigate protein expression of two genes, E-cadherin, CDH1 and beta-catenin, CTNNB1, in a series of superficial spreading and nodular malignant melanomas.

E-cadherin is one of the most important molecules of cell-cell adhesion in epithelial tissues. This 120 kDa transmembrane glycoprotein is generally localized on the surfaces of epithelial cells in a region of cell-cell contact that is known as the adherens junction<sup>1, 2</sup>. Besides its role in normal cells, this gene can play a major role in malignant cell transformation, and especially in tumor development and progression. Most tumors have abnormal cellular architecture, and loss of tissue integrity can lead to local invasion. Thus, loss of function of E-cadherin tumor suppressor protein correlates with increased invasiveness and metastasis of tumors, resulting in it being referred to as the «suppressor of invasion» gene<sup>3, 4</sup>.

The other gene investigated, beta-catenin, **is a 92-kDa protein first identified** as a molecule associated with the cytoplasmic tail of E-cadherin<sup>5</sup>. It shows great homology to Armadillo of *Drosophila melanogaster*<sup>6</sup> 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 GSK3beta. In the absence of a WNT signal, beta-catenin is phosphorylated and targeted for degradation by the ubiquitin/proteasome machinery<sup>7</sup>. In response to WNT signalling, 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)<sup>8</sup>. Together they activate new gene expression programs. One of the target genes for beta-catenin/TCF encodes c-MYC protein<sup>9</sup>, explaining why constitutive activation of the wnt pathway can lead to cancer.

Although several molecular studies have examined alterations in oncogenes and tumor suppressor genes in malignant melanoma<sup>10</sup>, 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 CDKN2A and CDK4 genes residing on chromosomes 9p21 and 12q14, as well as MC1R gene located at 16q24<sup>11, 12</sup>. These genes together with signal transduction pathways in which they are implied are primarily changed in hereditary melanoma. Sporadic forms of melanoma

characterize changes of genes: BRAF, RAS, c-MET and PTEN <sup>10</sup> but findings on their involvement are inconsistent and inconclusive.

There are several reasons why we propose studying E-cadherin and beta-catenin protein expression in malignant melanoma. It has been well documented that wnt genes, together with other components of wnt signalling pathway, are implicated in cancer <sup>13</sup>, 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 in the epidermis. In normal human epidermis melanocytes reside at the basement membrane and through their dendritic processes contact multiple keratinocytes, the predominant cell type in the epidermis. Keratinocytes play essential role in regulating melanocyte proliferation and phenotype <sup>14</sup> and this regulation is mediated by a major adhesion molecule between these two cell types – E-cadherin.

And finally, melanoma shows a very high tendency of metastasis, a process preceded by adherens junction disassembly, and both molecules that interested us are important indicators of invasiveness.

The aim of our investigation is to detect and quantify expression changes of two proteins, E-cadherin and beta-catenin, in order to identify molecular targets involved in malignant melanoma development. We also aimed to correlate the level of immunohistochemical staining to clinicopathological parameters.

## **Materials and Methods**

### **Melanoma samples**

A total of 70 primary melanoma tissues were collected from the Department of Pathology Clinical Hospital Centre Rebro, Zagreb. The patients had no family history of melanoma. The 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=57.79; s.d.=15.22; median 62 years), and there were 36 females and 34 males. The mean ages at diagnosis for both sexes were similar (M=57.52; F=58.03).

The samples were formalin fixed, paraffin embedded and 4 µm thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). Of the

primary tumors a pathologist classified 58.6% as superficial spreading and 41.4% as nodular. The pathohistological classification was assessed according to Clark <sup>15</sup>. The distribution of different Clark stages in our sample showed that prevailing stages were 4 and 3, 44.3% and 31% respectively. Clark 5 represented 12.9%, Clark 2 -11.4% of the total sample, while Clark 1 was not present. **The local Ethical committee approved our study.**

### **Immunohistochemistry**

The 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% H<sub>2</sub>O<sub>2</sub>. 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 : 100 (E-cadherin) and 1 : 200 (beta-catenin), respectively, were applied for 30 minutes at room temperature. The antibodies used were: Monoclonal Mouse anti-human E-cadherin NCH-38, and Monoclonal Mouse anti-human Beta-catenin – 1, both Dako Corporation, U.S.A.

After incubation the 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, Denmark.

To discriminate melanin pigments from specific staining products we counterstained the samples with Giemsa. 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 **using image analysing software manufactured by Vamstec (Zagreb, Croatia)**. 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. 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 E-cadherin and 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 E-cadherin and beta-catenin expression in tumor tissues to the levels of their expression in normal adjacent skin. To test the correlation between the levels of E-cadherin and beta-catenin expression in tumor and corresponding skin to patients' sex Mann-Whitney test was used. Mann-Whitney was also used to evaluate the relationship of expression intensity of beta-catenin to its cellular location. Variance analysis (a one-way ANOVA test) was used to correlate the intensity of expression of investigated proteins to the patients' age at diagnosis.

All statistical evaluations were performed according to the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

## **Results**

### **E-cadherin expression levels**

Seventy primary melanomas (41 superficial spreading, 29 nodular), as well as 30 normal skin sections were analyzed by immunohistochemistry for protein expression of E-cadherin. In normal skin as well as in morphologically normal skin adjacent to melanoma E-cadherin showed homogenous membranous staining of the epidermis. However, in both superficial spreading and nodular melanomas E-cadherin expression decreased significantly as compared to normal skin expression. No difference in distribution was observed in those two groups. In a proportion of samples (26/70, 37%) a total loss of E-cadherin protein was observed. Figure 1A shows melanoma sample where distinctive decrease of E-cadherin protein in melanoma cells was observed, and B - immediate adjacent skin of the same patient.

### **Beta-catenin expression levels**

Same melanoma samples were immunohistochemically stained for protein expression of beta-catenin. Normal skin and skin adjacent to melanomas showed homogenous membranous staining of the epidermis, similar to the picture of E-cadherin staining. 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 30.6% of analyzed patients, in 24.2% in the cell membrane, in 21% in both the cytoplasm and membrane, in 1.6% in the membrane and nucleus, in 4.8% in the cytoplasm and nucleus, while in 17.7% of patients beta-catenin could not be observed. Figure 2 shows beta-catenin staining in normal skin (A), and different locations of the protein in melanoma (B, C, D).

### **Image analysis and the correlations to the collected parameters**

The obtained results were then evaluated by Image analysis as staining density, i.e. light permeability (LP). To further specify the role that E-cadherin and beta-catenin play 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 E-cadherin protein levels were slightly greater in normal skin of non-melanoma patients than protein levels of E-cadherin in the skin adjacent to melanoma. Beta-catenin levels were lower in normal skin of non-melanoma patients than levels in the skin adjacent to melanoma. Mean values for light permeabilities for E-cadherin in normal skin of non-melanoma patients was 65, and for beta-catenin 147. These numbers enabled us to calculate relative LP increase or decrease of E-cadherin and beta-catenin proteins in tumor tissue and adjacent skin. We introduced variables relative increase of protein expression in tumor tissue and adjacent skin defined as:

$Etmrel = etm/exnorms \times 100$  and  $Eskinrel = eskin/exnorms \times 100$ , for E-cadherin, and  $Btmrel = btm/bxnorms \times 100$  and  $Bskinrel = bskin/bxnorms \times 100$ , for beta-catenin. Etmrel and Btmrel represented relative increase or decrease of LP in tumor tissue for E-cadherin/beta-catenin; etm and btm denoted measured LP in tumor tissue; exnorms and bxnorms values of LP in normal skin, Eskinrel and Bskinrel – relative increase or decrease of LP in the adjacent skin; and eskin and bskin measured values of LP in the adjacent skin.

Comparison of mean values of relative LP for E-cadherin and beta-catenin in tumor tissue **showed** that values for E-cadherin **were** significantly greater than those for beta-catenin (259,67 to 116,23;  $t=22.70$ ;  $P=0.000$ ). Since greater LP indicated less protein, our results showed that in the same patients levels of E-cadherin protein **were** significantly lower than levels of beta-catenin.

Image analysis confirmed reduced staining of E-cadherin in all of the 70 melanoma samples, i.e. LP in grey scale pixels (reversely proportional to the protein level) was greater in all melanoma tissues to the LP of the adjacent skin. The comparison of mean values of the relative LP of E-cadherin in melanoma samples to the LP in the immediately adjacent normal skin showed significantly greater values in tumor tissue when compared to adjacent skin (256.06 to 169.87;  $t=11.55$ ,  $P=0.000$ ). It **was** obvious that the expression of E-cadherin in tumor tissue **was** significantly lower than that recorded in the adjacent skin.

To our surprise comparison of LP for beta-catenin in tumor tissue and the adjacent skin did not show any relevant differences (115.30 compared to 115.24;  $t=0.04$ ,  $P=0.968$ ). The results of Image analysis showing difference in light permeabilities are demonstrated in table 1.

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 membrane. Comparison of mean values of the LP of samples in which beta-catenin was visually determined in the cell membrane (105.68) to that of cases where beta-catenin was not visually present in the cell membrane (123.72) showed a statistically significant difference ( $Z= -2.525$ ;  $P= 0.012$ ) indicating that higher levels of beta-catenin **were** correlated with the presence of the protein in the cell membrane. **No significant correlation of E-cadherin expression to beta-catenin location in the cell was observed ( $Z= -1.291$ ;  $P= 0.197$ ).**

The pathohistological Clark stages (stages 2–5 were present in our sample) of melanoma progression were not correlated with expression of the E-cadherin protein ( $\chi^2 = 5.124$ ;  $P=0.163$ ). However, a significant difference in beta-catenin expression was noted in the different Clark stages ( $\chi^2 = 12.854$ ;  $P=0.005$ ). Patients with Clark 4 and 5 **had** significantly less beta-catenin in melanoma cells than patients with Clark 2 and 3 (tables 2 and 3).

To determine and explain how age influenced expression of the investigated proteins we divided our sample in two groups: patients younger than 62 and patients older than 62 years of age (62 is the age median at diagnosis – 49% of analysed patients are younger than 62 and 50.8% of patients are older). Variance analysis (ANOVA) showed that patients older than 62 had significantly higher mean values of LP for E-cadherin protein in tumor tissue in comparison to patients younger than 62 (270.75 to 235.70,  $F=7.303$ ,  $P=0.009$ ). E-cadherin in tumors of older patients was therefore significantly lower than in patients younger than 62. Age at diagnosis of those two subgroups was not correlated to the E-cadherin expression in the adjacent skin, nor to beta-catenin expression in melanoma and adjacent skin.

Although the correlation of E-cadherin expression in tumor tissue to the patient sex showed no significant difference (Mann-Whitney test,  $Z=-1.416$ ,  $P=0.157$ ), and there was no difference in the E-cadherin expression in the adjacent skin in males and females ( $Z = -0.378$ ,  $P = 0.705$ ), the analysis of beta-catenin according to the sex of the patient showed significant differences between men and women. Beta-catenin levels in melanoma were significantly lower in males, than its protein levels in females (Mann-Whitney test,  $Z = -3.167$ ,  $P = 0.002$ ). Similar discrepancy was observed in the adjacent skin, beta-catenin levels were also significantly lower in men (Mann-Whitney test,  $Z = -2.829$ ,  $P = 0.005$ ).

Four metastatic melanomas together with their lymph node metastasis were also analyzed. In all four metastatic melanoma there was drastic decrease in E-cadherin expression (mean LP value 143) to the adjacent skin (mean LP value 82), and the similar values were observed for their lymph node metastasis. Beta-catenin levels were very low in lymph node metastatic lesions (mean measured LP 192) as well as in metastatic tumors. Only one lymph node metastasis differed from its melanoma in beta-catenin levels and had LP of 194 to the LP of 131 in melanoma. All metastatic lesions had beta-catenin located in the cytoplasm and the one with different LPs in cytoplasm and membrane.

## Discussion

Our first finding on significantly lower E-cadherin protein levels in tumor tissue to the levels of beta-catenin, which may suggest that E-cadherin's expression is inferior to beta-catenin's in the same melanoma patients, indicates for the first time

the relation of quantities of those proteins. Since the proteins are bound to each other in adherens junctions one would expect similar decrease in image analysis values.

It is possible that the disruption of adherens junctions changes the levels of those two proteins in melanoma which is consistent to beta-catenin's dual cellular role- in signaling and structure. Although E-cadherin is not directly involved in the wnt signaling, loss of its expression indirectly promotes the positive wnt signal, since the disruption or nonformation of adherens junctions generates free cytoplasmic beta-catenin. The question whether downregulation of E-cadherin precedes expression of beta-catenin in melanocyte transformation and therefore unables its binding, or whether the quantities of the two proteins are expressed independently still remains.

We should also consider the different quantities of the two proteins in our normal skin samples (unrelated to melanoma). In normal skin homeostasis the quantities of E-cadherin protein are higher than beta-catenin quantities. It is striking that the quantities of those two molecules are inverse to their relation in melanoma.

We found significantly less E-cadherin protein in all melanomas examined to its levels in the skin adjacent to tumors. This result indicates once again that the integrity of intercellular adhesion is disrupted, since the main mediating molecule is E-cadherin. Reports by other authors using melanoma cell lines are in accordance with our results <sup>16, 17</sup>. Since mutations of E-cadherin gene (CDH1) have not been discovered in melanoma cells, an epigenetic cause for the decrease in E-cadherin expression has been postulated <sup>18</sup>.

The results that we obtained on beta-catenin expression were not so straightforward. Quantification of beta-catenin protein expression showed that image analysis values in melanoma and adjacent skin are almost identical in the samples we analyzed. Nevertheless, the location of the protein was quite different in melanoma than in the adjacent skin. In the biggest percentage of our sample (30.6%) the protein was localized exclusively in the cytoplasm which also indicates beta-catenin's signalling role. Twenty-four percent of our melanoma sample had beta-catenin localized in the membrane. Other authors <sup>19</sup> have also found membranous staining in a bigger part of their melanoma sample (83%). This finding could be explained by the fact that instead of E-cadherin, 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<sup>20</sup>. We can speculate that membranous localization of beta-catenin in melanoma is the result of its binding to N-cadherin. **It would be interesting for future studies to investigate N-cadherin expression as well.**

When we subdivided total sample in two groups: one with beta-catenin present in the membrane and the other group where the protein was absent from the membrane, the observed different quantities were found to be statistically significant. Significantly stronger expression of beta-catenin was observed in the subgroup of melanomas that had membranous staining. When beta-catenin acts in both membranous location and signaling, its quantities are greater than when the junctions are disrupted and solely signaling role remains.

Many investigators have reported on cytoplasmic localization of beta-catenin in melanoma cells. Mælandsmo and coworkers<sup>21</sup> 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.<sup>22</sup> 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 21%. Mælandsmo and coworkers<sup>21</sup> also found that beta-catenin is totally absent in 5% of their cases, a number a bit lower than our finding of 17.7%. 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.

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, pathohistological diagnosis, age at diagnosis and sex of the patients, have demonstrated interesting although difficult to interpret 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 ( $p=0.005$ ) than patients with Clark 2 and 3. **We also observed very low levels of beta-catenin in a small sample of metastatic**

melanomas as well as in their lymph node metastasis. These findings are in accordance to other authors. Maelandsumo et al.<sup>21</sup> and Kageshita et al.<sup>22</sup> point out in their work that beta-catenin is lost or reducedly expressed in advanced lesions, while Krenkel and coworkers<sup>16</sup> 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 potential marker of melanoma progression.

The results of our study show that E-cadherin and beta-catenin may have important roles in melanoma development and progression. Changes in E-cadherin and especially beta-catenin levels are correlated with melanoma progression and could be used as molecular markers of disease evolution. It would be interesting to obtain data on wnt target genes, such as c-myc, in future studies.

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**Figure legends**

Figure 1. The figure is showing immunohistochemical staining of melanoma sample (A) and immediate adjacent skin (B). Distinctive decrease of E-cadherin protein in melanoma cells is demonstrated.

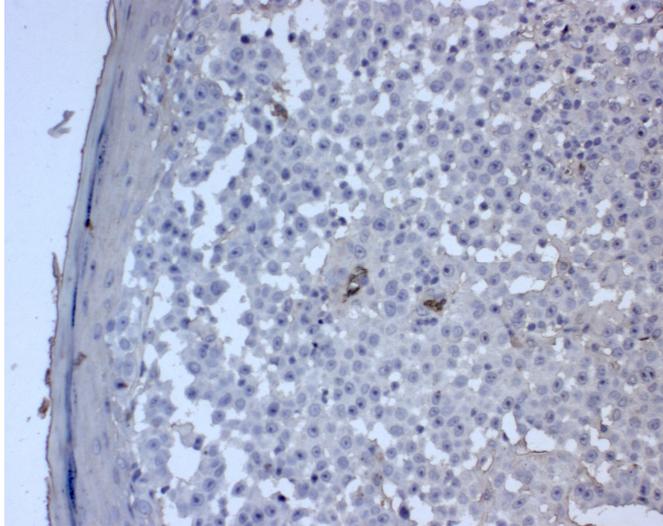


Figure 1 A

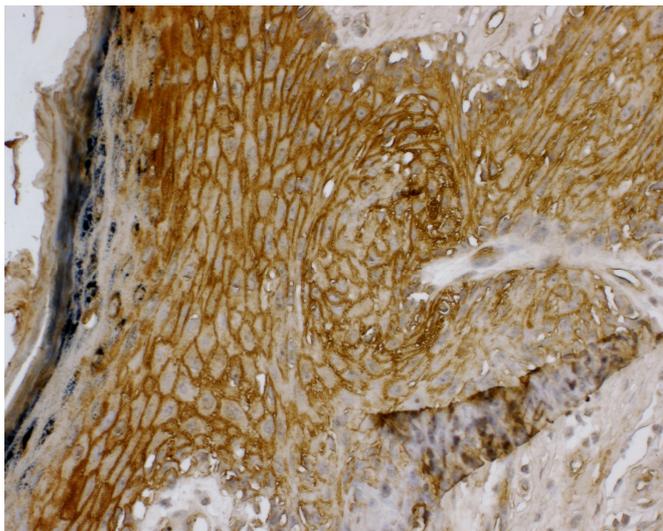


Figure 1 B

Figure 2. Same melanoma samples were immunohistochemically stained for protein expression of beta-catenin. Normal skin and skin adjacent to melanomas showed homogenous membranous staining of the epidermis (A). The figure shows beta-catenin staining in normal skin (A), and different locations of the protein in melanoma (B-cytoplasmic, C- membranous and cytoplasmic, D- cytoplasmic and nuclear).

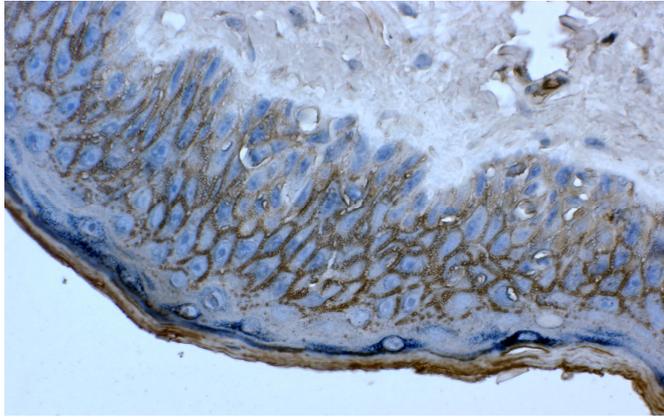


Figure 2 A

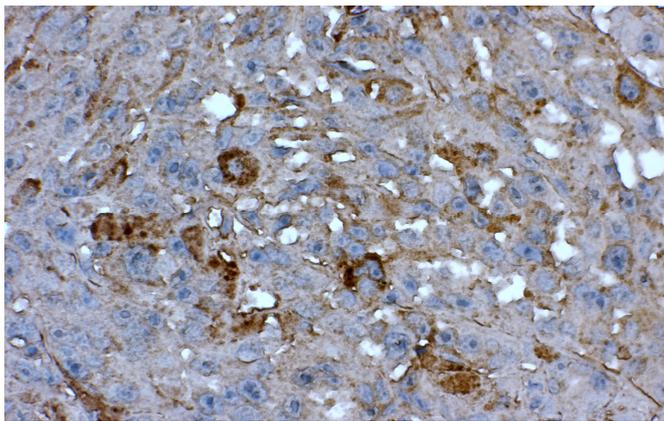


Figure 2 B

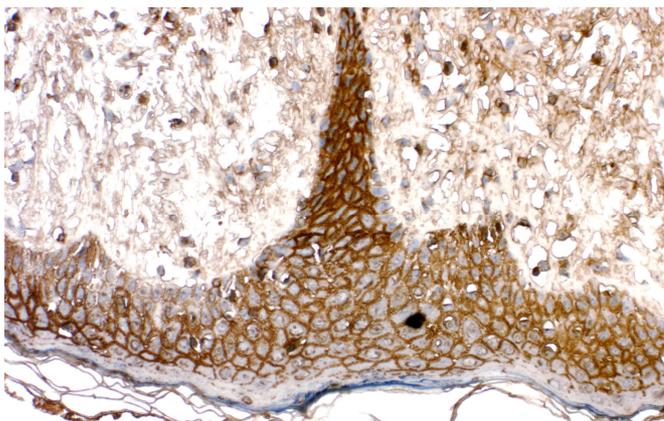


Figure 2 C

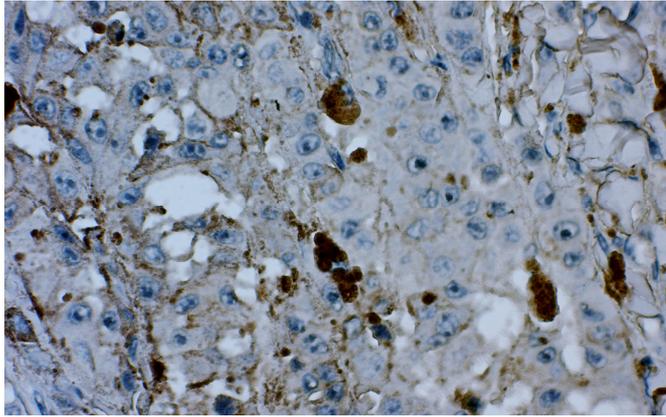


Figure 2 D

Table 1. Comparison of pairs: relative increase of the E-cadherin and beta-catenin proteins in melanoma tissue; E-cadherin protein in melanoma and adjacent skin, and beta-catenin protein expression in melanoma and the adjacent skin.

<b>Difference between pairs</b>			
	pair Etmrel <sup>1</sup> – Btmrel <sup>2</sup>	pair Etmrel – Eskinrel <sup>3</sup>	pair Btmrel – Bskinrel <sup>4</sup>
m. v. d. <sup>5</sup>	143,44	86,18	0,06
st. dev. <sup>6</sup>	47,27	42,20	12,36
m. st. er. <sup>7</sup>	6,31	7,46	1,52
t statistics	22,70	11,55	0,04
d. f. <sup>8</sup>	55	31	65
sign <sup>9</sup>	0,000	0,000	0,96

<sup>1</sup> relative light permeability increase in tumor for E-cadherin protein

<sup>2</sup> relative light permeability increase in tumor for beta-catenin protein

<sup>3</sup> relative light permeability increase in the skin adjacent to melanoma for E-cadherin protein

<sup>4</sup> relative light permeability increase in the skin adjacent to melanoma for beta-catenin protein

<sup>5</sup> difference between mean values for Etmrel i Btmrel, <sup>6</sup> standard deviation

<sup>7</sup> mean value of the standard error, <sup>8</sup> degrees of freedom, <sup>9</sup> statistical significance

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

	<b>Clark</b>	<b>N</b>	<b>Rank<sup>1</sup></b>	
<u>Etmrel<sup>2</sup></u>	2	7	17,71	
	3	19	29,37	
	4	29	33,38	
	5	5	36,00	
	Total	60		
	$\chi^2$			5,124
	D. fr. <sup>3</sup>			3
	St sign <sup>4</sup>			0,163
<u>Btmrel<sup>5</sup></u>	2	8	20,31	
	3	20	25,80	
	4	29	41,98	
	5	9	35,00	
	Total	66		
	$\chi^2$			12,854
	D. fr.			3
	St. sign			0,005

<sup>1</sup> Mean value of the rank

<sup>2</sup> Relative light permeability increase in tumor for E-cadherin protein

<sup>3</sup> Degrees of freedom

<sup>4</sup> Statistical significance

<sup>5</sup> Relative light permeability increase in tumor for beta-catenin protein

Table 3. Mean values of relative light permeabilities for E-cadherin and beta-catenin distributed to Clark stages.

Clark		Etmrel <sup>1</sup>	Btmrel <sup>2</sup>
2	M. v. <sup>3</sup>	229,28	94,87
	St. dev. <sup>4</sup>	39,73	27,05
	Minimum	183,00	46,00
	Maximum	286,00	135,00
	N	7	8
3	M. v.	254,78	106,7
	St. dev.	49,97	24,97
	Minimum	171,00	67,00
	Maximum	309,00	135,00
	N	19	20
4	M. v.	264,13	126,13
	St. dev.	46,86	16,76
	Minimum	191,00	67,00
	Maximum	308,00	136,00
	N	29	29
5	M. v.	290,20	117,66
	St. dev.	11,09	25,88
	Minimum	272,00	60,00
	Maximum	300,00	135,00
	N	5	9
Total	M. v.	259,28	115,30
	St. dev.	46,67	24,33
	Minimum	171,00	46,00
	Maximum	309,00	136,00
	N	60	66

<sup>1</sup> Relative light permeability increase in tumor for E-cadherin protein

<sup>2</sup> Relative light permeability increase in tumor for beta-catenin protein

<sup>3</sup> mean value; <sup>4</sup> Standard deviation