IMMUNOHISTOCHEMICAL ASSESSMENT OF E-CADHERIN AND β -CATENIN IN TRICHOFOLLICULOMAS AND TRICHOEPITHELIOMAS

Michala Bezdekova^{a*}, Svetlana Brychtova^a, Eva Sedlakova^a, Jana Steigerova^a, Alice Hlobilkova^a, Martina Bienova^b, Renata Kucerova^b, Tomas Brychta^c, Veronika Krejci^a, Zdenek Kolar^a

^a Laboratory of Molecular Pathology and Department of Pathology, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic

^b Department of Dermatovenereology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, I. P. Pavlova 6, 775 20 Olomouc, Czech Republic

^c Department of Internal Medicine, SPEA Olomouc, nam. Hrdinu 2, 772 00 Olomouc, Czech Republic
e-mail: mfiuraskova@seznam.cz

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Background: Trichofolliculomas and trichoepitheliomas are benign skin neoplasms originating from hair follicle cells. They result from defects in the signaling pathways that regulate hair follicle morphogenesis and regeneration. Thus they seem to be an excellent model of these processes. It is known that the E-cadherin/ β -catenin system of adhesion molecules plays a crucial role in the maintenance of tissue architecture.

Aim: The aim of the present study was to investigate their involvement in benign hair follicle tumor development.

Methods: Semiquantitative intensity of expression were examined in formalin-fixed and paraffin-embedded tissue sections of 53 trichoepitheliomas, 15 trichofolliculomas and 19 normal skin samples by indirect immunohistochemistry.

Results: The intensity of E-cadherin/ β -catenin expression in tumor cells did not differ from controls. However, normal hair follicles cells exhibited membranous E-cadherin/ β -catenin expression, whereas both types of tumors, particularly trichoepitheliomas, showed E-cadherin/ β -catenin expression with a predominantly cytoplasmic localization.

Conclusions: We suggest that this dystopic distribution of the E-cadherin/ β -catenin complex in hair follicle tumor cells may be a marker of cell-cell adhesion disruption which may contribute to the tumor formation.

INTRODUCTION

Trichofolliculomas (TF) and trichoepitheliomas (TE) are benign tumors derived from the hair follicle (hair follicle tumors, HFT) (ref.¹). According to recent data, disruption of normal dermal-epidermal interactions in HFT resulting in altered hair follicle cell morphogenesis is assumed².

In normal epithelial structures, cell-cell junctions play an important role in the maintenance, integrity and morphology of the epithelium³. It has been reported that the E-cadherin/ β -catenin system of adhesion molecules plays a crucial role in this processes⁴. E-cadherin is a cell adhesion transmembrane molecule which is a member of a family of functionally related transmembrane glycoproteins that mediate Ca^{2+} -dependent intercellular cellular adhesion⁵. E-cadherin is important for epidermal intercellular adherence because it is required for the adhesive properties of keratinocytes and skin differentiation and loss of E-cadherin-mediated cell adhesion is one rate-limiting step in the progression from adenoma to carcinoma⁶. Furthermore, it determines the morphogenesis and hair follicle cycling⁷. The adhesion is mediated via interaction

with adjacent cells through their N-terminal ectodomains and the cytoplasmic terminal tail of E-cadherin links specifically to β -catenin that binds directly to the cytoskeletal actin⁵. A deficiency in E-cadherin causes loss of adherent junctions which leads to impaired intercellular signaling, but not to direct tumor transformation⁸. It has been reported that dysfunction or disruption of cell adhesion molecules accompany the invasiveness and metastatic behavior of malignant cells⁹ and that a loss or decreased expression of E-cadherin is frequently observed mainly in advanced, poorly differentiated carcinomas¹⁰.

β -Catenin is a molecular sensor that integrates cell-cell junctions and cytoskeletal dynamics with signaling pathways affecting morphogenesis, tissue homeostasis, and intercellular communication within tissues¹¹. It has been shown that differentiation of the hair follicle is regulated and linked to the same pathways that initiate its development¹². Generally, β -catenin has a dual function. It plays a key role in cell-cell adhesion by linking cadherins to α -catenin and cytoskeletal actin⁴. In the absence of Wnt signal, β -catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3 β (glycogen synthase kinase 3 β), axin

and a tumor suppressor APC (adenomatous polyposis coli). These proteins promote the phosphorylation of serine and threonine residues in the NH₂-terminal region of β -catenin. β -Catenin is then degraded by casein kinase CK1 and protein phosphatases PP2A and PP2C through the ubiquitin proteasome pathway. Wnt signaling inhibits this process, leading to an accumulation of β -catenin in the nucleus which promotes the formation of transcriptionally active complexes with members of the Tcf/lef family (T-cell factor/lymphoid enhancer factor). The Tcf/ β -catenin heterodimers act as bipartite transcription factors and activate expression of the specific Wnt responsive genes that encode proteins regulating cell cycle, e.g. c-Myc, Cyclin D1 and Pitx2¹³⁻¹⁵. Alterations in β -catenin mediated regulation have been demonstrated mainly during cancer development, where mutations of the β -catenin gene *CTNNB1* result in disruption of a large number of cellular functions leading to loss of growth control and neoplastic change¹⁶⁻¹⁹. However, β -catenin mutations also induce benign tumor growth, as has been demonstrated for example in pilomatricomas⁷.

This study is focused on study of the E-cadherin/ β -catenin expression in trichofolliculomas (TF) and trichoepitheliomas (TE), benign adnexal neoplasms originating from the hair follicle^{20, 21}. We hypothesize that aberrant activation of these cell adhesion molecules may be implicated in hair follicle tumor development and can contribute to better understanding of hair follicle cell regulation.

MATERIAL AND METHODS

Samples

Formalin-fixed, paraffin-embedded, routine biopsy specimens were taken from the tissue archive of Department of Pathology, Faculty of Medicine and Dentistry, Palacký University, Olomouc. Each sample was reexamined by an experienced pathologist in order to confirm the diagnosis. Fifty-three trichoepitheliomas and fifteen trichofolliculomas were analyzed. Nineteen specimens of healthy scalp skin taken from autopsy (9 samples) and from biopsy (10 samples) were used as controls.

Immunohistochemistry

Indirect immunohistochemical staining of 5 μ m thick tissue sections was performed using the immunostainer Benchmark (Ventana Medical Systems S.A.). Sections were incubated with mouse monoclonal antibodies anti-E-cadherin (clone 36B5, dilution 1 : 25, Novocastra, UK), anti- β -catenin (clone E-5, dilution 1 : 50, Santa Cruz Biotechnology, CA, USA). Colon carcinoma and ductal breast carcinoma tissues were used as a positive control for β -catenin and E-cadherin, respectively. The primary antibodies were omitted in the case of negative controls. Semiquantitative assessment of protein expression was performed using a modified H-score in which both intensity and proportion of staining was categorized. Category A indicated the proportion of positive cell stain-

ing throughout the section and was assigned a scale from 0 to 3 (0 = 0-4 %; 1 = 5-24 %; 2 = 25-49 %; 3 = 50-100 %). Category B represented the average intensity; the presence of negative, weak, intermediate and strong staining was given a score from 0 to 3. Category A was multiplied by category B to form a multiplicative score. The cases were sorted into three subgroups; H-score 0 referred to negative expression; H-score 1-2 to weak expression; H-score 3-9 to moderate/strong expression.

Statistical analysis

The protein expressions were evaluated using a Chi-square test ($p < 0.05$). The cellular localizations of proteins were statistically analyzed using Fisher's exact test with Yates correction ($p < 0.05$).

RESULTS

E-cadherin

E-cadherin positivity was found in the membranes of epidermal keratinocytes and normal hair follicle cells and sebaceous cells (Fig. 1a) where moderate to strong intensity of the protein expression prevailed (Fig. 2a). When comparing normal epithelial/hair follicle/sebaceous cells to tumorous ones, marked differences in protein expression or localization were found. In TF, subcellular relocalization of the protein was observed (Fig. 1b); apart from typical membranous, also cytoplasmic (42.9 %) or both cytoplasmic and membranous localization of protein (21.4 %) were seen ($p = 0.003$) (Fig. 2b). The intensity of E-cadherin expression in tumor cells did not differ from control cells.

TE exhibited total loss of purely membranous E-cadherin localization; both cytoplasmic (68.4 %) and combined membranous and cytoplasmic (28.9 %) expression was observed ($p = 0.0001$) (Fig. 2b). The protein expression in tumor cells was significantly decreased in comparison to normal control keratinocytes ($p = 0.01$). Furthermore, TE expressed E-cadherin in lower levels than TF ($p = 0.05$).

β -catenin

The protein β -catenin was present in high to moderate levels in membranes of epidermal keratinocytes and in normal hair follicle cells (Fig. 3a, 4a). In TF, significant changes in protein localization were observed in contrast to control epithelial cells and cells of hair follicle and sebaceous glands ($p = 0.001$); 50 % of the tumor samples had cytoplasmic only or combined membranous/cytoplasmic coexpression (Fig. 3b). TF showed similar intensities of β -catenin expression as control skin cells.

In TE, a significant loss of membrane-type expression was observed in contrast to control epithelium ($p < 0.0001$); 81.3 % of tumor samples demonstrated cytoplasmic and 9.4 % membranous/cytoplasmic β -catenin staining. The difference in β -catenin localization was apparent between TE and TF tumor cells ($p = 0.002$) whereas a membranous pattern of staining prevailed in

Fig. 1. Membranous staining of E-cadherin in the hair follicle of normal skin, original magnification 200 $\times$ (a); Strong cytoplasmic staining of E-cadherin in benign trichofolliculoma cells, original magnification 400 $\times$ (b).

Fig. 2. Comparison of E-cadherin expression in the epithelium, adnexa and tumor of trichoepitheliomas (TE), trichofolliculomas (TF) and control skin (C) (a); E-cadherin distribution in the tumor samples (M=membranous, C=cytoplasmic, N=nuclear) (b).

Fig. 3. Membranous staining of β -catenin in the control normal epidermis, original magnification 400 $\times$ (a); β -catenin positivity on the membrane and in the cytoplasm of trichoepithelioma tumor cells, original magnification 320 $\times$ (b).

Fig. 4. Comparison of β -catenin expression in the epithelium, adnexa and tumor of trichoepitheliomas (TE), trichofolliculomas (TF) and control skin (C) (a); β -catenin localization in trichofolliculomas and trichoepitheliomas (M=membranous, C=cytoplasmic, N=nuclear) (b).

TF (Fig. 4b). No nuclear β -catenin staining was found either in TE or TF. TE had only slightly reduced β -catenin expression compared to control cells. However, TE revealed a significant decrease in β -catenin in comparison to TF ($p = 0.021$).

DISCUSSION

In tumors, we revealed a loss of the membranous β -catenin immunostaining, which is usually observed in the normal keratinocytes, hair follicles cells and sebaceous cells, accompanied by translocation of the protein to the cytoplasmic compartment. Trichoepitheliomas exhibited mainly cytoplasmic, less frequently combined membranous/cytoplasmic staining. On the other hand a third of trichofolliculomas showed preserved membranous protein expression. Membranous localization of the protein is known to be associated with its physiological function as a modulator of intercellular communication, tissue morphogenesis and homeostasis in different cell types.

It has been proposed that β -catenin nuclear and/or cytoplasmic localization may correlate with β -catenin gene mutation^{22, 23}. In general, changes in β -catenin regulation have been demonstrated mainly during cancer development, where mutations of the β -catenin gene *CTNNB1* result in disruption of a number of its cellular functions leading to a loss of growth control. Recent studies have found that aberrant activation of β -catenin is also involved in the regulation of benign tumor growth. Exon 3 β -catenin-activating mutations were found in all cases of pilomatricoma; the tumors display both nuclear and cytoplasmic, but only focal membranous protein expression in the basaloid cells^{24, 25}. The nuclear protein accumulation has been demonstrated to be associated with its stabilization which is induced by inhibition of GSK3 β and results in reduced β -catenin phosphorylation. However, in our

study we demonstrated no nuclear β -catenin distribution. On this basis, we suggest that the Wnt signaling pathway is not involved in the trichofolliculoma and trichoepithelioma development. These data are paralleled by Demirkan et al. who detected no β -catenin gene mutation in other benign trichogenic tumors except of pilomatricomas²⁶. To support this hypothesis, c-myc and cyclin D1 expressions were analyzed in tumors (data not shown). The levels of c-myc and cyclin D did not differ from the protein expression in control hair follicle and sebaceous cells. Therefore, we consider cytoplasmic distribution of β -catenin to be important for the TE and TF formation.

Similarly, E-cadherin showed loss of membrane-type expression with relocalization into the cytoplasm of tumor cells, predominantly in trichoepitheliomas. No changes in intensity of expression however were found. While loss of E-cadherin expression has been observed in many malignancies as an indicator of the protein mutation associated with its enhanced transcriptional activity^{9, 27–29}, the cytoplasmic or nuclear protein localization is considered to be a marker of alterations in its intercellular signaling. It has been reported that the decreased tyrosine phosphorylation of E-cadherin may reflect a loss of the E-cadherin/ β -catenin complex function and may be implicated in tumor invasion³⁰.

When we compared TE and TF, a difference in protein localization was noted. In a large percentage of TF, membranous staining was preserved. This suggests that normal intercellular communication is, at least partly maintained. From a histogenetic point of view, trichofolliculomas are considered to be hamartomas of hair follicles whereas trichoepitheliomas demonstrate classical benign adnexal neoplasms, whose tumor cells, like in many other tumors, proliferate in the absence of stimulating normal signals³¹. We conclude that a disturbance in the normal cell-cell adherent junction is probably the key event in the trichofolliculoma and trichoepithelioma formation.

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