Alterations of β -Catenin Pathway in Non-Melanoma Skin Tumors

Loss of α -ABC Nuclear Reactivity Correlates with the Presence of β -Catenin Gene Mutation

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To determine the role of β -catenin pathway in human skin carcinogenesis, 135 non-melanoma skin tumors were analyzed for β -catenin expression and gene mutations. Intense nucleocytoplasmic immunoreactivity for C terminus β -catenin antibodies was observed in all pilomatricomas and in single cases of trichoepithelioma and squamous cell carcinoma showing peculiar signs of matrical differentiation. Moderate increase of β -catenin nuclear staining was detected in a significant proportion of basal cell carcinomas, Bowen disease, spiroadenomas, and occasionally also in squamous cell carcinomas, but in these neoplasms only a limited fraction of tumor cells accumulated β -catenin. Molecular analysis revealed that β -catenin gene mutations are a peculiar feature of skin tumors with matrical differentiation and correlate with a pattern of intense and diffuse β -catenin nuclear expression. In contrast, adenomatous polyposis coli (APC) and AXIN2 mutations were not involved in skin tumorigenesis. Analysis of Wnt pathway revealed that TCF-1 and MITE-M were selectively induced in the tumor types harboring β -catenin mutations, indicating that a Wnt/ β -catenin pathway involving TCF-1 and MITE-M is activated in these tumors. Interestingly, high expression levels of TCF-3 were found in basal cell carcinomas and spiroadenomas. TCF-3 is reported to act as a negative modulator of β -catenin degradation pathway. Thus, the moderate increase of β -catenin nuclear staining detected in these tumor types might, at least in part, be due to a TCF-3-dependent mechanism. Finally, we found that the presence of β -catenin mutations significantly correlated with loss of nuclear immunoreactivity for an antibody raised against the N termi-

nus of β -catenin (α ABC). Thus, a combined analysis with C terminus- β -catenin antibodies and α ABC Ab may represent a powerful investigative approach for the detection of β -catenin structural alterations. (*Am J Pathol* 2003, 163:2277–2287)

The skin, the largest and more specialized organ of the body with a wide range of cell lineages, is the site of origin of a complex array of different tumor types. Epithelial tumors of the skin are usually classified into epidermal tumors, which account for over 90% of all skin cancers [eg, basal cell carcinomas (BCC) and squamous cell carcinomas (SCC)], and neoplasms deriving from skin adnexae such as hair follicle tumors (HFT), sebaceous gland tumors (SGT), apocrine gland tumors (AGT) and eccrine gland tumors (EGT).^{1,2} The histopathological diagnosis of these tumors is often difficult, as reflected by the existence of different classification schemes. In fact, all skin cell types can undergo neoplastic transformation, giving rise to a vast and complex potential for benign and malignant development. Moreover, the frequent finding of composite aspects further complicates the picture, with divergent cell lineages within the same neoplasm.^{1,2}

Diagnostic difficulties are paralleled by gaps in the knowledge of genetic mechanisms of neoplastic transformation of skin structures and appendages. In fact, although skin carcinogenesis has been widely investigated, particularly in the murine model, our knowledge is mostly limited to the genetic alterations involved in epidermal tumors, particularly SCC and BCC.³ More limited is our knowledge about molecular mechanisms underlying tumorigenesis in other skin structures such as hair follicle, sweat, and sebaceous glands.

Recent studies have suggested a central role for β -catenin in the development of certain types of human pilar tumors.^{4–6} β -catenin is a multifunctional protein that

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controls a number of cell activities, both at the membrane and the nuclear level.⁷ As a membrane protein, β -catenin bridges between the cytoskeleton and cadherins, thus acting as a structural component of adherens junctions.⁸ In the nucleus, β -catenin mediates the Wnt/TCF signaling.^{9–12} Wnt pathway is extremely complex and the plethora of components and interactors increases continuously. These include the Wnt receptors of the Frizzled family, the cytosolic proteins Dishevelled, glycogen synthase kinase 3 β (GSK3 β), casein kinase 1 (CKI), axin, APC, β -catenin itself, and the Lef/TCF family of transcription factors.^{7,12–14} The underlying principle of this complex set-up is to prevent nuclear accumulation of β -catenin in the absence of Wnt signals. In fact, in the absence of Wnt activation, β -catenin is essentially sequestered by E-cadherin at the plasma membrane, while β -catenin cytosolic pool undergoes rapid proteasome-mediated degradation. This degradation is triggered by phosphorylation of specific residues of β -catenin operated by GSK3 β . Axin, APC, and CKI are also relevant components of β -catenin demolition complex, as they appear to facilitate GSK3 β -dependent phosphorylation of β -catenin. On activation of the Wnt pathway, GSK3 β is inactivated. This results in a block of proteasome-mediated degradation of β -catenin which then accumulates in the cytoplasm, moves to the nucleus and here interacts with the Lef/TCF family of transcription factors. Among TCF/ β -catenin target genes are cyclin D1^{15,16}, metalloproteinase matrilysin,¹⁷ survivin,¹⁸ and MITF,¹⁹ as well as components of the same Wnt/ β -catenin pathway such as TCF-1^{20,21} and AXIN2.²²

By regulating both cell-cell interactions and gene transcription, β -catenin exerts a unique role in the control of cell proliferation and differentiation. Mouse models have shown that lack of β -catenin expression results in embryonic lethality, due to failure of ectodermal cell layer development.²³ In contrast, constitutive activation of β -catenin produces aberrant tissue proliferations that eventually lead to neoplastic transformation.^{4,24–26} Accordingly, abnormal stabilization and nuclear accumulation of β -catenin have been implicated in various neoplasias. In particular, nuclear overexpression of β -catenin, due to either mutations in the GSK3 β phosphorylation sites of β -catenin or alterations of APC or AXIN negative regulators, have been reported in a number of human epithelial cancers such as colon, liver, prostate, and ovarian carcinomas as well as in endometrial tumors and medulloblastoma.^{7,13,27}

The involvement of β -catenin in skin carcinogenesis has emerged from a study in which a constitutively active form of β -catenin was expressed under the control of the keratin-14 promoter in transgenic mice²⁴. These mice exhibited a high propensity for development of two types of skin tumors, trichofolliculoma (TF) and pilomatricoma (PMX), both originating from the hair matrix and hair germ, structures where the keratin-14 promoter is particularly active.²⁸ The involvement of β -catenin in human hair follicle tumorigenesis was subsequently confirmed by the finding of β -catenin activating mutations in a series of human PMX.⁴ However, in the K14- β -catenin mouse model high levels of constitutively active β -catenin were selectively expressed in the basal layer of the epidermis

and follicle outer root sheath, where the development of TF and PMX eventually takes place. This mouse model did not address other structures where the keratin-14 promoter is less active. Thus, the spectrum of skin neoplasms affected by alterations of β -catenin could be broader than originally suggested by the K-14 transgenic model, and whether β -catenin plays a role in human non-melanoma skin tumors other than PMX has not been fully investigated until now.

To shed light on this issue, we have analyzed a large number of human cutaneous neoplasms, including the most representative histotypes, for alterations of the β -catenin signaling pathway. This is the first extensive study on the role of β -catenin pathway in human non-melanoma skin tumors.

Our data indicate that, among human skin neoplasms, β -catenin gene mutations are a peculiar feature of the tumors of matrical origin like PMX. Intense and diffuse β -catenin nuclear accumulation correlated with β -catenin mutations and with aberrant activation of TCF-1 and MIFT-M. β -catenin nuclear accumulation in a limited percentage of nuclei was featured by other tumor types such as BCC, BD, and SA but was not associated to mutation in either β -catenin or β -catenin negative regulators APC and AXIN2. Instead, TCF-3 was found overexpressed in these tumors. Overall, our data indicate that abnormalities of Wnt/ β -catenin signaling pathway play a role in a large spectrum of skin neoplasms, although mutations of β -catenin seem to be a specific hallmark of tumors with matrical differentiation.

Finally, we provide evidence that α ABC antibody²⁹ is a powerful investigative tool for the detection of β -catenin mutations in skin tumors.

Materials and Methods

Samples

The study was carried out on a selected series of 135 neoplasms, representing the most common epithelial skin tumors, with the exclusion of melanocytic and mesenchymal neoplasms (Table 1). All of the samples were archival formalin-fixed, paraffin-embedded specimens obtained from the Belluno City Hospital.

Immunohistochemical Analysis

All cases were immunostained with a StreptABC technique, using two different C terminus β -catenin monoclonal antibodies (MoAb clone 14, 1:500, Transduction Laboratories, Lexington, KY; MoAb clone 15B8, 1:250, Sigma-Aldrich, St. Louis, MO). Most cases were also tested with α ABC (MoAb clone 8E7, 1:250, Upstate Biotech, Lake Placid, NY). The majority of cases showing nucleocytoplasmic accumulation of β -catenin in a fraction of cells greater than 10%, as well as a significant number of β -catenin-negative tumors, were also tested

Table 1. β -Catenin Expression and Gene Alterations in Skin Tumors

Histologic diagnosis	Total cases analyzed	β -Catenin expression (C terminus β -catenin Ab)				β -Catenin mutations
		<10% Reactive nuclei	10–40% Reactive nuclei	40–80% Reactive nuclei	>80% Reactive nuclei	
Hair Follicle Tumors (HFT)	55	27	1	1	26	19
Pilar proliferative lesions (Inverted Follicular Keratosis, Fibrofolliculoma, Pilar Cyst, Pore of Wiener, Pilar seath acanthoma, Pilar Hamartoma, Comedonic nevus)	12	12	0	0	0	—
Trichofolliculoma	4	4	0	0	0	—
Tricholemmoma	1	1	0	0	0	—
Trichoadenoma	1	1	0	0	0	—
Trichoepithelioma (TE)	10	9	1	0	0	NO
Trichoepithelioma with matrical differentiation (TE-MD)	1	0	0	1	0	YES (1/1)
Pilomatricoma (PMX)	26	0	0	0	26	YES (18/26)
Epidermal Tumors (ET)	51	31	19	0	1	1
Follicular and seborrheic keratosis	2	2	0	0	0	—
Verrucous diskertoma	1	0	1	0	0	—
Viral wart	4	4	0	0	0	—
Keratoacanthoma	4	4	0	0	0	—
Ca <i>in situ</i> Bowen	6	2	4	0	0	NO
Basal cell carcinoma (BCC)	16	8	8	0	0	NO
BCC with follicular differentiation (BCC-FD)	6	2	4	0	0	NO
Squamous cell carcinoma (SCC)	11	9	2	0	0	NO
SCC with matrical differentiation (SCC-MD)	1	0	0	0	1	YES (1/1)
Apocrine (AGT), Sebaceous (SGT), and Eccrine (EGT) Gland tumors	29	23	5	1	0	0
Apocrine cystoadenoma	3	3	0	0	0	—
Apocrine carcinoma	1	1	0	0	0	—
Nevous sebaceous	4	4	0	0	0	—
Sebaceous adenoma	1	1	0	0	0	—
Sebaceous carcinoma	1	1	0	0	0	—
Eccrine carcinoma	1	1	0	0	0	—
Hydradenoma	5	5	0	0	0	—
Syringoma	2	2	0	0	0	—
Microcystic carcinoma	2	2	0	0	0	—
Spiroadenoma (SA)	4	0	3	1	0	NO
Poroma	4	3	1	0	0	NO
Porocarcinoma	1	1	0	0	0	—
Total skin tumors analyzed	135	82 (61%)	24 (18%)	2 (1%)	27 (20%)	20/135 (15%)

YES, mutation detected, (tumors harboring mutations/tumors analyzed). See Table 2 for details; NO, no mutation detected; —, not done.

for the expression of Lef/TCF family of transcription factors: TCF-1 (MoAb clone 7H3, 1:100, Upstate Biotech), TCF-3 (polyclonal goat IgG SC8635, 1:300, Santa Cruz Biotechnology, Santa Cruz, CA), TCF-4 (MoAb clone 6H5-3, 1:250, Upstate Biotech), co-expression of TCF-3 and TCF-4 (MoAb clone 6F12-3, 1:200, Upstate Biotech) and Lef-1 (goat polyclonal IgG SC8592, 1:250, Santa Cruz Biotechnology).

The expression of a number of β -catenin downstream targets and regulators, including cyclin D1 (MoAb clone DCS-6, 1:100, NeoMarkers, Fremont, CA), p53 (MoAb clone DO7, 1:200, NeoMarkers), p63 (MoAb clone 4A4, 1:200, NeoMarkers) and the melanocyte isoform of microphthalmia transcription factor MITF-M (MoAb clone 34CA5, 1:100, Novocastra, Newcastle upon Tyne, United Kingdom), was also evaluated.

Heat-induced antigen retrieval was performed using the microwave oven and citrate buffer (pH 6.0, 0.01 mol/L) for C terminus β -catenin antibodies, p53 and p63.

Microwave-oven treatment and citrate buffer (pH 7.0, 0.01 mol/L) were used for all of the members of Lef/TCF family of transcription factors. N terminus β -catenin ABC Ab, cyclin D1, and MITF-M immunostaining were in TE buffer (pH 9.0).

Primary antibodies were detected using a sensitive strept-ABC technique with diaminobenzidine development.³⁰ All stainings were performed on a Labvision automatic immunostainer. Appropriate positive and negative controls were run simultaneously.

Molecular Analysis

Most human cancers that involve β -catenin alterations carry mutations in amino acid residues of the N-terminal segment corresponding to exon 3 (<http://www.stanford.edu/~mnusse/arm/bcatmut.html>).^{4,31–34} Exon 3 encodes for the aminoterminal portion of the protein, which harbors the

GSK3 β phosphorylation sites. Mutations in this region render β -catenin resistant to phosphorylation-dependent ubiquitin-mediated degradation, thus resulting in β -catenin nucleocytoplasmic accumulation.^{35–37} Mutations at exon 3 are also characteristic of human pilomatricomas.⁴ On this ground, β -catenin mutations involving exon 3 were analyzed by polymerase chain reaction (PCR)-direct sequencing in 83 skin tumors, including all of the cases showing β -catenin nucleocytoplasmic accumulation in a fraction of cells greater than 10% (53 cases) plus 30 tumors displaying accumulation of β -catenin protein in scattered cells or no nuclear accumulation at all. The 53 cases with nucleocytoplasmic accumulation of β -catenin in more than 10% of neoplastic cells were also analyzed for mutations of AXIN2 and APC.

Exon 3 of β -catenin was amplified using the following set of primers: β -cat-1s: 5'-ATTGGA TGGAGTTGGACATGGC-3'; β -cat-2a: 5'-CCAGCTACTTGTCTTGAGTGAAG G-3'. Fragment size was 223 bp.

AXIN2 mutation analysis was focused on exon 7, where most of the mutations detected so far have been mapped.^{38–41} The primers used to amplify AXIN2-exon 7 were as follows: AXIN2-sense 5'-TTCTAACCAGTTTCTTTCCT-3'; AXIN2-anti 5'-CTCCACCCAAACCAATCCCT-3'. Fragment size, 340 bp.

APC mutation analysis was focused on exon 15 (codons 1256–1499), which includes the mutation cluster region (MCR).⁴² PCR was performed using the following two sets of primers which amplify two overlapping portions of APC exon 15: APC-Gn 5'-AAGAAACAATACAGACTTATTGT-3'; APC-Gc 5'-ATGAGTGGGGTCTCCTGAAC-3'; APC-Hn 5'-ATCTCCCTCCAAAAGTGGTGC-3'; APC-Hc 5'-TCCATCTGGAGTACTTTCCGT G-3'. Fragment G size, 382 bp; fragment H size, 352 bp.

PCR was performed in a final volume of 50 μ l in the presence of 200 ng of DNA, 25 pmol of each primer, 200 μ mol/L dNTPs, 1.5 mmol/L MgCl₂ and 2 units of TaqDNA polymerase (Promega, Madison, WI).

For β -catenin and AXIN2 PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds.

For APC amplification PCR conditions were: initial denaturation at 95°C for 3 minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds and elongation at 72°C for 1 minute.

PCR fragments were gel-purified and sequence-analyzed with a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). All sequences were confirmed in both sense and antisense orientation.

Results

Expression of β -Catenin in Normal Skin and Skin Tumors

Immunohistochemical analysis was performed with β -catenin antibodies recognizing the C terminus of the protein (clone 14, Transduction Laboratories and clone

15B8, Sigma-Aldrich). Immunostaining showed that β -catenin was mainly localized to the membrane of epithelial cells of epidermis, eccrine, apocrine, and sebaceous glands of normal human skin. Differentiating structures of hair follicles displayed a membrane reaction pattern. However, in hair matrix cells, together with a membranous pattern, an intense nucleocytoplasmic staining for β -catenin was observed. β -catenin nucleocytoplasmic localization was also occasionally observed in basal cells of the epidermis, in scattered differentiated keratinocytes of the upper layer of the epidermis and in luminal cells of sebaceous glands. Dermal fibroblasts, endothelial cells, and other mesenchymal structures showed mainly a membranous reaction pattern with some nuclear reactivity, especially in mesenchymal cells of the dermal papilla.

A total of 135 non-melanoma skin neoplasms were analyzed with C terminus β -catenin antibodies. A normal β -catenin membranous pattern was detected in all of the cases analyzed. Some tumors, mostly BCC, SCC, and SGT, showed a reduced membrane expression of β -catenin, in the absence of evident cytoplasmic relocalization of the protein. Loss of β -catenin membranous expression, not related to mutation of the gene, has been reported in several tumor types^{36,43,44} and, although the molecular mechanism of this phenomenon is unclear, it is thought to be associated to impaired catenin-cadherin interaction and increased invasiveness.^{45–47}

A strong nucleocytoplasmic accumulation of β -catenin was observed in hair follicle tumors, mainly in PMX. Occasional β -catenin nuclear staining was observed also in other skin tumor histotypes but always involving a more limited fraction of cells.

Results of immunohistochemical analysis are detailed below and summarized in Table 1.

Hair Follicle Tumors (HFT)

Intense nucleocytoplasmic immunoreactivity for β -catenin was observed in all of the cases of PMX analyzed. Essentially all basal cells (80% to 95%) showed an aberrant nucleocytoplasmic accumulation of β -catenin, while in transitional cells β -catenin immunoreactivity was restricted to the cytoplasm (Figure 1). No reactivity for β -catenin antibodies was detected in ghost cells and in rare tumors composed exclusively of ghost cells (not included in Table 1). Apart from PMX, and with the exception of only a single case of trichoepithelioma (TE), no other hair follicle tumor showed an aberrant nuclear accumulation of β -catenin. Interestingly, the single case of TE, in which about 40% of neoplastic nuclei were immunoreactive, showed peculiar aspects of matrical differentiation and pilomatricoma-like areas (TE-MD) (Figure 1).

Epidermal Tumors (ET)

Nucleocytoplasmic β -catenin staining was observed in the majority of BCC, BD, and in a fraction of SCC but this was usually weaker than that observed in PMX and limited to 10 to 25% of neoplastic cells (Figure 1). Only in a

Figure 1. Immunohistochemical analysis with C terminus β -catenin Abs. **a:** Pilomatricoma with intense β -catenin nucleocytoplasmic immunoreactivity. This tumor harbored a mutation at codon 34 of β -catenin. Original magnification, $\times 200$. **b:** Trichoepithelioma with peculiar aspects of matrical differentiation (TE-MD). Nucleocytoplasmic β -catenin reactivity was observed in pilomatricoma-like areas. This tumor carried a mutation at codon 41 of β -catenin gene. Original magnification, $\times 200$. **c:** Eccrine spiroadenoma with nucleocytoplasmic β -catenin immunoreactivity in a large fraction of neoplastic cell and preserved membranous staining. Original magnification, $\times 200$. **d:** Basal cell carcinoma with follicular differentiation showing β -catenin down-regulation and scattered positive nuclei. Original magnification, $\times 400$.

single case of SCC aberrant accumulation of β -catenin involved over 80% of the neoplastic population and, interestingly, this tumor displayed peculiar features of matrical differentiation (SCC-MD).

Eccrine, Apocrine, and Sebaceous Gland Tumors (EGT, AGT, SGT)

Among eccrine, apocrine, and sebaceous gland tumors an abnormal nuclear accumulation of β -catenin was observed in particular in spiroadenomas (SA), where up to 40% of the nuclei were immunoreactive for β -catenin antibodies (Table 1; Figure 1).

Lef/TCFs, MITF-M, Cyclin D1, p63, and p53 Expression in Normal Skin and Skin Tumors

TCF-4 was widely expressed in many normal skin structures. Intense TCF-4 expression was observed in outer root sheath cells and, to a lesser extent, in matrical and inner root sheath cells. Reactivity for anti-TCF-4 antibodies was also observed in epithelial cells of the adnexes and in stromal cells of the dermis, including the follicular

papilla. Basal and suprabasal cells of the epidermis showed weak expression of TCF-4.

Similar to normal skin, TCF-4 was widely expressed in all skin tumor types (Figure 2).

A widespread expression in skin tumors was observed for Lef-1, which, in normal skin, is mostly expressed in matrical and mesenchymal cells of the follicular papilla, while a weaker reactivity was observed in the other skin epithelial structures.

TCF-3 expression in normal skin was restricted to the nuclei of epithelial cells of the outer root sheath. No other normal epithelial structure showed reactivity for anti-TCF-3 antibodies. To confirm these results an antibody that recognizes both TCF-3 and -4 (6F12-3) was used. This reagent displayed a reaction pattern that was the sum of the reactivity observed with single anti-TCF-3 and -TCF-4 specific antibodies.

In skin tumors, a strong TCF-3 immunoreactivity was detected in BCC (9 of 11 cases tested), and in SA (4 of 4 cases tested), with a number of reactive nuclei ranging between 20% and 80% (Figure 2).

No immunoreactivity for TCF-1 antibodies was observed in any epithelial structure of normal skin; nuclear staining was observed only in few lymphocytes of the

Figure 2. TCFs and MITF-M expression in non-melanoma skin tumors. TCF-3 expression in a representative case of Spiroadenoma (a) and basal cell carcinoma (b). TCF-4 immunoreactivity in a case of basal cell carcinoma (c). TCF-1 (d) and MITF-M (e) expression in Pilomatricomas. Original magnification, $\times 200$.

tumor samples with inflammatory infiltrate, serving thus as an internal positive control.

Among human skin neoplasms, activation of TCF-1 expression, in a variable percentage of cells (10% to 40%), was almost selectively observed in tumors showing strong and diffuse expression of β -catenin. These included PMX (23 of 24 cases tested) (Figure 2), and the two tumors with peculiar signs of matrical differentiation overexpressing β -catenin (TE-MD and SCC-MD). The only exception being the single case of sebaceous carcinoma included in this series in which TCF-1 expression was not associated to β -catenin accumulation. All of the other skin tumor types, similar to normal skin structures, were negative for TCF-1 expression.

Similar to TCF-1, also MITF-M expression was associated to strong nuclear immunoreactivity for β -catenin. MITF-M expression was evaluated using an antibody originally raised against the melanocyte specific isoform of the microphthalmia transcription factor. In normal skin, MITF-M expression was observed in the nuclei of inner root sheath cells (Huxley's layer and cuticle of the inner sheath), in addition to melanocytes scattered in the basal epidermis and in the bulb.

While most types of cutaneous neoplasms showed only isolated positive nuclei, all PMX (Figure 2) and the two tumors with matrical differentiation (TE-MD and SCC-MD) showed a strong nuclear accumulation of MITF-M, which involved 20% to 70% of the nuclei.

Cyclin D1 is indicated as a transcriptional target of β -catenin,^{15,16} while alterations of p53 and p63 are suggested to influence β -catenin turnover.^{48,49} Nevertheless, analysis of cyclin D1, p53, and p63 was not particularly informative on the activation of the Wnt/ β -catenin pathway since immunoreactivity was detected in most tumors, in a variable percentage of cells (20% to 60% for cyclin D1; 10% to 60% for p53; 40% to 90% for p63), without any apparent relation with β -catenin expression or mutation.

β -Catenin, APC, and AXIN2 Mutation Analysis

Mutational analysis was focused on those cases in which C terminus β -catenin antibodies detected an intense nucleo-cytoplasmic reactivity in a fraction of neoplastic cells greater than 10% (53 out of 135 skin tumors). In addition, 30 tumors displaying accumulation of β -catenin protein in scattered cells or no nuclear accumulation at all were also analyzed. Results of molecular analysis are summarized in Table 1 and Table 2.

Twenty out of 83 cases analyzed carried missense mutations in the third exon of β -catenin gene. All of the tumors with β -catenin mutations displayed nuclear accumulation of the protein in over 40% of neoplastic cells. These included 18 of 26 PMX analyzed (70%) and the two tumors with peculiar aspects of matrical differentiation, namely TE-MD and SCC-MD (Table 2). The mutational spectrum involved residues previously described as target of mutation in different tumor types (see <http://www.stanford.edu/~rnusse/arm/bcatmut.html>).

No mutation in either APC or AXIN2 was found in any of the 53 cases in which the fraction of β -catenin reactive nuclei was greater than 10%.

Analysis of β -Catenin Expression with α ABC Antibody

α ABC (anti-active β -catenin) monoclonal antibody was originally raised by van Noort and colleagues²⁹ against the N terminus of β -catenin. Van Noort and colleagues demonstrated that this antibody reacted against dephosphorylated, active forms of the protein and was capable of visualizing the activation of Wnt signaling *in vivo*. The minimal epitope of α ABC was deduced to span residues 36–44 of β -catenin. This antibody was proven effective in recognizing non-phosphorylated Ser-37 and Thr-41,

Table 2. *β-Catenin Gene Mutations in Skin Tumors Correlate with Matrical Differentiation and TCF-1 and MITF-M Activation*

Histologic diagnosis	C terminus <i>β</i> -catenin Abs % reactive nuclei	<i>α</i> ABC pattern	<i>β</i> -Catenin mutations	TCF-1 % reactive nuclei	MITF-M % reactive nuclei
PMX	80	Loss	D32N (GAC-AAC)	10	30
PMX	85	Loss	G34V (GGA-GTA)	30	60
PMX	85	N+	D32Y (GAC-TAC)	0	40
PMX	85	N+	D32Y (GAC-TAC)	20	30
PMX	90	Loss	G34R (GGA-AGA)	20	50
PMX	90	Loss	33 nt insertion after codon 32	5	40
PMX	90	Loss	D32E (GAC-GAG) T41A (ACC-GCC)	10	30
PMX	90	Loss	D32N (GAC-AAC)	20	40
PMX	90	N+	D32G (GAC-GGC)	20	50
PMX	90	Loss	I35S (ATC-AGC)	40	40
PMX	90	N+	D32G (GAC-GGC)	30	40
PMX	90	N+	D32Y (GAC-TAC)	25	50
PMX	90	N+	D32Y (GAC-TAC)	30	20
PMX	90	Loss	T41I (ACC-ATC)	30	40
PMX	90	Loss	S37F (TCT-TTT)	30	40
PMX	90	N+	S33F (TCT-TTT)	30	50
PMX	90	Loss	D32N (GAC-AAC)	20	40
PMX	90	Loss	D32N (GAC-AAC)	40	70
TE-MD (TE with matrical differentiation)	40	Loss	T41A (ACC-GCC)	10	30
SCC-MD (SCC with matrical differentiation)	80	Loss	S37F (TCT-TTT)	10	20

Among 83 skin tumors tested, only 20 harbored *β*-catenin gene mutations.

These tumors included 18 pilomatricomas and 2 tumors showing peculiar signs of matrical differentiation (1 trichoepithelioma, TE-MD, and 1 squamous cell carcinoma, SCC-MD).

while phosphorylation or substitution of either residue abrogated binding of *α*ABC.

Immunostaining with *α*ABC revealed that in normal skin structures the pattern of reactivity for *α*ABC was overlapping that of C terminus *β*-catenin antibodies, although the intensity of nuclear staining in hair matrix cells was weaker. A concordant pattern for *α*ABC and C terminus *β*-catenin antibodies was also observed in all of the tumors in which *β*-catenin expression was restricted to the membrane as in normal skin, and in the tumors in which only scattered cells showed nucleo-cytoplasmic accumulation of *β*-catenin. Surprisingly, 14 of 53 cases in which C terminus *β*-catenin Abs revealed a strong nucleo-cytoplasmic immunoreactivity in over 10% of the cells failed to show such an accumulation when probed with *α*ABC, while maintaining a normal membranous expression (Figure 3). Interestingly, loss of nuclear immu-

noreactivity for *α*ABC correlated with the presence of *β*-catenin mutations in 13 out of 14 cases (Fisher's Exact Test, *P* < 0.001) (Table 3). Analysis of *β*-catenin alterations indicated that 8 of 11 different types of amino acid substitutions were scored as loss of ABC signal. Three types of amino acid substitutions did not affect ABC binding: D32Y, D32G, and S33F (Tables 2 and 4). These mutations were detected in the 7 tumors (of the 20 of our series harboring *β*-catenin mutations) which, despite the presence of mutations, maintained nuclear reactivity for *α*ABC (Table 2).

The ability of *α*ABC Ab to score mutant forms of *β*-catenin (as a discordant nuclear reactivity for C terminus *versus α*ABC Ab) on formalin-fixed, paraffin-embedded samples was confirmed also in two colon cancer cell lines. Nuclei of HCT116 cell line, which harbors a 3-bp deletion of *β*-catenin,³² were positive for C terminus

Figure 3. Comparison of the nuclear immunohistochemical pattern for C terminus β -catenin Abs and α ABC Ab. Serial sections of two representative cases of pilomatricoma immunostained with C terminus β -catenin Abs (**a** and **c**) and with α ABC Ab (**b** and **d**). Original magnification, $\times 200$. **Inserts** in **b** and **d** show a further $\times 4$ magnification of the field delimited by a frame. Panels **a** and **b** show a case of pilomatricoma which overexpresses wild-type β -catenin; **c** and **d** show a case carrying a D to N amino acid substitution at codon 32. Nuclear staining for α ABC is maintained in wild-type (**b**), whereas it is lost in mutated (**d**) pilomatricoma.

β -catenin Abs but negative for α ABC. In contrast, the LoVo cell line, which accumulates at nuclear level wild-type β -catenin due to APC abnormalities,⁵⁰ showed a concordant pattern of nucleo-cytoplasmic immunoreactivity with α ABC and C terminus β -catenin Abs (data not shown).

Table 3. Loss of Nuclear Immunoreactivity for α ABC Correlates with β -Catenin Mutations

	β -Catenin mutation	No β -catenin mutation	Totals
C Terminus +/ α ABC + (concordant pattern)	7	32	39
C Terminus +/ α ABC loss (discordant pattern)	13	1	14
Totals	20	33	53

Fisher's exact test, $P < 0.001$.

Fifty-three tumors showing a nuclear immunoreactivity for C terminus β -catenin antibodies in a fraction of cells greater than 10% were probed with α ABC antibody. Among 14 tumors showing a discordant pattern with C terminus β -catenin antibodies versus α ABC (C terminus +/ α ABC loss), 13 carried mutation of β -catenin. Among the cases showing a concordant pattern of reactivity for C terminus β -catenin antibodies and α ABC (C terminus +/ α ABC +, 39 cases), only 7 carried mutations. These mutations corresponded to D32Y, D32G, and S33F amino acid substitutions (see Table 4).

Discussion

Skin neoplasms represent a heterogeneous and complex group of tumors deriving from the various structures that constitute the human skin. These proliferative lesions are a composite puzzle of entities perplexing to many pathol-

Table 4. β -Catenin Amino Acid Substitution and α ABC Nuclear Reactivity

β -Catenin mutation	α ABC versus C term- β -catenin
D 32 Y	N+/N+
D 32 G	N+/N+
D 32 N	Loss/N+
S 33 F	N+/N+
G 34 V	Loss/N+
G 34 R	Loss/N+
I 35 S	Loss/N+
S 37 F	N+/N+
T 41 I	Loss/N+
T 41 A	Loss/N+
33 bp insertion after codon 32	Loss/N+

N+/N+, α ABC, and C terminus β -catenin Abs detected a similar pattern of nuclear reactivity; Loss/N+, in the presence of this amino acid substitution of β -catenin the nuclear reactivity detected by C terminus β -catenin Abs was lost with α ABC Ab.

ogists and dermatologists. Difficulties in correlation of these lesions with their normal counterparts, combined with gaps in knowledge about molecular aspects of normal and neoplastic transformation of the different components of human skin, are the major reasons of different interpretations and classifications of cutaneous proliferations.^{1,2} A deeper knowledge of the genetics of human skin tumors would provide important supports for a better classification and management of these particular forms of neoplasia.

Recently, the involvement of β -catenin in human cutaneous neoplastic transformation has been suggested by the finding of β -catenin gene mutations in PMX, a tumor arising from hair follicle matrix cells.⁴ However, the role of Wnt/ β -catenin pathway in the development of the different types of skin neoplasm has not been fully elucidated yet. To evaluate the relevance of β -catenin signaling in human skin tumorigenesis we analyzed 135 cases of common tumors of the epidermis and adnexae for alterations of Wnt/ β -catenin pathway.

Immunohistochemical analysis with C terminus β -catenin antibodies revealed that β -catenin was aberrantly accumulated in the nuclear compartment in all of the cases of PMX analyzed. In these tumors almost all nuclei of tumor cells were positive for β -catenin antibodies. A similar pattern of intense and widespread nucleo-cytoplasmic reactivity was detected also in a single case of TE (TE-MD) and in one case of SCC (SCC-MD). Interestingly both tumors showed peculiar features of matrical differentiation.

A moderate nuclear immunoreactivity for β -catenin was also observed in a significant fraction of BCC, BD, and SA, and occasionally in other tumor types, but in these cases β -catenin accumulation always involved a limited number of nuclei.

Molecular analysis revealed that nucleo-cytoplasmic accumulation of β -catenin significantly correlated with the presence of gene mutation selectively in PMX and in the single cases of TE and SCC showing peculiar signs of matrical differentiation. About 70% of PMX analyzed carried mutation of β -catenin gene. This frequency was similar to that reported originally for PMX by Chan and co-workers.⁴

With the only exception of PMX and the single cases of TE-MD and SCC-MD, all of the other skin tumors in which a variable percentage of cells showed a moderate nuclear accumulation of β -catenin failed to show stabilizing mutation of β -catenin gene. Large deletions in the N-terminal portion of the protein, which may also be responsible for aberrant stabilization of β -catenin (see <http://www.stanford.edu/~rnusse/arm/bcatmut.html>), were investigated through an immunohistochemical analysis with an antibody, which recognizes the N terminus of β -catenin (α ABC).²⁹ The maintenance in these cases of a pattern of immunoreactivity for N terminus α ABC Ab similar to that for C terminus β -catenin Ab allowed us to rule out the expression of N terminus-truncated, phosphorylation-resistant forms of β -catenin.

To determine whether other components of the Wnt/ β -catenin pathway were involved in β -catenin stabilization in skin tumors, we focused our attention on APC and

AXIN2, alterations of which have been reported to be associated with β -catenin nuclear accumulation. APC gene is mutated in a large fraction of colorectal, thyroid, and ovarian carcinomas, desmoid tumors and synovial sarcomas.²⁷ Mutations of AXIN2 have been described for colorectal, ovarian, endometrial, and hepatocellular carcinomas.^{38–41}

No mutation of APC or AXIN2 was detected in our series of skin tumors showing accumulation of β -catenin in a fraction of nuclei greater than 10%, including PMX, BCC, BD, SCC, poroma, and SA. Therefore, other molecules affecting β -catenin protein turnover are likely to play a role in these tumors. A number of proteins have been described to interfere with β -catenin activation. In particular, the aberrant stabilization of β -catenin observed in BCC could be related to abnormalities in the Hedgehog/Patched pathway. In fact, Wnt is considered a downstream target of Hedgehog/Patched signaling^{51–53} and Patched gene is frequently mutated in BCC.^{54–56} Other proteins involved in the control of β -catenin expression include mutant forms of E-cadherin,^{27,35,57–59} loss of Presenilin expression^{60–62} and recently also alterations of p53-tumor suppressor gene or expression of deltaN p63^{48,49}; however, no correlation between β -catenin, p53 or p63 immunoreactivity was found in our series of skin tumors.

To clarify the role of β -catenin in skin tumorigenesis, we analyzed the expression of a number of components of Wnt/ β -catenin pathway. We found that, while Lef-1 and TCF-4 were diffusely expressed in skin tumors, irrespective of the tumor histotype and the presence of β -catenin nuclear accumulation, TCF-3 was selectively expressed in a large fraction of BCC and EGT, namely SA. In normal human skin TCF-3 was found expressed in outer root sheath cells, similar to what was originally reported in the mouse.⁶³ Interestingly, it has been shown that TCF-3 inhibits β -catenin degradation by competing with AXIN and APC for β -catenin binding.⁶⁴ Thus, a deregulated TCF-3 expression might account for the reduced turnover of β -catenin observed in a fraction of skin tumors showing moderate nuclear accumulation of β -catenin in the absence of gene mutations and could contribute, together with Patched gene, to neoplastic transformation of BCC.

Furthermore, we found that TCF-1 and MITF-M significantly correlated with constitutive activation of β -catenin pathway, being expressed in the tumor types carrying β -catenin gene mutations, including PMX and the two tumors (TE-MD and SCC-MD) with peculiar aspects of matrical differentiation. Both TCF-1 and MITF are reported to be Wnt transcriptional targets.^{19–21} This suggests that TCF-1 and MITF are important mediators of Wnt/ β -catenin pathway in these types of skin neoplasms. MITF-M is a transcription factor implicated in melanocyte development and it has been proposed as a specific melanocytic marker.⁶⁵ Our finding of MITF-M expression in the nuclei of inner root sheath cells and in matrical skin tumors supports a role for this protein also in the control of cell growth and transformation of cells of non-melanocytic lineage.

Finally, we found that loss of nuclear immunoreactivity for α ABC, an antibody raised against the N terminus of

β -catenin and proven able to detect non-phosphorylated, active forms β -catenin,²⁹ significantly correlates with the presence of mutations of β -catenin gene. Among 53 tumors which showed intense nucleo-cytoplasmic immunoreactivity for C terminus β -catenin antibodies in over 10% of the cells, 14 cases failed to show such a nuclear accumulation when probed with α ABC Ab. Interestingly, 13 of these 14 cases (92%) carried mutations of β -catenin gene. However, not all of the tumors carrying β -catenin gene mutations lost their reactivity for α ABC. In fact, while 8 of 11 types of amino acid substitutions found in our series of tumors were associated to loss of nuclear reactivity for α ABC Ab (73%), three types of mutations did not affect α ABC Ab binding. These involved amino acid substitutions at codon 32 from aspartate to glycine or tyrosine (D32Y and D32G) and amino acid substitution at codon 33 from serine to phenylalanine (S33F). Indeed, Van Noort and colleagues²⁹ had previously shown that amino acid substitutions at codon 33 do not alter α ABC binding.

Thus, we argue that a discrepancy between nuclear reactivity with C terminus β -catenin antibodies and negativity for α ABC Ab can be considered bona fide suggestive of β -catenin gene mutations, thus pointing at α ABC as a powerful investigative tool.

In conclusion, this is the first extensive analysis of the role of alterations of β -catenin pathway in human skin carcinogenesis. Our results indicate that β -catenin gene mutations are a hallmark, among non-melanoma skin neoplasms, of the tumors with matrical differentiation and a TCF-1/MITF-mediated pathway is likely activated in these neoplasms. Moreover, our data support the notion that TCF-3 could be involved in the accumulation of wild-type forms β -catenin in skin tumors such as BCC and SA.

We finally provide evidence that a combined analysis with C terminus β -catenin/ α ABC Ab may represent a powerful approach for the detection of mutant forms of β -catenin.

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