

Frequent β -catenin overexpression without exon 3 mutation in cutaneous lymphomas

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β -Catenin is a ubiquitously cytoplasmic protein that has a critical role in embryonic development and mature tissue homeostasis through its effects on E-cadherin-mediated cell adhesion and Wnt-dependent signal transduction. Mutations that alter specific β -catenin residues important for GSK-3 β phosphorylation, or increase the half-life of the protein, were identified in human cancer. However, the role of the Wnt pathway in B- and T-cell oncogenesis has not been extensively investigated. To assess the role of β -catenin defects in primary cutaneous lymphomas, we examined the expression pattern and the genetic alteration of β -catenin on 79 samples from 74 patients with primary cutaneous lymphomas from B- and T-cell origin. Immunohistochemical analysis revealed β -catenin deregulation in five primary cutaneous B-cell lymphomas (21%) and in 21 primary cutaneous T-cell lymphomas (42%) without nuclear accumulation suggesting that activation and accumulation of β -catenin may play an important role in the development of skin lymphomas. Mutation analysis of β -catenin exon 3, which included the responsible element for Wnt signaling, was therefore done in 19 samples. However, genetic alterations of β -catenin exon 3 were not detected in any of these cases suggesting that other regulatory mechanisms may be relevant in activating β -catenin signaling in cutaneous lymphomas. *Modern Pathology* (2004) 17, 1275–1281, advance online publication, 11 June 2004; doi:10.1038/modpathol.3800181

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Primary cutaneous lymphomas comprise a spectrum of diseases characterized by infiltration of the skin by malignant lymphocytes. The clinical manifestations of cutaneous lymphomas vary and can mimic benign.¹ Cutaneous T-cell lymphomas include mycosis fungoides, Sézary syndrome, lymphomatoid papulosis, CD30+ large cell lymphoma and adult T-cell leukemia/lymphoma. Primary cutaneous B-cell lymphomas are less common than T-cell lymphomas and account for less than 20% of all primary cutaneous non-Hodgkin's lymphomas and are classified according to their cell of origin.² The precise mechanisms of lymphoma genesis are still obscure. It appears that various factors are involved including environmental, infectious agents, chromosomal translocations, insertions, or point mutations resulting from genetic instability of tumor cells.³ The microenvironment in the skin contributes to the peculiar behavior of these neoplasms by

providing various signals from adhesion molecules and cytokines.

β -Catenin is a multifunctional protein that acts as a component of the homotypic cell–cell adhesion apparatus and as a coactivator of the transcription of T-cell transcription factor/lymphoid enhancer binding factor (TCF/LEF) target genes. In the epithelial cells, β -catenin is localized in the cytoskeletal complex that regulates cell–cell adhesion.^{4,5} Further, β -catenin is a critical member of the Wnt signaling pathway, which plays an important role in embryonic development and tumorigenesis.^{6,7}

Without stimulation, β -catenin is degraded by the ubiquitin–proteasome pathway, which depends upon β -catenin phosphorylation. The current model suggests that Axin and APC serve as scaffolds to facilitate the phosphorylation of β -catenin by GSK-3 β at several specific serine and threonine residues encoded by exon 3. Phosphorylated β -catenin, but not the unphosphorylated form, is rapidly degraded by the ubiquitin–proteasome degradation complex.⁸ Hence, because Wnt activation inhibits the GSK-3 β activity, Wnt activation promotes accumulation of the unphosphorylated β -catenin molecule in the cytoplasm.⁹ Subsequently, free cytoplasmic β -catenin enters the nucleus, forms a complex with members of T-cell factor/lymphoid enhancer factor

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(TCF/LEF) family of transcription factors, and upregulates the expression of target genes.^{10,11} Recently many important genes, including *c-myc*, *cyclin D1* and *matrilysin* (matrix metalloproteinase-7), which are involved in oncogenesis have been identified as target genes for β -catenin/Tcf-induced transcription.^{12–14} Several studies have also shown that β -catenin can be oncogenically activated by direct genetic mutation of the phosphorylation sites in exon 3, by the inactivation of GSK-3 β , Axin and APC or by the activation of Wnt signaling pathways.^{15–17}

Recently, Wnt family members have been demonstrated to contribute to lymphopoiesis at early stages of both B- and T-cell development. In fact, Wnt signaling through β -catenin has a positive role in the control of T-cell development, such that an absence or reduction in the Wnt signal leads to a reduction in cell proliferation rate and differentiation to the CD4⁺CD8⁺ double-positive stage, whereas increased Wnt signaling through stabilization of β -catenin enables differentiation of thymocytes in the absence of a pre-TCR signal.^{18–20}

The role of the Wnt pathway in B- and T-cell oncogenesis has not been extensively investigated. β -Catenin is not detectable in normal peripheral blood T cells but is expressed in Jurkat cells, in several other leukemic cell lines and in the human T-cell lymphoma/leukemia cell line Hut102 derived from a patient with cutaneous T-cell lymphoma.^{21,22}

In this work, in order to increase our understanding of the molecular mechanisms underlying the development of cutaneous lymphomas, we investigated the potential involvement of β -catenin signaling in human primary cutaneous lymphomas. We determined the presence and the localization of β -catenin protein, and screened for mutation of β -catenin exon 3.

Materials and methods

Patients

The present study was based on a selection of 74 cases of human primary cutaneous lymphomas (56 men, 18 women) with a median age of 56.7 years (range, 21–91 years) collected from the archives of Istituto Dermatologico dell'Immacolata of Rome. Cases were classified according to REAL classification and included: 35 mycosis fungoides (MF), 10 patients with Sézary Syndrome (SS), three anaplastic large cell lymphoma (ALCL), 1 NK-cell lymphoma, 1 angiocentric T-cell lymphoma (ATCL), 16 large B-cell lymphoma (LBCL), 3 marginal zone B-cell lymphoma (MZBL) and 5 follicular B-cell lymphoma (FL). These cases were characterized immunophenotypically with antibodies against the B-cell markers (CD20 and CD79a), the T-cell markers (CD3, CD45RO) and CD30 (additional immunostaining for CD56 was performed for the diagnosis of NK-cell lymphoma). All cases selected, with the

exception of the NK-cell lymphoma case had a monoclonal origin as determined by polymerase chain reaction amplification of TCR- γ gene. As negative controls 10 non-neoplastic skin biopsies with lymphocyte-rich infiltrates such as non specific dermatitis, psoriasis and eczema were used.

Immunohistochemical Staining of β -Catenin

Representative sections from formalin-fixed, paraffin-embedded tumor tissue were processed for immunohistochemical staining. Briefly, 3 μ m sections were deparaffinized in xylene for 20 min and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked for 10 min with 3% hydrogen peroxidase. Sections were heated in pH 6.0 citrate buffer for 8 min in a microwave at 600 W to retrieve the antigen. Nonspecific antibody binding was blocked for 10 min by incubation with protein block serum free (Dako, Santa Barbara, CA, USA). An anti- β -catenin monoclonal antibody (Zymed) was diluted 1:100 and incubated in a humidity chamber for 2 h. The primary antibody was visualized using an anti-mouse HRP system with AEC as the chromogen (Dako). Sections were then counterstained by hematoxylin. Positive immunoreactivity of keratinocytes was used as an internal positive control. Phosphate-buffered saline instead of the primary antibody was used as a negative control.

Using cytomorphologic criteria, tumor cells were differentiated from smaller reactive cells. Staining intensities were classified semiquantitatively as follows: 4: strong expression 75–100% of the tumor cells, 3: 50–75%, 2: 25–50%, 1: 25–10% and 0: less than 10% of the tumor cells.

Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) Analysis of β -Catenin Exon 3

Based on the immunostaining results of the β -catenin protein, a total of 19 cases were selected for mutational analysis in the GSK- β phosphorylation consensus motif of the β -catenin gene by PCR-SSCP. Genomic DNA was extracted from the same block evaluated for β -catenin expression. Eight 10 μ m embedded sections were deparaffinized using two successive washes of xylene, hydrated through 80% ethanol solution and air-dried. The DNA for PCR amplification was extracted and purified using Dneasy Tissue Kits (Qiagen, Hilden, Germany).

PCR-SSCP analysis for β -catenin exon 3 was performed using the following primers: forward: 5'-ATGGAACCAGACAGAAAAGC-3' and reverse 5'-GCTACTTGTCTTGTAGTGAAG-3'. The amplification product included the coding sequence for GSK-3 β phosphorylation consensus region of β -catenin, which has been found to contain activating mutations. The PCR mixture contained: 1 μ l of

genomic DNA as template (100–200 ng), 5 μ l of 10 \times PCR buffer I (Perkin-Elmer), 250 μ M of each dNTP, 25 pmol of each primer and 2.5 U of Ampli-Gold Taq (Perkin-Elmer) in a final volume of 50 μ l. The reaction was run in a thermal cycler (GeneAmpTM PCR System 2400, Applied Biosystems). The PCR conditions were: 6 min of initial denaturation at 94°C and 35 cycles consisting of 1 min of denaturation at 94°C followed by 1 min at 55°C annealing step and 1 min at 72°C elongation step. The final 7 min incubation at 72°C assured a complete extension of the PCR products. The presence of amplified products were checked by electrophoresis in 1.5% agarose gel containing 2 μ g/ml ethidium bromide in TBE buffer.

Nonradioactive SSCP was performed as follows. Reaction mixtures consisting of 20 μ l containing 10 μ l of PCR product (about 0.8 μ g of DNA), 6 μ l of 1 M urea and 4 μ l of loading buffer (0.25 mg bromophenol blue, 0.25% xylene cyanol and 2.5 mM EDTA) were heated to 94°C for 5 min, chilled on ice and then subjected to electrophoresis in 10% polyacrylamide (29:1 acrylamide:bisacrylamide) TBE gels containing 20 mM HEPES at 200 V in 1 \times TBE buffer for 2 h in a cold room maintaining the electrophoretic chamber in an ice bath. Human genomic DNA from a healthy donor was used as a negative control in order to evaluate the mobility pattern of the bands in SSCP analysis. A single base substitution from a melanoma patient was used to establish that a point mutations mobility shift could be detected when mutant DNA comprised as little as 6% of the total gene copies in a PCR mixture (data not shown). Mutational analysis was performed only in the cases with more than 20% of positive cells considering the whole amount of cells present in the tissue.

Gel Staining

Gels were stained with a 0.5 μ g/ml solution of ethidium bromide in 1 \times TBE buffer for 20 min, and then destained in distilled water for 5 min. Ethidium bromide-stained bands were visualized using a 340 nm UV-viewing box and photographed.

Statistical Analyses

To evaluate the statistical correlation between β -catenin expression and both CD30 and the large cell transformation, Fisher's exact probability test was used. A *P*-value <0.05 was considered significant.

Results

Immunohistochemical Expression of β -Catenin in Primary Cutaneous Lymphomas

Formalin-fixed, paraffin-embedded tissues from 79 biopsies derived from 74 patients were analyzed by

immunohistochemistry for the protein expression of β -catenin. Immunohistochemical results were graded independently by two investigators according to the location and intensity of the signal. Staining of β -catenin was considered to be positive when at least 10% of tumor cells within the whole infiltrate were reactive for β -catenin antibody.

The results of β -catenin expression are summarized in Table 1. β -Catenin-positive staining of tumor cells was detected in five of 24 (21%) cases of B-cell lymphomas, including 3 of 16 (19%) diffuse large B-cells lymphomas, 1 of 3 (33%) marginal cells lymphomas, 1 of 5 (20%) follicular center lymphomas. In T-cells lymphomas we observed β -catenin accumulation in 21 of 50 (42%) case, including 3 of 3 (100%) anaplastic large cell lymphomas, 11 of 35 (31%) mycosis fungoides and 7 of 10 (70%) of Sézary Syndrome. Angiocentric lymphoma and NK-cell lymphoma did not show β -catenin antibody reactivity. The expression pattern observed was always cytoplasmic with a particular distribution in the perinuclear area (Figure 1). In all cases, we observed a dot-like positivity with the exception of one case of Sézary Syndrome patient that had a diffuse cytoplasmic strong positivity with few cells having a dot-like signal.

Correlation between β -Catenin and CD30 Expression

CD30 antigen is a characteristic cell surface receptor for activated T-cell and the malignant cells of Hodgkin's disease (HD), anaplastic large cell lymphoma (ALCL), mycosis fungoides (MF) evolving into large cell lymphoma and a few other non-Hodgkin's lymphomas. In order to investigate the relationship between β -catenin accumulation and CD30 expression in T-cell lymphomas, we performed a Fisher's exact probability test. Our results demonstrated that 11 of 14 (78%) CD30+ cases exhibited staining for β -catenin, whereas only 10 of 36 (27.7%) patients with CD30- T-cell lymphoma showed β -catenin positivity, demonstrating a significant association (*P*=0.0016) between β -catenin staining and CD30 immunoreactivity (Table 2).

Correlation between β -Catenin Expression and Large Cell Transformation in Mycosis Fungoides

Our immunohistochemical results in MF revealed an important association between β -catenin expression and disease progression (*P*=0.00041). In particular, we reported that 9 of 13 (69%) patients that developed the morphologic appearance of a large cell lymphoma showed a strong β -catenin staining. More importantly, this positivity was predominantly seen in large transformed cells. In contrast, only 2 of 22 (9%) of low-grade MF expressed β -catenin (Table 3).

Table 1 Expression of β -catenin in cutaneous NHL

Case no.	Sex/age	Diagnosis	β -catenin+	Mutation
<i>Primary cutaneous B-cell lymphoma</i>				
1	M/72	DLBCL	0	ND
2	M/39	DLBCL	0	ND
3	F/48	DLBCL	0	ND
4	F/67	DLBCL	0	ND
5	M/32	DLBCL	0	ND
6	M/70	DLBCL	2	WT
7	M/69	DLBCL	0	ND
8	M/59	DLBCL	0	ND
9	M/49	DLBCL	0	ND
10	F/55	DLBCL	0	ND
11	M/53	DLBCL	0	ND
12	M/54	DLBCL	0	ND
13	M/53	DLBCL	0	ND
14	M/49	DLBCL	0	ND
15	M/51	DLBCL	1	ND
16	M/41	DLBCL	3	WT
17	M/71	MZBCL	3	WT
18	M/79	MZBCL	0	ND
19	F/55	MZBCL	0	ND
20	M/37	FL	0	ND
21	M/63	FL	2	WT
22	M/83	FL	0	ND
23	M/61	FL	0	ND
24	M/49	FL	0	ND
<i>Primary cutaneous T-cell lymphoma</i>				
25	M/50	MF	2	WT
26	M/41	MF	0	ND
27	F/28	MF	0	ND
28	M/28	MF	0	ND
29	M/40	MF	0	ND
30	M/72	MF	0	ND
31	M/62	MF	0	ND
32	M/27	MF	0	ND
33	F/74	MF	0	ND
34	M/42	MF	0	ND
35	M/35	MF	0	ND
36	F/37	MF	0	ND
37	M/26	MF	0	ND
38	M/70	MF	1	ND
39	M/40	MF	0	ND
40	M/71	MF*	4	WT
41	M/63	MF	0	ND
42	F/68	MF	0	ND
43	M/67	MF	0	ND
44	M/32	MF	0	ND
45	M/44	MF	0	ND
46	F/66	MF	0	ND
47	M/65	MF	0	ND
48	M/91	MF*	0	ND
49	F/80	MF**	2	WT
50	M/38	MF**	2	WT
51	F/53	MF**	0	ND
52	M/38	MF**	2	WT
53	M/72	MF**	0	WT
54	F/71	MF**	1	WT
55	F/54	MF**	4	WT
56	M/37	MF**	0	ND
57	F/36	MF**	2	ND
58	M/28	MF**	1	WT
59	F/69	MF**	1	ND
60	M/52	ALCL	2	WT
61	M/56	ALCL	3	ND
62	M/52	ALCL	2	ND
63	M/27	ATCL	0	ND
64	F/59	NK/T	0	ND
65	M/62	SS	0	ND
66	M/72	SS	1	WT

Table 1 Continued

Case no.	Sex/age	Diagnosis	β -catenin+	Mutation
67	M/59	SS	0	ND
68	F/59	SS	2	ND
69	M/48	SS	3	WT
70	M/63	SS	0	ND
71	M/66	SS	3	WT
72	M/52	SS	2	WT
73	M/86	SS	1	ND
74	F/59	SS	4	WT
<i>Control</i>				
1	F/29	Dermatitis	0	ND
2	F/41	Dermatitis	0	ND
3	M/60	Dermatitis	0	ND
4	F/60	Dermatitis	0	ND
5	M/39	Dermatitis	0	ND
6	M/66	Psoriasis	0	ND
7	M/71	Psoriasis	0	ND
8	F/46	Eczema	0	ND
9	M/68	Eczema	0	ND
10	M/65	Eczema	0	ND

DLBCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; MZBCL = marginal zone B-cell lymphoma; MF = mycosis fungoides; ALCL = anaplastic large cell lymphoma; NK = NK-cell lymphoma; ATCL = angiocentric T-cell lymphoma; SS = Sézary Syndrome. *In evolution to a large T-cell lymphoma CD30(-). **In evolution to a large T-cell lymphoma CD30+. Staining intensities were classified semi-quantitatively as follows: 4: strong expression in 75–100% of the tumor cells; 3: 50–75%; 2: 50–25%; 1: 25–10% of the tumor cells. ND = not done; WT = wild type.

Mutational Analysis of β -Catenin Exon 3

In order to investigate a possible cause of β -catenin deregulation in primary cutaneous lymphomas, we performed a mutational analysis in 19 of the 26 samples that exhibited marked accumulation of β -catenin in the neoplastic lymphoid cells. A portion of exon 3 coding for the GSK-3 β phosphorylation consensus region of β -catenin was selected for analysis because it harbors the majority of β -catenin mutations identified in other human malignancies. The technique is based on the principle that when heated and then cooled mutated β -catenin will form secondary conformers different from those seen with WT β -catenin. These conformers can be detected as an extra or abnormal band when the PCR product is run on a polyacrylamide gel. Aberrant mobility shift, indicating a genetic mutation, was never detected in any of our samples (Figure 2).

Discussion

In this study, we demonstrated that β -catenin is expressed by tumor cells in cutaneous lymphoproliferative disorders at various frequencies. Interestingly, mutations of β -catenin exon 3 were not detected in any of the analyzed samples, indicating that while deregulation of β -catenin expression is frequent, mutations in its gene are rare in cutaneous lymphomas.

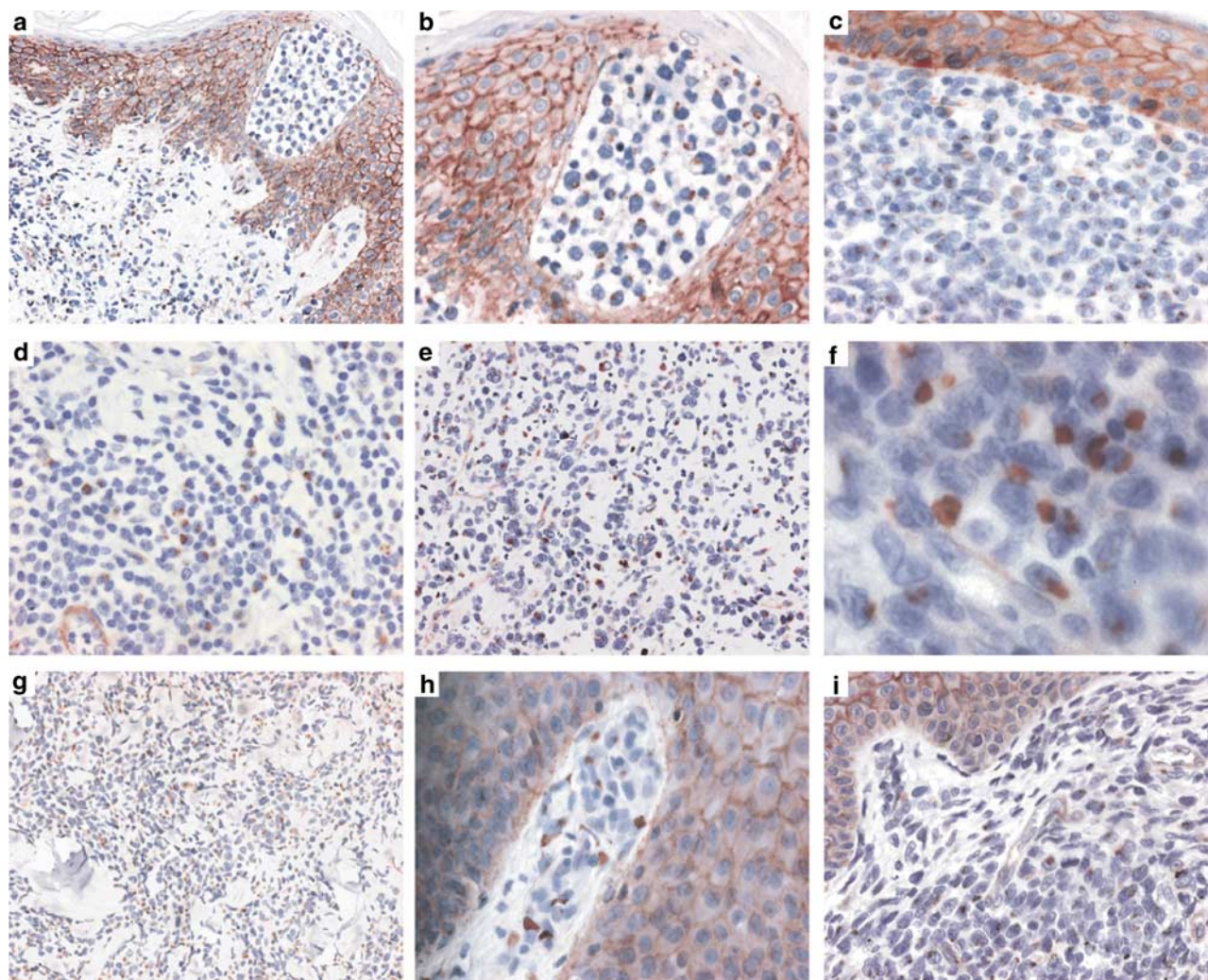


Figure 1 Representative immunohistochemistry analysis of β -catenin in human primary cutaneous lymphomas. (a) Sézary Syndrome demonstrating β -catenin positive cells in the dermal and epidermal infiltration of neoplastic lymphoid cells. (b) Same case of SS as (a) at high magnification showing strong expression in Pautrier microabscess. (c) Large B-cell lymphoma. (d–f) Three different cases of anaplastic large cell lymphoma. (h) A case of *Mycosis fungoides*. (g–i) *Mycosis fungoides* in large cell transformation.

Table 2 Significant relationship between β -catenin and CD30 expression in T-cell lymphoma

		<i>CD30</i>		<i>Total</i>
		–	+	
β -Catenin	–	26	3	29
	+	10	11	21
Total		36	14	50

Table 3 Significant relationship between β -catenin expression and large cell transformation in *Mycosis fungoides*

		<i>MF</i>		<i>Total</i>
		<i>LTC</i> –	<i>LTC</i> +	
β -Catenin	–	20	4	24
	+	2	9	11
Total		22	13	35

MF = mycosis fungoides; LTC = large T cell.

Normal resting peripheral blood mononuclear cells do not contain detectable levels of β -catenin protein. In contrast, neoplastic lymphoid cells showed a cytoplasmic distribution with a particular perinuclear dot-like aspect. Our observation suggests the possibility that the cellular localization of β -catenin is in the Golgi apparatus. The absence of β -

catenin in the nucleus correlates with no detectable expression of target genes for β -catenin/Tcf-induced transcription such as cyclin D1 (data not shown). The absence of nuclear distribution of β -catenin could be correlated with the loss of expression of lymphoid enhancer factor-1 (LEF-1) and T-cell factor-1 (TCF-1) in the neoplastic cells. TCF-1 and

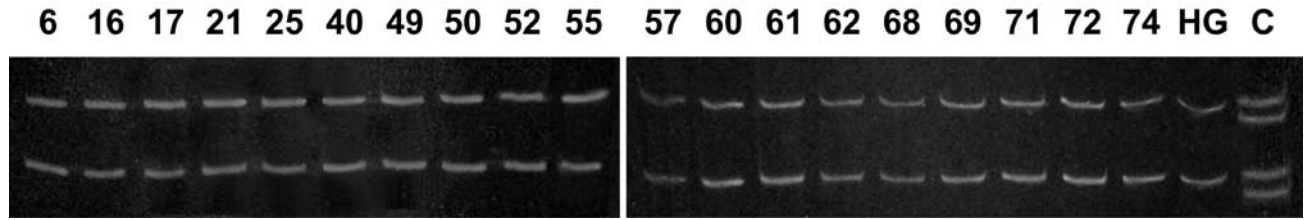


Figure 2 'Cold' single-strand conformation polymorphism analysis of β -catenin exon 3 in tissue of cutaneous lymphomas. No mobility shifted band was observed compared to human genomic DNA (HG). A mutant carries single base substitution was used to control the migration of aberrant bands compared with wild-type bands (C).

LEF-1 are expressed in thymocytes and mature T cells in mice and human, LEF-1 is also expressed in B-cell precursors, but not in mature B cells.^{23,24} When β -catenin is absent, the transcription factor TCF-1/LEF-1 acts in the nucleus as a transcriptional repressor interacting with the co-repressors (TLE/Groucho and/or CtBP) of the target gene promoters.²⁵

Actually, it has been previously reported that β -catenin nuclear retention is mediated by the LEF-1/TCF-1 transcription factors.²⁶ β -Catenin protein contains no recognizable nuclear localization signal (NLS) and it was proposed that β -catenin distribution is determined by its interaction with APC or by anchorage to LEF-1/TCF-1 in the nucleus or to cadherin at junctions. Dorfman and coworkers found that TCF-1 and LEF-1 in peripheral T-cell lymphomas are expressed in a coordinate manner, and that this expression correlates with that of the marker of Th1 T-cell activation. In contrast, cases of peripheral T-cell lymphomas expressing marker of Th2 T-cell activation failed to exhibit staining for LEF-1 and TCF-1.²⁷ Several studies have suggested that MF in large cell transformation, primary cutaneous anaplastic large cell lymphomas and Sézary Syndrome correlate with a specific Th2 cytokines pattern subtype and that, in contrast, primary cutaneous T-cell lymphomas CD30⁻, MF and angiocentric lymphoma express the Th1 differentiation-associated marker.²⁸⁻³⁰ It is interesting to note that our β -catenin positive cases are mainly Sézary Syndrome, anaplastic large cell lymphomas and MF in large-cell transformation, which are forms of T-cell lymphomas with aggressive behavior and Th2 profile. One could to explain the absence of β -catenin in the nucleus with the loss of LEF-1 and TCF-1 expression, a finding also reported in Th2 subtypes. It remains to be explained why β -catenin deregulation occurs preferentially in Th2 subtype of T-cell lymphomas.

Mutations of β -catenin exon 3 were not detected in any of the analyzed samples indicating that deregulation of β -catenin signaling may be caused by other mechanisms. These findings are consistent with similar studies in other human tumors, in which β -catenin accumulation was a frequent event but β -catenin mutations were infrequent.^{31,32} The role of many proteins that work in regulating β -catenin including Axin, Axin2, GSK-3 β ,

β -TrCP, PP2a and FRAT (frequently rearranged in advanced T-cell lymphomas) are currently being investigated. Preliminary data show wild-type Axin2 in most cases. However, we cannot exclude that there is an increase of β -catenin transcript and of the amount of the protein produced. This point is being tested on established and primary cell lines.

In summary, in our study, we found β -catenin expression in neoplastic tumor cells in 26 of 74 (35%) cases of primary cutaneous lymphoma, but unlike previous studies concerning other human tumors, we did not see any nuclear activity of the protein. The deregulation of β -catenin, that is a common occurrence in cutaneous lymphomas, appears to correlate with CD30 expression. CD30 is involved in the regulation of apoptosis and proliferation of activated lymphoid cells suggesting that the outcome of CD30 engagement may depend on the target cell, its state of differentiation, its transformation status and environmental cofactors.³³ The expression of this molecule can be induced on normal peripheral blood B- and T cells by mitogens and viruses, indicating that CD30 represents a differentiation antigen whose expression is associated with activation of lymphoid cells. β -Catenin activation has a positive role in the control of T-cell development and it is possible to speculate that the coexpression of β -catenin and CD30 might be a signal of the lost proliferative control by lymphoid cells. Even if there is no evidence of the usefulness of β -catenin accumulation as a clinical prognostic marker because it is present in both low- and high-grade lymphomas, it may be relevant within the MF group. This point will be proven after a longer follow-up.

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