

INVESTIGATIVE REPORT

Low Frequency of β -Catenin Gene Mutations in PilomatrixomaSEOG-JUN HA¹, JUNG-SOO KIM¹, EUN-JOO SEO², KYUNG-HO LEE¹, HYUN-JEONG LEE¹ and JIN-WOU KIM¹*Departments of ¹Dermatology and ²Clinical Pathology, College of Medicine, The Catholic University of Korea, Korea*

We investigated β -catenin and adenomatous polyposis coli (APC) gene abnormalities in human pilomatrixoma, in which a high incidence of β -catenin gene mutations has been reported. Nucleated tumour cells were microdissected from 20 paraffin-embedded pilomatrixomas. Exon 3 of the β -catenin gene was amplified using polymerase chain reaction and sequencing analysis was performed. Immunostaining for β -catenin and lymphoid-enhancer factor-1 was performed using the avidin-biotin-peroxidase method. Dinucleotide repeat markers D5S409 and D5S299 were used for polymerase chain reaction-based microsatellite analysis of the APC gene. The mutation cluster region of the APC gene was amplified using polymerase chain reaction and sequenced. Sequencing analysis revealed β -catenin gene mutations in 30%. All studied samples showed nuclear lymphoid-enhancer factor-1 and cytoplasmic/nuclear β -catenin expression. Loss of heterozygosity was observed in the APC gene, but no mutations in the mutation cluster region were found in seven tumours without β -catenin mutations. The frequency of β -catenin gene mutations was remarkably low, thus suggesting (i) the presence of mutations in other than exon 3 of the β -catenin gene, (ii) a possible role of APC gene abnormalities, or (iii) involvement of other components of the Wingless-type MMTV integration site family pathway. **Key words:** adenomatous polyposis coli gene; exon 3; loss of heterozygosity.

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Pilomatrixomas are benign skin tumours consisting of three types of cells: basophilic proliferating cells, shadow cells and transitional cells. They are reported as developing from activating mutations in β -catenin (1, 2), which is involved in signal transduction in the Wingless-type signalling pathway and suggested to have important functions in the morphogenesis of hair follicles and the formation of hair follicle-related tumours (1, 2). Transgenic mice with truncated β -catenin mutations and stable constitutive β -catenin expression develop hair follicle tumours histologically similar to pilomatrixomas in

humans (1). In human pilomatrixomas, a high incidence of β -catenin gene mutations was confirmed with nuclear expression of lymphoid-enhancer factor-1 (Lef-1) in proliferating pilomatrixoma cells (2). Cytoplasmic β -catenin is stabilized by the activating mutations in β -catenin, and this enables downstream transcriptional activation by members of the Lef family (2). β -catenin expression in pilomatrixoma has been reported in recent studies showing conflicting results on the pattern of expression (3, 4). In this study, we investigated β -catenin expression by immunohistochemistry and β -catenin gene mutations in pilomatrixomas. Since we found an unexpectedly low incidence of mutations in exon 3 of the β -catenin gene, a previously known mutational hotspot in pilomatrixoma, we also studied the possible role of adenomatous polyposis coli (APC) gene abnormalities in pilomatrixomas without β -catenin gene mutations.

MATERIAL AND METHODS

Tissues

This study included 20 formalin-fixed, paraffin-embedded pilomatrixomas in Koreans selected for the presence of enough basophilic cells for DNA extraction. The patients had no personal history of benign or malignant gastrointestinal tumours. The history for familial adenomatous polyposis was negative in all patients. Patient 7 had a family history of colon and stomach cancer in her first-degree relatives, father and uncle. Patient 10 had a brother who died from colon cancer.

Microdissection and DNA extraction

Basophilic nucleated tumour cells in the matrix cell zone were microdissected under light microscopic examination from haematoxylin and eosin-stained sections without contamination of normal cells. As normal controls, infiltrating inflammatory lymphocytes were microdissected from the same histologic specimens. Approximately 400 cells were microdissected from 6- μ m thin sections of each sample. Microdissection was performed as described previously with a simple and practical device, SPEMII (Simple, Precise and Economical Microdissection Device; BM Korea Co., Seoul, Korea) (5). The tissue affixed to the needle tip was resuspended in 10 μ l of DNA extraction buffer containing 0.05 M Tris-hydrochloric acid (HCl), 10 mM ethylenediamine tetra-acetic acid (EDTA), 1% Tween 20 (Sigma, St. Louis, MO, USA), and 0.1 mg/ml proteinase K and incubated overnight at 52°C. The mixture was boiled for 10 min to inactivate proteinase K and 1 μ l of this solution was used as a DNA template for the polymerase chain reaction (PCR).

Sequencing analysis of exon 3 of the β -catenin gene

Exon 3 of β -catenin was amplified from tissue genomic DNAs using PCR and specific oligonucleotide primers

as described (4). PCR products were gel-purified (Qiagen gel-extraction kit, Qiagen, Chatsworth, CA, USA) and sequenced using an ABI sequencing kit on an ABI 377 Prism sequencer (Perkin-Elmer, Norwalk, CT, USA). All sequencing reactions were performed in duplicate.

Immunohistochemistry

Immunostaining for β -catenin and Lef-1 was performed on 6- μ m formalin-fixed, paraffin-embedded tissue sections by the avidin-biotin-peroxidase method. Sections were deparaffinized and rehydrated. Mouse anti-human β -catenin monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) diluted 1:200 and goat anti-Lef-1 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:100 were applied overnight and for 1 h, respectively. The primary antibody was omitted in negative controls.

Screening of loss of heterozygosity in the APC gene

Dinucleotide repeat markers D5S409 and D5S299 were used for PCR-based microsatellite analysis of the APC gene. The sequences of the primers were forward (F) 5' GGGATGAAGTGTGGATAAAC 3' and reverse (R) 5' TAGGATGGCAGTGCTCTTAG for D5S409 and F 5' GCTATTCTCTCAGGATCTTG 3' and R 5' GTAAGCCAGGACAAGATGACA for D5S299. PCR was performed using 1 μ Ci of 32 P-labelled dCTP incorporated into 10 μ l of reaction mixture. PCR reactions were carried out in a Perkin-Elmer Thermocycler 2400 for 32 cycles under the following conditions in order: 95°C for 50 sec, 50–58°C for 50 sec and 72°C for 60 sec. The PCR products were denatured in 95% formamide and electrophoresed on denaturing polyacrylamide gels. The products were visualized by autoradiography. Loss of heterozygosity (LOH) was defined by a visible change in allele:allele ratio in tumours compared with matching normal tissue (7). Microsatellite instability was defined by the presence of bands in tumour DNA that were not visible in corresponding normal DNA (7).

PCR and sequencing analysis of the mutation cluster region (MCR) of the APC gene

The MCR region between codon 1260 and 1547 was divided into two segments, each separately amplified using PCR (8). The primer pairs have been described previously (9). PCR was performed with 38 cycles for 0.5 min at 95°C, 2 min at 51°C and 2 min at 70°C, as described (10). PCR products were used as a template for each DNA sequencing reaction and sequenced using the ABI sequencing kit on an ABI 377 Prism sequencer (Perkin-Elmer). All sequencing reactions were performed in duplicate.

RESULTS

β -catenin gene mutations

Sequencing analysis revealed 6 mutations in β -catenin in 6 (30%) of 20 pilomatricomas (Table I). Four samples had mutations in codon 37 (S37Y, S37C and two S37F) and two samples in codon 33 (S33C and S33F).

Immunohistochemistry

An accumulation of β -catenin protein was found in cytoplasm and nuclei of basaloid cells in 19 (100%) of 19 studied samples (Fig. 1a and Table I). Transitional cells showed β -catenin expression mainly in cytoplasm

and cytoplasmic membrane, and a few cells had nuclear β -catenin expression. Nuclear Lef-1 expression was observed in basaloid cells in 10 (100%) of 10 pilomatricomas studied (Table I). Lef-1 expression was localized to the nucleated matrix cell zone (Fig. 1b).

Loss of heterozygosity of the APC gene

LOH analysis was performed in 10 tumours (patient nos. 1 to 10). LOH was observed in 3 (75%) out of 4 informative samples and in 2 (50%) out of 4 informative samples from microsatellite markers D5S299 and D5S409 analysis, respectively (Table I and Fig. 2).

APC gene mutations

Sequencing analysis could be performed in 7 pilomatricomas that did not possess β -catenin mutations (patient nos. 1 to 6 and 8). No mutations were found in the MCR region (between codons 1260 and 1547 in exon 15 of the APC gene) in any of the 7 tumours.

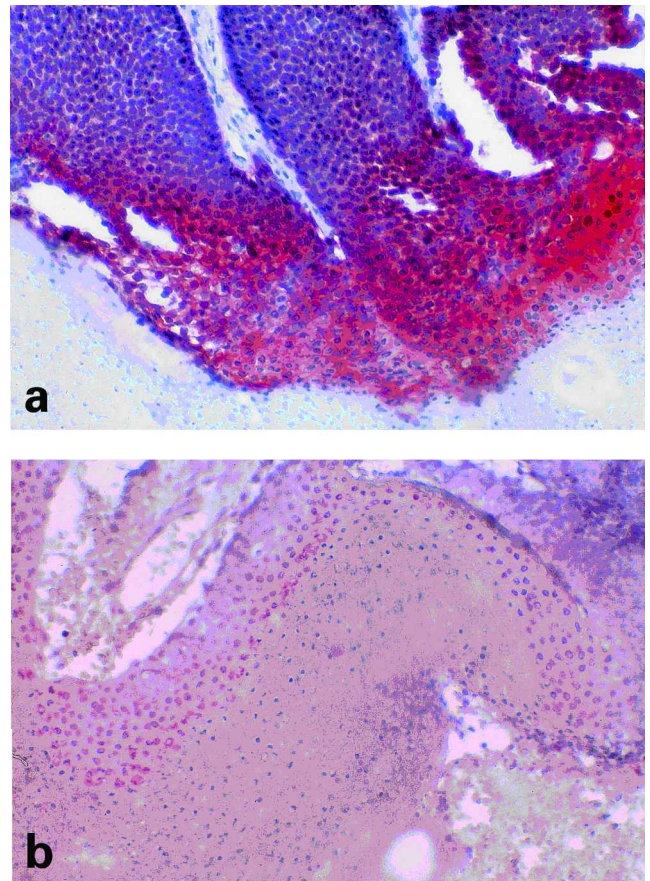


Fig. 1. Immunohistochemical analysis of β -catenin expression shows nuclear and cytoplasmic staining in basaloid cells of pilomatricoma, and cytoplasmic and membranous staining with scattered nuclear staining in transitional cells (original magnification $\times 100$) (a). Nuclear staining of Lef-1 protein is observed in nucleated cells of pilomatricoma (original magnification $\times 100$) (b).

Table I. Summary of β -catenin mutations and immunohistochemical results

No.	Sex/age	Site	β -catenin mutations	Lef-1 staining	β -catenin staining	LOH at D5S299	LOH at D5S409
1	F/8	Face	-	N	N,C	+	NI
2	M/1	Face	-	N	N,C	+	-
3	F/27	Shoulder	-	N	N,C	+	NI
4	M/7	Face	-	N	N,C	NI	+
5	F/16	Face	-	N	N,C	NI	NI
6	F/19	Arm	-	N	N,C	NI	+
7	F/12	Face	-	N	N,C	-	NI
8	F/23	Arm	-	N	N,C	NI	NI
9	M/20	Shoulder	-	N	N,C	NI	NI
10	F/64	Face	S37Y (TCT→TAT)	N	N,C	-	-
11	F/23	Face	-	ND	N,C	ND	ND
12	F/16	Neck	S37F (TCT→TTT)	ND	N,C	ND	ND
13	F/29	Arm	S37F (TCT→TTT)	ND	N,C	ND	ND
14	F/19	Forearm	-	ND	N,C	ND	ND
15	M/8	Neck	-	ND	ND	ND	ND
16	M/8	Face	-	ND	N,C	ND	ND
17	M/9	Neck	S37C (TCT→TGT)	ND	N,C	ND	ND
18	M/27	Forearm	S33C (TCT→TGT)	ND	N,C	ND	ND
19	F/9	Neck	S33F (TCT→TTT)	ND	N,C	ND	ND
20	F/11	Face	-	ND	N,C	ND	ND

C= cytoplasmic staining; Lef-1= lymphoid enhancer factor-1; LOH= loss of heterozygosity; N= nuclear staining; ND= not done; NI= not informative.

DISCUSSION

It has been claimed that β -catenin mutations play a causal role in the development of pilomatricomas (1, 2). In a recent study, 75% of pilomatricomas had β -catenin mutations involving the amino-terminal segment of the protein; this percentage was greater than in all other tumours examined (2). The authors observed missense mutations in 12 out of 16 samples affecting the

amino-terminal segment, normally involved in phosphorylation-dependent, ubiquitin-mediated degradation of the protein. It has been suggested that these mutations cause stabilization or constitutive activation of β -catenin. β -catenin mutations were not observed in the remaining 25% of the pilomatricomas examined. As evidenced also by strong nuclear expression of Lef-1 in the proliferating cells of the tumour, β -catenin/Lef misregulation has been suggested as being the major cause of hair matrix tumorigenesis in mice and humans (1, 2). The development of pilomatricomas in transgenic mice expressing the amino-terminally truncated, activating form of β -catenin implies that this protein has a causal role (1). In another recent study (11), β -catenin gene mutations were found in 7 of 7 (100%) pilomatricomas with basophilic nucleated cells.

In our study, β -catenin gene mutations were remarkably low (30%) also when compared to the high incidence of the mutations previously reported by Chan et al. (2). Six missense mutations were found in the N-terminal segment of β -catenin in this study. These mutations have been described in pilomatricomas (2, 11) and other human carcinomas and cell lines (6, 12–15). In our study, mutations were found only in codons 33 and 37, and none were found in other previously reported residues including codons 32, 34 and 31. These serine residues are known to be essential for glycogen synthase kinase-3 β -dependent phosphorylation (16–18). We could exclude contamination of normal cells by microdissecting each basophilic nucleated tumour cell under light microscopic examination, a previously verified method (5). The lower frequency of β -catenin gene mutation in our study might not be entirely representative given that we have analysed

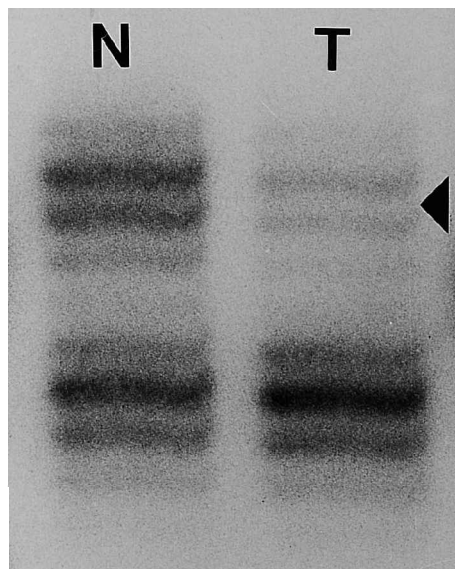


Fig. 2. Pilomatricoma from patient no. 2 shows loss of heterozygosity at adenomatous polyposis coli microsatellite locus D5S409 (N= normal, T= tumour). The arrowhead indicates the allele loss with a clear reduction in signal intensity in the tumour as compared with the corresponding normal alleles.

only 20 tumours. Another possibility is that the mutations could be present in other exons of the gene that have never been screened (1, 2, 4, 6, 11). A racial difference might be suggested in the development of β -catenin or APC-related tumours.

The absence of β -catenin mutations in 70% of our cases may also suggest APC inactivation, or involvement of other components of the wingless type pathway, because all of the studied pilomatricomas revealed expression of nuclear Lef-1 and nuclear and cytoplasmic β -catenin, and these immunohistochemical results imply the abnormalities in wingless type pathway resulting in stabilization or constitutive expression of β -catenin. We studied the possible involvement of APC gene abnormalities in pilomatricomas without β -catenin gene mutations. MCR of the APC gene within the 5' end of exon 15, between nucleotide 3000 and 4800 (codons 1000 to 1600), represents approximately 60% of reported somatic mutations (9). This region encodes β -catenin binding and degradation sites, and the location of MCR suggests the importance of APC-mediated β -catenin gene degradation. In this study, we studied a major portion of this MCR (codons 1260 to 1547). In some of the studied cases, we observed LOH of the APC gene; however, we could not detect APC gene mutations in the MCR region in exon 15. The presence of LOH does not directly imply the presence of mutations, or APC gene mutations in pilomatricomas might be involved outside the MCR we have studied.

Recent studies demonstrate different results on the expression pattern of β -catenin (3, 4, 11). Moreno-Bueno et al. (4) reported the same pattern of β -catenin expression as in our study. Kajino et al. (11) also reported that basophilic cells of seven cases of pilomatricoma with β -catenin gene mutation showed nuclear and cytoplasmic immunostaining of β -catenin. Park et al. (3) showed only membranous or cytoplasmic staining of β -catenin with no nuclear immunoreactivity. Our results confirm nuclear and cytoplasmic expression of β -catenin in basophilic cells and some transitional cells, and these expression patterns imply involvement of β -catenin stabilization in development of pilomatricoma.

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