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Gene Mutations in Lymphoproliferative Disorders of T and NK/T Cell Phenotypes Developing in Renal Transplant Patients

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SUMMARY: Post-transplantation lymphoproliferative disorder (PT-LPD) is characterized by lymphoid proliferation after organ or bone marrow transplantation. In Western countries, most cases of PT-LPD are B-cell–derived and Epstein-Barr virus–associated, in which alterations of *c-myc*, *p53,* and N-*ras* genes might play a role in disease progression. In Japan, PT-LPD of T- and NK/T-cell types are not uncommon in renal transplant patients. Mutations of *p53* (exons 4 through 8), K-*ras* (exons 1 and 2), *c-kit* (exons 11 and 17), and *β-catenin* genes (exon 3) in 12 cases of these diseases were analyzed by PCR single strand conformation polymorphism and then by direct sequencing. *p53* gene mutations were detected in 5 of 5 cases of peripheral T-cell lymphoma, 3 (60%) of 5 cases of adult T-cell leukemia/lymphoma, and 1 of 2 cases of NK/T cell lymphoma. Twenty-five percent of T and NK/T cell lymphomas showed K-*ras* mutations. Mutations of *c-kit* and *β-catenin* genes were found in 33% of cases. Among a total of 42 substitution mutations, 40 were transitions with involvement of CpG sites in 20 to 30% of cases. Most cases had at least one mutation that changed an amino acid, which might have provided the selection pressure for expansion. These findings suggested that *p53* gene mutations might play a central role in development of peripheral T-cell lymphoma including adult T-cell leukemia/lymphoma in renal transplant patients. (*Lab Invest 2002, 82:257–264*).

Post-transplantation lymphoproliferative disorders (PT-LPD) are characterized by lymphoid proliferation occurring after organ or bone marrow transplantation (Nalesnik, 1998; Pen et al, 1969). Most are B-cell–derived, Epstein-Barr virus–associated, and show poor prognosis. Spontaneous regression of the lesions is occasionally seen when immunosuppressive agents are reduced (Nalesnik, 1998; Starzl et al, 1984). PT-LPD of B-cell type are categorized into four groups: reactive plasmacytic hyperplasia, polymorphic PT-LPD, monomorphic PT-LPD, and others including plasmacytoma-like PT-LPD (Harris et al, 1997). Clinical outcomes of plasmacytic hyperplasia and polymorphic PT-LPD are significantly better than those of monomorphic PT-LPD (Chadburn et al, 1998). Molecular genetic studies of B-cell–type PT-LPD have suggested that alterations of *c-myc*, *p53,* and N-*ras* genes might play a role in progression of polymorphic PT-LPD to monomorphic PT-LPD (Knowles et al, 1995).

Most PT-LPD cases are B-cell–derived in Western countries (Dockrell et al, 1998; Leblond et al, 1995), whereas a recent study revealed that PT-LPD of T- and NK/T-cell type are not uncommon in Japan (Hoshida et al, 2001). Characteristically, half of the T-cell cases are adult T-cell leukemia/lymphoma (ATL) with proven human T-lymphotropic virus type I (HTLV-1) genome in the tumor tissues and seropositivity for HTLV-1. This high frequency of ATL might be caused by transmission of HTLV-1 via blood transfusion during hemodialysis, because Japanese patients usually receive various periods of hemodialysis before renal transplantation (Hoshida et al, 2001). Little information is available regarding the molecular genetic characteristics of PT-LPD of T-cell and NK/T-cell type. In the present study, mutations of *p53*, *c-kit*, K-*ras,* and *β-catenin* genes in PT-LPD of T-cell and NK/T-cell type in renal transplant patients were analyzed by PCR single strand conformation polymorphism (PCR-SSCP) and then by direct sequencing.

p53 is a well-known tumor suppressor gene that causes cells with damaged DNA to arrest at the G1 phase of the cell cycle or stimulates the expression of the *bax* gene encoding a protein that promotes apoptosis (Levine et al, 1991). *p53* gene mutations have been detected in a wide variety of human cancers, mainly in exons 5 through 8 (Hollstein et al, 1991). A high incidence of malignant lymphoma in p53 knockout mice has been reported (Donehower et al, 1992), suggesting an important role of *p53* gene mutations in lymphomagenesis.

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The *c-kit* proto-oncogene encodes a receptor tyrosine kinase, which plays a crucial role in proliferation and differentiation of hematopoietic stem cells, mast cells, and interstitial cells of Cajal. Development of acute leukemia or malignant lymphoma was reported in transgenic mice expressing KIT ^{V814} (Asp 814→VAL) (Kitayama et al, 1996). Recently, we reported the high frequency of *c-kit* gene mutations in sinonasal NK/T-cell lymphoma (Hongyo et al, 2000).

The K-*ras* gene encodes 21-KD ras protein, a GTP- and GDP-binding protein, which plays a role in signal transduction through transmembrane signaling systems. Mutations of the *ras* gene, mostly involving codon 12, stabilize ras protein in its GTP-bound active form and cause constitutive signal transduction that might result in malignant transformation of the mutated cells (Barbacid, 1987). Indeed, K-*ras* mutations are frequently observed in pancreatic, colorectal, and lung adenocarcinoma (Brentnall et al, 1995; Jackson et al, 1997).

The *β-catenin* gene is a homolog of the *Drosophila Armadillo* gene and plays an important role in cell-cell adhesion and signal transduction. *β-catenin* links the cytoplasmic domain of E-cadherin to α-catenin and anchors this complex to the actin cytoskeleton (Huber et al, 1996). *β-catenin* forms heterodimers with DNA-binding proteins, which function as downstream transcriptional activators in the Wnt signaling pathway (Behrens et al, 1996). Without signaling stimulus, β-catenin is degraded in association with adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) (Aberle et al, 1997). Therefore, either mutations in *APC* or activating mutations at crucial regulatory sites in exon 3 of the *β-catenin* gene result in accumulation of β-catenin protein in the cytoplasm (Morin et al, 1997). Then, *β-catenin* complex leads to overexpression of c-myc, which is related to cell proliferation and differentiation, thus leading to tumorigenesis (He et al, 1998). Indeed, mutations of the *β-catenin* gene have occasionally been found in several kinds of cancers (Fukuchi et al, 1998; Hsu et al, 2000; Sparks et al, 1998; Voeller et al, 1998). Although there have been no reports describing mutations of the *β-catenin* gene in malignant lymphomas, Knowles et al (1995) reported a crucial role of overexpression of the *c-myc* gene in progression from polymorphic to monomorphic PT-LPD of B-cell type. Therefore, we examined *β-catenin* gene mutations in T- and NK/T-cell PT-LPD.

Results

Mutations of **p53***,* **c-kit***,* **K-ras***, and* **β-catenin** *Genes*

By direct sequencing of SSCP products, 24 single-nucleotide substitution mutations in the *p53* gene were detected in 9 of 12 cases with T- and NK/T-cell lymphoma (Table 1). *p53* gene mutations were found in all cases of peripheral T-cell lymphoma, 60% of ATL, and 50% of NK/T-cell lymphomas (Fig. 1). In peripheral T-cell lymphoma, not specified, three cases had three mutations and one case had five mutations. Mutations were located in exon 5 (12 of 24 cases, 50%) and exon 4 (6 of 24 cases, 25%). Sixteen mutations were missense mutations leading to amino acid substitutions, and eight were silent mutations resulting in no amino acid changes. G:C to A:T transitions were the predominant mutations (16 mutations), followed by A:T to G:C transitions (6 mutations). Two were transversion mutations. Three mutations found at codons 175 (Case 8), 196, and 273 (Case 2) with CpG sites were reported previously as hot spots (Donehower et al, 1992).

In the *c-kit* gene, seven single-nucleotide substitution mutations were detected in 4 (33.3%) of 12 cases (Table 2). These were located at exon 11 (six mutations, 85.7%) and exon 17 (one mutation, 14.3%). One mutation was detected in two cases and two and three mutations were detected in one case each. Four mutations were missense and three were silent. Six mutations were G:C to A:T transitions and one was an A:T to G:C transition.

Four mutations were detected in the K-*ras* gene in three cases: all were G to A transitions. Three of the four were missense mutations. There were three mutations in the *β-catenin* gene, and all were G to A transitions.

Discussion

Most cases of PT-LPD in Western countries are B-cell–derived, and information regarding the genetic mechanism in B-cell PT-LPD has been accumulating. Mutation analysis of the *p53* gene has been confined principally to exons 5 through 8 because 90% of the mutations in human tumors occurred in this region (Hollstein et al, 1991). Monomorphic PT-LPD might have a genetic alteration including *p53*, whereas polymorphic PT-LPD does not, suggesting that *p53* mutations are important for progression of B-cell PT-LPD (Knowles et al, 1995). *p53* gene mutations are also frequent in AIDS-related B-cell lymphoma (Gaidano et al, 1998). These findings suggested that *p53* mutations are common findings in lymphomas developing in immunocompromised hosts, which are frequently Epstein-Barr virus–associated (Edwards and Raab-Traub, 1994).

All of the cases in our study that had peripheral T-cell lymphoma other than ATL showed *p53* mutations, which is a striking contrast to that in T-cell lymphoma developed in immunocompetent individuals (8.3%; $p < 0.01$; Fisher's exact test) (Gaidano et al, 1998). Imamura et al (1994) reported that approximately 30% to 50% of ATL showed *p53* mutations and suggested that *p53* gene mutations play a role in the late stage of tumorigenesis; that is, evolution from a polyclonally or oligoclonally expanded population of cells to monoclonal expansion might result from *p53* gene mutations. The frequency of *p53* mutations in the patients in our study who had ATL was 60%, which was similar to that in ATL in immunocompetent individuals. A case of nasal NK/T-cell lymphoma showed *p53* mutations. We recently reported the relatively high frequency of *p53* mutations in cases of nasal NK/T-cell lymphoma (Li et al, 2000).

Table 1. ***p53*** **Gene Mutation in Post-Transplantation Lymphoproliferative Diseases with T- and NK/T-Cell Phenotype**

No.	Age/Sex	Histology	Primary site	Stage	*p53* Exon/codon	*p53* Nucleotide	*p53* Amino acid
1	32/M	PT	Liver	III	5/156	CGC → GGC	Arg → Gly
2	40/M	PT	Small intestine	IV	4/107	TAC → TAT	Tyr → Tyr
					6/196	CGA → TGA	Arg → stop
					8/273	CGT → TGT	Arg → Cys
3	37/M	PT	Cervical LN	III	5/152	CCG → CCA	Pro → Pro
					6/194	CTT → TTT	Leu → Phe
					7/247	AAC → GAC	Asn → Asp
4	44/M	PT	Muscle	IV	4/56	GAA → GAG	Glu → Glu
					4/107	TAC → TAT	Tyr → Tyr
					5/176	TGC → TAC	Cys → Tyr
					5/177	CCC → TCC	Pro → Ser
					6/215	AGT → AGC	Ser → Ser
5	34/M	PT	Liver	II	4/101	AAA → AAT	Lys → Asn
					5/142	CCT → CCC	Pro → Pro
					5/179	CAT → CGT	His → Arg
6	32/M	ATL	Undetermined	IV	–	–	–
7	32/M	ATL	Inguinal LN	III	4/84	GCC → GCT	Ala → Ala
8	43/M	ATL	Skin	I	5/175	CGC → CAC	Arg → His
					8/269	AGC → AAC	Ser → Asn
9	56/M	ATL	Spleen	IV	4/84	GCC → GTC	Ala → Val
					5/179	CAT → CGT	His → Arg
10	47/F	ATL	Undetermined	I	–	–	–
11	27/M	Nasal NKTCL	Nasal cavity	I	5/140	ACC → ACT	Thr → Thr
					4/144	CAG → CGG	Gln → Arg
					5/166	TCA → TTA	Ser → Leu
					5/171	GAG → AAG	Glu → Lys
12	35/M	Nodal NKTCL	Cervical LN	III	–	–	–

PT, Peripheral T-cell lymphoma, not specified; ATL, Adult T-cell leukemia/lymphoma; NKTCL, NK/T-cell lymphoma.

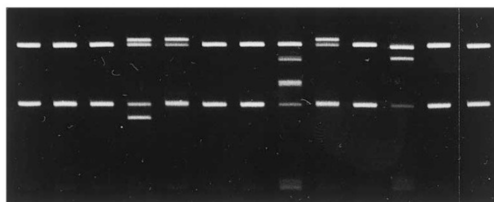

Figure 1.
"Cold" single strand conformation polymorphism analysis of *p53* exon 5-a in the tissue of lymphoma developing in renal transplant patients. Aberrant migration bands compared with wild-type (*WT*) were seen in Cases 4, 5, 8, 9, and 11.

Transitions at CpG dinucleotide sites were the predominant substitutions in non-Hodgkin's lymphoma (NHL) cases, and G:C to T:A transversion was uncommon (Hollstein et al, 1991). In this series of PT-LPD of T- or NK/T-cell type, 40 of 42 substitution mutations in *p53*, *c-kit*, *β-catenin,* and K-*ras* genes were transitions: CpG sites were involved in 20% to 30% of cases. The predominance of transition mutations in our series suggested that some "endogenous" mutagens act in lymphomagenesis. Most cases in the current series had at least one mutation that changed an amino acid, which might have provided the selection pressure for expansion. The predominant sites for *p53* mutation were not presented in previous reports on NHL (Adamson et al, 1995; Ichikawa et al, 1992). In our series of PT-LPD of T- and NK/T-cell type, exons 4 and/or 5 of the *p53* gene were involved in 75% of cases. Codon 179 in exon 5 was involved in two cases, but no distinct mutational "hot spots" in the *p53* gene were found. Mutation at codon 825 of the *c-kit* gene found in one case of ATL was reported to be a hot spot in sinonasal NK/T-cell lymphoma (Hongyo et al, 2000).

Ras activation represents a widespread oncogenic event, and is found in 10% to 15% of human neoplasms (Barbacid, 1987). *Ras* (H, K, and N-*ras*) mutations were reported to be absent in lymphomas of immunocompetent individuals (Neri et al, 1988) and infrequent in AIDS-related lymphoma (3.7% to 11.1%) (Ballerini et al, 1993). Thyroid lymphoma, a lymphoma that develops in autoimmune thyroiditis, showed relatively frequent mutations in the K-*ras* gene (25% of cases) (Takakuwa et al, 2000). K-*ras* mutation was reported in cancers in renal transplant patients who received immunosuppressive agents (Arakawa et al, 1990). Thus, we examined K-*ras* mutations in T- and NK/T-cell lymphomas in patients receiving renal transplantation. Twenty-five percent of T- and NK/T- cell lymphomas in our cases showed K-*ras* mutations, a proportion that was similar to that in thyroid lympho-

Table 2. **K-*ras*, *c-kit*, and *β-catenin* Gene Mutation in Post-Transplantation Lymphoproliferative Disorders with T- and NK/T-Cell Phenotype in Japan**

			K-*ras*			*c-kit*			*β-catenin*		
No.	Age/Sex	Histology	Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid
1	32/M	PT	1/13	GGC → GAC	Gly → Asp	–	–	–	–	–	–
2	40/M	PT	–	–	–	–	–	–	3/20	GCG → GCA	Ala → Ala
3	37/M	PT	–	–	–	11/585	CCC → CCT	Pro → Pro	–	–	–
4	44/M	PT	–	–	–	–	–	–	–	–	–
5	34/M	PT	–	–	–	11/566	AAC → AAT	Asn → Asn	3/57	GTG → ATG	Val → Met
						11/585	CCC → CCT	Pro → Pro	–	–	–
6	32/M	ATL	–	–	–	11/559	GTT → AAT	Val → Ile	–	–	–
						11/561	GAG → GAA	Glu → Glu	–	–	–
						17/825	GTT → GCT	Val → Ala	–	–	–
7	32/M	ATL	–	–	–	–	–	–	–	–	–
8	43/M	ATL	1/15	GGC → GAC	Gly → Asp	11/577	CCT → TCT	Pro → Ser	–	–	–
9	56/M	ATL	–	–	–	–	–	–	–	–	–
10	47/F	ATL	–	–	–	–	–	–	–	–	–
11	27/M	Nasal NKTCL	1/13	GGC → GAC	Gly → Asp	–	–	–	3/56	GAT → AAT	Asp → Asn
			1/22	CAG → CAA	Gln → Gln	–	–	–	–	–	–
12	35/M	Nodal NKTCL	–	–	–	–	–	–	–	–	–

PT, Peripheral T-cell lymphoma, not specified; ATL, Adult T-cell leukemia/lymphoma; NKTCL, NK/T-cell lymphoma.

mas. Knowles et al (1995) reported that N-*ras* was the only *ras* gene altered and that 75% of monomorphic PT-LPD of B-cell type had N-*ras* gene mutations.

Seven mutations in the *c-kit* gene were found in our cases: four were missense and three were silent. Mutations in the β-*catenin* gene were occasionally seen in cancer of the colon (16%) (Sparks et al, 1998), endometrium (13.2%) (Fukuchi et al, 1998), prostate (4.8%) (Voeller et al, 1998), and liver (13.1%) (Hsu et al, 2000). Mutations usually involved the serine/threonine residues of the GSK-3β region of β-*catenin*. In our cases, three mutations were detected, but none of the mutations involved serine/threonine residues.

In conclusion, the present study suggested that mutations of the *p53* gene are key phenomena for development of peripheral T-cell lymphoma including ATL in renal transplantation patients.

Patients and Methods

Patient Characteristics

Twelve patients in whom T-cell and NK/T-cell lymphoproliferative disorders developed after renal transplantation were selected for the present study: two patients through a review of the records of renal transplantation during the period from 1970 to 1995 at three hospitals in Japan (Osaka University Hospital, Hyogo Prefectural Nishinomiya Hospital, and Kinki University Hospital), six patients through a review of Japanese journals, three patients through the "Annual of Pathological Autopsy Cases in Japan (1977–1993)," and one patient through consultation case files of our department. Histologic specimens obtained by biopsy or autopsy were fixed in 10% formalin and routinely processed for paraffin-embedding. Histologic sections 4 μm thick were stained with hematoxylin and eosin and immunoperoxidase (ABC method) as previously described (Hsu et al, 1981). Cases were classified according to the revised European American Lymphoma (REAL) classification (Harris et al, 1994), and five patients (Cases 1 through 5) were categorized as having peripheral T-cell lymphoma, not specified (CD20^{-}, CD56^{-}, CD3ϵ^{+}, CD45RO^{+}, CD4^{+}, CD8^{+}, and/or TIA-1^{+}). Tumor cells in four of these five cases were positive for β–F1^{+}, which is known to be reactive with TCRβ chain. Another five cases (Cases 6 through 10) were categorized as ATL with proven HTLV-1 genome in the tumor tissue and seropositivity for the virus. Four of these patients (Cases 7 through 10) were born in ATL-endemic area in Japan, and presented with skin eruption typical for ATL in two patients. The remaining patient (Case 6) showed a leukemic blood picture with ATL cells occupying 48% of peripheral leukocytes. Tumor cells in these five cases were similar to those in cases with peripheral T-cell lymphomas, except for CD 56 positive in one of the ATL cases. The remaining two cases (Cases 11 and 12) with a polymorphous pattern of proliferation consisting of medium to large cells with irregular nuclei and macrophages were categorized as NK/T-cell lymphoma because the proliferating cells were CD16^{+}, CD56^{+}, CD3ϵ^{+}, CD4^{-}, CD8$^{+\text{ or }-}$, CD20^{-}, TIA-1^{+}. One of NK/T-cell lymphomas arose in the nasal cavity and one arose in the lymph node. Rearrangements of the T-cell receptor β- and γ-chain genes were examined in one case of nasal NK/T-cell lymphoma, showing the germline configuration. This case was negative for β–F1 by immunohistochemistry. Details of clinical and histologic information of these cases were reported previously (Hoshida et al, 2001). Briefly, the age of patients at the time of transplantation ranged from 17 to 47 (median, 30) years and that at diagnosis of PT-LPD ranged from 27 to 56 (median, 36) years. The interval between renal transplantation and tumor development ranged from 1 to 264 (median, 64) months. All patients, except one, were male. The primary site of PT-LPD was extranodal in seven cases, nodal in three, and undetermined in two. The Ann Arbor staging scheme was applied in all cases on the basis of physical examination records, surgical notes, and pathologic examination of the specimens: three patients had Stage I, one Stage II, four Stage III, and four Stage IV disease.

Detection of p53*, K*-ras*,* c-kit*, and* β-catenin *Gene Mutations*

DNA for PCR amplification was extracted using chelating resin (Sigma Chemical, St. Louis, Missouri). Briefly, three paraffin sections 10 μm thick were cut, transferred into sterile distilled water containing 20% chelating resin, and boiled for 15 minutes. After centrifugation, the supernatant was transferred to a sterile 500-μl tube and stored at $-20°$ C. The PCR primer pairs for amplification of exon 4 through 8 of the *p53* gene, exon 1 and 2 of K-*ras* gene, exon 11 and 17 of *c-kit* gene, and exon 3 of β-*catenin* gene are listed in Table 3. DNA amplification and nonradioactive SSCP (cold SSCP) analysis were carried out to detect mutations, as described previously (Hongyo et al, 1993). Briefly, hot start PCR for DNA amplification was performed as follows: 45 cycles of denaturation at 95° C for 30 seconds; annealing at each temperature shown in Table 3 for 30 seconds; extension at 72° C for 1 minute, and final extension at 72° C for 7 minutes. Paraffin blocks containing no sample were cut and used as negative controls throughout the procedures. The amplified products were subjected to electrophoresis in 1.5% agarose gels containing 2 μg/ml ethidium bromide in TBE buffer (90 mM Tris, 92 mM boric acid, 2.5 mM EDTA). After electrophoresis, the gels were examined on an ultraviolet transilluminator.

Nonradioactive SSCP was performed as follows. Reaction mixtures consisting of 20 ml containing 5 μl of PCR product (20–200 ng of DNA), 0.2 μl of 1 M methylmercury hydroxide, 3 μl of loading buffer (15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol), and TBE buffer were heated to 90° C for 4 minutes, put on ice, and then subjected to electrophoresis in 18% polyacrylamide TBE gels at 300 volts, while maintaining the temperatures at those shown in Table 3. The bands that migrated apart from that of wild-type were determined as SSCP-positive. The bands

Table 3. Oligonucleotide Primers Used for PCR

Gene	Sequences		Annealing temperature for DNA amplification	Maintaining temperature for PCR-SSCP
p53				
Exon 4a	5′-TTTTCACCCATCTACAGTCC-3′	Upstream	58° C	20° C
	5′-CAAGAAGCCCAGACGGAAAC-3′	Downstream		
b	5′-CCTGGCCCCTGTCATCTTCT-3′	Upstream	58° C	20° C
	5′-AAGAAATGCAGGGGGATACG-3′	Downstream		
Exon 5a	5′-TCTGTCTCCTTCCTCTTCCTA-3′	Upstream	57° C	35° C
	5′-CATGTGCTGTGACTGCTTGT-3′	Downstream		
b	5′-TGTGCAGCTGTGGGTTGATTC-3′	Upstream	62° C	25° C
	5′-CAGCCCTGTCGTCTCTCCAG-3′	Downstream		
Exon 6	5′-TTGCTCTTAGGTCTGGCCCCT-3′	Upstream	64° C	35° C
	5′-TAGGGAGGTCAAATAAGCAG-3′	Downstream		
Exon 7	5′-TAGGTTGGCTCTGACTGTACC-3′	Upstream	60° C	25° C
	5′-TGACCTGGAGTCTTCAGTGT-3′	Downstream		
Exon 8	5′-TCTTGCTTCTCTTTTCCTAT-3′	Upstream	56° C	10° C
	5′-CGCTTCTTGTCCTGCTTGCT-3′	Downstream		
K-*ras*				
Exon 1	5′-CATGTTCTAATATAGTCACA-3′	Upstream	48° C	25° C
	5′-CTCTATTGTTGGATCATATTCGTCC-3′	Downstream		
Exon 2	5′-ACTGTGTTTCTCCCTTCTCA-3′	Upstream	48° C	5° C
	5′-CACAAAGAAAGCCCTCCCCA-3′	Downstream		
c-kit				
Exon 11	5′-GATCTATTTTTCCCTTTCTC-3′	Upstream	56° C	20° C
	5′-AGCCCCTGTTTCATACTGAC-3′	Downstream		
Exon 17	5′-CATGGTCGGATCACAAAGAT-3′	Upstream	54° C	15° C
	5′-ATTATGAAAGTCACGGAAAC-3′	Downstream		
β-catenin				
Exon 3	5′-GCTGATTTGATGGAGTTGGA-3′	Upstream	56° C	25° C
	5′-GCTACTTGTTCTTGAGTGAA-3′	Downstream		

determined as possibly mutated by SSCP were extracted from the gels and amplified by 25 cycles of PCR to enrich the mutated alleles.

Sequencing was performed by the dideoxy chain termination method using a Big Dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, California). Sequencing primers were the same as those used for PCR. Sequencing was performed according to the manufacturer's protocol: 30 cycles of denaturation (95° C for 30 seconds), annealing (54° C for 30 seconds), and extension (72° C for 3 minutes), and then cooling at 20° C after the final cycle. After ethanol precipitation, the samples were analyzed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). PCR-SSCP analyses and sequencing of mutated bands were repeated three times for each sample to exclude the possibility of contamination and PCR fidelity artefacts.

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