

Frequent β -Catenin Abnormalities in Bone and Soft-tissue Tumors

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We have screened mutations of the β -catenin gene by using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method in 62 malignant bone and soft-tissue tumors, including malignant fibrous histiocytomas (MFHs), osteosarcomas, synovial sarcomas, liposarcomas, malignant schwannomas, and other types of tumors, as well as 11 benign tumors. β -Catenin-activating missense mutations were found in two malignant tumors. One found in MFH occurred at codon 45 and caused an amino acid substitution from serine (one of the GSK3 β -targeted phosphorylation sites) to phenylalanine. The other, detected in synovial sarcoma at codon 32, resulted in an amino acid change from aspartic acid (located adjacent to the phosphorylation target, serine, encoded by codon 33) to tyrosine. Furthermore, we found accumulation of β -catenin by western-blotting analysis in 12 of 19 malignant tumors in which we found no mutation involving exon 3. Our results suggested the possible involvement of β -catenin activation, by β -catenin gene mutation or alteration of other factor(s), in the formation and/or progression of various types of bone and soft-tissue tumors.

Key words: β -Catenin — Mutation — Bone tumor — Soft-tissue tumor

Soft-tissue tumors are classified into various histopathological types that include malignant fibrous histiocytoma (MFH), synovial sarcoma, liposarcoma, malignant schwannoma, fibrosarcoma, rhabdomyosarcoma, and leiomyosarcoma for malignant tumors, and desmoid tumors, fibroma, lipoma, and schwannoma for benign tumors.¹⁾ In these tumors, *p53* and *Rb* gene alterations are found at the frequency of 10 to 40%.²⁻⁴⁾ In addition, amplifications of *c-sis*, *c-myc*, and *mdm2*, point mutations of *H-ras* and *K-ras*, or adenomatous polyposis coli (APC) mutations have been reported in some types of tumors.⁴⁻¹²⁾

Dysfunction of the *APC* gene was shown to contribute to the development of desmoid tumor, one of the commonest extra-colonic manifestations in patients with familial adenomatous polyposis (FAP),^{13, 14)} as well as sporadic cases.¹⁰⁻¹²⁾ Hence, the functional activation of β -catenin caused by APC mutation is considered likely to be a key step in desmoid formation.¹⁵⁾ In addition to APC alterations, mutations of the β -catenin gene itself were identified in several percent of colorectal cancers,¹⁶⁻¹⁸⁾ in which stabilized and accumulated mutant β -catenin induces increased Wnt/Wingless signaling through Tcf/

Lef-mediated transcriptional activation.^{19, 20)} In desmoid tumors, the same mechanism might be involved. However, it is not known whether or not β -catenin activation is involved in other types of bone and soft-tissue tumorigenesis.

Oncogenic transformation induced by β -catenin gene transfer into NIH3T3 fibroblasts was demonstrated,²¹⁾ and serum-independent cellular proliferation and morphological change were produced in Rat-1 fibroblasts by Wnt-1, which induces β -catenin activation.²²⁾ These results suggest that β -catenin activation is associated with the formation of tumors of fibroblast origin. Furthermore, cytoplasmic staining of β -catenin was reported in a rhabdomyosarcoma-derived cell line.²³⁾ When β -catenin is activated in tumor cells, it exists in the cytoplasm,^{18, 24)} whereas in normal cells it is present in the membrane, in a bound form with cadherin. This raises the possibility that β -catenin is also activated in rhabdomyosarcoma. These observations suggest that β -catenin mutation may be present in tumors of fibroblast or myocyte origin, as well as desmoid tumors.

To examine this point, we screened the β -catenin gene by using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method in addition to measuring its protein level by western-blotting analysis. Here we discuss the association between β -catenin activation and tumorigenesis of bone and soft-tissue tumors other than desmoid tumors.

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The abbreviations used are: PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; MFH, malignant fibrous histiocytoma; APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis.

MATERIALS AND METHODS

Tissue samples and DNA extraction Seventy-three bone and soft-tissue tumors consisting of 62 malignant and 11 benign tumors were obtained from patients who underwent surgical resection at Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka) or Osaka University Medical School Hospital (Osaka), with informed consent. Diagnoses were based on the histopathology. The 62 malignant tumors included 21 MFHs, eight osteosarcomas, seven each of synovial sarcomas, liposarcomas, and malignant schwannomas, three rhabdomyosarcomas, two each of epithelioid sarcomas, neurofibrosarcomas, and chondrosarcomas, and one each of fibrosarcoma, leiomyosarcoma, and alveolar soft-part sarcoma. The 11 benign tumors consisted of three each of giant cell tumors, lipomas, and schwannomas, one desmoplastic fibroma, and one hemangioma (Table I). Fresh materials of each tumor and corresponding normal tissue (muscle or subcutaneous tissue) were stored at -80°C until DNAs were extracted. High-molecular DNAs of each tumor were extracted from frozen materials as described elsewhere.¹⁸⁾

Table I. Histological Type and Summary of β -Catenin Alterations in Bone and Soft-tissue Tumors

Histological type	Number of tumors with β -catenin mutation	Number of tumors with elevated expression of β -catenin
Malignant		
MFH	1/21	5/8 ^{a)}
Osteosarcoma	0/8	4/6
Synovial sarcoma	1/7	1/3 ^{b)}
Liposarcoma	0/7	
Malignant schwannoma	0/7	
Rhabdomyosarcoma	0/3	1/2
Epithelioid sarcoma	0/2	0/1
Neurofibrosarcoma	0/2	
Chondrosarcoma	0/2	
Fibrosarcoma	0/1	
Leiomyosarcoma	0/1	
Alveolar soft-part sarcoma	0/1	
Total	2/62	11/20
Benign		
Giant cell tumor	0/3	
Lipoma	0/3	
Schwannoma	0/3	
Desmoplastic fibroma	0/1	
Hemangioma	0/1	
Total	0/11	
Total	2/73	11/20

a) One tumor with mutant β -catenin is included.
 b) The tumor with mutant β -catenin is not included.

PCR-SSCP analysis Using a pair of intronic primers, G-F (5'-CCAATCTACTAATGCTAATACTG-3') and G-R (5'-CTGCATTCTGACTTTTCAGTAAGG-3'), which flank exon 3, 0.1 μ g of genomic DNA of each tumor was amplified. The PCR reaction was performed in a volume of 20 μ l using Ex Taq polymerase (Takara, Otsu) as follows; 4 min at 94°C for initial denaturing followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, using a GeneAmp PCR system 9600 (Perkin Elmer-Cetus, Norwalk, CT). After PCR amplification, an aliquot of PCR product from each sample was mixed with SSCP-loading buffer and electrophoresed for 12 h in a 5% polyacrylamide gel containing 10% glycerol at 500 volts, with cooling at 4°C. The gel was stained with SYBR GREEN II (FMC Bioproducts, Rockland, ME) and bands were quantitated with the FMBIOII Multiview fluorescent image analyzer (Takara). Aberrant bands were isolated from the gel and recovered DNAs were used for the second PCR reaction.

Sequence analyses of aberrant bands We determined DNA sequences by using an Applied Biosystems model 373 or 377 DNA sequencer (Perkin Elmer-Cetus) and a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA). Mutations were confirmed by repeated experiments and Southern hybridization with oligonucleotides corresponding to the mutated DNA sequence.

PCR amplification of the genomic region Exon 3 and surrounding introns were amplified by PCR with primer pairs C-F (5'-CCAGCGTGGACAATGGCTAC-3') and C-R (5'-TGAGCTCGAGTCATTGCATAC-3') which are located at exons 2 and 4, respectively. The conditions were as follows; 4 min at 94°C for initial denaturing followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min.

Protein analysis for β -catenin Western-blotting analysis was performed to determine the protein level in tumors for which frozen materials were available, as described previously.¹⁸⁾ After electrophoresis of 20 μ g of total protein from each tumor tissue in 7.5% polyacrylamide gel, proteins were transferred to polyvinylidene difluoride (PVDF) membrane. β -Catenin and actin were visualized with anti- β -catenin (C19220, Transduction Laboratories, Lexington, KY) and anti-actin (CP01, CALBIOCHEM, Cambridge, MA) monoclonal antibody, respectively, and ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, UK).

RESULTS

We screened mutations of exon 3 of the β -catenin gene by PCR-SSCP analysis. Among the 62 malignant and 11 benign tumors tested, aberrant migrations were observed in two tumors, one MFH (T17) and one synovial sarcoma

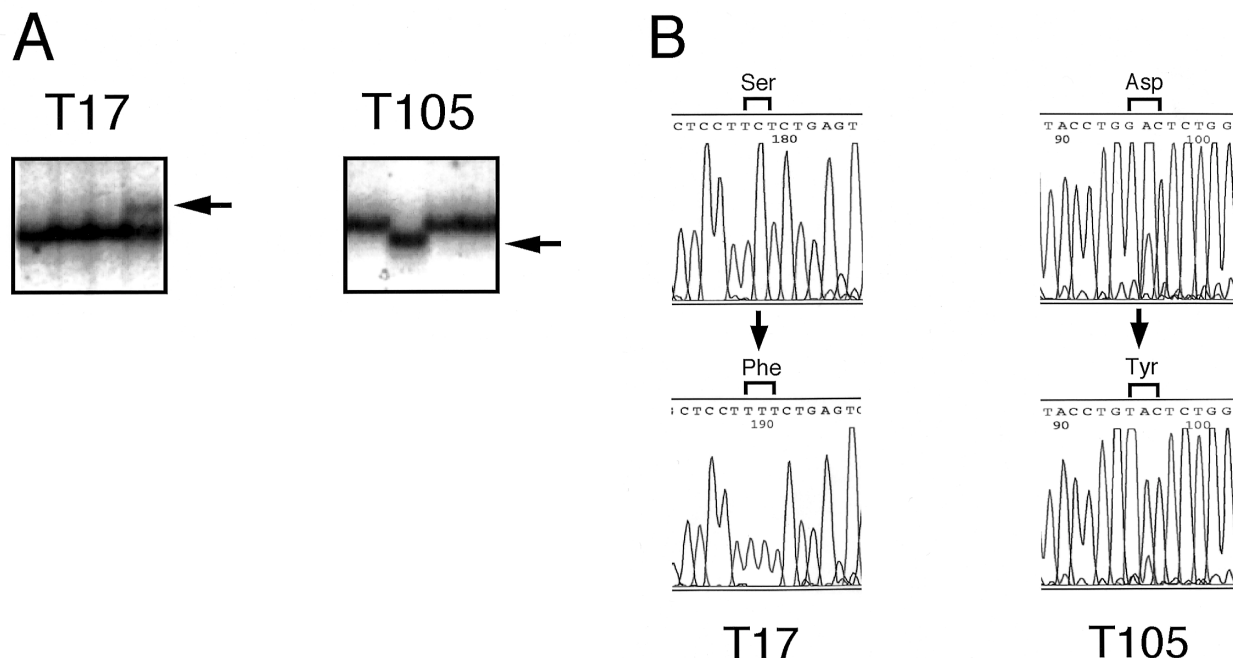


Fig. 1. (A) The results of SSCP analysis. Aberrant bands are seen in T17 (MFH) and T105 (synovial sarcoma), as indicated by arrows. (B) Sequence analysis of DNA corresponding to the aberrant fragment (T17 and T105) detected by the SSCP analysis. The nucleotide C (the second letter of codon 45) is changed to T, resulting in an amino acid substitution of Phe (TTT) for Ser (TCT) in T17. In T105, the nucleotide T (the first letter of codon 32) instead of G results in an amino acid substitution from Asp (GAC) to Tyr (TAC).

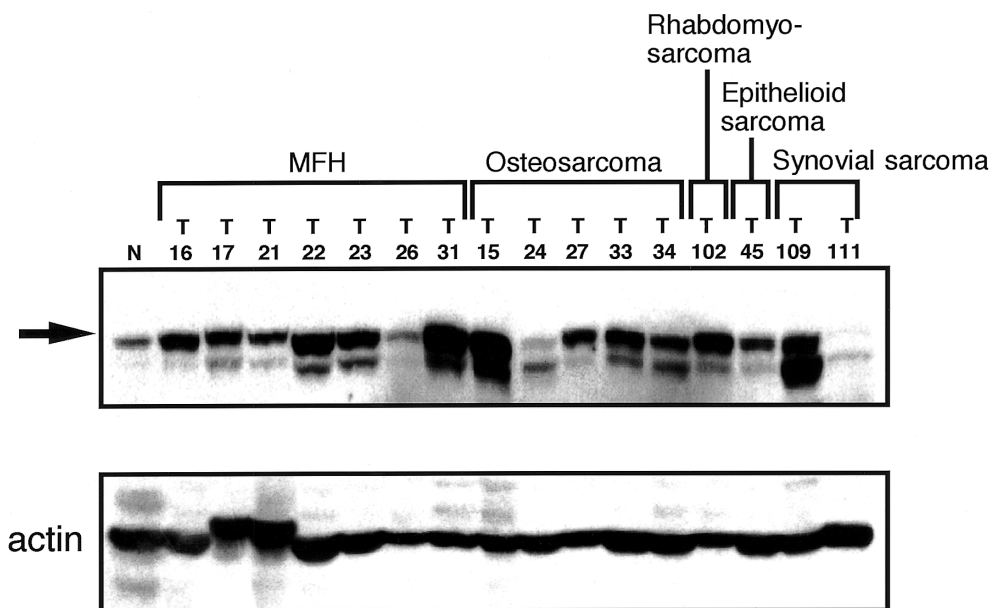


Fig. 2. Western-blotting analysis of tumors. The histological types are shown in the upper column. Accumulation of β-catenin at the position of full size (MW 92,000), indicated by an arrow, is observed in all tumors except T21, T26, T24, T45, and T111. N, normal control. The blot was controlled for protein loading by stripping the blot and reprobng with an antibody against actin.

(T105), in the SSCP analysis (Fig. 1A). Sequencing analyses of the aberrant band of T17 showed a nucleotide change at codon 45 (TTT for TCT), resulting in amino acid substitution from serine to phenylalanine, and that of T105 revealed an amino acid substitution at codon 32 of tyrosine (TAC) for aspartic acid (GAC) (Fig. 1B). In addition, we screened for interstitial deletions involving exon 3, such as are frequently observed in colorectal cancer without APC mutation,¹⁸⁾ by PCR amplification between exon 2 to 4, but no abnormality was identified (data not shown).

Although the frequency of oncogenic β -catenin gene mutations that lead to accumulation of β -catenin was low, we also performed western-blotting analysis for 20 tumors for which frozen materials were available. We normalized β -catenin expression with respect to actin. Among the 20 tumors (eight MFHs, six osteosarcomas, three synovial sarcomas, two rhabdomyosarcomas, and one epithelioid sarcoma), we detected accumulation of β -catenin in 11 tumors as shown in Fig. 2 and Table I.

DISCUSSION

β -Catenin activated by mutation of APC or β -catenin itself is known to form a complex with Tcf/Lef transcriptional factors and to be translocated to the nucleus.^{19, 20)} Binding of the β -catenin-Tcf/Lef complex to specific DNA sequences of the downstream genes transactivates genes involved in the Wnt/Wingless signal transduction pathway.²⁵⁾ β -Catenin mutations reported so far in several types of tumors are missense mutations of potentially phosphorylatable residues, i.e., serine (at codons 33, 37, and 45) and threonine (at codon 41), or neighboring sites where amino acid changes may influence the efficiency of phosphorylation or alter the protein structure. In addition, interstitial deletions that cause skipping of exon 3 in the transcript have been found in colorectal tumors without APC mutation.¹⁸⁾ Through coupling with the tumor suppressor APC, which is suspected to function as ubiquitin ligase, degradation of β -catenin starts. Phosphorylation by

GSK3 β of these serine/threonine residues is important for this coupling.^{16, 26)} Hence, β -catenin mutated at these residues is supposed to escape degradation, being accumulated in the cytoplasm.^{18, 24, 26)} The two mutations found in this study are thus considered to lead to oncogenic β -catenin activation that is likely to contribute to the tumor development.

Although the frequency of genetic alterations of the β -catenin gene is low, our western-blotting analysis revealed accumulation of β -catenin in more than half of malignant bone and soft-tissue tumors. Since no known β -catenin-activating mutation was found in these β -catenin-accumulated tumors, our results suggest the presence of an unknown oncogenic activation mechanism(s) of β -catenin that plays a significant role in these tumors. Although the frequency of the β -catenin gene mutation is as low as several percent in colorectal tumors,¹⁸⁾ APC gene mutations, which cause β -catenin stabilization and accumulation, are present in the majority of tumors.^{18, 27)} In endometrioid ovarian carcinomas, the frequency of accumulation of β -catenin in the tumor was also much higher than that of mutation of the β -catenin gene itself.²⁸⁾ However, in these tumors, the contribution of APC mutations is likely to be very low. Taken together with the results of previous studies, our results suggest that an unknown mechanism(s) that plays an important role in β -catenin regulation may be involved in the development of a relatively large proportion of bone and soft-tissue tumors.

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