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Mutations of *Adenomatous Polyposis Coli* and *β-Catenin* Genes during Progression of Lung Tumors Induced by *N*-Nitrosobis(2-hydroxypropyl)amine in Rats[1]

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ABSTRACT

In the present study, we investigated mutations of the *adenomatous polyposis coli* (*APC*) and *β-catenin* genes to clarify possible molecular mechanisms underlying development of lung tumors induced by *N*-nitrosobis(2-hydroxypropyl)amine (BHP) in rats. Male Wistar rats, 6 weeks of age, were given 2000 ppm BHP in drinking water for 12 weeks and then maintained without further treatment until sacrifice at week 25. DNA was extracted from paraffin-embedded tissues, and PCR-single-strand conformation polymorphism analysis, followed by nucleotide sequencing, was performed. No *APC* mutations were detected in 17 hyperplasias, but 2 of 15 adenomas (13.3%) and 8 of 20 adenocarcinomas (40.0%) showed changes within exon 1 to the mutation cluster region in exon 15. For *β-catenin*, no mutations were detected in 17 hyperplasias, but 3 of 15 adenomas (20.0%) and 5 of 20 adenocarcinomas (25.0%) had alterations within or flanking codons corresponding to important phosphorylation sites. Immunohistochemical staining showed β-catenin protein localized in the cell membranes in the surrounding normal-appearing lung and 216 hyperplasias and localized mainly in the cytoplasm and/or nucleus in 10 of 37 adenomas (27.0%) and 21 of 40 adenocarcinomas (52.5%). These results suggest that the APC-β-catenin-T-cell factor signaling pathway is involved in the acquisition of growth advantage from adenomas to adenocarcinomas in BHP-induced rat lung carcinogenesis.

INTRODUCTION

The majority of lung cancers, one of the most common forms of cancer in humans, are NSCLCs[3]. It is now widely accepted that carcinogenesis is a multistep process involving sequential accumulation of changes at the genome level (1). Although there are a number of reports on gene alterations in human lung cancers, rate-limiting events have yet to be established. Previously, we described a model for the development of NSCLCs in rats given BHP in drinking water with high yields of adenomatous lesions, including adenocarcinomas (2, 3). This model is useful for investigation of molecular mechanisms involved in step-by-step development of lung adenocarcinomas from adenomatous preneoplastic lesions. The molecular events demonstrated thus far are a high frequency of Ki-*ras* mutations, but no mutations of Ha-*ras* and *p53*, in relatively early lesions (4). We also found overexpression of VEGF to be related to up-regulation of VEGF receptor-1/fms-like tyrosine kinase-1 and VEGF receptor-2/fetal liver kinase-1 expression (5) and that of midkine (6) during BHP-induced lung carcinogenesis.

The protein β-catenin, a submembranous component of the cadherin-mediated cell-cell adhesion system, has been demonstrated to be the downstream activator of Wnt signal transduction (7, 8). The amounts of cytoplasmic β-catenin are mainly regulated by interaction with APC protein, the adenomatous polyposis coli gene product, and by phosphorylation at serine and/or threonine residues through the action of GSK-3β (9). Mutations of APC or β-catenin affect the degradation of β-catenin protein by the ubiquitin/proteasome system and result in its stabilization and accumulation within the cell (10, 11). Association with members of the Tcf family (12, 13) and their complexes then causes transactivation of growth-promoting genes, such as c-*myc* and *cyclin D1* (14, 15). It has been suggested that the APC-β-catenin-Tcf signaling pathway plays a major role in colon cancer of humans, through mutational inactivation of APC or activation of β-catenin (10, 11, 16). With regard to human lung cancers, several reports have documented deletions at 5q of the *APC* gene (17–19) without evidence of *APC* point mutations (19, 20), and it has been suggested that APC itself is unlikely to play a significant role in human lung cancer (20). In rodents, decreased expression of the *APC* gene in mouse lung neoplasias has been reported, whereas mutation and loss of heterozygosity of APC were not found (21). Recently, *β-catenin* mutations have been described in several cancers of humans and rodents (10, 11, 22–25). However, to our knowledge there have been no reports of such alteration in lung cancer. When studied by immunohistochemistry, β-catenin showed no evidence of oncogenic activation in human lung cancer (26).

In this present study, to clarify the involvement of APC-β-catenin-Tcf signaling pathway in lung carcinogenesis, we therefore investigated *APC* and *β-catenin* gene mutations in lung lesions induced by BHP in rats.

MATERIALS AND METHODS

Animals and Treatment. Male Wistar rats, 5 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed three to five to a plastic cage in an air-conditioned room, with a constant temperature of 25°C with a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the study. After a 1-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Co. Ltd., Tokyo, Japan), the animals received 2000 ppm BHP (Nakalai Tesque Co. Ltd., Kyoto, Japan) in their drinking water for 12 weeks and then drinking water without BHP. The animals were killed under ether anesthesia 25 weeks after the beginning of the experiment.

Tissue Preparations. The lungs were immediately removed, fixed in formalin at 4°C, and routinely processed for paraffin embedding. Three serial thin sections were made. Two cut at 3-μm thickness were stained with H&E for histological examination and for immunohistochemical analysis of β-catenin. The other section at 5-μm thickness was used for DNA extraction. Lung lesions were classified according to the diagnostic criteria described previously (2, 3).

PCR-SSCP Analysis of *APC* and *β-Catenin* Genes. DNA extraction from paraffin-embedded sections of 17 hyperplasias, 15 adenomas, 20 adenocarcinomas, and 1 normal lung tissue was performed as described previously (27), followed by PCR-SSCP analysis for *APC* and *β-catenin* mutations.

For the *APC* gene, PCR-SSCP and PCR-restriction-SSCP analysis were carried out with methods reported previously (28, 29). The primers used in this study were chosen to amplify exon 1 through the MCR in exon 15 of the *APC* gene with intron sequences flanking coding exons as described previously

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; BHP, *N*-nitrosobis(2-hydroxypropyl)amine; SSCP, single-strand conformation polymorphism; MCR, mutation cluster region; Tcf, T-cell factor; VEGF, vascular endothelial growth factor; GSK-3β, glycogen synthase kinase 3β.

(Refs. 28 and 29; Table 1). Briefly, PCR for SSCP was performed in 5 μl of reaction mixture containing 4 pmol of each primer, 1× PCR buffer (Perkin-Elmer Corp., Applied Biosystems Division, Foster City, CA), 200 μM of each deoxynucleotide triphosphate, 68 nM of [α-^{32}P]dCTP, 0.5 unit of AmpliTaq (Perkin-Elmer), and 50 ng of template DNA. The mixture was subjected to 35 cycles of amplification, each consisting of 0.5 min at 94°C for denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for extension. Amplified products longer than 300 bp were digested with restriction enzyme before electrophoresis (Refs. 28 and 29; Table 1). To rule out the PCR artifacts, PCR amplification was repeated from individual original DNA at least once. The samples were applied to 6 or 10% polyacrylamide gels with or without 5% glycerol after denaturation at 90°C for 3 min. Electrophoresis was performed at 40 W for 2–4 h at 20°C. The gels were dried on filter paper and used to expose X-ray film at −80°C.

For the *β-catenin* gene, primers of appropriate oligonucleotide sequences, 5′-GCTGACCTGATGGAGTTGGA-3′ and 5′-GCTACTTGCTCTTGCGTGAA-3′, were used for specific amplification of the consensus sequence for GSK-3β phosphorylation as described previously (Table 1; Ref. 23). PCR amplification for SSCP was performed under the following reaction conditions: denaturation step for 5 min at 95°C, 35 cycles for 1 min at 95°C, 1 min at 60°C and 2 min at 72°C, and extension for 10 min at 72°C (25). The resultant PCR products were applied to 6% polyacrylamide gels with or without 5% glycerol. Electrophoresis was performed at 40 W for ~2.5 h at 20°C, and gels were subjected to autoradiography.

Cloning and DNA Nucleotide Sequencing. The DNA fragments of mobility-shifted bands in the polyacrylamide gel were extracted and reamplified. The PCR products obtained were cloned with a TOPO TA cloning kit (Invitrogen, San Diego, CA), and recombinant plasmid DNA clones were sequenced using Sequencing Pro (TOYOBO Co. Ltd., Tokyo, Japan). In each experiment, 5–10 clones from different bacterial colonies were investigated.

Immunohistochemical Staining for *β-Catenin*. Immunohistochemical staining for *β-catenin* was studied for 216 hyperplasias, 37 adenomas, and 40 adenocarcinomas, including the samples examined for mutation analysis of *β-catenin* and Ki-*ras*. Demonstration of anti-β-catenin binding was performed according to a standard protocol for DAKO ENVISION + (Dako Japan, Kyoto, Japan). Briefly, deparaffinized tissue sections were incubated in 3% H_2O_2 dissolved in methanol for 30 min and then autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min twice. Normal goat serum (Sigma Chemical Co., St. Louis, MO) was diluted at 4% in Tris-buffered saline and used to block nonspecific cross-reactions by incubation for 30 min at room temperature. The mouse monoclonal anti-β-catenin antibody (Transduction Laboratories, Lexington, KY) was used at the concentration of 0.05 μg/ml in 4% goat serum added to Tris-buffered saline and incubated for 30 min at 37°C. 3,3′-Diaminobenzidine tetrahydrochloride (Nacalai Tesque Co. Ltd., Kyoto, Japan) was used for visualization of binding. The specificity of the binding was confirmed by negative control staining using mouse nonimmune serum instead of the primary antibody (data not shown).

RESULTS

***APC* and *β-Catenin* Mutations.** Representative results of PCR-SSCP and PCR-restriction-SSCP analysis and sequencing analysis for *APC* mutations are shown in Fig. 1. None of 17 hyperplasias showed bandshifts in exon 1 through the MCR in exon 15. Two of 15 adenomas showed bandshifts in exons 12 and 15A, respectively (13.3%), whereas 8 of 20 adenocarcinomas showed bandshifts in exons 3, 8, 9, 10, 11, 14, 15BD, and 15FH. Among those 8, 2 cases had bandshifts in both exons 3 and 9, and exons 15BD and 15FH, respectively. Two adenomas showed GCT to CGT (Ala to Arg) transversions at codon 515 and TGT to TGC (Cys to Cys) transition at codon 679, respectively, the former leading to a base substitution and the latter to no amino acid alteration. In the 8 adenocarcinomas, one showed a GAA to AAA (Glu to Lys) transition at codon 292 and a TGC to TGT (Cys to Cys) transition at codon 415, and another a GTT to GTC (Val to Val) transition at codon 819 and an AGT to AAT (Ser to Asn) transition at codon 1392. One of the two mutations in both cases resulted in no amino acid alteration. The other 5 cases showed a GTT to ATT (Val to Ile) transition at codon 440, a GGG to AGG (Gly to Arg) transition at codon 469, a TGG to TGA (Trp to Stop) transition at codon 591, an ATG to GTG (Met to Val) transition at codon 817, and a GAC to GGC (Asp to Gly) transition at codon 1422. That involving codon 298 was without amino acid alteration. No deletions or LOH were found for the 17 hyperplasias, 15 adenomas, and 20 adenocarcinomas.

Results of PCR-SSCP analysis and sequencing analysis for *β-catenin* mutations are illustrated in Fig. 2. In 17 hyperplasias, no fragments showed bandshifts. However, in 3 of 15 adenomas (20.0%) and 5 of 20 adenocarcinomas (25.0%), bandshifts were apparent indicative of *β-catenin* mutations. In the 3 adenomas, there were two GAT to GTT (Asp to Val) transversions at codon 32 and one GGA to GAA (Gly to Glu) transition at codon 34. The 5 adenocarcinomas had two GAT to GTT (Asp to Val) transversions at codon 32, one TCT to TTT (Ser to Phe) transition at codon 33, one ATC to GTC (Ile to Val) transition at codon 35, and one ACC to ATC (Thr to Ile) transition at codon 41. The patterns and incidence of *APC* and *β-catenin* mutations in adenomas and adenocarcinomas are summarized in Table 2.

Table 1 *The primer sequences used for PCR-SSCP analysis*

Genes	Exon	Forward	Reverse	Restriction enzyme
APC[a]	1	5′-CACGCAGCTACTCCATTTTT-3′	5′-TTACAAGAGGAAACACTGAA-3′	
	2	5′-GGTTATTTAGCATTTCATGC-3′	5′-TCTCTAAAGTCCTTTAAAAA-3′	
	3	5′-GGCATAAGCAGTTACTACAT-3′	5′-GAGCTCCCGTACACAAGAGT-3′	
	4	5′-GCTCTCACAAGTCCATTGCT-3′	5′-AAGTCCCCAAATCTAAAAGC-3′	
	5	5′-AAATTAAGTGCTCTTTGTGG-3′	5′-GGCAGAGCTGTCAGTGTGCA-3′	
	6	5′-ATCTGACTTGATTCTCTTTT-3′	5′-CACACGCATGTAACAAAAGG-3′	
	7	5′-CGGTTTTGTACATAACGATA-3′	5′-TTTCCAGGGGCTCACATTCT-3′	
	8	5′-CTTCTAGACTGAGAGTGTCA-3′	5′-CATAAAGACCGAGTGAGAAC-3′	
	9	5′-GATCTGGCTTTTGGGTATTA-3′	5′-TTGAAACATGCATTGTGATG-3′	*Pvu*II, *Hin*fI
	10	5′-AGCATTATGGCTTAGGTGAC-3′	5′-ATTATCTGTGGCATTACAAA-3′	
	11	5′-CAGTTTATTCGATTATGGAT-3′	5′-AGCACCTTAGGAAGTAAATC-3′	
	12	5′-GCCCCTTGATACTACTCT-3′	5′-AAAAGCCACATCAGAAAT-3′	
	13	5′-AAAAAGGCAGTTAGTATGAA-3′	5′-TTAATACCAGCTCAGAGAGG-3′	
	14	5′-TTTATAGAAACAGCACTGAA-3′	5′-CGTTCTAAAGAAACAAACTC-3′	*Msp*I
	15A	5′-TGTCACTAACCACATTTCCT-3′	5′-AGCTGCTGCGCTCCCCATAG-3′	
	15B	5′-CAGGAGGCTCTGTGGGACAT-3′	5′-TGCTTACTGCGATGAGATGC-3′	
	15BD	5′-TTTATCAGAAACCTTTGACA-3′	5′-TTACCATAGCCATCACTACT-3′	*Hae*III, *Mva*I
	15DE	5′-GACATGCTCCATGCCTTATG-3′	5′-CATGGTGTTTCTCTTCATTA-3′	*Rsa*I, *Tag*I
	15EG	5′-GAAGATGACAAGCCTACCAA-3′	5′-AGAGTCTGCCTCCTGTGTTG-3′	*Rsa*I
	15FH	5′-TTTCTCAAGGTGTAGTTCCT-3′	5′-TTGCTTCTGCTTGGTGGCAT-3′	*Msp*I
	15HI	5′-TTCTGTCAGCTCCCTGGACA-3′	5′-TCGGAATCATCTAATAAGTC-3′	*Msp*I
β-Catenin[b]		5′-GCTGACCTGATGGAGTTGGA-3′	5′-GCTACTTGCTCTTGCGTGAA-3′	

[a–b] These sequences were previously reported in: [a] Refs. 28 and 29; and [b] Ref. 23.

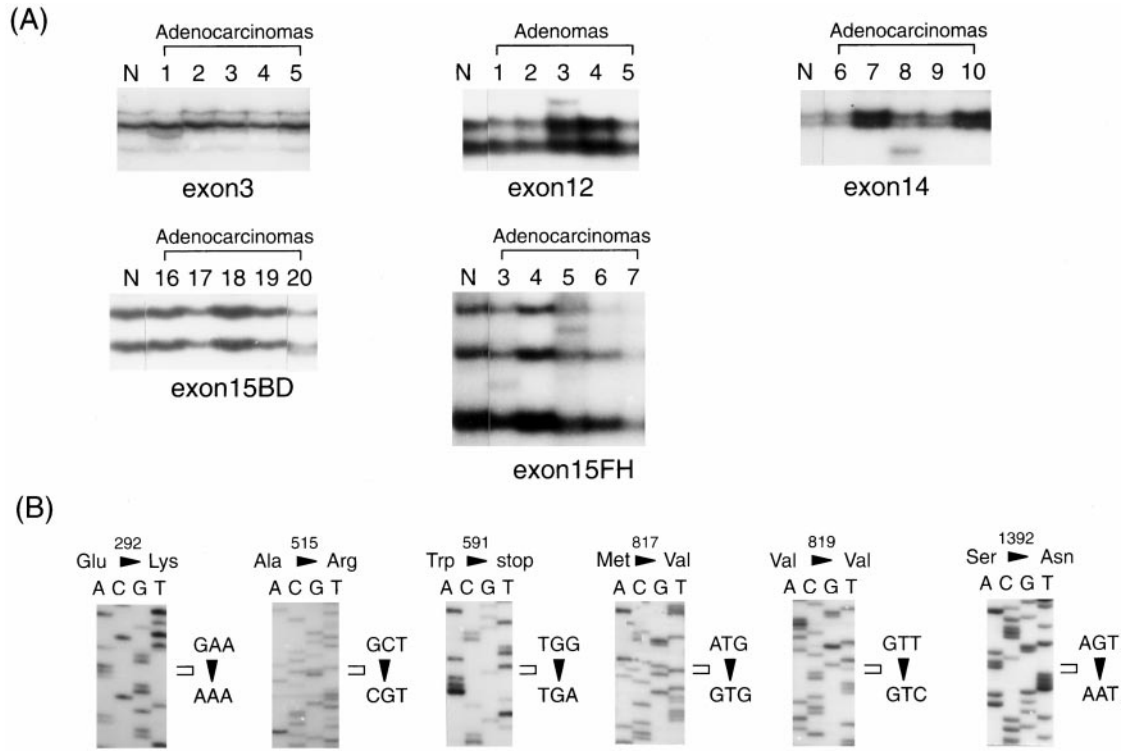

Fig. 1. Representative results of PCR-SSCP and sequencing analysis for *APC* gene mutations in lung lesions induced by BHP in rats. *A*, representative results of SSCP analysis in exon 12 in adenomas and exons 3, 14, 15BD, and 15FH in adenocarcinomas. Bandshifts are shown for sample 1 (*exon3*), sample 3 (*exon12*), sample 8 (*exon14*), sample 20 (*exon15BD*), and samples 3 and 5 (*exon15FH*). *N*, normal lung. *B*, representative results of sequencing analysis for *APC* mutations. These results and the corresponding lesion numbers are summarized in Table 2.

Immunohistochemistry. Representative micrographs of lesions stained with anti-β-catenin antibody are shown in Fig. 3. β-Catenin protein was found to be localized in cell membranes in background tissue and in hyperplasias of the lungs of rats. In adenomas and adenocarcinomas, in contrast, staining was mainly in the cytoplasm and/or nucleus. Data for the localization of β-catenin protein are summarized in Table 3. β-Catenin protein was localized at the cell membranes in all hyperplasias, whereas it was localized in the cytoplasm in 5 of 37 adenomas (13.5%) and 6 of 40 adenocarcinomas (15.0%) and in the cytoplasm and nucleus in 5 of 37 adenomas (13.5%) and 15 of 40 adenocarcinomas (37.5%). All cases that demonstrated *APC* and/or *β-catenin* mutations with amino acid substitution also featured localization of β-catenin protein in the cytoplasm and/or nucleus (Table 2).

DISCUSSION

Previously, we reported relatively high frequencies of Ki-*ras* mutations in lung lesions induced by BHP in rats; 40.0% of hyperplasias, 35.7% of adenomas, and 72.0% of adenocarcinomas were positive, suggesting that Ki-*ras* mutation is an early event of BHP-induced lung carcinogenesis in rats (4). In this study, we found *APC* mutations in 13.3% (2 of 15) of adenomas and 40.0% (8 of 20) of adenocarcinomas, along with *β-catenin* mutations in 20.0% (3 of 15) of adenomas and 23.0% (5 of 20) of adenocarcinomas. However, no mutations of *APC* and *β-catenin* were detected in 17 hyperplasias. Therefore, the present results suggest that the APC-β-catenin-T-cell factor signaling pathway is involved in the acquisition of growth advantage from adenomas to adenocarcinomas in BHP-induced rat lung carcinogenesis.

In human colon tumors, >95% of the *APC* mutations are frameshift or nonsense mutations resulting in a truncated protein (30, 31). There have been few reports of *APC* mutations in rodents (28, 29, 32). In rat colon tumors induced by heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine caused 5′-GGGA-3′ to 5′-GGA-3′ frameshift mutations of *APC* (50.0%), whereas 2-amino-3-methylimidazo[4,5-*f*]quinoline was associated with missense (7.7%) and nonsense mutations (7.7%; Ref. 28). In contrast, no *APC* mutations were apparent in ulcerative colitis-associated rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate (29). Recently, missense mutations have been reported to be a feature of rat colorectal carcinogenesis induced by dimethylhydrazine (32).

In the present study, among 2 adenomas and 8 adenocarcinomas with *APC* mutations, 9 cases were missense or silent and only 1 case was nonsense, with no frameshift mutations being found. Therefore, it seems that the frequency and the mode of APC mutation may depend on the inducing carcinogen or species. It is unknown whether missense mutations of the *APC* gene have any significance. In this study, the nonsense mutation at codon 591 would be expected to cause truncation of the ACP protein. A repeated 20-amino acid sequence in the central region of APC protein includes the phosphorylation site and binding of β-catenin to this region is dependent on phosphorylation by GSK-3β (33, 34). This region also includes the binding site for Axin and coductin (35, 36). Missense mutations at codons 1392 and 1422 in this region may therefore have influenced phosphorylation by GSK-3β or the binding of these proteins. The first 1000 amino acids of APC protein contain heptad repeat motifs, including the Armadillo repeat (33, 37). The heptad repeats are believed to be capable of facilitating protein-protein interaction through formation of extended α-helices that present a series hydrophobic residues extending along one side. These hydrophobic regions can stabilize the

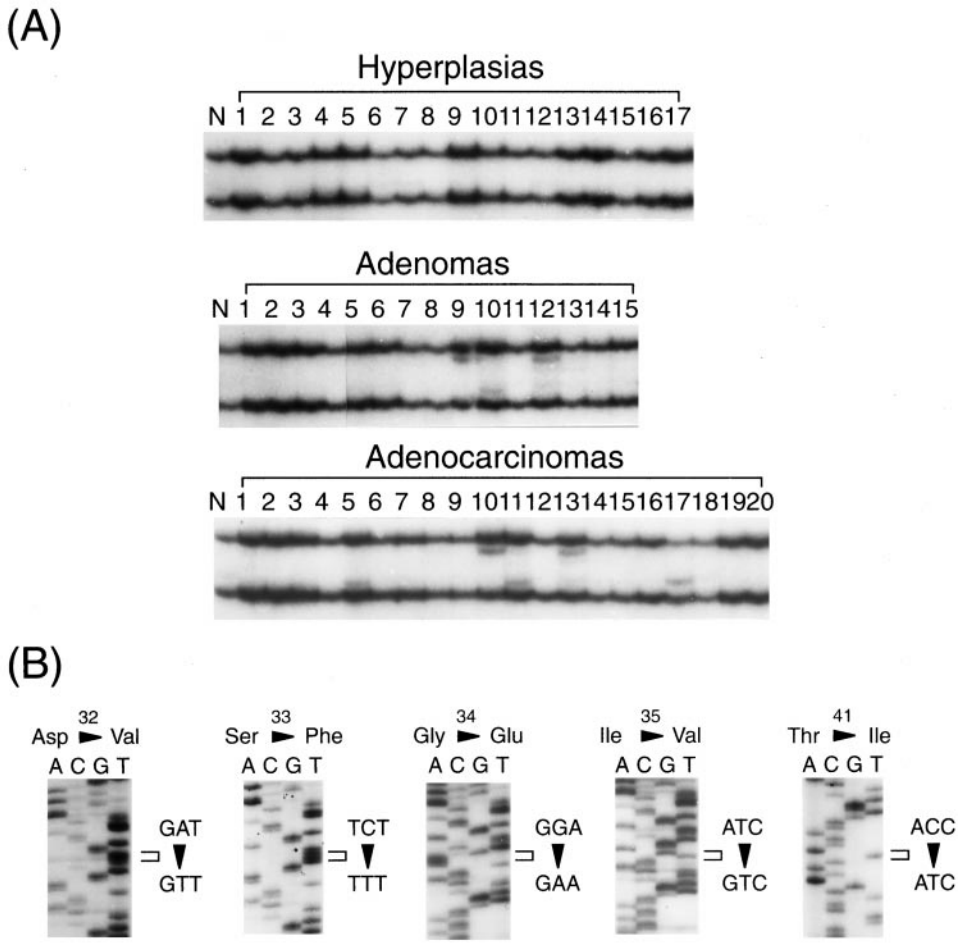

Fig. 2. Representative results of PCR-SSCP and sequencing analysis for *β-catenin* gene mutations in lung lesions induced by BHP in rats. *A*, SSCP analysis for *β-catenin* gene mutations in hyperplasias, adenomas, and adenocarcinomas. Bandshifts are shown for samples 9, 10, and 12 of adenomas and samples 5, 10, 11, 13, and 17 of adenocarcinomas. *N*, normal lung. *B*, representative results of sequencing analysis for *β-catenin* mutations. These results and the corresponding lesion numbers are summarized in Table 2.

interaction of two such α-helices to form either homo- or heterodimers through generation of a coiled coil (33, 37). Codons 292, 440, 469, 515, 591, and 817 are included in this region, and missense mutations in this region may affect protein-protein interactions. We have showed a correlation between *APC* mutations with amino acid substitution and the accumulation of β-catenin protein. No accumulations were found in the cases without *APC* or *β-catenin* mutations (Table 2). These findings provide support for the possibility that some missense mutations, though presumably not all cases, may contribute to accumulation of β-catenin protein. In this study, we did not inves-

Table 2 *Incidence and patterns of APC and β-catenin gene mutations and the status[a] of β-catenin protein localization in lung lesions induced*

	Incidence and patterns of mutations[b]				
No. of samples	APC		β-catenin		Location of β-catenin protein
Adenomas	(2/15; 13.3%)		(3/15; 20.0%)		
3	515 GCT to CGT (Ala to Arg)				Cytoplasm
4	679 TGT to TGC (Cys to Cys)	M[c]			Cell membrane
9			32 GAT to GTT (Asp to Val)	M	Nucleus and cytoplasm
10			34 GGA to GAA (Gly to Glu)	M	Cytoplasm
12			32 GAT to GTT (Asp to Val)	M	Nucleus and cytoplasm
Adenocarcinomas	(8/20;40.0%)		(5/20; 25.0%)		
1	292 GAA to AAA (Glu to Lys)				Cytoplasm
	415 TGC to TGT (Cys to Cys)	M			
2	469 GGG to AGG (Gly to Arg)	M			Nucleus and cytoplasm
3	819 GTT to GTC (Val to Val)	S			Nucleus and cytoplasm
	1392 AGT to AAT (Ser to Asn)	M			
5	1422 GAC to GGC (Asp to Gly)	M	35 ATC to GTC (lle to Val)	M	Nucleus and cytoplasm
6	298 GCT to GCC (Ala to Ala)	S			Cell membrane
8	591 TGG to TGA (Trp to Stop)	N			Nucleus and cytoplasm
10			32 GAT to GTT (Asp to Val)	M	Nucleus and cytoplasm
11			33 TCT to TTT (Ser to Phe)	M	Nucleus and cytoplasm
13			32 GAT to GTT (Asp to Val)	M	Nucleus and cytoplasm
16	440 GTT to ATT (Val to lle)	M			Cytoplasm
17			41 ACC to ATC (Thr to IIe)	M	Nucleus and cytoplasm
20	817 ATG to GTG (Met to Val)	M			Cytoplasm

[a] Staining patterns were divided into three groups: cell membrane, cytoplasm, and nucleus and cytoplasm.
[b] codons, base substitutions, and amino acid alterations.
[c] Type of mutation: M, missense mutation; S, silent mutation; N, nonsense mutation.

tigate the expression levels of *APC* and *wnt* genes in lung lesions induced by BHP in rat. Decreased expression of APC (20) or activation of Wnt signaling (7, 8) could also affect the stabilization and accumulation of β-catenin protein. Whereas 50–60% of the somatic mutations of *APC* in human colon tumors are clustered in a 700-bp region, designated as the MCR, in the middle part of exon 15 (30), in this study, such APC changes accounted for only 2 of 10 in our genetic alterations (adenocarcinomas 3 and 5; 20%).

Mutations of β-catenin have been reported at codons 32, 33, 34, 35, 37, and 41 in colon tumors and hepatocellular carcinomas induced by various carcinogens in rats (23–25). It is considered that the serine and threonine sites located in codons 33, 37, and 41 in *β-catenin* are important for GSK-3β phosphorylation, and codons 32, 34 and 35, which neighbor a serine, are also supposed to be necessary for the ubiquitin-dependent proteolysis system (9–11). Therefore, *β-catenin* mutations at these sites result in the stabilization and accumulation of β-catenin protein. In the present study, *β-catenin* mutations were detected at the same codons, with the exception of codon 37, but no specific site was evident.

Table 3 *Intrecellular localization of β-catenin protein studied by BHP in rats*[a]

Lesions	No. of samples examined	Location of β-catenin protein (%)		
		Cell membrane	Cytoplasm	Nucleus and cytoplasm
Hyperplasias	216	216 (100)	0 (0)	0 (0)
Adenomas	37	27 (73.0)	5 (13.5)	5 (13.5)
Adenocarcinomas	40	19 (47.5)	6 (15.0)	15 (37.5)

[a] Staining patterns were divided into three groups: cell membrane, cytoplasm; and nucleus and cytoplasm.

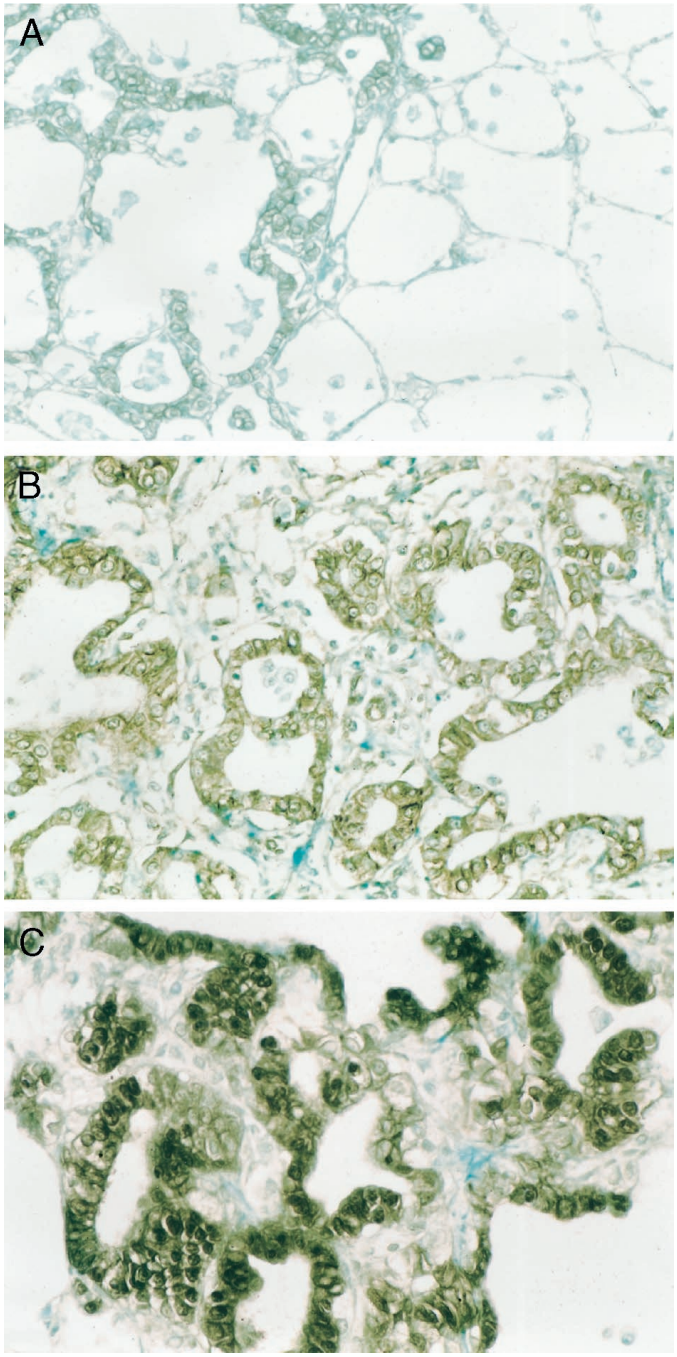

Fig. 3. β-Catenin protein expression demonstrated immunohistochemically. *A*, β-catenin protein is located in cytoplasmic membranes in a hyperplasia of rat lung (×270). *B*, β-catenin protein is stained in cytoplasmic membranes and the cytoplasm in an adenocarcinoma (sample 1) of rat lung (×285). *C*, β-catenin protein is stained in cytoplasmic membranes, the cytoplasm, and the nucleus in an adenocarcinoma (sample 11) of rat lung (×290).

It is considered that the G/C to A/T transition is a common mutation induced by ethylating *N*-nitroso compounds (38). However, in this study, neither G/C to A/T nor C/G to T/A transitions of APC were found in 2 adenomas and 5 adenocarcinomas with *APC* mutations. Among 8 *β-catenin* mutations, G/C to A/T or C/G to T/A transitions were detected in 1 of 3 adenomas and 2 of 5 adenocarcinomas. The Ki-*ras* mutations in contrast were all G/C to A/T transitions at codon 12 (4). Therefore, it seems that the Ki-*ras* mutations were caused by BHP *per se* and that *APC* and *β-catenin* mutations might have been attributable to some other factors, such as DNA damage caused by chronic oxidative stress, acting during lung carcinogenesis initiated by BHP.

The present immunohistochemical study revealed a frequent shift in β-catenin protein localization from the cell membranes to the cytoplasm and nucleus in adenomas and adenocarcinomas but not in hyperplasias, suggesting accumulation in tumor cells. The incidence of protein localization in the cytoplasm and/or nucleus generally corresponded with a finding for *APC* and/or *β-catenin* mutations, providing supporting evidence for a causal role in the stabilization and accumulation of β-catenin protein within the cells. Accumulated β-catenin could interact with Tcf family members in the nucleus, resulting in acquisition of growth advantage by activation of target genes.

In human lung cancers, no mutations of *APC* or oncogenic activation of *β-catenin* have been reported (19, 20, 26). Therefore, the molecular pathways underlying human NSCLCs and BHP-induced lung tumors appear to be quite different. Additional studies are now necessary to determine the involvement of specific growth-promoting genes regulated by β-catenin protein complexes and members of the Tcf family.

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