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Advances in Brief

High Frequency of β-*Catenin* (*Ctnnb1*) Mutations in the Colon Tumors Induced by Two Heterocyclic Amines in the F344 Rat[1]

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Abstract

Activating mutations in the β-*catenin* (*CTNNB1*) gene corresponding to N-terminal phosphorylation sites in the protein have been implicated in the development of human colon cancer. To determine the possible involvement of such mutations during chemically induced colon carcinogenesis, we examined the corresponding region of *Ctnnb1* in colon tumors induced in the F344 rat by two cooked meat heterocyclic amines, 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). All of the colon tumors induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline that were examined (5 of 5) and 4 of 7 PhIP-induced colon tumors had mutations within or flanking codons corresponding to important phosphorylation sites in β-catenin. None of the colon tumors bearing *Ctnnb1* mutations had genetic changes in the *Apc* gene, and those that contained wild-type *Ctnnb1* were known from our previous work to contain *Apc* mutations. The results provide evidence for a major role of the β-catenin/Apc pathway in the development of heterocyclic amine-induced colon tumors and give further weight to the view that regulation of β-catenin is critical to the tumor suppressive effects of Apc during colon carcinogenesis. In contrast, *Ctnnb1* mutations were completely absent in 23 PhIP-induced mammary tumors, in accordance with recent work showing that human breast carcinomas lack mutations in *CTNNB1*.

Introduction

Colon cancer is believed to arise via the accumulation of genetic alterations that activate oncogenes and inactivate tumor suppressor genes (1). Genes that have received particular interest in human colorectal carcinogenesis include Ki-*ras*, *DCC*, *p53*, and *APC*,[4] plus those associated with DNA mismatch repair (1, 2). However, in the colon tumors induced in experimental animals by heterocyclic amines from cooked meat, such as IQ[5] and PhIP, no Ki-*ras* or *p53* mutations were detected (3). In contrast, 15% (2 of 13) of IQ-induced colon tumors and 50% (4 of 8) of PhIP-induced colon tumors had mutations in *Apc*, the so-called "gatekeeper" of colorectal cancer. In previous work, *Apc* exons 1–14 plus the mutation cluster region of exon 15 were examined (4), leaving the possibility that mutations might exist in the remainder of exon 15 (accounting for approximately half of the Apc protein). Therefore, one aim of the present study was to confirm the lack of additional mutations in the remainder of *Apc* exon 15. In addition, we sought to screen the same IQ- and PhIP-induced colon tumors for possible mutations in the β-*catenin* (*Ctnnb1*) gene, because recent evidence has shown that regulation of β-catenin is critical to the tumor suppressor activity of Apc (5).

β-Catenin is a cytosolic protein with an NH_2-terminal region of about 130 amino acids, a central region of 550 amino acids, which comprise the armadillo repeat region, and a COOH-terminal region of 100 amino acids (5–9). The armadillo repeat region contains a positively charged superhelical groove that interacts with APC, cadherin cell adhesion molecules, the Tcf/LEF-1 transcription factor, and several other proteins (6). The NH_2-terminal region contains functionally significant phosphorylation sites, which play a role in targeting cytosolic β-catenin for degradation (5, 9). Notably, human colorectal cell lines and primary colon tumors that lacked mutations in the *APC* gene recently were shown to harbor activating mutations in *CTNNB1* (5). Because PhIP produces colon tumors in the male F344 rat but mammary gland tumors in the female (4, 10), we also screened PhIP-induced mammary tumors for mutations in *Ctnnb1*.

Materials and Methods

Colon tumors were induced in the male F344 rat by chronic administration of PhIP and IQ at dietary levels of 0.04 and 0.03%, respectively (4). Mammary tumors were induced in (SD×F344)F1 rats by administration of PhIP (10). The DNA samples used here were the same as those in previous studies (4, 10). A total of 12 colon tumors (5 IQ-induced and 7 PhIP-induced) and 23 mammary tumors were used for *Ctnnb1* or *Apc* mutation analysis. In our previous work on these tumors, exons 1–14 plus the mutation cluster region of exon 15 of the *Apc* gene were studied; *Apc* mutations were detected in 3 of 7 PhIP-induced colon tumors and none of the IQ-induced colon tumors (4). To complete our examination of *Apc* exon 15, DNA was amplified by the PCR using the seven sets of primers listed elsewhere (11). Alternatively, DNA was amplified with primers designed to produce a 150-bp product of *Ctnnb1*, corresponding to functionally important phosphorylation sites in β-catenin (5). The primers b1 (forward; 5′-GGAGTTGGACATGGCCATGG-3′) and b4 (reverse; 5′-TCCACATCCTCTTCCTCAGG-3′) were included in the following PCR reaction mixture (total volume, 10 μl): 200 ng of genomic DNA, 1 μM of each primer, 80 μM dNTPs, 0.65 μl of ^{32}P-labeled dCTP (370 MBq/ml; 110 TBq/mmol, Amersham Corp.), 0.5 units of Taq polymerase in 1× reaction buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; Perkin-Elmer Corp.). After initial denaturation at 94°C for 3 min, 35 cycles of PCR were conducted as follows: 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, ending with 72°C for 1 min. PCR products were subjected to SSCP analysis, cloned, and sequenced as detailed previously (4). Conditions for reamplification of SSCP bands prior to cloning were as follows (reaction volume, 50 μl): 50 ng of template DNA in 2 μl of TE buffer, 0.8 μM b1 and b4 primers, 2 mM dNTPs, and 5 units of Taq in 1× reaction buffer, with 35 cycles of PCR as above.

Results and Discussion

Screening of the PhIP- and IQ-induced colon tumors by SSCP analysis indicated a high frequency of *Ctnnb1* mutation. DNA samples from all of the IQ-induced tumors (5 of 5) and 4 of 7 of the PhIP-induced tumors demonstrated band shifts in the SSCP gels (Fig. 1). Notably, the samples in *Lanes 1*, *4*, and *6* that lacked *Ctnnb1*

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[4] *Uppercase*, human; *lowercase*, rat or mouse.

[5] The abbreviations used are: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SSCP, single-strand conformation polymorphism; GSK3, glycogen synthase kinase-3β.

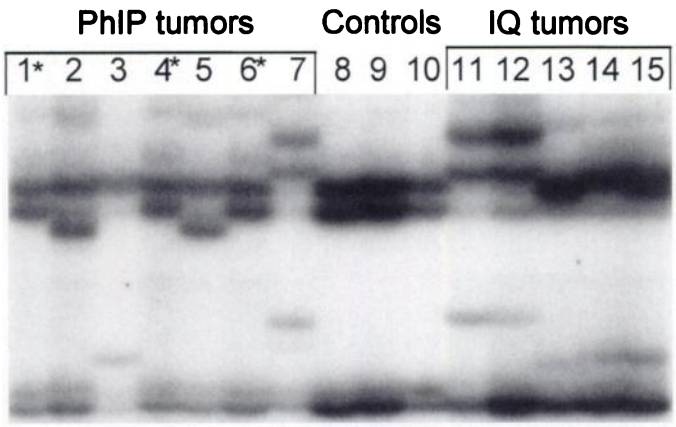

Fig. 1. PCR-SSCP analysis of *Ctnnb1* in PhIP- and IQ-induced colon tumors of the F344 rat. *Lanes 1–7*, PhIP-induced colon tumors; *Lanes 8–10*, normal rat colon; *Lanes 11–15*, IQ-induced colon tumors. Band shifts were observed in *Lanes 2, 3, 5, 7*, and *11–15* under different conditions of PCR-SSCP analysis. *, samples in *Lanes 1, 4*, and *6* that did not show different mobilities from the controls were those reported previously to contain *Apc* mutations (4). Bands with altered mobilities were excised from the gel for sequencing analysis (see Fig. 2).

mutations were from PhIP-induced colon tumors that previously had been shown to contain mutations in *Apc* (4). Using several primer sets, we completed the screening of *Apc* codon 15; no further mutations were detected in any of the colon tumor samples (data not shown). Thus, the results from this study indicate that 100% of the colon tumors contain mutations either in *Apc* or *Ctnnb1*. Although genetic changes in these two genes need not be mutually exclusive, none of the PhIP- or IQ-induced tumors presently examined contained mutations in both *Apc* and *Ctnnb1*.

In human colorectal cancer cell lines and primary tumors, *CTNNB1* mutations were detected in codons that corresponded to phosphorylation sites in the NH_2-terminal domain of the β-catenin protein, within the consensus sequence for GSK3 binding (5). Specifically, Ser^{33}→Tyr, Thr^{41}→Ala, and Ser^{45}→Phe changes occurred as a result of point mutations within the corresponding codons, and one additional case involved a 3-bp deletion that removed codon 45 in half the clones, causing loss of Ser^{45} (5). In PhIP- and IQ-induced colon tumors, sequence analysis revealed only two instances of mutation that directly changed a phosphorylation site in the GSK3 consensus sequence (Fig. 2). Both cases involved a C→G missense mutation that changed Ser^{37}→Cys (Fig. 2*a*). In the human colon cell lines and primary colon tumors examined to date, Ser^{37} remained intact (5), but several melanoma cell lines contained a TCT→TTT mutation that replaced Ser^{37} with Phe (8). All but one of the remaining PhIP- and IQ-induced tumors had mutations that altered the amino acid residues immediately adjacent to Ser^{33} (Fig. 2, *b–d*). In accordance with the known preference of both IQ and PhIP for forming DNA adducts at guanines (12, 13), G→A (codons 32 and 34) and G→T (codon 34) mutations were predominant (Fig. 2). These mutations substituted small amino acids (Asp^{32} and Gly^{34}) for more bulky or charged residues (Asn^{32}, Val^{34}, and Glu^{34}), which probably interfere with the phosphorylation of Ser^{33}. In addition, one of the tumors induced by IQ had a mutation in codon 35 (ATC→AGC), which substituted Ile^{35} with Ser.

Table 1 summarizes these results in terms of the mutations detected within specific codons of *Ctnnb1* and the corresponding amino acid substitutions in the protein. It is noteworthy that several PhIP- and IQ-induced colon tumors contained mutations in codons 32 and 34 of *Ctnnb1*, because these codons also were mutated in a number of azoxymethane-induced rat colon tumors (14).

The present study has identified for the first time a gene that is mutated with high frequency in both PhIP- and IQ-induced colon tumors. Precisely because these tumors lack *p53* or Ki-*ras* mutations and have low or undetectable levels of microsatellite mutations but contain 15–50% mutation frequencies in *Apc*, the results imply that Apc/β-catenin deregulation might play a major role during heterocyclic amine-induced colon carcinogenesis. Mutation of *Ctnnb1* may activate β-catenin-Tcf/LEF-1 signaling pathways,

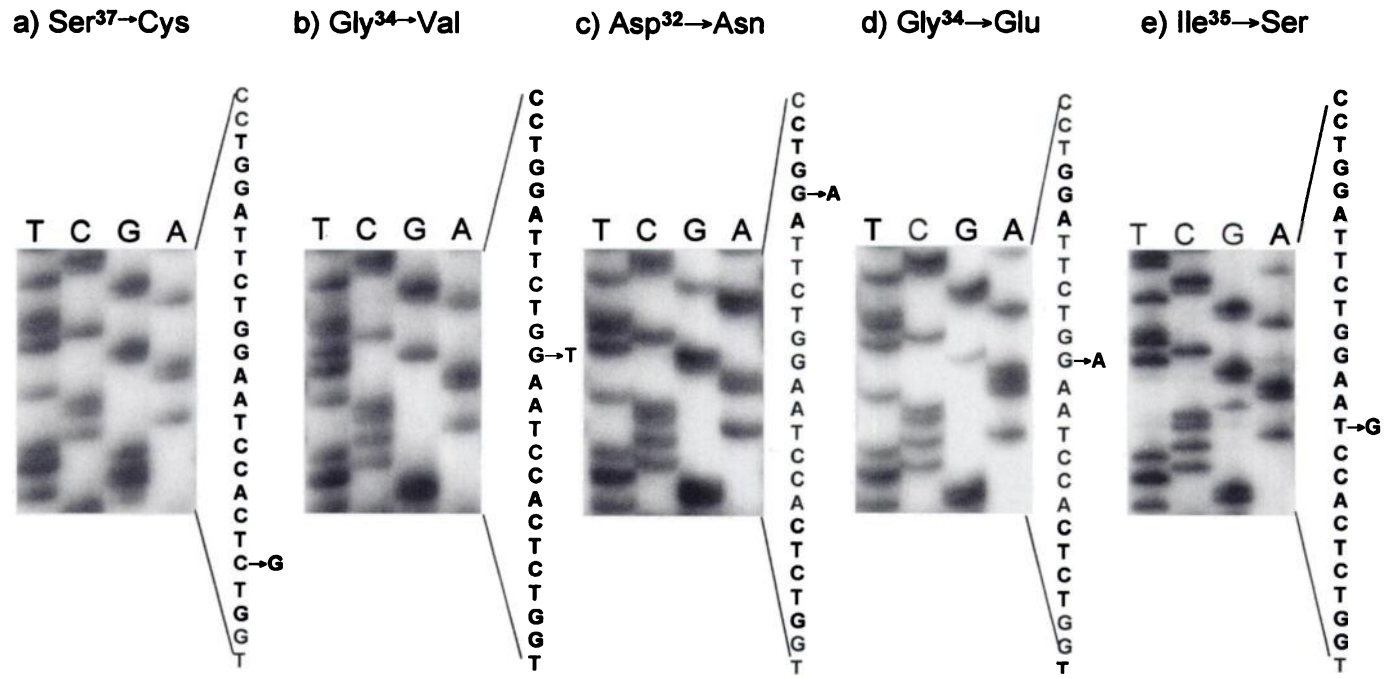

Fig. 2. Sequence of *Ctnnb1* codons 31–39 showing the types of mutation observed in IQ- and PhIP-induced colon tumors. One mutation (*a*) directly affected Ser^{37}, which fits the consensus sequence for GSK3 phosphorylation, but others (*b–d*) directly flanked a second putative GSK3-phosphorylation residue, Ser^{33}. Midway between these two serines, a mutation was detected (*e*) that converted Ile^{35} to Ser. These results and the corresponding tumor numbers are summarized in Table 1.

Table 1 *Summary of Ctnnb1 mutations in IQ- and PhIP-induced colon tumors and the corresponding amino acid substitutions in the β-catenin protein*[a]

	Codon					
	32	33	34	35	36	37
Wild-type	GAT	TCT	GGA	ATC	CAC	TCT
Mutations:	AAT		GTA	AGC		TGT
			GAA			
Protein (wild type)	Asp	SER	Gly	Ile	His	SER[b]
Substituted residues	Asn		Val	SER		Cys
			Glu			
Tumor samples[c]	P2-1, Q31, Q4-1		P7, Q8, Q12	Q5		P2-2, P18-1

[a] Initial studies showed that the amino acid sequence in the NH_2-terminal domain of human β-catenin was conserved in the rat protein, and that the corresponding region of rat *Ctnbb1* contains 91% sequence homology with the human gene sequence.[6] Only the region that contained mutations in PhIP- or IQ-induced colon tumors is shown here.

[b] The two SER residues are within the consensus sequence for GSK3 phosphorylation (5).

[c] PhIP- and IQ-induced tumors are designated P and Q, respectively.

which alter the levels of expression of proliferation- or apoptosis-related genes (5), but the specific genes remain to be identified. An attractive hypothesis might involve the bcl-2 family of proteins, because IQ-induced colon tumors express increased levels of the antiapoptotic protein bcl-2 and decreased amounts of proapoptotic bax protein (15). However, β-catenin interacts upstream with several other proteins in addition to Apc and Tcf-family transcription factors, including the epidermal growth factor receptor tyrosine kinase domain and cadherin cell adhesion molecules (5–9). The next step will be to determine which protein partners and downstream targets play a role in the mechanism by which heterocyclic amines trigger colon carcinogenesis.

Finally, none of the 23 PhIP-induced mammary tumors examined in this study had *Ctnnb1* mutations (results not shown). Recent evidence indicates that human breast carcinomas lack mutations in both the α- and β-*catenin* genes (16). However, it has been reported that Apc^{Min}, a mutation in the murine *Apc* gene, predisposes *Min/+* mice to mammary carcinomas (17). Mutations in the *Apc* gene remain to be analyzed in the PhIP-induced mammary tumors to better understand the role of the Apc/β-catenin pathway in mammary carcinogenesis (17).

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