More Frequent $\beta$-Catenin Gene Mutations in Adenomas than in Aberrant Crypt Foci or Adenocarcinomas in the Large Intestines of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-treated Rats

Tetsuya Tsukamoto,¹, ⁴ Harunari Tanaka,¹ Hiroko Fukami,¹ Manami Inoue,² Mami Takahashi,¹ Keiji Wakabayashi¹ and Masae Tatematsu¹

¹Laboratory of Pathology and ²Laboratory of Epidemiology, Aichi Cancer Center Research Institute, Nagoya 464-8681 and ³Cancer Prevention Division, National Cancer Center Research Institute, Tokyo 104-0045

Alteration of adenomatous polyposis coli (APC) is known to be an early event in neoplasia, causing activation of the $\beta$-catenin/Tcf pathway. Although it is thought that alterations in APC and $\beta$-catenin may complement one another, the contribution of $\beta$-catenin mutations to colorectal carcinogenesis remains unclear. We therefore performed PCR-single strand conformation polymorphism analysis and direct sequencing of exon 3 of $\beta$-catenin gene in adenomas, adenocarcinomas, and aberrant crypt foci (ACF), considered to be putative precursor lesions of colorectal neoplasias, in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) treated F344 rats. $\beta$-Catenin mutations were identified in all of 7 adenomas (100%) and 6 of 12 (50%) adenocarcinomas. All of the mutations were found in codons 32 through 34, the serine encoded by codon 33 being an important phosphorylation site by glycogen synthase kinase-3β. Regarding ACF, 14 of 46 (30.4%) were found to be mutated, eleven (78%) in codon 34, and the others in codon 45 (frequently altered in human colon cancer), and codons 47 and 56 (which have not been previously reported). The frequency of $\beta$-catenin mutations in adenomas was significantly higher than in ACF (P<0.001) and adenocarcinomas (P<0.05). Thus, $\beta$-catenin mutations may have more importance in the genesis of adenomas than ACF or adenocarcinomas in rat colon carcinogens by PhIP.

Key words: $\beta$-Catenin — Rat — Colorectal tumor — ACF — PhIP

Aberrant crypt foci (ACF) are generally considered to be putative preneoplastic lesions for colorectal carcinomas in man¹ as well as in experimental animals.² Molecular analysis has revealed alteration of the adenomatous polyposis coli (APC)/$\beta$-catenin pathway to be one of the earliest events in colorectal carcinoma development,³ being found in dysplastic ACF.⁴ In colon cancers with wild-type APC,⁶ $\beta$-catenin may harbor mutations in exon 3, where serine and threonine residues are physiologically phosphorylated by glycogen synthase kinase (GSK)-3β. Mutations of serine/threonine residues prevent degradation of $\beta$-catenin protein in an APC-dependent manner⁶ and cause activation of the $\beta$-catenin/Tcf-4 signal transduction pathway.⁶, ⁷

In a rodent model, Takahashi et al.⁸ found frequent mutations (6 of 8, 75%) in $\beta$-catenin exon 3 in azoxymethane (AOM)-induced rat colon adenocarcinomas. Furthermore, Dashwood et al.⁹ demonstrated, although the number of samples was limited, all of 5 colon adenocarcinomas induced by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 4 of 7 (57%) by 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) to have mutations in exon 3. While Kakiuchi et al.¹⁰ reported Apc to exhibit a specific one-base deletion in PhIP-induced rat colon carcinomas, these tumors did not have Apc mutations and it was concluded that Apc and $\beta$-catenin mutations are mutually exclusive. Suzui et al.¹¹ recently reported frequent (18 of 20, 90%) $\beta$-catenin mutations in colon cancers induced by methylazoxymethanol acetate plus 1-hydroxyanthraquinone.

However, the situation with regard to earlier lesions such as ACF, in which APC changes are believed to be involved in human cases,¹² remains unclear. Recent evidence suggests that $\beta$-catenin mutations are more frequent in small benign lesions than in large adenomas and invasive carcinomas,¹³ so that adenomas in some cases may be end points in themselves. We recently analyzed the morphology and localization of ACF, adenomas and adenocarcinomas sequentially in PhIP-treated F344 rats and obtained a sufficient number of samples for statistical analysis.¹⁴ PhIP is the most abundant heterocyclic amine in the human environment,¹⁵ being produced in broiled meat and fish during cooking.¹⁶ and is considered one of the most important carcinogens taken in the daily diet. We here utilized the same samples (46 ACF, 7 adenomas and 12 adenocarcinomas) to evaluate the importance of $\beta$-catenin mutations during the progression of ACF through to
adenocarcinomas. The findings indicate that β-catenin gene mutations may occur early in ACF but that adenomas exhibit a higher frequency of this genetic alteration than adenocarcinomas.

**MATERIALS AND METHODS**

**Sample and DNA isolation** Three-week-old male Fischer 344 rats were treated with PhIP-HCl (Nard Institute, Osaka) as recently reported. ACF were isolated and DNAs were extracted with InstaGene Purification matrix (Bio-RAD, Hercules, CA). Colon tumors appearing at weeks 50 and 75 were cut into two pieces. One half of each was histologically analyzed for classification into adenomas and adenocarcinomas as described. The other was employed for DNA isolation with a QIAamp Tissue Kit (Qiagen Co., Ltd., Tokyo).

**PCR-single strand conformation polymorphism (SSCP) analysis** PCR-SSCP analysis was performed basically as described. For the PCR, a 5 μl reaction mixture was used containing 1 μl of genomic DNA, 200 nM each of 5′ and 3′ primers, 200 μM each dNTP, 167 nM [α-32P]dCTP (NEG-513H, NEN Life Science Products, Boston, MA), 0.025 units/μl AmpliTaq Gold DNA polymerase and its provided buffer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). The 5′- and 3′-primer sequences for amplification of the GSK-3β phosphorylation consensus sequence in exon 3 of β-catenin gene were 5′-GCTGACTTGCCTGATGGAGTTGGA-3′ (B1U) and 5′-GCTACTTGCCTTGTGCTTGGAA-3′ (B1D), respectively.

The PCR for tumor DNA samples was run as follows: 95°C-10 min ×1, 94°C-1 min: 55°C-1 min: 72°C-1 min ×30, 72°C-10 min ×1 with a PCR Thermal Cycler MP (TaKaRa, Ohtsu). For ACF, the cycle number was 35 instead of 30 to obtain a comparable sensitivity. Five-microliter aliquots of PCR products were diluted with 40 μl of loading dye (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol FF), denatured at 90°C for 5 min and chilled quickly on ice. Then the samples were electrophoretically separated in a 0.5× nondenaturing MDE polyacrylamide gel (FMC BioProducts, Rockland, ME) containing 5% glycerol at 40 W for 6 h using a power supply (Power Pac 3000, Bio-RAD) with a temperature probe set at 20°C in a cold room, dried, and applied to an imaging plate, which was then analyzed with a BAS 2500 (Fuji Film).

**Statistical analysis** The proportions of ACF and tumors

![Image](image_url)

**Fig. 1.** Representative results of PCR-SSCP analysis of β-catenin exon 3 in tumors (A) and in ACF (B). A, Tumors #4, 5, and 8 are adenomas and #1, 2, 3, 6, 7, 9, 10, 11, and 12 are adenocarcinomas. Mutant bands were observed in tumors #1, 2, 4, 5, 8, and 12. #1 had a GAT-to-AAT mutation at codon 32. Tumors #2, 8 and 12 had GGA-to-GTA mutations at codon 34, #4 and 5 had GAT-to-GCT mutations at codon 32, and #3, 6, 7, 9, 10, 11 and 11 were wild type. B, ACF #7 had a TCC-to-TAC mutation at codon 45, #9 had a GAT-to-TAT mutation at codon 56, #11 had a GGA-to-GTA mutation at codon 34, and #12 through 14 had GGA-to-GAA mutations at codon 34. N, normal control; M1 and M2, mutant controls harboring a GAT-to-AAT mutation in codon 32 and a GGA-to-GAA mutation in codon 34, respectively.
harboring mutations of $\beta$-catenin, with 95% confidence intervals obtained by the exact method, were compared with the use of Fisher’s exact probability method, as described elsewhere.

RESULTS

$\beta$-Catenin gene mutations in tumors  Representative PCR-SSCP results are shown in Fig. 1A, tumors #4, 5, and 8 being adenomas and #1, 2, 3, 6, 7, 9, 10, 11, and 12 being adenocarcinomas. All of 7 adenomas (100%) were revealed to have mutations restricted to codons 32 and 34 (gray cells in the top panel of Fig. 2). In codon 32, the mutations found were GAT (Asp) to GCT (Ala) (see tumors #4 and 5). In codon 34, they were GGA (Gly) to GTA (Val) as in tumor #8. In contrast to adenomas, only 6 out of 12 carcinomas (50%) harbored $\beta$-catenin mutations, found in codons 32 through 34, including GAT (Asp) to AAT (Asn) at codon 32 like tumor #1, TCT (Ser) to TGT (Cys) at codon 33, and GGA (Gly) to GTA (Val) as in tumors #2 and 12 and to GAA (Glu) (black cells in the top panel of Fig. 2). Summarizing the types of mutations, 6 were G:C-to-A:T transition mutations and 7 were transversions, including 2 A:T to C:G, 1 C:G to G:C, and 4 G:C to T:A. The remaining carcinomas like tumors #3, 6, 7, 9, 10, and 11 in Fig. 1A, migrating like the wild-type control (lane N), were confirmed to be wild type by sequencing analysis.

$\beta$-Catenin gene mutations in ACF  ACF could be microscopically isolated without contamination with surrounding cells. Genomic DNAs were successfully isolated with InstaGene Purification Matrix (BIO-RAD) and analyzed with PCR-SSCP (see Fig. 1B). Mutant bands were further analyzed by direct sequencing of the PCR products. Out of 46 ACF, 14 (30.4%) had mutations in exon 3

Fig. 2. Sequence of $\beta$-catenin exon 3 with the codon numbers and deduced amino acids (middle horizontal bar), and $\beta$-catenin mutations found in tumors (above) and in ACF (below). All 7 adenomas (gray cells in top panel) had mutations in codons 32 and 34. Six out of 12 adenocarcinomas (black cells in top panel) harbored mutations in codons 32, 33, and 34. Fourteen ACF (gray cells in bottom panel) out of 46 contained mutations localized mainly in codon 34, but also at other sites. The substituted amino acids are: GCT (Ala) and AAT (Asn) in codon 32; TGT (Cys) in codon 33; GAA (Glu) and GTA (Val) in codon 34; TAC (Tyr) in codon 45; AAT (Asn) in codon 47; and TAT (Tyr) in codon 56.

Fig. 3. Comparison of frequency of $\beta$-catenin mutations in ACF, adenomas and adenocarcinomas. Mutation rates were 30.4% (14 out of 46), 100% (7 out of 7), and 50% (6 out of 12) in ACF, adenomas, and adenocarcinomas, respectively.
of \(\beta\)-catenin. Included were changes of GGA (Gly) to GAA (Glu) (ACF #12, 13, and 14) and to GTA (Val) (ACF #11) in codon 34, TCC (Ser) to TAC (Tyr) in codon 45 (ACF #7), AGT (Ser) to AAT (Asn) in codon 47, and GAT (Asp) to TAT (Tyr) in codon 56 (ACF #9). There were 9 G:C-to-A:T transitions, and 4 G:C-to-T:A and 1 C:G-to-A:T transversions. ACF #1–6, 8, and 10 migrated like the wild-type control (lane N). The mutations are summarized in the bottom panel of Fig. 2.

Comparison of frequency of \(\beta\)-catenin gene mutations in ACF, adenomas and adenocarcinomas

The mutation frequencies were 14 out of 46 (30.4%) in ACF, 7/7 (100%) in adenomas, and 6/12 (50%) in adenocarcinomas (shown in Fig. 3, mean±95% confidence intervals). Statistical analysis (Fisher’s exact probability method) revealed significant differences between ACF and adenomas \((P<0.001)\) and between adenomas and adenocarcinomas \((P<0.05)\) in \(\beta\)-catenin mutation rates.

**DISCUSSION**

We have recently reported a sequential study of PhIP-induced ACF, adenomas and adenocarcinomas in terms of morphology and localization in the rat large intestine using a crypt isolation technique.\(^{14}\) The advantage of crypt isolation is in eliminating contamination with surrounding normal crypts as well as pericryptal stromal and inflammatory cells. Furthermore, single colonic crypts can be used as a maximum monoclonal unit for molecular analysis, as proved by our previous study of C3H↔BALB/c chimeric mice.\(^{20}\) In the present case, we demonstrated more frequent \(\beta\)-catenin gene mutation in adenomas than in ACF and adenocarcinomas in the large intestine of PhIP-treated F344 rats.

In rat models, \(\beta\)-catenin mutations have been reported in colon tumors induced by azoxymethane,\(^{8}\) heterocyclic amines\(^9\) and methylazoxymethanol acetate plus 1-hydroxyanthraquinone.\(^{11}\) Mutations were found in codons 33, 37, and 41 encoding serine and threonine that are direct targets for phosphorylation by GSK-3\(\beta\). Also reported were alterations of codons 32, 34, and 35, encoding neighboring serine and threonine residues, possibly resulting in conformational changes and abnormal phosphorylation of \(\beta\)-catenin protein. In the present study, mutations were present in codons 32 through 34 in adenomas and adenocarcinomas, in accordance with the previous reports.\(^{8,9,11}\) Our data suggest the possibility that specific mutations found in adenomas vs. adenocarcinomas could be correlated with the degree of degradation of \(\beta\)-catenin protein and consequently the transformation capability. Ten out of 13 changes involved guanine, consistent with a previous report that PhIP preferentially forms DNA adducts at guanines.\(^{21}\)

ACF are considered to be precancerous lesions because of their correlation with carcinogen exposure in animal models.\(^{20}\) Dysplastic ACF are also thought to be precursors of human colorectal carcinomas.\(^{12}\) In the present study, mutations were found in 30.4% (14 out of 46). Most (11 out of 14, 78.6%) were in codon 34, and involved guanine, as in adenomas/adenocarcinomas. While mutations in codons 47 and 56 have not been reported earlier to our knowledge, crypt cells could be transformed by an amino acid substitution of serine at codon 47, which might be an additional phosphorylation site, and the GAT (Asp)→TAT (Tyr) change at codon 56 could activate the \(\beta\)-catenin/Tcf signal transduction pathway by influencing phosphorylation or degradation by altering the complex tertiary structure.

Our finding that the \(\beta\)-catenin mutation rate was significantly higher in adenomas than in ACF \((P<0.001)\) or adenocarcinomas \((P<0.05)\) is consistent with the data of Samowitz et al.,\(^{13}\) showing a greater frequency in small colorectal adenomas than in larger adenomas and invasive carcinomas isolated from human patients. However, Suzui et al.\(^{13}\) found 3 of 4 adenomas (75%) but 15 of 16 adenocarcinomas (94%) to harbor \(\beta\)-catenin mutations. This discrepancy may be explained by the difference in initiating carcinogen, methylazoxymethanol acetate being applied in combination with 1-hydroxyanthraquinone in their study. We used PhIP, one of the heterocyclic amines, since it is contained in the diet,\(^{14}\) and this may better mimic the human case.

In conclusion, \(\beta\)-catenin mutations occurred in ACF and the existence of the same mutations in adenomas and adenocarcinomas suggests that they are early events during colorectal tumorigenesis. Various mutations, however, might have limited transforming potential as indicated by the findings (i) that some mutations in ACF were not found in tumors and (ii) that adenomas harbored \(\beta\)-catenin mutations more commonly than adenocarcinomas did. An explanation of this phenomenon requires further analysis of the transforming potential of individual \(\beta\)-catenin mutations.

**ACKNOWLEDGMENTS**

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, by Grants-in-Aid from CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation, by a grant from the Society for Promotion of Pathology of Aichi, Japan, and by Aichi Cancer Research Foundation.

(Received January 19, 2000/Revised April 17, 2000/2nd Revised May 22, 2000/Accepted June 5, 2000)
REFERENCES

1) Roncucci, L., Medline, A. and Bruce, W. R. Classification of aberrant crypt foci and microadenomas in human colon. Cancer Epidemiol. Biomarkers Prev., 1, 57–60 (1991).
2) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett., 37, 147–146 (1987).
3) Powell, S. M., Zitz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B. and Kinzler, K. W. APC mutations occur early during colorectal tumorigenesis. Nature, 359, 235–237 (1992).
4) Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Morris, P. J., Vogelstein, B. and Kinzler, K. W. Molecular determinants of dysplasia in colorectal lesions. Cancer Res., 54, 5523–5526 (1994).
5) Otori, K., Sugiyama, K., Hasebe, T., Fukushima, S. and Esumi, H. Emergence of adenomatous aberrant crypt foci (ACF) from hyperplastic ACF with concomitant increase in cell proliferation. Cancer Res., 55, 4743–4746 (1995).
6) Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science, 275, 1787–1790 (1997).
7) Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC−/− colon carcinoma. Science, 275, 1784–1787 (1997).
8) Takahashi, M., Fukuda, K., Sugimura, T. and Wakabayashi, K. β-Catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. Cancer Res., 58, 42–46 (1998).
9) Dashwood, R. H., Suzui, M., Nakagama, H., Sugimura, T. and Nagao, M. High frequency of β-catenin (ctnnb1) mutations in the colon tumors induced by two heterocyclic amines in the F344 rat. Cancer Res., 58, 1127–1129 (1998).
10) Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J. H., Sugimura, T. and Nagao, M. Specific 5′-GGGA-3′→5′-GGA-3′ mutation of the Apc gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Proc. Natl. Acad. Sci. USA, 92, 910–914 (1995).
11) Suzui, M., Ushijima, T., Dashwood, R. H., Yoshimi, N., Sugimura, T., Mori, H. and Nagao, M. Frequent mutations of the rat β-catenin gene in colon cancers induced by methylazoxymethanol acetate plus 1-hydroxyanthraquinone. Mol. Carcinog., 24, 232–237 (1999).
12) Kinzler, K. W. and Vogelstein, B. Lessons from hereditary colon cancer. Cell, 87, 159–170 (1996).
13) Samowitz, W. S., Powers, M. D., Sprioo, L. N., Nollet, F., van Roy, F. and Slattery, M. β-Catenin mutations are more frequent in small colorectal adenomas than in larger adenomas and invasive carcinomas. Cancer Res., 59, 1442–1444 (1999).
14) Tsukamoto, T., Kozaki, K., Nishikawa, Y., Yamamoto, M., Fukami, H., Inoue, M., Wakabayashi, K. and Tatematsu, M. Development and distribution of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced aberrant crypt foci in the rat large intestine. Jpn. J. Cancer Res., 90, 720–725 (1999).
15) Selton, L. S., Knize, M. G., Shen, N. H., Lewis, M. R., Andresen, B. D., Happe, J. and Hatch, F. T. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Carcinogenesis, 7, 1081–1086 (1986).
16) Nagao, M. and Sugimura, T. Carcinogenic factors in food with relevance to colon cancer development. Mutat. Res., 290, 43–51 (1993).
17) Tsukamoto, T., Takahashi, T., Ueda, R., Hibi, K., Saito, H. and Takahashi, T. Molecular analysis of the protein tyrosine phosphatase γ gene in human lung cancer cell lines. Cancer Res., 52, 3506–3509 (1992).
18) Swanson, S. M., Guzman, R. C., Tsukamoto, T., Huang, T. T., Dougherty, C. D. and Nandi, S. N-Ethyl-N-nitrosourea induces mammary cancers in the pituitary-isografted mouse which are histologically and genotypically distinct from those induced by N-methyl-N-nitrosourea. Cancer Lett., 102, 159–165 (1996).
19) Rosner, B. “Fundamentals of Biostatistics,” 4th Ed. (1995). Duxbury Press, Wadsworth Publ. Co., Belmont, CA.
20) Tatematsu, M., Masui, T., Fukami, H., Yamamoto, M., Nakanishi, H., Inada, K., Kusakabe, M. and Sakakura, T. Primary monoclonal and secondary polyclonal growth of colon neoplastic lesions in C3H/HeN→BALB/c chimeric mice treated with 1,2-dimethylhydrazine immunohistochemical detection of C3H strain-specific antigen and simple sequence length polymorphism analysis of DNA. Int. J. Cancer, 66, 234–238 (1996).
21) Nagaoka, H., Wakabayashi, K., Kim, S.-B., Kim, I.-S., Tanaka, Y., Ochiai, M., Tada, A., Nukaya, H., Sugimura, T. and Nagao, M. Adduct formation at C-8 of guanine on its phosphate esters. Jpn. J. Cancer Res., 83, 1025–1029 (1992).
22) Bird, R. P. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett., 93, 55–71 (1995).