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## Mutational Analysis of *Ctnnb1* and *Apc* in Tumors from Rats Given 1,2-Dimethylhydrazine or 2-Amino-3-methylimidazo[4,5-*f*]quinoline: Mutational ‘Hotspots’ and the Relative Expression of $\beta$ -Catenin and c-jun

Carmen A. Blum<sup>1</sup>, Tomoko Tanaka<sup>1</sup>, Xiaoying Zhong<sup>1</sup>, Qingjie Li<sup>1</sup>, Wan-Mohaiza Dashwood<sup>1</sup>, Clifford Pereira<sup>2</sup>, Meirong Xu<sup>1</sup>, and Roderick H. Dashwood<sup>1,\*</sup>

<sup>1</sup> Linus Pauling Institute, Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR

<sup>2</sup> Department of Statistics, Oregon State University, Corvallis, OR

### Abstract

There is growing interest in  $\beta$ -catenin and its role in various human cancers. We recently reported that 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)- and 1,2-dimethylhydrazine (DMH)-induced colon tumors in the rat contain mutations in *Ctnnb1*, the gene for  $\beta$ -catenin, but the mutation spectrum was influenced by postinitiation exposure to chlorophyllin (CHL) and indole-3-carbinol (I3C) [Blum et al., *Carcinogenesis* 2001;22:315–320]. The present paper describes a follow-up study in which all of the target organs for IQ- and DMH-induced tumorigenesis were screened; *Ctnnb1* mutations were found in 44 of 119 DMH-induced colon tumors, six of 13 IQ-induced colon tumors, 28 of 81 DMH-induced small intestine tumors, none of five IQ-induced small intestine tumors, four of 106 IQ-induced liver tumors, none of 14 DMH-induced Zymbal’s gland tumors, none of 24 IQ-induced Zymbal’s gland tumors, and none of 29 IQ-induced skin tumors. In tumors from rats given carcinogen alone, or carcinogen plus CHL or I3C, *Ctnnb1* mutations frequently substituted amino acids adjacent to Ser33, a critical Ser/Thr residue in the glycogen synthase kinase-3 $\beta$  regulatory domain of  $\beta$ -catenin. However, substitution of critical Ser/Thr residues themselves was detected in only three of 24 (12.5%) of the tumors from rats given carcinogen alone, compared with 23 of 58 (40%) of the tumors from rats given carcinogen and treated postinitiation with I3C or CHL ( $P < 0.02$ ). More than 50 of the colon tumors with wild-type  $\beta$ -catenin were examined further for their *Apc* status; the overall frequency of *Apc* mutations was  $< 10\%$ , and these genetic changes occurred exclusively in the ‘Mutation Cluster Region’ of *Apc*. A subset of colon tumors also was examined for expression of  $\beta$ -catenin and c-jun; these proteins were overexpressed in all tumors containing *Ctnnb1* mutations, but the expression was highest in tumors with *Ctnnb1* mutations affecting Thr41 and Ser45 residues in the glycogen synthase kinase-3 $\beta$  region of  $\beta$ -catenin. Thus, *Ctnnb1* mutations occurred more frequently than *Apc* mutations in colon and small intestine tumors of the rat, and certain mutations upregulated  $\beta$ -catenin/T-cell factor target genes more effectively than others, perhaps influencing the response to phytochemicals administered postinitiation.

### Keywords

*CTNNB1*; *APC*; Wnt signaling; TCF/LEF target genes; chlorophyllin; indole-3-carbinol

\*Correspondence to: Dashwood, Weniger Hall 503, Linus Pauling Institute, Oregon State University, Corvallis, OR 97331-6512.

## INTRODUCTION

There has been a surge of interest recently in the Wnt signaling pathway and its role in human cancer development [1–4]. Much of this interest evolved from studies of the molecular changes in oncogenes and tumor suppressor genes in colorectal and other human cancers [5]. In human colorectal cancers, the *APC* tumor suppressor gene is a common target for mutation, but colon tumors with wild-type *APC* typically have genetic changes in the human  $\beta$ -catenin gene (*CTNNB1*) [6–8]. The product of the latter gene,  $\beta$ -catenin, is a cadherin-binding protein involved in cell-cell adhesion [9–11], but also functions as a transcriptional activator when complexed in the nucleus with members of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family of proteins [1]. Control of the cytosolic levels of  $\beta$ -catenin occurs via a multi-protein complex, in which *APC*, axin/conductin, and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) negatively regulate  $\beta$ -catenin expression [1]. In this complex, GSK-3 $\beta$  phosphorylates key Ser/Thr residues in the N-terminal region of  $\beta$ -catenin and the protein is targeted for ubiquitination and proteosomal degradation [12]. In primary human colon tumors and colorectal cancer cell lines, mutations in *CTNNB1* substitute critical Ser/Thr residues in the GSK-3 $\beta$  region and stabilize  $\beta$ -catenin, leading to accumulation of  $\beta$ -catenin/TCF complexes in the nucleus [6–8], and activation of target genes, such as *c-MYC*, *c-JUN*, and *CYCLIN D1* [13–15].

Mutations in  $\beta$ -catenin also have been detected in colon tumors of animals treated with chemical carcinogens, such as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 1,2-dimethylhydrazine (DMH), azoxymethane, and methylazoxymethanol acetate plus 1-hydroxyanthraquinone [16–19]. Indeed, there are several similarities between human and rat colon tumors with respect to the  $\beta$ -catenin/*Apc* pathway. First, as in the human situation, colon tumors in the rat contain mutations in *Apc* or *Ctnnb1*, but not in both of these genes [17]. Second,  $\beta$ -catenin/TCF target genes frequently are overexpressed, including *c-myc*, *c-jun*, and cyclin D1 [16]. Third, genetic changes in human *CTNNB1* or murine *Ctnnb1* typically substitute amino acids within the N-terminal GSK-3 $\beta$  regulatory domain of  $\beta$ -catenin. However, one interesting species difference is in the spectrum of genetic changes in  $\beta$ -catenin. Thus, whereas the vast majority of  $\beta$ -catenin mutations in human colon cancers substitute critical Ser/Thr residues directly [6–8], in rat colon tumors a high frequency of the mutations substitute amino acids adjacent to key Ser/Thr residues [16–19]. For example, the combined data from studies in which rats were treated with azoxymethane, PhIP, IQ, or 1-hydroxyanthraquinone plus methylazoxymethanol acetate revealed only six of 35 tumors (17%) with mutations that substituted critical Ser/Thr residues directly, whereas 28 of 35 (80%) had genetic changes affecting residues immediately adjacent to Ser33 in  $\beta$ -catenin [19]. Moreover, these genetic changes were localized to two CTGGA sequences in close proximity to one another, suggesting the presence of possible mutational ‘hotspots’ in *Ctnnb1*.

To complicate matters further, the spectrum and frequency of  $\beta$ -catenin mutations can be influenced by other factors, including exposure to dietary phytochemicals. This was demonstrated in a recent study in which rats were given IQ or DMH and treated postinitiation with chlorophyllin (CHL) or indole-3-carbinol (I3C); in rats given carcinogen alone, virtually all of the mutations resulted in substitutions adjacent to Ser33, whereas in rats given carcinogen followed by CHL or I3C treatment, almost half of the mutations substituted critical Ser/Thr residues directly [16]. The latter report focused almost exclusively on the colon tumors, and suggested the need for further investigation of the other target organs, namely the small intestine, liver, skin, and Zymbal’s gland [20]. Therefore, the present study sought to provide a comprehensive analysis of  $\beta$ -catenin mutations in the major target organs for DMH- and IQ-induced tumorigenesis in the rat. In addition, a subset of colon tumors containing wild-type  $\beta$ -catenin was examined further in order to determine the mutation status of *Apc*. Finally, the relative expression of  $\beta$ -catenin and  $\beta$ -catenin/TCF target genes was assessed as a function of

each specific codon affected (i.e., amino acid substituted) within the GSK-3 $\beta$  regulatory region of  $\beta$ -catenin. The hypothesis was that certain *Ctnnb1* mutations might activate  $\beta$ -catenin/TCF target genes more effectively than others, thus influencing the response to phytochemicals administered postinitiation.

## MATERIALS AND METHODS

### Source of Tumors

Tumors were from a 1-yr study in which male F344 rats were initiated with DMH or IQ during the first 5 wk of the experiment, and 1 wk later the animals were treated with CHL or I3C until termination. Further details, including histopathological findings, were presented in the original report [21]. For information on doses of the test compounds and their routes of administration, see Table 1.

### Mutation Screening and Sequencing

Tissue samples were extracted with DNAzol genomic isolation reagent (Molecular Research Center, Inc., Cincinnati, OH), and initially a 150-bp region corresponding to the GSK-3 $\beta$  region of  $\beta$ -catenin was amplified by the polymerase chain reaction (PCR), as described before [16, 17]. Previous studies of carcinogen-induced rat colon tumors established that mutations in  $\beta$ -catenin were localized exclusively to the GSK-3 $\beta$  region, and no genetic changes were detected in the armadillo region or C-terminal domain [16–19,22]. Nonetheless, for tumors that lacked a mutation in the GSK-3 $\beta$  region we performed comprehensive screening of all 14 exons and the intron-exon boundaries of the entire *Ctnnb1* gene, with the PCR primers and conditions reported elsewhere [16,17,22]. In addition, colon tumors with wild-type  $\beta$ -catenin were screened for mutations in *Apc*, as detailed previously [17,20,23]. In brief, single-strand conformation polymorphism (SSCP) analysis was conducted by means of the GenePhor electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ). Six ng of DNA were loaded per well, run at 15°C, 600 V, 25 mA, and 15 W for 1.5 h, using the optimal conditions for detecting  $\beta$ -catenin or *Apc* mutations, based on preliminary studies at different temperatures and in the presence and absence of glycerol [16,22,23]. The gel was silver stained, and bands with altered migration were cut from the gel, reamplified, and cleaned using Wizard PCR Preps (Promega, Madison, WI). All of the samples reported here to contain mutations were confirmed by sequencing in both directions on an ABI Prizm™ 3.3 sequencer.

### Reverse Transcriptase-PCR (RT-PCR)

The Micro-FastTrack 2.0 kit (Invitrogen, Carlsbad, CA) was used to isolate mRNA from freshly thawed tissues, and cDNA was synthesized via the Promega RT system. Primers and PCR conditions for the genes of interest, namely *cyclin D1*, *c-myc*, and *c-jun*, were as reported previously [16].

### Western Blotting

Tissues were homogenized at room temperature in RIPA lysis buffer (Qiagen, Valencia, CA) and centrifuged at 14,000 g for 2 min through the QIAshredder (Qiagen). Fifteen to twenty micrograms of protein were run on a 4–12% Bis-Tris Gel (Invitrogen) and transferred onto nitrocellulose membrane. After overnight blocking, the membrane was incubated for 1 h with primary antibody, washed, incubated with secondary antibody conjugated with horse-radish peroxidase (BioRad), and developed using ECL reagents 1 and 2 (Amersham Corp.). Equal loading and transfer was confirmed by amido black staining and by comparison with glyceraldehyde-3-phosphate dehydrogenase. Sources and dilutions of the antibodies used, as well as other experimental details, were reported previously [16,24]. Imaging and

quantification of the blots was performed on an AlphaInnotech photodocumentation system (AlphaInnotech Corp., San Leandro, CA).

### Statistical Analyses

Comparisons were made among treatment groups, and for specific 'hotspots' within  $\beta$ -catenin, by means of the Fisher's Exact Test and ANOVA followed by Fisher's PLSD, respectively.

## RESULTS

DNA was successfully amplified from a total of 391 tumors, and *Ctnnb1* mutations were screened by SSCP analysis and confirmed by direct sequencing (Figure 1 and Table 1). All of the mutations were localized within the GSK-3 $\beta$  region of  $\beta$ -catenin, coded by exon 2 of rat *Ctnnb1*, which corresponds to exon 3 of human *CTNNB1* [22].

In all but one case, primers designed to amplify the region encoding the GSK-3 $\beta$  domain of  $\beta$ -catenin gave a single PCR product of 150 bp. One IQ-induced liver tumor gave two PCR products, one with the expected size of 150 bp and the second approximately 50 bp shorter (Figure 1a). Bands with shifted mobility in the SSCP gel (Figure 1b) were removed and sequenced, revealing an in-frame deletion originating in codon 32 and extending through codon 49 (Figure 1c). This deletion would be expected to remove all four of the critical Ser/Thr residues within the GSK-3 $\beta$  regulatory domain, without affecting other important domains in  $\beta$ -catenin, such as the armadillo repeats that bind TCF, LEF, Apc, and other protein partners [1].

Typical SSCP results are shown in Figure 1d; single base pair substitutions were detected in codons 32, 33, 34, 37, 41, and 45 of *Ctnnb1*. Sequencing data for the more common mutations in this region of *Ctnnb1* have been reported previously [16].

Table 1 summarizes the results for each specific target organ examined, namely colon, small intestine, liver, Zymbal's gland, and skin. In the colon tumors from groups given DMH or IQ, the overall frequency of  $\beta$ -catenin mutations was 44/119 (37%) and 6/13 (46%), respectively. In the small intestine tumors induced by DMH, 28/81 (35%) harbored mutations in  $\beta$ -catenin, whereas none of the IQ-induced small intestine tumors had such mutations, perhaps due to the small sample set available for analysis (none of five). Screening of more than 100 IQ-induced liver tumors revealed four cases of  $\beta$ -catenin mutation (4/106 = 3.8%; Table 1). The mutation screening also included 14 DMH-induced Zymbal's gland tumors, 24 IQ-induced Zymbal's gland tumors, and 29 IQ-induced skin tumors, but mutations in  $\beta$ -catenin were detected in none of these tumors. Thus, taken together, 82 of 391 (21%) of the tumors examined here had mutations in  $\beta$ -catenin, with low or undetectable frequencies in liver, skin, and Zymbal's gland tumors, but higher frequencies in colon and small intestine tumors.

Inspection of the data in Table 1 reveals that G  $\rightarrow$  A transitions were common within two CTGGA sequences around codons 31–34 (45/82 = 56%). Of the remainder, many involved C  $\rightarrow$  T transitions in codons 33, 37, 41, and 45 (21/37 = 57%). The findings are consistent with the known preference of IQ and DMH for forming adducts with guanine bases, leading to a preponderance of G  $\rightarrow$  A or C  $\rightarrow$  T transitions (66/82 = 80%).

Colon tumors with wild-type  $\beta$ -catenin were screened further for their Apc status. None of the IQ-induced colon tumors examined had mutations affecting Apc (none of five), consistent with prior studies that showed few *Apc* mutations in IQ-induced colon tumors, but a relatively high frequency of such mutations in colon tumors initiated by PhIP [20]. In groups given DMH, the frequency of Apc mutations was as follows: DMH alone, none of five; DMH + CHL, none of four; DMH + I3C, five of 43. In the latter group, four of five of the mutations involved a C  $\rightarrow$

T transition, three of which produced a premature stop codon and one a change from A to V. One tumor had a G → A transition that did not change the corresponding amino acid (Q) (Table 2). Genetic changes were detected only in the central Mutation Cluster Region of *Apc*, and no mutations were found elsewhere in *Apc*, such as the N-terminal armadillo repeat region or the C-terminal basic domain [1]. For all groups combined, the overall frequency of mutations in *Apc* was five of 57 (8.8%).

Previously, colon tumors from rats given carcinogen alone were compared with those from animals given carcinogen plus CHL or I3C, and this revealed a shift in the  $\beta$ -catenin mutation spectrum [16]. After completing the mutational analysis of all target organs of IQ- and DMH-induced tumorigenesis in the present study, the shift in the spectrum of  $\beta$ -catenin mutations was equally striking (Figure 2). Thus, in the tumors from rats given carcinogen alone, most of the amino acid substitutions were immediately adjacent to Ser33 (21 of 24 = 87.5%), and there were only three cases in which a critical Ser/Thr residue was substituted directly, two affecting Ser33 and one substituting Ser45 (three of 24 = 12.5%). However, in groups given IQ or DMH and posttreated with I3C or CHL, 23 of 58 (40%) of the tumors had mutations that substituted critical Ser/Thr residues directly. The increased frequency of mutations affecting critical Ser/Thr residues was highly significant following phytochemical treatment ( $P < 0.02$ ).

An important observation from our previous study was that the expression of  $\beta$ -catenin/TCF target genes appears to be higher in colon tumors with certain  $\beta$ -catenin mutations versus others [16]. Specifically, five colon tumors with wild-type  $\beta$ -catenin had low or undetectable levels of c-jun and c-myc, three tumors with mutations in codons 32 or 34 of *Ctnnb1* had intermediate expression, and two tumors with mutations in codon 41 of *Ctnnb1* (i.e., directly substituting a critical Thr41 residue in  $\beta$ -catenin) had high expression levels of these proteins. In the present investigation, as before [16], RT-PCR analyses confirmed the high expression of *cyclin D1*, *c-myc*, and *c-jun* in colon tumors with  $\beta$ -catenin mutations (data not presented). Consequently, the relative expression of  $\beta$ -catenin and c-jun proteins was examined in greater detail for each specific codon of *Ctnnb1* affected; triplicate samples were analyzed in each case in order to generate data that was amenable to statistical analysis. Representative blots are shown for three tumors with mutations in codon 32 and three tumors with mutations in codon 41 of *Ctnnb1*, as well as three tumors containing wild-type  $\beta$ -catenin (Figure 3, inset).  $\beta$ -Catenin and c-Jun proteins were elevated in all tumors with *Ctnnb1* mutations, but they were fourfold to sixfold higher in tumors with mutations in codons 32, 33, 34, or 37 compared with eightfold to tenfold higher in tumors with mutations in codons 41 or 45 ( $P < 0.05$ ).

In the case of the small intestine tumors from this study, many were of a size that allowed for histological examination and mutation screening but not Western blotting. However, for samples that were amenable to immunoblot analysis,  $\beta$ -catenin and c-Jun proteins were expressed at highest levels when mutations involved codons 41 and 45 of *Ctnnb1* (data not presented). Results for colon and small intestine tumors suggested that mutations in *Ctnnb1* may not have been functionally equivalent, and that certain mutations might have activated  $\beta$ -catenin/TCF target genes more effectively than others. Future studies will examine the protein expression by immunohistochemical methods to determine which population of cells (e.g., anaplastic cells versus less anaplastic cells) are associated with these changes in  $\beta$ -catenin and its downstream targets.

## DISCUSSION

This report describes the most comprehensive screening to date of  $\beta$ -catenin mutations in the major target organs of DMH- and IQ-induced tumorigenesis in the rat, and provides the first attempt to quantify the relative expression in vivo of  $\beta$ -catenin and one of its target genes, *c-jun*, according to the specific site of mutation in *Ctnnb1*. The results confirm and extend

previous work in which  $\beta$ -catenin mutants are expressed by transient transfection in cultured cells and are shown to activate gene expression in a reporter assay in vitro [25]. However, data from the present in vivo study revealed that  $\beta$ -catenin and c-jun proteins were more highly elevated in tumors with mutations affecting codons 41 and 45 compared with tumors harboring other types of mutation in *Cttnb1* (Figure 3). The significance of this finding might relate to the recent discovery that GSK-3 $\beta$  does not phosphorylate all four Ser/Thr residues in  $\beta$ -catenin to the same extent, and that a 'priming' kinase (possibly casein kinase-1) is required for initial phosphorylation of Ser45 [26]. It is believed that GSK-3 $\beta$  subsequently phosphorylates other Ser/Thr residues in a sequential fashion (Thr41  $\rightarrow$  Ser37  $\rightarrow$  Ser33); thus, a mutation in either codon 45 or codon 41 of *Cttnb1* would likely interfere with phosphorylation of multiple Ser/Thr residues in  $\beta$ -catenin, and thereby effectively interfere with subsequent ubiquitination and proteosomal degradation [12].

These results also might help to explain the response to phytochemicals given postinitiation. Screening of colon and small intestine tumors from rats treated with carcinogen alone showed hotspots for mutation around codon 33, and a high preponderance of the substitutions affected residues on either side of Ser33 (Figure 2). Virtually none of the tumors from rats given DMH or IQ alone had mutations affecting Ser37, Thr41, or Ser45 of  $\beta$ -catenin, yet these mutations were quite common in tumors from rats given carcinogen and treated with I3C or CHL postinitiation.

Our current working hypothesis is that mutations affecting Thr41 and Ser45 so severely upregulate  $\beta$ -catenin expression and  $\beta$ -catenin/TCF target genes that the cells normally respond by undergoing early apoptosis (i.e., these cells would be deleted from the intestinal mucosa rather than progressing to tumors). However, chronic exposure to exogenous agents, including certain phytochemicals, might interfere in the apoptotic mechanisms, thereby enabling a population of cells to progress to tumor formation that might otherwise be deleted by programmed cell death. Inhibition of apoptosis is an important mechanism driving colorectal cancer development in humans [27], as well as in animals treated with chemical carcinogens such as IQ [28], and chemo-preventive agents that are effective against colon cancer frequently enhance the rate of apoptosis in the colonic mucosa [29]. In this context, any phytochemical that overrides the apoptotic mechanism according to the hypothesis stated above would be predicted to act as a tumor promoter rather than a chemopreventive agent, although this could be influenced by the dose administered and the duration of postinitiation exposure. It is noteworthy, therefore, that most of the mutations in Ser37, Thr41 and Ser45 were from I3C and CHL groups in which tumor promotion, not suppression, was previously reported [21]. Whether natural chlorophyll as opposed to synthetic CHL, or I3C as it is present in cruciferous vegetables, acts as a tumor promoter under the same experimental conditions remains to be determined, but a pilot study showed suppression rather than promotion of colonic aberrant crypts by natural chlorophyll [30]. It is also noteworthy that in the original study [21], CHL acted in a concentration-dependent manner to suppress IQ-induced liver tumors. The ability of CHL and I3C to promote in one tissue but suppress in another might be related to the relative importance of the  $\beta$ -catenin signaling pathway in those tissues, but further study is required in order to confirm this hypothesis.

One apparent discrepancy between the working hypothesis and the results presented here is the relatively high frequency of mutations affecting Ser37 (I3C and CHL groups, Figure 2) and the 'intermediate' expression of  $\beta$ -catenin and c-jun proteins in tumors bearing those mutations (Figure 3). It is possible that other target genes of  $\beta$ -catenin/TCF signaling may be more strongly overexpressed, such as *c-myc*, cyclin D1, peroxisome proliferator activator receptor- $\delta$ , or matrix metalloproteinase-7 [1], but additional studies are necessary. Nonetheless, the findings from the present investigation clearly indicated that the spectrum of  $\beta$ -catenin mutations in tumors can be influenced by exogenous factors, including certain phytochemicals

in the diet. This is consistent with the well-established relationship between diet and cancer, and the overall importance of gene-diet interactions in determining human cancer risk [31–34].

In summary, this report described the mutational analysis of *Ctnnb1* in the major target organs of DMH- and IQ-induced tumorigenesis in the rat. Mutations were found at higher frequency in the small intestine tumors and colon tumors, and at low or undetectable frequency in liver, skin, and Zymbal's gland tumors. More than 50 of the colon tumors with wild-type  $\beta$ -catenin also were screened for genetic changes in *Apc*; the overall frequency of such mutations was <10%, and they were localized to the Mutation Cluster Region of *Apc*.  $\beta$ -catenin and c-jun proteins were more strongly overexpressed in tumors with mutations affecting codons 41 and 45 of *Ctnnb1* compared with other mutations in  $\beta$ -catenin. Against this background of genetic changes, phytochemicals such as CHL and I3C might select for or against specific  $\beta$ -catenin mutations, and thereby act as suppressing agents or tumor promoters in different tissues [35, 36].

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#### Abbreviations

<b><i>APC</i></b>	human adenomatous polyposis coli gene
<b><i>Apc</i></b>	murine <i>Apc</i> gene
<b><i>CTNNB1</i></b>	human $\beta$ -catenin gene
<b><i>Ctnnb1</i></b>	murine $\beta$ -catenin gene
<b>TCF</b>	T-cell factor
<b>LEF</b>	lymphocyte enhancer factor
<b>GSK-3<math>\beta</math></b>	glycogen synthase kinase-3 $\beta$
<b>DMH</b>	1,2-dimethyl-hydrazine
<b>IQ</b>	2-amino-3-methylimidazo[4,5- <i>f</i> ]quinoline
<b>PhIP</b>	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i> ]pyridine
<b>CHL</b>	chlorophyllin

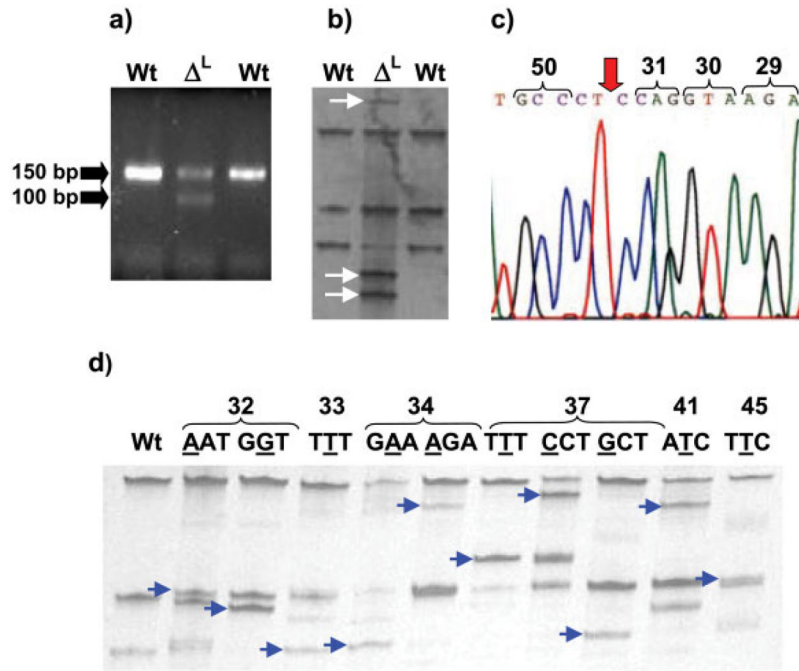
<b>I3C</b>	indole-3-carbinol
<b>PCR</b>	polymerase chain reaction
<b>SSCP</b>	single-strand conformation polymorphism

## References

- Polakis P. Wnt signaling and cancer. *Genes Dev* 2000;14:1837–1851. [PubMed: 10921899]
- Oving IM, Clevers HC. Molecular causes of colon cancer. *Eur J Clin Invest* 2002;32:448–457. [PubMed: 12059991]
- Clements WM, Wang J, Sarnaik A, et al.  $\beta$ -Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. *Cancer Res* 2002;62:3503–3506. [PubMed: 12067995]
- Lustig B, Jerchow B, Sachs M, et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* 2002;22:1184–1193. [PubMed: 11809809]
- Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 1997;386:761–763. [PubMed: 9126728]
- Morin PJ, Sparks AB, Korinek V, et al. Activation of  $\beta$ -Catenin-Tcf signaling in colon cancer by mutations in  $\beta$ -Catenin or APC. *Science* 1997;275:1787–1790. [PubMed: 9065402]
- Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/ $\beta$ -Catenin/Tcf pathway in colorectal cancer. *Cancer Res* 1998;58:1130–1134. [PubMed: 9515795]
- Ilyas M, Tomlinson IP, Rowan A, Pignatelli M, Bodmer WF.  $\beta$ -Catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci USA* 1997;94:10330–10334. [PubMed: 9294210]
- Hajra KM, Fearon ER. Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer* 2002;34:255–268. [PubMed: 12007186]
- Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest* 2002;109:987–991. [PubMed: 11956233]
- Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998;153:333–339. [PubMed: 9708792]
- Aberle H, Bauer A, Stappert J, Kispert A, Kemler R.  $\beta$ -catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* 1997;16:3797–3804. [PubMed: 9233789]
- He T-C, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. *Science* 1998;281:1509–1512. [PubMed: 9727977]
- Mann B, Gelos M, Siedow A, et al. Target genes of  $\beta$ -catenin-T-cell factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci USA* 1999;96:1603–1608. [PubMed: 9990071]
- Tetsu O, McCormick F.  $\beta$ -Catenin regulates expression of cyclin D1 in colon carcinoma cell. *Nature* 1999;398:422–426. [PubMed: 10201372]
- Blum CA, Xu M, Orner GA, et al.  $\beta$ -Catenin mutation in rat colon tumors initiated by 1,2-dimethylhydrazine and 2-amino-3-methylimidazo[4,5-f]quinoline, and the effect of post-initiation treatment with chlorophyllin and indole-3-carbinol. *Carcinogenesis* 2001;22:315–320. [PubMed: 11181454]
- Dashwood RH, Suzui M, Nakagama H, Sugimura T, Nagao M. High frequency of  $\beta$ -catenin (Ctnnb1) mutations in the colon tumors induced by two heterocyclic amines in the F344 rat. *Cancer Res* 1998;58:1127–1129. [PubMed: 9515794]
- Takahashi M, Fukuda K, Sugimura T, Wakabayashi K.  $\beta$ -catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Res* 1998;58:42–46. [PubMed: 9426055]

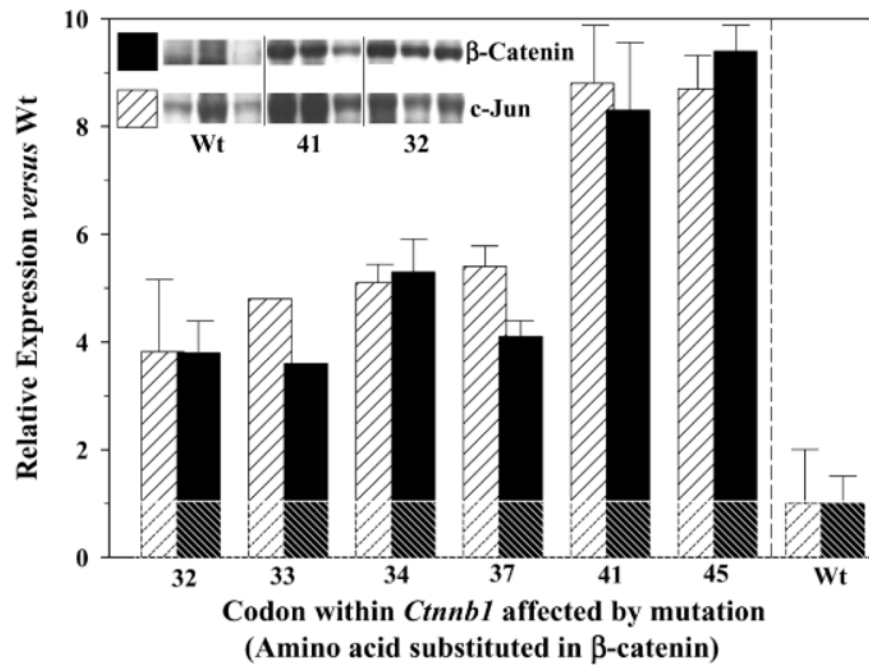


19. Suzui M, Ushijima T, Dashwood RH, et al. Frequent mutations of the rat  $\beta$ -catenin gene in colon cancers induced by methylazoxymethanol acetate plus 1-hydroxyanthraquinone. *Mol Carcinog* 1999;24:232–237. [PubMed: 10204808]
20. Nagao M, Ushijima T, Toyota M, Inoue R, Sugimura T. Genetic changes induced by heterocyclic amines. *Mutat Res* 1997;376:161–167. [PubMed: 9202752]
21. Xu M, Orner GA, Bailey GS, Stoner GD, Horio D, Dashwood RH. Post-initiation effects of chlorophyllin and indole-3-carbinol in rats given 1,2-dimethylhydrazine or 2-amino-3-methylimidazo[4,5-f]quinoline. *Carcinogenesis* 2001;22:309–314. [PubMed: 11181453]
22. Li Q, Dixon BM, Al-Fageeh M, Blum CA, Dashwood RH. Sequencing of the rat  $\beta$ -catenin gene (*Ctmb1*) and mutational analysis of liver tumors induced by 2-amino-3-methylimidazo[4,5-f]quinoline. *Gene* 2002;283:255–262. [PubMed: 11867232]
23. Suzui M, Ushijima T, Yoshimi N, et al. No involvement of APC gene mutations in ulcerative colitis-associated rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate. *Mol Carcinog* 1997;20(4):389–393. [PubMed: 9433483]
24. Orner GA, Dashwood W-M, Blum CA, Díaz D, Li Q, Dashwood RH. Suppression of tumorigenesis in the *Apc*<sup>min</sup> mouse: Down-regulation of  $\beta$ -catenin signaling by a combination of tea plus sulindac. *Carcinogenesis* 2003;24:263–267. [PubMed: 12584176]
25. Porfiri E, Rubinfeld B, Albert I, Hovanes K, Waterman M, Polakis P. Induction of a  $\beta$ -catenin-LEF-1 complex by wnt-1 and transforming mutants of  $\beta$ -catenin. *Oncogene* 1997;15:2833–2839. [PubMed: 9419974]
26. Hagen T, Vidal-Puig A. Characterisation of the phosphorylation of  $\beta$ -catenin at the GSK-3 priming site Ser45. *Biochem Biophys Res Commun* 2002;294:324–328. [PubMed: 12051714]
27. Bedi A, Pasricha PJ, Akhtar AJ, et al. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995;55:1811–1816. [PubMed: 7728743]
28. Hayashi R, Luk H, Horio D, Dashwood R. Inhibition of apoptosis in colon tumors induced in the rat by 2-amino-3-methylimidazo[4,5-f]quinoline. *Cancer Res* 1996;56:4307–4310. [PubMed: 8813112]
29. Chan TA. Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol* 2002;3:166–174. [PubMed: 11902503]
30. Blum CA, Xu M, Orner GA, et al. Promotion versus suppression of rat colon carcinogenesis by chlorophyllin and chlorophyll: Modulation of apoptosis, cell proliferation, and  $\beta$ -catenin/Tcf signaling. *Mutat Res*. 2003in press
31. Go VL, Wong DA, Butrum R. Diet, nutrition and cancer prevention: Where are we going from here? *J Nutr* 2001;131:3121S–3126S. [PubMed: 11694657]
32. Rock CL, Lampe JW, Patterson RE. Nutrition, genetics, and risks of cancer. *Annul Rev Public Health* 2000;21:47–64. [PubMed: 10884945]
33. Patterson RE, Eaton DL, Potter JD. The genetic revolution: Change and challenge for the dietetics profession. *J Am Diet Assoc* 1999;99:1412–1420. [PubMed: 10570679]
34. Mathers JC, Burn J. Nutrition in cancer prevention. *Curr Opin Oncol* 1999;11:402–407. [PubMed: 10505780]
35. Dashwood RH. Indole-3-carbinol: Anticarcinogen or tumor promoter in brassica vegetables? *Chem Biol Interact* 1998;110:1–5. [PubMed: 9566721]
36. Dashwood RH. Modulation of heterocyclic amine-induced mutagenicity and carcinogenicity: an 'A-to-Z' guide to chemopreventive agents, promoters, and transgenic models. *Mutat Res* 2002;511:89–112. [PubMed: 12052429]



**Figure 1.** Single-strand conformation polymorphism (SSCP) screening and sequencing of exon 2 of rat *Ctnnb1*, the region that corresponds to exon 3 of human *CTNNB1*, and codes for the GSK-3 $\beta$  domain of  $\beta$ -catenin [22]. (a) Ethidium bromide-stained agarose gel and (b) SSCP gel, showing two IQ-induced liver tumors that were wild-type (wt) and a third with a deletion (middle lane,  $\Delta^L$ ); (c) sequencing of  $\Delta^L$  with the reverse primer revealed an in-frame deletion involving codons 32 to 49; (d) representative SSCP patterns for the more common mutations in IQ- and DMH-induced tumors; bands shown with arrows were removed and sequenced. For examples of typical sequencing results, see [16].





**Figure 3.**

Expression in rat colon tumors of  $\beta$ -catenin (filled bars) and c-jun (hatched bars). Total tissue lysates were prepared from three separate colon tumors for each of the codons mutated in *Ctnnb1*, and these were subjected to SDS-polyacrylamide gel electrophoresis and probed with antibodies to  $\beta$ -catenin and c-Jun proteins, as reported before [16]. Inset, representative blots from three tumors containing wild-type (wt)  $\beta$ -catenin, three tumors with mutations in codon 41 of *Ctnnb1*, and three tumors with mutations in codon 32 of *Ctnnb1*. Normal colonic mucosa and positive controls for the proteins of interest were included in each gel (not shown). Densitometry measurements were obtained with a gel documentation system and associated software; results were first normalized to expression levels of glyceraldehyde-3-phosphate dehydrogenase and then expressed relative to the protein levels in tumors with wild-type  $\beta$ -catenin (assigned an arbitrary expression value of 1.0). Data represent mean  $\pm$  SD,  $n = 3$  (except for codon 33, which was from a single tumor). The horizontal line is a reference guide indicating the relative expression of 1.0 for c-jun and  $\beta$ -catenin proteins in tumors with wt  $\beta$ -catenin; all of the bars above this line were significantly different from wt controls, and the bars labeled 41 and 45 were significantly different from all others ( $P < 0.05$ ).

**Table 1**  
Summary of  $\beta$ -Catenin Mutations in DMH and IQ Tumors (All Target Organs\*)

Codon <sup>†</sup> →	31	32	33	34	37	41	45	Totals <sup>‡</sup>
Group ↓	CTG	GAT	TCT	GGA	TCT	ACC	TCC	
<b>COLON TUMORS</b>								
DMH alone		<u>AAT</u> (4 <sup>§</sup> )	TIT(2)	G <u>AA</u> (4)				10/30
+0.1% CHL		<u>AAT</u> (2)			TIT			3/13
+0.01% CHL		<u>GGT</u> (2), <u>AAT</u>				ATC		4/9
+0.001% CHL		<u>AAT</u> (2)			TIT(3)	<u>AIC</u> (3)	<u>TIC</u> (2)	10/20
+0.1% I3C		<u>AAT</u> (2)		GAA (2)		ATC	<u>TIC</u>	6/19
+0.01% I3C		<u>AAT</u> , <u>GGT</u>		GAA (3)		ATC		6/12
+0.001% I3C		<u>GGT</u> (3)			TIT(2)			5/16
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IQ alone				GAA				1/3
+0.1% CHL		<u>GIT</u>						1/1
+0.01% CHL		<u>GIT</u>						1/2
+0.001% CHL		<u>AAT</u>			<u>CCT</u>			2/3
+0.1% I3C								0/0
+0.01% I3C								1/1
+0.001% I3C					<u>GCT</u>			1/3
<b>SMALL INTESTINE TUMORS</b>								
DMH alone		<u>AAT</u> (8)		GAA(4)			<u>TIC</u>	13/30
+0.1% CHL				GAA				1/2
+0.01% CHL				GAA				1/6
+0.001% CHL								0/7
+0.1% I3C		<u>GGT</u>	TIT	GAA(6), <u>AGA</u>	TIT			10/22
+0.01% I3C		<u>GIT</u>						1/5
+0.001% I3C				GAA	TIT			2/9
IQ - all groups								0/5
<b>LIVER TUMORS</b>								
IQ alone								0/16
+0.1% CHL								0/7
+0.01% CHL		<sup>32</sup> G <u>Δ</u> AG <sup>49</sup>						1/16
+0.001% CHL								0/20
+0.1% I3C								0/14
+0.01% I3C				GAA	<u>TGT</u>			2/14
+0.001% I3C				AGA				1/19
<b>ZYMBAL'S GLAND TUMORS</b>								
DMH - all groups								0/14
IQ - all groups								0/24
<b>SKIN TUMORS</b>								
IQ -all groups								0/29
<b><math>\beta</math>-CATENIN MUTATIONS/TOTAL TUMORS SCREENED</b>								<b>82/391</b>

\* Rats were initiated with carcinogen for 5 wk; IQ was given in the diet (AIN-93) at a concentration of 0.03%, whereas DMH was injected s.c. once/wk (20 mg/kg body wt). Starting one wk after the last carcinogen treatment until the end of the study at 1 yr, rats were given CHL in the drinking water or I3C in the diet, at concentrations shown in the table. For details on the pathology of each tumor type, see [21].

<sup>†</sup> Only codons affected by mutation are shown (except codon 31, in gray, to highlight the two CTGGA sequences commonly affected, dotted boxes).

<sup>‡</sup> Number of mutations confirmed by sequencing/total tumors successfully amplified.

<sup>§</sup> Total number of tumors with a mutation in the codon indicated.

// All groups combined for each carcinogen within the target organ shown. Colon tumor data were from a previous report [16].

**Table 2**

## Summary of Apc Mutations in Colon Tumors\*

Codon affected	Treatment	Nucleotide change detected	Consequence
1094	DMH + 0.001% I3C	CAA → <u>T</u> AA	Q → stop
1149	DMH + 0.1% I3C	CAG → <u>T</u> AG	Q → stop
1234	DMH + 0.001% I3C	CAA → <u>T</u> AA	Q → stop
1307	DMH + 0.1% I3C	GCC → <u>G</u> TC	A → V
1722	DMH + 0.001% I3C	CAG → <u>C</u> AA	Q → Q

\* Colon tumors that contained wild-type  $\beta$ -catenin (Table 1) were screened further for mutations in the *Apc* gene. Genetic changes were identified exclusively within the region spanning nt 1094–1722, which encompasses the so-called ‘Mutation Cluster Region’ of *Apc* [1].