

Genetic Analysis of Colon Tumors Induced by a Dietary Carcinogen PhIP in CYP1A Humanized Mice: Identification of Mutation of β -catenin/Ctnnb1 as the Driver Gene for the Carcinogenesis

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Replacing mouse *Cyp1a* with human *CYP1A* enables the humanized CYP1A mice to mimic human metabolism of the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), by N²-hydroxylation to a proximate carcinogen. Our previous study demonstrated that PhIP, combined with the dextrin sulfate sodium (DSS)-induced colitis, induces colon carcinogenesis in hCYP1A mice. Here, we employed whole exome sequencing and found multiple gene mutations in PhIP/DSS-induced colon tumors. Mutations in the exon 3 of *Ctnnb1*/ β -catenin, however, were the predominant events. We further sequenced the key fragments of *Apc*, *Ctnnb1*, and *Kras*, because mutations of these genes in the humans are commonly found as the drivers of colorectal cancer. Mutations on either codon 32 or 34 in the exon 3 of *Ctnnb1* were found in 39 out of 42 tumors, but no mutation was found in either *Apc* or *Kras*. The sequence context of codons 32 and 34 suggests that PhIP targets +3G in a TGGA motif of *Ctnnb1*. Since mutations that activate Wnt signal is a major driving force for human colorectal cancers, we conclude that the mutated β -catenin is the driver in PhIP/DSS-induced colon carcinogenesis. This result suggests that the colon tumors in hCYP1A mice mimic human colorectal carcinogenesis not only in the dietary etiology involving PhIP, but also in the aberrant activation of the Wnt signaling pathway as the driving force. © 2014 Wiley Periodicals, Inc.

Key words: colorectal cancer; carcinogenesis; exon sequencing; driver mutation

INTRODUCTION

Colorectal cancer (CRC) is among the top three cancers in both women and men that cause morbidity and mortality. In the US, about 150 000 new cases are diagnosed every year. Even though the survival rate for CRC has improved during the last decade, about one third of patients die from this disease, which accounts for about 10% of cancer associated death [1]. The conversion of normal intestinal epithelial cells to microadenoma and adenocarcinoma, and further progression to metastatic cancer involves a series of activation of oncogenes and inactivation of tumor suppressors [2]. The prominent genetic events include mutations in key components of the Wnt signaling (e.g. APC), the proliferation signaling (e.g. PI3K, AKT, K-ras), the TGF β signaling, and DNA damage response and repair (e.g. p53, mismatch repair MMR genes MMRs) [2]. About 90% human CRCs are found harboring dominant active mutations in the Wnt signaling pathway components; among them, mutations in APC gene account for the majority (~70–80%) [2]. An alternative to APC mutation is the active mutation of β -catenin/CTNNB1 gene, which is found in \leq 5% human CRCs but at a much higher frequency

in hereditary nonpolyposis colorectal cancer (HNPCC) [3]. Mutations in other components (e.g. AXIN1, AXIN2, Dickkopf-1) that lead to the activation of Wnt signaling are also reported [2]. Recent study on human CRC using the next generation sequencing technology further documents the importance of these mutations in human CRC [4]. Epigenetic alterations are also demonstrated to play important roles in colorectal carcinogenesis [5]. These alterations, especially the genetic mutations, are

Abbreviations: AOM, azoxymethane; CRC, colorectal cancer; CYP, cytochrome P450; DSS, dextrin sulfate sodium; MCR, mutation cluster region; MNU, N-methyl-N-nitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

Grant sponsor: National Institutes of Health; Grant numbers: RO1CA120915; RO1 CA122474; RO1 CA133021; Grant sponsor: National Institutes of Health; Grant numbers: CA72720; ES05022

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Received 19 March 2014; Revised 23 May 2014; Accepted 6 June 2014

DOI 10.1002/mc.22199

Published online 17 August 2014 in Wiley Online Library (wileyonlinelibrary.com).

recognized for their roles in the different stages of CRC development [6]. For instance, APC plays the gatekeeper role in normal colon epithelial cells and the loss of APC results in the activation of Wnt signaling and the subsequent imbalance of cell proliferation over cell death that leads to the earlier lesion such as microadenoma [7]. Overall, the findings of these disease-associated genetic/epigenetic events reveal molecular mechanism of colorectal carcinogenesis, and these events provide potential biomarkers for diagnosis and prognoses as well as potential targets for therapy.

It is well recognized that the risk of cancer is associated with life style and environment factors [8–10]. Diet and nutrition are estimated to be related to up to 50% of human CRC worldwide [10]. Some dietary factors are known to have carcinogenic activity. One group of such chemical is heterocyclic amines (HCAs), which are found in cooked meat and fish [11,12]. HCAs are listed in the US Department of Health and Human Services (2005) as chemical “reasonably anticipated to be a human carcinogen”. Human exposure to HCAs is in a wide range (0.1–12 µg/d) [13]. PhIP is the most common and the most abundant HCAs [13,14]. Daily intake of PhIP by Americans is estimated to be 280–460 ng/d per person [15].

HCAs are metabolically activated in vivo to proximate and ultimate carcinogens. In human, PhIP as well as other HCAs are mainly metabolized through N-hydroxylation by cytochrome P450 (CYP) 1A2 and then conjugated by N-acetyltransferase or sulfotransferase [12,16,17]. The acetoxo or sulfate metabolite is spontaneously converted to aryl nitrenium ion (R-NH⁺), which can react with DNA to form adduct at the 8-position carbon of deoxyguanine base, dG-C8-PhIP. In human colon samples, dG-C8-PhIP is detected at approximately 3 adducts per 10⁸ nucleotides or about 20 adducts per cells [18]. Indeed, a fast acetyltransferase polymorphic trait of N-acetyltransferase NAT2 (i.e. NAT2*4) has been associated with an increased risk of CRC [19]. PhIP-DNA adduct was also reported in human prostate [20]. In the experimental studies using rodents, PhIP-DNA adduct has been found in different organs (e.g. liver, colon, prostate, lung) [12,13,17,21,22]. In mouse, PhIP is mainly metabolized by Cyp1a2 through 4'-hydroxylation, which leads to detoxification [16], and a higher dose of PhIP is required to induce tumor and the induction is independent of the activation of PhIP by Cyp1a2 [23]. Thus, mouse is not a suitable experimental model to mimic PhIP-induced carcinogenesis in humans. To overcome this limitation, we have used the hCYP1A mice, in which the mouse *Cyp1A* gene was replaced by human *CYP1A* gene [24,25] and demonstrated that PhIP induces colon and prostate carcinogenesis, recapitulating several key features of human cancers [26,27]. Given the fact that high temperature cooked meat and fish are very common in the western diet, the PhIP-induced colon cancer in hCYP1A mice

would share the dietary etiology of certain types of human CRC.

In this study, we used exon-sequencing technology and selected fragments for Sanger sequencing to analyze the genetic alterations in the exome of the PhIP-induced colon tumors. Our result demonstrated β -catenin/*Ctnnb1* gene as the predominant target of PhIP, and the observed mutations of this gene are expected to activate the Wnt signaling pathway which drives colon carcinogenesis.

MATERIALS AND METHODS

Chemicals

PhIP-HCl was purchased from Wake Chemicals USA, Inc. (Richmond, VA) and dissolved in milliQ water. Dextran sulfate sodium (DSS; molecular weight 35 000–44 000) was purchased from MP Biomedicals (Solon, OH) and dissolved in milliQ water to 1.2% (w/v).

Animals and Procedures

Humanized CYP1A mice (hCYP1A), carrying human *CYP1A* gene to replace mouse *Cyp1A* gene, were generated as described previously [26]. All animal procedures were in accordance with the animal study protocol (No. 02-027) approved by the Rutgers University Institutional Animal Care and Use Committee. Mice were maintained in a sterile room of animal facility under standard 12 h light/12 h dark cycle with water and diet provided ad libitum unless otherwise specified. At the age of week 5, mice were switched from a normal chow diet to the AIN93M diet (Research Diets, New Brunswick, NJ) throughout the experimental study and body weights were measured weekly. At week 6, the mice were treated with a dose of PhIP (200 mg/kg body weight) by oral gavage. After 1 wk, the mice were administered 1.5% (w/v) DSS in drinking water for a week. During this time period, mice were monitored daily for changes in body weight and rectal bleeding. Mice were then returned to regular drinking water and continuously fed on AIN93M diet without any other treatment until sacrifice at 10 wk after the PhIP treatment. After mice were sacrificed by CO₂ asphyxiation, the colon was excised, flushed with saline, the length measured, cut longitudinally, and flattened on filter paper. The number, size, and location of visible tumors were recorded under dissecting microscope. The tumors were dissected under dissecting microscope and stored in RNAlater solution (Qiagen, Valencia, CA) at –80°C. After the tumors were removed, the mucosal epithelium layer in the descending colon were separated from the muscle layer by a plastic scratcher and stored in RNAlater solution to serve as the normal matching control.

DNA/RNA Extraction and Exome Sequencing

To extract total RNA and genomic DNA from colon tumors and its matching normal colon tissue, the

tissues stored in RNAlater were homogenized in the lysis buffer of AllPrep DNA/RNA Mini Kit (Qiagen) using Omni Bead Raptor 24 (Omni International, Kennesaw, GA). DNA and RNA were then purified according to the manufacturer protocol. The quality of DNA and RNA were examined by gel electrophoresis.

For genetic analysis, the medium size tumors from two mice were used: two tumors and one matching normal control samples for each mouse. Exome sequencing of these six samples was performed using Agilent Mouse Exome Sequencing Kit on the Applied Biosystems SOLiD system done by Shanghai Bio Co. at USA (North Brunswick, NJ). The captured sequence was then analyzed by Extensible Genomics Toolbox of DNAnexus (Mountain View, CA).

PCR and DNA Sanger Sequencing

The N-terminal half (6 kb) of the coding sequence of *Apc* was amplified by RT-PCR in five 1.2–1.5 kb overlapping fragment using RNA samples with SuperScript First-Strand reverse transcription kit (Life Technologies, Grand Island, NY) and Advantage 2 PCR kit (Clontech, Mountain View, CA). *Apc* fragment (equal to position 3600 bp to 5600 bp in coding region) containing mutation cluster region (MCR), *Ctnnb1* exon 3, and *Kras* exon 1 were directly amplified using genomic DNAs with Advantage 2 PCR kit (Clontech). The sequence information of all primers is listed in Table 1. The amplified products were separated by electrophoresis and then purified using the QIAquick Gel Extraction Kit (Qiagen). The purified products were sequenced using Sanger sequence method from both directions with PCR primers by Genewiz Inc. (South Plainfield, NJ).

RESULTS

Somatic Genetic Signature of PhIP/DSS-Induced Colon Tumor as Determined by Exome Sequencing

We sequenced the exome of four PhIP-induced colon tumors and two corresponding nontumorous colon tissue samples as controls. These samples were from two randomly selected male mice: one mouse had four tumors and the other had seven tumors. We took two tumors and one control from each mouse. The carcinogenesis experiment consisted of 10 mice treated with PhIP and DSS, and the average tumor number in this experiment was about four, which was in the normal range [26]. The tumors at this stage were tubular adenocarcinoma, showing tubular architecture featured with the surface epithelial dysplasia that extends downward in the base, serrated crypt lumens with stratified pencil-like nuclei in some crypts and nuclear atypia in the majority of the tumor cells, as well as some microinvasive features, as described in our previous report [26]. We completely sequenced 4.3–6.310⁹ bases, and over 90% of reads were mapped to the mm9 mouse (*Mus musculus*) genome (<http://genome.ucsc.edu>) for all samples (Table 2). These results covered completely over 98.2% exons; for each sample, only about 1.2% exons were partial covered and only less than 0.2% exons were not covered (Table 2). The 14 exons in *Cyp1a* (*Cyp1a1* and *1a2*) gene, which were knocked out in this transgenic mouse, were completely not detected in all six samples, whereas the exons of adjacent genes were well fully covered (Supplemental Figure S1). This data support the high confidence of this exome sequencing result.

Table 1. Sequence Information of Primers Used in PCR to Characterize *Apc*, *Ctnnb1*, and *Kras*

Gene and Target Region		PCR Primers		Product Size (bp)
<i>Apc</i>				
mRNA -30 to 1333	Forward	GTCTGGCAGGTCCAAGGGTA		1356
	Reverse	CTGATGCTCAACAGGAGCTG		
mRNA 1200 to 2600	Forward	TCCGAGTCCTTCATCTTTTGAAC		1470
	Reverse	TTTCTGTTGTTGGATGGTAAGCACTGAGGC		
mRNA 2450 to 3900	Forward	TGACTGTTCTTTCACCATATTTAAATACTA		1338
	Reverse	CTTCTGCTGTCTGCAGAGTATTAG		
mRNA 3600 to 5000*	Forward	CATCAGCACAAAGCACTAAACCTG		1474
	Reverse	CCAATTCATTTGGAGGGGACTCTA		
mRNA 4850 to 6135	Forward	CAGCACAGAATAGGCTGCAGGCAC		1568
	Reverse	GGCAGAACTTATACACTCCTGTAACA		
<i>Ctnnb1</i>				
Partial intron 2 - exon 3		CTAACATACTCTGTTTTACAGCTGAC		250
		CAGCTACTTGCTCTTGCGTGA		
<i>Kras</i>				
Exon 1 (G12 region)		GCCTGCTGAAAATGACTGAG		116
		ATCGTAGGGTCATACTCATCCAC		

*Since this fragment contains MCR (~3750–4500 bp), this set of primers were also used to amplify genomic DNA for Sanger sequence.

Table 2. Whole-Exome Sequencing summary

	Mouse No.1			Mouse No. 2		
	Control	Tumor 1	Tumor 2	Control	Tumor 1	Tumor 2
Total bases	4,356,463,400	4,814,310,600	6,367,888,800	4,816,666,800	4,973,358,000	6,003,544,400
Total read	43,564,634	48,143,106	63,678,888	48,166,668	49,733,580	60,035,444
MAPed read	40,706,535	45,059,045	58,463,734	44,731,090	46,600,323	55,529,103
% of MAPed	93%	93.59%	91.81%	92.87%	93.70%	92.49%
Exon coverage						
Completely	204,240	204,863	204,990	204,665	205,045	204,978
Partial	3,117	2,625	2,560	2,811	2,482	2,587
Unsequenced	443	312	250	324	273	235

By examining the tumor-normal pairs, we identified 1110 to 1482 somatic mutations, including nucleotide substitutions, insertions, and deletions in the exome (Table 3). Among them, over 80% were single nucleotide substitutions. The majority of mutations were heterozygous. There were more mutations on G/C than on A/T. The mutation rates in the exon area in tumors were 20–27 per 10^6 bases. According to the exome data of human CRCs [4], tumors bearing mutation rates over 8.2 per 10^6 bases are considered as hypermutated; thus, PhIP/DSS-induced tumors are hypermutated tumors. In terms of protein coding, these mutations included 695–885 non-synonymous mutations and 23–31 reading frame shifts; a few of them are expected to be cancer-drivers, while the others are the passenger mutations.

Common Mutations in PhIP/DSS-induced Tumors

We identified a group of 43 genes carrying non-synonymous mutations or reading frame shift in all four tumors (Supplemental Table S1). Most of these genes carry multiple mutations in each tumors. In total, these 43 genes carried 120 non-synonymous mutations or reading frame shift in each tumors. Since it has been suggested that cancer driver gene

does not need more than one alteration to initiate/promote cancer [28], it is likely that most of these genes were mutated by PhIP but not drivers or mutated at the later stages of carcinogenesis. There were five genes (*Fam129a*, *Fcnb*, *V1rc1*, *Zbtb2*, and *Dusp14*) in which only one mutation was observed in each tumor. According to the literature, however, *Zbtb2* and *Dusp14* have only minimum involvement in signaling regulation, and no involvement in carcinogenesis. Mutation frequencies of these genes in human CRCs are also extremely low [4]. Therefore, it is less likely that these mutations play significant roles in PhIP/DSS-induced colon carcinogenesis. We next reduced the searching creatio to the genes having mutations in “at least in three tumors” and identified additional 43 genes carrying non-synonymous mutations or reading frame shift in at least three tumors (Table 4). Within this group, there were 26 genes that carry no more than one mutation in each tumor. Among these genes, *Ctnnb1*, *Hoxa10*, and *Maml3* are potential candidates as the driver genes. Mutant *Ctnnb1* is a well-documented cancer driver, especially in CRC [2]. Deregulated expression of *Hoxa10* has been reported in gastric cancer [29]. *Maml3* is essential for Notch signaling [30], which plays critical role in the development of human CRC. No literature

Table 3. Genetic Mutations in the Whole-Exome of PhIP-Induced Colon Tumors

	Mouse 1		Mouse 2	
	Tumor 1	Tumor 2	Tumor 1	Tumor 2
In coding DNA sequence	1110	1198	1321	1482
Single nucleotide substitution	927	991	1155	1276
Multiple nucleotide substitution	126	158	121	144
Insertion	29	23	26	31
Deletion	28	26	19	31
Deletion in GC	23	23	13	28
Mixed	0	0	0	0
Heterozygous	963	1059	928	1046
Mutation in A or T	428	492	549	607
Mutation in G or C	702	752	800	906
In Codon				
Synonymous	357	412	517	534
Non-Synonymous	695	722	748	885
frameshift	23	30	24	31

Table 4. Genes Carrying Non-Synonymous Mutation in Three Tumors

Chromosome No.	Gene	Numbers of Variations			
		Mouse 1		Mouse 2	
		Tumor 1	Tumor 2	Tumor 1	Tumor 2
chr1	Rrs1	1	6		4
chr1	Ptpv	1		4	4
chr2	Crb2	1	1		1
chr2	Fmn1	1		1	1
chr2	Sirpa	3		2	3
chr2	Nsfl1c	4		2	1
chr2	Olf1261		1	2	3
chr2	Mageb3		2	1	1
chr3	Gm5150	1	1	1	
chr3	Med12l	1	1	1	
chr3	Maml3	1		1	1
chr4	Ftl2	1		3	2
chr5	Vmn2r14	1	1	1	
chr5	Speer4b		1	1	1
chr6	Olf213	1	3	1	
chr6	Hoxa10	1	1		1
chr6	Zfml	1		1	1
chr6	Ppp4r2		1	2	2
chr6	Prp2		1	5	3
chr7	Hbb-b1	1		2	1
chr7	Hbb-b2	1		2	1
chr7	Lrrc68		1	1	1
chr8	Csmd1	1	1	1	
chr8	Thap1	1		1	1
chr8	Cdh11	1		1	1
chr8	Cdt1	2		1	1
chr9	Ddx6	2	1	1	
chr9	Crtap	1	1	1	
chr9	Mfrp	1		1	1
chr9	Ctnnb1	1		1	1
chr9	Apoa4		4	3	1
chr9	Atr		2	1	1
chr11	Nlrp1a	2	1		3
chr11	Olf463	1	1		1
chr11	Elac2	1		1	1
chr11	Cog1		1	1	1
chr12	Syne2	1	1		1
chr13	Heatr1	1	1		1
chr13	Nlrp4f	2	1		3
chr14	Vmn2r89	4	1		1
chr14	Mycbp2	1	1		1
chr15	Larp4		1	1	1
chr15	Krt81		1	1	1
chr16	Cd200r2	4		2	1
chr17	Vmn2r115	3	3	2	
chr17	Slc22a7		1	1	1
chr19	Cyp2c39	1	2		1
chr19	Cyp2c50		1	1	1
chrX	Las1l	2	1	2	
chrX	Diap2	1	2	1	
Total (Genes)	43				
Total (Mutations)		44	42	48	41

supports the role of other genes involved in carcinogenesis.

We then focused on the location of mutations. We found 29 mutations in the coding regions of 19 genes

that lead to non-synonymous substitution or reading frame shift in all four tumors (Supplemental Table S2). As expected, these common mutations were among the genes identified in Supplemental Table S1; but

they localized in the genes carrying multiple mutations, suggesting that these mutations are not the drivers. Then, we again reduced the searching critic to the genes having mutations in “at least in three tumors” and identified 61 mutations in the coding regions of 41 genes (Supplemental Table S3). The majority of mutations in this list were in the genes that were identified in Supplemental Table S1 and Table S4. The only mutation with known impact in carcinogenesis is a common mutation in *Maml3* found in three tumors. However, this mutation was a deletion of a CAG from a stretch of ~70 CAG repeats, which is unlikely to significantly impact the function of *Maml3* protein. Thus, to narrow the searching list by mutation position did not provide more insightful information. These common mutations may represent the fragile sites in mouse genome when mice were treated in our experimental conditions.

Nevertheless, the above analyses on the exomes of four tumors reveal that three out of four tumors carry somatic mutations in *Ctnnb1*, of which the role is well recognized in CRC. The mutations were on codon 32 in tumor one and three, and codon 34 in tumor four. The amino acid residues encoded by codon 32 and 34 are the sites mediating the recognition and binding of ubiquitin ligase; mutations on codons 32 and 34 display stronger transformation activity than other mutations, such as codon 33 [31,32]. These mutations have been reported in human CRC [2,33,34]. Thus, somatic mutation in *Ctnnb1* is the candidate for the driver event in PhIP/DSS-induced colon carcinogenesis in hCYP1A mice.

Fragment Sequencing Reveals the Dominant Active Mutations in *Ctnnb1* in Majority of PhIP/DSS-induced Tumors

Above, we identified the dominant activation mutations in *Ctnnb1* in three out four PhIP/DSS-induced tumors. However, we did not find any alteration in other well-known CRC genes such as *APC* and *Kras*. To validate this result in more tumors, we next selected 11 mice (six males and five females) from two experiments, dissected all visible tumors (a total of 42, including the four samples used for exome sequencing) and the adjacent normal control epithelial tissues. The DNA samples were used for Sanger-sequencing to examine the key regions of *Ctnnb1*, *Apc*, and *Kras* genes, of which human counterparts are CRC drivers. We focused on the N-terminal half of the coding sequence of *Apc*, because human *APC* gene mutations are predominately in the N-terminal half and the majority of somatic mutations locate in the so-called mutation cluster region (MCR) within N-terminal half [2,33,35]. For *Ctnnb1*, we focused on Exon 3, encoding the motif consisting of phosphorylation and ubiquitin ligase recognition sites, in which mutations cause dominant active Wnt signaling and are frequently found in human CRC [2,33,34]. For *Kras*, we focused on exon 1 containing codon 12 of

which the mutation is a well-characterized oncogenic event in a wide range of human cancers including CRC [2]. The sequencing results in 42 tumors plus 11 matching controls showed that there was no mutation in *Apc* and *Kras*, but there were high frequent mutations on *Ctnnb1* codons 32 and 34. In 42 tumors, we found mutations on *Ctnnb1* in 39 tumors, no mutation in two tumors and inconclusive for one tumor (Table 5). Among the 39 mutant *Ctnnb1* tumors, there were 36 tumors carrying only one mutation, on either codon 32 and 34; while two tumors carrying both codon 32 and 34 mutations. All mutations on codons 32 and 34 are on G. On codon 32, there was no preference in the conversion of G to A/C/T resulting in the substitution of Asp by Asn, Tyr or His; whereas, on codon 34, the majority of mutation was the conversion of G to T, resulting in the substitution of Gly to Val (Figure 1A). Presumably, cells carrying the oncogenic events resulted from these substitutions acquired growth advantage. The context sequences in codon 32 and 34 imply that it is the +3 G substitution in a TGGA motif (Figure 1B). Overall, oncogenic mutations on *Ctnnb1* were found in the majority (>92%) of PhIP/DSS induced colon tumors in hCYP1A mice.

Interestingly, Sanger sequencing result revealed that all four tumor samples used for exome sequencing carry *Ctnnb1* codon 32 and 34 mutations, although exome sequence analysis result indicated no mutations in tumor No. 3. We examined the original exome sequencing data in tumor No. Three for *Ctnnb1*, and found that this tumor actually carried codon 34 mutation, but the frequency of the DNA fragment carrying this mutation was low and it was recognized as false signal by analysis software (Supplemental Figure S2). Indeed, this mutation, a conversion of GG to TT in exome sequence, was also true in Sanger sequencing result (Table 5). The Sanger sequencing result, however, could not determine whether the substitution was a GGA to TTA or a mixture of GGA to GTA and TGA (a stop codon). The re-examination of original exome sequencing data excludes the possibility that the mutation of codon 34 generated a Stop codon.

There were three tumors, tumors 2 and 4 in mouse No.7 and tumor 1 in mouse No. 11, carrying more than one mutations (Table 5). Possibly, they were tumors that appeared as single tumors but consisted of tumor cells from different progenies in nearby location. Tumor 4 in mouse 1 carried C to T mutation on codon 33. This mutation might not be induced by PhIP since PhIP-DNA adduct is only on G. Because the DSS-induced colitis is necessary for this model, it is possible that mutation on codon 33 was caused by oxidative stress associated with colitis.

In addition, we sequenced the regions where the mutations of *Dusp14* and *Zbtb2* locate in 20 tumors in 5 male mice plus the adjacent normal colonic epithelium. For *Dusp14*, the results of 20 tumors

Table 5. Mutations in *Ctnnb1* Codons 32, 33, and 34 in PhIP-Induced hCYP1A Mouse Colon Tumors

Mouse No. and Sex	DNA ID	Codon 32		Codon 33		Codon 34	
		Mutation*	AA change	Mutation	AA change	Mutation	AA change
1 (M)	Control ^{†, ‡}			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2 [‡]	GAT→ <u>C</u> AT	Asp→His			GGA→ <u>I</u> I A [§]	Gly→Leu/stop/Val [§]
	Tumor 3 [‡]						
2 (M)	Tumor 4			TCT→T <u>A</u> T	Ser→Tyr		
	Control [†]			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2					GGA→G <u>I</u> A	Gly→Val
	Tumor 3 [‡]	GAT→ <u>A</u> AT	Asp→Asn				
	Tumor 4	GAT→ <u>I</u> AT	Asp→Tyr				
	Tumor 5	GAT→ <u>I</u> AT	Asp→Tyr				
3 (M)	Tumor 6	GAT→ <u>A</u> AT	Asp→Asn				
	Tumor 7 [‡]					GGA→G <u>I</u> A	Gly→Val
	Control			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
4 (M)	Tumor 2	GAT→ <u>A</u> AT	Asp→Asn			GGA→G <u>I</u> A	Gly→Val
	Tumor 3						
	Control			wt			
5 (M)	Tumor 1	GAT→ <u>I</u> AT	Asp→Tyr				
	Tumor 2	GAT→ <u>I</u> AT	Asp→Tyr				
	Tumor 3	GAT→ <u>I</u> AT	Asp→Tyr				
6 (M)	Tumor 4					GGA→ <u>C</u> GA	Gly→Arg
	Control			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2			wt			
7 (F)	Tumor 3	GAT→ <u>A</u> AT	Asp→Asn				
	Control			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2	GAT→ <u>I</u> / <u>A</u> AT	Asp→Tyr/Asn				
	Tumor 3	GAT→ <u>A</u> AT	Asp→Asn				
8 (F)	Tumor 4	GAT→ <u>A</u> AT	Asp→Asn			GGA→G <u>I</u> A	Gly→Val
	Tumor 5					GGA→G <u>I</u> / <u>A</u> A	Gly→Val/Glu
	Control			wt			
	Tumor 1	GAT→ <u>I</u> AT	Asp→Tyr				
9 (F)	Control			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2	GAT→ <u>A</u> AT	Asp→Asn				
	Tumor 3	GAT→ <u>C</u> AT	Asp→His				
	Tumor 4	GAT→ <u>A</u> AT	Asp→Asn				
	Tumor 5	GAT→ <u>A</u> AT	Asp→Asn				
	Tumor 6			nd			
	Tumor 7	GAT→ <u>C</u> AT	Asp→His				
10 (F)	Tumor 8	GAT→ <u>C</u> AT	Asp→His				
	Control			wt			
	Tumor 1					GGA→G <u>I</u> A	Gly→Val
	Tumor 2					GGA→G <u>I</u> A	Gly→Val
11 (F)	Tumor 3			wt			
	Control	wt					
	Tumor 1	GAT→ <u>C</u> AT	Asp→His			GGA→G <u>I</u> A	Gly→Val
	Tumor 2					GGA→G <u>I</u> A	Gly→Val

M-male; F-female.

*The mutated base is underlined.

†All controls were the DNAs extracted from the normal adjacent colon epithelial tissues of the same mice.

‡These samples were also used for exome sequencing.

§This mutation could generate a few possible amino acid substitution.

||There was no enough DNA or bad quality of DNA extracted for sequencing.

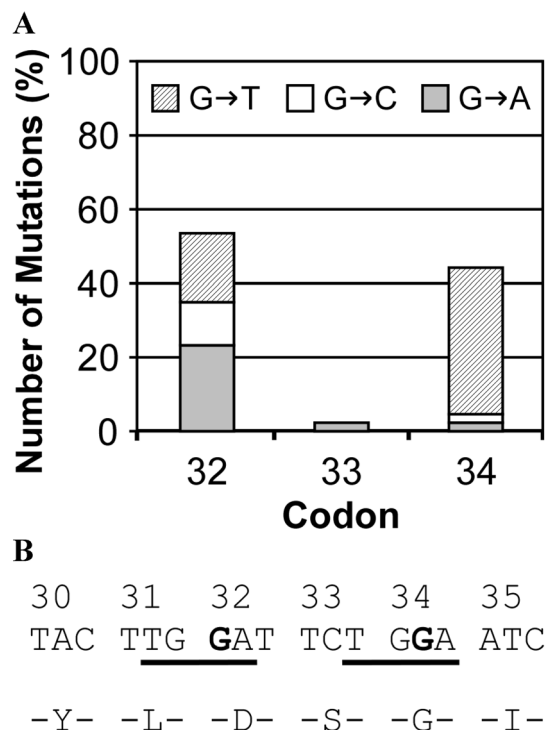


Figure 1. Summary of the mutations on the codon 32, 33 and 34 of *Ctnnb1* in the PhIP-induced colon tumors in hCYP1A mice. (A) The nucleotide substitution/mutation in codon 32–34. (B) The nucleotide and amino acid sequences of *Ctnnb1* from codon 30 to 35. The common nucleotide sequences context in codon 32 and 34 are underlined, and the mutated positions are in bold.

and 5 matching controls showed that there was no mutation (Supplemental Table S4), indicating that the *Dusp14* mutation found by exome-sequencing is a sequencing error. For *Zbtb2*, the result showed that the A to C mutation found in exome-sequencing were in all 20 tumors and 5 normal controls, suggesting that the hCYP1A mice used in our experiment carry a single nucleotide polymorphism (Supplemental Table S5) and this “mutation” is not associated with the PhIP/DSS-induced carcinogenesis. Indeed, the original exome-sequencing data showed that the sequencing coverage in this region of *Zbtb2* in two normal control samples was very low (by only one sequenced oligonucleotide) and the C in the control samples was recognized as the sequencing error because the reference genome (mm9) is A at this position; thus, the C in 4 exome-sequenced tumors were picked as the mutation.

DISCUSSION

Mutations in *APC* and *CTNNB1*, resulting active Wnt signaling, are the most common events in the initiation of human CRC [2,6]. In this study, we characterized PhIP/DSS-induced colon tumors in hCYP1A mice using exon sequencing and revealed that the activation mutation in *Ctnnb1* is the most prevalent event. Mutations in *Ctnnb1* are also found

in azoxymethane (AOM)/DSS-induced colon tumors in rodents [36,37]. AOM also induces mutations in *Kras* in rats (~70%) and mice (0–10%) as well as mutations in *Apc* in rats (~8%) [36]. The progression of tumorigenesis in our model is compatible to that of DSS treatment-induced colon tumor development in *Apc^{min/+}* mouse; in which adenocarcinoma is induced in colon in about 5 wk [38]. In the PhIP/DSS treated hCYP1A mice, colon adenocarcinoma also develops in about 5 wk after DSS treatment [26]. This similarity suggests that PhIP treatment initiates colon carcinogenesis by attenuating the “gate-keeper” role of a controlled Wnt signaling.

Previous studies using different models established several potential mechanism of PhIP-induced carcinogenesis. Using cultured cells and rats, it was found that PhIP metabolite attacks DNA, forms dG-C8-PhIP adduct, and subsequently induces G to C mutation or G deletion, which was suggested to result in the expression of truncated APC protein or dominant active mutant *Ctnnb1* and lead to the activation of Wnt signaling [39–42]. Notably, a -1G deletion in a G stretch such as 5'-GGGA-3' in *Apc* gene was found in 4 out of 8 PhIP-induced F344 rat colon tumors causing reading frame shift and truncation [43]. Such a -1G deletion was also reported in PhIP-induced rat mammary gland tumor [42]. Mutations in *Ctnnb1* gene were also reported in PhIP-induced rat and mouse colon tumors. An earlier report found *Ctnnb1* gene mutations in 4 out of 7 PhIP-induced F344 rat colon tumor on codons 32, 34, 35, and 37 [44]. A more detailed study showed PhIP treatment in F344 rats results in *Ctnnb1* mutations in 30% of aberrant crypt foci (ACF), 100% of adenoma (7 out of 7), and 50% of adenocarcinoma (6 out of 12); the mutations included those on codons 32, 33, and 34 that are found in human CRC and on codons 45, 47, and 56 that are not known in human CRC [45]. However, induction of tumor in rats require repeat intake of higher doses of PhIP (0.04% in diet for 40 wk) [46]. A single dose of PhIP (i.e. 200 mg/kg BW) combined with DSS-induced colitis was reported to induce colon tumors in mouse [47,48]. By targeted sequencing the exon 3 of *Ctnnb1*, mutations on codon 32 and 34 were found in 7 out of 7 PhIP/DSS-induced CD-1 mouse tumors [48]. In the present study using exome-sequencing, a genome-wide approach, we did not find any G deletion in the sequenced region of *Apc* which was found in the PhIP-induced rat colon tumors. The overall incident with base deletion was very low (Table 3). Thus, the G-deletion may not play a significant role in PhIP-induced colon carcinogenesis in hCYP1A mice. Regarding *Ctnnb1* mutations, we found mutations in over 92% of hCYP1A mouse colon tumors, consistent with previous finding that the activation mutations in *Ctnnb1* are high frequent events in the PhIP/DSS-induced CD-1 mouse tumors [48].

In our study, a single dose of PhIP (200 mg/kg BW) followed by 7 d of 1.5% DSS treatment in the hCYP1A

mice resulted in a tumor incidence of 100% with an average of 6 tumors per mouse [26]. The same treatment in the wildtype C57BL/6J did not produce any tumor in 24 wk [26]; this result is different from the induction of low frequent tumorigenesis by the same dose of PhIP in the wildtype CD-1, C57BL/6J, and MSM/Ms mice without the human CYP1A gene reported previously [47,48]. The reason for this discrepancy is not known; it may be related to the dose of DSS and the diet used. In combination, these results suggest that the human CYP1A transgene in hCYP1A mice enhanced the activation of PhIP to the ultimate carcinogen, but it does not alter the mutation spectrum (i.e. mutations in *Cttnb1*).

The consumption of high temperature cooked meat and fish containing PhIP and other HCA compounds increases the CRC risk [10,49,50]. PhIP-DNA adducts are commonly found in human colon biopsy samples [51]. Our result provides a molecular mechanism supporting the importance of PhIP in initiating human CRC. However, to what degree that PhIP contribute to human CRC or other cancers still needs be evaluated. First, active mutations of β -catenin/CTNNB1 are found in $\leq 5\%$ of human CRCs [2,34], and the mutations on codons G32 and G34 of *Cttnb1* found in the present study may be similar to those in this type of CRC. Second, colitis-associated CRCs which the DSS-induced colitis models [52,53] are mainly driven by p53 mutation and loss of heterozygosity (LOH), found in ~ 50 – 80% human cases; in contrast, APC associated events are much less frequent ($<33\%$) and seems not to be the earlier driver events [54]. Data of CTNNB1 mutation in colitis-associated CRC is not available because the prevalence is very low. It suggests that only a small fraction of human CRCs is caused by PhIP. Thus, the etiological role of PhIP in human CRC should be carefully evaluated with specific context. PhIP-DNA adducts are also found in other organs in human such as prostate [20,55] and the level is associated with the consumption of grilled meat [20,55]. In a rat model, PhIP-DNA adducts are also found in several organs such as colon, liver, pancreas, and lung [12,13,17,21,22]. Therefore, it is reasonable to suggest that the intake of PhIP and other HCAs could induce the similar mutation in other organs. CTNNB1 mutations were reported in 5% prostate cancer [56], 16–54% endometrial ovarian cancer [57], 3–44% hepatocellular carcinoma [58], 16–38% adrenal tumors [59], and 85% desmoid tumors [60]. CTNNB1 was also found as one of the 13 significant mutated genes in a recent genome wide sequencing study on 183 lung cancers and its mutation seems not to co-occur with KRAS mutation [61]. We recently reported that PhIP induces prostate lesions (i.e. high-grade prostatic intraepithelial neoplasia) in hCYP1A mice [27]. Thus, it is highly likely that PhIP also induces carcinogenesis in other organs.

In summary, our study revealed that over 92% of PhIP/DSS-induced colon tumors in hCYP1A mice carry the dominant active mutation in *Cttnb1* gene. This result demonstrated that PhIP/DSS-induced colon carcinogenesis in hCYP1A mice is caused by the aberration of the gate-keeper of Wnt signaling, a cancer driver event in majority of human CRCs. The results indicate that our model in hCYP1A mice mimics human colorectal carcinogenesis is not only in the dietary etiology involving PhIP, but also in the aberrant activation of the Wnt signaling pathway as the driver.

ACKNOWLEDGMENT

We thank Dr. Guangxun Li for technical support and discussion. This work was supported by the National Institutes of Health (RO1CA120915, RO1CA122474 and RO1CA133021) as well as the Shared facilities funded by CA72720 and ES05022.

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