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SYSTEMATIC REVIEWS

Integrative analysis of aberrant Wnt signaling in hepatitis B virus-related hepatocellular carcinoma

Shan-Long Ding, Zi-Wei Yang, Jie Wang, Xiao-Lei Zhang, Xiang-Mei Chen, Feng-Min Lu

Shan-Long Ding, Zi-Wei Yang, Jie Wang, Xiao-Lei Zhang, Xiang-Mei Chen, Feng-Min Lu, Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China
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Correspondence to: Feng-Min Lu, PhD, Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, China. lu.fengmin@bjmu.edu.cn
Telephone: +86-10-82805136
Fax: +86-10-82805136
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Abstract

AIM: To comprehensively understand the underlying molecular events accounting for aberrant Wnt signaling activation in hepatocellular carcinoma (HCC).

METHODS: This study was retrospective. The HCC tissue specimens used in this research were obtained from patients who underwent liver surgery. The Catalogue of Somatic Mutations in Cancer (COSMIC) database was searched for the mutation statuses of *CTNNB1*, *TP53*, and protein degradation regulator genes of *CTNNB1*. Dual-luciferase reporter assay was performed with TOP/FOP reporters to detect whether *TP53* gain-of-function (GOF) mutations could enhance the transcriptional activity of Wnt signaling. Methylation sensitive restriction enzyme-quantitative PCR was used to explore the methylation status of CpG islands located in the promoters of *APC*, *SFRP1*, and *SFRP5* in HCCs with different risk factors. Finally, nested-reverse transcription PCR was performed to examine the integration of *HBx* in front of *LINE1* element and the existence of *HBx-LINE1* chimeric transcript in Hepatitis B virus-related HCC. All results in this article were analyzed with the software SPSS version 19.0 for Windows, and different groups were compared by χ^2 test as appropriate.

RESULTS: Based on the data from COSMIC database, compared with other solid tumors, mutation frequency of *CTNNB1* was significantly higher in HCC ($P < 0.01$). The rate of *CTNNB1* mutation was significantly less frequent in Hepatitis B virus-related HCC than in other etiologies ($P < 0.01$). Dual-luciferase reporter system and TOP/FOP reporter assays confirmed that *TP53* GOF mutants were able to enhance the transcriptional ability of Wnt signaling. An exclusive relationship between the status of *TP53* and *CTNNB1* mutations was observed. However, according to the COSMIC database, *TP53* GOF mutation is rare in HCC, which indicates that *TP53* GOF mutation is not a reason for the aberrant activation of Wnt signaling in HCC. *APC* and *AXIN1* were mutated in HCC. By using methylation sensitive restriction enzyme-quantitative PCR, hypermethylation of *APC* was detected in HCC with different risk factors, whereas *SFRP1* and *SFRP5* were not hypermethylated in any of the HCC etiologies, which indicates that

the mutation of *APC* and *AXIN1*, together with the methylation of *APC* could take part in the overactivation of Wnt signaling. Nested-reverse transcription PCR failed to detect the integration of *HBx* before the *LINE1* element, or the existence of an *HBx-LINE1* chimeric transcript, suggesting that integration could not play a role in the aberrant activation of Wnt signaling in HCC.

CONCLUSION: In HCC, genetic/epigenetic aberration of *CTNNB1* and its protein degradation regulators are the major cause of Wnt signaling overactivation.

Key words: β-catenin; CTNNB1; Hepatitis B virus; Hepatocellular carcinoma; TP53; Wnt signaling

Core tip: Abnormal activation of Wnt/β-catenin signaling can be detected in approximately 50%-70% of hepatocellular carcinoma (HCC). It is necessary to take the analysis about the cause of Wnt/β-catenin signaling pathway aberration with the etiologic differences into consideration. In this review, the suggested genetic/epigenetic aberrations and their involvement in the abnormal Wnt/β-catenin overactivation in HCC were comprehensively analyzed, with focus on the cause of hepatitis B virus-related HCC. We suggest that genetic/epigenetic aberration of *CTNNB1* and its protein degradation regulators are the major cause of Wnt signaling overactivation. *TP53* gain-of-function mutation is seldom involved, and *HBx-LINE1* chimeric transcripts created by viral integration may not be present.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most deathly human malignancy[1]. The leading causative factors of HCC include chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, exposure to aflatoxin-contaminated food and alcohol consumption. Chronic HBV carriers have a 5-15-fold increased risk of HCC compared with the general population[2]. China alone accounts for more than half of the world's annually diagnosed new HCC cases, predominately due to high prevalence of HBV infection and consequent cirrhosis[3].

The development of HCC is a multistage process, during which numerous genetic/epigenetic abnormalities are involved. The Wnt/β-catenin signaling pathway, which plays key roles in development and adult tissue homeostasis, is highly conserved throughout evolution. Abnormal activation of this pathway could induce different diseases, especially tumors[4]. When Wnt signaling is activated, canonical Wnt signals are transduced through Frizzled family receptors and LRP5/LRP6 co-receptors located on the cell membrane, initiating the β-catenin signaling cascade[5]. β-catenin is the core component of the Wnt/β-catenin pathway and is sequestered in the cytoplasm by the "destruction complex", which includes Axin, glycogen synthase kinase 3, and adenomatous polyposis coli (APC). This multi-protein destruction complex could target the proto-oncogene β-catenin for ubiquitin-mediated proteolysis[6,7]. Activation of the wnt signaling could prevent glycogen synthase kinase 3β (GSK-3β)-mediated β-catenin degradation, leading to accumulation and nuclear translocation of β-catenin[8]. The nuclear accumulated β-catenin could then combine with T-cell factor/lymphoid enhancer factor, and thereby promote the transcription of downstream target genes, including *FGF20*, *DKK1*, *WISP1*, *MYC*, *CCND1*, and so on. It has been shown that 50%-70% of HCC tissues have abnormal β-catenin protein accumulation[9,10]. Furthermore, β-catenin expression, especially in poorly differentiated tumors, is an indicator of poor prognosis, as HCC patients with β-catenin positive grade Ⅲ tumors have a significantly poorer prognosis[11]. Therefore, β-catenin could play important roles in the development and prognosis of HCC.

Many mechanisms have been shown to be involved in the aberrant activation of Wnt signaling. First, mutation of the β-catenin coding gene *CTNNB1* causes aberrant activation of Wnt signaling in many tumors, including sporadic colorectal cancer[12], anaplastic thyroid carcinoma[13], gastric cancer[14], and HCC[15]. Second, aberration of several constitution molecules such as APC, AXIN, secreted Frizzled related protein (SFRP) 1 and SFRP5 in Wnt signaling could also affect the activation of Wnt signaling. In addition, *TP53* gain-of-function (GOF) mutations were reported to activate Wnt signaling[16]. However, whether all these mechanisms play important roles in HCC, especially in the HBV-related HCC, have not been fully understood. Furthermore, a recent study reported that *HBx* could integrate into human genome and form an *HBx-LINE1* chimeric transcript, and this transcript could activate Wnt signaling as an long noncoding RNA[17]. Through literature review and experimental detection, the possible presence of *HBx-LINE1* in primary live tumor was also addressed. In this study, by integrative analysis of these potential factors, we summarize the known molecular mechanisms of aberrant activation of Wnt signaling in HCC.

The aim of the study was to understand the underlying molecular events accounting for aberrant Wnt signaling activation in HCC, particularly in the HCC with background of chronic HBV infection.

Table 1 *CTNNB1* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
9635572	14/75	NA/NA	NA/NA	NA/NA	NA/NA
9671767	8/31	NA/NA	NA/NA	NA/NA	NA/NA
10487827	14/32	2/5	2/13	0/0	10/13
10595907	13/22	0/0	13/22	0/0	0/0
10665646	9/38	NA/NA	NA/NA	NA/NA	NA/NA
10700176	13/100	NA/NA	NA/NA	NA/NA	NA/NA
10980116	57/421	27/265	23/95	3/44	4/17
11282485	6/32	NA/NA	NA/NA	NA/NA	NA/NA
11375957	26/137	3/33	12/31	0/9	11/41
11429783	0/22	0/0	0/0	0/0	0/22
11443619	7/60	5/48	0/2	0/0	2/10
11477549	1/14	0/1	1/12	0/0	0/1
11570580	15/34	NA/NA	NA/NA	NA/NA	NA/NA
12101426	14/73	NA/NA	NA/NA	NA/NA	NA/NA
12375019	10/57	0/0	10/57	0/0	0/0
12439747	184/1123	NA/NA	NA/NA	NA/NA	NA/NA
12845670	24/100	5/26	16/51	0/0	3/23
14999698	3/61	0/29	0/7	0/1	0/0
15067328	10/89	NA/NA	NA/NA	NA/NA	NA/NA
15151624	1/61	NA/NA	NA/NA	NA/NA	NA/NA
15288479	2/16	0/0	0/4	0/0	2/6
15305374	44/781	NA/512	NA/151	NA/52	NA/66
15814635	37/265	NA/161	NA/53	NA/26	NA/25
17187432	34/120	NA/NA	NA/NA	NA/NA	NA/NA
17393110	7/29	NA/NA	NA/NA	NA/NA	NA/NA
17510384	13/81	2/13	8/44	1/2	2/18
17531558	4/42	0/7	4/23	0/3	0/9
18171349	1/36	0/21	0/4	0/0	1/11
18282277	11/52	NA/NA	NA/NA	NA/NA	NA/NA
18358501	4/54	3/43	0/1	0/2	1/8
18467159	47/223	NA/NA	NA/NA	NA/NA	NA/NA
18701503	28/81	0/0	28/81	0/0	0/0
19101982	10/32	0/0	3/6	0/0	7/26
20347502	1/1	0/0	1/1	0/0	0/0
20923573	7/15	1/4	4/10	0/0	2/2
20963515	2/20	2/20	0/0	0/0	0/0
21457159	19/44	3/11	10/23	1/2	5/8
21822264	28/139	5/50	13/43	1/2	9/44
22561517	42/149	3/32	7/24	1/4	31/89
25021421	53/176	13/78	NA/NA	NA/NA	NA/NA

Data are presented as the number of mutation cases/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

MATERIALS AND METHODS

Database resources

Catalogue of Somatic Mutations in Cancer (COSMIC) database (http://cancer.sanger.ac.uk/) was searched for summarizing the mutation statuses of *CTNNB1*, *APC*, *AXIN1*, *AXIN2*, and *TP53*. *TP53* GOF mutants were defined including *S127Y*, *P151S*, *R156P*, *Y163N*, *Y163C*, *V173L*, *R175H*, *C176Y*, *H179R*, *L194R*, *Y205C*, *H214R*, *Y220C*, *Y234C*, *M237I*, *S241F*, *G245C*, *G245S*, *G245V*, *G245D*, *R248W*, *R248G*, *R248Q*, *R273C*, *R273L*, *R273H*, *R273P*, *C275Y*, *D281G*, and *R282W,* as suggested[16]. For investigating the link between gene mutation and etiology, we also consulted the articles referred by the COSMIC database, and determined the etiology of each case. The mutation data of *CTNNB1*, *AXIN1*, and *TP53* is listed in Tables 1-9.

HCC tissue samples

HCC tissue samples were obtained from patients who underwent routine curative surgery at Henan Oncology Hospital in Zhengzhou, Henan Province of China. This study was retrospective, and all tissues were obtained during surgeries. All patients were HBV-positive, which was indicated with serum HBsAg or HBV-DNA presence. For detecting the methylation status of *APC*, *SFRP1*, and *SFRP5*, seven pairs of tissue specimens from HCC patients with serum anti-HCV-positive and ten pairs of tissue specimens from HCC patients without HBV/HCV viral infection were collected. Six tumor-free tissues from patients with hepatic hemangioma were used as controls. All tissues were snap frozen in liquate nitrogen until use. The study was approved by the Ethics Committee in the university, and the informed consents were obtained

Table 2 *AXIN1* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
10700176	9/100	NA/NA	NA/NA	NA/NA	NA/NA
11375957	12/112	8/32	0/31	2/9	2/40
12101426	4/56	0/9	0/5	0/2	4/40
15067328	25/89	NA/NA	NA/NA	NA/NA	NA/NA
18171349	9/36	5/21	3/4	0/0	1/11
21499249	1/1	0/0	1/1	0/0	0/0
22561517	19/149	5/32	5/24	1/4	8/89
25021421	25/176	13/78	NA/NA	NA/NA	NA/NA

Data are presented as the number of mutation cases/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

from all patients and donors before the start of the study.

DNA methylation detection

DNA was extracted from tissues by digestion of frozen samples with 1% proteinase K, followed by standard phenol/chloroform and ethanol precipitation. The DNA methylation status was detected by DNA methylation-sensitive restriction endonuclease digestion, followed by subsequent quantitative (q)PCR assay as described previously[18]. In brief, 2 μg DNA was treated with *Hha*I methylation-sensitive enzyme at 37 ℃ for 16 h. *Hha*I can digest the GCGC sequence if the cytosine is not methylated. Then, qPCR was performed to amplify the target template with primers between which *Hha*I cutting sites were located. The reaction was performed in a 96-well plate on Roche Lightcyler 480 Ⅱ Real-Time PCR System (Roche, Basel, Switzerland). Methylation intensity was quantified between 0% and 100% by calculating Ct values of tissues treated either with or without *Hha*I digestion. Primers used for *APC*, *SFRP1*, and *SFRP5* methylation detection are: *APC*, F-CGGACCAGGGCGCTCCCCATTCC and R-TGACACCCTGGCGGGCTGCACCAA; *SFRP1*, F-TCGCCCCGCCGGGAGCTGATTG and R-GGCTGGAGTGCGCGGGGCTCCT; *SFRP5* F-CCAGTGCAGCGCCCCCAGCAGCA and R-CGCGGCGCGCACCTGGAGAG.

Reverse transcription-PCR assay

Total RNA was extracted with TRI-Reagent (Invitrogen of Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol, and reverse transcription was then performed using Reverse Transcription Kit (Thermo Fish Scientific). Nested-PCR was performed to detect *HBx-LINE1* transcript (primers: first round: F-TCCCCGTCTGTGCCTTCTC and R-TAGTGCTGCAATAAACATGGGA; second round: F-ACGCGGTCTCCCCGTCTGT and R-GCTGGATCATATGGAAGCTCTGG). β-actin was used as the calibrator gene (primers: F-CTACAGCTTCACCACCACGG and R-TCAGGCAGCTCGTAGCTCTTC).

Plasmid construction

PcDNA3.1-TP53 expression vector was kept in our lab, which covers the coding sequence of *TP53* and contains a myc tag. Using this vector as a template, three different GOF mutation sites were precisely mutated. The primers used: R273C, F-AACAGCTTTGAGGTGTGTTTGTGCCTGTCCTGGG and R-GACAGGCACAAACACACACCTCAAAGCTGTTCCGTC; R273H, F-AACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG and R-GACAGGCACAAACATGCACCTCAAAGCTGTTCCGTC; Y220C, F-GTGTGGTGGTGCCCTGTGAGCCGCCTGAGGTTGGCT and R-ACCTCAGGCGGCTCACAGGGCACCACCACACTATGT.

Luciferase reporter assays

The TOP/FOP reporter plasmids were co-transfected with Renilla luciferase vector into HEK 293T, SMMC 7721, and Huh-7 cell lines using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were washed twice with PBS and lysed in passive lysis buffer. PGL3-basic vector was used as a negative control. Luciferase activity was analyzed using a luminometer and a dual luciferase assay kit according to the manufacturer (Promega Corp., Madison, WI, United States). Luciferase counts were normalized using Tk-Renilla-luciferase (Promega).

Statistical analysis

All analyses were performed with the software SPSS version 19.0 (IBM, Armonk, NY, United States). Different groups were compared by χ^2 tests as appropriate. All statistical tests were two-sided, and $P < 0.01$ was considered as statistically significant.

RESULTS

CTNNB1 mutation is a major causative factor of aberrant Wnt signaling activation in HCC

CTNNB1 mutation, especially mutation at the phosphorylation sites in N-terminal domain, could affect β-catenin protein stability and its combining capability with APC and AXIN. Firstly, we summarized the mutation frequency of *CTNNB1* in different tumors, including HCC, stomach, lung, ovary, colon tumor and esophageal squamous cell carcinoma, based on the information collected from COSMIC database. As shown in Figure 1A, the *CTNNB1* mutation rate

Table 3 *TP53* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
1311638	0/21/36	0/21/36	0/0/0	0/0/0	0/0/0
1327523	3/20/61	3/12/37	0/4/17	0/3/4	0/1/3
1655254	2/9/43	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
1672732	0/5/10	0/5/8	0/0/NA	0/0/NA	0/0/NA
1849234	0/8/16	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
7903205	2/20/35	2/15/28	0/NA/NA	0/NA/NA	0/NA/NA
8093350	1/17/53	0/3/7	NA/NA/NA	NA/NA/NA	NA/NA/NA
8093978	0/3/20	0/NA/17	0/NA/NA	0/NA/NA	0/NA/NA
8100480	1/3/20	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8108145	2/6/15	2/6/15	0/0/0	0/0/0	0/0/0
8261444	5/12/16	1/2/3	4/8/10	0/1/2	0/1/1
8290606	0/19/80	0/8/28	0/NA/NA	0/NA/NA	0/NA/NA
8302580	1/6/22	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8380058	0/2/15	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8382111	0/12/38	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8384081	0/4/45	0/4/28	0/0/11	0/0/2	0/0/4
8389246	0/11/34	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8390289	0/10/15	0/8/13	0/NA/NA	0/NA/NA	0/NA/NA
8390407	0/1/18	0/0/3	0/0/5	0/0/1	0/1/9
8393166	1/1/7	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8407553	3/19/63	2/10/16	1/4/15	0/0/0	0/5/32
8565124	0/2/12	0/0/3	0/0/2	0/0/1	0/2/6
8655704	3/10/38	NA/NA/19	NA/NA/10	NA/NA/5	NA/NA/4
8655958	2/2/20	1/1/4	NA/NA/NA	NA/NA/NA	NA/NA/NA
8672994	0/1/18	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8895490	0/9/12	0/0/0	0/1/3	0/2/3	0/3/6
9012469	0/8/16	0/1/8	0/NA/NA	0/NA/NA	0/NA/NA
9270015	2/26/105	2/24/78	0/NA/NA	0/NA/NA	0/NA/NA
9463584	0/2/8	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
9699537	2/6/97	NA/NA/16	NA/NA/NA	NA/NA/NA	NA/NA/NA
9781942	1/2/15	0/0/0	1/2/15	0/0/0	0/0/0
10389750	0/17/31	0/11/17	0/NA/NA	0/NA/NA	0/NA/NA
10389978	0/12/24	0/10/19	0/NA/NA	0/NA/NA	0/NA/NA
10564952	2/9/11	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
10662591	0/5/27	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
10699891	4/11/18	0/0/0	0/0/0	0/0/0	4/11/18
10743047	0/35/40	0/33/38	0/NA/NA	0/NA/NA	0/NA/NA
11051249	2/21/83	0/14/50	1/6/16	0/0/0	1/1/17
11191353	0/14/55	0/NA/45	0/NA/NA	0/NA/NA	0/NA/NA
11282486	2/6/22	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
11375957	1/36/137	1/15/33	0/10/31	0/3/9	0/8/41
11704835	8/30/71	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
12483005	3/7/34	2/5/27	0/1/3	0/0/0	1/1/4
12640682	0/2/18	0/1/5	0/1/8	0/0/4	0/0/1
12759240	2/16/33	2/16/33	0/0/0	0/0/0	0/0/0
12845670	0/14/100	0/4/26	0/7/51	0/0/0	0/3/23
14499690	0/18/68	0/NA/52	0/NA/2	0/0/0	0/NA/14
14675778	0/5/22	0/0/0	0/5/22	0/0/0	0/0/0
14687797	3/9/80	2/7/NA	0/1/NA	0/0/NA	1/1/NA
15017592	4/22/50	NA/NA/13	NA/NA/33	NA/NA/3	NA/NA/1
15126338	3/27/48	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
15943041	8/8/20	8/8/20	0/0/0	0/0/0	0/0/0
16078640	2/7/41	NA/NA/1	NA/NA/26	NA/NA/NA	NA/NA/NA
16570275	1/11/40	1/4/22	0/2/5	0/2/5	0/3/8
16685387	0/28/55	0/NA/49	0/NA/NA	0/NA/NA	0/NA/NA
16697535	0/7/23	0/7/23	0/0/0	0/0/0	0/0/0
17066440	0/5/51	0/0/14	0/4/30	0/0/0	0/1/7
17266182	1/28/50	1/24/41	0/NA/NA	0/NA/NA	0/NA/NA
17350822	4/16/83	NA/2/13	NA/13/64	NA/0/2	NA/1/4
17510384	0/25/81	0/5/13	0/15/44	0/2/2	0/3/18
17531558	2/7/42	1/1/7	1/4/24	0/1/2	0/1/9
21499249	0/1/1	0/0/0	0/1/1	0/0/0	0/0/0
21760996	0/9/26	0/6/13	0/2/4	0/0/0	0/0/4
21822264	4/39/139	2/20/50	1/10/43	0/2/2	1/7/44
22561517	0/26/149	0/9/32	0/7/24	0/2/4	0/8/89
22634756	3/14/27	3/7/11	0/7/14	0/0/0	0/0/2
22922871	1/1/10	1/1/10	0/0/0	0/0/0	0/0/0

Data are presented as the number of gain-of-function mutation cases/number of mutations/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

Table 4 Mutation case numbers and rates of *CTNNB1* in different tissues

Cancer type	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HCC	671	3720	18.04	
Colon Adenocarcinoma	126	1318	9.56	< 0.001
Ovary carcinoma	102	1599	6.38	< 0.001
Stomach Adenocarcinoma	54	1137	4.75	< 0.001
Lung Adenocarcinoma	38	1219	3.12	< 0.001
ESCC	5	347	1.44	< 0.001

[1]The *P* value *vs* HCC. ESCC: Esophageal squamous cell carcinoma; HCC: Hepatocellular carcinoma.

Table 5 Mutation case numbers and rates of CTNNB1 in hepatocellular carcinoma etiologies

Viral background	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HBV	74	686	10.79	
HCV	155	554	27.98	< 0.001
HBV and HCV	7	69	10.14	0.999
Non-viral	90	348	25.86	< 0.001

[1]The *P* value *vs* HBV. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

in HCC was 18.04%, which was significantly higher than the mutation rates in other tumors (Table 4), implicating that *CTNNB1* mutation could be one of the major reasons for aberrant activation of Wnt signaling commonly seen in HCC. Next, the HCC patients were classified into different groups according to the background of viral infection, and then we compared the rates of *CTNNB1* mutation among different HCC groups. As shown in Figure 1B, the *CTNNB1* mutation rate in chronic HBV-related HCC was 10.79%, which was similar to that in HBV/HCV coinfection-related HCC, but significantly lower than those with HCV-related HCC or non-viral HCC (Table 5). Nevertheless, the *CTNNB1* mutation rate in chronic HBV-related HCC was still higher than that in several other human tumors such as esophageal squamous cell carcinoma, lung cancer, and gastric cancer.

TP53 GOF mutants may not contribute to aberrant Wnt signaling activation in HCC

Tumor suppressor gene *TP53* is the most frequently mutated gene in cancer. Among these p53 mutants, some mutations not only lose the tumor suppressive functions, but also gain novel oncogenic activities, including promotion of tumor cell proliferation, survival, metabolic changes, angiogenesis, and metastasis, which were defined as p53 GOF activities[19]. It has been reported that β-catenin expression and the Wnt signaling pathway are highly activated in tumors harboring GOF p53 mutants[16]. To investigate whether *TP53* GOF mutant could activate the Wnt signaling

Table 6 Mutation case numbers and rates of *AXIN1* in different tissues

Cancer type	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)
Hepatocellular carcinoma	75	872	8.60
Colon adenocarcinoma	20	513	3.90
Ovary carcinoma	3	749	0.40
Stomach adenocarcinoma	18	377	4.77
Lung adenocarcinoma	6	697	0.86
Esophageal squamous cell carcinoma	3	130	2.31

Table 7 Mutation case numbers and rates of *AXIN1* in hepatocellular carcinoma etiologies

Viral background	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HBV	31	172	18.02	
HCV	9	65	13.85	0.964
HBV and HCV	3	15	20.00	0.999
non-viral	15	180	8.33	0.122

[1]The *P* value *vs* HBV. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

pathway in HCC, we performed a TOP/FOP luciferase assay by overexpressing Y220C, R273C, and R273H *TP53* GOF mutants in HEK 293T, SMMC7721, and Huh-7 cell lines. Compared with wild-type p53, Y220C enhanced the TOP/FOP value in Huh-7 cells, whereas R273H and R273C enhanced the TOP/FOP value in SMMC 7721 cells. As a positive control, all three GOF mutations enhanced TOP/FOP values in HEK 293T cells (Figure 2A). The effect of p53 GOF mutants in those hepatic origin cells was insignificant and inconsistent. Consistent with the results of the *in vitro* analysis, searching of the COSMIC database revealed that, though the total *TP53* mutation rate in HCC was as high as 29.33%, the rate of *TP53* GOF mutation in HCCs was only 4.27%, substantially lower than that observed in other tumors (Figure 2B and Table 8). Furthermore, such a low rate of *TP53* GOF mutation was constantly present among HCCs of different etiologies (Figure 2B). Taken together, although *TP53* GOF mutants activate Wnt signaling in HCC cell lines *in vitro*, they seldom occur in HCC and likely do play a major role in aberrant activation of Wnt signaling commonly present in HCC.

Frequent genetic/epigenetic aberrations in negative regulators involved in Wnt signaling activation in HCC

Since APC and AXINs can form a degradation complex with GSK-3β to prompt the ubiquitination-dependent degradation of β-catenin protein, aberration of either APC or AXINs might affect the activity of the Wnt/β-catenin signaling pathway. Based on the COSMIC database, the mutation rates of *AXIN1*, *AXIN2*, and *APC* in HCC were 8.60%, 0.42%, and 1.33%, respectively (Figure 3A). The high frequent mutation rate of *AXIN1* suggests that it commonly contributes

Table 8 Mutation case numbers and rates of TP53 in different tissues

Cancer type	GOF mutation cases (*n*)	Mutation cases (*n*)	Total cases (*n*)	GOF mutation rate (%)	Non-GOF mutation rate (%)	Total mutation rate (%)	*P* value[1]
HCC	91	825	2813	3.23	26.09	29.33	
Colon adenocarcinoma	331	1867	3596	9.20	42.71	51.92	< 0.001
Ovary carcinoma	392	1604	3329	11.78	36.41	48.18	< 0.001
Stomach adenocarcinoma	270	1160	3459	7.81	25.73	33.54	< 0.001
Lung adenocarcinoma	421	2414	6394	6.58	31.17	37.75	< 0.001
ESCC	224	972	1858	12.06	40.26	52.31	< 0.001

[1]GOF mutation rate *vs* HCC. ESCC: Esophageal squamous cell carcinoma; GOF: Gain-of-function; HCC: Hepatocellular carcinoma.

Table 9 Mutation case numbers and rates of TP53 in hepatocellular carcinoma etiologies

Viral background	GOF mutation cases (*n*)	Mutation cases (*n*)	Total cases (*n*)	GOF mutation rate (%)	Non-GOF mutation rate (%)	Total mutation rate (%)
HBV	35	321	819	4.27	34.92	39.19
HCV	9	101	398	2.26	23.12	25.38
HBV and HCV	0	18	41	0.00	43.90	43.90
non-viral	7	60	346	2.02	15.32	17.34

GOF: Gain-of-function; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

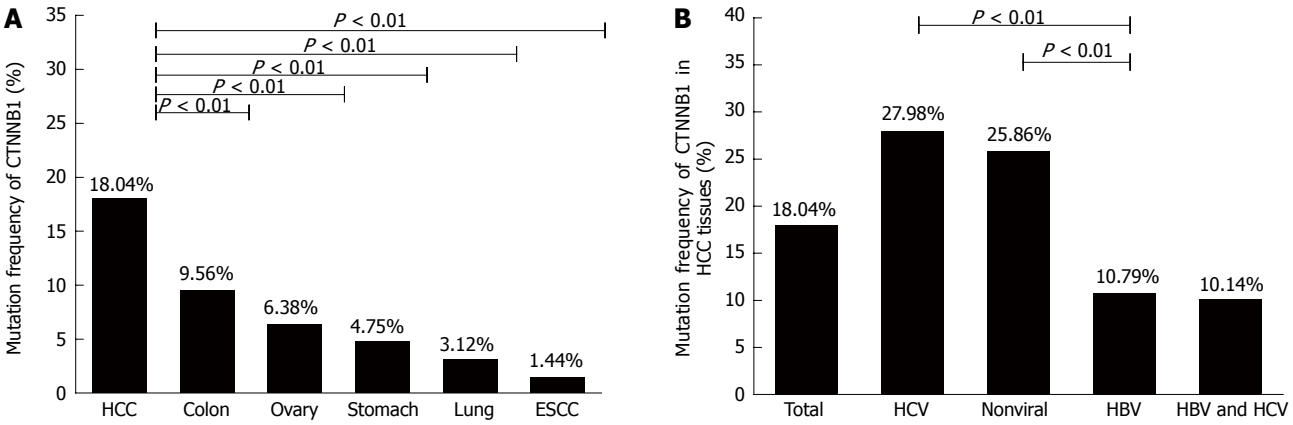

Figure 1 *CTNNB1* mutation rate in tumors based on the information collected from COSMIC database. *CTNNB1* mutation rates in A: Different human tumors; and B: Hepatocellular carcinoma tissues with different risk factors. ESCC: Esophageal squamous cell carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

to the aberrant Wnt signaling activation in HCC.

SFRP1 and *SFRP5* encode SFRP, which is the antagonist of the Wnt signaling pathway. The epigenetic downregulation of SFRP has been shown to be involved in hepatocarcinogenesis[20]. We previously demonstrated that the promoter CpG island of *APC* is hypermethylated in HCC with HBV infection[21]. To further demonstrate the involvement of the negative regulators in Wnt/β-catenin overactivation in HCC, methylation sensitive restriction enzyme-qPCR was performed to detect the methylation statuses of *APC*, *SFRP1*, and *SFRP5* in HCC with different risk factors, including between HCV-related HCC and those without viral infection. The results showed extensive hypermethylation of the *APC* promoter in all HCC of different etiologies (Figure 3B). As shown in Figure 3C, hypermethylation of *SFRP1* was present in 15% (3/20) of HBV-infected tumor tissues, 0% (0/7) of HCV-infected tumor tissues, 20% (2/10) of non-infected tumor tissues, and 0% (0/6) of hepatic hemangioma tissues, whereas hypermethylation of *SFRP5* was present in 5% (1/20) of HBV-infected tumor tissues, 28.6% (2/7) of HCV-infected tumor tissues, 0% (0/10) of non-infected tumor tissues, and 0% (0/6) of hepatic hemangioma tissues. These results suggest that *SFRP1* and *SFRP5* are not primary causes of aberrant Wnt signaling activation in HCCs.

HBx-LINE1 transcripts are not detected in tissues of HBV-related HCC

A recent report described that *HBx* frequently forms a chimeric transcript (*HBx-LINE1*) after integrating into the *LINE1* element in 8p11.21 of the host genome, and the authors further suggested that *HBx-LINE1* could activate the Wnt signaling pathway as a long noncoding RNA[17]. To explore whether *HBx-LINE1*

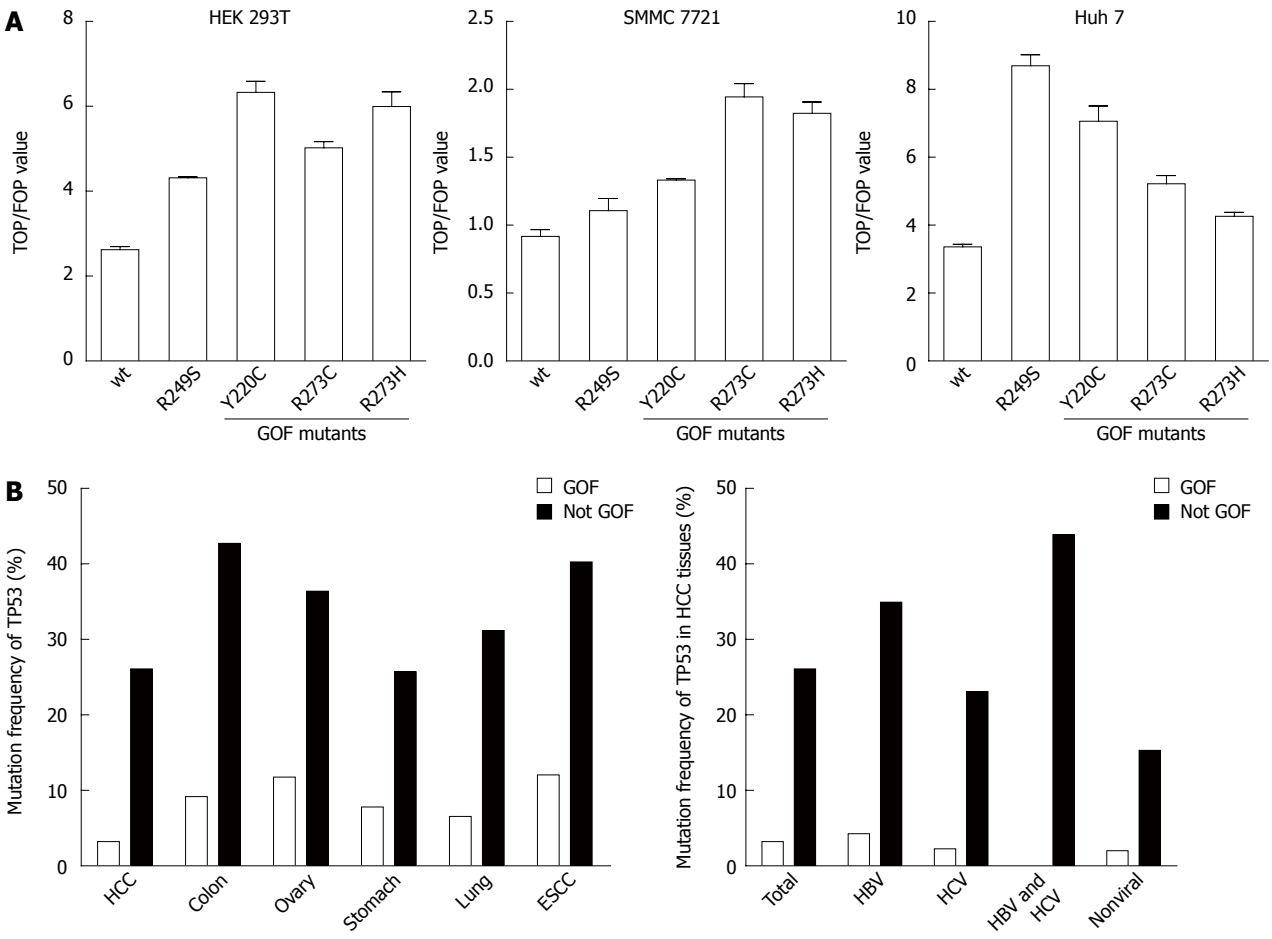

Figure 2 ***TP53*** **GOF mutation does not likely contribute to the aberrant activation of Wnt signaling.** A: *TP53* GOF mutants activate Wnt signaling *in vitro;* B: *TP53* mutation rate in different tumors and different etiologies of HCC. Although *TP53* mutation was commonly detected in HCC, GOF mutation was a rare event in HCC with different etiologies. ESCC: Esophageal squamous cell carcinoma; GOF: Gain-of-function; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

could take part in the aberrant activation of Wnt signaling in HBV-related HCC, the expression of this transcript was measured in up to 30 HCC tissues with a chronic HBV infection background. Unfortunately, even by nested-PCR, no *HBx-LINE1* chimeric transcript was detected. To exclude the possibility that the absence was due to an experimental failure, PCR primers were designed to detect the *HBx-LINE1* viral-host junction sequences at the DNA level. Still, no *HBx-LINE1* integration was detected (data not shown). Moreover, we carefully reviewed the available viral integration information from published data. In a total of 1115 HBV integration sites derived from 299 HBV-HCC patients, only one was found mapped at chromosome 8p11.21. However, further precise analysis excluded the possibility to form an *HBx-LINE1* transcript[22], because the integration site was away from the *LINE1* site. Collectively, *HBx* was not expected to exactly integrate at such an accurate site to form the *HBx-LINE1* chimeric transcript, at least not at the high frequency as described by the report[17]. As a result, *HBx-LINE1*, the suggested viral-host junction transcript, may not commonly present in HBV-infection related HCCs.

DISCUSSION

Abnormal activation of Wnt/β-catenin signaling is detected in 50%-70% of HCC cases, making it the most common signaling pathway aberration in this cancer[10]. However, it is necessary to analyze the cause of Wnt/β-catenin signaling pathway aberration while considering the different etiologic causes of HCC. In the present study, we summarized all the suggested factors relevant to the aberrant activation of the Wnt signaling pathway in HCC with different causative etiologies. Those genetic/epigenetic events include *CTNNB1* gene mutation, *TP53* GOF mutation, the presence of an *HBx-LINE1* chimeric transcript, and aberrations of other genes within this signaling pathway.

CTNNB1, which encodes β-catenin, has been recognized as one of the most frequently mutated genes in primary HCC. Indeed, according to the COSMIC database, the mutation of *CTNNB1* was detected in 671/3720 HCC cases, with detailed

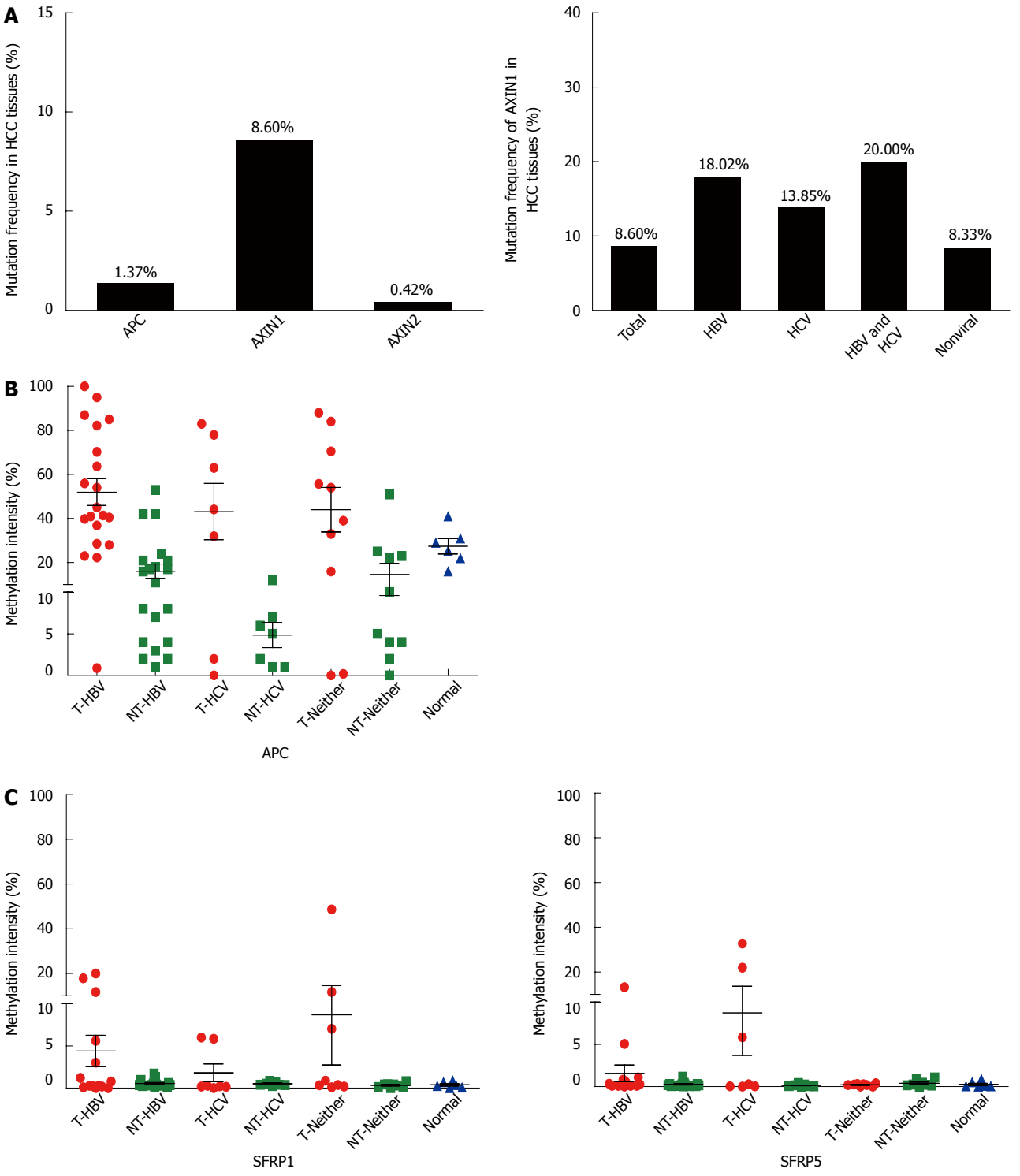

Figure 3 Frequent genetic/epigenetic aberrations of the negative regulators in Wnt signaling in hepatocellular carcinoma. A: *AXIN1* was frequently mutated in HCCs with different etiologies; B: *APC* promoter hypermethylation was frequently found in HCCs with different etiologies; C: Neither *SFRP1* nor *SFRP5* was frequently hypermethylated in HCCs with different risk factors. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

mutation information provided for 543 of these cases (http://cancer.sanger.ac.uk/). It is worthwhile to note that among the 543 identified mutations, 477 were point mutations located at N-terminal domain, 309 of which were at β-catenin phosphorylation sites (serines 33, 37, and 45 and threonine 41). This result is consistent with the fact that the stability of β-catenin relies on the phosphorylation of its N-terminal domain.

The large percentage (18.04%; 671/3720) of *CTNNB1* mutations makes it one of the major causative mechanisms contributing to the aberrant activation of Wnt signaling in HCC. However, the mutation rate of *CTNNB1* in chronic HBV-related HCC was only 10.79%, much lower than in HCV-related

cases or of other etiologic backgrounds. The lower rate of *CTNNB1* mutation in HBV-related HCC has been reported previously by other laboratories, in which relatively small patient cohorts were used[1,23]. The other gene with a high frequency of mutation in HCC is *AXIN1*, another component of the "destruction complex". Interestingly, in contrast to *CTNNB1*, the rate of *AXIN1* mutation was higher in HBV-related HCC compared to HCC of other etiologic backgrounds.

The above results suggest that mutation of genes comprising the "destruction complex", together with their target *CTNNB1* mutation, are common events contributing to the altered Wnt signaling pathway activation in HCC. In addition to genetic mutations, epigenetic modulation can also cause aberrant gene expression. We previously reported that in HBV-related HCCs, the CpG islands in the promoter regions of *APC* and *AXIN2* were frequently hypermethylated[20]. Frequent *APC* hypermethylation in HBV- and HCV-related HCC tissues has also been reported by Feng *et al*[24]. In this study, we compared the methylation status in HCC with different etiologies. However, there was no noticeable difference among HCCs with different etiologic backgrounds. Taken together, these results suggest that *APC* promoter hypermethylation is a causative factor for aberrant Wnt signaling activation in HCC tissues. In addition, our data and those from other laboratories suggest that *SFRP* methylation may not be a primary factor for aberrant Wnt signaling activation in HCC[24].

The recent report that *HBx-LINE1* chimeric transcripts can activate Wnt signaling has attracted some recognition[17,25,26]. However, we doubted the possibility of such a high frequency of identical integration (present in 23% of primary HCC tissues, as described by the author[17]). As a matter of fact, the integration of HBV into the host cellular genome is generally a random event, and it is hard to imagine that HBV integrates precisely at chr.8p11.21 in close to one-quarter of HCC tumor tissues. Moreover, in order to make an *HBx-LINE1* junction, the HBV genome must also be broken exactly at the same site of its genome. Our lab had searched up to 1115 HBV host genome-adjacent sequences from several articles, including our data, and none of them locate at the site of the *LINE1* element, which is essential for the formation of the *HBx-LINE1* chimeric transcripts[27-31]. In addition, the effort to detect the presence of *HBx-LINE1* chimeric transcripts by the powerful nested Reverse transcription-PCR, or PCR at the tumor genome level, failed. In addition, the previous report utilized Sanger sequencing to confirm the formation of *HBx-LINE1* transcripts[17]; we noticed that the viral nucleic acid sequence was exactly the same, not even a single nucleotide variant to this high mutation-rate virus. Therefore, we had sufficient cause to doubt that *HBx* can accurately integrate with *LINE1* with such an extremely high frequency.

Besides the above factors, some other mechanisms that can activate Wnt signaling should be further explored, such as the phosphorylation of extracellular signal-regulated kinase and protein kinase B by HBx, which may be the major mechanism for Wnt signaling activation in HBV-related HCC[32,33]. Additionally, it was reported that HBx can competitively combine with APC protein to release GSK-3β[34]. However, further experimental evidences are still needed to confirm this postulation.

Together with other previous reports, we propose that several possible mechanisms account for the aberrant Wnt signaling activation in HCC, including *CTNNB1* mutation, as well as hypermethylation of *APC* and *AXIN1* mutation. In contrast, hypermethylation-mediated silencing of *SFRP1* and *SFRP5* expression was not a common event. Additionally, although *TP53* GOF mutations have the potential to activate Wnt signaling, they rarely occur in HCC, and therefore, it should not be counted as a causative factor of aberrant Wnt signaling overactivation in HCC. Unfortunately, our results do not support the hypothesis that *HBx-LINE1* chimeric transcripts activate Wnt signaling in HCC, as none of the 1115 known HBV viral-host cellular genome junction sequences involved the *LINE1* sequence, and furthermore, the *HBx-LINE1* chimeric transcript was not detected at the mRNA or genomic DNA level.

As our understanding about Wnt signaling pathways continues to grow, the potential clinical value of our knowledge on Wnt signaling and HCC should be further studied.

COMMENTS

Background

The development of hepatocellular carcinoma (HCC) is a multistage process, during which numerous genetic/epigenetic factors could be involved. As one of the most important factors, the Wnt/β-catenin signaling pathway is frequently activated, about 50%-70% of HCC tissues show abnormal β-catenin protein accumulation, which predicts poor prognosis. It has become recognized that the Wnt signaling pathway plays an important role in the development and prognosis of HCC.

Research frontiers

Many mechanisms have been reported to be involved in the aberrant activation of Wnt signaling in HCC. Mutation of *CTNNB1*, which encodes β-catenin, could cause cytoplasmic accumulation of β-catenin. Genetic or epigenetic aberrations of several constitution molecules in the Wnt signaling pathway could also affect its activation. Furthermore, *TP53* gain-of-function mutations have the ability to upregulate the expression of *CTNNB1*. Finally, a recent report suggested a frequent integration of *HBx* into *LINE1* elements of the human genome and formation of *HBx-LINE1* chimeric transcripts, which enhance the transcriptional activity of Wnt signaling.

Innovations and breakthroughs

Previous reports have suggested the presence of different underlying mechanisms for the aberrant activation of Wnt signaling in HCC. However, whether all these mechanisms could really take part in this process is not known. In this article, by integrative analysis of the potential factors, the involvement of the following suggested mechanisms in the aberrant activation of Wnt signaling in HCC were investigated: the mutation rate of *CTNNB*1, *TP53*, *APC*, *AXIN1*, and *AXIN2* by searching in COSMIC database; and the epigenetic aberrations of the constituent molecules in Wnt signaling, such as *APC*, *SFRP1*, and *SFRP5*, by determining their CpG island methylation status using a methylation sensitive restriction enzyme-quantitative PCR technique developed in the laboratory. In addition, in order to judge whether *HBx* integration in *LINE1*

elements could activate Wnt signaling in HBV-related HCC, the proposed *HBx-LINE* integration was also examined at both the genome and RNA levels among HBV-related HCC tissue specimens by nested reverse transcription-PCR and PCR.

Applications

This integrative study provides a panoramic view of the underlying mechanisms relevant to the aberrant activation of Wnt signaling in HCC. The discovery will enhance our understanding of hepatocarcinogenesis.

Terminology

The Wnt/β-catenin pathway is highly conserved throughout evolution, and plays key roles in development in adult tissue homeostasis. β-catenin is the core component which is precisely regulated. β-catenin can be degraded by the destruction complex composed of APC, AXIN, and GSK-3β. Wnt signaling activation leads to nuclear translocation of β-catenin, where it promotes the transcription of several downstream target genes.

Peer-review

The authors present a comprehensive study. The methodology is correct. The conclusions are consistent with the results obtained. This study represents a significant contribution to advance our study on the process of hepatocarcinogenesis.

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