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Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/ β -catenin signaling pathway in hepatocellular carcinoma cells

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Abstract

Background/Aims—The canonical Wnt signaling is frequently activated in human hepatocellular carcinoma (HCC). We previously demonstrated that up-regulation of Frizzled-7 receptor (FZD7) in HCC was associated with nuclear accumulation of wild-type β -catenin. Here, we investigated Wnt ligand(s) that may activate the Wnt/ β -catenin pathway through FZD7 in HCC cells.

Methods—To identify Wnt ligand expression, RT-PCR was performed in HCC cells. To evaluate the function of Wnt3 and FZD7 in HCC, we utilized Wnt3 over-expressing FOCUS HCC cells (FOCUS-Wnt3) and human tumors.

Results—In hepatitis B virus (HBV)-induced HCC, Wnt3 was upregulated in tumor and peritumoral tissues compared to normal liver and downstream β -catenin target genes were also increased in these samples. Activation of the Wnt/ β -catenin pathway in FOCUS-Wnt3 cells was demonstrated by β -catenin accumulation, enhanced TCF transcriptional activity and proliferation rate. The activation of Wnt/ β -catenin signaling in FOCUS-Wnt3 was abolished by a knockdown of FZD7 expression by siRNA. More important, a specific Wnt3-FZD7 interaction was observed by co-immunoprecipitation experiments, which suggest that the action of Wnt3 was mediated via FZD7.

Conclusions—These findings demonstrate a functional interaction between Wnt3 and FZD7 leading to activation of the Wnt/ β -catenin signaling pathway in HCC cells and may play a role during hepatocarcinogenesis.

Keywords

Wnt3; FZD7; hepatocellular carcinoma; canonical Wnt pathway

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1. Introduction

The Wnts are a family of secreted glycoproteins that serve as extracellular signaling molecules involved in cell differentiation, migration, and proliferation during embryonic development and lead to tumor formation when aberrantly activated [1]. Wnt signaling is mediated by Frizzled receptors (FZD) and co-receptor low-density lipoprotein-related protein (LRP) and this interaction promotes nuclear accumulation of β -catenin characteristic of canonical Wnt pathway activation. Although activation of Wnt signaling depends on specific Wnt/FZD combinations, the specificity of the interaction remains largely unknown, particularly in vertebrates. Direct binding of human FZD has been demonstrated for some Wnt proteins, including Wnt3A and Wnt7. A Wnt7A ligand activates the canonical Wnt signaling via FZD5-LRP6 receptor complex in PC12 cells [2] and the Jun-N-terminal kinase (JNK) pathway through FZD9 in non-small cell lung cancer [3]. Wnt7B activates canonical signaling in epithelial and vascular smooth muscle cells through interactions with FZD1 and FZD10 [4]. In addition, FZD1 interacts with Wnt3A and Wnt5A in 293T cells [5]. Given that both Wnt and FZD families consist of multiple members and each member is capable of binding to multiple partners, how various Wnts achieve a specific interaction with FZDs is an important question to address in understanding the molecular mechanisms of Wnt signaling and particularly in HCC.

There is increasing evidence that aberrant activation of the Wnt/ β -catenin signaling is associated with tumor development and/or progression [6–9]. It has been established that the accumulation of β -catenin results from adenomatous polyposis coli (APC) or β -catenin gene mutations in approximately 90% of colorectal cancers [10]. However, many HCC with β -catenin nuclear and cytoplasmic accumulation do not have these gene mutations. This phenomenon raises the possibility of activation by upstream components of this cascade. HCC is the major primary malignant tumor of the liver and is a frequent cause of death worldwide [11,12]. Although chronic hepatitis B or C infection and exposure to hepatocarcinogens like aflatoxin B1 and alcohol are well known major risk factors [13], the molecular mechanisms that contribute to tumor initiation or progression remain largely unknown. Past studies revealed genetic involvement of at least three pathways, including p53, retinoblastoma and Wnt/ β -catenin signaling in the pathogenesis of HCC [14,15].

Our previous studies demonstrated that FZD7 was overexpressed in both transgenic mouse models of HCC and human tumors and associated with nuclear accumulation of wild-type β -catenin. In addition, overexpression of FZD7 was observed not only in tumors but also in matched surrounding dysplastic liver tissues. These observations suggest that it may be an early event during the development of this disease [16,17]. However, there is no information on which Wnt ligand(s) are involved in binding to FZD7 and result in subsequent activation of the Wnt/ β -catenin pathway during tumor development.

In this investigation, we determined which Wnt mRNAs were expressed in HCC cells using reverse-transcriptase polymerase chain reaction (RT-PCR) and found the expression of Wnt3, Wnt5A, Wnt6, and Wnt11 were exhibited among the 19 human Wnt ligands studied. Interestingly, Wnt3 was detected in all four HCC cell lines tested and the expression level was higher than that observed in fetal brain. In addition, Wnt3 has been found to induce strong transformation activity through activation of the canonical Wnt/ β -catenin signaling. In this context, we evaluated if Wnt3 could activate β -catenin signaling in HCC cells and explored the physical and functional relationships between Wnt3 and FZD7 interactions.

2. Materials and methods

2.1. Human HCC tissues and cell lines

Seventeen pairs of HCC and matched, peritumoral liver tissues were obtained from South Korean patients (Table 1). Exon 3 mutations of the β -catenin gene from these HCC and peritumoral tissues were analyzed by a previous method [18]. Four normal liver tissues were obtained from individuals who had undergone hepatic resection for single metastasis of colorectal cancer to the liver and served as controls. All were male subjects and the median age was 46 (range 37–57). Histological examination of the liver was normal. Use of these tissues was approved by Brown University Institutional Review Board. Huh7, FOCUS, Hep3B, and HepG2 HCC cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (BSA).

2.2. RT-PCR and real-time RT-PCR assays

Total cellular RNA was extracted using TRIzol[®] Reagent (Invitrogen[™]). RNA from human tissues was purchased from Stratagene (La Jolla, CA). To determine the levels of Wnt3, FZD7, c-Myc, cyclin D1, glutamine synthetase (GS), orphan G-protein-coupled receptor 49 (GPR49), and T-box protein 3 (Tbx3) mRNA expression, real-time RT-PCR was performed using iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA) as previously described [16]. The sequences of primer pairs for each Wnt ligand and target genes are available upon a request.

2.3. Generation of anti-Wnt3 antibody

A murine monoclonal anti-Wnt3 antibody (Wnt3 mAb) was generated against a synthetic peptide corresponding to amino acids 259-274 of human Wnt3 (²⁵⁹LRAKYSLFKPPTERDL²⁷⁴). The peptide sequence has no significant homology with other members of Wnt family or known proteins. The specificity of antibody was verified by Western blot and immunofluorescence staining after transfection with the Wnt3-myc plasmid (data not shown).

2.4. Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated with epitope retrieval, and incubated overnight at 4°C with anti-Wnt3 mAb or FZD7 antibodies [16]. Sections incubated with mouse-IgG or rabbit-IgG served as negative controls. The Histostain-Plus kits (Zymed Lab., San Francisco, CA) were used to localize visible staining.

2.5. Plasmids

Since human Wnt3 cDNA (a kind gift from R. Nusse, Stanford University Medical Center) was missing 22 amino acids at C-terminus [19], we constructed Wnt3-myc plasmid by extension to full-length Wnt3 using PCR based on the sequence of the human Wnt3 cDNA, and subcloning into pcDNA[™]3.1/myc-His vector (Invitrogen[™]). pSUPER8xTOPFlash and pSUPER8xFOPFlash [20] were kind gifts from R. Moon (University of Washington). To generate FZD7-EE plasmid, full-length FZD7 cDNA was cloned from HepG2 cells and subcloned into pcDNA[™]3.1/myc-His with EYMPME (EE) tag. For the construction of a cystein-rich domain (CRD; putative Wnt binding motif) deletion mutant of FZD7 (FZD7- Δ CRD-EE), we removed 104 amino acids (Cys⁴⁹ - Pro¹⁵²) using PCR reactions. All plasmids constructed were verified by sequencing.

2.6. Co-immunoprecipitation and western blot analysis

Cells were co-transfected with Wnt3-myc and either FZD7-EE or FZD7- Δ CRD-EE plasmids. Immunoaffinity purification was performed using anti-EE antibody immobilized onto

Sepharose Fast Flow™ beads (Covance, Berkeley, CA). Western blot analysis was carried out as previously described [16] using following antibodies: anti-myc-tag and anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti- β -catenin (Cell Signaling Technology Inc., Beverly, MA), anti-EE (Covance), anti-c-Myc (Abcam, Cambridge, MA), anti-GS (Transduction lab., San Jose, CA), anti-cyclin D1 (Upstate, Lake Placid, NY).

2.7. Immunofluorescence staining

FOCUS cells, transfected with Wnt3-myc, were grown on a chamber slide (Nalge Nunc, Naperville, IL). For double immunofluorescence staining, cells were incubated with anti-myc-tag and anti- β -catenin antibodies overnight at 4°C, and FITC-conjugated anti-rabbit IgG and Texas red-conjugated anti-mouse IgG (Vector Lab., Berlingame, CA) secondary antibodies were applied. Finally, coverslips were mounted with anti-fade Vectashield medium with DAPI (Vector Lab.) to counterstain nuclei and examined under an Olympus IX70 fluorescence microscope (DSC Optical Services, Newton, MA).

2.8. Stable transfection and TCF transcriptional activity assay

To establish FOCUS stably expressing Wnt3, we transfected Wnt3-myc or empty vector as a control, and colonies selected using 200 μ g/mL of G418 (Life Technologies, Gaithersburg, MD). For the TCF activity assay, pSUPER8xTOPFlash or pSUPER8xFOPFlash and β -galactosidase plasmids were co-transfected into HCC cells. Twenty-four hours after transfection, the cells were serum-starved for 24 h and stimulated with 1% FBS in DMEM. Luciferase activity was measured at 24 h after stimulation unless indicated using the Luciferase Assay System (Promega, Madison, MI). The assay was normalized with β -galactosidase activity as a transfection efficiency control.

2.9. Blocking experiments with anti-Wnt3 antibody or Wnt3 siRNA

For blocking experiments with anti-Wnt3 mAb, cells were incubated with 1% FBS in DMEM containing either anti-Wnt3 mAb or mouse IgG (DaKoCytomation, Carpinteria, CA) as a control at 24 hr after serum-deprivation.

Small interfering RNA (siRNA) for the β -catenin gene (Silencer Validated siRNA, CTNNB1; 5'-GGUGGUGGUAAUAAGGCtt-3') and Wnt3 (Silencer Pre-designed siRNA, Wnt3; 5'-GUGUAUUCGCAUCUACGACtt-3') were purchased from Ambion (Austin, TX). Control siRNA (siGLO CyclophilinB siRNA; a control for transfection efficiency and target gene silencing with fluorescence) and a siRNA specific for human FZD7 (ON-TARGETplus SMARTpool) were purchased from Dharmacon (Lafayette, CO).

2.10. Cell proliferation and wound healing assays

We used the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, FOCUS cells stably expressing Wnt3-myc or empty vector were plated in 96-well plates at a density of 2000 cells/well. Cell proliferation was measured with a combined MTS/PMS solution. Results are presented as the average absorbance of six wells in one experiment and reported as the mean of triplicate assays. For wound healing assay, confluent monolayer cells were scraped with a sterile micropipette tip. The cells were then treated with either anti-Wnt3 mAb (10 μ g/mL) or mouse IgG as a control and photographed at different time points.

2.11. Statistical analysis

Data analyses were performed using the statistical package SPSS for PC (version 9.0; SPSS, Chicago, IL). Data are expressed as mean $\pm$ SD or SE, or median (range). The Student's *t* test,

Wilcoxon signed-ranks test, or Fisher's exact test was used, as appropriate. A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Expression of Wnt mRNAs in HCC cell lines

To determine which Wnt ligands may be involved in the activation of the Wnt/ β -catenin signaling pathway in HCC, RT-PCR was performed in 4 HCC cells (HepG2, Hep3B, Huh7, and FOCUS) using pairs of primers specific for all known 19 human Wnt genes. The efficacy of these primers for PCR was evaluated using human fetal and adult tissues as positive controls (data not shown). All of the primers chosen for this study have been selected on the basis of PCR results obtained from at least 3 sets of test primers specific for each Wnt gene. Most of Wnt mRNAs were expressed in fetal tissues except for Wnt3A (expressed in placenta) and Wnt9A (expressed in adult skeletal muscle). In HCC cell lines, Wnt5A mRNA was detected only in FOCUS cells and Wnt11 was found in HepG2, Hep3B, and Huh7 (Fig. 1). Wnt3 and Wnt6 mRNA expression was present in all 4 HCC cell lines. Interestingly, the expression level of Wnt3 was higher in HCC cell lines compared to fetal brain. Additionally, Wnt3 has been found to induce strong transformation activity and morphological changes, whereas Wnt6 produced weak morphological changes in mammary epithelial cells [21,22], and for these reasons Wnt3 was selected for further study. Indeed, Wnt3 was initially identified as an oncogene activated in tumors arising from mouse mammary tumor virus infection [23].

3.2. Expression of Wnt3, FZD7, and β -catenin target genes in human HBV-related HCC tissues

Wnt3 mRNA expression level was determined in 17 pairs of human HCCs and corresponding peritumoral tissues, and in 4 normal liver samples by real-time RT-PCR. Fourteen of 17 (82%) samples were associated with HBV infection (Table 1). Since we have previously shown that FZD7 receptor was upregulated in HCC and peritumoral tissues, we reconfirmed the expression of FZD7 mRNAs in these samples as well (Fig. 2A). Thirteen of 17 (76%) HCC tumors and 9 of 17 (53%) peritumoral tissues showed increased Wnt3 mRNA expression above that found in normal liver. Both HCC and peritumoral tissues showed increased FZD7 mRNA expression in 11 of 17 (65%) paired samples compared with normal liver. Eleven of 17 (65%) showed increased expression of FZD7 mRNA in HCC tumor compared with peritumoral tissues ($P = 0.031$ by Wilcoxon signed-ranks test). It was interesting to note that 15 of 17 (88%) revealed upregulation of either Wnt3 and/or FZD7 in tumor tissue. We also evaluated if the expression levels of Wnt3 and/or FZD7 were associated with β -catenin gene mutations. Four of 17 (24%) HCC tumors revealed mutations in exon 3 of the β -catenin gene (Fig. 2A), they were single missense mutations and accompanied by nuclear accumulation of β -catenin using immunohistochemical staining (data not shown). Consistent with our previous findings, FZD7 expression was significantly higher in tumors with wild-type β -catenin compared to peritumoral tissues (Fig. 2B). The observation that both Wnt3 and FZD7 were upregulated in HBV-related HCC tumors prompted us to establish the cellular localization of these proteins using immunohistochemical staining. As shown in Fig. 2C, we observed that both Wnt3 and FZD7 proteins were present in the tumor cells; peritumoral areas, however, revealed reduced expression. Following secretion, Wnt proteins are hydrophobic and found associated with the extracellular matrix as well as on the cell surface where activation of signaling takes place [24]. These results suggest that Wnt3 could stimulate FZD7 via autocrine or paracrine mechanisms(s) in HBV-related HCC.

To evaluate the functional consequences of Wnt3 and FZD7 overexpression in HBV-related HCC, we analyzed the expression level of five β -catenin target genes in these tumor and peritumoral tissues. The five genes included two canonical, c-Myc and cyclin D1, and three

recently identified β -catenin target genes overexpressed in human HCC: GS [25], GPR49 [26], and Tbx3 [27]. We observed that GS, Tbx3 and c-Myc were expressed at significantly higher levels in tumors with both mutated and wild type β -catenin than peritumoral tissues (Fig. 2D). The level of GPR49 was significantly increased only in tumors with a mutated β -catenin gene compared to peritumoral tissues. There was no significant difference in cyclin D1 expression.

3.3. Overexpression of Wnt3 activates the Wnt/ β -catenin pathway in HCC cell lines

To characterize the functional properties of Wnt3 in HCC, we determined if exogenous expression of Wnt3 could activate the canonical pathway in FOCUS cells. Stable transfection with a Wnt3-myc in FOCUS cells (FOCUS-Wnt3) resulted in marked increases of both mRNA (data not shown) and protein levels compared to vector-transfected FOCUS cells (FOCUS-C) as revealed by both anti-Wnt3 and myc-tag antibodies (Fig. 3A). The cellular accumulation of β -catenin in FOCUS-Wnt3 cells was detected by Western blot and immunofluorescence staining (Fig. 3A and B). The enhanced expression of GS was also revealed which is a marker of activated Wnt/ β -catenin signaling in both normal and transformed hepatocytes [25]. In addition, the protein levels of c-Myc and cyclin D1 were determined which are known as downstream target genes of TCF/ β -catenin activation. Although these genes are commonly upregulated in HCC, a direct link between activation of β -catenin and induction of c-Myc and/or cyclin D1 in the liver is controversial [28–31]. In FOCUS-Wnt3 cells, overexpression of Wnt3 induced both cyclin D1 and c-Myc expression (Fig. 3A). To confirm if Wnt3 could activate the Wnt/ β -catenin signaling in HCC cells, we assessed downstream TCF activity. There was a 3-fold increase in FOCUS-Wnt3 cells as compared to FOCUS-C control cells (Fig. 3C) ($P < 0.01$, Student's *t* test). In addition, FOCUS-Wnt3 cells exhibited enhanced cell proliferation compared with FOCUS-C cells at day 3 and 4 (Fig. 3D). These results imply that ectopic expression of Wnt3 induced cell proliferation via activation of the Wnt/ β -catenin signaling.

3.4. Inhibition of the canonical Wnt signaling by either Wnt3 siRNA or anti-Wnt3 mAb

To further test the role of Wnt3 on the Wnt/ β -catenin pathway in HCC, we employed siRNA to reduce the expression of Wnt3 in FOCUS HCC cells. The endogenous expression level of Wnt3 in FOCUS cells transfected with the specific siRNA for Wnt3 was reduced by 62% compared with cells transfected with control siRNA (GLO). Reduction of Wnt3 expression led to a decrease of β -catenin levels by 43% (Fig. 4A). In addition, TCF activity was significantly reduced by 30% compared to the control (Fig. 4B). More important, the proliferation rate of FOCUS cells declined at day 4 and 5 following knockdown of Wnt3 by siRNA (Fig. 4C). Recently it has been reported that inhibition of β -catenin expression by siRNA reduced nuclear accumulation and suppressed pediatric hepatic tumor cell proliferation without changes observed in the apoptosis rate [32]. Consistent with their observations, reduction of endogenous level of β -catenin by siRNA led to a decrease in both TCF transcriptional activity as well as cell proliferation (Fig. 4). Additionally, the TCF transcriptional activities were also decreased by 50% in Huh7 ($P < 0.001$), 33% in Hep3B ($P < 0.05$) by Wnt3 siRNA in a dose-dependent manner (data not shown).

We also evaluated if blocking of Wnt3 action with an anti-Wnt3 mAb had effects on Wnt/ β -catenin signaling. Treatment with anti-Wnt3 mAb resulted in a decrease of TCF activity by 60% in Huh7 ($P < 0.001$) and 40% in FOCUS cells ($P < 0.001$), compared with cells incubated with a control mAb (Fig. 5A). The question of whether inhibition of Wnt/ β -catenin signaling would lead to functional changes in the phenotype of HCC cells was addressed. A wound-healing assay revealed delayed wound healing of FOCUS and Huh7 cells treated with anti-Wnt3 mAb. Most of the wound closed with migrating FOCUS cells exposed to the control antibody at 24 h as compared to cells treated with anti-Wnt3 mAb (Fig. 5B).

3.5. Activation of Wnt/ β -catenin signaling by Wnt3 was mediated through FZD7 receptor in HCC cells

Previous studies demonstrated that up-regulation of FZD7 was associated with activation of Wnt/ β -catenin cascade in HCC [16,17]. The present study suggests that Wnt3 also has a positive effect on canonical Wnt signaling in HCC. Therefore, we hypothesized that Wnt3 action may be through the FZD7 receptor. To test this possibility, the physical interaction between Wnt3 and FZD7 was examined by co-immunoprecipitation experiments. A full-length human FZD7 (FZD7-EE) and FZD7- Δ CRD-EE (deletion mutant of the putative ligand-binding motif in FZD7) were generated with an EE tag at C-terminus, followed by immunoblot analysis to confirm the expression of Wnt3-myc, FZD7-EE, and FZD7- Δ CRD-EE proteins in Huh7 and FOCUS cells (Fig. 6A; left panel). In this context, Huh7 and FOCUS cells were co-transfected with Wnt3-myc and FZD7-EE or FZD7- Δ CRD-EE expression plasmids. Cell lysates were subjected to an immunoaffinity purification using anti-EE antibody and immunoblotted with anti-EE or anti-myc-tag antibody. As shown in Fig. 6A (right panel), both FZD7-EE and FZD7- Δ CRD-EE were detected with anti-EE antibody. However, Wnt3 could only be identified by a co-immunoprecipitation from cells co-transfected with Wnt3-myc and FZD7-EE but not from cells co-transfected with Wnt3-myc and FZD7- Δ CRD-EE deletion mutant. These findings suggest that this interaction is specific through direct binding to the CRD of FZD7.

It was important to establish if Wnt3/ β -catenin signaling was mediated by FZD7. We used FOCUS-Wnt3 cells, which overexpress Wnt3 in these experiments. Levels of FZD7 and β -catenin were reduced by 65% and 80% in FZD7 siRNA transfected cells compared to control cells, respectively (Fig. 6B). In addition, TCF transcriptional activity was reduced as well by 63% compared to FOCUS-Wnt3 cells transfected with the control siRNA GLO (Fig. 6C). Cell proliferation was decreased principally after 4 days of culture (Fig. 6D). These experiments suggest that activation of the Wnt/ β -catenin pathway by Wnt3 is mediated in part through FZD7 in HCC cells.

4. Discussion

Nuclear and/or cellular β -catenin accumulation, a hallmark of the canonical Wnt signaling activation, is found in 30–70% of HCCs. However, mutations of β -catenin and/or APC genes are detected only in 20–30%, whereas axin1 mutations are rare in HCC [18,33–38]. Previous reports suggest that several signaling components upstream of β -catenin may be involved for wild-type β -catenin accumulation to occur in HCC. These events include overexpression of FZD7 or Dishevelled, or down-regulation of the human homologue of Dapper 1 and SFRP1 by promoter hypermethylation [16,17,39–41].

We have previously demonstrated a marked upregulation of FZD7 in HBV-related HCC. This event was associated with wild-type β -catenin accumulation in the cytoplasm and nucleus. The functional consequences of FZD7 upregulation were increased cell motility and migration [16,17]. Interestingly, FZD7 overexpression was already evident in peritumoral dysplastic liver tissue as compared to normal liver suggesting that upregulation was an early event during tumor formation. The present study confirms those previous observations. Indeed, 65% of HBV-related HCCs had higher levels of FZD7 expression in HCC compared to peritumoral tissues, which also had upregulation of FZD7 compared to normal liver. Enhanced FZD7 gene expression has also been reported in esophageal and gastric tumors [42,43]. These findings suggest that overexpression of FZD7 is closely related to activation of the canonical Wnt pathway in these cancers as well. However, the precise mechanism(s) for expression in gastric tumors are unknown. One interesting possibility is the potential role of Wnt ligand(s) since human teratocarcinoma (NCCIT) cells stimulated with Wnt3a produced increased transcription of the FZD7 gene [44]. Thus, there are various possible explanations for FZD7 overexpression

in HCC that include paracrine or autocrine induction by Wnt ligands, gene amplification and demethylation of FZD7 gene promoter sequences; further studies will be required to examine these possibilities.

Wnt3 belongs to the Wnt1 class of ligands and stimulates the canonical Wnt/ β -catenin pathway [45]. Enhanced Wnt3 gene expression has been reported in A549 (lung), MKN45 (gastric), and human breast and rectal tumors [46]. In this study, Wnt3 mRNA expression was increased in 76% of HBV-related HCC and 59% of peritumoral tissues compared with normal liver. Furthermore, 53% of HBV-related HCC showed increased Wnt3 expression compared with adjacent peritumoral tissues. Therefore, upregulation of Wnt3 may also be an early event during hepatocarcinogenesis. The localization of both Wnt3 and FZD7 proteins in transformed hepatocytes implies a possible autocrine or paracrine loop of β -catenin activation in HBV-related HCC. In this regard, a recent study also revealed that both Wnt3 and FZD7 are highly expressed in Paneth cells located in the lower portions of intestinal crypts of the small intestine which is known to be a highly proliferative tissue [47]. Taken together, these findings support the idea that both Wnt3 and FZD7 may be important in cell proliferation and migration via activation of the canonical Wnt signaling cascade.

There is little known about the direct interaction between Wnts and FZDs, although upregulation of Wnts and/or FZDs genes has been found in variety of tumor types. To address this issue, we performed co-immunoprecipitation experiments using Wnt3 and FZD7 plasmids. To rule out nonspecific bindings between the 2 proteins, a CRD-deleted FZD7 plasmid was employed as a control. The interaction between Wnt3 and FZD7 was absent when the CRD for Wnt ligand binding domain of the FZD7 was deleted. Thus, the interaction between Wnt3 and FZD7 is specific and occurred through the CRD motif of FZD7. In addition to a physical association between Wnt3 and FZD7, the findings of enhanced TCF transcriptional activity exhibited by Wnt3 was related to FZD7 expression as shown by knockdown experiments with siRNA. Such observations support the concept that Wnt3 action on the canonical Wnt signaling cascade is mediated through FZD7 in HCC cells.

There have been reports that Wnt ligands bind to multiple FZD receptors and *vice versa* [48, 49]. In particular, Wnt5a and Wnt11 also activate the non-canonical Wnt signaling upon binding to FZD7 [50,51]. These investigations provide support for our findings since expression of Wnt3, Wnt5A, and Wnt11 was detected in HCC cells all of which are known to have upregulation of FZD7 [17]. It will be of interest to further define the mechanisms of how FZD7 receptors may interact with different Wnt ligands during hepatocarcinogenesis.

In the present study, we documented upregulation of Wnt3 and FZD7 in HBV-related HCC tumors and corresponding matched peritumoral tissues compared to normal liver. The activation of Wnt/ β -catenin signaling pathway by Wnt3 in HCC cell lines was demonstrated by exogenous overexpression of Wnt3 or by blocking Wnt3 action with Wnt3 siRNA or anti-Wnt3 mAb, which revealed positive and negative influences on HCC cell proliferation and migration, respectively. More important, our experiments provide evidence that activation of the Wnt3/ β -catenin cascade was mediated by the FZD7 through binding of Wnt3 to the CRD of FZD7. These findings suggest that Wnt3 is one of ligands for FZD7, which is overexpressed in the majority of HBV-related HCC and subsequently leads to activation of the β -catenin signal transduction cascade as one of key elements in the pathogenesis of this disease.

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Fig. 1.

Expression of Wnt mRNAs in HCC cell lines. Wnt ligand mRNAs were detected in HCC cell lines using RT-PCR. Wnt3 and Wnt6 mRNAs were found in all HCC cell lines. Wnt5A was present only in FOCUS, whereas Wnt11 was detected in HepG2, Hep3B, and Huh7. The expression of glyceraldehydes-3-phosphate dehydrogenase gene (GAPDH) verified the quality of mRNA in each sample. * Wnt3, Wnt6, and Wnt11 from FB (fetal brain), whereas Wnt5A from FK (fetal kidney).

Fig. 2.

Expression of Wnt3, FZD7, and β -catenin target genes in human HBV-related HCC tissues. (A) The levels of Wnt3 and FZD7 mRNA measured by real-time RT-PCR. The black bars represent the mRNA levels in HCC tissues, and the white bars in corresponding peritumoral areas. Experiments were performed in duplicate, and data are expressed as a ratio to the mean value found in normal liver ($N = 4$). The Wnt3 mRNA (left panel) expression showed increased in HCCs (77%) and peritumoral tissues (59%). The FZD7 mRNA (right panel) expression was increased in both HCC and peritumoral tissues (59%) compared to normal liver. Within the paired samples, 11 of 17 (65%) showed increased expression of FZD7 mRNA in tumors compared with corresponding peritumoral tissues ($P < 0.05$). Tumors with a mutant β -catenin gene are marked with an asterisk. (B) Distribution of Wnt3 and FZD7 expression according to β -catenin status. T(mut); tumor with β -catenin mutation, T(WT); tumor with wild-type β -catenin, pT; peritumoral tissue. (C) Detection of Wnt3 and FZD7 proteins by immunohistochemistry. Representative example (case No. 6) of HCC (e–h) and peritumoral tissue (a–d) immunostained with either anti-Wnt3 Ab (b, f) or anti-FZD7 Ab (c, g). Panels (a, e) depict hematoxylin and eosin staining, and (d, h) represent negative controls (see Methods). Note that Wnt3 and FZD7 expression is evident in tumor cells whereas peritumoral tissues show reduced levels of staining. (D) Evaluation of Wnt/ β -catenin target gene expression by real-time RT-PCR in HCC according to β -catenin status. Values are expressed as multiples of the relative expression found in normal liver ($N = 4$). Mean expression values of GS, Tbx3 and c-Myc were significantly higher in HCCs with both wild type and mutant β -catenin gene compared to peritumoral tissues. There was significant increase in GPR49 expression in HCC carrying mutant β -catenin compared with peritumoral tissue.

Fig. 3. Overexpression of Wnt3 activated Wnt/β-catenin signaling in FOCUS HCC cells. (A) Western blot analysis in FOCUS-Wnt3 or FOCUS-C cells. Wnt3 was overexpressed in FOCUS-Wnt3 as demonstrated by anti-Wnt3 and anti-myc tag mAbs. Expression levels of Cyclin D1, GS, and c-Myc proteins were also increased. Note that the anti-c-Myc mAb does not recognize the myc-tag, but rather the c-Myc protein. Actin was used as a loading control. (B) Control (FOCUS-C) or FOCUS-Wnt3 cells were double-immunostained with anti-myc tag (red color) and anti-β-catenin (green color) Abs; the nucleus was counterstained with the DAPI (blue color). The bottom panel reveals the merged images indicating nuclear localization of β-catenin. (C) The TCF transcriptional activity was increased by 3 fold in FOCUS-Wnt3 cells compared with control (**P* < 0.01). (D) Wnt3 expression increased the FOCUS cell proliferation rate. The results are expressed as the mean ± SE of triplicate assays. **P* < 0.05 versus control

Fig. 4.

Inhibition of Wnt/β-catenin signaling by Wnt3 siRNA. (A) FOCUS cells were transfected with either siRNA GLO (a control for transfection efficiency and silencing), Wnt3 siRNA, or β-catenin siRNA and immunoblotted with either anti-Wnt3 or anti-β-catenin mAbs (left panel). Expression levels of Wnt3 and β-catenin were plotted as a ratio to actin (right panel). (B) Effects of Wnt3 siRNA on the TCF transcriptional activities in FOCUS HCC cell lines. Wnt3 siRNA, control siRNA (siRNA GLO), or β-catenin siRNA were co-transfected in the presence of a TCF reporter gene. The TCF transcriptional activity was decreased in FOCUS HCC cells (30% for Wnt3 siRNA, and 43% for β-catenin siRNA). (C) FOCUS cell proliferation was reduced by Wnt3 siRNA compared to control. The results are expressed as the mean ± SE of triplicate assays. * $P < 0.05$ versus control.

Fig. 5.

Anti-Wnt3 mAb treatment inhibits Wnt/ β -catenin signaling. (A) Effects of anti-Wnt3 mAb on TCF transcriptional activities in HCC cell lines. The TCF transcriptional activities were decreased in Huh7 (60%), and FOCUS (40%) cells by the anti-Wnt3 mAb. * $P < 0.001$ versus control. (B) Delayed wound healing exhibited by the anti-Wnt3 mAb. Both Huh7 and FOCUS cells treated with anti-Wnt3 mAb showed delayed wound closure. At 24 h, most of the wound was closed with migrating FOCUS cells treated with the control IgG, while it remained open in those treated with anti-Wnt3 mAb. Graph of the wound closure (percent) plotted against time (bottom panel).

Fig. 6.

Activation of Wnt/β-catenin signaling is mediated by a Wnt3 and FZD7 interaction. (A) Co-immunoprecipitation assay demonstrating the interaction between Wnt3 and FZD7. Huh7 and FOCUS cells were transfected with either Wnt3-myc and FZD7-EE or FZD7-ΔCRD-EE plasmids. Cell lysates were first immunopurified using monoclonal anti-EE antibody immobilized onto a Sepharose Fast Flow matrix, and immunoblotted with anti-EE or anti-myc antibody. (Left panel) Demonstration of Wnt3, FZD7, and FZD7-ΔCRD protein expression in Huh7 and FOCUS cells after transfection. (Right panel) Immunoblotting with anti-EE or anti-myc antibodies after immunoprecipitation. Note that Wnt3 was detected only in an immunoprecipitation derived from cells co-transfected with Wnt3-myc and FZD7-EE but not from cells transfected with Wnt3-myc and FZD7-ΔCRD-EE, which lacks the putative ligand-binding domain. (B) Western blot analysis to evaluate the knockdown of FZD7 expression by FZD7 siRNA. Transfection of FZD7 siRNA in FOCUS-Wnt3 reduced FZD7 expression (by 65%) as well as β-catenin accumulation (by 80%) (left panel). Expression levels of Wnt3,

FZD7 and β -catenin were plotted as a ratio to actin (right panel). (C) FZD7 siRNA abolished the high TCF transcriptional activity in FOCUS-Wnt3 mediated by Wnt3 overexpression. (D) FOCUS-Wnt3 cell proliferation was reduced by FZD7 siRNA compared to control. The results are expressed as the mean $\pm$ SE of triplicate assays. * $P < 0.05$ versus control.

Table 1Clinical and Pathologic Features and β -catenin Mutation in HCC Patients

Features	n (%)
Age (y)	Range, 22–67; mean age, 47
Sex	
Male	14 (82)
Female	3 (18)
Virus	
HBs Ag	14 (82)
HCV Ab	0
Differentiation	
Well	1 (6)
Moderate	8 (47)
Poor	6 (35)
Un	2 (12)
HCC staging	
T1 (solitary tumor without vascular invasion)	0
T2 (solitary tumor with vascular invasion or multiple tumors smaller than 5 cm)	4 (24)
T3 (any tumor with major vascular invasion or multiple tumors larger than 5 cm)	13 (76)
Liver Cirrhosis	10 (59)
β -catenin	
IHC (C and/or N)	8 (47)
mutation in exon 3	4 (24)

N, nucleus; C, cytoplasm;

IHC, immunohistochemical staining