

**Targeting Wnt/ β -catenin Signaling in
Liver Cancers**

Wenhui Wang

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Targeting Wnt/ β -catenin Signaling in Liver Cancers

Therapie gericht op Wnt/ β -catenine signalering in leverkankers

Thesis

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Wenhui Wang

born in Datong, Shanxi Province, China

Erasmus University Rotterdam



Doctoral Committee

Promoter:

Prof. dr. M. P. Peppelenbosch

Inner Committee:

Prof. dr. J.N.M. Ijzermans

Prof. dr. C.J.M. van Noesel

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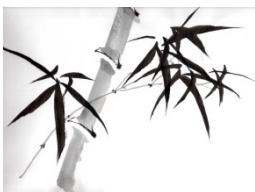
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Chapter 1

General Introduction and Outline of This Thesis



Liver cancers

Liver cancer is one of the leading global health care issues. Primary liver cancers include hepatocellular carcinoma (HCC), cholangiocarcinoma and hepatoblastoma, the latter which is mainly observed in children. Focal nodular hyperplasia and hepatocellular adenoma are benign hepatocellular tumors that develop most frequently in women without cirrhosis (1). Other tumors observed in the liver, such as fibrosarcoma, angiosarcoma, leiomyosarcoma and lymphoma, are rare but have malignant potential.

Around 90% of liver cancer patients are diagnosed with HCC, the fifth most frequent cancer and highly relevant to cancer related deaths worldwide (2, 3). Hepatitis B (HBV) and hepatitis C (HCV) viruses together with alcohol abuse, obesity-induced non-alcoholic steatohepatitis (NASH) and aflatoxin-B1 exposure are considered as the major causes for HCC, which show regional preferences. In parts of Asia and Africa, high incidence of HCC is linked to elevated HBV and aflatoxin-B1 prevalence (4). In western countries, the main causes are HCV, alcohol abuse and NASH (5, 6).

Hepatocarcinogenesis is a complex pathological process that can take decades from tumor cell initiation to the final malignant tumor. Etiological factors mentioned above lead to chronic hepatitis, which gives rise to fibrosis and progression to cirrhosis around 10 years later (7). Cirrhosis chronically alters the liver microenvironment which potentiates the initiation and progression of HCC. During this process, the accumulation of aberrant genetic and epigenetic modifications elicits the dysregulation of signaling pathways. This in turn facilitates the transformation from the precancerous dysplastic hepatocytes into early HCC that ultimately progresses to malignant phenotypes.

Wnt/ β -catenin signaling

The evolutionarily conserved Wnt signaling pathway is involved in both physiological and pathophysiological processes (8-10). Wnt signaling is triggered by Wnt ligands. These ligands are generated within the endoplasmic reticulum (ER), modified by palmitoylation by the Wnt acyl-transferase porcupine (PORCN) and shuttled by Wntless (WLS) from the Golgi to the plasma membrane where they can signal in an autocrine or paracrine manner (Figure.1) (11, 12). Nineteen Wnt ligands have been identified in the human genome. Based on the

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dependency of β -catenin to transduce the signal, Wnt signaling is subdivided into canonical Wnt/ β -catenin pathway, the noncanonical planar cell polarity pathway, or the noncanonical Wnt/calcium pathway. In this thesis, we mainly focus on the canonical Wnt/ β -catenin pathway, largely relevant to HCC.

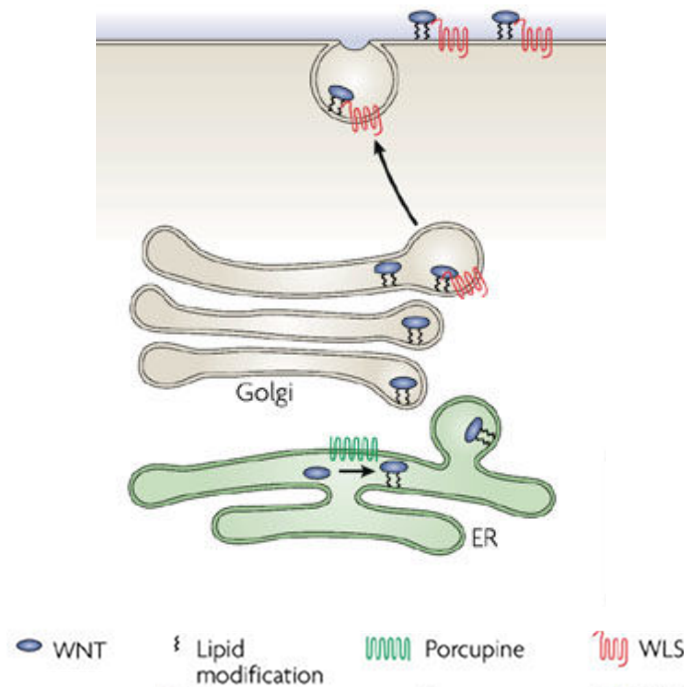


Figure.1 Wnts are lipid modified by PORCN in the ER and escorted by WLS from the Golgi to the plasma membrane for secretion. Figure adapted from reference Hausmann G, et al (11).

Canonical Wnt/ β -catenin signaling is normally turned off in tissues of adults with the exception of part of stem cell niches (13). Regulation of Wnt/ β -catenin signaling is fine-tuned at both extracellular and intracellular levels. Extracellularly, Wnt ligands are captured by Wnt antagonists, such as secreted Frizzled-related proteins (SFRPs), dickkopf (DKKs) and the Wnt inhibitory factor (WIF) (14). Intracellularly, the cytosolic transcription factor β -catenin is tightly regulated by a multiprotein complex composed of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1 α . Kinase CK1 α triggers the priming site at Ser45 of β -catenin allowing the subsequent phosphorylation at Thr41, Ser37 and Ser33 by GSK3. Then the β -transducin repeat containing protein (β TRCP) recognizes the phosphorylated β -catenin for subsequent proteolysis (14, 15). The overall effect is to maintain minimal levels of free cytosolic β -catenin (Figure.2).

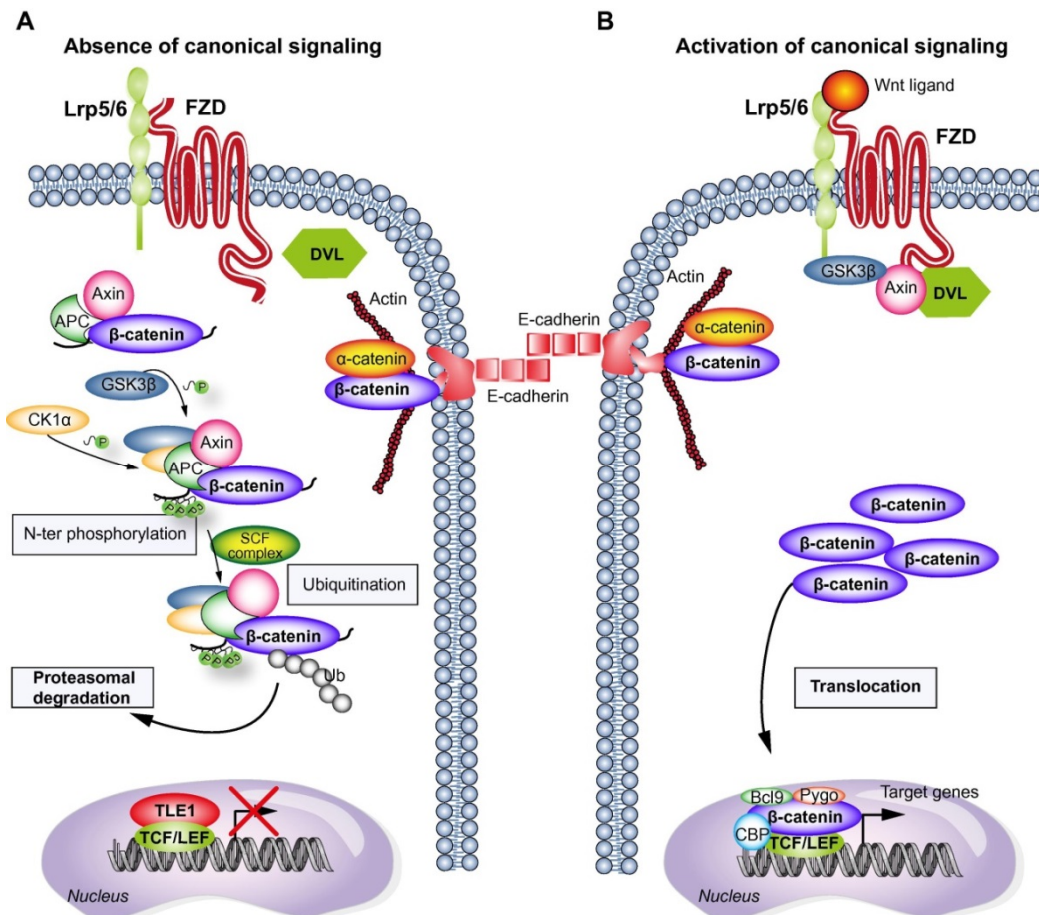


Figure.2 The canonical Wnt/β-catenin signaling pathway. (A) In the absence of Wnt ligands, β-catenin is phosphorylated by a degradation complex consisting of GSK3β, CK1α, APC and AXIN1/AXIN2. Phosphorylated β-catenin is targeted for proteasomal degradation after ubiquitination by the SCF protein complex. In the nucleus, the TCF/LEF transcription factor activity is repressed by TLE-1. (B) Activation of canonical Wnt/β-catenin signaling leads to the dissociation of the degradation complex. As a result, β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it promotes the expression of target genes *via* interaction with TCF/LEF transcription factors and other proteins such as CBP, Bcl9, and Pygo. Both figure and text adapted from reference Pez F, et al (16).

Wnt ligands initiate the signaling by binding with a member of the frizzled receptor (FZD) family and one of the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors. Then the scaffolding proteins disheveled (DVL) and AXIN are recruited to the membrane, leading to the disassembly of the multiprotein β-catenin destruction complex (17) and subsequent accumulation of unphosphorylated β-catenin (active β-catenin) in the cytoplasm. The active β-catenin translocates to the nucleus (18) and binds transcription factors of the T-cell factor (TCF7, TCF7L1 and TCF7L2)/lymphoid enhancer-binding factor (LEF) family, triggering the transcription of downstream Wnt/β-catenin target genes (16, 19) (Figure.2). Thus, the mechanism underlying the regulation of canonical Wnt/β-catenin

signaling is complicated. Functional mutations of these related proteins could lead to the dysregulation of target gene transcription.

Aberrant activation of Wnt/ β -catenin signaling in hepatocellular carcinoma

Inappropriate activation of Wnt/ β -catenin signaling is critical in HCC (14, 16, 20-22). The central component transcription factor β -catenin shows nuclear accumulation in around 40%-70% of HCC patients. Distinctive molecular or genetic alterations have been identified to stabilize β -catenin and to aberrantly trigger Wnt/ β -catenin signaling, such as elevated level of upstream Wnt ligands or cell surface receptors and decrease of extracellular inhibitors [26]. In addition, frequently gain-of-function mutations are observed in exon3 of the CTNNB1 gene (encoding β -catenin) (15-25%) at the phosphorylation residues (23), which result in the expression of mutant β -catenin resistant to proteolytic degradation. Loss-of-function mutations of negative regulators are reported in AXIN1 (10.4%), AXIN2 (3.3%) and APC (1.4%) (24), evidently contrasting with the situation in colorectal cancer (CRC) where up to 80% of cancers display mutated APC (25, 26). Frameshift mutations or genomic deletions in these genes elicit the compromised function of the multiprotein complex to degrade β -catenin and is thus also related to enhanced Wnt/ β -catenin signaling.

The relative mutation frequencies of these various Wnt/ β -catenin signaling elements are different in HCC as compared to other cancers, e.g. sporadic CRC. The reason underlying these disparities could be due to different etiology of HCC, such as HBV or HCV, which shows preference on the different type of mutations that arise in liver genomes as compared to other sites in the body (24, 27). It may also derive from the fact that in different organs, optimal cancer-driving Wnt/ β -catenin signaling mutations may be substantially different, resulting in selection pressure for different types of mutations (25).

Target Wnt/ β -catenin signaling

In light of the importance of aberrant activation of Wnt/ β -catenin signaling in HCC, components involved in this pathway could be promising therapeutic targets for HCC therapy. After decades of extensive research to identify these Wnt/ β -catenin signaling inhibitors, there have been numerous small molecules discovered that may possess this potential. In table 1,

we provide a summary of the reported Wnt/ β -catenin signaling inhibitors tested in different tumor types. Some of these inhibitors target the upstream components of Wnt/ β -catenin signaling including the porcupine protein, Wnt ligands, FZD receptors and co-receptors. Others aim at interfering with the intracellular signal transduction process by targeting DVL, AXIN, GSK3, CK1 α or β -catenin itself, or affect its interaction with co-activators or transcriptional factors. Some of these inhibitors are currently undergoing clinical trials.

Table.1 Wnt/ β -catenin signaling inhibitors undergoing preclinical and clinical evaluation

Targets	Compounds	Diseases	Stage	References
Porcupine	LGK974	Pancreatic adenocarcinoma, BRAF mutant colorectal cancer head and neck squamous cell carcinoma	Phase 1	(28)
	IWPs	Colon cancer	Preclinical	
	Wnt-C59	Mammary tumor Pancreatic cancer Colorectal cancer	Preclinical	(29, 30)
	ETC-1922159	Mammary tumor Teratocarcinomas Breast cancer	Phase 1	(31)
FZD1/2/5/7/8	OMP-18R5 (vantictumab)	Lung cancer Pancreas cancer Colon cancer	Phase 1	(32)
FZD7	sFZD7	HCC Liver cancers (HCC)	Preclinical	(33)
FZD8	OMP-54F28	Ovarian Cancer Pancreas cancer Solid tumor	Phase 1	(21)
LRP6	Niclosamide	Prostate; Breast	Preclinical	(34)
	Silibinin	Prostate; Breast	Preclinical	(35)
LRP5/6	Salinomycin	breast, prostate, lung, gastric, osteosarcoma, HCC	Preclinical	(36-41)
Wnt1	Anti-Wnt1	HCC , CRC, Lung cancer, sarcoma, breast cancer, head-neck squamous cell carcinoma	Preclinical	(42-46)
Wnt2	Anti-Wnt2	Melanoma, mesothelioma, lung cancer	Preclinical	(47-49)
Wnt10b	Anti-Wnt10b	head-neck squamous cell carcinoma	Preclinical	(46)
Wnt ligands	WIF-Fc/ SFRP-Fc	HCC	Preclinical	(50)
DVL	NSC668036			(51)
	3289-8625	Prostate cancer		(52)
	FJ9	Melanoma, lung cancer		(53)
Tankyrase/AXIN	XAV939	CRC, neuroblastoma, breast cancer	Preclinical	(54-56)
	IWR-1	CRC, prostate	Preclinical	(57)
	JW55	CRC	Preclinical	(58)
GSK3 β	DIFs	Leukemia	Preclinical	(59-63)

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		HeLa		
CK1 α	Pyridinium	CRC	Preclinical	(64, 65)
β -catenin phosphorylation	CGK062	CRC, HCC , prostate cancer	Preclinical	(66)
β -catenin ubiquitination	Hexachlorophene	CRC	Preclinical	(67)
	Isoreserpine	CRC	Preclinical	(68)
β -catenin	β -catenin siRNA	HCC	Preclinical	(69)
	BBI608	Glioblastoma, CRC, HCC , gastric cancer, pancreas cancer, lung cancer	Phase 1/2	(70)
β -catenin/CBP	ICG-001	CRC, breast cancer, pancreatic cancer, head-neck squamous cell carcinoma	Preclinical	(71-74)
	PRI-724	Pancreatic adenocarcinoma, leukemia, CRC, HCV-induced cirrhosis, solid tumor	Phase 1	
β -catenin/TCF	PKF115-548 PKF222-815 CGP049090 FH535	HCC , CRC, lung cancer	Preclinical	(75-78)
cyclooxygenases	NSAIDS	CRC	Preclinical	(79-82)
β -catenin/E-cadherin	Vitamin derivatives	CRC	Preclinical	(83)
β -catenin nuclear export	Peg-IFN	HCC	Preclinical	(84)

Aim of the thesis

During last decades, a tremendous progression of knowledge and understanding about liver cancer development has been witnessed. The progression in clinical technology has been instrumental for early detection. In addition, the Barcelona clinic liver cancer (BCLC) staging and treatment model that also takes into account remaining liver functionality and general health of the patient, has been broadly endorsed worldwide. These achievements facilitate the early diagnosis and enable more efficient treatment strategies to improve the outcome of HCC patients. Nevertheless, HCC related mortality is still high worldwide. Given that HCC individuals show extensive phenotypic and molecular heterogeneity, it is important to identify the molecular features and genomic traits of HCC patients, thus aiding reasonable stratification and optimal personal treatment decisions.

As one of the critical contributing factors to HCC growth, aberrant activation of Wnt/ β -catenin signaling derives from a variety of molecular alterations involved in this pathway, especially mutations of β -catenin and AXIN1. As illustrated in Table 1, a variety of drugs targeting Wnt/ β -catenin signaling have been developed, but most of them have not been

thoroughly tested for liver cancer. In the current thesis, our first aim is to explore the effectiveness of two classes of drugs in a panel of HCC cell lines, i.e. an extracellular Wnt secretion inhibitor and cytosolic tankyrase inhibitor. The second aim is to compare the response and sensitivity for these inhibitors depending on the specific β -catenin signaling defect present in these HCC cell lines, i.e. *CTNNB1* or *AXIN1* mutations.

Our third aim is to achieve a better understanding the mechanism through which *AXIN1* mutations contribute to HCC cell growth. As a critical negative regulator of Wnt/ β -catenin signaling, mutation or deletion of *AXIN1* is expected to support tumor growth by enhancing β -catenin signaling. However, *AXIN1* mutations were shown to not cause a robust induction of β -catenin target genes in liver cancers (3, 85). Hence, the specific role of *AXIN1* mutation in reprogramming Wnt/ β -catenin signaling in liver cancers remains under debate. Lastly, we also intend to find new potential molecular targets regulating Wnt/ β -catenin signaling in HCC.

Outline of this thesis

In **chapter 1**, we have provided a general introduction of Wnt/ β -catenin signaling and its role in the initiation and progression of liver cancers. In **chapter 2**, we review how aberrant activated Wnt/ β -catenin signaling interacts with the HCV viral components and potentiates the progression from hepatitis C to HCC. In **chapter 3**, we investigate the dependency of extracellular Wnt secretion to support growth in 9 HCC cell lines characterized by mutations in either *CTNNB1*, *AXIN1* or no obvious mutation in a β -catenin signaling related component, also in comparison with CRC cell lines. In **chapter 4**, we use the same cell line panel to explore whether the inhibition of tankyrase, by endorsed inhibitors XAV939 as well as IWR-1, stabilizes *AXIN1/2* and attenuates Wnt/ β -catenin signaling in HCC cells. We further compare the contribution of *AXIN1* and *AXIN2* in this process. In **chapter 5**, we test the expression levels of serine-threonine kinase receptor-associated protein (STRAP) in patient HCC tissues and investigate its function in regulating Wnt/ β -catenin signaling activity in our panel of HCC cell lines. In a related project (**chapter 6**), we describe a novel approach to support the identification of Familial Adenomatous Polyposis carriers among children with hepatoblastoma tumors employing β -catenin immunohistochemical staining and mutation analysis.

Chapter 1

The novel insights derived from this thesis will be summarized and discussed in **chapter 7** and **chapter 8**, which will provide a better understanding of Wnt/ β -catenin signaling in liver cancers and experimental evidence for future stratification and optimal treatment decision of patients carrying Wnt-driven liver cancers.

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Chapter 2

Action and function of Wnt/ β -catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma

Wenhui Wang, Qiuwei Pan, Gwenny M. Fuhler, Ron Smits, Maikel P. Peppelenbosch*

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center
and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands



Abstract

Hepatitis C virus (HCV) infection is one of the leading causes for hepatocellular carcinoma (HCC) worldwide, but the mechanistic basis as to how chronic HCV infection furthers the HCC process remains only poorly understood. Intriguingly, accumulating evidence indicates that HCV core and nonstructural proteins provoke activation of the Wnt/ β -catenin signaling pathway, whereas the evidence supporting a role of Wnt/ β -catenin signaling in the onset and progression of HCC is compelling. Convincing molecular explanations as how expression of viral effectors translate into increased activity of the Wnt/ β -catenin signaling machinery are still largely lacking, hampering design of rational strategies aimed at preventing HCC. Furthermore, how such increased signaling is especially associated with HCC oncogenesis in the context of HCV infection remains obscure as well. Here we review the body of contemporary biomedical knowledge on the role of Wnt/ β -catenin pathway in the progression from chronic hepatitis C, to cirrhosis and HCC and explore potential hypotheses as to the mechanisms involved.

Keywords: Hepatocellular carcinoma; Wnt/ β -catenin signaling; hepatitis C virus

Introduction

HCV is estimated to infect up to 2% of the global population (around 180 million people worldwide) [1] with approximately 3-4 million new infections each year [2, 3]. Following infection, 60%-80% of affected individuals eventually develop chronic hepatitis [4]. After around 10 years of infection, 5%-10% of these chronically infected patients progress to cirrhosis [5]. In addition to the high mortality associated with advanced cirrhosis *per se*, annually another 2.0%-6.6% of cirrhotic patients with HCV infection progress to HCC [6, 7]. Understanding the details as to how HCV infection can promote the HCC process is thus of critical importance for the rational design of novel avenues aimed at the prevention and treatment of HCC.

Distinct from hepatitis B virus (HBV), a DNA virus that can integrate into the human genome and thus directly provoke genomic alterations potentially leading to cancer [8], HCV is a RNA virus lacking a DNA intermediate phase in its life cycle and therefore its infection of liver cells is not associated with damage to the host genetic material *per se* [9]. Hence, the tumor promoting potential of HCV derives from indirect interaction with the hepatocyte genome. However, other pathogens with a similar infection route are less clearly associated with progression towards HCC, compare for instance hepatitis E virus infection. It thus appears that HCV has specific properties that promote further hepatocyte transformation.

The Wnt/ β -catenin pathway is an attractive candidate to mediate the HCV-specific effects leading to hepatocyte oncogenic transformation. Activation of this pathway is clearly contributing to hepatocarcinogenesis as indicated by the detection of recurrent genetic mutations of Wnt/ β -catenin signaling pathway components in HCC that appear especially frequent in HCV-related tumors. Intriguingly, HCV-derived viral proteins appear to be capable of autonomous activation of Wnt/ β -catenin signaling, although the underlying molecular mechanisms remain poorly understood. Here we explore potential hypotheses explaining these effects and summarize documented interactions of Wnt/ β -catenin signaling components in HCC patients with HCV infection. We propose that the Wnt/ β -catenin signaling pathway constitutes a rational target for the prevention and treatment of HCV-associated HCC.

Wnt/ β -catenin signaling

Wnt/ β -catenin signaling is a pivotal morphogenetic pathway and accordingly associated with a host of physiological and pathophysiological processes, including embryonic patterning, cell proliferation, differentiation, angiogenesis and especially cancer [10-12]. Wnt signalling is initiated by binding of Wnt ligands to their cognate receptors. These Wnt ligands are 40kDa cysteine-rich glycoproteins [13], which following synthesis and primary glycosylation on the endoplasmic reticulum are palmitoylated by Wnt acyl-transferase porcupine protein in the Golgi apparatus. Secretion of Wnts then requires the evenness interrupted/wntless/G protein-coupled receptor 177 (Evi/Wls/GPR177), which shuttles palmitoylated Wnts to the plasma membrane, where they are released by the cell and initiate autocrine or paracrine signaling. Hitherto, nineteen Wnts have been identified in the human genome [14], and because annotation of *Homo sapiens* DNA is now quite complete it is unlikely further Wnt paralogues will be discovered. Wnts can provoke different modes of cellular signaling, either mediated by β -catenin or independent of this protein. According to the dependence on β -catenin for provoking cellular effects, Wnts are classified into canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) subgroups [15, 16]. In this review we shall focus on the canonical Wnts, as these are most associated with HCC in general and HCV-infection associated HCC in particular.

Except for several stem cell niches, canonical Wnt/ β -catenin signaling is typically not active in tissues of adult individuals [17], despite constitutive production of Wnt ligands. This is a result of the action of a range of Wnt antagonists, such as secreted Frizzled-related proteins (SFRPs), dickkopf (DKKs) and the wnt inhibitory factor (WIF) [18]. In this non-signaling state, cytosolic β -catenin is continuously phosphorylated at Ser33, Ser37, Thr41 and Ser45 residues located in exon3 by a multiprotein complex consisting of adenomatous polyposis coli (APC), AXIN, glycogen synthase kinase-3 β (GSK3 β) and casein kinase 1 (CK1). These phosphorylations cause β -catenin to be recognized and poly-ubiquitinated by the β -transducin repeat containing protein (β TrCP) followed by β -catenin degradation in the proteasome [18, 19]. The overall effect is that minimal free cytosolic β -catenin is available for nuclear signaling and thus Wnt-mediated gene transcription is absent under normal conditions.

Upon binding of Wnt ligands to a complex consisting of the frizzled receptor (FZD) and co-receptors, which include the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the scaffolding protein disheveled (DVL) is recruited to the membrane, an event that in turn

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causes the disassembly of the multiprotein β -catenin destruction complex. This results in rescue of β -catenin from proteasomal degradation and thus the accumulation of β -catenin in the cytoplasm, eventually causing β -catenin translocation to the nucleus [20]. In the nucleus, β -catenin binds transcription factors of the T-cell factor (TCF7, TCF7L1 and TCF7L2) 4/lymphoid enhancer-binding factor (LEF) family, triggering transcription of downstream Wnt target genes, including *CYCLIND1*, *AXIN2*, *C-MYC*, *RING FINGER PROTEIN 43 (RNF43)* and *ZINC/RING FINGER PROTEIN 3 (ZNR3)* [21, 22]. RNF43 and ZNR3 are two closely related transmembrane E3 ligases, which remove surface FZD receptors by promoting their endocytosis [23]. This E3 ligase activity is in turn negatively modulated by R-spondins (RSPO) and the leucine-rich repeat-containing G-protein coupled receptor 4/5/6 (LGR4/5/6) that sequester RNF43 and ZNR3 from FZD receptors by forming a tripartite complex [24]. Hence regulation of Wnt target gene transcription is complex allowing for extensive regulation but also for mechanisms leading to deregulation of target gene transcription in pathophysiology.

Further complexity is added by the role of β -catenin in cell-cell adhesion where it acts, independent of its transcriptional activity, by forming a complex with cadherins and facilitating the formation of cellular junctions between adjacent hepatocytes. The β -catenin captured in these cell-adhesion complexes represents a dynamic pool of β -catenin capable of nuclear signaling following several stimuli. One of these stimuli is β -catenin tyrosine phosphorylation by receptor tyrosine kinases activated by growth factors produced by epithelial as well as stromal cells. In particular, phosphorylation of β -catenin residue Tyr654 results in its release from cadherins and an increase in TCF-mediated transcriptional activity [25-28]. Furthermore, the adherence pool of β -catenin also appears to be under indirect control of Wnt signaling itself. Upon activation of canonical Wnt/ β -catenin signaling, the suppression of GSK3 β leads to the upregulation of SNAIL [29]. As SNAIL is a repressor of the *CDH1* gene encoding E-cadherin [30, 31], this will lead to reduced E-cadherin production. Diminished E-cadherin causes the dissociation of the complex and subsequent internalization of β -catenin and accumulation of β -catenin in the perinuclear endocytic recycling compartment which promotes translocation to the nucleus to activate Wnt/ β -catenin signaling [32, 33]. Hence pathogens can also provoke β -catenin signaling by disrupting intercellular junctions, in addition to direct effects on elements of the Wnt signaling cascade involved in regulating β -catenin-mediated transcription.

Aberrant activation of Wnt/ β -catenin signaling during HCC

Important in the context of potential modulation by HCV infection in relation to HCC is that aberrant signal transduction in general and β -catenin signaling in particular, is one of the key characteristics of hepatocarcinogenesis [34]. Functional deregulation of Wnt/ β -catenin signaling is reported frequently in HCC strongly suggesting that this pathway is important in this tumor type. Various genetic and molecular alterations have been identified to be pro-oncogenic in a variety of settings, and have as a common denominator that they stabilize β -catenin thus provoking enhanced transcriptional activity of Wnt target genes. Table 1 summarizes the relative mutation frequency of Wnt/ β -catenin signaling elements in HCC patients. Employing HCC cohorts from different countries, the most prevalent are activating mutations in *CTNNB1* (encoding β -catenin) followed by loss-of-function mutations in *AXIN1*, *AXIN2* and *APC*. The relative mutation frequencies of these various Wnt/ β -catenin signaling elements are different in HCC as compared to other cancers, e.g. sporadic colorectal cancer. The reason why these differences emerge may result from different etiology of HCC and thus the type of mutations induced in liver genomes as compared to other sites in the body, but may also derive from the fact that in different organs, optimal cancer-driving Wnt/ β -catenin signaling mutations may be substantially different, resulting in selection pressure for different types of mutations [35, 36]. As indicated in Table 1, in HCCs around 22.1% harbor specific gain-of-function mutations of *CTNNB1*. Missense, insertion or partial deletions within *CTNNB1* exon 3 lead to the generation of a mutant β -catenin protein preventing the proper phosphorylation of amino acids Ser33, Ser37, Thr41 and Ser45 resulting in compromised degradation and thus stabilization of β -catenin in the cytoplasm. Less frequently, loss-of-function mutation of *AXIN1*, *AXIN2* or *APC* is found in 10.4%, 3.3% and 1.4% of HCCs respectively, evidently contrasting with the situation in colorectal cancer where up to 80 % of cancers display mutated APC [36, 37]. Frameshift or deletion in these genes yields impaired ability of the destruction complex to degrade β -catenin and is thus also associated with enhanced Wnt/ β -catenin signaling. Overexpression of upstream ligands or cell surface receptors and reduction of extracellular inhibitors have been reported to stimulate activation of this pathway in HCC as well [38]. Thus evidently, at some stage in the progression towards full-blown HCC, acquisition of increased Wnt/ β -catenin signaling provides liver cancer cells a relative advantage over cells not having

such mutations. Here we shall argue that especially HCV infections create the conditions which allow pre-carcinogenic cancer cells to exhibit such enhanced Wnt/ β -catenin signaling.

High frequency of *CTNNB1* mutation in HCV related HCC

HCV infection presents a substantial clinical challenge, for which only direct anti-viral medication appears a suitable solution [75]. If left untreated or not timely-recognized, persistent HCV infection causes immune-mediated chronic liver damage and compensatory hepatic regeneration by inducing cell proliferation and thus creates a microenvironment permissive for the induction of genetic alterations to the hepatocyte genome [76]. Following HCV infections, genetic abnormalities accumulate relatively slowly during the sequence of chronic hepatitis and increased cirrhosis that finally progresses to HCC. Consequently, the selective growth advantage provided to hepatocytes with a malignant phenotype eventually facilitates the development of phenotypically and genetically heterogeneous HCC [77]. As one of the principal proto-oncogenes in HCC development, the relatively high frequency of *CTNNB1* mutations in HCV-related HCC is especially striking, in the view of the relative absence of such mutations in HBV-related liver cancers but also in the view of their paucity in non-virally associated HCC (Table 2). Indeed, around 26.7% of HCV-related HCC harbor a *CTNNB1* mutation, which is much higher than that observed in HBV-associated HCC (11.6%) or that observed in total non-virally-associated HCC (21.2%). Furthermore, we noticed that, different from colorectal cancers which mainly show Thr41 and Ser45 mutations [36], HCV-related HCC shows a preference for *CTNNB1* mutations from Asp32 to Ser37 residues [45, 47, 49, 59, 68, 70, 71] (Fig.1). Recently, a genotype-phenotype correlation was shown for *CTNNB1* mutations, suggesting that activating mutations occurring at the Asp32 to Ser37 residues lead to higher signaling levels than mutations at Thr41 and Ser45 [39]. This may partially explain the preference. It also could be attributable to the mutagenic dose demanded to induce HCC.

Table 1. Genetic mutation in components of Wnt/ β -catenin pathway in HCC

Reference	Patient N	Mutant samples N(%)				Region
		CTNNB1	AXIN1	AXIN2	APC	
Rebouissou et al.[34]	373	146(39)	NA	NA	NA	France, Spain, Italy
Hirotsu et al.[35]	9	2(22.2)	NA	NA	NA	Japan
Schulze et al.[36]	243	95(37.4)	27(11.1)	3(1.2)	4(1.6)	France, Italy, Spain
Kan et al.[37]	88	14(15.9)	4(4.5)	2(2.3)	2(2.3)	China
Kitao et al.[38]	134	27(20.1)	NA	NA	NA	Japan
Ding et al.[39]	156	15(9.6)	NA	NA	NA	China
Tornesello et al.[40]	67	10(14.9)	NA	NA	NA	Southern Italy
Cleary et al.[41]	87	20(22.9)	NA	NA	NA	Canada, NC
Guichard et al.[42]	125	41(32.8)	19(15.2)	NA	2(1.6)	France
Lachenmayer et al.[43]	90	29(32.2)	NA	NA	NA	USA, Netherlands, Italy, Spain, Germany
Li et al.[44]	139	28(20.1)	NA	NA	NA	USA, Netherlands, China
Cieply et al.[45]	32	9(28.1)	NA	NA	NA	USA
Bengochea et al.[46]	62	16(25.8)	NA	NA	NA	Thailand, France
Austinat et al.[47]	40	10(25)	2(5)	NA	NA	Germany
Kim et al.[48]	36	1(2.8)	9(25)	NA	NA	Korea
Zucman-Rossi et al.[49]	45	18(40)	5(11.1)	NA	NA	France
Boyault et al.[50]	120	34(28.3)	13(10.8)	NA	NA	France
Zucman-Rossi et al.[51]	96	12(12.5)	NA	NA	NA	France
Park et al.[52]	81	13(16)	5(6.2)	NA	NA	Korea
Ishizaki et al.[53]	89	10(11.2)	13(14.6)	9(10.1)	NA	Japan
Cui et al.[54]	34	15(44.1)	NA	NA	NA	China
Edamoto et al.[55]	100	24(24)	NA	NA	0	Japan, Switzerland
Taniguchi et al.[56]	73	14(19.2)	7(9.6)	2(2.7)	NA	UK
Wong et al.[57]	60	7(11.7)	NA	NA	NA	China
Mao et al.[58]	262	37(14.1)	NA	NA	NA	Taiwan
Cui et al.[59]	34	15(44.1)	NA	NA	NA	China
Laurent-Puig et al.[60]	137	26(19)	12(8.8)	NA	NA	France
Devereux et al.[61]	62	5(8.1)	NA	NA	NA	China
Hsu et al.[62]	434	57(13.1)	NA	NA	NA	Taiwan
Satoh et al.[63]	87	0(0)	5(5.7)	NA	NA	Japan
Huang et al.[64]	22	9(41)	NA	NA	NA	Japan, Switzerland
Legoix et al.[65]	119	21(17.6)	NA	NA	NA	France
Terris et al.[66]	73	14(19.2)	NA	NA	NA	France
Kondo et al.[67]	38	9(24)	NA	NA	NA	Japan
Nhieu et al.[68]	35	12(34.3)	NA	NA	NA	France
Miyoshi et al.[69]	75	14(18.7)	NA	NA	NA	Japan
de La Coste et al.[70]	31	8(25.8)	NA	NA	NA	France
Total	3788	837(22.1)	121(10.4)	16(3.3)	8(1.4)	

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Mutations at Ser45 require the selective duplication of the mutated allele as second activating hit, whereas only one activating hit for mutations at the Asp32 to Ser37. Although *CTNNB1* mutation appear a late stage event in the progression to HCC [56], the high rate of *CTNNB1* mutations observed may be directly and causally related to the HCV infectious process as *in vitro* studies show that both acute and chronic HCV infections provoke specifically *CTNNB1* mutations, in hematological model systems and HCCs [78]. Evidently, clarifying the relationship between infection with a non-integrating virus and subsequent *CTNNB1* mutations may prove exceedingly useful for designing strategies aimed at preventing HCV-associated HCC.

Table 2. Comparison of *CTNNB1* mutation in subtypes of HCC

References	CTNNB1 mutant samples N(%)			Mutation type	Amino acid	Region
	HCV	HBV	NV			
Hirotsu et al.[35]	2/5(40)	0/1(0)	0/3(0)	Missense	Gly34, His36	Japan
Kitao et al.[38]	12/55(21.8)	4/34(11.8)	11/44(25)	NA	NA	Japan
Ding et al.[39]	NA	12/110(10.9)	3/46(6.5)	Missense	Asp32,Gly34, Ser37, Thr41,Ser45	China
Tornesello et al.[40]	10/57(17.5)	0/10(0)	NA	Missense	Asp32, Ser33,Gly34 Ile35, Ser37, Ser45 Asp32, Ser33,Gly34 Ile35, Ser37, Thr41, Ser45	Southern Italy
Kan et al. [37]	NA	12/81(14.8)	NA	Missense		China
Guichard et al.[42]	8/24(33.3)	4/35(11.4)	30/80(37.5)	Missense Insertion Deletion	Asp32, Ser33, Ser37, Thr41,Thr42 Ser45	France
Li et al.[44]	14/45(31.1)	6/52(11.5)	9/44(20.5)	Missense Deletion	Asp32, Ser33,Gly34, His36, Ser37, Thr41, Ser45 ,Asn387	USA, Netherlands, China
Bengochea et al.[46]	8/20(40)	3/18(16.7)	5/24(20.8)	Missense Insertion	Asp32, Ser33, Ser37, Thr41 Ser45	Thailand, France
Kim et al.[48]	0/4(0)	0/21(0)	1/14(7.1)	Missense	Ser33	China
Park et al.[52]	0/6(0)	13/78(16.7)	NA	Missense Deletion	Asp32, Ser33,Gly34 Ile35, His36, Ser37, Thr41, Ser45	Korea
Edamoto et al.[55]	16/51(31.4)	5/26(19.2)	3/23(13)	Missense	Asp32, Ser33, His36, Ser37, Thr41, Ser45	Japan, Switzerland
Wong et al.[57]	0/2(0)	5/48(10.4)	2/10(20)	Missense Deletion	Asp32, Ser33,Gly34 Ile35, Ser37, Thr41, Ser45	China
Hsu et al.[62]	23/92(25)	30/323(9.3)	4/19(21.1)	Missense Deletion	Asp32, Gly34, Thr41, Ser45	Taiwan
Huang et al.[64]	9/22(41)	NA	NA	Missense	Asp32, Ser33,Ser37, Thr41, Ser45	Japan, Switzerland
Legoix et al.[65]	7/30(23.3)	5/26(19.2)	13/64(20.3)	Missense Deletion	Asp32, Ser33,Gly34, Ser37, Thr41, Ser45	France
Terris et al.[66]	2/7(28.6)	3/14(21.4)	9/52(17.3)	Missense Deletion	Asp32, Ser33,Gly34, Ser37, Ser45	France
Kondo et al.[67]	7/22(31.8)	1/8(12.5)	1/9(11.1)	Missense Deletion	Asp32, Ser33,Gly34 Ile35, His36, Ser37, Thr41, Ser45	Japan
Total	118/442(26.7)	103/885(11.6)	91/432(21.1)			

	32	33	34	35	36	37				41				45	
CTG	GAC	TCT	GGA	ATC	CAT	TCT	GGT	GCC	ACT	ACC	ACA	GCT	CCT	TCT	CTG
L	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L
	GGC	TGT	GAA	AGC	CCT	TTT				GCC				CCT	
	G	C	E	S	P	F				A				P	
	(5)	(3)	(4)	(1)	(2)	(3)				(9)				(5)	
	TAC	TAT	GTA			TAT				ATC				TAT	
	Y	Y	V			Y				I				Y	
	(3)	(3)	(4)			(3)				(1)				(2)	
	CAC	GCT	AGA			TGT								TTT	
	H	A	R			C								F	
	(2)	(1)	(2)			(2)								(2)	
	GCC	CCT				CCT								TGT	
	A	P				P								C	
	(2)	(1)				(1)								(1)	
	AAC					GCT									
	N					A									
	(2)					(1)									
	GTC														
	V														
	(1)														

Figure 1. Summary of CTNNB1 exon 3 mutations in HCV-related HCC. Illustrated are the locations of the CTNNB1 mutations reported in 68 tumors from 65 HCC patients (one tumor with p.D32_G48del, not shown). N-terminal serine and threonine phosphorylation residues are indicated bold. Numbers in brackets are absolute number of tumors tested with given mutation.

HCV structural proteins activate Wnt/ β -catenin signaling

The HCV genome is a single-stranded positive sense 9.6kb RNA molecule, which includes a single open reading frame encoding a polyprotein of \approx 3,000 amino acids that following translation is cleaved into 10 mature proteins by both host and viral proteases. These proteins are the structural proteins (core, E1 and E2), the viroporin p7 and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The pro-oncogenic pathogenesis of HCV appears mainly mediated by the core protein and two of the non-structural proteins NS3 and NS5A [79]. These pro-oncogenic effects appear to depend largely on the potential of these proteins to mediate activation of Wnt/ β -catenin signaling.

Core protein

The 21kDa core protein is the major component of HCV. Despite lacking obvious organelle localization signals in the primary sequence, it is not only detected in the cytosol, but also in the Golgi apparatus, in lipid droplets and in the nucleus [80, 81]. Remarkably, in the latter

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organelle it serves as a regulator of hepatocyte transcription, facilitating Wnt/ β -catenin signaling. This is brought about by upregulation of canonical Wnts, FZD and LRP5/6 receptors [82, 83] while concomitantly inhibiting transcription of Wnt antagonists SFRP2 and DKK1 [84]. The latter effect is mediated by epigenetic silencing of the promoters involved by core protein-mediated recruitment of DNA methyltransferase-1 (DNMT1) and histone deacetylase-1 (HDAC1) to the transcription start site, an effect already detected early in hepatitis infection [84, 85]. In addition, the HCV core protein mediates hypermethylation of the *CDH1* (E-cadherin) gene promoter [86]. Reduced production of E-cadherin results in diminished sequestering of β -catenin in β -catenin/E-cadherin complexes and thus enhanced activation of Wnt/ β -catenin signaling (Fig.2). Hence, the core protein mediates a plethora of molecular events leading to increased Wnt/ β -catenin signaling and thus apparently HCV is under substantial selection pressure to provoke Wnt/ β -catenin signaling. Potential sources for this selection pressure are a necessity to counteract hepatocyte apoptosis, whereas Wnt/ β -catenin signaling-driven expansion of the HCV-infected compartment may be involved as well.

NS5A

The notion that HCV is under selection pressure to counteract apoptosis is further reinforced by observations that NS5A not only functions as a component of the HCV RNA replication complex [87], but also binds to the p85 regulatory subunit of phosphoinositide 3 kinase (PI3K) thus activating the downstream effector serine/threonine kinase Akt [88, 89]. Akt activation provides powerful anti-apoptotic signal and also mediates the inactivation of GSK3 β , stabilization of β -catenin and subsequent stimulation of β -catenin dependent transcription [90]. In addition, the NS5A protein binds and stabilizes β -catenin directly [91], apparently independent of its effects on Akt and GSK3 β [92] (Fig.2). Thus the multiple stimulatory effects of NS5A on Wnt/ β -catenin signaling are also testimony of the selection pressure of HCV to increase hepatocyte Wnt/ β -catenin signaling.

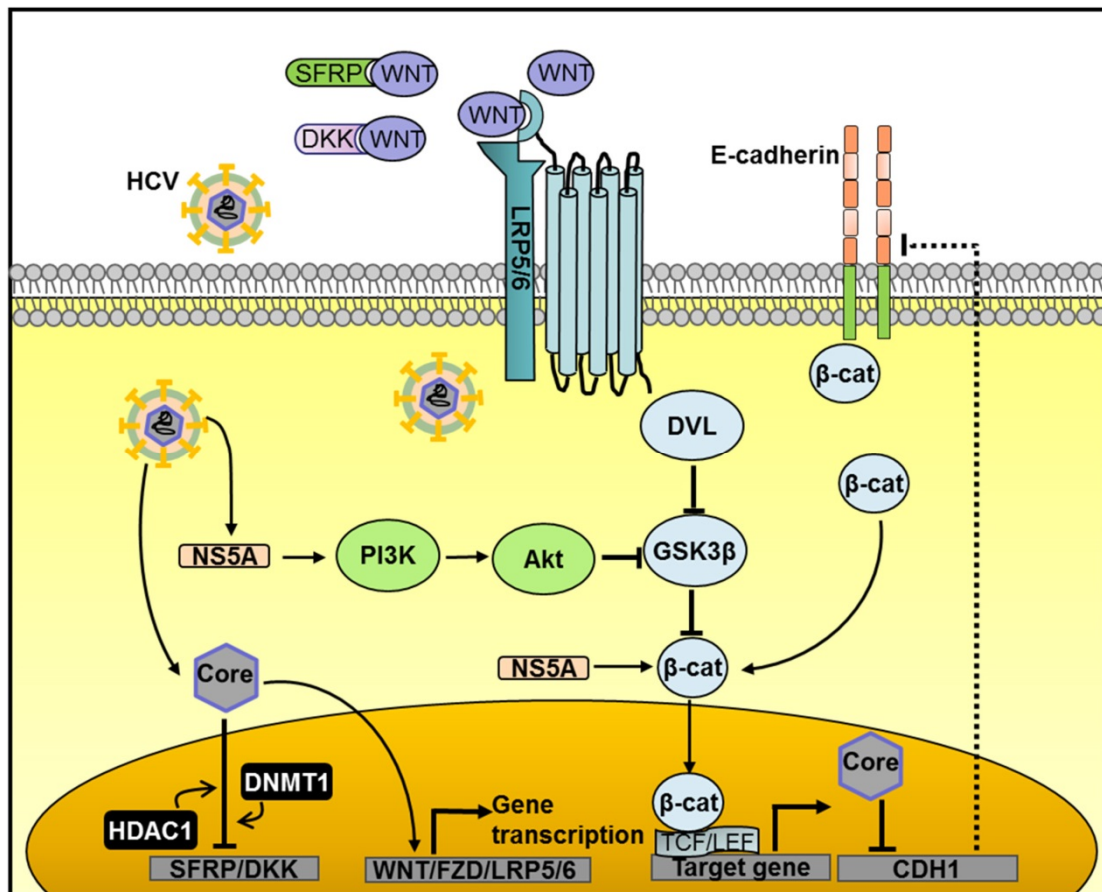


Figure 2. Wnt/ β -catenin signaling is activated by HCV virus proteins. HCV core protein elevates gene expression of Wnt ligands, FZD and LRP5/6 receptors but decreases the expression of Wnt antagonists DKK and SFRP by recruiting DNMT1 and HDAC1 to their transcription start sites. In addition, HCV core protein releases β -catenin from the β -catenin/E-cadherin complexes by suppression of the *CDH1* gene promoter encoding E-cadherin. NS5A protein activates PI3K/Akt signaling leading to the inactivation of GSK3 β and subsequent reduced breakdown of β -catenin, or directly stabilizes β -catenin protein. The overall effect is the cytoplasmic accumulation of β -catenin and stimulation of downstream transcription.

More Wnt/ β -catenin signaling-stimulating effects

The hypotheses that successful HCV-infection critically depends on its potential to stimulate Wnt/ β -catenin signaling is further supported by observations that, in addition to direct activation, HCV infection leads to elevation of microRNA-155 (miR-155) [93] and microRNA-199a-5p [94], in turn triggering Wnt/ β -catenin signaling. MiR-155 acts as an oncomiR by targeting the suppressor of the cytokine signaling 1 (SOCS1) gene [95] that directly inhibits APC expression, one of the major negative regulators of Wnt/ β -catenin signaling [93]. Moreover, both direct and indirect activation by HCV viral proteins may explain the notable dysregulation of Wnt/ β -catenin signaling in hepatitis C and related HCC subclass. Moreover, HCV core, NS3 and NS5A proteins may facilitate further oncogenic transformation of infected hepatocytes [79] by suppression of DNA repair mechanisms, potentially causing *CTNNB1*

mutations. Support for this idea could be found in the observation that in experimental animals hepatocarcinogenic nitrosamine diethylnitrosamine (DEN) provokes cancer by inducing *CTNNB1* mutations [96, 97] and thus increased mutagenic pressure through corrupting DNA repair may be preferentially associated with this mutation. Hence effects on the DNA repair machinery exerted by HCV core, NS3 and NS5A may link increased Wnt/ β -catenin signaling mediated by direct effects of these proteins early in infection to mutation-mediated activation of Wnt/ β -catenin signaling later in the progression to HCC.

Wnt/ β -catenin signaling paves the way for chronic hepatitis C to HCC

Inflammation

The HCV virus battles with the immune system. Thus negative modulation of inflammatory responses through enhanced Wnt/ β -catenin signaling could conceivably provide further selection pressure of HCV to acquire Wnt/ β -catenin signaling-activating properties. The effect of Wnt/ β -catenin signaling, however, on hepatocyte immune responses remains controversial. On one hand, Wnt/ β -catenin signaling could suppress the immune response by blunting T cell activation [98, 99], reducing TNF release [100] or stimulating the production of the chemokine-like chemotactic factor leukocyte cell-derived chemotaxin 2 (LECT2) and invariant NKT cells (iNKT) responses, both of which relay antiinflammatory response [101]. On the other hand, Wnt/ β -catenin signaling triggers inflammatory responses by activating the pro-inflammatory NF- κ B pathway, as evident from experimentation in a hepatocyte-specific APC and LECT2 knockout (*APC^{-/-}LECT2^{-/-}*) mouse model [101]. In potential agreement, germline genetic variations in Wnt/ β -catenin signaling elements were significantly associated with the risk for inflammation in HCV-infected males [102]. Thus the issue as to how HCV-elicited Wnt/ β -catenin signaling relates to HCV-provoked inflammation warrants further experimentation.

Fibrosis to cirrhosis and HCC development

Chronic inflammation evoked by HCV infection may culminate in liver fibrosis. Such fibrosis progresses gradually and disrupts liver physical structure and function over the course of several decades, finally resulting in fatal diseases such as cirrhosis and HCC [103]. Given HCV-stimulation of Wnt/ β -catenin signaling probably evolved to support the early phases of viral infection, emerging data suggest that activated Wnt/ β -catenin signaling by HCV participates

in the pathogenesis of liver fibrosis as well [102, 103], mainly by enhancing hepatic stellate cell (HSC) activation and survival [104]. The subsequent progression toward full-blown HCC is a complex process involving many various signaling pathways, but especially crosstalk between epidermal growth factor receptor (EGFR) signaling and fibroblast growth factor (FGF) receptor signaling and aberrant activation of Wnt/ β -catenin signaling appears important here.

The EGFR pathway controls a variety of signals ranging from cell proliferation, cell motility, apoptosis decrease, to epithelial mesenchymal transition, upregulation of matrix metalloproteinases (MMP), and even stem cell maintenance [105]. EGFR is highly expressed in the adult liver [106] and plays an essential role in the G1/S phase transition for hepatocyte proliferation [107]. EGFR pathway dysregulation has been reported in 60% to 80% of HCC patients [108], and associated with the late stages and the degree of tumor differentiation [109, 110]. EGFR favors HCV entry through co-internalization of a HCV-CD81-EGFR complex following binding of EGFR ligands to the receptor and subsequent endocytosis [111, 112]. Following clathrin-mediated endocytosis of the EGFR, the receptor is routed for eventual intracellular degradation [113]. The viral NS5A protein, however, perturbs EGFR trafficking and degradation, increasing EGFR signaling and contributing to HCV-mediated HCC development [114]. Binding of Wnt1 and Wnt5a to FZD transactivates EGFR signaling by MMP-mediated release of soluble EGFR ligands, such as TGF α [115]. Activated β -catenin might form heterodimers with EGFR to enhance EGFR pathway activation [116]. Conversely, EGFR signaling contributes to Wnt/ β -catenin signaling in various ways. Firstly, EGFR can directly induce tyrosine phosphorylation of β -catenin at residue Y654, thereby decreasing the binding with cell-adhesion complexes and releasing it for nuclear signaling [104, 117]. In fact, this phenomenon has been observed for a large number of growth factors signaling through receptor tyrosine kinases, such as HGF and FGFs that are produced in excess by the cirrhotic tissue adjacent to tumor tissue [28, 118-120]. Secondly, EGFR stimulates the PI3K/Akt and Ras/Raf/MEK/ERK cascades that both can promote β -catenin signaling through inhibiting GSK3 β activity [121-125] (Fig.3). Thus HCV-mediated activation of Wnt/ β -catenin signaling may initiate a vicious interaction between EGFR and Wnt signaling, promoting potentially pro-oncogenic hepatocyte proliferation.

Similar to the EGFR pathway, FGF-initiated signaling is a cardinal regulator of hepatocyte proliferation, differentiation, embryonic development and organogenesis as well as hepatic

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tumorigenesis [126, 127]. Especially in chronic hepatitis C-associated HCC, activation of FGF signaling is observed [128, 129] and increased FGF levels are associated with enhanced HCV replication and release of infectious particles [130]. Crosstalk of Wnt and FGF pathways in HCV-related HCC is supported by observations that FGF signaling leads to the release of β -catenin from the β -catenin/E-cadherin complexes due to the phosphorylation of Tyr654 as described above. Furthermore, FGF2 increases expression of β -catenin mRNA, upregulates β -catenin nuclear translocation and inactivates GSK3 β [131], probably mediated through activation of PI3K/Akt and Ras/Raf/MEK/ERK pathways. Conversely, Wnt/ β -catenin signaling is able to activate FGF signaling by increasing *FGF18* and *FGF20* expression [132] (Fig.3). Thus again, vicious interaction between Wnt/ β -catenin signaling and FGF signaling appears to occur.

Of interest, it has been reported that the Src homology region 2 domain-containing phosphatase-2 (SHP-2) can be activated by HCV structural E2 protein [133]. Thus conceivably SHP-2 may be an effector on EGFR and FGF signaling in HCV related HCC. Overexpression of SHP-2 promotes liver tumor cell growth and metastasis by coordinately activating not only PI3K/Akt and Ras/Raf/MEK/ERK pathways [121] but also Wnt/ β -catenin signaling [134]. The latter effect is due to tyrosine dephosphorylation of parafibromin/Cdc73, acting as a tumor suppressor inhibiting *CYCLIND1* and *C-MYC*, together with SUV39H1. As a result, parafibromin acquires the ability to bind β -catenin stably, overriding the repression effect and inducing the expression of Wnt target genes [134] (Fig.3). Together, these results suggest that SHP-2 is one of the critical molecules enhanced during early HCV infection and contributes to the later progression to final HCC, which needs further investigation.

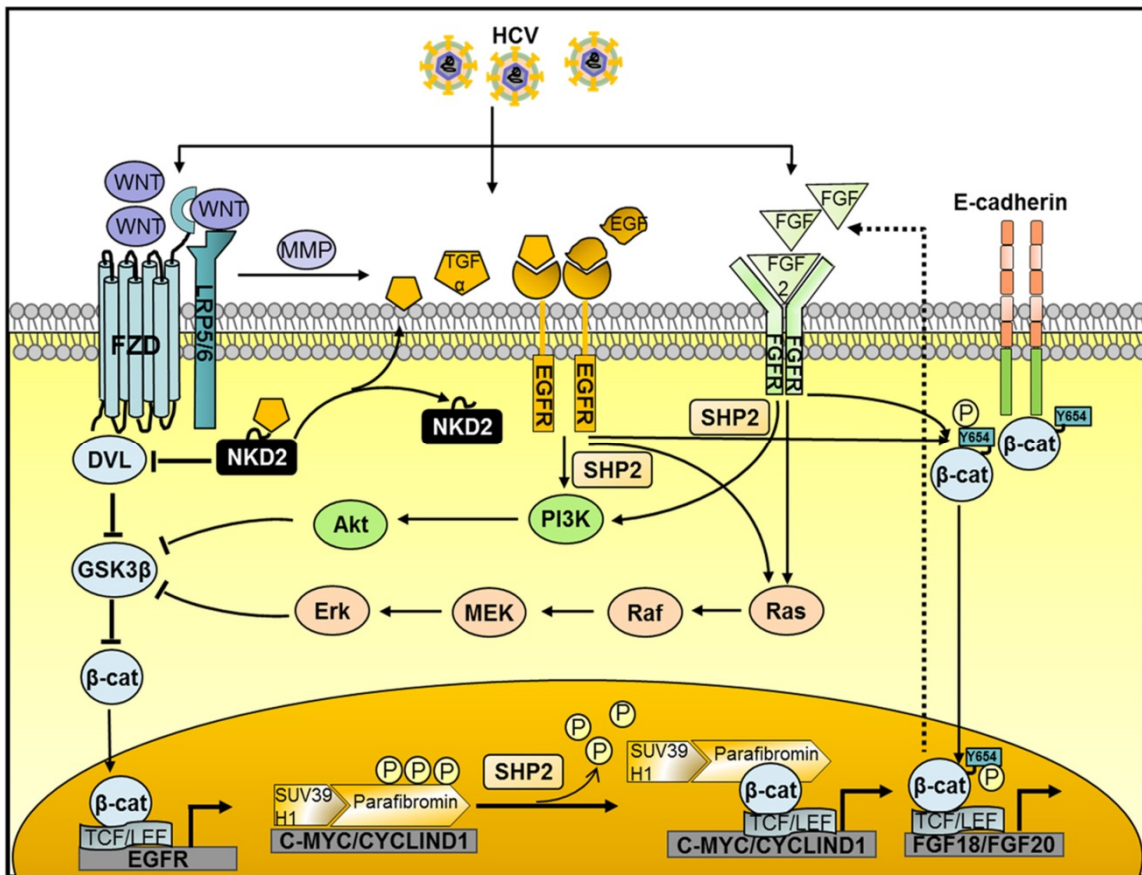


Figure 3. Crosstalk of Wnt/ β -catenin pathway with EGFR and FGF pathways in HCV related HCC. HCV promotes Wnt signaling as well as EGFR and FGF pathways. The Wnt/ β -catenin and EGFR pathways activate each other. Binding of Wnt ligands with FZD receptors transactivates EGFR signaling by MMP-mediated release of soluble EGFR ligands. EGFR signaling transactivates Wnt/ β -catenin signaling through PI3K/Akt and Ras/Raf/MEK/Erk pathways but also by releasing β -catenin from β -catenin/E-cadherin complexes due to residue Tyr654 phosphorylation. Activated β -catenin forms heterodimers with EGFR and in turn promote EGFR pathway. On the other hand, Wnt signaling stimulates FGF signaling by inducing FGF18 and FGF20 ligand expression. In turn, the association of FGF19 to FGFR leads to the release of β -catenin from the β -catenin/E-cadherin complexes. FGF2 signaling inhibits GSK3 β activity through PI3K/Akt and Ras/Raf/MEK/Erk pathways. Activated SHP-2 in both PI3K/Akt and Ras/Raf/MEK/Erk pathways dephosphorylates parafibromin which acquires the ability to bind β -catenin stably, overriding the repression effect on the *CYCLIND1* and *C-MYC* expression and triggering downstream signaling.

Conclusion

As one of the important cascades involved in HCV-related HCC initiation and development, Wnt/ β -catenin signaling is aberrantly activated by HCV viral core and NS5A proteins. In turn, stimulated Wnt/ β -catenin signaling promotes progression of hepatitis C during inflammation and fibrosis eventually promoting cirrhosis and HCC. This interaction is further aggravated by a vicious circle involving the EGFR and FGF pathways.

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Chapter 3

Blocking Wnt secretion reduces growth of hepatocellular carcinoma cell lines mostly independent of β -catenin signaling

Wenhui Wang, Lei Xu*, Pengyu Liu*, Kiran Jairam, Yuebang Yin, Kan Chen, Dave Sprengers,
Maikel P. Peppelenbosch, Qiuwei Pan, Ron Smits*

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center
and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands



Abstract

Aberrant activation of Wnt/ β -catenin signaling plays a key role in the onset and development of hepatocellular carcinomas (HCC), with about half of them acquiring mutations in either *CTNNB1* or *AXIN1*. However, it remains unclear whether these mutations impose sufficient β -catenin signaling or require upstream Wnt ligand activation for sustaining optimal growth, as previously suggested for colorectal cancers. Using a panel of nine HCC cell lines we show that siRNA mediated knock-down of β -catenin impairs growth of all these lines. Blocking Wnt secretion, either by treatment with the IWP12 porcupine inhibitor or knockdown of WLS, reduces growth of most of the lines. Unexpectedly, interfering with Wnt secretion does not clearly affect the level of β -catenin signaling in the majority of lines, suggesting that other mechanisms underlie the growth suppressive effect. However, IWP12 treatment did not induce autophagy or endoplasmic reticulum (ER) stress, which may have resulted from the accumulation of Wnt ligands within the ER. Similar results were observed for colorectal cancer cell lines used for comparison in various assays. These results suggest that most colorectal and liver cancers with mutations in components of the β -catenin degradation complex do not strongly rely on extracellular Wnt ligand exposure to support optimal growth. In addition, our results also suggest that blocking Wnt secretion may aid in tumor suppression through alternative routes currently unappreciated.

Keywords: Hepatocellular carcinoma; Wnt/ β -catenin signaling

Introduction

Hepatocellular carcinoma (HCC) is considered as the fifth most common cancer and the third main reason for cancer related death with 748,000 cases and 695,000 deaths each year [1, 2]. The etiology of HCC includes Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection, alcohol liver disease, non-alcoholic steatohepatitis (NASH) and aflatoxin-B1 exposure [3]. More than 80% of all HCCs occur in Eastern and Southeastern Asia where the main cause is HBV combined with exposure to aflatoxin-B1 [4]. In Europe, Japan and the United States, HCV represents the dominant risk factor, together with alcohol abuse and nonalcoholic fatty liver diseases [5, 6].

Wnt/ β -catenin signaling plays an important role in a wide range of biological processes, involving embryonic patterning, cell proliferation, differentiation, angiogenesis, carcinogenesis, metastasis and drug resistance [7-9]. Underscoring the relevance of this pathway, many tumor types including HCC, exhibit enhanced Wnt/ β -catenin signaling that strongly contributes to tumor growth [10]. Activation of Wnt/ β -catenin signaling starts with the secretion of Wnt ligands. Wnts produced within the endoplasmic reticulum (ER) are palmitoylated by the Wnt acyl-transferase porcupine (PORCN), which is essential for their secretion and signaling activity. Following this lipid modification, Wntless (WLS) is needed to shuttle the Wnt proteins from the Golgi to the plasma membrane where they can signal in an autocrine or paracrine manner [11]. In the absence of upstream Wnt signaling, β -catenin is phosphorylated at N-terminal Ser/Thr residues by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1 α . Phosphorylated β -catenin is then ubiquitinated, leading to its proteasomal degradation [12-14]. The overall effect is low β -catenin levels in the cytoplasm and nucleus of unstimulated cells. Upon binding of Wnt ligands to Frizzled and LRP5/6 co-receptors, Disheveled (DVL) becomes phosphorylated, subsequently resulting in the inhibition of the β -catenin destruction complex [15]. As a result, β -catenin is stabilized and is able to translocate to the nucleus and associate with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt/ β -catenin target genes thereby affecting cellular decisions [4]. In addition to this classical (canonical) Wnt signaling pathway, Wnts can also signal in an alternative (non-canonical) fashion independent of β -catenin through associating with Frizzled and ROR1/2 receptors instead of

LRP5/6. Activation of non-canonical Wnt signaling mainly affects cellular processes involved in migration and cellular polarity [8].

Hepatocarcinogenesis is a multistep process, progressing from a normal hepatocyte to a transformed phenotype as a result of the accumulation of aberrant genetic and epigenetic modifications and activation of various signaling pathways [16-18]. Increasing evidence indicates that activation of Wnt/ β -catenin signaling is critical in hepatic oncogenesis [19, 20]. About 40%-70% of HCCs are characterized by nuclear accumulation of β -catenin, the hallmark of active signaling. Various molecular and genetic alterations contribute to aberrant activation of Wnt/ β -catenin signaling. Mutations within components of the canonical Wnt/ β -catenin signaling enhance stabilization of β -catenin and transcriptional activity in the nucleus. Approximately one third of all HCCs carry oncogenic β -catenin mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation [21]. In another subset of tumors, loss-of-function mutations of negative regulators are observed in the *APC* and *AXIN1* genes, respectively in 1-3% and 8-15% of tumors [19], both causing compromised ability to degrade β -catenin [8]. In addition to mutations, various other mechanisms have been suggested to promote β -catenin signaling, including overexpression of Wnt ligands and/or their corresponding receptors, and reduced expression of extracellular inhibitors [22]. Given the importance of β -catenin signaling for hepatic oncogenesis, various treatments targeting this route have been evaluated [23].

Cancers harboring mutations within intracellular components of the β -catenin signaling pathway, i.e., mutation of *APC*, *AXIN* or β -catenin itself, were often considered to become largely independent of upstream regulation by extracellular Wnt ligands. This belief has however been challenged during the last years. For example, Wnt antagonists SFRPs and DKKs are reported to attenuate Wnt signaling in colorectal cancer (CRC) [24, 25]. Recently, it was demonstrated that interfering with Wnt secretion or reducing the expression of specific Wnt ligands impaired the growth of *APC* and β -catenin mutant CRC cell lines [26]. These results also indicated that interfering with Wnt secretion, for example using the newly developed PORCN inhibitors [27, 28], could be useful as an additive treatment option for tumors characterized by enhanced β -catenin signaling. Here, we have investigated whether this also holds truth for β -catenin or *AXIN1* mutant liver cancer cells.

Materials and methods

Cell lines

Human HCC cell lines Hep3B, Huh6, Huh7, PLC/PRF/5, SNU182, SNU398, SNU449 and CRC cell lines CACO2, DLD1, HT29, SW480, HCT116, LS174T, SW48 and RKO were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). The hepatoblastoma cell line HepG2 was cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in Williams E medium (Invitrogen-Gibco, Breda, The Netherlands) complemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. HepaRG was cultured in William's E medium supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin (Sigma-Aldrich, St Louis, MO), and 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich, St Louis, MO). Identity of all cell lines was confirmed by STR genotyping. *CTNNB1* mutation status was confirmed in all the nine HCC cell lines by Sanger sequencing and was consistent with those reported at COSMIC, the Catalogue Of Somatic Mutations In Cancer (<http://cancer.sanger.ac.uk>) [29].

For the preparation of conditioned medium, L-Control and L-Wnt3A cells were cultured in complete DMEM medium, followed by collection and filtration of medium according to standard procedures. HCC and CRC cell lines were stimulated with 25% L-Control or L-Wnt3A medium.

Reagents

IWP12 (Sigma-Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) with a final stock concentration of 10mM. Antibodies specific for β -catenin (Cat. #9561, Cell Signaling Technology), dishevelled adaptor protein (DVL2) (Cat. #3216, Cell Signaling Technology), WLS (Cat.#MABS87, clone YJ5 Millipore), LC3 I / II (Cat. #4108, Cell Signaling Technology), GPR177(Wls/Evi) (Cat.#MABS87, Millipore) and β -actin (sc-47778, Santa Cruz), Tubulin (sc-8035, Santa Cruz) anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were used for western blot analysis.

Table1. Gene mutations of Wnt/ β -catenin signaling components in HCC and CRC cell lines

Cell line	Gene	AA alteration	Zygoty
HCC			
HepG2	<i>CTNNB1</i>	p.W25_I140 del	Heterozygous
Huh6	<i>CTNNB1</i>	p.G34V	Heterozygous
SNU398	<i>CTNNB1</i>	p.S37C	Heterozygous
Hep3B	<i>AXIN1</i>	p.R146*	Homozygous
PLC/PRF/5	<i>AXIN1</i>	p.(R373_M418 del)	Homozygous
SNU449	<i>AXIN1</i>	p.R712*	Homozygous
Huh7			
HepaRG			
SNU182			
CRC			
SW480	<i>APC</i>	p.Q1338*	Homozygous
HT29	<i>APC</i>	p.T1556fs*3	Heterozygous
		p.E853*	Heterozygous
Caco2	<i>APC</i>	p.Q1367*	Homozygous
DLD1	<i>APC</i>	p.R2166*	Heterozygous
		p.I1417fs*2	Heterozygous
	<i>AXIN1</i>	p.L396M	Heterozygous
	<i>RNF43</i>	p.G659fs*41	Heterozygous
SW48	<i>CTNNB1</i>	p.S33Y	Heterozygous
	<i>RNF43</i>	p.G659fs*41	Heterozygous
		p.V299fs*143	Heterozygous
HCT116	<i>CTNNB1</i>	p.S45del	Heterozygous
	<i>RNF43</i>	p.R117fs*41	Heterozygous
LS174T	<i>CTNNB1</i>	p.S45F	Homozygous
RKO	<i>RNF43</i>	p.G659fs*41	Heterozygous

Gene knockdown by small interfering RNA (siRNA)

Smartpool ON-TARGETplus siRNAs targeting *CTNNB1* and *WLS* were obtained from Dharmacon. The ON-TARGETplus Non-targeting siRNA #2 was used as negative control. Cells were reverse-transfected in a 96-well plate using a total of 0.2 μ l DharmaFECT formulation 4 (Thermo Fischer Scientific) and 25nM of each siRNA per well. Following 72 hours incubation, the effect on knock-down was determined by western blotting.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). CDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC) and subjected to quantitative Real-Time PCR analyses. Analyses were performed using the StepOne Real-Time PCR System and the

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StepOnev2.0 software (Applied Biosystem, Darmstadt, Germany). Primer sequences are provided in supplementary Table1. All expression levels are depicted relative to the expression of *GAPDH*.

Western blot assay

Cells were lysed in Laemmli sample buffer with 0.1 M DTT and heated for 5-10 minutes at 95 °C, followed by loading and separation on a 8-15% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 ml Odyssey Blocking Buffer and 2.5 ml PBS containing 0.05% Tween 20 (PBS-T), followed by incubation with primary antibody (all 1:1000) overnight at 4 °C. The membrane was washed 3 times with PBS-T followed by incubation for 1.5 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for β -actin or Tubulin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantified with Odyssey 3.0 software.

β -catenin reporter assays

The β -catenin reporter assays were basically performed as previously described [30, 31]. In short, twenty hours before transfection, we plated 10^5 cells per well on 12-well plates. Each well was transfected with 500 ng Wnt Responsive Element (WRE) or Mutant Responsive Element (MRE) vectors and 20 ng TK-Renilla using polyethylenimine (PEI) (Sigma-Aldrich, St Louis, MO) or Fugene HD (Promega). We measured luciferase activities in a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany) and normalized the data for the transfection efficiency by using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. Transfections were performed in triplicate and the mean and standard error were calculated for each condition. The β -catenin reporter activities are shown as WRE/MRE ratios.

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates and incubated at 37°C with 5% CO₂ for 3 h. The medium

was removed and 100 μ l DMSO was added to each well. The absorbance of each well was read on a microplate absorbance reader (BIO-RAD) at wavelength of 490 nm. For siRNA mediated knockdown of genes, 4 independent wells were assayed for each cell line at least two times, whereas for IWP12 treatment 6 independent wells were used. The mean and standard error were calculated for each condition.

Cell cycle analysis

Around 60%-80% confluency, cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4°C. The cells were washed twice with PBS and incubated with 20ug/ml RNase at 37°C for 30 min followed by incubation with 50ug/ml Propidium Iodide (PI) at 4°C for 30min. Then samples were tested immediately by FACS. Cell cycle was analyzed by FlowJo_V10 software. For each treatment, two independent wells were tested for Huh6, SNU449 and Huh7 two times. The mean and standard error were calculated for each condition.

Statistical analysis

All results were presented as mean \pm SD. Comparisons between groups were performed with one sample t test. Differences were considered significant at a p value less than 0.05.

Results

β -catenin signaling activity of HCC cell lines

To investigate the importance of β -catenin signaling and Wnt secretion for sustaining cell growth, we employed 9 HCC cell lines, listed in Table 1 in which gene mutations related to Wnt/ β -catenin signaling are depicted. We also used 8 CRC cell lines for comparison in various assays, known to largely depend on β -catenin signaling for their growth. First, we determined the baseline β -catenin signaling activity for all these cell lines using a β -catenin reporter assay and qRT-PCR of *AXIN2*, a well-established β -catenin target gene. As indicated in Figure 1, in line with previous publications, all β -catenin mutant HCC lines (SNU398, HepG2 and Huh6) showed a robust induction of both reporter activity as well as high *AXIN2* expression. The *AXIN1* mutant lines (PLC/PRF/5, Hep3B and SNU449) also displayed enhanced reporter activity, albeit generally more modest, whereas the expression of *AXIN2* was low. Interestingly, among the HCC lines without an obvious mutation, SNU182 presented with high β -catenin signaling activity, both on reporter level and *AXIN2* expression. Huh7 and HepaRG showed low reporter

activity together with low *AXIN2* expression. All CRC lines, except RKO being wild type for *APC* and *CTNNB1*, showed the expected increase in reporter activity (Figure S1).

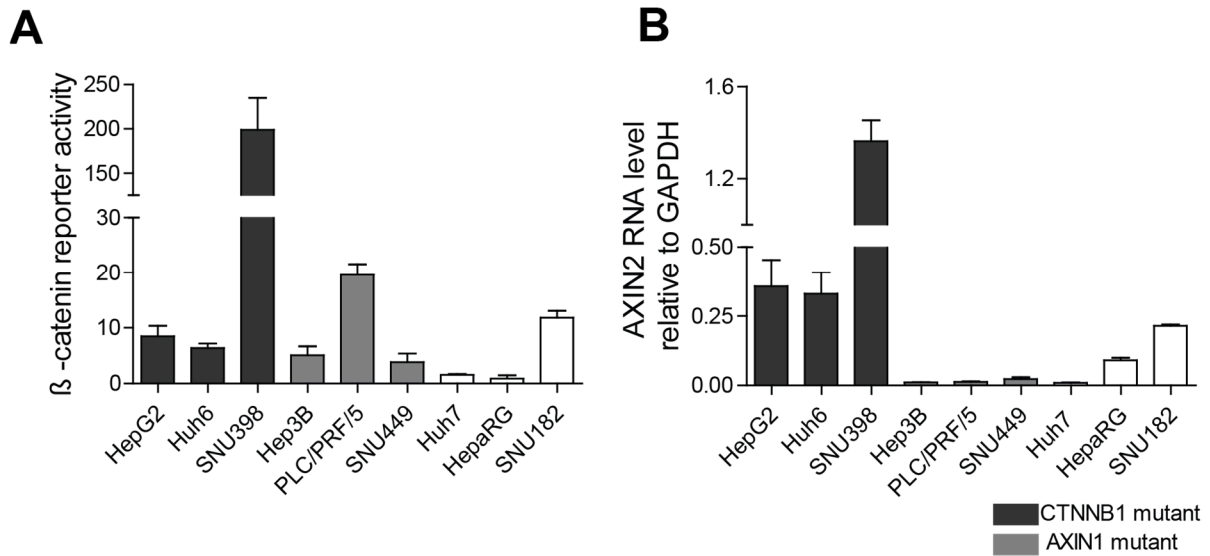


Fig.1. Baseline β -catenin signaling activity in HCC cell lines. (A) β -catenin luciferase reporter assay showing β -catenin signaling activity in HCC cell lines (mean \pm SD, n = 3). The β -catenin reporter activities are depicted as WRE/MRE ratios. (B) QRT-PCR assay showing expression of the β -catenin target gene *AXIN2* in HCC cell lines (mean \pm SD, n = 3). Expression levels are depicted relative to the housekeeping gene *GAPDH*.

Requirement for β -catenin signaling to sustain efficient cell growth

Next, we determined the dependence on β -catenin signaling for supporting efficient growth by transiently transfecting smartpool siRNAs targeting *CTNNB1* or a control siRNA, followed by a MTT assay to test cell numbers after 3 days of culture. For most cell lines we accomplished more than 80% knock-down of β -catenin at protein level as determined by quantitative western blot analysis, with the exception of HepaRG in which a 65% reduction was observed (see Figures 2A and S2). As indicated in Figure 2B, β -catenin signaling activity was clearly suppressed by siRNA mediated knockdown in the three lines tested for this purpose, i.e. Huh6, SNU449 and Huh7. All HCC cell lines were inhibited significantly in their growth (Figure 2C) suggesting that β -catenin signaling is important for the growth of these tumor cells, even in the ones that show only low to modest levels of signaling, such as Huh7 and HepaRG. Cell cycle analysis in three lines showed that β -catenin knockdown provoked a dramatic G0/G1 phase arrest in Huh6 (Fig 2D). Also in Huh7 a notable increase of cells in G0/G1 is observed with a significant reduction of cells in the G2/M phase, whereas for SNU449 a trend is observed towards more cells in G0/G1 and less cells in S-phase. As expected, all five tested CRC cell lines showed a significantly reduced growth upon β -catenin knockdown (Figure 2E).

Expression levels of WNT ligands in HCC cell lines

Taken together, the results above indicate that targeting the β -catenin signaling pathway represents an attractive route to suppress the growth of HCCs. This pathway has however been refractory to target in an efficient manner. More recently, inhibitors of Wnt secretion have been proposed as treatment options for malignancies dependent on Wnt-ligand secretion for their growth [26-28]. However, a prerequisite is that the tumor cells express sufficient levels of Wnt ligands capable of inducing β -catenin signaling. Hence, we investigated the expression profile of all 19 Wnt ligand genes in our HCC cell line panel by qRT-PCR. From the group of Wnt ligands more commonly associated with inducing β -catenin signaling (*WNT1, 2, 3, 3A, 8A, 8B, 10A* and *10B*), only *WNT3* was clearly expressed in all HCC cell lines, followed by high *WNT10A* expression restricted to the SNU182 and HepaRG cell lines, and high *WNT2* expression in SNU449 (Figure 3A). The remaining ligands of this group are barely detectable or expressed at least at 10-fold lower levels in all cell lines (Figure S3). When piling up the expression of all Wnt ligands from this group (Figure 3B), the SNU182 cell line clearly stands out as one with overall highest expression level, which may explain its high β -catenin signaling activity reported above. The β -catenin and AXIN1 mutant cell lines are among the low-to-intermediate ones.

Among the group of Wnt ligands more commonly associated with activating alternative pathways (*WNT4, 5A, 5B, 6, 7A, 7B* and *11*) depicted in Figure S4A, *WNT5A* was most prominently expressed, being very high in HepaRG and SNU182, and readily detectable in SNU398 and SNU449. The latter cell line also shows high expression of *WNT5B* and *WNT7B*. Other Wnt ligands of this group are detectable only at low levels or absent within

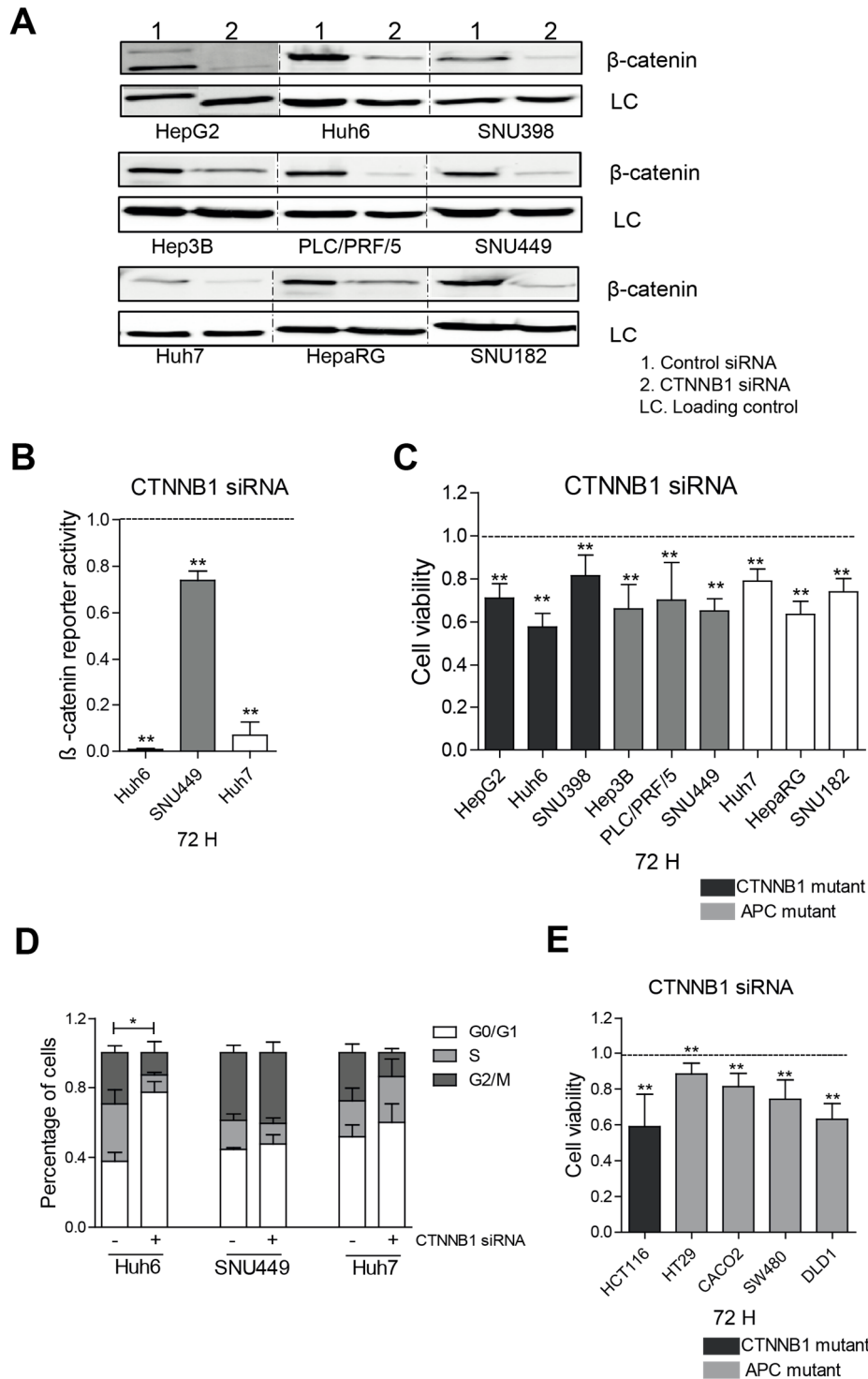


Fig.2. Requirement of β -catenin signaling for sustaining HCC cell growth. (A) HCC cell lines were transiently transfected with *CTNNB1* siRNAs for 72 hours. Cell lysates were collected for western blotting with indicated antibodies. Tubulin and β -actin served as loading control (LC). (B) Silencing of β -catenin caused the reduction of β -catenin signaling activity (mean \pm SD, n=2, two times). (C) Silencing of β -catenin inhibited the growth of HCC cell lines as determined by MTT assay (mean \pm SD, n=4, two times). (D) Silencing of β -catenin alters cell cycle progression in Huh6 (all phases significantly changed) and Huh7 cells (G2/M phase significantly reduced). SNU449 is less clearly altered, although the proportion of cells in S-phase is reduced (mean \pm SD, n=2, two times). (E) β -catenin knockdown reduced cell growth in colorectal cancer cell lines. Values depicted are relative to the ones obtained with the non-targeting siRNA that are arbitrarily set to 1. * p <0.05; ** p <0.01.

most cell lines. Overall, SNU182 is again the most prominent expressing cell line, whereas the β -catenin and AXIN1 mutant cell lines are among the low-to-intermediate expressers (Figure 3B). Of the remaining Wnt ligands (*WNT2B*, *9A*, *9B* and *16*), *WNT2B* was highly expressed in PLC/PRF/5, SNU182, SNU398, and SNU449, while *WNT9B* was clearly expressed in Hep3B (Supplemental Figure S4B).

Combining the expression of all Wnt ligands shows that SNU182 has again the highest overall levels, followed by HepaRG and SNU449 (Figure 3B, right panel). As all Wnt ligands trigger the phosphorylation of DVL2 upon binding of either the FZD_LRP5/6 or FZD_ROR1/2 receptor complexes [32, 33], we determined baseline pDVL2 levels in all cell lines. As shown in Figure 3C, highest levels of phosphorylated DVL2 are observed in the three cell lines with highest Wnt levels, whereas for the remaining six lines no clear correlation can be observed. In summary, although all HCC cell lines show a large variation in Wnt ligand expression, they all express Wnt ligands that can contribute to β -catenin signaling.

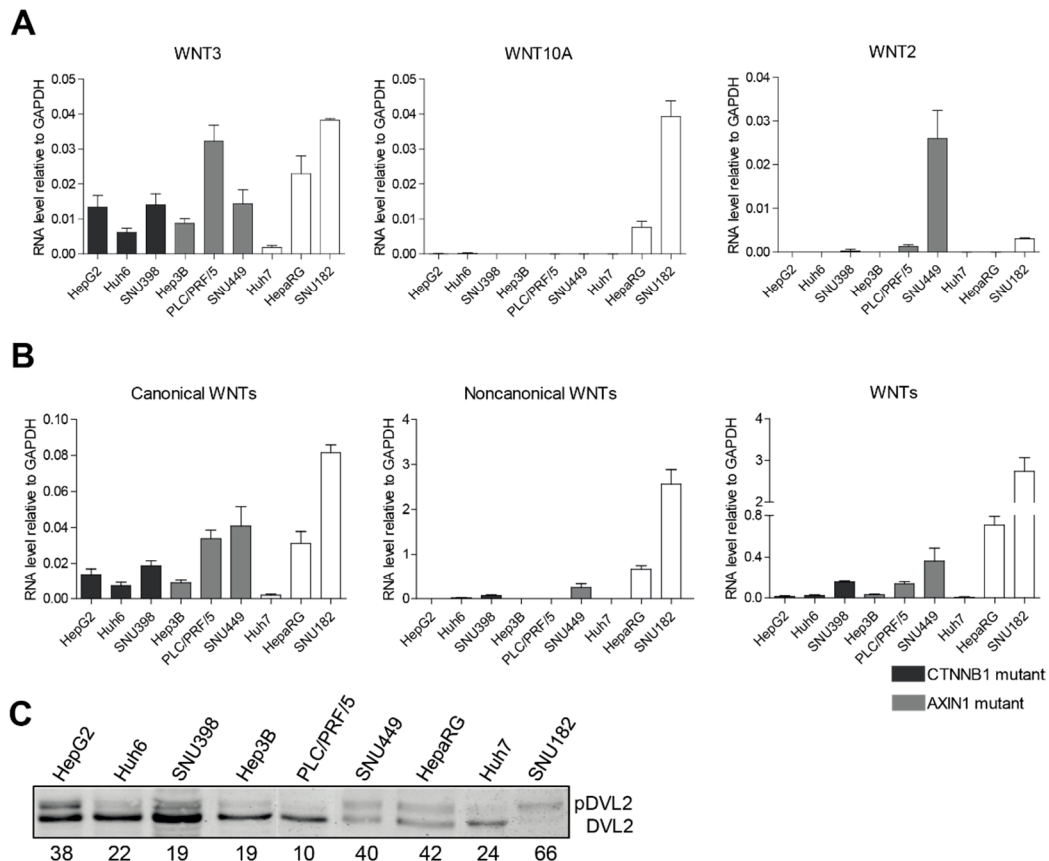


Fig.3. Expression levels of Wnt family members in HCC cell lines determined by qRT-PCR. (A) Expression levels of the top three expressed “canonical” Wnt ligands (mean \pm SD, n=3). (B) Piled-up expression levels of canonical, non-canonical and overall Wnt ligands, respectively. All expression levels are depicted relative to the housekeeping gene *GAPDH*. (C) Baseline levels of phosphorylated DVL2 (pDVL2) in all HCC cell lines. Values below the image represent percentage of total DVL2 that is in the phosphorylated form (upper band).

Wnt secretion blockage reduces growth of HCC cells

Given that most HCC cell lines show increased β -catenin signaling activity and expression of canonical Wnt ligands, we wished to investigate the consequences of suppressing the secretion of Wnt ligands. To this aim we used two methods, i.e., treatment with IWP12, an effective inhibitor of PORCN required for palmitoylation of Wnt proteins [34], and knockdown of *WLS*, which shuttles the palmitoylated Wnts from the Golgi to the plasma membrane. Both treatments are expected to reduce overall levels of secreted Wnt ligands. Following three days of IWP12 treatment, reduced cell numbers were observed for all β -catenin mutant cell lines (Figure 4A), ranging from 10% reduction (Huh6) to 35% (SNU398). Among the non-mutant lines, growth of HepaRG was strongly suppressed by IWP12, whereas Huh7 and SNU182 showed more modest reductions of their growth. The AXIN1 mutant lines were not clearly affected by IWP12 with the exception of PLC/PRF/5. Effects on growth following knockdown of *WLS* were largely in line with IWP12 treatment, with the exception of the AXIN1 mutant lines Hep3B and SNU449 that were significantly suppressed by *WLS* knockdown, and a less impressive growth reduction of HepaRG when compared with IWP12 (Figure 4B). Examples of efficient *WLS* knockdown are shown in supplemental Figure S5.

For comparison, the same assays were also performed on five CRC cell lines (Figure 4C,D). Both treatments showed the strongest growth suppression when applied to the β -catenin and RNF43 mutant HCT116 cell line. Intermediate effects were observed in DLD1, HT29, and SW480, whereas CACO2 was barely affected. In conclusion, most HCC and CRC cell lines are suppressed in their growth by both IWP12 treatment as well as *WLS* knockdown.

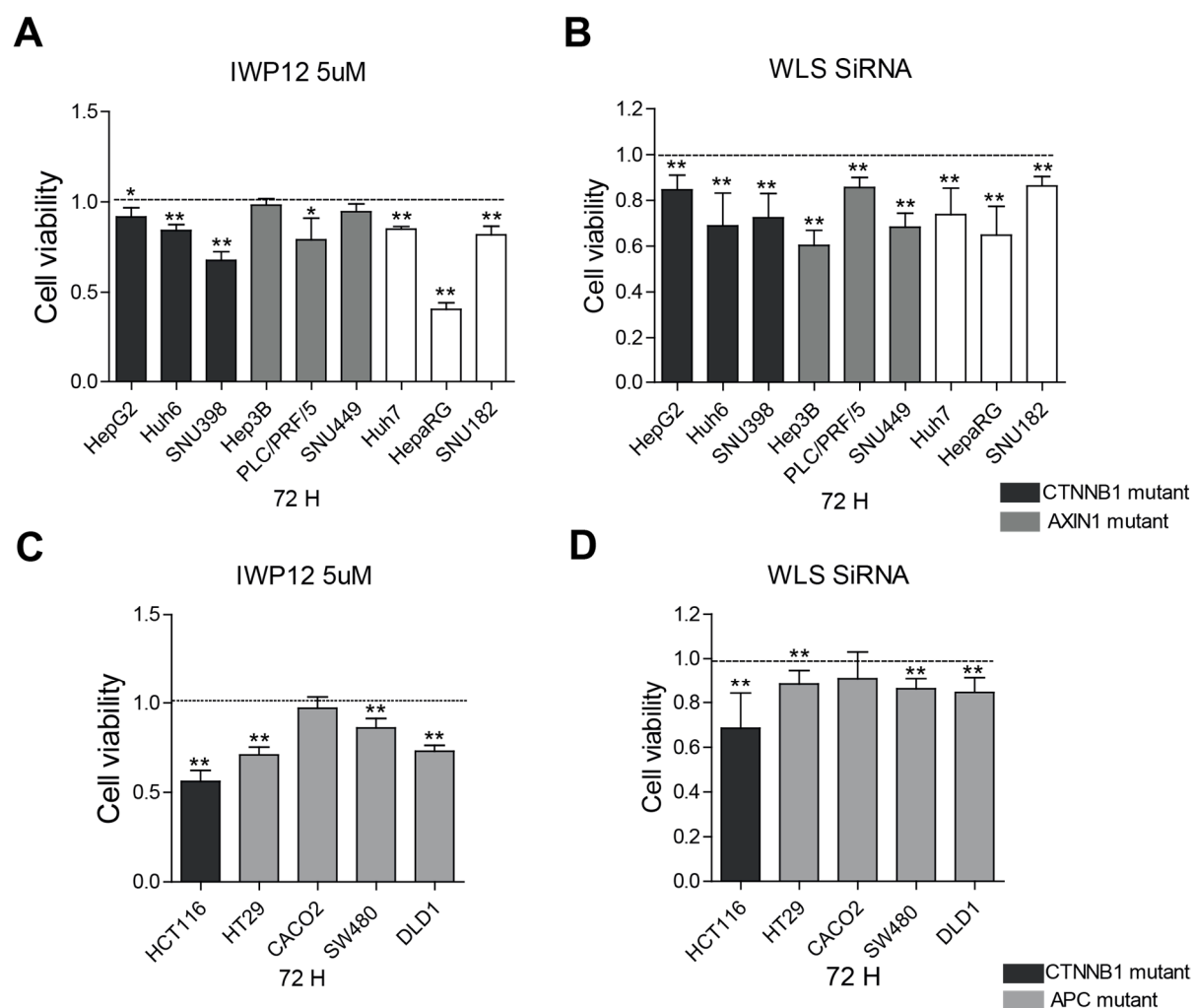


Fig.4. Inhibition of growth by Wnt secretion blockage. (A, C) IWP12 reduced cell growth of most HCC and CRC cell lines. Cell lines were incubated with 5 μ M IWP12 for 72 hours and tested by MTT assay (mean \pm SD, n=6, two times). Values depicted are relative to cell numbers obtained with the control DMSO treatment that are set to 1. (B, D) Cell lines were transiently transfected with *WLS* or non-targeting siRNAs for 72 hours, followed by MTT assay (mean \pm SD, n = 4, two times). Values depicted are relative to cell numbers obtained with the non-targeting siRNA that are set to 1. * p <0.05; ** p <0.01.

Altered exposure to extracellular Wnt ligands does not affect β -catenin signaling activity in most HCC cell lines

Previously, it was reported that Wnt secretion is required to maintain sufficiently high levels of canonical Wnt/ β -catenin signaling activity in both APC and β -catenin mutant CRC cell lines [26]. Here, we asked whether the β -catenin and AXIN1 mutant HCC cell lines were also dependent on Wnt secretion to sustain this pathway activity. Exposure to extracellular Wnt ligands was again reduced by treating all cell lines with IWP12, after which we measured β -catenin signaling activity using the reporter assay and *AXIN2* qRT-PCR. After 48 hours, β -catenin reporter activity was clearly suppressed only in SNU182 (Figure 5A), whereas the remaining eight HCC lines were not or only modestly inhibited in their reporter activity.

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Reduction of *AXIN2* expression confirmed the strong repressing effect of IWP12 in the SNU182 cell line, while no reduction was observed in the other cell lines (Figure 5B). Overall, this analysis shows that IWP12 treatment barely affects β -catenin signaling activity in most HCC cell lines, with the exception of SNU182.

In a true tumor setting, in addition to autocrine signaling, HCC cells are also exposed to Wnt ligands coming from the tumor microenvironment. Therefore, to determine the effects on β -catenin signaling of increased levels of extracellular Wnt ligands, we exposed them to L-Wnt3A conditioned medium. As shown in Figure 5C, β -catenin reporter activity was strongly enhanced in the Huh7 cell line and clearly activated in SNU182, both of which were confirmed by qRT-PCR for *AXIN2* (Figure 5D). Importantly, none of the β -catenin or AXIN1 mutant HCC lines showed enhanced β -catenin signaling following the addition of L-Wnt3A conditioned medium.

These results indicate that β -catenin and AXIN1 mutant HCC cell lines appear largely insensitive to the level of Wnt ligand exposure for sustaining intracellular β -catenin signaling, which could either mean that the expressed mutant β -catenin or AXIN1 protein determine overall signaling levels in a dominant fashion or, alternatively, that these cells have defects in their machinery to transduce Wnt signals. To test the latter option, we determined pDVL2 levels following treatment with IWP12 or L-Wnt3A conditioned medium (Figure 5E). Phosphorylation levels were not changed in Huh6, and only a modest reduction was observed in HepG2 following IWP12 treatment. In contrast, the SNU398 cell line showed a robust response in pDVL2 levels, decreasing from 21% to 7% by IWP12 treatment and upregulation to 59% following Wnt3A treatment. Thus, both options may hold true depending on the specific cell line under investigation. High variability in pDVL2 response was also observed in the remaining AXIN1-mutant and non-mutant HCC cell lines. The PLC/PRF/5 cell line showed a low baseline pDVL2 level, which was altered neither by IWP12 nor Wnt3A. The Huh7 and Hep3B cell lines also showed low baseline levels, which can however clearly be increased by the addition of Wnt3A. On the other hand, most DVL2 was phosphorylated at baseline in SNU182, which can be inhibited by IWP12, but can hardly be further stimulated by the addition of Wnt3A. Lastly, SNU449 and HepaRG showed intermediate pDVL2 levels at baseline that can both be reduced and activated by the respective treatments. Thus, all HCC cell lines show a

large variation in both their baseline levels of pDVL2 as well as responsiveness to Wnt ligand exposure, irrespective of their mutation status.

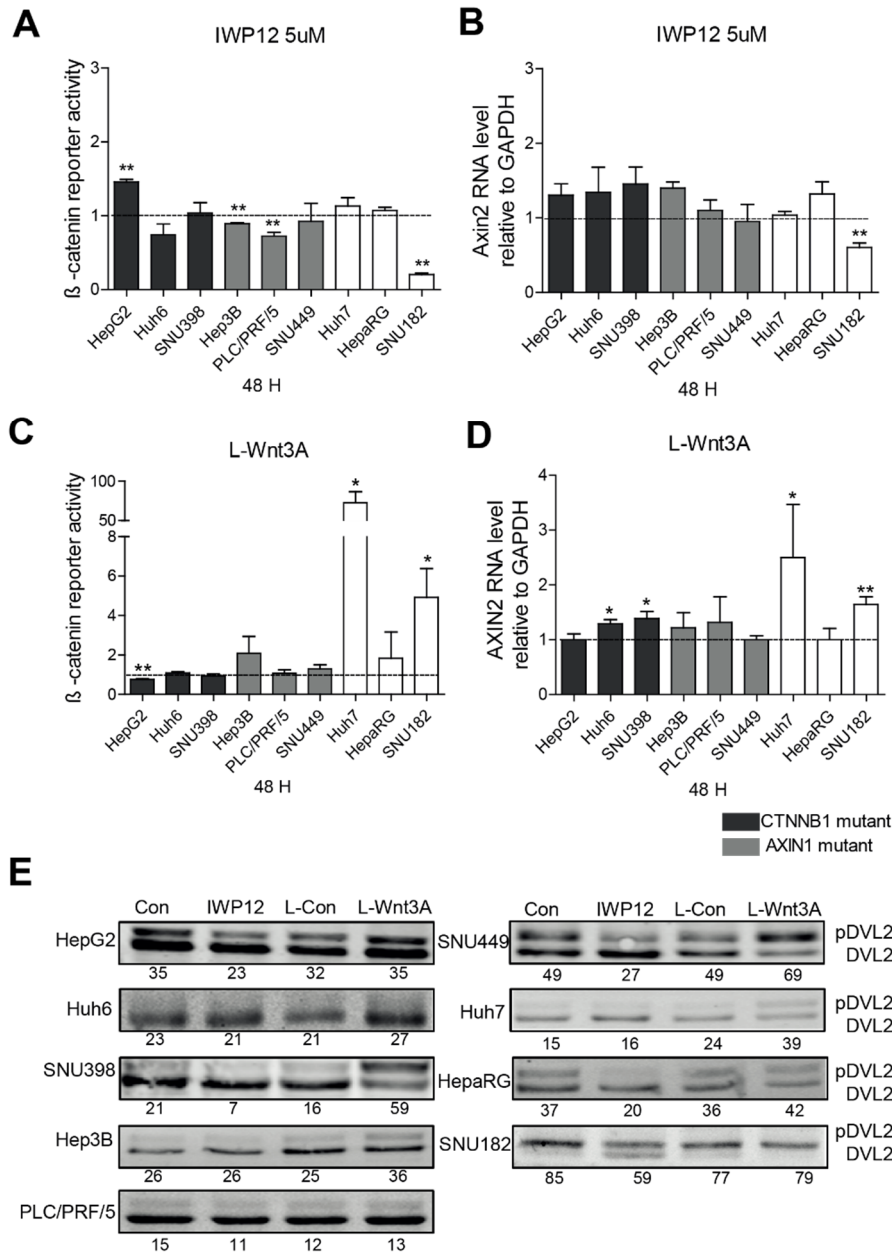


Fig.5. Growth reduction due to decreased Wnt secretion appears independent from β -catenin signaling. (A) β -catenin reporter activity was not clearly reduced by IWP12 in most HCC cell lines, except for SNU182 (mean \pm SD, n=3). (B) QRT-PCR for *AXIN2* showed that its expression following IWP12 treatment was only reduced significantly in the SNU182 cell line (mean \pm SD, n=3, two times). (C) L-Wnt3A conditioned medium significantly promoted β -catenin signaling activity in Huh7 and SNU182 cell lines. A significant albeit modest reduction of reporter activity was observed in HepG2. (mean \pm SD, n=3). (D) Increased β -catenin signaling in SNU182 and Huh7 following L-Wnt3A treatment was confirmed by *AXIN2* qRT-PCR (mean \pm SD, n=3, two times). All qRT-PCR and reporter values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 1. * p <0.05; ** p <0.01. (E) Phosphorylation level of DVL2 protein following treatment with IWP12 or L-Wnt3A conditioned medium (“Con” is DMSO only, “L-Con” is L-Control conditioned medium). Values below the images represent percentage of total DVL2 that is in the phosphorylated form (upper band).

Response of CRC cell lines to alterations in Wnt ligand levels

Among the eight CRC cell lines treated with IWP12, only HCT116 showed a significant reduction in β -catenin reporter activity as well as *AXIN2* expression (Figure 6A,B). *AXIN2* expression was slightly reduced in HT29 and SW480, while CACO2 showed a reduced reporter activity only. Interestingly, HCT116 was also the only cell line in which both reporter activity and *AXIN2* expression could be significantly stimulated by the addition of extracellular Wnt3A (Figure 6C,D). Analysis of pDVL2 levels in a selection of 5 CRC lines showed that overall baseline levels were low with the exception of HCT116 in which 60% of DVL2 is phosphorylated (Figure 6E). IWP12 treatment shows the expected decrease in pDVL2 in HCT116, whereas none of the other cell lines showed clear alterations in pDVL2 levels following treatment with either IWP12 or Wnt3A.

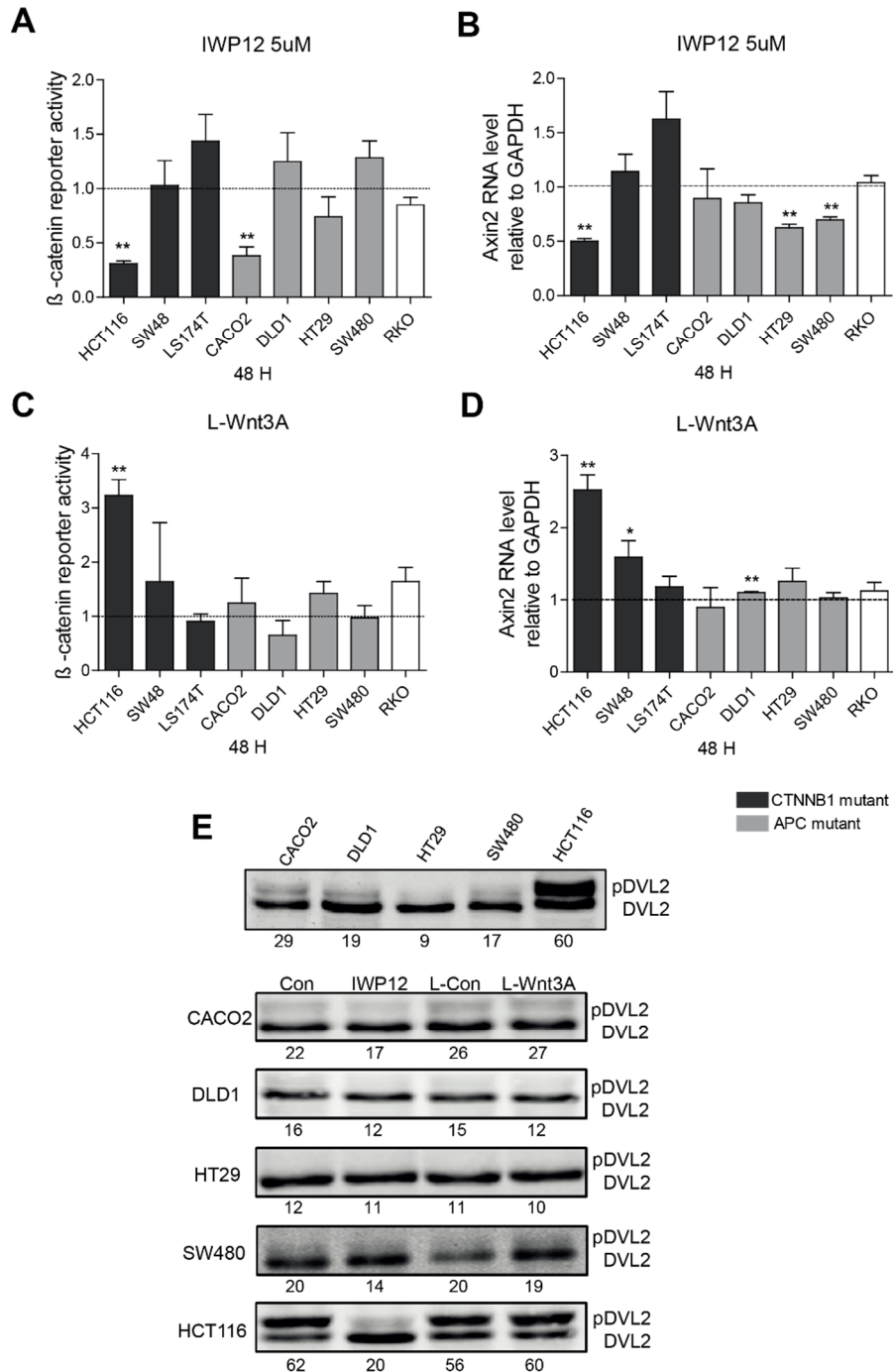


Fig.6. Responsiveness of CRC cell lines to alterations in Wnt ligand levels. (A) β-catenin reporter activity was significantly reduced by IWP12 in CACO2 and HCT116 cell lines (mean ± SD, n=3). (B) QRT-PCR for *AXIN2* showed that IWP12 treatment reduced its expression in HCT116. Modest but significant reductions were observed in HT29 and SW480 cell lines (mean ± SD, n=3, two times). (C) L-Wnt3A conditioned medium only increased β-catenin reporter activity significantly in HCT116 (mean ± SD, n=3). (D) *AXIN2* qRT-PCR confirmed upregulation of β-catenin signaling due to L-Wnt3A in HCT116. Significant but modest increases in *AXIN2* expression are seen in SW48 and DLD1 (mean ± SD, n=3). Reporter values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 1. **p*<0.05; ***p*<0.01. (E) Top image shows comparison of baseline pDVL2 levels within a selection of five CRC cell lines. Bottom images show pDVL2 levels following treatment with IWP12 or L-Wnt3A conditioned medium (“Con” is DMSO only, “L-Con” is L-Control conditioned medium). Values below the images represent percentage of total DVL2 that is in the phosphorylated form (upper band).

Blocking Wnt secretion does not lead to increased ER stress

Blocking Wnt secretion using IWP12 or *WLS* knockdown reduces growth of HCC cell lines, apparently largely independent of β -catenin signaling. These treatments however also predict the accumulation of Wnt ligands in the ER, which may lead to activation of an ER stress response thereby reducing proliferation or inducing apoptosis. Therefore, ER stress was evaluated after IWP12 treatment in HCC cell lines using the expression of the ER-stress induced genes *CHOP* and *GRP94* as a read-out. As shown in Figure 7A and 7B, expression of *CHOP* was clearly increased in SNU398 (4.5-fold), whereas *GRP94* expression was elevated in SNU182 (2.8-fold). However, none of the other HCC cell lines displayed strong signs of induction. Overall, these results suggest that blocking Wnt secretion is not associated with the induction of a strong ER stress response.

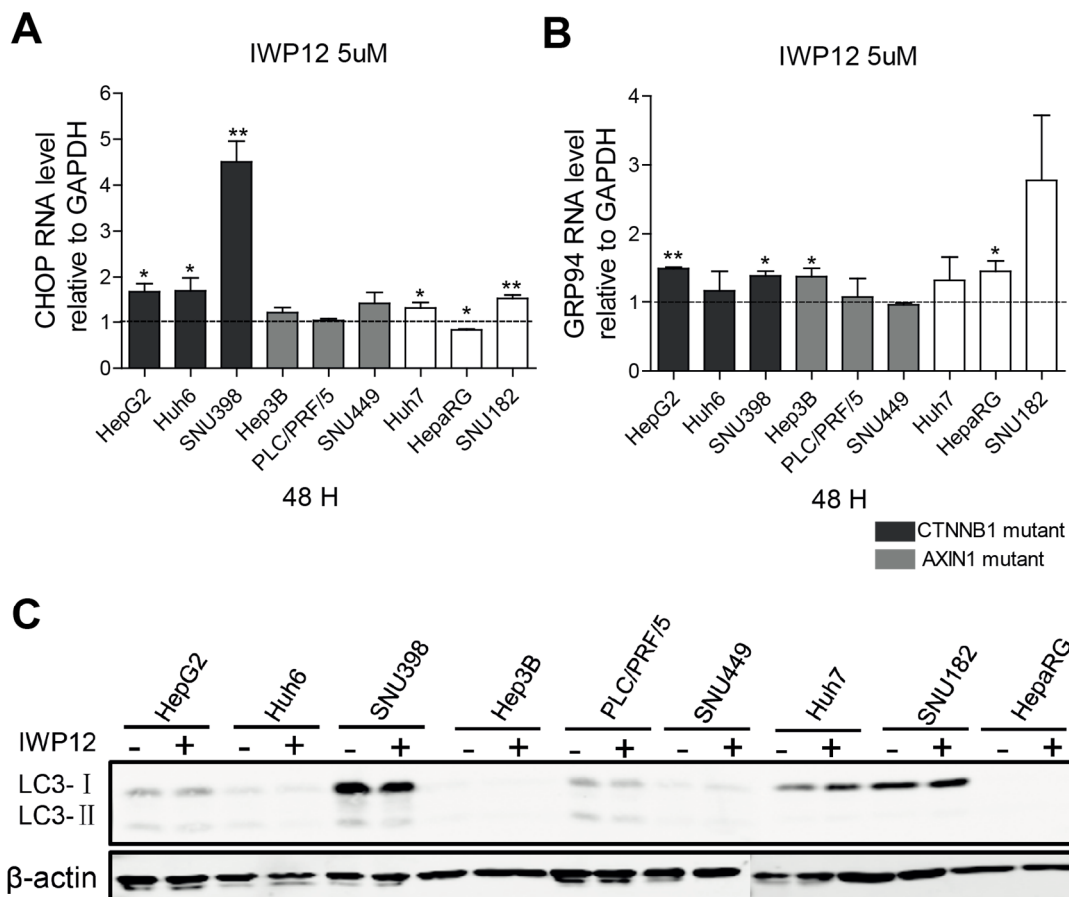


Fig.7. IWP12 treatment does not lead to the induction of a strong ER stress response or autophagy. Following 48 hours of IWP12 treatment, the expression of the ER stress induced response genes *CHOP* (A) and *GRP94* (B) was evaluated by qRT-PCR (mean \pm SD, n=3). Except for a clear induction of *CHOP* in SNU398 and *GRP94* in SNU182, IWP12 caused slight or no induction of an ER stress response in other cell lines. Values depicted are relative to those obtained for the untreated control samples that are arbitrarily set to 1. * p <0.05; ** p <0.01. (C) Wnt secretion inhibition does not enhance autophagy in HCC cell lines. After incubation with IWP12 for 48 hours, the expression of LC3- II was tested by western blot.

Wnt secretion inhibition does not induce autophagy

Since knockdown of β -catenin or suppression of β -catenin signaling induced autophagy and even autophagic cell death in head and neck squamous cell carcinoma cells [35] and breast cancer stem-like cells [36], we further hypothesized that blocking Wnt secretion could exert similar effects on HCC cell lines. During autophagy the microtubule-associated protein 1A/1B-light chain 3 (LC3) is converted through lipidation into a lower migrating isoform (LC3-II) detectable by western blot, which is used as an indicator of autophagosome formation. As indicated in Figure 7C, IWP12 treatment does not change the LC3 pattern in any of the HCC cell lines, showing that it does not induce autophagy.

Discussion

In this article we have investigated the importance of β -catenin signaling and Wnt secretion for sustaining hepatocellular carcinoma growth. Using a panel of 9 HCC cell lines we show that β -catenin signaling is required to support optimal growth in all of them, in line with other reports using a limited number of cell lines [37, 38]. This is to be expected for cell lines carrying oncogenic β -catenin mutations in which the activating mutation will have provided a selective growth advantage during tumor formation, but it also holds true for the non-mutant ones that show only low levels of baseline signaling, such as Huh7 and HepaRG, as well as the AXIN1 mutant lines. The latter observation is of relevance as it has been debated whether AXIN1 mutations lead to a significant enhancement of β -catenin signaling within liver cancers. This subset of tumors apparently lacks a robust nuclear β -catenin accumulation and shows no clear upregulation of target genes such as *AXIN2* or *GLUL* [39, 40]. Also in our hands, the AXIN1 mutant lines are among the lowest expressors of *AXIN2*. Nevertheless, in these lines *AXIN2* was readily detectable by qRT-PCR (Ct values below 28), in addition to β -catenin reporter activities approaching those of the β -catenin mutant ones. Given that also these lines are suppressed in their growth following β -catenin knockdown, it shows that the majority of HCCs independent of their mutational profile, rely on β -catenin signaling for optimal growth.

Besides its role in signaling, β -catenin is also involved in cell-cell adhesion by directly binding to cadherins [41]. As such, the siRNA mediated knock-down that we apply here, will also likely reduce the amount of β -catenin sequestered at these adherens junctions. However, several investigations have shown that complete loss of β -catenin does not automatically lead

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to alterations in cell adhesion, including hepatocytes and hepatocellular cancer cells [42-45]. In all cases, it was shown that γ -catenin compensates for its loss, thereby retaining normal cell adhesion. Importantly, these studies show that it is mainly the signaling function of β -catenin that is affected following knock-down.

Next we addressed the question to what extent extracellular exposure to Wnt ligands contributes to the observed levels of β -catenin signaling. Using qRT-PCR we tested the expression of all 19 Wnt ligands in our cell line panel. The Wnt expression profile that we observed largely corresponds with the semi-quantitative analyses performed by others [22, 46]. *WNT3* is the most abundantly expressed “canonical” Wnt ligand uniformly expressed in all cell lines, whereas all the others are expressed at low level or only in a subset of the cell lines. SNU182 clearly stands out as the overall highest expressor of Wnt ligands, likely explaining the high level of phosphorylated DVL2 that we observed in this cell line [33]. Among the non-mutant lines, SNU182 also showed the highest β -catenin reporter activity and level of *AXIN2* expression, comparable with the β -catenin mutant ones. As such, it is not unexpected that this cell line strongly relies on Wnt ligand secretion to retain increased β -catenin signaling. In fact, it is the only HCC cell line that shows a clear reduction following Wnt secretion blockage on both reporter activity as well as *AXIN2* expression level. On the other hand, the non-mutant Huh7 cell line expresses the lowest amount of Wnt ligands explaining its low baseline signaling levels, but it is highly responsive to Wnt ligand exposure for inducing strong β -catenin signaling. Within a true tumor setting it may represent a subtype of liver cancers that heavily depends on Wnt ligands expressed by cells within the tumor microenvironment, whose secretion would also be inhibited by the porcupine inhibitors employed here, whereas the SNU182 line is largely autonomous in this respect. Importantly, none of the β -catenin and *AXIN1*-mutant HCC cell lines are clearly affected in β -catenin signaling upon alterations in Wnt ligand exposure, irrespective of their source, suggesting that the expressed oncogenic β -catenin or mutant *AXIN1* proteins determine overall signaling levels in a dominant fashion.

In our hands this also holds true for most of the APC and β -catenin mutant CRC lines that we investigated. Among 8 CRC lines tested, only HCT116 shows a strongly reduced reporter activity and *AXIN2* levels following IWP12 treatment, while it is also the only one in which both β -catenin signaling readouts are clearly increased after Wnt3A exposure. Analysis of pDVL2

levels is largely in accordance with this lack of response, i.e. most cell lines tested show only low baseline levels that are barely changed by either treatment (DLD1, HT29, SW480), suggesting that these lines are not actively signaling through Wnt ligand receptors. These results also challenge the universal validity of the conclusions drawn by Voloshanenko et al. who proposed that colorectal cancers still strongly depend on Wnt ligand exposure for maintaining optimal β -catenin signaling levels [26]. Their overall well-performed study depended on a thorough analysis of the HCT116 cell line and to a lesser extent on other lines such as DLD1. Importantly, the β -catenin mutant HCT116 cell line is nowadays known to carry an inactivating mutation in the transmembrane E3 ubiquitin ligase RNF43, which strongly sensitizes these cells to exposure by Wnt ligands (see discussion below) [28, 47-49]. As such, their study may have unknowingly overstated the importance of Wnt ligand signaling for CRC growth in general, warranting a more extensive analysis in a larger cohort of CRC samples and cell lines.

In recent years, Wnt secretion inhibitors, such as the porcupine inhibitor used in our study, have emerged as candidate drugs for treating Wnt-driven cancers. Cancers that are considered to be especially responsive to these treatments are the ones carrying somatic mutations resulting in a persistent presence of Wnt receptors at the cell surface [28, 48]. In normal cells, the Wnt/Frizzled receptors are continuously endocytosed and degraded following ubiquitination by RNF43 or its close homolog ZNRF3. Both these ubiquitin ligases are inhibited in their action by one of four secreted R-spondin proteins [50]. Consequently, mutational inactivation of RNF43/ZNRF3 or a strongly increased production of R-spondins through the generation of aberrant fusion transcripts, both lead to tumor cells with high levels of Wnt/Frizzled receptor at their surface and hyper-responsiveness to Wnt ligands. These genetic aberrations have been identified in 10-20% of CRCs and in various other tumor types [28, 51, 52], but are to the best of our knowledge not present in HCCs, suggesting that these tumors are not prime candidates for treatment with Wnt secretion inhibitors. Nevertheless, our analysis shows that most of the HCC cell lines are reduced in their growth to varying extents, following both WLS knockdown as well as IWP12 treatment.

The mechanism of the growth suppression remains more elusive at present. Except for the SNU182 cell line, we do see little evidence of β -catenin signaling modulation, suggesting that other mechanisms are at play. One possibility is the induction of ER stress resulting from

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the aberrant accumulation of Wnt ligands in the ER. However, except from increased expression of the ER-induced genes *CHOP* and *GRP94*, in resp. SNU398 and SNU182, we do not see strong evidence that Wnt secretion blockage leads to high levels of ER stress. In addition, autophagy did not contribute to the growth suppression either as there was no visible change in the pattern of the autophagy marker LC3 following IWP12 treatment. An alternative explanation may reside in the reduced secretion of Wnt ligands more commonly signaling through β -catenin independent pathways. Activation of these alternative pathways has however mainly been shown to affect cellular processes involved in migration and cellular polarity and actually to counteract cell proliferation [8]. This “non-canonical” pathway has not been extensively studied in liver cancer, but the available literature does indeed support a growth suppressive effect [46, 53]. Therefore, interfering with the secretion of this subset of Wnt ligands is expected to support cellular growth, which is in contrast to the growth suppression that we observe. In line with our results, Covey et al. have shown that knocking-down PORCN in various tumor cell lines reduced their growth through a Wnt-independent pathway [54]. Also in their case no obvious explanation could be uncovered, but both studies highlight the importance of considering alternative roles for proteins involved in Wnt secretion and their role in regulating cell growth.

In conclusion, our study shows that the majority of HCC cell lines depend on β -catenin signaling for maintaining optimal growth. Extracellular exposure to Wnt ligands has a minor contribution to overall β -catenin signaling strength in the β -catenin and AXIN1-mutant cell lines. Despite this observation, interfering with Wnt secretion through WLS knockdown or inhibition of porcupine function results in reduced growth, indicating that these proteins may have alternative roles currently unappreciated.

Supplementary Figures and Tables

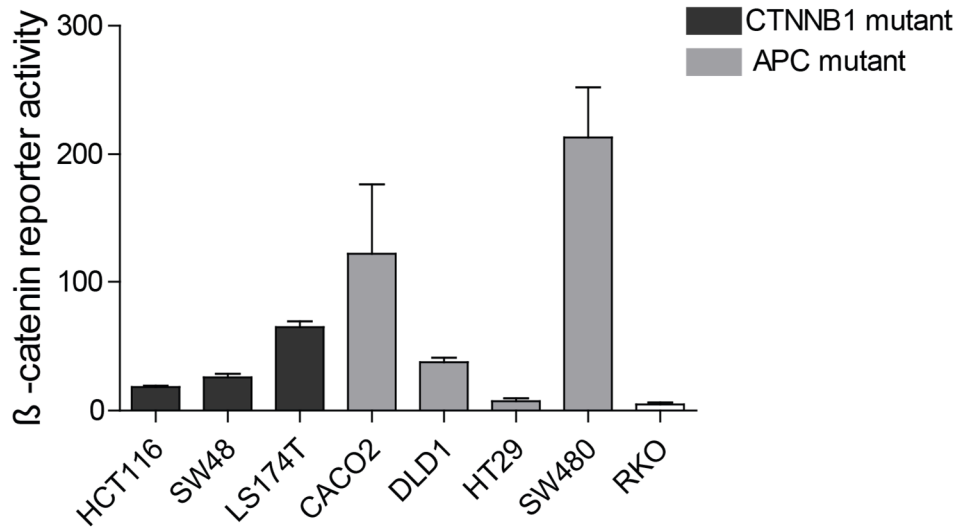


Fig.S1. Wnt/ β -catenin signaling activity in CRC cell lines determined by a β -catenin luciferase reporter assay (mean \pm SD, n=3).

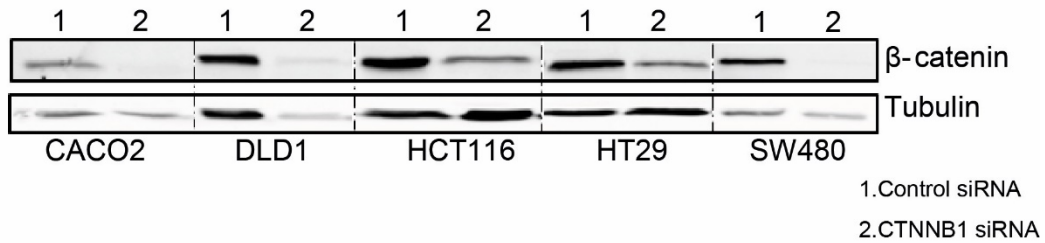


Fig.S2. siRNA mediated silencing of β -catenin in CRC cell lines confirmed by western blotting.

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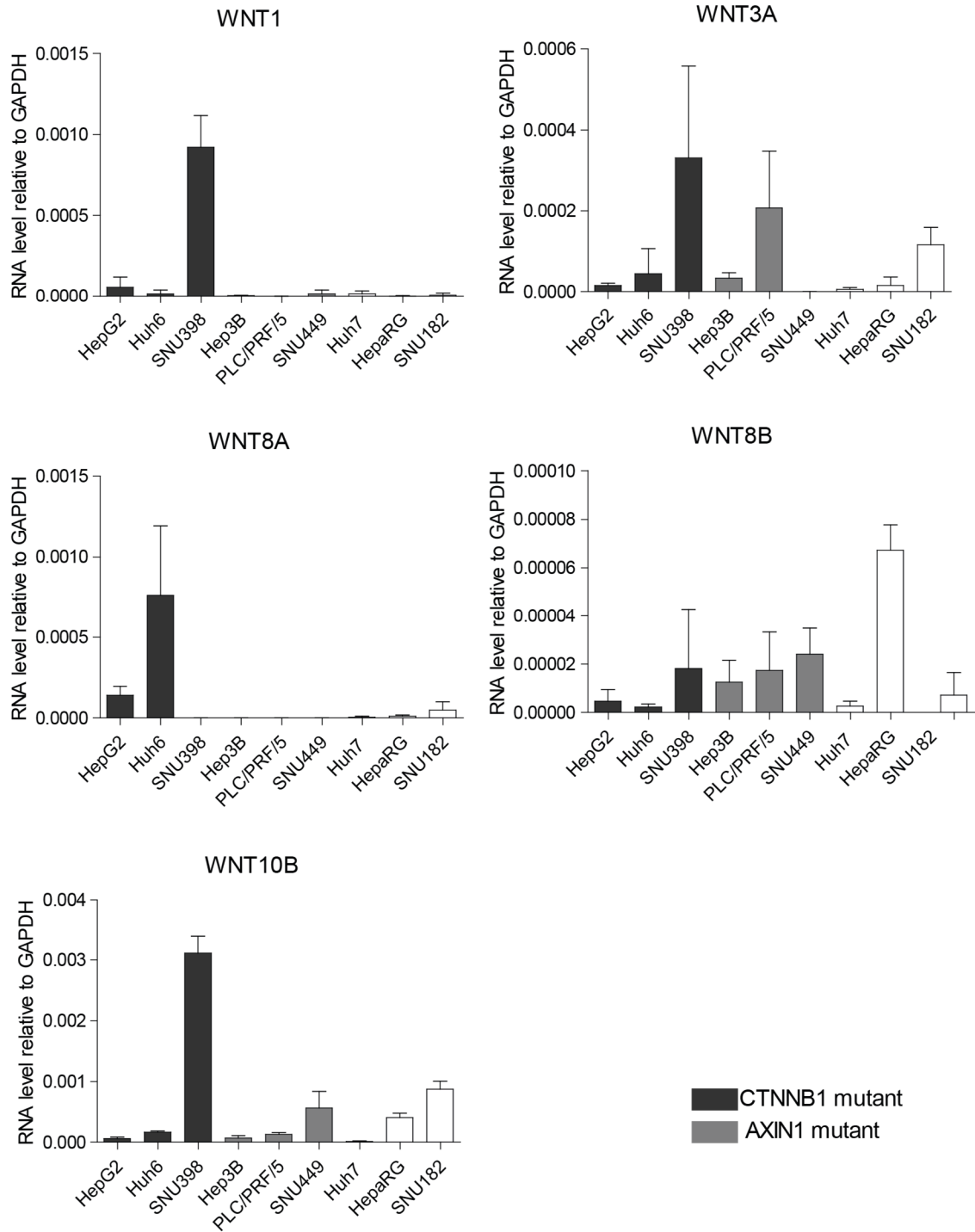


Fig.S3. Expression levels of remaining “canonical” Wnt ligands in HCC cell lines tested by qRT-PCR (mean \pm SD, n =3).

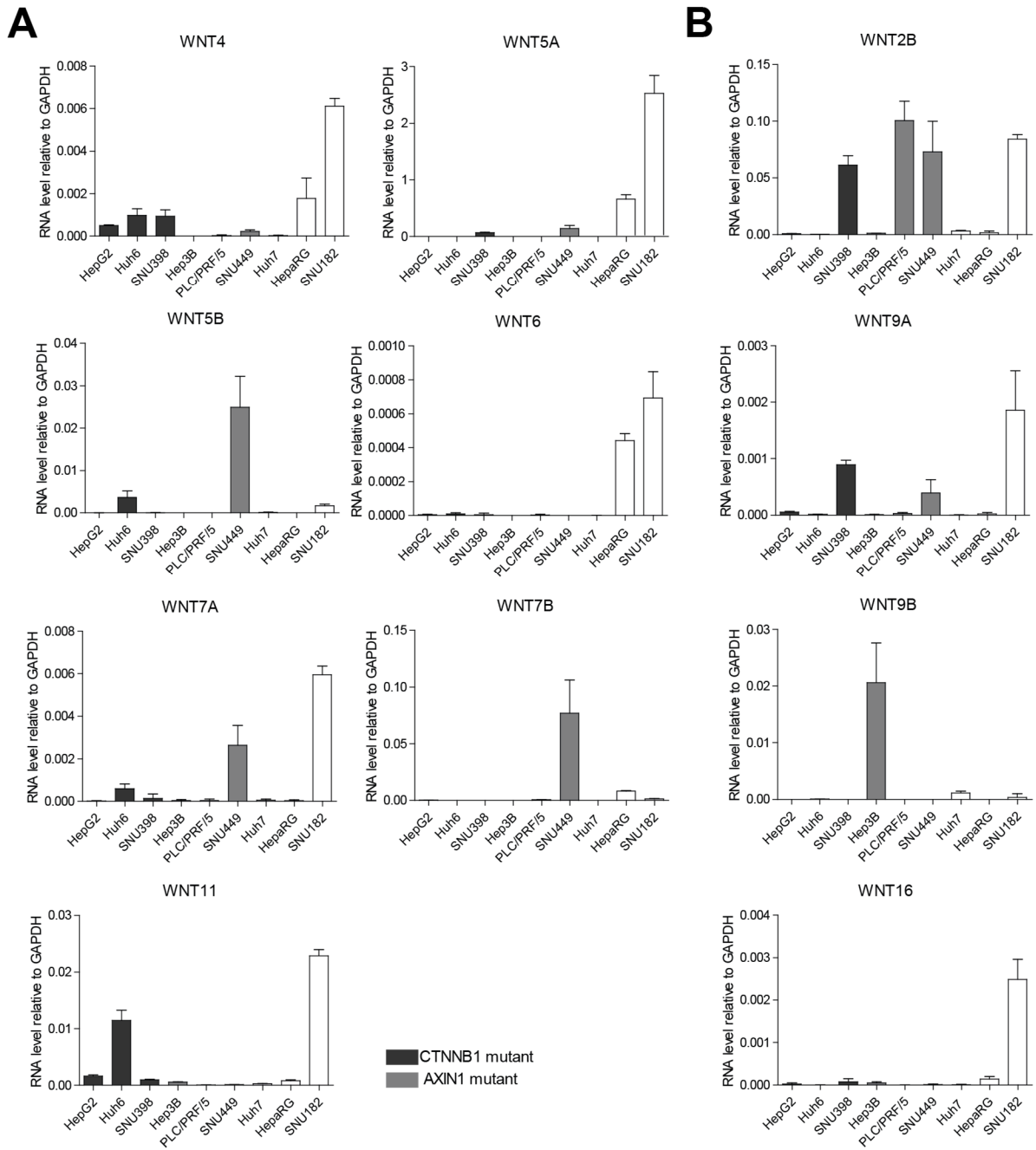


Fig.S4. Expression levels of “non-canonical” (A) and unclassified (B) Wnt ligands in HCC cell lines tested by qRT-PCR (mean ± SD, n =3).

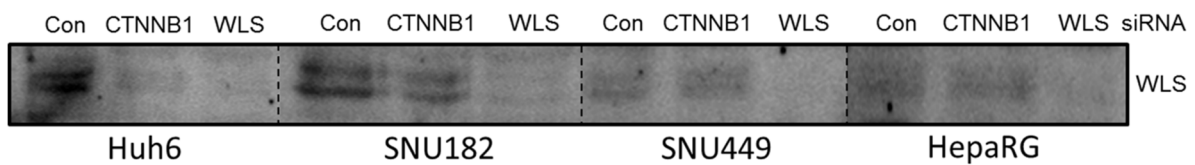


Fig.S5. SiRNA mediated silencing of WLS in HCC cell lines tested by western blotting.

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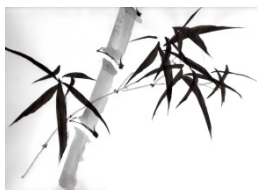
Chapter 6

A novel tissue-based β -catenin gene and immunohistochemical analysis to exclude Familial Adenomatous Polyposis among children with hepatoblastoma tumors

Hendrikus J. Dubbink^a, Iris H.I.M. Hollink^{b,*}, Carolina Avenca Valente^{a,*}, Wenhui Wang^{c,*}, Pengyu Liu^c, Michail Doukas^a, Max M. van Noesel^d, Winand N.M. Dinjens^a, Anja Wagner^b, Ron Smits^c

Departments of Pathology^a, Clinical Genetics^b, Gastroenterology and Hepatology^c, Erasmus MC Cancer Institute, University Medical Center, Rotterdam, The Netherlands. ^dPrincess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands.

*Authors contributed equally to this article



List of Abbreviations:

BWS	Beckwith-Wiedemann syndrome
DAB	3,3'-diaminobenzidine
FAP	Familial Adenomatous Polyposis
FFPE	formalin-fixed paraffin-embedded
HB	Hepatoblastoma
HCA	hepatocellular adenoma
HCC	hepatocellular carcinoma
IHC	Immunohistochemistry
LFS	Li-Fraumeni Syndrome
NF1	Neurofibromatosis type I
PBS	Phosphate-Buffered Saline
S/T	Serine/Threonine
STR	Short Tandem Repeats

Abstract

Background: The Wnt/ β -catenin pathway plays a central role in the pathogenesis of most hepatoblastomas, i.e. up to 60-80% carry activating *CTNNB1* mutations. Hepatoblastomas can however also be the first manifestation of familial adenomatous polyposis (FAP). As this is a severe disease, it is important for the patient and related family members to firmly exclude FAP at an early stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, which is associated with 10-20% false-negative results. Here, we establish and validate a tissue-based β -catenin gene and immunohistochemical analysis, which complements germline mutation screening to exclude the diagnosis FAP among HB patients.

Methods: Tumor tissues of 18 hepatoblastoma patients including 3 FAP cases were subjected to *CTNNB1* exon3 mutational analysis and immunohistochemistry comparing staining patterns for total and exon3 specific β -catenin antibodies.

Results: Our novel tissue-based method reliably identified all three FAP patients. Their tumors were characterized by a wild-type exon3 sequence and a comparable nuclear staining for both antibodies. In contrast, the non-FAP tumors carried missense *CTNNB1* mutations combined with a clearly reduced staining for the exon3 antibody, or complete loss of staining in case of lesions with exon3 deletions.

Conclusion: We have successfully established and validated a novel β -catenin gene and immunohistochemical diagnostic method, which, when combined with routine germline DNA testing, allows the exclusion of the diagnosis FAP among HB patients.

Key words: Hepatoblastoma, genetic counseling, familial adenomatous polyposis (FAP), Wnt/ β -catenin signaling, *CTNNB1*, *APC*

Introduction

Hepatoblastoma (HB) is the most common pediatric liver malignancy with an estimated incidence of 1 per 100,000 children, mainly affecting children during their first 3 years of life.^{1,2} HBs are believed to originate from hepatic progenitor cells that acquire malignant transformation during embryogenesis. Histologically they show similarity to immature hepatocytes of the developing liver.^{3,4} Most HBs are believed to be of sporadic nature, but about 10-20% have been associated with genetic defects such as Beckwith-Wiedemann syndrome (BWS) or familial adenomatous polyposis (FAP).^{2,5,6}

The Wnt/ β -catenin signaling pathway is recognized as having a central role in the pathogenesis of hepatoblastomas.⁴ The β -catenin protein is a key effector of the pathway, affecting cellular decisions such as stem cell maintenance and cell proliferation through modulating the expression of specific target genes. In normal cells, β -catenin is involved both in cell adhesion, when located at the cell membrane, and in transcriptional regulation, when present in the nucleus. In the absence of upstream Wnt signaling, β -catenin is phosphorylated at N-terminal Serine/Threonine (S/T) residues by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1 α .⁷⁻¹¹ Phosphorylated β -catenin is then ubiquitinated, leading to its proteasomal degradation. When cells are exposed to Wnt ligands, this β -catenin breakdown complex is temporarily inhibited, leading to the stabilization of β -catenin. As a result, it translocates into the nucleus and associates with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt/ β -catenin target genes.

In several tumor types, this pathway is constitutively activated due to 'loss of function' mutations of the *APC*, *AXIN1* or *AXIN2* genes leading to inefficient β -catenin degradation and its intracellular stabilization. Other tumors carry oncogenic β -catenin (*CTNNB1*) mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation. Important for our study, disease-causing mutations in the *APC* and *CTNNB1* genes have been shown to occur in a mutually exclusive manner in all tumor types studied so far.¹² These mutations lead to aberrant stabilization of β -catenin, which

constitutively activates downstream Wnt/ β -catenin target genes and triggers a genetic program resulting in tumor formation.

HBs are characterized by a high proportion (60-80%) of activating mutations within the *CTNNB1* gene, either by point mutation or deletions encompassing exon 3.¹³⁻²² The deletions represent more than 60% of the somatic *CTNNB1* mutations and vary in size from small intra-exonic deletions to larger ones extending up to part of exon 2 or 4. All of them have in common that they result in in-frame deletions leading to mutant β -catenin that is resistant to degradation.

Somatic APC mutations have been rarely reported in HBs.^{14,16,17,23} However, familial adenomatous polyposis (FAP) patients carrying a germline APC mutation, present with a 750-7500-fold increased risk of hepatoblastoma development in comparison to the general population.^{2,24-27} FAP is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. Untreated, the risk of colorectal cancer in FAP patients approaches 100%. The average age when colorectal polyps are detected is about 15 years.²⁸ Colonoscopy and surgery in adolescence prevent colorectal cancer formation in FAP. When FAP is diagnosed in time, health benefits and increased life expectancy can be achieved. Importantly, in children hepatoblastoma can be the first manifestation of FAP within a family, especially given the high proportion (10-25%) of de novo germline mutations occurring in the APC gene.²⁹⁻³¹ This is of importance for the management of the child itself and its possible future offspring, but might also have implications for yet asymptomatic parents and siblings. For this reason, it has been advocated that all HB patients with unknown family history, should be referred for APC germline mutation detection,^{6,32-34} using either combination of currently available methods such as next-generation or Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA). These methods will uncover up to 80-90% of disease-causing APC mutations,^{35,36} but inherent to any diagnostic method will lead to false-negative results. Examples include complex insertions and deletions, chromosomal translocations, or when the patient is a low-mosaic carrier of the APC mutation.^{37,38} Moreover, sequence analysis will identify genetic variants with uncertain disease-causing relevance. Given the severity of the FAP syndrome a thorough exclusion of carrier status is highly desirable. Here, we establish and validate a tissue-based β -catenin gene and immunohistochemical analysis, which complements the

germline mutation screening to exclude the diagnosis FAP among HB patients. We also discuss options how it can be implemented in current clinical practice.

Materials and Methods

Patients

From the period 1995-2013, in total 23 pediatric liver tumors were available for analysis from the pathology archive of the Erasmus MC. Tissues were obtained from the initial operative procedure and embedded in paraffin after formalin fixation. The medical records and family history of patients were analyzed. Patient and tumor characteristics are provided in Table 1. All samples were revised by a GI-pathologist (MD) according to a recently proposed consensus classification for pediatric liver tumors.³⁹

Immunohistochemistry (IHC)

Immunohistochemical analyses for total β -catenin (1:200, clone-14, BD-Transduction Laboratories) and non-phosphorylated Ser33/37/Thr41 β -catenin (1:400, #8814, Cell Signaling Technology) were performed in an automated stainer (Benchmark-Ultra, Ventana Medical Systems, Tucson, AZ). Sections were deparaffinized and pre-treated with standard cell conditioning 1 solution (CC1) at 100°C for 64 min, followed by incubation with the specified antibodies at 37°C for 60 min. The antibodies were visualized with the OptiView IHC DAB detection kit.

Tumor DNA and RNA isolation and *CTNNB1* mutation analysis

Tumor DNA was extracted by microdissection from formalin-fixed paraffin-embedded (FFPE) tissue fragments using proteinase-K and 5% Chelex[®]-100 Chelating Resin (BioRAD, #1432832), as previously described.⁴⁰ Sequence analysis of *CTNNB1* exon 3 was performed by bi-directional sequencing of PCR-amplified fragments using M13-tailed forward and reverse primers (Supplemental Material S1). To test for genomic deletions of *CTNNB1* exon3, two independent PCRs were performed using primers located within exon2 and exon4. Details of the PCR and sequencing reactions and RA isolation are provided in Supplemental Material S1.

Cell lines

Short Tandem Repeat-verified cell lines used in this study were cultured as previously described.⁴¹ Preparation of paraffin-embedded cell line blocks is described in Supplemental Material S1.

Generation of β -catenin variant expression vectors and transfection

Expression vectors for N-terminal FLAG-tagged β -catenin variants were generated using the pcDNA-5'UT-FLAG vector as basis (kindly provided by Dr. Veronique Lefebvre, Lerner Research Institute, Cleveland OH, USA). Wild-type, S33Y and exon3 deleted variants of human *CTNNB1* were cloned using the Gibson assembly method (NEB). The G34V, S37F, T41A and S45P variants were generated by Q5[®] site-directed mutagenesis (NEB), using the wild-type clone as basis. Primers are available upon request. All plasmids were fully sequence verified. Next, HEK-293 cells seeded in 6-well plates were transiently transfected with Fugene-HD (Promega) using 1 μ g of plasmid DNA. After two days, cells were lysed in Laemmli sample buffer with 0.1M DTT and heated for 5-10 minutes at 95°C.

Western blot assay

The western blot assay and quantification for the FLAG-tag, total and non-phosphorylated Ser33/37/Thr41 β -catenin were performed basically as previously described, using all antibodies at a 1:1000 dilution.⁴¹

Results

Patient and tumor characteristics

In total 23 pediatric liver tumors were included in the study (Table 1), of which 18 were hepatoblastomas (HB), 4 hepatocellular carcinomas (HCC) and one hepatocellular adenoma (HCA). In accordance with literature, most HB patients were diagnosed during their first three years of life. The youngest age at diagnosis was 0.5 years, whereas the oldest patient was 9.7 years. Hepatoblastoma patients HB-4, HB-14 and HB-29 had been diagnosed with FAP previously. The HCC detected in HB-15 was diagnosed in a child belonging to a Li-Fraumeni family (LFS), i.e. *TP53* germline mutation, whereas HB-32 was observed in a patient with Neurofibromatosis type I (NF1). Hypomethylation of LIT1 was reported for patient HB-19, although no other signs of BWS were observed.

Immunohistochemistry for total β -catenin

Immunohistochemistry for β -catenin showed nuclear accumulation of β -catenin in one HCC (HB-8), while in the remaining three HCCs and the HCA an exclusive membranous pattern was observed (Table 2). All HBs showed evidence of nuclear staining, including the ones derived from the FAP patients. No nuclear β -catenin was detected in normal pre-existing hepatocytes.

Table 1. Patient and tumor characteristics

Sample	Gender	Age (years)	HB/HCC	Pathology	Known genetic predisposition
HB-4	F	5.5	HB	Epithelial (too few tumor cells for proper evaluation)	Familial Adenomatous Polyposis
HB-6	M	0,6	HB	Epithelial mixed	
HB-7	M	2,1	HB	Epithelial fetal	
HB-8	M	5,9	HCC	Classic	
HB-11	F	1.2	HB	Epithelial mixed	
HB-13	F	0.5	HB	Epithelial mixed	
HB-14	M	3.5	HB	Epithelial fetal	Familial Adenomatous Polyposis
HB-15	F	9.7	HCC	Classic	Belonging to Li-Fraumeni family (TP53)
HB-16	F	15.8	HCC	Fibrolamellar	
HB-17	M	9.7	HB	Epithelial fetal with low mitotic activity	
HB-18	F	11.4	HCC	Fibrolamellar	
HB-19	F	1.7	HB	Epithelial fetal with low mitotic activity	Hypomethylation of LIT1 (no signs of BWS)
HB-20	M	1,1	HB	Mixed epithelial-mesenchymal, no teratoid	See note*
HB-21	M	0.8	HB	Epithelial mixed	
HB-22	M	1.0	HB	Epithelial mixed	
HB-23	M	9.4	HB	Epithelial fetal	
HB-24	M	1.8	HB	Epithelial fetal, mitotically active	
HB-25	F	16.3	HCA		See note*
HB-27	F	7.3	HB	Epithelial pleomorphic	
HB-28	F	1.1	HB	Mixed epithelial-mesenchymal, teratoid	
HB-29	M	1.6	HB	Mixed epithelial-mesenchymal, teratoid	Familial Adenomatous Polyposis
HB-30	F	0.8	HB	Mixed epithelial-mesenchymal	
HB-32	F	1.1	HB	Mixed epithelial-mesenchymal, no teratoid	neurofibromatosis type I

HCA; Hepatocellular Adenoma, HB; Hepatoblastoma, HCC; Hepatocellular Carcinoma

CTNNB1 mutation analysis

Sequence analysis of exon 3 of *CTNNB1* succeeded in 22/23 tumors. In 8/18 HBs point mutations were detected within or in close proximity to the region encoding the S/T residues required for proteolytic breakdown of β -catenin (Table 2). As expected, no mutation was detected in all three FAP-derived HBs and in the HCC and HCA samples without nuclear

staining. Also no mutation was detected within the single HCC with nuclear accumulation of β -catenin.

Hepatoblastomas are however characterized by a high proportion of genomic deletions partially or completely encompassing exon 3,¹³⁻²¹ most of which will be missed by the regular exon 3 sequence analysis. Therefore, we attempted two genomic PCRs on DNA isolated from the FFPE samples, using a common forward primer in exon 2 and two reverse primers, respectively at the 5'- and 3'-side of exon 4. The latter can detect the reported deletions.

Table 2. *CTNNB1* mutational status and β -catenin IHC pattern of pediatric liver cancers

Sample	HB or HCC	genetic predisposition	Nucleotide change detected in genomic DNA	RNA alteration	Amino Acid alteration	C-ter β -cat IHC	S33/37/T41 β -cat IHC
HB-15	HCC	LFS	None			Membrane only	Membrane only
HB-16	HCC		None			Membrane only	Membrane only
HB-18	HCC		None			Membrane only	Membrane only
HB-25	HCA		None			Membrane only	Membrane only
HB-4	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-14	HB	FAP p.R1114*	none			Nuclear	Nuclear
HB-29	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-6	HB		c.97T>C		p.S33P	Nuclear	Nuclear
HB-17	HB		c.86C>T + c.94G>T		p.S29F + p.D32Y	Nuclear	Moderate Nuclear
HB-22	HB		c.94G>A		p.D32S	Nuclear	Nuclear
HB-7	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-13	HB		c.101G>T		p.G34V	Patchy nuclear	Faintly nuclear
HB-20	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-21	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-28	HB		c.121A>G		p.T41A	Nuclear	Patchy nuclear
HB-8	HCC		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-11	HB		Not analyzable	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-19	HB	LIT1	None	\pm 215 bp del	p.?	Nuclear	Negative
HB-23	HB		None			Nuclear	Membrane only
HB-24	HB		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-27	HB		None			Nuclear	Membrane only
HB-30	HB		c.98_238del	r.98_238del	p.G34_A80del	Nuclear	Negative
HB-32	HB	NF1	c.14-202_241+69del	r.14_241del	p.A5_A80del	Nuclear	Negative

At large, four different staining patterns were observed using the C-terminal and S33/37/T43 β -catenin antibodies. Combined with the genomic mutation analysis the most likely β -catenin-related mechanism contributing to tumor formation is color-coded as follows:

No evidence of mutations leading to β -catenin activation, APC mutation or β -catenin D32/S33 amino acid alterations, β -catenin G34 amino acid alteration, β -catenin exon 3 deletion.

"Nuclear" means a combination of nuclear, cytoplasmic and membranous staining. "Negative" means no staining visible in tumor cells.

The following abbreviations are used: HCA-Hepatocellular Adenoma, HB-Hepatoblastoma, HCC-Hepatocellular Carcinoma, FAP-familial adenomatous polyposis, LFS-Li-Fraumeni syndrome, LIT1-LIT1 hypomethylation, NF1-Neurofibromatosis Type-1, IHC-Immunohistochemistry.

Immunohistochemical identification of β -catenin alterations

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Given the poor quality of DNA extracted from FFPE material, we sought for an alternative method to identify samples with genomic *CTNNB1* deletions. All these mutations generate mutant β -catenin proteins that are more resistant to proteolytic breakdown. Hence, the great majority of β -catenin protein present within a *CTNNB1* mutant cell is represented by the mutant protein. This will be especially the case for the nuclear compartment as most of the wild-type protein is prevented from entering the nucleus through degradation by the APC/AXIN breakdown complex. We hypothesized that an immunohistochemical analysis using a β -catenin antibody specifically recognizing the N-terminal S/T residues, could distinguish between APC- and β -catenin mutant tumors. In APC-mutant tumors wild-type β -catenin accumulates in the nucleus that can still be detected by such an antibody, whereas (partial) deletion or mutation of the β -catenin S/T residues would prevent detection.

To validate this approach, we first tested a panel of cell lines with known *CTNNB1* mutations using a C-terminal β -catenin antibody in comparison with one raised against a peptide corresponding to residues surrounding S37. Western blot analysis using the C-terminal antibody robustly demonstrated β -catenin in all cell lines, including the shortened mutant product present in HepG2 cells (Figure 1A). The non-phospho S33/37/T41 antibody readily detected the wild-type β -catenin present in the APC-mutant line SW480. Also the S45 mutant β -catenin present in HCT116 and LS174T was demonstrated at high intensity compared with the C-terminal antibody. In contrast, the exon 3 deleted product of HepG2 and S37/T41 alterations completely abrogated detection, while S33 and G34 alterations resulted in reduced detection. The latter may represent binding to wild-type β -catenin present in these cells and/or retained affinity for the mutant protein.

To investigate residual binding affinity for mutant β -catenin by the S33/37/T41 antibody, we transiently expressed N-terminal FLAG-tagged β -catenin variants in HEK-293 cells (Figure 1B). Deletion of exon 3, and S37F/T41A alterations completely abrogated binding. In contrast, the S33Y variant was identified at comparable levels to the wild-type protein, while the G34V variant is detected with $\pm 40\%$ reduced signal intensity. In conclusion, the S33/37/T41 antibody retains affinity for S33/G34 alterations when present in denatured form on western blot, while S37/T41 changes and deletion of exon 3 prevent its binding.

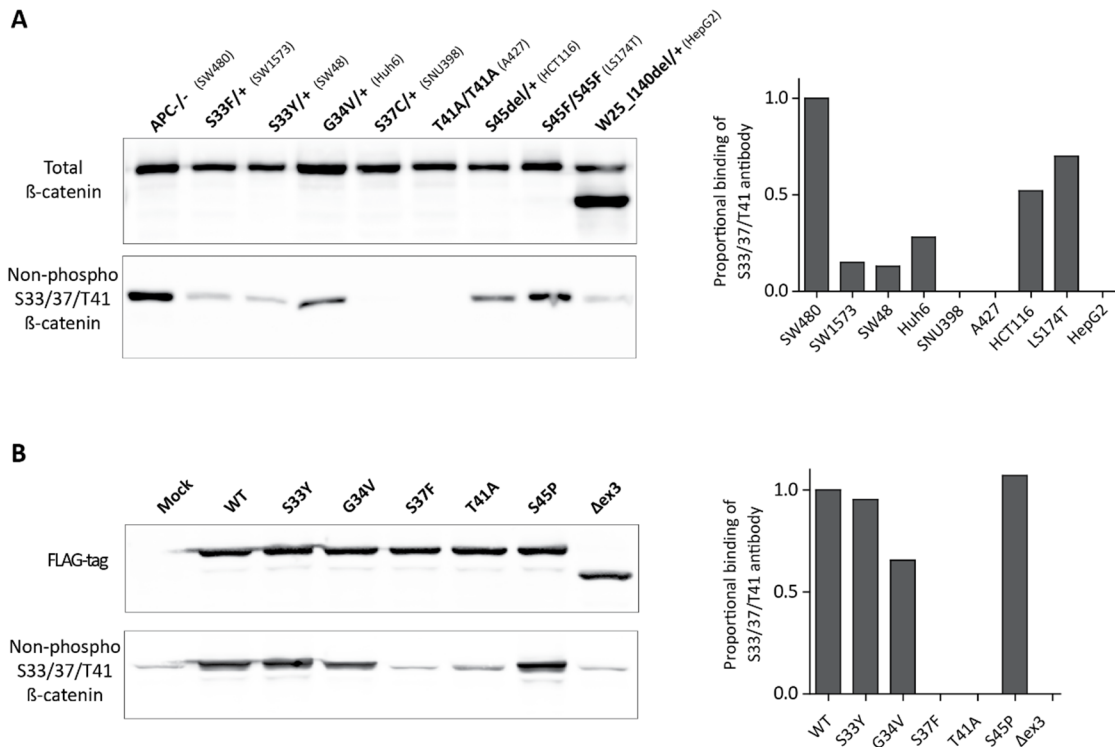


Figure 1. Western blot validation of the non-phospho S33/37/T41 β-catenin antibody. (A) Western blot analysis of one APC mutant and eight β-catenin mutant cell lines using a C-terminal β-catenin antibody (upper lanes) in comparison with one raised against a peptide corresponding to residues surrounding S37 (lower lanes). Amino acid alterations and zygosity status are depicted on top. Name of cell line is in brackets. Deletion of exon 3 and alterations of S37 and T41 abrogate binding. Reduced signal for S33 and G34 variants suggests lowered affinity, although residual binding to remaining wild type protein cannot be formally excluded. S45 changes are largely unaffected. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the APC-mutant SW480 cell line set arbitrarily to one. (B) Transient expression of FLAG-tagged wild type and S/T mutant β-catenin variants shows that S33Y β-catenin can still be recognized by the S33/37/T41 antibody. The G34V variant shows a ±40% reduced signal intensity. The other variants confirm the cell line analysis. The weaker band with lower molecular weight visible in all lanes is the endogenous β-catenin protein produced by HEK-293 cells. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the wild type protein set arbitrarily to one.

Next, we evaluated this antibody for immunohistochemical purposes using paraffin blocks containing formalin-fixed β-catenin mutant cell lines. In line with the western blot data, S45 mutant HCT116 and LS174T showed a comparable staining pattern for both antibodies (Figure 2). In strong contrast, all other β-catenin mutant lines showed a clearly reduced overall staining using the S33/37/T41 antibody. The S37C and T41A mutant lines are completely negative. In the S33 mutant lines SW48 and SW1573 nuclear staining can be observed in some cells, although with reduced intensity. The exon 3 deletion mutant HepG2 line showed an exclusive membranous staining, most likely resulting from detection of the remaining wild-type protein. Also in the G34V mutant Huh6 line no obvious nuclear staining was observed. Surprisingly, IHC staining of this variant is much weaker than anticipated from the western blot analysis, most likely resulting from reduced antibody affinity when present in its “native”

form in FFPE sections and/or partial phosphorylation of S37/T41 residues. In conclusion, the S33/37/T41 β -catenin antibody can reliably identify the cell lines with exon 3 *CTNNB1* mutations, with the exception of S33 and S45 alterations.

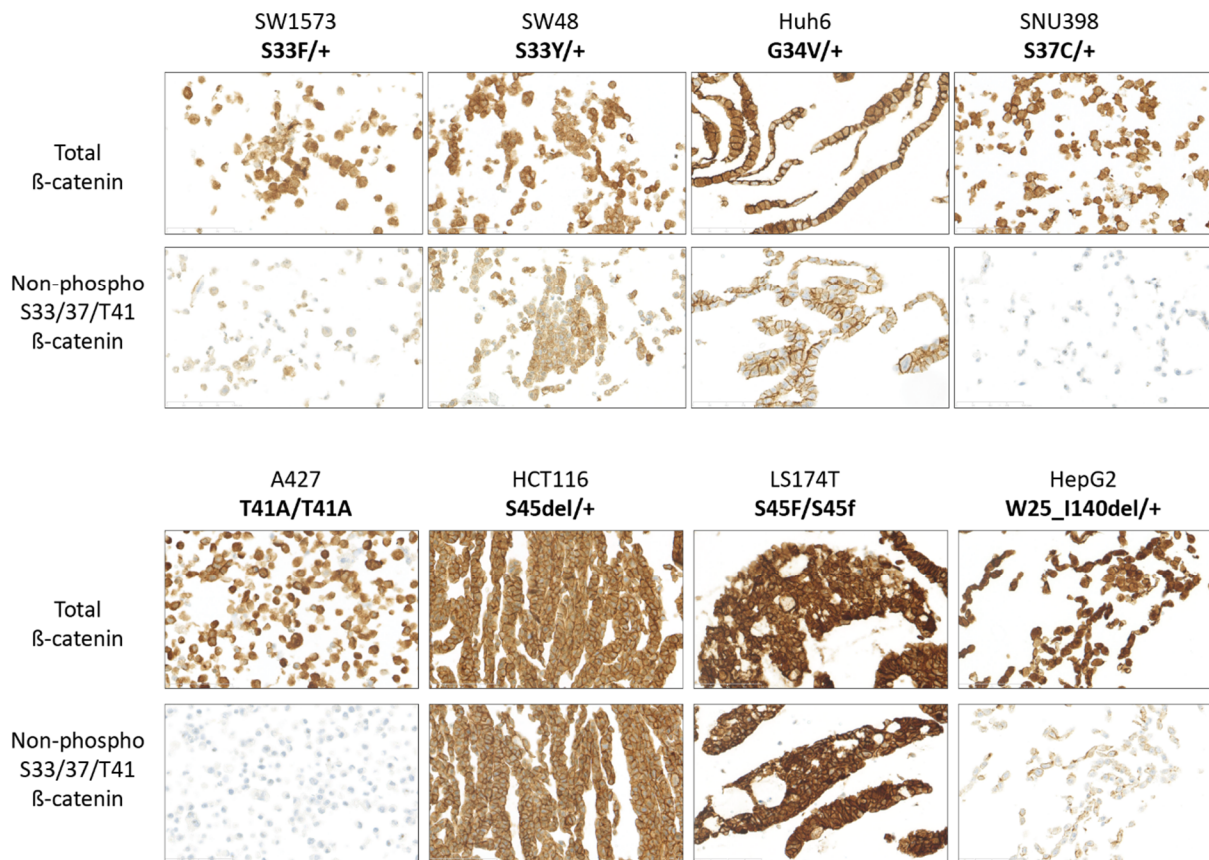


Figure 2. Immunohistochemical validation of the non-phospho S33/37/T41 β -catenin antibody. Formalin-fixed and paraffin-embedded cell pellets of the β -catenin mutant cell lines were stained with the C-terminal (top rows) and S33/37/T41 (bottom rows) β -catenin antibodies. Staining patterns are largely in line with western blot analysis with the exception of the relatively weak staining pattern observed for the G34V protein present in Huh6. HepG2 shows an exclusive membranous staining with the S33/37/T41 antibody, most likely reflecting the remaining wild type protein. Both S33 mutant cell lines retain some nuclear detection, albeit slightly reduced. S45 variant cell lines show identical staining patterns with both antibodies. Original magnification 400x.

Next, the S33/37/T41 antibody was applied to the tumor sections and compared with that of total β -catenin (Figure 3, Supplemental Figure S1). At large, four different staining patterns were observed. The tumors with exclusively membranous β -catenin were stained in an identical fashion with the S33/37/T41 antibody (Figure 3A,B and S1). Importantly, all three FAP-associated lesions showed basically identical staining patterns with both antibodies as well, i.e. a high proportion of tumor cells showing evidence of nuclear accumulation (Figure 3C,D and S1). This pattern was also observed in lesions with D32 and S33 mutations, albeit with slightly lower intensity for the S33/37/T41 antibody (Figure 3E,F and S1). The third pattern is observed in HBs with G34V mutation. These showed a strongly reduced overall

staining using the S33/37/T41 antibody. However, occasionally tumor cells with nuclear staining can be clearly observed, especially in regions with heavy staining for total β -catenin (Figure 3G,H and S1). This pattern was also observed in HB-28, the only lesion with a T41 mutation, in which a heavy overall β -catenin staining is accompanied by occasional nuclear-positive cells using the S33/37/T41 antibody (Figure S1). As the T41A alteration cannot be detected by this latter antibody, the nuclear staining most likely results from wild-type protein induced to enter the nucleus. Lastly, in both HBs with proven genomic exon 3 deletions virtually no tumor cell staining was detectable (Figure 3I,J and S1). A similar pattern was also identified in six additional samples with no identifiable point mutation, suggesting that in total 7 out of 18 HB samples and one HCC sample carry genomic exon 3 deletions. In two of these samples some residual membranous staining was visible (HB-23 and HB-27, Figure S1).

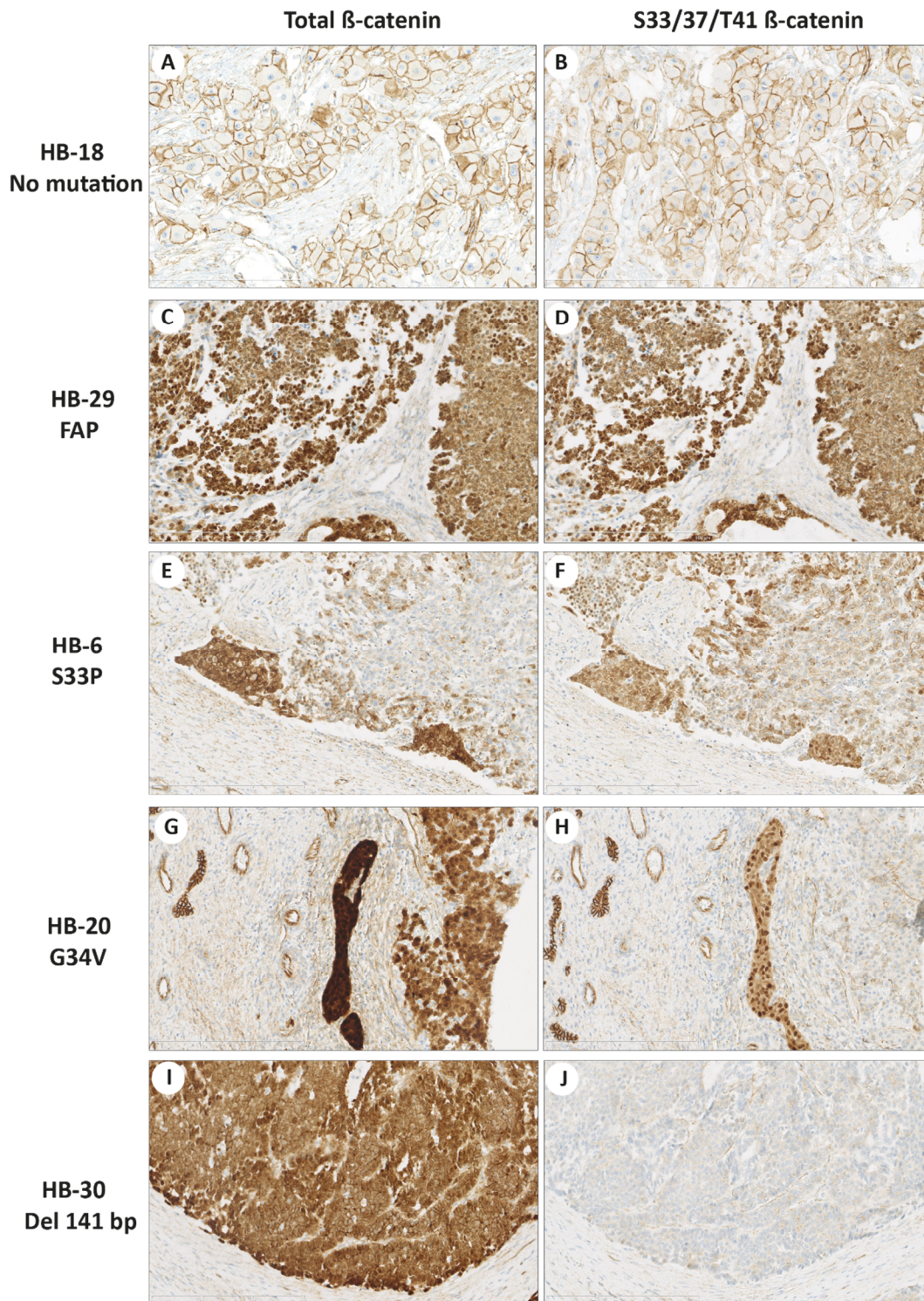


Figure 3. Immunohistochemical identification of β -catenin alterations in tumor samples.

FFPE sections of pediatric liver tumors were stained with the C-terminal (left column) and S33/37/T41 (right column) β -catenin antibodies. (A,B) Comparable membranous staining in the fibrolamellar hepatocellular carcinoma (HB-18) with no evidence of β -catenin mutation or activation. (C,D) HB-29 derived from a FAP patient shows identical nuclear and cytoplasmic accumulation of wild type β -catenin using both antibodies. (E,F) In sample HB-6 a p.S33P mutation was detected, which shows nuclear staining with both antibodies, albeit slightly weaker using the S33/37/T41 antibody. (G,H) Sample HB-20 carrying a p.G34V mutation shows clear nuclear β -catenin accumulation in tumor sections. Staining with the S33/37/T41 antibody is strongly reduced or entirely lost in tumor cells, while normal structures on the left are stained in an identical fashion. (I-L) The strong nuclear and cytoplasmic staining of sample HB-30 with genomic deletion of exon 3, is completely lost using the S33/37/T41 antibody. Original magnification 200x.

Confirmation of exon 3 deletion by RT-PCR

To confirm that these latter samples indeed express mutant *CTNNB1* mRNA (partially) lacking exon 3, we performed RT-PCR on RNA isolated from paraffin sections. Successful RNA isolation was possible in 6 samples. In 4 out of 6 samples a 127 bp product was observed indicative of a complete absence of exon 3 (Figure 4). In sample HB-30 a shortened 214 bp product was observed, which after sequencing was confirmed to carry the same intra-exonic 141 bp deletion already observed during the genomic DNA analysis. Sample HB-19 showed a shortened reproducible product of about 140 bp, indicating that an approximate 215 bp deletion is present in the cDNA. In conclusion, all 6 samples with negative S33/37/T41 β -catenin nuclear staining and absence of exon 3 point mutation, showed evidence of (partial) exon 3 deletion on RNA level.

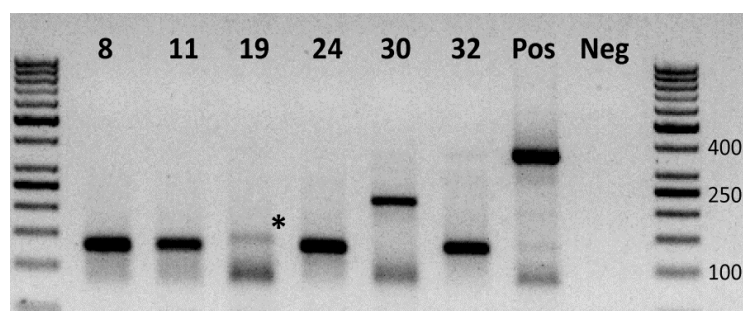


Figure 4. Confirmation of exon 3 deletion by RT-PCR.

RNA was isolated from 6 HBs with immunohistochemical evidence of (partial) exon 3 loss, and subjected to RT-PCR using primers in exon 2 and 4. In 4 out of 6 samples a 127 bp product was observed indicative of a complete absence of exon 3 (HB-8, HB-11, HB-24 and HB-32). Sample HB-19 showed a reproducible shortened product of approximately 140 bp (*). In sample HB-30 the intra-exonic 141 bp genomic deletion was confirmed on RNA level. Positive control is RNA isolated from normal tissue.

Discussion

Most HBs are believed to be of sporadic nature, but about 10-20% have been associated with genetic defects such as Beckwith-Wiedemann syndrome or familial adenomatous polyposis.^{2,5,6} For this reason, it has been advocated that all HB patients with unknown family history, should be referred for genetic counseling.⁶ Among the genetic syndromes associated with hepatoblastoma development, FAP is one of the most common with an estimated frequency between 5-10%.^{2,16} As this is a severe disease, it is important for the patient itself but also for yet asymptomatic family members to determine their carrier status at an early stage. Especially a firm exclusion of an underlying *APC* germline mutation will prevent the unexpected emergence of polyposis or colorectal cancer at a later stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, but inherent to any

diagnostic method this will occasionally lead to false-negative results. In case of classical polyposis phenotypes the *APC* mutation detection rate is around 80-90%,^{35,36} meaning that a possible 10-20% of FAP patients may be missed by routine genetic testing. This leads to the highly undesired scenario in which apparent *APC* mutation negative patients develop polyposis later. For this reason, Aretz et al. proposed for all *APC*-negative HB patients to undergo colonoscopy around age 15 and one at age 25, and to also offer colonoscopy to the parents.² The downside of this approach is that many genuine *APC*-negative individuals will be exposed to unneeded medical investigations, associated with anxiety and considerable costs. Here, we establish and validate a tissue-based β -catenin gene and immunohistochemical analysis that complements the germline mutation screening to exclude the diagnosis FAP among HB patients. Following successful confirmation in an independent series, its application will lead to a near-complete exclusion of FAP among HB patients.

Our method is based on the observation that tumor-driving *CTNNB1* and *APC* mutations occur in a mutually exclusive nature in all tumor types studied so far.¹² As such, the identification of a somatic activating *CTNNB1* mutation in a HB patient directly reduces the risk of carrying a germline *APC* mutation to that of the general population. By combining mutational analysis of *CTNNB1* exon3 and IHC comparing staining patterns for total and S33/37/T41 β -catenin, we could reliably identify all three FAP patients in our cohort of 18 HB cases. Their tumors are characterized by a comparable nuclear staining for both antibodies and wild-type exon 3 sequence. In fact, the IHC analysis by itself can already select a large proportion of HBs that carry an activating *CTNNB1* mutation, and thus have no increased risk of an underlying *APC* germline mutation. Our epitope and immunohistochemical analysis suggests that all amino acid alterations and genomic deletions encompassing the G34-T41 region would prevent or strongly reduce binding of the S33/37/T41 antibody. Reviewing the literature for the type of *CTNNB1* mutations reported in HBs, about 85% of mutations (198/234) fulfil this criterion.¹³⁻
²¹ The main exceptions are D32/S33 amino acid alterations, while S45 mutations are rarely observed in HB (3/234). Although the IHC analysis can already identify most HB patients with *CTNNB1* mutations, we nevertheless propose to also include the exon 3 mutation analysis on tumor DNA. This will identify point mutations outside the epitope of the S33/37/T41 antibody (D32, S33 and S45), and provides a confirmation of the *CTNNB1* mutational status.

As *CTNNB1* mutations are the predominant mutation in pediatric liver cancers (up to 80%), most patients are unlikely carriers of a germline *APC* mutation. A small subset of cancers arise without apparent β -catenin activation, i.e. the ones with exclusively membranous β -catenin staining and wild-type sequence. As it is highly unlikely that such a β -catenin nuclear-negative tumor arises in a FAP patient, these are also unlikely to carry a germline *APC* mutation. Anecdotally, somatic *APC* and *AXIN1/AXIN2* mutations have been reported in hepatoblastomas that also lead to tumors in which wild-type β -catenin accumulates in the nucleus.^{15,16,22} These rare tumors would lead to *APC* germline negative patients, whereas the tumor analysis indicates an underlying FAP syndrome. Below we discuss how to deal with this small subset of HB tumors.

How to implement our method in current clinical practice? As HBs can be a manifestation of several genetic syndromes, all patients should be offered genetic counseling and evaluated for syndromic features such as the macrosomia and organomegaly associated with BWS. In the absence of such features and a negative family history, a genetic mutation analysis is warranted. This should include at least the *APC* gene, but may also include other genetic cancer predisposition syndromes such as LFS and NF1.^{42,43} Simultaneous with or preceding the counseling process, we propose to combine a routine histopathological evaluation of the tumor tissue with both β -catenin stainings described here. In addition, tumor DNA should be isolated from the FFPE-material and evaluated for *CTNNB1* exon3 mutations, which can also be done as part of a larger next-gen sequencing panel.

In most cases the genetic and tumor tissue analyses will corroborate each other. Identification of a germline *APC* mutation will be accompanied by a comparable staining with both antibodies and wild-type *CTNNB1* sequence in the tumor tissue, while *APC* germline negative cases will show somatic activating *CTNNB1* mutations and reduced staining with the S33/37/T41 antibody. However, given the approximate 10-20% false-negative *APC* mutation analyses, occasionally tumors will be identified showing nuclear accumulation of wild-type β -catenin without an identifiable *APC* germline mutation. As these cases are at increased risk to develop FAP at a later stage, colonoscopies of the index patient and its parents can be performed, as suggested by Aretz et al.² Using our diagnostic method, this can however be restricted to the subset of *APC* germline negative patients that express wild-type β -catenin in the tumor. Alternatively, the somatic mutation analysis of these tumors can be extended to

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identify inactivating somatic *AXIN1/2* or *APC* mutations. When *AXIN1* or *AXIN2* mutations are identified this also excludes the diagnosis FAP. In the case of a “somatic” *APC* mutation more caution is needed as this may indicate a missed germline mutation that is present for example in a low-mosaic fashion in the patient.

Our approach can also be extended to other extracolonic FAP manifestations, such as pilomatricomas and desmoids. These lesions are also characterized by high frequencies of somatic β -catenin mutations, meaning that most are of sporadic nature.⁴⁴⁻⁵⁰ About one third of *CTNNB1* mutations reported for desmoids are at codon S45, which are missed by the IHC method described here, but if desired this analysis can be extended by including an antibody specifically recognizing this epitope (e.g. #19807, Cell Signaling technology).

In conclusion, we have successfully established and validated a novel β -catenin gene and immunohistochemical diagnostic method, which following confirmation in an independent series, allows the near-complete exclusion of the diagnosis FAP among HB patients, when combined with routine germline DNA testing.

Supplemental Methods

Detailed PCR and sequencing protocol for *CTNNB1* mutation analysis

PCR products from tumor DNA were generated in a 15µl reaction mixture including 1µl of each primer (10µM) and 7.5µl KAPA2G Robust Hot Start ReadyMix (KAPA-Biosystems, #KK5702). The PCR reaction was performed with a thermocycler (Biometra, Göttingen, Germany) with an initial denaturation step (95°C) for 3 min., followed by 35 cycles of denaturation (95°C) for 15 sec., annealing (60°C) for 15 sec. and extension (72°C) for 15 sec. PCR products were sequenced with M13-forward primer and M13-reverse primers using the BigDye® Terminator-v3.1 Cycle Sequencing kit (Applied Biosystems™, ThermoFisher Scientific, Waltham, MA, USA). Sequences were analyzed on an ABI-3730 DNA Analyzer (Applied Biosystems™). Data were analyzed with Mutation Surveyor® software (SoftGenetics) and compared with the public sequences of GenBank (NG_013302.1 or NM_001904.3)

Tumor RNA isolation and analysis

RNA was extracted by microdissection from FFPE tissue fragments using the RNeasy®-FFPE Kit (Qiagen, Cat No./ID: 73504) according to the manufacturer's instructions. cDNA was generated basically according to the manufacturer's instructions (Thermo Scientific, #K1612) using 20pmol of M13-tailed gene-specific reverse primers CTNNB1-Ex3del-R4.1 and CTNNB1-Ex3-4del-R4.2 (Supplemental Table 2). Next, to detect *CTNNB1* deletions at cDNA level, two independent PCRs were performed with the FAM-labeled CTNNB1-Ex2-F forward primer and M13R-CTNNB1-Ex3del-R4.1 and M13R-CTNNB1-Ex3-4del-R4.2 reverse primers. PCR conditions were as described above.

Cell lines

Cell lines SW1573, SW48, SW480, Huh6, SNU398, HCT116 and HEK-293 were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco) complemented with 10% fetal calf serum (FCS) (Hyclone, Lonan, Utah). A427 and LS174T were cultured in RPMI-1640 medium (Invitrogen-Gibco), respectively with 10% and 5% FCS. HepG2 was cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in Williams-E medium (Invitrogen-Gibco) complemented with 10% FCS. All media were supplemented with 100IU/ml penicillin and 100µg/ml streptomycin. Identity of all cell lines was confirmed by Short Tandem Repeat

genotyping. *CTNNB1* mutation status was confirmed in all cell lines by Sanger sequencing and was consistent with those reported at COSMIC (<http://cancer.sanger.ac.uk>).³⁸

Preparation of paraffin blocks from cell lines

For each cell line 2-5 15cm tissues culture dishes were grown to near-confluency. Next, the cells were scraped with a rubber policeman, collected and centrifuged. The resulting pellet was washed with PBS once, followed by the addition of 15ml of PBS-buffered 10% formalin. After at least 2 hrs fixation, cells were transferred to 2ml tubes and spun down for 3 minutes at 300G. The cell pellet was mixed with a 1% agarose solution in PBS, maintained at 50°C before use, by gently stirring with a pipette tip or flame-closed Pasteur pipette. Following a cool-down on ice, a small hole was burned in the bottom using a heated needle, after which the agarose plug was released by pressurized air. The agarose plug was placed in buffered formalin again and embedded in paraffin according to routine protocols. Fixation time for all cell lines was at least 6hrs and a maximum of 24hrs.

Supplementary Tables and Figures

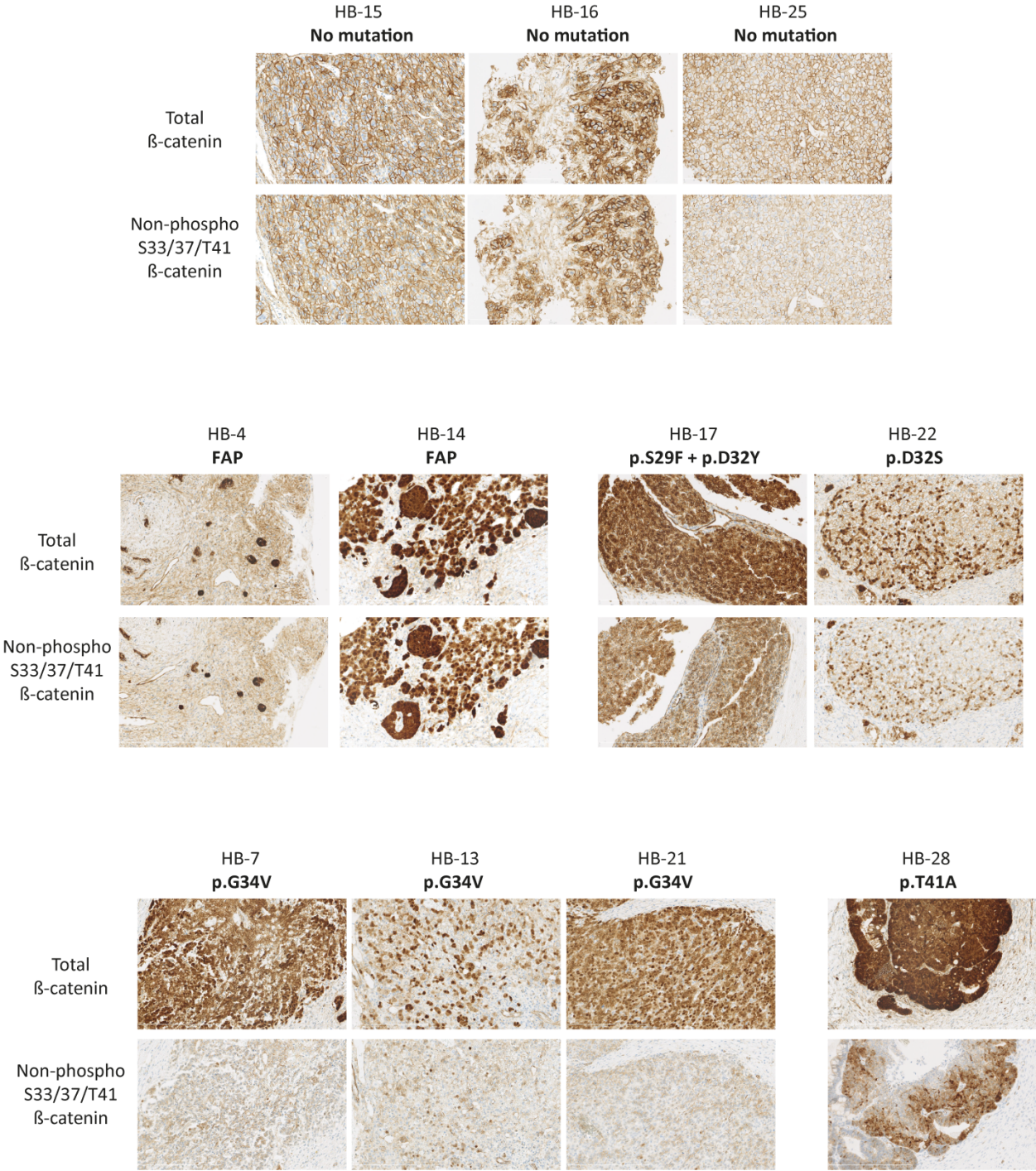
Supplemental Table 1 Primer sequences for amplifying genomic *CTNNB1*

Primer	Nucleotide sequence	Expected size (without M13)
CTNNB1-AMPL7153305149-Ex3-F	M13F-CTGATTTGATGGAGTTGGACATGG	160
CTNNB1-AMPL7153305149-Ex3-R	M13R-TCCACATCCTCTTCCTCAGGATT	
CTNNB1-Ex2F1	GAAAATCCAGCGTGGACAATG	980
CTNNB1-Ex4R2	TGCCCTCATCTAATGTCTCAG	
CTNNB1-Ex2F2	TGGACAATGGCTACTCAAG	945
CTNNB1-Ex4R1	AACATAGCAGCTCGTACCCTC	
M13F	TGTAAAACGACGGCCAGT	
M13R	CAGGAAACAGCTATGACC	

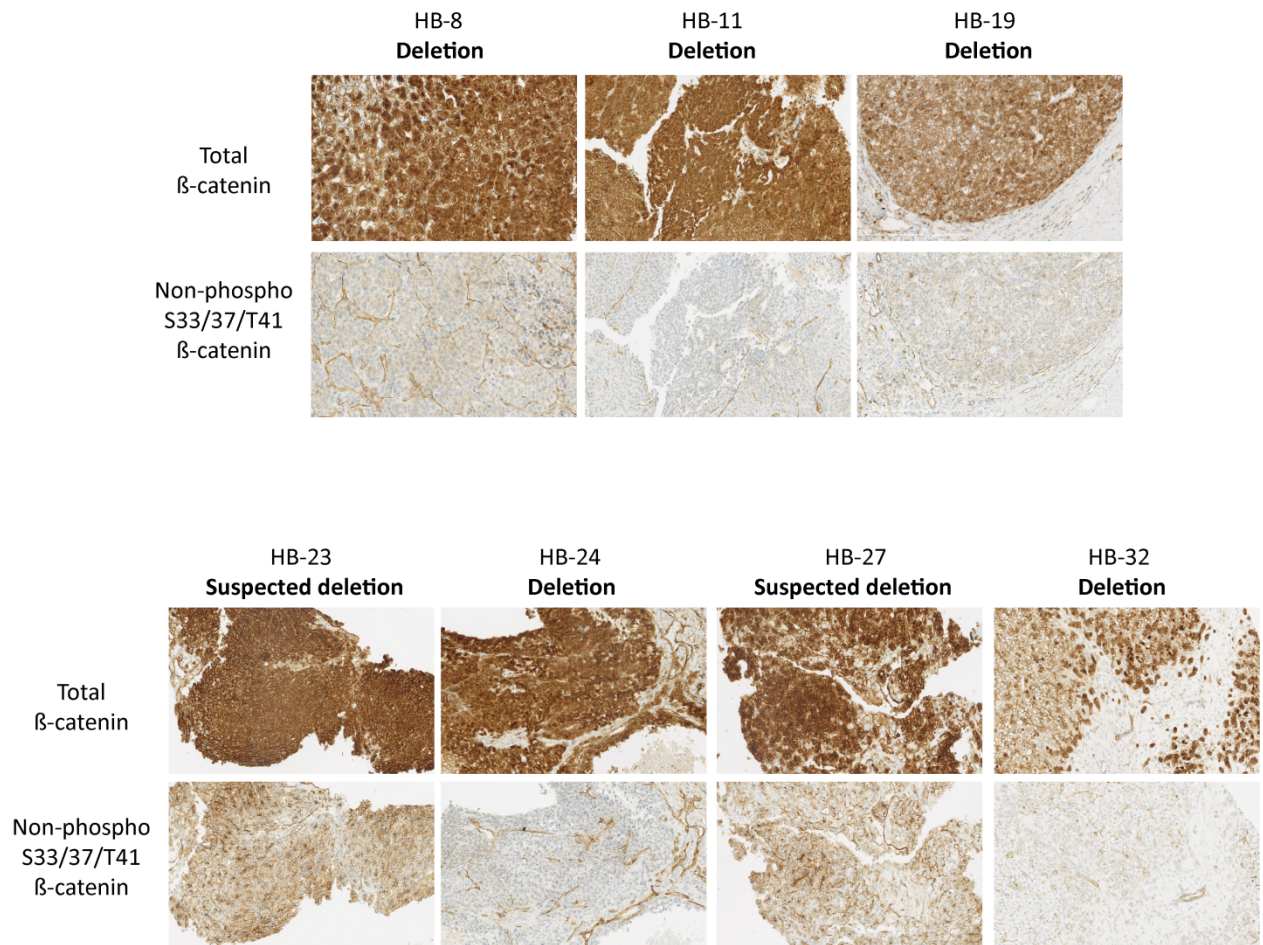
Supplemental Table 2 Primer sequences for analyzing *CTNNB1* cDNA

Primer	Nucleotide sequence	Expected wild type product size (bp)
CTNNB1-Ex2-F	GTTTTGAAAATCCAGCGTGGACAAT	
CTNNB1-Ex3del-R4.1	M13R-TCATCTAATGTCTCAGGGAACATAGCAGCTCGTACCCTC	355
CTNNB1-Ex3del-R4.2	M13R-AGTTTTGTCAGTTCAGGGATTGCA	517

Supplemental Figure 1, part 1



Supplemental Figure 1 continued



Supplemental Figure 1.

Immunohistochemical identification of β -catenin alterations in remaining tumor samples.

FFPE sections of remaining pediatric liver tumors were stained with the C-terminal (top rows) and S33/37/T41 (bottom rows) β -catenin antibodies. Identified mutation status is reported for each sample.

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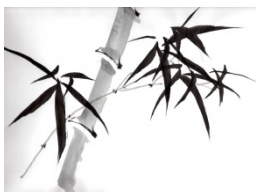
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Chapter 7

SUMMARY



Wnt/ β -catenin signaling is important for the proper development of all multicellular organisms and is a critical regulator for most types of epithelial stem cells. Therefore, it is not surprising that dysregulation of Wnt/ β -catenin signaling is observed in many cancers (1). Also, in hepatocellular carcinoma (HCC), frequently aberrant activation of this signaling pathway has been identified, for example by acquired activating β -catenin or inactivating *AXIN1* mutations. Besides direct mutational activation, β -catenin signaling can also be modulated through various other mechanisms. One example is provided by the viral proteins expressed during chronic HBV or HCV infection, both prominent risk factors for the development of HCC. In **Chapter 2**, we comprehensively reviewed the current biomedical knowledge on the role of the Wnt/ β -catenin signaling pathway in the progression from chronic hepatitis C infection to cirrhosis and HCC, and explored potential hypotheses as to the mechanisms involved. In this review, we first summarize the relative mutation frequencies of Wnt/ β -catenin signaling elements in HCC cohorts reported previously. Activating mutations in *CTNNB1* are the most prevalent in general and especially in HCV-related HCC (>25%). Next, we discuss the mutual interaction between HCV infection and Wnt/ β -catenin signaling. Both the HCV viral core and NS5A proteins provoke activation of Wnt/ β -catenin signaling. HCV infection is also associated with increased EGFR and FGF signaling, both contributing to the cirrhotic and tumorigenic process by enhancing the PI3K/AKT and Ras/Raf/MEK/ERK pathways, but also by further increasing β -catenin signaling. Thus, HCV infection exploits multiple mechanisms leading to enhanced β -catenin signaling. In turn, stimulated Wnt/ β -catenin signaling paves the way for progression of hepatitis C during inflammation and fibrosis to eventually cirrhosis and HCC.

Despite of the development of a large number of molecular inhibitors targeting Wnt/ β -catenin signaling during recent decades, little is known about the response to these inhibitors of HCC cells carrying different genetic mutations relevant to aberrantly activated Wnt/ β -catenin signaling. In **Chapter 3**, we tested the Wnt secretion inhibitor IWP12 in our HCC cell line panel. First, using siRNA mediated knock-down of β -catenin we showed that the majority of HCC cell lines were dependent on β -catenin signaling for optimal cell growth. Blocking Wnt secretion by treatment with the IWP12 porcupine inhibitor reduced growth of most HCC cell lines, which was confirmed by knock-down of WLS essential for Wnt secretion. However, none of the *CTNNB1* and *AXIN1* mutant HCC cell lines were affected at the β -catenin signaling level. This was shown both by a β -catenin reporter assay and RNA level of *AXIN2* (a well-known

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target gene of β -catenin signaling) upon alterations of Wnt ligand exposure, neither by blocking Wnt secretion using IWP12 nor by the addition of WNT3A ligand. This suggests that the expressed oncogenic β -catenin or mutant AXIN1 proteins determine overall signaling levels in a dominant fashion. Furthermore, IWP12 treatment did not induce autophagy or endoplasmic reticulum (ER) stress, which may have resulted from the accumulation of Wnt ligands within the ER. In accordance with our results, Covey et al. also have shown that knocking down porcupine in various tumor cell lines reduced their growth through a Wnt-independent pathway with no clear explanation revealed (2). Similarly, most CRC cell lines tested as a comparison in various assays, showed dependency on β -catenin signaling and inhibition of cell growth after Wnt secretion blockage, either by IWP12 or knockdown of WLS. In accordance with liver cancer cell lines, the growth inhibition was not associated with clear alterations in Wnt/ β -catenin signaling regardless of the underlying *APC* or *CTNNB1* mutations. Our results suggest that most colorectal and liver cancers with mutations in components of the β -catenin degradation complex do not strongly rely on extracellular Wnt ligand exposure to support optimal growth. Blocking Wnt secretion may however aid in tumor suppression through alternative routes currently unappreciated.

In **chapter 4**, we investigated a second class of molecular inhibitors of β -catenin signaling, that is the Tankyrase inhibitors, and we determined in more detail to which extent AXIN1 and AXIN2 are involved in the regulation of β -catenin signaling in HCC. Together with APC and the kinases GSK3 and CK1 α , these proteins form the core of the β -catenin break-down complex. Loss-of-function mutations in AXIN1 and AXIN2 are observed in 10.4% and 3.3% of HCC patients respectively (3). Mutated AXIN1 or AXIN2 are expected to lead to enhanced Wnt/ β -catenin signaling, although this has been debated (4-6). The AXIN proteins themselves are also under proteolytic control and are rapidly degraded following poly-ADP-ribosylation by Tankyrase enzymes. Hence, Tankyrases have emerged as therapeutic targets as their inhibition theoretically leads to AXIN accumulation, ultimately expected to result in reduced β -catenin signaling and tumor cell growth. In this chapter, we first investigated in more detail to what extent AXIN1 and AXIN2 are involved in regulating β -catenin signaling in our panel of HCC cell lines. Western blot analysis showed that both AXINs were expressed at low levels in the non-mutant and *AXIN1* mutant cell lines, while they were readily detectable in the *CTNNB1* mutant ones. Tankyrase inhibition for one day resulted in a clear accumulation of AXIN2

exclusively in the *CTNNB1* mutant lines. Surprisingly, no clear accumulation of AXIN1 was observed in any of the lines. Mechanistically, this may be explained by the RNA expression levels that we observed for both genes, as *AXIN1* was expressed at considerably lower levels than *AXIN2* in all HCC lines. Recently, Thorvaldsen et al. showed that sustained protein translation is required for AXIN accumulation (6). Indirectly this also implies that higher *AXIN2* RNA expression levels will lead to a more robust protein accumulation, which is in line with our results. Next, we investigated to what extent both AXIN proteins are involved in regulating β -catenin signaling in HCC. We knocked-down their expression using siRNA, with APC knock-down as a positive control. Simultaneous knock-down of both AXINs in the non-mutant lines indicates that they work in a complementary fashion to regulate β -catenin signaling. As expected, in the *AXIN1* mutant lines *AXIN1* knock-down had no clear effect, while *AXIN2* knock-down resulted in a similar activation of β -catenin signaling as APC knock-down. These results show that both AXIN proteins are involved in β -catenin regulation in HCC, arguing against some reports that suggest that *AXIN1* mutations do not contribute to HCC tumor growth through enhancing β -catenin signaling (4, 5). Lastly, we tested whether Tankyrases represent an attractive target to inhibit the growth of HCCs. β -catenin signaling was barely affected following Tankyrase inhibition in the *CTNNB1* mutant cell lines, while it was clearly diminished in all *AXIN1* and most non-mutant lines. Despite this inhibition in β -catenin signaling the growth and colony formation capabilities of these cell lines were at most slightly inhibited by Tankyrase inhibition. These results suggest that Tankyrase inhibitors are not likely going to efficiently affect the growth of HCCs.

In **chapter 5**, we uncovered the importance of oncogenic serine-threonine kinase receptor associated protein (STRAP) to sustain HCC cell growth through enhancing Wnt/ β -catenin signaling. STRAP harbors seven WD40-repeat domains and is considered to be a scaffolding protein without enzymatic function that exerts regulatory functions on a variety of cellular processes. Using immunohistochemistry we showed that STRAP was up-regulated in HCC, as previously reported in breast (7), lung (8) and colorectal cancers (8, 9). To study the function of STRAP in more detail, we applied siRNA mediated knock-down on our panel of HCC cell lines and generated knock-out clones using CRISPR/Cas9 gene editing. Loss of STRAP resulted in a strongly impaired growth, altered cell cycle progression and reduced capacity to form colonies from single cells. Furthermore, our findings indirectly (β -catenin target gene

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expression) and directly (β -catenin signaling activity using reporter assays) provide evidence that the abundance of STRAP stimulates Wnt/ β -catenin signaling activity, thereby supporting optimal HCC cell growth. Mechanistically, others have shown that STRAP binds to the catalytic site of GSK3 β via its WD40 domain region, eliciting the compromised ability of GSK3 β to phosphorylate the (N-terminal) serine/threonine residues encoded by exon3 of β -catenin (10, 11). Accordingly, we observed reduced levels of active β -catenin in STRAP knock-out clones of Huh6 and PLC/PRF/5. In these lines we also observed reduced levels of C-terminal phosphorylated β -catenin at S675, which is expected to result in more ubiquitination of β -catenin and less recruitment of transcriptional co-activators (12-14). Nevertheless, different from *CTNNB1* mutant Huh6 and *AXIN1* mutant PLC/PRF/5 cell lines, Huh7 apparently does not share any of the above rationales, despite showing reduced β -catenin signaling following STRAP loss. This indicates that STRAP may modulate β -catenin signaling through alternative routes that remain to be uncovered. RNA sequencing analysis of STRAP knock-out clones and controls thereof, also suggests that STRAP could facilitate stem cell self-renewal while simultaneously attenuating cell differentiation. The same analysis also revealed that besides Wnt/ β -catenin signaling, many other signaling pathways and metabolic processes were affected following STRAP loss, which is in line with reports demonstrating STRAP's role in regulating other signaling pathways (15-17). Taken together, our results show that STRAP expression is increased in hepatocellular cancers, and provides growth advantage to the tumor cells, among others by enhancing Wnt/ β -catenin signaling.

In **chapter 6**, we extended our study to hepatoblastoma (HB), a potentially malignant pediatric liver cancer. The Wnt/ β -catenin pathway plays a central role in the pathogenesis of these tumors, i.e. up to 60-80% carry activating *CTNNB1* mutations. Hepatoblastomas can however also be the first manifestation of familial adenomatous polyposis (FAP), which is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. FAP patients carry germline *APC* gene mutations leading to enhanced β -catenin signaling following somatic mutation of the remaining wild-type *APC* allele. As this is a severe disease, it is important for the patient and related family members to firmly exclude FAP at an early stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, which is associated with 10-20% false-negative results. In this chapter, we establish and validate a tissue-based β -catenin gene and

immunohistochemical analysis, which complements germline mutation screening to exclude the diagnosis FAP among HB patients. Important for our study, tumor-driving *CTNNB1* and *APC* mutations occur in a mutually exclusive fashion in all tumor types. Hence, the identification of a somatic activating *CTNNB1* exon3 mutation in a HB patient directly reduces the risk of carrying a germline *APC* mutation to that of the general population. *CTNNB1* mutation analysis in HBs is however complicated by a high proportion (>60%) of genomic deletions, varying in size from small intra-exonic deletions to larger ones extending up to part of exon 2 or 4, which can easily be missed using routine mutation analysis. Here we show that by combining *CTNNB1* exon3 mutational analysis and immunohistochemistry comparing staining patterns for total and exon3 specific β -catenin antibodies, we can faithfully diagnose the great majority of hepatoblastomas. Among 18 lesions, all three FAP tumors were characterized by a wild-type exon3 sequence and a comparable nuclear staining for both antibodies. In contrast, the non-FAP tumors carried missense *CTNNB1* mutations combined with a clearly reduced staining for the exon3 antibody, or complete loss of staining in case of lesions with exon3 deletions. When this method is combined with routine germline DNA testing, it allows the near-complete exclusion of the diagnosis FAP among HB patients.

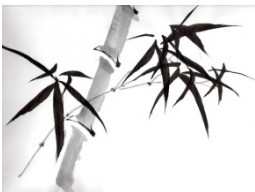
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Chapter 8

DISCUSSION



HCC is a heterogeneous cancer type in which many etiological factors, geographic differences and long-time pathological processes contribute to the development of a malignant tumor (1, 2). As one of the most important cascades facilitating HCC initiation and progression, aberrantly activated Wnt/ β -catenin signaling has become a focus for pharmacological intervention in recent decades. Although various drugs targeting this signaling pathway have been developed and studied in cancers, evidence about the efficiency of these drugs in different HCC subgroups is still lacking. In my thesis, using liver cancer cell lines, I aimed to identify subgroups of HCCs that may benefit from inhibitors that target β -catenin signaling at different cellular levels.

1. Contribution of mutant β -catenin and AXIN1 to aberrant activation of Wnt/ β -catenin-signaling pathway in HCC progression

As reported in Chapter 2 and by others, aberrant stimulation of Wnt/ β -catenin signaling in HCC patients is mainly due to mutations at the *CTNNB1* (20%-25%) or *AXIN1* (10%) genes (3-5). The oncogenic *CTNNB1* mutations consist of single mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation. For these activating mutations it is well-accepted that they support tumor growth by enhancing β -catenin signaling in a dominant fashion. However, whether *AXIN1* mutations also lead to HCC by enhancing β -catenin signaling is still under debate. In *AXIN1* mutant HCCs neither a clear nuclear β -catenin accumulation is observed nor a clear induction of β -catenin target genes (4). Similar observations have been made in mice with a liver-specific deletion of *AXIN1* in which also no clear nuclear β -catenin is detected (6). A potential explanation for this apparent lack of β -catenin signaling activation could be functional compensation by its homolog *AXIN2*. In addition, it becomes more complex by multi-function roles of *AXIN1* regulating the activity of c-Myc (7), p53 (8, 9), Smad3 (10) and interacting with other components of centrosome and mitotic spindles (11-14). However, based on our results we believe that *AXIN1* loss leads to enhanced β -catenin signaling relevant for tumor growth, albeit in a less dominant way. Firstly, we observe that all three *AXIN1* mutant lines we investigated show higher baselines of β -catenin signaling activity by a β -catenin reporter assay, indicating β -catenin is actively signaling to the nucleus. The signaling activity is however not as high as that in *CTNNB1* mutant lines. Secondly, all these *AXIN1* mutant lines are impaired in their growth following siRNA mediated knockdown of β -catenin, again suggesting that they

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require some β -catenin signaling to sustain growth. Additionally, another three lines without mutations relevant to β -catenin signaling, show induced β -catenin reporter activity resulting from siRNA mediated knockdown of AXIN1.

Hence, although β -catenin is a more powerful driver determining the β -catenin signaling activity, the mutated AXIN1 also contributes to the aberrant activation of β -catenin signaling, which is important for tumor initiation and progression in particular HCC subgroups.

2. Alternative treatment for HCC by targeting Wnt/ β -catenin-signaling pathway

Due to difficulties for early detection, most HCC patients are diagnosed at advanced stage. For these advanced HCC patients, the current treatment strategy is chemotherapy limited to the multi-kinase inhibitor sorafenib. However, treatment with sorafenib promises a minimal survival benefit of at most a few months in the majority of HCC patients. Hence, alternative treatment options are urgently needed and as explained above targeting the β -catenin signaling pathway may represent an attractive option.

In support of this we observe in our cell line panel a growth reduction of all 9 lines when β -catenin levels are reduced using siRNA mediated knock-down. Other studies have provided similar results in a limited number of cell lines (4, 15), and by reducing β -catenin levels in mouse hepatocarcinogenesis models (16, 17). Which method to choose for reducing β -catenin signaling in the clinical setting is however more challenging. Here we first investigated the Wnt-secretion inhibitor IWP12. This inhibitor was demonstrated to interfere with Wnt secretion and impair the growth of APC and β -catenin mutant CRC cell lines (18). Similarly, our study shows that IWP12 reduced growth of most HCC cell lines, which was confirmed by knock-down of WLS, a second protein also essential for Wnt secretion. Nevertheless, the growth reduction did not result from blocking β -catenin signaling and is also not due to alterations in autophagy or ER stress responses. A potential alternative explanation not investigated by us, could lie in other functions of Wnt ligands not linked to β -catenin signaling. This leads us to the “non-canonical” Wnt signaling pathway, which supports a growth suppressive effect (19, 20). Therefore, interfering with the secretion of this subset of Wnt ligands is expected to support cellular growth, in contrast to the growth suppression that we observed. Another explanation is the mTOR pathway important for cell growth by activating ribosome biogenesis and protein synthesis, which is validated to be stimulated by Wnt signaling depending on GSK3 instead of β -catenin (21). This suggests that mTOR signaling

could be the mechanism underlying the growth reduction following IWP12 treatment, which needs further investigation.

As a second method to reduce β -catenin signaling, we used tankyrase inhibition. β -catenin signaling was barely affected following tankyrase inhibition in the *CTNNB1* mutant cell lines, while it was clearly diminished in all *AXIN1* and most non-mutant lines. This indicates that in the latter cell lines, tankyrase inhibitor works through regulation of AXIN stability while the *CTNNB1* mutant lines do not respond, most likely because of the mutant β -catenin determining β -catenin signaling activity in a dominant fashion. However, no clear effect on cell growth was observed, even in the lines where some reduction in signaling was obtained.

Based on our data, reduction in β -catenin signaling obtained with either method is apparently insufficient to significantly affect the growth of HCC cell lines. This highlights the demand of other inhibitors or combinations thereof to achieve optimal effects in the clinic.

3. Other potential avenues to target Wnt/ β -catenin signaling

Conventional inhibitors targeting Wnt/ β -catenin signaling are conservatively designed to block the function or interaction of components specifically involved in this pathway. In Chapter 5, we identify STRAP as a new player in enhancing Wnt/ β -catenin signaling in HCC. STRAP has been defined as a scaffold protein participating in a variety of signaling pathways in addition to β -catenin. Thus, theoretically blocking the function of STRAP could impair tumor growth through multiple avenues. Whether the STRAP protein is druggable remains however to be seen, although it will most likely be challenging as there is no enzymatic function that can be easily blocked.

As an active pathway during the embryonic process and carcinogenesis, Wnt/ β -catenin signaling crosstalks with a number of other signaling pathways. In Chapter 2, we summarized that both EGF and FGF pathways are triggered by Wnt/ β -catenin signaling, which in turn stimulate β -catenin signaling through inhibiting GSK3 β activity in HCC development (3). For various other pathways including NOTCH (22), NF- κ B (23), TGF β (24) and so on, it has also been demonstrated that they interact with Wnt/ β -catenin signaling.

The function of STRAP in regulating Wnt/ β -catenin signaling as well as the mutual crosstalk between Wnt/ β -catenin signaling and other pathways imply a supplementary therapeutic approach that synergizes with conventional anti-cancer drugs, to hopefully benefit the therapeutic outcomes of HCC patients.

4. Future directions

Although Wnt/ β -catenin signaling has been widely investigated and targeted to cure tumors, a systematic study about the efficiency of the prevailing inhibitors for HCC treatment is still lacking. In this thesis, we started *in vitro* experiments (cell lines) with two inhibitors. In the future, this can also be extended to other reported inhibitors of the pathway, possibly in combination with drugs targeting other cellular targets. Based on the data obtained from these *in vitro* studies, *in vivo* HCC models will also be necessary to evaluate the effect of these inhibitors in more physiological conditions. Since the ultimate goal of this study is to ameliorate clinical practice for HCC patients, organoids directly derived from tumors will be an ideal model for large-scale drug screening to identify the sensitive subgroups to specific inhibitors (25).

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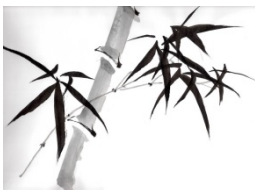
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Chapter 9

Nederlandse samenvatting

Dutch summary



Samenvatting

Wnt/ β -catenine signalering is belangrijk voor de ontwikkeling van alle meercellige organismen en is een essentiële regulator van de meeste epitheliale stamcellen. Daarom is het niet verrassend dat in vele kankers een foutieve regulatie van Wnt/ β -catenine signalering gezien wordt (1). Ook in leverkanker wordt veelvuldig een abnormale activatie van deze signaalroute gezien, bijvoorbeeld door het verkrijgen van een activerende β -catenine of inactiverende AXIN1 mutatie. Behalve door middel van mutaties, kan β -catenine signalering ook door diverse andere mechanismen beïnvloed worden. Een voorbeeld zijn de virale eiwitten die tot expressie komen tijdens een chronische infectie met HBV of HCV, beide belangrijke risicofactoren voor het ontstaan van leverkanker. In **hoofdstuk 2** beschrijven we uitgebreid de huidige biomedische kennis over de rol van Wnt/ β -catenine signalering in de progressie van chronische hepatitis C infectie naar levercirrose en leverkanker, en bespreken we in meer detail enkele van de mogelijke onderliggende mechanismen. Allereerst benoemen we de relatieve mutatie-frequenties van aan β -catenine signalering gerelateerde genen zoals die in leverkankercohorten gezien worden. Activerende mutaties in *CTNNB1* (coderend voor β -catenine) zijn de meest voorkomende mutaties resulterend in verhoogd β -catenine signalering en dit is vooral het geval in HCV-gerelateerde levertumoren (>25%). Vervolgens bespreken we de interactie tussen HCV infectie en Wnt/ β -catenine signalering. Zowel het virale HCV core als het NS5A eiwit dragen bij aan de activatie van β -catenine signalering. HCV infectie is ook geassocieerd met een verhoogde EGFR en FGF signalering. Beide dragen bij aan de vorming van cirrose en tumorvorming door middel van het activeren van de PI3K/AKT en Ras/Raf/MEK/ERK signaalroutes, maar leiden ook tot een toename in β -catenine signalering. Samenvattend, leidt HCV infectie dus via meerdere mechanismen tot een verhoogde β -catenine signalering. Op zijn beurt draagt de geactiveerde β -catenine signalering bij aan de progressie van de initiële hepatitis C infectie naar uiteindelijk cirrose en leverkanker.

Ondanks de ontwikkeling van een groot aantal moleculaire remmers van β -catenine signalering in de afgelopen decennia, is er nog weinig bekend over de effectiviteit van deze remmers op leverkankercellen met specifieke genetische mutaties leidend tot een verhoogde β -catenine signalering. In **hoofdstuk 3** testen we de Wnt secretie remmer IWP12 in ons panel van leverkankercellijnen. Allereerst laten we zien dat door middel van siRNA gemedieerde knockdown van β -catenine, het overgrote merendeel van de leverkankercellijnen afhankelijk

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is van β -catenine signalering voor een optimale groei. Het blokkeren van Wnt secretie door middel van een behandeling met de porcupine remmer IWP12, vermindert de groei van de meeste leverkankercellijnen. Dit effect wordt bevestigd met een knockdown van WLS, wat essentieel is voor uitscheiding van Wnt liganden. Geen enkele van de *CTNNB1* en *AXIN1* mutante cellijnen wordt echter beïnvloedt op het niveau van β -catenine signalering. Dit laten we zien met een β -catenine reporter analyse als ook onveranderde *AXIN2* RNA expressie niveaus (een betrouwbaar door β -catenine gereguleerd gen) volgend op veranderingen in Wnt ligand blootstelling, noch door de Wnt secretie te blokkeren met IWP12 noch met de toevoeging van WNT3A ligand. Dit suggereert dat de geëxprimeerde oncogene β -catenine en mutante *AXIN1* eiwitten het signaalniveau op een dominante manier beïnvloeden. Daarnaast leidt IWP12 behandeling ook niet tot de inductie van autofagie of endoplasmatisch reticulum (ER) stress die mogelijk zou kunnen ontstaan als gevolg van de opstapeling van Wnt ligand in het ER. In overeenstemming met ons resultaat hebben anderen laten zien dat knockdown van porcupine de groei van verschillende cellijnen verminderd op een Wnt-onafhankelijke manier, zonder dat een duidelijke verklaring werd gevonden (2). De meeste darmkankercellijnen die we voor diverse analyses ter vergelijking hebben gebruikt, laten een vergelijkbaar resultaat zien. Ze zijn afhankelijk van β -catenine signalering en laten een remming in groei zien naar het blokkeren van Wnt secretie, zowel m.b.v. IWP12 als WLS knockdown. In overeenstemming met de leverkankercellijnen is die groeiremming niet geassocieerd met een duidelijke verandering in β -catenine signalering en onafhankelijk van de onderliggende *APC* of *CTNNB1* mutatie. Onze resultaten suggereren dus dat de meeste darm- en leverkankercellijnen met mutaties in de β -catenine signaleringsroute, voor een optimale groei niet sterk afhankelijk zijn van extracellulaire blootstelling van Wnt liganden. Het blokkeren van Wnt secretie lijkt echter wel bij te dragen aan de remming van tumorgroei via nu nog onbekende mechanismen.

In **hoofdstuk 4** bestuderen we een tweede categorie van moleculaire remmers van β -catenine signalering, namelijk de Tankyrase-remmers, en bestuderen we in meer detail de bijdrage van *AXIN1* en *AXIN2* aan β -catenine signalering in leverkanker. Beide eiwitten vormen samen met *APC* en de kinases *GSK3* en *CK1 α* , de belangrijkste onderdelen van het β -catenine afbraakcomplex. Mutaties in *AXIN1* en *AXIN2* die tot verlies van functie leiden, worden gezien in respectievelijk 10.4% en 3.3% van de levertumoren (3)]. Van deze mutaties wordt verwacht dat die leiden tot een verhoogde β -catenine signalering, hoewel dit door enkele publicaties

wordt betwist (4-6). De AXIN eiwitten staan zelf ook onder proteolytische controle en worden snel afgebroken na poly-ADP-ribosylatie door Tankyrase enzymen. Om die reden zijn Tankyrase-remmers in het vizier gekomen als mogelijke therapie omdat hun remming in theorie leidt tot stapeling van AXIN, uiteindelijk resulterend in verlaagde β -catenine signalering en tumorgroei. In dit hoofdstuk bestuderen we eerst de expressie-niveaus van AXIN1 en AXIN2 in ons panel van leverkankerlijnen. Western blot analyse laat zien dat beide AXIN eiwitten laag tot expressie komen in de niet-gemuteerde en *AXIN1*-gemuteerde cellijnen, terwijl ze gemakkelijk aan te tonen zijn in de *CTNNB1* mutante lijnen. Remming van Tankyrase leidde alleen in de *CTNNB1* mutante lijnen tot een duidelijk opstapeling van AXIN2. Tot onze verrassing was er in geen enkele cellijn een duidelijke stapeling van AXIN1 eiwit aantoonbaar. Een mogelijke verklaring is te vinden in de RNA expressie niveaus van beide genen, omdat *AXIN1* duidelijk lager tot expressie kwam dan *AXIN2* in alle cellijnen. Onlangs hebben Thorvaldsen *et al* laten zien dat er een continue aanmaak van nieuw eiwit nodig is voor AXIN stapeling (6). Indirect betekent dit dat een hogere *AXIN2* RNA expressie zal leiden tot een meer robuuste eiwitstapeling, wat in overeenstemming is met onze resultaten. Vervolgens hebben we onderzocht in welke mate beide AXIN eiwitten betrokken zijn bij de regulatie van β -catenine signalering in leverkanker. Hun RNA expressie werd sterk verlaagd m.b.v. siRNA, waarbij we *APC* knockdown als positieve controle gebruikten. Gelijktijdige knockdown van beide AXIN genen in de niet-gemuteerde cellijnen liet zien dat beide complementair werken in de regulatie van β -catenine signalering. In de *AXIN1* mutante lijnen had *AXIN1* knockdown zoals verwacht geen effect, terwijl *AXIN2* knockdown leidde tot een vergelijkbare activatie van β -catenine signalering als *APC* knockdown. Deze resultaten tonen aan dat beide AXIN eiwitten betrokken zijn bij β -catenine regulatie in leverkanker, wat in tegenspraak lijkt met publicaties die suggereren dat *AXIN1* mutaties niet bijdragen aan de groei van leverkanker door het activeren van β -catenine signalering (5). Als laatste testen we in dit hoofdstuk of de remming van Tankyrases een goede benadering is voor de remming van leverkankergroei. Het niveau van β -catenine signalering wordt nauwelijks beïnvloed door Tankyrase remming in de β -catenine mutante lijnen, terwijl dit duidelijk het geval is in de overige lijnen. Ondanks deze remming resulteerde Tankyrase in geen of slechts een minimaal effect op de groei en kolonievormings-capaciteit van de behandelde leverkankercellijnen. Deze resultaten suggereren dat Tankyrase-remmers waarschijnlijk niet een effectieve behandeling van leverkanker zullen vormen.

In **hoofdstuk 5** ontdekken we het belang van het oncogene serine-threonine kinase receptor associated protein (STRAP) voor het ondersteunen van de groei van leverkanker d.m.v. het versterken van β -catenine signalering. STRAP bevat zeven WD40 domeinen en wordt beschouwd als een structureel eiwit zonder enzymatische functie die diverse regulatoire rollen vervult op een groot aantal cellulaire processen. Gebruikmakend van immunohistochemie tonen we een verhoogde STRAP expressie aan in leverkanker, zoals eerder ook al aangetoond is voor borst-, long- en darmkanker (7-9). Om de functie van STRAP in meer detail te onderzoeken, hebben we siRNA gemedieerde knockdown toegepast op ons panel van leverkankercellijnen en knock-out clones gegenereerd m.b.v. CRISPR/Cas9 genmodificatie. Verlies van het STRAP eiwit resulteerde in een sterk verminderde groei, een veranderd celcyclusprofiel en een verminderde capaciteit tot het vormen van kolonies vanuit een enkele cel. Daarnaast tonen we aan dat STRAP de β -catenine signalering en daarmee de groei van leverkankercellen stimuleert, zowel direct gemeten (verhoogde expressie van door β -catenine gereguleerde genen) als indirect (β -catenine signalering gemeten met een reporter-analyse). Anderen hebben laten zien dat STRAP aan het katalytische domein van GSK3 β bindt via zijn WD40 domein en daarmee de capaciteit van GSK3 β verminderd om β -catenine te fosforyleren op de door exon3 gecodeerde N-terminale serine/threonine residuen(10, 11). In overeenstemming daarmee zien we verlaagde niveaus van actief β -catenine in de STRAP knock-out clones van de Huh6 en PLC/PRF/5 cellijnen. Bovendien zien we in deze lijnen een vermindering van C-terminale β -catenine fosforylatie op S675, wat naar verwachting leidt tot een verminderde rekrutering van transcriptionele co-activatoren (12-14). Afwijkend van de β -catenine mutante Huh6 en AXIN1 mutante PLC/PRF/5 cellijn lijkt in Huh7 geen van deze mechanismen operationeel ondanks een verminderde β -catenine signalering na verlies van STRAP expressie. Dit suggereert dat STRAP β -catenine signalering ook via nu nog onbekende mechanismen kan beïnvloeden. Een RNA sequentie analyse van STRAP knock-out clones en controles suggereert dat STRAP de vernieuwing van stamcellen kan vergemakkelijken en tegelijkertijd de differentiatie van cellen kan afremmen. Dezelfde analyse laat zien dat behalve β -catenine signalering, diverse andere signaalroutes en metabole processen verstoord zijn na het verlies van STRAP expressie, wat in overeenstemming is met andere publicaties (15-17). Samenvattend laten onze resultaten zien dat STRAP verhoogd tot expressie komt in leverkanker en een groeivoordeel geeft aan de tumorcellen, onder andere door het versterken van het β -catenine signaal.

In **hoofdstuk 6** bestuderen we hepatoblastomas, een potentieel kwaadaardige vorm van leverkanker die vooral bij kinderen voorkomt. De Wnt/ β -catenine signaalroute speelt een belangrijke rol in het ontstaan van deze tumoren, d.w.z. tussen de 60-80% heeft een activerende *CTNNB1* mutatie. Hepatoblastomas kunnen echter ook de eerste manifestatie zijn van familiale adenomateuze polyposis (FAP), een erfelijke aanleg leidend tot duizenden dikke darmpoliepen en uiteindelijk darmkanker. FAP patiënten dragen een kiembaan *APC* genmutatie die resulteert in verhoogde β -catenine signalering zodra er een somatische mutatie ontstaat in het overblijvende wild-type *APC* allel. Dit is een ernstige ziekte waarbij het van groot belang is voor de hepatoblastoma patiënt en gerelateerde familieleden, om FAP in een vroeg stadium uit te sluiten. De huidige diagnose is voor een belangrijk deel gebaseerd op kiembaan *APC* mutatie analyse op genomisch DNA, die echter in 10-20% van de gevallen tot een fout-negatieve uitslag leidt. In dit hoofdstuk ontwikkelen we en valideren een weefsel-gebaseerde β -catenine gen en immunohistochemische analyse, die de kiembaanmutatie analyse aanvult om de diagnose FAP bij hepatoblastoma patiënten grondig uit te sluiten. Van belang voor onze studie is de bevinding dat *CTNNB1* en *APC* mutaties die leiden tot tumorvorming, nooit in dezelfde tumor gezien worden. De vondst van een somatische activerende exon3 β -catenine mutatie in een hepatoblastoma patiënt betekent dan ook meteen dat het risico op FAP sterk verlaagd wordt en gelijk is aan die van de gehele bevolking. *CTNNB1* mutatie analyse in hepatoblastomas wordt echter bemoeilijkt door het grote aandeel (>60%) van genomische deleties, die in grootte kunnen variëren van kleine intra-exonische deleties tot grotere, die een deel van exon 2 tot 4 kunnen beslaan. Deze deleties kunnen gemakkelijk gemist worden met een routinematige mutatie analyse. In dit hoofdstuk laten we zien dat door het combineren van exon3 *CTNNB1* mutatie analyse en een immunohistochemische vergelijking van totaal en exon3-specifieke β -catenine antilichamen, we een betrouwbare diagnose kunnen maken voor het overgrote merendeel van de hepatoblastomas. Van de 18 onderzochte monsters, lieten alle drie de FAP-tumoren een wild-type exon3 sequentie zien en een vergelijkbaar kleurpatroon met beide antilichamen. De overige niet-FAP tumoren werden echter gekenmerkt door de identificatie van een missense mutatie en een sterk verminderde kleuring met het exon3 specifieke antilichaam, of een volledig verlies van kleuring in het geval van exon3 deleties. Als deze methode gecombineerd wordt met de routinematige kiembaan *APC* mutatie analyse, dan kan deze leiden tot een nagenoeg volledige uitsluiting van een FAP-diagnose onder hepatoblastoma patiënten.

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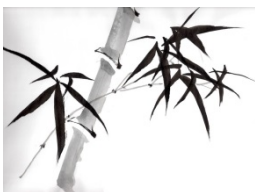
Appendix

Acknowledgements

Publications

PhD Portfolio

Curriculum Vitae



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PhD Portfolio

Name PhD Student	Wenhui Wang
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2013 - June 2018
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Copromotor	Dr. Ron Smits

General Courses

- 2013 Biomedical Research Techniques XIII
- 2013 International course on laboratory animal science (Art. 9)
- 2014 Photoshop and Illustrator CS6 workshop
- 2015 Workshop Writing Successful Grant Proposals
- 2016 Course on biomedical English writing course for MSc and PhD-students
- 2016 Basic and translational oncology
- 2016 Next generation sequencing training: CLC Workbench / Ingenuity Variant Analysis Workshop

National and International Conferences

- 2015, Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands. (Poster presentation)
- 2016, 9th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, The Netherlands (oral presentation)
- 2016 The 24th Biennial Congress of the European Association for Cancer Research, Manchester, United Kingdom (poster presentation)
- 2017, EASL HCC SUMMIT, Geneva, Switzerland (ePoster presentation)

Academic Awards

- 2013, China Scholarship Council (CSC) Scholarship (File No. 201306300027)
- 2016, Erasmus Trustfonds travel grant (Reference: III A 97095.78/16.0366/evt)
- 2017, Young Investigator Travel Awards (EASL HCC SUMMIT 2017)

Curriculum Vitae

Wenhui Wang was born on August 24, 1986, in Datong, Shanxi Province, China. She attended primary, middle and high school in Datong.

In 2005, she moved to Taiyuan in Shanxi Province and started her undergraduate study. She majored in Clinical Medicine (5 years) in the Shanxi Medical University. She obtained her Bachelor degree of Medicine in 2010 and started master research in the same year in Nanjing University, Jiangsu Province, China. She studied in the College of Medicine under the supervision of Prof. Fangyu Wang. Her main focus was to study the function of claudin proteins in the development of colorectal cancer. She obtained the Master degree of Medicine in 2013.

In 2013, with the support of China Scholarship Council, she got an opportunity to start her PhD research at the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, the Netherlands. Under the supervision of Prof. Maikel P. Peppelenbosch and Dr. Ron Smits, she mainly focused on the therapeutic improvement of hepatocellular carcinoma (HCC) by targeting Wnt/ β -catenin signaling. She was dedicated to elucidating the important role of Wnt/ β -catenin signaling in HCC therapy.

