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Author(s)	Fatima, S; Lee, NPY; Luk, JMC
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REVIEW

Dickkopfs and Wnt/β-catenin signalling in liver cancer

Sarwat Fatima, Nikki P Lee, John M Luk

Sarwat Fatima, Nikki P Lee, Department of Surgery, The University of Hong Kong, Hong Kong, China
John M Luk, Department of Pharmacology and Surgery, National University Health System, Singapore 117597, Singapore
John M Luk, Cancer Science Institute of Singapore, National University of Singapore, Singapore 117456, Singapore
John M Luk, Institute of Molecular and Cell Biology, A*STAR Singapore, Singapore 138673, Singapore
Author contributions: Fatima S designed and wrote the manuscript; Lee NP and Luk JM edited, advised and supervised this work.
Correspondence to: John M Luk, Professor, Cancer Science Institute of Singapore, National University of Singapore, Singapore 117456, Singapore. jmluk@nus.edu.sg
Telephone: +65-65164516 Fax: +65-68737690
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Peer reviewers: Steven A Rosenzweig, PhD, Professor, Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, United States; Eiji Miyoshi, MD, PhD, Professor, Department of Molecular Biochemistry and Clinical Investigation, Professor and Chairman, Osaka University Graduate School of Medicine, 565-0871 1-7 Yamada-oka Suita, Japan

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Abstract

Liver cancer is the fifth and seventh most common cause of cancer in men and women, respectively. Wnt/β-catenin signalling has emerged as a critical player in both the development of normal liver as well as an oncogenic driver in hepatocellular carcinoma (HCC). Based on the current understanding, this article summarizes the possible mechanisms for the aberrant activation of this pathway with specific focus on HCC. Furthermore, we will discuss the role of dickkopfs (DKKs) in regulating Wnt/β-catenin signalling, which is poorly understood and understudied. DKKs are a family of secreted proteins that comprise at least four members, namely DKK1-DKK4, which act as inhibitors of Wnt/β-catenin signalling. Nevertheless, not all members antagonize Wnt/β-catenin signalling. Their functional significance in hepatocarcinogenesis remains to be further characterized for which these studies should provide new insights into the regulatory role of DKKs in Wnt/β-catenin signalling in hepatic carcinogenesis. Because of the important oncogenic roles, there are an increasing number of therapeutic molecules targeting β-catenin and the Wnt/β-catenin pathway for potential therapy of HCC.

HEPATOCELLULAR CARCINOMA AND THE UNMET MEDICAL NEEDS

Liver cancer ranks the fifth most common cancer in men and the second leading cause of cancer-related death. In women, it is the seventh most frequent cancer and sixth leading cause of cancer death[1]. Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver. Men are three times more likely to develop HCC than women and the incidence increases with age[2]. HCC is prevalent in Asia and Africa, but recently it is on the rise in the Western world due to an increase in hepatitis C virus (HCV) infection[3]. Risk factors for HCC include chronic hepatitis B virus (HBV) and HCV infections, cirrhosis, chronic alcohol abuse, aflatoxin ingestion, non-alcoholic steatohepatitis and other metabolic liver diseases[4,5]. Much of HCC occurs in the background of cirrhosis. About 80%-90% of patients with cirrhosis go on to develop HCC eventually and the remaining 10%-20% of cases develop HCC without cirrhosis. Furthermore, HBV and HCV infections increase the risk of developing cirrhosis and later HCC. Among the HCC

cases with cirrhosis, HCV infection has been identified in 27%-73% and HBV infection in 12%-55%[6,7].

HCC suffers from a high mortality rate due to lack of effective diagnostic methods for early detection as well as lack of treatment options especially for those with advanced disease conditions. Despite vigorous attempts to screen for early HCC by common surveillance techniques using serum α-fetoprotein (AFP) and ultrasound examination, early HCC is asymptomatic and most HCC cases are presented late when surgical treatments are not amenable[8]. Although surgical resection remains the treatment of choice for patients with well-preserved liver function, it is associated with a high risk of post-operative complications and tumour recurrence[7]. Liver transplantation is another treatment option for early HCC but this is limited by the shortage of suitable liver grafts[9]. Other surgical treatments for HCC include radio-frequency ablation (RFA), microwave ablation (MWA) and transcatheter arterial chemoembolization (TACE). RFA and MWA techniques utilise high frequency radio-waves and micro-waves, respectively, to kill tumour tissues by heat. Although several studies have reported better disease-free survival and a lower frequency of recurrence after surgical resection compared to RFA[10,11], others report better overall survival and disease-free survival for HCC patients with multi-nodular tumours following RFA[12]. Recently, Simo *et al*[13] reported no difference regarding the efficacies of MWA and RFA procedures. TACE is routinely performed on HCC patients who are not eligible for surgical resection or tumour ablation techniques. However, the survival benefits of TACE depend on careful patient selection. Patients with multi-nodular HCC, without vascular invasion and no extrahepatic metastases show a better 2-year survival (63%) after TACE than HCC patients with vascular invasion undergoing TACE (31%)[14,15]. However, tumour recurrence is an important limitation to any of the HCC treatments, and thus, understanding the molecular biology of HCC is crucial for the development of novel therapies.

In recent years, studies have shed light on the clinical implications of signalling pathways in HCC, including the Ras/Raf/MEK/ERK pathway[16], the PI3K/Akt/mTOR pathway[17], the JNK pathway[18] and the NF-κB pathway[19]. A promising approach would be to identify molecular pathways responsible for initiating and sustaining HCC as targets for HCC therapy. The canonical Wnt/β-catenin signalling pathway is another such oncogenic pathway, which is frequently activated in HCC and is reported to play a pivotal role in tumourigenesis[20]. This article reviews the canonical Wnt/β-catenin signalling pathway and its involvement in HCC development. In addition, antagonists of this pathway and their implications in HCC are described and discussed. From this prior knowledge, we hope to identify members of this pathway that could serve as potential targets for HCC therapy.

OVERVIEW OF WNT/β-CATENIN PATHWAY

The Wnt/β-catenin pathway is a well-conserved pathway that is important in embryonic development, cell proliferation, survival, regeneration and self-renewal[21-23]. Likewise, a great deal of understanding has been achieved by studying pathological specimens and using mouse models of liver diseases to understand the aberrations of this pathway in liver diseases ranging from hepatitis to HCC[24-26]. Based on these earlier studies, the Wnt/β-catenin pathway is a central player in maintaining liver health and is dysregulated in hepatic cancers, which makes it an attractive candidate for potential therapies of HCC.

In an unstimulated cell, endogenous β-catenin is found at the adherens junctions, where it interacts with components of the cadherin-associated protein complexes to confer cell-cell adhesion functions[27,28]. On the other hand, surplus β-catenin in the cytoplasm is degraded by the action of a destruction complex which consists of glycogen synthase kinase 3β (GSK3β), Axin, adenomatous polyposis coli (APC) and casein kinase Iα (CKIα)[29]. β-Catenin is first phosphorylated at serine-45 (Ser45) by CKIα to further prime it for phosphorylation by GSK3β at Ser33, Ser37 and threonine-41 (Thr41). The phosphorylated β-catenin is then ubiquitinated by β-transducin repeat-containing protein (β-TrCP) and subsequently degraded by the proteasome[30] (Figure 1A). Maher *et al*[31] reported that β-catenin phosphorylated at Ser45 and not at Ser33/Ser37/Thr41 is predominantly located in the nucleus, whereas β-catenin phosphorylated at Ser33/Ser37/Thr41 is mostly localized to the cytoplasm. This spatial separation of β-catenin suggests that phosphorylation at Ser45 and at Ser33/Ser37/Thr41 is not necessarily coupled. It may also imply that phosphorylation at Ser45 by CKIα serves another function, yet to be delineated, other than priming β-catenin for further phosphorylation by GSK3β.

For diseased condition, the Wnt/β-catenin signalling pathway is activated upon binding of Wnt to one of the members of the frizzled (FZD) family and to low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6. The FZD recruits dishevelled (Dvl) to the plasma membrane, which in turn recruits Axin and GSK3β to LRP5/LRP6[32]. The intercellular domain of LRP5/LRP6 contains five reiterated PPPSPxS motifs, which are dually phosphorylated by GSK3β and CKIα[33]. The phosphorylation of LRP5/LRP6 disrupts the formation of the destruction complex, thereby preventing GSK3β from phosphorylating β-catenin. Therefore, β-catenin is not degraded and accumulates in the cytoplasm from where it translocates to the nucleus. In the absence of Wnt, T-cell factor (TCF)/lymphoid enhancer factor (LEF) represses gene expression by interacting with co-repressor Groucho, which promotes histone deacetylation and chromatin modelling in the nucleus[34]. Nuclear accumulation of β-catenin displaces Groucho from TCF/LEF and recruits other transcriptional co-activators, e.g. CREB binding protein (CBP), for upregulation of target genes that are implicated in cell proliferation, anti-apoptosis, and angiogenesis, such as cyclin D1[35] (Figure 1B). Recent studies have supported receptor-mediated endocytosis for Wnt induced signalling[36,37]. Specifically, Wnt3a was shown to induce caveolin-dependent internalization of

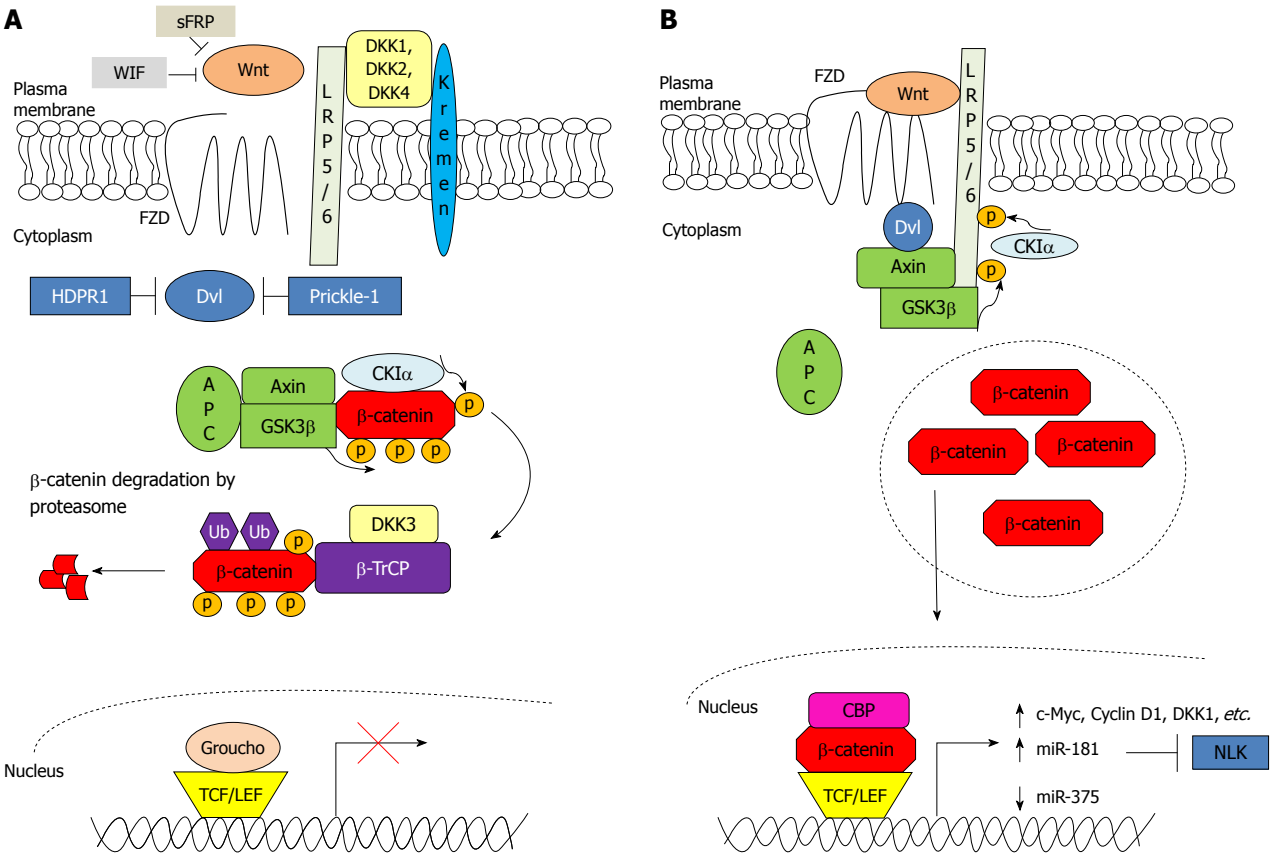

Figure 1 Wnt/β-catenin signalling in the absence and presence of Wnt stimulus. A: Wnt/β-catenin signalling is regulated by several antagonists to prevent the formation of frizzled (FZD)-Wnt-low-density lipoprotein receptor-related protein 5/6 (LRP5/6) complex. Secreted frizzled-related protein (sFRP) and Wnt inhibitory factor (WIF) bind directly to Wnt, whereas dickkopfs (DKKs) bind to LRP5/6. Furthermore, human homologue of Dapper (HDPR1) and Prickle-1 inhibit the action of dishevelled (Dvl). In the absence of Wnt stimulus, β-catenin is first primed for phosphorylation by casein kinase Iα (CKIα) followed by phosphorylation by glycogen synthase kinase 3β (GSK3β) at three residues. The phosphorylated β-catenin is targeted for ubiquitination by β-transducin repeat-containing protein (β-TrCP) and is subsequently degraded by the proteasome. In the nucleus, T-cell factor (TCF)/lymphoid enhancer factor (LEF) represses transcription of the Wnt/β-catenin pathway target genes by interacting with co-repressor Groucho; B: Wnt binds to and activates FZD and LRP5/6 receptors. Dvl is recruited to the plasma membrane and binds to FZD. This results in the recruitment of Axin and GSK3β to LRP5/6. LRP5/6 is then phosphorylated by CKIα and GSK3β, resulting in an inactivation of the destruction complex and leading to β-catenin accumulation in the cytoplasm. β-catenin then subsequently translocates to the nucleus where it binds with TCF/LEF and other co-activators e.g. CREB binding protein (CBP) to mediate transcription of genes and microRNAs responsible for proliferation and growth. APC: Adenomatous polyposis coli; NLK: Nemo-like kinase; p: Phosphorylated; Ub: Ubiquitinated.

LRP6, which would in turn recruit Axin to LRP6 phosphorylated by GSK3β and CKIα, and thereby lead to β-catenin accumulation. Thus, caveolin plays a critical role in activating Wnt/β-catenin signalling[37].

Very recently, Chairoungdua *et al*[38] showed a novel mechanism of reducing cytoplasmic β-catenin levels independently of GSK3β phosphorylation or ubiquitination by exporting β-catenin out of the cell *via* exosomes. Exosomes are vesicles that form inside endosomes and the vesicles are then secreted when the endosomes fuse with the plasma membrane[39]. These exosomes are enriched in E-cadherin and tetraspanin proteins (CD9 and CD82). Expression of these tetraspanins was shown to decrease β-catenin protein levels, but further experiments showed that E-cadherin was also necessary for β-catenin secretion in exosomes. The molecular mechanism for the inclusion of CD9, CD82 and E-cadherin in exosomes warrants further investigation. Furthermore, how these tetraspanins induce exosome formation remains to be characterized. Although much remains to be investigated, this important and novel mechanism offers an alternative route for the regulation of Wnt/β-catenin activity, further highlighting the significance of keeping the Wnt/β-catenin pathway under check.

ABERRANT WNT/β-CATENIN SIGNALLING IN HCC

Role of aberrant β-catenin activation in HCC

HCC is one of the cancers with a high rate of dysregulation in the Wnt/β-catenin pathway and although 40%-70%[40-43] of HCC patients have tumours with high levels of β-catenin accumulation, there is little agreement on the use of β-catenin in prognosis. Nuclear accumulation of β-catenin is strongly associated with β-catenin mutations[41]. A majority of β-catenin mutations in HCC are missense mutations occurring at exon 3. This region is responsible for phosphorylation and ubiquitination of β-catenin, and therefore, mutation in this region results

in stable β-catenin that consequently accumulates in the nucleus. Mao *et al*[44] associated nuclear β-catenin accumulation to β-catenin mutation, non-invasive form of tumour and good prognosis. HCC tumours with mutant nuclear β-catenin resulted in a better 5-year survival than HCC tumours with wild-type nuclear β-catenin accumulation. This is suggestive of the fact that wild-type β-catenin accumulation and mutant β-catenin accumulation are not equivalent. Furthermore, in this study of 37 HCC tumours with nuclear mutant β-catenin accumulation, 19 mutations occurred at the sites of GSK3β phosphorylation (Ser45, Ser33, Ser37 and Thr41), 3 tumours had β-catenin deletions, and 15 mutations were reported at other sites. However, several studies have correlated nuclear β-catenin accumulation to tumour progression and poor prognosis[43,45,46]. Kondo *et al*[46] reported that β-catenin accumulation and β-catenin mutation do not occur early in hepatocarcinogenesis, but could be associated with malignant progression of HCC. Similar to these findings, Inagawa *et al*[45] observed poor prognosis in HCC patients with nuclear β-catenin accumulation in grade Ⅲ HCC tumours and not in grade I or grade Ⅱ HCC tumours. Furthermore, nuclear β-catenin accumulation in HCC has also been correlated to Ki67 (a marker for tumour cell proliferation), suggesting that β-catenin promotes tumour progression[43]. The discrepancy in β-catenin accumulation and HCC prognosis could be due to the type of β-catenin mutations. Functional studies on the role of different mutations on β-catenin stability may offer insights into mechanisms involved in β-catenin regulation. Other reasons for the discrepancy may include tumour histology and the size of the tumour. Additionally, the presence of β-catenin mutations demonstrates different phenotypical features in HCC. Cieply *et al*[47] reported that HCC tumours harbouring a missense mutation at exon 3 exhibit a more aggressive phenotype and may develop HCC without cirrhosis compared to HCC with non-mutated β-catenin. Thus, β-catenin mutations may serve as an independent risk factor for the development of HCC in the absence of cirrhosis. Greater tumour size has also been reported in HCC tumours with β-catenin mutations as compared to those without mutation in β-catenin[48]. Some studies have correlated cytoplasmic β-catenin (non-nuclear β-catenin) with poor cellular differentiation, large tumour size (> 5 cm in diameter) and short disease-free survival[41]. For reasons not yet elucidated, HCV-associated HCC has a greater frequency of β-catenin mutations than the HBV-associated type[42].

Several studies on transgenic animal models have shown that overexpression of mutant or stable forms of β-catenin on its own is not sufficient to induce tumours in liver[49-51]. However, deletion of APC in mice results in hepatomegaly, hepatocyte hyperplasia and rapid mortality[52]. Thus, β-catenin mutations or accumulation may cooperate with other genes or signalling pathways to result in hepatocarcinogenesis. However, it is also important to take into account the functional roles of APC that are independent of β-catenin, e.g. APC maintains epithelial integrity in non-transformed mouse mammary epithelial cells[53] and it regulates cell cycle progression through the S phase by inhibiting DNA replication *via* direct interaction with DNA in colon cancer cell lines[54]. New mouse models are required that mimic irregular Wnt/β-catenin pathway to understand the role of this pathway as well as its therapeutic implications.

Table 1 Downstream molecules of the Wnt/β-catenin pathway with overexpression in hepatocellular carcinoma

Gene	Ref.
c-Myc	[159]
Cyclin D1	[57]
Dickkopf 1	[126]
Epidermal growth factor receptor	[51]
Glutamine synthetase	[63]
Glutamate transporter	[63]
Leukocyte cell-derived chemotaxin 2	[160]
Ornithine aminotransferase	[63]
Orphan G-protein-coupled receptor	[64]
Regenerating islet-derived 1 α	[161]
Regenerating islet-derived 3 α	[161]

Wnt/β-catenin pathway target genes in HCC

Several β-catenin target genes in association with liver carcinogenesis were identified by their high expression in chronic liver diseases and HCC. However, their specific role in hepatocarcinogenesis remains unknown. Frequent amplification and overexpression of c-Myc and cyclin D1 in HCC is associated with cytoplasmic and nuclear β-catenin accumulation along with poor prognosis[55-61]. However, there is little consensus on whether the overexpression of c-Myc and cyclin D1 is a result of mutations in β-catenin. Cadoret *et al*[49] did not report c-Myc or cyclin D1 induction in the liver of transgenic mice that express a mutant form of β-catenin, although such mice did exhibit hepatomegaly and marked hepatocellular proliferation. In contrast, de La Coste *et al*[62] reported activating somatic mutations in β-catenin in 50% of hepatic tumours in c-Myc transgenic mice. In addition to cyclin D1 and c-Myc, several other genes have been identified as downstream molecules of the Wnt/β-catenin pathway in HCC (Table 1). Expression of these genes was discovered in HCC transgenic mice or in HCC tissues exhibiting accumulation of wild type or mutated β-catenin. For example, glutamine synthetase and orphan G-protein-coupled receptor are frequently overexpressed in HCC with mutation in β-catenin[63,64]. Further studies are warranted to understand whether mutated or stable β-catenin results in transcription of different target genes, and if silencing these target genes affects aberrant Wnt/β-catenin pathway in a negative feedback manner. More importantly, can the expression of β-catenin target genes in HCC, e.g. glutamine synthetase or cyclin D1, be sufficient to identify β-catenin activation?

Table 2 Aberrant expression of Wnt/β-catenin pathway components in liver cancer

Component	Expression in tumour tissues *vs* non-tumour/ healthy tissues	Incidence (%)	Method	Ref.
β-catenin	High	40-70	IHC	[40-43]
Wnt1	High	41	PCR	[69]
Wnt3	High	39-76	PCR	[70,71]
Wnt4	High	20	PCR	[71]
Wnt5A	High	25	PCR	[71]
FZD3	High	41	PCR	[71]
FZD6	High	31	PCR	[71]
FZD7	High	33-90	PCR	[70-72]
phospho-GSK3β	High	52	IHC	[73]
Axin1	Low	67	IHC	[76]
Dvl	High	71	Western blotting	[82]
Prickle-1	Low	55	PCR	[82]
HDPR1	Low	58	PCR	[83]
PIN1	High	53	Western blotting	[65]
TCF-4	High	91	PCR	[87]
LEF-1	High	52	IHC	[88]
CDH17	High	72	IHC	[90]

CDH17: Cadherin-17; Dvl: Dishevelled; FZD: Frizzled; phospho-GSK3β: Phosphorylated glycogen synthase kinase 3β; HDPR1: Human homologue of Dapper; IHC: Immunohistochemistry; LEF: Lymphoid enhancer factor; PCR: Polymerase chain reaction; PIN1: Peptidyl-prolyl cis/trans isomerase; TCF: T-cell factor.

Alterations of Wnt/β-catenin pathway components in HCC

Accumulation of β-catenin can also occur in the absence of β-catenin mutation or due to aberrant expression of other members of the Wnt/β-catenin pathway[65]. Table 2 summarizes the aberrant expression of Wnt/β-catenin signalling components in HCC.

Wnt and FZD

The Wnt family is composed of nineteen secreted glycoproteins[66]. They bind to the extracellular domain of FZDs and activate the Wnt/β-catenin pathway[67]. Ten different FZD genes have been identified in mammals and all of them encode seven transmembrane receptors[68]. Wnt1 is upregulated in HCC tissues compared to adjacent non-tumour tissues and its expression has been associated with tumour recurrence[69]. Furthermore, three other Wnt genes (Wnt3, Wnt4 and Wnt5A), and three FZD genes (FZD3, FZD6 and FZD7) are also upregulated in HCC tissues and preneoplastic peritumoural tissues as compared with normal liver tissues, suggesting that their overexpression may be an early event in hepatocarcinogenesis. However, only the overexpression of FZD7 has been associated with nuclear and/or cytoplasmic accumulation of β-catenin in HCC[70-72].

GSK3β, CKIα, Axin and APC

There are no reports of mutations or aberrant expression of CKIα in HCC. A few studies have reported overexpression of phosphorylated GSK3β (phospho-GSK3β, inactive form of GSK3β) in HCC cells and tissues, but this has not been found to be associated with β-catenin accumulation[73,74].

There are two Axin genes. Axin1 is constitutively expressed, whereas Axin2 (also known as Axil or Conductin) is induced by Wnt/β-catenin signalling and takes part in a negative feedback loop[75]. Kim *et al*[76] recently observed reduced Axin1 expression only in HCC tissues and not in cirrhotic nodules, implying that its reduced expression is independent of cirrhosis. There are currently no reports of Axin2 expression in HCC. However, Axin1 and Axin2 mutations are rare in HCC occurring in only 10% and 3% of HCC cases, respectively[48,77,78]. A majority of these mutations are frameshift or nonsense mutations and more than half of HCC cases with Axin1 or Axin2 mutations have β-catenin accumulation in the cytoplasm or nucleus[77]. Interestingly, Zucman-Rossi *et al*[79] recently demonstrated that the loss of Axin1 function is not equivalent to gain-of-function of β-catenin, suggesting that Axin1 could also be participating in other pathways in HCC. APC mutations are very rare in HCC and promoter methylation has been reported to play an important role in its inactivation[80].

Dvl and Peptidyl-prolyl cis/trans isomerase

Dvl is a positive regulator of Wnt/β-catenin signalling and prevents GSK3β from phosphorylating β-catenin, leading to β-catenin stabilisation[81]. Its overexpression has been shown to be critical in Wnt/β-catenin signalling activation and β-catenin accumulation in various cancers including HCC[82]. Two inhibitors of Dvl have been identified including Prickle-1[82] and human homologue of Dapper[83] both of which are reduced in HCC and their reduced expression has significant association with β-catenin accumulation. Peptidyl-prolyl cis/trans isomerase (PIN1) is another positive regulator of the Wnt/β-catenin pathway and functions by inhibiting the interaction between β-catenin and APC[84]. It is also overexpressed in more than 50% of HCC cases and this has been correlated to increased β-catenin and cyclin D1 accumulation. Furthermore, β-catenin mutation and PIN1 overexpression are mutually exclusive events in HCC, suggesting that mechanisms other than β-catenin mutation also lead to β-catenin stabilisation and accumulation[65].

TCF/LEF

The human TCF/LEF family consists of four members, LEF-1, TCF-1, TCF-3 and TCF-4, and all members contain a conserved high mobility group box to bend DNA to allow binding of transcription factors, a β-catenin-binding domain to bind β-catenin and a transcription repression domain to recruit co-repressors like Groucho[85]. When β-catenin translocates to the nucleus, it binds to the β-catenin-binding domain of TCF/LEF and activates transcription of target genes. The TCF/LEF family has several spliced forms with unknown functional significance and it is suggested that they may activate preferential genes[86]. In HCC, mutations of TCF-4 are rare with

Jiang *et al*[87] reporting mutations in 2 of 32 HCC cases, whereas Cui *et al*[25] did not report any mutation in 34 HCC cases. However, overexpression of TCF-4 in HCC tissues has been correlated to c-Myc overexpression, tumour capsule status and intrahepatic metastasis[87]. LEF-1 is also overexpressed in HCC and associated with cyclin D1 overexpression, but it is not found to be correlated with any clinicopathological parameters[88]. Therefore, it is not known whether the aberrant expression of TCF-4 and LEF-1 is a primary event in HCC or a result of aberrant Wnt/β-catenin signalling in HCC. Expression of other TCF/LEF family members in HCC has not been reported.

Cadherin-17

Cadherin-17 (CDH17) belongs to the cadherin superfamily comprising transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion[89]. Since β-catenin plays an essential role in cadherin-mediated cell-cell adhesion as well as in Wnt/β-catenin signalling, our laboratory identified CDH17 to affect Wnt/β-catenin signalling in HCC. CDH17 is overexpressed in HCC cells and in more than 70% of HCC cases[90]. In addition, overexpression of an alternative splice variant of CDH17 (lacking exon 7) in more than 50% of HCC is correlated to tumour recurrence, venous infiltration and reduced overall survival[91]. Interestingly, the CDH17 splice variant is found to be triggered by two single nucleotide polymorphisms that are identified as genetic factors contributing to the high development of HCC in Asia[92]. Furthermore, RNA interference (RNAi)-mediated knockdown of CDH17 in metastatic HCC cells has been shown to inhibit tumourigenesis *in vivo* and reduce Wnt/β-catenin signalling by decreasing phospho-GSK3β and cyclin D1. This was accompanied by re-localisation of β-catenin to the cytoplasm[93].

Tetraspanins

Tetraspanins are transmembrane proteins known to affect a wide range of functions including cell-cell adhesion, cell growth and suppression of metastasis[94]. The recent involvement of tetraspanins CD9 and CD82 in a novel mechanism to antagonize Wnt/β-catenin signalling by exosomal release of β-catenin is an exciting avenue to explore in HCC. This exosomal release of β-catenin may be compromised in cancers with high Wnt/β-catenin signalling. CD9 and CD82 are suppressors of metastasis and their expression is reduced in HCC with portal vein invasion and/or intrahepatic metastasis[95]. Chairoungdua *et al*[38] demonstrated Wnt/β-catenin signalling inhibition in a metastatic cell line following restoration of CD82 expression. Thus, these tetraspanins may suppress metastasis by antagonizing Wnt/β-catenin signalling by targeting β-catenin for exosomal release. It will be important to investigate the correlation between CD9 and CD82 with β-catenin in HCC.

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression[96]. They are aberrantly expressed in HCC compared to their non-tumour liver tissues[97-99] and contribute to liver tumourigenesis[100,101]. Several miRNAs have been identified to affect the Wnt/β-catenin pathway[102]. Using a global microarray-based miRNA profiling approach, Ji *et al*[103] identified miRNA-181 (miR-181) to be upregulated in HCC tumours that were positive for epithelial cell adhesion molecule (EpCAM) and AFP ($EpCAM^+AFP^+$). Such tumours demonstrated cancer stem cell properties and an activation of Wnt/β-catenin signalling. *In vitro* studies showed a correlation between overexpression of miR-181 and β-catenin in HCC cells and further demonstrated that miR-181 promoted the stemness of $EpCAM^+AFP^+$ HCC cells by targeting CDX2 (caudal type homeobox transcription factor 2), GATA6 (GATA binding protein 6, a hepatic transcriptional regulator of differentiation) and nemo-like kinase (NLK, an inhibitor of Wnt/β-catenin signalling). These findings provide evidence that miR-181 is transcriptionally activated by Wnt/β-catenin signalling and in turn inhibits its regulators. In addition, miR-375 is another miRNA involved in the Wnt/β-catenin pathway and it is downregulated by β-catenin in HCC[104]. However, the function of miR-375 and the mechanisms by which it is regulated by β-catenin are not clear. Further research is needed to investigate the involvement of miRNAs in Wnt/β-catenin signalling in HCC.

Yes-associated protein

The Hippo signalling pathway controls organ size by regulating cell proliferation and apoptosis. The signalling cascade of this pathway ultimately leads to the phosphorylation of yes-associated protein (YAP), a downstream effector of this pathway. YAP is a transcriptional co-activator and its phosphorylation causes it to remain in the cytoplasm and prevent the transcription of genes responsible for cell proliferation and inhibition of apoptosis[105]. Recently, a few studies have described the Hippo pathway as a negative regulator of Wnt/β-catenin signalling[106,107]. Varelas *et al*[106] reported phosphorylated Taz (component of the Hippo pathway) to inhibit the activation of Dvl, thereby preventing β-catenin stabilisation and activation. Heallen *et al*[107] recently showed nuclear interaction of unphosphorylated YAP and β-catenin in cardiac cells of mice with dysregulated Hippo signalling. These mice had enlarged hearts and overexpressed Wnt/β-catenin target genes. Additionally, dysregulation of the Hippo signalling pathway and inhibition of β-catenin resulted in restriction of cardiomyocyte overgrowth. This study offered insights into the direct interaction between the downstream effectors of these two important pathways. In HCC, nuclear overexpression of YAP (unphosphorylated YAP) has been reported in 62% of HCC and has been associated with short disease-free survival and overall survival[108]. Since β-catenin is also found to accumulate in the nucleus of HCC patients[43,45], further studies are warranted to understand the clinical implications of YAP and β-catenin overexpression in HCC.

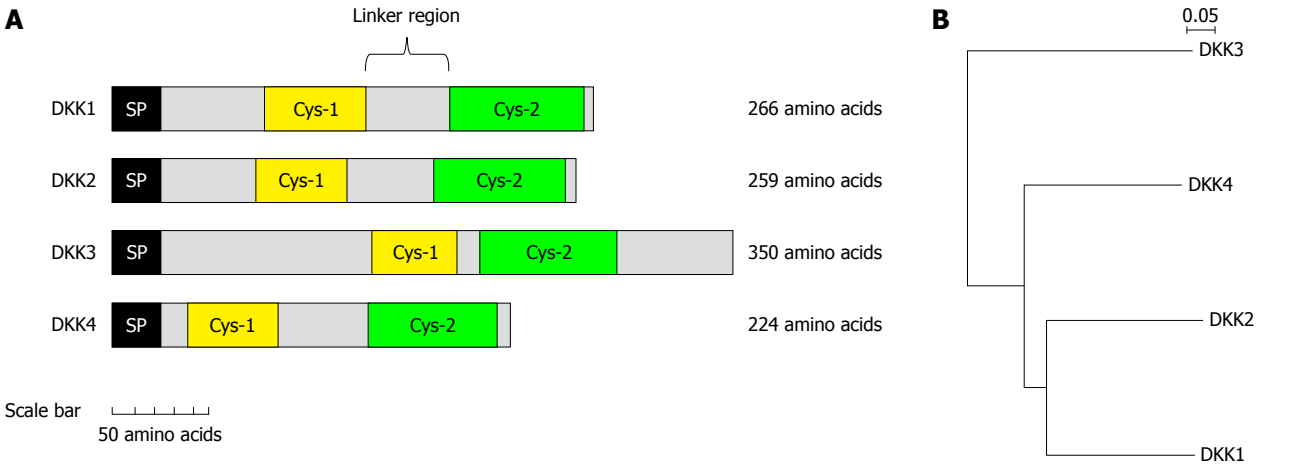

Figure 2 Domain structure and phylogenetic tree of human dickkopf proteins. A: Each dickkopf (DKK) contains two cysteine-rich domains, each of which is separated by a linker region of various lengths. The amino-terminal cysteine-rich domain (Cys-1) domains are unique to each DKK, whereas the carboxyl-terminal cysteine-rich domain (Cys-2) domains are conserved among all members of the DKK family; B: Phylogenetic tree of the DKK proteins. Amino acid sequences were aligned by the Clustal W program, and the phylogenetic tree was constructed by the neighbour joining method. The scale bar indicates the estimated number of substitutions per 50 amino acids. SP: Signal peptide.

WNT SIGNALLING ANTAGONISTS

As mentioned above, there are many factors affecting the Wnt/β-catenin pathway in HCC. However, the occurrence of these factors is not absolute in each clinical case of HCC. Therefore, the involvement of other factors in modulating the Wnt/β-catenin pathway is speculated. Factors upstream of GSK3β/Axin/APC protein complex, like those occurring at the extracellular level, may also be those involved. For this, secreted Wnt antagonists are distinguished examples. These antagonists can be divided broadly into two groups based on their different mechanisms in preventing the interaction of Wnt with LRP5/LRP6[109]. Group I consists of Wise, sclerostin and dickkopfs (DKKs) that bind directly to LRP5/LRP6. Group II comprises Wnt inhibitory factor and secreted frizzled-related protein that act by directly binding to Wnt proteins and prevent the formation of the Wnt receptor complex. This review will focus on the role of DKKs on the Wnt/β-catenin pathway in HCC.

Structure of DKKs

The DKK family consists of four members (DKK1 to DKK4). They are secreted glycoproteins of 225-350 amino acids with molecular weights between 25 and 29 kDa for DKK1, DKK2 and DKK4, and 38 kDa for DKK3. DKKs contain two conserved cysteine-rich domains, each of which is separated by a linker region of various lengths[110]. Each cysteine-rich domain contains ten cysteine residues. The amino-terminal cysteine-rich domain (Cys-1) is unique to each DKK, whereas the carboxyl-terminal cysteine-rich domain (Cys-2) is highly conserved among all members of the DKK family. The position of each cysteine residue in the Cys-2 domain closely resembles proteins in the colipase family[111]. Because of the role of colipases in lipid hydrolysis[112], the presence of this feature suggests the ability of DKKs to interact with lipids in regulating Wnt/β-catenin signalling[111]. Among all DKKs, DKK3 is the most divergent member[113]. It contains an extended amino-terminal domain preceding the Cys-1 region and an extended carboxyl-terminal domain following the Cys-2 domain. All DKKs possess several potential sites for proteolytic cleavage by furin-type proteases, indicating that they may be subjected to post-translational modifications[114]. Figure 2A and B illustrate the differences between different DKKs.

Functions of DKKs on the Wnt/β-catenin pathway

Members of the DKK family differ not only in their structures but also in their mRNA expression in HCC and in their ability to modulate Wnt/β-catenin signalling. Table 3 summarizes these differences between members of the DKK family.

DKK1 is the most studied member of the DKK family. It was originally identified as a head inducer when its mRNA was injected into *Xenopus* embryos[115]. Experiments inactivating *Xenopus* DKK1 with anti-DKK1 antibodies[115] and involving DKK1 knockout mice[116] show a lack of anterior head structures, highlighting its importance in head formation. DKK1 inhibits Wnt-induced stabilisation of β-catenin and two models have been proposed. The first model proposes that DKK1 binds to the extracellular domain of LRP5/LRP6 and prevents the formation of the FZD-Wnt-LRP5/LRP6 complex in response to Wnt, thereby attenuating Wnt activity[117]. The second model proposes that DKK1 inhibits Wnt signalling by inducing clathrin-dependent internalisation of LRP6[37]. However, contrary to the results of Yamamoto *et al*[37], Blitzer *et al*[118] used mouse fibroblast cells to show that clathrin-dependent internalisation of LRP6 is required to propagate Wnt/β-catenin signalling, and disturbing this clathrin-mediated endocytosis blocks Wnt activity. These recent findings suggest that mechanisms of DKK1 antagonistic activity may vary in different cell types.

Table 3 Differences between the DKK family members

Property	DKK1	DKK2	DKK3	DKK4	Ref.
Variants	Unknown	Unknown	2	Unknown	[130]
Location of Cys-1	85-138 amino acids	78-127 amino acids	147-195 amino acids	41-90 amino acids	[110]
Location of Cys-2	189-263 amino acids	183-256 amino acids	208-284 amino acids	145-218 amino acids	[110]
Length of linker region (number of amino acids)	50	56	12	54	[110]
Receptor	LRP5/LRP6	LRP5/LRP6	Not identified	LRP5/LRP6	[113]
Activity in Wnt/β-catenin signalling in HCC	Antagonist	Not reported	Not reported	Not reported	[126]
Expression in HCC tissues	Overexpressed	Reduced	Reduced	Not reported	[126,143]
Phenotype of knockout mice	Embryonic lethality	Viable, fertile, blindness, osteopaenia	Viable, fertile, hyperactive, increased IgM, natural killer cells and haematocrit levels	Not reported	[116,162-164]

Cys-1: Amino-terminal cysteine-rich domain; Cys-2: Carboxyl-terminal cysteine-rich domain; HCC: Hepatocellular carcinoma; LRP: Lipoprotein receptor-related protein; DKK: Dickkopf.

In addition to binding to LRP5/LRP6, DKK1 also binds to Kremen 1 (Krm1) and Krm2, which belong to another class of transmembrane receptors[119,120]. DKK1 binds to Krm1 and Krm2 with high affinity and this inhibits Wnt/β-catenin signalling[121]. Despite that, gene knockout studies in mice have shown that Krm1 and Krm2 are not universally required for DKK1-associated function[122]. It was demonstrated that DKK1 mutants are able to antagonize Wnt activity without binding to Krm1 or Krm2[123]. Therefore, Krms may not be essential for DKK1 function and further studies are needed to understand their involvement in Wnt/β-catenin signalling.

Among all DKKs, DKK4 demonstrates similar antagonistic activity towards the Wnt/β-catenin pathway as DKK1 by binding to LRP5/LRP6 and Krms[120]. While functioning upstream of the Wnt/β-catenin pathway, DKK1 and DKK4 are also downstream targets of the Wnt/β-catenin pathway, creating a negative feedback loop to regulate Wnt/β-catenin signalling[124,125]. However, this feedback mechanism is often abrogated in cancers including HCC[125,126].

Like DKK1 and DKK4, DKK2 also binds to LRP5/LRP6 and Krms. However, DKK2 may serve as an antagonist or an agonist to the Wnt/β-catenin pathway depending on cellular context. For instance, overexpression of DKK2 in 293 fibroblast cells results in Wnt/β-catenin pathway activation, whereas co-transfection of DKK2 and Krm2 in the same cell type inhibits this pathway[127]. To explain this phenomenon, Chen *et al*[128] identified YWTD β-propeller domains of LRP5/LRP6 as the docking sites for Cys-2 of mouse DKK2, while this Cys-2 domain also contains binding site for Krm1 and Krm2. Therefore, the expression of Krms serves as a switch for the dual role of DKK2 on the Wnt/β-catenin pathway.

Unlike other DKKs having a role in Wnt/β-catenin signalling by binding to LRP5/LRP6 and Krms, the receptor for DKK3 has not been identified and its effect on this pathway remains unclear. Earlier studies in *Xenopus* suggested that DKK3 is not involved in Wnt/β-catenin signalling[113,115], but recent studies have demonstrated that DKK3 is associated with a reduction in cytoplasmic and nuclear accumulation of β-catenin in Saos-2 osteosarcoma cells, lung cancer cells and cervical cancer cells[129-131]. Interestingly, Lee *et al*[130] identified β-TrCP as a binding partner to DKK3, and possibly DKK3 may reduce β-catenin levels *via* ubiquitination. Therefore, the potential role of DKK3 in Wnt/β-catenin pathway remains to be determined.

Since different DKKs exhibit different effects on Wnt/β-catenin activity, it will be important to study the mechanisms by which each member of the DKK family exerts its effect on the Wnt/β-catenin pathway. Furthermore, as reported by Yamamoto *et al*[37] and Blitzer *et al*[118], DKKs may have different mechanisms of action in different cell types. Additionally, sharing the same receptor may not imply exhibiting the same effect as in the case of DKK1 and DKK2. For instance, they both bind to LRP5/LRP6 and Krms, but DKK1 serves as an antagonist, whereas DKK2 serves as both an agonist and an antagonist to the Wnt/β-catenin pathway.

DKKs in HCC

As mentioned above, the Wnt/β-catenin pathway plays a critical role in HCC and not surprisingly, Wnt inhibitors, including DKKs, are involved. More research studies have been dedicated to studying the role of DKKs in HCC over the past few years.

DKK1

Several studies have reported overexpression of DKK1 in HCC cell lines and tissues[126,132,133], while Yu *et al*[126] was the first to demonstrate a correlation between DKK1 overexpression and cytoplasmic/nuclear β-catenin accumulation in HCC. It was demonstrated through *in vitro* assays that DKK1 failed to inhibit TCF-mediated transcriptional activity in HCC cells with cytoplasmic or nuclear β-catenin accumulation, suggesting an abrogation of the negative feedback loop of DKK1 in HCC. Survival analysis correlated overexpression of DKK1 with poor prognosis of HCC patients, and DKK1 was identified as an independent prognostic marker for overall survival and

disease-free survival. The 5-year overall survival and disease-free survival rates for HCC patients overexpressing DKK1 (43.4% and 34.2%, respectively) were significantly lower than HCC patients with reduced DKK1 expression (59.3% and 55.2%, respectively). Although elevated levels of AFP remain the gold standard for screening HCC, there are, however, a subgroup of patients who have HCC and normal levels of AFP. When patients were stratified according to AFP levels, DKK1 overexpression demonstrated worse prognosis for AFP-normal HCC patients, suggesting that DKK1 may serve as a prognostic marker for this group of patients. Furthermore, HCC patients with high DKK1 and β-catenin expression also showed poor prognosis. The 5-year overall survival and disease-free survival rates were 66.0% and 59.8%, respectively, for HCC patients without DKK1 and β-catenin expression, and 46.0% and 18.0% for HCC patients with high DKK1 and high β-catenin expression[126]. This study highlights the important role of DKK1 in Wnt/β-catenin signalling in HCC.

Other than total AFP levels, *lens culinaris* agglutinin–reactive AFP (AFP-L3) and prothrombin induced by vitamin K absence-Ⅱ (PIVKA-Ⅱ) have been reported as tumour markers for HCC. High serum AFP-L3 levels have recently been reported as a prognostic marker even in HCC patients with low AFP levels[134]. Additionally, high serum levels of PIVKA-Ⅱ have been associated with advanced HCC with portal vein invasion[135].

Because of its high expression in HCC tissues and its secretory nature, DKK1 is hypothesized to be present at high levels in the serum of HCC patients. However, there are no studies evaluating high DKK1 serum levels on HCC progression or prognosis and it would be important to conduct such a study to understand the clinical significance of DKK1 in HCC. On the other hand, high levels of DKK1 in patients' serum are associated with poor prognosis in various cancers including oesophageal squamous cell carcinoma[136], lung cancer[137], breast cancer[138] and cervical cancer[138], suggesting that the serum level of DKK1 may also reflect the prognosis of HCC patients. In multiple myeloma, high DKK1 serum levels are associated with osteolytic bone lesions[139] and patients responding to anti-myeloma treatment show a decrease in DKK1 serum levels[140], suggesting the involvement of DKK1 in this aspect. Recently, Fulciniti *et al*[141] evaluated the effect of anti-DKK1 monoclonal antibody (BHQ880) in a multiple myeloma mouse model and found that it induced bone formation and inhibited tumour-induced osteolytic bone lesions. Like multiple myeloma, HCC is also osteolytic in nature with 20% of HCC patients having bone metastasis[142], making it important to assess the role of DKK1 in bone metastasis in HCC.

DKK2

In HCC, a higher level of DKK2 methylation was detected in HCC tissues compared to corresponding non-cancerous cirrhotic tissues[143], suggesting its role in hepatocarcinogenesis. Epigenetic silencing of DKK2 has also been reported in gastric cancer[144], oesophageal squamous cell carcinoma[145] and renal cell carcinoma[146]. In renal cell carcinoma, no significant relationship was found between DKK2 methylation and β-catenin expression[146]. Although the effect of DKK2 on Wnt/β-catenin signalling depends on the expression of LRP5/LRP6 and Krms, DKK2 expression has not been studied in the context of these molecules. Furthermore, there are currently no reports on the effect of DKK2 on Wnt/β-catenin signalling in HCC.

DKK3

There are few reports on the clinical significance of DKK3 in HCC[143,147]. Reduction in DKK3 expression is associated with increased frequency of methylation in HCC tissues compared to corresponding non-cancerous cirrhotic tissues, implying that DKK3 methylation may not be an early event in HCC, but may function in the progression of HCC. Furthermore, DKK3 methylation has been significantly associated with short progression-free survival in HCC patients[143]. The effect of DKK3 on Wnt/β-catenin signalling has not been reported in HCC, but reduced DKK3 expression has been shown to correlate with β-catenin accumulation in lung cancer[131]. Although there are no reports of DKK3 level in HCC serum, reduced DKK3 serum levels in ovarian cancer are associated with the presence of lymph node metastasis, and high DKK3 serum levels have been associated with large tumours in cervical cancer[148]. Interestingly, multigene methylation status of a combination of Wnt antagonist genes, including DKK3 and others, in the serum have been proposed as markers for diagnosis, staging and prognosis of renal cell carcinoma[149]. Although different Wnt antagonists may function differently, their cumulative silencing may serve as a molecular marker for cancer detection.

DKK4

DKK4 is least studied in cancer. It is located on chromosome 8p11.2-p11.1 and this chromosomal region experiences frequent loss of heterozygosity in HCC[150]. This may explain reduced expression of DKK4 in HCC cell lines without detection of DKK4 methylation[144]. Currently there are no reports on DKK4 expression in HCC tissues or its effect on Wnt/β-catenin signalling in HCC. Recently, Hirata *et al*[151] reported a reduction of Wnt target genes after ectopic expression of DKK4 in renal carcinoma-derived Caki cells. However, overexpression of DKK4 also resulted in activation of the JNK pathway and enhanced tumour growth *in vivo*. This suggests that DKK4 may be involved in pathways other than Wnt/β-catenin signalling.

POTENTIAL THERAPEUTIC TARGETS FOR WNT/β-CATENIN SIGNALLING IN HCC

Mounting evidence suggests the role of β-catenin stabi-

lisation in promoting tumour proliferation in HCC patients[43,152]. Such observations make Wnt/β-catenin signalling an attractive target for cancer therapy. In line with this hypothesis, small molecule inhibitors have been developed to hinder Wnt/β-catenin signalling by disrupting protein-protein interactions of components of this pathway. These include the fungal derivatives, PKF115-584 and CGP049090, that function to impede the interaction between β-catenin and the TCF complex[153]. Emami *et al*[154] reported another molecule called ICG-001, which disrupts the interaction between β-catenin and CBP. More recently, pyrvinium pamoate, an anthelmintic drug, was shown to inhibit the Wnt/β-catenin pathway by allosteric activation of CKIα and subsequently increased levels of β-catenin destruction complex secondary to Axin stabilisation[155]. Therapeutic antibodies against Wnt1 and Wnt2 have also demonstrated Wnt/β-catenin signalling inhibition and suppression of tumour growth *in vivo*[156,157]. β-catenin suppression through chemoprophylaxis may offer another alternative therapy. R-Etodolac (enantiomer of the non-steroidal anti-inflammatory drug Etodolac) reduced proliferation and survival of two HCC cell lines (HepG2 and Hep3B) by decreasing the total and active forms (dephosphorylated at Ser33/Ser37/Thr41) of β-catenin[158]. Further studies will be important to access clinical implications of these potential targets in HCC.

PERSPECTIVES

Aberrant activation of Wnt/β-catenin signalling is an important event in HCC that leads to transcription of various target genes involved in carcinogenesis. It will be clinically relevant to target the Wnt/β-catenin pathway and regulate its activity. However, since the pathway can be aberrantly activated by the dysfunction of several genes, it is important to ensure that when one member is targeted the other functions of the same target, if any, are not disrupted. For instance, β-catenin is involved in the Wnt/β-catenin pathway and plays a critical role in cell-cell adhesion. Therefore, care should be taken in designing reagents for disruption of molecules with dual functions. For tumours with mutated β-catenin, one approach could be to utilize RNAi targeting mutant β-catenin for therapy, as this will target selectively for tumour cells, but not healthy cells. However, mutant β-catenin-negative tumours with aberrant Wnt/β-catenin signalling offer the greatest challenge as there could be a multitude of reasons for aberrant Wnt/β-catenin activation. Therefore, altering Wnt/β-catenin signalling may be advantageous for therapeutic means but there are caveats and limitations involved.

In line with these investigations, DKKs have been discovered over the past 12 years for their abilities to interfere in the Wnt/β-catenin pathway. Since then, much effort has been dedicated to exploring their functions and implications in liver development and disease. Different DKKs exhibit different expressions and functions in various cancers. Some important questions remain to be answered. For example, where are DKKs localised in HCC cells? Is their expression associated with cytoplasmic/nuclear β-catenin accumulation in HCC cells? Do DKKs have prognostic value in HCC patients? Do they exert their effects on both wild type and mutant β-catenin in HCC cells? Do DKKs degrade β-catenin *via* a ubiquitination-dependent mechanism? Are different DKKs involved in different stages of HCC development? What is the receptor for DKK3? Are DKKs redundant in their functions? Furthermore, the negative feedback mechanism of DKK1 also warrants further research. Although DKK1 inhibits Wnt/β-catenin signalling, its overexpression has no effect on HCC cells with cytoplasmic/nuclear β-catenin accumulation[126]. This may be suggestive of an abrogated negative feedback loop or it may also imply that the inhibitory effect of DKK1 is only functional until reaching a point of saturation beyond which it cannot exert its inhibitory effect. In summary, compared to the overwhelming body of evidence showing the functional role of Wnt/β-catenin signalling in HCC, the biological and physiological roles of DKKs in relation to this pathway remain to be discovered. DKKs provide a new avenue to explore and they add another level of complexity to the already complex Wnt/β-catenin signalling in HCC.

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