Review paper

Alterations of Wnt/β-catenin signaling pathway in hepatocellular carcinomas associated with hepatitis C virus

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The Wnt/Fzd/β-catenin signaling pathway plays a significant role in physiology and pathology of the liver. The role of β-catenin is linked mainly to the canonical pathway of the system. Phosphorylation of β-catenin and abnormalities in function of the E-cadherin-catenin unit lead to loss of intercellular junctions, progression in liver fibrosis, and development of cirrhosis and hepatocellular carcinoma (HCC). Progression of liver diseases is noted to be accompanied by disturbances in β-catenin expression (mainly with its overexpression), with its cytoplasmic or nuclear translocation and with lowered expression of E-cadherin. Increase in transcriptional activity of β-catenin is associated mainly with mutations of *CTNNB1*. Detailed mechanisms of HCC development are not known.

More β-catenin mutations are manifested in hepatitis C virus (HCV)-associated than in HBV-related HCC. In recent years the role of nonstructural proteins and of the core protein of HCV has been accentuated in induction of the Wnt pathway. HCV proteins affect in a double manner expression of E-cadherin, including modulation of the Wnt pathway and reduction of E-cadherin expression at the transcriptional level.

This review presents current data on mechanisms of hepatocarcinogenesis involving participation of the Wnt canonical pathway and, in particular, interaction of Wnt pathway components with HCV genome products in the process.

Key words: Wnt canonical pathway, β-catenin/E-cadherin complex, hepatitis C virus, hepatocarcinogenesis.

## Wnt/Fzd/β-catenin signaling pathway in liver

The Wnt/Fzd/β-catenin signaling pathway plays a significant role in liver physiology and pathology. Its involvement was demonstrated in development of liver and in morphogenesis of biliary ducts. It maintains correct homeostasis of liver cells in postnatal life [1], and it influences development of structure and metabolic activity of the hepatic acinus, allowing for growth and regeneration of the liver. It protects the liver from the effects of toxic agents and oxidative stress [2]. Also mechanisms of carcinogenesis, related both to hepatoblastoma and to primary hepatocellular carcinoma (HCC) involve the pathway {1, 3}. Interactions of the pathway have been described with other pathways accelerating hepatocyte proliferation. Detailed mechanisms of HCC development remain unknown and determination of a simple model of hepatocarcinogenesis with involvement of the Wnt signaling pathway has proven to be difficult [1]. Due to the fact that incidence of chronic hepatitis C
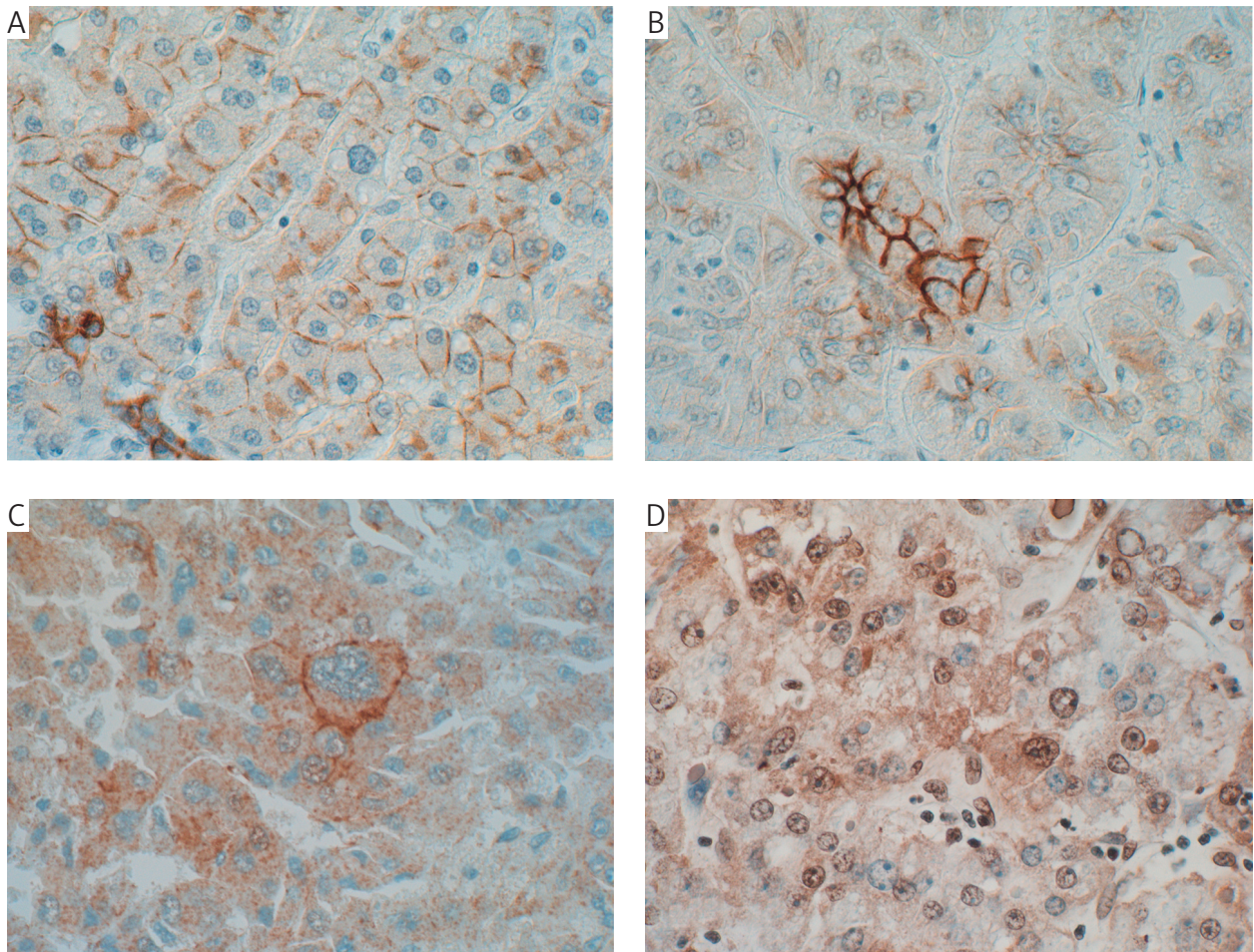
Fig. 1. Immunocytochemical and hybridocytochemical localization of β-catenin in normal and pathological liver. Membranous expression of β-catenin in control liver (A); mostly membranous (B) and cytoplasmic (C) expression of β-catenin in human hepatocellular carcinoma cells; mRNA for β-catenin in HCC (D). Immunohistochemistry (A-C) and hybridization *in situ* method (D). Hematoxylin counterstained. Objective magnification 40×(A-D)

is increasing, leading to development of end-stage liver disease including cirrhosis and HCC [4], it is important to define mechanisms of hepatitis C virus (HCV)-induced liver carcinogenesis with involvement of the Wnt/β-catenin signaling pathway. Already at the beginning of this century more numerous mutations of β-catenin were found to develop in HCV-associated HCC than with HBV-related HCC [5]. Recent years have brought the results of studies on direct interactions between oncogenic HCV proteins and components of the Wnt/β-catenin signaling pathway [6-10].

Double role of β-catenin

In its inactivated state, β-catenin is phosphorylated at its serine (SER)/threonine (THR) residues. It represents a component of a large cytoplasmic protein complex with glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1), the product of the APC (adenomatous polyposis coli) suppressor gene and axin/conductin [11, 12]. The complex controls intra-

cellular levels of β-catenin mainly through protein phosphorylation. Phosphorylation of the β-catenin N-terminus represents a pre-requirement for recognition by β-TrCP of an ubiquitin ligase E component, with its subsequent degradation in proteasomes. At the first stage phosphorylation of serine takes place in position 45 (SER^{45}) by CK1α/ε, and then SER^{33}, SER^{37} and THR^{41} by GSK3β [13]. Control of β-catenin phosphorylation also involves the Diversin protein: while CK1α binds directly to axin, CK1ε links the ankyrin fragment of the Diversin protein, forming a degradation complex [14]. Phosphorylation of β-catenin by GSK3β is much more effective in the presence of axin, and overexpression of conductin additionally augments degradation of β-catenin. In neoplastic tumors (including those in the liver) expression of conductin (but not axin) is frequently elevated and may represent an early diagnostic marker of certain tumors [15]. APC protein represents another protein involved in formation of the β-catenin destructive complex. The sites for β-catenin binding are located in its central portion [16]. The critical

variables for β-catenin activity involve nuclear export of APC (reducing its activity) or loss of the nuclear export signal (NES) sequence in the mutated APC (increasing transcriptional activity) [17].

β-catenin, together with the remaining catenins (α and γ) and E-cadherin, participates in formation of intercellular junctions of *zonulae adherens* type. It is located at the inner side of the cell membrane, and it secures the link between E-cadherin and cell cytoskeleton [18]. *Zonulae adherens* are also present between polarized epithelial cells, such as hepatocytes. Apical surfaces of the cells represent regions which generate bile canaliculi while membranes of basal hepatocyte portions adjoin sinusoidal endothelial cells. Junctions of *zonula adherens*, desmosome and nexus (gap junction) types are present on lateral surfaces of hepatocytes [19]. Tight junctions are areas of localized contact, found in the apical region of adjacent epithelial cells. In the liver they are situated close to capillary bile canaliculi, and they isolate the canalicular compartment from the intercellular space and hepatic sinusoids [19, 20]. A novel mechanism is suggested of cross-talk between specific components of tight and adherens junctions to regulate adhesion between hepatic cells [21]. β-catenin is encoded by the CTNNB1 gene (chromosome 3p21-p22), consisting of 16 exons (the first is a non-coding exon). It represents a highly conserved protein, formed of 781 amino acids (aa), and, together with plakoglobin, it belongs to the armadillo protein family [22].

Canonical pathway of Wnt/Fzd/β-catenin signaling

Stimulation of the canonical pathway induced by Wnt ligands takes place through one of the receptors belonging to the Frizzled (Fzd) family [23]. Out of the old two LRP (low density lipoprotein-receptor related protein) co-receptors, LRP-5 and -6, the latter is more important in formation of the Fzd-LRP complex [24]. The Fzd receptor, through its PDZ (P_{SD}-95/discs large/ZO-1 homologous) domain, recruits a cytoplasmic Dvl (disheveled) protein, which contains three conserved domains of DIX, PDZ and DEP. DIX domains and, probably, multimerization of Dvl protein, recruits the axin complex and activates GSK3β to phosphorylate the intracellularly located PPPSP motifs of the LRP co-receptor. This leads to inhibition of β-catenin phosphorylation and to its accumulation in cytoplasm [23]. The Dvl protein binds approximately 18 DAPs (Dvl-associated), including Nkd, Idax, Frodo, Dapper, GBP/Frat, Stbm, Daam1 and Pricle proteins, which may activate or inhibit Wnt signaling [25].

Activation of the Wnt canonical pathway results in inhibition of β-catenin phosphorylation and absence of the protein degradation. Its stabilization and accumulation in the cytoplasm facilitates transport of β-catenin to the cell nucleus. Through the development of a complex with LEF (lymphoid enhancer factor)/TCF (T cell factor), expression of various genes becomes intensified.

Individual Wnt ligands (19 cystein-rich glycoproteins) were qualified to form two groups. The first contains transforming glycoproteins with oncogenic properties, linked to the canonical pathway, including Wnt-1, -3a, -8 and -8b. The other group contains non-transforming proteins, activating the non-canonical pathway with activity opposite to that of first group ligands. They include Wnt-4, -5a and -11 [26]. In addition, it was demonstrated that certain non-canonical ligands (Wnt-4 and Wnt-5a) may induce β-catenin-dependent signals but only upon fusion with specific subtypes of Fzd receptors, and they manifest a selective dependence from LRP-5 and LRP-6 [24].

Receptors of Frizzled family

Fzd receptors represent a separate class (Frizzled) in the family of GPCRs (G-protein-coupled receptors), consisting of 10 isoforms of Fzd_{1-10} [27]. They are responsible for proliferation, differentiation and migration of cells, including hepatocytes [24]. Each of the receptors represents a protein with seven hydrophobic transmembrane domains, a *C*-terminal PDZ domain and an *N*-terminal extracellular cysteine-rich domain (CRD), which binds Wnt ligands [28]. Various Wnt ligands bind to distinct Fzd receptors [27]. For example, Fzd2 receptor, consisting of 565 aa (56% identical to a homologous receptor in *Drosophila*), is encoded on chromosome 17q21.1. [29, 30]. Human Fzd1 and Fzd7 receptors, with size of, respectively, 647 and 574 aa, have been mapped to chromosomes 7q21 and 2q33, respectively [30]. Recent reports indicate that the Fzd7 receptor undergoes overexpression in various tumors, including HCC. It plays a significant role in biology of stem cells, and in development and progression of malignant tumors [31].

In function of the canonical pathway of Wnt, transport of β-catenin to the cell nucleus has principal significance. The detailed mechanism of the transport (particularly in tumor cells) has not been fully clarified. Previously the process was suggested to involve the NLS, independently of involvement of the importin protein, as a result of a direct interaction with proteins of nuclear envelope pores [32]. Subsequent studies excluded presence of NLS in the β-catenin molecule [33, 34]. β-catenin undergoes translocation also in the reciprocal direction, from the cell nucleus to the cytoplasm. The export takes place in association with APC, axin [35] and RanBP3 (Ran binding protein 3) proteins [36]. Axin and APC augment cytoplasmic while TCF4 and BCL9/Pygopus augment nuclear expression of β-catenin, but this reflects its in-

creased accumulation in a given compartment rather than a stimulated transport [37]. Some investigators point to the need for activation of small GTPases and Rac-1 (Ras-related C3 botulinum toxin substrate 1) in the process of nuclear accumulation of β-catenin. Together with JNK2 (Jun N terminal kinase 2) and β-catenin it forms a triple cytoplasmic complex, causing phosphorylation of SER[191] and SER[605] in the β-catenin molecule, facilitating its transport to the cell nucleus [38]. Recent studies demonstrate that SER[23] undergoing glycosylation (O-GlcNAc modification) is responsible for subcellular localization and transactivation of β-catenin. Upon glycosylation of SER[23], β-catenin undergoes translocation from the cell nucleus to cell membranes. This is linked to amplification of β-catenin interaction with E-cadherin, a decreased β-catenin-TCF interaction, decreased transcriptional activity and Wnt target gene expression [39].

Following translocation to the cell nucleus, β-catenin binds to TCF/LEF transcription factors, belonging to HMG (High Mobility Group) box proteins. In mammals four genes encode TCF (TCF1, LEF1, TCF3 and TCF4). They associate with DNA sequences termed WRE (Wnt responsive element). In cases of absence of Wnt stimulation and upon absence of β-catenin in the cell nucleus, the TCF/LEF complex inhibits transcription of Wnt-dependent genes. It contains four domains, the *N*-terminal β-catenin-binding domain, the central domain, the HMG domain which binds to DNA, and also contains an NLS sequence as well as a long terminal C fragment [40]. Another element which co-operates with TCF in inhibiting transcription of Wnt-dependent proteins involves Groucho proteins (Grg-1, -2, -3, -4, -5) in *Drosophila* and homologous proteins in mammals, i.e. TLE-1, -2, -3, -4 (transducin-like enhancer split) and hAES (amine terminated enhancer split). The transcription-inhibiting mechanism employing Groucho/TLE is linked to histone deacetylase RPD3 from the HDAC-1 (histone deacetylase) protein group, responsible for development of a more compact chromatin structure and transcription repression [41].

E-cadherin/β-catenin complex in physiology

Catenins (including β-catenin) and E-cadherin (typical for epithelial cells) form a structural-functional E-cadherin-catenin unit (ECCU). Interactions between the proteins are not direct, and instead an allosteric switch in α-catenin may mediate actin cytoskeleton reorganization. The complex is controlled by processes of phosphorylation and endocytosis [42]. Cadherins are glycoproteins consisting of intracellular, transmembrane and extracellular portions. Apart from calcium ion-dependent control of cellular adhesion, they participate in tissue morphogenesis, recognition and grouping of appropriate cells,

maintenance of tissue coherence and coordination of cell translocation [43]. They are included in the superfamily of cell adhesion molecules, which in their extracellular portions contain cadherin repeats EC1-EC5. Within hepatic E-cadherin (liver-cadherin, LI-cadherin) DXNDN and DXD motifs were identified, responsible for binding calcium ions. LI-cadherin is localized to the basolateral domain of hepatocytes and enterocytes [44]. The cytoplasmic domain of classical cadherins is highly conserved, while its catenin-binding site has been mapped to 72 aa of the C-terminal portion of the E-cadherin molecule. This fragment of E-cadherin participates in interactions with cytoplasmic proteins and controls functions of cadherins [18, 45]. Six subfamilies of cadherins are distinguished, including the classical ones (type I), atypical ones (type II), present in desmosomes – desmocollin and desmoglein, protocadherins and Flamingo cadherin [46]. Epithelial E-cadherin was the first identified cadherin. It forms adherens junctions between epithelial cells, and belongs to classical cadherins, along with N-cadherins in nervous tissue, P-cadherins in placenta and R-cadherin in retina. β-catenin binds to a cytoplasmic domain of E-cadherin and through linkage with α-catenin it anchors it to actin of the cytoskeleton. The membranous domain of cadherin binds to p120 protein. It is indispensable for stabilization of E-cadherin and it fulfils functions controlling junctions between cadherin and the cytoskeleton through interactions with small GTPases of the Rho family. Also p120 protein represents a factor controlling the cadherin cycle [47]. Linkage between cadherin and β-catenin and between β-catenin and α-catenin is controlled by numerous kinases and phosphatases [42]. The process of E-cadherin degradation starts with phosphorylation of TYR within its molecule, followed by recognition and binding of Hakai protein (ubiquitin ligase E3) in a Src phosphorylation-dependent manner [48].

E-cadherin/β-catenin complex in pathology

Disturbances in structure and function of ECCU were detected in the process of organ fibrosis, including liver fibrosis [49]. The process is closely linked to decreased expression of E-cadherin and overexpression of β-catenin with its cytoplasmic translocation, which results in a loss of intercellular junctions [45]. Such alterations were detected in cells of biliary duct epithelium in patients with primary biliary cirrhosis, primary sclerosing cholangitis and in alcohol-induced hepatitis [50]. Also in hepatic stellate cells (HSCs) involvement of Wnt/β-catenin pathway components was demonstrated in mechanisms of liver cirrhosis. As compared to resting cells, activated HSCs were demonstrated to contain 3- to 12-fold increased quantities of mRNA for representatives of the canonical (Wnt-3a and -10b) and non-canonical (Wnt-4 and -5a) pathway

of Wnt, receptors Fzd-1 and -2 and for co-receptors LRP-6 and Ryk. This was accompanied by markedly increased nuclear expression of β-catenin. Activity of TCF-dependent genes was stimulated by Wnt-1 and inhibited by inhibitors of the Wnt pathway – small proteins of Chibby (blocking interactions of β-catenin with TCF) and Dkk-1 (blocking interactions of Wnt with LRP). Presence of Dkk-1 reduced agonist-stimulated activation of HSCs, while a high concentration of Dkk-1 intensified apoptosis in activated cultures of HSCs [51]. In another study, activation of HSCs proliferation was demonstrated and inhibition of TRAIL-induced apoptosis under the effect of Wnt-3a. The reciprocal relationship was also detected, or inhibition of activity and increased apoptosis in HSCs under the effect of an inhibitor of the Wnt pathway, i.e. SFRP 1 (Secreted frizzled-related protein 1) [52].

Disturbances in cadherin/catenin complex and epithelial-mesenchymal transition

The cadherin/catenin complex actively participates in epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET), which are important both in physiology (embryonic development) and in pathology (fibrosis of organs, carcinogenesis) [45]. The EMT process is characterized by de-differentiation of epithelial cells to fibroblasts and myofibroblasts, which produce components of extracellular matrix. Epithelial cells lose their marker proteins, such as E-cadherin, ZO-1 (*zonula occludens-1*) and cytokeratins, gaining phenotypic markers of mesenchymal cells, such as vimentin, α-smooth muscle actin (α-SMA) or fibroblast-specific protein-1 (FSP1). The cells of altered phenotype begin to produce mainly collagen type I and fibronectin [45]. EMT leads to a loss of intercellular junctions. A decrease in E-cadherin level results in release of β-catenin from its associations and facilitates EMT, while the restored presence of E-cadherin re-establishes the altered cell phenotype. Hakai protein participates in the dynamic recycling of E-cadherin, which modulates cell adhesion and is involved in EMT [48]. Intercellular junctions with E-cadherin also provide a target for ADAM 10 (A disintegrin and metalloproteinase 10). The protein cuts the extracellular domain of cadherin close to its transmembrane domain, releasing in parallel β-catenin. It may increase its transcriptional activity, augmenting expression of the gene encoding cyclin D1 [53]. Also the intracellular domain of cadherin may provide a target for proteolytic cuts exerted by presenilin, which results in a loss of cellular adhesion and increase in amounts of free β- and α-catenin [54]. Epigenetic alterations of E-cadherin are also described (methylation of the gene promoter), which may lead to lowered expression of the protein, progression of disease and development of neoplastic metastases [55]. Control proteins, containing zinc-finger proteins, coded by the gene families of Snail and Slug and SIP-1 (Smad interacting protein-1) represent negative controllers of the E-cadherin gene [56]. β-catenin is also involved in the TGF-β-dependent EMT [57]. In the absence of TGF-β, both E-cadherin and β-catenin undergo degradation, with the resulting loss of intercellular junctions. At the same time, cytoplasmic accessibility of β-catenin becomes augmented and its transport to the cell nucleus becomes possible [58].

Disturbances in Wnt/β-catenin pathway in liver carcinogenesis

Involvement of the canonical and non-canonical Wnt pathway in liver oncogenesis has been described by various investigators [59-67]. One of the most frequently described mechanisms for activation of the canonical signaling pathway in HCC involves activation of β-catenin through mutations in the CTNNB1 gene. This is accompanied by overexpression/repression of other genes involved in transmission of signals to the cell nucleus, with the resulting intensification of proliferation, migration and cellular invasion. At the molecular level, a characteristic trait described in hepatocellular tumors involves nuclear or cytoplasmic accumulation of β-catenin, detected in a higher proportion of cells in cases of hepatoblastoma (50-80%) than in HCC (8-40%) [60, 62-64, 68]. Taniguchi *et al*. detected *CTNNB1* mutations in 19% of HCC and in 70% of hepatoblastoma cases. They included mainly point mutations, and more than half of hepatoblastomas contained deletions. Approximately 50% of HCC with mutations of axin and conductin manifested accumulation of β-catenin in the cell nucleus, cytoplasm or on cell membranes [69]. In HCC a relationship was detected between nuclear location of the protein and more pronounced proliferative activity of hepatocytes and shorter survival of the patients [64], or the opposite: lower invasiveness of HCC and more frequent 5-year survival of the patients [62]. Relatively early, another role was suggested for wild-type β-catenin as compared to its mutated form. The mutated form of the protein was supposed to be linked to HCC subtypes with a better prognosis [62]. Nuclear localization of β-catenin may also be induced by the TGF-β signaling pathway, in response to trans-differentiation of neoplastic hepatocytes to immature liver progenitor cells. Nuclear expression of β-catenin was correlated with tumor invasion or relapses of HCC following liver transplantation [66].

Certain investigators detected a relatively high proportion of patients (62%) with non-nuclear accumulation of β-catenin (in cytoplasm/cell membranes), pointing to heterogeneous mechanisms of the protein accumulation in HCC [63]. Most of the observations point to the fact that mutations with-

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in *CTNNB1* are manifested in a later stage of liver cancer development while nuclear accumulation of β-catenin is detected in early stages of HCC development, suggesting other (distinct from mutations) mechanisms of stabilization involving the protein [63, 70, 71]. Using a model of transgenic mice, nuclear localization of β-catenin was demonstrated only in *adenoma* and in highly differentiated cancers of eosinophil phenotype, which also pointed to the fact that activation of the Wnt/β-catenin pathway with protein translocation of the cell nucleus represents an early stage of carcinogenesis [72]. Following years of investigations, two HCC subtypes were distinguished, depending on molecular alterations related to the Wnt/β-catenin pathway. The first one, with a mutation in *CTNNB1*, is characterized by increased expression of liver-specific targets. HCC of this subgroup represents well-differentiated tumors of a low histological malignancy, with stable chromosomes and a good prognosis. In the other subtype of HCC, also with the Wnt/β-catenin pathway activated, no β-catenin mutations are detected. The tumors are characterized by extensive dysregulation of the classical Wnt pathway, a significant degree of chromosome instability, aggressive phenotype, and they are preferentially linked to HBV infection [71, 73]. Interestingly, even if involvement of β-catenin is of key importance to embryonic development of liver and for processes of liver regeneration [2], activation of β-catenin itself remains insufficient to initiate *per se* the process of liver carcinogenesis [67, 74]: a transient hepatocyte hyperplasia was noted only, with no neoplastic transformation [74]. However, the activated β-catenin may cooperate with other pathways of oncogenesis, such as insulin/IGF-1/IRS-1/MAPK, H-RAS, MET, AKT or with chemical compounds which initiate carcinogenesis [74, 75]. Even if the mutated form of β-catenin is insufficient to trigger the process of HCC development, it promotes the process in another manner (increasing chromosome instability, amplifying action of other oncogenes) [67]. Amazingly, a phenomenon of sevenfold increase in development of liver tumors was detected in mice with *CTNNB1* knockout, as compared to control mice [76]. It seems paradoxical that both presence of the mutated β-catenin form and absence of wild type β-catenin amplifies the DEN (diethyl nitrosamine)-induced liver carcinogenesis in mice [67]. Mechanisms of HCC development in mice with a knockout of the β-catenin gene remain unknown.

Recent studies indicate involvement of the Wnt/β-catenin pathway in processes of self-renewal and expansion of liver cancer stem cells (CSCs), which may initiate HCC. The evidence is available for preferential activation of the Wnt/β-catenin pathway also within the pool of stem cells within a mature, regenerating liver, termed oval cells or hepatic pro-

genitor cells (HPCs) [77]. As progenitor cells, they manifest uninhibited growth, which makes them similar to cancer cells and suggests that disturbed control over their division may provide a cause for development of HCC. This has been corroborated in studies on animal models [78]. Liver diseases leading to development of cancer also frequently lead to activation of HPCs, which may suggest that it is precisely this group of cells which provides a starting point for HCC development [79]. In a significant proportion of HCC, one or more markers of HPCs can be detected, which are absent in normal mature hepatocytes [80, 81]. In the oval cells, stimulated to proliferation, an increase was detected in Wnt-3-induced dephosphorylated β-catenin in the cell nucleus and augmented transcriptional activity in the Wnt/β-catenin/TCF pathway, with activation of the cell cycle [82]. In another investigation, increased amounts of total and active (dephosphorylated) β-catenin forms were detected in the cytoplasm and the cell nucleus. The increased expression of β-catenin was accompanied by increased amounts of Wnt-1 in the neighboring hepatocytes and augmented expression of the Fzd-2 receptor in oval cells, in parallel with reduced expression of WIF-1, an inhibitor of Wnt. An additional proof for involvement of the Wnt/β-catenin pathway in proliferation of oval cells was provided by the dramatic reduction in the number of the cells in livers of rodents devoid of the β-catenin gene [83]. Signals of the Wnt/β-catenin pathway may also affect the microenvironment of HCC and in this way may affect survival and growth of neoplastic cells [67].

β-catenin and E-cadherin, as components of the Wnt signaling pathway, have been placed on the list of serum markers of liver carcinogenesis [84]. In sera of HCC patients (etiologically linked to infection with HCV genotype 4) with liver cirrhosis, significantly higher levels of four proteins were detected, including β-catenin and E-cadherin, as compared to sera of patients with chronic HCV infection with no cancer and sera of control individuals [84]. Summing up the above, it may be accepted that β-catenin probably plays a role in initiation of hepatic oncogenesis and, at subsequent stages, the non-canonical pathway of Wnt becomes mobilized [67].

Wnt/β-catenin pathway in HCV-associated liver carcinogenesis

Studies on involvement of HCV in liver carcinogenesis developing through modulation of the Wnt/β-catenin signaling pathway have been conducted since the 1990s. At the beginning, nuclear accumulation of β-catenin was demonstrated in HCC, on the background of HCV infection and in association with mutations in the β-catenin gene, which were detected in 26-41% of patients with HCC [61, 85]. Activation of the Wnt/β-catenin signaling path-

way and its involvement in liver carcinogenesis were also linked to axin mutations [86], inactivation of GSK-3β [87], dephosphorylation of β-catenin [59] and up-regulation of Fzd-7 [88]. Zhang *et al*. demonstrated that also the up-regulated microRNA-155 (miR-155), markedly increased in HCV-infected patients, activates the Wnt signaling pathway with nuclear accumulation of β-catenin and the accompanying increase in cyclin D1, c-Myc, and survivin. It was also determined that a direct and functional target of miR-155 involved APC [89]. However, it was not until *in vitro* studies were conducted that interactions between HCV proteins and the Wnt/β-catenin signaling pathway were clarified. In HepG2 cell lines both NS5A protein and the entire HCV polyprotein were demonstrated to be responsible for the increase in β-catenin level (protein accumulation and stabilization, decreased degradation in proteasomes) in cells with expression of the HCV genome products. This was developing in the mechanism of a reduced activity manifested by the FKHR (forkhead transcription factor) and increased phosphorylation of GSK-3β [6]. Thus, the elevated cellular level of β-catenin resulted from activation of the PI3K/Akt signaling pathway. This caused augmented transcription of β-catenin-dependent genes and was supposed to facilitate neoplastic transformation of HCV-infected hepatocytes. Involvement of NS5A protein in activation of the Wnt/β-catenin signaling pathway was confirmed in subsequent studies [7], documenting direct activation of endogenous, unphosphorylated wild-type β-catenin by NS5A protein and co-localization of the two proteins in cytoplasm of HepG2 cells. The mechanism of β-catenin accumulation at the protein level, also through inactivation of GSK-3β, was confirmed. In addition, the investigators proved that NS5A protein may directly interact with β-catenin through its N-terminus and the ARM 1-6 region of β-catenin [7]. The authors also succeeded in demonstrating that the N terminus of NS5A affects TCF-4-dependent transcriptional activity. In other studies, evidence was provided for a role of NS5A in binding of the p85 regulatory subunit of phosphoinositide-3 kinase (PIK3) and, in consequence, in stabilization of β-catenin, independently of effector kinases for PIK3, i.e. Akt and GSK-3β. Both ends of the NS5A protein (N and C) were found indispensable for the direct binding of β-catenin and for full activation of the protein within the Wnt pathway [8]. Recent studies of Higgs *et al*. demonstrated a direct role for NS5A protein in β-catenin-dependent c-Myc expression [90].

Direct activation of the Wnt/β-catenin pathway was demonstrated in an *in vitro* model also separately for the core (C) protein of HCV [9, 10, 91]. HCV-core transfected Huh7 cells up-regulated Wnt-1 and WISP-2 transcription [91]. The cells demonstrated intensified proliferation, DNA synthesis and pro-

gression of the cell cycle [91]. In both studies by Liu *et al*., core protein of HCV amplified the TCF-dependent transcriptional activity, intensified expression and stabilized β-catenin at the protein level in Huh7 cells through inactivation of GSK-3β. It proved to be responsible for amplification of cell proliferation and promotion of tumor growth following action of one of the Wnt pathway ligands, the Wnt-3a protein [9, 10]. Core protein of HCV increases active β-catenin and nuclear accumulation in SMMC-7721 cells. Up-regulation of gene expression involving many Wnt ligands (Wnt-2, -3, -3a, -10a, -10b, Fzd-1, -2, -3, -6, -7, -9, and LRP5/6 co-receptors) was demonstrated [10]. HCV also affects in a twofold way expression of E-cadherin, indirectly by modulation of the Wnt/β-catenin pathway and directly with mediation of HCV core protein. C protein diminishes expression of E-cadherin at the transcriptional level, through methylation of CpG islands in the promoter of the CDH1 gene [92, 93].

Recent studies brought proof for HCV involvement also in EMT [94-96]. In cultures of HCC cells infected with genotype 1b or 2a of HCV, increased expression of numerous EMT markers (including vimentin, snail, slug and twist proteins) was demonstrated and a decrease in E-cadherin expression, as well as an altered phenotype of hepatocytes, with higher expression of fibroblast-specific protein 1 (FSP-1) and elevated levels of β-catenin phosphorylated at Ser[552] [94]. Grégoire *et al*. suggested that neither Hedgehog nor β-catenin is required for NS5A-mediated EMT [96]. The study of Quan *et al*. strongly suggests that the HCV core-induced epigenetic silencing of SFRP (secreted frizzled-related protein) family may lead to activation of the Wnt signaling pathway and increase HCC aggressiveness through induction of EMT [97].

Clinicopathological role of β-catenin and E-cadherin expression in hepatocellular carcinomas

β-catenin represents a recognized oncogene, and both qualitative (pattern of expression) and quantitative evaluation of tissue expression of the protein permitted genetically distinct subsets of HCC to be distinguished [5, 62, 71, 73]. In most HCCs, a variable percentage of cells is noted with abnormal localization of β-catenin (i.e. cytoplasmic, nuclear, or C/N) [59, 61-66, 68, 69, 101]. Nuclear localization of the protein most frequently correlated with somatic mutations of β-catenin [5, 59, 62, 102], although descriptions of nuclear accumulation of the protein are available in cases free of the gene mutation [63]. The percentage of cells with β-catenin mutation in HCC is quantitatively quite variable (from a few to a few dozen percent) [59, 63, 64, 68, 103]. Mutations

in the β-catenin gene seem to be more frequent in HCC with the background of HCV than HBV infection [5].

In HCC most frequently tissue overexpression of the protein is noted [63, 101, 103], but studies are also available which manifest lower expression of the protein in cancer than in the control [62, 104, our own unpublished data]. Recently, a subgroup of patients with HCC has been distinguished (~15%) with complete absence of tissue β-catenin expression [105].

Most positive correlations between invasive character of HCC, high metastatic potential of HCC, poorer cellular differentiation, and shorter survival of patients involve manifestation of nuclear expression or overexpression of β-catenin, independently of localization of the protein [63, 64, 103]. On the other hand, individual studies describing reduced expression of β-catenin [62, our own unpublished data], or even its absence in HCC in a proportion of the patients [105], document absence of significant correlations between the expression on one hand and invasiveness and prognosis of HCC on the other [62], and in the case with complete absence of the protein significantly lower fibrosis and inflammation, but unremarkable differences in proliferation [105]. At present, attempts are being undertaken to evaluate numerous immunohistochemical markers (in parallel with β-catenin) of a high negative predictive value in HCC, such as glutamine synthase (one of the transcriptional targets of β-catenin) [105].

Changes in expression of the other ECCU component, i.e. E-cadherin, in HCC are more frequently linked to epigenetic alterations in the *CDH1* promoter than to gene mutations [55, 102]. In HCC mainly a decrease in tissue expression of E-cadherin used to be described, as compared to the control [104, our own unpublished observations]. However, also variable (both decreased and augmented) expression of the protein was described in the studied group of HCC [102]. Individual studies documented increased accumulation of the protein in HCC cells [106]. No nuclear localization of E-cadherin was described. In cases with parallel examination of both ECCU proteins the decreased expression of E-cadherin and overexpression of β-catenin was found to be correlated with lymph node invasion, poor pathological stage, TNM stage, and worse prognosis [101]. Correlations were demonstrated between lowered expression of E-cadherin (or its loss) on one hand

and advanced stage, poorly differentiated histology and relapse of HCC following operation on the other [107].

Until now, the variability of tissue expression manifested by β-catenin and E-cadherin in the entire HCC group has not permitted the proteins to be recognized as independent prognostic indices in HCC [104, our own unpublished observations]. Examination of the proteins' expression is not recommended in the routine histopathological diagnosis of HCC. Nevertheless, the quoted results of studies point to complex relationships between tissue expression of the principal representative of the Wnt canonical pathway (β-catenin) and E-cadherin on one hand and histopathological indices of HCC invasion or clinical data of the patients on the other. In our opinion, further studies should be devoted to developing a more uniform scale for quantitative evaluation of the proteins in tissue material which would allow one to draw more reliable conclusions from meta-analysis of the data. In cases of HCV-associated HCC in parallel to expression of β-catenin and E-cadherin, it would be important to examine tissue expression of HCV viral proteins (core, non-structural proteins) [our own unpublished data].

In HCC treatment using therapy targeted at the Wnt/β-catenin pathway, inhibitors of the pathway remain in preclinical evaluation, and only a few compounds have started to reach the phase I clinical trials [review of the topic: 67]. In the opinion of the authors, an ideal antagonist of the Wnt pathway would involve a drug which would exert its action in the cell nucleus. In Poland the only registered systemic drug for HCC targeted therapy involves the multikinase inhibitor sorafenib [108]. Targeted therapy in HCC requires analysis of multiple serum and tissue biomarkers. Uniform quantitative analysis in cases of tissue expression manifested by Wnt/β-catenin pathway proteins may prove to be an invaluable tool in classification for treatment. The individualized targeted therapeutic strategies in HCC should also take into account molecular interactions between the Wnt pathway and fragments of the HCV genome.

The most important *in vitro* and *in vivo* studies on Wnt/β-catenin signaling pathway components in HCV-related hepatocellular carcinomas are summarized in Table I.

The authors declare no conflict of interest.

Table I. Wnt/β-catenin signaling pathway components in human HCV-associated hepatocellular carcinoma

Model of the study	Method of detection	Summary of the findings	Ref.
HCV-related HCCs (n = 22)	RT-PCR; IHC; SSCP analysis and direct DNA sequencing for mutations	β-catenin mutations in 9 (41%) cases; nuclear accumulation of β-catenin in all 9 tumors with a β-catenin mutation and 2 additional tumors without a mutation	Huang et al., 1999 [61]
HCCs associated with HCV (n = 51); HBV (n = 26) or excess alcohol intake (n = 23)	Methylation-specific PCR; differential PCR; SSCP analysis and direct DNA sequencing for mutations	Mutations (single nucleotide substitutions at different putative phosphorylation sites of contiguous residues) of β-catenin in 13-31% of cases; the frequencies of mutations were not significantly different between HCV-related HCCs, those with HBV (19%) and associated with alcohol (13%)	Edamoto et al., 2003 [98]
Raji cells and Ramos cells	Cloning and sequencing of PBMC, cellular, and tumor DNA; cell culture; plasmids; ligation-mediated PCR; gene knock-down by using small interfering RNA (siRNA)	Acute and chronic HCV infection caused a 5- to 10-fold increase in mutation frequency in Ig heavy chain, BCL-2, p53, and β-catenin genes of in vitro HCV-infected B cell lines and HCV-associated PBMC, and HCCs	Machida et al., 2004 [99]
HepG2 cells	Plasmid constructs; cell culture; generation and propagation of recombinant baculoviruses; luciferase assays; IF; immunoblotting analyses	NS5A protein expressed either alone or in the context of complete HCV polyprotein mediated increase in GSK-3β phosphorylation, an increase in the overall levels of β-catenin and resulted in a concomitant increase of β-catenin-dependent transcription within the cells. The effects were dependent on the Akt pathway	Street et al., 2005 [6]
Huh-7, HepG2, Hep3B, and FOCUS cells	Plasmids; cell culture and transfection; immunoblot; IF; cell proliferation assays; cell cycle analysis; microarray analysis; RT-PCR and real-time PCR	HCV core protein induces Huh-7 cell proliferation whether alone or in the context of HCV replication, which was at least partly mediated by transcriptional upregulation of growth-related genes, in particular Wnt-1	Fukutomi et al., 2005 [91]
HepG2 cells	Plasmids; transfection and luciferase assay; RNA interference system; RT-PCR and real-time PCR analysis; western blot analysis; methylation specific PCR; indirect IF; cell aggregation assay; transwell cell migration assay	HCV core protein represses E-cadherin expression via upregulation of both DNA methyltransferase 1 (DNMT1) and DNMT3b enzymes, which leads to hypermethylation and inactivation of E-cadherin promoter; core-expressing cells exhibit altered morphology, reduced cell-to-cell adhesion, and increased cell invasion ability	Arora et al., 2008 [92]
CH-C or CH-B (n = 19); HCC (n = 48) (protein assay group); CH-C or CH-B (n = 22) and HCC (n = 23) (validation group)	Antibody array platform and immunoassay; data extraction; bioinformatics analysis	7 proteins significantly differentiated HCC patients from hepatitis patients, which include CTNNB; 8 proteins significantly differentiated HCC patients with "normal" levels of AFP from hepatitis patients, which include CTNNB; plasma levels of CTNNB were significantly higher in the HCC group	Sun et al., 2008 [100]
Liver specimens; Huh7, HepG2, Cos7 cells	Plasmids; cell culture; transfection, and HCV infection; luciferase reporter gene assays; immunoblot analysis; Glutathione S-transferase pull-down assays and co-immunoprecipitation; confocal microscopy	NS5A protein directly interacts with endogenous β-catenin and colocalizes with β-catenin in cytoplasm; NS5A protein inactivates GSK-3β and increases subsequent accumulation of β-catenin in HepG2 cells and in HCV patients' liver tissues	Park et al., 2009 [7]
Cos-7 and Huh-7 cells	DNA manipulation and constructs; cell culture; luciferase assays; western blotting; immunoprecipitations; IF	N and C termini of NS5A are required for full activation of β-catenin; NS5A either alone or in complex with p85 is able to bind directly to β-catenin; this interaction is augmented by PI3K	Milward et al., 2010 [8]
Huh-7, Huh-7.5 cells	Cell culture and transfection; quantitative methylation specific PCR analysis; immunoblot; indirect IF; real-time PCR	E-cadherin (CDH1) promoter was hypermethylated in genotype 1b HCV core protein-positive cells; genotype 1b HCV core protein expression induces sirtuin 1 (SIRT1) upregulation	Ripoli et al., 2011 [93]
HEK293 cells; HepG2, Huh-7 and SMMC-7721 cells	Cell culture; plasmids; luciferase assay; IF; western blotting; RT-PCR analysis; MTS proliferation assay; crystal violet cell viability assay; cell cycle analysis; xenograft tumor model of human HCC in athymic nude mice; IHC	HCV core protein enhances Tcf-dependent transcriptional activity induced by Wnt3A in HCC cell lines; increases and stabilizes β-catenin levels in Huh-7 cell line through inactivation of GSK-3β; core protein also increases cell proliferation rate and promotes Wnt3A-induced tumor growth in the xenograft tumor model of human HCC	Liu et al., 2011 [9]

Table I. Cont.

Model of the study	Method of detection	Summary of the findings	Ref.
HEK293 cells and the human hepatoma cell line SMMC-7721	Cell culture; plasmids; luciferase assay; RT-PCR; western blotting; IF; MTS proliferation assay; crystal violet staining	HCV core protein plays role in activating β-catenin/Tcf-4-dependent transcriptional activity and increases β-catenin expression and nuclear accumulation of the protein; core protein upregulates gene expression of canonical Wnt ligands (Wnt-2, -3, -3a, -8b, -10a, -10b, Fzd-1, -2, -5, -6, -7, -9, and LRP5/6 co-receptors)	Liu et al., 2011 [10]
Patient's serum samples with HCV genotype 4-associated HCC patients (n = 32); CH-C patients (n = 28; asymptomatic carriers (ASC) with non-cirrhotic CH-C (n = 11)	ELISA	Serum β-catenin levels were significantly elevated in patients with HCC compared to those with CH, ASC and healthy controls. Among the six studied markers, β-catenin was also found to be the only marker that can discriminate between patients with HCC and those with chronic hepatitis	Zekri et al., 2011 [84]
Human hepatic tissues: controls; CH-C (n = 34), HCV-associated HCC (n = 10); Huh-7 cell line	Cell culture; RT-PCR, real-time PCR; cell transfection and stimulation; western blot analysis, cell proliferation assay; flow cytometric analysis	HCV infection resulted in NF-κB-dependent up-regulation of miR-155 expression, which promoted tumorigenesis by increasing Wnt signaling; APC, which negatively regulates Wnt signaling, was identified as the functional target of miR-155	Zhang et al., 2012 [89]
IHH, Huh-7 cells; biopsy specimens from HCV-infected patients (n = 10)	Generation of cell culture-grown HCV; EMT arrays; western blot analysis; IF; β-galactosidase staining for cellular senescence	HCV infected hepatocytes (HCV genotype 1a or 2a) displayed a fibroblast-like shape and an extended life span. Increased mRNA and protein expression levels of vimentin, snail, slug, and twist; loss of the epithelial cell marker E-cadherin; primary human hepatocytes infected with HCV display EMT via activation of the Akt/β-catenin signaling pathway	Bose et al., 2012 [94]
BMEL cells; HCV-infected primary human hepatocytes	Cell culture; TGF-β reporter assay; retroviral constructs and RNA interference; Western blot and IF; qPCR; cell tracking by time-lapse microscopy; wound-healing assay; invasion assay; xenograft model	Expression of NS5A HCV in primary hepatic precursors and in IHH cell lines gave rise to profound modifications of cell polarity, leading to EMT; the effects of NS5A were additive to those of TGF-β; NS5A cooperates with oncogenic Ras, giving rise to transformed, invasive cells that are highly tumorigenic in vivo	Akkari et al., 2012 [95]
Hepatocyte cell lines harboring an HCV replicon and the infectious HCV strain JFH1; transgenic murine model expressing the entire HCV ORF	Cell culture	Increased c-Myc expression; activation of Akt by NS5A, and the subsequent stabilization of β-catenin; β-catenin-dependent c-Myc expression led to increased production of ROS, mitochondrial perturbation, enhanced DNA damage and aberrant cell-cycle arrest	Higgs, et al., 2013 [90]
Huh-7, HepG2 cells	Cell culture	HCV core protein downregulates SFRP1 expression by inducing hypermethylation of the SFRP1 promoter, which may lead to activation of the Wnt signaling pathway and contribute to HCC aggressiveness through induction of EMT; core protein markedly increases the expression level and binding of DNA methyltransferase-1 and histone deacetylase-1, resulting in epigenetic silencing of SFRP1 expression	Quan et al., 2013 [97]
BMEL cells; HCV-infected primary human hepatocytes	Cell culture; TGF-β reporter assay; retroviral constructs and RNA interference; Western blot and IF; qPCR; cell tracking by time-lapse microscopy; wound-healing assay; invasion assay; xenograft model	No evidence either of increased expression of components of Wnt/β-catenin pathway or, more significantly, of sustained transcriptional activation of axin2 in BMEL-NS5A cells undergoing EMT; their results suggest that β-catenin signaling is not required for NS5AC-mediated EMT	Grégoire et al., 2013 [96]

APC – adenomatous polyposis coli; BMEL – bipotential mouse embryonic liver; CH – chronic hepatitis; CH-C/B – chronic hepatitis C/B; ELISA – enzyme-linked immunosorbent assay; EMT – epithelial to mesenchymal transition; FOCUS – (Friendship of China and United States) – human hepatocellular carcinoma cell line; HCGs – human hepatocellular carcinomas; IHC – immunocytochemistry; IHH – immortalized human hepatocytes; miR-155 – microRNA-155; ORF – open reading frame; PBMC – peripheral blood mononuclear cell; RT-PCR – reverse transcription polymerase chain reaction; SFRP1 – secreted frizzled-related protein 1; ʼTGF-β – transforming growth factor β; SSCP – single-strand conformation polymorphism

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