Downregulation of GSK3β and Upregulation of URG7 in Hepatitis B-Related Hepatocellular Carcinoma

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Abstract

Hepatitis B virus (HBV) is the leading cause of hepatocellular carcinoma (HCC). The exact molecular contributors to the development of HBV-related HCC are not yet completely understood. Recent studies demonstrated that the deregulation of the Wnt pathway is highly associated with the development of HCC. Besides, HBV is known to have roles in the deregulation of this pathway. The present study evaluated the molecular aspects of the Wnt pathway in HBV-related HCC in liver tissue samples. Viral characterization was done by identifying the HBx mutations and the assessment of intrahepatic viral load. The expression of Wnt pathway genes was assessed using real-time PCR and methylation-specific PCR. The intrahepatic viral load was significantly higher in tumor samples than in normal tissues (P = 0.0008). Aberrant expression was observed in Wnt-1, Wnt-7a, FZD2, FZD7, β-catenin, URG7, c-Myc, SFRP5, and Gsk3β, among which Wnt1, FZD2, SFRP5, Gsk3β, and URG7 were associated with HBV. HBx mutations at positions I88, L116, and I127 + F32 were associated with the decreased expression of GSK3β and overexpression of URG7 and Wnt. Alterations in the expression level of β-catenin, as well as some mutants of HBx, were correlated with the level of c-Myc. HBV-related HCC seems to be mostly coordinated with epigenetic behaviors of HBx, such a multi-functional peptide with suppressing/trans-activating functions.

Keywords: HBV, HCC, Wnt Pathway, HBx, Mutation, Gene Expression

1. Background

Hepatocellular carcinoma (HCC) is the most prevalent primary liver malignancy in adults and is the third main cause of cancer-related deaths in humans. Viral infections, including hepatitis B virus (HBV) and hepatitis C virus (HCV), are the leading causes of HCC (1). The underlying mechanisms of HCC include a complex of multistep molecular signaling pathways that handle alterations in the genetic figures of the virus and/or the host (2). Nevertheless, the precise molecular mechanisms by which HBV contributes to the development of HCC are not yet completely understood. However, recent studies have identified that some biological pathways are more involved in the underlying mechanisms of HCC development, including the wingless-related integration site (Wnt) pathway, EGF-R, Ras/MAPK, and PI3K/Akt (3).

The Wnt pathway is a highly conserved critical pathway that is important in the regulation of cell polarity and differentiation and plays a role in several tumors such as HCC (4, 5). β-catenin is the key element of the Wnt pathway that may be mutated and/or overexpressed in an aberrant state of this pathway (6-8). In most adult tissues, the Wnt pathway remains inactive or steady. This physiologic state is regulated through phosphorylation of β-catenin by the Gsk3β and CK enzymes that lead to degrading β-catenin, thus preventing its transportation to the nucleus (9). However, in an active Wnt state, the Wnt ligands interact with the Frizzled class receptors (FZD) receptors, thereby sta-
bilizing cytoplasmic \( \beta \)-catenin, leading to nuclear trans-
portation, which, in turn, induces transcription through TCF/LEF and target genes involved in cell proliferation (10).

Hepatitis B virus is a major leading cause of HCC, with
887,000 deaths in 2015, largely due to cirrhosis and HCC
(11). Moreover, HBV-related HCC has shown an increasing
trend in the last decades in developed countries (12). Al-
though the involvement of HBV-related mechanisms in the
development of HCC is not yet completely understood,
some features of HBV are proposed, including higher viral
loads in chronic HBV infection (13), HBV genotypes C and
F (14), integration of viral genes into the human genome,
viral proteins and last but not least, mutations in viral pro-
teins (HBx, HBsAg) (13-15). As a pleiotropic protein, HBx is
known for the transcriptional regulation and activation of
a variety of host genes involved in epigenetic modification
and control of cell proliferation. Therefore, the investi-
gation of its oncogenic potential has been hyped recently as
an attractive field of interest (16). Recent studies demon-
strated that HBx has potential roles in the regulation of
a variety of signaling transduction pathways, including
Wnt/\( \beta \)-catenin, Ras/MAPK, SAPK/JNK, and NF-\( \kappa \)B pathways
(17, 18). Several lines of studies indicated that HBV also pro-
duces mutant forms of HBx that are involved in carcino-
genesis through the epigenetic regulation of several path-
ways and/or tumor suppressor genes (19, 20).

2. Objectives

In this work, we attempted to study the Wnt pathway
with a comprehensive gene expression analysis along with
the virological characteristics for the elucidation of poten-
tial interactions between HBV and Wnt pathway status.

3. Methods

3.1. Patients and Samples

A cross-sectional study was conducted using fresh
frozen (FF) samples taken of surgical resection by fine need-
le biopsy in hospitals affiliated to Iran University of Medi-
cal Sciences (IUMS). The FF samples included HCC tissues
and some other tissues that were later identified as his-
tologically normal. The study retrospectively investigated
formalin-fixed paraffin-embedded (FFPE) samples, includ-
ing HCC and non-tumor tissues, as well. Indeed, the control
group included liver samples routinely gathered for med-
ical reasons and later determined as histologically normal.
The stored samples had been collected from March 2010
to August 2019, and FF samples from May 2017 to Septem-
ber 2019. The FF and FFPE samples were histologically ex-
amined by an expert pathologist for the confirmation of
HCC or normal histology. The serologic data including the
level of liver enzymes, HBV, and HCV seromarkers, were ob-
tained from the medical records of patients in the hospit-
als. All participants signed informed consent forms, and
the proposal of this study was approved by the Research
Ethics Committee of IUMS (ethics code: IR.IUMS.FMD.REC
1396.9321540004).

3.2. DNA Extraction

One milligram (mg) of each tissue sample was sub-
jected to DNA isolation using DNA extraction kits (MN,
Macherey-Nagel GmbH & Co. KG) according to kit in-
structions with minor modifications. The FFPE samples
were first cut in 15-\( \mu \)m thicknesses, followed by paraffin
removal using xylene and washing with 100% ethanol. Sub-
sequently, 1 mg of the obtained tissue was used for DNA ex-
traction, as mentioned above. The concentration and pu-
urity of isolated DNA were checked by BioPhotometer and
agarose gel electrophoresis, and DNA isolation was con-
ﬁrmed by performing a PCR for \( \beta \)-globin, as described pre-
viously (21).

3.3. RNA Extraction and cDNA Synthesis

A tissue RNA extraction kit (NucleoSpin® total RNA,
MN, Macherey-Nagel GmbH & Co. KG) was used for the ex-
traction of RNA from FF samples. The FFPE samples were
first cut in 15-\( \mu \)m thicknesses, followed by paraffin removal
using xylene and washing with 100% ethanol. For the im-
provement of RNA isolation from FFPE samples, we used a
further processing method, and then the processed sam-
ple were subjected to the above-mentioned kit, as de-
scribed in the Supplementary section. The purity and con-
centration of obtained RNA were checked by a BioPho-
tometer (ratio of 260/280 was optimized between 1.7 and
2). The concentration and integrity of RNA were observed
with agarose gel electrophoresis.

For cDNA synthesis, extracted RNA was treated with
DNase I (Yekta Tajhiz Azma, YTA, Iran) to remove any
contaminating genomic DNA. Next, the cDNA synthesis
was done by using random hexamer primers according
to the instruction of the cDNA synthesis kit (Fermentas,
Thermo Fisher Scientific TR Limited, Waltham, Mas-
sachusetts, United States).

3.4. Determination of HBx Mutations

The HBV infection was investigated by partial ampli-
fication of the small S gene, as described previously (22-
24). A conventional nested-PCR was performed to amplify
the HBx gene, as described previously, with some modifi-
cations (24). For the determination of HBx mutations, PCR
products were sequenced and aligned with an attributed
reference sequence in BioEdit software (GQ183486).
3.5. Assessment of Intrahepatic Viral Load

Five microliters ($\mu$L) of the extracted DNA were used for the determination of the viral load in liver tissues according to a previously described quantitative method with minor modifications (23). The primers and probe sequences were as follows: BSF: 5'-TCGGGGCCTCGTCTCTGAA-3', BSR 5'-AGGACAAACGGGCAACATA-3', probe: 5'-FAM-ACTACCGTGTGTCTTGGCC-TAMRA-3'. These primers correspond to a highly conserved region of the S gene that covers all HBV genotypes. The reaction was performed in a three-step program: Initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 15 s, 58°C for 15 s, and 72°C for 20 s. A cloned segment of the small S gene was serially diluted tenfold and used for the construction of standard curves and quantification in each run. The concentration of intrahepatic HBV load in the samples was expressed as copies/$\mu$L of extracted DNA (for all samples, $1\ mg$ of tissue was used for DNA extraction and eluted from the spin filter with $50\ \mu$L elution buffer).

3.6. Investigating the Expression Level of Wnt Pathway Genes

Among the key elements of the Wnt pathway, 14 genes were selected for the assessment of gene expression using real-time PCR. The selection of these genes was based on a literature review of previous in-vitro and/or in-vivo studies that demonstrated the deregulation of different Wnt genes (primers designed and used for the gene expression assay are provided in the supplementary method). Due to the baseline degradation of RNA in FFPE samples (Supplementary File), there was a minor shift in the raw Ct data between paired FF and FFPE samples. Although there was a substantial correlation between the Cts of paired FF-FFPE samples, the mean Pearson’s correlation coefficient was 0.86 $\pm$ 0.11. The amplification program was carried out in a Rotor-Gene 3000 instrument using SYBR-PCR master 2X (Yekta Tajhiz Azma, YTA, Iran). Samples were tested in duplicate, and the mean cycles of threshold were considered. Values were normalized relative to the expression of two internal housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hydroxymethylbilane synthase (HMBS)). The $2^{\Delta\Delta Ct}$ formula was used for the calculation of relative gene expression levels. In this way, $\Delta\Delta Ct$ is equal to the Ct of target gene minus the mean Ct of GAPDH and HMBS, and $\Delta\Delta Ct$ is equal to tumor tissue $\Delta Ct$ minus normal sample $\Delta Ct$ (25).

3.7. Methylation Analysis

Downregulated genes were analyzed further by methylation specific-PCR (MSP). In this method, 2 $\mu$g of extracted DNA underwent bisulfite genomic treatment (MaxSpin Bisulfite Modification Genomic DNA, Maxcell Company, Iran). The MSP analysis was applied for SFRP1, SFRP5, and CDH1, as described previously (18, 26). The hypermethylation and suppressed status of SFRP1 had already been established among different cancer cell lines such as HeLa and Caco-2 (27, 28); thus, we used HeLa and Caco-2 cells as positive controls of methylated DNA.

3.8. Statistical Analysis

All qualitative and quantitative variables were analyzed by the chi-square ($\chi^2$) test or Fisher’s exact tests, Mann-Whitney test, and student t-test using SPSS version 25. The measurement of $\Delta\Delta Ct$ and folding of expression levels was conducted in Excel. The graphical analysis of gene expression was performed by Graph Pad Prism 6.

4. Results

4.1. Baseline Characteristics of the Population

A totally of 161 samples were examined including HBV-HCC ($n = 79$), non-viral HCC ($n = 41$), and tissues with normal histology ($n = 41$). Demographic and clinical data of the patients are summarized in Table 1. The mean ALT enzyme level was 66.6 $\pm$ 43.2 IU/L, and the mean AST level was 96.4 $\pm$ 70.1 in the tumor group that were significantly higher than the normal range ($P = 0.009$ and $P = 0.005$, respectively). In the control group, samples with normal results in terms of liver enzymes, blood sugar, LDL, and bilirubin levels were selected for the gene expression assay. Besides participants of the control group of gene expression analysis, all had a negative result for viral markers.

| Table 1. Demographic and Clinical Information of the Population Studied* |
|---------------------------------------------------------------|
| Characteristics | HBV-HCC ($N = 79$) | Non-Viral HCC ($N = 41$) | Normal Liver ($N = 41$) |
| Gender | | | |
| Male | 61 (77.2) | 28 (68.3) | 22 (52.8) |
| Female | 18 (22.8) | 13 (31.7) | 13 (37.1) |
| Age | | | |
| Mean ± SD | 50.7 ± 14.2 | 59.2 ± 14.2 | 50.5 ± 14.5 |
| < 40 | 4 (5.1) | 5 (12.2) | 7 (20) |
| 40 - 50 | 9 (11.4) | 5 (12.2) | 9 (25.7) |
| 51 - 60 | 23 (29.1) | 6 (14.6) | 11 (31.4) |
| > 60 | 43 (54.4) | 25 (61) | 8 (22.8) |
| HBV+ | 79 (100) | 0 | 16 (39) |
| Cirrhosis | 23 (29.1) | 7 (17) | - |

*Values are expressed as Mean ± SD or No. (%).
4.2. Viral Characteristics

Tissue samples were subjected to DNA isolation and then tested for HBV-DNA. Overall, 59% of the samples were found to be positive for HBV, while the rate was 79/120 (65.8%) among tumor samples. All the HBV-positive tissues were investigated for the determination of intrahepatic viral load. Among 79 HBV-positive tumor samples, 14 (17.7%) had no detectable liver viral load based on real-time PCR. The mean intra-hepatic viral load among HCC cases was $12 \times 10^6 \pm 11 \times 10^6$ copies/$\mu$L of extracted DNA while it was $4.2 \times 10^6 \pm 5.6 \times 10^6$ copies/$\mu$L among HBV-positive tissues with normal histology ($P = 0.0008$, Figure 1). None of the participants had a positive co-infection result for seromarkers of HCV, HDV, and HIV.

Figure 1: Comparison of intrahepatic viral load of tumor samples and normal cases (Mann Whitney test).

4.3. Expression Level of Wnt Pathway Key Elements

The results of real-time PCR showed an aberrant expression pattern in genes that were assessed in the Wnt pathway (Figure 2). The results showed an increased expression level for Wnt-1, Wnt-7a, FZD2, FZD7, β-catenin, URG7, and c-Myc among the HCC cases. Although there was a significantly decreased expression level for SFRP5 and GSK3β, the observed decreases for CDH1 and SFRP1 were not significant (Figure 2). This result was coherent with the result of MSP analysis, which showed the hypermethylation of SFRP5, SFRP1, and CDH1 genes (Figure 2).

4.4. Expression Level of Wnt Genes in Association with HBV

The association of HBV infection with the deregulation of Wnt genes was investigated. There was a more aberrant expression level among several studied genes in HBV-HCCs than in non-viral ones (Figure 3). The expression levels of Wnt-1, FZD2, and URG7 were much higher in HBV-infected HCC tumors, and there was a significant down-expression for SFRP1 and GSK3β (Figure 3).

4.5. Correlation Between the Level of β-catenin and c-Myc

The higher expression of β-catenin leads to cytoplasmic accumulation and further nuclear translocation of this protein, which induces TCF/LEF-mediated transcription of targeted genes. Downstream target genes of the pathway include c-Myc, Cyclin D1, CCN2, connective tissue growth factor (CTGF), (CCN5) Wnt1 inducible signaling pathway protein subfamily 2 (WISP2), c-jun, and c-fus, whose upregulation causes transformation and cancer progression in various tumors (5, 9, 29, 30). The cellular proto-oncogene c-Myc is widely accepted as an upregulated tumor factor in liver malignancies (31, 32). Therefore, we assessed the expression level of c-Myc as one of the downstream targets of the Wnt/β-catenin pathway. Tumor samples with higher expression of β-catenin were significantly correlated with the higher level of c-Myc expression (Figure 4). Therefore, the evaluation of the c-Myc level can be used as a reliable consequence of Wnt pathway activation, as indicated previously (31, 32).

4.6. Wnt Pathway in Association with HBx Mutants

To check the potential effect of different HBx mutants on the Wnt pathway, the HBx region of the HBV genome was sequenced in both HCC-HBV and normal groups. The results revealed that F30V, T36D, I88F/M, and F132I were the most prevalent HBx mutations observed in HBV-HCCs (Table 2). The prevalence of F30V, T36D, I88F/M, and I127T/L mutations was much higher in tumor samples than in normal HBV cases, among which I127T/L was statistically significant.

We also surveyed which mutations were more likely associated with deregulations that were in association with HBV-HCCs (deregulated genes found in Figure 3). Comparative analysis between the expression levels of c-Myc among samples harboring different HBx mutants revealed that C69R, I88F/M, S101Y, L116V, and a double mutation at P = 0.0008 (Figure 3). The prevalence of F30V, T36D, I88F/M, and I127T/L mutations was much higher in tumor samples than in normal HBV cases, among which I127T/L was statistically significant.

We also surveyed which mutations were more likely associated with deregulations that were in association with HBV-HCCs (deregulated genes found in Figure 3). Comparative analysis between the expression levels of c-Myc among samples harboring different HBx mutants revealed that C69R, I88F/M, S101Y, L116V, and a double mutation at I127 and F132 had roles in the aberration of the β-catenin pathway (Figure 3B). Then, the deregulated genes were analyzed in terms of mRNA expression among cases with different HBx mutations. It was determined that mutations at the protein binding domain of HBx (aa 51 - 151) had a significant impact on deregulation. Overall, URG7 and glycogen synthase kinase 3 beta (GSK3β) were the most prominent genes whose expression was affected by HBx. Mutations, including I88F/M and L116V, were independently associated with the significant depression of GSK3β. There was also a higher expression of Wnt1 among samples with S101Y mutation. The double mutation I127L + F132I was associated with the overexpression of URG7 (Figure 5). The significance of all mutations of HBx in association with the Wnt genes is illustrated in the Supplementary section (Supplementary File).
5. Discussion

Chronic HBV infection is now determined as a principal risk factor responsible for the development of HCC. However, the molecular mechanisms of HBV involvement are not yet clarified. This may be due to different etiologies and several risk factors contributing to HCC, such as aflatoxin, alcohol, other viruses, heterogeneity, and geographical distribution of patients. Meanwhile, the deregulation of some critical pathways, such as the Wnt pathway, is now known as distinctive in the development of HCC. Therefore, the gene expression profile of the Wnt pathway and HBV characteristics were investigated in this research.

In this study, the profiling of mRNA expression was applied for Wnt pathway genes and downstream targets. There was an aberrant Wnt pathway among HCC tumors. We also observed the overexpression of Wnt-1, Wnt-7a, FZD2, URG7, and β-catenin, and down-regulation of SFRP5 and GSK3β. The deregulation of different Wnt pathway genes has been repeatedly described in the recent decade (1, 31, 32), which supports the result of the current study.

Mutations in the CTNNB1 gene are major activators
of the Wnt pathway with an elevated figure of cytoplasmic β-catenin accumulation. Tumor samples of this study had been previously surveyed for CTNNB1 mutations; cases with mutations in the hot spots of β-catenin had elevated levels of β-catenin and had correlations with the increased expression of cell-myelocytomatosis (c-Myc) \((38)\). Nevertheless, previous studies showed nuclear accumulation of β-catenin in the absence of activating mutations in hot spots of the CTNNB1 gene \((39, 40)\). Therefore, there might be other external or internal factors disrupting the pathway and contributing to the degrading complex (adenomatous polyposis coli (APC), protein phosphatase, component of the beta-catenin destruction complex required for regulating CTNNB1 levels (Axin), GSK3β, and casein kinase 1 family (CK1)) and cytoplasmic stabilization of β-catenin. HBV is a potential external factor in modulation of the Wnt pathway with the benefit of having proteins with some protein binding activities such as HBx, S, and/or Core pro-
teins, which can dysregulate the canonical Wnt pathway (14, 16, 18, 19).

There is a consensus that a higher viral load of HBV is correlated with a greater risk of exacerbation of liver diseases such as liver cirrhosis (LC) and HCC (14, 41, 42). Generally, active HBV infection is measured by serum viral loads for therapeutic objectives; however, hepatocytes are the main replicative targets of HBV, and the liver is the most important tissue for identifying the course of HBV infection (43). Our results showed higher intrahepatic viral loads in the tumor group than in the non-tumor group, as described in some previous studies (43-45). Moreover, previ-
ous studies found a direct correlation between serum and hepatic viral load (44, 45).

The activation of the Wnt pathway causes the activation and overexpression of downstream target genes such as c-Myc that can be assessed for the determination of Wnt pathway status. In the current study, tumor samples with elevated levels of β-catenin were significantly correlated with the increased expression of c-Myc; this finding is in line with previous reports (31, 32, 46). Thus, the surveillance of c-Myc can be used as an authentic consequence of active Wnt pathway and overexpression of β-catenin, as described previously (31, 32). However, the result of the current study is based on the expression level of mRNA and needs further assays with the assessment of protein expression levels.

Moreover, it has been found that HBx is a binding partner of APC that can displace β-catenin from its degradation complex (16), thus elevating the cytoplasmic accumulation of β-catenin and subsequently leading to the overexpression of c-Myc. In this study, F30V was the most prevalent HBx mutation; however, it has been previously described concerning HCC and reduced replication efficiency.
of HBV (19). Gene expression analysis determined that tumor samples with I88F/M and L116V were suppressed in the level of GSK3β, and the double mutation of I127L + F132I was associated with the overexpression of URG7. Moreover, URG7 is a recently discovered spliced variant of the ABCG6 gene, which is upregulated by the induction of HBx (47). This protein has been shown to activate the β-catenin pathway through either inhibiting GSK3β or transactivating the promoter of the CTNNBI gene (10). Besides, in this study, there was a positive correlation between the mRNA level of URG7 and β-catenin.

The basal Core promoter (BCP) region of HBV harbors most mutations that highly increase the risk of HCC (14, 48). It is noteworthy to mention that most of the regulatory elements of HBV such as enhancer II, Core promoter, BCP, and direct repeat 1 (DR1) are completely or partly within the HBx coding region (48). Therefore, mutations in HBx can affect the HCC progression through the regulation of HBV replication and/or via direct interaction of HBx with host regulatory proteins, as discussed above. In the BCP region, there were some mutations among which, I127T/L was most prevalent; it is a well-known HBx mutation related to a higher risk of HCC (20). This mutation, along with F132I, was associated with the overexpression of URG7.

The protein binding capability of HBx has been shown to target DNA methyltransferases and subsequently downregulate the host genes (48, 49). This phenomenon has been identified for HBx-mediated suppression of CDH1 and SFRP1 genes (18, 26, 50). Furthermore, our MSP analysis represented the hypermethylation of CDH1, SFRP1, and SFRP5 in HBV-HCCs, demonstrating an epigenetic silencing event directed by HBx. The repression of SFRP1 and CDH1 leads to the subcellular localization of β-catenin that results in more cell proliferation.

In conclusion, HBV has significant roles in the activation of the Wnt pathway. Among the studied genes, it seems that URG7 and GSK3β are the most prominent molecular targets of HBx in the Wnt pathway that can be elegant choices for future investigations. Regarding the transactivating and oncogenic potential of HBx that has significant roles in HBV replication and regulation of the Wnt pathway, it’s targeting as a therapeutic approach might have a promising perspective.

**Supplementary Material**

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

**Footnotes**

**Authors’ Contribution:** Idea, design, and experiments: Davod Javanmard and Seyed Hamidreza Monavari. Methodology and technical support: Davod Javanmard, Mohammad Najafi, Mohammad Hadi Karbalaei Niya, Farah Bokharai-Salim, Maryam Eshghezai, and Ma sood Ziaee. Sampling and patient data: Davod Javanmard, Fahimeh Safarnezhad Tameshkel, Mahshid Panahi, Maryam Mehrabi, and Mohammad Reza Babaie. Drafting of the manuscript, analyzing data, and critical revision: Davod Javanmard, Davod Kalafkhany, and Seyed Jalal Kiani.

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**References**

1. Lin X, Wang Q, Cao Z, Geng M, Cao Y, Liu X. Differential expression of wnt pathway genes in sporadic hepatocellular carcinomas infected with hepatitis B virus identified with OligoGE Arrays. Hepat Mon. 2013;13(1):e6092. doi: 10.5812/hepatmon.6092. [PubMed: 23483081]. [PubMed Central: PMC3589875].

2. Pezzuto F, Izzo F, Buonaguro L, Annunziata C, Tatangelo F, Botti G, et al. Tumor specific mutations in TERT promoter and CTNNBI gene in hepatitis B and hepatitis C related hepatocellular carcinoma. Oncotarget. 2016;7(34):54253-62. doi: 10.18632/oncotarget.9801. [PubMed: 27276731]. [PubMed Central: PMC5342139].

3. M IS, Uribe D, Jaramillo CM, Osorio G, Perez JC, Lopez R, et al. Wnt/β-catenin signaling pathway in hepatocellular carcinomas cases from Colombia. Ann Hepatol. 2015;14(1):64-74. doi: 10.1016/s1665-2681(19)30802-6.
4. van Zuylen WJ, Rawlinson WD, Ford CE. The Wnt pathway: A key network in cell signalling dysregulated by viruses. Rev Med Virol. 2016;26(5):534–55. doi: 10.1002/rmv.1892. [PubMed: 27273590].

5. Schweigert A, Fischer C, Mayr D, von Schweinitz D, Kappler R, Hubertus J. Activation of the Wnt/beta-catenin pathway is common in wilms tumor, but rarely through beta-catenin mutation and APC promoter methylation. Pediatr Surg Int. 2016;32(12):1241–6. doi: 10.1007/s00380-016-0370-6. [PubMed: 27769598].

6. Chen SP, Wu CC, Huang SY, Kang JC, Chiu SC, Yang KL, et al. beta-catenin and Kras mutations and RASSF1A promoter methylation in Taiwanese colorectal cancer patients. Genet Test Mol Biomarkers. 2012;16(1):1277–81. doi: 10.1089/gtmb.2012.0126. [PubMed: 23009572].

7. Pentheroudakis G, Kotteas EA, Koutoula V, Papadopoulou K, Charalambous E, Cervantes A, et al. Mutational profiling of the RAS, PIK3, MET and b-catenin pathways in cancer of unknown primary: A retrospective study of the Hellenic Cooperative Oncology Group. Clin Exp Metastasis. 2004;21(7):769–9. doi: 10.1023/B:CEMM.0000034992.03664.e6. [PubMed: 24997756].

8. Ding SL, Yang ZW, Wang J, Zhang XI, Chen XM, Lu FM. Interaclonal interaction between Wnt3 and Frizzled-7 leads to activation of Wnt signaling. Cancer Lett. 2019;470(2):340–55. doi: 10.1016/j.canlet.2010.09.018. [PubMed: 20971552].

9. Tai D, Wells K, Arcaroli J, Vanderbilt C, Aisner DL, Messersmith WA, et al. Targeting the WNT signaling pathway in cancer therapeutics. Clin Exp Metastasis. 2010;27(5):319–28. doi: 10.1007/s10447-009-9964-x. [PubMed: 20441510].

10. van Zuylen WJ, Rawlinson WD, Ford CE. The Wnt pathway: A key network in cell signalling dysregulated by viruses. Rev Med Virol. 2016;26(5):534–55. doi: 10.1002/rmv.1892. [PubMed: 27273590].

11. Al-Qahtani AA, Al-Anazi MR, Nazir N, Gahi R, Abdo AA, Sanai FM, et al. Hepatitis B virus (HBV) X gene mutations and their association with liver disease progression in HBV-infected patients. Oncotarget. 2017;8(36):50115–25. doi: 10.18632/oncotarget.22428. [PubMed: 29285338].

12. Javanmard D, Behravan M, Ghanadkafci M, Salehabadi A, Ziae M, Namaei MH. Detection of chlamydia trachomatis in pap smear samples from South Khorasan Province of Iran. Int J Fertil Steril. 2018;12(3):11–6. doi: 10.22074/ijfs.2018.5064. [PubMed: 29314204]. [PubMed Central: PMC5767929].

13. Ziae M, Javanmard D, Sharifzadeh G, Hasan Namaei M, Azarkar G. Genotyping and mutation pattern in the overlapping MHR region of HBV isolates in Southern Khorasan, Eastern Iran. Hepat Mon. 2016;16(10). e7806. doi: 10.5812/hepatmon.37806. [PubMed: 27882062]. [PubMed Central: PMC511392].

14. Azarkar Z, Ziae M, Ebrahimzadeh A, Sharifzadeh G, Javanmard D. Epidemiology, risk factors, and molecular characterization of occult hepatitis B infection among anti-hepatitis B core antigen alone subjects. J Med Virol. 2019;91(4):665–22. doi: 10.1002/jmv.25343. [PubMed: 30345528].

15. Javanmard D, Namaei MH, Farahmand M, Ziae A, Amini E, Ziae M. Molecular and serological characterization of occult hepatitis B virus infection among patients with hemophilia. J Med Virol. 2019;91(8):3519–27. doi: 10.1002/jmv.25899. [PubMed: 30908666].

16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8. doi: 10.1016/S1046-2023(01)00030-9. [PubMed: 11486609].

17. Lee JW, Kwon HW, Jung JK, Choi KH, Min DS, Jang KL. Hepatitis B virus X protein represses E-cadherin expression via activation of DNA methyltransferase 1. Oncogene. 2005;24(44):6655–27. doi: 10.1038/sj.onc.1208487. [PubMed: 16007168].

18. Vincent KM, Postovit LM. A pan-cancer analysis of secreted Frizzled-related proteins: re-examining their proposed tumour suppressive function. Sci Rep. 2017;7:42719. doi: 10.1038/srep42719. [PubMed: 28282991]. [PubMed Central: PMC516967].

19. Shin H, Kim JH, Lee YS, Lee YC. Change in gene expression profiles of secreted-frizzled-related proteins (SRFPs) by sodium butyrate in gastric cancers: induction of promoter demethylation and histone modification causing inhibition of Wnt signaling. Int J Oncol. 2012;40(5):1333–42. doi: 10.3892/ijol.2012.1327. [PubMed: 22245241].

20. Ding F, Wang M, Du Y, Du S, Zhu Z, Yan Z. BHx inhibits the wnt signaling pathway by suppressing beta-catenin transcription in the nucleus. Sci Rep. 2016;6:38313. doi: 10.1038/srep38313. [PubMed: 27910912]. [PubMed Central: PMC513598].

21. Liu XL, Meng J, Zhang XT, Liang XH, Zhang F, Zhao GR, et al. ING5 inhibits lung cancer invasion and epithelial-mesenchymal transition by inhibiting the WNT/beta-catenin pathway. Thorac Cancer. 2019;10(4):848–55. doi: 10.1111/1759-7743.13053. [PubMed: 30802861]. [PubMed Central: PMC6449268].

22. Cui J, Zhou X, Liu Y, Zhang T, Remeih M. Wnt signaling in hepatocellular carcinoma: Analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes. J Gastroen- terol Hepatol. 2003;18(3):280–7. doi: 10.1046/j.1440-1746.2003.02971.x. [PubMed: 12605282].

23. Kim M, Lee HC, Tasedosnorn O, Hartley R, Lim YS, Yu E, et al. Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/beta-catenin signaling pathway in hepatocellular carcinoma cells. J Hepatol. 2008;48(5):780–91. doi: 10.1016/j.jhep.2007.12.020. [PubMed: 18137877]. [PubMed Central: PMC239089].

24. Shahrornadi S, Yahyapour Y, Mahmodi M, Alavian SM, Fazeli Z, Jazayeri SM. High prevalence of occult hepatitis B virus infection in...
children born to HBsAg-positive mothers despite prophylaxis with hepatitis B vaccination and HBIG. J Hepatol. 2012;57(3):515–21. doi: 10.1016/j.jhep.2012.04.021. [PubMed: 2267152].

34. Kim W, Lee S, Son Y, Ko C, Ryu WS. DDB1 stimulates viral transcription of hepatitis B virus via HBx-independent mechanisms. J Virol. 2016;90(20):10364–53. doi: 10.1128/JVI.00977-16. [PubMed: 27535046]. [PubMed Central: PMC5068577].

35. Kim H, Lee SA, Kim BJ. X region mutations of hepatitis B virus related to clinical severity. World J Gastroenterol. 2016;22(24):5467–78. doi: 10.3748/wjg.v22.i24.5467. [PubMed: 27350725]. [PubMed Central: PMC4917607].

36. Zhang Q Yin J, Zhang Y, Deng Y, Ji X, Du Y, et al. HLA-DP polymorphisms affect the outcomes of chronic hepatitis B virus infections, possibly through interacting with viral mutations. J Virol. 2013;87(22):12176–86. doi: 10.1128/JVI.02073-13. [PubMed: 24006435]. [PubMed Central: PMC3807892].

37. Lin CL, Chu YD, Yeh CT. Emergence of oncogenic-enhancing hepatitis B virus X gene mutants in patients receiving suboptimal entecavir treatment. Hepatology. 2019;69(5):2292–6. doi: 10.002/hep.30423. [PubMed: 30552766]. [PubMed Central: PMC6644294].

38. Javanmard D, Najafi M, Babaei MR, Karbalaei Nyia MH, Eghaei M, Panahi M, et al. Investigation of CNVNI genes mutations and expression in hepatocellular carcinoma and cirrhosis in association with hepatitis B virus infection. Infect Agent Cancer. 2020;5(3):2170-0. doi: 10.1186/s13027-020-00297-5. [PubMed: 32514293]. [PubMed Central: PMC7268124].

39. Austiniat M, Dunsch R, Wittenkind C, Tannapfel A, Gebhardt R, Gautzsch F. Correlation between beta-catenin mutations and expression of Wnt-signaling target genes in hepatocellular carcinoma. Mol Cancer. 2008;7(2):118. doi: 10.1186/1476-4598-7-21. [PubMed: 18282277]. [PubMed Central: PMC2287886].

40. Okabe H, Kinoshita H, Imai K, Nakagawa S, Higashi T, Arima K, et al. Diverse basis of beta-catenin activation in human hepatocellular carcinoma: Implications in biology and prognosis. PLoS One. 2016;11(4), e0152695. doi: 10.1371/journal.pone.0152695. [PubMed: 2700093]. [PubMed Central: PMC4839611].

41. Chen C, Yang HI, Hu J, Chen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295(13):65-73. doi: 10.1001/jama.295.1.65. [PubMed: 1639218].

42. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: Special emphasis on disease progression and prognostic factors. J Hepatol. 2008;48(2):333-52. doi: 10.1016/j.jhep.2007.11.011. [PubMed: 18096267].

43. Biazar T, Yahyapour Y, Hasanjani Roushan MR, Rajabnia R, Saedeghi M, Taheri H, et al. Relationship between hepatitis B DNA viral load in the liver and its histology in patients with chronic hepatitis B. Caspian J Intern Med. 2015;6(4):209-12. doi: 10.3748/cjm.1561.1.6(4).209. [PubMed Central: PMC4649269].

44. Bayram A, Ertikli S, Ozkur A, Bayram M, Sari I. Quantification of intrahepatic total hepatitis B virus DNA in chronic hepatitis B patients and its relationship with liver histology. J Clin Pathol. 2008;61(3):338-42. doi: 10.1136/jcp.2007.050765. [PubMed: 17693576].

45. Wong DK, Yuen MF, Tse E, Yuan H, Sum SS, Hui CK, et al. Detection of intrahepatic hepatitis B virus DNA and correlation with hepatic necroinflammation and fibrosis. J Clin Microbiol. 2004;42(9):3920-4. doi: 10.1128/JCM.42.9.3920-3924.2004. [PubMed: 15664969]. [PubMed Central: PMC356337].

46. Elmileik H, Paterson AC, Kew MC. Beta-catenin mutations and expression, 249serine p53 tumor suppressor gene mutation, and hepatitis B virus infection in southern African Blacks with hepatocellular carcinoma. J Gastroenterol. 2005;40(4):58-61. doi: 10.1002/jso.20304. [PubMed: 16121349].

47. Pan J, Lian Z, Wallet S, Feitelson MA. The hepatitis B x antigen effector, URG7, blocks tumour necrosis factor alpha-mediated apop- tosis by activation of phosphoinositol 3-kinase and beta-catenin. J Gen Virol. 2007;88(Pt 12):3275-85. doi: 10.1099/vir.0.83214-0. [PubMed: 18024896].

48. Meier-Stephenson V, Bremner WR, Dalton CS, van Marle G, Coffin CS, Patel TR. Comprehensive analysis of hepatitis B virus promoter region mutations. Viruses. 2018;10(11). doi: 10.3390/v10110603. [PubMed: 30388827]. [PubMed Central: PMC6265984].

49. Wei X, Xiang T, Ren G, Tan C, Liu R, Xu X, et al. miR101 is down- regulated by the hepatitis B virus x protein and induces aberrant DNA methylation by targeting DNA methyltransferase 3a. Cell Signal. 2013;25(8):1476-85. doi: 10.1016/j.cellsig.2012.10.013. [PubMed: 23124077].

50. Takagi H, Sasaki S, Suzuki H, Toyota M, Nojima M, Nojima M, et al. Frequent epigenetic inactivation of SFRP genes in hepatocellular carcinoma. J Gastroenterol. 2008;43(5):378-89. doi: 10.1007/s00535-008-2170-0. [PubMed: 18592156].

51. Shen Z, Tang J, Cai X, Huang Y, Gao Q, Liang L, et al. HBx mutations promote hepatoma cell migration through the Wnt/beta-catenin sig- naling pathway. Cancer Sci. 2016;107(10):1380-9. doi: 10.1111/cas.13014. [PubMed: 27420728]. [PubMed Central: PMC5084678].