The emerging role of epigenetics (DNA methylation) in hepatitis C virus-induced hepatocellular carcinoma

Ayma Aftab[1], Humera Naveed[1], Habiba Idrees[2], Liaqat Ali[3], Muhammad Idrees[1] and Samia Afzal[1*]

[1] *Division of Molecular Virology and Infectious Diseases, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore 54590, Pakistan*
[2] *CMH Lahore Medical College & IOD, Lahore 54810, Pakistan*
[3] *Department of Biological Sciences, National University of Medical Sciences (NUMS), Rawalpindi, Punjab 46000, Pakistan*
* Corresponding author, E-mail: samiaraza@live.com

Abstract

Epigenetics has enthralled humanity as a form of genetic alteration that may underlie different human cancers. Among these cancers, hepatocellular carcinoma (HCC) is a threatening disease and one of the leading causes of cancer-related death worldwide. This review attempts to summarize the scientific work examining the hypermethylation of promoters for tumor suppressor genes to hepatitis C virus (HCV) infection and the development of HCC. Data shows that the development of HCC is a multistep phenomenon where varying promoter methylation profiles can be observed. Moreover, HCV-mediated HCC has been linked to hypermethylation of tumor suppressor genes. Evidence suggests that HCV Core, NS3, NS4b, and NS5a can potentially inhibit tumor suppressor genes. Therefore, HCV genes disrupt the function of host tumor suppressor genes by inducing epigenetic modifications that result in tumor progression.

Background

Epigenetics has been reported as a major factor in diseases like diabetes[1], genomic imprinting[2], and various cancers[3]. Increasing recognition of the role of epigenesis has altered scientific approaches to understanding cancer-related genes. Large-scale scrutiny of these aberrations indicates that the reduced transcription of major regulatory genes leads to malignant phenotypes[4]. Subtle modifications alter the DNA, or the associated histones can be passed on to future progeny. The reversible nature of these modifications makes them a striking area of research for epi-drug development[5].

Genetic instability and mutations have been considered the critical factor in most cancers. Current studies propose that epigenetic alteration is an underlying cause of clonal expansion in tumors. Over the last three decades, research investigating epigenetic defects identified several potential mechanisms, including acetylation, phosphorylation, ubiquitylation, sumoylation, DNA methylation, histone modification, nucleosome remodeling, and non-coding RNA-mediated epigenetics[4]. Scientists have prominently recognized methylation and histone modification as key processes that drive genomic imprinting through interdependent pathways[6].

DNA methylation and histone modifications are crucial epigenetic mechanisms driving hepatocellular carcinoma (HCC) by altering gene expression[7]. DNA methylation occurs at CpG islands which are rare throughout the human genome but are often found in the proximity of promoters. 50-70% of all CpG islands in the heterochromatic region are methylated, while unmethylated CpG islands in euchromatin regions ensure the transcriptional activation of the regulatory genes[6,8–10]. The promoters of malignancy suppressor or tumor activator genes associated with 5' CpG islands are methylated by DNA methyltransferases (DNMT3a and DNMT3b)[11]. S-adenosyl methionine acts as a methyl group supplier to methyltransferases[12] and therefore mediates cancer development. Hypermethylation, and hypomethylation can hamper the cell cycle by either silencing or activating regulatory genes. In particular, hypomethylation plays a major role in DNA replication leading to genetic disruption and chromosomal instability[13]. On the other hand, hypermethylation of 5' CpG islands in the vicinity of tumor-suppressor gene promoters impedes transcription, thus, leading to enhanced tumor formation[14].

Histone modifications are a post-translational modification that involves acetylation and methylation of lysines at the histone tails in both euchromatin and heterochromatin[15]. There are four core histones H2A, H2B, H3, and H4, that form an octamer around which DNA is coiled. During acetylation, histone acetyltransferase enzymes (HATs) catalyze histone N-terminal lysine residues, while deacetylation involves histone deacetylases (HDACs)[16]. The opposite charges on the positive lysine in histones and negative DNA allow them to form a compact chromatin structure that can conceal regions of DNA from transcription factors. Acetylation of H3 and H4, therefore, abolishes the charge from lysine and loosens the compact bundle of DNA and histones. This exposes different promoter regions and allows the binding of transcription factors. Lysine methyltransferases perform mono-, di-, and tri-methylation of histone lysine residues. In cancer cells, the methylation pattern is altered in H3K9 and H3K27[17]. Current studies have disclosed that H3K9, H3K27, and H4K20 mono-methylation stimulate gene transcription[18,19], whereas H3K9, H3K27, and H4K20 tri-methylation leads to epigenetic silencing[19]. Now, epigenetic research has shown that the centromeric histone H3 variant leads to the formation of CENP-A stable chromatin, which gives the centromere an epigenetic label. During the cell cycle, the presence of CENP-A ensures a balance between cell division and centromere propagation[20].

The main difference between DNA methylation and histone modification is the relative stability of the interaction. DNA methylation is a more stable modification, while on the other hand, histone modifications are more transient[14], and strenuous to analyze. Later, scientists observed that DNA methylation and histone modification complement each other during developmental stages[21].

Notably, epigenetic pathways are involved in the main type of primary liver cancer, hepatocellular carcinoma (HCC)[22]. Different geographical areas show variations in the rate of occurrence and etiological factors for HCC. Several environmental factors involved in HCC development have been linked to epigenetic changes, including hepatitis B virus (HBV), and hepatitis C virus (HCV) infection, chronic alcohol intake, and aflatoxin[23]. Research to understand the mechanisms involved in liver cirrhosis revealed many pathways that showed association with HCC. These pathways include the p53 pathway, the Rb pathway, the transforming growth factor-beta (TGF-β) pathway, and the Wnt/beta-catenin pathway. The irregular signaling in these pathways might be due to the activation of oncogenes and silencing of tumor suppressor genes[24].

HCV is the cause of widespread chronic liver disease. Almost 58 million people are infected with HCV worldwide[25]. HCV is a member of the Flaviviridae family. This 9.6 kb RNA virus was first reported in 1989[26,27]. The genome comprises a single open reading frame that encodes a polyprotein of 3,000 amino acids and non-translated regions situated at the 5' and 3' terminus[28]. Various studies have reported adverse effects of HCV infection on both oncogenes and tumor suppressor genes that in turn disturb cellular

pathways. This would lead to chromatin remodeling, RNA-associated silencing, silencing of tumor suppressor genes by hypermethylation, and oncogene activation by hypomethylation[29].

Several notable tumor suppressor genes are *p53, Rb, p21, p27, p14ARF, E-cadherin, Axin 1, APC, SOCS1/3, RASSF1A, NORE1, KLF6,* and *FHIT,* which have been studied in a plethora of research. HCV proteins hypermethylated CpG islands within the promoters of these genes, which silences their transcription[29] and allows an E2F transcription factor-mediated increase in unwanted cell growth. Figure 1 depicts a general concept of HCV-mediated HCC. It also shows various tumor suppressor genes that play a vital role at different stages of the cell cycle.

In this review, HCV-mediated methylation of tumor suppressor genes will be discussed to provide an abridged overview of this fatal disease.

Retinoblastoma gene and HCV

The retinoblastoma gene, *Rb*, is one of the most well-studied tumor suppressor genes that obstruct the cell cycle at G1 and that entry into the S-phase. *Rb* silencing has been observed in various cancers such as soft tissue carcinoma, retinoblastoma, osteosarcoma, lung cancer, breast cancer, and prostate cancers[30]. *Rb*
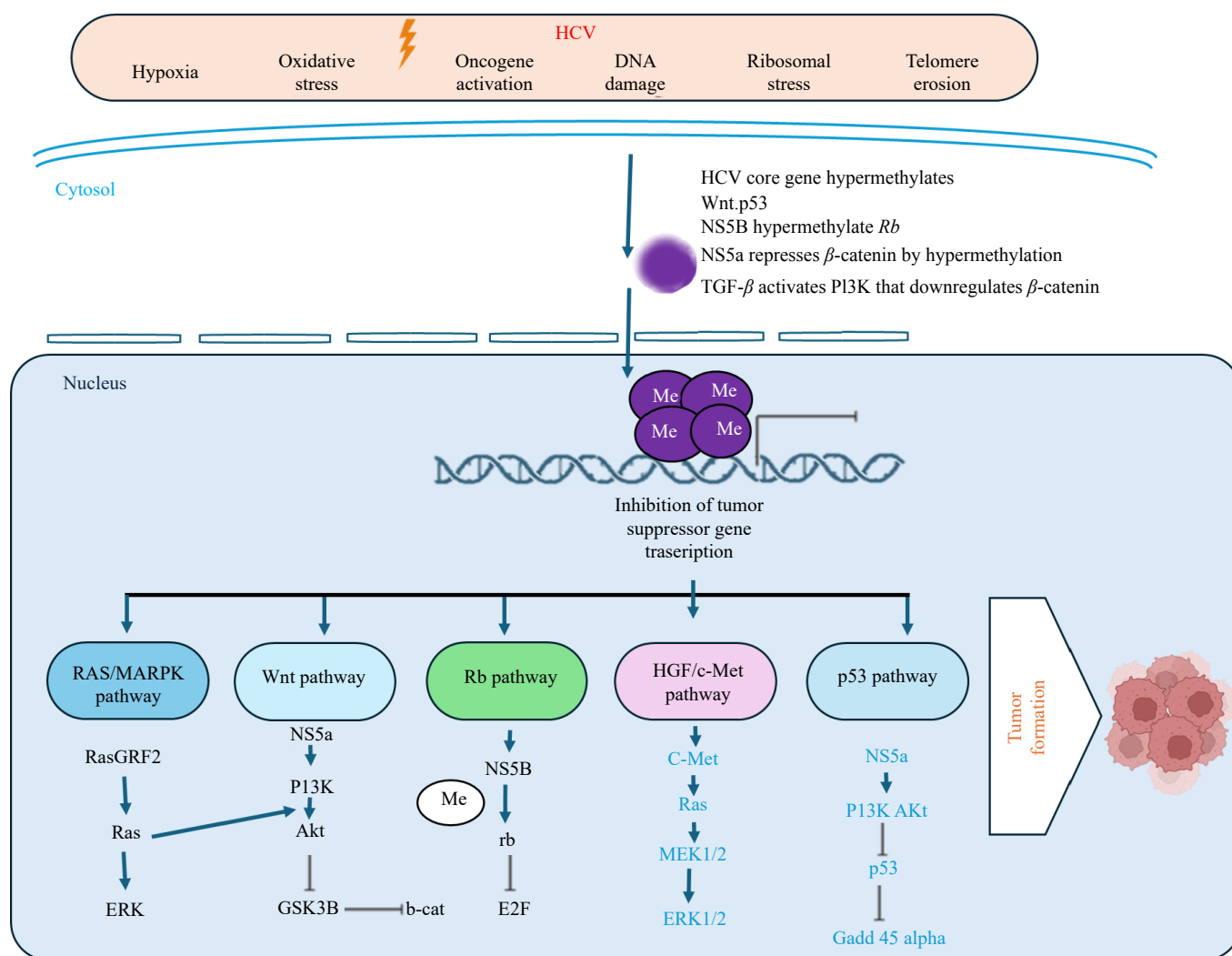
Fig. 1 HCV infection hypermethylates promoter CpG island of Tumor Suppressor Gene and silences its activity. Different HCV genes control p53, rb, TGF-β, HGF/c-Met, RAS/MAPK, and Wnt pathways and disrupt the functionality of tumor suppressor genes Dysfunctional TSGs allow defective DNA to sneak out cell cycle checkpoints. Consequently, leads to the proliferation of cells with damaged DNA and develops tumorigenesis. Created with BioRender.com.

promoters contain a CpG Island of 600 bp that is normally unmethylated[31], whereas infectious agents can cause hypermethylation of CpG islands within this promoter[30].

Earlier it was thought that methylation of only specific CpG sites could lead to cancer. In 1993 however, Ohtani-Fujita et al.[32] proved that the entire promoter of *Rb* underwent hypermethylation in the presence of CpG methylase, which down-regulated its expression. Complete promoter methylation reduced its activity to 8%, whereas HpaII or FnuDII methylase partially methylates it and thus partially inactivates it. At that time, it was not clear whether the reduced activity of the *Rb* gene could cause cancer or if it was by random chance[33]. To explore this mystery, Stirzaker et al.[34] used bisulfite sequencing to investigate methylation of the Rb promoter in 1997. They found varying levels of hypermethylation in all tumor cases. The results indicated different hypermethylation patterns in the promoter region that were not limited to specific sites[35]. Other data about hypermethylation of the *Rb* gene promoter in HCV patient samples revealed reduced transcription and increased tumor induction[36]. Zhang et al. analyzed the *Rb* gene methylation status in HCC patients who were coinfected with HBV and HCV, and they witnessed a trend of hypermethylation[37,38].

p16, p14, p15 genes and HCV

p16, a tumor suppressor gene, is essential for the phosphorylation of the Rb protein that maintains the integrity of DNA during the cell cycle checkpoint G1-S phase[39]. It is located on chromosome 9p21 and is involved in various human cancers. Data shows that *p16* undergoes genetic and epigenetic modifications which promote oncogenic activity in normal cells. *p16* hypermethylation was observed along with an increased level of α-fetoprotein in the serum of HCC patients, making it an attractive biomarker for tumor detection[40]. In 2001, a study was conducted to detect the methylation status of the *p16* gene promoter in different cases of HCC. It was found that hypermethylation silenced *p16* expression in premature hepatocarcinogenesis only in patients who were infected with HCV or HBV. This study paved a new path toward identifying and treating liver cancer in its early stages[41]. Another study confirmed the efficacy of early malignancy diagnosis by identifying hypermethylation of the *p16* gene and the *DAPK* gene in HCC patient blood samples[42]. Later, *p16* down-regulation was observed in HCC patients due to promoter hypermethylation of *CDKN2A*[43]. Recently, *p16* promoter methylation was found to be more prevalent in HCV or HBV-positive HCC patients as compared to cirrhotic liver diseases caused by non-viral agents[44].

Before 2002, it was thought that hypermethylation of a single gene caused cirrhosis; however, it was shown that multiple hypermethylated genes could be observed in HCC[45]. That same year, researchers in Taiwan (China) confirmed the prevailing notion that higher methylation levels are seen with increasing age during HCV infection, and all tumors from elderly patients showed reduced *p16* expression[46]. They also identified malfunctions in the p14, p16, p53, and Rb pathways. The p14 protein prevents the degradation of the p53 protein. Hypermethylation of the *p14* promoter was observed in far fewer HCV cases than in the *p16* promoter[47,48]. The deleterious effect of promoter methylation of *p14, p15,* and *Rb1* genes on the Rb pathway was confirmed by research conducted in the subsequent year, as shown by the absence of *Rb1* expression among patients with HCV-induced HCC[49]. *p16* hypermethylation was observed to be one of the most impactful alterations in HCV-induced HCC patients, yet hypermethylation was also detected in *p15, p14, Rb,* and *PTEN* (encodes a phosphatase that prevents tumor formation) genes[48]. This suggests that multiple hypermethylated genes may be involved in the development of liver cancer in the presence of HCV.

The *p14* and *p15* (the mediator of TGF-β-induced cell arrest) tumor suppressor genes are of interest due to their role in the cell cycle and their relation to *p16*. Interestingly, the *p14* gene is within the *CDKN2A* locus and plays a role in monitoring the integrity of the G1-G2 phase. The *p14* gene contains three exons (1-β, 2, and 3), where exons 2 and 3 are shared by the *p16* gene. A study conducted on an Egyptian population suffering from HCV-induced HCC showed hypermethylation of the *p14* promoter that can be specifically used as an early tumor detection marker[50]. *p16* is also significantly homologous to the p15 protein and exists 25 kilobases from the *p15INK4B/MTS2/CDKN2* gene[51]. *p15* is expressed in response to transforming growth factor-β (TGF-β). *p15* inactivates *CDKN4* and *CDKN6*, which then stops the G1-S phase transition. Therefore, the absence of TGF-β leads to *p15* silencing. There are doubts that *p15* acts as an independent tumor suppressor gene[51], but it is clear that the methylation status is altered as detected by methylation-specific PCR in HCV-mediated HCC. Hypermethylation of the *p14, p15,* and *p16* promoters was reported in cancer cases[52].

In 2009, a cohort of Thai patients suffering from HCV were tested for promoter methylation. Methylation-specific PCR data showed hypermethylation of the *P16INK4a* promoter in 41.4% of tumors and 6.9% of normal liver tissues[53]. Throughout these studies, methylation-specific PCR has been used as the primary method in establishing the relationship between disease symptoms and methylation status[37,50].

p53 gene and HCV

p53 is a major tumor suppressor gene that controls apoptosis, maintains genome integrity and plays a vital role in angiogenesis. Very few studies on the *p53* promoter hypermethylation status have been conducted during HCV infection[24]. HCV or HBV-mediated HCC cases showed hypermethylation in *p53* and *Rb* promoters[37] though most of the research reported mutations in the *p53* gene[54]. The *core* gene of HCV has been reported as a positive as well as a negative regulator of *p53* in different cell lines[55].

Various (*CDKN2A, CDKN2B, CDH1, GSTP1, SOCS1, APC*) genes and HCV

SOCS-1 negatively regulates cytokines that signal through the JAK/STAT3 pathway, whereas *APC* inhibits tumor formation through the Wnt/β-catenin signaling pathway. *SOCS-1, APC,* and *p15* promoters were found to be significantly hypermethylated in HCV-induced HCC patients as compared to HCV-negative HCC patients[56]. The *SOCS-1* gene promoter has been reported to be hypermethylated during HCV-mediated chronic liver disease and cirrhosis. Studies have shown that the frequency of hypermethylated tumor suppressor genes increases from the early stages of fibrosis to the later stage of liver cirrhosis[57].

CDKN2A blocks MDM2-induced degradation of p53, whereas *CDKN2B* prevents the activation of the CDK kinases, and thus controls cell cycle G1 progression[58]. Epigenetic silencing of the tumor suppressor gene is initiated by viral infections that cause the development of HCC. A study based on an Indian cohort found that six tumor suppressor gene (*CDKN2A, CDKN2B, CDH1, GSTP1, SOCS1,* and *APC*) promoter regions showed that the *CDKN2B* promoter was more frequently hypermethylated compared to *APC* and *GSTP1* (plays a role in detoxification and antioxidant system). These findings showed that hypermethylated *CDKN2B, SOCS1, CDH1*, and *GSTP1* play a prime role in the pathogenesis of HCC. Thus, the development of HCC in response to HCV may be linked to methylation profiles of tumor suppressor gene promoters[59].

The *APC* gene plays a prime role in promoting proper signaling in the Wnt pathway. The frequency of *APC* promoter methylation was observed in almost 80% of HCV-induced HCC cases[59,60].

The immunoreactivity score (IRS) of expressed proteins revealed that *APC* methylation in HCV-infected cells is comparatively more than in normal cells[61]. A high-throughput methylation technique was employed to investigate methylated CpG sites that are associated with HCV-induced HCC and demonstrated that the *APC* promoter was hypermethylated[62].

p27 and HCV

p27, a cyclin-dependent kinase inhibitor, monitors cyclin-dependent kinases during the G1-S phase transition. In HCV-associated HCC, immunohistochemical analysis showed down-regulated expression of *p27* in HCV-mediated HCC patients. Furthermore, immunoprecipitation, western blot analysis, and MSP data indicated that *p27* expression was silenced due to *p16* promoter hypermethylation[63].

SPINT2 and HCV

SPINT2, a serine protease inhibitor, inhibits the HGF activator, which prevents the formation of active hepatocyte growth factor. *SPINT2* was reported to be hypermethylated during HCV infection for the first time in 2009[63]. The study presented *SPINT2, CCND2*, and *RASSF1A* as potent, highly sensitive, and specific markers to differentiate between HCC and non-HCC samples. Another study conducted on *SPINT2* confirmed a strong link between HCV infection and HCC development via hypermethylation[64].

NORE1A and HCV

The potential of NORE1A as a prognostic and diagnostic biomarker is highlighted by the correlation between its downregulation in HCC and its poor prognosis[65]. The sensitivity and specificity of HCC detection, especially in the early stages, may be improved by combining NORE1A with methylation and protein markers. Compared to AFP, the HepaClear panel, which combines protein markers and hypermethylated CpG sites, showed a sensitivity of 84.7% for early-stage HCC[66].

EFEMP1 and HCV

Epidermal growth factor containing Fibulin-like Extracellular Matrix Protein 1 (*EFEMP1*) is located at chromosome 2p16 and regulates angiogenesis. One case study observed that the *EFEMP1* gene was hypermethylated in tumorous tissue sampled from a woman of 68 years. Double expression array analysis showed down-regulated expression of the gene in this patient with HCV-associated HCC[67]. According to Zhao et al.[68] and Ma[69], 130,512 CpG sites across the whole genome have been analyzed for the association between HCV infection and the development of HCC. They found that 228 CpG sites were associated with HCV infection, 17,207 with cirrhosis, and 56 with HCV infection and cirrhosis collectively. They also found

that 2,109 (53.8%) CpG sites within promoter regions of the entire genome were significantly hypermethylated. Table 1 shows the role of tumor suppressor genes within different phases of the cell cycle. Furthermore, the development of HCC induced by HCV infection was comparatively more than HBV-induced HCC[70].

The role of HCV genes in tumor suppressor gene hypermethylation

Core protein

HCV Core protein has been observed to down-regulate *p53* expression and promote unattended cell growth. It has been reported as a positive regulator of the *p53* gene that prevents unattended cell propagation[89]. Another group of researchers observed that the HCV Core of genotype 1a was a negative regulator of the *p53* gene in COS-7 and HeLa cells. These contradictory activities of the HCV Core may be associated with different genotypes and cell cultures[90]. HCV Core-induced *p53* silencing HepG2 cells. A study conducted in 2018 showed HCV Core-mediated down-regulation of the *p53* gene using a luciferase reporter in HepG2 cells. The doxycycline-regulated Rat1 cell line transfected with HCV Core showed reduced *Rb* expression and unregulated cell expansion[91].

A study conducted on the effects of HCV Core on *p16* gene methylation and expression proved that its promoter was hypermethylated and expression was significantly down-regulated, this further disrupted Rb-mediated apoptotic activity[92]. Using a 3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay, one study explored down-regulation of the *PTEN* gene in the presence of the HCV Core gene, which in turn activated NF-kB and caused exponential cell growth[55]. Together this body of research highlights the essential role of HCV Core protein in the hypermethylation of tumor suppressor genes and its association with HCC progression.

NS3 and NS5a

Earlier serological studies showed significant down-regulation of *p16* expression in the presence of antigens of the HCV proteins, NS3 and NS5, in patient serum samples[93]. Down-regulated *p53* expression was observed when *NS3* and *NS5a* were transfected in the hepatoma cell line[94]. Experiments conducted in HepG2 and Saos-2 cells showed that NS5a forms a complex with the *p53* gene and, therefore, down-regulates *p21* expression[84].

HCV NS5b is associated with the *Rb* gene of the host cell and down-regulates its expression[80]. It can be inferred from available data that the presence of HCV genes hinders *p53* and *Rb* gene expression disrupting tumor suppressor gene expression. Table 2 summarizes the HCV genes responsible for down-regulating expression through epigenetic alteration of tumor suppressor gene

Table 1. Tumor suppressor genes regulating cell cycle phases.

Cell cycle phases	Tumor suppressor genes	Effect of hypermethylation	Function	Refs
G1-S	*PTEN*[71], *p15*[72], *p16*[73], *p18*[74], *p19*[75], *p27*[76], *SOCS1*[77], *CDKN2A*[78], *CDKN2B*[78], *RASSF1A*[79], *Rb*[71]	Loss of cell cycle control, uncontrolled proliferationCyclin D-CDK4/6 activation, bypass of G1 checkpoint	HCV NS5b is associated with *Rb* gene of the host cell and down-regulates its expression. *p27*, a cyclin-dependent kinase inhibitor.*SOCS-1* down-regulates cytokines of JAK/STAT3 pathway *CDKN2A* degrades *p53 CDKN2B* controls G1phase progression. *RASSF1A* is a potent, highly sensitive, and specific marker to differentiate between HCC and non-HCC samples.	[58,64,80,81]
S	*p53*[82], *p21*[83]	Impaired DNA repair, apoptosis, and cell cycle arrest	NS5a forms a complex with the *p53* gene and, therefore, down-regulates *p21* expression.	[84]
G2-M	*PTEN*[85]	Inactivation of tumor-suppressive phosphatase activity	*PTEN* encodes a phosphatase that prevents tumor formation.	[48]
M	*p53, p21*[86], *APC*[87]	Impaired DNA repair, apoptosis, and cell cycle arrest	*APC* gene of the Wnt pathway was found to be hypermethylated in almost 80% of HCV-induced HCC cases	[88]

Table 2. HCV genes down-regulate tumor suppressor genes' expression in HCC.

HCV genes	Tumor suppressor genes	Function	Refs
Core	P53[99], rb[99], p16[100], PTEN[55]	By means of methylation, the aberration core affects the cell cycle. It hypermethylates the p16 promoter and mitigates its expression. P16 is suggested as a negative regulator of the G1 checkpoint that inactivates during HCC pathogenesis.	[92]
		Core-mediated Rb phosphorylation that releases E2F1 and activates DNMT-1 which is mandatory for HCV infection.	[101,102]
		HCV Core diminishes the activity of ATRA to upregulate p53 and apoptosis.	[103]
		The core gene down-regulates the *PTEN* gene.	[104]
NS3	P21[105], p53[99]	NS3 down-regulates p53 and p21.	[94]
NS5a	PTEN[106], p53[99], p21[107]	Down-regulated *p53* expression was observed when *NS3* and *NS5a* were transfected in hepatoma cell line. Experiments conducted in HepG2 and Saos-2 cells showed that NS5a forms a complex with the *p53* gene and, therefore, down-regulates *p21* expression.	[94,108]
		NS5a down-regulates *PTEN* and inhibits the PI3K-Akt pathway.	[106]
NS5b	Rb[106]	HCV NS5b associates with *Rb* gene of the host cell and down-regulates its expression	[80]

promoters that have been studied, according to the best of our knowledge.

ATP-dependent chromatin remodelers, such as SWI/SNF complexes, are prevented from recruiting and activating by HCV Core and NS5A proteins[95,96]. The hypermethylation and silencing of tumor suppressor genes result from chromatin compaction caused by the loss of function of these complexes. Hepatocellular carcinoma result from epigenetic changes that facilitate carcinogenic pathways[97]. Receptively, these epigenetic modifications persist as 'epigenetic memory' following virus eradication, as evidenced by hypercohesion and elevated HCC risk. This suggests that HCV has long-term effects on chromatin[98].

The role of RNA-binding proteins (RBPs) in HCV-induced HCC epigenetics

RNA-binding proteins (RBPs) participate in several biological functions, including transcription regulation and RNA processing. Dysregulation of RBPs can affect the expression of tumor-promoting genes, accelerating cancer growth[109]. The interaction between (RBPs) and hepatocellular carcinoma (HCC) is significant in HCV infection. RBPs, such as heterogeneous nuclear ribonucleoproteins (hnRNPs), are critical for chromatin organization, and transcriptional control[109]. HCV infection can activate certain RBPs, which may interact with DNMTs to increase methylation at tumor suppressor gene promoters and cause HCC[110].

The role of repetitive elements in HCV-induced HCC epigenetics

Specific Repetitive Elements (REs) methylation is lost during HCV infection. This process can lead to the development of liver cancer[111]. Res, including long interspersed element-1 (LINE-1) and Alu element (Alu), promotes HCC development[112]. LINE-1 and Alu are found abundantly within the genome. They can mobilize in the human genome, which leads to genetic instability[111,112] and contribute to cancer development[113]. Genomic integrity is maintained by DNA methylation of Res[114]. Thus, RE hypomethylation allows them to mobilize within the genome and lead to HCC development[111]. It has been shown that during HCV infection, certain Res (LINE-1) are hypermethylated that initiate the process of HCC[111].

The role of epigenetics in HCV-induced HCC

Cell proliferation, cell differentiation, and apoptosis are controlled by the Wnt/β-catenin signaling pathway[115]. The binding of the Wnt ligand to the low-density-lipoprotein-related protein 5/6 co-receptor complex initiates β-catenin accumulation and nuclear translocation. This canonical pathway activates target genes c-Myc and cyclin D1[116]. Epigenetic changes can result in improper activation of the Wnt pathway and promote cancer development[117]. Wnt extracellular antagonist, secreted frizzled-related proteins (SFRPs), has an N-terminal cysteine-rich domain that can block Wnt–receptor interactions and obstruct Wnt signaling[118]. HCC cells expressed high levels of Dnmt1, HDAC1, and methyl-CpG-binding domain (MBD) proteins that bind near the transcriptional start site (TSS) of the *SFRP1* gene. When Dnmt1 was knocked down, HCV Core-induced epithelial-mesenchymal transition (EMT) was inhibited and obstructed Wnt/β-catenin signaling. Hypermethylation of the SRFP1 promoter induces EMT during HCV infection and leads to HCC progression[119].

The role of Dnmt in epigenetic events

DNA methylation process involves the methyl group donor, S-adenosyl methionine, and Dnmt (1, 3a, 3b) enzymes that transfer the methyl group to DNA. Promoter hypermethylation of tumor suppressor genes was observed in several cases of cancer where Dnmt enzymes were over-expressed[120]. Dnmt1 has been reported to hypermethylated the tumor suppressor genes E-cadherin[121], PARP-1[101], and GSTP1[122]. Using a Huh7 cell model, it was found that Dnmt1 is over-expressed in cells transfected with the HCV-*Core* gene, leading to epigenetic silencing of *SFRP-1*. Knockdown of Dnmt1 restored *SFRP-1* expression[116].

Epigenetic changes induced by HCV are inherited by their daughter hepatocytes that ultimately lead to the development of HCC. The many epigenetic alterations that change gene expression are also inheritable to future generations. Methylation of chromosomes 3 and 16 has been observed in hepatocarcinogenesis in regions that suppress tumor suppressor genes *RASSF1A*, BLU, and *FHIT*[123]. Alterations in miRNA expression that target methyltransferases and hypermethylation of *INK4a* and *INK4b* tumor suppressor genes also influence hepatocarcinogenesis[123]. Epigenetic changes during HCC have been reported in the Ras/Raf/MAPK, and mTOR pathways. Methylation-associated epigenetic changes are observed in G-protein receptor signaling[124].

The impact of HCV-induced epigenetic changes on the immune system

Few patients with acute HCV infection successfully clear the virus from the body. Often, patients with a chronic infection develop HCC[125]. HCV induces epigenetic alteration in the host genome. During HCV infection, HCV pathogen-associated molecular patterns

(PAMPS) activate the toll-like receptor (TLRs), and retinoic acid-inducible gene I (*RIG-I*) pathways, which stimulate Interferon (IFN) type I signaling[126]. HCV infection triggers the secretion of inter-leukins (IL) and activates inflammasomes. Liver cells infected with HCV activate macrophages that produce NALP3 inflammasomes and IL-8. This molecular mechanism activates natural killer cells (NK) of the disease, the host body fails to fight against the HCV infection, and immune factors (IFN signaling, activation of NF-κB, TNF-α, and IL-6) become dysregulating to the development of HCC[127]. T-cells become exhausted, meaning they lose their functionality and show increased expression of inhibitory receptors such as programmed cell death 1 (PD-1). It has been reported that high PD-1 expression can persist even after direct-acting antiviral (DAA) therapy[128,129]. This may be due to epigenetic alterations resulting from chronic HCV infection that contribute to the progression of liver cancer.

Hepatocyte apoptosis is affected by HCV Core protein. Thus, H_2O_2 upregulates p14 expression that degrades MDM2 in the proteasome. This stabilizes p53. Core hypermethylates the promoter of p14 and inhibits its expression, which in turn hinders p53 by MDM2[54]. Thus, p14 and p53 inhibition lead to HCC progression. The repressed p14 stops the function of trans-retinoic acid (ATRA) that prevents hepatocyte apoptosis. ATRA hypomethylates the promoter of p14 that activates the p53-dependent apoptotic pathway. HCV Core diminishes the activity of ATRA to upregulate p53 and apoptosis. Core mediates increased expression of DNMT1, DNMT3A, and DNMT3B that hypomethylate the p14 promoter[130] of using methylation aberration core affects the cell cycle. It hypermethylates the p16 promoter and mitigates its expression. P16 is suggested as a negative regulator of the G1 checkpoint that inactivates during HCC pathogenesis[92].

HCV-induced DNA methylation leads to DNA damage and cell cycle arrest. The Gadd45 gene family encodes the Gadd45β protein that repairs DNA excision. During genotoxic stress, this protein inhibits G2/M progression due to HCV-mediated promoter hypermethylation of the Gadd45 gene. Higgs et al. observed the down-regulation of Gadd45β in HCV-infected cells. Moreover, a noticeable decrease in Gadd45β expression was observed in liver biopsies of HCV patients and non-tumoral tissues from transgenic mouse models[131]. There exist two unique mechanisms that govern the Core-mediated up-regulation of DNMT expression. Park et al. suggested that intracellular transducers (ERK1/2, JNKs, and p38 MAP kinases) play a role in core mediated activation of AP-1 expression and DNMT-1 promoter that has binding motifs of the AP-1 complex. JNK inhibitor reduced the expression of DNMT-1 in core-expressing cells[92]. Arora et al. suggested another mechanism that showed stimulation of the Rb-E2F pathway by core-mediated Rb phosphory-lation that releases E2F1 and activates DNMT-1[101]. mRNA and protein expression levels of DNMT-1 and DNMT-3B are different in different HCV genotypes[91]. DNMT-1 and DNMT-3B are essential for HCV infection, RNA synthesis[132], and its expression.

Core hypermethylates and represses E6-associated protein (E6AP) and evades poly-ubiquitination by E6AP and its proteasomal degradation[103]. Promoter methylation of SPINT2/HAI-2 was found in HCC patients. Hypermethylation of SPINT2/HAI-2 inhibits the HGF activator enzyme and the HGF/c-Met receptor pathway. Thus, down-regulates a urokinase plasminogen activator that degrades extracellular matrix, migration, and metastasis. HCV-mediated aberrated methylation leads to inflammation and ROS generation in HCV-infected cells[27]. Another tumor suppressor gene, RASSF1A, was reported to be hypermethylated during HCC progression. IFNγ expression increased in HCV-infected mice. Natural killer (NK) cells express IFNγ,

and NK cell inhibitor reduces its expression. Thus, the function of NK cells in HCV-infected mice induces DNA methylation[133]. Global DNA methylation in enhancers containing binding sites of FOXA1, FOXA2, and HNF4A, was observed in liver biopsies from HCV-infected patients. Nishida et al. observed that persistent HCV infection increased methylation in CpG loci. Thus, the aberrated methylation profile of HCC patients leads to HCC progression[134]. All the suggested pathways reflect the involvement of epigenetics in the immune system.

Persistent epigenetic changes after DAA treatment

Tumor recurrence in patients treated with DAAs has been reported by various epidemiological studies. I*n vitro*, HCV infection models showed persistent epigenetic signatures even after the removal of the virus by DAAs treatment. Eight gene signatures have been persistently reported in HCV-infected and post-SVR liver biopsies[135]. Cytoskeleton remodeling pathways are epigenetically altered during HCV infection. These pathways are involved in the invasion and metastasis of tumorous cells[135]. This epigenetic scarring persists within the genome even after DAAs-mediated HCV eradication[135]. Recent research has shown that the epidermal growth factor receptor (EGFR) inhibitor drug, Erlotinib, inhibits the epigenetic-inducing enzymes and reverses the epigenetic signature *in vitro*[135]. Potentially, such drugs can be employed to diminish HCV-induced epigenetic signatures from the genome to decrease the risk of liver cancer[135].

Clinically, long-lasting effects are required, it reprograms the epigenetic changes to fight chronic diseases. Therefore, sustained effects cannot be expected if one epigenetic enzyme is targeted to use epigenetic editing, several epigenetic 'writers and erasers' could be targeted. Treatments should be done in a way that guarantees the persistence of these effects over several cell replications[136].

Role of flavonoids in HCC treatment

Worldwide, the commonly consumed epigenetic regulators include flavonoids. Flavonoids are strong cancer inhibitors, as they have been proven in different studies. They restore dysfunctional epigenetics and intervene in DNA methylation, histone modification, and non-coding RNA expression. Flavonoids always showed significant efficacy either used alone or in adjuvant therapy[137]. The efficacy of flavonoids is unchallengeable. While flavonoids produce anti-cancerous effects through multiple mechanisms, they can simultaneously cause problems for drug administration. Efficacy and results may vary while modulating epigenetic aberrations, and this is quite common[137]. Insufficient bioavailability is the main hindrance to its clinical application. The researchers now have begun to improve the pharmacokinetic properties of flavonoids by using them in adjuvant therapy (other plant compounds, Nano-drug carrier system). By blocking DNMTs and histone-modifying enzymes, natural substances—in particular, flavonoids like quercetin, genistein, and epigallocatechin gallate (EGCG) from green tea—have demonstrated potential in restoring epigenetic changes in HCC[138]. These substances trigger apoptosis, control the cell cycle, and support anti-cancer pathways. Furthermore, it has been shown that curcumin and resveratrol can change the methylation state of tumor suppressor genes such as **RASSF1A,** which slows the growth of HCC[139]. The effectiveness of treatment may be further increased by combining flavonoids with synthetic medications, especially when it comes to addressing preneoplastic cells[140].

Epigenetic modifiers

It has been noted that various protein targets respond to epigenetic modifiers such as HMTs, KDMs, and HDACs. Depending upon different target genes of the same cell, the epigenetic modulator, G9a/KMT1C, can act as a coactivator or a corepressor[141]. EZH2/KMT6 epigenetic modulator can also inhibit or activate gene expression depending on the tumor type when over-expressed[142]. Pathophysiological implications are better understood while monitoring the whole impact of the epigenetic machinery. Advanced epi-drugs, such as DNMT inhibitors like guadecitabine, which demethylates hypermethylated tumor suppressor genes like CDKN2A and SOCS1, and HDAC inhibitors, which encourage gene reactivation through increased histone acetylation, are the mainstay of therapeutic strategies to reverse HCV-induced epigenetic changes[143]. With ongoing clinical trials examining their effectiveness across different cancer types, combining these medicines suggests synergistic promise in reactivating suppressed genes and enhancing outcomes in HCV-related HCC[144]. Advancements in scientific research have revealed promising inhibitors of epigenetic modifiers. Recent data shows that these inhibitors do not antagonize the modifiers but bind to the enzymes that regulate the epigenome (Table 3).

Conclusions

Epigenetic changes in the promoters of tumor suppressor genes progress with the advancement of liver disease associated with HCV infection. Since epigenetic modifications are reversible by nature, it is an attractive area for epi-drug development. Thus, understanding the mechanism behind specific HCV genes that promote the development of cancer may open new horizons for the treatment of HCC patients. This review is an effort to summarize data on the promoter methylation status of tumor suppressor genes in the presence of HCV-induced HCC. This review focuses on discoveries about how hepatitis C virus (HCV) genes affect epigenetic modifications and HCC. The effect of NS5a on histone modifications, which change chromatin structure and silence tumor suppressor genes, and the function of the HCV Core protein in causing DNA methylation, which silences important regulatory genes including p16, p53, and PTEN, are remarkable results. Additionally, even after viral clearance, residual epigenetic scars following HCV treatment, such as hypermethylation and histone alterations, point to a risk of HCC recurrence. Additionally, the study compiles evidence of extensive hypermethylation across tumor suppressor genes, including CDKN2A/B, SOCS1, and EFEMP1, which impacts pathways essential for apoptosis and cell cycle regulation. These results highlight the necessity of long-term post-treatment monitoring and the promise of designed epigenetic therapy.

Future perspectives

Focusing on HCV-induced epigenetic changes in tumor suppressor genes during HCC development may prove to help design potent epi-drugs. It may aid in devising strategies that can undo these modifications in the promoters of tumor suppressor genes and allow the normal cell cycle to continue. Certain HCV genes could be targeted to disrupt their function, thus allowing the tumor suppressor genes to function properly. Additional tumor suppressor genes should be examined in HCV-induced HCC tissue samples. Further studies should concentrate on improving tumor suppressor gene methylation patterns as non-invasive biomarkers for early HCC identification, with a particular emphasis on integration with multi-omics techniques and liquid biopsy technologies. The diagnostic and prognostic value of markers such NORE1A, RASSF1A, SPINT2, CDKN2A (p16), APC, SOCS1, and EFEMP1 should be investigated.

The accuracy of diagnosis may be improved by looking into population-specific methylation patterns and how they interact with environmental and genetic factors. Furthermore, these methylation markers may be combined with newly developed protein panels and epi-drug response monitoring to produce all-inclusive tools for individualized HCC screening and prognosis.

Executive summary

Approximately 58 million people worldwide are infected with HCV. Scientists rarely focus on HCV-induced HCC that can involve epigenetic modifications to tumor suppressor genes and their expression. To date, there is only a minute amount of data regarding the epigenetic profile of HCV-induced HCC. Researchers historically explored epigenetic profiles of HCC irrespective of its cause (alcohol consumption, HCV, HBV, or any other environmental factors). The literature referenced in this review shows that more studies are needed to focus on the HCV-induced epigenetic changes in tumor suppressor genes to decipher the mechanisms underlying the development of HCC. *EFEMP1, SPINT1, Rb, p53, CDKN2A, CDKN2B, CDH1, GSTP1, SOCS1, APC, p27 p16, p14,* and *p15* are tumor suppressor genes that have been found to have hypermethylated promoters during HCV-induced HCC. The methylation profiles of these promoters can potentially be used as early detection biomarkers for reducing deaths from HCC. HCV-*NS3a*, HCV-*NS5a*, and HCV-*Core* have been reported to down-regulate *p53, p16, p21, PTEN,* and *Rb* genes in different cell lines. Thus, furthering the concept that HCV-induced hypermethylation and silencing of tumor suppressor genes promote HCC development. The impacts of these findings are extensive. Because HCV-induced epigenetic changes are reversible, they are desirable targets for therapeutic intervention. In addition to understanding the molecular processes underlying the development of HCC, methylation patterns of tumour suppressor gene promoters serve as a basis for the creation of innovative epigenetic treatments. Drugs that target histone-modifying enzymes and DNA methyltransferases (DNMTs) may be able to stop the spread of tumors and restore normal gene expression. Furthermore, non-invasive screening and monitoring of HCV-associated HCC may be made possible by detecting hypermethylation patterns in circulating tumor DNA (ctDNA).

Table 3. HCV-induced epigenetic changes can be inhibited by epigenetic modifiers inhibitors. These inhibitors and their modifiers are given in the table.

Epigenetic modifiers	Description	Inhibitors of modifiers	Ref.
Epidermal growth factor receptor (EGFR)	HCV infection activates EGFR that epigenetically modify cytoskeleton remodeling	Erlotinib	[107]
Unfolded Protein Response (UPR)	HCV genome induces UPR. UPR inhibitor has been reported to partially recover the dysregulated gene expression.	UPR inhibitor	[107]
Lysophosphatidic acid (LPA) pathway	LPA initiates the HCC-inducing pathways. The efficacy of the inhibitors was observed in the diethylnitrosamine (DEN) rat model. These inhibitors suppress Rho and ERK pathways.	ATX (AM063), LPAR1 (AM095)	[107]

Ethical statements

Not applicable.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Afzal S; draft manuscript preparation: Aftab A; data acquisition, figures and tables preparation: Naveed H, Idrees H; manuscript review: Ali L, Idrees M, Afazal S. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

References

1. Ling C, Groop L. 2009. Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes* 58:2718–25

2. Kierszenbaum AL. 2002. Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. *Molecular Reproduction and Development* 63:269–72

3. Montalvo-Casimiro M, González-Barrios R, Meraz-Rodriguez MA, Juárez-González VT, Arriaga-Canon C, et al. 2020. Epidrug repurposing: discovering new faces of old acquaintances in cancer therapy. *Frontiers in Oncology* 10:605386

4. Kanwal R, Gupta S. 2012. Epigenetic modifications in cancer. *Clinical Genetics* 81:303–11

5. Ganesan A, Arimondo PB, Rots MG, Jeronimo C, Berdasco M. 2019. The timeline of epigenetic drug discovery: from reality to dreams. *Clinical Epigenetics* 11:174

6. MacDonald WA. 2012. Epigenetic mechanisms of genomic imprinting: common themes in the regulation of imprinted regions in mammals, plants, and insects. *Genetics research international* 2012:585024

7. Lee HT, Oh S, Ro DH, Yoo H, Kwon YW. 2020. The key role of DNA methylation and histone acetylation in epigenetics of atherosclerosis. *Journal of lipid and atherosclerosis* 9:419

8. Laird PW. 2003. The power and the promise of DNA methylation markers. *Nature Reviews Cancer* 3:253–66

9. Feinberg AP, Tycko B. 2004. The history of cancer epigenetics. *Nature Reviews Cancer* 4:143–53

10. Jones PA, Baylin SB. 2007. The epigenomics of cancer. *Cell* 128:683–92

11. Rodríguez-Paredes M, Esteller M. 2011. Cancer epigenetics reaches mainstream oncology. *Nature Medicine* 17:330–39

12. Xu TH, Liu M, Zhou XE, Liang G, Zhao G, et al. 2020. Structure of nucleosome-bound DNA methyltransferases DNMT3A and DNMT3B. *Nature* 586:151–55

13. Yin H, Zhou Y, Xu Z, Chen L, Zhang D, et al. 2013. An electrochemical assay for DNA methylation, methyltransferase activity and inhibitor screening based on methyl binding domain protein. *Biosensors and Bioelectronics* 41:492–97

14. Phillips T. 2008. The role of methylation in gene expression. *Nature Education* 1:116

15. Esteller M. 2002. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 21:5427–40

16. Zhang Y, Reinberg D. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes & Development* 15:2343–60

17. Seto E, Yoshida M. 2014. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harbor Perspectives in Biology* 6:a018713

18. Ranjan S, Sharma PK. 2016. Effect of environmental chemical exposures on epigenetics of diseases: a systematic review. *International Journal of Life Sciences Scientific Research* 2(4):531–38

19. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, et al. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* 129:823–37

20. Silva MC, Bodor DL, Stellfox ME, Martins NM, Hochegger H, et al. 2012. Cdk activity couples epigenetic centromere inheritance to cell cycle progression. *Developmental Cell* 22:52–63

21. Cedar H, Bergman Y. 2009. Linking DNA methylation and histone modification: patterns and paradigms. *Nature Reviews Genetics* 10:295–304

22. Fernández-Barrena MG, Arechederra M, Colyn L, Berasain C, Avila MA. 2020. Epigenetics in hepatocellular carcinoma development and therapy: The tip of the iceberg. *JHEP Reports* 2:100167

23. Edamoto Y, Hara A, Biernat W, Terracciano L, Cathomas G, et al. 2003. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *International Journal of Cancer* 106:334–41

24. Martin J, Dufour JF. 2008. Tumor suppressor and hepatocellular carcinoma. *World Journal of Gastroenterology* 14(11):1720–33

25. Cui F, Blach S, Mingiedi CM, Gonzalez MA, Alaama AS, et al. 2023. Global reporting of progress towards elimination of hepatitis B and hepatitis C. *The lancet Gastroenterology & hepatology* 8:332–42

26. Butt S, Idrees M, Akbar H, ur Rehman I, Awan Z, et al. 2010. The changing epidemiology pattern and frequency distribution of hepatitis C virus in Pakistan. *Infection, Genetics and Evolution* 10:595–600

27. Rehman IU, Idrees M, Ali M, Ali L, Butt S, et al. 2011. Hepatitis C virus genotype 3a with phylogenetically distinct origin is circulating in Pakistan. *Genetic Vaccines and Therapy* 9:2

28. Chevaliez S, Pawlotsky JM. 2006. HCV genome and life cycle. In *Hepatitis C viruses: genomes and molecular biology*, ed. Tan SL. Norfolk, UK: Horizon Bioscience. www.ncbi.nlm.nih.gov/books/NBK1630/

29. Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F, et al. 2006. A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Research* 66:8342–46

30. Zhou M, Tang J, Fan J, Wen X, Shen J, et al. 2024. Recent progress in retinoblastoma: pathogenesis, presentation, diagnosis and management. *Asia-Pacific Journal of Ophthalmology* 13:100058

31. Joseph B, Mamatha G, Raman G, Shanmugam MP, Kumaramanickavel G. 2004. Methylation status of RB1 promoter in Indian retinoblastoma patients. *Cancer Biology & Therapy* 3:184–87

32. Ohtani-Fujita N, Fujita T, Aoike A, Osifchin N, Robbins P, et al. 1993. CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* 8(4):1063–67

33. Reis AH, Vargas FR, Lemos B. 2012. More epigenetic hits than meets the eye: microRNAs and genes associated with the tumorigenesis of retinoblastoma. *Frontiers in Genetics* 3:284

34. Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, et al. 1997. Extensive DNA methylation spanning the *Rb* promoter in retinoblastoma tumors. *Cancer Research* 57:2229–37

35. Janostiak R, Torres-Sanchez A, Posas F, de Nadal E. 2022. Understanding retinoblastoma post-translational regulation for the design of targeted cancer therapies. *Cancers* 14:1265

36. Rotchell JM, Ulnal E, Van Beneden RJ, Ostrander GK. 2001. Retinoblastoma gene mutations in chemically induced liver tumor samples of Japanese medaka (*Oryzias latipes*). *Marine Biotechnology* 3:S44–S49

37. Zhang C, Li Z, Cheng Y, Jia F, Li R, et al. 2007. CpG island methylator phenotype association with elevated serum α-fetoprotein level in hepatocellular carcinoma. *Clinical Cancer Research* 13:944–52

38. Zhang C, Guo X, Jiang G, Zhang L, Yang Y, et al. 2008. CpG island methylator phenotype association with upregulated telomerase

activity in hepatocellular carcinoma. *International Journal of Cancer* 123:998–1004

39. Hayslip J, Montero A. 2006. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. *Molecular cancer* 5:44

40. Hahne JC, Mirchev M, Kotzev I, Lampis A, Valeri N. 2017. Biomarkers for monitoring response to therapies and detection of acquired resistance in advanced gastrointestinal cancers. In *Frontiers in Clinical Drug Research - Anti-Cancer Agents*, ed. Atta-ur-Rahman. Vol. 4. pp. 1–73. doi: 10.2174/9781681084817117040003

41. Kaneto H, Sasaki S, Yamamoto H, Itoh F, Toyota M, et al. 2001. Detection of hypermethylation of the *p16INK4A* gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. *Gut* 48:372–77

42. Lin Q, Chen LB, Tang YM, Wang J. 2005. Promoter hypermethylation of p16 gene and DAPK gene in sera from hepatocellular carcinoma (HCC) patients. *Chinese Journal of Cancer Research* 17:250–54

43. Csepregi A, Ebert MP, Röcken C, Schneider-Stock R, Hoffmann J, et al. 2010. Promoter methylation of CDKN2A and lack of p16 expression characterize patients with hepatocellular carcinoma. *BMC Cancer* 10:317

44. Lv X, Ye G, Zhang X, Huang T. 2017. p16 Methylation was associated with the development, age, hepatic viruses infection of hepatocellular carcinoma, and p16 expression had a poor survival: a systematic meta-analysis (PRISMA). *Medicine* 96:e8106

45. Pogribny IP, Rusyn I. 2014. Role of epigenetic aberrations in the development and progression of human hepatocellular carcinoma. *Cancer Letters* 342:223–30

46. Wang LH, Wu CF, Rajasekaran N, Shin YK. 2019. Loss of tumor suppressor gene function in human cancer: an overview. *Cellular Physiology and Biochemistry* 51:2647–93

47. Tannapfel A, Busse C, Geißler F, Witzigmann H, Hauss J, et al. 2002. INK4a-ARF alterations in liver cell adenoma. *Gut* 51:253–58

48. Hayashi T, Tamori A, Nishikawa M, Morikawa H, Enomoto M, et al. 2009. Differences in molecular alterations of hepatocellular carcinoma between patients with a sustained virological response and those with hepatitis C virus infection. *Liver International* 29:126–32

49. Yu M, Xu W, Jie Y, Pang J, Huang S, et al. 2021. Identification and validation of three core genes in p53 signaling pathway in hepatitis B virus-related hepatocellular carcinoma. *World Journal of Surgical Oncology* 19:66

50. Zekri AN, Bahnasy AA, Shoeab FEM, Mohamed WS, El-Dahshan DH, et al. 2014. Methylation of multiple genes in hepatitis C virus associated hepatocellular carcinoma. *Journal of Advanced Research* 5:27–40

51. Graham C, LeBrun DP. 2015. Tumor suppressors in follicular lymphoma. *Leukemia & Lymphoma* 56:1981–88

52. Xu G, Zhou X, Xing J, Xiao Y, Jin B, et al. 2020. Identification of RASSF1A promoter hypermethylation as a biomarker for hepatocellular carcinoma. *Cancer Cell International* 20:547

53. Saelee P, Petmitr S, Wongkham S, Chariyalertsak S, Homcha-cm P. 2010. Detection of DNA methylation of p16INK4a in hepatocellular carcinoma by methylation specific-polymerase chain reaction

54. Seo YL, Heo S, Jang KL. 2015. Hepatitis C virus core protein overcomes H_2O_2-induced apoptosis by downregulating p14 expression via DNA methylation. *Journal of General Virology* 96:822–32

55. Zhang Y, Li RQ, Feng XD, Zhang YH, Wang L. 2014. Down-regulation of PTEN by HCV core protein through activating nuclear factor-κB. *International Journal of Clinical and Experimental Pathology* 7:7351

56. Pasha HF, Mohamed RH, Radwan MI. 2019. RASSF1A and SOCS1 genes methylation status as a noninvasive marker for hepatocellular carcinoma. *Cancer Biomarkers* 24:241–47

57. Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, Yasukawa H. 2012. SOCS, inflammation, and autoimmunity. *Frontiers in Immunology* 3:20

58. Zhu Y, Ning Z, Li X, Lin Z. 2024. Machine learning algorithms identify target genes and the molecular mechanism of matrine against diffuse large B-cell lymphoma. *Current Computer-Aided Drug Design* 20:847–59

59. Kiran M, Chawla YK, Kaur J. 2009. Methylation profiling of tumor suppressor genes and oncogenes in hepatitis virus-related hepatocellular carcinoma in northern India. *Cancer genetics and cytogenetics* 195:112–19

60. Han TS, Ban HS, Hur K, Cho HS. 2018. The epigenetic regulation of HCC metastasis. *International Journal of Molecular Sciences* 19:3978

61. Baro L, Islam A, Brown HM, Bell ZA, Juanes MA. 2023. APC-driven actin nucleation powers collective cell dynamics in colorectal cancer cells. *iScience* 26:106583

62. Bao J, Geng X, Hou C, Zhao Y, Huo D, et al. 2018. A simple and universal electrochemical assay for sensitive detection of DNA methylation, methyltransferase activity and screening of inhibitors. *Journal of Electroanalytical Chemistry* 814:144–52

63. Matsuda Y, Ichida T, Genda T, Yamagiwa S, Aoyagi Y, et al. 2003. Loss of p16 contributes to p27 sequestration by cyclin D1-cyclin-dependent kinase 4 complexes and poor prognosis in hepatocellular carcinoma. *Clinical Cancer Research* 9:3389–96

64. Moribe T, Iizuka N, Miura T, Kimura N, Tamatsukuri S, et al. 2009. Methylation of multiple genes as molecular markers for diagnosis of a small, well-differentiated hepatocellular carcinoma. *International Journal of Cancer* 125:388–97

65. Liu LL, Zhang MF, Pan YH, Yun JP, Zhang CZ. 2014. NORE1A sensitises cancer cells to sorafenib-induced apoptosis and indicates hepatocellular carcinoma prognosis. *Tumour Biology* 35:1763–74

66. Bai Y, Xu J, Li D, Zhang X, Chen D, et al. 2023. HepaClear, a blood-based panel combining novel methylated CpG sites and protein markers, for the detection of early-stage hepatocellular carcinoma. *Clinical Epigenetics* 15:99

67. Inokawa Y, Nomoto S, Hishida M, Hayashi M, Kanda M, et al. 2013. Detection of doublecortin domain-containing 2 (DCDC2), a new candidate tumor suppressor gene of hepatocellular carcinoma, by triple combination array analysis. *Journal of Experimental & Clinical Cancer Research* 32:65

68. Zhao P, Malik S, Xing S. 2021. Epigenetic mechanisms involved in HCV-induced hepatocellular carcinoma (HCC). *Frontiers in Oncology* 11:67792

69. Ma L. 2014. Pathology features and molecular genetic mechanisms of hepatocellular carcinoma development in patients with hepatitis C associated liver cirrhosis. *Hereditary Genetics: Current Research* 3:e109

70. Song MA, Kwee SA, Tiirikainen M, Hernandez BY, Okimoto G, et al. 2016. Comparison of genome-scale DNA methylation profiles in hepatocellular carcinoma by viral status. *Epigenetics* 11:464–74

71. Khalid A, Hussain T, Manzoor S, Saalim M, Khaliq S. 2017. PTEN: a potential prognostic marker in virus-induced hepatocellular carcinoma. *Tumour Biology* 39:1

72. Chang CN, Feng MJ, Chen YL, Yuan RH, Jeng YM. 2013. p15^PAF is an Rb/E2F-regulated S-phase protein essential for DNA synthesis and cell cycle progression. *PLoS One* 8:e61196

73. Saleh T, Khasawneh AI, Himsawi N, Abu-Raideh J, Ejeilat V, et al. 2022. Senolytic therapy: a potential approach for the elimination of oncogene-induced senescent HPV-positive cells. *International Journal of Molecular Sciences* 23:15512

74. Zhu S, Cao J, Sun H, Liu K, Li Y, et al. 2016. p18 inhibits reprogramming through inactivation of Cdk4/6. *Scientific Reports* 6:31085

75. García-Cruz R, Camats M, Calin GA, Liu CG, Volinia S, et al. 2015. The role of p19 and p21 H-Ras proteins and mutants in miRNA expression in cancer and a Costello syndrome cell model. *BMC Medical Genetics* 16:46

76. Sun C, Wang G, Wrighton KH, Lin H, Zhou S, et al. 2016. Regulation of p27^Kip1 phosphorylation and G1 cell cycle progression by protein phosphatase PPM1G. *American Journal of Cancer Research* 6:2207–20

77. Ding J, Xu K, Sun S, Qian C, Yin S, et al. 2020. SOCS1 blocks G1-S transition in hepatocellular carcinoma by reducing the stability of the CyclinD1/CDK4 complex in the nucleus. *Aging* 12:3962–75

78. Ponomarev AV, Solodovnik AA, Mkrtchyan AS, Finashutina YP, Turba AA, et al. 2018. Relationship between deletion and point mutations of p53 and drug resistance to aranoza in human melanoma cell lines. *Russian Journal of Biotherapy* 17:64–69

79. Ghazaleh HA, Chow RS, Choo SL, Pham D, Olesen JD, et al. 2010. 14-3-3 mediated regulation of the tumor suppressor protein, RASSF1A. *Apoptosis* 15:117–27

80. Munakata T, Nakamura M, Liang Y, Li K, Lemon SM. 2005. Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C virus

NS5B RNA-dependent RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* 102:18159–64

81. Ye C, Zhu D, Lu J, Zhang X, Li L. 2016. The diagnostic value of suppressor of cytokine signaling-1 (SOCS-1) methylation for human hepatocellular carcinoma (HCC): a meta-analysis based on data from 15 studies. *International Journal of Clinical and Experimental Medicine* 9:15146–56

82. Barr AR, Cooper S, Heldt FS, Butera F, Stoy H, et al. 2017. DNA damage during S-phase mediates the proliferation-quiescence decision in the subsequent G1 via p21 expression. *Nature Communications* 8:14728

83. Zhu H, Zhang L, Wu S, Teraishi F, Davis JJ, et al. 2004. Induction of S-phase arrest and p21 overexpression by a small molecule 2[3-(2,3-dichlorophenoxy)propyl] amino] ethanol in correlation with activation of ERK. *Oncogene* 23:4984–92

84. Bittar C, Shrivastava S, Bhanja Chowdhury J, Rahal P, Ray RB. 2013. Hepatitis C virus NS2 protein inhibits DNA damage pathway by sequestering p53 to the cytoplasm. *PLoS One* 8:e62581

85. Zhang X, Jia S, Yang S, Yang Y, Yang T, et al. 2012. Arsenic trioxide induces G2/M arrest in hepatocellular carcinoma cells by increasing the tumor suppressor PTEN expression. *Journal of Cellular Biochemistry* 113:3528–35

86. Ticli G, Cazzalini O, Stivala LA, Prosperi E. 2022. Revisiting the function of p21CDKN1A in DNA repair: The influence of protein interactions and stability. *International Journal of Molecular Sciences* 23:7058

87. Alfieri C, Zhang S, Barford D. 2017. Visualizing the complex functions and mechanisms of the anaphase promoting complex/cyclosome (APC/C). *Open Biology* 7:170204

88. Lala V, Zubair M, Minter D. 2023. *Liver function tests*. Treasure Island (FL): StatPearls Publishing. www.ncbi.nlm.nih.gov/books/NBK482489/

89. Dharel N, Kato N, Muroyama R, Taniguchi H, Otsuka M, et al. 2008. Potential contribution of tumor suppressor p53 in the host defense against hepatitis C virus. *Hepatology* 47:1136–49

90. Kwak J, Jang KL. 2018. Hepatitis C virus core protein activates p53 to inhibit E6-associated protein expression via promoter hypermethylation. *Journal of Life Science* 28:1007–15

91. Banerjee A, Ray RB, Ray R. 2010. Oncogenic potential of hepatitis C virus proteins. *Viruses* 2:2108

92. Park SH, Lim JS, Lim SY, Tiwari I, Jang KL. 2011. Hepatitis C virus core protein stimulates cell growth by down-regulating p16 expression via DNA methylation. *Cancer Letters* 310:61–68

93. Ni Q, Gao Y, Yang X, Zhang Q, Guo B, et al. 2022. Analysis of the network pharmacology and the structure-activity relationship of glycyrrhizic acid and glycyrrhetinic acid. *Frontiers in Pharmacology* 13:1001018

94. D'souza S, Lau KC, Coffin CS, Patel TR. 2020. Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma. *World Journal of Gastroenterology* 26:5759

95. Bieluszewski T, Prakash S, Roulé T, Wagner D. 2023. The role and activity of SWI/SNF chromatin remodelers. *Annual Review of Plant Biology* 74:139–63

96. Mittal P, Roberts CWM. 2020. The SWI/SNF complex in cancer — biology, biomarkers and therapy. *Nature Reviews Clinical Oncology* 17:435–48

97. Nagaraju GP, Dariya B, Kasa P, Peela S, El-Rayes BF. 2022. Epigenetics in hepatocellular carcinoma. *Seminars in Cancer Biology* 86:622–32

98. Jancewicz I, Siedlecki JA, Sarnowski TJ, Sarnowska E. 2019. BRM: the core ATPase subunit of SWI/SNF chromatin-remodelling complex—a tumour suppressor or tumour-promoting factor? *Epigenetics & Chromatin* 12:68

99. Aftab A, Afzal S, Idrees M, Shahid AA. 2021. *p53* and *rb* promoter methylation in hepatitis C virus-related chronic hepatitis and hepatocellular carcinoma. *Future Virology* 16:15–25

100. Cha S, Park I, Jang KL. 2021. Hepatitis C virus core protein activates proteasomal activator 28 gamma to downregulate p16 levels via ubiquitin-independent proteasomal degradation. *Heliyon* 7:e06134

101. Arora P, Kim EO, Jung JK, Jang KL. 2008. Hepatitis C virus core protein downregulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b. *Cancer letters* 261:244–52

102. Chen X, Ma J, Wang XA, Zi T, Qian D, et al. 2022. CCNB1 and AURKA are critical genes for prostate cancer progression and castration-resistant prostate cancer resistant to vinblastine. *Frontiers in Endocrinology* 13:1106175

103. Kwak J, Shim JH, Tiwari I, Jang KL. 2016. Hepatitis C virus core protein inhibits E6AP expression via DNA methylation to escape from ubiquitin-dependent proteasomal degradation. *Cancer Letters* 380:59–68

104. Zhang Y, Li Z, Chen M, Chen H, Zhong Q, et al. 2019. Identification of a new eight-long noncoding RNA molecular signature for breast cancer survival prediction. *DNA and Cell Biology* 38:1529–39

105. Choudhary HB, Mandlik SK, Mandlik DS. 2023. Role of p53 suppression in the pathogenesis of hepatocellular carcinoma. *World Journal of Gastrointestinal Pathophysiology* 14:46

106. Vescovo T, Refolo G, Vitagliano G, Fimia GM, Piacentini M. 2016. Molecular mechanisms of hepatitis C virus–induced hepatocellular carcinoma. *Clinical Microbiology and Infection* 22:853–61

107. Domovitz T, Gal-Tanamy M. 2021. Tracking down the epigenetic footprint of HCV-induced hepatocarcinogenesis. *Journal of Clinical Medicine* 10:551

108. Ma J, He Y, Li F, Han L, You C, et al. 2024. Segment anything in medical images. *Nature Communications* 15:654

109. Garbulowski M, Mosca R, Gallardo-Dodd CJ, Kutter C, Sonnhammer ELL. 2024. Comprehensive analysis of the RBP regulome reveals functional modules and drug candidates in liver cancer. *bioRxiv* Preprint

110. Lin Y, Zheng J, Mai Z, Lin P, Lu Y, et al. 2024. Unveiling the veil of RNA binding protein phase separation in cancer biology and therapy. *Cancer Letters* 601:217160

111. Zheng Y, Hlady RA, Joyce BT, Robertson KD, He C, et al. 2019. DNA methylation of individual repetitive elements in hepatitis C virus infection-induced hepatocellular carcinoma. *Clinical Epigenetics* 11:145

112. Shukla R, Upton KR, Muñoz-Lopez M, Gerhardt DJ, Fisher ME, et al. 2013. Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. *Cell* 153:101–11

113. Cordaux R, Batzer MA. 2009. The impact of retrotransposons on human genome evolution. *Nature Reviews Genetics* 10:691–703

114. Zheng Y, Joyce BT, Liu L, Zhang Z, Kibbe WA, et al. 2017. Prediction of genome-wide DNA methylation in repetitive elements. *Nucleic Acids Research* 45:8697–711

115. Li VSW, Ng SS, Boersema PJ, Low TY, Karthaus WR, et al. 2012. Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex. *Cell* 149:1245–56

116. Clevers H, Nusse R. 2012. Wnt/β-catenin signaling and disease. *Cell* 149:1192–205

117. Jiang X, Tan J, Li J, Kivimäe S, Yang X, et al. 2008. DACT3 is an epigenetic regulator of Wnt/β-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. *Cancer Cell* 13:529–41

118. Malcomson FC, Willis ND, McCallum I, Xie L, Ibero-Baraibar I, et al. 2017. Effects of supplementation with nondigestible carbohydrates on fecal calprotectin and on epigenetic regulation of SFRP1 expression in the large-bowel mucosa of healthy individuals. *The American Journal of Clinical Nutrition* 105:400–10

119. Quan H, Zhou F, Nie D, Chen Q, Cai X, et al. 2014. Hepatitis C virus core protein epigenetically silences SFRP1 and enhances HCC aggressiveness by inducing epithelial–mesenchymal transition. *Oncogene* 33:2826–35

120. Li KK, Li F, Li QS, Yang K, Jin B. 2013. DNA methylation as a target of epigenetic therapeutics in cancer. *Anti-Cancer Agents in Medicinal Chemistry* 13:242–47

121. Yoon H, Jang KL. 2022. Hepatitis B virus X protein and hepatitis C virus core protein cooperate to repress E-cadherin expression via DNA methylation. *Heliyon* 8

122. Chiam K, Centenera MM, Butler LM, Tilley WD, Bianco-Miotto T. 2011. GSTP1 DNA methylation and expression status is indicative of 5-aza-2′-deoxycytidine efficacy in human prostate cancer cells. *PLoS One* 6:e25634

123. Dong Y, Wang A. 2014. Aberrant DNA methylation in hepatocellular carcinoma tumor suppression. *Oncology Letters* 8:963–68

124. Bhat V, Srinathan S, Pasini E, Angeli M, Chen E, et al. 2018. Epigenetic basis of hepatocellular carcinoma: A network-based integrative meta-analysis. *World Journal of Hepatology* 10:155

125. Heim MH, Thimme R. 2014. Innate and adaptive immune responses in HCV infections. *Journal of Hepatology* 61:S14–S25

126. Olaniyan MF, Olaniyan TB. 2024. Hepatitis C virus infection: innate and adaptive immunity, risk factors, genotypes and prevalence in Nigeria – a systematic review. *Microbes and Infectious Diseases* 5(2):551–70

127. Serti E, Werner JM, Chattergoon M, Cox AL, Lohmann V, et al. 2014. Monocytes activate natural killer cells via inflammasome-induced interleukin 18 in response to hepatitis C virus replication. *Gastroenterology* 147:209–220.e3

128. Casey JL, Feld JJ, MacParland SA. 2019. Restoration of HCV-specific immune responses with antiviral therapy: a case for DAA treatment in acute HCV infection. *Cells* 8:317

129. Wieland D, Kemming J, Schuch A, Emmerich F, Knolle P, et al. 2017. TCF1$^+$ hepatitis C virus-specific CD8$^+$ T cells are maintained after cessation of chronic antigen stimulation. *Nature Communications* 8:15050

130. Kwak J, Choi JH, Jang KL. 2017. Hepatitis C virus Core overcomes all-trans retinoic acid-induced apoptosis in human hepatoma cells by inhibiting *p14* expression via DNA methylation. *Oncotarget* 8:85584

131. Higgs MR, Lerat H, Pawlotsky JM. 2010. Downregulation of Gadd45β expression by hepatitis C virus leads to defective cell cycle arrest. *Cancer Research* 70:4901−11

132. Chen XW, Lin X. 2014. Big data deep learning: challenges and perspectives. *IEEE Access* 2:514−25

133. Okamoto Y, Shinjo K, Shimizu Y, Sano T, Yamao K, et al. 2014. Hepatitis virus infection affects DNA methylation in mice with humanized livers. *Gastroenterology* 146:562−72

134. Nishida N, Iwanishi M, Minami T, Chishina H, Arizumi T, et al. 2015. Hepatic DNA methylation is affected by hepatocellular carcinoma risk in patients with and without hepatitis virus. *Digestive Diseases* 33:745−50

135. Perez S, Kaspi A, Domovitz T, Davidovich A, Lavi-Itzkovitz A, et al. 2019. Hepatitis C virus leaves an epigenetic signature post cure of infection by direct-acting antivirals. *PLoS Genetics* 15:e1008181

136. Cano-Rodriguez D, Gjaltema RAF, Jilderda LJ, Jellema P, Dokter-Fokkens J, et al. 2016. Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. *Nature Communications* 7:12284

137. Jiang W, Xia T, Liu C, Li J, Zhang W, et al. 2021. Remodeling the epigenetic landscape of cancer—application potential of flavonoids in the prevention and treatment of cancer. *Frontiers in Oncology* 11:705903

138. Gholap AD, Chavda VP, Vaghela DA, Sawant SD, Hatvate NT, et al. 2024. A benefaction of flavonoids as epigenetic modulators for effective cancer treatment. *Preprint*

139. Aanniz T, Bouyahya A, Balahbib A, El Kadri K, Khalid A, et al. 2024. Natural bioactive compounds targeting DNA methyltransferase enzymes in cancer: mechanisms insights and efficiencies. *Chemico-Biological Interactions* 392:110907

140. Heidor R, Vargas-Mendez E, Moreno FS. 2019. Epigenetic aspects of hepatocellular carcinoma chemoprevention. In *Epigenetics of Cancer Prevention*, eds. Bishayee A, Bhatia D. Amsterdam: Elsevier. pp. 231-49. doi: 10.1016/b978-0-12-812494-9.00011-1

141. Chaturvedi CP, Somasundaram B, Singh K, Carpenedo RL, Stanford WL, et al. 2012. Maintenance of gene silencing by the coordinate action of the H3K9 methyltransferase G9a/KMT1C and the H3K4 demethylase Jarid1a/KDM5A. *Proceedings of the National Academy of Sciences* 109:18845−50

142. Chen QW, Zhu XY, Li YY, Meng ZQ. 2014. Epigenetic regulation and cancer. *Oncology Reports* 31:523−32

143. Patnaik E, Madu C, Lu Y. 2023. Epigenetic modulators as therapeutic agents in cancer. *International Journal of Molecular Sciences* 24:14964

144. Costa PMdS, Sales SLA, Pinheiro DP, Pontes LQ, Maranhão SSA, et al. 2023. Epigenetic reprogramming in cancer: from diagnosis to treatment. *Frontiers in Cell and Developmental Biology* 11:1116805