REVIEW ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Promoter methylation of certain tumor regulatory genes and hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy of adults and the second responsible cause of cancer-related death around the world. Hepatocellular carcinoma (HCC) accounts for about 90% of primary liver malignancies. In Egypt chronic hepatitis C virus (HCV) is a major health burden and a major risk factor for HCC. Alteration of DNA Methylation at promoter regions has a recognized role during early evolution and development of human diseases, including cancers. It can be in the form of hypermethylation, hypomethylation, and loss of imprinting. RUNX3 and p16 are tumor suppressor genes that may be inactivated by hypermethylation which is a key epigenetic mechanism that contributes to the initiation and progression of various types of human carcinomas including HCC.

Keywords: RUNX3; p16; Hepatocellular carcinoma; Gene Methylation.

1. Hepatocellular Carcinoma

Hepatocellular Carcinoma (HCC) is a serious public health issue and the fourth leading cause of cancer mortality worldwide **(Kudo et al., 2014; Suresh et al., 2020)**. HCC accounts for about 80% of the primary liver cancers while the other types include cholangiocarcinoma (10–20%) and angiosarcoma (1%) **(Biswas et al., 2015)**. There is a striking variation in HCC incidence rate across geographic regions and at the global level. Over 800,000 people are diagnosed with liver cancer each year **(Ferlay et al., 2019; Thylur et al., 2020)**.

HCC predominantly affects men more than women (two to four times higher in men) with its highest incidence in the age group of 45–65 years **(Wands, 2007; Mittal et al., 2018)**. HCC is the fifth most common cancer in men and the ninth most occurring cancer in women **(Bray et al., 2018)**. The overall ratio of mortality to incidence is 0.95, which reflects the poor prognosis of HCC **(Njei et al., 2015)**.

HCC is an extremely complex condition and there are multiple factors involved in the etiology of HCC. The HCC malignant progression is related to genetic, lifestyle and environmental factors **(Younossi and Henry, 2016)**. The major risk factors for HCC include hepatitis B virus (HBV) and hepatitis C virus (HCV), diabetes, obesity, alcoholic fatty liver disease (AFLD), and non-alcoholic fatty liver disease (NAFLD). Additional risk factors that are also known to increase the incidence of HCC are tobacco smoking, food contaminants such as aflatoxins, familial or genetic factors, and various environmental toxins that act as carcinogens **(Sanyal et al., 2010; Jindal et al.,**

2019; Yang et al., 2019) (Figure 1).

1.1. Risk Factors

1.1.1. Viral hepatitis

Chronic liver disease and cirrhosis remain the most important risk factors for the development of HCC of which viral hepatitis and excessive alcohol intake are the leading risk factors worldwide. Chronic viral hepatitis can lead to cirrhosis and/or HCC. HBV and HCV are the most common causes of chronic hepatitis in the world **(Constantin et al., 2010)**.

HBV is a double-stranded, circular DNA molecule with eight genotypes (A to H). Geno- types A and D are more common in Europe and the Middle East, while genotypes B and C are more common in Asia **(Bruix and Sherman, 2011)**. HCV is a small, single-stranded RNA virus, which exhibits high genetic variability **(Choo et al., 1991)**. There are six different genotypes of HCV isolated. Genotypes I, II, and III are predominant in the Western countries and the Far East, while type IV is predominant in the Middle East **(Suresh et al., 2020)**. Once infected with HCV, 80% of patients progress to chronic hepatitis, with ~20% developing cirrhosis **(Asham et al., 2013).** In hepatitis C, the development of HCC occurs almost exclusively in the liver with established cirrhosis; however, in the HALT-C trial, 8% of HCC occurred in patients with only advanced fibrosis **(Lok et al., 2009)**. A synergistic effect with alcohol increases the incidence of HCC between 1.7-fold and 2.9-fold when compared to HCV–HCC alone **(Puoti et al., 2004; Singal and Anand, 2007)**. The risk of HCC was reduced significantly in patients who obtained a sustained viral response after treatment of HCV with a 54% reduction in all-cause mortality **(Morgan et al., 2013)**.

1.1.2. Alcoholic and non-alcoholic Fatty Liver Disease

Over the last decade, fatty liver disease is emerging as one of the leading etiologies for chronic liver disease progressing to HCC **(Pocha and Xie, 2019)**. The growing inclination towards western dietary pattern, sociocultural changes and the lifestyle with limited or no physical activity has sharply increased the incidence rates of NAFLD- and AFLD-associated HCC across the continents **(Romero-Gómez et al., 2017; Wandji et al., 2020)**.

The pathological spectra of liver injury in promoting HCC development are similar in these two fatty liver diseases despite having divergent pathogenic origin with yet some key distinct features **(Figure 2)**. Furthermore, a high-calorie diet and ethanol act synergistically at multiple levels potentiating hepatocarcinogenesis **(Younossi and Henry, 2016)**.

AFLD is attributed to excessive alcohol consumption that causes hepatic injury by the build-up of fats, inflammation, and scarring leading to HCC, which could be fatal. Globally, the prevalence of AFLD is increasing and has become a significant contributor to the liver disease burden accounting for 30% of HCC related deaths **(Pennisi et al., 2019)**. By contrast, excessive alcohol consumption (more than 14 drinks/week and 7 drinks/week for men and women, respectively) is considered to cause AFLD **(Wandji et al., 2020)**. The threshold level of alcohol intake causing hepatotoxic effect varies and it depends on a variety of factors such as gender, ethnicity, and genetics **(Gramenzi et al., 2006)**.

1.1.3. Diabetes and obesity

Sixty percent of patients older than 50 years with diabetes or obesity are thought to have non-alcoholic steatohepatitis (NASH) with advanced fibrosis **(Rinella, 2015)**. Chronic medical conditions such as diabetes mellitus and obesity increase the risk of HCC. Diabetes mellitus directly affects the liver because of the essential role the liver plays in glucose metabolism. It can lead to chronic hepatitis, fatty liver, liver failure, and cirrhosis. Diabetes is an independent risk factor for HCC **(Wang et al., 2012; Gao et al., 2013)**. Patients with diabetes have between 1.8- and 4-fold increased risk of HCC. When compared to HCV, NASH-related HCC liver transplants increased by nearly four times in the decade from 2002 to 2012 **(Wong et al., 2014)**.

It is well-known that obesity is associated with many hepatobiliary diseases, including NAFLD, steatosis, and cryptogenic cirrhosis; all of which can lead to the development of HCC **(Calle et al., 2003; Reddy and Rao, 2006)**. Obesity itself increases the risk of HCC by 1.5- to 4-fold. The relative risk of HCC is 117% for overweight subjects and 189% for obese patients **(Larsson and Wolk, 2007)**.

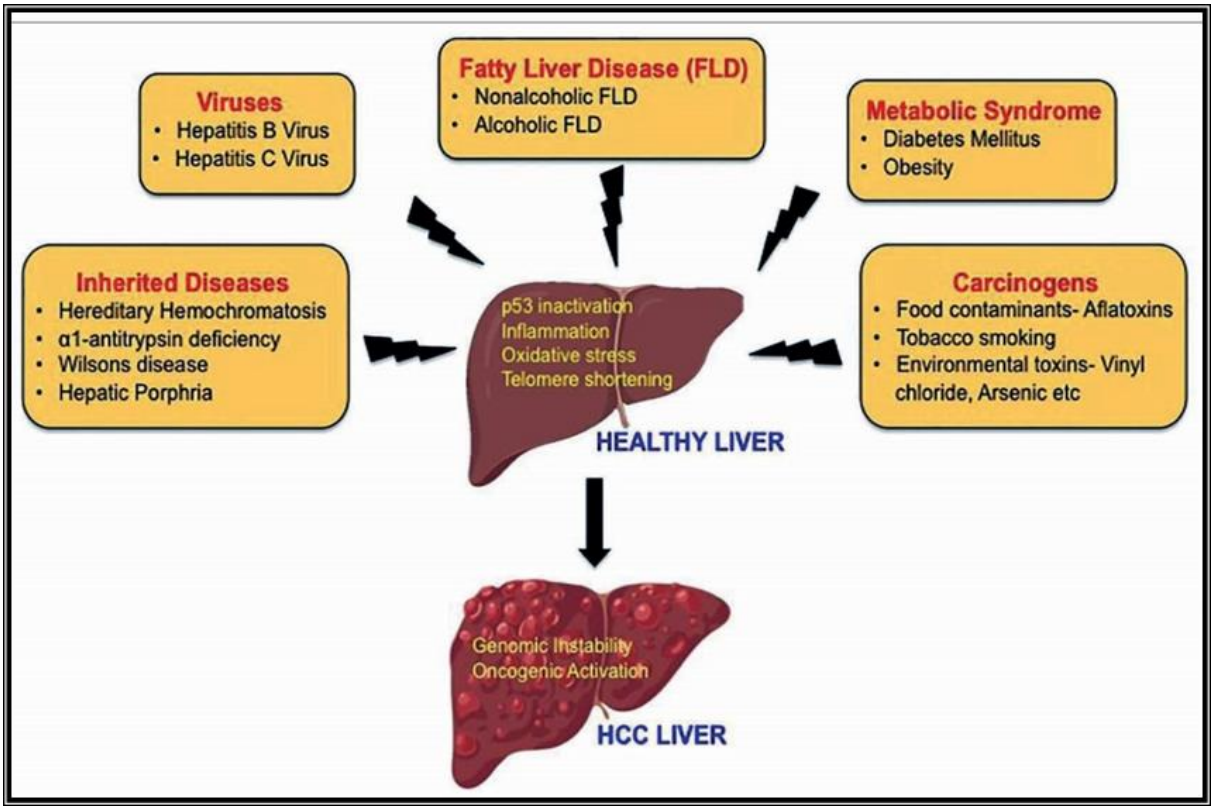

Figure 1. The etiology of hepatocellular carcinoma. A variety of risk factors have been associated with the development of HCC, including hepatitis viruses, carcinogens, heredity diseases, metabolic syndrome, and fatty liver disease. The mechanisms by which these etiological factors may induce hepatocarcinogenesis mainly include p53 inactivation, inflammation, oxidative stress, and telomere shortening leading to genomic instability and activation of multiple oncogenic signaling pathways **(Suresh et al., 2020)**.

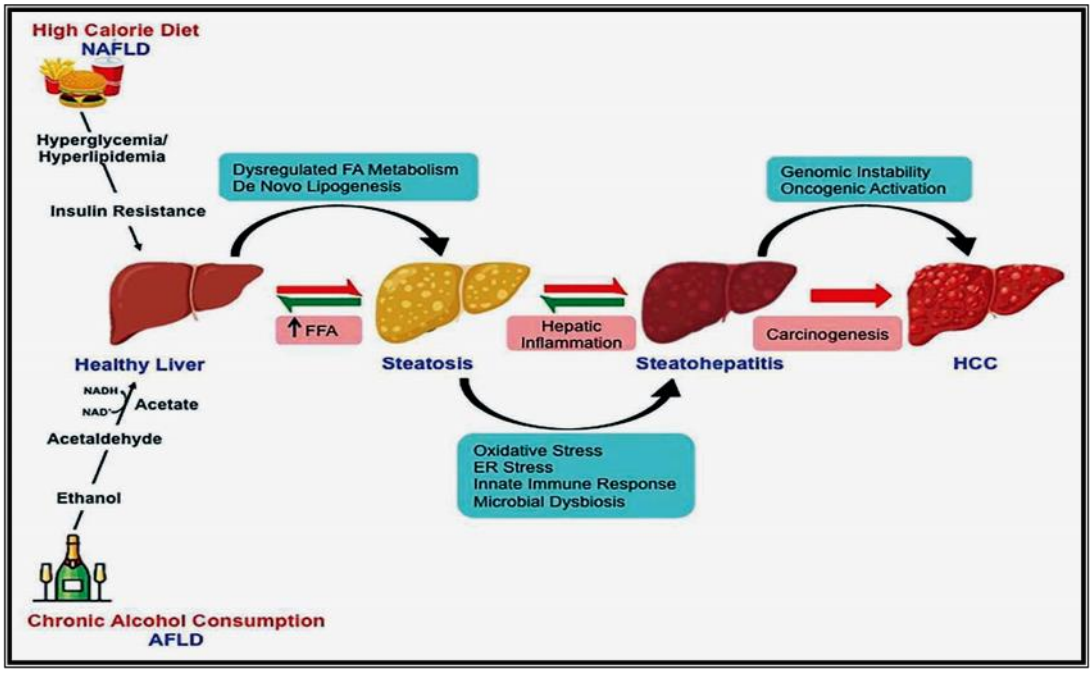

Figure 2. Molecular mechanisms involved in nonalcoholic- and alcoholic-associated HCC. High-calorie diet and excessive alcohol consumption are the major risk factors for the development of NAFLD and AFLD, respectively. Despite the divergent pathogenic origin, the pathological spectra of liver injury in promoting HCC development in NAFLD and AFLD share common molecular pathways **(Suresh et al., 2020)**.

1.1.4. Other predisposing conditions:

Males are more likely to be infected with viral hepatitis, consume greater quantities of alcohol, smoke cigarettes, and have a higher body mass index than women. Androgens (AR) are male hormones that have been increasingly reported in male-predominant HCC **(Montella et al., 2015)**. They are mainly involved in various physiological and pathological activities by combining with androgen receptors (ARs) **(Beato and Klug, 2000)**. A study by Wu et al **(Wu et al., 2010)**, identified that overexpression of ARs enhanced HCC cell growth and invasion in vitro, and HCC initiation in vivo. Previous studies have reported higher androgen levels and more active androgen response elements (AREs) in liver tumor tissues, compared with control tissues **(Barone et al., 2009; Wang et al., 2009)**. AR binding to ARE of the cell cycle related kinase promoter region controls activation of the β-catenin/T-cell fac tor signaling pathway and has been identified as a major carcinogenic event and described in animal models and up to 90% of HCC cases **(Feng et al., 2011)**.

Ligand-stimulated AR upregulated miR-216a, resulting in tumorigenesis, and AR and miR-216a were concordantly over-expressed in clinical specimens **(Chen et al., 2012)**. Both activity and secretion of aromatase, an enzyme which converts androgens to estrogens, was markedly increased in human HCC tissues and HepG2 cells **(Koh et al., 2011)**.

Aflatoxin produced by Aspergillus species (molds) found on grains, corn, peanuts, or soybeans stored in warm humid conditions is a potent hepatocarcinogen. The risk of HCC with aflatoxin is dependent on the dose and duration of exposure. Aflatoxin exerts a synergistic effect on HVB- and HCV-induced liver cancer, the risk being 30 times greater with chronic HBV plus aflatoxin exposure than with aflatoxin exposure alone **(Liu and Wu, 2010)**. The most potent aflatoxin, AFB1, when removed from the environment has resulted in a reduction of the incidence of HCC **(Chen et al., 2013)**.

The risk of HCC with hereditary hemochromatosis is estimated to be between 100- and 200-fold (**Ko et al., 2007)**. Other iron over- load states such as thalassemia have not only been associated with HCC but also have a high prevalence of HCV that may contribute to the increased risk of primary liver cancer. South African blacks who consume beer brewed in nongalvanized steel drums have increased iron stores leading to an increase in the risk of HCC 10 times that of people with normal iron stores **(Gandini et al., 2008)**.

Studies investigating the use of oral contraceptive pills and the risk for development of HCC have been inconclusive; however, a review of six studies showed a significant increase in HCC risk with a longer duration (>5 years) of exposure to oral contraceptives **(Maheshwari et al., 2007)**.

2. Epigenetics

The term “epigenetics” was coined by Conrad Hal Waddington, a British developmental biologist in 1942, to describe the “whole complex of developmental processes” linking genotype and phenotype **(Waddington, 2012)**. Since then, this concept has changed several times (**Deichmann, 2016)**, and recent studies on epigenetics at the molecular level mainly cover changes in DNA methylation, histone modifications, non-coding RNAs (ncRNAs), and higher-order chromatin structure. Epigenetic mechanisms define mitotically heritable differences in gene expression potential without altering the primary DNA sequence. These mechanisms are highly regulated by a large number of proteins that establish, read, and erase specific epigenetic modifications, thereby defining where and when the transcriptional machinery can access the primary DNA sequences to drive normal growth and differentiation in the developing embryo and fetus. Several types of epigenetic marks work in concert to drive appropriate gene expression **(Inbar-Feigenberg et al., 2013) (Figure 3)**. Even though the role of epigenetics was first recognized in development, an increasing amount of evidence has shown that it is also related to the development and progression of many common disease **(Jin and Liu, 2018)**.

2.1. Epigenetic marks

DNA methylation, histone modifications, and ncRNAs are described below as independent mechanisms, but it is important to note that there is cross-talk between the different epigenetic marks to regulate the epigenome **(Weber et al., 2007; Otani et al., 2009)**.

2.1.1. Histone modifications

The basic unit of chromatin consists of an octamer of histone proteins, two each of H2A, H2B, H3, and

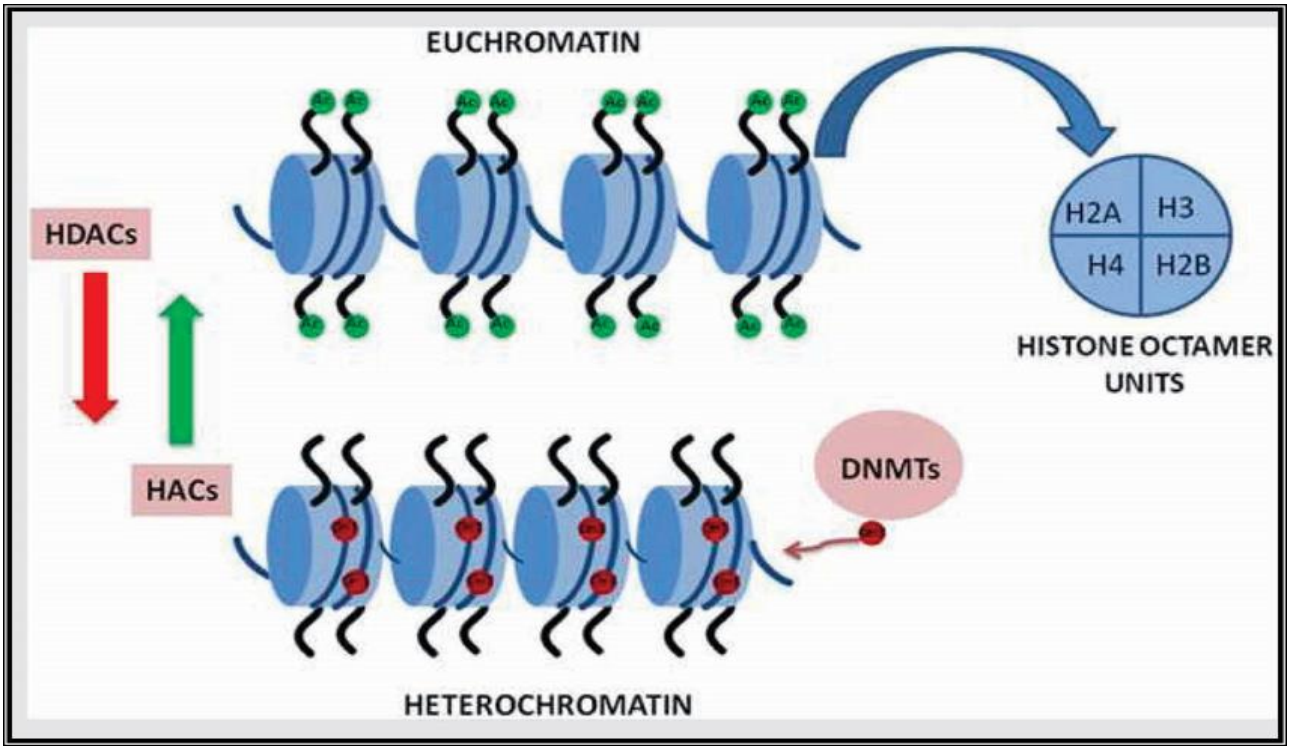

Figure 3. Epigenetic mechanisms affecting gene expression. Epigenetic patterns are established by a number of mechanisms. Epigenetic marks include DNA methylation and covalent modifications of histone proteins. DNA methylation is established and maintained by the DNMT enzymes. DNA is wrapped around histone protein cores composed of an octamer containing two copies of each core histone: H2A, H2B, H3, and H4. Together, these form the basic unit of chromatin, the nucleosome. Histone modifications are regulated by several enzymes including histone acetyltransferases (HATs) and deacetylases (HDACs). Acetylation of histone proteins by HAT commonly found in euchromatin (relaxed state of chromatin) and is associated with active transcription. Deacetylation of histone proteins by HDAC and methylation of DNA by DNMTs is a hallmark of heterochromatin (condensed state of chromatin), which is associated with transcriptional repression **(Inbar-Feigenberg et al., 2013)**.

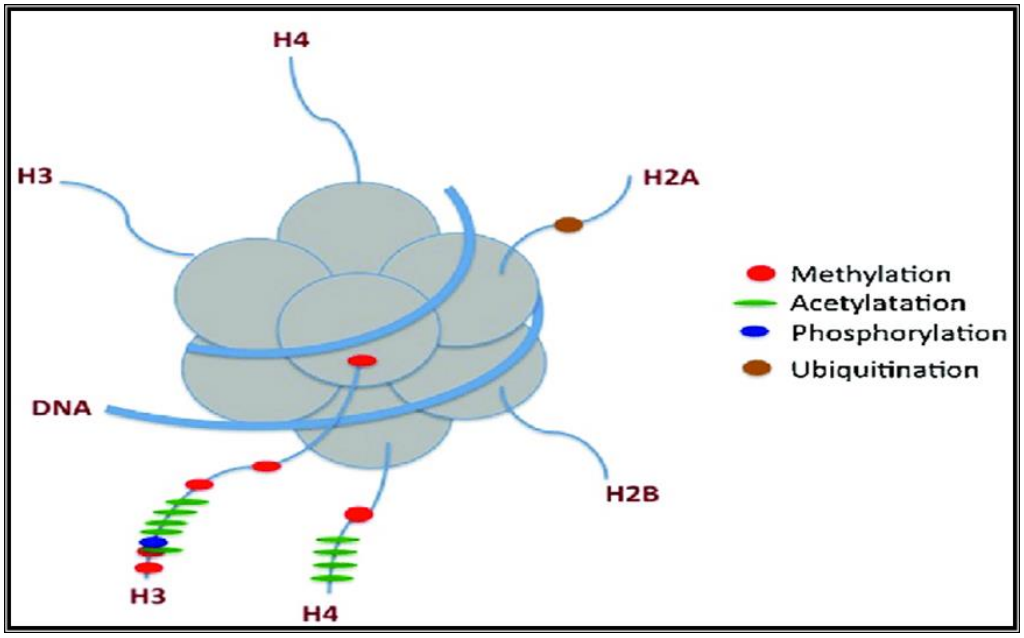

Figure 4. Schematic representation of histone modifications. The methylation sites are represented in red color at H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20. The acetylation sites are shown in green color at amino acid H3K9, H3K14, H3K18, and H3K23 and H4K5, H4K8, H4K12, and H4K16. The phosphorylation site is indicated in blue color at H3S10. 82 A ubiquitination site is randomly designated in brown color or H2A **(Nayan et al., 2015)**.

H4. DNA wraps around this core, which provides structural stability and the capacity to regulate gene expression. Each core histone within the nucleosome contains a globular domain and a highly dynamic N-terminal tail extending from the globular domains. Histone proteins have tails that can have a number of post-translational modifications including acetylation, methylation, phosphorylation, ubiquitylation, simulation, ADP-ribosylation, proline isomerization, citrullination, butyrylation, phosphorylation, and glycosylation **(Fraga et al., 2005) (Figure 4)**.

2.1.2. Regulatory ncRNAs

Non-coding RNAs (ncRNAs) are also required for epigenetic regulation of gene expression. Although eukaryotic genomes transcribe up to 75% of genomic DNA, approximately 3% of these transcripts encode for proteins; the majority are ncRNAs, which can be classified according to size and function **(Consortium, 2011; Djebali et al., 2012)**. Regulatory ncRNAs; including small interfering RNAs (siRNAs), microRNAs (miRNAs), and long ncRNAs (lncRNAs); play important roles in regulation of gene expression at several levels: transcription, mRNA degradation, splicing, and translation **(Kaikkonen et al., 2011)**.

siRNAs are double-stranded RNAs that mediate post-transcriptional silencing, in part by inducing hetero- chromatin to recruit histone deacetylase complexes **(Grewal, 2010)**.

miRNAs comprise a class of endogenous, small (18–24 nucleotides in length); single-stranded RNAs that can control gene expression by targeting specific mRNAs for degradation and/or translational repression **(Hutvágner and Zamore, 2002; Lee et al., 2003)**. They can also control gene expression by recruiting chromatin-modifying complexes to DNA through binding to DNA regulatory regions, thereby altering chromatin conformation **(Chuang and Jones, 2007; Carthew and Sontheimer, 2009)**.

Long interfering non-coding RNAs (LincRNAs), a subset of lncRNA, exhibit high conservation across different species. They have been shown to guide chromatin-modifying complexes to specific genomic loci, thereby participating in the establishment of cell type–specific epigenetic states **(Guttman et al., 2009)**.

2.1.3. DNA methylation

One of the best studied epigenetic mechanisms is DNA methylation **(Maunakea et al., 2010)**. DNA methylation is typically associated with gene silencing through binding of methylation-sensitive DNA binding proteins and/or by interacting with various modifications of histone proteins that modulate access of gene promoters to transcriptional machinery. In eukaryotic species, DNA methylation involves transfer of a methyl group (CH_3) to the cytosine at the carbon 5 position **(Figure 5)** of the CpG dinucleotide **(Lande-Diner et al., 2007)**. The vast majority of mammalian DNA methylation occurs at CpG dinucleotides **(Ibrahim et al., 2006; Lande-Diner et al., 2007)**.

3. Epigenetics in liver cancer

The role of epigenetic deregulation in HCC is being increasingly recognized **(Pogribny and Rusyn, 2014)**. In addition to changes in DNA methylation, microRNA expression, mutations affecting epigenetic regulatory genes have recently been discovered in HCC **(Fujimoto et al., 2012)**. HCC cells display global hypomethylation as well as promoter hypermethylation of a large set of genes **(Poungpairoj et al., 2015)**. Promoter hypermethylation appears to affect mainly tumour suppressor and antiproliferative genes resulting in downregulation of gene expression. Aberrations in microRNA expression have also been observed with several of them being linked to metabolic and phenotypic changes in HCC cells **(Sandoval and Esteller, 2012)**. Several genes encoding epigenetic regulatory proteins are involved in hepatocellular malignancy. Expression of histone deacetylases (HDACs) is deregulated in different cancers **(Weichert, 2009)**, and some of them are also deregulated in HCC. HDACs-1, -2 and -3 are over-expressed in HCC (**Quint et al., 2011)**.

3.1. The runt domain-related transcription factor (RUNX) family genes

The RUNX family genes, which are composed of RUNX1, RUNX2, and RUNX3, are essential regulators of cell fate in the development and regulation of p53-dependent DNA damage response and/or tumorigenesis **(Blyth et al., 2005; Ito, 2008; Ozaki et al., 2013)**. RUNX3 gene is one of the most critical members of the runt domain family and plays a critical role in the regulation of cell proliferation, apoptosis, angiogenesis, as well as cell adhesion and invasion **(Lund and Van Lohuizen, 2002; Subramaniam et al., 2009)**.

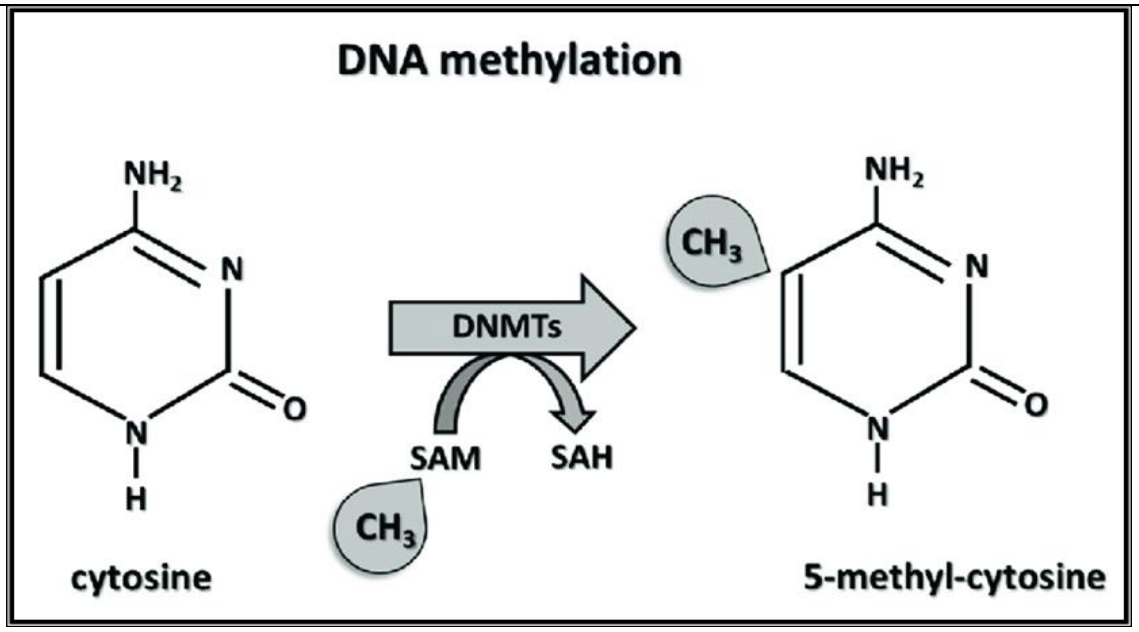

Figure 5. The mechanism of DNA methylation. DNA methylation is exerted by DNA methyltransferases (DNMTs) (DNMT3a- DNMT3b-DNMT1). DNMTs at the 5 -position of cytosine residues in CpG dinucleotides transfer methyl groups from SAM (S-adenosylmethionine) to SAH (S-adenosylhomocysteine); thus, 5-methylcytosine is formed **(Ciechomska et al., 2019)**.

RUNX3 gene, localized in chromosome 1p36, a region that exhibits frequent loss of heterozygosity events in breast, colon, gastric, and ovarian cancers, is considered as a tumor suppressor gene involved in the transforming growth factor-beta (TGF-β) signaling pathway (Levanon et al., 1994). RUNX3 and p53 are both principal responders of the p14 (ARF)-MDM2 cell surveillance pathway that prevents pathologic consequences of abnormal oncogene activation (Chi et al., 2009). Its precise function has been intensively studied in several tumors, with upregulation of inducing cell cycle arrest, apoptosis, and downregulation of cyclin D1 expression **(Li et al., 2002; Chi et al., 2005; Shiraha et al., 2011; Chen, 2012)**.

Lack of protein expression of RUNX3 by promoter methylation (hypermethylation) has been found to play an important role in liver epithelial tumorigenesis and epithelial-mesenchymal transition of HCC **(Li and Jiang, 2011; Shiraha et al., 2011; Tanaka et al., 2012)**. Decreased levels of RUNX3 mRNA have been observed in 50–92 % of HCC cases **(Mori et al., 2005; Miyagawa et al., 2006)**. Hypermethylation of the RUNX3 promoter was found in 41–76 % of HCC cases **(Kim et al., 2004; Mori et al., 2005; Nishida et al., 2008; Moribe et al., 2009)**. Hypermethylation of the RUNX3 promoter was also found to be more frequent in HCV-related HCC (81.8 %) **(Nishida et al., 2008)**.

Recently, the hypermethylation of RUNX3 was shown to be associated with HCC and significantly correlated with higher serum levels of alpha fetoprotein (AFP) in an Egyptian sample **(El‑shaarawy et l., 2022)**.

3.2. p16 gene

The INK4 family is a family of cyclin-dependent kinase inhibitors that includes four members: $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, which show analogous biological characteristics involved in inhibition of cell growth and in tumor suppression **(Serrano, 1997; Komata et al., 2003)**. The tumor suppressor p16 gene encodes proteins involved in the regulation of two fundamental cell cycle pathways, the p53 and the RB1 pathway. The INK4A locus is localized in short arm of chromosome 9 at position band 21.3 (9p21.3) **(Donovan and Slingerland, 2000)**. Using alternative exons, the p16 gene generates four transcriptional variants: $p16^{INK4A}$, a cyclin-dependent kinase inhibitor, $p14^{ARF}$ (alternative reading frame), which binds to MDM2 (**Robertson and Jones, 1999)**, p12 and p16 (Li et al., 2011). The structure of the p16 gene includes exons E1β, E1α, E2, E2, and E3 **(Figure 6)**. p16 is a tumor suppressor gene and goes by several names: MTS-1 (major tumor suppressor 1), INK4a (inhibitor of cyclin-dependent kinase 4a)

or $p16^{INK4}$ and CDKN2A (cyclin-dependent kinases inhibitor 2A). Its location at 9p21 is the site of loss of heterozygosity in several malignancies and unsurprisingly, p16 is thus implicated in several tumors **(Serrano, 1997)**.

p16 is a major inactivation target in HCC **(Baek et al., 2000)**. Promoter hypermethylation of INK4A, resulting in the loss of INK4A RNA expression in HCC tissue specimens **(Wang et al., 2012)**, has been attributed to the eventual loss of p16 expression in 60–80 % of HCC specimens **(Kaneto et al., 2001; Harder et al., 2008)**.

Hepatitis virus-positive HCC specimens have higher p16 methylation levels compared to HCC specimens without hepatitis virus infection **(Jicai et al., 2006; Feng et al., 2010)**. Hypermethylation patterns of p16 in HCC liver specimens also correlate with its hypermethylation in plasma and serum **(Wong et al., 1999)**. In a recent study on Egyptian HCC patients, p16 promotor methylation showed association with HCC incidence. Additionally, it showed also a positive correlation with higher serum AFP and AST levels in the patients **(El‑shaarawy et l., 2022)**.

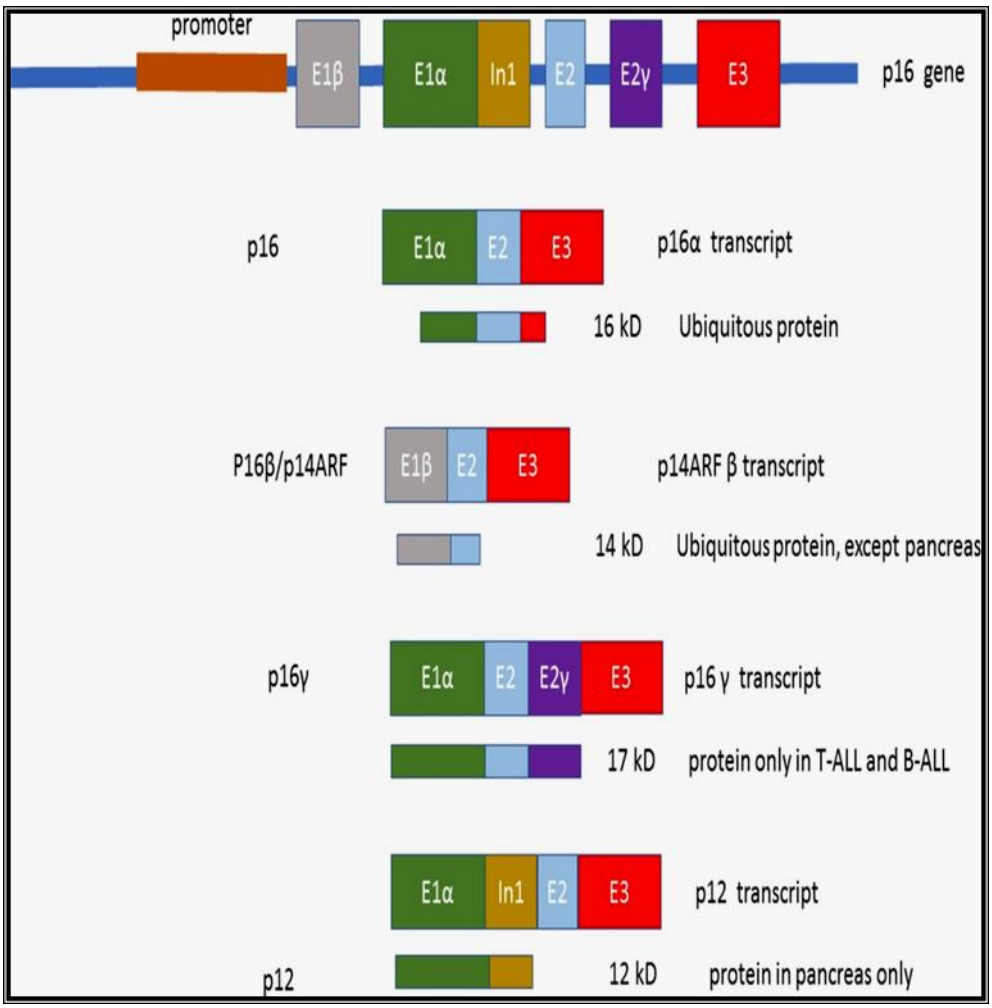

Figure 6. Schematic representation of the p16 gene **(Li et al., 2011).**

4. Conclusion

This review outlines the role of the epigenetic markers, especially the hypermethylation of tumor regulatory genes such as RUNX3 gene and p16 gene, with the development and prognosis of hepatocellular carcinoma.

Conflict of Interest

None of the authors have any conflicts of interest.

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