DISCUSSION

Infection with Hepatitis C Virus (HCV) is a major risk factor for the development of HCC. Despite the fact that HCV is a RNA virus without a DNA intermediate, recent studies demonstrate that HCV viral proteins may actively participate in epigenetic regulation of hepatic cancer stem cell phenotypes and induce HCC-specific epigenetic changes. Identification of host epigenetic alterations induced by HCV infection and epigenetic differences between hepatic cancer stem cells and the bulk non-tumorigenic cancer cells, may yield potential biomarkers for early detection, as well as therapeutic targets for HCV associated HCC. [78]One short-term goal of diagnostic technologies is to reduce invasiveness and refine disease-forecasting. If specific epigenetic markers can be established for a specific disease, it will be feasible to use blood samples to analyze epigenetic signatures. The analysis of methylation in plasma or whole blood DNA could allow identifying aberrant methylation patterns before the onset of the symptoms. [61,113, 114]

Distinct epigenetic marks decide which sets of genes may be expressed and which genes are kept silent. Epigenetic marks can be grouped into four main categories: DNA methylation, histone modifications, chromatin remodeling and non coding RNA. [21]

DNA can be modified by methylation of cytosine bases. The enzymes that methylate DNA are called DNA methyltransferases. In humans the *de novo* DNA methyltransferases DNMT3A and DNMT3B methylate the genome during embryonic development, whereas the maintenance DNA methyltransferase DNMT1 methylates hemimethylated DNA following mitosis. Methylated DNA inhibits gene expression, as it attracts methylcytosine binding proteins that promote chromatin condensation into transcriptionally repressive conformations. In humans, only cytosines preceding guanines (CpG dinucleotides) are known to be highly methylated. Although the majority of CpGs are located in non-coding regions and are typically methylated, most remaining CpG dinucleotides are found in clusters upstream of gene coding sequences. These clusters, called CpG islands, are typically nonmethylated so as to allow gene expression.[6]

Cancer is caused by failure of checks and balances that control cell numbers in response to the needs of the whole organism. Inappropriate function of genes that promote or inhibit cell growth or survival can be caused by errors introduced into the genetic code itself or by faulty epigenetic mechanisms deciding which genes can and cannot be expressed. [115]

Since the disclosure of epigenetic regulation in key genes, many studies have shown the clinical efficacy of measuring promoter hypermethylation in various specimens such as tumor tissue, feces, and urine for determining the diagnosis and prognosis of cancer patients.Most studies measuring methylated DNA in the blood stream of HCC patients have reported positive results .[104] Unlike mutations , DNA methylation usually occurs at a fixed location in the promoter region of the gene , facilitating development of suitable assays .In addition,DNA methylation changes can be detected noninvasively in blood or other body fluids ,making them ideal biomarker.[78]

Aberrant DNA methylation is a major epigenetic mechanism of gene silencing and is observed in many cancers including HCC, Several studies support the potential role of promoter hypermethylation in HCC-related gene silencing, and this has been shown to be positively correlated with tumor progression. [29,88]Epigenetic changes on Ras association domain family member 1 (RASSF1A), p16, and p15 tumor suppressor genes in DNA have been shown to be potential biomarkers for early detection in populations at high risk for HCC.[114]

Serine protease inhibitor gene (Kunitz type 2, SPINT2) encodes a transmembrane protein with two extracellular Kunitz domains that inhibits a variety of serine proteases. The protein inhibits hepatocyte growth factor (HGF) activator, which prevents the formation of active hepatocyte growth factor. This gene is a putative tumor suppressor. The SPINT2 gene is located on the long (q) arm of chromosome 19 at position 13.1(19q13.1).[91]

The present work aimed at studying the methylation status of the promoter of serine peptidase inhibitor, kunitz type 2 (SPINT2) gene in chronic hepatitis-C virus (HCV) infected Egyptian patients with and without hepatocellular carcinoma.

In the current study, the methylation status of SPINT2 gene promoter was studied in the peripheral blood by using Methylation-specific PCR (MSP) technique, in which two primer sets was designed to bisulphite-converted DNA. One primer set is complementary to methylated DNA (M-MSP reaction) while the other primer set was specifically complementary to unmethylated DNA (U-MSP reaction). Bisulphite-converted DNA was amplified in two separate reactions and the products analysed in parallel by agarose gel electrophoresis. This is a sensitive method of detecting methylation allowing detection of methylated alleles in an otherwise unmethylated sample.[116]

In the present study 130 Egyptian adults were included. They were divided into two groups: group I (80 HCV infected patients) and group II (50 apparently healthy control subjects). Group I was further subdivided into two subgroups: group I a (50 HCV infected patients without HCC) and group I b (30 HCV infected patients with HCC).

In the present study ,for all patients (group I) HCV RNA was estimated by Real Time PCR and it was also found that there was no statistically significant difference between HCV PCR viral load between HCV (group Ia) and HCC (group Ib).(Table XIII)

About 200 million people are infected with hepatitis C viruses worldwide .More than two thirds of people with acute HCV infection will develop persistent HCV infection, leading to chronis hepatitis , liver cirrhosis and ultimately HCC .[79,117]

According to child pugh classification of patients (group I) in the current study , patients with chronic HCV (group Ia) showed different classes of liver insult; class A was found in 46.5% ,class B in 41.9% and class C in 11.6 % of HCV patients .(Table VIII)

In the current study the methylation status of SPINT -2 in HCV group (Ia) showed that 60 % of cases were hemimethylated , 4 % were hypermethylated and 36 % were unmethylated.While in the control group (II) , 40 % of cases showed hemimethylation and 60 % of cases were unmethylated.None of cases showed hypermethylation. (Table XVa)

HCV core protein was reported to down regulate expression of E-cadherin correlated with CpG island methylation of E-cadherin promoter through activation of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3B (DNMT3B) in the HCV core protein-expressing HepG2 cell line.Frequent methylation may occur through activation of DNMT by HCV core protein ,or it may be explained by longer infection period.[87]

As for many other tumors, development of hepatocellularcarcinoma (HCC) must be understood as a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSG). In the last decades, in addition to genetic alterations, epigenetic inactivation of tumor suppressor genes by promoter hypermethylation has been recognized as an important and alternative mechanism in tumorigenesis. In HCC, aberrant methylation of promoter sequences occurs not only in advanced tumors, it has been also observed in premalignant conditions just as chronic viral hepatitis B or C and cirrhotic liver. [79]

DNA methylation patterns undergo complex changes in cancer. The total amount of methylated cytosine is usually decreased resulting in global hypomethylation. Besides global hypomethylation, most cancers also show focal hypermethylation in distinct subsets of promoter-associated CpG islands. [118] Affected genes are permanently silenced, since methylation marks are propagated through mitosis and are maintained in the malignant clone. Hundreds to thousands of genes can be epigenetically silenced by CpG island hypermethylation in human cancer, suggesting a general disturbance of epigenetic memory.[119] Methylation affects individual cancer patients with varying extent. While some patients have minimal changes, others show concordant hypermethylation of multiple genes. Epigenetic DNA methylation changes in cancer appear to be considerably more frequent events than genetic mutations. [120] Epigenetic alterations are easier to reverse than mutations affecting the genetic code. [121,122] Two inhibitors of DNA methyltransferases; azacytidine and deoxyazacytidine, have already been approved by the Food and Drug Administration as effective drugs for treatment of patients with myelodysplastic syndromes.[123,124]

In the present sudy, patients with HCV and superimposed HCC showed a methylation status as follows: 6.7% hypermethlated , 60 % hemimethylated and 33.3% unmethylated .(Table XVa)

In the current study, comparison was made between the 3 groups regarding the methylation status and results were divided into 3 categories: unmethylated and hemimethylated or hypermethylated. It was found that there was a statistically significant difference within the groups regarding the methylation status(p=0.028). (Table XV a), when the hemimethyalted subjects and hypermethylated subjects were combined in one group (aberrantly methylated) there was a statistically significant difference within the groups regarding the methylation status.The aberrant methylation in control subjects was significantly less frequent than HCV (p= 0.01) as well less frequent than HCC (p=0.021) , however there was no significant difference between HCV and HCC as regards the methylation status . (Table XV b)

Meta analysis done by Feng et al ,2013 [78]studied changes of DNA methylation with HCC progression and concluded that methylation of several genes occurred not only in HCC and its precursor lesions, but also in chronic hepatitis and liver cirrhosis, suggesting

that these changes are early events during HCC progression; DNA methylation of four genes (Col1A2, IGFBP2, CTGF, fibronectin increased from normal liver, chronic hepatitis, liver cirrhosis to hepatoma .[125] Frequency of E-cadherin promoter methylation increased from dysplastic nodules to early stage and late stage HCCs.[126]Similarly, methylation of p16, p15 and SFRP1 was not only present in HCC, but was also present at low frequencies in chronic hepatitis and liver cirrhosis samples (Fukai *et al*., 2005; Shih *et al*., 2006). [127,128]Further, methylation analysis in various liver tissues demonstrated that the number and frequency of genes methylated progressively increased in liver cirrhosis, dysplastic nodules and HCC, supporting the hypothesis that CpG island methylation of tumor-related genes is an early and frequent event and methylation changes accumulate during a multistep hepatocarcinogenesis.[129]

Archer *et al*. (2010)[130] used a high throughput single nucleotide polymorphism genotyping system with assay probes (Illumina Golden Gate assay)to study methylation status on 76 liver tissues, including 20 HCV positive HCC and adjacent non-tumorous liver tissues, 16 HCV positive cirrhotic liver tissues and 20 normal liver tissues and they identified specific methylation changes in both cirrhotic and HCC. Deng *et al*. (2010)[87]analyzed DNA methylation changes by methylated DNA immunoprecipitation-on-chip (MeDIP-chip) on 3 HBV-HCC, 3 HCVHCC and 3 normal liver tissues and showed that DNA methylation preferentially occurred in HCV-related HCC cases.

Fukai et al 2003, [96]studied the methylation status of the promoter of SPINT2 gene in liver tissues using the same technique, Methyl Specific PCR, in Japanese population and concluded that there is hypermethylation in primary HCC tumors, in 21 of 26 (80.8%) tumors, whereas in corresponding nontumorous liver tissues, *SPINT 2* hypermethylation was detected in 7 of 26 (26.9%) samples In addition, all of the surrounding non tumorous liver samples with SPINT-2 hypermethylation exhibited cirrhosis moreover, in terms of hepatitis virus association with HCC, they found a higher incidence of *SPINT2* methylation in hepatitis C virus-positive tumors than hepatitis B virus surface antigen-positive tumors.

A study used the methyl specific PCR on HCC tissue level was performed by Sua et al (2008),[131] found more aberrant methylation, either complete hypermethyaltion (28.6%) or partial hypermethylation (42.9%) in cancerous tissues and to lesser extent they found abnormal methylation, in the surrounding neighboring cirrhotic tissue. They reported that 2% of non cancerous tissues were hypermethlated, 18.4 % were partially hypermethylated while 79.6 % of tissues were unmethylated.

Moreover Kwok-Kwan et al (2009),[132] conducted a study on HCC cell lines and HCC liver tissues from 50 patients in Hong Kong with bisulphite DNA sequencing and methylation-specific PCR and found that the promoter of the SPINT2 gene was frequently hypermethylated in both HCC cell lines and human HCCs.

Moribe T et al (2009),[133]used quantitative methylation-specific PCR and showed that SPINT2 was frequently methylated in small HCC tissues but unmethylated in non-HCC liver tissues, promising a high specificity for methylation patterns of SPINT2 circulating in the blood stream. Moreover, the frequency of aberrant promoter methylation increased during the progression from precancerous lesion to HCC.

Another study done by Iizuka et al (2011),[104] studied the promoter of the SPINT2 gene in the sera of HCV hepatitis, HCV liver cirrhosis and HCC patients in Japan by using

quantitative methyl specific PCR and there was hypermethylation of SPINT 2 promoter of these subjects (100 % of both HCV hepatitis and HCV liver cirrhosis patients and 16 % of HCC subjects) cut off for methylation level was methylation level more than 0.2 picogram/ml and for this finding it was concluded that SPINT2 had the highest accuracy in detecting non-HCC patients with chronic hepatitis or cirrhosis. Methylated SPINT2 was also detectable in sera from 2 HCC cases negative for both AFP and PIVKA-II.

In the present study, methylation status did not show any significant correlation with AFP, HCV viral load and child pugh score. (TablesVIII-XX)

A growing body of research has reported associations between age and the state of the epigenome. In particular, DNA methylation associates with chronological age over long time scales, and changes in methylation have been linked to complex age-associated diseases such as cancer, [34,38] however in the current study there was no statistically significant difference observed regarding age,(Table VII).In the current study it was concluded that there was no significant correlation between age and methylation status in the studied groups .(TablesVIII-XX)

In the present study we found increased risk to develop aberrant methylation in the SPINT 2 gene promoter IN HCV patients(group I) when compared to the normal subjects (group II), as crude odd's ratio was 2.52 with 95 % confidence interval (1.23-5.14). To exclude the effect of aging as confounder that might affect the methylation status adjustment was made for odd's ratio and it was 2.4 with 95 % confidence interval (1.13-5.26). (Table XVII)

Although further studies are required to determine the functional significance of aberrant methylation of SPINT 2 gene promoter ,the present study of qualitative MSP reveals a global picture of DNA methylation changes in HCV and HCC .Thus providing better understanding of the epigenetic mechanism that might underlie the stepwise progression of hepatocarcinogenesis .