AIM OF THE WORK

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.

SUBJECTS AND METHODS

SUBJECTS

After approval of the Ethical Committee of the Medical Research Institute, 130 subjects were included in the present study, informed consents were taken from all subjects who participated in the study. The subjects were divided into two groups:

- **Group 1:** It consisted of 80 chronic hepatitis-C virus infected patients recruited from medical research institute hospital ,they were subdivided into 2subgroups:
  - **Group** 1 (a): 50 chronic hepatitis-C virus infected patients without HCC.
  - **Group** 1 (b): 30 chronic hepatitis-C virus infected patients with superimposed HCC.
- **Group 2:** consisted of 50 apparently healthy volunteers of matched age, sex and socioeconomic status to the patient group.

Exclusion criteria included any chronic liver disease not related to hepatitis-C virus and alcohol intake as well as any other malignancy other than HCC.

METHODS

To all the studied subjects, the following was done:

I. Detailed history taking:

Detailed history was taken from all subjects including bleeding, jaundice and any surgical intervention or blood transfusion.

II. Complete physical examination:

Complete physical examination was done to all subjects with special stress on:

1) Abdominal examination for signs of hepatic failure as ascites.
2) Abdominal ultrasound.

The liver echo pattern was recorded to confirm the presence or absence of cirrhosis and any liver mass if present .

3) Child-Pugh classification. [105]

Table II: Child-Pugh scoring

	1	2	3
Encephalopathy	Nil	Slight to moderate	Moderate to severe
Ascites	Nil	Slight	Moderate to severe
Serum albumin (g/dL)	> 3.5	2.8 to 3.5	< 2.8
Serum total bilirubin (mg/dL)	< 2	2-3	> 3
Prothrombin activity (% of normal)	> 70	40 to 70	< 40

According to the Child-Pugh classification, patients with chronic liver diseases were classified into 3 risk groups (a, b, c) according to the numerical score obtained from the provided table (Table III).

Table III: Child-Pugh classification. [105]

Risk group	Numerical score
1- Child-Pugh class a	5 to 6
2- Child-Pugh class b	7 to 9
3- Child-Pugh class c	10-15

III. Laboratory investigations:

Blood sampling:

- Following eight hours fasting period 8 milliliters whole venous blood were withdrawn in vacutainers from each subject.
- 1 mL of whole blood was added in a purple capped vacutainer tube for the molecular studies.
- 1.8 ml of whole blood was mixed with 0.2 ml of 3.2 % Na citrate in blue capped vacutainer for coagulation studies
- 1 mL of the blood was added in a purple capped vacutainer tube to perform complete blood count.
- The rest 4.2 mL whole blood were left to clot in a red capped vacutainer and the obtained serum was used for the determination of the concentrations of serum levels of glucose, creatinine, albumin, total and direct bilirubin, as well as serum activities of the enzymes gamma glutamyl transferase, alanine and aspartate aminotransferases using fully automated chemistry analyzer Olympus AU400. Internal (Beckman coulter) and external (Biorad) quality controls were performed.

1. Complete Blood Count (CBC): [106]

It was performed on the Hemastar-II cell counter. The haemoglobin concentration, haematocrit value, red and white cell counts as well as platelet count were measured, along with automatic calculation of the hematological indices. A blood smear was spread on a glass slide, left to dry, and stained with Leishmann stain for the determination of differential white cell count.

2. Plasma prothrombin activity (PT): [106]

It was performed on the Dade Behring fibrin timer II, by incubating citrated plasma with excess tissue thromboplastin and calcium at 37°C, then, the clotting time of plasma was reported. The conversion of prothrombin time to "percentage of normal" was used to measure the prothrombin activity of a plasma sample.

3. Fasting serum glucose (FSG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin, direct bilirubin, gamma glutamyl transferase (GGT). [107]

These assays were performed on Olympus AU400 Chemistry Autoanalyzer, then the final absorbance of (fasting serum glucose, albumin, total billirubin and direct billirubin) and delta abosorbance of (AST,ALT and GGT) was compared to standard (auto calibrator) of known concentration

4- Serodetection of hepatitis C virus IgG antibody by sandwich enzyme linked immunosorbant assay (ELISA) technique [108](Murex Diagnostic Limited. Darford, England):

The diluted samples were incubated in micro-wells coated with highly purified HCV antigen (HCV-Ag) that contained sequences from the putative C, NS3, NS4, and NS5

regions of HCV that were bound to any anti-HCV antibody (Ab) in the sample during the first incubation. Unbound material was removed by washing.

The formed Ag-Ab complexes were then incubated with peroxidase conjugated monoclonal anti-human IgG. An antigen-antibody-anti human IgG antibody-enzyme complex was developed after the second incubation. Excess unbound enzyme conjugate was removed by washing.

The bound enzyme conjugate developed a purple color on addition of a suitable substrate, 3,3',5,5' tetramethyl benzidine (TMB) which was converted to orange when the enzyme reaction was terminated with sulphuric acid (2M). The color developed was read photometrically at λ450. The color developed in the wells was directly related to the concentration of the antibodies in the sample. All readings above the cutoff value (0.6 + mean negative control) were considered positive.

5- Serodetection of hepatitis B virus surface antigen (HBs-Ag) by sandwich enzyme linked immunosorbant assay (ELISA) technique[109] (Murex Diagnostic Limited. Darford, England)

The samples were pre-incubated in microwells coated with a mixture of mouse monoclonal antibodies specific for different epitopes on HBsAg. Then, affinity purified goat antibody to HBsAg conjugated to horse-raddish peroxidase was added and second incubation was done.

During the two incubation steps, any HBs-Ag in the sample would bind to the well in an antibody-antigen-antibody-enzyme complex. Excess unbound enzyme conjugate was removed by washing. Wells that contained HBs-Ag and hence the bound enzyme conjugate developed a purple color on addition of a suitable substrate, 3,3',5,5' tetramethyl benzidine (TMB).Then the color was converted to orange when the enzyme reaction was terminated with sulphuric acid (2M). The color developed was read photometrically at λ450. All readings above the cutoff value (0.05 + mean negative control) were considered positive.

6- Estimation of AFP by chemiluminometric method:[107]

The assay was performed using Immulite 1000, which is a solid phase, two-site sequential chemiluminescent immunometric assay.

Principle:

It involved a competitive binding between the labeled (with alkaline phosphatase) and non-labeled antigens to the limited amount of antibody binding sites using a chemiluminescent substrate.

7- HCV RNA Real time PCR: [110]

Real time PCR was performed with the Mx3000P ™ (Stratagene) real time PCR system.

Principle:

TaqMan probe real-time PCR,The probe consists of two types of fluorophores the quencher (Q) fluorophore (usually a long wavelength colored dye) at the 3' end (TAMRA) and the reporter (R) fluorophore (usually a short-wavelength colored dye) on the 5' end (FAM) of the probe. While the probe is intact the quencher (Q) dye reduces the fluorescence from the reporter (R) dye by the use of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. If the target sequence is present, the probe anneals downstream of the primer sites on the template DNA after denaturation by high temperature, then the primers anneal to the DNA. *Taq* polymerase then adds nucleotides and so displace the TaqMan probe from the template DNA and cleave it by the 5' nuclease activity of Taq DNA polymerase. This separates the quencher from the reporter, and allows the reporter to give off and emits its energy. This is then quantified using a special software on a computer attached to the device. The more times the denaturing and annealing takes place, the more opportunities there are for the TaqMan probe to bind and, in turn, the more emitted light is detected. So, the fluorescent signal increases in direct proportion to the amount of amplified product in the reaction tube.

8- Molecular studies :methyl specific PCR for SPINT 2 gene: [58]

These studies included:

I. DNA extraction from peripheral blood leucocytes.
II. Bisulfite conversion of the extracted DNA.
III. Methyl specific Polymerase chain reaction amplification using 2 different sets of primers: one for the methylated DNA and the other for the unmethylated DNA for SPINT2 gene.
IV. Agarose gel electrophoresis for the PCR products.

I- DNA extraction from peripheral blood leucocytes :

DNA was purified from whole blood using Gene JET™ Genomic DNA purification (Fermentas – Thermo, USA).

Principle:

Samples were digested with Proteinase K in the lysis solution. The lysate was then mixed with ethanol and loaded on the purification column where the DNA bound to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the Elution Buffer.

Reagents: for 50 preparations

1) Proteinase K solution: 1.2 ml
2) Lysis solution: 24 ml
3) Wash buffer I (concentrated): 10 ml
4) Wash buffer II (concentrated): 10ml
   The amount of ethanol (96-100%) specified on the bottle label was added to wash buffer I (concentrated) and wash buffer II (concentrated) prior to first use

5) Elution buffer (10 mM Tris-Cl, pH 9.0, 0.5 mM EDTA): 30 ml
6) GeneJET^{TM} Genomic DNA purification columns pre-assembled with collection tubes: 50.
7) Collection Tubes: 50 in number.

Sample collection and storage:

DNA was freshly extracted.

Storage conditions:

Proteinase K solution was stable at room temperature as long as it is not opened. After being opened it was stored at -20°C. Other components of the kit were stored at room temperature (15-25°C).

Steps:

1) 400 µL of Lysis solution were added and 20 µl of Proteinase K Solution were also added in a microfuge tube to 200 µl of whole blood, and then were mixed thoroughly by vortexing to obtain a uniform suspension.
2) The samples were incubated at 56°C using thermomixer until the cells were completely lysed (10 minutes).
3) 200 µL of ethanol (96-100%) were added to each sample and was mixed by vortexing.
4) The prepared lysates were transfered to Gene JET^{TM} Genomic DNA Purification Columns inserted in collection tubes, then the columns were centrifuged for 1 minute at 6000 x g. The collection tubes containing the flow through solution were discarded, and then the Gene JET^{TM} Genomic DNA Purification Columns were placed into new 2 mL collection tubes.
5) 500 µL of wash buffer I were added, then the columns were centrifuged for 1 minute at 8000 x g. The flow-through was discarded and the purification columns were placed back into new collection tubes.
6) 500 µl of wash buffer II were added to the Gene JET^{TM} Genomic DNA Purification Columns, then the columns were centrifuged for 3 minutes at maximum speed (≥12000 x g).
7) Optional step: If residual solution was seen in the purification columns, the collection tubes were emptied and the columns were re-spined for 1 minute at maximum speed, then the collection tubes containing the flow-through solutions were discarded and the GeneJET Genomic DNA purification columns were transfered to a sterile 1.5 mL microcentrifuge tubes.
8) 100 µl of elution buffer were added to the centre of the Gene JETTM Genomic DNA Purification Column membranes to elute genomic DNA, then the columns were incubated for 2 minutes at room temperature then centrifuged for one minute at 8000 x g.
9) The purification columns were discarded, and the purified DNA was collected immediately after centrifugation in the collection tubes, then was divided into two aliquots and stored at -20°C till PCR amplification.
10) Integrity of genomic DNA was detected by agarose gel electrophoresis of the yield on 1 % agarose gel for 30 minutes at 90 V (5µL extracted DNA + 2µL gel loading dye). (Figure 14)

11) The concentration and purity of the genomic DNA was measured on a nanodrop 1000 spectrophotometer (Thermo scientific, USA) at 260 and 280 nm.

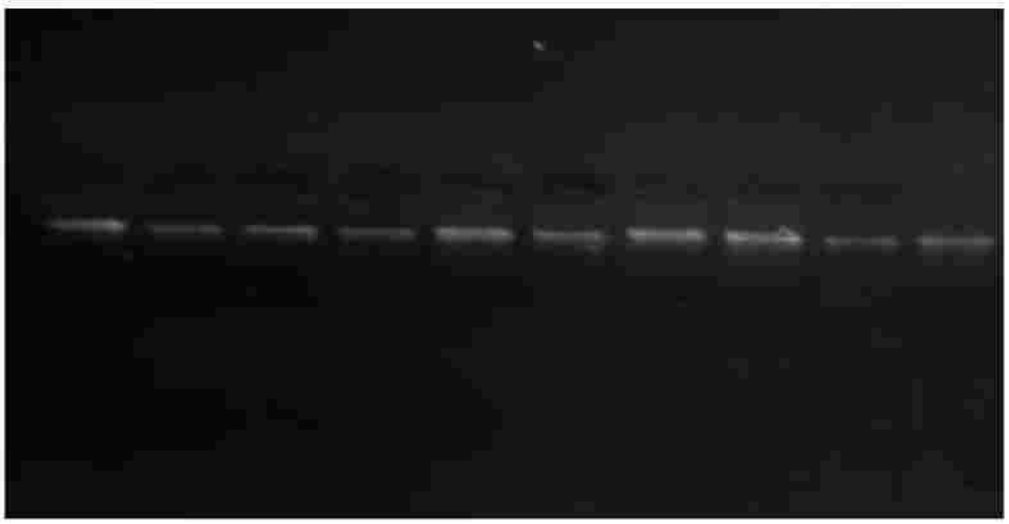

Figure (14): Genomic DNA run on 1% agarose gel.

II- Bisulfite conversion of the extracted DNA

Bisulfate conversion for the extracted DNA was done by the use of **EZ DNA Methylation™ Kit** (zymo research USA)

Principle of action:

Bisulfite modification step creates sequence differences in the DNA. Unmethylated cytosines of a CpG dinucleotide were converted to uracils, while methylated cytosines of a CpG dinucleotide were protected from change

The **EZ DNA Methylation™ Kit** (D5001) features a simplified procedure that streamlines bisulfite conversion of DNA. The kit is based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine was converted into uracil. The product's innovative in-column desulphonation technology eliminates otherwise cumbersome precipitations. The kit was designed to reduce template degradation, minimize DNA loss during treatment and clean-up, while ensuring complete conversion of the DNA. Purified, converted DNA is ideal for PCR amplification.

Reagents:

EZ DNA Methylation™ Kit (D5001) is enough for 50 reactions and **consists of the following reagents:**

CT Conversion Reagent
M-Dilution Buffer
M-Binding Buffer
M-Wash Buffer
M-Desulphonation Buffer
M-Elution Buffer

Zymo-Spin ™IC Columns
Collection Tues

Strorage:

Room temperature.

Reagent preparation:

CT Conversion Reagent:

750 µl water and 185 µl of **M-Dilution Buffer** added to a tube of **CT Conversion Reagent** then mix at room temperature with frequent vortexing or shaking for 10 minutes .

Storage : The **CT Conversion Reagent** is light sensitive, so minimize its exposure to light. For best results, the **CT Conversion Reagent** should be used immediately following preparation. If not used immediately, the **CT Conversion Reagent** solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored **CT Conversion Reagent** solution must be warmed to 37°C, then vortexed prior to use .

M-Wash Buffer:

24 ml of 100% ethanol added to the 6 ml M-Wash Buffer concentrate (D5001) before use.

Protocol:

1 . 7.5 µl of M-Dilution Buffer were added to the DNA sample (30 µl form HCV patients and 15 µl from control group) and the total volume was adjusted to 50 µl with water. The sample was mixed by flicking or pipetting up and down .
2- The sample was incubated at 42°C for 15 minutes .
3- After the above incubation, 97.5 µl of the prepared CT Conversion Reagent added to each sample and mixed .
4- The sample was incubated in the dark at 50°C for 16 hours
5- The sample was incubated at 0-4°C (e.g., on ice) for 10 minutes
6- 400 µl of M-Binding Buffer were added to a Zymo-Spin ™IC Column and place the column into a provided Collection Tube .
7- The sample (from Step 5) was loaded into the Zymo-Spin ™IC Column containing the M-Binding Buffer.,the cap closed and mixed by inverting the column several times .
8- Centrifugation at full speed (>10 000‹x *g*) for 30 seconds. the flow-through was discarded .
9- 100 µl of M-Wash Buffer were added to the column. Centrifugation at full speed for 30 seconds .
10- 200 µl of M-Desulphonation Buffer were added to the column and standed at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifugation at full speed for 30 seconds .
11- 200 µl of M-Wash Buffer were added to the column. Centrifugation at full speed for 30 seconds. another 200 µl of M-Wash Buffer were added and centrifugation for an additional 3 0seconds .

12- The column was placed into a 1.5 ml microcentrifuge tube. 15 µl of M-Elution Buffer were added directly to the column matrix. Centrifugation for 30 seconds at full speed to elute the DNA. Then the sample stored at or below -20°C for later use.

III- Methyl specific Polymerase chain reaction amplification using specific primers for SPINT2 gene:

Using 2 different sets of primers: one for the methylated DNA and the other for the unmethylated DNA for SPINT2 gene.

Principle:

Methylation- specific PCR, the sequence differences were detected by amplification using primers that distinguish between the methylated and unmethylated bisulfite-modified DNA and unmodified DNA. In the design of primers specific for methylated DNA, cytosines that were conserved because of their methylation are placed in the 3′ end. In the design of primers that only amplify unmethylated DNA after bisulfite modification‹ thymidines, derived from converted cytosines, are placed in the 3 end.

The anti sense primers annealed with the target sequence then the sense primers annealed with the anti sense primers extention.

Reagents:

1) ZymoTaq ™DNA Polymerase premix (E2004, 200 Reactions) (zymoresearch, USA)

ZymoTaq ™DNA polymerase premix contained all the reagents needed to perform "hot start "PCR. The inclusion of a heat-activated, thermal-stable DNA polymerase reduces primer dimer and non-specific product formation that can occur when performing conventional PCR. This unique product was specifically designed for the amplification of bisulfite-treated DNA for methylation detection. The product generated specific amplicons with little or no by-product formation. Heating at 95° C for 10 minutes to initiate polymerization

Contents of the kit:

Zymo*Taq* ™PreMix :8 x 625 µl concentration 2X (to be diluted 1+1)
DNase/RNase-Free H2O: 5 x 1 ml (ready to use)
Storage:-20 c

2) EpiTect ®Control DNA (human), methylated and unmethylated bisulfite converted (100 reactions):

DNA control was applied with each run, two different types of ready to use bisulfite converted DNA control one for the methylated PCR and the other for the unmethylated

Control reactions were performed when undertaking methylation analysis (e.g., methylation specific PCR [MSP]) to ensure that the PCR primers are specific for the detection of methylated bisulfite converted or unmethylated bisulfite converted DNA.

The methylated and bisulfite converted DNA was stored in EB Buffer (10 mM Tris·Cl) in a ready-to use 10 ng/µl solution.

Complete in vitro methylation of the methylated control DNA was achieved using *Sss*I *methylase* .Bisulfite conversion of control DNA was achieved using the EpiTect Bisulfite Kit.

Storage:

The control DNA was stored at-20° c immediately upon receipt. When stored under these conditions and handled correctly, this product can be kept for at least 6 months without showing any reduction in performance.

Procedure:

1 µl (10 ng) of each control DNA was used for every PCR reaction.

3) Primers:

SPINT 2 gene (chr19:43447037) contains a highly dense CGs area (13%).

2 pairs of lyophilized primers for SPINT2 gene (methylated and unmethylated) were reconstituted by addition of sterile nuclease free water.

Table IV: Primers for methylated SPINT2 gene

Forward sense	5′-GTTGAGTGTCGTAGGCGGC-3′
Reverse antisense	5′- ACCTTCAATATAAAAACGCCCG-3′

Table V: Primers for unmethylated SPINT2 gene

Forward sense	5′-GTTGAGTGTTGTAGGTGGT-3′
Reverse antisense	5′-ACCTTCAATATAAAAACACCCA-3′

Protocol of amplification:

In a 0.2 mL eppendorf tube the following were mixed:

Table VI: Reagents of PCR amplification

ZymoTaq DNA Polymerase premix (2X)	12.5 uL
Converted DNA	2.5 uL
nuclease-free Water	9.4 uL
Forward Primer	0.3 uL
Reverse Primer	0.3 uL
Total volume	25uL

Tubes were transferred to the thermal cycler (Quanta Biotech, UK) where the PCR conditions were adjusted as follows:

A- Steps for MSP methylated Run:

Phases	Cycle number	Temperature(°C)	Time (Minutes)
Initial Denaturation	1	95	10
Amplification	35		
• Denaturation		95	0.5
• Annealing		57	0.5
• Extension		72	0.45
Final extension	1	72	7

B- Steps for MSP unmethylated Run:

Phases	Cycle number	Temperature(°C)	Time (Minutes)
Initial Denaturation	1	95	10
Amplification	35		
• Denaturation		95	0.5
• Annealing		50	0.5
• Extension		72	0.45
Final extension	1	72	7

Agarose gel electrophoresis for the digested PCR product

Reagents:

1) GeneRuler™ 50 bp DNA Ladder (Fermentas– Thermo, USA), the DNA fragments range from 1000 bp to 50 bp. It contains two reference bands: 500 and 250 bp.
2) Tris borate EDTA (TBE) buffer (10X) stock solution: working TBE (1X) buffer was prepared by measuring 100 mL stock solution and completed to one liter of distilled water.
3) Agarose gel (Promega).

Steps:

– Agarose gel (wt/vol) 2.0% was prepared by weighing 2.0 gram agarose powder and dissolving it in up to 100 mL of diluted 1X TBE buffer, followed by boiling until dissolution of the agarose. Then 10µL ethidium bromide was added to the gel while still hot for staining. Finally, the gel was poured into the assembled electrophoresis plate and allowed to solidify.

- The gel was poured into the mould to which the combs were perpendicularly placed and properly spaced.
- After the gel was completely set (30-45 minutes at room temperature), the combs were carefully removed and the gel was mounted in the electrophoretic tank.
- TBE buffer was poured in the electrophoresis basin, just enough to cover the gel.
- 10µL of the products were slowly loaded into the slots of the submerged gel using a micropipette.
- 10 µL of the Gene Ruler^{TM} 50 bp ladder were slowly loaded into one of the slots of the submerged gel using a micropipette.
- The lid of the tank was closed and the electrical leads were attached, so that the DNA will migrate towards the anode. The voltage applied was 120 volts for about 45minutes.
- A 302 nm ultraviolet transilluminator was used for visualization of the DNA bands.

Results:

Amplification was successful if a band appeared with the same size as that of the control (100 base pair). If amplification was successful in the unmethylated run and failed in the methylated run, this was considered unmethylated status of SPINT2 promotor. If the reverse occurred, this was hypermethylation. And if amplification was successful in both runs, this was hemimethylation and this could suggest methylation heterogeneity where methylation is not inclusive of all CpG sites between the primers, hereby allowing amplification with both primer sets. Hypermethylation and hemimethylation were considered aberrant methylation status. (Fig.11,12).

Figure (15): Amplified bisulfite converted DNA after MSP Using methylated primers on 2% agarose gel.

The first lane for 50 base pair genomic ladder ,the second lane for the negative control (nuclease free water containing no genomice DNA) with no product seen

The third lane for the positive control (methyalted DNA control) with the product seen ;its size 100 bp .

Samples 1-4 are bisulfite converted DNA for samples which contain methylated allele and show successful amplification with metylated primers methylation specific PCR run ,their products seen with same size as the methyalted DNA control (100bp)

Sample 5 is bisulfite converted DNA which does not contain methylated alleles and show failed amplification with methylated primers (No PCR product).

Figure (16): Amplified bisulfite converted DNA after MSP Using unmethylated primers on 2% agarose gel.

The first lane for 50 base pair genomic ladder ,the second lane for the negative control (nuclease free water containing no genomice DNA) with no product seen The third lane for the positive control (unmethyalted DNA control) with the product seen ;its size 100 bp .

Samples 1-9 and samples 11-17 are bisulfite converted DNA for samples which contain unmethylated allele and show successful amplification with ummetylated primers methylation specific PCR run ,their products seen with same size as the unmethyalted DNA control (100bp)

Sample number 10 is bisulfite converted DNA which does not contain unmethylated alleles and shows failed amplification with the unmethylated primers(No PCR product).

Technical considerations:

- Extracted DNA was quantitated by NanoDrop 1000 to determine concentration and purity.
- The in-column desulphonation technology of the methylation kit eliminated undesired burdensome precipitations. The kit was designed to reduce template degradation, minimize DNA loss during treatment and clean-up.
- The inclusion of heat-activated, thermal-stable DNA polymerase ZymoTaq™ DNA polymerase premix (zymoresearch, USA) specifically designed for the amplification of bisulfite-treated DNA for methylation detection. This premix not only contained all the reagents needed to perform" hot start "PCR, but also generated specific amplicons with little or no by-product formation that could occur when performing conventional PCR.
- Negative control, DNase/RNase free distilled water without DNA, was included in each amplification protocol.
- The use of two different types of DNA controls; one for the methylated and another for the unmethylated, ensured the specificity of the used PCR primers and helped in results interpretation

Statistical analysis of the data:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. [111] Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it revealed normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between more than two population were analyzed using F-test (ANOVA). For abnormally distributed data, comparison between two independent population were done using Mann Whitney test while Kruskal Wallis test was used to compare between different groups and pair wise comparison was assessed using Mann-Whitney test. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level. [112]

Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level. Odd's ratio (OR) and 95% Confidence Interval were used to calculate the ratio of the odds of an event occurring in one patient group to the odds of it occurring in the control group.

RESULTS

Table VII shows comparison between studied groups regarding age and gender, percent of males were (23) 46%,(21) 70% and (29)58 % in the HCV, HCC and control group, respectively.

There was no statistical significant difference between the groups regarding the age and gender.

Table (VII): Statistical analysis between the studied groups according to age and gender:

	HCV (N=50)		HCC (N =30)		Control (N =50)		Test of Sig.	p
	No.	%	No.	%	No.	%		
Gender (%)								
Male	23	46.0	21	70.0	29	58.0	χ^2=4.499	0.105
Female	27	54.0	9	30.0	21	42.0		
Age (Years)								
Min. – Max.	39 – 67		45.0 – 71.0		40 – 65		$^{KW}\chi^2$= 1.533	0.465
Median	50.5		53.5		48.0			

χ^2: Chi square test
$^{KW}\chi^2$: Chi square test for Kruskal Wallis test

Table VIII shows comparison between HCV group (Ia) and HCC group (Ib) as regards the clinical data , regarding the focal lesions and Ascites ; these findings were more frequent in HCC rather than HCV, on the other hand as regards the splenomegaly there was no statistically significant difference between HCV and HCC patients.

Table **VIII** shows also comparison of child pugh score between HCV and HCC groups.

In HCV patients (group Ia)Child Pugh class A was found in 46.5 %, class B in 41.9 % and class C in 11.6 % of patients, while in HCC patients (group Ib) class A was found in 16 % , class B in 52% and class C in 32 % of patients .

Child pugh score was calculated for only 43 HCV patients and 25 HCC patients for whom all clinical data were available.

Table (VIII): Comparison between the studied groups as regards some clinical data

	HCV Ia (n=50)		HCC Ib (n=30)		Test of Sig.	p
	No.	**%**	**No.**	**%**		
Splenomegaly						
No	20	40.0	8	26.7	χ^2=1.465	0.226
Yes	30	60.0	22	73.3		
Ascites						
No	34	68.0	10	33.0		
Mild	4	8.0	6	20.0	Z=2.817*	0.005*
Moderate	5	10.0	5	16.7		
Massive	7	14.0	9	30.0		
Focal Lesions						
No			0	0.0		
One			13	43.3		
Two			13	43.3		
>Two			4	13.3		
	HCV Ia (N =43)		**HCC Ib (N =25)**			
	No.	**%**	**No.**	**%**		
Child Pugh						
A	20	46.5	4	16.0	Z=2.800*	0.005*
B	18	41.9	13	52.0		
C	5	11.6	8	32.0		

χ^2: Value for Chi square test
Z: Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table IX shows the comparison between the studied groups regarding Hb level, platelets; in HCV and HCC patients they were significantly lower than the control group ($p<0.001$), however, there was no statistically significant difference between HCV (group Ia) and HCC (group Ib) patients regarding Hb and platelets.The same table shows the comparison between studied groups regarding INR; in HCV (group Ia) and HCC (group Ib) patients which was significantly higher than control group ($p<0.001$), but there was no statistically significant difference between HCV and HCC patients.

On the other hand there was no statistically significant difference between different groups regarding the WBCs count.

Table (IX): Statistical analysis between the studied groups as regards Complete blood count and Prothrombin studies

	HCV Ia (N =50)	HCC Ib (N =30)	Control II (N=50)	$^{KW}\chi^2$	p
Hb (g/dl)					
Min. – Max.	6.10 – 16.40	6.40 – 14.20	11.10 – 17.10	$^{KW}\chi^2$=52.880*	<0.001*
Median	10.30	10.70	14.45		
P1	0.14				
P2	<0.001				
P3		<0.001			
WBCs (*1000 /ul)					
Mean ± SD.	6.84 ± 2.45	7.30 ± 3.49	6.87 ± 1.42	F=0.397	0.673
Plateletes (*1000 /ul)					
Min. – Max.	14.0 – 440.0	6.0 – 229.0	155.0 – 420.0	$^{KW}\chi^2$=59.516*	<0.001*
Median	141.50	109.50	262.50		
P1	0.12				
P2	<0.001				
P3		<0.001			
INR					
Min. – Max.	1.0 – 3.26	1.09 – 3.82	0.91 – 1.17	$^{KW}\chi^2$=74.916*	<0.001*
Median	1.35	1.52	1.02		
P1	0.08				
P2	<0.001				
P3		<0.001			

$^{KW}\chi^2$: Chi square test for Kruskal Wallis test
F: F test (ANOVA)
Sig. bet. grps was done using Mann Whitney test
P : Significance within the groups ,P1:significane between group Ia and group Ib ,P2:significane between group Ia and group II ,P3:significane between group Ib and group II
Hb=hemoglobin
WBCs= White Blood Cells

Table X shows the comparison between studied groups regarding fasting serum glucose, serum creatinine, total bilirubin and ALT; in HCV (group Ia) patients and HCC (group Ib) patients they were significantly higher than control group II ($p < 0.001$), but there was no statistically significant difference between HCV and HCC patients. Also table X shows the comparison of direct bilirubin, GGT, AST; they were in HCV (group Ia) and HCC (group Ib) patients significantly higher than control group II ($p<0.001$), moreover their levels were significantly higher in HCC (group Ib) than HCV (group Ia) .

On the other hand albumin in HCV and HCC patients was significantly lower than control group ($p<0.001$), moreover it was significantly lower in HCC (group Ib) than HCV (group Ia) patients .

Table (X): Statistical analysis of some biochemical parameters in the studied groups.

	HCV Ia (N=50)	HCC Ib (N=30)	Control II (N=50)	$KW\chi^2$	p
Albumin (g/dl)					
Min. – Max.	1.80 – 4.40	1.15 – 4.10	3.70 – 5.20	86.166*	<0.001*
Median	2.85	2.40	4.70		
P1	<0.001				
P2	<0.001				
P3		<0.001			
GGT (U/L)					
Min. – Max.	11.0 – 300.0	11.0 – 469.0	6.0 – 51.0	45.364*	<0.001*
Median	40.50	70.0	26.0		
P1	0.004				
P2	<0.001				
P3		<0.001			
AST(U/L)					
Min. – Max.	16.0 – 203.0	32.0 – 450.0	6.0 – 32.0	93.059*	<0.001*
Median	49.0	87.50	17.0		
P1	<0.001				
P2	<0.001				
P3		<0.001			
ALT(U/L)					
Min. – Max.	10.0 – 180.0	12.0 – 350.0	4.0 – 35.0	51.505*	<0.001*
Median	35.50	47.50	19.50		
P1	0.19				
P2	<0.001				
P3		<0.001			
FSG(mg/dl)					
Min. – Max.	77.0 – 294.0	67.0 – 230.0	75.0 – 99.0	21.218*	<0.001*
Median	150	98.50	85.0		
P1	0.21				
P2	<0.001				
P3		<0.001			
Creatinine(mg/dl)					
Min. – Max.	0.51 – 4.70	0.70 – 4.20	0.60 – 1.40	17.480*	<0.001*
Median	1.10	1.25	0.90		
P1	0.29				
P2	<0.001				
P3		<0.001			

Table (X): Statistical analysis of some biochemical parameters in the studied groups (Cont.)

	HCV Ia (N=50)	HCC Ib (N=30)	Control II (N=50)	$^{KW}\chi^2$	p
Total bilirubin (mg/dl)					
Min. – Max.	0.20 – 33.55	0.20 – 29.50	0.20 – 0.80	76.233*	<0.001*
Median	1.40	2.30	0.50		
P1	0.29				
P2	<0.001				
P3		<0.001			
Direct bilirubin (mg/dl)					
Min. – Max.	0.10 – 22.80	0.10 – 26.10	0.10 – 0.20	76.485*	<0.001*
Median	0.85	1.80	0.10		
P1	<0.001				
P2	<0.001				
P3		<0.001			

$^{KW}\chi^2$: Chi square test for Kruskal Wallis test
Sig. bet. grps was done using Mann Whitney test
P : Significance within the groups
P1:significane between group Ia and group Ib
P2:significane between group Ia and group II
P3:significane between group Ib and group II
FSG=Fasting serum glucose
ALT=Alanine aminotransferase
AST=Aspartate aminotransferase (AST)
GGT= Gamma Glutamyl transferase

Table (XI): Serum alpha feto protein (µg/L) in the studied groups .

Case No.	HCV(group Ia) N=50 AFP	HCC (group Ib) N=30 AFP	Control (group II) N=50 AFP
1	88	460	3
2	63	3450	4
3	87	7980	2
4	2.2	9050	6
5	8.6	80	6
6	7.4	3087	7.1
7	15.8	677	5.6
8	3.99	19	3.4
9	6.9	984	5.9
10	61	1100	2.1
11	39	670	5.2
12	32	4570	4.2
13	12	780	1.5
14	66	6900	3.3
15	5.4	6790	2.9
16	22	2340	3.5
17	6	3900	2.4
18	7.8	1350	3.5
19	9.8	8900	4.9
20	36	2300	5.8
21	21	6700	7.2
22	30	3450	5.4
23	132	5000	3.2
24	6	890	1.7
25	5.9	2900	0.9
26	9	89	0.8
27	45	6070	0.8
28	15.8	2390	1.2
29	117	879	1.2
30	13	520	1.3
31	70		1.4
32	8.9		2.2
33	65		3.3
34	7.8		2.4
35	69		2.5
36	12.8		3.3
37	79		4.2
38	120		4.3
39	15.5		2.2
40	39		6.9
41	9		7.3
42	79		5.3
43	19.9		3.5
44	8.5		5.1
45	23		3.2
46	3.9		1.5
47	8.2		1.6
48	4.8		1.8
49	6.9		2.2
50	30		3.2
Median	15.8	2365	3.25
Min. – Max.	2.20 – 132.0	19.0 – 9050.0	0.80 – 7.30

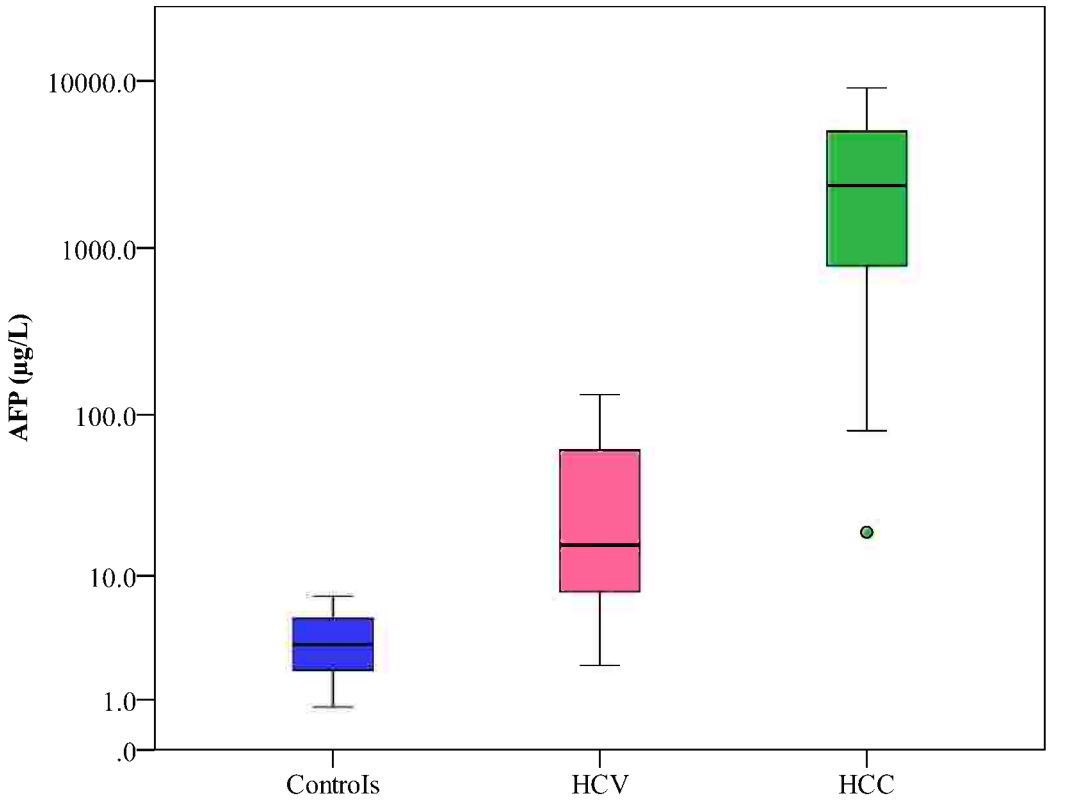

Figure (17): Box blots representing the median values of AFP in the studied groups

Table (XII): HCV PCR viral load (*10^4 IU/ml) in the the patients subgroups.

Case No.	HCV(group Ia) N=50 HCV PCR	HCC (group Ib) N=30 HCV PCR
1	224.67	12.44
2	8.94	345.70
3	389.50	345.66
4	244.67	11.90
5	13.45	34.57
6	36.70	344.87
7	34.82	56.77
8	66.20	8.86
9	66.79	245.48
10	255.67	7.82
11	148.50	77.78
12	134.52	123.70
13	23.48	234.56
14	556.00	123.46
15	32.44	234.47
16	155.50	23.46
17	51.42	44.46
18	44.55	234.00
19	88.76	345.56
20	199.80	234.51
21	11.14	234.89
22	133.54	123.46
23	356.78	124.45
24	23.88	9.80
25	22.67	112.20
26	55.59	119.92
27	45.10	123.99
28	92.12	239.80
29	39.98	59.80
30	278.28	0.40
31	125.56	
32	127.97	
33	237.70	
34	109.38	
35	198.87	
36	134.49	
37	23.49	
38	30.98	
39	56.89	
40	122.99	
41	234.99	
42	98.84	
43	46.67	
44	390.88	
45	77.80	
46	55.58	
47	112.24	
48	122.40	
49	34.49	
50	55.88	
Median	92.12	123.46
Min. – Max.	8.94-556	0.4—345.7

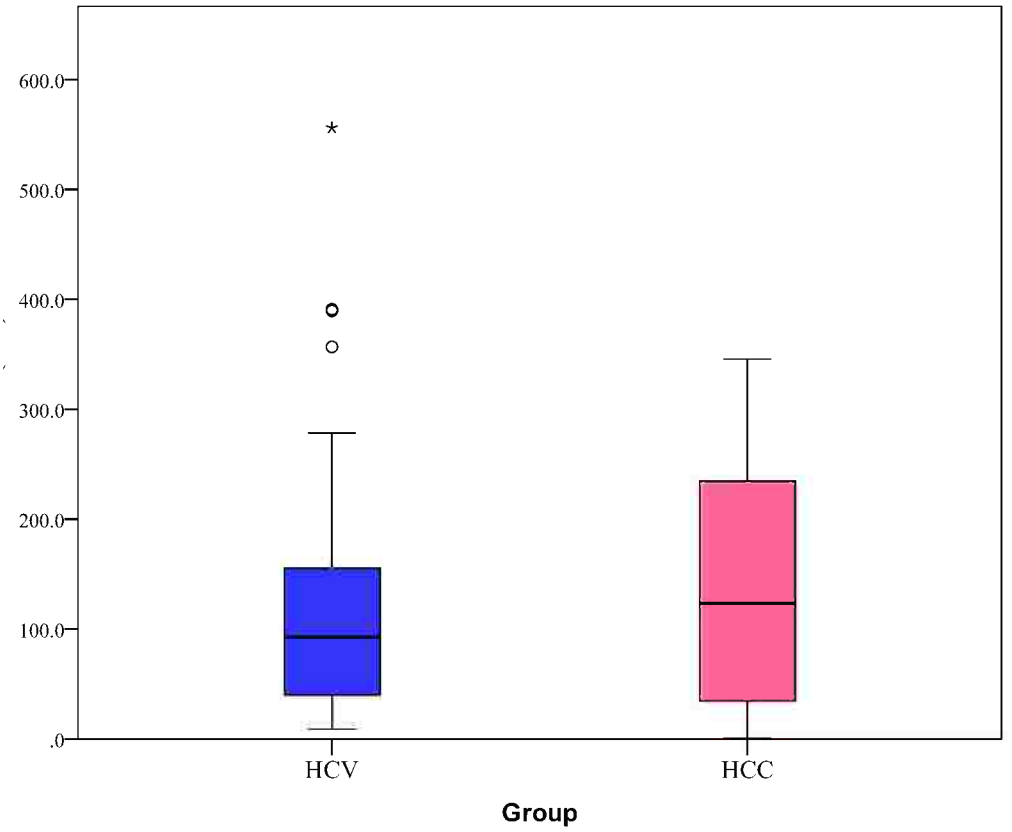

Figure (18): Box blots representing the median values of HCV PCR in the patients subgroups

Tables XI-XIII show the comparison between groups regarding AFP; it was significantly higher in HCV (group Ia) patients and HCC (group Ib) patients when compared to control group II ($p<0.001$), and its level was significantly higher in HCC (group Ib) than HCV (group Ia). The same tables show the comparison between HCV viral load by PCR between HCV (group Ia) and HCC (group Ib) and there was no statistically significant difference.

Table (XIII): Statistical analysis of AFP and HCV PCR in the studied groups.

	HCV Ia (N =50)	HCC Ib (N =30)	Control II (N =50)	Test of Sig.	p
AFP (µg/L)					
Min. – Max.	2.20 – 132.0	19.0 – 9050.0	0.80 – 7.30	$^{KW}\chi^2=102.995^*$	$<0.001^*$
Median	15.80	2365.0	3.25		
P1	<0.001				
P2	<0.001				
P3		<0.001			
HCV PCR (10^4 IU/mL)					
Min. – Max.	8.94 – 556.0	0.4 – 345.7	-	Z=0.338	0.735
Median	92.12	123.46	-		

χ^2: Chi square test
$^{KW}\chi^2$: Chi square test for Kruskal Wallis test
Z: Mann Whitney test
Sig. bet. grps was done using Mann Whitney test
P1:significane between group Ia and group Ib
P2:significane between group Ia and group II
P3:significane between group Ib and group II
AFP= Alpha feto protein

Table (XIV): Methylation status of SPINT2 gene promoter in the studied groups

Case No.	HCV(group Ia)	HCC (group Ib)	Control (group II)
1	2	2	2
2	2	1	1
3	1	2	2
4	2	2	1
5	2	2	1
6	2	2	1
7	2	2	2
8	1	1	1
9	2	2	1
10	1	2	1
11	2	2	2
12	2	2	1
13	1	2	1
14	1	2	1
15	2	3	1
16	2	2	2
17	1	1	1
18	1	1	2
19	2	1	2
20	1	3	1
21	1	1	2
22	2	2	2
23	1	2	1
24	2	1	1
25	1	2	2
26	2	2	2
27	2	1	2
28	2	1	1
29	2	1	1
30	2	2	2
31	2		2
32	2		1
33	1		2
34	2		1
35	3		1
36	3		1
37	1		2
38	1		1
39	1		1
40	1		1
41	1		2
42	2		2
43	1		1
44	2		1
45	2		1
46	2		1
47	2		1
48	2		2
49	2		2
50	2		1

1= unmethylated 2=hemimethylated 3=hypermethylated

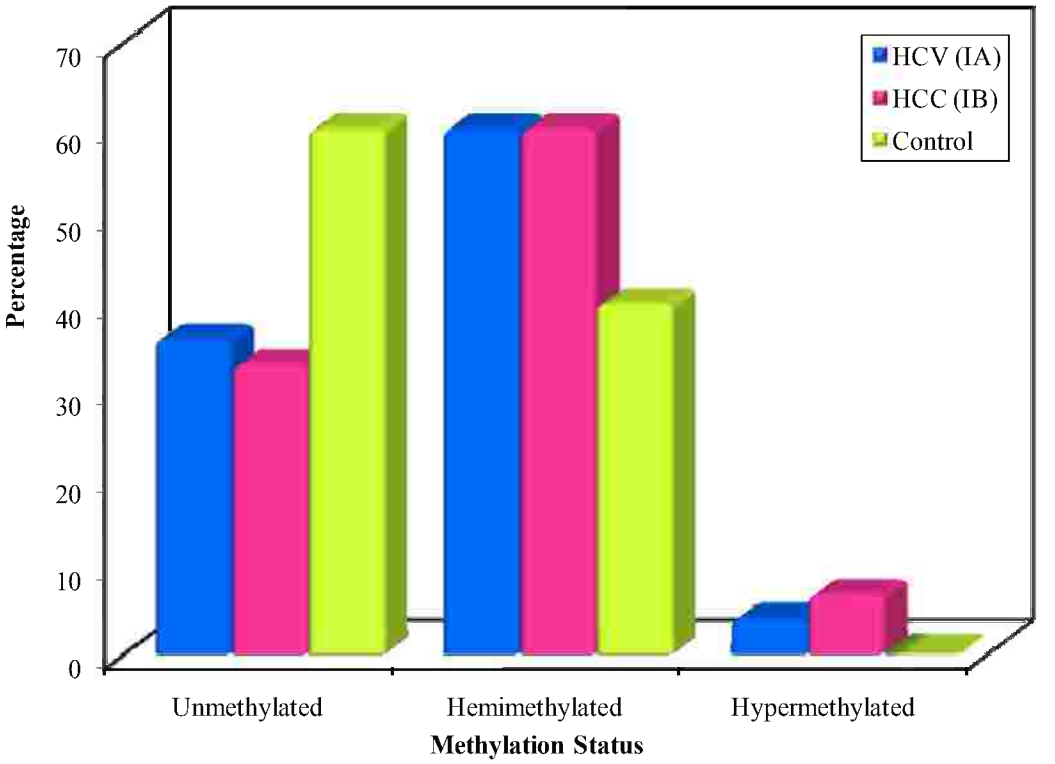

Figure (19): Comparison between the studied groups according to methylation status of SPINT 2 gene promoter.

Table XVa shows comparison between the studied groups according to the methylation status, and it was found that in HCV group 60 % of subjects were hemi ethylated, also in HCC group 60 % of subjects were hemimethylated, while in control goup only 40 % of cases were hemimethylated. Regarding the hypermethylation, there was no hypermethylated cases in the control group, on the other hand 4 % subjects in the HCV group and 6.7 % of subjects in the HCC group were hypermethylated.

The same table shows that there was a statistically significant difference within the groups regarding the methylation status(p=0.028).

Table (XV a): Statistical analysis of the methylation status of SPINT2 gene promoter in the studied groups

	HCV (Ia) (Number =50)		HCC (Ib) (Number =30)		Control (II) (Number =50)		χ^2	^{MC}p
	No.	%	No.	%	No.	%		
Methylation Status								
Unmethylated	18	36.0	10	33.3	30	60.0		
Hemimethylated	30	60.0	18	60.0	20	40.0	9.627*	0.028*
Hypermethylated	2	4.0	2	6.7	0	0.0		

χ^2: Value for Chi square test
MC: Monte Carlo test

Table XV b shows comparison between the studied groups according to the methylation status after they are categorized into unmethylated and aberrantly methylated (hemimethylated and hypermethlated cases) and it was found that in HCV group 64 % of subjects were aberrantly methylated, in HCC group 66.7 % of subjects were aberrantly methylated, while in control goup only 40 % of cases were aberrantly methylated. The same table shows that there was a statistically significant difference within the groups regarding the methylation status. Aberrant methylation was more frequent in HCV(p= 0.01) and HCC (p=0.021) when compared to the control group.

On the other hand there was no statistical significant difference as regards methylation status between HCV patients and HCC patients

Table (XV b): Statistical analysis between the three studied groups according to methylation status (unmethylated vs aberrantly methylated)

	HCV (Ia) (Number =50)		HCC (Ib) (Number =30)		Control (II) (Number =50)		χ^2	p
	No.	%	No.	%	No.	%		
Methylation Status								
Unmethylated	18	36.0	10	33.3	30	60.0	7.837*	0.021*
Aberrantly methylated	32	64.0	20	66.7	20	40.0		
P1	0.52							
P2	0.01							
P3			0.021					

χ^2: Chi square test
P : Significance within the groups
P1:significane between group Ia and group Ib
P2:significane between group Ia and group II
P3:significane between group Ib and group II
*: Statistically significant at $p \leq 0.05$

Table XV c shows comparison between the studied groups according to the methylation status after being categorized into unmethylated and aberrantly methylated (hemimethylated and hypermethlated cases), it was found that in control subjects (group II) 40% showed aberrant methylation of SPINT2 gene promoter ,while in HCV and HCC patients (group I) aberrant methylation of SPINT2 gene promoter was observed in 65 % of patients ,which is significantly higher than the control subjects (p=0.005).

Table (XV c): Statistical analysis between the studied two major groups according to methylation status (unmethylated vs aberrantly methylated)

	Patients (group I) (Number =80)		Control (group II) (Number =50)		χ^2	^{MC}p
	No.	%	No.	%		
Methylation Status						
Unmethylated	28	35.0	30	60.0	7.783*	0.005*
Aberrantly methylated	52	65.0	20	40.0		

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

Table XVI shows the Relation between methylation status with AFP and HCV PCR in the patients (group I), the group was divided according to the methylation status to unmethylated and aberrantly methylated subgroups and there was no statistical difference between these subgroups as regards AFP level and HCV viral load

Table (XVI): Relation between methylation status with AFP and HCV PCR in patients (group I)

	Unmethylated (number =28)	**Aberrant methylated (number =52)**	**Z**	**p**
AFP (µg/L)				
Min. – Max.	3.99 – 8900.0	2.20 – 9050.0		
Mean ± SD.	1261.93±2377.60	1165.30±2255.35	0.514	0.607
Median	65.50	54.0		
HCV PCR (10^4 IU/mL)				
Min. – Max.	8.87 – 556.0	0.4 – 390.88		
Mean ± SD.	156.35± 146.61	117.27 ± 94.57	0.585	0.559
Median	94.59	110.79		

Z: Z for Mann Whitney test

Table XVII shows the odd's ratio between the cases and control and it was found that the crude odd's ratio was 2.52 with 95 % confidence interval (1.23-5.14, p=0.05), to exclude the effect of aging on the methylation status of the SPINT2 gene adjustment for the odd's ratio was done; adjusted odd's ratio was 2.4 with 95 % confidence interval (1.13-5.26, p=0.012)

Table (XVII): Odd's ratio between the patients and control groups

	Cases (group I) (Number =80)		**Control (group II) (Number =50)**		**Crude odd's ratio**	**adjusted for age odd's ratio**
	No.	**%**	**No.**	**%**		
Methylation Status					P=0.05	P=0.012
Unmethylated	28	35.0	30	60.0	O.R=2.52	O.R=2.4
Abberantly methylated	52	65	20	40	C.I =(1.23-5.14)	C.I= (1.13-5.26)

O.R odd's ratio calculated using chi square test.
C.I : 95 % confidence interval

Tables (XVIII-XX) show no significant correlation between some studied parameters and methylation status in each of the studied groups.

Table (XVIII):Correlation between different parameters in HCV group

		FSG	Creatinine	AFP	HCV-PCR	Child pugh	Methylation status
Age	r_s	-0.075	0.164	-0.015	-0.068	0.061	0.242
	p	0.607	0.254	0.915	0.638	0.697	0.091
FSG	r_s		0.184	-0.242	0.255	0.083	0.189
	p		0.201	0.090	0.073	0.595	0.188
Creatinine	r_s			0.128	0.035	0.011	0.151
	p			0.375	0.809	0.943	0.296
AFP	r_s				0.265	0.049	-0.192
	p				0.063	0.755	0.182
HCV-PCR	r_s					0.223	-0.006
	p					0.151	0.968
Child pugh	r_s						-0.225
	p						0.147

r_s: Spearman coefficient

Table (XIX): Correlation between different parameters in HCC group

		FSG	Creatinine	AFP	HCV-PCR	Child pugh	Methylation status
Age	r_s	0.178	-0.006	0.089	0.149	0.252	0.127
	p	0.346	0.974	0.640	0.431	0.225	0.504
FSG	r_s		0.207	0.519*	0.252	0.048	0.029
	p		0.274	0.003	0.179	0.820	0.881
Creatinine	r_s			0.028	0.335	0.197	-0.188
	p			0.882	0.070	0.345	0.319
AFP	r_s				0.489*	0.094	-0.102
	p				0.006	0.654	0.591
HCV-PCR	r_s					0.370	-0.139
	p					0.069	0.464
Child pugh	r_s						-0.172
	p						0.411

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

Table (XX): Correlation between different parameters in control group

		FSG	Creatinine	AFP	Methylation status
Age	r_s	0.050	0.102	-0.137	0.173
	p	0.728	0.483	0.343	0.230
FSG	r_s		-0.111	-0.113	-0.014
	p		0.443	0.436	0.922
Creatinine	r_s			-0.208	0.030
	p			0.148	0.836
AFP	r_s				-0.018
	p				0.899

r_s: Spearman coefficient