The Fundamental Role Played by Cell Cycle Proteins in Controlling Cell Proliferation in Chronic Hepatitis C Virus Infection.

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ABSTRACT
Background: examining the alteration of cell cycle genes in early hepatitis C virus (HCV) found that altered expression of mitotic checkpoint genes, MAD2L1, KNTC1, CDC16 and CDC34, KNTC1 known as "rough deal protein" (ROD) is part of a complex involved in elaborating an inhibitory signal due to improper chromosomal aligment during cell division.
Aim of the work: attempt for the identification of proteins (genes), which act as predictive factors to identify patients with high risk of cell transformation and HCC development.
Patients and methods: fifty three patients with chronic HCV infection, age ranged between 18 and 58 years, time of assessment was before starting therapy of hepatitis C at the National Hepatology and Tropical Medicine Research Institute. Ten healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography, and collection of blood samples for routine laboratory investigation; CBCs. Liver biopsy was done to all patients and controls, patients revealed mild fibrosis (Metavir fibrosis scores from F1 to F3). Also, we used freshly frozen liver biopsies mRNA levels with perspective protein levels of four genes: P27, P15, KNTC1, MAD2L1. **Results:** significant association of P27, P15, KNTC1 and MAD2L-1 with the progression of liver fibrosis in chronic HCV liver biopsy was found. **Conclusion:** there is altered gene expression in HCV-associated liver disease. **Recommendations:** the emerging interest of hepatologists in the influence of genetic factors in HCV. Evaluation of the expression of key proteins related to the cell cycle and apoptosis in chronically infected patients with HCV would be of significance to understand disease pathogenesis, and will help in identifying novel prognostic indicators.
Key words: Cell cycle proteins P27, P15, KNTC1 and MAD2L-1, cell division, mitosis, HCV.

INTRODUCTION

Hepatitis C virus (HCV) infection is a national and global public health concern, affecting up to 4 million individuals in the United States and 200 million individuals worldwide.[1] The role of genetic factors in spontaneous clearance of HCV and fibrosis progression need to be identified. Documented evidence indicates that inter-individual genome variability contributes considerably to the observed differences in natural resistance or susceptibility to specific micro-organism and to the phenotype once infection is established. [2] Defects in chromosomal segregation are a common feature of liver tumor cells suggesting a possible role of mitosis deregulation in the pathogenesis of hepatocellular carcinoma HCC. [3]

In chronic HCV infection, rounds of hepatic cell destruction and regeneration along with fibrosis occur as a result of persistent inflammation. This phenomenon provides the pathogenic basis of

HCV associated liver disease. Together these events keep the dividing hepatocytes susceptible to

cellular insult and hence putting them at a greater risk of acquiring mutations. Liver injury is generally believed to be initiated by the death of infected afflicting problems of inflammation, regenerative hepatocyte proliferation and fibrosis in the surrounding. [4] The development of a subgenomic HCV RNA replicon capable of replication in the human hepatoma cell line, Huh 7, was a significant advance. [5, 6] Further, complete replication of HCV in cell culture has been achieved. [6-8]

The molecular events during proliferation are related closely to the cycle and its regulation. When stimulated to proliferate, hepatocytes first enter the G1 phase of the cell division cycle which is followed by DNA synthesis, or the S phase. Progression through each phase of the cell cycle involves periodic activation of phase-specific protein kinase complexes comprising of cyclins and cyclin dependent kinase (CDKs). Therefore,

cyclin D-CDK4/CDK6 complex is activated in the G1 phase and cyclin A-CDK2 is activated in the S phase. [9, 10] Cyclin-CDK complexes are known to be regulated negatively by CDK inhibitors (CKIs), which are induced in response to different stimuli including DNA damage and oxidative stress. [11, 12]

PATIENTS AND METHODS

Fifty three patients with chronic HCV infection, their age between 18 and 58 years, and time of assessment was before starting therapy of hepatitis C at the National Hepatology and Tropical Medicine Research Institute. Ten healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation. Liver biopsy was done to all patients and controls, patients revealed mild fibrosis (Metavir fibrosis scores from F1 to F3). Also, we used freshly frozen liver biopsies mRNA levels with perspective protein levels of four genes: P27, P15, KNTC1, and MAD2L1.

Detection of gene expression by quantitative real time PCR (qRT-PCR):

RNA isolation and reverse transcription:

RNA was extracted from tissues homogenate by a Mini RNA Isolation Kit (quiagene cat. No. 74104, USA) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (ThermoFisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. 1 µg of RNA was used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Applied Biosystem cat. No. 205311, USA). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl2 (25mM), RT buffer (10X), dNTP mixture (10mM), oligod(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1 h.

Quantitative real time PCR:

qPCR was performed in an optical 96-well plate with an ABIPRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions(10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C) reaction contained SYBR Green Master Mix (Applied Biosystems cat. No. 4368577), gene-specific forward and reverse primers (10 µM), cDNA and nuclease-free water. The sequences of PCR primer pairs used are shown in Table 1. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which was used as the control housekeeping gene. [13]

Ethical consideration:

Informed consent was obtained from each patient and controls at the time of taking liver biopsy sample. The Research Ethical Committee of the General Organization for Teaching Hospitals and Institutes approved the study protocol. Liver biopsy was taken from healthy subjects during liver donation.

Statistical analysis:

Analysis of data of all patients was done by IBM computer using SPSS (Statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 22. Data were statistically described in terms of mean, SD, median, minimum and maximum. Comparison of quantitative variables was done using the non-parametric Mann-Whitney test. Receiver operated characteristic (ROC) curve was constructed with area under curve analysis done to detect the cutoff value of different genes to detect patients. A probability value (P value) less than 0.05 was considered statistically significant. [14]

RESULTS

The study included 53 patients with CHCV infection before receiving treatment and 10 controls.

We found that the mean level of P27 gene in the liver biopsy=6.83, which show highly statistically significant difference between CHCV patients and the controls (p<0.001) (table 2 and graph 2 with sensitivity = 96.2 % and 100 % specificity with cutoff value of 1.25 detected by ROC curve graph 1, table 3 and 4).

The mean level of P15 gene in the liver biopsy = 6.27 which was significantly higher in the CHCV patients than the healthy controls ($p<0.001$) (table 2 and graph 3) with sensitivity 83% and specificity 100%, the cutoff value 1.55 detected by ROC curve (graph 1, table 3 and 4).

The mean level of KNTC1 gene in the liver biopsy = 3.68 with highly statistically significant difference between CHCV patients and the healthy volunteers ($p<0.001$) (table 2 and graph 4) with sensitivity = 90.6% and specificity 100%, the cutoff value 1.5 detected by ROC curve (graph 1, table 3 and 4).

The mean level of MAD2L-1 gene in the liver biopsy = 4.24, which was significantly higher in the CHCV patients than the healthy controls ($p<0.001$) (table 2 and graph 5) with sensitivity 83% and specificity 100%. The cutoff value 1.35 was detected by ROC curve (graph 1, table 3 and 4). Figures 1-4 illustrated the Real-Time Amplification plots curves for multiple quantitative PCR genes of P27, P15, KNTC1 and MAD2L-1.

DISCUSSION

Proliferative responses of hepatocytes to HCV infection are particularly important in subsequent pathogenesis as hepatocytes are the primary site of chronic HCV replication and receive different cellular stresses from lymphocytes and kupffer cells. [15-17] Impaired proliferation of hepatocytes has been reported in chronic HCV infection. Considering the fundamental role played by cell cycle proteins in controlling cell proliferation altered regulation of these proteins could significantly contribute to HCV disease progression and subsequent HCC. [18] A disrupted cell cycle progression of hepatocytes was reported in chronic HCV infection, which can contribute significantly with associated pathogenesis. The altered expression of these cell cycle proteins in hepatocytes is suggestive of an impaired cell cycle progression that limit the regenerative response of the liver to ongoing injury, leading to the progression of disease. [19]

Cyclin-dependent kinase inhibitor 1B (P27^{kip1}) is an enzyme inhibitor that in humans is encoded by the CDKN1B gene. [20] It encodes a protein which belongs to the CIP/KIP family of cyclin dependent kinase (Cdk) inhibitor proteins. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cycle progression at G1. It is often referred to as a cell cycle inhibitor protein because its major function is to stop or slow down the cell division cycle. Increased levels of the P27^{kip1} protein typically cause cells to arrest in the G1 phase of the cell cycle. Likewise, P27^{Kip1} is able to bind other Cdk proteins when complexed to cyclin subunits such as Cyclin E/Cdk2 and Cyclin A/Cdk2. [20]

P15^{PAF}, a proliferating cell nuclear antigen (PCNA) associated factor with increased expression in tumor tissues is an essential protein in both DNA replication and DNA damage repair. A 15 kD protein, P15^{PAF} was identified as a PCNA-associated factor in a yeast two-hybrid screen. P15^{PAF} is localized primarily in the nucleus. P15^{PAF} shares the conserved PCNA binding motif with several other PCNA binding proteins including CDK inhibitor P21. Overexpression of P15^{PAF} competes with P21-PCNA binding. Mutation of this motif in P15^{PAF} abolished its PCNA- binding activity. Notably, P15^{PAF} expression in several types of tumor tissues was significantly increased. Like PCNA, P15^{PAF} may possess prognostic significance in a broad array of human cancers. [21]

Kinetochore-associated protein 1 (KNTC1) is essential component of the mitotic checkpoint, which prevents cells from prematurely exiting mitosis. Also, it is required for the assembly of the dynein-dynactin and MAD1-MAD2 complexes onto kinetochores and dynamic pattern of localization during the cell cycle. At interphase, it is uniformly distributed throughout the cytoplasm and nucleus. By prophase and until late stages of prometaphase, a fraction of the total pool is concentrated at kinetochores. By metaphase, it is detected at kinetochores, along spindle fibers and most prominently at the poles. By late anaphase until the end of telophase, no longer detectable on kinetochores or along spindle fibers, but still present at the spindle poles. [22]

MAD2 Mitotic Arrest Deficient-Like 1 (Yeast) is a component of the spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. It is required for the execution of the mitotic checkpoint which monitors the process of kinetochore-spindle attachment and inhibits the activity of the anaphase promoting complex by sequestering CDC20 until all chromosomes are aligned at the metaphase plate. MAD2L1 is related

to the MAD2L2 gene located on chromosome 1. A MAD2 pseudogene has been mapped to chromosome 14. [23-25] In the present study the expression levels of the four cycle genes P27, P15, KNTC1 and MAD2L1 were significantly elevated. Sarfraz *et al*. [18] discovered that in the early fibrosis group, increased mRNA levels of cell proliferation genes as well as cell cycle inhibitor genes were observed. Increased expression of CDK inhibitor protein P27 was consistent with its mRNA level detected in early fibrosis. CDK inhibitor protein P15 was highly expressed in both early and advanced fibrosis. Among the mitotic checkpoint regulators, expression of KNTC1 was significantly reduced in advanced fibrosis while MAD2L1 showed a non-significant decrease.

Persistent inflammation and cell destruction during HCV infection has been suggested to induce proliferation of normally quiescent hepatocytes [26-28], which helps in restoring liver mass and maintaining hepatic function. Moreover, an arrested cell cycle in hepatocytes, during HCV infection [19,29] there was 3 fold increase in the mRNA levels of proliferation related genes Mcm-2, -3, -5, CDC2 and cyclin in early HCV group while the mRNA levels of cell cycle inhibitors P27, GADD45A, MAD2L1 and KNTC1 were also found to be significantly increased. These transcriptional changes suggest a mixed population of cells in the infected livers of chronic hepatitis C some of which are proliferating state while others in an arrested state.

At protein level of four differentially expressed genes, selected on the basis of their crucial role in cell cycle regulation confirms the modulations in the expression of CDK inhibitor P27 and mitotic checkpoint genes MAD2L1and KNTC1. It is important to note that these cell cycle proteins have not been implicated previously in chronic liver disease. Moreover, the association of P27 and KNTC1 expression with liver fibrosis suggests a role of these markers in the progression of liver disease. [18] In current study, analysis at protein level by immunohistochemistry in HCV-infected hepatic biopsies showed a significant increase in the expression of P27 protein that belongs to the same KIP family as P21. This protein is involved in inhibiting cell cycle progression at both G1 and S phases and is also defined as a tumor suppressor protein. [30] Mitselou *et al*. [31] added that P27 protein expression was more frequent in hepatitis and HCC when compared with normal tissue. Fang *et al*. [32] showed that a member of MAD2 family, MAD2L1, participates in inhibiting anaphase promoting complex (APC) from ubiquitinating securin, whose degradation is a prerequisite for sister chromatid separation and mitosis. Ohkawa *et al*. [33] and Yang *et al*. [34] postulated, that a number of ex vivo studies revealed that the expression of HCV proteins in cultured cells modulates normal cell cycle regulation and apoptosis .Similarly, studies of liver specimens from patients with HCV infection have also shown impaired hepatocyte proliferation [35] and enhanced apoptosis [36] that may play a role in the subsequent pathogenesis. Feldman added that, apoptosis normally occurs in liver cells during development and in renewal of hepatocytes in the adult liver. However, programmed cell death can also be triggered in various viral, immunological, malignant or drug-induced liver diseases. [37]

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Table (1): Primer sequences used for RT-PCR

Primer	Sequence
P27	Forward primer 5′-CCTCTTCGGCCCGGTGGAC -3' Reverse primer 5′- TCTGCTCCACAGAACCGGC-3'
P15	Forward primer 5′- GGAATGGGCGAGGAGAACAAGGGCATG-3' Reverse primer5′-ATAAGCTTGGCGTCAGTCCCCCGTGGCT-3'
KNTC1	Forward primer 5′-GATATCTGGCCCCTCTTCCTGA Reverse primer 5′-AGCGGCGCCTCCACTTGTCG
MAD2L1	Forward primer 5′-GCGCGTGCTTTTGTTTGTG-3' Rerverse primer 5′-AGTAAAGGTTTCAGATGGATAT-3'
GAPDH	Forward primer 5′-CCATGGAGAAGGCTGGGG-3' Reverse primer 5′-CAAAGTTGTCATGGATGACC-3'
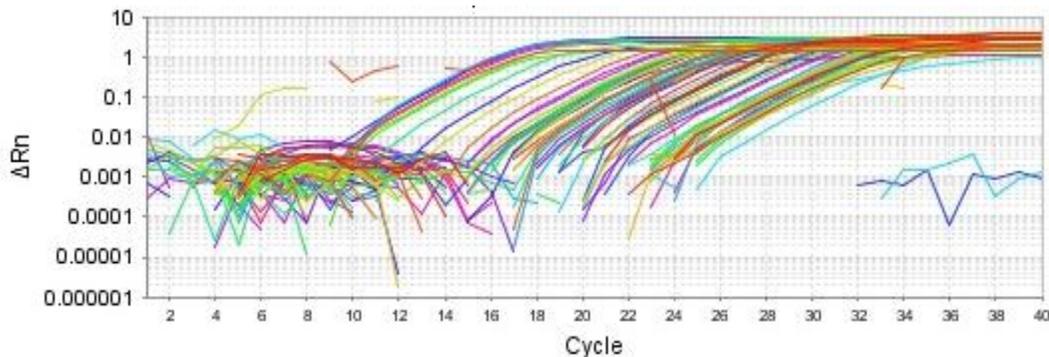
Fig (1): The Real-Time Amplification Plots quantitative PCR curve of P27 Gene.
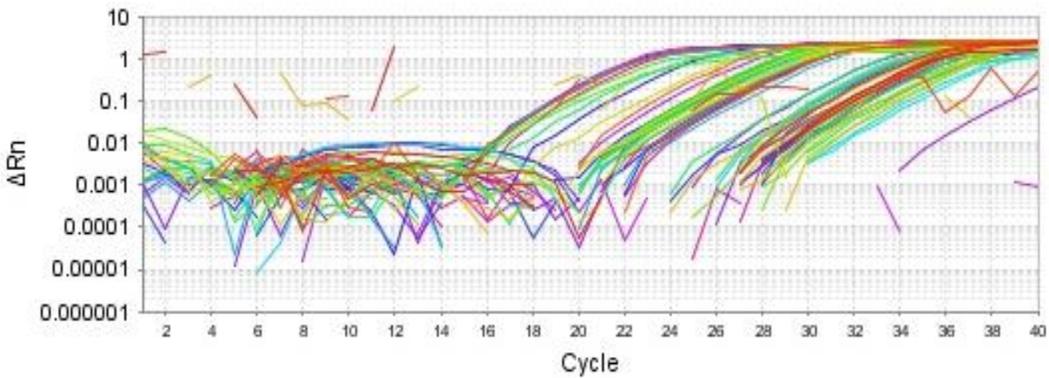
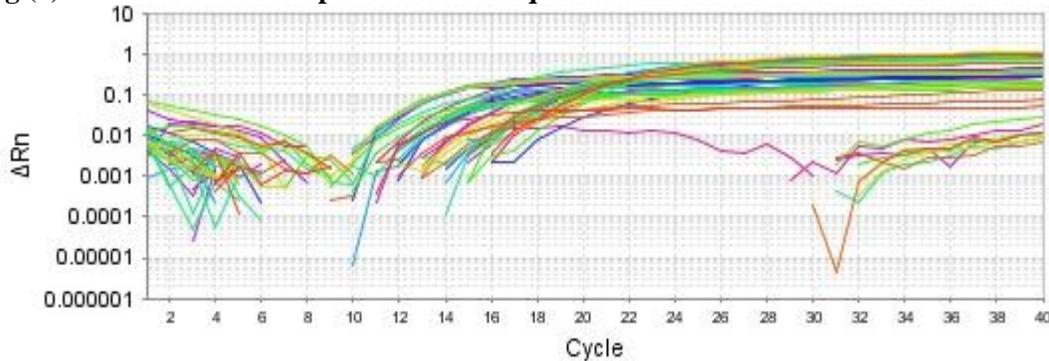**Fig (2): The Real-Time Amplification Plots quantitative PCR curve of P15 Gene.**

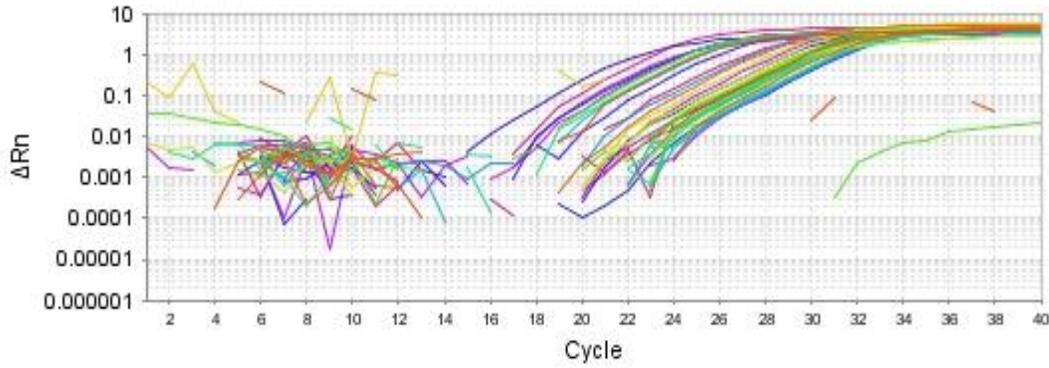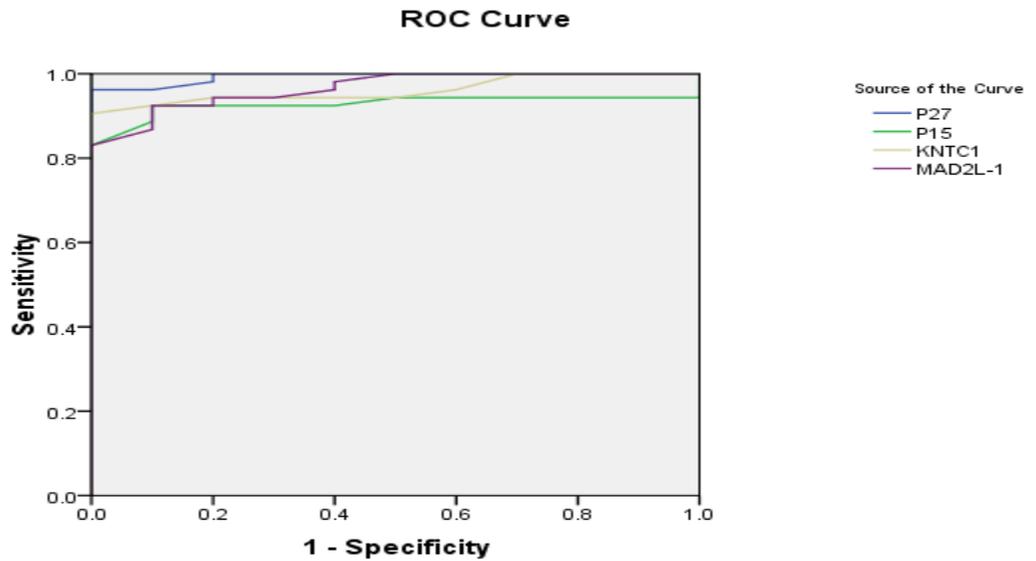**Fig (3): The Real-Time Amplification Plots quantitative PCR curve of KNTC1 Gene.**

Fig (4): The Real-Time Amplification Plots quantitative PCR curve of MAD2L1 Gene.

Table (2): Comparison between CHCV patients and healthy controls as regard P27, P15, KNTC1 and MAD2L-1 genes.

	Patients					Control					P value
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	
P27	6.83	3.08	6.70	1.07	13.50	1.04	.07	1.02	.96	1.20	<0.001
P15	6.27	3.71	6.30	.70	10.90	1.09	.12	1.04	1.00	1.40	<0.001
KNTC1	3.68	2.74	2.50	1.03	13.10	1.10	.14	1.05	1.00	1.40	<0.001
MAD2L-	4.24	3.23	2.90	1.03	10.80	1.06	.09	1.02	.99	1.30	<0.001

Table (2) shows highly statistically significant difference between both groups as regard different genes.


Graph (1): Roc curve for detection of sensitivity and specificity of each gene in CHCV patients samples.

Table (3): The positive and negative predictive values (PPV, NPV) of each gene.

| Test Result Variable(s)	Area under curve	P value	95% Confidence Interval	
			Lower Bound	Upper Bound
P27	.993	<0.001	.980	1.007
P15	.928	<0.001	.862	.994
KNTC1	.961	<0.001	.917	1.006
MAD2L-	.966	<0.001	.925	1.007

Table (4): Shows the best cut off values, sensitivity and specificity percent of each gene.

| Test Result Variable(s)	Cutoff value	Sensitivity (%)	Specificity (%)
P27	1.25	96.2	100
P15	1.55	83	100
KNTC1	1.5	90.6	100
MAD2L-	1.35	83	100

Values are presented as mean ±SD
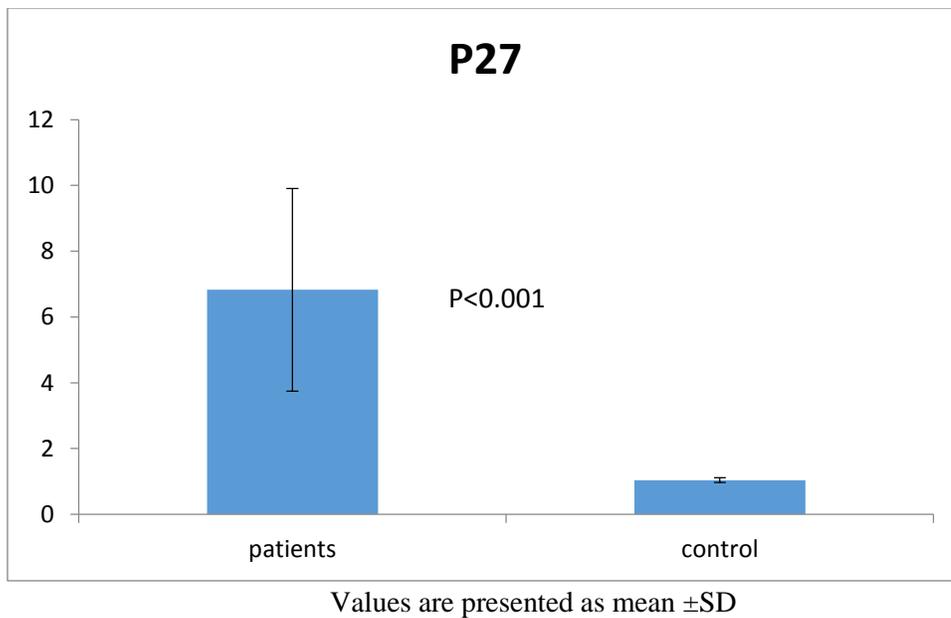
Graph (2): The comparison between CHCV patients and controls as regarding P27 gene.

Graph (2) illustrates highly significant difference between both groups as regarding Cyclin-dependent Kinase inhibitor IB.
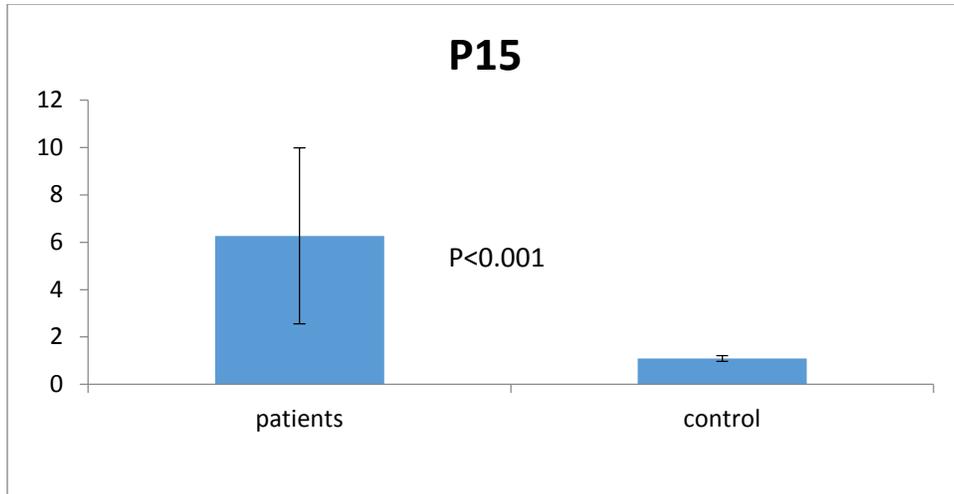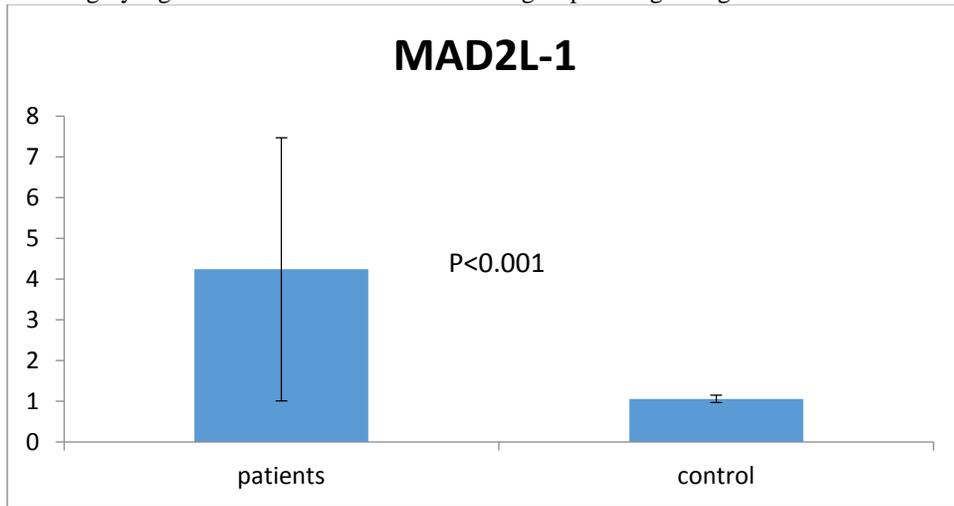
Graph (3): The comparison between CHCV patients and controls as regarding P15 gene.
Graph (3) illustrates highly significant difference between both groups as regarding proliferating cell nuclear antigen (PNCA).
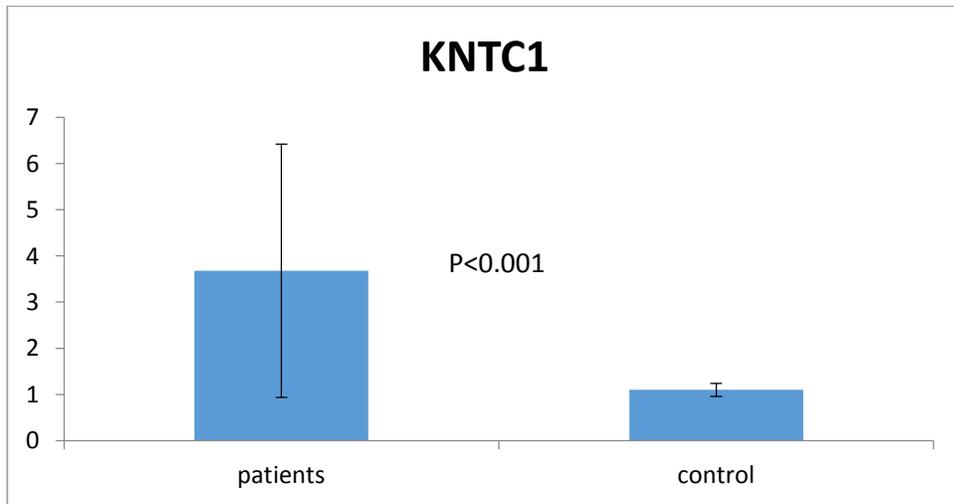
Graph (4): The comparison between CHCV patients and controls as regarding KNTC1 gene.
Graph (4) illustrates highly significant difference between both groups as regarding the kinetochore-associated protein 1.

Graph (5): The comparison between CHCV patients and controls as regarding MADL1 gene.
Graph (5) illustrates highly significant difference between both groups as regarding the (Mitotic arrest deficient, yeast, homolog)-like1