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IFN-Stimulated Genes Upregulation Pattern in Chronic Hepatitis C

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ABSTRACT

Background: The development of effective tools for the large-scale analysis of gene expression has provided new insights into the involvement of gene networks and regular pathways in various disease processes. The chemokine receptor CXCR3 is a G protein-coupled receptor found predominantly on T cells that is activated by three ligands as follow: CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC), and play a key role in immune and inflammatory responses by promoting recruitment and activation of different subpopulations of leukocytes. **Aim of the work:** The study is a logical functional approach for the development of serum markers chemokines that bind to CXC chemokine receptor 3 to determine whether they play a role in the future of immune system to clear HCV, these chemokines: CXCL9, CXCL10 and CXCL11. **Patients and methods:** 131 male and female patients with chronic hepatitis C virus (CHCV) infection, their age ranges between 22 and 55 years, selected from the National Hepatology and Tropical Medicine Research Institute. The included patients were divided to two groups, the first group: 80 patients were investigated for the predictive values of CXCL9,10,11 and CXCR3 chemokines in peripheral blood mononuclear cells (PBMCs), the second group were fifty one patients analyzed for the expression of surface markers on CD8+T cells. Twenty healthy individuals were included to serve as controls for each group. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation and serological assay. **Results:** Chemokine CXCL9, CXCL10, CXCL11 and their receptor CXCR3 expression levels are induced in PBMCs during CHCV infection, associated with increased the expression levels of CD8+T cells in CHCV patients. **Conclusion:** The interaction between chemokines and their receptors is essential in recruiting HCV-specific T cells to control the infection. **Recommendations:** The regulation of chemokines and their receptors could be a future potential therapeutic target to decrease liver inflammation and to increase specific T cell migration to the infected liver, the blocking of chemokines and chemokine receptor engagement is a therapeutic strategy that should be explored in the near future for non-responders to current anti-HCV therapy.

Key words: Chemokines CXCL 9, 10, 11, Chemokine receptor CXCR3, Hepatitis C virus infection, immunoregulation pattern of genes.

INTRODUCTION

Hepatitis C is a major cause of chronic liver disease worldwide. An important and striking feature of hepatitis C is its tendency toward chronicity. In > 70% of infected individuals, hepatitis C virus (HCV) establishes a persistent infection over decades that may lead to cirrhosis and hepatocellular carcinoma. [(1)] The estimated worldwide prevalence of HCV infection is 2% to 3%, which translates to an estimated 170 million infected individuals. [(2)] Successful eradication of the virus is achieved in only 15-20% of newly infected individuals, while the remainder develops chronic infection.

The ability of the virus to persist within a host is astonishing, and is attributed to its efficient ability to evade the adaptive and innate components of the host immune System. [(3)] Chemokines represent a target for modulation by viruses including the HCV. HCV is known to modulate chemokine expression in vitro and may therefore enable its survival by subverting the immune response in vivo through altered leukocyte chemotaxis resulting

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in impaired viral clearance and the establishment of chronic low-grade inflammation. [4]

The selective CXC chemokine receptor 3 (CXCR3) agonists, monokine induced by interferon-(IFN-)\VCXC chemokine ligand 9 (CXCL9), IFN-inducible protein 10\V CXCL 10, and IFN-inducible T cell chemoattractant (I-TAC)\V CXCL11, attract CXCR3 cells such as CD45RO T lymphocytes, B cells, and natural killer cells. Further, all three chemokines are potent, natural antagonists for CXCR3 and feature defensin-like. [5]

Chemokines are small heparin-binding proteins that direct the movement of mononuclear cells through the body to contribute to the development of an adaptive immune response and to the pathogenesis of inflammation. These proteins are 8-10 kDa in size with 20%-70% amino acid sequence homology and are secreted by resident cells at the inflammatory site. [6] The CXC family has been subdivided into two groups depending on the presence of the ELR motif (Glu-Leu-Arg). [7, 8]

Interactions between chemokines and enzymes are vital in immunoregulatin. Structural protein citrullination by peptidylarginine deiminase (PAD) has been associated with autoimmunity. [9] CXCL10 binds CXCR3 receptor to induce chemotaxis, apoptosis, cell growth and angiostasis. Altrations in CXCL10 expression levels have been associated with inflammatory diseases including infectious diseases, immune dysfunction and tumor development. CXCL10 is also recognized as a biomarker that predicts severity of various diseases. [10] Fibrosis is a hallmark of chronic diseases, yet many aspects of its mechanism remain to be defined. Chemokines are ubiquitous chemotactic molecules that mediate many acute and chronic inflammatory conditions, and CXC chemokine genes colocalize with a CXCL9 locus shown to include fibrogenic genes. [11]

PATIENTS AND METHODS

131 male and female patients with chronic hepatitis C virus (CHCV) infection, their age ranges between 22 and 55 years, selected from the National Hepatology and Tropical Medicine Research Institute. The included patients were divided to two groups, the first group: 80 patients were investigated for the predictive values of CXCL9,10,11 and CXCR3 chemokines in peripheral blood mononuclear cells (PBMCs), the second group were fifty one patients analyzed for the expression of surface markers on CD8 + T cells. Twenty healthy individuals were included to serve as controls for each group. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation and serological assay.

Protocol: Purification of Total Cellular RNA from Human Whole Blood:

Procedure

1. Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube, add 5 ml of Buffer EL to 1 ml of whole blood, and mix in a tube which has a total volume of ≥ 8 ml. **Note**: Use an appropriate amount of whole blood. Up to 1.5 ml of healthy blood (typically 4000–7000 leukocytes per microliter) can be processed. Reduce amount appropriately if blood with elevated numbers of leukocytes is used.

2. Incubate for 10–15 min on ice. Mix by vortexing briefly 2 times during incubation. The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time can be extended to 20 min.

3. Centrifuge at 400 x g for 10 min at 4°C, and completely remove and discard supernatant. Leukocytes will form a pellet after centrifugation. Ensure supernatant is completely removed. Trace amounts of erythrocytes, which give the pellet a red tint, will be eliminated in the following wash step.

4. Add Buffer EL (Erythrocyte lysis) to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step 1). Resuspend cells by vortexing briefly. For example, add 2 ml of Buffer EL per 1 ml of whole blood used in step 1.

5. Centrifuge at 400 x g for 10 min at 4°C, and completely remove and discard supernatant. **Note**: Incomplete removal of the

supernatant will interfere with lysis and subsequent binding of RNA to the QIAamp ® RNA blood mini Kit (50) Cat. No.: 52304, Lot No.: 148019701 spin column, resulting in lower yield.

6. Add Buffer RLT to pelleted leukocytes according to the table 1 below. Vortex or pipet to mix.

When not using healthy blood, refer to number of leukocytes to determine the volume of Buffer RLT required. Buffer RLT disrupts the cells. No cell clumps should be visible before you proceed to the homogenization step. Vortex or pipet further to remove any clumps.

7. Pipet lysate directly into a QIAshredder spin column in a 2 ml collection tube (provided) and centrifuge for 2 min at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate. To avoid aerosol formation, adjust pipet to ≥750 µl to ensure that the lysate can be added to the QIAshredder spin column in a single step. If too many cells have been used, after homogenization the lysate will be too viscous to pipet.

8. Add 1 volume (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge. A precipitate may form after the addition of ethanol. This will not affect the QIAamp procedure.

9. Carefully pipet sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim. Centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm). Maximum loading volume is 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge as above. Discard flow-through* and collection tube. Optional: If performing optional on-column DNase digestion.

10. Transfer the QIAamp spin column into a new 2 ml collection tube (provided). Apply 700 µl Buffer RW1 to the QIAamp spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash. Discard flow-through* and collection tube.

11. Place QIAamp spin column in a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE into the QIAamp spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard flow-through* and collection tube. **Note**: Ensure ethanol is added to Buffer RPE.

12. Carefully open the QIAamp spin column and add 500 µl of Buffer RPE. Close the cap and centrifuge at full speed (20,000 x g, 14,000 rpm) for 3 min. **Note**: Some centrifuge rotors may distort slightly upon deceleration, resulting in flow-through, containing Buffer RPE, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column. * Flow-through contains Buffer RW1 or RLT and is therefore incompatible with bleach.

13. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer RPE carryover.

14. Transfer QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at ≥ 8000 x g (≥10,000 rpm) to elute. Repeat if >0.5 ml whole blood (or >2 x 106 leukocytes) has been processed. [12]

cDNA Reaction Preparation

The kit contains reagents that when combined, form a 2X Reverse Transcription (RT) Master Mix. An equal volume of RNA sample should be added. To avoid RNase contamination, RNase-free reagents and consumables must be used.

To prepare the 2X RT Master Mix (per 20 µL reaction):

1. Allow the kit components to thaw on ice.
2. Referring to the table 2 below, calculate the volume of components needed to prepare the required number of reactions. **Note:** Prepare the RT master mix on ice.

Immunophenotyping analysis by flow cytometry

PBMCs were isolated by Ficoll-Histopaque (Sigma Chemical Co., St Louis, MO) density centrifugation. Cells were stained by

fluorescent antibodies per manufacturer's instructions and analyzed with Flow Jo (Tree Star Inc., San Carlos, CA) 17, PD-1 positivity was determined by an isotype control-defined cutoff (99.5%). [13]

Detection of PD-1 gene expression using real time PCR (RT–PCR)

RNA extraction:

Total RNA was isolated from whole blood using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically, concentrations of the RNA were assessed using the OD 260/280 ratio. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide.

cDNA synthesis:

First-strand cDNA synthesis was performed with cDNA synthesis kit supplied by Qiagene. Briefly, 2 μg total RNA was mixed with 0.5 μg of oligo (dT) 12-18 primer in a total volume of 12 μL. After the mixture was heated at 70°C for 10 min, a solution containing 50 mmol/L Tris Hcl (PH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 μL RNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5 μL. This mixture was incubated at 42°C for 1 h.

Real-time quantitative polymerase chain reaction (PCR):

For real-time quantitative PCR, 5 μL of first-strand cDNA was used in a total volume of 25 μL, containing 12.5 μL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer (shown in table 1). PCR reactions consisting of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism 7500 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 beta actin genes. [14]

Table (1):

Buffer RLT* (μl)	Healthy whole blood (ml)	No. of leukocytes
350	Up to 0.5	Up to 2 x 106
600	0.5 to 1.5	2 x 106 to 1 x 107

* Ensure β-ME is added to Buffer RLT.

Table (2):

Component	**Volume (μL)/Reaction Kit**	
With RNase Inhibitor Kit	**Without RNase Inhibitor**	
10X RT Buffer	2.0	2.0
25X dNTP Mix (100 mM)	0.8	0.8
10X RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	--
Nuclease-free H2O	3.2	4.2
Total per Reaction	10.0	10.0

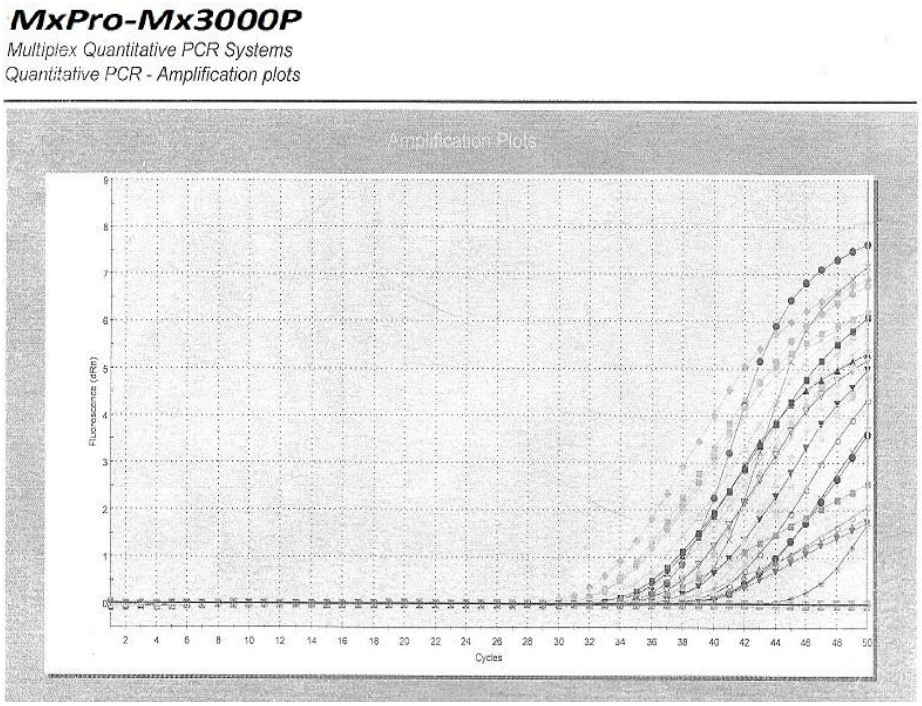

Fig (1): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

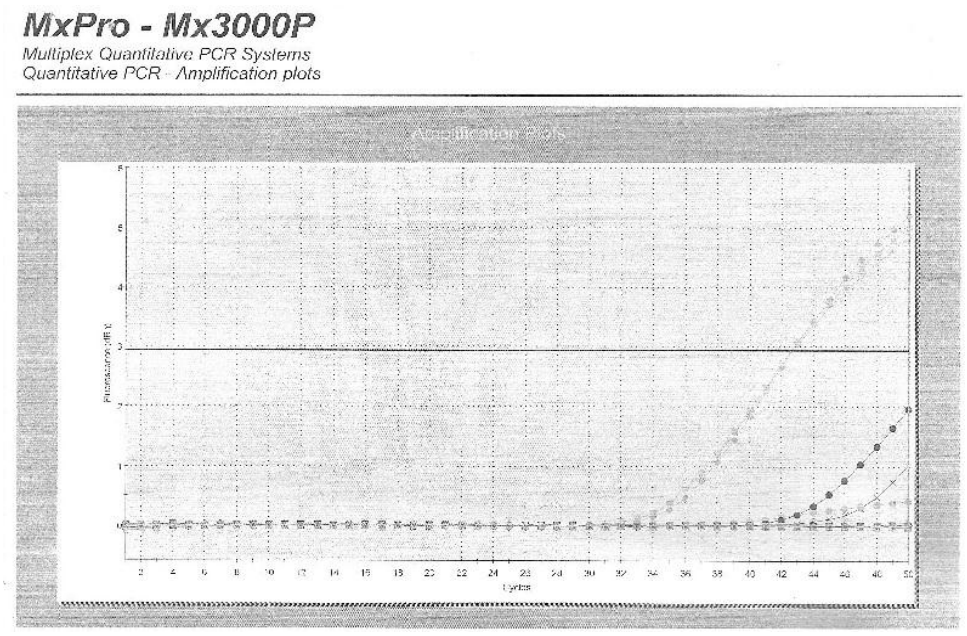

Fig (2): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

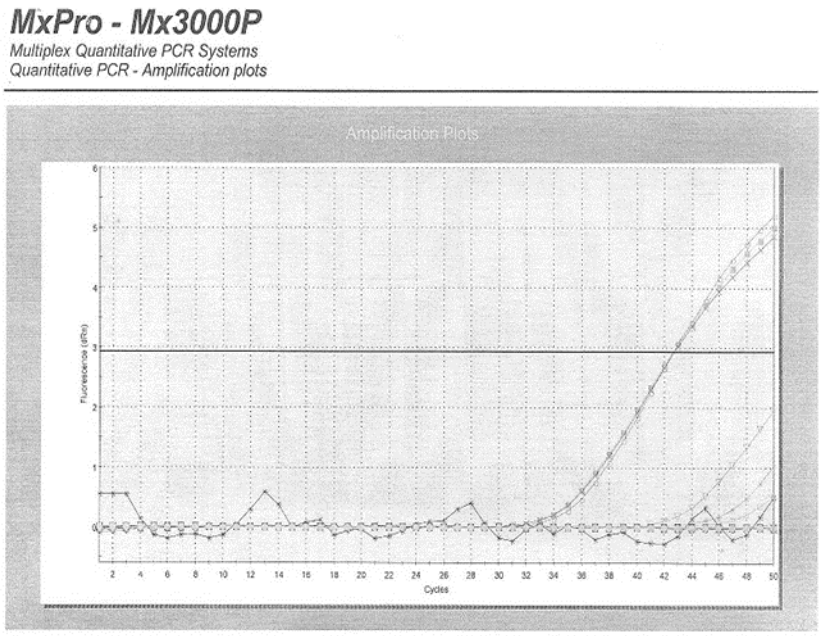

Fig (3): Illustrates the CXCL9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

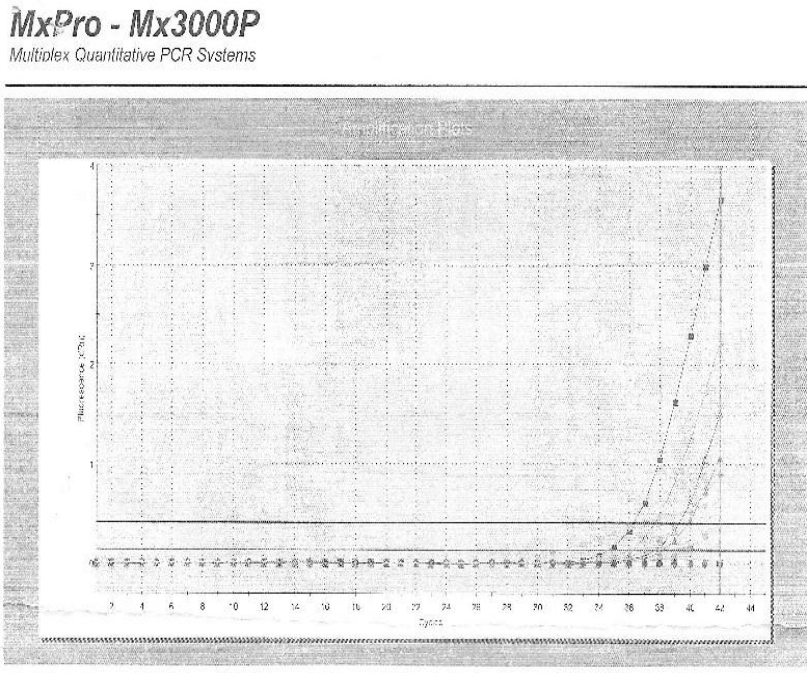

Fig (4): Illustrates the CXCL9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

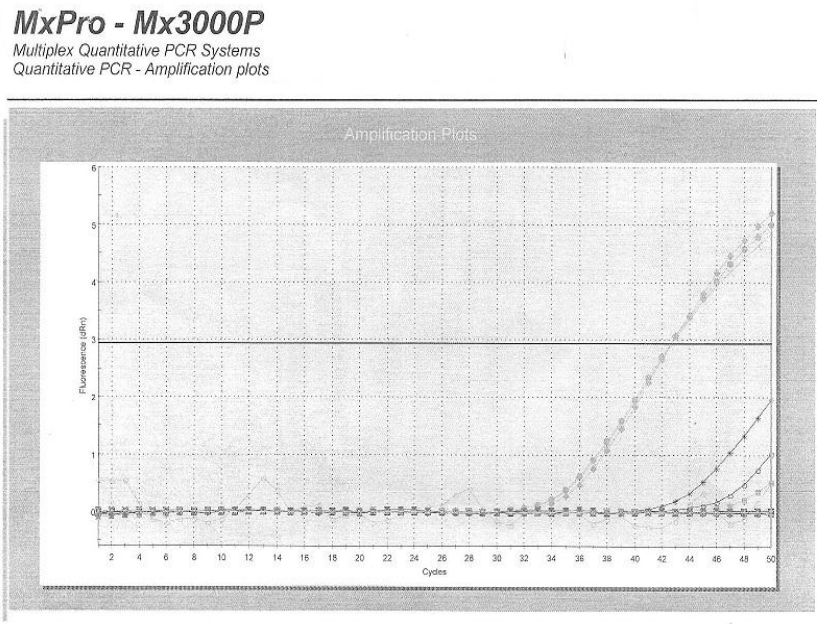

Fig (5): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

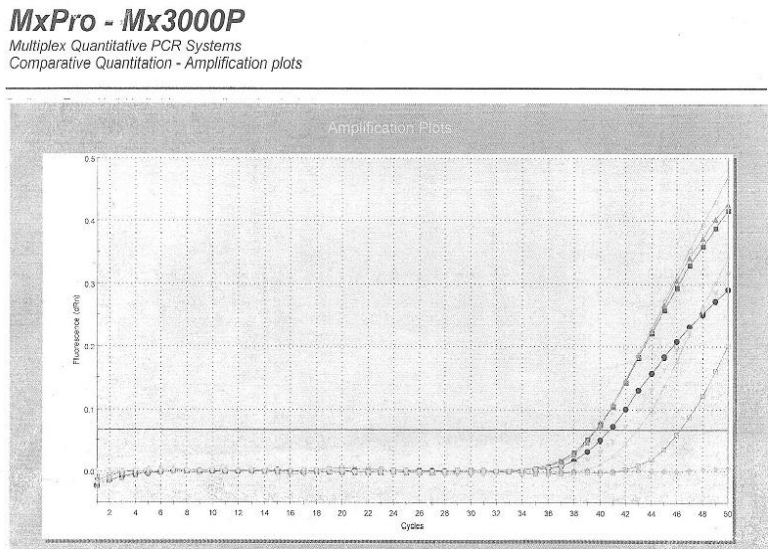

Fig (6): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

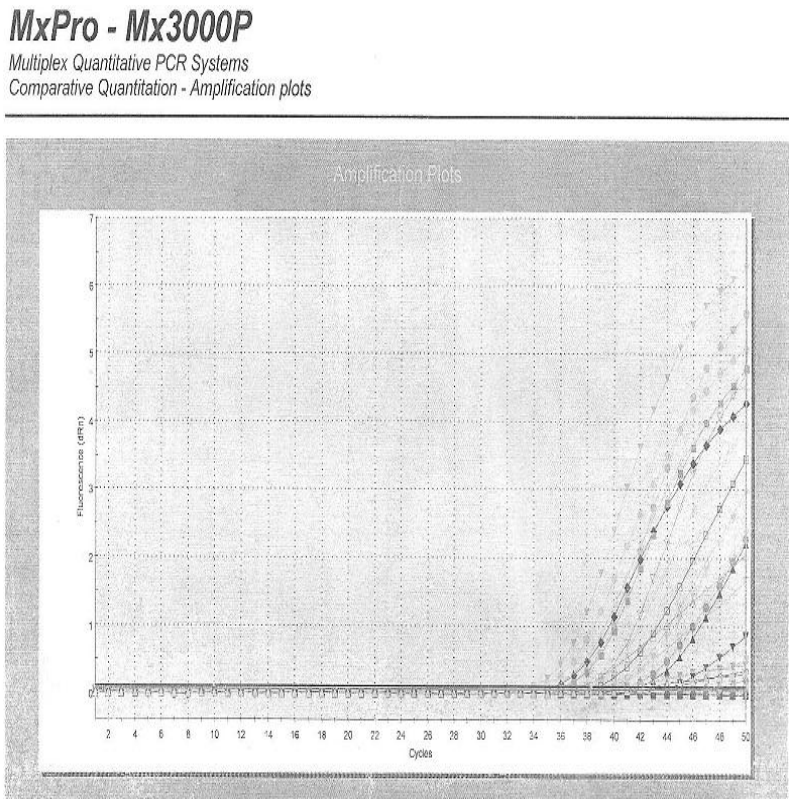

Fig (7): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

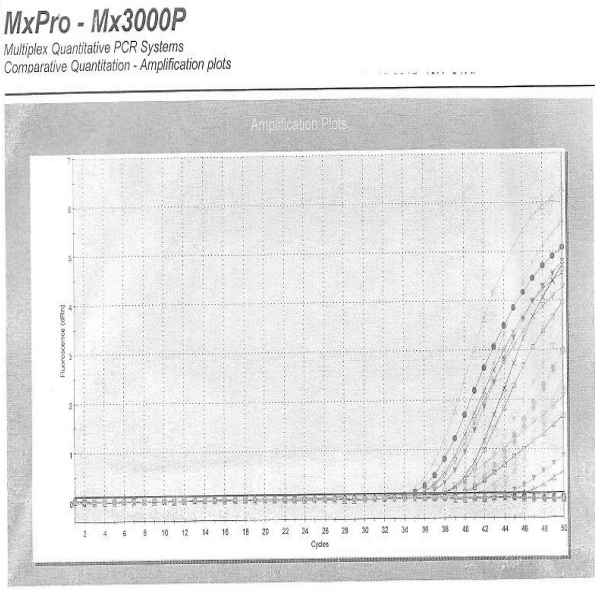

Fig (8): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

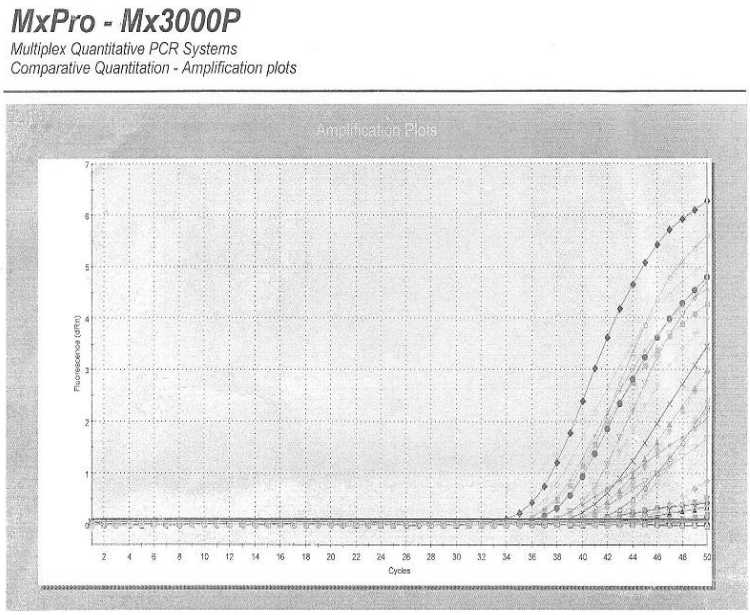

Fig (9): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

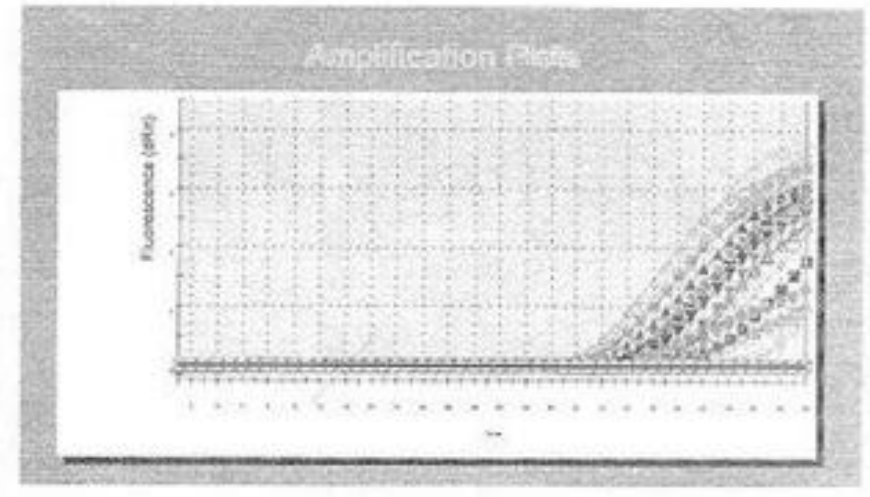

Fig (10): Illustrates the CXCL 9, 10, 11 and CXCR3 genes, Real-time Amplification plots quantitative PCR.

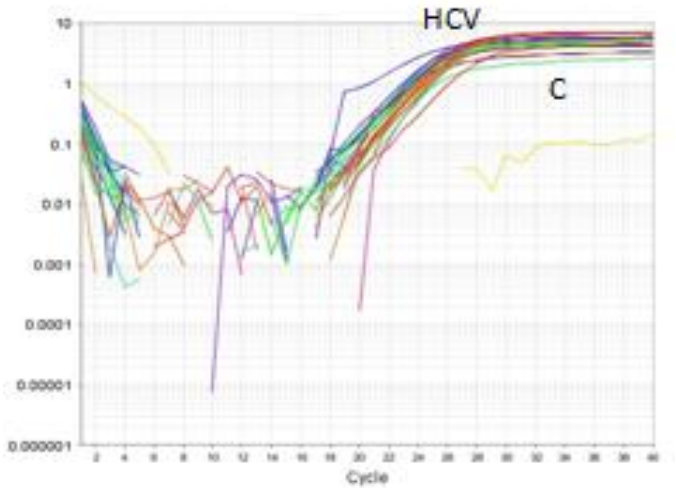

Fig (11): Amplification plots curve of quantitative real time-PCR illustrated increased programmed death cell-1 (PD-1) expression in circulating HCV-specific CD8+T-cells from patients with hepatitis C.

Ethical consideration: Informed consent was obtained from each patient at the time of drawing blood samples. The Research Ethical committee of the central Organization for Teaching Hospitals and Institutes approved the study protocol.

Statistical analysis: Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 21. Data was summarized using mean and standard deviation. Comparisons between quantitative variables in cases were done using one sample T test to compare against control value 1. [15] Unpaired t-test was used to compare two groups as regard quantitative variables. [16] P-values less than 0.05 were considered as statistically significant.

RESULTS

The quantitative expression levels of the Real time-PCR activation by HCV chemokines CXCL 9, 10, 11 and chemokine receptor CXCR3 were increased with statistically significant difference in CHCV infection against control value (1) as shown by table (3) using unpaired t-test, graph 1, 2, 3 and 4.

The Real time –PCR expression of activation by HCV level of CD8+T cells in PBMCs of CHCV patients was higher level as compared to the healthy controls with statistically highly significant difference ($p < 0.001$) by using unpaired t-test table (4), figure (11), graph (5,6).

Figure (1) to figure (10) illustrated the Real time – Amplification plots for multiple quantitative PCR genes CXCL 9, 10, 11 and CXCR3 receptor.

DISCUSSION

The ligands for CXCR3 are interferon (IFN)-ð inducible protein 10 (IP-10, CXCL 10), monokine induced by IFN-ð (Mig, CXCL9), and IFN-inducible T-cell α chemoattractant (1-TAC, CXCL 11). CXCR3 is expressed on activated T cells and natural killer cells. [17] The migration of lymphocytes to the liver is a complex process involving adhesion, rolling, triggering, and transendothelial migration. Chemokines and their receptors play an essential role in this multistep pathway. [18, 19] Therefore, chemokines and their receptors are associated with viral control but are also associated with immune-mediated liver inflammation.

Moreover, in a hepatotropic viral infection in humans, a huge intrahepatic non-specific mononuclear infiltrate during viral persistence was noticed, while this was not present in subjects with control. [20]

The intrahepatic chemoattraction of non-specific T cells perpetuated the liver damage. Consequently, also in humans, chemokines and their receptors develop an important role in viral clearance and in the development of chronic tissue inflammation. Obviously, the modulation of these pathways is important for generating an efficient immune response, and for participating in the inflammatory process during the chronic infection phase, but pathway modulation could also be a viral strategy used by HCV to escape from immune control. [21] Persistent HCV infection is characterized by a non-specific inflammatory infiltrate in the liver, mainly composed of CD8+T cells [22, 23], and responsible for liver damage. [24] These cells are attracted by the interaction between the intrahepatic secreted chemokines and the chemokine receptors expressed on T cells, a correlation between liver inflammation and liver infiltrating CXCR3 expressing T cells. [25, 26]

In the present work the quantitative expression levels of the Real time-PCR activation by HCV chemokines CXCL 9, 10, 11 and chemokine receptor CXCR3 were increased with statistically significant difference in CHCV infection. **Larrubia *et al.*** [27] observed that intrahepatic and peripheral blood levels CXCL 9, 10, 11 and chemokine receptor CXCR3 are increased during chronic hepatitis C. The interaction between chemokines and their receptors is essential in recruiting HCV-specific T cells to control the infection. In chronic hepatitis C, the expression of different chemokines in the liver has been described. CXCL10 is increased in the liver and peripheral blood during hepatitis C. [28-30, 25] This molecule is produced by hepatocytes and sinusoidal endothelial cells. [29, 30] CXCL9 and CXCL11 are also increased in the serum and liver of subjects with chronic hepatitis C. [29, 31] CXCL9 is detected primarily on sinusoidal endothelial cells, while CXCL11 is produced mainly by hepatocytes. [29, 26] The expression of all these chemokines in the liver can be induced directly by HCV. High hepatocyte synthesis of CXCL10, CXCL9 induced by some HCV proteins such as NSSA and core. [32] Although in vitro study suggests that HCV proteins could also decrease the expression of CXCL10. [33] Intrahepatic CXCL10 mRNA levels are associated with intralobular inflammation. [34] Similarly, CXCL9 and CXCL11 correlate with the grade of liver inflammation. [26, 35]

Zeremski *et al.* [36] postulated that a study, examining a large sample of chronic hepatitis C patients showed a positive correlation between CXCL9, CXCL10 and CXCL11 intrahepatic levels and the grade of fibrosis. Indicating that persistent liver inflammation produced by the mononuclear cells attracted by these chemokines could finally induce the activation of a liver fibrosis cascade. It is possible to speculate that chemokines are secreted in the infected liver to attract an adaptive immune response able to clear the virus. Unfortunatley, when the specific response fails these chemokines also attract non-specific T cells, when are not able to remove the virus but produce liver inflammation. Therefore, as chemokines are nonspecific chemoattractants, the intrahepatic inflammatory infiltrate produced during chronic infection is mainly non-HCV-specific and consequently unable to eliminate HCV. [37]

In the present study the Real time – PCR expression of activation by HCV level of CD8+T cells in PBMCs of CHCV patients was higher level as compared to the healthy controls with statistically highly significant difference. **Kastenmuller *et al.*** [38] discovered that after an infection, the immune system generates long-lived memory lymphocytes whose increased frequency and altered state of differentiation enhance host defense against re-infection. Recently, the spatial distribution of memory cells was found to contribute to their protective function. Effector memory CD8+T cells reside in peripheral tissue sites of initial pathogen encounter, in apparent anticipation of re-infection. The memory CD8+ T cells were concentrated near peripheral entry portals of lymph-borne pathogens, promoting rapid engagement of

infected sentinel macrophages. A feed-forward CXCL9-dependent circuit provided additional chemotactic cues that further increase local memory cell density.

Chemokines produced in the liver during hepatitis C virus (HCV) infection induce migration of activated T cells the periphery of infected parenchyma. The milieu of chemokines secreted by infected hepatocytes is predominatly associated with the T-helper/T-cytotoxic type-1 cell (Th1/Tc1) response. [27] Persistent HCV infection is characterized by a non-specific inflammatory infiltrate in the liver, mainly composed of CD8 + T cells. [22, 23], and responsible for liver damage. [24] These cells are attracted by the interaction between the intrahepatic secreted chemokines and the chemokine receptors expressed on T cells. Moreover, it was shown that the increase in CXCR3 expressing CD8 + T cells during treatment is associated with SVR. [25]

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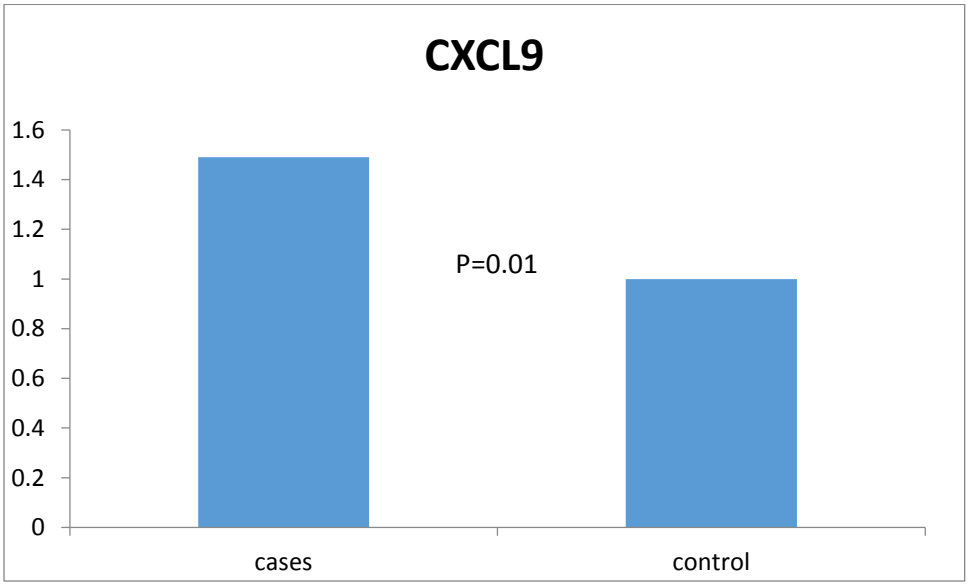
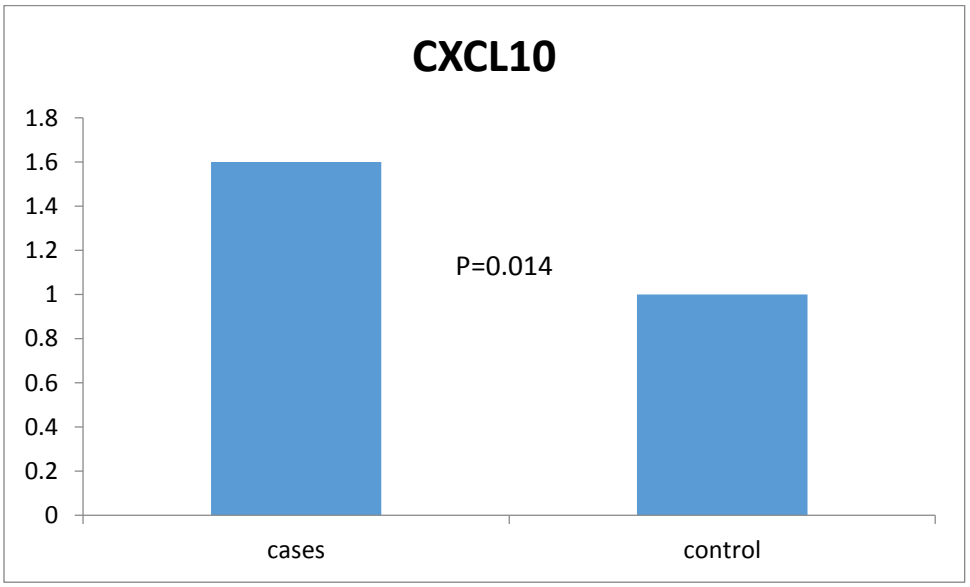
Table (3): The CHCV patients as regarding CXCL9, CXCL10, CXCL11and CXCR3 genes.

Variable Genes	Mean	Stand. Deviation	P Value
CXCL9	***1.49***	***1.25***	***.010***
CXCL10	***1.60***	***1.14***	***.014***
CXCL11	***1.7***	***1.24***	***<0.001***
CXCR3	***1.31***	***1.13***	***.089***

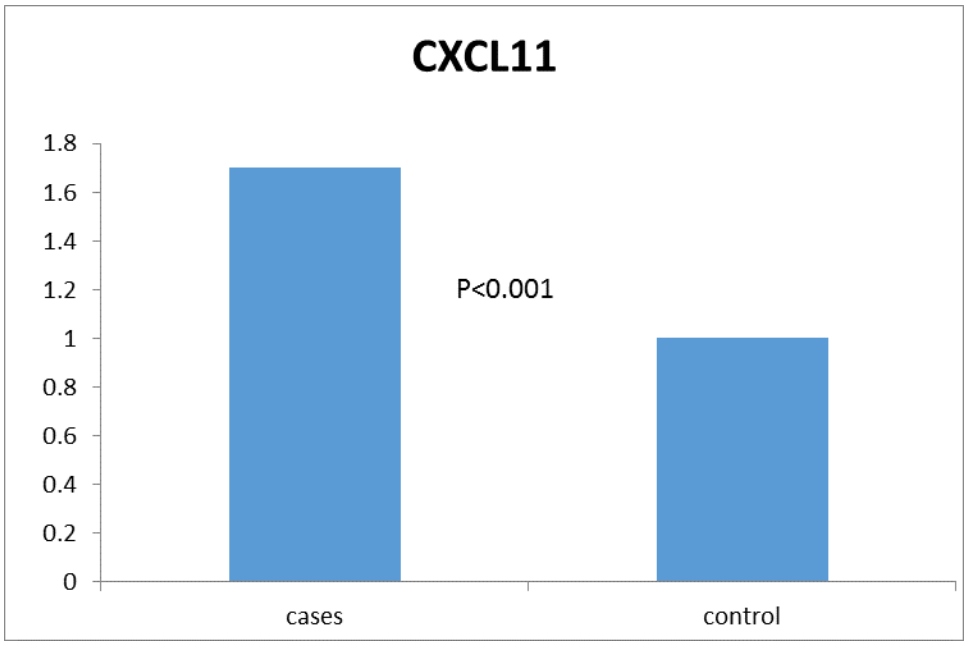
This table shows that the variable genes were increased in CHCV patients with statistically significant value by using T-test ($p < 0.05$).



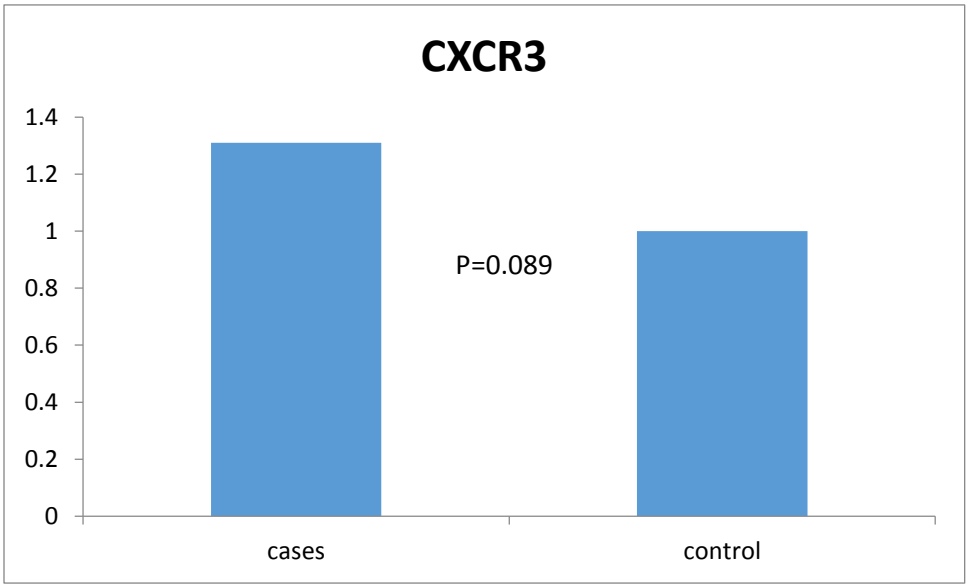
Graph (1): Quantitative expression levels of CXCL 9 gene in CHCV patients as compared to healthy controls.



Graph (2): Quantitative expression level of CXCL 10 gene In CHCV patients as compared to healthy controls.



Graph (3): Quantitative expression level of CXCL 11 gene In CHCV patients as compared to healthy controls.

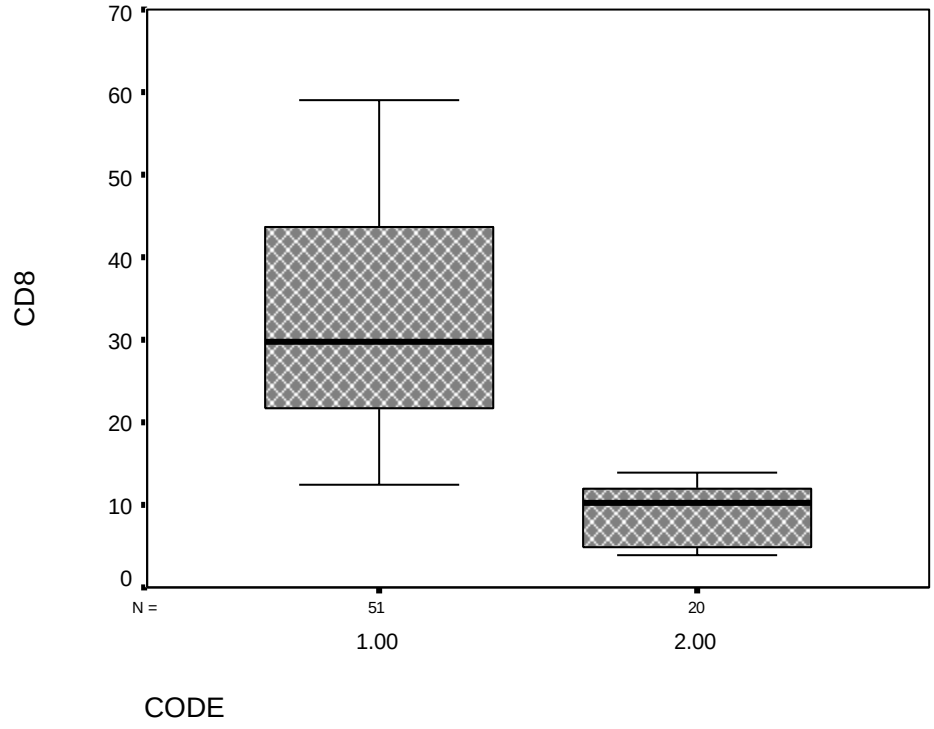


Graph (4): Quantitative expression level of CXCR3 gene In CHCV patients as compared to healthy controls.

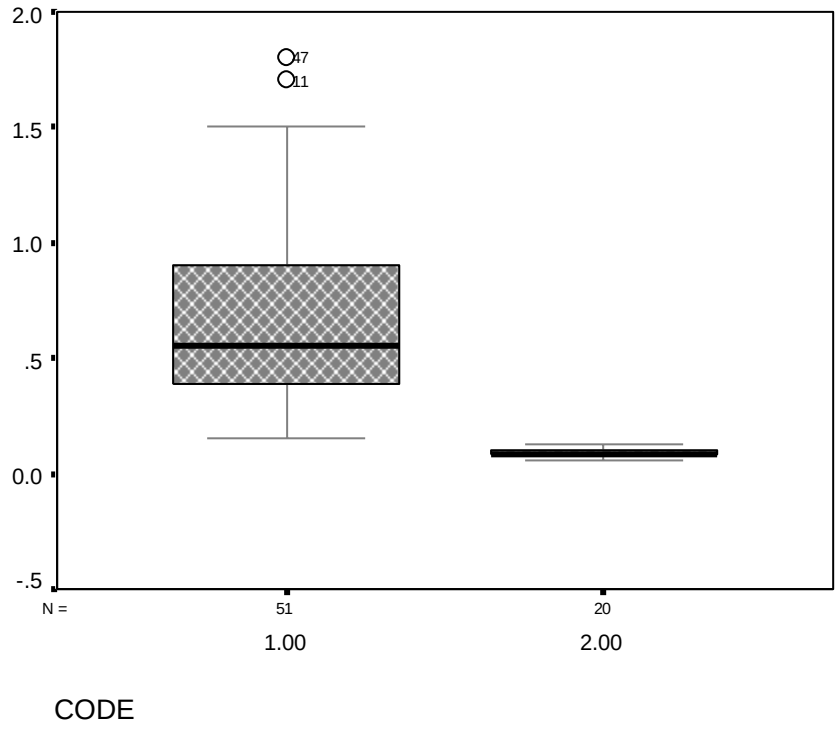
Table (4): Comparison between CHCV patients and control group as regard lab data.

Variables	Cases N=51	Controls N=20	T	P
PD1% expression in CD8+T	**32.7±12**	**9±3.5**	**8**	**<0.001** HS
PD1 gene	**0.65±0.3**	**0.09±0.02**	**6**	**<0.001** HS
Viral load	**9,101,35±179,566**	**---**	**---**	**---**

This table shows statistically significant difference between both groups as regard programmed death-1 (PD-1) gene and expression levels of CD8+T cells by using unpaired t-test.



Graph (5): Quantitative Expression level of CD8 + T cell in PBMCs from patients with CHCV in comparison to the healthy controls (Code: 1.00 = patients & 2.00 = controls).



Graph (6): Quantitative expression level of PD-1 % in PBMCs from patients with CHCV in comparison to the healthy controls (Code: 1.00 = patients & 2.00 = controls).