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Expression of IL-1b, IL-12, IL-17a, TNF-a and Fox-P3 in Patients with Low, Medium and High-Hepatitis Viral Load

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Abstract:

Hepatitis B virus infection(HBVI) is a major causing of concern world wide causing significant mortality. Poor diagnosis and prognostic factors remain one of crucial factors responsible for poor management of the disease. Here in this study we show that the inflammatory cytokine such as IL-1b, IL-17a, TNF-A have a major prognostic value in HBV patients. In addition to these cytokine immune-suppressing FoxP3 can also have a vital implication in the prognosis and overall survival of HBV patients. As such we analyzed the mRNA expression of IL(1b;17a ;12); TNF-A; and FoxP3 in HBV patients with high, medium and LVLpatients. We here found that the expression of IL-1beta and TNF-alpha were high in HVLgroups indicating the high immune response against HBV and similarly suppressive effect by FoxP3 in LVLgroup in response to the overall suppression of inflammation. Therefore, supplementation of IL-1beta and /or TNF alpha can support clearance of HBV at initial stages of infection but also important here is to monitor the expression of immune suppressinf FoxP3 which may prevent in eliciting a potent immune response against HBV.

Keywords:IL-1b, IL-12, IL-17a, TNF-a and Fox-P3, Hepatitis Viral Load, Expression.

Introduction :

Poor diagnosis and prognostic factors remain one of crucial factors responsible for the ill management of the HBVI , Its a major cause of concern worldwide causing significant mortality. Individuals with chronic hepatitis_B(CHB) were increasing risk of developing liver cirrhosis;hepatic de-compensation; and other progressive diseases [1]. There is no particular treatment that completely eliminates HBVI. Current drugs aresignificant side effects,expensive,and limiting efficacy. Current guidelines try to standardize the clinical practice and drugs to increase patient compliance to the regimen. During chronic liver damage,, adaptive immune cells are crucially involved in the pathogenesis of hepatic inflammation particularly T-cells playing important role in antiviral defense [2].Immune cells are activated by some inflammatory cytokinesfor the wound heal promotion or immune response initiation; they are releasing via many different cell types and activate both immunity (adaptive and innate) system cells [3]. However, this immune response is somewhat hindered by Foxp3 expressing Treg cells which suppress the immune system, aprimary mechanism in immunological self-tolerance, preventing autoimmune disease development.Cytokines are thought to participate in non cytolyticHBVclearance during the acute infections and during T-cell mediated virus controlling, and their antiviral impactswere established in differentmodels of experimental [4]. Antiviral effect of cytokine-mediated are multifunctional and targetsmanyfundamental viral replication steps [5].In earlier studies its reporting that the expression of IL-12, TNF-α was found to be lowerin patients suffering from HBV [6]. He *et al* in a study analyzed the cytokine profile of about 30 cytokines related to anti-HBV viral immunity and found that the increase of IL(12p70; 23p19, and 29) promoted H.B.V clearance [7]. IL-1β, IL-12, TNF-α target the HBV virus at

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different phases of life cycle and their expression as such can be an important marker of disease progression [8]. Therefore, wecanpropose that the serum cytokine expression patterns and Foxp3 might be related tovarious HBV infection stages and can result in better prognosis and fabrication of individualized immunotherapy in HBV patients.In this study we analyzed the mRNA expression of IL(12;17a;1β); TNF-α as well asFoxP3 in blood samples of HBV patients with high, medium and LVLpatients.

Material & Methods:

Viral Load Estimation: Fresh EDTA-Blood samples were collected in the lab for routine HBV quantification. The total HBV-DNA was isolated using magnetic particle based kit from Promega .

Briefly, added 2.9ml of Lysis Buffer to a 5ml tube and 100ul uniformly suspended magnetic particles and added 50ul of Proteinase K (5mg/ml) to tube,thenmixed properly. After mixing, added of 200ul plasma to the tube and mixed completely; the tube was incubated at 50C for 30min. in a dry bath. After incubation the tube was put on a magnetic stand for 2 minutes and the supernatant was removed by pipetting. Then the tube was put on a non magnetic stand andadde (700ul) from wash buffer_1 ,mixed properly and the contents were shifted to a new (1.5ml)microcentrifuge tube. Then the tube was put on a magnetic stand and the supernatant was removed carefully by pipetting. Then placed the tube on non-magnetic stand and added 700ul of wash buffer 2 and mixed properly. Again put the tube on magnetic stand and after 2 minutes and remove the supernatant. Then the tube was put ona nonmagnetic stand again and the washing by wash buffer 2 was repeated as above. After removal of all wash buffer 2, tube was put on a non magnetic stand and added 25ul of Elution buffer to tube and incubation at 75C for 20minutes, the tube has been removed from dry bath and 65ul of wash buffer 2 was added and mixed thoroughly to remove all DNA from the magnetic particles. Then placed the tube at magnetic stand for twominutes and collected the supernatant containing DNA in a fresh microcentrifuge tube.

From the freshly prepared DNA sample 50ul per reaction was used for HBV DNA Quantification using Abbot's HBV Quantification Kit () as per the instructions.

Isolation of RNA from whole blood:The remaining blood of the HBV patients was used for isolation of total RNA from whole blood. The 300ul of EDTA blood sample was used for isolation of total RNA using Ambion-Invitrogen Kit (RNA 4PCR). Briefly, RBCs were dissolved in RBC lysis buffer (0.45M Ammonium chloride), and the tubes were centrifuged at 2500rpm to pellet the WBCs. The pellet was dissolved in 300ul cell lysis buffer and then was added the 64% Ethanol solution to precipitate the RNA. The total solution was passed through a column and washed using washing buffer 1 and twice with washing buffer 2/3. Spin the empty column to remove the entire remaining wash buffer. Then added 45ul of preheated Elution Buffer, kept at RT for 1 min and then centrifuge to get the RNA into an RNase-free fresh tube given in the kit. The RNA was treated with DNase by adding 1/10 volume of DNaseBuffer and 1ul of DNase enzyme and kept the tube at 37C for 30 min. After incubation and 2ul of DNase inactivating agent, mix properly and kept for 2 min at RT. After which centrifuge the tube at 12,000rpm to remove the DNase inactivating agent and saved the supernatant in a fresh RNase-Free microcentrifuge tube.

cDNASynthesis:The total RNA isolated from whole blood was the quantified using Nanodrop2000 (ThermoFisher). A total of 500ng of RNA from each sample was used to convert into cDNA using Applied Biosystems Kit (High Capacity cDNA kit). Briefly, in a 20ul reaction 2.0ul of 10x RT Reaction

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buffer was added, along with 1.0ul of dNTPs, 1.0ul of Random Hexamers, 1.0ul of RNase Inhibitor, sufficient amount of RNA carrying 500ng of total RNA and the final volume was made up with sterile RNase free water. The reaction was mixed and spin shortly and was incubated in a thermal cycler at one cycle of at 25C for 10min, one cycle at 37C for two hours followed by incubation at 85C for 10min to deactivate the enzyme. The cDNA was placed at -20C until used.

Real-Time PCR(RT-PCT) for Gene Expression of Immunological Parameters:The expression of immunological parameters, namely, IL-1beta, IL-12, IL-17, TNF-alpha and Fox-P3 was studied on RT-PCTusing SYBR Select(Applied Biosystems) on StepOne plus RT-PCTSystem. Briefly, an amount of 10ng of total RNA per well was used for in a 20ul reaction in duplicates to study the levels of mRNA expression of these parameters. The PCR cycles were put as one cycle of 2min at 52C, one cycle of 2 min at 95C and 45 cycles of 95C for 15Sec. 60C for 1min and data was collected at 60C step. The Primers of all these factors used are given below and the oligos were synthesized from IDT-DNA Technologies, USA.

Immunological Parameters	Forward and reversal	Primers
GAPDH	F	CTTTTGCGTCGCCAG
	R	TTGATGGCAACAATATCCAC
IL-1b	F	GAAGCTGATGGCCCTAAACA
	R	AAGCCCTTGCTGTAGTGGTG
IL-12	F	AGGGCCATTGGACTCTCC
	R	GTTCCCATATGGCCACGAG
IL-17a	F	TGTGATCTGGGAGGCAAAGT
	R	ACTTTGCCTCCCAGATCACA
TNF.a	F	CAGCCTCTTCTCCTTCCTGA
	R	AGATGATCTGACTGCCTGGG
Fox-P3	F	CCAGGCTGATCCTTTTCTGT
	R	CAAGGGCCAAGGAAGGG

F: fwd;R:REV.

The Ct Values determined in RT-PCTwas used to calculate the Delta-Delta CT Value which was further used to determine the RQ values to find out the fold change in the expression of immunological parameters when GAPDH is used as endogenous control. The groups of controls, High;medium and low viral load (HVL; MVL and LVL) were compared to find the immunological parameter to show any significant information.

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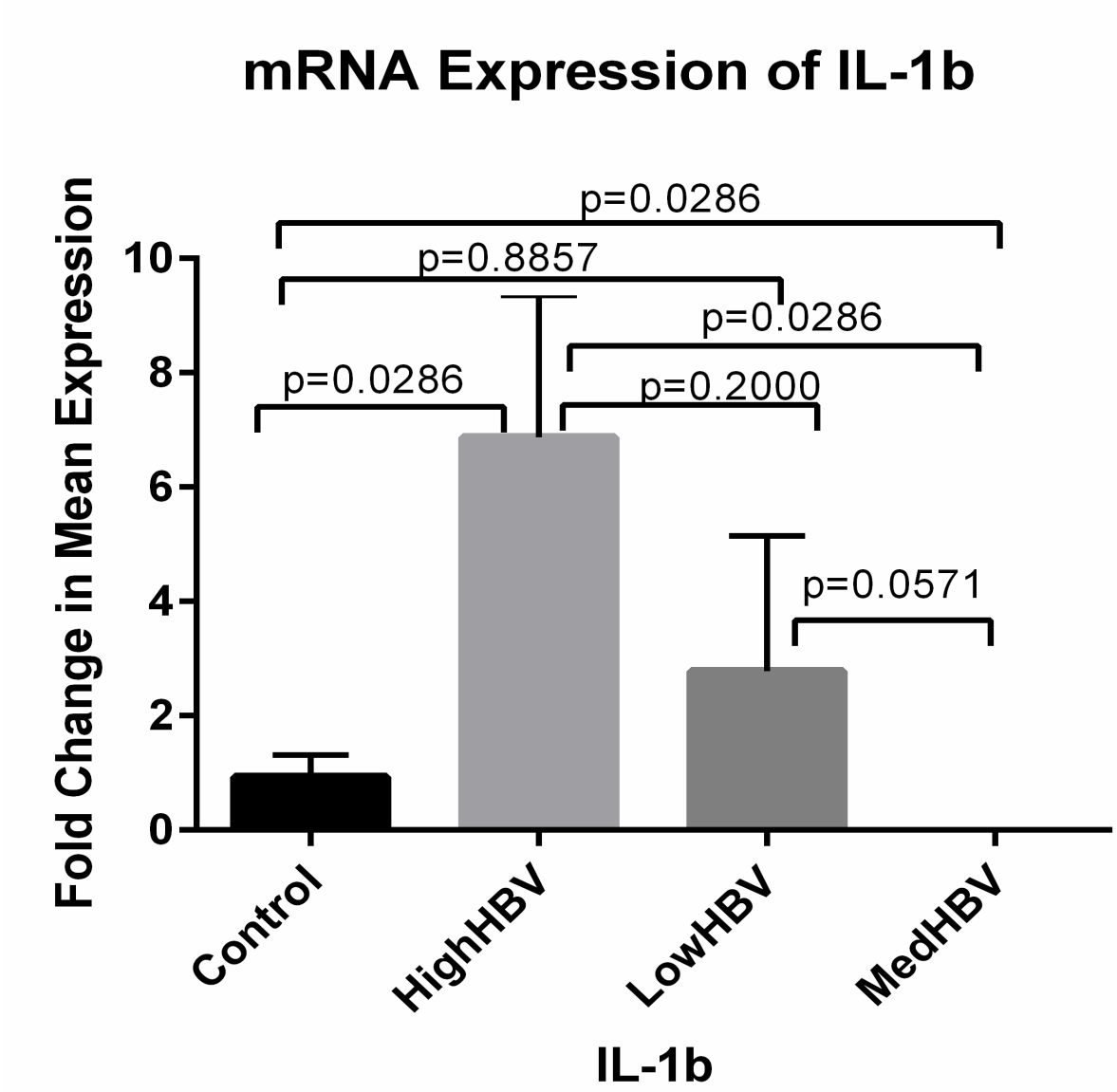
Results :



The cytokine messenger RNA expressionswere studied by RT-PCTand plotted as the change in fold of the mRNA transcript as compared to the endogenous gene GAPDH in control subjects. It was found that the expression of IL-1b is high in patients with High-viral load and is significantly different from medium viral load patients and healthy controls but not significantly different from low-viral load group. The increase in expression of IL-1b transcript in LVLwas found to be non-significant when compared between medium-viral load patients and with healthy controls. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.

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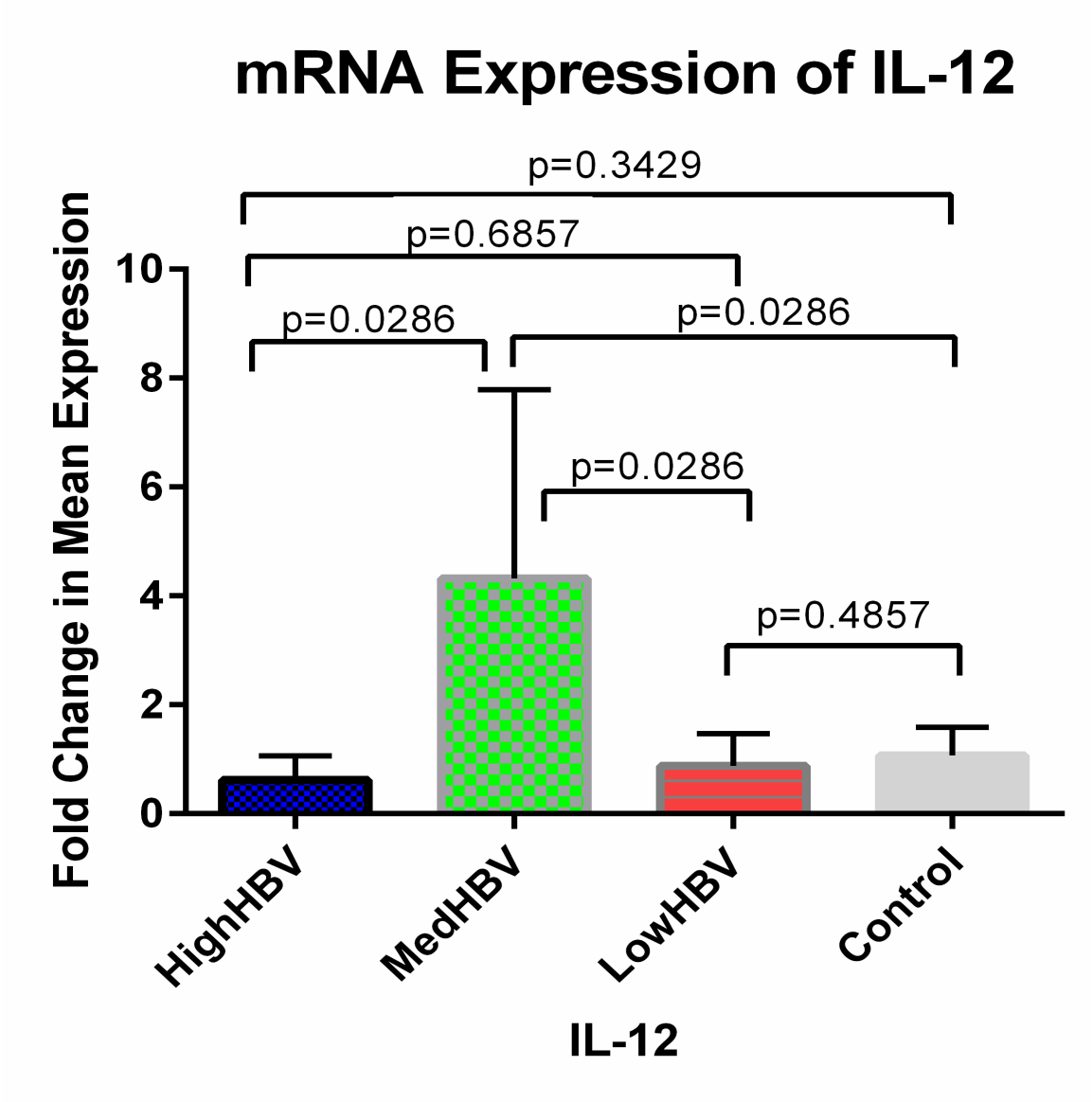

The cytokine messenger RNA expressionswere studied by RT-PCTand was plotted as the change in fold of the mRNA transcript as compared to the endogenous gene GAPDH in control subjects. It was found that the expression of IL-12 is prevalent in patients with medium viral load and is significantly higher than low and HVL (H.V.L) patients and healthy controls. The increase in expression of IL-12 transcript was to be insignificant when compared between high and LVLpatients and also with healthy controls. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.

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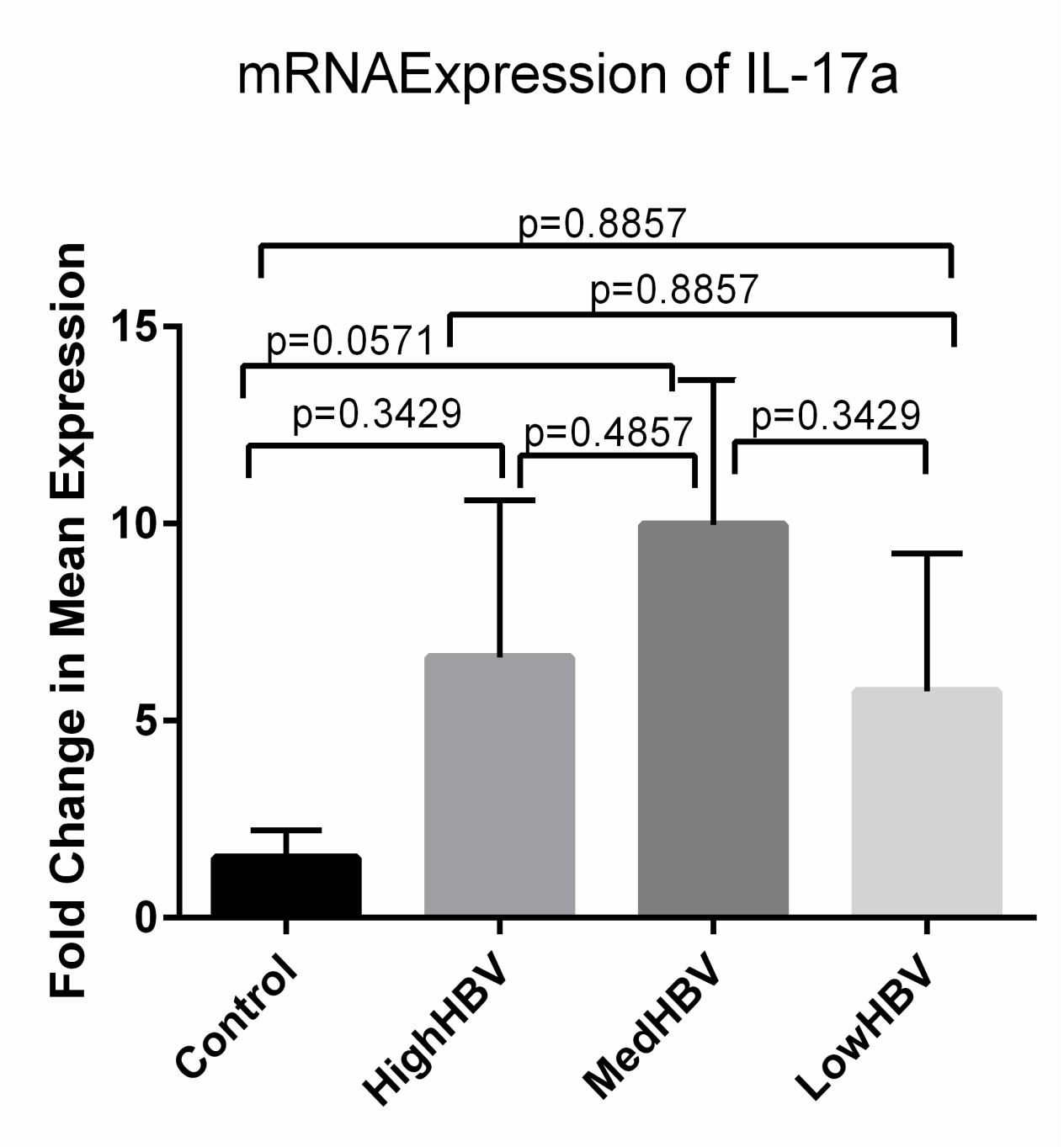

While studying the change in the expressions of messenger RNA of cytokines by RT-PCT in HVL, M.V.L and LVLgroupandresult was plotted in comparison to the expression of endogenous gene GAPDH in control subjects. It was found that the expression of IL-17a was present in all groups and also in controls. Although an increase in expression was found in all group of patients but the change was insignificant in comparison with healthy controls and as

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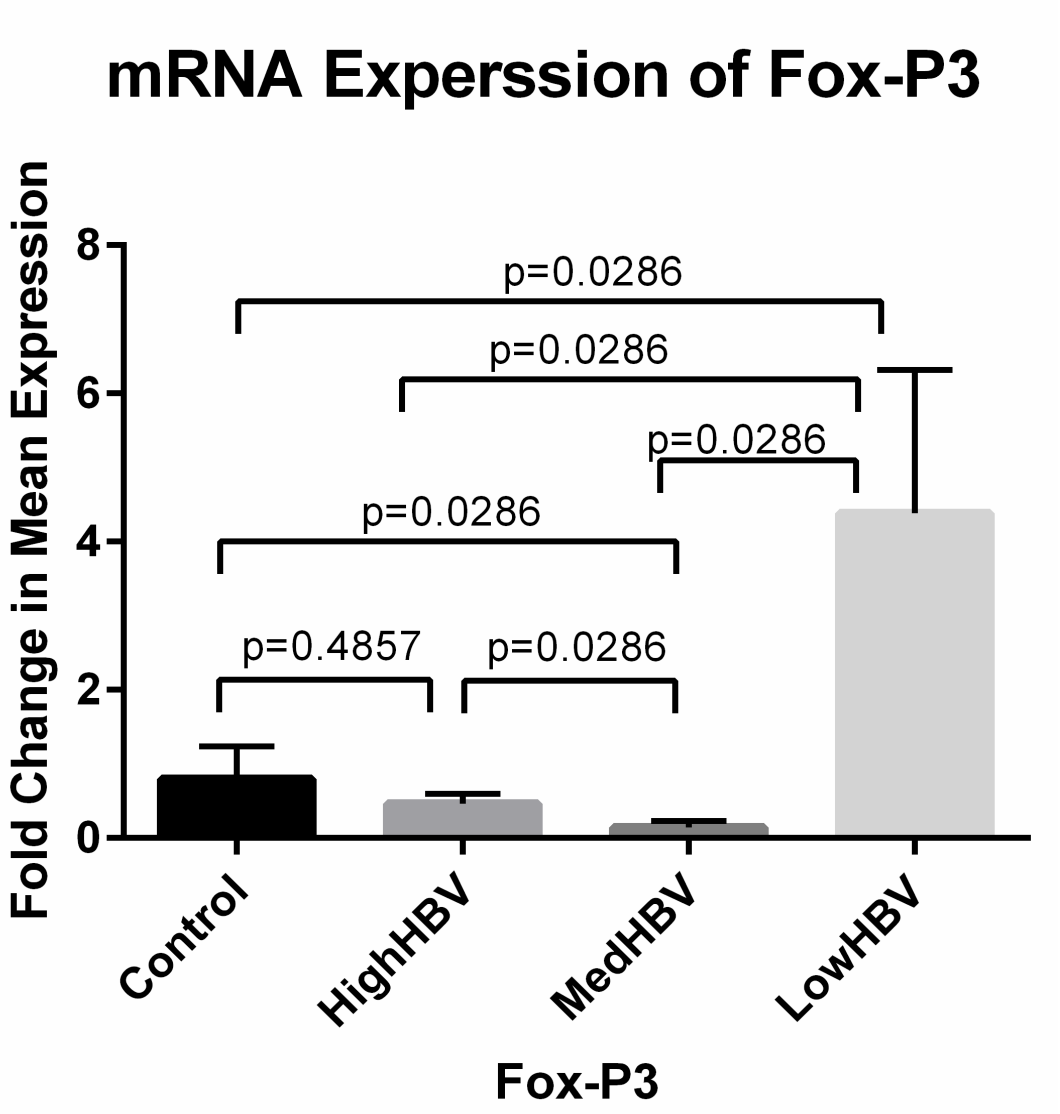
well as within groups. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.



The analysis of the expressions of messenger-RNA of the cytokines by RT-PCThas been done and the results were plotted as the change in fold of the mRNA transcript as compared to the endogenous gene GAPDH in control subjects. The expression of Fox-P3 was found to be prevalent in patients with LVL and its significantly higher than medium and HVLpatients as well as healthy controls. A decrease in expression of Fox-P3 in high and medium-viral load groups. Although insignificant decrease in expression was found in high-viral group but this decrease

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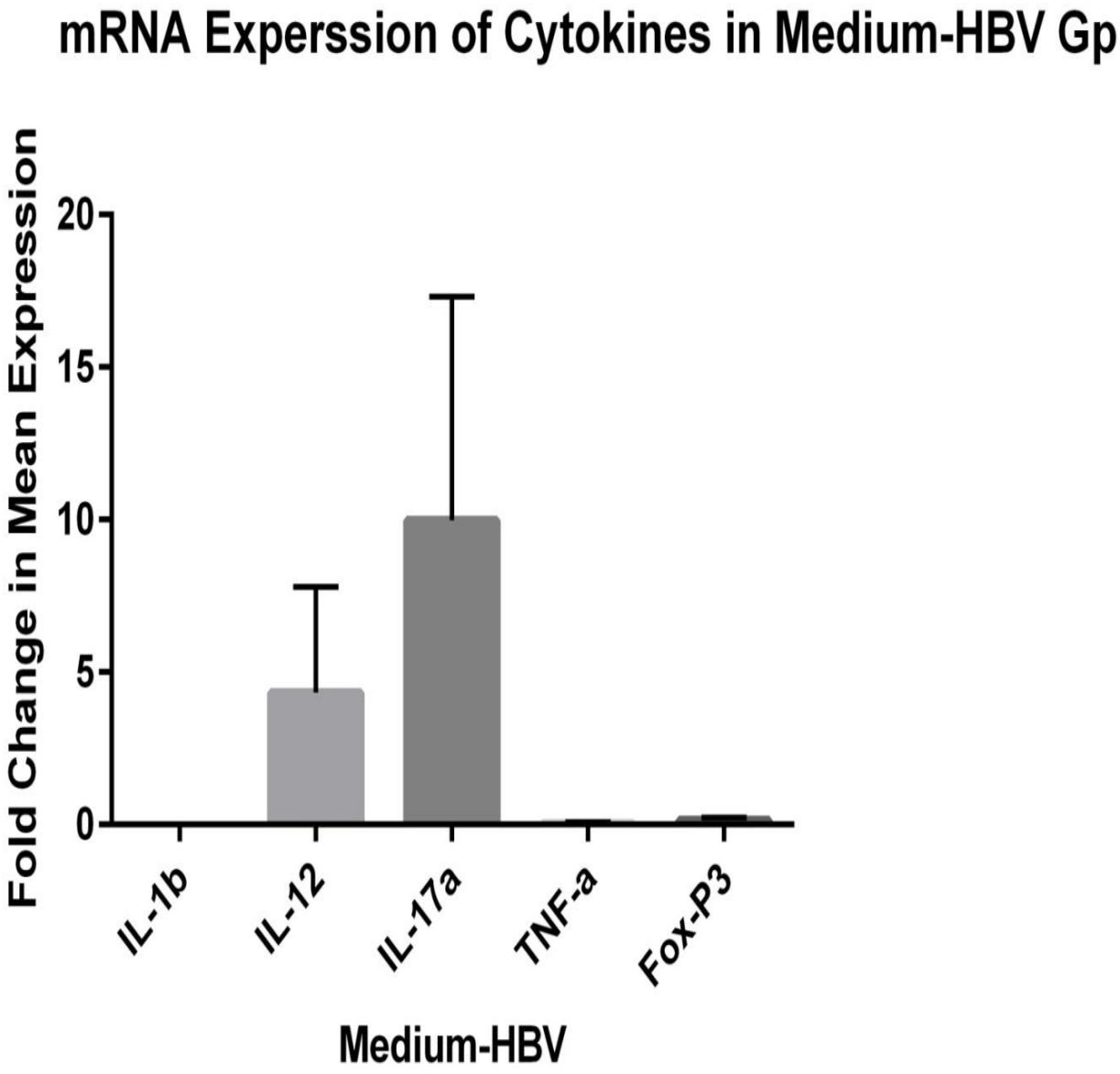
was significant medium-viral load patients when compared with healthy controls. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.



The messenger-RNA expression of cytokines was analyzed by group-wise as cytokine- genes were plotted and in case of medium-viral load it was found that the expression of IL(12& IL-17a) were founded increase but the expression of IL-1b, TNF-a and Fox-P3 was found to be

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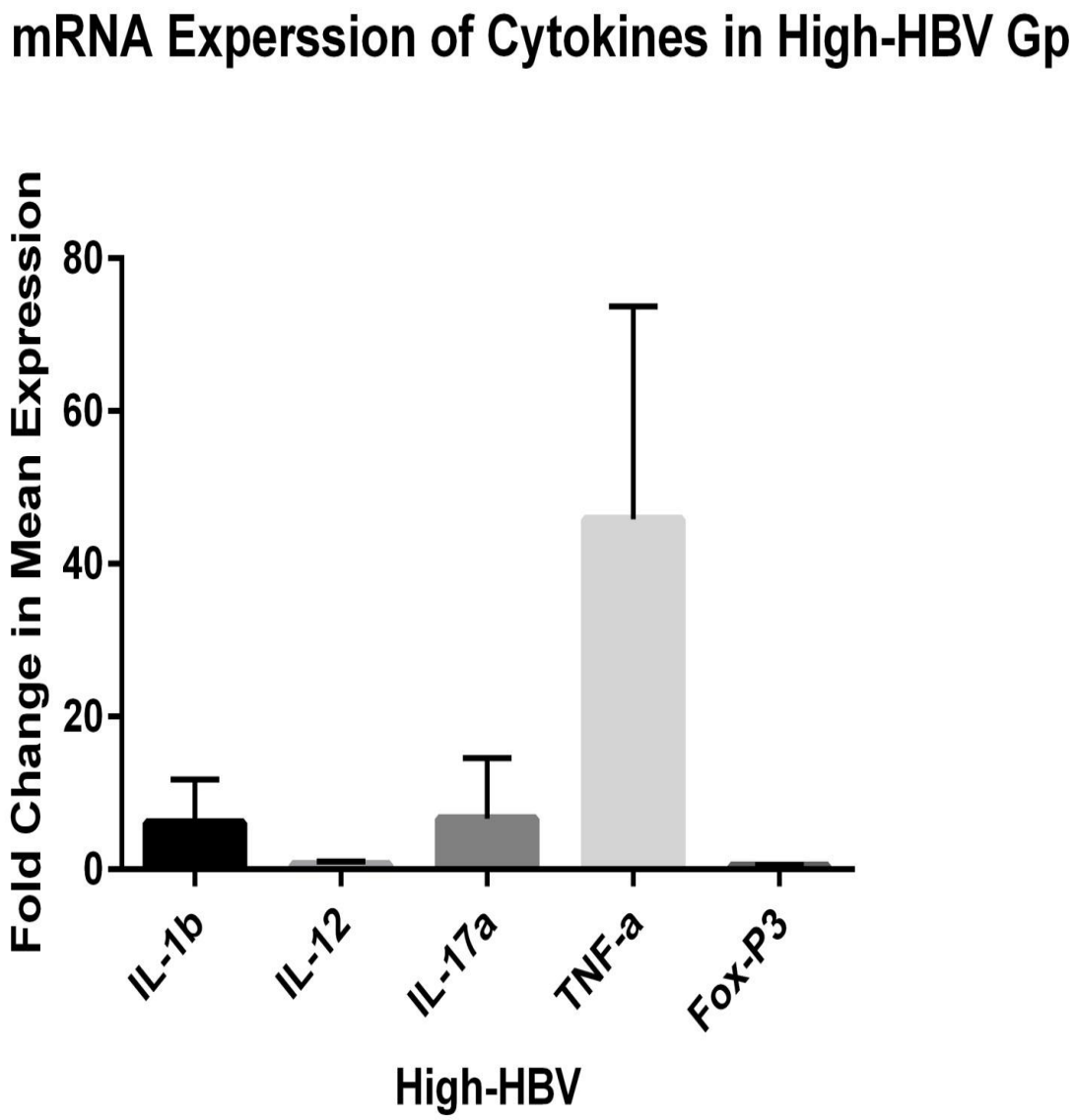
decreased in comparison to former cytokines. this group of patients. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.



The messenger-RNA expression of cytokines was analyzed by group-wise as cytokine- genes were plotted and in case of high-viral load the expression of TNF-a was found to be very high followed by the expression IL-17a and then IL-1b and the expression of IL-12 and Fox-P3 was found to be decreased as compared to the former cytokines. All data was analyzed by GraphPad (v6.0) and the graph was plotted with error bars.

Discussion:

It has been known that the imbalance in the Th17/Treg immune response and the cytokine environment has big influence on the pathogenesis of various autoimmune and infectious diseases [9]. Here we have studied the immune responses that are affected and may playing important role in the pathogenesis of

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Hepatitis B viral infection. We included incurent study, three groups of patients with low, medium and H.V.Ls as determined by RT- PCR and having no treatment at the time of sample collection. The samples were received after approval of the study from Institutional Ethics Committee and we also obtained written consent of the patients. The expression of mRNA transcripts for various cytokines as determined by RT-PCR revealed that there is big difference between various pathogenic conditions in patients with H.B.V.I. The significant increased expression of TNF-alpha and IL-1beta,were founded in groups of patients with high viral indicated that the cells release more IL-1 beta andTNF-a as protective immune response [10] to severe infection as both of these factors are needed to suppress the replication of HBV by oxidative stress as reported by Togashi and team [11]. The LVLand medium viral load patients didn't show much expression of TNF-a and IL-1beta as these patients may be in the progressive stage of infection and the effective immune response was not build till the time of their diagnosis as all patients are freshly diagnosed and treatment naïve. The comparatively low expression of TNF-a in patients with medium viral load could be due to the suppressive effect of viral to establish infection in the body. The increased expression of IL-1beta could be of a protective nature as reported earlier but the increased expression of TNF-alpha can also be seen to be stimulated due to viral X-protein as supported by the study of Lara-Pezzi and group [12]. The increased expression of IL-1beta could be the result of down regulation of Fas/Fas-L (out of the scope of this study) during the protective mechanism of the hepatic cells to keep entering into chronic stage leading to cirrhosis [13]and could be induced by HBV-X protein [14-15]. We have found increased expression of IL-1beta and TNF-alpha irrespective of the infective stage whether acute or chronic as didn't differentiate between these groups as our goal was to observe the cytokine in response to viral load. It could also be determined that the expression of IL-1beta and TNF-alpha together is known to restrict the in-vitro replication of HBV [16]. Increased expression TNF-alpha can also be seen in conjunction with its suppressive effect on regulatory T-cell which could further inhibit the immune response elicited in the body against HBV [17] and is known to inhibit growth of HBV in transgenic mice [18]. In contrary to the expression of IL-1beta and TNF-alpha the expression of IL-12 was found to be significantly reduced in HVLgroup while it was upregulated in patients with medium viral loads. The increased expression of IL-17a although insignicant, could be correlated with the increased expression of IL-12 which could be in a phase when HBV is under the effective control of body's immune system. This is also known that HBV surface antigen can also inhibit the expression of IL-12 [19,20] as is seen in cases of Low and H.V.Ls where infective viral protein could be supposedly more as in comparison to the group carrying medium viral load. Similarly, FoxP3 was also found to be increased in the group with LVL indicating the overall suppressive effect of immune system on any kind of inflammatory reactions including the HBV infection. Thus, we here found that the expression of IL-1beta and TNF-alpha were high in HVLgroups indicating the high immune response against HBV and similarly suppressive effect by FoxP3 in LVL group in response to the overall suppression of inflammation. Therefore, supplementation of IL-1beta and /or TNF alpha can support clearance of HBV at initial stages of infection but we need to study this on a big cohort of patients to get more convincing results as we had only few patients and this was the limitation of this study due to which we could not further differentiate the patients on the basis of acute of chronic infection.

Conclusion :

Inefficient prognostic factors and inadequate immune therapies result in poor management of HBV. Here we have shown that IL(1b;17a ;12); TNF-A; and FoxP3 can be efficient prognostic factor for predicting better outcomes in HBV patients. Our results were in accordance with earlier reports showing that

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IL(1β;12) and TNF-α together restrict the in-vitro replication of HBV. Increased expression TNF-alpha can also be seen in conjunction with its suppressive effect on regulatory T-cell which could further inhibit the immune response elicited in the body against HBV. Similarly, FoxP3 was also found to be increased in the group with LVL indicating the overall suppressive effect of immune system on any kind of inflammatory reactions including the HBV infection. It could be concluded that the expression of both (TNF-α andIL-1β) were high in HVLgroups indicating a strong immune response against HBV and similarly high FoxP3 expression could be implicated as a result of suppressive effect by FoxP3 in LVL group in response to the overall suppression of inflammation.Thuscytokine-based therapies could provide interesting approaches in designing effective immunotherapy against HBV infection with least adverse effects.

Recommendation :

A larger cohort of patients need to be taken to further validate the results of present study. In addition to this a study of larger pool of cytokines could be more effective in understanding the relationship between thecytokine profile and the viral load in the HBV patients.

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