Association of Interleukin-18 genotypes (-607 C>A) and (-137 G>C) with the hepatitis B virus disease progression to hepatocellular carcinoma

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Research Article

Keywords: Hepatitis B Virus, HCC, IL-18, SNPs, genotype

Posted Date: February 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-209571/v1>

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Abstract

Chronic infection with HBV has been reported to be associated with the development of HCC. The inflammation mounted by cytokine mediated immune system plays an important role in the pathogenesis of HBV associated HCC. IL-18 is a pro-inflammatory cytokine whose role in the development of HBV associated chronic to malignant disease state has not been much studied. The present study was conceived to determine the role of genetic polymorphisms in *IL-18*, serum levels of IL-18 and expression level of its signal transducers in the HBV disease progression. A total of 403 subjects were enrolled for this study including 102 healthy subjects and 301 patients with HBV infection in different diseased categories. Polymorphism was determined using PCR-RFLP. Genotypic distributions between the groups were compared using odd's ratio and 95% CI were calculated to express the relative risk. Circulating IL-18 levels were determined by ELISA. Expression level of pSTAT1 and pNF- κ B were determined by western blotting. In case of *IL-18*(-607C > A), the heterozygous genotype (CA) was found to be a protective factor while in case of *IL-18*(-137G > C) the heterozygous genotype (GC) acted as a risk factor for disease progression from HBV to HCC. Moreover, serum IL-18 levels were significantly increased during HBV disease progression to HCC as compared to controls. Also the levels of activated signal transducers (pSTAT1 and pNF- κ B) of IL-18 in stimulated PBMCs were significantly increased during HBV to HCC disease progression. These findings suggest that IL-18 has the potential to act as a biomarker of HBV related disease progression to HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common type of primary malignancy of liver and occurs primarily in chronic liver disease and cirrhotic patients. HCC, at present is the third most common cause of deaths related to cancer globally, affecting more than 500,000 people annually [1]. The incidence of HCC shows a lot of geographic variability [2]. Sub-Saharan Africa or eastern Asia has more than 80% of liver cancer cases and China accounts for 50% of the cases [3]. There are approximately 19,000 new cases of HCC and around 17,000 deaths occur due to HCC per year in India. The risk factors that lead to the hepatocellular carcinoma development include infections with hepatitis C virus (HCV) and hepatitis B virus (HBV), aflatoxin B1 chronic exposure, oxidative stress, excessive alcohol consumption and multiple genetic alterations etc. [5]. The prevalence of two main risk factors for HCC in India is ~2.4-4.0% for active hepatitis B virus and ~1.0–1.9% for chronic hepatitis C virus [4]. It is believed that chronic inflammation of the liver mediated by immune system can lead to the development of HCC because of constant cell death that results in proliferation of cells and thus increased incidence of genetic alterations.

Cytokines are a family of proteins produced in response to an enormous range of cellular stresses that include inflammation, infection, and injury induced by carcinogen. These processes result in the initiation of signal transduction pathways that are cytokine specific. Cytokine release is of primary importance in the regulation of immune system as these play a vital role in the defense against viral infections and carcinogenesis. Cytokines help in immune cell proliferation, differentiation, and inactivation or apoptosis

[6]. Moreover, among the cytokines, the IFN- γ mainly regulates the response of immune system against intracellular pathogens. IFN- γ triggers macrophages via the formation of reactive oxygen intermediates (ROIs), nitric oxide (NO), and increased class I and II MHC molecule expression and other co-stimulatory molecules implicated in antigen presentation [7]. IL-18, which is relatively a newly discovered cytokine, and is a potent inducer of IFN- γ , has a vital role in HCC development [8]. In addition to IFN-gamma, IL-18, also stimulates the production of TNF- α , IL-1 and has synergistic action with IL-12. When IL-12 is not present, it induces Th2 response by inducing the synthesis of IL-4 and IL-10 [9]. Hence, this cytokine might have a role in HBV disease outcome. There are a couple of studies which have analyzed the association of *IL-18* genotypes with HBV disease [10-12]. However, there is very limited knowledge in the literature specifically regarding genotype distribution of *IL-18* and susceptibility to the progression of HBV associated chronic to malignant disease. Moreover, there is no report studying the association of *IL-18* gene polymorphism and its circulating levels with HCC in HBV patients in Indian population. Also, there is no previous study correlating IL-18 signaling components with progression of disease from chronic hepatitis to HCC in Indian population, in spite of an increasing incidence of HBV related HCC in India. It becomes important to carry out such studies in a population based manner as it has been observed that there is high intricacy of multivariate interlink between the genomic information and the phenotype that manifests. HCC development is a multiple stage conglomerate process which relates to causative factors such as gender, age, environmental toxins, drug and alcohol abuse, elevated HBV DNA levels, and HBV genotype [13]. HBV genotype B and C are found to be widespread in Han population [14] whereas genotypes A, C and D are mostly found in Indian population with genotype D being the most predominant [15]. Hence, genetic diversity of genotypes of HBV exist that exhibits distinct geographical and ethnic distributions. Recently, a study by Sawai et al [13] using four distinct cohorts including Japanese, Korean and Hongkongese observed no association between rs17401966 and HBV-derived HCC which was earlier observed by Zhang et al [16] in southern, eastern and northern Chinese population. Hence this study concluded that such observations might be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Their study further highlighted the need to carry out the polymorphism-based studies in different ethnic groups because of the observation that genotypic distributions can vary in different regions belonging to even the same continent. Therefore, we hypothesize that polymorphism in IL-18 gene might contribute to the increased susceptibility to the HBV disease progression by altering the protein expression of various components of cell signaling pathways.

Materials And Methods

Patients

We recruited 102 healthy subjects and 301 patients (HbsAg +ve) in total 318 males and 85 females with mean age (43.77 $\pm$ 16.43) that were clinically categorized as inactive hepatitis [carriers], chronic-active HBV infection, HBV associated cirrhosis and HCC, in the Liver Clinic of the Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Healthy volunteers, age and gender matched were enrolled as controls with no chronic liver or kidney diseases as well as no HIV, HBV or HCV infection. All subjects enrolled in this study were of Indian ethnicity and living in northern part

of India. The protocol was approved by the institutional ethics committee, Chandigarh, India and informed consent written by all the participants was taken. The sample size in our study is small, but this was the convenient sample size based on the frequency of HBV-HCC patients attending the hepatology clinic in our institute.

Genotyping for SNPs *IL-18*[-137]and *IL-18* [-607]genes

Primers for PCR-RFLP of *IL-18* (-137) and *IL-18* (- 607) were synthesized by Sigma Aldrich Bangalore, India. Thermo F501L DNA polymerase II and NEB 50bp DNA ladder were used. dNTPs mix [10mM] were obtained from MBI Fermentas. Restriction endonucleases used for RFLP study i.e. [*Bst*ul and *Fau*l,] were obtained from New England Biolabs. Isolation of genomic DNA from whole blood was done using Qiagen mini kit for blood using manufacturer's protocol. Nanodrop spectrophotometer was used to check the purity and quantity of DNA. The DNA having the absorbance ratio at 260/280 nm of greater than 1.8 was used for the study. Moreover, integrity of DNA was checked through 1% agarose gel electrophoresis. The genotyping analysis at position -137 and -607 of *IL-18* was done by PCR with primers that were sequence specific (PCRSSP): *IL-18*(-137) (fwd: CACAGAGCCCAACTTTACGGCAAGAA) (rev:GACTGCTGTCGGCACTCCTTGG) with product size of 155bp, for *IL-18*(-607) (fwd: GCCCTCTTACCTGAACCTTTACGGCAGAGAA)(rev:AGAATTTACTTTTCAGTGGAACAGGAGTCC) with product size 171bp. The PCR conditions were initial 2mins of denaturation at temperature of 94 °C , followed by 35 cycles of denaturation at 94 °C for 20s , annealing at 59 °C for 50s, and extension at 72°C for 20s. Identification of each allele was done according to its size.

Measurement of circulating IL-18 levels

Collection of blood samples was done by venipuncture from patients and healthy controls and blood was allowed to clot at room temperature for approximately 30-minutes. Then, the serum was isolated by centrifugation at 3,000 rpm for 5 minutes. Aliquots of serum were made and refrigerated at -80°C till the use for further analysis. Levels of IL-18 in the patients (n=190) and control sera (n=30) were measured using commercially available human IL-18 ELISA Kit (RayBio, Georgia, United States) as per the instructions of manufacturer.

Analysis of expression levels of signal transducers (STAT1 and NF-κB) of IL-18 in stimulated PBMCs

A total of 25 patients were recruited five each from aforementioned categories, 10mL of venous blood was collected from each patient under aseptic conditions using heparinized vials and samples were subjected to PBMC isolation procedure. Standard FicollPaque procedure was used to isolate PBMCs from freshly collected blood. Cells were stimulated with 10µg/mL of IL-18. PBMCs were then suspended in 300µL lysis buffer, centrifuged at constant speed of 12,000xg for 30min at 4°C. Supernatant was aspirated and pellet was discarded and proteins were then stored at -20°C till further use for western blotting. For western blotting proteins from PBMCs were separated on SDS-PAGE gel (12%) and then transferred to a PVDF membrane. Blocking of membrane was performed with 3% BSA in phosphate buffer saline (PBS) overnight at 4°C. Incubation of membranes was done with anti pSTAT-1 human

polyclonal antibody in 1% BSA (1:200 Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-pNF- κ B (1:100 Santa Cruz Biotechnology, Santa Cruz, CA). After washing alternatively with PBS and PBS-T, the membranes were incubated with HRP labeled secondary antibody (dilution 1:10000) for both pSTAT-1 and pNF- κ B at room temperature for 1hr. Enhanced chemiluminescence (ECL) western blotting detection reagent (biorad) was used for immune-detection. Image J software was used for determining relative band density.

Statistical Analysis

Genotype frequency was derived as the number of subjects with a specific genotype divided by the total number of subjects screened. Test for Hardy-Weinberg equilibrium was also done. Genotype association with the risk of developing HCC is represented as odds ratios (Ors) with 95 per cent confidence intervals [CIs]. OR analysis indicates the risk association of a factor (OR>1: risk factor or positive association; OR<1: protective factor or negative association; OR=1: no association). Categorical data is shown as numbers and percentages and were analyzed by χ^2 test. Continuous data is shown as mean $\pm$ SD or median with quartiles and analyzed by unpaired t-test and Mann-Whitney U test. For more than two groups, data was analyzed by ANOVA or Kruskal Wallis test. The normality of the continuous data was checked by Shapiro Wilk test. *P* values <0.05 are considered significant. Statistical analysis was performed using SPSS software version 22.0 (IBM, Chicago, IL) and Graphpad prism version 6.0 (La Jolla, California).

Results

To analyze the influence of polymorphisms at *IL-18* gene promoter on the course of HBV disease progression, gene and allele frequency were analyzed in various categories of HBV infection.

As evident from Fig. 1a the presence of 171 bp and 101bp bands in all the lanes correspond to heterozygous genotype (CA), while wild genotype CC is depicted by 171bp band in case of *IL-18*(-607). The presence of 155bp band depicting wild genotype (GG) and that of 155bp and 116 bp bands show heterozygous genotype (GC) of *IL-18* (-137) as depicted in Fig. 1b.

Genotype distribution of *IL-18* genotypes [-607C > A] and [-137 G > C]

Susceptibility to HBV disease progression might be influenced by SNPs in the cytokine genes. So, we analyzed genotypic distribution of *IL-18* genotypes in different categories of subjects. Genotypic distribution for *IL-18* (-607) showed that genotype CA was the most common among all groups. Genotype CC of *IL-18* (-607) was present maximally (67%) in HBV-cirrhotic subjects and found to be the least in inactive carrier subjects. HBV -HCC subjects had maximum distribution of AA genotype as compared to other groups (Table 1).

Table 1.Genotype and allele distribution of among different categories of subjects of *IL-18* (-607)

genotypes and its association with HBV-HCC risk

Groups	Healthy Controls (n=102)	Inactive carrier (n=81)	Hepatitis (n=74)	Cirrhosis (n=67)	HCC (n=72)
Genotypic frequency					
CC	45 (44.12)	22 (27.16)	30(40.55)	44 (67)	40 (55.56)
CA	57 (55.88)	57 (70.37)	42 (56.75)	19 (29.23)	27(44.82)
AA	NIL	2 (2.47)	2 (2.70)	2 (3.07)	5(6.94)
Allele Frequency					
C Allele	147(72.05)	101(62.34)	102(68.92)	107(82.31)	107(74.31)
A Allele	57(27.95)	61(37.66)	46(31.08)	23(17.69)	37(25.69)
OR (CA)					
	1 (REF)	----	1.1 (0.63- 1.94)	0.34*** (0.19- 0.62)	0.64 (0.36- 1.11)
		1 (REF)	0.54* (0.3-0.98)	0.17*** □ (0.09-0.31)	0.31***□ (0.17-0.56)

Data represented as number of subjects (genotypic distribution. %).Data represented as Odd's ratio (95% confidence interval) ;*** p<0.001 w.r.t control ;□ ***p<0.001 w.r.t inactive carrier; *p<0.05 w.r.t inactive carrier.

Genotypic distribution for *IL-18*(-137) revealed that GG genotype was the most prevalent one among all groups. *IL-18*(-137) GC genotype was present maximally in Chronic active hepatitis (50%) subjects and found to be the least in HBV-cirrhotic subjects. However, HBV cirrhotic subjects showed the highest distribution of CC genotype as compared to other groups [Table 2].

Table 2

Genotype and allele distribution of among different categories of subjects of *IL-18* (-137) genotypes and its association with HBV-HCC risk

Groups	Healthy Controls (n = 72)	Inactive carrier (n = 72)	Hepatitis (n = 78)	Cirrhosis (n = 70)	HCC (n = 61)
Genotypic frequency					
GG	47 (65.27)	42 (58.33)	35 (44.87)	46(65.71)	34(55.74)
G C	23 (31.94)	28 (38.88)	39 (50)	20(28.57)	25(40.99)
CC	2 (2.7)	2 (2.77)	4 (5.12)	4 (5.71)	2 (3.28)
Allele Frequency					
G Allele	117(81.25)	112(77.78)	109(69.87)	112(80)	93(76.23)
C Allele	27(18.75)	32(22.22)	47(30.13)	28(20)	29(23.77)
	1(REF)	—	2.3**	OR(GC)	1.5
	1 (REF)	1(REF)	(1.3–4.1)	0.89 (0.48–1.68)	(0.84–2.7)
		—	1.67	0.659	1.1
		1(REF)	(0.94–2.9)	(0.36–1.18)	(0.62–1.95)
			2.76	OR (CC)	1.42
			(0.6-12.64)	2.1	(0.27–7.4)
			2.5	(0.47–9.3)	1.27
			(0.54–11.320)	1.9	(0.24–6.63)
				(0.42–8.33)	
Data represented as number of subjects (genotypic distribution.%). Data represented as Odd's ratio (95% confidence interval) ; ** p < 0.01 w.r.t control					

Association of *IL-18* genotypes (-607C > A) and (-137 G > C) with HBV- HCC risk

As presented in table 1, the odd's ratio for *IL-18* (-607) genotype association with HBV disease progression showed genotype CA as a protective factor for HBV-cirrhotic subjects among controls. Also, this genotype was found to be protective for hepatitis [1.8 fold], cirrhosis [5.8

fold] and HBV-HCC (3.2 fold) subjects among HBV inactive carriers. However, no significant association for AA genotype was noticed. The odd's ratio for the association between *IL-18* (-137) genotypes and the

advancement in disease showed that genotype *GC* as a significant risk factor for the chronic active hepatitis with control as a reference. Also, *CC* genotype was found to be a 2.7- and 2.5- fold risk for the HBV-hepatitis development among controls and carriers

respectively albeit non-significantly as shown in Table 2.

Association between IL-18 genotypes (-607C > A) and (-137 G > C) and HBV DNA load

Further exploration for association between *IL-18* genotypes and HBV-DNA load was carried out. All the cases were categorized into two sub-groups based on HBV DNA load ($> 10^{-3}$ and $< 10^{-3}$ IU/dL). The distribution characteristics of gene polymorphisms in the two groups are summarized in Table 3 .None of the genotypes studied showed any significant association with HBV DNA load.

Table 3.Association of *IL-18*(-137 & -607) genotypes with HBV DNA load

Polymorphisms	HBV-DNA LOAD		χ^2	p-value	OR	95%CI
	/(IU/DL)					
	>10 ⁻³	<10 ⁻³				
IL-18(- 607)						
CC	37	54	0.510	0.510	1.202	0.695-2.079
CA	55	59	0.117	0.177	0.647	0.375-1.116
AA	1	9	0.030	<.05	7.327	0.912-58.901
IL -18(-137)						
GG	44	69	0.462	0.462	1.217	0.721-2.005
CG	46	61	0.645	0.645	0.884	0.524-1.493
CC	6	6	0.534	0.534	0.692	0.216-2.215

HBV-DNA $<1 \times 10^{-3}$ IU/mL; HBV-DNA $\geq 1 \times 10^{-3}$ IU/mL. p-value obtained from χ^2 test. AA compared with both CC and CA;* p<0.05.

Association between IL-18 genotypes [-607C > A] & [-137G > C] and HBeAg status

The status of HBeAg is very important in disease development from CHB to HCC. So we investigated association between genotype of *IL-18* with HBeAg status. As shown in Table 4, the genotypes *CC* and *CA* of *IL-18* (-607) showed significant ($p < 0.001$) positive association and significant ($p < 0.001$) negative association respectively with HBeAg status whereas *AA* genotype did not show any association ($p = .663$). Also, *GG* and *GC* genotypes of *IL-18* (-137) showed significant ($p < 0.001$) positive association and significant ($p < 0.001$) negative association respectively with HBeAg status and *CC* genotype showed no association.

Table 4
Association between *IL-18* (-607C > A) & (-137G > C) genotypes and HBeAg status

HBeAg						
Genotypes	Neg. (n)	Pos. (n)	χ^2	p-value	OR	95% CI
IL-18(-607)						
CC	13	83	0.000	< 0.000	7.485	3.786–14.798
CA	65	51	0.000	< 0.000	0.139	0.073–0.226
AA	3	7	0.633	0.633	1.358	0.341–5.404
IL-18(-137)						
GG	65	47	0.000	< 0.000	7.882	3.729–16.660
GC	100	7	0.000	< 0.000	0.104	0.044–0.241
CC	9	3	0.979	0.979	1.019	0.266–3.899
OR, odd's ratio : CI, Confidence interval ; χ^2 test for p-Value; *** $p < 0.001$ means statistically significant. Neg. (HbeAg negative) and Pos. (HbeAg Positive)						

Analysis of association between genotypes of IL-18 gene [-607C > A] and [-137 G > C] and biochemical parameters:

Analysis of associations between *IL-18* (-137) genotypes and biochemical markers showed that levels of ALT (alanine transaminase), AST (aspartate transaminase) and ALP (alkaline phosphatase), TP [total protein] and ALB (albumin) had no significant difference among patients having *CC*, *GC*, *GG* genotypes. Significant association ($p < 0.05$) was, however, found among *IL-18* (-607) *CA* and *CC* genotypes with AST and TP levels as shown in Table 5.

Table 5
Association between genotypes of *IL-18* (-137 & -607) and biochemical parameters

IL-18 (137-genotype)	AST	ALT	ALP	TP	Albumin
CC	27.4 (19–46)	37(11–52)	88(56–153)	-	4.9(0.1-8)
GC	43(3-1850)	43(9-1563)	143(1.1–564)	7.5(0.3–22)	4.6(2.3–8.5)
GG	45(17-2440)	53.5(3-1320)	132(1-830)	6.8(3.7–28)	
P value	0.23 ^a	0.42 ^b	0.16 ^b	0.56 ^a	0.72 ^a
IL-18 (607-genotype)					
CA	37(13-1850)	48.4(3-1319)	133(1.1–564)	11.2(4.6–111)	5(1.65–11.2)
CC	110(15-2440)	36(10-1320)	139(1.0-352)	6.9(3.0–28)	4(2.3–8.10)
P value	0.001 ^{a***}	0.37 ^a	0.83 ^a	0.01 ^{a**}	0.09 ^a
p-value obtained by ^a Kruskal wallis test (significance between 3 groups) ;***p < 0.001, and ^bMann-Whitney test (significance between 2 groups). Data represented as a median value (q1-q2).					

Analysis of circulating IL-18 levels and its association with IL-18 genotypes

Inappropriate production of IL-18 contributes to the pathogenesis in various types of cancers and may affect the clinical outcome of patients [17]. So, serum IL-18 levels may have some prognostic significance. Thus, accordingly levels of IL-18 were quantified in serum samples of patients at different stages of infection of HBV and comparison with normal healthy controls was done. The mean levels of circulating IL-18 expressed as pg/ml were significantly increased in inactive carrier (58.98 ± 24.49 ; $P < 0.05$), hepatitis (61.90 ± 37.89 ; $P < 0.01$), HBV-related cirrhosis (70.51 ± 32.10 ; $P < 0.001$), and HBV-HCC patients (76.44 ± 22.22 pg/mL; $P < 0.001$) as compared to the normal healthy controls (43.23 ± 12.35). Also, significant difference was observed in IL-18 levels in inactive carrier ($P < 0.001$) and hepatitis ($P < 0.05$) patients when compared to HCC group. The representation of data of IL-18 as median $\pm$ interquartile range is shown in Fig. 2a. Further we investigated correlation between serum *IL-18* levels with its genotypes, we observed no association between circulating cytokine levels (*IL-18*) and their associated genotypes as presented in Fig. 2b.

Analysis of levels of signal transducers (pSTAT1 and pNF- κ B) in IL-18 stimulated PBMCs

Protein expression of pSTAT1 in isolated PBMCs stimulated by IL-18 in various categories revealed a consistent and significant increase in the expression level of pSTAT as the disease progressed from

inactive carrier to HCC. Approximately 2-fold increase in the activated STAT was observed in HCC and cirrhosis categories as compared to control subjects as depicted in fig .3a. Moreover, a significant elevation in the levels of expression of pNFkB in stimulated PBMCs in hepatitis, cirrhosis and HCC as compared to healthy control was observed as shown in Fig. 3b.

Discussion

India, the second highest populated country has nearly 50 million people afflicted with chronic HBV infection [prevalence ~ 4%]. The disease is mostly acquired horizontally, but the role of vertical transmission should not be underestimated [18]. In clinical subjects, infection with HBV leads to a variety of clinical conditions ranging from asymptomatic status to cirrhotic liver, and further progression towards HCC [19]. Inflammation is the initial step in development of HCC, and chronic hepatitis may promote hepato-oncogenic potential via inducing cirrhosis, although there are certain studies which depict that inflammation-mediated HCC can develop even in the absence of cirrhosis [20]. Cytokines are the key molecules in immune-modulation and are reported to play a pivotal part in HCC progression [21]. It has been stated that genotypes of cytokines serve a critical role in genetic regulation of immune responses against HBV and also determine an individual's risk of developing HCC. Several studies have confirmed that SNPs associated with cytokine genes appear to be related with risk of HCC development [22–24]. Earlier studies from our lab have reported single-nucleotide polymorphisms in cytokine affect the production of cytokines greatly, thus modulate the immune balance outcome [25]. Also, a positive association of *IL-10* (-819/-592) CC/TA genotype with HBV related disease progression and risk of HCC development was observed [26]. A role of *IL-6* (-572/-597) in the pathogenesis HBV disease was also found in Indian population [27]. In other study, the levels of IL-1B, a pro-inflammatory cytokine was determined to be significantly increased with the progression of disease to HCC thus indicating IL-1 has a fundamental role in the hepatitis B virus mediated disease chronicity in the Indian inhabitants [28].

IL-18 is a potent inducer of IFN- γ and believed to have a vital role in HCC development. It is secreted from macrophages and liver Kupffer cells [29]. The main function related to this cytokine is stimulation of IFN- γ secretion from CD4⁺ T_H1 cells, T-cells, B-cells as well as natural killer cells, particularly in association with IL-12 [30, 31]. IL-18 induces NK cells as well as T_H1 cells to express Fas ligand and stimulate perforin mediated cytotoxicity [32, 33]. Furthermore, IL-18 stimulates T_H1 cells to divide thus leading to IFN- γ , granulocyte macrophage colony stimulation factor, IL-2 and IL-2R α chain production, whereas no such action of IL-18 was observed on T_H2 cells [34, 35]. Moreover, IL-18 also induces the production of TNF- α , IL-1 and has a synergistic action with IL-12. When IL-12 is absent however, it induces Th2 response by inducing the synthesis of IL-4 and IL-10. Considering its immune modulatory functions, we hypothesized that this cytokine might have a role in the disease progression of HBV.

The present study evaluated how the *IL-18* (-607/-137) genotypes, the circulating blood levels of IL-18 and also levels of signal transducers of IL-18 signaling pathway are associated with the progression of hepatitis B infection from the inactive carrier state to the malignancy. Genotypic distribution for *IL-*

18(-137) showed genotype *GG* was the most common among all the groups and this study was found to be consistent with the study by Yelcin et al in which they observed genotype *GG* was the highest in CCL[Chronic lymphocytic leukemia] as well as healthy subjects [36]. Genotypic distribution for *IL-18(-607)* genotypes showed *CA* genotype was most prevalent among all the groups. Earlier study by Farjadfar et al [37] also showed that genotype *CA* as the most frequently distributed in both controls and lung cancer patients. Texeria et al[38] have observed different results in which they found higher frequency of the *IL-18-607(AA)* genotype among HCC patients compared to healthy individuals in Brazilian population while Zhang et al [39] found same in Chinese Han population. This difference might be due to the genetic diversity of IL-18 genotypes in different regions and stresses upon the fact that genetic polymorphism studies should be encouraged to be carried out in different regions among different ethnic groups. When the association of the genotypes with HBV progression and subsequent HCC risk was studied, the *IL-18(-607) CA* genotype was found to be a significant protective factor in Hepatitis B virus disease progression. This observation differed from the study conducted by Hasan et.al [40] who found no significant difference for *CA* genotype of *IL-18(-607)* in HBV infection. A study by Chang et al showed an association of the *CA* and *CC* genotypes of *IL-18(-607)* with a significant decrease in the risk of renal cell carcinoma [RCC] compared with the *AA* genotype [41]. *CC* genotype of *IL-18(-137)* was a risk though the results were not significant. A study conducted by Zhao et al found similar results, *CC* genotype of *IL-18-137G/C* had a 2.9 fold risk of breast cancer as compared to the *CC* genotype, and the *CC* genotype also showed an increased risk of breast cancer compared with the *GG + GC* genotype[42]. This study was also somewhat similar to the study conducted by Lau et al [43] that patients carrying the *IL-18-137G/C* polymorphism genotype have a high risk of HCC in Chinese Han population. The authors in this study had HCC patients only unlike our study where we have enrolled patients with different stages of disease pathology during the progression to HCC.

Upon analyzing the levels of circulating IL-18 in various categories, we found increased serum levels of IL-18 in all disease categories as compared to controls signifying a progressive increase during the progression of the disease. Parallel to this, a study conducted by Haghshenas et al. [44] also revealed increased serum levels of this cytokine in gastro-intestinal cancer patients when matched up with the controls. No significant association could be observed between circulating cytokine levels and their associated genotypes in our study. Contrary to this, Hassan et al. [45] have found the IL-18 levels and its associated genotypes were significantly associated in Iraqi population. This discrepancy may be due to the small sample size of our study.

HBeAg is a protein of hepatitis B virus which signifies virus is undergoing active replication and infected person with Hepatitis B can possibly spread the virus on to other person. HBeAg positivity has been found to be linked with higher risk of HCC [46, 47]. We carried out analyses in which we stratified subjects according to HBeAg positive and HBeAg negative groups and association between *IL-18* genotypes and HBeAg status was observed. The *(-607)-CC* genotype showed a positive association (7-fold) with HBeAg positivity while *CA* genotype was negatively associated. Similarly, in case of *IL-18(-137)* genotypes, *GG* was found to be a highly negative factor [9.6 -fold] for HBeAg status while *GC* showed around 8- fold positive association. These observations corroborated our finding that the *IL-18 (-607) CA* genotype was

a significant protective factor for HBV disease development. This is a novel finding and needs to be evaluated further. We also analyzed association between *IL-18* (-137 & -607) genotypes with HBV-DNA load in all the patients and no significant association was observed. This is in parallel with the study conducted by Hua Jiang et al. [48] who observed that *IL-18* (-137) genotype has no association with HBV DNA load. Analysis of association between *IL-18*(-137 & -607) genotypes and various biochemical parameters related to liver function tests were also carried out and we observed *IL-18*(-607) genotypes *CA* and *CC* showed significant association with AST and TP levels. Contrary to this study conducted by Lau et al found no significant association between *IL-18* genotypes (-137) & (-607) with any of the biochemical parameters.

Most of the cytokine and chemokine production are mainly regulated at the transcriptional level via the expression of particular groups of transcription factors that are being controlled by NF- κ B, IRFs and MAPKs [49, 50]. Free IL-18 gets bound to its specific cell surface receptor which is heterodimeric in nature belonging to superfamily of IL-1 receptor/Toll like receptor. It consists of two subunits namely IL-18Ra and IL-18Rb [51], comprising of three Ig-like domains extracellularly and one intracellular TIR (Toll/IL-1R domain) of IL-18 and IL-18Ra interaction alleviate its binding with IL-18Rb and with the MyD88 [myeloid differentiation factor] which is a adaptor protein through TIR domain [52, 53]. Thus signal transduction pathway is initiated by the employment of the IL-1 receptor activating kinase (IRAK). Auto-phosphorylation and dissociation of IRAK occurs from the receptor complex and it subsequently binds to TNFR-associated factor-6 (TRAF6) thus gradually leading to translocation of the nuclear factor κ B (NF- κ B) to nucleus. Interaction of the IL-18R complex can also cause STAT3, the mitogen-activated protein kinase (MAPK) p38, ERK and JNK activation [54]. The components of STAT family play a key role in production of the cytokines. As we have observed the elevated levels of circulating IL-18, it was imperative to check the status of its downstream signal transducers, we analyzed the expression of activated NF- κ B and STAT1 in PBMCs in various disease categories. We found significant augmentation in the expression levels of pSTAT1 and pNF- κ B in the HBV-HCC disease progression. There is no report available regarding the IL-18 induced activation of pSTAT1 and pNF- κ B in HCC. However, our observation is in consistent with the study done by Zakiet al in which they have observed that IL-18 levels activate the expression levels of STAT1 in colorectal tumor [55]. Hirotada et al. observed that signaling mediated by IL-18/IL-18R activate NF- κ B via IRAK/TRAF6 pathway in murine lymphoblast EL-4 cells [56].

The major limitation of the study was the small sample size, which demands further investigation on a larger group of population to find the association of said genotypes to stratify HBV- patients on low and high risk categories. Also biomarker potential of IL-18 can be validated further in larger cohort studies among different populations.

Conclusions

Our study demonstrated *IL-18*(-607) *CA* genotype to be a protective factor for the progression of HBV-HCC disease. Levels of circulating IL-18 were found to be elevated with the progression of HBV-disease to HCC and hence should be evaluated for potential biomarker for HBV disease chronicity to malignancy. We

demonstrated that IL-18 has a key role in the HBV-HCC disease pathogenesis through activation of signal transducers; STAT1 and NF- κ B.

Abbreviations

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ROIs, reactive oxygen intermediates; NO, Nitric oxide, PBMCs, peripheral blood mononuclear cells; pSTAT 1, phosphorylated signal transducer and activator of transcription; NF- κ B, phosphorylated signal transducer and activator of transcription; PCR-RFLP, Polymerase chain reaction- restriction fragment length polymorphism; IFN- γ , interferon gamma; SNPs, Single nucleotide polymorphisms; HBeAG, hepatitis B e-antigen; CHB, chronic hepatitis B.

Declarations

Acknowledgements

We express our immense gratitude to the participants and their families. The contribution of Mr Chander Mohan and Mr Gurpreet Singh, laboratory technologists is duly acknowledged. We acknowledge the financial support (grant number: SR/S0/HS-0043/2012) from Department of Science and Technology (DST), New Delhi

Authors' contribution

Dr Jyotdeep Kaur and Dr Roli Saxena planned the study. Dr Yogesh Kumar Chawla enrolled and diagnosed the patients. Sample collection was done by Taqveema Ali and Roli Saxena. Taqveema Ali, Roli Saxena, Isha Rani, Renuka Sharma, Deepti More, Rajendra Ola, Stuti Agarwal performed experiments and collected data. Dr. Jyotdeep Kaur and Taqveema Ali analyzed and interpreted data. Dr Jyotdeep Kaur and Taqveema Ali drafted manuscript and all the authors approved the draft of the submitted manuscript.

Conflicts of Interest: Authors declare no conflict of interest

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article.

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Figures

Fig 1.

Figure 1

PCR-RFLP analysis of IL-18 polymorphism 1a. Genotyping for the IL-18 position -607 1b. Genotyping for the IL-18 position -137 *DM stands for DNA Marker

Fig 2.

Figure 2

Dot Plot analysis of IL-18 levels in different categories of HBV-infected patients and its correlation with genotypes 2a. The results are expressed as median $\pm$ interquartile range in all groups. * $p < 0.01$ w.r.t control, ** $p < 0.05$ w.r.t control, # $p < 0.001$ w.r.t control, ## $p < 0.001$ w.r.t CHB [inactive], \$ $p < 0.001$ w.r.t CHB [active]. The bar represents the median in all categories. 2b. IL-18 levels in control, carrier and diseased groups in different genotypes.

Fig.3

a.

b.

Figure 3

Levels of signal transducers [pSTAT1 and pNF-κB] of IL-18 in stimulated PBMCs depicted by blot and densitometry. 3a. Lanes 1,2,3,4 and 5 show Control, Inactive carrier, active CHB [hepatitis] and HCC respectively. Bar graphs show the protein expression levels of pSTAT1 in all categories 3b. Lanes 1,2,3,4 and 5 show Control, Inactive carrier, active CHB [hepatitis] and HCC respectively. Bar graphs show the protein expression levels of pNF-κB in all categories