Association of TNF- α Promoter Polymorphism with HBV Associated Disease Outcome Among HBV Infected Patients from Orissa, Southern Part of East India

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Background: TNF- α promoter polymorphism has been known to be a potential predictive factor in patients with HBV infection. We therefore tried to investigate whether the TNF- α promoter polymorphism at position -238, -857 and -863 was associated with the outcome of HBV infection in a population from Orissa, southern part of East India. **Methods:** A total of 195 patients recruited for the study were classified into 85 controls and 110 HBV infected cases, which included 34 IC, 30 CLD, 32 LC and 14 HCC patients. The polymorphisms at the respective sites were detected by a PCR-RFLP followed by statistical analysis. **Results:** The frequency of the genotype -238 GG and the allele -238G in the cases (89.0% and 92.7% respectively) was significantly higher than that in the controls (68.2% and 82.2% respectively) ($P < 0.001$, OR = 3.8 and $P = 0.001$, OR = 2.73). Whereas the -238 GA genotype was significantly high in the control group (28.2%) when compared to the cases (7.2%) ($P < 0.001$, OR = 0.2). Similarly, the frequency of -863CC and the allele -863C was significantly higher among the cases (24.5% and 49.5%) compared to controls (1.17% and 34.7%), ($P < 0.001$, OR = 27.32 and $P = 0.003$, OR = 1.85), whereas the -863CA genotype was significantly high in the controls (67.0%) when compared to the cases (50.0%) ($P = 0.01$, OR = 0.49). Haplotype -863C/-857C/-238G in cases was significantly higher than controls ($P = 0.002$). Multivariate logistic regression analysis indicates that the genotype -863CC bears a negative association with liver disease progression. **Conclusion:** The present study established an association of polymorphisms at site -238 and -863 of the TNF- α promoter with the outcome HBV infection and disease progression. (J CLIN EXP HEPATOL 2014;4:202-208)

Hepatitis B virus (HBV) infection in adults is usually clinically unapparent, and the virus is cleared after infection. Only about 5-10% of adults become persistently infected and develops chronic liver disease with varied severity,¹ which could not be explained completely by the virus itself or the environmental factors. Progress of HBV infection might be affected by host genetic susceptibility.² Due to the non-cytolytic nature of HBV, the hepatocellular injuries caused by HBV infection are principally immune mediated.³⁻⁶ Host Immune

attacks against HBV are mainly facilitated by a cellular reaction. Cytokines play an important role in defense against viral infection, indirectly through determination of the predominant pattern of the host response, and directly through inhibition of viral replication.⁷ With the implication of host immune response against HBV and increase in viral pathogenesis, several researchers presently pay more attention to special clinical manifestation in relation to alleles responsible for antigen-presenting system and cytokines, that show polymorphisms and can also influence susceptibilities and outcomes of HBV infection.

Tumor necrosis factor-alpha (TNF- α) is produced by macrophages, monocytes, neutrophils, T-cells and NK-cells. It is synthesized as a membrane protein, which is cleaved to produce its soluble 17 Kd form. TNF- α , a principal mediator of cellular immune response and inflammation can stimulate cytokine secretion, increase the expression of adhesion molecules as well as activate neutrophils. There are several reports pertaining to the role TNF-alpha in HBV infection. It might have an important function in non-cytolytic and cytolytic clearance of

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Abbreviations: CLD: chronic liver disease; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; IC: inactive carrier; LC: liver cirrhosis; TNF- α : tumor necrosis factor alpha

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HBV.⁸⁻¹⁰ Reports indicate that an elevated serum TNF- α level in chronic HBV-infected patients before the seroconversion of HBeAg was associated with fatal outcome in hepatic failure.^{8,11} Moreover, the expression of human leukocyte antigen (HLA)-II molecules is also affected by TNF- α , and viral antigen presentation.¹²

Human TNF- α gene is known to be located within the class-III region of HLA in chromosome 6, and is closely associated to HLA gene clusters. It is well known that HLA polymorphisms are related with HBV infection outcomes.¹³ Therefore, the TNF- α gene is vindicated as a candidate gene in the study of genetic susceptibility in HBV infection. There are many SNPs within the TNF gene which can influence the capacity of cytokine production in an individual. In the promoter, these are at positions (relative to the transcription start site) -1031 (T $\rightarrow$ C), -863 (C $\rightarrow$ A), -857 (C $\rightarrow$ A), -851 (C $\rightarrow$ T), -419 (G $\rightarrow$ C), -376 (G $\rightarrow$ A), -308 (G $\rightarrow$ A), -238 (G $\rightarrow$ A), -162 (G $\rightarrow$ A), and -49 (G $\rightarrow$ A).¹⁴

Literature studies on various diseases have shown that specific TNF genotypes resulting from these SNPs in the TNF- α promoter were associated with high or low levels of TNF- α secretion,¹⁵⁻²³ but ethnic difference could lead to different results. This association has earlier been investigated in various case control studies across the world, yet such reports from India are still scanty. Earlier two independent studies, one from North India²⁴ and the other from south India²⁵ have found that TNF polymorphisms were associated with the outcome of HBV infection. Therefore, the aim of the present study was to investigate whether the TNF- α promoter polymorphism at position -238, -857 and -863 was associated with the clinical outcome in an HBV infected population of Orissa, the southern part of East India.

MATERIALS AND METHODS

Study Subjects

A total of 195 samples enrolled in this study were collected from Berhampur Medical College, Berhampur and Sriram Chandra Bhanj (SCB) Medical College & Hospital, Cuttack, Orissa, India. Subjects were divided into two different groups according to serologic tests, HBV virological index, liver functional indexes and symptoms of hepatitis B infection, 85 controls (82 male and 3 female) and 110 cases (98 male and 12 female). The cases were further divided into inactive carrier (IC, $n = 34$), Chronic liver disease (CLD, $n = 30$), Liver cirrhosis (LC, $n = 32$), and hepatocellular carcinoma (HCC, $n = 14$). The criteria selected for grouping subjects into 'controls' and 'cases' were as follows. The control subjects were definitely negative for hepatitis B surface antigen (HBsAg) and HBV DNA, but positive for anti-HBc antibody, had normal liver functional tests. Subjects who served as cases were defined as explained before.²⁶ All

HBV infected patient recruited for the study harbored HBV/D genotype. We excluded subjects who were positive for anti-HCV or anti-HIV. Patients with any other types of liver disease such as autoimmune hepatitis, toxic hepatitis, primary biliary cirrhosis or Budd-Chiari Syndrome and NASH were also excluded. Signed informed consents were obtained from all subjects recruited in the study.

Serological Testing

Commercial ELISA kits were used for HBsAg [Biomerieux, Boxtel, The Netherlands], anti-HCV [Ortho-Clinical Diagnostics, Raritan, N.J., USA] and anti-HIV [Biomerieux] detection.

Genotyping

Genomic DNA was extracted from blood clots using QIAamp genomic DNA kits (Qiagen, Netherlands) following the manufacturer's instructions. The SNPs at the positions -238(G/A) (rs361525), -857(C/T) (rs1799724) and -863(A/C) (rs1800630) (Table 1) were detected by Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP).^{20,21,27} In brief a 152-bp, a 131-bp and a 133-bp fragment were amplified by PCR for positions -238 G/A, -857C/T and -863 A/C respectively. PCR was performed in a 25 μ l reaction using 0.3 units of Chromous Taq polymerase (London, UK), 0.2 pmol of forward/reverse primers and 50 μ g of DNA in a Perkin Elmer thermocycler (2720, Applied Biosystems). The PCR products of -238G/A [GG: 132 + 20 bp, GA: 152 + 132 + 20 bp, AA: 152 bp], -857C/T [CC: 106 + 25 bp, CT: 131 + 106 + 25 bp, TT: 131 bp] and -863C/A [CC: 108 + 25 bp, CA: 133 + 108 + 25 bp, AA: 133 bp] region were digested overnight at 37 °C with MspI, HincII and StyI restriction enzymes respectively. The digestion products were separated on 3% agarose gel and visualized directly under UV light with ethidium bromide staining. Genotyping of around 50% of the samples, chosen randomly were repeated in order to confirm the results. Viral genotyping was done as discussed earlier.²⁸

Statistical Analysis

The Epi Info v 3.5.0 (Centers for Disease Control & Prevention, USA and World Health Organization, Geneva,

Table 1 Selected Single Nucleotide Polymorphisms Located at the Promoter Area of the Tumor Necrosis Factor- α Gene.

rs number	Gene	Location	Substitution	Minor allele
rs1800630	TNF- α	Promoter (-863)	A/C	A
rs1799724	TNF- α	Promoter (-857)	C/T	T
rs361525	TNF- α	Promoter (-238)	A/G	A

Table showing the SNPs included in the study, their position in the TNF- α promoter and the polymorphism type.

Switzerland) was used to estimate chi-squared values, odds ratios and 95% confidence intervals. Multivariate logistic regression analysis was performed to study the association of the outcome of HBV infection with gender, age and TNF- α promoter polymorphisms. Statistical software MINITAB 15 (Pennsylvania, USA) was used for the purpose, and P -value < 0.05 was taken to indicate statistical significance. Haploview 4.2 (Broad Institute, Cambridge, Massachusetts, USA) was used to calculate linkage disequilibrium and haplotypes between pair wise combinations of the SNPs at the 3 positions. PLINK 1.07 was used to calculate the HWE- P values (<http://pngu.mgh.harvard.edu/purcell/plink/>).

RESULTS

Genotype Distributions and Allele Frequencies

Control vs Cases

The genotypic frequencies of the two groups (control and cases) at the three sites of TNF- α promoter are shown in Table 2. The frequency of the -238 GG genotype in the cases (89.0%) is significantly higher than that in the control group (68.2%) ($P < 0.001$, OR = 3.8, 95% CI = 1.69–8.6).

Whereas the -238 GA genotype was significantly high in the control group (28.2%) when compared to the cases (7.2%) ($P < 0.001$, OR = 0.2, 95% CI = 0.08–0.5). Carrier frequency -238 (GA/AA) was found to be significantly high in the control group (31.7%) compared to the cases (10.9%). The frequency of G allele among the cases was significantly higher than that in control group (92.7% vs. 82.3%, $\chi^2 = 9.92$, $P = 0.001$, OR = 2.73; 95% CI = 1.38–5.4).

Similarly, we observed that the frequency of -863CC was significantly higher among the cases compared to controls (24.5% Vs 1.17%, $P < 0.001$, OR = 27.32, 95% CI = 3.82–5.52) (Table 2). Whereas the -863CA genotype was significantly high in the controls (67.0%) when compared to the cases (50.0%) ($P = 0.01$, OR = 0.49, 95% CI = 0.26–0.9). Carrier frequency (-863CA/AA) was found to be significantly higher among the controls (98.8%) compared to cases (75.4%) ($P < 0.001$) as well as the variant allele C was found to be significantly higher among the cases (49.5%) as compared to controls (34.7%) ($P = 0.003$, OR = 1.85, 95% CI = 1.20–2.85).

No significant differences in genotype frequencies of -857C/T were found between the two groups and also between the cases when analyzed individually.

Table 2 Distribution of Genotypes and Allele Frequencies Among Controls and Cases at Sites -863, -857 & -238 of the TNF- α Promoter.

TNF- α position	Genotypes	Control (<i>n</i> = 85) (%)	Cases (<i>n</i> = 110) (%)	HWP	χ^2 -value	<i>P</i> -value	OR	95% CI
-863 (C/A) (rs1800630)	CC	1 (1.17)	27 (24.5)	6.54E-06	21.29	<0.001*	27.32	3.82–552.71
	CA	57 (67.0)	55 (50.0)		5.71	0.01*	0.49	0.26–0.92
	AA	27 (31.7)	28 (25.5)		0.94	0.3	0.73	0.37–1.44
	Carrier frequency (CA/AA)	84 (98.8)	83 (75.4)		21.29	<0.001*	0.04	0.00–0.26
Variant allele (%)	C	59 (34.7)	109 (49.5)		8.61	0.003*	1.85	1.20–2.85
	A	111 (65.2)	111 (50.4)		8.61	0.003*	0.54	0.35–0.83
-857 (C/T) (rs1799724)	CC	67 (78.8)	79 (71.8)	0.027	1.25	0.26	0.68	0.33–1.40
	CT	14 (16.4)	27 (24.5)		1.88	0.17	1.64	0.76–3.61
	TT	4 (4.7)	4 (3.6)		0.14	0.7	0.76	0.15–3.77
	Carrier frequency (CT/TT)	18 (21.1)	31 (28.1)		1.25	0.26	1.46	0.71–3.00
Variant allele (%)	C	148 (87.0)	185 (84.0)		0.68	0.4	0.79	0.42–1.45
	T	22 (12.9)	35 (15.9)		0.68	0.4	1.27	0.69–2.35
-238 (G/A) (rs361525)	GG	58 (68.2)	98 (89.0)	0.715	13.03	<0.001*	3.8	1.69–8.67
	GA	24 (28.2)	8 (7.2)		15.36	<0.001*	0.2	0.08–0.50
	AA	3 (3.5)	4 (3.6)		0.00	0.96	1.03	0.19–5.99
	Carrier frequency (GA/AA)	27 (31.7)	12 (10.9)		13.03	<0.001*	0.26	0.12–0.59
Variant allele (%)	G	140 (82.3)	204 (92.7)		9.92	0.001*	2.73	1.38–5.47
	A	30 (17.6)	16 (7.2)		9.92	0.001*	0.37	0.18–0.73

Genomic DNA from controls and HBV infected cases were extracted from blood clots and subjected to PCR-RFLP using specific primers and enzymes for genotyping followed by statistical analysis. *Represent statistically significant values. HWP represents Hardy–Weinberg P value.

Table 3 Distribution of Genotypes and Allele Frequencies Among Controls and Various Clinical Categories.

	TNF- α position	Control (n = 85)	IC (34)	CLD (30)	LC (32)	HCC (14)
–863 (C/A) (rs1800630)	CC	1 (1.17)	12 (35.2) ^{b*}	7 (23.33)	7 (21.87) ^{b*}	1 (7.14) ^{b*}
	CA	57 (67.0)	12 (35.2) ^{c*}	16 (53.33)	20 (62.5) ^{c*}	7 (50)
	AA	27 (31.7)	10 (29.4)	7 (23.33)	5 (15.62)	6 (42.85)
Variant allele (%)	C	59 (34.7)	36 (52.9)	30 (50.0)	34 (53.1)	9 (32.1)
	A	111 (65.2)	32 (47.0)	30 (50.0)	30 (46.8)	20 (71.4)
–857 (C/T) (rs1799724)	CC	67 (78.8)	24 (70.5)	23 (76.6)	22 (68.7)	10 (71.4)
	CT	14 (16.4)	7 (20.5)	7 (23.3)	9 (28.1)	4 (28.5)
	TT	4 (4.7)	3 (8.8)	0	1 (3.1)	0
Variant allele (%)	C	148 (87.0)	55 (80.8)	53 (88.3)	53 (82.8)	21 (75.0)
	T	22 (12.9)	13 (19.1)	7 (11.6)	11 (17.1)	4 (14.2)
–238 (G/A) (rs361525)	GG	58 (68.2)	33 (97.0) ^{a*}	24 (80.0)	30 (93.7)	11 (78.5) ^{a*}
	GA	24 (28.2)	1 (2.9)	4 (13.3)	2 (6.2)	1 (7.1)
	AA	3 (3.5)	0	2 (6.6)	0	2 (14.2)
Variant allele (%)	G	140 (82.3)	67 (98.5)	52 (86.6)	62 (96.8)	23 (82.1)
	A	30 (17.6)	1 (1.4)	8 (13.3)	2 (3.1)	5 (17.8)

The HBV infected cases were further classified into Inactive carriers (IC), Chronic Liver Disease (CLD), Liver cirrhosis (LC) and Hepatocellular carcinoma (HCC). Genotyping results among the classified cases and controls were analyzed statistically. *Represent statistically significant values. (IC^a Vs. HCC^a: $P = 0.03$; IC^b Vs LC^b: $P = 0.02$; IC^b Vs HCC^b: $P = 0.04$; IC^c Vs LC^c: $P = 0.02$).

Cases (IC vs CLD vs LC vs HCC)

When the TNF positions were analyzed individually among the cases group (i.e., IC, CLD, LC, HCC) (Table 3) it was found that the frequency of –238 GG genotype and the –863CC genotype was highest in the ICs (97.0% and 35.2% respectively). Consequently, the –238 G allele and the –863C allele was highest among them. The –238 GG genotype was significantly high among the ICs compared to the HCC patients ($P = 0.03$). The –863CC genotype was also significantly high in the ICs when compared to the LC as well as the HCC patients ($P = 0.02$ and 0.04 respectively). On the other hand, the –863CA genotype was significantly high in the LC patients compared to the ICs ($P = 0.02$).

Haplotype Analysis

Haplotype analysis as summarized in Table 4 revealed that –863A/–857C/–238G was the major haplotype in both controls and cases. Frequency of haplotype –863C/–857C/–238G in cases was significantly higher than that of control group ($P = 0.002$). The minor allele frequency as calculated by haploview for –238, –863, and –857 was observed to be 0.07, 0.4, and 0.1 respectively.

Linkage Disequilibrium

There was no clear LD between any of the 3 positions (–863/–857/–238) of the TNF- α promoter included in our study.

Table 4 Frequencies of TNF- α –238/–857/–863 Haplotype Among Controls and Cases.

Haplotype	Control (%)	Cases (%)	P-value (χ^2 value)	Odd's ratio	95% CI
ACG	52.2	40.8	0.10 (2.65)	0.63	0.34–1.14
CCG	18.7	36.8	0.002 (9.13)*	2.64	1.33–5.27
CTG	7.3	9.6	0.44 (0.58)	1.48	0.49–4.52
ATG	4.2	5.5	0.73 (0.12)	1.26	0.28–5.81
ACA	7.4	3.3	0.10 (1.73)	0.41	0.08–1.82
CCA	8.8	3.2	0.07 (3.19)	0.31	0.06–1.31
ATA	1.4	0.8	0.56 (0.34)	0.49	0.02–7.10

The haplotype frequencies derived from the 3 SNPs of interest were calculated and examined by Haploview followed by statistical analysis. *Represent statistically significant values.

Table 5 Multivariate Logistic Regression Analysis Showing Predictive Factors of an Infected Individual Towards Increased Disease Severity.

Variable	β	χ^2 -value	P-value	OR	95% CI
–863 CC	–1.4294	8.7616	0.003*	0.24	0.09–0.62
–857 CC	–0.2497	0.3600	0.552	0.78	0.34–1.77
–238 GG	–0.7498	1.7424	0.570	0.47	0.15–1.44

Clinical stages of hepatitis B infection were ordered according to the increasing level of disease severity i.e. as level 1 (IC), level 2 (CLD), level 3 (LC) and level 4 (HCC) and the frequency of the homozygous genotype –863CC, –857CC and –238 GG were analyzed by a multivariate logistic regression analysis with respect to increased disease severity.

*Represent statistically significant values. Note: the table includes analysis of only the infected cases excluding the controls.

Multivariate Logistic Regression Analysis

The data sets were subjected to multivariate logistic regression analysis, in order to study the association of factors with the different clinical stages and increasing level of disease severity of hepatitis B infection.

Our study population followed a normal distribution with a median age of 35 years. Thus, in the analysis, according to their age the patients were grouped into two categories, viz. age less than 35 years and age greater than or equal to 35 years. The analysis showed that, after adjusting for confounding effects of gender and age, the GG and CC genotype resulting from the polymorphisms at positions –238 and –863 respectively of TNF- α gene promoter were independently significantly associated with the cases (Supplementary Table 1). In both cases, the association was positive, indicating that –238 GG and –863CC tend to be associated more with cases as compared to control. However, TNF- α –857C/T showed no significant association with cases group. Again while analyzing the effect of polymorphism at position –863, CC genotype showed significantly high association with the ICs compared to the LC patients. No other significant association in respect of TNF- α promoter polymorphisms was noted in the study.

Table 5 represents a multivariate logistic regression analysis pertaining to the TNF polymorphisms among different clinical groups under the HBV infected cases. Here ordinal logistic regression was performed as it was possible to order the clinical stages of hepatitis B infection according to the increasing level of disease severity; such as level 1 (IC), level 2 (CLD), level 3 (LC) and level 4 (HCC). The analysis shows that after adjusting for the confounding effects of gender and age, the polymorphisms at position –863 of TNF- α gene promoter significantly affects the course of HBV infection which indicates that –863CC genotype tends to be associated less with increasing disease severity (IC $\rightarrow$ CHB $\rightarrow$ LC $\rightarrow$ HCC) i.e. the clinical category with the highest level of disease severity shows least association with the –863CC genotype

and vice versa. Table 5 also shows that the significant predictive factors for higher infection in a patient are attributable to the presence of A allele at the –863 position of the TNF-promoter.

DISCUSSION

Till date, among several studies conducted in relation to various cytokines, TNF- α happens to be one of the most important in terms of viral infection. TNF- α is a pro-inflammatory cytokine that is also a key transcriptional factor of other pro-inflammatory cytokines and chemokines, which may augment the inflammatory cascade against various infectious pathogens. Previous animal models have demonstrated the effects of TNF- α on the suppression of HBV protein expression and virus replication.²⁹

Earlier studies indicate that the polymorphism in its promoter affects the amount of cytokine production.³⁰ And the amount of cytokine production in turn may be associated with different clinical outcomes in humans infected with HBV. SNPs in the promoter of TNF- α influence its production due to differential binding capabilities of allele specific binding elements in the TNF- α promoter with the transacting factors.

In the current study, we have analyzed the polymorphism in three positions of the TNF promoter, viz –238, –857 and –863. The TNF-238 lies in a putative regulatory box (y box) of the TNF promoter which binds to the regulatory DNA binding proteins and the variation of this site will affect the activities of the promoter.³¹ Our results indicate that the –238 GG genotype is significantly high in the cases when compared to healthy controls. This is in concordance with independent studies from China^{21,27} where they speculate that the reason behind this might be that the presence of G allele decreased the expression of TNF- α and inhibited clearance of HBV. Whereas, the –238A allele was significantly high among the controls (OR = 0.37) signifying that it probably provides protection against the infection. A study by Lu²⁰ also exhibit similar results. However, other studies^{20,32} have demonstrated contradictory results. Ethnic difference could play a major role in explaining these conflicting results. Additionally, our study also shows a significant high GA genotype in controls as compared to cases. This indicates a high carrier state prevalence in the –238 position among the control group in our study population.

Our findings also suggested the predominance of –863CC among the cases than controls. This is in accordance to some other independent studies from different parts of the world.^{19,33} Another study from India showed no significant relation of –863CA/AA genotype with cases and control groups.³⁴ The binding efficiency of NF- κ B complexes (p50-p65/p50-p50) at the –863 position was ruled by allelic variations at this position of the

TNF- α promoter. Evidences suggest that the binding of p50-p65 complex of NF- κ B can augment the downstream transcriptional activity of TNF while the p50-p50 may exert inhibitory effects on transcriptional activation by competing with the p50-p65 complex.³⁵ The A allele of TNF- α -863 SNP is reported to bind the p50/p65 complex of the downstream nuclear factor- κ B (NF- κ B) only, while the C allele can bind both the p50/p50 and the p50/p65 complex.³⁵ Thus, the -863 A allele shows increased promoter activity in transient transfection experiments compared to the C-allele during LPS stimulation.³⁵ This clearly explains why, in our study, the presence of AA genotype is more prevalent among the controls whereas the CC genotype is highest in the cases group.

In addition to case control analysis we have also evaluated these polymorphisms in different clinical categories pertaining to HBV infection ranging from Inactive carriers (IC), Chronic Liver Disease (CLD), Liver cirrhosis (LC) and Hepatocellular carcinoma (HCC) which to the best of our knowledge was formerly not done. It is extremely interesting to note that both the -238 GG genotype as well as the -863CC genotype (associated with the decrease expression of TNF- α) were found to be highest among the ICs. In fact, statistical analysis among cases also exhibit similar results. HBV being a non-cytolytic virus, the generation of symptoms is principally immune-mediated. Obviously the decreased TNF- α expression might be one of the reasons why the ICs fail to manifest symptoms.

If a disease can be categorized into more than two categories according to its severity, ordinal logistic regression analysis is used. MLR analysis for determinants of cases (IC, CLD, LC and HCC) reveal that genotype -863CC is negatively associated with increased disease severity, where as it was observed that -863CA/AA significantly showed positive association with increased disease severity. MLR analysis reveals that the significant predictive factors towards increasing disease severity in an HBV infected patient are attributable the presence of A allele at the -863 position of the TNF-promoter. To the best of our knowledge, this finding is exclusive in our study among the subjects from Orissa. It is noteworthy, that at the -863 position, the wild type CC genotype is higher in the cases compared to controls indicating that possibly the presence of the C allele will increase the risk of HBV infection, however when only the cases were analyzed (excluding controls), the CC genotype is associated less with disease progression. This signifies that probably the presence -863AA genotype (which is associated with increased TNF production) helps the host to raise an immune response which either clears the infection or leads to symptomatic disease progression (i.e. CLD $\rightarrow$ LC $\rightarrow$ HCC) as generation of symptoms are principally immune mediated.

However, the presence of -863CC (associated with less TNF production) in an infected individual culminates in an inactive carrier unable to generate symptoms.

However, a major limitation of the study is the small sample size in each sub group. Therefore, though our study reveals encouraging trends towards association of TNF polymorphism with disease progression, the results need to be further confirmed in a larger cohort of patients.

Another key contradictory result at this locus is the predominance of the heterozygous CA genotype among all the study subjects. Heterozygosity at any position in a gene indicates an increased Darwinian fitness in the population.

Though a number of studies have demonstrated a significant role of the polymorphic -857 promoter region of the TNF- α gene in relation to the outcome of HBV infection,^{27,32} no significant association in its respect was noted in this study. Ethnic difference could play a vital role in explaining these conflicting results, because the results from several studies suggested that the distribution of TNF promoter polymorphisms in the study subjects was different from those with other racial origins.^{8,20}

In contrast to Du et al,²⁷ our study indicated that the frequency of haplotype -863C/-857C/-238G in cases was significantly higher than that of control group. No significant linkage disequilibrium was found between any two sites among the 3 sites selected in our study. We also observed that rs1800630 (-863) has a Hardy-Weinberg *P*-value of 8.23E-03 which suggests that this SNP should not be included in association testing. The lower sample size might be a probable reason. We are, thus, not discussing about any association significance of this SNP, whereas, we are just reporting the genotyping result.

In conclusion, the present study established a clean association of polymorphisms at site -238 and -863 of the TNF- α promoter and HBV related disease progression. A further study with larger cohorts and combined *in vitro* studies will help know how gene variation determines the constitutive and inductive expression of TNF- α , and provide a theoretical basis for discovery of HBV infection mechanisms and find new strategies for diagnosis and therapy.

CONFLICTS OF INTEREST

All authors have none to declare.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jceh.2014.08.002>.

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