TGF-β1 gene − 509C>T promoter polymorphism modulates TGF-β1 levels in hepatitis E patients

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Elevated levels of transforming growth factor-β1 (TGF-β1) and its positive correlation with Foxp3 expression in hepatitis E patients have indicated involvement of TGF-β1 in hepatitis E pathogenesis. The current study determined polymorphisms in TGF-β1 gene, plasma TGF-β1 levels and T effector (Teff) cell proliferation and explored their association in a case control study. Polymorphisms in three selected sites (− 509C→T, +869T→C and +915G→C) of TGF-β1 gene by PCR & restriction fragment length polymorphism methods, plasma TGF-β1 quantitation by ELISA and Teff (CD4+CD25−) cell proliferation by CFSE method were carried out in 277 hepatitis E patients (HE) with self-limiting infection and 233 ethnically matched healthy controls (HCs) from western India. Frequency of CT genotype of −509C>T site was significantly higher in hepatitis E patients compared to healthy controls (p = 0.017; OR 1.53, 95% CI 1.07–2.17). Plasma TGF-β1 levels were significantly higher in HE compared to HCs. TGF-β1 level of patient having CT genotype of −509C>T site was significantly higher compared to those having CC or TT genotypes. Teff cell proliferation was negatively correlated with plasma TGF-β1 levels in hepatitis E patients (r = −0.568; p = 0.014). Influence of TGF-β1 promoter (−509C→T) polymorphism on plasma TGF-β1 levels and inverse correlation of Teff cell proliferation with plasma TGF-β1 levels in self-limiting hepatitis E patients suggest key role of TGF-β1 in augmentation of reported T regulatory cell mediated pathogenesis in hepatitis E.

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1. Introduction

Hepatitis E, an epidemic as well as sporadic disease, prevalent in the developing countries is mostly characterized by a self-limiting course and a severe course, i.e. fulminant hepatitis with high mortality, in pregnant women (Taneja et al., 2009; Navaneethan et al., 2008). Pathogenesis of a severe course, i.e. fulminant hepatitis with high mortality, in pregnant women also clear the infection, it is important to understand the factors determining differential outcomes of HEV infection. Understanding the immune response (Smith, 1996). Polymorphisms in cytokine genes affect gene transcription and cause inter-individual variations in cytokine production (Turner et al., 1997). The TGF-β1 gene is located on chromosome 19q13 (Lawrence, 1996; Clark and Coker, 1998). Seven genetic polymorphisms of TGF-β1 have been identified; 3 in the upstream region at −988, −800 and −509; 1 in a non-translated region at +72; 2 in the signal peptide sequence of exon one at codon 10 (+869C>T) and 25 (+915G>C); and 1 in the protein coding region at codon 629 (Awad et al., 1998). It is proposed that the TGF-β1 production is under genetic control (Granger et al., 1999; Shah et al., 2006). TGF-β1 gene polymorphisms at codon 10 and 25 regulate the TGF-β1 production in vivo and in vitro (Blobe et al., 2000; Luedeking et al., 2000; Meng et al., 2005). The −509C>T is located

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within an YY1 consensus binding site and — 509T allele has been associated with increased TGF-β1 plasma level (Shah et al., 2006; Luedeking et al., 2000) and reduced T cell proliferation (Meng et al., 2005).

Our group has previously shown polymorphism in TNF-α to be associated with susceptibility and polymorphism in IFN-γ to be associated with the clinical outcome of hepatitis E infection (Mishra and Arankalle, 2011). In a similar line, Devi et al. have shown association of TNF-α — 308 AA, IFN-γ + 874 AA, TGF-β1 codon 10 + 869 TT & codon 25 GC genotypes with preterm delivery, TNF-α — 308 GG & IL-6 — 174 CC genotypes with low birth weight, IL-6 — 174 CC geno-type with fetal loss and IL-6 — 174 CC & TGF-β1 codon 10 + 869 TC genotypes with small-for-dates in HEV infected pregnant women compared to uninfected controls (Devi et al., 2014).

In the absence of data on the association of specific mutations in the TGF-β1 gene with a given pattern of secretion of the same, in the current study, we have estimated plasma TGF-β1 levels, genotyped the SNPs in the TGF-β1 gene, measured T effector (Teff) cell proliferation and have evaluated their correlation in a Western Indian populations of hepatitis E patients and matched healthy controls.

2. Subjects & methods

2.1. Ethics statement

All procedures performed in current study involving human partici-pants were in accordance with the ethical standards of the Institutional Ethical Committee for Research on Humans.

2.2. Informed consent

Written informed consents were taken from all individual partici-pants included in the study.

2.3. Study population

A total of 498 study subjects including 277 hepatitis E patients (168 males and 109 females) (mean age ± standard deviation; 29.8 ± 12.7) and sex, age and ethnicity matched healthy controls, 221 (126 males and 95 females) (mean age ± standard deviation; 31.4 ± 9.74) were enrolled in the current study. The patients as well as controls enrolled were from Western India.

2.4. Serology

Hepatitis E diagnosis was based on the presence of IgM antibodies to HEV (IgM-anti-HEV) by ELISA (Arankalle et al., 2007). The control group consisted of age and sex-matched apparently healthy individuals. All the study subjects were screened for IgG and IgM-anti-HEV antibodies, IgM-anti-HAV antibodies, HBSAg, IgM-anti-HBC, anti-HCV and anti-HIV antibodies (ELISA, Abbott, USA). The patients were negative for the serological markers for HAV, HBV, HCV and HIV, while the controls were negative for these markers as well as for IgM and IgG anti-HEV antibo-dies. The acute patients were confirmed to be positive for serum anti-HEV IgM antibody and had elevated ALT levels. The recovered individuals having a recent history of acute hepatitis E had normalized ALT levels, positive for anti-HEV IgG antibody and were positive/negative for anti-HEV IgM antibody. Together, the patient group is referred as HE. Exclusion criteria for study group included infection with viral co-infections, past history of pre-existing liver disease and any other asso-ciated diseases.

2.5. Genotyping of TGF-β1 polymorphism

2.5.1. DNA extraction

Genomic DNA was extracted from blood/PBMCs using QIAamp DNA Blood Mini extraction kit (Qiagen, Germany). DNA was quantified by spectrophotometer (ND-1000, Nanodrop Technologies) and the quality was assessed by running on an agarose gel.

2.5.2. Genetic analysis

Genotyping of the 3 SNPs in the TGF-β1 gene (— 509C>T, + 869T>C and +915G>C) reported to be associated with alteration in plasma TGF-β1 levels (Fig. 1a) was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Romani et al., 2011).

2.5.3. PCR amplification

The PCR conditions for + 869T>C and +915G>C SNPs were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58.4 °C for 30 s, and 72 °C for 30 s, with an extension at 72 °C for 10 min. The PCR conditions for the — 509C>T SNP were as follows: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 62 °C for 45 s, and 72 °C for 30 s, with an extension at 72 °C for 10 min.

For genotyping of — 509C>T SNP, the PCR product was digested using the EcoR1I restriction enzyme (RE) (New England Biolabs, USA). For genotyping of + 869T>C and +915G>C SNPs, a pair of indigenously designed primers that amplified both polymorphic sites were used. This PCR product was used as a template for two RFLP reactions with MspA1I for + 869T>C and BglII for +915G>C (New England Biolabs, USA). The primer sequences, RE, digestion sites and fragments for the detection of each SNPs are depicted in Table 1. Amplification of 100 ng (5 μL) of template DNA for each of the 2 PCR reactions was performed using a total volume of 25 μL containing final concentration of forward and reverse primers 10 pmol (2.5 μL each), 1.25 U of Taq polymerase (2.5 μL) (Applied Biosystems, USA), 200 μM of each dNTP (2.5 μL) (Fermentas, Latvia), 37 mM of MgCl2 (2.5 μL), dimethyl sulfoxide (1.25 μL) (Sigma Aldrich, Germany), and distilled water (6.25 μL).

2.5.4. Restriction enzyme digestion

10 μL of the PCR amplification product for studied SNPs + 869T>C, +915G>C and —509C>T were digested with MspA1I, BglII and Eco81I RE respectively in a 20 μL volume mixture containing 2 μL CutSmart Buffer for MspA1I, BglII and 3.1 buffer for Eco81I and 1 μL RE and 7 μL Milli Q water. The reaction mixture was incubated at 37 °C for 45 min. The RFLP products for + 915G>C and —509C>T SNPs were run on a 2.5% agarose gel electrophoresis at 100 mV for 1 h in 100 mL TAE buffer, and RFLP products for +869T>C were separated and run by using 16% polyacrylamide gel electrophoresis at 80 mV for 5 h in 100 mL TBE buffer. The size of the restriction fragments for three RFLP reactions products were determined using 100 bp DNA ladder (Invitrogen, USA).

2.6. Direct DNA sequencing

To confirm the RFLP genotyping results, 20% of the samples were ge-notyped to match the product sequence with the established TGF-β1 se-quence. The PCR product was purifed using MinElute PCR Purification Kit and both the strands were sequenced using Big Dye Terminator cycle sequencing Ready Reaction Kit (ver. 3.1; Life Technologies, USA) and automatic sequencer (ABI 3130 XL, USA) (Fig. 1b–d).

2.7. TGF-β1 quantitation

Using standard ELISA kits (R&D Systems, USA) and following the manufacturer’s instructions, TGF-β1 plasma levels were estimated in duplicate from HE patients (n = 95) and HCs (n = 69).

2.8. Teff cell proliferation assay

Isolation of Teff cells and CFSE (carboxyfluorescein succinimidyl ester) (Invitrogen, USA) based proliferation was carried out in 18 acute hepatitis E patients as previously described by Rathod et al. (2014).
2.9. Calculation of gene frequencies and statistical analysis

Allele and genotype frequencies were calculated by direct counting. Chi square test was used for comparison of allele and genotype frequencies between different study groups. p values, odds ratio (OR) with 95% confidence limits (CI) were calculated using MedCalc software. Statistically significant differences between groups was further assessed using ANOVA with Bonferroni post-hoc corrections. Statistical analyses were performed with SPSS 20 software (SPSS Inc., USA).

3. Results

3.1. Position −509C>T

Seventy three (26.35%) of HE and 68 (29.18%) of HCs were homozygote CC, 153 (55.23%) of HE and 104 (44.63%) of HCs were heterozygote CT, whereas homozygote TT was found in 51 (18.41%) of HE and 49 (21.03%) of HCs at this position. The frequency of only CT genotype was significantly higher in HE group.

Table 1

| Polymorphism site in TGFβ1 gene | Restriction enzyme | Primer sequences | Restriction site | Genotype | Restricted fragments, base pairs size |
|---------------------------------|---------------------|------------------|-----------------|----------|-------------------------------------|
| −509C>T (promoter)             | Eco8I               | F: 5-CAGTAAATGTATGGGGTCGCAG-3′<br>R: 5-GGTGTCAGTGGGAGGAGGG-3′ | 5′-CC▼INAGG..3′<br>3′-GGAN1▲CC..5′ | TT       | 153                                |
|                                 |                     |                  |                 | CT       | 153, 117, 36                         |
|                                 |                     |                  |                 | CC       | 117, 36                             |
| +869C>T (codon 10)             | MspAI               | F: 5-GTTATTTCCGTGGGATACTGAGAC-3′<br>R: 5-GACCTCCTTGGCGTAGTAGTCG-3′ | 5′-CMG▼CKG..3′<br>3′-GKC▲GMC..5′ | CC       | 12, 40, 67, 108, 230                 |
|                                 |                     |                  |                 | CT       | 12, 40, 67, 108, 230, 242            |
|                                 |                     |                  |                 | TT       | 40, 67, 108, 242                     |
| +915G>C (codon 25)             | BglII               | As codon 10      | 5′-GCCNNNN▼NGGC..3′<br>3′-CGGNN▲NNNNCGG..5′ | GG       | 212, 252, 60                         |
|                                 |                     |                  |                 | GC       | 212, 252, 312                        |
|                                 |                     |                  |                 | CC       | 212, 312                             |
compared to the HC group \((p = 0.017; \text{OR} 1.53; 95\% \text{CI} 1.07–2.17)\) (Table 2).

3.2. Position +869T>C

Eighty three (29.96\%) of HE, and 67 (28.75\%) of HCs were homozygote CC, 142 (51.26\%) of HE and 111 (47.63\%) of HCs were heterozygote CT and homozygote TT was found in 52 (18.77\%) of HE and 43 (18.45\%) of HCs at this position. No significant difference in the genotype distribution and allele frequency was found (Table 2).

3.3. Position +915G>C

Eighty seven (31.40\%) of HE and 72 (30.90\%) of HCs were homozygote GG, 145 (52.34\%) of HE and 110 (47.21\%) of HCs were heterozygote GC and homozygote CC was found in 45 (16.24\%) of HE and 39 (16.73\%) of HCs at this position. No significant difference in the genotype distribution and allele frequency was found (Table 2).

3.4. Plasma TGF-β1 levels

Irrespective of the genotype, plasma TGF-β1 level in the HE group was significantly higher than HCs (42.74 ± 22.57 pg/mL vs. 11.74 ± 8.10 pg/mL, \(p = 0.0001\)) (Fig. 2a). At +509C>T site, in HE group plasma TGF-β1 level was significantly higher in CT genotype compared to homozygous CC \((p = 0.0003)\) or TT \((p = 0.013)\) genotypes, while the same was comparable in HCs (Fig. 2b). At +869T>C and +915G>C SNPs no significant difference was observed among the genotypes with respect to plasma TGF-β1 level (Fig. 2c–d).

3.5. Spearman correlation analysis

In a set of 18 acute hepatitis E patients, concentration of plasma TGF-β1 was correlated with their Teff cell proliferation. A negative correlation of TGF-β1 level and Teff cell proliferation was observed \((r = -0.568, p = 0.014)\) (Fig. 2e).

4. Discussion

Participation of TGF-β1 with Treg cells to mediate immunosuppression, its function in maintaining peripheral tolerance and in restoration of suppressive activity of Treg cells are established phenomena (Nakamura et al., 2004; Bommireddy and Doetschman, 2007). Increased protein level of TGF-β1 in hepatitis E patients, elevation of TGF-β1 on both unstimulated and HEV antigen stimulated PBMCs of acute hepatitis E patients and recovered individuals at both phenotypic and gene expression levels have suggested TGF-β1 to be a pivotal cytokine in the pathogenesis of hepatitis E (Rathod and Tripathy, 2014; Rathod et al., 2014; Gewaltig et al., 2002).

An association of SNPs in the TGF-β1 gene – 509C>T with rate of progression of HCV-induced fibrosis has been reported in a study from Germany (Gewaltig et al., 2002). In an Italian study, Falleti et al. have shown CT and TT genotypes of TGF-β1 – 509C>T to be higher in cirrhotic group than control group indicating its detrimental association (Falleti et al., 2008). Egyptian study by Hanafy and Abdo reporting different genotype and allele distributions of TGF-β1 – 509C>T site in chronic HCV patients and controls and similar scenario again on Taiwanese HCV patients by Dai et al. further established involvement of this genotype in hepatitis C (Hanafy and Abdo, 2011; Dai et al., 2008). In a different tune, Kimura et al. have demonstrated – 509C allele to be associated with a higher clearance rate of HCV as well as with lower promoter activity (Kimura et al., 2006). However, in the contest of hepatitis B and Iranian patients, absence of association between TGF-β1 – 509C>T and +915G>C polymorphisms with chronic hepatitis B indicated that these changes do not play a significant role in increasing the risk of chronic infection in Iranian patients (Razavi et al., 2014; Romani et al., 2011). Our finding of TGF-β1 polymorphism at position – 509 with increase in C>T genotype in the patient group suggests its protective role, since all the patients recovered.

Changes in the production of TGF-β1 have been linked to numerous disease states. Elevated levels of circulating TGF-β1 in acute viral hepatitis and in its fulminant form have been demonstrated (Nakamura et al., 2000; Koulentaki et al., 2002). TGF-β1 is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and HCC (Bissell, 2001). Serum levels of TGF-β1 are elevated in fibrosis and in patients with chronic HCV infection, viral core protein up-regulates TGF-β1 transcription exacerbating liver fibrosis progression (Falleti et al., 2008; Kopp et al., 1996; Taniguchi et al., 2004). Miwa et al. detected increased levels of plasma TGF-β1 in patients with fulminant viral hepatitis B (Miwa et al., 1997). Plasma TGF-β1 has been suggested as a useful serologic marker in the diagnosis of small HCCs (Song et al., 2002). Higher plasma TGF-β1 of the current hepatitis E patients needs further evaluation.

Elevated plasma TGF-β1 levels associated with the – 509C>T polymorphism have been suggested to suppress immune activation and higher risk of allergy (Kim et al., 2007). Polymorphisms of TGF-β1 gene at – 509C>T and 869T>C are not related to plasma TGF-β1 levels and are not associated with increased susceptibility to development of chronic obstructive pulmonary disease from cigarette smoking in Hong Kong Chinese population (Mak et al., 2009). Report that – 509C>T polymorphism is significantly associated with a high plasma concentration of TGF-β1 (Grairgner et al., 1999) goes in parallel with our observation of higher TGF-β1 plasma level in – 509C>T site of HE patients with a CT genotype compared to patients with either CC or TT genotypes.

Genotype distribution and allele frequencies at position +869T>C and +915G>C SNPs of the current study were not significantly different in the patient and control groups. However in HEV infected pregnant women, TGF-β1 cytokine polymorphism at +869T&T & +915GC genotypes are reported to be associated with preterm delivery and TGF-β1 +869TC genotype with small-for-dates compared to not infected with HEV and controls (Devi et al., 2014). Since, the same group has previously shown higher TGF-β1 levels in HEV infected pregnant women, carrying out polymorphism at – 509C>T site might have yielded significant results (Kumar et al., 2014).

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Table 2

| Genotype distribution and allele frequencies of studied TGF-β1 polymorphisms. |
|---|
| **TGF-β1 genotype** | **HE (n = 277) %** | **HCs (n = 221) %** | **OR (95% CI)** | **p-Value** |
| Genotype: – 509 (C>T) | | | | |
| CC | 73 (26.35) | 68 (29.18) | 0.80 (0.54–1.19) | 0.27 |
| CT | 153 (55.23) | 104 (46.33) | 1.53 (1.07–2.17) | 0.017 |
| TT | 51 (18.41) | 49 (21.03) | 0.79 (0.55–1.22) | 0.29 |
| Allele | | | | |
| C | 299 (61.78) | 240 (59.26) | 1.11 (0.84–1.45) | 0.44 |
| T | 185 (38.22) | 168 (40.72) | 0.79 (0.68–1.17) | 0.44 |
| Genotype: +869 (T>C) | | | | |
| CC | 83 (29.96) | 67 (28.75) | 0.98 (0.66–1.44) | 0.93 |
| CT | 142 (51.26) | 111 (47.63) | 1.04 (0.73–1.44) | 0.81 |
| TT | 52 (18.77) | 43 (18.63) | 0.95 (0.61–1.49) | 0.84 |
| Allele | | | | |
| C | 308 (61.72) | 245 (60.79) | 0.92 (0.78–1.33) | 0.86 |
| T | 191 (38.28) | 158 (39.21) | 0.97 (0.74–1.27) | 0.86 |
| Genotype: +915 (G>C) | | | | |
| GG | 87 (31.40) | 72 (30.90) | 0.94 (0.64–1.38) | 0.78 |
| GC | 145 (52.34) | 110 (47.21) | 1.12 (0.78–1.60) | 0.51 |
| CC | 45 (16.24) | 39 (16.73) | 0.90 (0.56–1.41) | 0.67 |
| Allele | | | | |
| C | 90 (22.56) | 78 (23.49) | 0.91 (0.65–1.29) | 0.63 |
| G | 319 (77.44) | 254 (76.51) | 1.08 (0.77–1.53) | 0.63 |
Suppressive activity of the Treg cells towards Teff cells in hepatitis E patients could be attributed to TGF-β1, known to promote human peripheral Treg cells development (Nakamura et al., 2004). Negative correlation of plasma TGF-β1 levels with Teff cell proliferation in the acute patients of the current study confirms the suppressing effect of TGF-β1 and goes in parallel with previously reported (Meng et al., 2005).
5. Conclusion

We identified (a) higher frequency of C to T base change at −509C−T site of TGF-β1 gene (b) higher plasma TGF-β1 levels in patients having CT genotype of −509C−T site and (c) negative correlation between Tc cell proliferation and plasma TGF-β1 levels indicating the importance of the interaction of three factors in limiting HEV infection. Future studies with severe fulminant form of hepatitis E patients will be needed to ratify the results of the present study.

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Conflict of interest

No conflict of interest existed.

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