ESPS manuscript NO: 26623

Title: Tumor necrosis factor-α –G308A polymorphism is associated with liver pathological changes in HCV patients

Response to reviewer (1) Comments:

We valued the precious response to our article made by you and by the eminent reviewers, we learned a lot from your comments indeed. In the next few lines we will respond to your comments point by point:

COMMENTS TO AUTHORS

This study added new information regarding a subtyping (4) of HCV and its relation with TNF-alfa cytokine.

- A limit of the study is the technical approach of RFLPs to detect the mutation. Please confirm in series of cases the mutation by another technique.

We added a photo of the PCR-RFLP analysis in the result section. Also, we confirmed in series of cases the RFLP analysis results by sequencing of the TNFα PCR products. We added two paragraphs in the manuscript; one paragraph in the Material and Methods and the other one in the Results as shown below:

Sequence analysis of TNF PCR products

To confirm the results of TNFα –308 PCR-RFLP analysis, some TNFα –308 PCR products were sequenced using Sanger dideoxynucleotide chain termination method. The TNFα –308 PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Then, the PCR products were sequenced with the TNFα reverse primer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc, CA, USA). After the cycle sequencing reaction, the products were purified using BigDye XTerminator purification Kit (Applied Biosystems Inc, CA, USA) and analyzed on ABI 3500 Genetic Analyzer.

The TNFα –308 RFLP and Sequence Analysis

The amplified TNFα –308 PCR products were digested with NcoI restriction enzyme, and run on 4% agarose gel, as shown in Figure 1A. The homozygote AA genotype at TNFα –308 showed the original 107 bp fragment intact (lacking the NcoI site) while the homozygote GG genotype showed two fragments of 87 and 20 bp. The heterozygote GA genotype showed three fragments of 87, 20, and 107 bp. Moreover, the sequence results confirmed the integrity of NcoI restriction site surrounding area and verified the results of TNFα –308 PCR-RFLP analysis. The sequence results of TNFα –308 different genotypes were shown in Figure (1B).

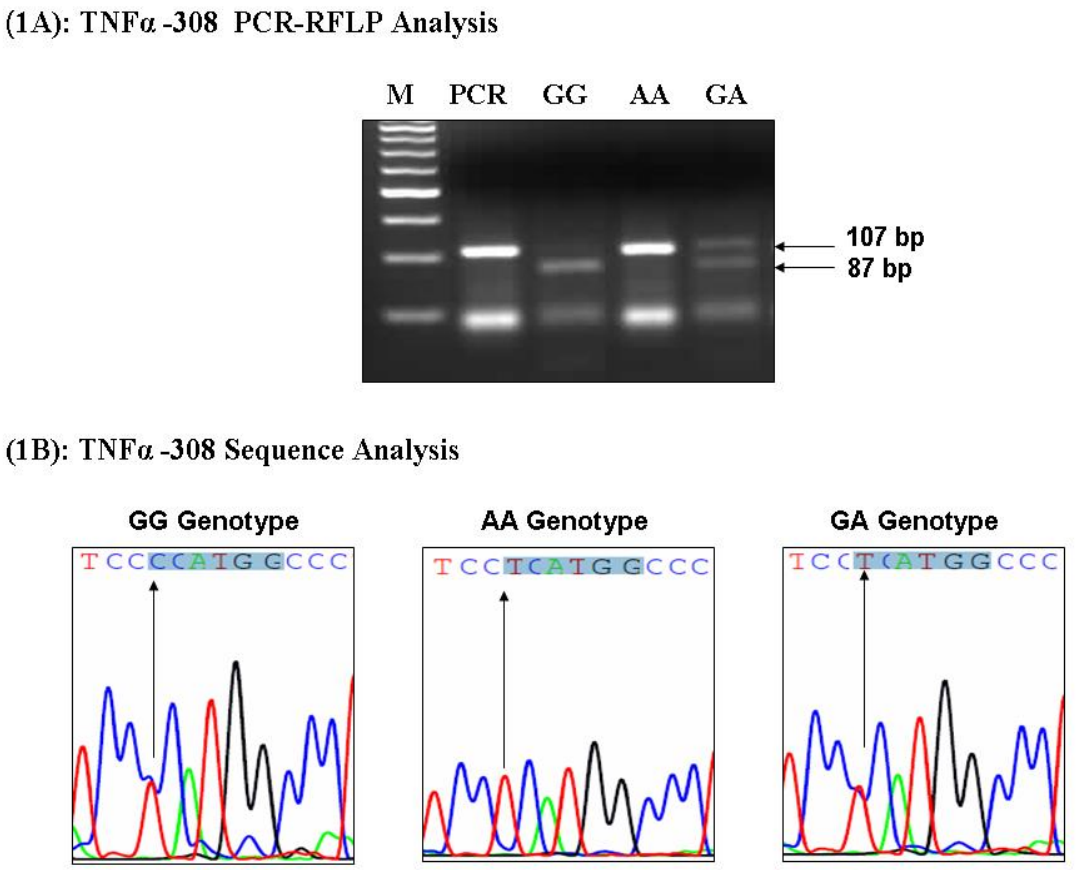

Figure 1 TNFα –G308A polymorphism analysis. (1A): TNFα –308 (PCR-RFLP) analysis, genomic DNA was extracted, amplified by PCR, digested with NcoI restriction enzyme, and run on 4% agarose gel. Lanes 1, 2, 3 and 4 correspond to PCR product before digestion, GG homozygote (87 bp), AA homozygote (107 bp), and GA heterozygote (107 and 87 bp), respectively. (1B): TNFα –308 PCR sequence analysis, the PCR products of different TNFα –308 genotypes were

purified and sequenced using the reverse primer. The Nco1 restriction site is highlighted and the arrow points to the single nucleotide polymorphism.

- Anti-TNFα therapy is generally effective and well tolerated in the setting of HCV-patients please added a comment regarding this clinical aspect in your paper.

We added a paragraph at the ending of the discussion as shown below:-

Several experimental studies showed that the inhibition of TNFα signaling by anti-TNFα antibodies or compounds could reduce inflammation, liver injury and improve fibrosis[40,41]. However, complete neutralization of TNFα in alcoholic hepatitis patients was associated with serious infectious complications[42]. Therefore, it was recommended to use pentoxifylline (PTX) which partially attenuate TNF-α level and has lower infectious complication rates. It was proved that PTX therapy effectively reduces the liver biochemical parameters and improves the histological injury in NASH patients[43].

References:

40. **Bahcecioglu** IH , Koca SS, Poyrazoglu OK, Yalniz M, Ozercan IH, Ustundag B, Sahin K, Dagli AF, Isik A. Hepatoprotective effect of infliximab, an anti-TNF alpha agent, on carbon tetrachloride-induced hepatic fibrosis. *Inflammation* 2008; **31**: 215-221 [PMID: 18427963 DOI: 10.1007/s10753-008-9067-1]

41. **Koca SS** , Bahcecioglu IH, Poyrazoglu OK, Ozercan IH, Sahin K, Ustundag B. The treatment with antibody of TNF-alpha reduces the inflammation, necrosis and fibrosis in the non-alcoholic steatohepatitis induced by methionine- and choline-deficient diet. *Inflammation* 2008; **31**: 91-98 [PMID: 18066656 DOI:10.1007/s10753-007-9053-z]

42 **Frazier TH** , Stocker AM, Kershner NA, Marsano LS, McClain CJ. Treatment of alcoholic liver disease. *Therap Adv Gastroenterol* 2011; **4**: 63-81 [PMID: 21317995 DOI:10.1177/1756283X10378925]

43. **Satapathy SK**, Sakhuja P, Malhotra V, Sharma BC, Sarin SK.Beneficial effects of pentoxifylline on hepatic steatosis, fibrosis and necroinflammation in patients with non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2007; **22**: 634-638 [PMID: 17444848 DOI: 10.1111/j.1440-1746.2006.04756.x]