

Dear Editor,

Thank you for carefully reviewing our manuscript titled "Effects of hepatitis E virus infection on interferon production via ISG15" for possible publication in the World Journal of Gastroenterology. We are grateful to you and your reviewers for their constructive critique. I have been in accordance with the requirements of the Journal changed my manuscript. We have revised the manuscript, highlighting our revisions in red and have attached point-by-point responses detailing how we have revised the manuscript in response to the reviewers' comments below. Thank you for your consideration and further review of our manuscript. Please do not hesitate to contact us with any further questions or recommendations.

Yours Sincerely,

Deying Tian

For Reviewer #1: In this experimental study, entitled “Effects of hepatitis E virus infection on interferon production via ISG15”, Min Wang et al demonstrated that HEV leads to over-production of interferon (IFN) alpha and beta in a liver cell line. Moreover, they showed that this process could be mediated by ISG15, an interferon-induced protein. The article is well written and easy to read. Moreover, the design of the study is clear and results have been illustrated effectively in the text as well as in the figures. Only few minor criticisms could be moved.

1) Authors designed 3 different siRNA to silence ISG15. They should report more details about them, for example their sequence in a supplementary table.

Thanks for the suggestion. In the revised manuscript, we added a Table 1 to explaining 3 different siRNA sequence.

2) Authors did not report the concentration of plasmids, siRNA and ISG15 in their experiments. This detail is of major importance for ISG15 since, as Authors themselves hypothesized (page 11 lines 1-3), it could act through a negative feedback mechanism. In this case, it would be interesting to assess which ISG15 concentration may elicit this feedback.

Thanks for the suggestion. The details about the concentration of plasmids, siRNA and ISG15 were added in the revised manuscript. In our study, C3A cells were transfected with 2ug ISG15 plasmids. It is of great significance to determine whether the other ISG15 concentration that you proposed could also play a negative regulatory role, and we will focus on the following experiments.

3) An aspect that was not investigated was cell replication. Indeed, it would be remarkable to evaluate whether HEV could induce cell apoptosis or replication and if those events could be mediated by IFN or ISG15.

Thanks for the suggestion. In our study, HEV-infected C3A cells retained the morphology characteristics of hepatocyte-like cells, and no obvious cytopathic effect was observed by immunofluorescence. In addition, C3A is widely used as an HEV cell infection model, and there is currently no report that HEV could affect its replication.

For Reviewer #2: Wang M et al. submitted the manuscript entitled "Effects of hepatitis E virus infection on interferon production via ISG15" for peer review. The Authors study the potential effects of hepatitis E virus (HEV) on the production of Type I interferons (IFNs) and its associations with production of ISG15, one of many interferon stimulated genes. The manuscript is well written , seems accurate and well organized. My major comments mainly concern the descriptions of the techniques used by authors.

1) Why did the authors use the C3A cells. A study conducted on other liver cell lines would have greater cognitive value.

In our previous study, we have investigated the impact of HEV on PLC/PRF/5 and A549 cells and found C3A cells have better infectious efficiency than the two cells during HEV infection.

2) Whether in the transfection processes different concentrations of HEV construct and plasmids for ORF3 and ISG15 were used. What concentrations were used for the results presented in manuscript.

2ug of ORF3 or ISG15 plasmid was used in the manuscript. The concentrations of HEV construct was 30copies per cell. The concentration of HEV construct and plasmids for ORF3 was used according our previous study (He M, Wang M, et al. The ORF3 Protein of Genotype 1 Hepatitis E Virus Suppresses TLR3-induced NF-kappaB Signaling via TRADD and RIP1. Sci Rep, 2016. 6: p. 27597). The impact of different concentrations of ISG15 plasmid on IFN will be investigated in the following experiments.

3) The authors should describe in more detail or place references on the construction of constructs.

Thanks for the suggestion. More details about construction of constructs were added in the section of reagents and plasmids in the revised manuscript.

4) Real time PCR; What housekeeping gene was used in the study. Please put the primer sequences for the test gene and housekeeping normalization gene in the manuscript. How authors removed residual genomic DNA in RNA samples obtained by TRIzol extraction.

Thanks for the suggestion.  $\beta$ -actin was used for housekeeping gene. We added a Table 1 to show the primer sequences for the test gene and housekeeping normalization gene in the revised manuscript. The cloning process and the PCR process are performed separately at different time periods to make sure that there is no DNA contamination, and the residual genomic DNA in RNA samples could be removed by DNase.

5) Legends for figures should be described in more detail - developing Con, GFP or NC abbreviations.

The details about Con, GFP or NC abbreviations were added in the revised manuscript. C3A cells were transfected with pEGFP-N1 empty vector (GFP), and untreated cells were used as the control (Con). Normal control (NC) indicates the untreated cells.

For Reviewer #3: Acute hepatitis E is caused by infection of hepatitis E virus (HEV) that likes the hepatitis A virus, it is orally transmitted, the clinical symptoms are similar to hepatitis A, and transient infection without chronicity except in special cases such as immunodeficiency. Hepatitis E is a constantly sporadic disease occurring in developing countries, but it is sometimes known to cause a large-scale epidemic via drinking water. Hepatitis E has been considered as a disease in areas where hygienic

environments are not well established. In this paper, using HEV 's infectious clone, it was revealed that HEV replication causes interferon production and at the same time induces ISG 15 that brakes interferon production. Major point

1. What is the role of ORF3 in HEV replication?

In HEV replication, ORF3 protein is associated with HEV egress and spreading to other cells (Nagashima S et al. A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells. J Gen Virol 2011).

2. Previous studies revealed that the ORF3 protein plays an important role in releasing HEV particles from infected cells as a result of analysis using ORF3 deficient mutant clones. In this paper, why does ORF3 deficient mutant clone not induce in interferon?

In our study, we found HEV ORF3 protein could enhance the production of IFN- $\alpha/\beta$ , and the ORF3 products of genotype 1 HEV have also been reported to enhance the production of IFN, so the ORF3 deficient mutant clone could not induce in interferon.

For Reviewer #4: The authors investigated interferon production with HEV infection to hepatoma cells C3A. They found that ORF3 suppressed the interferon production. The infection model of HEV to C3A was useful.

C3A is a derivative of HepG2. Brief introduction of C3A would be necessary for readers to understand the rationality of C3A.

Thanks for the suggestion. In the revised manuscript, we added a Brief introduction of C3A in the section of cells and virus.

Did the authors analyzed cell lines other than C3A?

Thanks for the suggestion. PLC/PRF/5 cells and A549 cells were also analyzed in our previous study.

Titration of the HEV was not clear. How did the authors determine the titer of HEV? This information is necessary for the other researchers to perform experiments with HEV.

Thanks for the suggestion. HEV RNA was first separated by gradient density centrifugation, and real-time PCR was used to detect the copies of HEV RNA. It was not clear how the authors avoid infection of HEV. How did the authors achieve biological safety to them?

First of all, the relevant personnel were injected with HEV vaccine. The HEV is sterilized by autoclaving and operated in a biological safety cabinet. As the HEV is spread through the fecal-oral transmission, the chance of being infected will be little if HEV do not enter mouth. If the liquid of HEV virus is splashed on the body, flush it with plenty of water immediately.

Plasmid construction of ORF3 was absent. How the plasmid was constructed should be clearly presented so that the other researchers could follow the experiments.

Thanks for the suggestion. More details about construction of constructs were added in the section of reagents and plasmids in the revised manuscript.

Construct of HEV infectious cDNA was absent. The virus production procedure was followed the manufactures instruction. But this procedure should be clearly stated.

The full length cDNA is synthesized by a bio-engineering company and added to the vector. More details about the virus production procedure were added in the revised manuscript.

Western blot analysis. Dilution or concentration of antibodies was not clear.

Thanks for the suggestion. More details about dilution or concentration of antibodies were added in the section of reagents and plasmids in the revised manuscript.

Real-time PCR. PCR machine was not described.

Thanks for the suggestion. A Roche Light Cycler 480 II Real-Time PCR System was used in the present study.

Primer sequences were absent. What internal control did the authors use?

Thanks for the suggestion. Primer sequences were added in a Table 1 in the revised manuscript. The internal control was  $\beta$ -actin.

What did the authors have in mind regarding the application of this experiments?

In the present study, we found HEV could inhibit the level of type I IFN through regulating the expression of ISG15, so ISG15 could be the target for the development of new antiviral drugs.