Biomedicine and Chemical Sciences 2(2) (2023) 131-138

10-BCS-1808-442

Content lists available at:
www.journals.irapa.org/index.php/BCS/issue/view/23

Biomedicine and Chemical Sciences

Journal homepage: https://www.journals.irapa.org/index.php/BCS

The Effect Of Hepatitis E Virus Open Reading Frame 3 Protein on The Expression Levels of IFN-β,IL6,TNF-α and RANTES in SW480 Colon Cancer Cells

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ARTICLEINFO

Article history:
Received on: January 17, 2023
Revised on: February 5, 2023
Accepted on: February 15, 2023
Published on: April 01, 2023

Keywords:
Hepatitis E virus
HEV immune response
HEV ORF3
HEV ORF3 plasmid
Transfection

ABSTRACT

The Hepatitis E virus (HEV) could induce chronic hepatitis and liver failure with high mortality through unclear mechanisms. The purpose of this study was to determine the effect of (recombinant fusion protein, ORF3 of genotype 1 HEV) on the expression levels of cytokine or chemokine genes in colon cancer cells (SW480) using a quantitative assay of "SYBR green" real-time PCR. An ORF3 protein expression system (Orf3-GFP plasmid) was synthesized in the company of GenScript (USA) and used as an expression vector, SW480 cells were infected by (Orf3-GFP plasmid) or GFP- control vector. Result of this study was approved after assessing the expression level of the ORF3 gene in ORF3 expressing cells and in control groups using ORF3 specific primer to ensure that the transfection was performed successfully, The expression levels of three genes (IFN-β, IL-6, and TNF-α) were estimated in ORF3-expressing cells using specific primers for all genes. In addition, the role of HEV Orf3 in upregulating RANTES expression levels was revealed. In conclusion: In SW480 cells, this study found that the ORF3 protein of genotype 1 HEV is capable of regulating the expression of multiple immune response genes including Interferon- β (IFN-β), *Interleukin-6 (*IL-6*)* Tumor necrosis factor-α (TNF-α) and regulated on activation, normal T cell expressed and secreted (RANTES) which may enhance viral replication and pathogenicity.

1. Introduction

Hepatitis E virus V (HEV) outbreaks are increasingly reported. Infection with HEV can cause acute hepatitis or, in rare cases, fulminant hepatic failure, although the disease is typically sporadic and self-limiting. Throughout the second and third months of pregnancy, HEV infection is also associated with high mortality rates of around 20 percent (Wu et al., 2020). The virus is small, non-enveloped (27-34 nm in diameter) and belongs to the family Hepeviridae, genus Orthohepevirus3, It has a linear, single-stranded, plus-sense RNA genome with three open reading frames: ORF1, ORF2, and ORF3 (Ledesma et al., 2019). ORF1 encodes a non-structural polyprotein associated with HEV replication (Cierniak et al., 2022), while ORF2 encodes a capsid protein involved in a virion assembly and immunogenicity (Boumaiza et al., 2021). ORF3 encodes a multifunctional phosphoprotein that suppresses host inflammation, protects virus-infected cells, and links intracellular signal transduction pathways (Maurer et al., 2022). The virus has four different genotypes but only one serotype. Genotypes 1 and 2 are restricted to human transmission, but genotypes 3 and 4 are zoonotic. There is a higher prevalence of HEV genotype 1 in young people with acute hepatitis (Wu et al. 2020). To replicate and cause pathogenicity, The HEV, like the majority of other viruses, modifies the machinery of the host cell. It would suggest that the ORF3 protein (pORF3) reduced the inflammatory response of the host cell (Chandra et al. 2011). This study aimed to analyze the effect of HEV ORF3 protein on the

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How to cite:

Abdulnabi, R. M., Othman, R. M., & Sada, H. K. A. (2023). The Effect Of Hepatitis E Virus Open Reading Frame 3 Protein on The Expression Levels of IFN-β,IL6,TNF-α and RANTES in SW480 Colon Cancer Cells. *Biomedicine and Chemical Sciences, 2*(2), 131–138.

DOI: **https://doi.org/10.48112/bcs.v2i2.442**

innate immune response of colon cancer cells represented by its regulatory effect on Interferon- β (IFN-β), *Interleukin-6 (*IL-6*),* Tumor necrosis factor-α (TNF-α) and regulated on activation normal T cell expressed and secreted (RANTES) genes expression.

2. Materials and Methods

ORF3_pcDNA3.1 (+)-P2A-eGFP was the plasmid (Orf3-plasmid) synthesized in the company of GenScript (USA) which was used as an expression vector. The open reading frame three encodings the ORF3 protein of the hepatitis E virus (genotype 1) retrieved from GenBank (Accession no.LC061267). pcDNA3.1 (+)-P2A-eGFP was the empty vector used as Control (GFP vector).The study used the Human colon cancer cell line (SW480) which in the previous report showed 59% efficiency for the transfection (Gray et al. 2018). The cell line was obtained from a company of Rawafid al-Eloom for scientific and research training in Hilla/Iraq. SW480 cell line was cultured in a Roswell Park Memorial Institute 1640 medium (RPMI 1640, Capricon Scientific GmbH, German)) supplemented with 10% fetal bovine serum (FBS), 80 mg/2 mL gentamycin (BRAWN, India). The optimal split ratio was 1:8 (seeding density 2-3x10, 000 cells/cm²). Cells were incubated at 37°C with a relative 5% CO2 humidified atmosphere.

Transfection of Cell Lines

When the monolayer density reached more than 80% confluence, the cells were passage. In a 24-well plate containing 500 µl of RPMI 1640 culture for each well, cells were seeded at a density of 0.5 x 10^6 cells per well. Transfection protocol achieved according to the supporting protocol attached to the kit of DNAfectin™ Plus Transfection Reagent (Applied Biological Materials Inc., Cat. No. G2500). After 24 hours, the plate was divided into 4 groups or rows (each group including three wells).Cells of the first row of plate transfected with Orf3- GFP plasmid (Orf3 group) while the second row transfected with GFP-vector (GFP group). for both the two rows, the complexes were prepared as follows: Solution a: Dilute 0.9 µg of DNA into 150µl of the serum and antibiotic-free medium. Solution b: 2.7µl of transfection reagent (lipofectamine) added to 150µl of serum and antibiotic-free medium, then both Solution a and b were incubated at room temperature (R.T) for 5 minutes. The solutions were mixed gently and then incubated for 20 min. at R.T. After that, 100 µl of the last solution was injected into each well. On the other hand, cells of the third and fourth rows used as control when the transfection reagent (lipofectamine) alone added to the third one (lipofectamine group) while the fourth row remained mock cells. After transfection, a 24-well plate was incubated at 37°C for 24 hours.

Evaluate Gene Expression

Harvesting

RNA isolation from a cell line was performed using GENEzol™ TriRNA Pure Kit Taiwan (Cat. No.: GZXD100) according to the manufacture instructions.

Converting RNA to cDNA

Before converting RNA to cDNA, The concentration of purified RNA was determined by using a Nanodrop spectrophotometer (NanoVue, USA). Total RNA extracted from each of the four groups was measured to ensure concentrations were comparable. The 2x Add Script RT Master kit from (Addbio, Korea) Ref 22101, Lot 2001A was used in reverse transcribed to cDNA. According to the manufacturer's instructions, mixed oligo-dT and random hexamers were utilized as primers for first-strand cDNA synthesis Table (1).

Gene Expression

Quantitative Polymerase Chain Reaction

Only one type of cell line used in the study was (SW480) to assess the gene expression of the ORF3, IFN, IL-6, TNF-α, RANTES, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (which was used as endogenous calibrator genes) in transfected and control treatments (ORF3-GFP plasmid, GFP vector, transfection Reagent and Mock cells). The primers that were used to determine gene expression are displayed in table 2 .For gene expression, The cDNA sample concentrations adopted in the reactions were between 5 and 15 ng/µl. The Real-Time PCR reaction was performed using Add SYBR Master (2x conc.) kit, Addbio, Korea (Cat.No.REF70201) according to the instruction enclosed with the mentioned kit, Briefly, the qPCR reaction mixture (20µl) was composed of 5µl of Template cDNA, 10 µl Add SYBR Master Mix (2x conc.), 1 µl Forward primer (10 µM), 1 µl Reverse primer (10 µM), 3µl Nuclease-Free D.W. The qPCR experiments were achieved by denaturation at 95°C for 5min, then 45 cycles at 95 °C (10s),58 °C(the 20s), and 72°C(15s). The average and standard deviation were extracted. In addition to Cycle threshold and baseline values were determined using the machine software and the results were compared to the control samples. All qPCR products were subjected to the melting curve analysis to guarantee primer specificity, the melting and amplification curves of the target genes were examined to qualify and quantify gene expression. ($2^{-\Delta\Delta Ct}$) was the method used to perform relative quantification.

Table 1
Thermal cycling of cDNA synthesis (Reverse transcriptase)

NO	Step	Temperature ºC	Time/min
1	Priming	25	10
2	Reverse transcription	50	60
3	RT inactivation	80	5

Statistical Analysis

In this study, the overexpression of the Orf3 gene was represented as the mean standard deviation (mean SD). All statistical analyses employed the ANOVA test; IBM SPSS was utilized (version 26.0). When the p-value was less than 0.05, we considered statistical differences or connections to be significant.

Table 2
Primers were used in this study to detect the gene expression.

Gene	sequences	Reference
GAPDH	F 5-GGTCGGAGTCAACGGATTT-3	(Atwan 2016)
	R 5-CCAGCATCGCCCCACTTG-3	
ORF3	F 5-GGTGGTTTCTGGGGTGAC-3	(Abravanel et al. 2012; Rivero-Juarez et al., 2017)
	R 5-AGGGGTTGGTTGGATGAA-3	
IFN-β	F 5-ATTGCCTCAAGGACAGGATG-3	(Xu et al., 2021)
	R 5-GGCCTTCAGGTAATGCAGAA-3	
TNF-α	F 5-CTCTTCTGCCTGCTGCACTTTG-3	(An et al., 2018)
	R 5-ATGGGCTACAGGCTTGTCACTC-3	
IL6	F 5-CCAGCTATGAACTCCTTCTC-3	(Wu et al., 2020)
	R 5-TACACCAGTGGCAAGTGCTC-3	
RANTES	F 5-TGGCTCGGACACCACTCCCTG-3	(Paroha et al., 2019)
	R 5-GTTGGCACACACTTGGCGGT -3	

3. Results and Discussion

Primer and Amplification Curve Specificity

Analysis of melting curve was performed on all PCR amplicons. RANTES , IFN-β, IL-6, TNF-α, ORF3, and GABDH the control gene, all showed only one peak as in Figure (1). SYBR green emission produced a quantifiable fluorescence signal, which was discovered from reaction tubes of all genes mentioned above and their appearing at early cycles, it was sufficient to be differentiated from the negative group. The ORF3 qPCR-specific primers were used to detect the ORF3 amplification curve, Expression levels of all genes were measured using their specific primers then the amplification curves were examined, and the thresholds were automatically adjusted for all genes as shown in Figure 2. All relative expression was determined by subtracting to the GAPDH CT value. The data are reported as the mean and standard deviation of the three technical replicates after being normalized to the control values. The gene expression profile was generated in each case using ΔΔCT analysis.

Relative Gene Expression of HEV ORF3

RNA that was extracted from cells and reverse transcribed to create the first strand of DNA was used as the template to assess the HEV ORF3 gene expression in SW480 cells. cDNA template has been mixed with specific primers to amplify the HEV ORF3 and subsequently measure its gene expression via qPCR SYBR green. HEV ORF3 gene expression was conducted in groups transfected by ORF3-GFP plasmid, GFP vector and treated with lipofectamine or left as mock cells. The results showed that HEVORF3 expression in the ORF3-GFP group increased 60fold (P-value =0.0001) compared to other treated samples. The ΔΔ CT analysis has been used for concluding the expression of the HEV ORF3 gene and CT value of GAPDH which is withdrawn as the housekeeping gene as in Figure 3 .To determine the fold change difference, the value of the control groups were normalized as shown in Figure 4 . The final battleground for survival is the host-pathogen interaction. Understanding the functions that viral and cellular proteins play in determining the host's susceptibility to infection and illness has advanced significantly.

It has been proposed that the pathogenic mechanism of HEV is primarily mediated by the immune system and does not need direct viral replication (Lhomme et al., 2020). This occurs in acute and chronic infections with a variety of extra-hepatic symptoms linked with altered immunological responses (Sooryanarain et al., 2021). Transcription factors that bind to particular places in the gene's promoter/enhancer regions control how much a gene is expressed (Chandra et al., 2011). In this study, specific primers were used in real-time RT-PCR analyses of selected genes to confirm the effects of pORF3. Expression of the ORF3 gene was highly significant (P-value=0.0001) which increased to more than 60 folds in the ORF3 group. While there were no CTs of ORF3 behind the negative ones, and the real values of the expression were obtained by ΔΔCT analysis which was also adopted in (Hameed, et al., 2022; Mohammed et al. 2020; Ridha & Atwan 2022; Sangour et al., 2021). Results of expression levels of ORF3 were necessary to confirm the success of the transfection using a specific ORF3 primer.

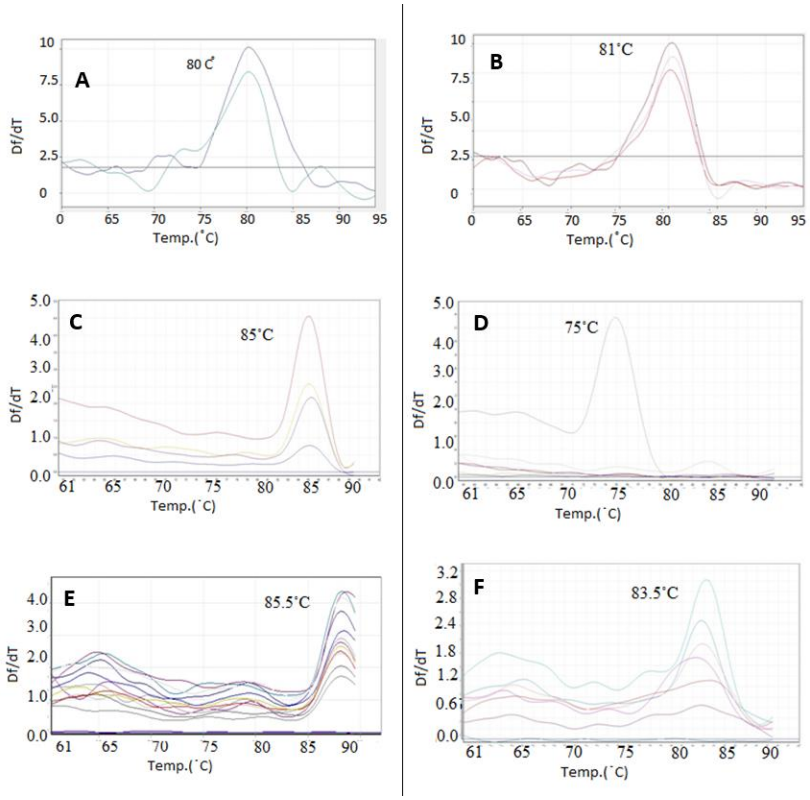

Fig. 1. Melting curve analysis of all genes. Specificity plot was gained from each gene amplified by absolute qPCR SYBR green which shows one peak at (81˚C) for GAPDH (A), also 81˚C for ORF3 (B), 85˚C for IFN-β (C), 75˚C for IL6 (D), 85.5˚C for TNF-α (E) and(83.5˚C) for RANTES (F) respectively.

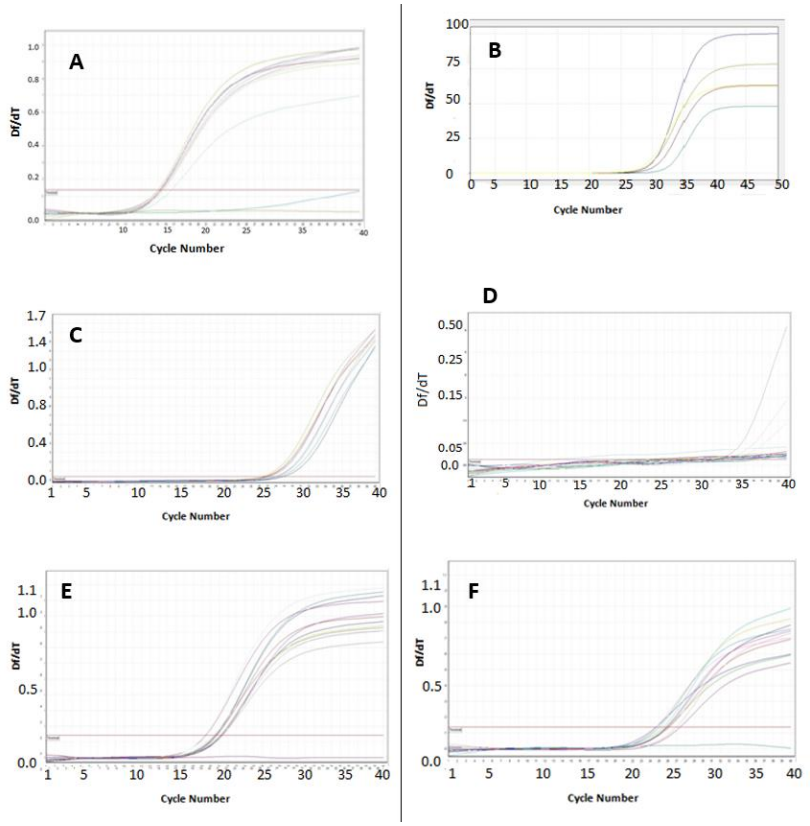

Fig. 2. Clear Amplification curves of genes GAPDH (A), ORF3 (B), IFN-β (C), IL6 (D), and TNF-α (E) and RANTES (F) respectively. The threshold was set automatically by the machine.

Relative Expression of IFN-β Gene

By measuring the mRNA expression of the genes mentioned above in SW480, it was possible to assess how the HEV ORF3 affected the stimulation of some inflammatory cytokines and chemokine. IFN-β gene expression was measured by qPCR using SYBR green dye and IFN-specific primers. The outcomes demonstrated that the expression level of IFN-β in comparison to GFP group and lipofectamine group decreased to 5 fold and one fold respectively. The differences in expression levels and fold change between groups are determined in Figures 5 and 6.

According to our findings, the ORF3 product (pORF3) is the cause of the decrease of the expression level of IFN-β in ORF3-expressing cells (1.5 fold) comparison with cells of the GFP group (5 fold) which transfected with empty GFP vector, that is primarily attributable to the mechanisms developed by many viruses to combat the innate immunity to replicate effectively inside the cell without any restriction by the body defenses, such as IFNs (Salman et al. 2021). The IFN response is initiated during viral infection by recognizing viral DNA, RNA, or other products by receptors in infected or neighboring cells (Koyama et al. 2008). He et al., (2016) observed that ORF3 protein downregulates Toll-like receptors 3-mediated NF-κB (Nuclear factor kappa B) signaling via TRADD (Tumor necrosis factor receptor 1-associated death domain protein) and RIP1 (*Receptor-interacting serine/threonine-protein kinase 1),* which is consistent with our findings and that explains the low levels of IFN since NFκB is the primary factor that stimulates IFN production. These viral proteins affect the activation of certain transcription factors in the cytoplasm of infected cells, which not only induce an antiviral state in adjacent uninfected cells but also serve as crucial regulators in the induction of an adaptive immune response (He et al., 2016). There is evidence that HEV inhibits IFN-β signaling in A549 cells through an ORF3-mediated reduction of STAT1 phosphorylation (Lei et al. 2019).

Relative Expression of the IL6 and TNF-α Genes

qPCR was performed using SYBR green dye and IL6-specific primers. Our results showed the expression level of the IL6 gene was reduced in ORF3 group 15 folds compared with the GFP group. The differences in expression levels and fold change between groups are determined in Figures 7 and 8. TNF-specific primers and SYBR green dye were used in qPCR to analyze TNF-α gene expression. Although there is a slight increase of TNF-α gene expression level (1 fold) in the ORF3 group compared to both Lipofectamine and Mock cells groups, there is a 2fold decrease in comparison to the GFP group. The differences in expression levels and fold change between groups are determined in Figure 9 and 10. In ORF3-expressing cells, the pORF3 dramatically reduced the levels of the inflammatory cytokines IL-6 and TNF-α compared with GFP group. This is consistent with the findings of He et al., 2016 that HEV ORF3 protein inhibited the phosphorylation of IκBα and interfered with the expression, phosphorylation, and nuclear translocation of NF-κB p65, hence inhibiting the production of (IL-6 and TNF- α). Multiple physiological activities, including the immunological response, protection against apoptosis, and inflammation, are regulated by the transcription factor NF-κB (Fitzgerald & Kagan 2020).

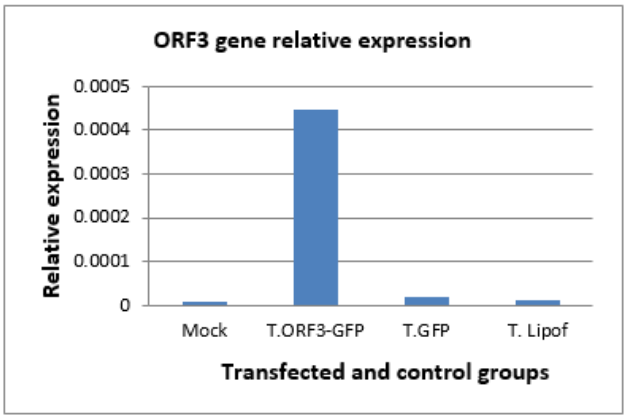

Fig. 3. HEV ORF3 relative expression level in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.ORF), GFP (T.GFP), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

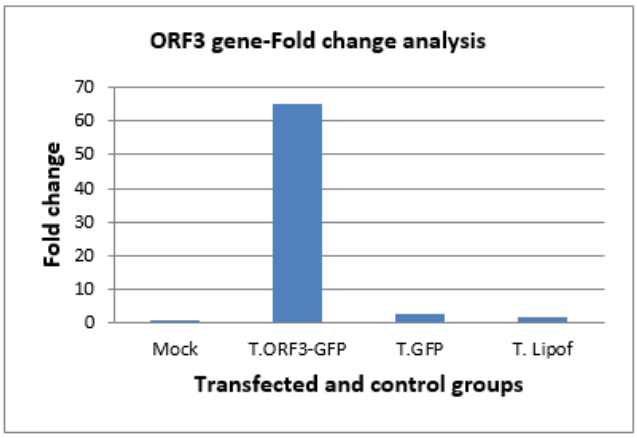

Fig. 4. HEV ORF3 fold change analysis in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.ORF3-GFP), GFP (T.GFP), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs. (P-value =0.0001).

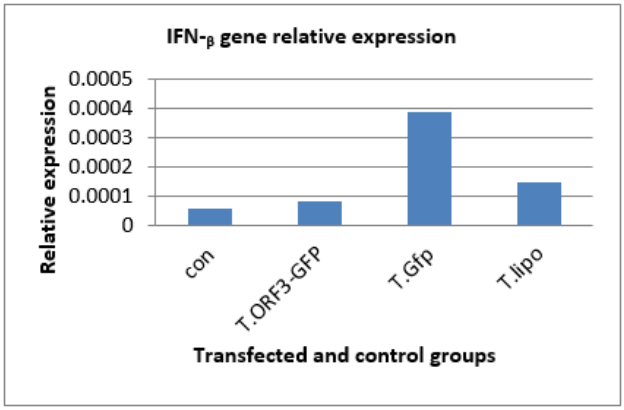

Fig. 5. IFN-β relative expression level with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

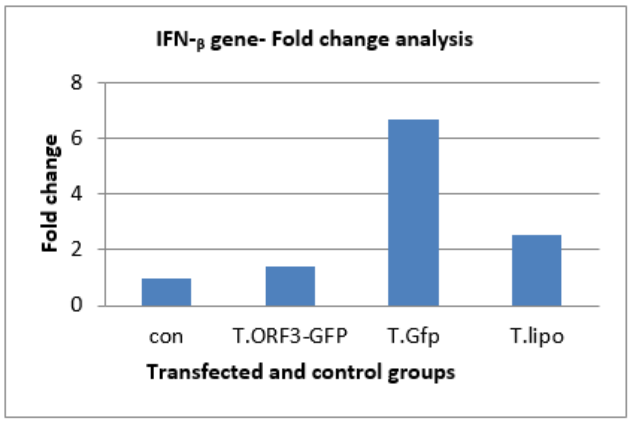

Fig. 6. IFN-β folds change analysis with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

Relative Expression of RANTES gene

In groups transfected with ORF3-GFP, and GFP, and treated with lipofectamine or left as mock cells, qPCR was used to measure the expression of the RANTES gene. RANTES expression levels were highly significant (P-value= 0.01) more than 1.5 folds increase in the ORF3 group compared to Lipofectamine or Mock cells groups. The differences in expression levels and fold change between groups are determined in figures 11 and 12. At 24 hours. The expression level of RANTES was elevated significantly in both ORF3 and GFP groups (3 folds) where the pORF3 was not caused reduction of RANTES and remained at the same level as in the GFP group. This finding is comparable to previous report (Devhare et al. 2013), when RANTES levels increased in cells infected with both the HEV-UV and HEV-live viruses. In viral infections, fibroblasts and epithelial cells are known to release RANTES, resulting in increased leukocyte recruitment. In chronic hepatitis C, intra-hepatic RANTES expression is strongly linked with the severity of hepatic inflammation (Aoki et al. 2015).

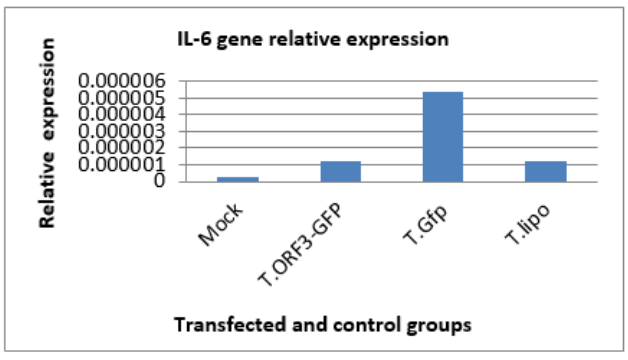

Fig. 7. IL6 relative expression level with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

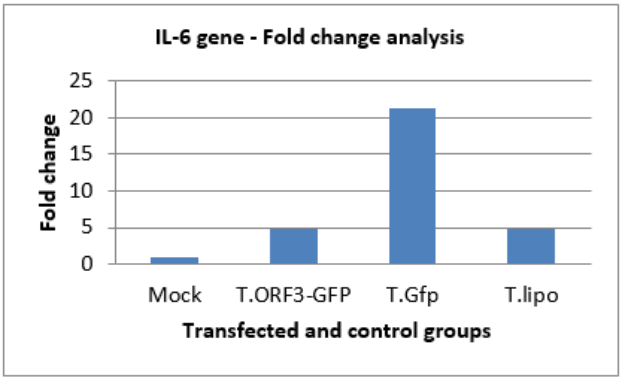

Fig. 8. IL6 fold change analysis with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

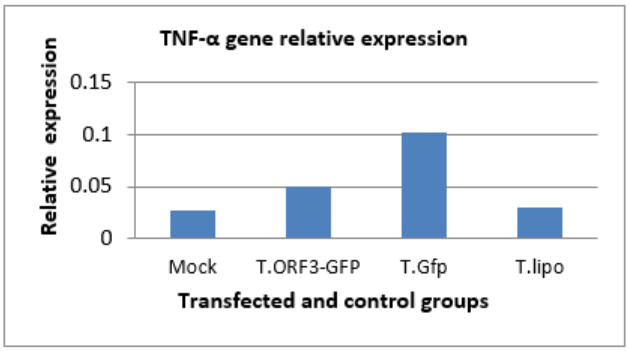

Fig. 9. TNF-α relative expression level with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels, the data was analyzed by ΔΔ CTs.

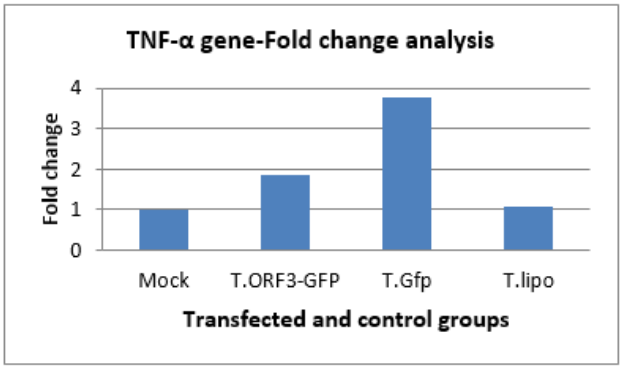

Fig. 10. TNF-α folds change analysis with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs.

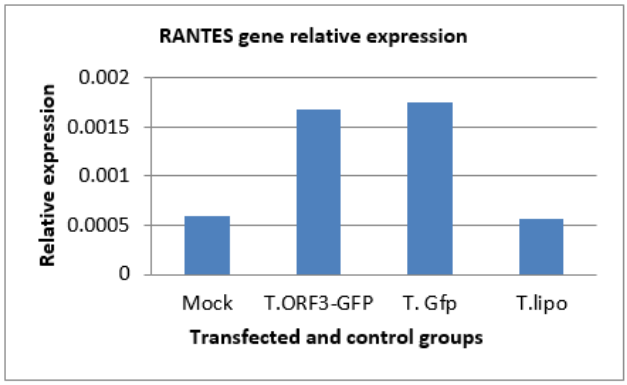

Fig. 11. RANTES relative expression level with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels, the data was analyzed by ΔΔ CTs.

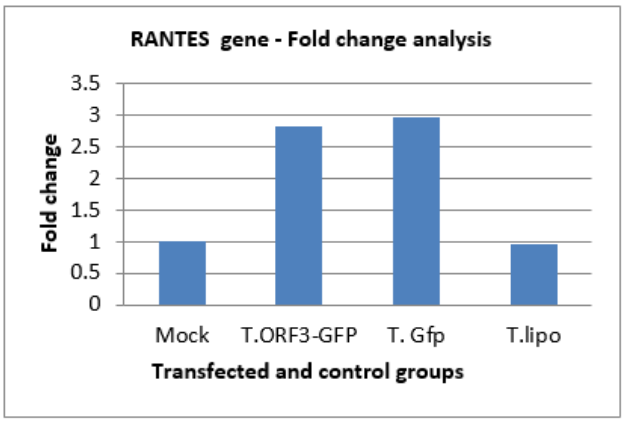

Fig. 12. RANTES fold change analysis with or without the overexpression of ORF3 in SW480. Equivalent cultures of cells were transfected with ORF3-GFP plasmid (T.Orf3-GFP), GFP (T.Gfp), and treated with a transfection reagent (T.lipo) or left as Mock cells (con.). Transcript levels were measured by qPCR and normalized to (GAPDH) mRNA levels. Finally the data was analyzed by ΔΔ CTs. (P-value= 0.01).

4. Conclusion

The HEV ORF3 product alters immunological response in ORF3-expressing cells causing persistent infection. These findings give justification for targeting ORF3 as a treatment for HEV infection.

Competing Interests

The authors have declared that no competing interests exist.

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