

ORF3 as a sensitive and specific diagnostic index for hepatitis E

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Abstract. We explored the significance of the expression of hepatitis E virus (HEV) open reading frame 3 (ORF3) in hepatitis E rat models. We also investigated its diagnostic value. Forty Sprague-Dawley (SD) rats were infected with HEV and 10 uninfected rats were selected for the control group. Rats were sacrificed at 14, 21, 35 and 70 days after infection. They were divided into 4 groups: Model group 1, model group 2, model group 3 and model group 4. ORF3 protein expression level in liver tissue, level of adipokines [fatty acid synthase (FAS), tissue inhibitor of metalloproteinase-2 (TIMP-2) and angiotensin-converting enzyme inhibitor 2 (ACE-2)], Th1/Th2 cells balance [interferon (IFN), interleukin-4 (IL-4) and Th1/Th2] and the level of immune outcome (levels of CD4⁺, CD8⁺ T lymphocytes and CD4⁺/CD8⁺) were measured and compared among groups. Our results showed that HEV IgG and HEV RNA levels in the model group 3 were higher than those in the other 3 groups. Compared with the control group, expression level of ORF3 protein in the liver tissue as well as Fas and TIMP levels were significantly higher in the model group 3. ACE-2 level was significantly lower than that of the control group ($P < 0.05$). In the model group 3, IFN- γ , IL-4 and Th1/Th2 levels were meaningfully higher than those of the control group. CD4⁺ T lymphocytes and CD4⁺/CD8⁺ ratio were obviously lower than those in the control group ($P < 0.05$). The expression level of ORF3 was positively correlated with levels of Fas, TIMP-2 and Th1/Th2. It was negatively correlated with ACE-2 and CD4⁺/CD8⁺ levels ($P < 0.05$). We concluded that ORF3 expression level was directly related to severity and prognosis, and that ORF3 protein can be considered as a sensitive and specific diagnostic index.

Introduction

Hepatitis E virus (HEV), a single-stranded, positive-sense RNA virus, is responsible for hepatitis E. HEV genome contains three open reading frames (ORFs). ORF1 encodes the

nonstructural proteins, ORF2 encodes the capsid protein and ORF3 encodes a small multifunctional protein (1-3). ORF3 protein plays an important role in viral signal transduction and immune suppression, and some believe that ORF3 may even play a role in virus pathogenicity (2,3). ORF3 protein can damage the host cell function and accelerate the release of viral particles from the infected cells (4). In this study, we explored the significance of ORF3 in the progression of the disease. We also investigated its diagnostic value. SD rat models with hepatitis E were established and the expression of ORF3 in liver tissue was studied. The effect of ORF3 on the imbalance of hepatitis E adipokines, inflammatory cytokines and immune function was also investigated.

Materials and methods

Animal sources. Fifty adult male Sprague-Dawley (SD) rats of SFP grade with gestational age of 3-5 weeks weighing 180 ± 10 g, were purchased from Shanghai Sangon Animal Experiment Center (Shanghai, China) and fed conventionally. Experiments were conducted when rats adapted to their new environment. Before the experiment, HEV IgG level in serum and liver function indexes were evaluated and results were all negative. This study was approved by the Medical Ethics Committee of Dali University.

Hepatitis E model and experimental grouping. HEV-positive pig feces were purchased from Lanzhou Veterinary Institute of Chinese Academy of Agricultural Sciences. Supernatant was collected from the feces suspension and inoculated intravenously into 40 SD rats (1 ml/rat). Daily activities of the rats were monitored. SD rats were sacrificed at 14, 21, 35 and 70 days after infection (10 rats were sacrificed at each time-point). Sacrificed rats were divided into 4 groups (10 rats/group): Model group 1, model group 2, model group 3 and model group 4. SD rats in the control group ($n=10$) were injected intravenously with 1 ml of normal saline and were sacrificed after 1 week. Fresh blood was collected and the separated serum was stored at -20°C . Liver tissues were also collected and stored at -20°C . The level of HEV IgG antibody in serum was measured using double-antigen sandwich enzyme-linked immunosorbent assay (ELISA) (Kehua, Shanghai, China), and HEV mRNA expression level was measured in serum using fluorescence quantitative PCR method.

ORF3 protein expression. ORF3 protein expression level was measured using western blot analysis. The expression of ORF3

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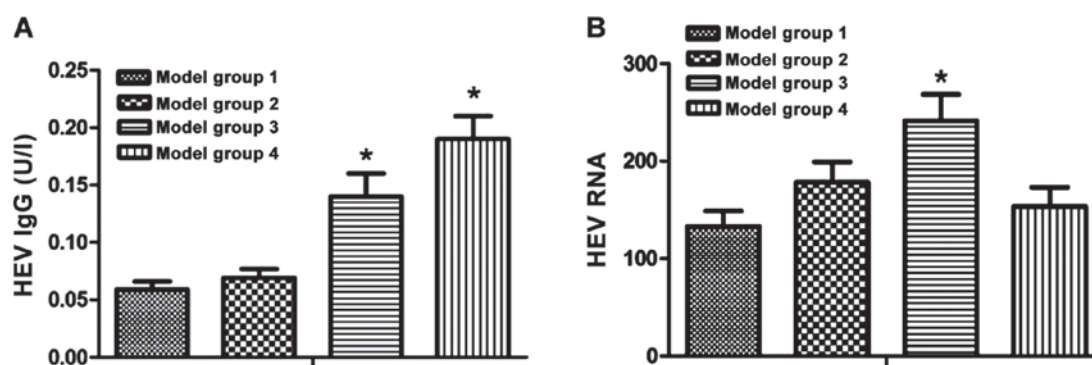


Figure 1. Comparison of expression levels of HEV IgG and HEV RNA in each model group. *P<0.05.

protein in the control group was set as 100, and the levels in each model group were calculated.

Detection of factors related to state of disease. Liver tissue homogenate was centrifuged and supernatant was collected, and adipocytokines levels were measured using ELISA. ELISA method was used to detect balance and immune outcomes of Th1/Th2 cells, levels of interferons (IFNs) and interleukin-4 (IL-4). Flow cytometry was used to determine CD4⁺ and CD8⁺ T lymphocyte levels and calculate the CD4⁺/CD8⁺ ratio.

Statistical analysis. SPSS 20.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were expressed by mean \pm standard deviation (SD). Comparisons were analyzed by one-way analysis of variance (ANOVA) and comparisons between groups were evaluated by LSD method. Comparisons within groups were conducted using independent sample t-test, and Pearson's test was used for the correlation analysis after the normal test. P<0.05 was considered to indicate a statistically significant difference.

Results

HEV IgG levels in the model group 3 and 4 were significantly higher than those of the model group 1 and 2. HEV RNA expression level in the model group 3 was significantly higher than that in other groups (P<0.05). Model group 3 was the most successful group in terms of HEV infection (Fig. 1). In the model group 3, ORF3 expression level in liver tissue was significantly higher than that of the control group, and the difference was statistically significant (P<0.05) (Fig. 2).

In the model group 3, fatty acid synthase (Fas) and tissue inhibitor of metalloproteinase (TIMP) levels in liver tissue were significantly higher than those in the control group, while angiotensin-converting enzyme inhibitor 2 (ACE-2) level was obviously lower than that of the control group. Differences were statistically significant (P<0.05) (Table I).

In the model group 3, IFNs, IL-4 and Th1/Th2 levels in the liver tissue were significantly higher than those in the control group. CD4⁺ T lymphocytes and the CD4⁺/CD8⁺ ratio were lower than those of the control group. Differences were statistically significant (P<0.05) (Table II). Pearson's test concluded that ORF3 protein expression level in the model group 3 was

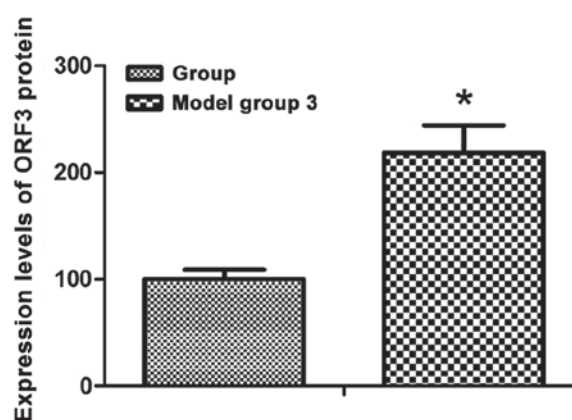


Figure 2. Comparison of expression levels of ORF3 protein in liver tissues of rats. ORF3, open reading frame 3. *P<0.05.

Table I. Adipocytokine levels in liver tissue.

Groups	Fas (mg/ml)	TIMP-2 (μg/l)	ACE-2 (ng/ml)
Model group 3	34.28±4.19	8.29±0.91	0.23±0.04
Control group	15.72±2.13	3.76±0.45	0.79±0.09
t-test	5.723	5.293	5.382
P-value	0.012	0.017	0.014

Fas, fatty acid synthase; TIMP, tissue inhibitor of metalloproteinase; ACE-2, angiotensin-converting enzyme inhibitor 2.

positively correlated with Fas, TIMP-2 and Th1/Th2 levels. It was negatively correlated with ACE-2 and CD4⁺/CD8⁺ levels (P<0.05) (Table III).

Discussion

Hepatitis E is a liver disease caused by a virus known as HEV. HEV infection can be serious in some cases and may lead to fulminant hepatic failure and death in a significant proportion of patients (5).

Early diagnosis of hepatitis E can significantly improve the prognosis. It has been reported that the three ORFs in HEV genome can be used for hepatitis E diagnosis.

Table II. Th1/Th2 cells balance and immune outcome.

Groups	Balance of Th1/Th2			Cellular immunity (%)		
	IFN- γ (ng/l)	IL-4 (ng/l)	Th1/Th2	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺
Model group 3	132.17 \pm 15.88	67.62 \pm 7.13	2.17 \pm 0.34	45.72 \pm 5.15	25.84 \pm 2.93	1.51 \pm 0.17
Control group	89.63 \pm 9.42	45.81 \pm 5.27	1.58 \pm 0.18	58.34 \pm 6.12	21.28 \pm 2.76	2.18 \pm 0.27
t-test	5.231	4.783	5.382	4.693	4.382	5.394
P-value	0.014	0.021	0.012	0.024	0.031	0.010

IFN- γ , interferon- γ ; IL-4, interleukin-4.

Table III. ORF3 protein expression level.

Indexes	Coefficient of determination r	P-value
Fas	0.393	0.021
TIMP-2	0.312	0.027
ACE-2	-0.383	0.024
Th1/Th2	0.339	0.026
CD4 ⁺ /CD8 ⁺	-0.397	0.019

ORF3, open reading frame 3; Fas, fatty acid synthase; TIMP, tissue inhibitor of metalloproteinase; ACE-2, angiotensin-converting enzyme inhibitor 2.

ORF3 which is involved in virus pathogenicity (3), encodes a protein with more than four epitopes (6). Preliminary studies discovered that ORF3 protein may be involved in MAPK/ERK transduction signal pathway and could be related to hepatocyte apoptosis (7). ORF3 protein is expressed in early phase and can trigger cell proliferation and differentiation (8). Our results suggested that the expression level of ORF3 protein in the model group 3 was significantly higher than that of other groups. Higher level of ORF3 protein expression can be used as an index for disease diagnosis (9). Our results also revealed that Fas and TIMP levels in the liver tissue in the model group 3 were significantly higher than other groups, while ACE-2 level was relatively lower. It has been shown that adipocytokines are closely related to the occurrence of infection, inflammation and fibrosis (10). Results obtained from previous studies revealed an increased level of Fas expression in cells expressing ORF3 protein (11). Other studies reported an overexpression of TIMP in animal model with chronic liver disease (12). It has been shown that ACE-2 has an inhibitory effect on the release of pro-inflammatory factors as well as growth factors. Significant decline in ACE-2 expression has been reported in cells expressing ORF3 protein (13). It was suggested that ORF3 protein may be involved in the process of fibrosis in hepatic cells.

In acute hepatitis E, cytokines are involved in Th1/Th2 imbalance. Balance disorder of Th1/Th2 is an important internal mechanism in various diseases. Th1 type factors that can lead to tissue damage mainly include IL-1, IL-12 and IFN. Th2 type factors mainly include IL-4 and IL-10. Th2 cells

mainly mediate humoral immunity and are involved in the secretion of anti-inflammatory factors.

Imbalance of Th1/Th2 in liver tissues is one of the important mechanisms in disease progression (14). A prior study (15) showed that HEV ORF3 protein in association with AMBP can suppress the proliferation as well as the migration of antigen induced T lymphocytes. It has been confirmed that cellular immunity plays an extremely important role in hepatitis A. Epidemiological and clinical characteristics of hepatitis E are very similar to those of hepatitis A, therefore it can be speculated that inhibition of cellular immune function is also an important mechanism leading to occurrence and development of hepatitis E (16). We showed that in the model group 3, CD4⁺ T lymphocytes and CD4⁺/CD8⁺ ratio in the liver tissue were relatively low. CD4⁺/CD8⁺ ratio directly reflects the state of cellular immune function. There is evidence showing that the cellular immune function suppression in hepatitis E may be related to the release of HEV particles, which is promoted by ORF3 protein (17).

Expression level of ORF3 is positively correlated with the level of Fas, TIMP-2 and Th1/Th2. It is negatively correlated with ACE-2 level and CD4⁺/CD8⁺ ratio. This suggested that ORF3 protein was directly involved in the occurrence and development of hepatitis E. We concluded that ORF3 expression level was directly related to severity and prognosis of hepatitis E. ORF3 protein can be considered as a sensitive and specific diagnostic index.

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