Relationship between sex hormones and RIG-I signaling in peripheral blood mononuclear cells of patients infected with hepatitis C virus

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Abstract. It has previously been suggested that men and women demonstrate differing immune responses to hepatitis C virus (HCV) infection, resulting in the investigation of the role of sex hormones and if they influence the anti-HCV response. The present study aimed to examine if hormone levels were associated with interferon (IFN) signaling pathways in peripheral blood mononuclear cells of 131 patients infected with HCV and 113 healthy controls. HCV infection was diagnosed based on the presence of anti-HCV antibodies and HCV RNA in serum. Expression of testosterone and estrogen was measured at the protein level using a competitive chemiluminescence immunoassay, and at the mRNA level using reverse transcription-quantitative polymerase chain reaction. HCV-infected males had increased levels of estrogen and a decreased ratio of testosterone to estrogen compared with healthy male controls (all P<0.001). HCV-infected patients demonstrated a significantly decreased expression of IFN and retinoic acid-induced gene protein I (RIG-I), RIG-I mRNA compared with controls. Pearson correlation analysis revealed that among males, levels of RIG-I correlated with levels of IFN-β mRNA (r=0.460), testosterone (r=-0.500), and the ratio of testosterone to estrogen (r=-0.477; all P<0.001). However, levels of RIG-I did not correlate with levels of IFN-α mRNA (r=0.158) or estrogen (r=0.173; both P>0.05). These results suggested that testosterone or the ratio of testosterone to estrogen may inhibit RIG-I signaling and thereby influence immune responses to HCV infection.

Introduction

Hepatitis C virus (HCV), a single-strand RNA virus of the Flaviviridae family, infects approximately 170 million people worldwide (1). HCV replication in infected individuals triggers an innate immune response that is often ineffective: As many as 80% of HCV-infected patients cannot eliminate the virus and develop chronic hepatitis, cirrhosis, and even liver cancer (2). Innate immune responses to HCV infection differ between men and women (3-5), leading to the question of whether sex hormones may influence these responses.

Innate immune responses are induced when pattern recognition receptors (PRRs), which are expressed on immune cells, especially antigen-presenting cells, recognize viral genetic material (6). PRRs include Rig-like helicase receptors, NOD-nucleotide binding oligomerization domain-like receptors and Toll-like receptors. PRRs trigger signaling pathways that induce the production and release of cytokines such as the type I interferons IFN-α and IFN-β

The central PRR activated in response to RNA virus infection is retinoic acid-induced gene protein I (RIG-I), a 5'-triphosphorylated, uncapped RNA-binding DexD/H-box helicase (7). After binding to viral RNA, RIG-I induces a host response in which the amino-caspase activation and recruitment domains (CARDs) interact with the amino-terminal CARD of the IFN-β promoter-stimulator 1 (IPS-1) (7).

The present study explored whether differences in sex hormone levels between men and women may lead to differences in RIG-I signaling in peripheral blood mononuclear cells (PBMCs) during HCV infection. If so, this may help explain why men and women differ in innate immune responses to such infection.

Materials and methods

Patients and controls. A total of 131 patients with chronic HCV infection (mean age, 53.72 years; range, 22-88) treated at Renmin Hospital of Wuhan University were enrolled in the study. HCV infection was diagnosed based on the presence in serum of anti-HCV antibodies and HCV-RNA, in accordance with guidelines from the Chinese Society of Hepatology and the Chinese Society of Infectious Disease (8). The viral strain in all patients was determined to be genotype 1b. As controls, 113 healthy aged-matched volunteers (mean age, 53.19 years; range, 17-78) were also enrolled in the study.

The study protocol was approved by the Renmin Hospital Ethics Committee [(2013) E-lun word (KY-035)], and all study participants gave written informed consent.

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Blood collection and analysis. Peripheral venous blood (3 ml) was collected from all study participants using procoagulant tubes and analyzed for sex hormone levels using a chemiluminescence immunoassay autoanalyzer (ADVIA Centaur XP, Siemens). Peripheral venous blood (2 ml) was also collected into anticoagulant (EDTA) tubes. All blood samples were processed within a few hour of collection. PBMCs were isolated by Ficoll density gradient centrifugation, and total RNA was extracted using TRIzol (Bao Biotechnology, Dalian, China). RNA showing an absorbance ratio (260/280 nm) of 1.8-2.1 was used in subsequent analysis.

This RNA template was mixed with 1 μl of oligo-dT primers, immediately centrifuged at 1,006 x g for 30 sec and then incubated at 65°C for 5 min. This mixture was combined with 4 μl of reaction buffer, 2 μl of dNTP mix, 1 μl of ribonuclease inhibitor, and 1 μl of reverse transcriptase, and then incubated at 42°C for 60 min followed by 72°C for 10 min. The resulting cDNA product was stored at -20°C until use.

This cDNA was then analyzed by real-time reverse transcription-PCR in reactions (20 μl) containing 2 μl of cDNA, 0.5 μl of each primer (Table I), 10 μl of SYBR Premix Ex Taq II (Bao Biotechnology), 0.4 μl of ROXII, and 6.6 μl of ddH$_2$O. Primers (Shanghai Biological Technology) showed good specificity based on primer dissolution curves and the amplification of a single band with the expected length. Reactions were performed on a thermal cycler (Thermo Fisher Scientific) under the following conditions: 94°C for 30 sec; 94°C for 20 sec; 50 cycles at 95°C for 15 sec, 60°C for 20 sec and 72°C for 35 sec. Reaction results were analyzed on a quantitative fluorescence analyzer (ABI VII7; Applied Biosystems). Amplification was considered efficient because the efficiency of all target gene reactions was 98-102%, and the difference in efficiency between target genes (RIG-I, IFN-α, IFN-β) and the internal reference gene (GAPDH) was <5%. This efficiency was assessed using five concentrations of 10-fold serial dilutions. Gene expression was expressed in relative units (RQ=2$^{-\Delta CT}$).

HCV RNA was quantified using an automatic nucleic acid extraction device and supporting reagents (Qiagen QIAcube), together with a LightCycler1.2 quantitative fluorescence PCR instrument (Roche). Amplicons were sequenced using an ABI 3130 (Applied Biosystems). HCV RNA load was determined using a Qiagen care HCV RT-PCR Assay V2 kit, which has a minimum detection limit of 5x10^2 IU/ml.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM, Chicago, IL, USA). Results showing a normal distribution were reported as mean ± standard error (SD), while results showing a skewed distribution were expressed as median and interquartile range (IQR). Inter-group differences in continuous variables were assessed for significance using independent t testing, while differences in categorical variables were assessed using χ^2 testing. Correlations between continuous variables were explored using Spearman correlation analysis. All p-values were two-sided, and the threshold of significance was defined as P<0.05.

Results

Characteristics of the study population. Patients chronically infected with HCV and healthy controls differed significantly

in liver function (Table II): The functional indices ALT, AST, ALP, GLB, DBIL, and hs-CRP were significantly higher in patients, while PA, TP, ALB, LDL, and PT-act were significantly lower (all P<0.01).

Serum levels of sex hormones in patients. Female patients with HCV showed similar levels of estrogen (E$_2$) and testosterone (T) as healthy female controls, as well as similar T/E$_2$ ratios (all P>0.05). Male patients with HCV showed significantly higher E2 levels than healthy males (P<0.001) and significantly lower T/E$_2$ ratio (P=0.041; Fig. 1).

Expression of mRNAs encoding IFN-α, IFN-β and RIG-I in PBMCs. Levels of mRNAs encoding IFN-α, IFN-β, or RIG-I were significantly lower in patients than in controls (P<0.001; Fig. 2).

Correlation of RIG-I mRNA levels with levels of sex hormones and IFN mRNA in males infected with HCV. RIG-I mRNA levels correlated negatively with levels of testosterone and T/E$_2$ (both P<0.001) and positively with levels of IFN-β mRNA (P<0.001; Fig. 3 and Table III).

Discussion

Approximately 170 million around the world are infected with HCV (1), more than half of whom live in China as well as a few other Asian countries (9). In China, the primary cause of HCV infection is transfusion with contaminated blood (10). Men and women show important differences in the pathogenesis of liver diseases related to HCV infection. Following HCV infection, men are at higher risk than women of progressing to chronic hepatitis, cirrhosis or hepatic carcinoma (11). Women may be more able to clear HCV virus than men of the same age, and this difference disappears after menopause (12,13). These observations suggest that sex hormones may influence outcomes of HCV infection.

Consistent with this idea, HCV-related liver fibrosis progresses faster in postmenopausal women than in premenopausal women, and estrogen replacement therapy can significantly delay this progression in postmenopausal women (14). Co-administering the estrogen analog Raloxifene with IFN and ribavirin leads to higher sustained virological response in women (61.3%) than using IFN and ribavirin (34.4%) (15). In HCV-infected patients, progesterone inhibits IFN signaling in PBMCs as well as TLR7 and MxA gene expression (16). These studies led us to ask whether sex hormones may also influence RIG-I signaling in PBMCs during HCV infection. If so, this may help explain the observed gender differences in HCV-related pathogenesis and disease outcomes.

Our results suggest that levels of mRNAs encoding IFN-α, IFN-β, and RIG-I are significantly lower in patients infected with HCV than in healthy controls (all P<0.001). This association between reduced levels of RIG-I expression and HCV infection is consistent with the ability of RIG-I to bind to viral double-stranded RNA within cells and induce an IFN-based antiviral immune response (17,18).

Among women in our study, levels of estrogen and testosterone as well as the T/E$_2$ ratio were similar between patients

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Target gene	Upstream primer (5'-3')	Downstream primer (5'-3')	Product length (bp)
RIG-I (NM_014314.3)	CCAGGGATCCCAGCAATGAG	TGTTTTGCGACGTCCAGTCA	200
IFN-α (NM_002176.2)	TGATCTCCCTGAGACCCACA	AGCAGGGGTGAGAGTCTTTGA	591
IFN-β (NM_002176.2)	AGTAGGCGACACTGTTCGTG	TGCTCATGAGTTTTCCCCTGG	446
GAPDH (NM_002046.5)	AACGGATTTGGTCGTATTGG	AGATGATGACCCTTTTGGCT	340

RIG-I, retinoic acid-induced gene protein I; IFN, interferon.

Table II. Clinical characteristics of healthy controls and patients infected with HCV.

Characteristic	Controls (n=113)	Patients (n=131)	P-value
Age, years	53.19 (12.69)	53.72 (15.35)	0.774
Male/female	61/52	62/69	0.266
ALT, U/l	17.04 (12.00-20.0)	53.67 (17.30-59.40)	0.000
AST, U/l	19.77 (17.00-23.00)	53.94 (22.00-52.00)	0.000
ALP, U/l	69.57 (55.50-79.00)	87.31 (57.25-97.00)	0.001
GGT, U/l	18.73 (12.00-24.50)	43.60 (15.75-55.15)	0.000
PA, mg/l	300.20±43.49	175.66±85.18	0.000
TP, g/l	72.06±4.50	66.87±12.32	0.000
ALB, g/l	43.05±2.85	37.31±8.34	0.000
GLB, g/l	24.91±9.89	29.08±7.20	0.000
TBIL, umol/l	11.94 (8.40-15.20)	17.56 (8.82-18.33)	0.004
DBIL, umol/l	3.56 (2.55-4.40)	8.82 (3.05-7.15)	0.001
BUN, mmol/l	4.92 (4.4.-5.60)	11.17 (3.93-6.90)	0.089
LDH, U/l	188.57±32.18	187.74±74.79	0.914
TC, mmol/l	4.22 (3.96-4.57)	43.06 (3.27-4.57)	0.126
TG, mmol/l	1.45 (0.86-1.69)	1.32 (0.72-1.42)	0.350
HDL-CH, mmol/l	1.24±0.30	1.12±0.46	0.025
LDL-CH, mmol/l	2.39±0.50	2.03±0.80	0.000
CK, U/l	94.31 (63.00-112.00)	102.89 (40.25-91.50)	0.025
hs-CRP, mg/l	1.20 (0.10-0.96)	20.69 (0.15-8.47)	0.000
PT, sec	10.98 (10.50-11.30)	13.13 (10.90-12.20)	0.047
PT-act, %	105.09 (95.65-113.90)	89.17 (77.90-104.60)	0.000
PT-INR	0.95 (0.90-0.98)	1.35 (0.94-1.05)	0.195
APTT, sec	28.20 (26.20-30.05)	30.41 (26.00-32.30)	0.025
TT, sec	18.07 (17.60-18.50)	19.05 (17.40-19.90)	0.017
FIB, g/l	2.69±0.49	2.40±0.85	0.004
D-dimer, mg/l	0.24 (0.17-0.31)	2.21 (0.15-1.01)	0.072

Values shown are mean ± SD or median (IQR). ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptadase; PA, prealbumin; TP, total protein; ALB, albumin; GLB, globulin; TBIL, total bilirubin; DBIL, direct bilirubin; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglyceride; HDL-CH, high density lipoprotein cholesterol; LDL-CH, low density lipoprotein cholesterol; CK, creatine kinase; hs-CRP, hypersensitive C-reactive protein; PT, prothrombin time; PT-act, prothrombin time-act; PT-INR, prothrombin time international normalized ratio; APTT, activated partial thromboplastin time; TT, thrombin time; FIB, fibrinogen.

and healthy controls. Among men, in contrast, levels of estrogen were higher (P<0.001) and the T/E$_2$ ratio was lower (P=0.041) in patients than in controls. Our results contrast with the proposed ability of estrogen to protect against HCV-related

liver pathogenesis, based on its ability to inhibit progression of HCV-related fibrosis (19).

We found that RIG-I mRNA levels correlated negatively with testosterone levels and T/E$_2$ ratios (both P<0.001) and
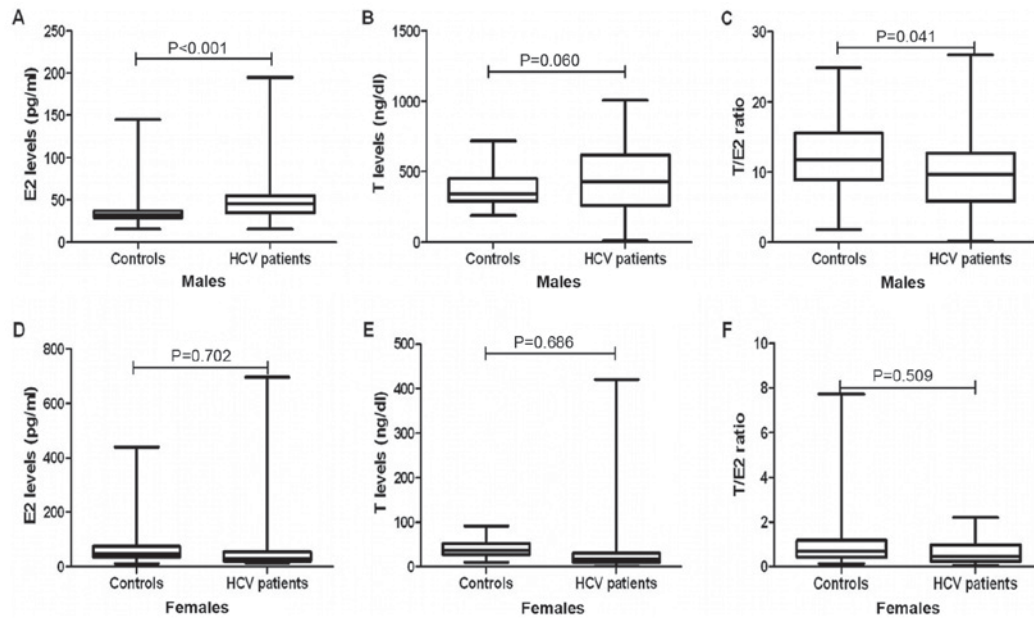
Figure 1. Comparison of sex hormone levels in healthy controls and patients infected with HCV, stratified by sex. Levels are shown as medians and interquartile ranges because data were not distributed normally. (A-C) Comparisons between male patients and male controls. (D-F) Comparisons between female patients and female controls. T, testosterone; E_2, estrogen; T/E_2, ratio of testosterone to estrogen.
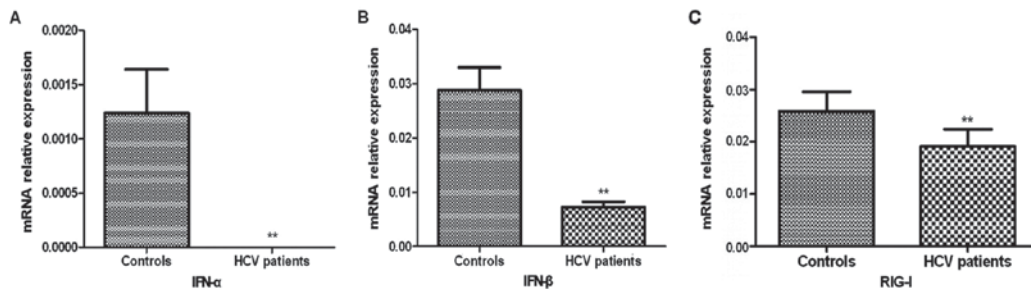

Figure 2. Expression of (A) IFN-α, (B) IFN-β, and (C) RIG-I mRNAs in PBMCs from healthy controls and patients infected with HCV. **$P<0.001$ vs. control.
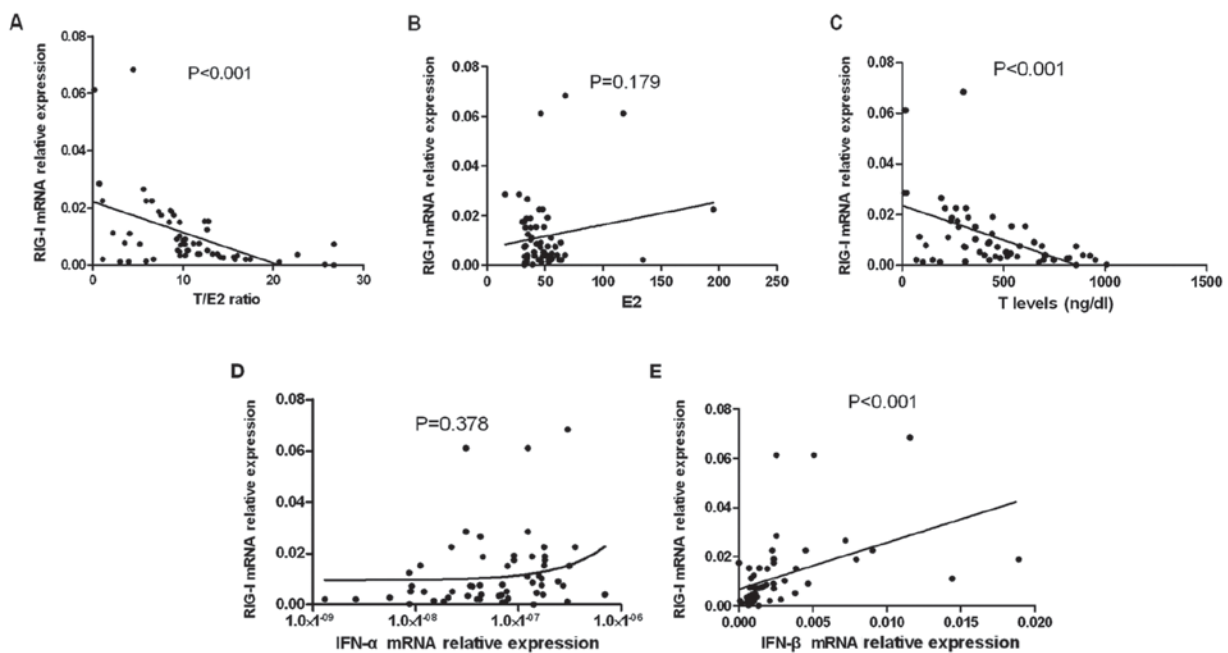

Figure 3. Correlation of RIG-I mRNA levels with levels of (A) ratio of testosterone to estrogen, (B) estrogen, (C) testosterone, (D) IFN-α mRNA or (E) IFN–β mRNA in males infected with HCV.

Table III. Correlation of RIG-I mRNA levels with levels of sex hormones and IFN mRNA in males infected with HCV.

RIG-I	T	E2	T/E2	IFN-α	IFN-β
Spearman correlation coefficient	-0.500	0.173	-0.477	0.158	0.460
P-value	<0.001	0.179	<0.001	0.221	<0.001

RIG-I, retinoic acid-induced gene protein I; IFN, interferon.

positively with IFN-β mRNA levels ($P<0.001$). These results are consistent with studies in which men with elevated serum testosterone levels exhibited the lowest levels of antibody and inflammatory cytokines following viral infection (20,21). Among men infected with HCV, each 1 ng/ml increase in total serum testosterone has been associated with a 27% increase in risk of advanced fibrosis and 16% increase in risk of advanced inflammatory activity (22). One possible explanation for these 'proviral' effects of testosterone is that it alters lipoprotein production in a way that promotes HCV replication (23). Another possibility, for which the present study provides evidence, is that testosterone inhibits immune responses to HCV infection.

Our results add to an already sizeable literature demonstrating gender-based differences in innate and adaptive immune responses (24-29); these differences influence the pathogenesis and outcomes of infectious and autoimmune diseases. Our study also provides one of the few analyses of testosterone and HCV infection. Our results suggest that, following HCV infection, estrogen promotes immune hyper-response, while testosterone inhibits immune responses. This implies that optimal immune response requires a dynamic balance between the two hormones. Future work should investigate this possibility by manipulating sex hormone levels and examining the resulting changes in RIG-I signaling in PBMCs.

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