Estrogen-related MxA transcriptional variation in hepatitis C virus-infected patients

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Sex has been reported to influence the rates of viral clearance in hepatitis C virus (HCV)-infected patients. However, little is known regarding the influence of sex on the host genetic response to HCV, which is mediated by the expression of interferon (IFN)-stimulated genes (ISGs) after the activation of janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway by IFN. Thus, we investigated gender differences in MxA genetic profile, which is a downstream reliable marker for JAK/STAT pathway activation. In all, 40 untreated HCV-infected patients were subclassified into premenopausal, postmenopausal, and male patients. The peripheral blood mononuclear cells (PBMCs) from premenopausal women showed the highest MxA gene expression compared to both postmenopausal females and males before and after IFN stimulation. The prestimulation of PBMCs with 17beta-estradiol prior to IFN treatment resulted in a decrease of MxA expression in all groups of patients. That was confirmed by the reversal of this effect using estrogen antagonist ICI182/780. This study demonstrates for the first time the presence of gender variations in the genetic response to chronic HCV infection and to interferon treatment. It also clarifies that estrogen is not the key player in enhancing the JAK/STAT pathway. (Translational Research 2012;159:190–196)

Abbreviations: cDNA = complementary DNA; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HCV = hepatitis C virus; IFN = interferon; ISG = interferon-stimulated gene; JAK = janus kinase; mRNA = messenger RNA; PEG = pegylated; PIAS = protein inhibitors of activated STATs; PKR = RNA-dependent protein kinase; RBV = ribavirin; SEM = standard of the mean; STAT = signal transducer and activator of transcription; SVR = sustained virologic response; TLR = toll-like receptor

Men and women differ in their susceptibility to viral infections. Women have been found to have an increased immune response which renders them less liable to acquire viral infections.[1] Among viral infections, chronic hepatitis C virus (HCV) infection still represents a major burden worldwide with high prevalence in Egypt.[2] The current standard treatment of chronic HCV infection consists of pegylated interferon alpha (PEG-IFNα) in combination with ribavirin (RBV).[3] Unfortunately, a response to this regimen is not achieved in all patients. This diversity in response to treatment is related to several factors, mainly the viral genotype where genotypes 2 and 3 show higher response rates compared with genotypes 1 and 4.[3] In addition, certain genetic polymorphisms near the IL28B gene recently have been found to be

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Submitted for publication May 9, 2011; revision submitted August 4, 2011; accepted for publication August 4, 2011.

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1931-5244/$ - see front matter

doi:10.1016/j.trsl.2011.08.002

AT A GLANCE COMMENTARY

Mekky R, et al.

Background

Little is known about the impact of sex on host genetic response to chronic hepatitis C infection (HCV) as well as interferon treatment. Sex-specific genetic response to IFN through sex hormones should be investigated. Thus, we aimed to investigate the effect of estrogen on genetic host response.

Translational Significance

This study obtained a clear finding that host genetic response to HCV varies between sexes with premenopausal women having the highest MxA expression. The suggested role of estrogen in enhancing genetic response to interferon treatment in HCV infection was denied in our study by demonstrating the attenuation of JAK/STAT pathway by estrogen.

associated strongly with the response to treatment.[4] Recently, sex has been suggested to be an additional factor affecting response to therapy. Female patients have higher viral clearance rates compared with male patients.[5] Furthermore, it has been demonstrated that the female sex is correlated positively with sustained virologic response (SVR), which is the goal of PEG-IFN/RBV therapy.[6] Recently, sex hormones have been demonstrated to play a critical role in sex variation in immune response to viral infections; where ovarectomy and gonadectomy have affected the interferon signaling in murine models in response to viral infections.[7] Moreover, the age-related decrease in full response to IFNα treatment in older women versus younger women has been attributed to a decrease in estrogen level.[8] However, little is known regarding the impact of sex hormones on host response to HCV on the molecular level. Host response to HCV is mediated by the production of endogenous type 1 IFN. Binding of IFN to its receptor leads to the activation of the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway and, consequently, the transcription of interferon-stimulated genes (ISGs) with antiviral activity. Among ISGs, the MxA gene was demonstrated to have a strong antiviral effect even in the absence of any other ISG.[9] In addition, MxA is regulated tightly by type 1 IFN, and thus, it is a reliable marker for the activation of the JAK/STAT pathway.[10]

In the current study, we aimed at assessing the sex differences in the genetic profile of MxA in response to both endogenous and exogenous interferon in chronically HCV-infected patients. Furthermore, the impact of estrogen on MxA gene expression was studied among different sexes to investigate the impact of estrogen on JAK/STAT pathway activation on the molecular level. The current study was performed on peripheral blood mononuclear cells (PBMCs) given that PBMCs are strong IFN producers.[11] They are even stronger IFN producers than hepatocytes.[12] Furthermore, PBMCs were shown to be a reservoir for HCV replication.[13]

MATERIALS AND METHODS

Subjects. A total of 40 patients (16 premenopausal patients, 14 postmenopausal patients, and 10 male patients) chronically infected with HCV and 29 healthy volunteers (12 premenopausal patients, 7 postmenopausal patients, and 10 male patients) were included in this study. HCV infection was diagnosed in all patients by the presence of anti-HCV antibodies and HCV RNA in the serum. All patients had a liver biopsy performed within 1 year before treatment. The histologic findings were classified according to the Metavir scoring system. The patients were all untreated but were candidates for PEG-IFN/RBV therapy. All patients were negative for the hepatitis B surface antigen. The patients were not on hormone replacement therapy or oral contraceptives. All patients and controls gave their written informed consent. All experiments were performed in compliance with the guidelines of the Institutional Review Board of Kasr El Aini Medical School in Cairo University and in accordance with the ethical standards of the declaration of Helsinki.

METHODS

Samples. A 4-mL sample peripheral venous blood was collected in the presence of an anticoagulant (ethylenediaminetetraacetic acid) from patients and healthy controls for the isolation of PBMCs. The samples were taken before starting IFN treatment. All samples were processed on the same day and within few hours after collection.

Isolation of PBMCs. The PBMCs were isolated using the Ficoll density gradient centrifugation method. Cell counting and cell viability were performed using Trypan blue. The PBMCs were either processed directly for gene expression or cultured prior to the stimulation study.

Cell culture of PBMCs. The PBMCs from each group of patients (premenopausal, postmenopausal, and male patients) were pooled and cultured in 24-well plates at a concentration of 1×10^6 mL in each well. The culture medium was composed of RPMI

supplemented with 1% L glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. The cells were cultured at 37°C in a 5% CO_2 atmosphere and 100% humidity for 24 h prior to stimulation.

Preparation of drugs and *ex vivo* stimulation of cultured PBMCs. The steroid sex hormone 17 beta-estradiol (Sigma Aldrich, Schnelldorf, Germany) and the receptor antagonist ICI 182,780 (Sigma Aldrich) were prepared initially as ethanol and DMSO stock solutions, respectively, at a concentration of (10^{-2} mol L^{-1}). Then, the samples were diluted to obtain an appropriate working solution concentration.

For each group of patients (premenopausal, postmenopausal, and male patients), the pooled PBMCs were either left untreated or were treated with interferon (3 μL mL^{-1}) (Reiferon, Minapharm, Egypt) alone, or were prestimulated with 17 beta-estradiol (10^{-8} mol L^{-1}) prior to Reiferon stimulation. To investigate whether the effect of 17 beta-estradiol is mediated through estrogen receptors, PBMCs from all groups of patients were treated with ICI 182/780 (10^{-6} mol L^{-1}) prior to stimulation with 17 beta-estradiol and Reiferon. Each experiment was performed in triplicate for each group of patients.

Total RNA extraction and reverse transcription. The total cellular RNA was extracted from PBMCs using the QIAamp RNA mini kit (Qiagen, Valencia, Calif). The total cellular RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit (Qiagen).

Quantification of gene expression. The messenger RNA (mRNA) expression level of MxA was quantified using TaqMan real-time quantitative polymerase chain reaction (ABI 7000; Applied Biosystems, Foster City, Calif). The amount of MxA mRNA expression was calculated relative to the amount of mRNA expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample. To confirm our results, the same experiment was done on another ISG, which is RNA-dependant protein kinase (PKR).

Statistical analysis. Gene expression is expressed in relative quantitation ($RQ = 2^{-\Delta\Delta CT}$) and data are expressed as the mean ± standard error of the mean (SEM). The Student *t* test was used for statistical analysis. The calculations were performed using the GraphPad Prism 5.00 software (GraphPad Software Inc., La Jolla, Calif). A P value less than 0.05 was considered statistically significant.

RESULTS

Baseline MxA gene expression in PBMCs. To investigate whether IFN-α/β is activated in the PBMCs of chronically HCV-infected patients, baseline expression level of MxA gene in patients before therapy initiation was compared to MxA gene expression level in healthy controls. MxA mRNA expression was found to be upregulated significantly in premenopausal patients (3.473 ± 0.766, N = 16) compared with sex- and age-matched healthy controls (1.320 ± 0.287, N = 12) (P = 0.027). In postmenopausal and male patients, MxA mRNA expression (1.414 ± 0.463, N = 14) (0.7416 ± 0.3890, N = 10) showed no significant difference compared with sex- and age-matched healthy controls (1.463 ± 0.530, N = 7) (0.9163 ± 0.1807, N = 10) (P = 0.9485) (P = 0.7986), respectively. Moreover, MxA mRNA expression was found to be unregulated significantly in premenopausal patients compared with postmenopausal patients (P = 0.034) and male patients (P = 0.0243). All MxA baseline gene expression of patients and controls are presented in Tables I and II respectively. Gene expression is expressed in relative quantitation ($RQ = 2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM (Fig 1).

MxA gene expression in cultured PBMCs. MxA gene expression in untreated pooled PBMCs was compared among premenopausal women, postmenopausal women. and male patients. Premenopausal patients showed a significant upregulation (7.140 ± 0.743, N = 16) when compared with both postmenopausal (1.626 ± 1.055, N = 14) (P = 0.0102) and male patients (1.566 ± 0.0165, N = 10) (P = 0.0212). Postmenopausal patients showed a no significant difference when compared with male patients (P = 0.9597). All values of MxA expression in unstimulated cultured pooled PBMCs of HCV-infected patients are presented in Table I. Gene expression is presented in relative quantitation ($RQ = 2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM (Fig 2).

Impact of estrogen and its blocker on IFN signaling pathway. MxA expression was compared among different groups of patients after stimulation of pooled PBMCs with interferon. Premenopausal patients showed significant increase in MxA expression (7089 ± 943.0, N = 16) compared with both postmenopausal (67.56 ± 13.24, N = 14), (P = 0.0104) and male patients (400.8 ± 77.32, N = 10), (P = 0.0021). On the one hand, the data showed that the MxA relative expression is magnified in male patients after interferon stimulation when compared with postmenopausal patients (P = 0.0450). On the other hand, MxA gene expression was assessed, in each group, after prestimulation with estrogen prior to IFN stimulation compared with IFN stimulation alone. The results showed a significant downregulation of MxA mRNA expression level in premenopausal (2633 ± 867.4, N = 16), postmenopausal

Table I. MxA gene expression in HCV patients after different stimulations

MxA Expression	Premenopausal (HCV)	Postmenopausal (HCV)	Males (HCV)
Baseline from individual samples	3.47 ± 0.766	1.41 ± 0.463	0.7416 ± 0.389
Cultured PBMCs with no IFN	7.14 ± 0.743	1.62 ± 1.055	1.56 ± 0.016
Cultured PBMCs with IFN	7089.30 ± 943.0	67.56 ± 13.24	400.84 ± 77.32
Cultured PBMCs with estrogen+ IFN	2633 ± 867.4	13.55 ± 4.258	12.06 ± 3.423
Cultured PBMCs with ICI+ estrogen + IFN	6452 ± 353.5	46.71 ± 10.18	86.30 ± 1.700

The relative MxA mRNA expression in PBMCs of premenopausal, postmenopausal, and male patients. MxA expression level was assessed on individual samples as well as pooled PBMCs. The PBMCS was either left untreated or treated with interferon alone, E + IFN and ICI + E + IFN. Gene expression is expressed in relative quantitation (RQ = $2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM.

Table II. Baseline MxA expression in healthy controls

MxA expression	Premenopausal	Postmenopausal	Male patients
Baseline from individual samples	1.32 ± 0.287	1.46 ± 0.5301	0.9163 ± 0.1807
Cultured PBMCs with no IFN	1.172 ± 0.152	1.53 ± 0.435	1.037 ± 0.286

The relative MxA mRNA expression in PBMCs of premenopausal, postmenopausal healthy controls and male controls. MxA expression level was assessed on individual samples as well as pooled PBMCs. Gene expression is expressed in relative quantitation (RQ = $2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM.

(13.55 ± 4.258, N = 14), and male patients (12.06 ± 3.423, N = 10) when compared with IFN stimulation only ($P = 0.0254$, $P = 0.0029$, and $P = 0.0074$, respectively). The stimulation of pooled PBMCs from all groups of patients (premenopausal, postmenopausal, and male patients) with ICI 182/780 prior to estrogen and IFN stimulation resulted in a significant increase in MxA expression (6452 ± 353.5, N = 16; 46.71 ± 10.18, N = 14; and 86.30 ± 1.700, N = 10, respectively) when compared with estrogen + IFN stimulation ($P = 0.0451$, $P = 0.0122$, and $P = 0.0005$, respectively; Fig 3). The amount of MxA mRNA expression was calculated relative to the amount of mRNA expression of the housekeeping gene GAPDH in the same sample. The values of MxA expression after different stimulation of PBMCs of HCV-infected patients are presented in Table I. Gene expression is expressed in relative quantitation (RQ = $2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM.

PKR gene expression in cultured PBMCs. To investigate whether estrogen downregulates the MxA directly or through the interaction with the JAK/STAT pathway, we assessed the effect of 17-beta estradiol on another important ISG, which is the PKR. Intriguingly, we found that PKR and MxA give the same pattern of expression after estrogen stimulation. IFN stimulation of PBMCs from premenopausal patients upregulates PKR expression significantly (36.34 ± 3.690, N = 16) when compared with untreated PBMCs from the same group of patients (6.146 ± 1.235, N = 16, $P = 0.0162$). 17-beta estradiol significantly downregulates the PKR expression (20.20 ± 1.804, N = 16) when compared with interferon stimulation alone ($P = 0.0207$). Gene expression is expressed in relative quantitation (RQ = $2^{-\Delta\Delta CT}$). The results are expressed as mean ± SEM (Fig 4).

DISCUSSION

Although several studies reported the impact of sex on virologic response among HCV patients, little is known regarding the influence of sex on the host genetic response to HCV, which is represented by the expression of ISGs. Whether this sex-specific genetic variation is mediated through sex hormones remained unquestioned. In the current study, sex variation in MxA expression, being a downstream gene of the JAK/STAT pathway and an indicator of interferon action,[14] has been assessed. MxA expression has been quantified both in peripheral blood of individual premenopausal/postmenopausal patients and male patients, as well as in cultured, pooled PBMCs from the 3 groups of patients. Baseline MxA expression in naïve individual patients has been found to be upregulated in premenopausal patients compared with their healthy, age-matched controls, postmenopausal patients, and male patients. Postmenopausal patients and male patients showed no difference in MxA expression compared with their age-matched controls (Fig 1). Pooled, cultured PBMCs from the same patients (premenopausal/postmenopausal patients) confirmed the same pattern of results (Fig 2). These results suggest that the IFN signaling pathway is more active in premenopausal

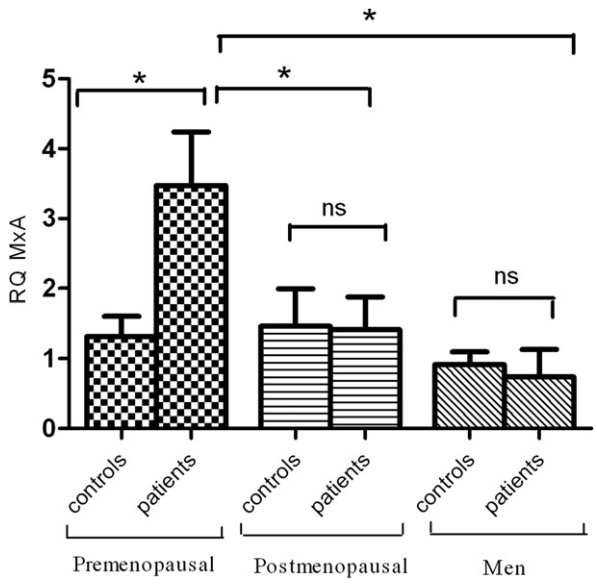

Fig 1. Baseline MxA expression. MxA mRNA was upregulated significantly in premenopausal HCV-infected patients (3.473 ± 0.766, N = 16) compared with age-matched healthy controls (1.320 ± 0.287, N = 12, $P = 0.0277$), postmenopausal patients (1.414 ± 0.463, N = 14, $P = 0.0348$) and male patients (0.7416 ± 0.3890, N = 10, $P = 0.0243$). MxA mRNA expression showed no significant difference between postmenopausal patients and postmenopausal healthy controls (1.463 ± 0.5301, N = 7, $P = 0.9485$). Furthermore, MxA mRNA expression showed no significant difference between male patients and male controls (0.9163 ± 0.1807, N = 10, $P = 0.7986$). * = $P < 0.05$ and ns = non significant difference.

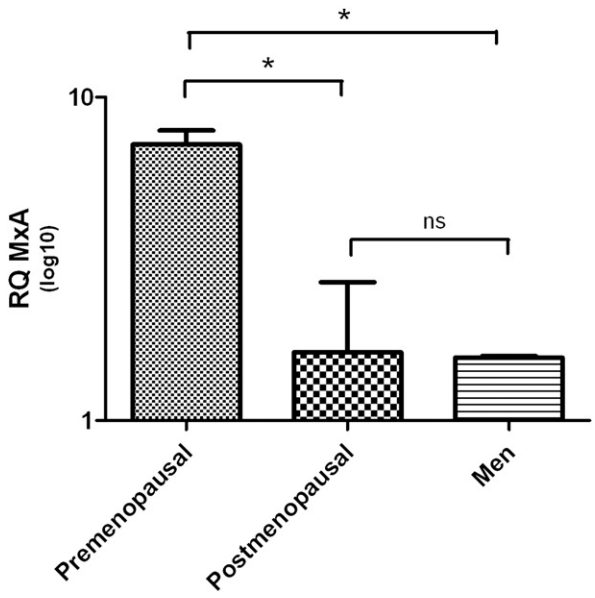

Fig 2. Sex differences in MxA baseline expression. Baseline MxA expression showed a significant upregulation in premenopausal patients (7.140 ± 0.743, N = 16) compared with both postmenopausal (1.626 ± 1.055, N = 14) and male patients (1.566 ± 0.016, N = 10; $P = 0.0212$, $P = 0.0102$, respectively). Baseline MxA expression showed no significant difference between postmenopausal and male patients ($P = 0.9597$). * = $P < 0.05$ and ns = non significant difference.

patients compared with postmenopausal patients and male patients. These differences could be related to a higher endogenous interferon release as a consequence of a higher induction of viral recognition receptors. This goes along with a previous study that has reported a sex difference in the production of interferon after stimulation with toll-like receptor 7 (TLR7) agonists with women being stronger producers of IFNα after TLR7 stimulation.[15] Furthermore, a decrease in cytokine production in postmenopausal women was been reported, which eventually lowers their immune response.[16] An important finding of the current study is the variation observed in the JAK/STAT pathway activation by exogenous IFN stimulation of pooled PBMCs among the 3 groups of patients with premenopausal having the highest MxA gene expression and postmenopausal having the lowest MxA gene expression (Fig 3). To elucidate the role of estrogen on MxA expression, pooled PBMCs were subjected to 17 beta-estradiol stimulation prior to IFN stimulation. We obtained a clear finding that estrogen prestimulation led to the downregulation of MxA expression in all groups of patients when compared with interferon stimulation alone (Fig 3). The incubation of PBMCs from all groups of patients with the pure estrogen receptor antagonist ICI182/780 prior to estrogen and interferon stimulation reversed the effect of estrogen significantly by increasing MxA expression to a comparable level after IFN stimulation alone. The findings obtained after estrogen stimulation could be explained by certain interactions between estrogen and JAK/STAT pathway. It was mentioned previously that estrogen receptor alpha upregulates suppressors of cytokine signaling 2 and 3 in the hepatic cells both *in vivo* and *in vitro*, which consequently inhibit the JAK/STAT pathway.[17] In contrast, another study demonstrated an inductive effect of estrogen on the expression of other inhibitors of JAK/STAT pathway such as protein inhibitors of activated STATs (PIAS) in multiple myeloma cells.[18] Moreover, the tyrosine protein kinase jak1, which is associated with IFN receptor, was found to have a decreased expression in breast cancer tissue when compared with noncancer tissue. This has been found to be correlated positively with the estrogen receptor status.[19] Furthermore, estrogen was reported to mediate the increase in tyrosine-dephosphorylation of STATs.[20] To rule out a unique effect of estrogen on MxA and to confirm that estrogen downregulates the MxA through attenuation of the JAK/STAT pathway, we assessed the effect of 17-beta estradiol on PKR expression. Expectedly, PKR was also downregulated

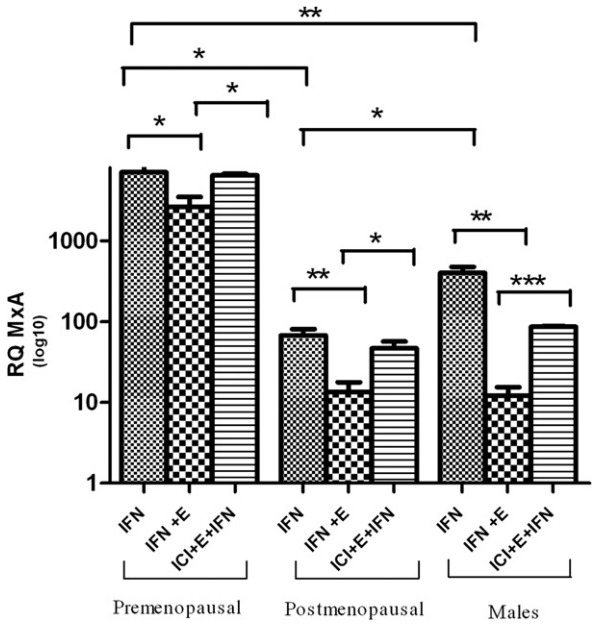

Fig 3. Impact of estrogen and its blocker on the IFN signaling pathway. After stimulation of cultured PBMCs with interferon, premenopausal patients showed marked upregulation of MxA expression (7089 ± 943.0, N = 16) when compared with both postmenopausal patients (67.56 ± 13.24, N = 14) and male patients (400.8 ± 77.32, N = 10) ($P = 0.0104$ and $P = 0.0021$, respectively). Male patients showed higher MxA expression (400.8 ± 77.32, N = 10) when compared with postmenopausal patients (67.56 ± 13.24, N = 14, $P = 0.0450$). Prestimulation of PBMCs with estrogen (E) prior to interferon treatment resulted in a significant decrease in MxA expression in premenopausal (2633 ± 867.4, N = 16), postmenopausal (13.55 ± 4.258, N = 14), and male patients (12.06 ± 3.423, N = 10) when compared with IFN stimulation only ($P = 0.0254$, $P = 0.0029$, $P = 0.0074$, respectively). In contrast, PBMCs were stimulated with ICI 6 h prior to estrogen treatment and then treated with IFN. This resulted in significant upregulation of MxA expression in premenopausal patients (6452 ± 353.5, N = 16) postmenopausal patients (46.71 ± 10.18, N = 14), and male patients (86.30 ± 1.700, N = 10) when compared with estrogen + IFN stimulation ($P = 0.0451$, $P = 0.0122$, and $P = 0.0005$, respectively). *** = $P < 0.001$,** = $P < 0.01$,* = $P < 0.05$.

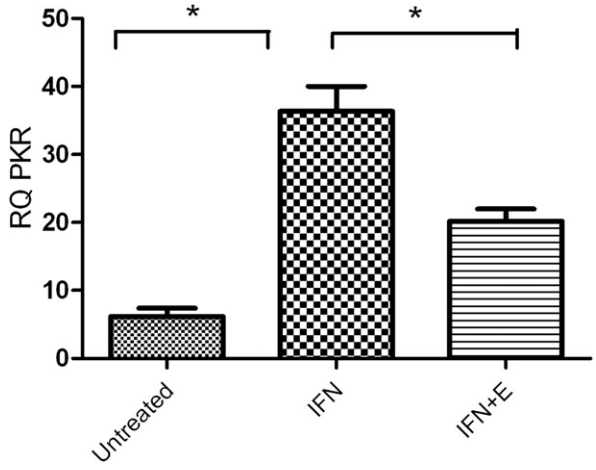

Fig 4. PKR expression in PBMCs after IFN Vs E+IFN. PBMCs of premenopausal patients were stimulated with IFN. This resulted in significant upregulation in the PKR expression (36.34 ± 3.690, N = 16) compared with unstimulated PBMCs from the same pool of PBMCs (6.146 ± 1.235, N = 16, $P = 0.0162$). E+IFN stimulation of PBMCs downregulated PKR expression significantly (20.20 ± 1.804, N = 16) when compared with IFN stimulation alone ($P = 0.0207$). * = $P < 0.05$.

after estrogen stimulation of PBMCs prior to interferon treatment. These data exclude bias of estrogen effect on MxA and stand as a proof that estrogen posses a weakening effect on the JAK/STAT pathway.

The downregulating effect of estrogen on MxA expression found in our study does not contradict the idea that women could be better clearers of the virus than men because estrogen has been demonstrated recently to inhibit the production of HCV infectious particles.[21] Furthermore, the higher rate of clearance among women may be the result of the increased level of other sex hormones such as progesterone, which needs to be investigated.

Finally, the current study obtained a clear finding that host genetic response to HCV varies among sexes, with premenopausal women having the highest MxA expression. Moreover, JAK/STAT pathway activation after IFN treatment was enhanced in premenopausal females. The suggested role of estrogen in enhancing genetic response to interferon treatment in HCV infection was denied in our study by demonstrating the attenuation of JAK/STAT pathway by estrogen.

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