Serum Levels of Endothelial Monocyte-Activating Polypeptide-II in Hepatitis C Patients

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Abstract

Objectives: Endothelial monocyte-activating polypeptide-II (EMAP-II) is a cytokine with pro-inflammatory and immune-suppressive properties. The goal of this study was to assess serum EMAP-II in treated and untreated Hepatitis C virus (HCV) patients. Furthermore, we determined the relationship between serum EMAP-II levels with the clinic pathological and laboratory parameters in patients with HCV.

Methods: 25 control patients (Group I), 25 treated HCV patients (Group II) and 25 newly diagnosed, untreated patients with HCV (Group III) were included in this study. Serum EMAP-II levels were detected by Enzyme linked immunosorbent assay (ELISA), and HCV RNA was assessed by real time-PCR (RT-PCR). The results were evaluated against clinical and laboratory data.

Results: Serum EMAP-II levels were significantly elevated in newly diagnosed, untreated HCV patients compared to treated HCV and control patients (p<0.001). We found that serum EMAP-II levels correlated positively with HCV RNA in untreated HCV patients (p<0.001). While serum albumin and platelet count correlated negatively with serum EMAP-II levels (p<0.001), a positive correlation was observed between EMAP-II and serum bilirubin (p<0.001).

Conclusions: Increased serum EMAP-II levels are present in newly diagnosed HCV patients compared to treated HCV and control patients, suggesting EMAP-II as a novel biomarker for HCV diagnosis.

Keywords: Hepatitis C virus (HCV); Endothelial monocyte-activating polypeptide-II (EMAP-II)

Introduction

Hepatitis C Virus (HCV) strongly contributes to the development of chronic hepatitis, liver cirrhosis and liver cancer [1-3]. As HCV replicates in the cytoplasm of hepatocytes, fibrosis or cirrhosis can result from immune-mediated mechanisms [4]. In spite of the detection of many antivirals to help combat the virus [5], little is known about suitable biomarkers for HCV virulence, patient adherence to therapy, or the development of therapy resistance.

Immunoregulatory cytokines in HCV have previously been studied [6-10], but reports on elevated levels of cytokines are inconclusive [9-12] with one study even showing decreased cytokine levels in HCV patients [13]. The standard treatment for patients with HCV is pegylated interferon plus ribavirin administration [14], which results in the reduction of serum cytokine levels. Indeed, interactions between HCV and the immune system are important for efficient treatment and elimination of the virus [6].

Endothelial monocyte activating polypeptide-II (EMAP-II) is a cytokine with anti-angiogenic and pro-inflammatory activities based on its ability to activate monocytes/macrophages, endothelial cells, and neutrophils [15,16]. Although EMAP-II was originally detected when released from cultured methylcholanthrene A (meth A) murine fibrosarcoma cells [17], more recent reports suggest apoptosis and cellular stress also cause the release of the mature EMAP-II protein [18,19]. Importantly, in addition to its pro-inflammatory properties, EMAP-II may act as an immunosuppressive cytokine through lymphocyte apoptosis [20].

The present work was designed to assess the abundance of EMAP-II in serum as a new biomarker for patients with active HCV.

Subjects and Methods

Patients and controls

A total of 75 subjects were selected for this study. They included 25 healthy controls negative for HCV, HBV, HAV markers and HIV antibodies (Group I). Furthermore, this study included 25 treated patients who were negative for HCV RNA and positive for HCV antibodies (Group II). Lastly, the survey included 25 untreated HCV patients who were positive for HCV RNA and for HCV antibodies (Group III). All subjects' ages ranged from 21 to 55 years old. The Hepatitis B surface antigen virus, HIV antibodies were negative in groups II and III. Patients with infections were taken out from the study. Informed written consents were obtained from all participants.

Laboratory methods

Complete blood counts were performed using cell counter Sysmex KX-21N (TAO Medical incorporation, Japan). Serum AST, ALT, bilirubin, albumin, creatinine and blood urea were assessed using clinical chemistry auto-Analyzer system Konelab 20i (Thermo Electron Incorporation, Finland).

HCV antibodies were assessed by ELISA kit using anti-HCV antibodies (Dia-Pro Diagnostic Bioprobes Srl, Italy). EMAP-II concentrations were measured with a commercially available ELISA kit (Sunred Biological Technology Co., Ltd, Shanghai).

Total RNA was extracted from all cases using QiAmp kit (Qiagen GmbH, Germany). The *HCV* gene was amplified using one-step real-time RT-PCR (Applied Biosystems, USA) with gene-specific primers- Forward primer: 5'-GAG CAR TTC AAG CAG AAG G-3' and the Reverse primer: 5'-TCC ACA TGG CTT CGC CCA RAA-3'.

Statistical analysis

SPSS program version 20 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Mean ± SD, one-way ANOVA test and chi-square test were performed in this study. A p value ≤ 0.05 was considered significant. The Pearson correlation test was used for assessing the relationship between variables.

Results

Patients characteristics

Control, treated, and untreated patient characteristics are listed in Table 1. Serum ALT, AST and bilirubin levels showed statistically significant differences in the untreated HCV group compared to those in treated HCV and control groups (p<0.001). Serum albumin was significantly decreased in the control group versus the treated and untreated patient groups (p<0.001) (Table 1).

Groups / Variables	Controls (Group I) (n=25)	Treated HCV (Group II) (n=25)	Untreated HCV (Group III) (n=25)	P value		
Age Range Mean ± SD	(22-55) 38.08 ± 10.03	(21-55) 39.56 ± 10.25	(21-55) 38.24 ± 9.82	0.849		
				I *vs* II	I *vs* III	II *vs* III
				0.861	0.998	0.888
Sex Male Female	13(52%) 12(48%)	13(52%) 12(48%)	14(56%) 11(44%)	0.948		
				I *vs* II	I *vs* III	II *vs* III
				1	0.777	0.777
Hepatomegaly No Yes	25(100%) 0(0%)	22(88%) 3(12%)	2(8%) 23(92%)	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.074	<0.001**	<0.001**
Hb Range Mean ± SD	(12.2-17) 14.23 ± 1.27	(12.2-17) 14.06 ± 1.41	(9.8-14.1) 11.73 ± 1.13	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.878	<0.001**	<0.001**
WBCs Range Mean ± SD	(4.5-9.4) 6.83 ± 1.49	(4.2-9.7) 6.75 ± 1.62	(2.5-6.4) 4.19 ± 1.13	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.979	<0.001**	<0.001**
Platelets Range Mean ± SD	(187-382) 273.4 ± 59.98	(185-402) 296.6 ± 65.67	(67-303) 169.92 ± 77.53	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.455	<0.001**	<0.001**
AST Range Mean ± SD	(12-34) 22.84 ± 6.47	(11-33) 21.44 ± 7.15	(44-129) 81.64 ± 22.17	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.933	<0.001**	<0.001**
ALT Range	(14-33) 23.16 ± 5.19	(14-33) 22.44 ± 5.37	(51-184) 102.44 ± 38.68	<0.001**		
				I *vs* II	I *vs* III	II *vs* III

Mean ± SD				0.993	<0.001**	<0.001**
Total bilirubin Range Mean ± SD	(0.5-0.9) 0.68 ± 0.13	(0.5-1) 0.69 ± 0.15	(0.6-1.6) 1.13 ± 0.3	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.990	<0.001**	<0.001**
Direct bilirubin Range Mean ± SD	(0.1-0.2) 0.14 ± 0.05	(0.1-0.2) 0.14 ± 0.05	(0.1-0.4) 0.25 ± 0.08	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				1	<0.001**	<0.001**
Albumin Range Mean ± SD	(4-4.8) 4.41 ± 0.23	(4-4.8) 4.46 ± 0.21	(3.1-4.5) 3.69 ± 0.45	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.858	<0.001**	<0.001**
Creatinine Range Mean ± SD	(0.8-1.4) 1.09 ± 0.17	(0.8-1.4) 1.09 ± 0.17	(0.8-1.4) 1.11 ± 0.16	0.943		
				I *vs* II	I *vs* III	II *vs* III
				0.996	0.942	0.967
Urea Range Mean ± SD	(23-40) 29.24 ± 4.56	(21-37) 29.48 ± 4.46	(21-37) 28.32 ± 5.11	0.659		
				I *vs* II	I *vs* III	II *vs* III
				0.982	0.771	0.662

Table 1: Comparison between patients and controls regarding demographic and laboratory data; N, number; ** p-value ≤ 0.001 HCV, hepatitis C virus; ALT, alanine transaminase; AST, aspartate transaminase; WBCs, white blood cells; Hb, hemoglobin.

There was a decrease in WBC, Hb and platelet counts in newly diagnosed HCV patients in comparison to the other groups (p<0.001). However, for the other parameters, no significant differences were detected between the untreated HCV patients compared to the control patients (p>0.05).

Levels of EMAP-II in serum

Newly diagnosed patients from Group III showed serum EMAP-II levels ranging from 370 – 27700 pg/ml with a mean of 8888.16 ± 8085.93 pg/ml. Patients in Group II with treated HCV had EMAP-II values ranging from 15 to 114 pg/ml with a mean of 52.68 ± 28.12 pg/ml. These values were comparable to the mean EMAP-II levels of 44.4 ± 27.96 pg/ml (ranging from 7-115 pg/ml) in control patients from Group I. Thus, serum EMAP-II levels in untreated HCV patients were significantly higher than other groups (p<0.001) (Table 2).

Groups Variables	Controls (Group I) (n=25)	Treated HCV (Group II) (n=25)	Untreated HCV (Group III) (n=25)	P value		
EMAP-II pg/ml Range Mean±SD	(7-115) 44.4±27.96	(15-114) 52.68±28.12	(370-27700) 8888.16±8085.93	<0.001**		
				I *vs* II	I *vs* III	II *vs* III
				0.678	<0.001**	<0.001**

Table 2: Comparison between patients and control groups regards EMAP-II; N, number; ** p-value ≤ 0.001; HCV, hepatitis C virus; HCV Ab, hepatitis C virus antibodies; EMAP-II, endothelial monocyte activating polypeptide.

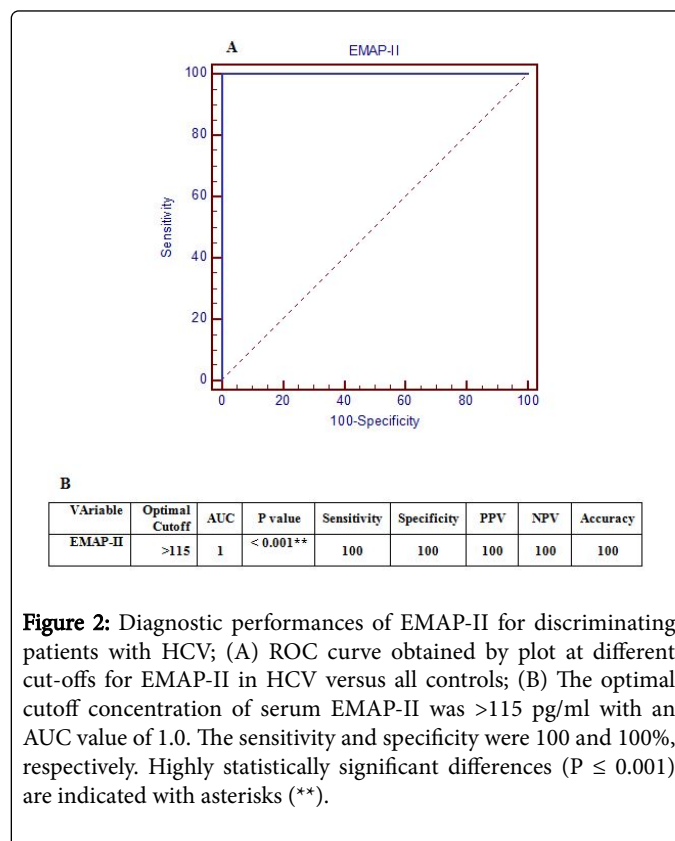|	EMAP-II pg/ml			P value			
	150-1000 (a) (n=7)	1000-10000 (b) (n=9)	>10000 (c) (n=9)				
Platelets Range Mean±SD	(189-303) 250±45.29	(84-265) 177.33±66.71	(67-141) 100.22±30.14	<0.001**			
				a vs b	a vs c	b vs c	
				0.023*	<0.001**	0.010*	
Albumin Range Mean±SD	(3.6-4.5) 4.11±0.31	(3.2-4.5) 3.76±0.43	(3.1-3.5) 3.28±0.11	<0.001*			
				a vs b	a vs c	b vs c	
				0.097	<0.001**	0.011*	

Table 3: Correlation of high serum EMAP-II with features in untreated HCV patients; N, number; * p-value ≤ 0.05; ** p-value ≤ 0.001. EMAP-II, endothelial monocyte activating polypeptide; HCV, hepatitis C virus.

Correlation of EMAP-II levels with HCV RNA and other laboratory parameters: To gain further insight into the role of EMAP-II in untreated HCV patients, we correlated serum levels of EMAP-II with other laboratory parameters. Importantly, serum EMAP-II values correlated strongly and highly significantly (p<0.001) with HCV RNA (Figure 1).
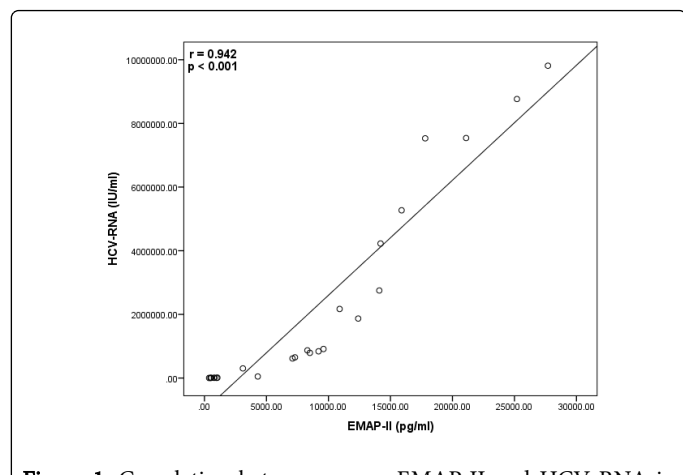
Figure 1: Correlation between serum EMAP-II and HCV RNA in untreated HCV patients. Shown are EMAP II serum levels plotted against HCV RNA. Note the highly significant (p<0.001) and strongly positive correlation (R = 0.942) between serum levels of EMAP-II and HCV RNA in HCV patients; r=0.75-1 (strong correlation); ** p-value ≤ 0.001; EMAP-II, endothelial monocyte activating polypeptide –II.

For further analysis, newly diagnosed patients were divided into 3 subgroups according to EMAP-II levels in their sera. These subgroups included EMAP-II levels 150 - 1000 pg/ml, 1000 – 10000 pg/ml and levels>10000 pg/ml. High serum EMAP-II concentrations showed a substantial negative correlation with platelet counts and albumin levels (p<0.001) (Table 3). Serum EMAP-II levels significantly correlated with bilirubin (p<0.001) (data not shown).

VAriable	Optimal Cutoff	AUC	P value	Sensitivity	Specificity	PPV	NPV	Accuracy
EMAP-II	>115	1	< 0.001**	100	100	100	100	100

Figure 2: Diagnostic performances of EMAP-II for discriminating patients with HCV; (A) ROC curve obtained by plot at different cut-offs for EMAP-II in HCV versus all controls; (B) The optimal cutoff concentration of serum EMAP-II was >115 pg/ml with an AUC value of 1.0. The sensitivity and specificity were 100 and 100%, respectively. Highly statistically significant differences (P ≤ 0.001) are indicated with asterisks (**).

ROC curve analysis

ROC analysis (Figure 2A and 2B) showed that EMAP-II could discriminate HCV patients from controls (AUC: 1.0, p<0.001). The sensitivity and specificity were 100% with optimal cutoff >115 pg/ml.

For differentiating treated HCV from healthy controls (data not shown), the optimal cutoff was>35 pg/ml with an AUC of 0.597. The optimal sensitivity and specificity were 80% and 44% respectively.

Discussion

Cytokines play a significant part in controlling HCV infection and fibrosis [21]. Previous studies observed that cytokines were significantly elevated in HCV and that IFN-alpha treatment decreased their levels [7,22]. However, other cytokine levels in HCV were similar to those observed in healthy controls. During HCV treatment, cytokine levels do not change significantly in patients who achieve a sustained virological response in comparison to patients resistant to treatment [23]. Our study is the first to have demonstrated increased serum levels of EMAP-II in newly diagnosed, untreated HCV patients and a significant positive correlation with HCV RNA. Of note, HCV RNA is one of the most important biomarkers for the diagnosis of HCV [24-26].

Our finding of high serum EMAP-II levels in patients with HCV may result from the secretion of EMAP-II by hepatocytes in response to the virus [27]. Of note, this study is also the first description of EMAP-II serum levels in patients with a viral infection. It may be possible that EMAP-II is a biomarker for viremic infections in general. In this context, we have previously shown in tissue culture studies that HIV-envelope protein gp120 causes the release of EMAP-II from endothelial cells, ultimately leading to endothelial apoptosis [28].

Previous studies reported high levels of serum IFN-γ in HCV patients and decreased significantly after treatment [29]. This may imply the correlation between EMAP-II and Th1 cells and both of them are directed against HCV core protein [30]. After HCV treatment, we detected a significant decrease in serum EMAP-II levels, so EMAP-II could be a biomarker of achieving virological response and can be measured before HCV treatment as an index of treatment efficacy. Further studies may be needed to show the presence of EMAP-II in HCV patients under treatment and check both of EMAP-II, and IFN-γ in different times in treatment.

In previous studies, we have also shown that both hypoxia and apoptosis increase the expression of EMAP-II and its release *in vivo* and *in vitro* [18,31]. HCV stabilizes hypoxia-inducible factor 1 alpha (HIF-1-α) [32] and thus may enhance EMAP-II gene expression. Moreover, apoptotic hepatocytes play a significant role in the pathogenesis of Hepatitis C [33], causing EMAP-II release in HCV. Reportedly, HCV E2 can cleave pro-EMAP-II (also known as AIMP1/p43) [34], which may lead to mature EMAP-II to detach from its multi-tRNA-synthetase complex to be released from cells. Importantly, we have demonstrated in the past that EMAP-II is not only produced as a result of apoptosis but can also induce apoptosis. This intensified effect may cause the increased, possibly compensatory, production of pro-EMAP-II, which constitutes the vicious cycle [35]. Furthermore, pro-inflammatory EMAP-II could enhance inflammatory exudates containing activated infiltrating mononuclear cells in the liver [36]. Finally, the proposed immunosuppressive effects of EMAP-II may play a major part in the development and exacerbation of the HCV infection by inducing lymphocyte apoptosis.

Taken together, it is possible that hypoxia and apoptosis are responsible for the increased serum EMAP-II levels in HCV. These increased levels may also play a part in the pathogenicity of HCV.

In our results, we observed a strong significant correlation between serum EMAP-II and HCV RNA levels in untreated HCV patients. EMAP-II may be produced in response to HCV replication; thus, EMAP-II could be considered as a surrogate marker for HCV. Importantly, this study showed that EMAP-II has the highest discriminatory power (AUC= 1.0), optimal sensitivity and specificity to differentiate HCV from controls. This finding suggests that EMAP-II has competitive advantages against other diagnostic biomarker of HCV including IL-10, VEGF, IL-18 and IL-12p40 [37]. Furthermore, decreasing serum levels of EMAP-II were important for predicting the complete remission in HCV with cutoff >35 pg/ml (AUC=0.597).

Conclusion

This study introduces EMAP-II as a biomarker for early diagnosis of HCV patients, to differentiate untreated HCV patients from treated HCV patients and to monitor HCV treatment efficacy. Future studies are required to address a possible involvement of EMAP-II in HCV pathogenesis.

Acknowledgements

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