

STUDY THE EXPRESSION OF TETRASPANIN (CD81) ON B CELLS IN RESPONSE TO INTERFERON THERAPY IN CHRONIC HCV PATIENTS

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ABSTRACT

Hepatitis C virus (HCV) infection causes life threatening disease due to its persistence in the liver, over time leading to cirrhosis and hepatocellular carcinoma. Therapeutic intervention with interferon (INF) - alpha combined with ribavirin is aimed at viral clearance, Interferon and ribavirin might discontinue the virus induced hepatocytes damage, thus slowing down the progress to end- stage liver disease. It has been demonstrated that HCV virion, via major structure envelop (E) protein 2 or E1E2 complex could specifically bind to human CD81 molecule, thereby altering the cellular activities in B cells, T cells and natural killer (NK) cells. CD81, a surface protein belonging to the tetraspanin family, facilitates B-T cell interaction in the process of antigen presentation Evidence has revealed that CD81 on B and T cells substantially enhanced T helper IL-4 secretion and NK-cell CD81 enabled E2-mediated reduction of the IFN-gamma levels Those data suggest that CD81 might activate the pathway leading to a predominant type-2 immune response, and as such be prone to cause a strong antibody production but weak or insufficient cytotoxic activities to clear the. The aim of this work was to investigate the clinical relevance and the possible mechanism of CD81 down-regulation under IFN-alpha treatment to detect possible role of CD 81 in response to INF therapy of chronic HCV patients.

Subjects and Methods: The study included: **Group I:** 10 healthy subjects. And 60 HCV patients classified into 3 groups: **Group II:** 20 chronic HCV patients have not received treatment **Group IIIa:** 20 chronic HCV patients have received treatment responsive to treatment **Group IIIb:** 20 chronic HCV patients have received treatment not responsive to treatment .the patients were subjected to: (CBC) .liver and kidney function tests and special investigation CD 81, CD19, CD 22.

Results: TLC and PLT higher in group I while ALT and AST were significantly higher in group II, while T. bilirubin was significantly higher in group IIIb, Direct bilirubin was significantly higher in group II ,and Alb was significantly higher in group I ,ASMA and ANA more higher in group IIIb, AFP and TSH were significantly higher in group IIIb , there was negative correlation between CD81 and TLC positive correlation between ALT, AST and CD81 were positively correlated to T Bilirubin , D Bilirubin and CD81. There was also significant negative correlation between Albumin and CD81. CD81, CD19, CD22 and Lymphocytes were significantly higher in group IIIb.

Conclusion: In conclusion, CD81 down-regulation is a primary host response to interferon-alpha-based therapy and an immunophenotype associated with anti-HCV sustained virological response (SVR)

Key words: HCV, CD81 ,interferone

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INTRODUCTION

Hepatitis C virus (HCV) infection causes life threatening disease due to its persistence in the liver, over time leading to cirrhosis and hepatocellular carcinoma. Therapeutic intervention with interferon (INF) - alpha combined with ribavirin is aimed at viral clearance⁽¹⁾

Interferon and ribavirin might discontinue the virus induced hepatocytes damage, thus slowing down the progress to end- stage liver disease ⁽²⁾ .The combination they could exert apparent eradication of HCV viraemia in 46- 78% of the treated patients ⁽³⁾ .

There are many virological (viral genotype and initial viral load) and host factors (HCV specific T-cell function to elicit intrahepatic cytotoxicity) affecting therapeutic efficiency ⁽¹⁾ . T cell produces type-1 cytokines, such as INF- gamma, and to suppress type-2 cytokines such as interleukin

(IL-4 and IL-10) and most likely favor a successful response to treatment ⁽⁴⁾ . Also genetic polymorphism affect function or exdpression of cytokines may be associated with therapeutic response against chronic HCV infection ⁽⁵⁾ .

It has been demonstrated that HCV virion, via major structure envelop (E) protein 2 could specifically bind to human CD81 molecule, thereby altering the cellular activities in B cells, T cells and natural killer (NK) cells ⁽⁶⁾ . CD81, a surface protein belonging to the tetraspanin family, facilitates B-T cell interaction in the process of antigen presentation ⁽⁷⁾ . Evidence has revealed that CD81 on B and T cells substantially enhanced T helper IL-4 secretion and NK-cell CD81 enabled E2-mediated reduction of the IFN-gamma levels ⁽⁷⁾ . Those data suggest that CD81 might activate the pathway leading to a predominant type-2 immune response, and as

such be prone to cause a strong antibody production but weak or insufficient cytotoxic activities to clear the virus ⁽⁸⁾. Down-regulation of CD81 has been demonstrated in association with a decrease in the HCV viral load in IFN-alpha recipients. This gives rise to the possibility for evaluating the CD81 status in correlation with the HCV pathogenesis and therapeutic responsiveness ⁽⁹⁾.

PATIENTS AND METHODS

This study was conducted in Clinical and Chemical pathology department and internal medicine, Zagazig University Hospitals during the period May 2012 to September 2014 ,all participants were informed adequately about the aim of the study and consented to donate samples for research purposes. Samples were obtained after informed consent and in accordance with procedures approved by the human ethics committee. The study included **group I:** 10 healthy subjects (control group) 5 male and 5 female with mean age 38.1 ± 10.8 yrs. And 60 HCV patients classified into 3 groups **group II:** 20 chronic HCV patients have not received treatment, 14 male and 6 female with mean age 46.7 ± 8.6 yrs **group IIIa:** 20 chronic HCV patients have received treatment responsive to treatment 14 male and 6 female with mean age 44 ± 10 years.

group IIIb: chronic HCV patients have received treatment not responsive to treatment 12 male and 8 female with mean age 43.8 ± 6.8 years.

The selected subjects had the following:

- A. **Detailed history:** with special emphasis on age, sex, presence of other medical diseases.
- B. **Through clinical examination:** laying stress on pallor, purpuric eruptions, size of liver and spleen, lymphadenopathy.
- C. **Routine Laboratory investigations:**
 1. **Complete blood count (CBC)** using sysmex Kx 21, with examination of Leishman stained PB smears for differential leucocytes count.
 2. **Liver function tests** (Bilirubin total and direct, Total protein, Albumin, ALT, AST, Alkaline phosphatase. (COBAS INTEGRA 400 plus, Roche Diagnostic Ltd)

3. **Kidney function tests** (Blood urea and Serum creatinine) (COBAS INTEGRA 400 plus, Roche Diagnostic Ltd)

D. Special investigation:

CD81, CD19, CD22 by flowcytometry

E. Other tests:

- ANA, ASMA by ELISA (Stat Fax 303)
- RF by turbidimetry (COBAS INTEGRA 400 plus, Roche Diagnostic Ltd)
- TSH, AFP by ELFA (minividas , Biomerieux)
- HCV RNA by PCR (real time PCR, Roche Diagnostic Ltd).

*Method of CD81, CD19, CD22:

Immunophenotyping of B- lymphocytes in peripheral blood samples using Becton-Dickinson (BD, USA), FACS Calibur Flow Cytometry.:

A- Sampling:

- 5mL of venous blood were aseptically collected from each patient, divided into:
 - 2mL blood dispensed into a tube containing K-Ethylene Diamine Tetra Acetate (K-EDTA) at a concentration of 1.2 mg/ml, to be used for CBC and preparation of Leishman stained smears PB smears and immunophenotyping of B-lymphocytes.
 - 3mL blood dispensed into plain tube to be used for liver and kidney function tests, ANA, ASMA, RF, AFP, TSH

B- Immunophenotyping of B lymphocytes:

Principle of Flowcytometry(FCM):

FCM is the measurement of numerous cell properties (cytometry) as the cells move in single file resulting in light scattering. Antibodies specific for various cellular antigens can be labeled with different fluorochromes that can absorb and emit light, allowing simultaneous multicolor flow cytometric analysis of two or more cell-associated antigens.

Reagents:

- Phosphate buffered saline (PBS) (8.0 g/L NaCl, 0.2 g/L kcl, 1.15 g/L NaH₂PO₄ and 0.2 g kH₂PO₄) added to 100 mL of distilled water with pH adjusted at 7.3 ± 0.2 .
- Lysing solution (1.5 mmol/L NH₄Cl, 100 mmol/L KHCO₃ and 10 mmol/L tetra Na-EDTA) made up to 1 liter with distilled water, pH adjusted at 7.2.

- Negative isotypic control (appropriately labelled according to the MoAbs used) for determining the non-specific binding of MoAbs.
 - MoAbs supplied by Beckman Coulter, France.
11. Cells were suspended in 500 μ L PBS to be ready for acquiring data by the FCM.
 12. 0.5mL of fixative (4g paraformaldehyde in 100mL PBS with 0.1% Na azide, pH 7.4 ± 0.2) was added and the tubes were kept at 4°C until analyzed on FCM within 24hr.

The panel of fluorescein isothiocyanate (FITC)/ phycoerythrin (PE) conjugated MoAbs (CD 81,CD19,CD22)

Procedure:

Surface marker analysis:

1. Blood was prepared with PBS so that TLC was adjusted between 5 and $10 \times 10^3/\text{mm}^3$.
2. For each sample, sets of tubes were labeled for all the MoAbs to be used, including 1 tube for the appropriate negative isotypic matched control MoAb.
3. $100\mu\text{L}$ of blod samples were delivered in each tube.
4. $10 \mu\text{L}$ of each MoAb as well as of the isotypic negative control MoAb were added to the respective tubes.
5. The tubes were vortexed and incubated in the dark at room temperature for 15 minutes.
6. 2 ml of PBS, as a wash buffer, were added to each tube and mixed thoroughly.
7. The tubes were centrifuged at 1500rpm for 5min and the supernatant was discarded.
8. 1mL of 1×10 lysing solution was added to each tube.
9. The tubes were vortexed and incubated for 5-10 minutes in the dark at room temperature.
10. The tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded.

Results: All data were analyzed using SPSS 22 for windows(IBM Corp., 2013). Continuous variables were expressed as the mean \pm SD & median (range), and the categorical variables were expressed as a number (percentage). Independent sample Student's test was used to compare two groups of normally distributed data while Mann Whitney U test was used for non-normally distributed data. Spearman's rank correlation coefficient was calculated between CD81 and other variables.

The study showed:

CD81, CD19, CD22 and Lymphocytic count were significantly higher in group IIIb.

There was highly significant negative correlation between CD81 and total leucocytic count. There were highly significant positive correlation between liver enzymes (ALT, AST) and CD81. There was highly significant positive correlation between Total Bilirubin, Direct Bilirubin and CD81. There was also significant negative correlation between Albumin and CD81. There was highly significant positive correlation between AFP, TSH, Lymphocytes count and CD81.

Table (1): CBC findings in the studied groups:

	I	II	IIIa	IIIb	F	P
TLC(count/ul)						
$\bar{X} \pm \text{SD}$	$7.4 \pm 1.3^*$	4.6 ± 1.6	4.4 ± 1.2	4.0 ± 1.1	16.09	$<0.001^{**}$
(Range)	5.5-9.8	2.3-7.7	1.9-6.4	2.4-5.9		
HB(g/dl)						
$\bar{X} \pm \text{SD}$	13.2 ± 1.3	12.3 ± 1.3	12 ± 1.1	12.6 ± 1.2	2.27	0.08
(Range)	11.4-15.6	9.3-14.8	9.6-13.5	10.0-14.7		
PLT(count/ul)						
$\bar{X} \pm \text{SD}$	$272 \pm 67^*$	155.8 ± 74	135.7 ± 39	144.2 ± 37.3	15.3	$<0.001^{**}$
(Range)	188-392	62-344	74-199	90-212		

Table 2 : Comparison of CD81 and lymphocytic count

	I	II	IIIa	IIIb	F	P
CD81 $\bar{X}\pm SD$	11.9 \pm 2	52.3 \pm 10.9*	19.7 \pm 4.2	56.8 \pm 6.9*	153.3	<0.001**
Range	8-14	34-76	12-29	44-70		
Lymphocytic percentage $\bar{X}\pm SD$	31.1 \pm 5.4	49.4 \pm 4.8	51 \pm 7	52.3 \pm 10.4	20.3	<0.001**
Range	20-38	39-57	44-66	38-78		

Table (3): Correlation between CD81 and other parameters.

	R	P	Sig.
ALT	0.41	<0.001**	HS
AST	0.5	<0.001**	HS

Table (4) : Correlation between CD81 and other parameters.

	R	P	Sig.
Bilirubin T.	0.62	<0.001**	HS
Bilirubin D	0.46	<0.001**	HS
Alb	-0.28	<0.05*	Sig.
TP	-0.11	>0.05	NS

Table (5): Correlation between CD81 and PCR in different groups:

	R	P	Sig.
Group II	0.55	<0.001**	HS
Group IIIa	-0.20	>0.05	NS
Group IIIb	0.41	<0.001**	HS

Table (6): Correlation between CD81 and other parameters.

	R	P	Sig.
Creatinine	-0.18	>0.05	NS
AFP	0.47	<0.001**	HS
TSH	0.33	<0.001**	HS
Lymph	0.41	<0.001**	HS

DISCUSSION

the present study aimed to investigate the clinical relevance and the possible mechanism of CD81 down-regulation under IFN-alpha treatment. In the present study, there were no significant differences between all groups regarding gender, age this was in accordance with ⁽¹⁰⁾ who founde that there was no significant difference between their patients regarding gender and age but their patients over 60 years showed higher percentage of the disease.

There were significant differences between all groups regarding TLC and PLT higher in group I this was in accordance with ⁽¹¹⁾ this could be explained by bone marrow depression due to treatment with interferone, in their study they also founded that hemoglobin level was significantly reduced in their patient this was in contrary to our stud as we found no significant difference in our patients . ASMA and ANA more higher in group IIIb this was in agreement with ⁽¹²⁾ this could be explained by the role of infectious

agent which may induce a variety of different auto-immune diseases or different agents may play a role in the pathogenesis of a single auto-immune condition⁽¹²⁾.

Our study showed that ALT and AST were significantly higher in group II, this was in agreement with⁽¹³⁾⁽¹¹⁾ while T. bilirubin was significantly higher in group IIIb, direct bilirubin was significantly higher in group II due to lack of treatment. In our study, CD81, CD19, CD22 and lymphocytic count were significantly higher in group IIIb this was in accordance with⁽¹³⁾, and also there were a highly significant positive correlation between CD81 and PCR findings, also CD81 and ALT,AST ,total and direct bilirubin level and we noticed a significant negative correlation between albumin and CD81 level. This was due to lower levels of cell surface-associated CD81 are associated with HCV and the initial decline of HCV RNA after initiation of antiviral therapy. Peripheral B-cell CD81 expression was shown to be increased in HCV-infected patients and was downregulated by antiviral therapy to a level comparable to that for normal controls. This effect may be directly related to antiviral therapy or indirectly to decrease of viral load⁽¹³⁾⁽¹¹⁾

As suggested by⁽⁶⁾ the up-regulation of CD81 mRNA in this study indicates that alfa interferon-induced down-regulation of CD81 protein probably occurs at the posttranscriptional or translational level. The decrease in cell surface-associated CD81 expression induced by alfa interferon could therefore reduce the attachment of HCV, and if CD81 is required for uptake of HCV virions, down-regulation of CD81 could also diminish de novo infection of susceptible cells. Additionally, mice lacking CD81 have enhanced T-cell proliferation and immune responses following T-cell antigen receptor engagement. The decreased expression of CD81 may thus enhance the immune response and facilitate virus elimination. Another possible effect of antiviral treatment on B-cell CD81 expression is an indirect effect via reducing the viral load.

Pretreatment B-cell CD81 expression is increased and that this increase is correlated with the HCV RNA level. In addition,

patients with a rapid initial decline of HCV RNA after initiation of antiviral therapy showed a decline of cell surface-associated CD81 and had lower levels of CD81 than patients without initial virological response⁽¹⁴⁾.

In this study we found that there were highly significant positive correlation between CD81 and PCR level in patients who did not receive treatment and patients who received treatment and did not respond this was in agreement with⁽¹⁴⁾, and this could be explained by the interferon-alpha-induced alteration of lymphocytic CD81. The CD81 levels in healthy subjects and naïve chronic HCV patients were compared, with the results showing that the two groups had comparable surface CD81 levels for total peripheral blood lymphocytes, subpopulation-B, -T, and -NK cells. In vitro interferon-alpha treatment could suppress the CD81 expression from both groups. Subsequently, they compared the in vitro interferon-alpha modulatory effects on lymphocytic CD81 from patients having received anti-HCV therapy with either sustained virological response (SVR) or without SVR. There was a significant down-regulation of the B-cell's CD81 only in the SVR group. The CD81 modulation was further investigated using Daudi lymphoid cell line, showing declined surface CD81 levels following treatment with interferon-alpha, interferon-beta or polyI:C. Thus, interferons could directly decrease CD81 expression. The interferon-alpha effect could be restored by 2-aminopurine, suggesting that double-stranded RNA activated kinase might be involved in the suppression of CD81. In conclusion, CD81 down-regulation is a primary host response to interferon-alpha-based therapy and an immunophenotype associated with anti-HCV SVR⁽¹⁵⁾.

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