Effect of interleukin 10 gene polymorphisms -1082 G/A and -592 C/A on response to antiviral therapy in children and adolescents with chronic hepatitis C virus Infection

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Abstract

Background: Egyptian rural villages have a high prevalence of hepatitis C virus (HCV) infection in children younger than 10 years. Interleukin 10 (IL-10) is an anti-inflammatory cytokine that serves to dampen inflammation that could be deleterious to the host, thus limiting potential tissue damage. High production of IL-10 can facilitate viral evasion by down regulating the protective inflammatory response and adversely affecting the response to antiviral treatment. The IL-10 promoter is highly polymorphic, two single nucleotide polymorphisms (SNPs) at positions-1082 G/A and -592 C/A that form three haplotypes (AA, AC, and GC) have been shown to be associated with differential IL-10 expression in humans. **Aim of work:** determine the prevalence of the 2 SNPs -1082 G/A and -592 C/A in the IL-10 promotor region and their effect on response to antiviral therapy in a cohort of children and young adults with HCV infection. **Patients and methods**: forty HCV patients underwent baseline quantitation of HCV-RNA by polymerase chain reaction (PCR) and baseline biochemical testing and were followed up for one year, both clinically and via laboratory assessment HCV-RNA viral load and liver function tests. The genotype status of IL-10 was assessed by real time polymerase chain reaction. **Results and conclusion:** there was no significant association between polymorphisms in the IL-10 gene (-1082G/A and -592C/A) or cytokine haplotype as regards response to therapy or severity of HCV infection in children. As for the SNP-592 C/A; there was a statistically significant association between the score of fibrosis and different genotypes ($P < 0.004$), concluding that the (A) allele is risky. HCV RNA-count and gamma glutamyl transferase pretreatment levels were found to be predictors of response to interferon therapy in HCV infected children in this study.
Key words: Interleukin 10, RT_PCR, SNP -1082 G/A , SNP -592 C/A, HCV

Introduction

According to the world Health Organization (WHO) report, at least 170 million people – more than 3 percent of the world's population are chronically infected with hepatitis C virus (HCV) (41).Egypt has the highest prevalence of HCV infection of any country in the world. It is estimated to be 8% in urban and 25% in rural areas with 8 to 10 million inhabitants having HCV antibodies (anti-HCV) and 5 to 7 million having active infections (i.e. HCV-RNA positive) (34)

Many cytokines secreted by T-helper 1(Th1) and T-helper 2 (Th2) cells are involved in the immune response to HCV infection and progression of HCV-related liver disease. Generally, two distinct patterns of cytokine production occur. Helper T-cell type 1 (Th1) cells release TNF-α, INF-γ and IL-2, which prime and maintain antigen-specific cellular immunity and are important in defense against viruses (46) thus causing inflammation and necrosis (26) while, helper T-cell type 2 (Th2) release IL-4 and IL-10, which modulate hepatic injury by suppressing the Th1 response and counteracting the fibrogenic effects of TNF-α, INF-γ and IL-2 (8). An imbalance in Th1 and Th2 cytokine is suggested to play an important role in the pathogenesis of chronic hepatitis C (6).

Interleukin 10 (IL-10) is an anti-inflammatory cytokine most readily associated with macrophages, both as a source of IL-10 and as the population most impacted by its action (32). Numerous other cells however, have been shown to secrete IL-10, including dendritic cells (DCs), T cells, B cells, neutrophils, eosinophils, and mast cells (32). The secretion of IL-10 is mediated by several cytokines, including IL-12, IL-6, transforming growth factor-β(TGF-β), and IL-27. Although the exact pathways that lead to IL-10 secretion are unclear, the action of IL-10 on target cells is more clearly described and is mediated by the IL-10 receptor (25, 37). It results in the down regulation of major histocompatibility complex class II (MHC II) proteins and co-stimulatory molecules, such as CD80 and CD86, on the surfaces of target macrophages (5, 13). IL-10 also suppresses the production of reactive oxygen and nitrogen intermediates in activated macrophages (17).

The action of IL-10 leads to inhibition of secretion of inflammatory cytokines, including IFN-Ɣ, TNF, IL-1, IL-2, and granulocyte-monocyte colony-stimulating factor (GM-CSF), as well as several chemokines (12,28). Therefore, after the generation of a proinflammatory immune response, IL-10 serves to dampen inflammation that could be deleterious to the host, limiting potential tissue damage.

Regulatory mechanisms that control the production of IL-10 include genetic polymorphism particularly in the promoter region (39,10). In humans IL-10 is encoded by the IL10 gene on chromosome 1q 31-32 (4, 22). The IL-10 promoter is highly polymorphic with three frequent point mutations -1082(G/A), -819(C/T), and -592(C/A) (16). Of particular note, two commonly studied single nucleotide polymorphisms in the promoter region of IL-10 gene (at positions-1082 G/A and -592 C/A relative to the transcription start site) that form three haplotypes (AA, AC, and GC) have been shown to be associated with differential IL-10 expression in humans (7).

These findings may suggest that heterogeneity in the promoter region of the IL-10 gene may have a role in determining the initial response of chronic hepatitis C to IFN-α therapy. Patients who are genetically predisposed to high IL-10 production have a poor response to IFN-α and may benefit from additional treatment strategies designed to enhance a Th1 response (9).
The current study aimed at testing the hypothesis that inheritance of polymorphisms of the IL-10 promoter gene might influence response to antiviral treatment. The single nucleotide polymorphism -1082 G/A and -592 C/A in the promoter region of IL-10 gene were assessed in all the studied subjects and the predictive value for these polymorphisms to the response to HCV therapy was analyzed.

Subjects and Methods

This study was approved by the Ethical Committee of Faculty of Medicine, Cairo University. It was done over years 2010-2012 on 40 children attending the Pediatric Hepatology Unit in Cairo University Pediatric Hospital, receiving treatment for chronic hepatitis C (CHC) using Pegylated-IFN α 2b (1.5 µg /kg weekly) and ribavirin(15mg/kg/day) for one year (48weeks).
Patients were screened for their eligibility to participate in the study. According to the Helsinki Declaration (42), purpose of the study was explained to all the subjects included in the study and parents of eligible patients signed an informed consent before joining the study and before physical examination and drawing of blood samples.

Chronically infected HCV patients were included in this study. Diagnosis was done based on serological, virological and histological testing. Elevated ALT level above the upper limit of normal within 6 months prior to entry to the study was a requisite to join the study. Patients had not been previously treated with interferon-based therapy.

Exclusion criteria were as follows: (1) Patients with decompensated liver disease. (2) Patients with anemia,(<13 g/dL for males and <12 g/dL for females), leucopenia (<3,000/mm3), neutropenia (<1500/mm3), or thrombocytopenia (< 100,000/mm3).(3) Patients with high serum creatinine. (4) Existence of autoimmunity, human

immunodeficiency virus (HIV), active schistosomiasis, hepatitis B infection, uncontrolled thyroid disorder, poorly controlled diabetes mellitus, hypertension, or psychiatric diseases.

Sampling:

Five ml of blood were collected in a plain sterile vaccutainer and freshly assayed for chemistry investigations, HCV antibodies as well as HCV-RNA titers. Three ml of blood in a sterile EDTA vaccutainer for the genotyping technique. DNA extraction was done using fresh samples, and then the extracted DNA was stored at – 20 ºC till amplification.

The following laboratory work up was done for all patients:

I-Standard laboratory tests including:

1-Routine Tests: Complete blood picture (on CELL-Dyn 3700, USA). Routine chemistry including random blood glucose, serum creatinine, and liver function tests including determination of total and direct serum bilirubin, serum albumin, aspartate amino transferase (AST), Alanine amino transferase (ALT), gamma-glutamyl transferase(GGT) and alkaline phosphatase (ALP) were all done on Hitachi 911*(Roche, GmbH Mannheim Germany)
Routine laboratory tests were done at base line, weeks 1, 2, 4 and monthly thereafter during treatment till week 24 to detect the development of any adverse side effects to the drugs necessitating dose modification, temporary or permanent stoppage of treatment.

2-Markers of Hepatitis virus: HBsAg and Anti-HCV were assessed on AXSYM (Abbott Laboratories, Abbott Park, IL) using kits supplied by Abbot.

3- HCV-RNA titer: Serum was tested for HCV RNA by using a quantitative real time polymerase chain reaction (PCR) at baseline and 12 weeks after start of therapy on Applied Biosystems 7500 Real time PCR System using kits supplied by Qiagen (Qiagen GmbH (Hoffmann-La Roche AG) Max-Volmer-Strabe 4-40724-Hilden-Germany).

Children whose HCV RNA became negative or achieved a 2 log decrease in their viral load continued with antiviral therapy. If not, the child was considered as non-responder and therapy was discontinued.

For responders, another HCV RNA was repeated after 24weeks of therapy and if positive the child was considered as non-responder and therapy was discontinued. For responders at 24 weeks, therapy was continued till 48weeks, and at the end of therapy the HCV RNA was repeated to assess the end of therapy response (ETVR).

II- Specific Investigations:

Genotyping of IL-10 gene -1082 G/A and -592 C/A polymorphisms by real time PCR.

DNA extraction from whole blood was done using QIAamp DNA blood Mini kit- Qiagen. The extracted DNA was then amplified according to the protocol proposed by Turner et al, 1997 (38). Genotype was determined by real-time PCR.

Amplification and Real-time PCR allelic discrimination assays:

PCR with sequence-specific primers was used to define the IL-10 promotor SNPs at the -1082 and -592 positions as previously described (38).

Genotyping was done on Applied Biosystem step one™ Real-Time PCR System. Allelic discrimination assays were designed using Taq- Man® SNP Genotyping Assays (Applied Biosystems)*****. Assays perform genotyping of the G →A®1082 (dbSNP ID: rs1800896, TaqMan® SNP Genotyping Assays ID: C_1747360_10) and C →A ®592 (dbSNP ID: rs1800872, TaqMan® SNP Genotyping Assays ID: C_1747363_10).

PCR reaction mix consisted of the following: Taqman universal PCR master mix (2X) 12.5µL, 20X working stock of SNP genotyping assay 1.25µL, patient DNA 5µL and that was completed to 25µL with.5 DNase-free water

Real Time PCR setup

A denaturation step with sample denaturation and enzyme activation at 95°C for 10 minutes, cycling: 50 cycles of PCR amplification of target DNA, at the following temperature profile, 92°C for 15 seconds then at 62°C for 60 seconds and finally allelic discrimination plate reading and analysis using the Sequence Detection System (SDS) Software. VIC dye and Fam-dye were used for allele discrimination. (Figures 1& 2)

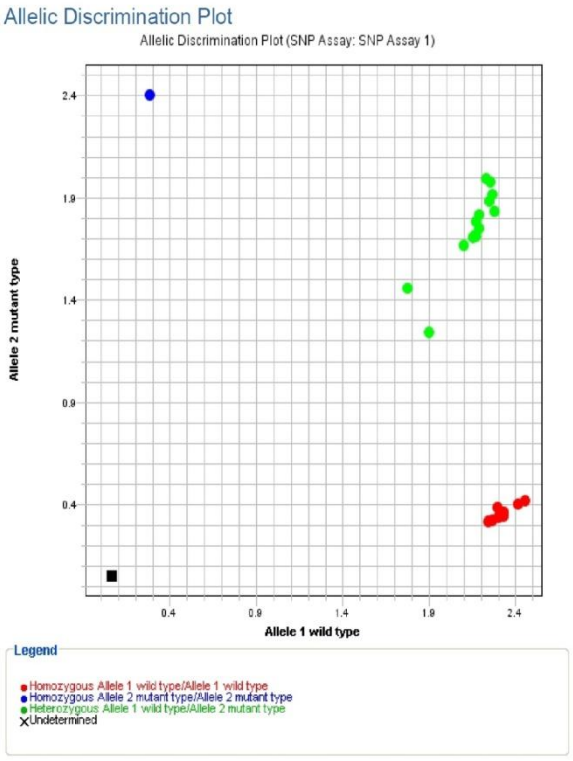

Figure 1*Allelic discrimination plot SNP assay1 G/A-1082 done on the Applied Biosystem Step One™ Real-Time PCR System*.

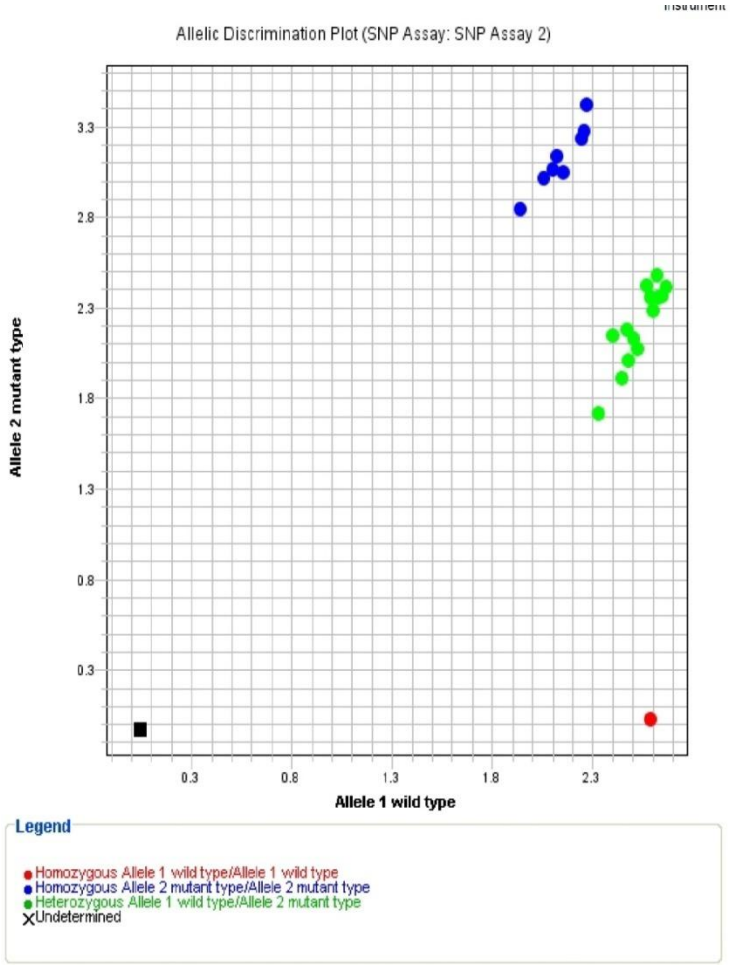

Figure 2*Allelic discrimination plot SNP assay2 C/A-592 done on the Applied Biosystem Step One™ Real-Time PCR System*.

Statistical Methods

The results were analyzed using the SPSS computer software package, version 15.0 (Chicago, IL, USA). Qualitative data were expressed as frequencies. Quantitative data were expressed as mean and SD and differences between the two groups were compared by Student t test when data were normally distributed. The quantitative data were expressed as median and interquartile range and differences between the two groups were compared by Mann Whitney test when data were not normally distributed. Multivariate logistic regression analysis was done to detect independent factors predicting response to interferon therapy in CHC patients. (23).
Differences were considered significant at $P \leq 0.05$.

Results:

Patients were divided into two groups according to their response to therapy:

1- Group I: Responders

Patients who had initial response to treatment with normalization of aminotransferases (ALT and AST) levels and clearance of the virus denoted by negative HCV RNA by PCR after 24 weeks of receiving treatment and completed treatment course.
This group included **23** patients, 14 males and 9 females, whose age ranged between 6 and 16 years.

2- Group II: Non-Responders.

This group included **17** patients, 14 males and 3 females, whose age ranged between 5 and 17 years.

On comparing the age, height, weight and BMI between the 2 groups there was no statistically significant difference observed. Concerning laboratory tests done, they all showed no statistically significant difference on comparing the 2 groups. Only the enzyme Gamma-Glutamyl-transferase was significantly higher in the non-responders and WBC count was higher in responders (P 0.001, P 0.008 respectively), whereas all other biochemical markers showed no statistically significant difference. Concerning the quantitative PCR, there was a statistically significant difference between the 2 groups being higher in the non-responder group (P 0.04).(Table 1)

Table 1: Base line data in responders and non-responders to interferon therapy.

	Responder(n=23)	Non responder(n=17)	P value
Age (mean±SD)	11 ± 3.2	10.4 ± 3.89	0.64
Gender Males Females	 14 (60.9%) 9 (39.1%)	 14 (82.4%) 3 (17.6%)	(0.179)
ALT(U/L) (25^{th}-75^{th} percentile)	60(47 – 103)	68(41 – 97)	0.83
GGT(U/L)	20(15 – 31)	56(30 – 126)	**0.001**
WBCs (x1000mm³)	7.59(5.7 – 9.4)	5.6(4.2 – 6.8)	**0.008**
Quantitative HCV RNA-PCR (IU/ml)	62.41(17.31 – 187.9)	226.5(59.7-618.9)	**0.04**

All the biochemical markers were presented as the median value and interquartile range P value was considered significant at ≤ 0.05.

Hepatitis activity index (HAI) from the liver biopsy done to the tested subjects showed no statistically significant difference as well as no statistically significant difference in fibrosis score

There was no statistically significant difference in the genotype distribution in both SNPs between the two studied groups (Table 2)

Table (2) Genotype distribution of the two SNPs in the two studied groups

	Responder (n = 23)	Non responder (n = 17)	P value
SNP1082:			
GG:	11(47.8%)	4(23.5%)	
GA:	8 (34.8%)	11(64.7%)	0.166
AA:	4 (17.4%)	2(11.8%)	
SNP 592:			
CC:	3 (13%)	1 (5.9%)	0.755
CA:	11(47.8%)	9(52.9%)	
AA:	9 (39.1%)	7(41.2%)	

When classifying the genotypes as risky genotypes (GA, AA) and (CA, AA) and non risky genotypes (GG) and (CC) for SNP-1082 and SNP -592respectively to predict response to interferon therapy in HCV infected patients; no statistically significant difference was found (Table 3)

Table (3) = Association between response to interferon therapy in HCV infected patients as regards genotype: risky genotypes (GA, AA) and (CA, AA) and non risky genotypes (GG) and (CC).

	Responder (n=23)	Non responder (n=17)	OR	95% CI interval	P value
SNP1082:					
GA and AA:	12 (52.17%)	13(76.47 %)	2.97	(0.74-11.9)	0.11
GG:	11 (47.8%)	4 (23.5%)			
SNP 592:					
CA and AA:	20 (86.9%)	16 (94.1%)	2.4	(0.23-25.34)	0.42
CC:	3 (13 %)	1 (5.8%)			

*Results were presented as number (n) of patients and percentage.**
P value was considered significant at ≤ 0.05.
0R= Odds ratio. 95th% CI interval= 95% Confidence interval.

When examining alleles and their association with response to therapy, no statistically significant difference was found as well as no statistically significant difference on examining the different haplotypes.

Multivariate logistic regression to assess the influence of different factors on response to therapy showed that only pretreatment levels of HCV-RNA were significantly associated with treatment outcome (P= 0.04 O.R 1.004 and 95th % CI = 1-1.008) where a lower count was observed in the responders group.

As for the second SNP-592 C/A; there was a statistical significant association between the score of fibrosis and different genotypes ($P < 0.004$), concluding that the (A) allele is risky

DISCUSSION

HCV infection remains a high-priority problem that needs to be addressed utilizing many different approaches. In this sense, discovery of genes associated with the HCV clearance is a crucial step that can result in novel strategies for patient treatment and recovery (36).

Part of the problem relates to the fact that the current standard of care treatment for HCV i.e., a combination PegIFNα-2b and ribavirin, is expensive, associated with significant side effects, and results in only 50–70% sustained virologic response (SVR) Thus predicting the likelihood of response of treatment before initiating therapy would be very useful.
The results of this study revealed that, the end of treatment virological response (ETVR) to PegIFN α-2b plus ribavirin therapy was 57.5% (23 responders versus 17 non responders).

The main target in this study was assessing the role of the SNPs in the promoter region of IL-10 gene (-1082 G/A and -592 C/A) in the response to HCV therapy and viral clearance, it was found that: regarding the frequency distribution of different genotypes of the two studied SNPs, there was no statistically significant difference in the genotype distribution or allele frequency between the two studied groups and neither SNP with different allele combination was detected as a significant predictor of response to treatment in HCV infected children ($p = 0.05$).

This is in agreement with previous reports (21, 10, 16) where IL-10 gene promoter polymorphisms were not associated with response to HCV treatment.

As regards percentage distribution of different haplotypes, no statistically significant difference between the studied groups was detected.
An analysis using the well-studied proximal and distal haplotypes in the promoter region by Olksyk et al, (33), was in agreement with the results concluded by the present study, where no positive associations was observed between the proximal IL-10 promoter SNPs of -1082 and -592 defining the GC, AC or AA haplotypes with response to HCV treatment.
The work by Mangia and coworkers (29) concluded that inheritance of -1082A, -819T, and -592A alleles as an extended haplotype of the IL-10 gene appeared to be associated with HCV eradication. The protective factor seems to be due to inheritance of the extended haplotype rather than to inheritance of a single polymorphism of the IL-10 gene promoter and no single polymorphism was more frequent in patients who spontaneously cleared the virus than in those who did not.
Another study (38) showed that AA combination at positions -1082 and -592 of IL-10 promoter gene positively correlated with initial response to standard interferon-α monotherapy in CHC patients.

Of special interest to us was the large-scale study done by Chuang et al, 2009 (10), which indicated that out of the three identified haplotypes (AA, AC, and GC), AA haplotype was commonly observed in their studied subjects (70.3%in CHC patients and 69.8% in healthy controls vs. 23.0% in Caucasian CH-C patients and 24.8% in healthy controls). Since AA haplotype has been shown to be associated with a sustained virologic response (SVR) of HCV infection and a better response to standard interferon-based antiviral therapy (14,29), the higher frequency of this haplotype in the Taiwanese population in that study may partly explain the higher SVR rate in previous Taiwanese reports (2,43,45), as well as this one, as compared to that in Western studies (30, 19, 20)as well as higher SVR rate in Taiwanese patients who received only 24 weeks of combination therapy compared to Caucasian ones who received 48 weeks of combination therapy (30,15,19).

Considerable interethnic variation in the distribution of genotypes may explain the discrepancy in the results between the various studies. (10)

In the current study, factors other than genotype were also analyzed (host and viral factors) that assess the therapeutic efficacy of INF treatment. It was found that patients with ETVR had significantly lower pretreatment PCR levels (p =0.04), this agrees with some studies (3,31) where there was a close relationship between baseline viral concentration and response to PEG-IFN alpha-2b plus ribavirin and SVR rates are increased 1.5-fold after 24 weeks of treatment in patients with <2 million copies/mL, compared with patients with higher viral loads.

As regards fibrosis stages and its relation to response treatment, in the current study it was found that fibrosis scoring was not associated with therapeutic efficacy of INF treatment. This finding agrees with the studies (47, 40) that found that a high baseline fibrosis was not associated with a lack of SVR.
On the other hand a study (3) demonstrated that absence of cirrhosis, low HCV RNA level (<500,000 IU/ mL) are predictors of sustained virological response to treatment for Chronic HCV infection.
A pilot study of a short course (14 weeks) of therapy with peg-IFN alpha-2b and ribavirin demonstrated that the lack of bridging fibrosis/cirrhosis was an important factor associated with SVR (11).

Multivariate logistic regression identified that only pretreatment HCV RNA levels (p= 0.04) is the most significant predictor of response. This is comparable with the work presented by Rauch et al, 2010 (35) who found that IL10 variants remained highly associated with response to HCV therapy after adjusting for known predictors of treatment response (HCV RNA levels, fibrosis scores).

This study failed to demonstrate any relation between SNP -1082G/A in IL10 gene and the degree of fibrosis or HAI. These findings were in concordance with the studies (14,24,2) where no association was found between hepatic inflammatory grading or fibrosis staging and IL-10 promoter gene polymorphisms.

As for the second SNP-592 C/A, there was a statistical significant association between the score of fibrosis and different genotypes (P < 0.004), concluding that the (A) allele is risky. However there was no statistical significant relation between HAI and the same SNP (0.07). These results agree with an earlier study (38) that assumed that hepatitis C patients who produce high levels of IL-10 have less hepatocellular injury and less ability to control infection and patients with low secretion of IL-10 have a better ability to eliminate the hepatitis infection. Individuals who were homozygous for IL-10 AA at position -592 had a higher fibrosis score (1).

Conclusion

In Conclusion, there is a significant relation between SNP -592 and degree of fibrosis in HCV infected children, and that HCV RNA-PCR and GGT pretreatment levels are predictors of response to interferon therapy in HCV infected children.
However there is no significant association between polymorphisms in the IL-10 gene (-1082G/A and -592C/A) or haplotype as regards response to therapy or severity of HCV infection in children

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