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Sequence Heterogeneity in the Core and NS5A Region of Hepatitis C Virus (HCV) and IL-28B Polymorphisms in Predicting Treatment Response among Chronic HCV Genotype 4 Infected Egyptian Patients

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A B S T R A C T

Egypt has the highest prevalence of HCV specially genotype 4. Both host and viral factors are important predictors of the efficacy of PEG-IFN/RBV therapy. The aim of this study was to investigate the mutational diversity of HCV core and NS5A regions and variation of*IL28B* gene to predict virological response. Thirty HCV patients were included in this study. Detection of viral load, amplification and direct sequencing of the core and NS5A genes, determination of *IL28B* SNPs rs12979860 and rs8099917 polymorphisms were done. Equal percentage 3.3% of patients were carrying core mutations at position 70, and 91, while 6.7% had mutations at position 71. The mean number of aa mutations in IRRDR (8.4 ± 5.5 vs. 8.3 ± 5.8) and in ISDR (6.9 ± 6.2 vs. 4.1 ± 1.8). This difference did not reach the statistical significant difference between SVR and non-responders. Regarding *IL28B* polymorphism for rs12979860 CC alleles achieved SVR compared to 90.9% with CT and 50% with TT alleles; for rs8099917 66.7% of patients with either GT or GG genotype achieved SVR. *IL28B* rs12979860 polymorphism, severity of fibrosis, and NS5A ISDR ≥2 are useful markers for predicting the outcome of PEG-IFN/RBV therapy.

Introduction

Hepatitis C virus (HCV) infection is a major global health problem, considered a major cause of chronic liver diseases. Its ability to evade host defense mechanisms services to establish persistent infection, producing a wide spectrum of pathogenicity (El-Shamy *et al.,* 2014). Based on phylogenetic analysis, HCV has been classified into seven different genotypes and diverse subtypes (Smith *et al.,* 2014). It is estimated that about 170 million people, 3% of the world's population, are infected with HCV.

Egypt has the highest prevalence of HCV worldwide, with an average of 14.7%. HCV genotype 4 (HCV-GT4) is responsible for 90% of the total HCV infections in Egypt (Lavanchy *et al.,* 2011; Miller *et al.,* 2010).

Pegylated interferon (PEG-IFN) and ribavirin (RBV) are likely to stay the HCV backbone therapy. The addition of direct acting antivirals (DAAs), their combination therapy without IFN and RBV as well as the evolution of resistance-associated variants that may

attenuate their efficacy do not permit the exclusion of RBV without bargaining rapid and early viral response rates, viral breakthrough, sustained viral response, and relapse rates (Nguyen *et al.,* 2015; Asselah *et al.,* 2008; Suzuki *et al.,* 2012).

In the European Association for the Study of the Liver (EASL) Recommendations on Treatment of Hepatitis C 2015, six treatment options are available in 2015 for patients infected with HCV genotype 4, including two IFN-containing regimens and four IFN-free regimens. In settings where none of these options is available, the combination of PegIFN-a and ribavirin remains acceptable. (EASL Recommendations on Treatment of Hepatitis C, 2015)

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (Kau *et al.,* 2008). Several factors were identified, including age, race, liver fibrosis, HCV genotype and HCV RNA levels (Shirakawa *et al.,* 2008). Viral factors in the form of genetic heterogeneity in the HCV genome were frequently the focus for investigation of IFN responsiveness, and amino acid substitutions in the core and NS5A regions were reported as markers that could be used to predict the response to IFN therapy. However, these relationships were controversial to clarify IFN responsiveness (El-Shamy *et al.,* 2012; Hayashi *et al.,* 2011; Nakagawa *et al.,* 2010).

Host genetic factors, as well, contribute to IFN treatment outcomes. Therefore, several genome-wide association study (GWAS) were performed to understand the host factors that were associated with IFN responsiveness; such as *interleukin-28B* (*IL28B*) gene polymorphisms. The single nucleotide polymorphisms (SNPs) of *IL28B*, rs12979860 and rs8099917 genotypes are significantly associated with the outcome of IFN therapy (Rauch *et al.,* 2010; Domagalski *et al.,* 2013; Itakura *et al.,* 2015).

The aim of this study was to identify the pretreatment factors including viral (amino acid substitutions in the HCV core gene and NS5A) and host-related factors (genetic variation near the *IL28B* gene) and the interplay between them that could predict sustained virological response.

Materials and Methods

Patients

This study was carried out on thirty HCV-infected treatment-naïve Egyptian patients; of them 24 (80%) males and 6 (20%) females. Their age ranged from 27 to 58 years, they were eligible for combined pegylated interferon and ribavirin therapy Patients with renal, cardiac, neoplasic disease, immunological disorders and cirrhotic patients as well as patients with HBV and HIV co-infection were excluded from the study. Baseline characteristics of the patients involved were illustrated in table 1. Liver biopsy was performed for each patient. Grading and staging for activity and fibrosis was done according to the METAVIR criteria. Patients received subcutaneous injections of pegylated- IFN-a 2b (1.5 mg/kg) once each week plus oral ribavirin (1000-1200 mg/day) daily. The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from each patient prior to enrollment in the study.

Blood samples (5ml) were collected from each patient, and divided into 3ml in plain tubes for some biochemical investigations and molecular investigations as pretreatment detection of HCV-RNA viral load by Real Time PCR. And 2ml in 0.5 M EDTA tubes for determination of *IL28B* SNPs rs12979860

and rs8099917 polymorphisms by Real Time PCR.

Sequence analysis of hepatitis C virus NS5A and the core regions of the hepatitis C virus genome

Viral RNA was extracted from patients' sera using QIAamp viral RNA mini spin Kit (Qiagen®) according to manufacturer's instructions.

The extracted viral RNA was used as a template for viral load determination by real time PCR using Artus HCV QS-RGQ-PCR Kit (Qiagen®).

Transcription of RNA into cDNA was done using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamers. RT was performed at 25°C for 5 min followed by 60 min at 42°C and then terminated by heating at 70ºC for 5 minutes.

Amplification of NS5A and the core regions of the HCV genome were performed. Primers used for amplification of the NS5A region of HCV were as follows: NS5A/F1 (5'-CTCAAYTCGTTCGTRGTGGGATC-3'; sense) and NS5A/R1 (5'-CGAAGGTC ACCTTCTTCTGCCG-3'; antisense) for one-step RT-PCR; and NS5A/F2 (5'-ATGCGA GCCYGAGCCGGACGT-3'; sense) and NS5A/R2 (5'-GCTCAGGGGGYT RATTG GCAGCT-3'; antisense) for the second-round PCR. Primers used for amplification of the core region of HCV were core/F1 (5'-GCTAGCCGAGTAGTGTTG-3'; sense) and core/R1 (5'-GATGTGRTGRTCGGCCTC-3'; antisense) for one-step RT-PCR; and core/F2 (5'-GGAGGTCTCGTAGACCGTGC-3'; sense) and core/R2 (5'-ATGTACCCCA TGAGGTCGGC-3'; antisense) for the second-round PCR.

For the amplification of NS5A and the core regions, nested PCR was used. The first run amplification was performed in 50 µL final reaction volume containing 2X DreamTaq Green PCR Master Mix (Thermo Scientific), 5 picomoles of each of the outer primers and 2 µL of copy DNA. The thermal cycling consisted of one cycle of initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing step at 50°C for 30 seconds, and extension step at 72°C for 60 seconds, each with a final extension cycle at 72°C for 10 minutes. The second-round PCR was performed under the same conditions as the first run.

The purified NS5A and core PCR products were sequenced using the BigDye Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) and the automated sequencer ABI prism 310 genetic analyzer (Applied Biosystems).

For determination of HCV genotypes and subtypes, NS5A and the core sequencing results was analyzed using HCV BLAST free online tool from HCV sequence database

Bio Edit Sequence alignment editor version 7.2.5 and ClustalW multiple alignment tool were used to undergo align NS5A and core Nucleotide and amino acid sequences from different patient isolates in order to determine the presence of mutations (Figs. 2, 4 and 5).

SNP genotyping

The two IL28B SNPs rs12979860 and rs8099917 were genotyped for each patient. Genomic DNA was extracted from peripheral blood samples (2ml) collected in 0.5 M EDTA tubes of all patients using PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocol. Polymorphisms rs12979860 (C > T) and rs8099917 (T > G) in gene *IL28B* were genotyped by Real-Time PCR, TaqMan real-time allelic discrimination assay using a Step

One PLUS Sequence Detector (Applied Biosystems, Foster City, CA, USA),2 µl DNA was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems).

Statistical analysis

Data were collected, coded and fed to statistical software SPSS version 20. The given graphs were constructed using Microsoft excel software.

Means and Standard deviations were estimated for quantitative data. For correlations, Spearman correlation coefficient was applied. Groups were compared by independent samples t-test, Mont Carlo exact probability or Mann-Whitney U test as appropriate. P value ≤ 0.05 was considered to be significant.

To evaluate the optimal threshold of the number of amino acid mutations in ISDR and IRRDR for prediction of treatment outcomes, the receiver operating characteristic (ROC) curve was constructed. Multiple stepwise logistic regression analysis was performed to identify variables that independently predicted the treatment outcome.

Results and Discussion

Out of the 30 HCV patients included in this study, 20 patients (66.7%) showed sustained viral response (SVR), while 10 patients (33.3%) were non-responders.

The relation between viral load and SVR was illustrated in table 2.

Twenty-three (76.7%) of the studied cases had liver biopsy showing established fibrosis (F2, F3) before starting treatment according to METAVIR scoring system, while 23.3% (7/30) have minimal fibrosis. Out of the 7 cases with minimal fibrosis, 5 (71.4%) have SVR to antiviral treatment, on the other hand, 65.2% (15/23) of those with established fibrosis display SVR (Table 3).

Regarding the distribution of core mutations among the 30 patients was shown in table 4. Patients carried core mutations at position 70, and 91 did not respond to treatment, while the 2 cases carried mutations at position 71 responded to treatment.

The mean number of aa mutations within IRRDR, ISDR of HCV NS5A obtained from pretreatment sera of 27 HCV GT-4-infected patients with SVR and non-responders (NS5A was not amplified in 3 cases) was calculated. The mean number of aa mutations in IRRDR (SVR= 8.4 ± 5.5 vs. Non-responders = 8.3 ± 5.8; p = 0.995), however, no significant difference was observed between SVR and non-responders. The mean number of aa mutations in ISDR (6.9 ± 6.2 vs. 4.1 ± 1.8; p = ^{U}p = 0.118); this difference did not reach the statistical significance (Fig. 1B, 3, 4, 5)

On performing ROC curve analysis to estimate the optimal cutoff number of ISDR and IRRDR mutations for SVR prediction. The analysis estimated 2 mutations for ISDR and 5 mutations for IRRDR as the optimal number to predict SVR (Fig. 3A, B). 83.3% of patients with SVR had HCV with ISDR mutations of ≥2. In contrast, 66.7% of the patients with non-responders had ISDR mutations < 2 (*P*=0.048).

On the other hand, the sequence heterogeneity within IRRDR is closely associated with the SVR in Egyptian patients infected with HCV GT-4 with a high degree of sequence variation in IRRDR, *i.e.*, (IRRDR ≥ 5) (*P*=0.086), while a low degree of sequence variation in this region (IRRDR <5) had no effect on treatment outcome (Fig. 3C, D).

IL-28B polymorphism (rs12979860 and rs8099917 alleles)

For rs12979860 alleles, 18/30 cases (60%) of the studied cases possess TT alleles, while CT alleles were found in 11/30 cases (36.7%) and CC allele profile was only found in 1 case (3.3%). For rs8099917 alleles, no case displays TT alleles, on the other hand, GG and TG represented by 60 and 40%, respectively. Regarding relation between rs12979860 and response to treatment, the case with CC alleles was viral responder compared to 90.9% with CT and 50% with TT alleles; these differences were found to be statistically significant. On the other hand, for rs8099917, TT genotype was not displayed, and 66.7% of patients with either GT or GG alleles were achieved SVR (Table 5).

The case having CC alleles for *IL-28B* SNP (rs12979860) was found to have no core mutations compared to 90.9% of cases having CT alleles. Interestingly, the HCV cases carrying core mutations at position 70, and 91, having TT alleles, similarly, In case of *IL 28B* SNP (rs8099917), it was found also that the HCV cases carrying core mutations at position 70, and 91, having GG alleles. However, these differences did not reach statistical significance.

The identification of independent predictive factors for SVR by multivariate logistic regression analysis revealed that alpha fetoprotein (AFP), *IL-28B*rs1297986060, Liver biopsy, and NS5A ISDR were correlated with the treatment outcome; representing independent predictive factors of PEG-IFN/RBV treatment outcome (Table 6).

HCV infection is a major global health problem, considered a major cause of chronic liver disease, HCC, and deaths from liver disease and is the most common indication for liver transplantation. PEG-IFN/RBV combination therapy is standard treatment for patients with chronic hepatitis C. The addition of DAA, their combination therapy without IFN and RBV and resistance developed does not permit the exclusion of RBV without compromising RVR and SVR rates, viral breakthrough, and relapse rates (Asselah *et al.*, 2011; Fusco *et al.*, 2011; Bronowicki *et al.*, 2012).

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (Kau *et al.*, 2008). The aim of this study was to identify the pretreatment factors that could predict sustained virological response, including viral (aa substitutions in the hepatitis C virus core gene and NS5A) and host-related factors (genetic variation near the *IL28B* gene).

The present study included 30 HCV naïve patients, 24 (80%) were males and 6 (20%) were females. Their age ranged from 27 to 58 years, with the highest percentage (50%) of the patients were in the age range between 40-<50 years. Our results are consistent with previous studies reporting higher prevalence of HCV in age groups older than 30 years. Darwish *et al., 1992* reported that anti-HCV among those 20 to 30 years old was 6%, as compared with 37.5% among those older than 30 years.

Genotype identification is clinically important for prediction of responses to, and in determining the duration of, antiviral therapy (Zein *et al.*, 1996). HCV genotype 4 has the highest prevalence among Egyptians, with a predominance of the subtype 4a (HCV-4a) (Lavanchy *et al.*, 2011; Miller *et al.*, 2010).

Sequencing and phylogenetic analysis of the core/E1 or NS5B region were considered to be the gold standard for HCV genotyping since they accurately identified the subtype

(Cai *et al.*, 2013). In the present study, core sequence analysis was evaluated for its effectiveness in identifying HCV genotypes/subtypes in the studied Egyptian patients. All the 30 isolates were successfully genotyped and sub-genotyped; 27 patients (90%) of them were of sub-genotype 4a, while the remaining 3 cases (3.3%) were equally distributed within 4n, 4o, 4l genotypes.

In the present work, 76.7% of the HCV patients had liver biopsy showing established fibrosis before starting treatment, while 23.3% have minimal fibrosis. With logistic regression analysis, state of liver injury was among the predicting factors of SVR In agreement with our results, Powis *et al.*, (2008) in their study on chronic HCV infections with GT-3 exploring a significant poor antiviral response in such patients with advanced liver injury.

In the present study, 63.3 % had a viral load ranging between 10^5 and 10^7 IU/ml; viral load was associated with SVR since 52.6% with viral load above 10^5 IU/ml achieved SVR compared to 90.9% of patients with viral load $<10^5$ IU/ml. Our results agreed with Magrin *et al.*, (1996) who found in his study that low pre-treatment serum HCV-RNA level, was important predictor of response to IFN therapy. On the other hand, Fallows *et al.*, 2000 found that pre-treatment viral load did not affect the outcome of treatment.

The difference in responses among patients infected with different HCV genotypes suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, the correlation between IFN-based therapy outcome and sequence polymorphisms within the viral core and NS5A proteins has been investigated.

Table.1 Baseline characteristics of the HCV patients (n = 30) according to gender

Parameter	Total		Gender				t (P)
			Male (24)		Female (6)		
	Mean	±SD	Mean	±SD	Mean	±SD	
Age (Years)	43.8	±10.3	42.3	±10.6	50.0	±6.6	1.6 (0.103)
BMI	26.8	±2.1	26.7	±2.2	27.2	±1.8	0.49 (0.627)
Glucose	91.1	±15.3	93.4	±15.8	81.7	±8.6	1.7 (0.092)
Creatinine	0.8	±0.2	0.8	±0.2	0.7	±0.2	1.4 (0.163)
Albumin	4.4	±0.9	4.2	±0.5	5.0	±1.8	1.0 (0.353)
Alkaline phosphatase	77.2	±34.3	77.6	±31.9	75.7	±46.1	0.12 (0.903)
AST	15.0	±9.2	15.6	±10.1	12.8	±4.0	0.65 (0.524)
ALT	18.1	±12.3	19.3	±13.3	13.3	±6.0	1.1 (0.294)
T.Bilirubin	0.8	±0.2	0.8	±0.2	0.8	±0.1	0.31 (0.760)
WBC*10^3	6.2	±1.7	6.3	±1.8	6.0	±1.2	0.33 (0.737)
Hb	13.9	±1.2	14.3	±1.1	12.4	±0.3	8.0 (0.001)*
Platelets*10^3	203.6	±73.6	178.9	±41.5	302.5	±94.2	3.1 (0.023)*
Prothrombin	94.4	±7.1	94.2	±7.4	95.2	±6.3	0.30 (0.763)
ANA titer	3.7	±6.1	3.3	±5.7	5.7	±8.5	0.63 (0.452)
TSH	1.9	±1.3	1.9	±0.9	2.3	±2.5	0.39 (0.706)
AFP	3.5	±2.0	3.8	±2.1	2.5	±1.6	1.4 (0.168)
ANC	2436.8	±541.3	2511.7	±564.8	2100.0	±241.5	1.4 (0.175)

t: independent samples t-test; * P < 0.05 (significant); Values are mean ± standard deviation (SD);
BMI body mass index, ALT alanine aminotransferase, AST aspartate aminotransferase

Table.2 Viral load in relation to sustained virological response among the studied cases

Viral load(IU/ml)	SVR		Non-responders		Total	*MCP*
	No	%	No	%		
$> 10^2 \leq 10^3$	2	100.0	0	0.0	2	
$> 10^3 \leq 10^4$	2	100.0	0	0.0	2	
$> 10^4 \leq 10^5$	6	85.7	1	14.3	7	
$> 10^5 \leq 10^6$	8	80.0	2	20.0	10	
$> 10^6 \leq 10^7$	0	0.0	6	100.0	6	0.007*
$> 10^7$	2	66.7	1	33.3	3	
Total	**20**		**10**		**30**	

MCP: Mont Carlo exact probability * P < 0.05 (significant)

Table.3 Distribution of the 30 HCV cases considering their response to Treatment in relation to liver biopsy (METAVIR score)

Liver Biopsy	Response				Total (n=30)	
	Non responder (n=10)		Responder [SVR](n=20)			
	No	%	No	%	No	%
A1F1	2	50.0	2	50.0	4	13.3
A1F2	2	100.0	0	0.0	2	6.7
A2F1	0	0.0	3	100.0	3	10.0
A2F2	5	26.3	14	73.7	19	63.3
A2F3	1	50.0	1	50.0	2	6.7

F1: portal and periportal fibrosis with no septum, F2: portal and periportal fibrosis with rare septum, F3: portal and periportal fibrosis with many septum, A1: minimal necroinflammatory activity, A2: moderate necroinflammatory activity

Table.4 Distribution of Core mutations among the studied cases and The irrelation to response to treatment

HCV mutation	Response				Total	*MCP*
	Non responder		Responder			
	No	%	No	%		
Core mutation						0.166
▪ Non / other mutations	8	30.8	18	69.2	26	
▪ 70	1	100.0	0	0.0	1	
▪ 71	0	0.0	2	100.0	2	
▪ 91	1	100.0	0	0.0	1	

MCP: P value based on Mont Carlo exact probability

Table.5 Relation of *IL 28B* polymorphism to response to treatment

IL 28B SNP	Response				Total	*MCP*
	Non- responder		SVR			
	No	%	No	%		
rs12979860						
▪ CC	0	0.0	1	100.0	1	0.050*
▪ CT	1	9.1	10	90.9	11	
▪ TT	9	50.0	9	50.0	18	
rs8099917						
▪ GG	6	33.3	12	66.7	18	1.000
▪ TG	4	33.3	8	66.7	12	

MCP: P value based on Mont Carlo exact probability, * P < 0.05 (significant)

Table.6 Multiple stepwise logistic regression analysis for predictors of response to Treatment with PEG-IFN/RBV for thirty HCV infected naive patients

Variables	B	S.E.	Sig.	Exp (B)	95.0% C. I. for Exp (B)	
					Lower	Upper
AFP	-.877	.361	**.015**	**.416**	.205	.844
rs1297986060	-1.928	.256	**.047**	**.145**	.070	.452
Liver biopsy(fibrosis)	-.007	.224	**.044**	**.850**	.375	.980
NS5A(ISDR)	.301	.102	**.046**	**1.351**	1.114	2.037

Fig.1 PCR products of core gene and NS5A genes: A) PCR products (core gene) were analyzed on a 2% agarose gel stained with ethidium bromide, compared to a 100 bp DNA ladder and visualized by ultraviolet light showing a band of 573 bp length. B) PCR products (NS5A gene) were analyzed on a 2% agarose gel stained with ethidium bromide, compared to a 100 bp DNA ladder and visualized by ultraviolet light showing a band of 675bp length

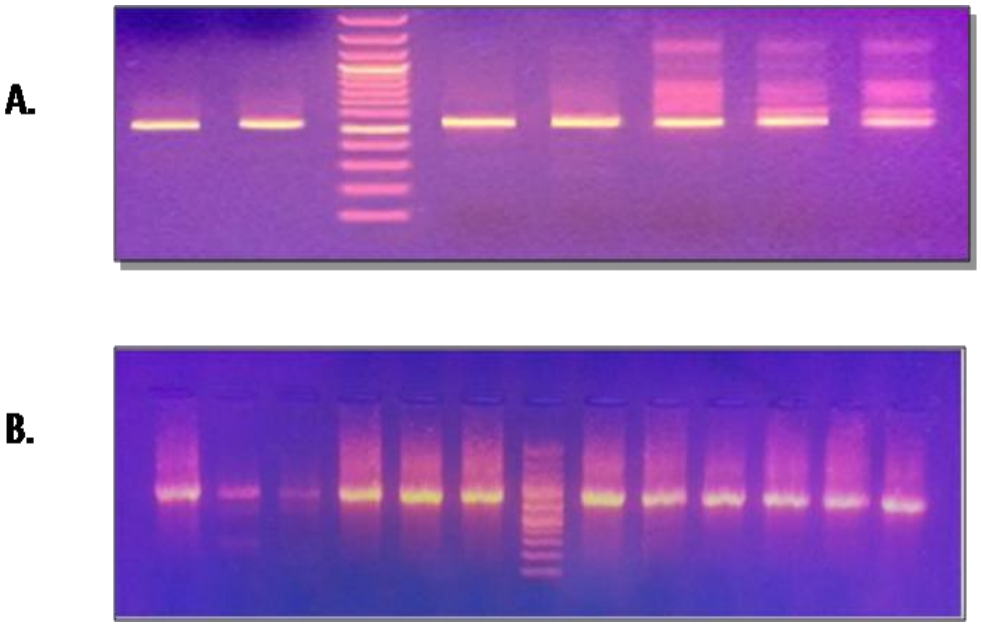


Fig.2 Amino acid sequence alignment in the core region obtained from sera of the 30 HCV GT-4 cases showing amino acid substitutions at positions 70, 71 and 91 in the Core region. Amino acid sequences were aligned with Bio Edit.
Reference Sequence with accession number (DQ988078) is shown at the top

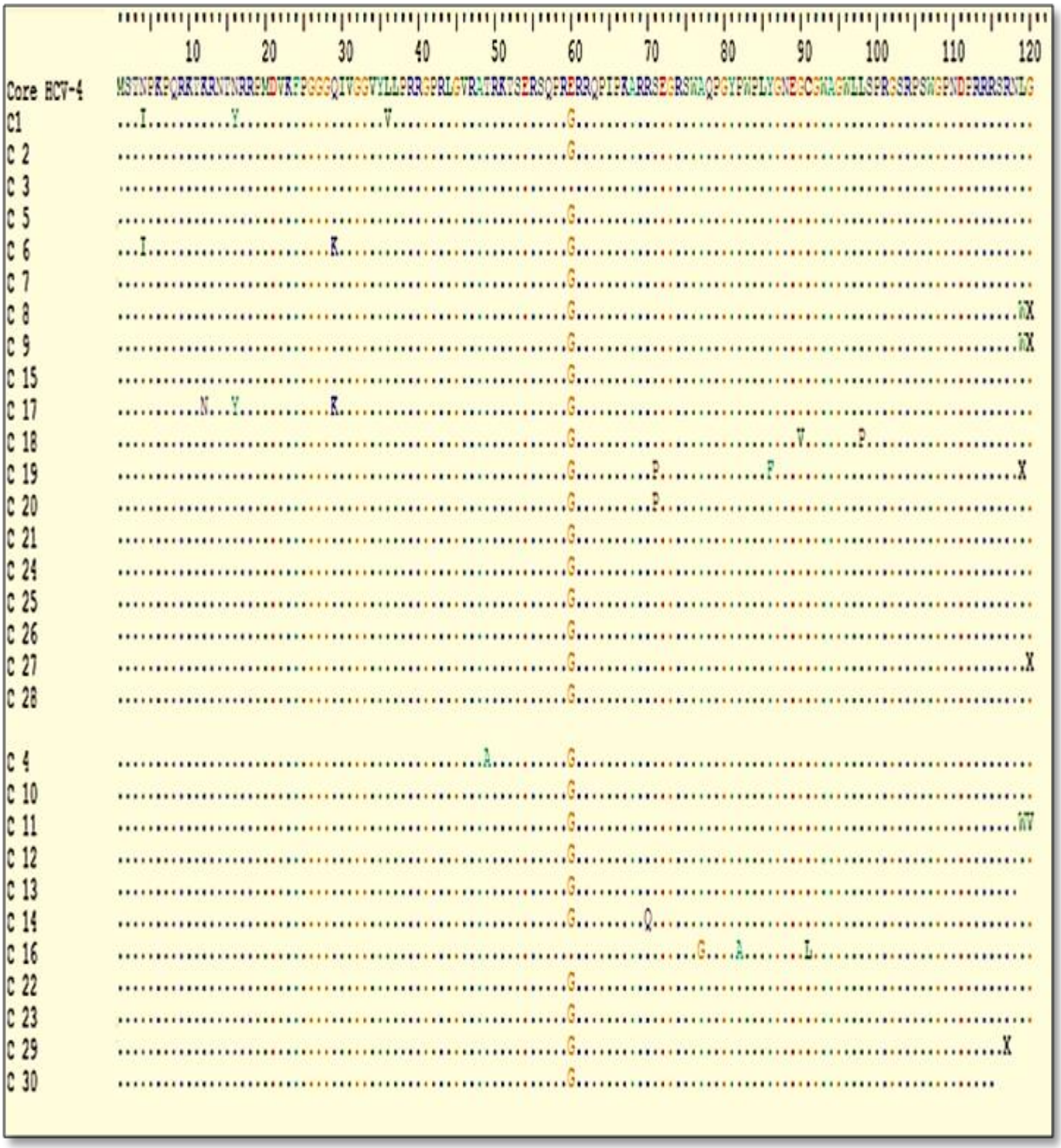
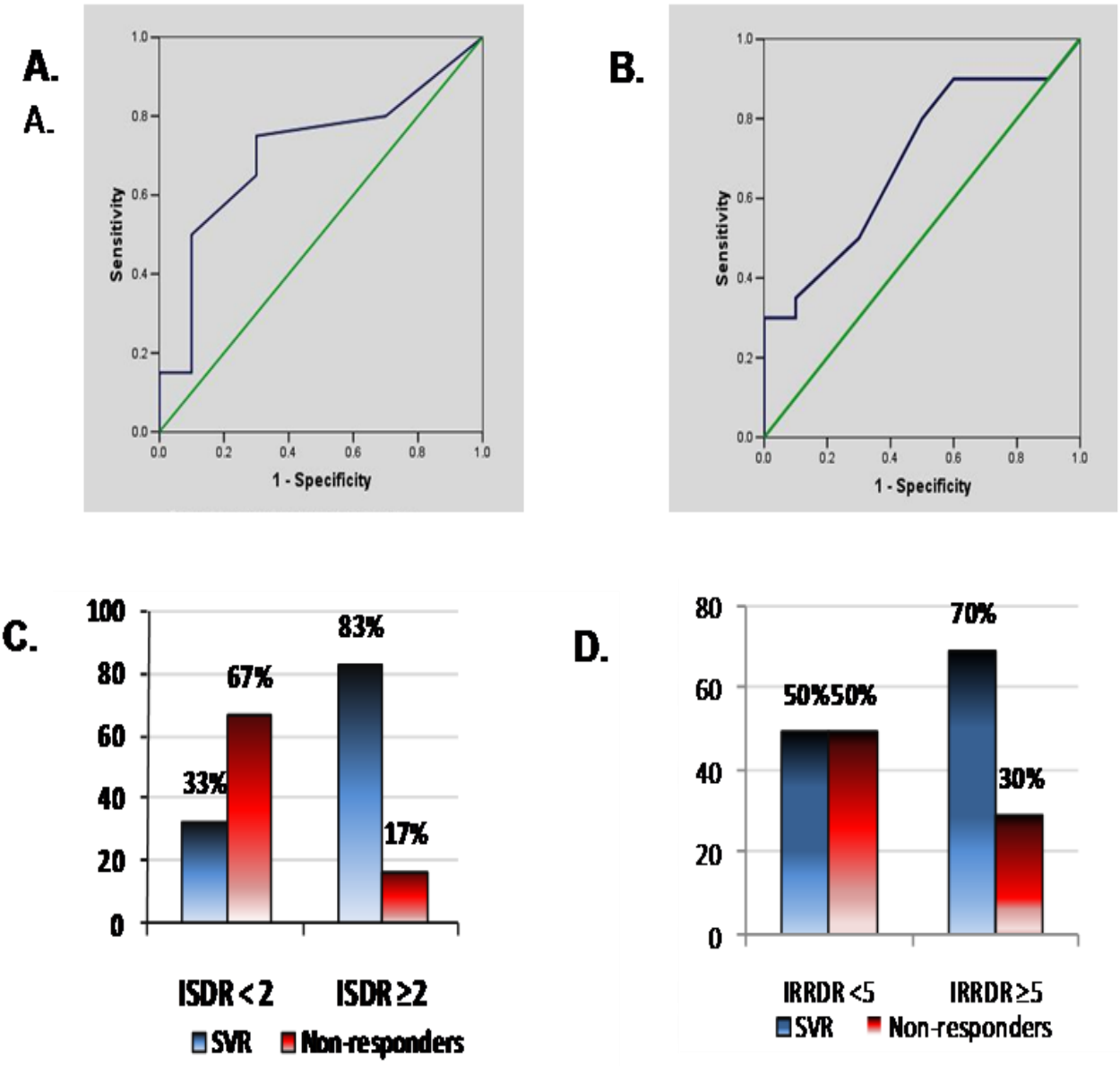


Fig.3 A) ROC curve analysis of NS5A ISDR [HCV GT-4] sequence heterogeneity for SVR prediction. The solid blue line curve shows the AUC. The optimal number of ISDR [HCV GT-4] mutations for SVR prediction, which yields the highest sensitivity (75%) and the highest specificity (75%) was 2.00. B) ROC curve analysis of NS5A IRRDR [HCV GT-4] sequence heterogeneity for SVR prediction. The solid blue line curve shows the AUC. The optimal number of IRRDR [HCV GT-4] mutations for SVR prediction, which yields the highest sensitivity (80%) and the highest specificity (70%) was 5.00. C) Sustained virologic response among 27 studied patients according to the optimal number of ISDR [HCV GT-4] mutations. D) Sustained virologic response among 27 studied patients according to the optimal number of IRRDR [HCV GT-4] mutations



SVR; Sustained virologic response, ISDR; Interferon sensitivity-determining region., IRRDR; Interferon sensitivity-determining region

Fig.4 Bio Edit amino acid sequence alignment in the NS5A ISDR region showing amino acid substitutions in the NS5A ISDR region of the 27 HCV GT-4 cases.
Reference Sequence with accession number (DQ988078) is shown at the top

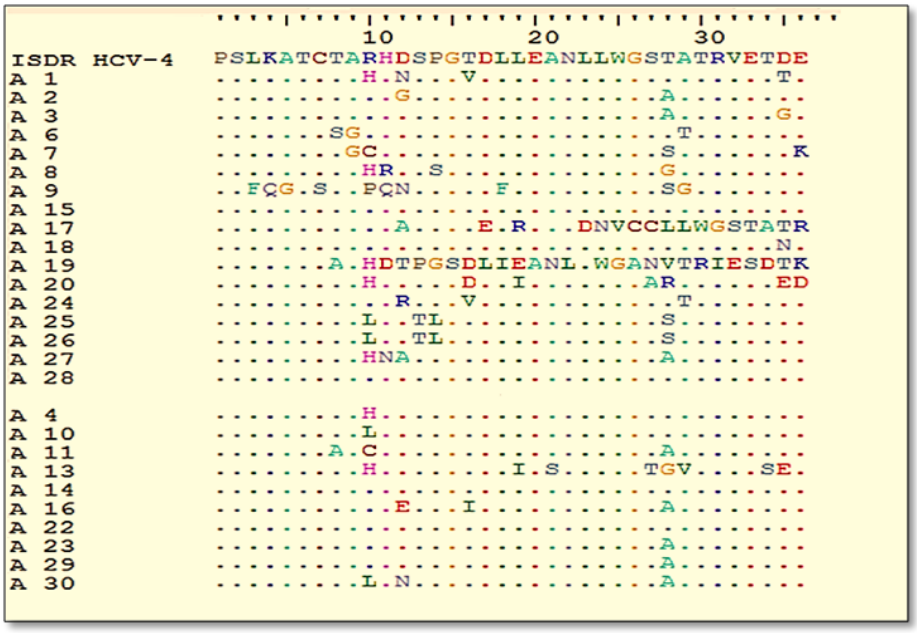
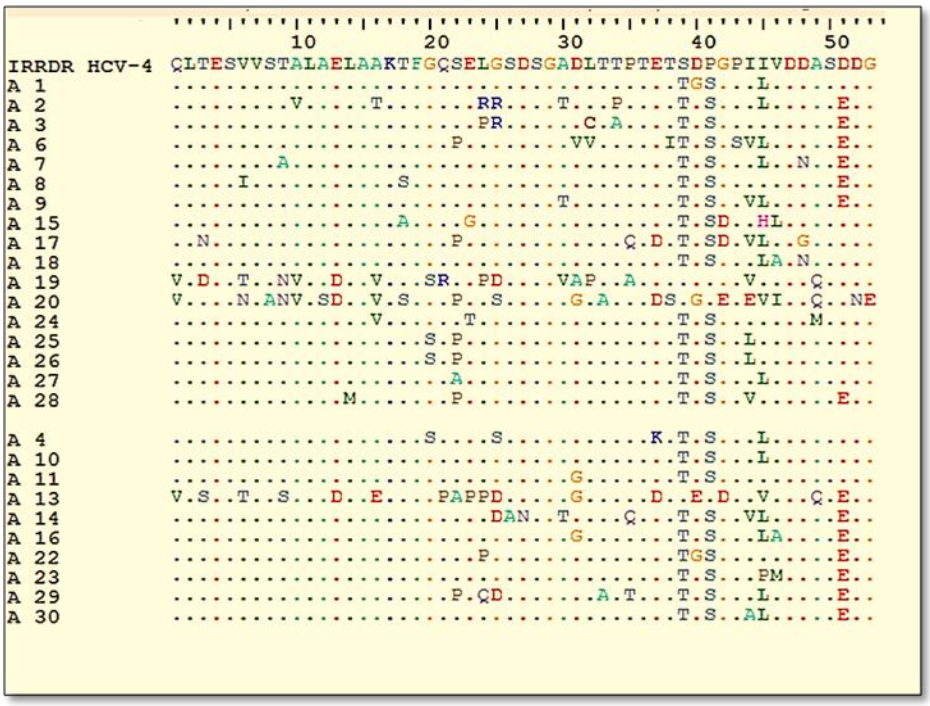


Fig.5 Bio Edit amino acid sequence alignment in the NS5A IRRDR region showing amino acid substitutions in the NS5A IRRDR region of the 27 HCV GT-4 cases
Reference Sequence with accession number (DQ988078) is shown at the top



HCV NS5A and core protein polymorphisms in particular at positions 70 and 91, was proposed as a pretreatment predictor of poor virological response. Minimal researches handled information regarding the correlation between sequence heterogeneity in the NS5A and core proteins of HCV GT-4 and PEG-IFN/RBV treatment outcome. Therefore, in the present study, we aimed to probe this concern in Egyptian patients infected with HCV GT-4.

As regards core variability as the residues at positions 70 and 91 were both well conserved among the sequences analyzed, and as there are only two cases one for each mutation 70 and 91, no evident correlation with treatment outcome was observed for these residues. On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-responders. Compared to our results, El-Shamy *et al.,* (2012) found no significant correlation between core gene polymorphism and treatment outcome in HCV GT-4 infection. In concordance with our study, the residue at position 70 of the core protein of all but two HCV GT-4 isolates analyzed in their study was Arg. They concluded that, this high degree of sequence conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-GT4 infection. Previous studies (Hayashi *et al.,* 2011; Okanoue *et al.,* 2009) carried out on genotype 1b HCV infection, showed that the mutations in aa 70 and 91 in the core region correlated with SVR during PEG-IFN/RBV combination treatment. Conversely, Enomoto *et al.,* 2010 reported that core 70Q substitution was not associated with SVR.

Viral genetic polymorphisms, in the NS5A especially (ISDR and IRRDR) regions, among HCV isolates have been linked to the difference in SVR rates to PEG-IFN/RBV therapy (El-Shamy *et al.,* 2012; Okanoue *et al.,* 2009).

In the present study the sequence heterogeneity within ISDR was closely associated with the SVR in the studied patients. A sequence variation of ≥ 2 significantly correlated with SVR. The majority of patients with SVR (83.3%) had HCV with ISDR of ≥2. In contrast, two-thirds (66.7%) of the non-responders had HCV with ISDR < 2 (*P*=0.048). Our present results suggest that the degree of sequence heterogeneity within ISDR would be an independent marker for prediction of SVR in HCV GT-4 infection.

The number of mutations in the ISDR was shown to be associated with the viral response to PEG-IFN/RBV combination treatment in Japan (Hayashi *et al.,* 2011; Nakagawa *et al.,* 2010). On the other hand, Enomoto *et al.,* 2010 reported that mutations in NS5A ISDR are associated with no SVR during PEG-IFN/RBV combination therapy.

Our result was coincident with previous studies (Hayashi *et al.,* 2011; 31.El-Shamy *et al.,* 2011) reporting that the original criterion of ISDR ≥ 4 to predict SVR was substituted by ISDR ≥ 2. This might result from the selective impact of IFN monotherapy, whereby the prevalence of sensitive isolates with ISDR ≥ 4 was decreased while that of HCV isolates of ISDR ≤ 3 was increased.

The molecular mechanism of ISDR-mediated IFN resistance is still unclear. Some studies have revealed that NS5A binds to and suppresses the function of the IFN induced PKR. PKR is known to inhibit viral replication by inhibiting viral protein synthesis through phosphorylation of eIF-2 (Gale *et al.,* 1998). It has also been demonstrated *in vitro* that NS5A induces the expression of IL-8 at both the mRNA and

protein levels. IL-8 is known to inhibit IFN-α signaling pathway (Polyak *et al.*, 2001).

In the present study the sequence heterogeneity within IRRDR was closely associated with the SVR in patients with a high degree of sequence variation in IRRDR (IRRDR ≥ 5), while a low degree of sequence variation in this region (IRRDR <5) had no effect on treatment outcome. However, with logistic regression analysis in our study, IRRDR was not among the predicting factors of SVR.

Previously, IRRDR ≥ 6 was identified as a viral genetic polymorphism that independently predicts SVR to PEG-IFN/RBV treatment. Kim *et al.*, 2012 suggested the clinical usefulness of the sequence heterogeneity of NS5A in HCV-2a (IRRDR ≥4) for determining viral sensitivity to PEG-IFN/RBV therapy. El-Shamy *et al.*, (2012) demonstrated that IRRDR >4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV GT-4 infection when treated with PEG-IFN/RBV.

El shamy *et al.*, (2014) described the importance of the cutoff number of mutations in IRRDR that is associated with treatment outcome that might possibly vary with different geographical regions: In certain geographical regions where HCV isolates with a high degree of sequence heterogeneity are predominant, a higher cutoff number of IRRDR mutations (such as 6 mutations) is applicable, whereas a lower cutoff number of IRRDR mutations (such as 4 mutations) is better applicable in regions where HCV isolates with a low degree of sequence heterogeneity are predominant.

IL-28B host genotype as a host factor considered one of the strongest predictors of peg-IFN/RBV therapy outcome. Two biallelic SNPs rs12979860 (C/T) and rs8099917 (T/G), located upstream of *IL-28B* gene may explain differences in the results of the treatment and can be useful as therapy response marker. Previous studies (Rauch *et al.*, 2010; Suppiah *et al.*, 2009) found that individuals with two copies of the C allele (CC genotype) for the rs12979860 SNP, were two fold more likely to respond to treatment.

Conversely, individuals carrying the CT or TT genotype were less likely to respond to treatment. Other studies (Rauch *et al.*, 2010; Thomas *et al.*, 2009) demonstrated that two copies of the T allele (TT genotype) for the rs8099917 SNP were strongly associated with natural HCV clearance and SVR. Similar to the rs12979860 pattern, the rs8099917 TG or GG genotype was less responsive to treatment.

In the present study, the frequency of *IL-28B* rs12979860 genotype in our HCV GT-4 Egyptian patients showed that 60% of them were of the TT genotype, followed by 36.7% (CT) while CC had the least expression (3.3%). Previous study found different distribution of *IL 28 B* genotype. Shaala *et al.*, (2014) revealed that in their GT-4 Egyptian patients, almost half of them were of the CT genotype (56.7%) followed by CC (30%) while TT had the least expression (13%). As well, Khairy *et al.*, (2013) reported that the frequency of *IL28* genotype in their 263 chronic HCV Egyptian patients receiving PEG-IFN/RBV therapy was CT, CC and TT represented 56 %, 25 % and 19% of the patients, respectively

In the present study, SVR was achieved in the case with CC genotype of the rs12979860 SNP and in 90.9% of the CT profile while only achieved in 50% of those patients with TT genotype. Our results were in agreement with that of Thompson *et al.*, (2010) and De Nicola *et al.*, (2012) who found in their study that in, the CC *IL-28B* type was associated

with improved viral kinetics and greater likelihood of SVR compared with CT and TT.

Regarding the genetic variation in rs8099917, in the present work, 66.7% of patients with either GT or GG genotype achieved SVR. TT genotype was not displayed in our cases. However the presence of T allele did not affect the response to treatment. These findings were supported by Antaki *et al.*, (2013) and Hayashi *et al.*, (2015) who found no significant impact of *IL-28B* on SVR in their patients concerning rs8099917. In contrast, earlier study stated that rs8099917 is strongly associated with response to PEG-IFN/RBV treatment (Akuta *et al.*, 2010; Rosso *et al.*, 2014).

In conclusion, substitutions of core residue either 70 or 91 were related to treatment response in the studied patients with HCV-GT4 infection, this observation needs to be confirmed in studies with larger number of patients with HCV-4 infection taking *IL-28B* polymorphisms into consideration. AFP, *IL-28B*rs12979860 polymorphism, severity of fibrosis, and NS5A ISDR in particular ISDR ≥2 are useful markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C GT-4 and may be used collectively to improve prediction of treatment response.

Hence, the patients who do not achieve sustained virological response need to be identified as early as possible, in order to free them of unnecessary side effects and high costs. Evaluation of the liver to measure the degree of fibrosis as it is considered one of the predicting factors for SVR rates. This could be done either by liver biopsy or better by using the Fibro Scan. Further understanding of the complex interaction between virus- and host-related factors should facilitate the development of more effective therapeutic regimens.

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