Identification of Single Nucleotide Polymorphisms in Myxovirus Resistance-1 (MxA) Gene Promoter (G/T at nt -88) Correlated With the Response of Hepatitis C Patients to Interferon

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Abstract

AIM: To investigate the association between the single nucleotide polymorphisms(SNPs) in -88 locus of myxovirus resistance-1 (MxA) gene promoter region and the treatment response to IFN-α.

METHODS: Genotypes of -88(G/T) locus of the MxA promoter region was examined by the polymerase chain reaction in hundred Egyptian patients with chronic hepatitis C and sixty healthy controls.

RESULTS: The T-alleles of MxA-88 locus may be associated with the response to interferon.The frequency of the G/Tgenotype was significantly higher in the responder group of patients (63.50%) than in the non-responder group (21.60%)

CONCLUSION: The results of the current work implied that heterozygosity for the –88 polymorphism of the MxA gene may be important host factors that influence the response to IFN-α therapy in patients with chronic HCV infection.

KEY WORDS: Myxovirus resistance-1(MxA),Single nucleotide polymorphisms(SNPs), Chronic hepatitis C and IFN-α.

INTRODUCTION

According to World Health Organization data, 3% of the human population (approximately 170 million people) is infected with HCV and the prevalence of chronic HCV is 0.1-26% and varies in different regions ***[1]***.Egypt is among the countries with the highest prevalence of hepatitis C virus (range 6%-15%) ***[2]***. That prevalence is higher in the Nile Delta than elsewhere in the country ***[3]***.

Treatment of chronic hepatitis C is a combination of pegylated interferon alpha and the antiviral drug ribavirin for a period of 24 or 48 weeks depending on genotype ***[4].***

Mx proteins are key mediators of the interferon (IFN) 2-induced antiviral response in vertebrates and hence of great biological interest and medical importance ***[5]***.The SNPs of the MxA gene is one of the important host factors that independently influence the response to IFN in patients with chronic HCV infection, especially those with a low viral load ***[6].***

The aim of the present work was to investigate the association between the single nucleotide polymorphisms (SNPs) in -88 locus of

myxovirus resistance-1 (MxA) gene promoter region and the treatment response to pegylated interferon in HCV-infected Egyptian patients.

MxA protein Polymorphisms was significantly associated with susceptibility to enterovirus 71 infection**[7]**, prostate cancer**[8],** Alzheimer disease **[9]**, the outcomes of HBV infection **[10]**, severe acute respiratory syndromes**[11]**, sub-acute sclerosing pan-encephalitis **[12]**, and that the SNP of the MxA gene is one of the important host factors that independently influences the response to IFN in patients with chronic HCV infection, especially those with a low viral load ***[6]***.

Subjects and Methods

Ethics statement: All human studies have been reviwed by ethics committee in faculty of medicine,cairo university which approved this research.

This study was conducted on 160 Egyptian subjects in adult age group (20-54 years); they were classified into two groups:

Group (I): Included 100 HCV chronically infected Egyptian patients; 38 females and 62 males. Patients were treated with peg-interferon (PEG-IFN) alpha 2b 1.5 µg /kg weekly and ribavirin (800-1000mg /day) for one year (48 weeks). According to their response to treatment patients were classified into two subgroups:

1-Responders (n= 63): who had normalization of aminotransferases (ALT and AST) levels and clearance of the virus denoted by negative HCV-RNA by PCR after 6 months of receiving treatment and remain negative after completion of the treatment course for 72 weeks.

2- Non-Responders (n= 37): who received treatment for 6 months and failed to clear the virus and give positive HCV-RNA by real time PCR.

Group (II): Included 60 healthy donors volunteered for the study and served as controls. They were 33 females and 27 males.

Patients Selection: All patients attended the liver unit of Tropical Medicine Department, at Kasr El-Aini Hospital, Cairo University Outpatient's Clinic as naive patients to receive combined treatment of Interferon and Ribavirin. All subjects were recruited in the period from August 2011 to February 2013. Patients were screened for their eligibility to participate in the study. Eligible patients signed an informed consent. Medical history and possible routes of acquiring HCV infection were taken.They had clinical examination and ultrasonography before treatment, then six months until the end of study. Pretreatment and post-

treatment histopathological examination of percutaneous needle liver biopsy.

Patients Inclusion Criteria: Chronically infected HCV patients aged 20 to 54 years old. Serological, virological and histological diagnosis of chronic HCV. Elevated ALT level above the upper limit of normal within 6 months prior to entry to the study. Patients had not been previously treated with interferon based therapy.

Patients Exclusion Criteria: Decompensated liver disease. Patients with hepatitis B surface antigen (HBsAg) seropositive or infected with the human immunodeficiency virus (HIV). Hemoglobin <13 g/dL for men and <12 g/dL for women, white blood cell count of <3,000/mm3, neutrophil count of <1500/mm3, or platelet count of < 100,000/mm3. Presence of ANA titre > 1/160. TSH out of normal range. Active schistosomiasis. Serum creatinine above upper normal limit. Poorly controlled diabetes mellitus, hypertension, or psychiatric diseases.

Blood sample collection and storage: Five ml peripheral blood sample were withdrawn from each patient by venipuncture in 2 divided dry sterile 2 vacotainer tubes. Two mL were taken on EDTA tube and stored at -800C to be used for DNA extraction and detection of polymorphism MxA gene. The other three mL were taken in plain tube and left for 10

minutes to clot and then centrifuged at 2000 Xg for 5 minutes. The serum was then separated to be used in:

(a) All serological markers for HCV, Interleukins, HBV and standard laboratory tests.(b) Viral RNA quantitation by real time PCR.

I- Standard laboratory tests:

1-Liver Function Tests: Including prothrombin time and concentration, serum bilirubin (direct and total), serum albumin, aspartate transaminase (AST), alanine transaminase (ALT), alpha pheto protein (AFP),complete blood picture (CBC) and alkaline phosphatase (ALK).

2- Anti-HCV ,HBsAg, and Anti-HBc.

3- HCV-RNA titer: by real time PCR before and after treatment.

4- Thyroid function tests: T3, T4 and TSH using Immulite.

5- Autoantibodies: ANA was done by Immunofluorescence kits.

6- Random blood glucose level: using glucose oxidase kits.

II- Molecular Biology Tests: MxA (position -88) genotypes was determined using a polymerase chain reaction (PCR)-restriction fragmentlength polymorphism (RFLP) assay.

DNA Extraction: DNA was extracted from whole blood using DNA extraction kit and stored at -80°C in aliquots until required. This was done using Qia-amplification extraction kit (Qiagene, USA)

B) Quantitation and assessment of DNA purity: DNA samples were subjected to DNA quantitation and purity assessment using the Nano-Drop® (ND)-1000 spectrophotometer (Nano-Drop Technologies, Inc. Wilmington, USA).

PCR amplification and detection of MxA gene polymorphism:

Detection of polymorphisms in MxA gene was carried out using PCR amplification using Taq polymerase enzyme and T-Gradient thermal cycler (Biometra, Germany). Identification of the 2 alleles at each polymorphic site was performed by incubating the PCR product with a restriction enzyme, followed by electrophoresis on agarose gels.

(1) **Primer sequences:** Table (1): The sequence of primers used for amplification of MxA gene

Primers	Oligonucleotide sequence	product size
MxA (-88 & -123)	F-5ACACACCCGTTTCCACCCTGGAGAGGCCAG-3 R- 5-TGCGCAGTGCTGGAGTGCGGCCTCCGCTCT-3	599bp

(2) PCR amplification: PCR mixture (total volume 50µl)**.**5 µl of 10X reaction buffer with MgCl2 (Amersham Pharmacia Biotech, Piscataway, NJ, USA)**.**0.5 µM of each primer (forward and reverse).0.2 mM/L dNTPs (Perkin-Elmer Corporation, Foster City, CA, USA) **.** 2 units Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) **.** 0.05 ug genomic DNA template.

Cycling Conditions: Denaturation at 95°C for 10 min. PCR reaction was carried out for 35 cycles under the following conditions: Denaturation at 95°C for 10 sec.Annealing at 65°C for 10 sec**.** Extension at 72°C for 60 sec**.**Then final extension cycle of 72°C for 7 minutes was done. Identification of the 2 alleles at each polymorphic site was performed by incubating the PCR product with a restriction enzyme, followed by electrophoresis on 3% agarose gels. Samples were prepared for loading by adding 2µl loading buffer to 10µl of the PCR reaction mixture. The PCR marker was also loaded into one of the wells

Performing the Electrophoresis: The power supply was programmed to give 60 volts for about 20 minutes. The gel was taken for viewing on ultra-violet trans-illuminator.

Gel electrophoresis for MxA-88: Similar to the previous steps, but 2% agarose gel was prepared by adding 1g agarose to 50ml of the 1X TBE buffer.

Detection of MxA-88 Polymorphism*:* For detection of the MxA-88 polymorphism, the bands generated on the gel wereseen. In homozygous wild genotype (GG) one band at 599 bp appeared, in homozygous mutant genotype (TT) 2 bands at 482bp &117 bp, while in heterozygous genotype (GT) 3 bands at 599bp, 482bp &117 bp appeared.

Statistical methods

Comparison of quantitative variables was done using krauscal-wallia and mann- whitney test for unpaired samples and Wilcoxon signed ranks test for paired samples. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Genotype and allele frequencies were compared between the disease and the control groups using chi-square tests. Odds ratio (OR) with 95% confidence intervals was calculated. Spearman rank correlation coefficients were estimated to assess potential relationships between variables of interest. Stepwise multivariate logistic regression analysis for detection of multiple factors that could affect and so predict the response of chronic hepatitis C patients to interferon treatment was

done. A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS version 16.

Results

The current study was conducted on one hundred and sixty Egyptian subjects in adult age group with average age (19- 54 years), they were classified into two groups

Group (I): Included 100 HCV chronically infected Egyptian patients; they were 62 males and 38 females with average age (20- 54 year), all are genotype 4a and patients were treated with PEG-IFN alpha 2b 1.5 μg /kg weekly and ribavirin (800-1000mg /day) for six months. According to response to treatment, patients were classified into two subgroups: responders and non responders.

1-Responders: Who had normalization of aminotransferases (ALT and AST) levels and clearance of the virus denoted by negative HCV-RNA by PCR after 6 months of receiving treatment and remain negative after completion of the treatment course for 72 weeks. They were 63 patients [23 (36.5%) females and 40 (63.5%) males], aged 38.40 ± 8.783 (mean ± SD) years.

2- Non responders: who received treatment for 6 months and failed to clear the virus and give positive HCV-RNA by PCR. They were 37 patients [15 (40.5%) females and 22 (59.5%) males], aged 39.35 ± 7.462 (mean ± SD) years.

Group (II): Included sixty healthy donors (age and sex matched) volunteered for the study and served as controls.

In the present study we estimate the serum levels of myxovirus resistance-1 (MxA) protein and detect the polymorphisms of its gene at point -88 G/T in all studied groups.

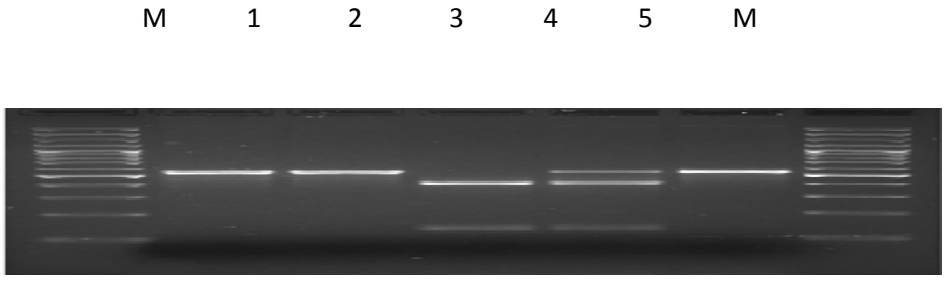

Figure (1): Agarose gel electrophoresis of MxA(-88) after restriction by HhaI

Lane M: Ladder (100 bp)

Lanes 1, 2&5: Homozygous wild GG genotype showed one band at (599 bp)

Lane 3: Homozygous mutant TT genotype showed 2 bands at (482 &117 bp)

Lane 4: Heterozygous GT genotype showed 3 bands at (599, 482 &117 bp)

Table (2): Distribution of HCV study participants according to treatment response

	No.	%
Non responders	37	37
Responders	63	63
Total	100	100

Table (3): Biochemical characteristic of hepatitis C patients among studied groups

Variables	HCV patients n=100	Controls n=60	p-value
Age (years)	38.75 ± 8.29	35.62 ± 7.73	> 0.05
ALT (U/L)	84.51 ± 54.37	27.62 ± 3.63	0.000*
AST (U/L)	102.13 ± 72.39	28.65 ± 4.52	0.000*
T-bilirubin (mg/dl)	1.25 ± 0.66	0.73 ± 0.19	0.000*
D-bilirubin (mg/dl)	0.39 ± 0.27	0.14 ± 0.05	0.000*
ALK (U/L)	115.26 ± 42.38	43.57 ± 7.31	0.000*
ALB (g/dl)	3.63 ± 0.50	3.84 ± 0.21	0.003*
AFP (ng/ml)	15.07 ± 12.81	5.92 ± 2.06	0.000*
PT (seconds)	12.67 ± 1.59	11.22 ± 0.59	0.000*
MxA (ng/ml)	8.26 ± 3.92	70.13 ± 26.41	0.000*

*Values were expressed as mean ± SD, P <0.05 was considered significant **

(Table 3) The age of HCV patients & controls showed no statistically significant difference from each other (P value > 0.05).But The level of

ALT, AST , T-bilirubin , D-bilirubin , ALK , AFP , PT in HCV patients were significantly higher than control group .Also,The level of albumin, MxA protein in HCV patients were significantly lower than controls .

Table (4): Genotypes and allele frequencies for SNP (-88 G/T) of MxA gene in hepatitis C patients and controls

MxA (-88)		**HCV patients** (100)	**Controls** (60)	**p value**
(Genotypes)	**GG**	43 (43%)	31 (51.7%)	0.4
	GT	48 (48%)	26 (43.3%)	
	TT	9 (9%)	3 (5%)	
(Alleles)	**G**	134 (67%)	88 (73.3%)	0.23
	T	66 (33%)	32 (26.6%)	
(Genotypes)	**GG** (wild)	43 (43%)	31 (51.7%)	0.20
	GT+TT (mutant)	57 (57%)	29 (48.3%)	

*p <0.05 was considered significant **

(Table 4) shows that there was a non-significant difference observed between hepatitis C patients and controls as regard the distribution of SNP (-88) alleles (G/G, G/T and T/T) (p = 0.4), (G/G vs. G/T, T/T) (p = 0.20) and (G alleles, T alleles) (p = 0.23).

Table (5): Genotypes and allele frequencies for SNP (-88 G/T) of MxA gene among responders and non-responders

MxA (-88)		Responders (63)	Non-responders (37)	p value
(Genotypes)	**GG**	16 (25.4%)	27 (73%)	0.000*
	GT	40 (63.5%)	8 (21.6%)	
	TT	7 (11.1%)	2 (5.4%)	
(Alleles)	**G**	72 (57.1%)	62 (83.8%)	0.001*
	T	54 (42.9%)	12 (16.2%)	
(Genotypes)	**GG** (wild)	16 (25.4%)	27 (73%)	0.000*
	GT+TT (mutant)	47 (74.6%)	10 (27%)	

*p <0.05 was considered significant **

(Table 5 and Figure 2) showed significant difference observed in the distribution of (G/G, G/T and T/T) (P = 0.000), also significant statistical distribution between responders and non-responders between mutant genotypes (G/T, T/T) as well as wild genotype (GG) (P = 0.000).Also,The allele frequencies between the two groups showed statistical significant difference (G allele (wild): 72 (57.1%), 62 (83.8%) and T allele (mutant): 54 (42.9), 12 (16.2%) respectively (p = 0.001)

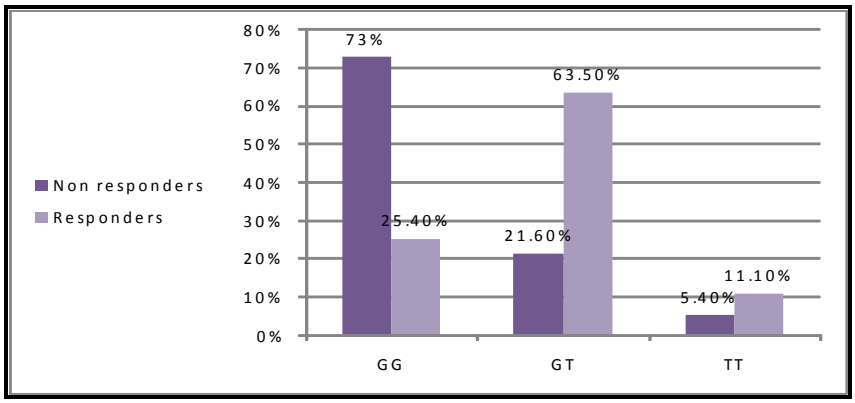

Figure (2): Genotype distribution of MxA (-88 G/T) gene among responders and non-responders

DISCUSSION

The hepatitis C virus (HCV) infection is presently a major public health problem, with approximately 170 million people worldwide persistently infected with this virus **[13]**. Egypt has the highest prevalence of antibodies to HCV in the world, estimated nationally at 14.7% **[14].**

The standard of care (SOC) for HCV infection consists in the combination of pegylated interferon (PEG-IFN) plus ribavirin. Several viral and host factors have been implicated in response to therapy including age, sex, ethnicity, steatosis, obesity, insulin resistance, HCV RNA levels and viral genotypes. Numerous studies have also indicated that specific single nucleotide polymorphisms (SNPs) of certain host genes are probably involved **[15].**

The current study aims to investigate the association MxA polymorphism at positions –88 with the response to IFN-α therapy in HCV-infected Egyptian patients; and also searched for potential interactions of different factors associated with response to treatment with IFN-α.

The present study showed that the pretreatment laboratory data including the mean serum levels of ALT, AST, total and direct bilirubin, ALP, AFP and PT were significantly higher in the group of HCV patients as compared to the control group, while the mean serum level of albumin was significantly lower. Abbas et al. (2009) **[16]** have shown similar results to the present work, in which the mean serum levels of ALT and AST were significantly higher in the HCV patients than in the healthy controls.

The interferon system is a crucial component of the innate immune response to infectious agents **[17]**; its biological activity is mediated by the induction of intracellular antiviral proteins, such as the myxoresistence A (MxA) protein, 2′–5′ oligoadenylate synthetase-1 (OAS-1) and the double stranded RNA (dsRNA)-dependent protein kinase (PKR). Moreover, the MxA protein is assumed to be the most specific surrogate parameter for IFN action **[6].**

Some reports have shown that increasing the MxA protein or mRNA levels were related to response to IFN-α therapy. However, the levels of the MxA protein or mRNA during IFN-α therapy differ between individuals. One cause of these differences may be associated with genomic factors **[6].**

Hijikata et al. (2000) **[18]** identified a SNP at nucleotide position –88 (G/T) in the MxA gene, in association with different responses of HCV-infected Japanese patients to IFN-α therapy. This SNP lies within an interferon-stimulated response element (ISRE)-like sequence in the promoter region of the MxA gene. These SNP was most likely associated with the levels of IFN-induced expression of the MxA protein, and thus further with the response of the hepatitis C patients to IFN-α therapy**[19].**

According to the present study, the genotype and allele frequencies at position –88 of the MxA promoter failed to show any significant difference between HCV patients and control subjects.

Previous studies have shown similar results to the present work, in which the distribution of genotypes at positions–88 **[18]** and between HCV patients and control subjects was not significantly affected. Moreover, the study of Suzuki et al., (2004) **[6],** was in agreement with the previous observations, where the allele frequency of the MxA

promoter gene at position –88 in HCV patients was the same as in healthy controls.

As regards to the MxA gene at position –88, the current results showed that the rate of G/G homozygosity was significantly lower in sustained responders to IFN-α therapy than in non-responders. These findings may be attributed to that G/G genotype expressed lower amount of MxA mRNA than G/T or T/T genotypes in IFN-treated peripheral blood mononuclear cells in vitro **[20]** Conversely, heterozygotes (G/T) were more likely to have sustained response with high significance. Inheritance of the T allele was significantly correlated with sustained responders than non-responders to IFN-α therapy.

It is quite possible that the SNP of the MxA promoter might influence the expression of MxA, and that carriers of MxA-T-positive might express the protein more efficiently when treated with IFN-α than those who are MxA-T-negative **[6].** This polymorphic site (MxA promoter at nucleotide –88) is involved in a genetic element with high homology to the ISRE consensus sequence, and the G-to-T change at this position increases this similarity **[21;18].** This suggests that the SNP of the MxA promoter might affect the expression of MxA. Consequently, patients who possess the G/G genotype at position –88 may produce a suboptimal MxA response when given IFN-α **[17].** The apparent effect of

the T-allele has also been shown to play a role in regulation of a downstream reporter gene by a luciferase reporter assay, suggesting that this variant has higher transcriptional activity than the G allele when stimulated with IFN-α **[19].**

The findings of the current work were highly supported by the work of Hijikata et al. (2000) **[18]**, in which the G/G genotype was significantly expressed in IFN-α non-responder patients , as opposed to the heterozygosity G/T, which was more frequently expressed among responders to IFN-α. Our findings were also comparable with those obtained by Knapp et al. (2003) **[17]**, who found that the G/T genotype was found more frequently in sustained responders to IFN-α therapy than in non-responders.

The results of the current study were in accordance with the work of Huang et al. (2008) **[22]**, who provided evidence that patients with MxA–88 T/T or G/T genotype infection have better therapeutic effectiveness than those with G/G genotype when treated with IFN-α. Moreover, the recent study of Lyra et al. (2011) **[23]** reported that the MxA gene –88G/G genotype was more frequent among non-responders compared to patients with sustained response, while –88G/T was more frequent among sustained responders.

However, the results of Maouzi et al. (2005) **[24]**, Welzel et al. (2009) **[25]** and Mohamed et al. (2011) **[26]** apparently contrast with the former results, in which they found no association of the –88 MxA SNP with response to IFN-α therapy.

In the present study, representative genotyping results for HCV patients with each genotype of the MxA gene at position -88 was demonstrated. The frequency of –88 G/G homozygotes in sustained responders (25.4%) was significantly lower than in non-responders (73%); moreover, the frequency of –88 G/T heterozygote was significantly higher in responders than in non-responders (63.5% vs. 21.6%). The T allele frequency was significantly higher in sustained responders than in non-responders (42.9% vs. 16.2%). This was consistent with the results obtained by Hijikata et al. (2000) **[18]**.

The only significant interaction between different combined genotypes of the MxA promoter at position –88 and the response to IFN-α therapy.that patients inheriting the wildtype G(–88) / G(–88) genotype was significantly non-responders, while heterozygotes G(–88) / T(–88) was more likely sustained responders. These findings were in agreement with those reported by Hijikata et al. (2001) **[19]**.

Conclusion

Data from the current study concluded that heterozygosity for both –88 and –123 polymorphisms of the MxA gene may be important predictors of sustained response to IFN-α therapy.

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الملخص العربى

الالتهاب الكبدى الوبائى بفيرس سى يمثل تهديدا رئيسيا للصحه العامه على الصعيد العالمى حيث يقدر عدد المصابين و المعرضين لخطر تليف و سرطان الكبد بنحو ١٧٠ مليون شخص فى جميع انحاء العالم .

يعتبر مكسو فيرس بروتين احد البروتينات المضاده للفيروسات ذات الحامض النووى الريبى عن طريق منع انتقالها الى داخل النواه .

شملت الدراسه الحاليه ١٦٠ فرد من الجنسين تم تقسيمهم الى مجموعتين ، المجموعه الأولى تتكون من ١٠٠ فرد مصاب بالالتهاب الكبدى الوبائى سى والاخرى تتكون من ٦٠ فرد سليم

تم اخد عينات دم لاستخراج الحمض النووى الجينى و تحليله بواسطه التفاعلات المتسلسله للبوليميز و انزيمات الاقتطاع الداخليه و ذلك لتحديد انماط المكسو فيرس جين عند النقطه -٨٨

أظهرت النتائج ان النوع الجينيG/G متماثل الالائل عند النقطه -٨٨ للمكسو فيرس جين اقل بكثير فى المستجيبين للعلاج بالانترفيرون مقارنه بغير المستجيبين بينما كان النوع الجيني G/T

مختلفه الالائل أكثر فى المستجيبين بكثير و كذلك تردد الاليل T ارتبط بشكل كبير مع استجابه متواصله لعلاج الانترفيرون .

تبين من الدراسه الحاليه ان الأختلاف الزيجوتي عند النقطه -٨٨ للمكسوفيرس قد يكون تنبؤ هام للاستجابه بالعلاج بالانترفيرون ومع ذلك فهى تفسر جزء من القابليه الوراثيه للاستجابه للعلاج . االكلمات داله: لالتهاب الكبدى الوبائى- مكسو فيرس-الانترفيرون