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Genetic study of I kappa B alpha gene promoter polymorphism associated with hepatitis C virus in Egyptian patients

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ABSTRACT

Background: Host genetic polymorphism is one of the major unalterable major factors for HCV infection, NF-κB proteins play multiple roles in immune response and involve in HCV infection and progression.

Aim of the study: To investigate the associations between single nucleotide polymorphism (SNPs) in NF-Kb and the susceptibility as well as resolution of HCV infection. **Patients and Methods:** This prospective case-control study was conducted at the physical examination center on 150 Egyptian population, including 50 uninfected control cases, 50 cases with spontaneous viral clearance, and 50 cases with persistent HCV infection, they are genotyped for four SNPs (rs11820062, rs230530, rs1056890 and rs3774963) using a Taq Man assay. **Results:** The current study revealed that the mutation in rs_11820062 of the I kappa B alpha gene significantly increased the risk for HCV infection with a p-value <0.05. **Conclusion:** This study revealed that genetic variants of the *NF-κB* pathway genes (rs11820062 T allele) are associated with an increased risk of HCV susceptibility.

Keywords: Recombinant immune blot assays, Single nucleotide polymorphisms, Nuclear factor of Kappa light polypeptide gene enhancer in B-cell.

INTRODUCTION

Over 185 million people worldwide suffer from hepatitis C virus (HCV) infection, it is usually asymptomatic and will develop some hepatic and extrahepatic disorders, including liver cirrhosis, hepatocellular carcinoma, non-Hodgkin's lymphoma, and cryoglobulinemia, etc ***(Kao et al., 2017).***

In recent years, due to the high effectiveness of clinical applications of novel direct-acting antiviral drugs (DAAs), the expectation for a cure for hepatitis C has increased dramatically, however, many significant challenges remain, including undetected most HCV cases, resistance-associated variants (RAVs) of HCV protease inhibitors, and HCV reinfection after cure of chronic hepatitis C, etc ***(Falade-Nwulia et al., 2017).***

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Nuclear factor κB (NF-κB) was first discovered as a nuclear factor binding to a κ enhancer of the immunoglobulin κ light chain gene of activated B cells, it can be found in almost all animal cell types and is an important key regulator of the anti-pathogen immune response, inflammatory reaction, cell proliferation and survival ***(Pawlotsky et al., 2016).***

The NF-κB transcription factor family is composed of five DNA binding proteins: NF-κB1 (p50, p105), NF-κB2 (p52, p100), RelA (p65), RelB, and c-Rel, these proteins can form homo dimers or heterodimer with widely different transcriptional activities and their expression levels show differences in the event and tissue-specific expression patterns in response to a different stimulus ***(*Taniguchi *et al., 2018).***

A variety of external stimulants can activate the NF-κB signaling pathway, including viruses, bacterial lipopolysaccharide, pro-inflammatory cytokines, and stress-inducing agents, followed by the promotion of the expression of hundreds of genes ***(* Barnabei *et al., 2021).***

NF-κB also can be activated by HCV and stimulates the production of IL-1, IL-6, lymphotoxin, and IFN-γ, due to its extensive influence, NF-κB can be regarded as the central mediator of the immune response ***(Saleh et al., 2021).***

The immune response to HCV is mainly regulated by NF-κB and interferon-signaling pathways, therefore, abnormal activation or regulation of NF-κB signaling pathway genes are associated with various immune-related diseases, including some infectious diseases, autoimmune diseases, chronic obstructive pulmonary disease, and cancer. ***(Park et al., 2016)***

PATIENTS AND METHODS

This prospective case-control study was conducted at the physical examination center during a period between December 2015 to September 2021 on 150 Egyptian population, including 50 uninfected control cases, 50 cases with spontaneous viral clearance, and 50 cases with persistent HCV infection, they were genotyped for four SNPs (rs11820062, rs230530, rs1056890 and rs3774963) using a Taq Man assay.

Inclusion criteria:

- The age of the studied cases ranged from 30 to 75 years.
- ***They were divided into three groups depending on the results of anti-HCV antibodies and HCV RNA:***

Group A: Included 50 cases of the HCV-uninfected control with:

- Sero-negative HCV antibodies.
- Sero-negative HCV- RNA.

Group B: Included 50 cases of spontaneous clearance with:

- Sero-negative HCV antibodies.
- Sero-negative HCV- RNA.

Group C: Included 50 cases with persistent HCV infection with:

- Sero-positive HCV antibodies.
- Sero-positive HCV- RNA.

NB: Group B&C, individuals were considered as infected cases.

Exclusion criteria:

1. Subjects that were co-infected with hepatitis B virus or human immunodeficiency virus (HIV).
2. Subjects that were suffering from other liver diseases (Including autoimmune, alcoholic, or metabolic liver diseases).
3. Subjects, that were on or treated with any antiviral medications before or during the study.

All patients were subjected to:

Informed consent: A written informed consent was obtained from all participants regarding the benefits and hazards of the study.

Viral serological testing:

All serological tests for HCV antibodies and HCV RNA were verified by three separate experiments within 12 consecutive months.

The technique of viral testing:

1. A 10 mL venous blood sample was collected from each participant after the interview.
2. White blood cells were isolated by centrifugation and subsequently stored at −80 °C until use.
3. Anti-HCV antibodies were tested using a third-generation enzyme-linked immune sorbent assay (ELISA).
4. Diagnostic Kit for Antibody to HCV 3.0 ELISA, Intec Products Inc, Xiamen, China) according to the manufacturer's instructions.
5. HCV RNA was extracted from patient serum using Trizol LS Reagent (Takara Biotech, Tokyo, Japan), and reverse transcription
6. PCR (Takara Biotech) was performed.
7. The Murex HCV Serotyping 1–6 Assay ELISA (Abbott, Wiesbaden, Germany) was used to determine the type-specific antibodies of various HCV genotypes.

SNPs selection:

Tag SNPs were selected, using the Haplo view software (version 4.2; Broad Institute, Cambridge, MA, USA) based on the linkage disequilibrium (LD) data of Hap Map Phase II.

SNPs located on 5′-UTR, 5′ flanking regions (rs11820062), 3′-UTR (rs1056890), and exons with missense substitutions were considered and combined with SNPs with some connection to liver disorders.

Utilizing the above strategies, four candidate SNPs, rs1056890, rs11820062, rs230530, and rs3774963, were selected for the study.

Genomic DNA Extraction:

Protease K digestion was used to extract genomic DNA from subject peripheral blood leukocytes, followed by phenol-chloroform extraction and ethanol precipitation.

1. Genomic DNA was extracted using the QIA amp DNA blood mini kits (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.
2. We dissolved DNA in TE buffer (10 m M Tris at pH 7.8 and 1 m M EDTA).
3. Quantification was done by measuring the optical density at 260 nm.
4. The final preparation was stored at 220uC and used to create templates for the polymerase chain reaction (PCR).

Genotyping:

Genotyping of the four SNPs was performed with a Taq Man allelic discrimination assay on an ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The following criteria were used to identify tagging SNPs:

- SNPs are located in the genes or within the 5 kb flanking region.
- A minor allele frequency ≥ 0.05.
- Other unselected SNPs could be captured by one of the tagging

SNPs, with a linkage disequilibrium of r2 ≥ 0.90.

A total of four SNPs were selected for genotyping which were:

- rs11820062 G >A.
- rs230530 A > G.
- rs3774963 G > C.
- rs1056890 C > T

Table 1: Descriptions of genetic polymorphisms of NF-kB genes under investigation.

SNPs (genotype	Gene	Region	MAFa/b	Taq Man-MGB probe/primers sequences
rs11820062 G > A	RelA	Intron 1 or nearGene-5	0.392/0.427	Probe-G: FAM-TCCCTCAGTTTTC-MGB
				Probe-A: VIC-TCCCTCAATTTTC-MGB
				Forward primer: CTTGACTCAGTTTCCCTCCACAC
				Reverse primer: GAGGGAAAACGGGGTAAGGAATC
rs230530 A > G	NF-κB1	Intron 3	0.473/0.476	Probe-A: FAM-CAAACATCTTAATTTACATTC-MGB
				Probe-G: HEX-AAACATCTTAATTTGCATTC-MGB
				Forward primer: AAAATGGACATACAAGCATTCTCCT
				Reverse primer: TGCAATAAATAAAGGCATATGGTGGT
rs3774963 G > C	NF-κB1	Intron 15	0.377/0.409	Probe-G: FAM-ATGTTCGACTCCCAC-MGB
				Probe-C: HEX-ATGTTCCACTCCCAC-MGB
				Forward primer: TGGAAGGCATGGTGTTTGG
				Reverse primer: TGTGACTGCTCCAGCCCATA
rs1056890 C > T	NF-κB2	3′-UTR	0.196/0.171	Probe-C: FAM-CACCTCCGAGAGC-MGB
				Probe-T: VIC-CACCTCTGAGAGCC-MGB
				Forward primer: TGGGCCTCAGGAGCCTAG
				Reverse primer: ATCAAAAGTTCAGGGGCGCTAG

The quality and potential misclassification of the genotyping results were assessed, by evaluating 5% of duplicated DNA samples, that were randomly selected from the whole samples, their replicates were 100% concordant.

Follow up:

- All serological results were confirmed by three separate experiments within the 6 month follow-up period.
- The control subjects in group A were matched by age and gender with infected subjects belonging to group B or group C.
- The associations between SNPs with the susceptibility to HCV infection were estimated by comparing group A v .s. group (B + C) or group B v. s. group C, respectively.

Ethical committee

Permission from the Molecular Biology Department, Biogenetic engineering Research Institute Sadat City University ethical committee was also obtained and approval from the institutional review board was taken. The title and objectives of the study were explained to them to ensure their cooperation.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0**.** **(**Armonk, NY: IBM Corp**)** qualitative data were described using numbers and percentages. The Kolmogorov-Smirnov test was used to verify the normality of distribution quantitative data were described using range (minimum and maximum), mean, standard deviation, median, and interquartile range (IQR).

The significance of the obtained results was judged at the 5% level. Qualitative variables were compared using the chi-square (x2) test or Fishers Exact test when frequencies were below five. P value < 0.05 was considered significant.

RESULTS

Table 1 showed that the mean age among the studied groups A, B, and C were (53.90 ± 13.34, 51.82 ± 13.41, and 52.54 ± 12.91) respectively, and the majority of studied cases were of female sex 29 (58%) out of 50 in group A, 32 (64%) out of 50 in group B and 27 (54%) out of 50, this table also showed that there was no statistically significant difference as regards the correlation between demographic data among the studied groups and risk of HCV infection, p-value > 0.05.

Table (1): Comparison of the demographic data in the three study groups

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Test of sig.
Age (Years)	53.90 ± 13.34	51.82 ± 13.41	52.54 ± 12.91	F = 0.319 P = 0.727
Sex				
Males	21 (42%)	18 (36%)	23 (46%)	χ^2= 1.045 P = 0.593
Females	29 (58%)	32 (64%)	27 (54%)	

χ2=Chi-square test
F = One-way ANOVA test
P= Intergroup significance

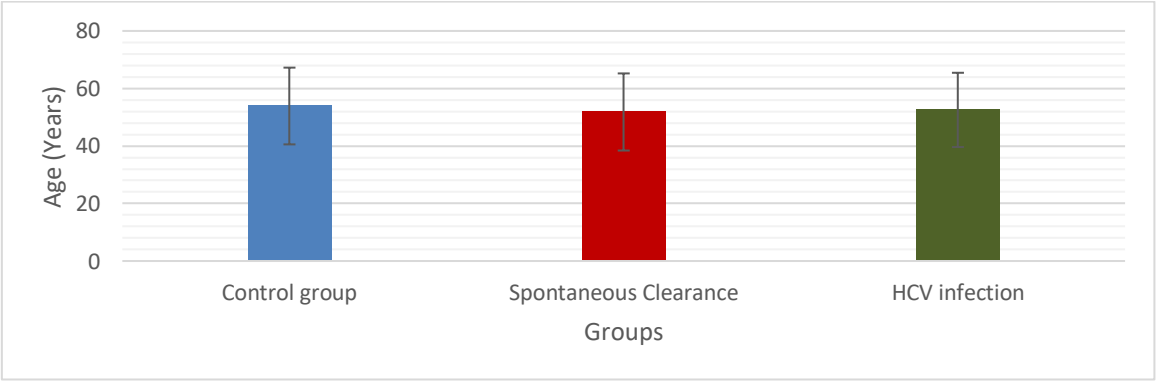

Figure (1): Age distribution among the studied groups

Table (2): Comparison of clinical data among the studied groups

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Test of sig.
Disease duration (Years)				
Mean ± SD	0	7.98 ± 4.12	8.72 ± 4.64	Z= -0.648 P= 0.534
Median (Range)		8 (1-15)	8 (1-17)	
Source of infection				
Occupational (health care workers)		6 (12%)	7 (14%)	MC = 1.146 P = 0.436
Hemodialysis (HD)		10 (20%)	9 (18%)	
Blood transfusion		19 (38%)	18 (36%)	
Drug abuse		5 (10%)	4 (8%)	
Previous surgical/dental procedure		10 (20%)	12 (24%)	
HCV RNA load (10^3)				
Mean ± SD	0	0	792.13 ± 888.23	NA
Median (Range)			561.6 (36.5 – 3650.2)	
HCV antibodies				
Negative	50 (100%)	0 (0%)	0 (0%)	NA
Positive	0 (0%)	50 (100%)	50 (100%)	

MC=Monte-Carlo test
KW = Kruskal Wallis test
P= Intergroup significance

Table 2 showed that there was no statistically significant difference between the three studied groups as regards the patient's clinical data and risk of HCV infection, with a p-value > 0.05.

Table (3): Genotypic distributions of rs_11820062 of I kappa B alpha gene among the three studied groups.

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Group (B+C)/ Group A		Group B/ Group C	
				OR (95% CI)	P value	OR (95% CI)	P value
Genotypes							
GG	17 (34%)	16 (32%)	18 (36%)	1		1	
GA	24 (48%)	24 (48%)	22 (44%)	0.923 (0.468-1.822)	**0.041*7**	0.851 (0.387-1.870)	0.868
AA	9 (18%)	10 (20%)	10 (20%)	1.139 (0.476-2.724)	**0.002***	1 (0.375-2.664)	1
Additive model				1.084 (0.369-1.794)	**0.002***	0.759 (0.240-1.126)	0.903
Dominant model				0.878 (0.367-2.100)	**0.008***	1 (0.613-1.632)	1
Recessive model				0.850 (0.394-2.467)	**0.014***	1 (0.346-1.776)	1

Table 3 showed that mutation in rs_11820062 of the I kappa B alpha gene significantly increased the risk for HCV infection with a p-value <0.05.

Table (4): Genotyping distributions of rs_230530 of I kappa B alpha gene among the three studied groups.

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Group (B+C)/ Group A		Group (B+C)/ Group A	
				OR (95% CI)	P value	OR (95% CI)	P value
Genotypes							
AA	13 (26%)	12 (24%)	13 (26%)	1		1	
AG	24 (48%)	27 (54%)	25 (50%)	1.174 (0.595-2.315)	0.644	0.852 (0.388-1.868)	0.689
GG	13 (26%)	11 (22%)	12 (24%)	0.850 (0.388-1.864)	0.685	1.120 (0.441-2.844)	0.812
Additive model				0.997 (0.428-2.625)	0.885	0.936 (0.347-1.946)	0.923
Dominant model				1.176 (0.537-2.579)	0.840	0.893 (0.352-2.269)	0.878
Recessive model				0.874 (0.407 -2.007)	0.625	1.059 (0.655-1.712)	0.804

Table 4 showed that mutation in rs_230530 of the I kappa B alpha gene had no statistically significant increased risk for HCV infection with a p-value > 0.05.

Table (5): Genotyping distributions of rs_3774963 of I kappa B alpha gene among the three studied groups.

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Group (B+C)/ Group A		Group (B+C)/ Group A	
				OR (95% CI)	P value	OR (95% CI)	P value
Genotypes							
GG	18 (36%)	19 (38%)	20 (40%)	1		1	
GC	23 (46%)	22 (44%)	23 (46%)	0.960 (0.486-1.899)	0.908	1.048 (0.493-2.384)	0.841
CC	9 (18%)	9 (18%)	7 (14%)	0.868 (0.354-2.130)	0.818	0.742 (0.253-2.176)	0.585
Additive model				0.758 (0.310-1.962)	0.920	0.944 (0.357-2.007)	0.862
Dominant model				1.152 (0.470-2.829)	0.757	1.348 (0.460-3.951)	0.785
Recessive model				0.788 (0.372-2.220)	0.796	0.855 (0.742-1.547)	0.587

Table 5 showed that mutation in rs_3774963 of the I kappa B alpha gene had no statistically significant increased risk for HCV infection with a p-value > 0.05.

Table (6): Genotyping distributions of rs_1056890 of I kappa B alpha gene among the three studied groups.

Variable	Group A (Control group) (N=50)	Group B (Spontaneous clearance) (N=50)	Group C (HCV infection) (N=50)	Group (B+C)/ Group 1		Group (B+C)/ Group A	
				OR (95% CI)	P value	OR (95% CI)	P value
Genotypes							
CC	34 (68%)	35 (70%)	34 (68%)	1		1	
CT	14 (28%)	12 (24%)	13 (26%)	0.857 (0.399-1.843)	0.693	1.113 (0.450-2.753)	0.817
TT	2 (4%)	3 (6%)	3 (6%)	1.532 (0.298-7.879)	0.607	1 (0.192-5.210)	1
Additive model				0.748 (0.267-1.854)	0.831	1.049 (0.337-1.950)	0.973
Dominant model				0.653 (0.127-3.357)	0.719	1 (0.486-2.059)	1
Recessive model				0.883 (0.582-1.339)	0.599	1 (0.236-4.241)	1

Table 6 showed that mutation in rs_1056890 of the I kappa B alpha gene had no statistically significant increased risk for HCV infection with a p-value > 0.05.

DISCUSSION

Regarding the demographic data of the three studied groups, the current study showed that the mean age among the studied groups A, B, and C were (53.90 ± 13.34, 51.82 ± 13.41, and 52.54 ± 12.91) respectively, the majority of studied cases were of female sex 29 (58%) out of 50 in group A, 32 (64%) out of 50 in group B and 27 (54%) out of 50.

The current study revealed that there was no statistically significant difference as regards the correlation between demographic data (age and sex) among studied groups and risk for HCV infection, with a p-value > 0.05.

Ting et al., 2017 in their study were in accordance with the previous findings, the author revealed that there was no statistical significance difference between patient's age and sex in studied groups and risk for HCV infection with a p-value > 0.05.

The current study showed that there was no statistically significant difference between the studied groups as regards, the correlation between patient's clinical data and risk for HCV infection, with a p-value > 0.05.

Ting et al., 2017 in their study were in dis accordance with the previous findings, the author found that blood donation, significantly affects the risk of infections, however, he did not find a statistically significant difference as regards other clinical data and risk for HCV infection.

The current study revealed that the mutation in rs_11820062 of the I kappa B alpha gene significantly increased the risk for HCV infection with a p-value < 0.05.

Ting et al., 2017 in their study were in agreement with the previous findings, the author found no associations between rs_11820062 mutation and HCV susceptibility.

The current study declared that the mutation in rs_230530 of the I kappa B alpha gene had no

statistically significant increased risk for HCV infection with a p-value > 0.05.

The current study documented that, the mutation in rs_3774963 of the I kappa B alpha gene had no statistically significant increased risk for HCV infection with a p-value > 0.05.

The current study showed that the mutation in rs_1056890 of the I kappa B alpha gene had no statistically significant increased risk for HCV infection with a p-value > 0.05.

Ting et al., 2017 in their study were in agreement with the previous findings, the author found no associations between rs230530, rs1056890, rs3774963, and HCV susceptibility.

CONCLUSION

- Polymorphisms within *NF-κB* pathway genes may be linked to hepatitis C virus infection susceptibility and outcomes.
- Nuclear factor-κB (NF-κB) is a key regulator of inflammations in many hepatic cell populations and is required for hepatocyte survival and liver homeostasis
- Survival and activation of hepatic stellate cells and hepatic myofibroblasts are regulated by NF-κB
- The key roles of NF-κB in the regulation of cell death, inflammations, and wound healing make it an important modulator of hepatic disease progression.
- NF-κB is a potential link between chronic liver injury, fibrosis, and hepatocellular carcinoma.

RECOMMENDATION

- Since its discovery more than 20 years ago, the NF-κB pathway has emerged as one of the best-characterized signaling pathways.
- Cell-type-specific functions of NF-κB need to be taken into account when designing therapies, that target this transcription factor.
- The central role of NF-κB in liver homeostasis and regulation of inflammation, fibrosis, and carcinogenesis is of high clinical relevance for chronic liver diseases, so further investigation is needed to explore the comprehensive mechanisms and functions of NF-κB.

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