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Role of signal transducer, and activator of the transcription 4 (STAT4) gene polymorphism as predictive marker for type-1 autoimmune hepatitis

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Abstract---Introduction: Autoimmune hepatitis (AIH) is one of autoimmune disorder with complex pathophysiology. Many genes involved in lethal autoimmune hepatitis disorder including *STAT4* gene. The aim of this study is to determine *STAT4* gene polymorphisms and relation of SNPs with disease severity among AIH patients. Method: One hundred AIH patient which diagnosed by clinically, ANA and ASMA and 100 control age matched healthy children were enrolled in this study. Patients' blood samples (5mL) were taken and split into two tubes, one tube used for immunological and clinical parameters and the second containing EDTA for PCR analysis. The first tubes were then left at room temperature for 10 minutes before being centrifuged (3500 rpm). After that, the serum was isolated the both tubes stored at -70°C until analysis, biochemical parameters were measured in full automated biochemistry analyzer while, IgG, ANA and ASMA was investigated using the enzyme-linked immunosorbent assay (ELISA) commercially available kits (Bioneer, Korea), while the *STAT4* (rs7574865, rs7582694) gene was

investigated using RFLP PCR How, and the commercially available kits (Bioneer, Korea). Results: The gender distribution and mean age of the AIH-type 1 and control groups were not statistically different ($p \leq 0.05$). In AIH-type I patients, among the type-1 AIH patients included, 86 % tested positive for ANA and 63 % tested positive for ASMA. Immunological results that presented by serum level of IgG revealed increasing in IgG level in AIH patients comparison to healthy with significant differences at ($p \leq 0.05$) (3759.21 ± 1821.43 , 901.85 ± 201.47) respectively. Furthermore the Biochemical markers levels there was a decreasing in level concentration of serum albumin, serum protein and total bilirubin in patients comparison to the healthy group, while there was increasing in concentration level mean of ALT, AST, Serum creatinine, ALP (235.2 ± 142.2 , 243.3 ± 133.7 , 253.3 ± 142.6 and 0.72 ± 0.17) respectively comparison to control groups (20.41 ± 5.86 , 26.78 ± 6.94 , 101.74 ± 6.78 and 0.34 ± 0.17) respectively with significant differences at ($p < 0.05$). On the other side of the current study STAT4 rs7574865 and rs7582694 genotype frequencies in both the patient and control populations conforms to the Hardy-Weinberg law of genetic equilibrium showed the distribution of STAT4 SNP (rs7574865) genotypes there are three were three genotypes at this locus: GG, GC, and TT and was distributed equally in both AIH and healthy people as in table (3) but for distribution of STAT4 SNP (rs7582694) the results reported different distribution which there are three genotypes at this locus: GG, GC, and CC and the CC genotype among patients was 12(12%) when compared with healthy controls 4(4%). However, the results of distribution of allele's frequency recorded the frequencies of the rs7574865- T allele in patients higher than healthy people with significant difference (at $p \leq 0.05$) respectively 0.35, 0.19 % respectively as well the frequencies of the rs7582694- C patients higher than healthy people with significant difference (at $p \leq 0.05$) 0.32, 0.19% respectively. Conclusion: This study showed an association between a STAT4 polymorphism and type-1 autoimmune hepatitis, suggesting that the alleles of both STAT4 SNPs (rs7574865 T allele, rs7582694 C allele) are associated with an increased risk of type-1 AIH. The disease could be predicted with AIH disease severity what??? by the dominant model of either SNPs and by the recessive model of rs7574865.

Keywords---Autoimmune hepatitis type I, gene Polymorphism, transcription 4 (STAT4).

Introduction

Autoimmune hepatitis (AIH) consider as one of liver chronic associated with hypergamma-globulinemia and production of autoantibodies (Czaja and Manns, 2010). According to some research on pediatric patients, the incidence is (about 0.4 case per 100,000 youngsters) (Deneau *et al.*, 2013). previous study in Poland found a prevalence of (3 to 4 per 100 000 children) (Li *et al.*, 2017). AIH disease is characterized by a female predominance and is uncommon in children, though it

can occur in very young children (Floresani *et al.*, 2013). The pathogenesis of AIH is unknown, however both genetic and environmental variables may have a role (Longhi *et al.*, 2010). Several earlier investigations have found a link between STAT4 and autoimmune illnesses Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are two examples. (SLE) (Remmers *et al.*, 2007, Sugiura *et al.*, 2012). What studies link AIH with studied SNPs. STAT4 polymorphism was found to be positively linked with type-1 AIH in a recent study (Li *et al.*, 2017) .

The missense mutation (SNP) has been linked to autoimmune illnesses such as RA and SLE (Begovich *et al.*, 2004, Hinks *et al.*, 2005, Orozco *et al.*, 2005, Orru *et al.*, 2009), the SNPs play a crucial influence in liver illness, according to a Japanese population-based study (Umemura *et al.*, 2016). Given the minimal number of studies on the STAT4 and genes and the risk of AIH, especially in children (Behairy *et al.*, 2021). The present study wanted to explore how STAT4 gene SNPs, as well as other gene interactions and haplotype combinations, increase the incidence of type 1 AIH in a Iraqi population and determination the association of AIH severity and gene polymorphism.

Material and Methods

Collection and selection the samples

Two hundred sample collected from Form Al-Hussain Medical City/Karbala between April 17th, 2016 and April 1st, 2021 which included 100 AIH patient which diagnosed by clinically, ANA and ASMA (with exclusion the patients with Fatty liver diseases ,nonalcoholic liver disease ,viral hepatitis) and 100 healthy object as a control group who were free of signs and symptoms of liver disease with same age and gender as the patients matched , and had no history of liver disease, Patients' blood samples (5mL) were taken and split into two tubes, one containing EDTA for PCR analysis and gel tube for serum collection sample ,After that, the serum was isolated and stored at -70°C until analysis.

Evaluation of Biochemical markers levels

The serum has separated by centrifugation at 3000 rpm for 25 min then was tested spectrophotometrically for the biochemical parameters were measured in full automated biochemistry analyzer (Chemray 240. USSR).

Evaluation of Immunological markers levels

IgG, Antinuclear antibodies (ANA), Anti-smooth muscle antibodies (ASMA) Were investigated using the enzyme-linked immunosorbent assay (ELISA) commercially available kits (Bioneer, Korea).

The molecular aspect

The STAT4(rs7574865,rs7582694) genes were investigated using RFLP PCR using commercially available kits (Bioneer, Korea), and performed according to the instructions in the kit brochure. rs7574865, F:59 AAAGAAGTGGGATAAAAAGAAGTTTG-39, R:59CCACTGAAATAAGATAACCACTGT-39, and rs7582694, rs/582094, F:59 ATCCAACCTCTTCTCAGCCCTT-39,

R:59TCATAATCAGGAGAGAGGAGT-39 were employed in the PCR reaction (Xu *et al.* 2011).

The endonuclease restriction enzyme

RFLP analysis was used for single nucleotide polymorphisms (STAT4), the endonuclease restriction enzyme *HpyCH4111* (*Helicobacter pylori* CH4III). Restriction digestions of all amplified DNA samples were performed using HpyCH4III restriction enzyme at 37°C for 4 hours to determine the genotype of STAT4 (rs7582694). The restriction digested PCR products, coupled with a 50bp molecular DNA size marker, were electrophoresed on a 2.5 percent TAE agarose gel for 40 minutes. Under ultraviolet light, the RFLP patterns were then determined. STAT4's digested product had three genotypes (GG), (GC), and (CG) (CC). The 338-bp-long PCR-amplified fragments of STAT 4 were extracted and digested using the endonuclease *HpyCH4*.

Statistical analysis

Statistical Analysis System- SAS (2012) program was used Least significant difference - LSD test (analysis of variance-ANOVA) was used for the significant comparison between means in this study (SAS,2012).

Results and Discussion

One hundred AIH patients and 100 healthy people, had different demographic and immunological characteristics. The gender distribution and mean age of the AIH-type 1 and control groups were not statistically different at ($p \leq 0.05$). In AIH-type I patients, Among the type-1 AIH patients included, 86 % tested positive for ANA and 63 % tested positive for ASMA.

Moreover, the immunological results that presented by serum level of IgG revealed increasing in IgG level in AIH patients comparison to healthy with significant differences at ($p \leq 0.05$) (3759.21 ± 1821.43 , 901.85 ± 201.47) respectively as well as ANA and ASMA (86%, 63%) respectively.

Table 1. General and immunological characteristics of Patients and healthy groups

Variables	AIH-type I	Healthy group	P.value
Age	6.9±4.1	6.5±4.5	0.31
Female	79(79%)	80(80%)	0.8
Male (n.%)	21(21%)	20(20%)	0.8
IgG(mg\dl)	3759.21±1821.43	901.85±201.47	<0.001
ANA + (n.%)	86(86%)	Negative	-
ASMA + (n. %)	63 (63%)	Negative	-

The results agreed with Xue *et al.*,2021 who found the IgG4, IgG, and GLB levels in the group with elevated serum IgG4 levels were higher than those in the normal group, Abe *et al.*(2011) had reported that type 1 AIH patients with elevated serum IgG4 levels had a higher rate of cirrhosis progression than

patients with low serum IgG4 levels (80.0% vs. 24.1%), suggesting a potential correlation between the increase in serum IgG4 levels and the occurrence of cirrhosis.

Considering that studies on the clinical features and pathogenesis of AIH with elevated serum IgG4 levels are lacking, and some existing research (Sucher et al.,2019) have pointed out that the degree of TH17 cells infiltration into the AIH liver is related to the degrees of liver inflammation and fibrosis TH17-related cytokines in some patients and found that serum IL-17 and IL-22 levels were significantly increased in AIH patients with elevated serum IgG4 levels, and these results were also supported by immunohistochemically evaluation of liver histology.

On other side, the current study also recorded a highly significant differences comparison with healthy people, this finding was agreed with (Özdoğan and Yaraş, 2020), this may indicate that the majority of AIH cases tend to progress insidiously. AIH is classified as type 1 or type 2, according to differences in their autoantibodies. Type 1 is the major type of AIH and presents with antinuclear antibodies (ANAs) and/or antismooth muscle antibodies (ASMAs), whereas the less common type 2 AIH is characterized by antiliver kidney microsome 1 (LKM 1)(Aizawa and Hokari,2017).

Furthermore the Biochemical markers levels that represented by the serum level of biochemical markers includes: ALT, AST, ALP, serum Creatinine serum albumin, serum protein and total bilirubin revealed various findings which there was a decreasing in level concentration of serum albumin , serum protein and total bilirubin in patients comparison to the healthy group, while there was increasing in concentration level mean of ALT, AST, Serum creatinine, ALP (235.2±142.2, 243.3±133.7, 253.3±142.6 and 0.72±0.17) respectively comparison to control group (20.41±5.86, 26.78±6.94, 101.74±6.78 and 0.34±0.17) respectively with significant differences at ($p \leq 0.05$) as shown in table (2).

Table 2. Biochemical markers levels among patients and healthy group

Bio-chemical markers	AIH-type I (n=100) Mean±SD	Health group (n=100) Mean±SD	P-value
ALT (IU\L)	235.2±142.2	20.41±5.86	<0.001
AST(IU\L)	243.3±133.7	26.78±6.94	<0.001
ALP(IU\L)	253.3±142.6	101.74±6.78	<0.001
Serum creatinine (mg\dl)	0.72±0.17	0.34±0.17	<0.001
Serum albumin (g\dl)	2.33±0.38	5.86±0.67	<0.001
Total bilirubin (mg\dl)	1.4±0.41	0.58±0.54	<0.001

These findings are agreed with (Ferrari *et al.*,2004) An increased AST/ALT ratio (normal value <1) has been claimed to be a marker of more severe disease in chronic hepatitis C.(Pohl *et al.*,2001) A similar prognostic relevance has been reported also in acute viral hepatitis In the light of these observations, it can be

speculated that the higher AST/ALT ratio detected in 'acute' AIH might reflect the more aggressive clinical course of this disease, compared to viral hepatitis (Ferrari *et al.*,2004).

Association between *STAT4* (rs7574865) polymorphisms and AIH type-1.

The results showed the products of successful binding between the *STAT4* (rs7574865) and specific primers for *STAT4* gene were detected by gel electrophoresis and product size was 147 bp for both patients and control group, figure 1.

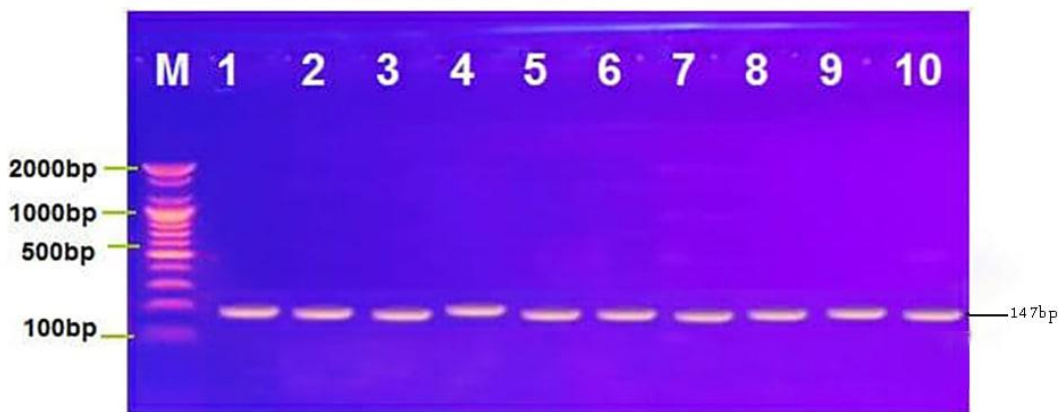


Figure 1. PCR product analysis on an agarose gel electrophoresis of *STAT4* rs7574865 gene from blood samples of AIH patients and healthy control on 1.5% agarose gel.

The distribution of *STAT4* rs7574865 gene polymorphism was detected by PCR-RFLP technique. T allele yielded two fragments (122 bp and 25 bp), but the G allele remained intact (147 bp). In the gel, the tiny band (25 bp) was lost (Figure 1 and 2).

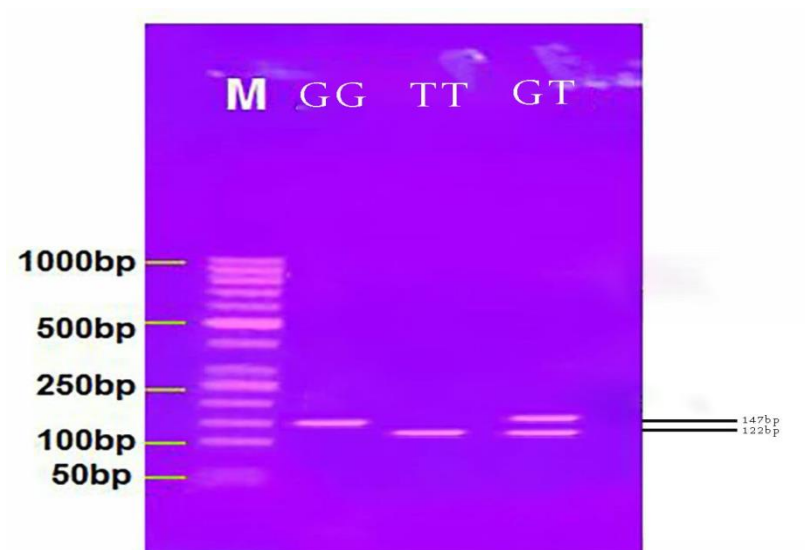


Figure 2. Agarose gel electrophoresis image of STAT4 rs7574865 RFLP-PCR product analysis employing HpaI restriction enzyme on a 2.5 percent agarose gel. The STAT 4 rs7582694 was split into 258 and 80 base pairs. The STAT 426 G allele was cleft into 258 bp and 80 bp fragments, however the STAT 4 C allele was left intact (Figure 3 and 4).

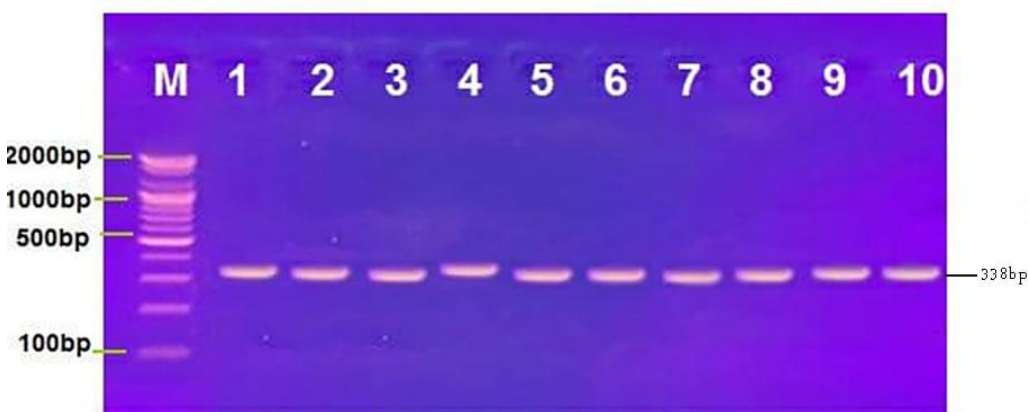


Figure 3. PCR product analysis on an agarose gel electrophoresis of STAT4 rs7582694 gene from blood samples of AIH patients and healthy control on 1.5% agarose gel.

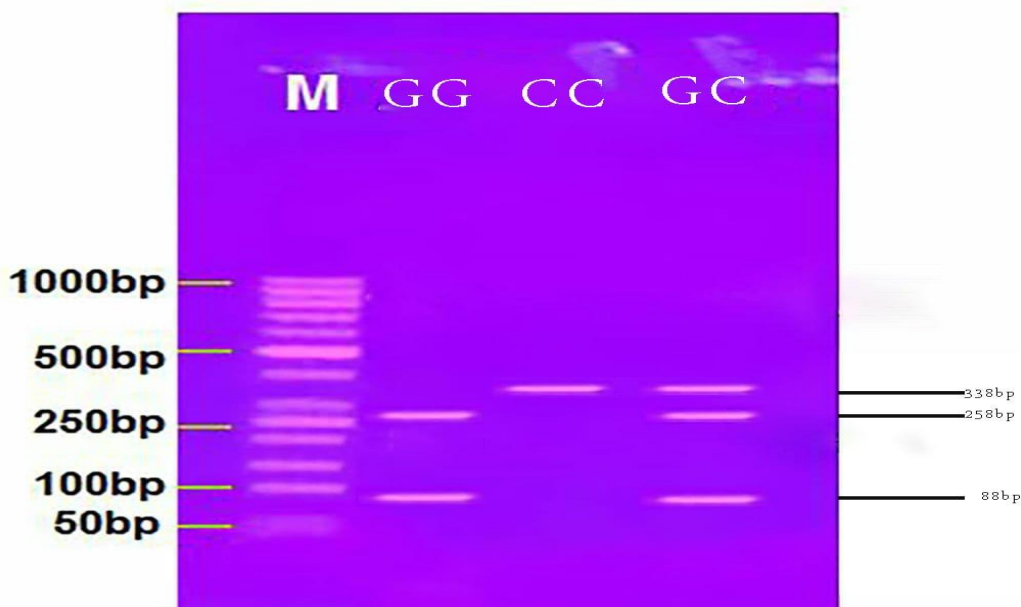


Figure.4. Agarose gel electrophoresis for product analysis of STAT4 rs7582694 by using HpyCH4 restriction gel at 80Vol/cm for 30 m, show the enzyme on RFLP-PCR 2.5% agarose.

On the other side of the current study STAT4 rs7574865 and rs7582694 genotype frequencies in both the patient and control populations conforms to the Hardy-Weinberg law of genetic equilibrium showed the distribution of STAT4 SNP

(rs7574865) genotypes there are three were three genotypes at this locus: GG, GC, and TT and was distributed equally in both AIH and healthy people as in table (3) but for distribution of STAT4 SNP (rs7582694) the results reported different distribution which there are three genotypes at this locus: GG, GC, and CC and the CC genotype among patients was 12(12%) when compared with healthy controls 4(4%) (table 4).

Table 3. Genotype distribution of STAT4 SNP (rs7574865) among patients and control group

STAT4 SNP (rs7574865)		Autoimmune hepatitis type-1	Control group	P Value
Additive genotypes	GG	50(50%)	72(72%)	0.04
	GT	31(31%)	18(18%)	0.018
	TT	19(19%)	10(10%)	0.001
Dominant	GG	50(50%)	72(72%)	0.04
	GT+TT	50(50%)	28(28%)	0.001
Recessive	GG+GT	81(81%)	90(90%)	0.04
	TT	29(29%)	10(10%)	0.003

Table 4. Genotype distribution of STAT4 SNP (rs7582694) among patients and control group

STAT4 SNP (rs7582694)		Autoimmune hepatitis type-1	Control group	P-Value
Additive genotypes	GG	48(48%)	66(66%)	
	GC	40(40%)	30(30%)	0.02
	CC	12(12%)	4(4%)	0.01
Dominant	GG	48(48%)	66(66%)	0.02
	GC+CC	52(52%)	34(34%)	0.004
Recessive	GG+GC	88(88%)	96(96%)	0.03
	CC	12(12%)	4(4%)	0.02

However, the results of distribution of allele's frequency recorded the frequencies of the rs7574865- T allele in patients higher than healthy people with significant difference (at $p \leq 0.05$) 0.35, 0.19 % respectively as well the frequencies of the rs7582694- C patients higher than healthy people with significant difference (at $p \leq 0.05$) 0.32, 0.19% respectively as shown in table 5.

Table 5. Distribution of allele's frequency between patients and control group

SNPs	Alleles	AIH type I	Control	P Value
STAT4(rs7574865)	G	G 131(0.65)	162(0.81)	0.03
	T	T 69 (0.35)	38(0.19)	0.04
STAT4SNP (rs7582694)	G	G 136(0.68)	162(0.81)	0.03
	C	C 64(0.32)	38(0.19)	0.04

The goal of this study was to see if there was a link between STAT4 gene polymorphisms and type-1 AIH vulnerability. These results matched by Li *et*

al.(2017), who discovered that both genotypes in controls are distributed according to HWE (both p values > 0.05). In type-1 AIH patients, the frequencies of the rs7574865 T allele and the rs7582694 C allele were considerably higher than in the control group (30.6 percent vs 19.3 percent, 32.5 percent vs 20.0). Furthermore, they discovered that people with the rs7582694 genotype GC or CC had the highest type-1 AIH risk compared to those with the rs7582694 genotype GG. They concluded that haplotypes containing the rs7582694-C and rs7574865-T alleles within the STAT4 gene were linked with a significantly higher type-1 AIH risk after adjusting for variables.

Furthermore, a meta-analysis by Liang *et al.* (2012) discovered that the STAT4 rs7574865 T variant was associated with susceptibility to various autoimmune illnesses, showing a link between the STAT4 gene polymorphism and autoimmune diseases. STAT4 polymorphism was previously proposed to be positively associated with type-1 AIH risk by Migita *et al.*(2017). STAT4 was one of the main transcription factors involved in regulating the Th1/Th2 cytokine balance (Watford *et al.*,2004). The link between STAT4 and autoimmune illnesses such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) has been identified in various prior investigations (Sugiura *et al.*,2012). STAT4 was a key genetic component in the synthesis of IL-22, which has a pathogenic role in IL17-dependent hepatitis, according to the pathophysiology of AIH (Xu *et al.*,2011).

The association between *STAT4* and some autoimmune diseases, including rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) has been reported in several previous studies (Sugiura *et al.*,2012). Taylor *et al.* [2008] concluded that STAT4 polymorphism played an important role on susceptibility to SLE, also suggested that STAT4 is a common autoimmune diseases related genetic risk factor, including RA and SLE. A recent meta- analysis (Tong *et al.*,2013) demonstrated a significant association between rs7574865- T allele within *STAT4* gene and susceptibility to SLE, RA, T1D and so on.

STAT4 has a crucial role in a pathogenesis of several diseases, including Irritable bowel disease (IBD) is an example of an autoimmune and inflammatory disease, according to recent in vivo and in vitro research. STAT4, which is triggered by certain cytokines via the Janus kinase (JAK)-STAT signaling cascade, controls innate and adaptive immune responses in a variety of ways SNPs have a minor impact on the STAT4 gene, and more research is needed(Yang *et al.*,2020) The etiology of AIH was complicated, implying STAT4 is a transcription factor that plays a role in Th1 and Th17 differentiation(Chaouali *et al.*,2018).STAT4, was found to be a key genetic component in the production of IL-22 which has a pathogenic role in IL-17-dependent hepatitis(Xu *et al.*,2011).

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Conflict of Interest

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