Short communication

Association of interleukin (IL)-4 variable number of tandem repeats (VNTRs) and IL-4-590 promoter polymorphisms with susceptibility to pediatric autoimmune hepatitis type 1

Amira Ibrahim Mansour\(^a\)*, Ola Galal Behairy\(^b\), Eman Rateb Abd Almonaem\(^b\), Rasha Mahmoud Abd-Rabuh\(^c\), Inas Abd Elmonem Ahmed\(^d\)

\(^a\) Clinical and Chemical Pathology Department, Benha Faculty of Medicine, Benha University, 13511, Egypt
\(^b\) Pediatric Department, Benha Faculty of Medicine, Benha University, 13511, Egypt
\(^c\) Pathology Department, Benha Faculty of Medicine, Benha University, 13511, Egypt
\(^d\) Medical Biochemistry Department, Benha Faculty of Medicine, Benha University, 13511, Egypt

ARTICLE INFO

Keywords:
Autoimmune hepatitis type 1
Children
Interleukin-4
Restriction Fragment Length Polymorphism (RFLP)
Single Nucleotide Polymorphism (SNP)

ABSTRACT

Objective: Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease mediated by an autoimmune reaction to hepatocytes, the present study aimed to assess the possible associations between interleukin-4 (IL-4) variable number of tandem repeats (VNTRs) and IL-4-590 promoter polymorphisms and susceptibility to autoimmune hepatitis type 1 in children.

Subjects and methods: The study was performed on 101 children diagnosed with AIH and 104 apparently healthy, age and sex-matched control children, diagnosis of AIH was based on the simplified score for the diagnosis of AIH. Genotyping for the IL-4 VNTR and IL-4-590 were performed using PCR-RFLP.

Results: The distribution of genotype frequencies of IL-4 gene intron 3 VNTR polymorphism were not significantly different between AIH patients and controls for 3R/2R and 2R/2R genotypes, while the 2R allele distribution was significantly higher among AIH patients than the control group. The frequency of IL-4-590 single nucleotide polymorphism (SNP) CT and TT genotypes was statistically higher among AIH patients than controls.

Conclusion: This study revealed the presence of an association between IL-4 -590 TT genotype and T alleles with increased AIH risk in pediatric patients, also assess its severity as they were detected with Child Plugh scores B and C.

1. Introduction

Autoimmune hepatitis (AIH) is a serious chronic inflammatory liver disease mediated by an autoimmune reaction to hepatocytes, characterized by autoantibodies, hypergammaglobulinemia, and interface hepatitis on histological examination in the absence of a known etiology [1]. Two types of AIH are recognized according to the nature of the serum autoantibodies: type 1 (AIH-1), positive for ANA and/or anti-smooth muscle antibody (SMA), and type 2 (AIH-2), positive for anti-liver kidney microsomal type 1 antibody (anti-LKM-1) or for anti-liver cytosol type 1 antibody (anti-LC-1) [2]. AIH-1 represents about 80% of AIH cases worldwide [3], with more predominance in females (about 75% of affected persons) [4]. The prevalence of AIH in an adult about 10 to 15 cases per 100,000 [5], while its real prevalence in children is still unknown as the presentation of childhood AIH is often insidious [6].

The immunopathogenesis of AIH is still unknown, but it involves dysregulation of Th1 and Th2 cells with dense infiltrate of lymphocytes, plasma cells, and macrophages which suggests an auto-aggressive cellular immune attack [7]. Most studies support the evidence that adaptive immune system (cellular and humoral) is the main player in the pathogenesis of AIH [8]. To date, few studies are available regarding the Th1/Th2 balance especially, in pediatric autoimmune hepatitis. Therefore, the aim of this study was to assess the possible associations between the IL-4 (the prototype cytokines Th2-type immune response) VNTRs and IL-4-590 promoter polymorphisms and susceptibility to autoimmune hepatitis type 1 in Egyptian children.

* Corresponding author at: 5 Alzahraa st., Benha, Qaluopia, Egypt.

E-mail addresses: AMIRA.MANSOUR@fmed.bu.edu.eg, amiraww2005@yahoo.com (A.I. Mansour), olaped99@yahoo.com (O.G. Behairy), rashahamaza32@yahoo.com (R.M. Abd-Rabuh), inas.ahmed@fmed.bu.edu.eg (I.A.E. Ahmed).

https://doi.org/10.1016/j.cyto.2018.01.009
Received 10 July 2017; Received in revised form 3 January 2018; Accepted 9 January 2018
2. Subject and methods

This case-control study was conducted at Pediatric Hepatology department, Benha University Hospital, Egypt, from December 2015 to February 2017. The study was approved by the Ethical Scientific Committee of Benha University and was carried out according to the guidelines of the Helsinki Declaration [9]. An informed consent was obtained from one of the parents before enrollment of their children in the study.

Two hundred and five children were included in the study. They were divided into 2 groups:


Inclusion criteria: all AIH patients < 18 years.

Exclusion criteria: children with other chronic liver diseases, viral hepatitis (hepatitis A, B, E and C, recent infection by EBV, herpes simplex virus and CMV, by negative specific IgM), drug-induced hepatitis, fatty liver disease, and metabolic disorders.

II. Control group: 104 apparently healthy, age and sex-matched children.

The study participants were subjected to full history taking with special attention to consanguinity of parents and history of autoimmune diseases in the patient or one of his first-degree relatives. Complete clinical examination including height, weight measurements and calculation of body mass index via dividing weight (in kg) by the square of height (kg m⁻²). Ultrasonographic assessment of the liver (span and texture), spleen span and for the presence of ascites.

Laboratory investigations were done for all enrolled subjects including:

- Liver function tests: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin and serum albumin: using Biosystem A15 auto-analyzer by appropriate chemical principles.

- Complete blood count: using automated hematolysis system, (Sysmex XE 5000).

- Serum Immunoglobulin G concentration (IgG): by radial immunodiffusion (IgG – NL RID, RN004.3, Binding Site, Birmingham, UK).

- Serum autoantibodies: by indirect immunofluorescence technique using NOVA Lite™ Rat Liver, Kidney, Stomach, for detection of ANA, ASMA, antibodies to liver kidney microsome type 1 (anti-LKM-1) and AMA (INOVA Diagnostics, Inc, Germany).

- To exclude other causes of liver diseases, all the patients had the following:

  1. Serological tests for viral hepatitis A, B and C virus excluded the presence of these infections: using elisa technique: HAV IgM: MONOLISA™ Anti-HAV IgM EIA (Bio-Rad, France). HCV Ab: INNOTEST® HCV Ab (InnoGenetics N.V., Belgium). HBsAg: GS HBsAg EIA 3.0 #32591(Bio-Rad, France).

  2. Work up for Wilson’s disease: serum ceruloplasmin (Colorimetric, Sigma-Aldrich Co., USA, Catalog Number MAK177) 24-h urinary copper before and after penicillamine (Colorimetric, Sigma-Aldrich Co., USA, Catalog Number MAK127).

  3. Alpha-1 antitrypsin deficiency was excluded by the absence of characteristic clinical features and determination of plasma levels of the alpha-1 antitrypsin level and phenotype. (Nephelometry, BN prospec, Siemens, Erlangen, Germany).

- Histopathological examination:

  Ultrasonography-guided liver biopsies were done for all patients using a Tru-Cut needle. (Hepafix luer Lock, Braun Melsungen AG, 3409 Melsungen, Germany). The adequate core contains at least 5 portal tracts. Biopsy specimens were fixed in formalin and embedded in paraffin. Five-μm thick sections were cut, mounted on a glass slide and stained with Hematoxylin and Eosin to evaluate the histological activity of hepatitis using Knodell's HA1 index. Also stained with Mason-Trichrome to assess fibrosis stage, Perls’ Prussian blue stain reveal iron deposits and PAS stain to exclude Alpha-1–antitrypsin deficiency.

- Detection of IL-4 VNTR and IL-4-590 promoter polymorphisms were done by PCR and restriction fragment length polymorphism (PCR-RFLP) [11]. DNA was extracted from peripheral blood leukocytes by Quick-g DNA MiniPrep (50 Preps) (ZYMO RESEARCH) (Catalog no D3024), according to manufacturer’s instructions.

- IL-4 VNTR genotyping: enzymatic amplification of the extracted DNA was performed by PCR; using PCR thermal cycler (PikoReal 24), the PCR was conducted in a reaction volume of 50 μL using 25 μL MyTaq Red Mix(2X) (BIOLINE, UK) (Bio-25043). IL-4 VNTR, was genotyped using primer (Biosearch technologies, USA): 1 μL of each primer, forward primer: 5‘ AGGCTGAAAGGAAAAACG-3’, and reverse primer: 5‘CTGTTGCACCTCACTGCTCC-3’ and 200 ng of the template. Amplification was carried out as follows: initial denaturation:5 min at 94 °C, 30 cycles consisting of 50 s of denaturation at 94 °C, 30 s of annealing at 61 °C, 45 s of extension at 72 °C with and a final extension of 5 min at 72 °C, the resultant PCR products were examined by gel electrophoresis. Alleles were named as follows: allele 3R = three repeats (254 bp), and allele 2R = two repeats (184 bp).

- IL-4-590 polymorphisms were genotyped by PCR–RFLP: The region surrounding this polymorphism was amplified with the following forward primer 5‘ACTAGGGCTCAGCTGTAGC-3’ and reverse primer 5‘CTGTTGACCTCACTGCTCC-3’. PCR was carried out in a total volume of 50 μL containing 200 ng of genomic DNA, 1 μL of each PCR primer (Biosearch technologies, USA) and 25 μL MyTaq Red Mix(2X) (BIOLINE, UK) (Bio-25043), thermal cycling conditions were: 94°C for 5 min followed by 32 cycles at 95°C for 30 s, at 57°C for 30 s and at 72°C for 30 s. A final extension step was carried out at 72°C for 5 min. The resultant PCR products showed a single fragment at 252 bp by gel electrophoresis. 10 μL of the amplified targeted DNA was digested by 1 μL of Bsm FI restriction enzyme (New England Biolabs, Beverly, MA, USA) at 37°C for one hour then separated on 2% agarose gel, stained with ethidium bromide and visualized by UV light. Homozygous CC genotype gives two bands at 192 and 60 bp. Homozygous TT genotype gives one band at 252 bp. Heterozygous CT genotype gives three bands at 192, 60 and 252 bp.

2.1. Statistical analysis

The collected data were summarized in terms of mean ± Standard Deviation (SD) and range for quantitative data and frequency and percentage for qualitative data. Comparisons between the different study groups were carried out using the Chi-square (χ²) and Fisher’s Exact Test (FET) to compare proportions as appropriate. The Odd’s Ratio (OR) and 95% Confidence Interval (95% CI) were also calculated. The student t-test (t) and Mann-Whitney test (z) were used to compare two groups regarding parametric and non-parametric data respectively. While one-way Analysis Of Variance (ANOVA; F) and Kruskal Wallis test (χ²) were used to compare more than two groups as appropriate. Statistical significance was accepted at P value < 0.05. All statistical analyses were carried out using STATA/SE version 11.2 for Windows (STATA Corporation, College Station, Texas).

3. Results

The present study included 101 pediatric patients with AIH, 57 (56.4%) were females and 44 were males (43.5%), their mean age was 7.5 ± 3.4 years, together with 104 healthy control subjects, 52 females (50%) and 52 males (50%), and their mean age was 7.1 ± 2.6 years. Positive consanguinity was detected in 28 patients (27.7%) and 31 of
control subjects (29.8%). Four female patients had concurrent autoimmune diseases (3 females had systemic lupus erythematosus and one had insulin dependent diabetes mellitus), also the frequency of a family history of autoimmune diseases in 1st-degree relatives was 3.9%, among the 101 patients, (in the form of vitiligo and AIH).

The most common clinical presentations were jaundice in 72.2% of patients, fatigue in 49.5% of patients, acute hepatitis-like illness was the first presentation in 45 (44.5%), abdominal pain in 44 patients (43.5%) bleeding in 29 patients (28.7%) and fever in 28 patients (27.7%) hepatomegaly 80 (79.2%), splenomegaly 30 (29.7%), lower limb edema 4(3.9%) and ascites 36 (35.6%) [mild ascites was 32(31.6%) and moderate in 4(3.9%)].

At the time of presentations, all patients had increased levels of AST (1.4–24 folds), ALT (0.4–20 folds) and total IgG (1.1–3.5 folds) above upper limits of normal. Serum alkaline phosphatase, GGT, and creatinine levels were normal. The mean values of PT, PTT, total, direct bilirubin, and INR were significantly higher among AIH patients compared to the control (P < .001). According to the autoantibody profile, all patients were type 1 AIH as none of our patients were positive for anti-LKM. Regarding other autoantibodies including ANA and ASMA, they were positive in 16/101 (15.8%) and 84/101 (83.1%) respectively and their titer ranged from 1:20 to 1:80, while AMA was negative in all patients.

Liver biopsies were performed for all cases, interface hepatitis was present in 85(84.16%), rosette formation in 61 (60.4%) patients, and regards types of cells; plasma cell infiltrates were detected in 81 (80.2%) and lymphocytic infiltrates were detected in 44(43.5%) patients. By applying scoring system for diagnosis AIH, we found that the frequency of definite diagnosis of AIH by revised original scoring system of the IAIHG compared to simplified scoring system was (37.6% versus 36.6% respectively), and probable diagnosis of AIH was (52.4% versus 29.2% respectively) AIH, while non-diagnostic was in (9.9% versus 33.6% respectively) (Table 1).

All patients received steroids as the initial therapy and in most of them, azathioprine was added to start withdrawal. 32 (31.6%) patients were on prednisone alone and 69(68.3%) patients were on prednisone and azathioprine. Complete response to treatment was observed in 55(54.4%) patients while 46(45.5%) had relapses (Table 1).

Different genotypic and allele frequency distributions for IL 4 are shown in Table 2. Genotype distributions of IL-4 VNTR and IL-4-590 promoter polymorphisms in cases and controls were consistent with the Hardy–Weinberg equilibrium.

3.1. Genotype distributions of IL-4 Intron 3 VNTR polymorphism

The three genotypes of intron 3 VNTR of IL-4 gene were classified as 2R/2R (184 bp—two 70 bp repeat allele), 3R/3R (254 bp—three 70 bp repeat allele) and 2R/3R (both 184 and 254 bp fragments). The distribution of genotype frequencies of IL-4 gene intron 3 VNTR polymorphism were not significantly different between AIH patients and controls for 2R/3R genotype (OR = 1.71, P = .17), and 2R/2R genotype (OR = 3.51, P = .15), while the 2R allele distribution was significantly higher in AIH patients than control group (P = .02).

3.2. Genotype distributions of IL-4-590 polymorphism

The frequencies of TT genotype and T alleles were significantly increased in AIH patients compared to the control group. Also, the frequency of CT and TT genotypes was statistically different between AIH patients and control (P = .003). Subjects with IL-4-590 TT genotype and carriers of T allele were significantly more likely to develop AIH (OR = 2.92, 95% CI = 0.84–11.48, P = .05) and (OR = 2.07, 95% CI = 1.26–3.4, P = .002) respectively.

Regarding IL-4 Intron 3 VNTR genotypes, no statistically significant associations were found between IL-4 Intron 3 VNTR genotypes and response to treatment or relapse rates (P = .38). In addition, we didn’t find any significant associations between hepatitis activity index (HAI) or histologic fibrosis index (HFI) and IL-4 Intron 3 VNTR genotypes distribution. There were no statistically significant differences between different genotypes regarding Child Plugh score.

Regarding IL-4-590 genotypes, no statistically significant associations were found between IL-4-590 genotypes and response to treatment or relapse rates (P = .50). In addition, no statistically significant differences between IL-4-590 genotypes regarding histologic activity index (HAI) or histologic fibrosis index (HFI). The Child Plugh score showed significant differences between IL-4-590 genotypes (P = .05) as 68.3% of CT genotype and 50% of TT genotype were scored B.

4. Discussion

Autoimmune hepatitis (AIH) is a serious autoimmune liver disease in both children and adults with a more aggressive course in children and more predominance in females [12].

As regarding presenting manifestations, 72.2% of our patients presented with jaundice, 44.5% with acute hepatitis-like illness and abdominal pain in 43.5% patients, these results agree with Czaia who reported that an acute presentation occurs in 25–75% of patients with autoimmune hepatitis [13].

Despite extensive studies trying to approach autoimmune hepatitis pathogenesis for a long time, the exact mechanisms are still unknown [14].

In this study, we investigated the possible associations between IL-4 variable number of tandem repeats (VNTRs) and IL-4-590 promoter polymorphisms and susceptibility to autoimmune hepatitis type1 in children, previous studies investigated these polymorphisms with several common diseases, especially autoimmune diseases, but to our knowledge, no previous studies have been carried out to determine the association between these polymorphisms with autoimmune hepatitis type1 in children.

In AIH, there is a shift from T helper 2 dominated (Th) to Th1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (no. = 101)</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver biopsy</td>
<td>Type of cell</td>
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<td></td>
</tr>
<tr>
<td>(No. &amp; %)</td>
<td>Eosinophils</td>
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<tr>
<td></td>
<td>Lymphocytes</td>
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<td></td>
<td>Plasma cells</td>
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<td>76.0%</td>
</tr>
<tr>
<td></td>
<td>Rosette formation</td>
<td>N = 61</td>
<td>60.4%</td>
</tr>
<tr>
<td></td>
<td>Interface hepatitis</td>
<td>N = 85</td>
<td>84.16%</td>
</tr>
<tr>
<td></td>
<td>Hepatitis activity index (HAI) ( \text{Mean} \pm \text{SD}; \text{range} )</td>
<td>7.3 ± 3.5 (1-15)</td>
<td></td>
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<tr>
<td></td>
<td>Fibrosis index (FI,6) ( \text{Mean} \pm \text{SD}; \text{range} )</td>
<td>2.6 ± 0.1 (0–5)</td>
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<td>Revised original scoring system of AIH</td>
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<tr>
<td></td>
<td>Definite AIH</td>
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<td>37.62</td>
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<tr>
<td></td>
<td>Probable AIH</td>
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<td>52.48</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>10</td>
<td>9.9</td>
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<td></td>
<td>Definite AIH</td>
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<tr>
<td></td>
<td>Probable AIH</td>
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<td>29.70</td>
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<tr>
<td></td>
<td>No</td>
<td>34</td>
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<td>Child Plugh score</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>26</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>59</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>16</td>
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<td>Prednisone alone</td>
<td>32</td>
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<tr>
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<td>Relapse</td>
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dominated immune response, which predispose to the inflammation in AIH [8]. IL-4 is a potent anti-inflammatory Th2 cytokine that regulates many immune functions and promote differentiation of naive helper T cells to Th2 cells, it also, have an important role in the development of autoimmune disorders [15]. Cytokine gene polymorphisms can affect cytokine levels and the Th1 and Th2 cells balance, which predispose to many autoimmune diseases, including AIH [7].

The present study revealed that IL-4-590 TT genotype and T alleles significantly associated with increased AIH risk (OR = 2.92, 95% CI = 0.84–11.48, P = .05) and (OR = 2.07, 95% CI = 1.26–3.4, P = .002) respectively. As well, there was no significant difference in IL-4 gene intron 3 VNTR gene polymorphism between AIH patients and control group.

On the contrary, Yousefi et al. [16] studied the possible associations of AIH in Iranian patients with IL4 gene variants and they found a higher frequency of IL-4-590C allele and CC genotype in AIH patients compared to healthy controls. Such contradiction may be attributed to the differences in study design, study population, as well as to different sample size [16].

In our study Child Plugh scores B and C were associated with both type I AIH than normal control subjects [17]. Also, Chernavsky et al. [18] investigated the cytokine profile in liver biopsies obtained from 25 patients with untreated pediatric autoimmune hepatitis (PAH), and they observe that IL-4 was upregulated in PAH samples when compared with both control liver biopsies (CL) and HCV-related chronic hepatitis. and they found also, a positive correlation between IL-4 mRNA and the hepatitis activity index of the disease suggesting its potential role in the breakdown of immune self-tolerance in autoimmune hepatitis [18].

Kawashima et al. [19] measured serum IL-4 and IL-4 mRNA levels in the peripheral mononuclear cells in children with autoimmune hepatitis, and they demonstrated non-significant difference in IL-4/ β actin ratio between the patients with AIH and the healthy controls, and patients showed lower serum IL-4 than healthy controls [19].

5. Conclusion

This study revealed the presence of an association between IL-4-590 TT genotype and T alleles with increased AIH risk in pediatric patients, also assess its severity as they were detected with Child Plugh score B and C. Further studies are needed to explore the IL-4 expression and mechanism of action in AIH pediatric patients.

References

Simultaneous expression of Th1 cytokines and IL-4 confers severe characteristics to Type I autoimmune hepatitis in children, Human Immunol. 65 (2004) 683–691.