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Association of the killer immunoglobulin-like receptor genes KIR2DS1 and KIR2DS4 with pediatric autoimmune hepatitis type I in Egypt

Ola Galal Behairy[1], Ola Samir El-Shimi[2*], Naglaa Hamed Shalan[3] and Shaymaa Mohamed Baghdady[4]

Abstract

Background: Killer cell immunoglobulin-like receptors (KIR) are considered to be the key receptors that control the development and function of human natural killer cells which play complex mechanisms in autoimmune diseases. We aimed in this study to assess possible associations between killer cell immunoglobulin-like receptors (KIR2DS1 and 2DS4) genes and susceptibility to autoimmune hepatitis type I in Egyptian children.

Results: In the case-control study conducted on eighty children diagnosed as autoimmune hepatitis (AIH) type I and eighty apparently healthy age and sex-matched control, we found that KIR2DS1, -2DS4, KIR2DS4-full length allele, and homozygous KIR2DS4-full/full variant were significantly associated with AIH-I, while the KIR1D allele and homozygous KIR2DS4-del/del variant were significantly observed in controls ($P < 0.05$ each). Absence of KIR2DS4 gene was significant among ANA positive AIH-I patients, patients on steroid therapy alone, and patients showing complete disease remission ($P < 0.05$ each). Higher activity and fibrosis indices were found significantly in patients lacking one or both studied genes.

Conclusions: Children carrying KIR2DS1, -2DS4 genes, KIR2DS4-full length allele, and homozygous KIR2DS4-full/full variant could be more susceptible to develop autoimmune hepatitis type I.

Keywords: Autoimmune hepatitis, KIR, KIR2DS1, KIR2DS4, Egypt

Background

Autoimmune hepatitis (AIH) is a chronic progressive inflammatory disease of the liver, characterized by elevated serum aminotransferases, hypergammaglobulinemia, non-organ-specific autoantibodies, and lymphoplasmacytic interface hepatitis and responded to immunosuppressive therapy. It affects all ethnicity, any age, and both sexes, with female predominance [1]. According to the autoantibodies profile, AIH is classified into two main types (type I and type II), and whether AIH is treated or not, it might end up with liver cirrhosis [2]. The etiology of AIH remains largely unresolved. Hence, the interaction between background genetic architecture, environmental factors, and defective immune system can lead to AIH via the induction of a T cell-mediated immune response against liver antigens [3].

Natural killer (NK) cells have central roles in the innate immune response to viruses, bacteria, and tumor cells [4]. They can mediate direct killing of viral infected and transformed target cells as well as secreting immune regulatory cytokines and chemokines that bridge both innate and adaptive immune systems and activating the adaptive immune response [5]. NK cell-mediated responses have been implicated in the pathogenesis of AIH and other autoimmune diseases [6–8]. In peripheral blood, NK cells (CD16+ and CD56+) constitute only 5–10% of the

* Correspondence: ola.samer@fmed.bu.edu.eg
[2]Clinical and Chemical Pathology Department, Faculty of Medicine, Benha University, Benha 13511, Egypt
Full list of author information is available at the end of the article

circulating lymphocytes. However, they represent about 30% of the total lymphocytes in hepatic tissue and may reach up to 50% in case of liver pathologies such as viral, immune-mediated liver diseases and allograft rejection [9].

NK cells express a broad spectrum of inhibitory and activating killer cell immunoglobulin-like receptors (KIRs) that regulate their activity via detection of the expression levels of MHC class I on normal and diseased cells in humans [10].

Human KIR gene family is encoded by highly homologous sequences located on chromosome 19q13.4 within the leukocyte receptor complex (LRC) [11]. A total of 17 *KIR* genes have been discovered and named based on the molecule structure they encode (KIR2DL1–5A and B, KIR2DS1–5, KIR3DL1–3, KIR3DS1, KIR2DP1, and KIR3DP1). The first digit after the KIR acronym represents the number of Ig-like domains "D" in the molecule. The "L," "S," or "P" letters indicate a "long," "short," cytoplasmic tails, or a pseudogene respectively. The number of the gene encoding the protein with this structure is represented by a final digit. When two or more genes have very similar structures and sequences, a final letter is given after the same number to distinguish them (*KIR2DL5A* and *KIR2DL5B*) [12].

Early population studies of KIR genotypes demonstrated variations in KIR gene content from individual to individual (49–55). Based on these studies, two major KIR haplotype groups, the A and B haplotypes, have emerged (49). The A haplotype has traditionally been defined as containing KIR3DL3, -2DL3, -2DL1, -2DL4, -3DL1, -2DS4, and -3DL2. In contrast, B haplotypes are more variable and characterized by the presence of more than one activating KIR gene. Based on population studies of KIR genotypes, two major KIR haplotype groups have been described, the A and B haplotypes. Group A haplotypes contain fixed number of genes (the inhibitory *KIR3DL3*, *-2DL1*, *-2DL3*, *-2DL4*, *-3DL1*, and *-3DL2* genes and the activating *KIR2DS4* gene) while the Group B haplotypes contain different combinations of both activating and inhibitory KIR genes including one or more of the following genes: *KIR2DS1*, *-2DS2*, *-2DS3*, *-2DS5*, *-3DS1*, *-2DL2*, *-2DL5A*, and *-2DL5B* genes [13, 14].

Few studies have been reported the association of KIR gene complexes with AIH susceptibility [15–17]. Therefore, we aimed to study the possible associations of KIR2DS1 and 2DS4 genes with AIH-I susceptibility in a group of Egyptian children.

Methods

Study population

This case-control study included 160 children divided into 2 groups; an AIH group included 80 children with AIH diagnosed according to the International Autoimmune Hepatitis Group (IAIHG) revised original scoring system and the simplified diagnostic scoring system of the IAIHG [18], referred to pediatric hepatology clinics in Benha University Hospitals and National Liver Institute—Menoufia University, between May 2015 and January 2019. Any child with other chronic liver diseases and/or comorbidity as cardiovascular, renal, or central nervous systems affection was excluded. The second group is the control group, which included 80 apparently healthy children selected from general population of matched age and sex with the patient group.

Methodology

Each patient underwent full history taking, comprehensive clinical examination, abdominal ultrasonography, and routine laboratory investigations. In order to diagnose AIH, patients were tested for anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), liver-kidney microsomal antibody type 1 (LKM-1), and anti-mitochondrial antibody (AMA) by indirect immunofluorescence technique using Nova Lite® ANA KSL (Mouse Kidney/Stomach/Liver) (Inova Diagnostics, Barcelona, Spain). According to seropositivity for autoantibodies, patients with ANA and/or ASMA with titer ≥ 1:20 were classified as AIH-I and patients with LKM-1 titer ≥ 1:40 were classified as AIH-II [19]. To exclude other causes of liver disease before patients assigned for liver biopsy, hepatitis viruses (B, C, E) as well as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were screened for their serological markers (HBs-Ag, anti-HBc, anti-HCV, anti-HEV, anti-CMV-IgM, anti-EBNA-IgM, and anti-VCA-IgM) and confirmed with RT-PCR. Wilson's disease was excluded by estimating serum ceruloplasmin, 24 h urinary copper before and after penicillamine and the presence of Keyser-Fleischer rings. An ultrasound-guided liver biopsy was performed for all patients using Menghini aspiration needle to obtain an adequate core containing at least 11 portal tracts (Hepafix Luer Lock Braun Melsungen AG, Melsungen, Germany). Formalin-fixed, paraffin-embedded biopsy specimens were cut and stained with hematoxylin and eosin to evaluate histological activity of hepatitis using Ishak hepatitis activity index (HAI), Mason-Trichrome stain to assess degree of fibrosis, Perls' Prussian blue stain to detect iron deposition, and periodic acid-Schiff (PAS) stain to exclude α1 anti-trypsin deficiency. HAI ranges from 0 to 18 and the total activity scores are defined as follows: 1–3 minimal, 4–8 mild, 9–12 moderate, and 13–18 severe activity, while the degree of fibrosis is defined as follows: 0 no fibrosis, 1 fibrous expansion of some portal areas, with or without short fibrous septa, 2 fibrous expansion of most portal areas, with or without short fibrous septa, 3 fibrous expansion of most portal areas with occasional portal to portal bridging, 4 fibrous expansion of most portal areas with marked bridging

(portal to portal as well as portal to central), 5 marked bridging with occasional nodules (incomplete cirrhosis), and 6 cirrhosis, probable or definite [20].

KIR genotyping

Genomic DNA from all participants was extracted from whole blood collected on EDTA tubes using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo-Fisher Scientific, Germany) according to the manufacturer's instructions. The isolated DNA concentration and purity were evaluated by NanoDrop™ 2000 (Thermo-Fisher Scientific, USA). Genotyping of KIR2DS1 and the full-length KIR2DS4 (KIR2DS4-FL) with its variant KIR1D (it is identical to 2DS4 except for a 22 bp deletion in the sequences encoding the second Ig domain; D2) were performed separately by PCR with sequence-specific primers (PCR-SSP) using MyTaq™ Red Mix (2X) (BIOLINE, UK) and primers [21] (Biosearch technologies, UK) on Veriti Dx Thermal Cycler (Thermo-Fisher Scientific, USA) programmed with a 2-min denaturation step at 94 °C, followed by 30 cycles of 92 °C for 10 s, 65 °C for 30 s, and 68 °C for 1 min 30 s, followed by 72 °C for 10 min. Annealing temperatures were modified for primers amplifying *KIR2DS4* (61 °C) and *KIR1D* (63 °C). The PCR products along with 100 bp marker were resolved by electrophoresis in 2.5% agarose gel stained with 0.3 μg/ml ethidium bromide. The bands were visualized using UV trans-illuminator at 254 nm (Fig. 1).

KIR haplotype group assignment

Depending on the two studied KIR genes, the presence of *KIR2DS4* gene is indicating KIR haplotype A, while the KIR haplotype B could contain *KIR2DS1* gene or not. Thus, the homozygous individuals for KIR haplotype A (KIR AA) could be differentiated from heterozygous or homozygous individuals for KIR haplotype B (KIR AB or BB, referred together as KIR Bx) [22].

Statistical analysis

Data were summarized in terms of mean ± standard deviation (SD) or median (range) for quantitative data and frequency (percentage) for qualitative data. Student's *t* test and the Mann-Whitney (*Z*) test were used to test differences between two groups regarding parametric and non-parametric data respectively, while the chi-square (χ^2) and Fisher exact (FE) tests were used to compare frequencies as appropriate. To quantify the strength of the association of studied genotypes, odds ratio (OR) and 95% confidence interval (CI) were calculated. 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).

Results

Study population characteristics

The mean age of the eighty studied patients was 7.8 ± 3.62 years. They were 46 (57.5%) females and 34 (42.5%) males with female to male ratio 1.3:1, while the 80 control children had a mean age 7.2 ± 3.42 years with a female to male ratio 1:1. The AIH patients presented clinically with jaundice (78%), fatigue (35%), abdominal pain (48.5%), abdominal distention (50%), fever (15%), and gastrointestinal bleeding (10%). Abdominal ultrasonography of patients revealed hepatomegaly (70%), splenomegaly (25%), and ascites (25%). Only 6 patients (7.5%) had previous or concomitant immune-mediated diseases in the form of arthritis or eczema. Family history of autoimmune disease—rheumatoid arthritis or systemic lupus erythematosus—in first degree relatives was positive in 10 patients (12.5%). All studied patients were AIH type I (anti-LKM-1 or AMA negative). Sixteen patients (23.5%) were ANA positive and 68 patients (85%) were ASMA positive with titer ranged from 1:20 to 1:80. The baseline biochemical and histopathological characteristics of patients are listed in (Table 1). By applying scoring system for AIH diagnosis, we found that the frequency of definite diagnosis of AIH by revised

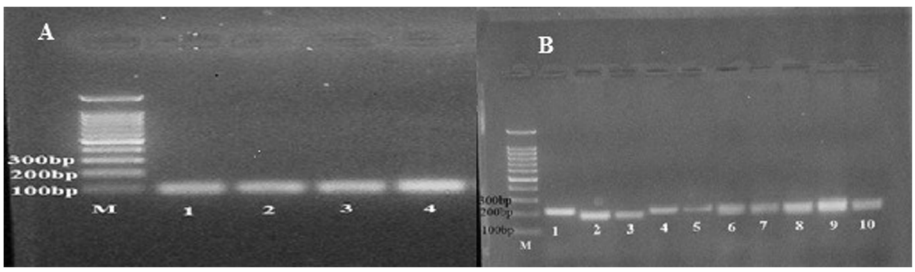

Fig. 1 KIR genotyping. **A** KIR2DS1 gene: samples 1–4 show the 102 bp fragment. **B** KIR2DS4 gene: samples 1, 4, and 5 are full/full (homozygous full length) at 219 bp, samples 2 and 3 are del/del (homozygous deletion variant) at 197 bp, and samples 6–10 are full/del (heterozygous). M: 100 bp marker. Primers sequences: KIR2DS1 [F: 5′-TCTCCATCAGTCGCATGAA/G-3′, R: 5′-AGGGCCCAGAGGAAAGTT-3′], KIR2DS4-FL [F: 5′-ATCCTGCAATGT TGGTCG-3′, R: 5′-CTGGATAGATGGTACATGTC-3′], KIR1D [F: 5′-ATCCTGCAATGTTGGTCG-3′, R: 5′-CTGGATAGATGGAGCTGCAG-3′] [21]

Table 1 Baseline characteristics of AIH-I patients (n = 80)

		AIH-1
Biochemical characteristics		
Aspartate aminotransferase (AST; U/L)		230 (57 –960)
Alanine aminotransferase (ALT; U/L)		230 (17 –825)
Alkaline phosphatase (ALP; U/L)		200 (55–613)
Gamma glutamyl transferase (GGT; U/L)		68 (22–100)
Prothrombin time (PT; sec)		16.3 (12–28)
Activated partial thromboplastin time (aPTT; sec)		44 (26–55)
International normalized ratio (INR)		1.6 (1–3.4)
Serum total bilirubin (μmol/L)		51.3 (5.13–353.97)
Serum direct bilirubin (μmol/L)		34.2 (1.71 –263.34)
Serum albumin (g/L)		32 (20–44)
Serum total proteins (g/L)		80 (48–100)
Serum total immunoglobulin G (IgG; μmol/L)[a]		220.15 (60.37–375.32)
Histopathological characteristics		
Infiltrating inflammatory cells	Eosinophil	8 (10)
	Lymphocytes	16 (45)
	Plasma cells	66 (82)
Hepatocytes and portal tracts changes	Ballooning and degeneration of hepatocytes	4 (5)
	Rosettes formation	16 (20)
	interface hepatitis (piecemeal necrosis and bridging)	64 (80)
Hepatitis activity index (HAI)		7 (1–15)
Degree of fibrosis		3 (0–5)

Data are given either median (range) or number (%)
[a]Normal value for serum total IgG 53.37–120.08 μmol/L

original scoring system of the IAIHG compared to simplified scoring system was 65% vs. 70%, respectively, and probable diagnosis of AIH was 35% vs. 30%, respectively. According to Child-Pugh classification, 23 (28.8%) of studied patients were class A, 49 (61.2%) class B, and 8 (10%) were class C. All our patients received steroid as an initial therapy (prednisolone 2 mg/kg/day; maximum 60 mg/day that was gradually tapered by 5 mg every 1–2 weeks based on the clinical symptoms, AST and ALT levels with 10 mg prednisolone for 2 years after improvement as a maintenance dose), and azathioprine (1–2 mg/kg/day; maximum 100 mg/day) was added to 54 patients who showed an increased AST or ALT levels during tapering steroid or who had significant side effects that necessitate reducing the dose of steroid (like severe cosmetic changes, duodenal ulcer, hypertension or corticosteroid-related osteopenia detected by bone densitometry). Complete remission (which is the normalization of liver enzymes and absence of clinical symptoms; normal ALT on a minimum of two occasions at least a month apart) was observed in 46 (57.5%) patients, and 34 (42.5%) patients had relapses (in which at least two-fold increase in AST or ALT in isolation or in combination with histological evidence of disease activity).

KIR frequencies in AIH-1 patients and controls

Both KIR2DS1 and 2DS4 genes were found to be significantly associated with the risk of development of AIH-I (85% in patients vs. 30% in controls; $P < 0.001$, OR 13.22 and 92.5% vs. 62.5%; $P < 0.001$, OR 7.4 respectively). As regards the KIR2DS4 alleles, KIR2DS4-full length allele was significantly detected in 75.7% of patients, whereas the KIR2DS4 22 bp deletion allele (KIR1D) was significantly observed in 80% of controls ($P = 0.002$, OR 12.44). The most significantly frequent KIR2DS4 variant in AIH-I patients was homozygous full length variant (KIR2DS4-full/full) (45.94%), while the homozygous deleted variant (KIR2DS4-del/del) was predominant among controls (68%) ($P = 0.003$) (Table 2). Studying KIR haplotypes revealed that the KIR Bx haplotype was more frequent in AIH-I patients (85%), while the KIR AA haplotype was more frequent in controls (70%) ($P < 0.001$, OR 13.22) (Table 2).

By studying the association between KIR2DS1 and KIR2DS4 genes with some disease characteristics in AIH-I patients, we observed that lacking the KIR2DS4 gene was significantly associated with positive ANA ($P < 0.001$), patients treated with steroid alone ($P = 0.008$), and patients had a complete remission ($P = 0.036$). We

Table 2 KIR frequencies in type I AIH and healthy individuals

KIR genes		AIH-I	Controls	*P*	OR (95% CI)
		(*n* = 80)	(*n* = 80)		
KIR2DS1	+	68 (85)	24 (30)	< 0.001	13.22 (6.07–28.79)
	–	12 (15)	56 (70)		
KIR2DS4	+	74 (92.5)	50 (62.5)	< 0.001	7.4 (2.87–19.08)
	–	6 (7.5)	30 (37.5)		
		(*n* = 74)	(*n* = 50)		
KIR2DS4 alleles					
KIR2DS4-FL		56 (75.7)	10 (20)	0.002	12.44 (5.2–29.79)
KIR1D		18 (24.3)	40 (80)		
KIR2DS4 variants					
Homozygous (full/full)		34 (45.94)	6 (12)	0.003	10.7 (3.79–30.25)
Heterozygous (full/del)		22 (29.73)	10 (20)		4.16 (1.62–10.65)
Homozygous (del/del)		18 (24.33)	34 (68)		R
KIR haplotypes		(*n* = 80)	(*n* = 80)		
KIR AA		12 (15)	56 (70)	< 0.001	13.22 (6.07–28.79)
KIR Bx (KIR AB and BB)		68 (85)	24 (30)		

Data are given as number (%)
+, present gene; –, absent gene; KIR1D, 22 bp deletion variant of KIR2DS4-full length; R, reference genotype

found that lacking both KIR2DS1 and KIR2DS4 genes were significantly associated with higher hepatitis activity index (HAI) (P = 0.006 and 0.012 respectively). Meanwhile, the absence of KIR2DS4 gene was significantly associated with higher degree of fibrosis (P = 0.043) (Table 3).

Discussion

AIH is a serious autoimmune liver disease affecting both children and adults with a female predominance and more aggressive course among children [2]. Despite extensive studies trying to approach AIH pathogenesis, the exact mechanisms are still unknown [23]. Many studies

Table 3 Comparison of studied KIR activating genes and some disease characteristics in AIH-I patients

	KIR2DS1			KIR2DS4		
	+ (*N* = 68)	– (*N* = 12)	*P*	+ (*N* = 74)	– (*N* = 6)	*P*
Anti-nuclear antibodies (ANA)						
Negative	52 (76.5)	12 (100)	0.32	64 (86.5)	0	< 0.001
Positive	16 (23.5)	0		10 (13.5)	6 (100)	
Anti-smooth muscle antibodies (ASMA)						
Negative	10 (14.7)	2 (16.7)	1.00	12 (16.2)	0	1.00
Positive	58 (85.3)	10 (83.3)		62 (83.8)	6 (100)	
Treatment regimen						
Steroid	22 (32.3)	4.(33.3)	1.00	20 (27)	6 (100)	0.008
Steroid and azathioprine	46 (67.7)	8 (66.7)		54 (73)	0	
Treatment response						
Complete remission	40 (58.8)	6 (50)	1.00	40 (54)	6 (100)	0.036
Relapse	28 (41.2)	6 (50)		34 (46)	0	
Hepatitis Activity Index (HAI)						
	7.06 ± 2.89	9.83 ± 4.44	0.006	7.22 ± 3.21	10.67 ± 2.31	0.012
Degree of fibrosis						
	2.94 ± 1.01	2.67 ± 0.52	0.369	2.84 ± 0.9	3.67 ± 1.53	0.043

Data are given either number (%) or mean ± SD
+, present gene; –, absent gene

have focused on the adaptive immune response mechanisms and tissue damage by CD4+ T helper and CD8+ T cytotoxic cells. The role of NK cells in AIH is not fully clarified, although NK cells might be attacking directly the liver parenchyma or contributing to damage through cytokine secretion and/or cell to cell contact [24]. We investigated the possible association between genetic variants of killer cell immunoglobulin-like receptors (KIR2DS1 and 2DS4) and the susceptibility to AIH-I in children. Previous studies have investigated these polymorphisms with several diseases, especially autoimmune diseases, but few researches have been carried out to determine these associations with pediatric onset AIH-I. KIR activating genes (KIR2DS1 and KIR2DS4) are the extensively studied KIR genes as they have been shown to be crucial in human immune response mechanisms. High frequencies of these genes have been described in various autoimmune disorders [15, 16].

In our study, we noticed that the KIR2DS1 and KIR2DS4 genes were significantly associated in with AIH-I patients than controls with 13.22- and 7.4-fold of increased risk to develop the disease. The frequencies of KIR2DS4-full length allele (KIR2DS4-FL) and KIR2DS4 22 bp deletion allele (KIR1D) were significantly higher in AIH-I patients and controls, respectively. Furthermore, the homozygous variant (KIR2DS4-full/full) predominate significantly in patients compared to the homozygous variant (KIR2DS4-del/del) among controls. The KIR Bx haplotype had a significant higher frequency in AIH-I patients with a 13.22-fold increased risk to develop AIH-I, while the KIR AA haplotype showed higher frequency among controls.

These findings were in agreement with Littera et al. [15], who found that the frequency of the activating KIR2DS1 gene in patients compared to controls was 57% vs. 43.9% [P = 0.028, OR 1.69 (95% CI 1.05–2.75)], but they recognized similar distribution of KIR AA and KIR Bx haplotypes between the AIH and control groups. These results support the hypothesis of the direct contribution of KIR2DS1-positive NK cells in AIH-I pathogenesis through shifting NK-cell receptor signaling towards the activating arm. Thus, the observed predictive potential of activating KIR gene KIR2DS1 makes it a suitable genetic biomarker for early onset AIH-I.

Our results were also in line with Podhorzer et al. [16], who reported higher frequency of the full length allele of KIR2DS4 (KIR2DS4-FL) in pediatric AIH (PAH). The KIR2DS4-FL allele was detected in 42% of the Argentinean Caucasian population carrying the KIR2DS4 gene. They reported the presence of KIR2DS4-FL in 70% of PAH patients with the homozygous KIR2DS4-FL that was significantly detected in PAH patients than controls (33% vs. 18%), while they found that homozygous KIR1D (KIR2DS4-del/del) was associated with a protective effect (P 0.0001, OR = 0.3, 95% CI 0.2–0.5). Moreover, Flores et al. [25] showed that the functional alleles of KIR2DS4 predominated in pediatric AIH patients while the KIR1D was common in most healthy Caucasian populations studied which support its protective effect against autoimmune hepatitis. Several studies have found an association between KIR activation genes and autoimmune diseases susceptibility. For instance, KIR2DS1 has been reported to be associated with psoriasis, systemic lupus erythematosus, and scleroderma [26–30].

Although KIR A haplotypes appear to provide a better immune response against viral infections and tumor cells, while KIR B haplotypes seem to have a favorable role during pregnancy [31], we found the homozygous KIR AA haplotype more associated with controls, whereas the KIR Bx haplotype was associated with AIH-1 patients. A possible explanation may be that the gene content of KIR A haplotypes with their highly polymorphic receptors provides a better immune surveillance against tumor cells, in comparison to the moderate polymorphism of the KIR B haplotypes. Such controversial role of homozygosity for KIR A haplotype in the immune system has been addressed previously [32, 33].

In the current work, we could detect an association between the absence of KIR2DS4 gene in AIH-I patients with positive ANA, patients on steroid therapy alone, and patients who showed complete remission, although, unexpectedly, a higher hepatitis activity index (HAI) was significant among patients lacking KIR2DS1 and KIR2DS4 genes and a higher degree of fibrosis in KIR2DS4 negative patients.

Conclusions

From this pilot study, we could conclude that children carrying KIR2DS1, -2DS4 genes, KIR2DS4-full length allele, and homozygous KIR2DS4-full/full variant may be more susceptible to develop AIH-I. Further big scale studies are required to confirm or disapprove our claim. Therefore, in the way to precision medicine, studying these genetic polymorphisms might help in tailoring management protocols and predicting outcomes in order to improve patients' survival and quality of life.

Abbreviations

AIH: Autoimmune hepatitis; AMA: Anti-mitochondrial antibody; ANA: Anti-nuclear antibody; ASMA: Anti-smooth muscle antibody; KIR: Killer cell immunoglobulin-like receptor; LKM-1: Liver-kidney microsomal antibody type 1; NK: Natural killer cells

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Authors' contributions
All authors have contributed to the current work. O.B. contributed substantially in the work idea, study design, data acquisition, analysis, and interpretation. O.E. and S.B. performed the laboratory experiments, analysis, and interpretation. N.S. performed the pathological experiments, analysis, and interpretation. O.E. and O.B. drafted and revised the manuscript. All the authors have read and approved the manuscript.

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Availability of data and materials
The authors declare the availability of all data and materials for the submitted work upon request.

Declarations

Ethics approval and consent to participate
This study was approved by the local ethical committee for research involving human subjects in Faculty of Medicine—Benha University (the approval number is not available). The study was carried out according to the guidelines of the Declaration of Helsinki. Ethical permission, and written consents were obtained from parents/guardians after being fully informed about the study and its procedures.

Consent for publication
Not applicable.

Competing interests
The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author details
[1]Pediatrics Department, Faculty of Medicine, Benha University, Benha, Egypt. [2]Clinical and Chemical Pathology Department, Faculty of Medicine, Benha University, Benha 13511, Egypt. [3]Pathology Department, Faculty of Medicine, Benha University, Benha, Egypt. [4]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Benha University, Benha, Egypt.

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