Polymorphisms in the cytotoxic T lymphocyte-associated protein-4 immune regulatory gene and their impact on inhibitor development in patients with hemophilia A

Aveen M. Raouf Abdulqader¹, Ali Ibrahim Mohammed¹ and Shwan Rachid²

Abstract
Objective: The development of inhibitors against infused factor VIII represents the most severe complication of substitution therapy in hemophilia A (HA) patients. Data on risk factors for inhibitor formation in Iraqi Kurdish patients with HA are unavailable. This study aimed to evaluate the impact of two single nucleotide polymorphisms (SNPs) in an immune regulatory gene in the emergence of inhibitors.

Methods: We focused on 126 patients with either severe or mild/moderate HA presenting with and without inhibitors. We analyzed the frequency of two polymorphisms in the cytotoxic T lymphocyte-associated protein-4 gene (CTLA-4; CTLA-4-318C>T and CTLA-4+49A>G). Genotyping was performed using restriction fragment length polymorphism–PCR and direct sequencing.

Results: We found no significant correlation between the CTLA-4-318 C>T T allele and inhibitor development among patients with severe or mild/moderate HA. However, a significantly high inhibitor risk was detected for the CTLA-4+49 A>G G allele (odds ratio [OR] = 3.1, 95% confidence interval [CI] = 1.383–7.024) and (OR = 4, 95% CI = 1.719–9.437) among patients with severe and mild/moderate HA, respectively.

Conclusion: We conclude that the CTLA-4+49 A>G SNP plays a substantial role as a potential risk determinant for inhibitor formation in Iraqi Kurdish patients with HA.
Introduction

Hemophilia A (HA) is an X-linked recessive bleeding disorder caused by a quantitative or qualitative deficiency in the factor VIII (FVIII) protein. HA is treated by replacing the deficient FVIII protein, but this therapy is often ineffective following the manifestation of neutralizing antibodies (inhibitors) against the infused FVIII protein; this is therefore the most burdensome complication of hemophilia management. In unselected patients with hemophilia, the prevalence of inhibitors is 5% to 7%, while its incidence is 25% to 35% in patients with severe disease and 3% to 13% in patients with mild/moderate disease.

As a typical multifactorial trait, risk factors for inhibitor formation in HA patients are classified into two main groups: modifiable (environmental factors) and non-modifiable (genetic factors). Environmental risk factors include treatment-related factors and immune system challenges. The main genetic predisposition for inhibitor development in HA patients is the causative FVIII genotype, but it also includes a group of auxiliary risk factors that are weaker than the FVIII genotype such as a family history of inhibitors, human leucocyte antigen haplotype, and polymorphisms of immune system-related genes including interleukin-10, tumor necrosis factor-α, and cytotoxic T lymphocyte-associated protein-4 (CTLA-4 on chromosome 2q33).

Human genetic data support the hypothesis that predisposing factors associated with autosomal genes, including those mentioned above, are ethnically divergent and thus not constant among populations worldwide.

HA is caused by several known gene defects, which accounts for the heterogeneity of disease phenotypes and inhibitor production. Patients with severe molecular defects (e.g. large deletions, inversions, and nonsense mutations) that result in the complete lack of the clotting protein appear to have a higher propensity to develop inhibitors than those with milder defects (e.g. missense and splice site mutations) in which some remnant FVIII antigen is present. Nevertheless, the discordance in inhibitor production observed in patients or siblings with similar mutations indicates that other genetic factors potentially function as modifiers.

The production of inhibitors to the infused FVIII protein is mediated by a T helper (T_H) cell-dependent process that also incorporates antigen-presenting cells (APCs) and B lymphocytes. Major histocompatibility complex class II molecules expressed on APCs present peptides of the infused factor to the T cell receptor expressed on T_H cells. However, a second co-stimulatory signal is needed to completely evoke the immune response. This signal is produced by the interaction between B7 (CD80/86) molecules on APCs and CD28 on T_H cells. CTLA-4 is a receptor primarily expressed on activated T cells, which competes with CD28 for the interaction with B7 molecules, leading to a decrease in T cell activity. Accordingly, blockade of this
interaction by CTLA-4-antibodies enhances T cell proliferation and B cell activity.\textsuperscript{15,16}

Two single nucleotide polymorphisms (SNPs) in \textit{CTLA-4} (CTLA-4-318 C \textgreater T in the promoter region and CTLA-4+49 A \textgreater G in coding sequence 1 encoding a threonine to alanine substitution in the leader peptide) have been found to activate the immune response in patients with antibody-mediated autoimmune diseases such as Graves’ disease, systemic lupus erythematosus, Hashimoto’s thyroiditis, Wegener’s granulomatosis, and multiple sclerosis.\textsuperscript{17–21} Furthermore, these two polymorphisms have also been shown to modify the propensity of HA patients to produce inhibitors.\textsuperscript{9,12}

Of the 5.7 million Iraqi Kurds, approximately 450 registered patients with HA have been identified. The frequency of patients with severe, moderate, and mild HA is 35.6\%, 51.1\%, and 13.3\%, respectively. The current study aimed to evaluate whether the two SNPs also influence the risk of inhibitor development in a case–controlled study of 126 Iraqi Kurd patients subdivided into those with severe HA and those with mild/moderate HA presenting with and without a history of inhibitor development.

\section*{Patients and methods}

This study was conducted according to the principles of the Declaration of Helsinki and with the approval of the local institutional ethical committee (College of Medicine, University of Sulaemaniyah approval no. 55; September 7 2017). All patients with hemophilia A (HA) are registered in local hemophilia treatment centers belonging to the Iraqi Society of Hemophilia, and written informed consent was obtained from all participants. Patients were diagnosed with HA according to World Federation of Hemophilia guidelines.\textsuperscript{4} All patients exhibited a prolonged activated partial thromboplastin time (aPTT) and reduced FVIII activity.

We performed a case–control study of inhibitor risk associated with two SNPs (CTLA-4-318 C \textgreater T and CTLA-4+49 A \textgreater G). One hundred twenty-six patients with HA, including 35 inhibitor-positive and 91 inhibitor-negative control patients, were included in the study and were subdivided into those with severe HA (n=60 cases; 20 with and 40 without inhibitors) and those with mild/moderate HA (n=66; 15 with and 51 without inhibitors). According to standard International Society on Thrombosis and Hemostasis definitions, patients were considered to express relevant inhibitors when they were documented on two separate occasions within a 1- to 4-week period and had a level of $\geq$0.6 Bethesda units (BU) per mL using the Nijmegen modification of the Bethesda assay.\textsuperscript{22} High-response inhibitors represent patients with an inhibitor titer $\geq$5 BU/mL at any time point, and low-response inhibitors were patients who persistently presented an inhibitor titer $<$5 BU/mL despite repeated challenge with FVIII replacement therapy.\textsuperscript{23} Clinical data included relevant patient information such as age, gender, ethnicity, age at first exposure to FVIII (recombinant or other blood products), and number of exposure days (ED).

\section*{FVIII level and inhibitor detection}

Blood was collected in tubes containing 3.18\% trisodium citrate at a 9:1 volumetric ratio. FVIII activity (FVIII:C) was determined using an aPTT-based one-stage clotting assay with an aPTT reagent sensitive to coagulation factor deficiency (\textit{STA}-C.K. PREST 5, \textit{STA}compact Max; Diagnostica Stago, Asnières sur seine, France) and FVIII-deficient plasma (\textit{STA}®-Deficient VIII, \textit{STA}compact Max; Diagnostica Stago) performed with the Stago (\textit{STA}compact Max) fully automated blood coagulation
analyzer which was calibrated and controlled according to the manufacturer’s instructions. The aPTT mixing study was performed to differentiate between a coagulation factor deficiency and the presence of inhibitors. Time-dependent inhibitors were assessed by measuring the aPTT of a mixture composed of one part patient plasma and one part normal plasma after incubation for 2 hours at 37°C. Time-independent inhibitors were determined by measuring the aPTT of an immediate mixture of patient and normal plasma that were incubated separately. The Nijmegen modification of the Bethesda assay, which is applied for more specific antibody detection in the lower range (cut-off point: 0.6 BU/mL) by virtue of the dilution of patient plasma with buffered normal pooled plasma, was performed to detect antibodies against FVIII. Normal pooled plasma was used as a negative control.24

**DNA extraction and genetic analysis**

High molecular weight genomic DNA was extracted from peripheral blood leucocytes anti-coagulated in K2EDTA using a salting-out procedure.25 The biallelic polymorphism CTLA-4-318 C > T (rs5742909) was analyzed using restriction fragment length polymorphism–PCR with the forward primer 5′-AAATGAATTGGACTG GATGGT-3′ and the reverse primer 5′-TTACGAGAAAGGAAGCGTG-3′ as described in previous publications.9,26 Amplification was performed in a final volume of 25 µl with 0.1 to 0.4 µg of genomic DNA, 0.4 µM of each primer (SinaClon Company, Tehran, Iran), 0.2 mM of each dNTP (Gen Fanavaran Co., Tehran, Iran), 1.5 U Super Taq DNA polymerase (Gen Fanavaran Co.), 0.8× PCR buffer, 2 µl dimethyl sulfoxide ≥99.9% (Sigma-Aldrich, St Louis, MO, USA), and 1.4 mM MgCl2 (Gen Fanavaran Co.). The PCR program was set to an initial denaturation of 94°C for 5 minutes, followed by 28 cycles of 1 minute denaturation at 94°C, 1 minute of annealing at 60°C, 1 minute of elongation at 72°C, and a final 10-minute extension at 72°C. The 247-bp PCR product (in 7 µl) was digested with 10 U Mse I restriction enzyme (Thermo Fisher Scientific Inc., Rockford, IL, USA) and 1× digestion buffer R at 65°C for 3 hours in a total volume of 25 µl. The digested PCR products were resolved on a 4% agarose gel. After Mse I digestion, the T allele produces two fragments (131 bp and 116 bp) while the C allele produces the 247 bp undigested fragment. CTLA-4-318 C > T was also analyzed by direct sequencing of the amplified PCR product.

The dimorphism in the CTLA-4+49 A > G allele (rs 231775) was determined by PCR amplification of genomic DNA using the in-house designed forward primer 5′-GTGTAATACATATCTGGGATCAA AGC-3′ and reverse primer 5′-CCC AGGTAGGAG AACACCTC-3′. The amplification mixture and PCR conditions were identical to those used to identify the previous polymorphism. CTLA-4+49 A > G was then detected by direct sequencing of the 300-bp PCR product. Amplified fragments were sequenced commercially using an ABI 3130 XL sequencer (Applied Biosystems, Foster City, CA, USA). The FinchTV sequence analysis software package (Geospiza Inc., Seattle, WA, USA) was used for sequence reading and analysis.

**Statistical analysis**

Statistical package IBM SPSS version 23 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Continuous data are presented as medians, means ± SD, and ranges. The Chi-squared test was used to compare differences in the following categorical data: genotypes, alleles, and phenotype frequencies between inhibitor-positive and inhibitor-negative patients. All


Results

Patient characteristics

A cohort of 126 male patients with HA from 88 unrelated families were enrolled in this study. The median age of all patients with inhibitors was 14 years (range, 5–30 years), while the median age in patients without inhibitors was 19 years (range, 5–51 years). Disease severity was determined by measuring the FVIII clotting activity. Of the 126 patients, 60 were diagnosed with severe HA (47.6%), 48 with moderate HA (38.1%), and 18 with mild HA (14.3%). Thirty-five patients were inhibitor-positive, of whom 80% (28/35) had a high titer of inhibitors (≥5 BU/mL) and 20% (7/35) had a low titer (<5 BU/mL). The frequency of inhibitors in patients with severe, moderate, and mild HA was 33.3% (20/60), 27% (13/48), and 11.1% (2/18), respectively. Patients exhibited a mean antibody titer of 67.27 BU/mL (range, 3–825 BU/mL).

The characteristics of patients with respect to inhibitors and the severity of HA are listed in Table 1. Among patients with severe HA, the mean age of inhibitor-positive patients was 15 ± 8 years (range, 5–30 years) and the mean age of inhibitor-negative controls was 22 ± 8 years (range, 5–38 years). Among patients with severe HA, 30% and 27.5% with and without inhibitors, respectively, received recombinant FVIII or other blood products before 6 months of age. The mean number of EDs among inhibitor-positive patients was 58 ± 27 SD (range, 9–120), while that in inhibitor-negative patients was 188 ± 82 (range, 35–450) (Table 2). The mean ages of inhibitor-positive and inhibitor-negative patients with mild/moderate HA were 16 ± 7 years (range, 6–28 years) and 18 ± 11 years (range, 5–51 years), respectively. Among patients with and without inhibitors, 13.3% and 15.7%, respectively, received FVIII before 6 months of age, while the mean ED was 68 ± 42 (range, 10–140) and 84 ± 42 (range, 30–200), respectively (Table 2).

Overall, our patients had no history of surgery. Further, apart from four patients with moderate HA who developed inhibitors after major gastrointestinal bleeds that required intensive treatment, the remaining patients suffered from joint and mucocutaneous bleeds or received prophylaxis therapy.

CTLA-4 genotype distribution

CTLA-4-318 C/T and CTLA-4+49 A/G polymorphisms were analyzed in 60 patients with severe HA (20 with and 40 without

Table 1. Characteristics of the study group with respect to inhibitor status

| Type of Hemophilia A | Mean FVIII level % | HR | LR | No Inhibitors | Total N |
|---------------------|--------------------|----|----|---------------|---------|
| Severe (<1%)        | 0.8                | 18 (30) | 2 (3.3) | 40 (66.7) | 60 |
| Moderate (1%–5%)    | 2.1                | 8 (16.6) | 5 (10.4) | 35 (73) | 48 |
| Mild (6%–30%)       | 13.6               | 2 (11.1) | 0 (0) | 16 (88.9) | 18 |
| Total N (%)         | –                  | 28 (22.2) | 7 (5.6) | 91 (72.2) | 126 |

HR: high responder; LR: low responder
inhibitors) and 66 patients with mild/moderate HA (15 with and 51 without inhibitors). No significant association was detected between a C/T substitution at position –318 in the promoter region and inhibitor development among both groups. Among patients with severe HA, the T allele (homozygous TT or heterozygous CT) was found in 50% of inhibitor-negative patients and in 35% of inhibitor-positive patients (Table 3). Thirteen (65%) patients with inhibitors were homozygous for the C allele (CC), three (15%) were homozygous for the T allele (TT), and four (20%) were heterozygous (CT), compared with 20 (50%), 10 (25%), and 10 (25%), respectively, of the control inhibitor-negative patients (Table 4). Total allele frequencies were 37.5% for the T allele and 62.5% for the C allele in the inhibitor-negative group compared with 25% and 75%, respectively, in the inhibitor-positive group. Based on our findings, there was no significant difference in the frequency of the T-positive phenotype in inhibitor-negative patients (50%) compared with inhibitor-positive patients (35%) (Table 4). Similarly, no significant correlation between CTLA-4-318 C/T SNP and inhibitor development was observed among patients with mild/moderate HA (Table 4).

Analysis of CTLA-4+49 A/G revealed that this polymorphism appeared at a significantly higher frequency in inhibitor-positive patients among both groups (severe and mild/moderate HA). A significantly higher inhibitor risk association was observed for the G allele. The frequency of the G allele was 47.5% in inhibitor-positive patients with severe HA while the frequency of the A allele was 52.5%, compared with 22.5% and 77.5%, respectively, in inhibitor-negative patients, corresponding to an OR of 3.1 (95% CI = 1.383–7.024, \(P = 0.005\) (Table 5). This significant association was persistent after considering the combination of genotypes, i.e., GG and AG, with a dominant effect revealing an OR of 3.5 (95% CI = 1.112–11.017, \(P = 0.028\) (Table 3). Five (25%) inhibitor-

| Table 2. Characteristics of the study group with respect to age, age at first exposure to FVIII, and exposure days |
|-----------------|------------------|------------------|------------------|
|                 | Mild/Moderate (n = 66) | Severe (n = 60) |
|                 | Inhibitor-positive (n = 15) | Inhibitor-negative (n = 51) | Inhibitor-positive (n = 20) | Inhibitor-negative (n = 40) |
| Age (years) Mean (±SD) | 16 (±7) | 18 (±11) | 15 (±8) | 22 (±8) |
| Range | 6–28 | 5–51 | 5–30 | 5–38 |
| Age at first exposure to FVIII N (%): <6 months | 2 (13.3) | 8 (15.7) | 6 (30) | 11 (27.5) |
| ≥6 months | 13 (86.7) | 43 (84.3) | 14 (70) | 29 (72.5) |
| Mean months (±SD) | 29 (±33) | 50 (±62) | 18 (±15) | 35 (±47) |
| Exposure days N (%): <150 | 15 (100) | 45 (88.2) | 20 (100) | 12 (30) |
| ≥150 | 0 (0) | 6 (11.8) | 0 (0) | 28 (70) |
| Mean exposure days (±SD) | 68 (±42) | 84 (±42) | 58 (±27) | 188 (±82) |
| Range | 10–140 | 30–200 | 9–120 | 35–450 |

SD, standard deviation.
Table 3. Relationship between CTLA-4 and inhibitor development based on division into CC, AA, and combined (CT, TT) and (AG, GG) genotypes

| CTLA-4 polymorphisms | Mild/Moderate (n = 66) | Severe (n = 60) |
|----------------------|------------------------|-----------------|
|                      | Inhibitor-positive (n = 15) | Inhibitor-negative (n = 51) | Inhibitor-positive (n = 20) | Inhibitor-negative (n = 40) |
| –318 C/T             |                        |                              |                            |                             |
| Genotype frequencies |                        |                              |                            |                             |
| CC                   | 10                     | 66.7                         | 37                          | 72.6                        |
| CT and TT            | 5                      | 33.3                         | 14                          | 27.4                        |
| +49 A/G              |                        |                              |                            |                             |
| Genotype frequencies |                        |                              |                            |                             |
| AA                   | 5                      | 33.3                         | 32                          | 62.7                        |
| AG and GG            | 10                     | 66.7                         | 19                          | 37.3                        |

aDifference between inhibitor-positive and -negative in mild/moderate group: OR = 1.3 (95% CI = 0.383–4.554).
bDifference between inhibitor-positive and -negative in severe group: OR = 0.5 (95% CI = 0.178–1.631).
cDifference between inhibitor-positive and -negative in mild/moderate group: OR = 3.4 (95% CI = 1–11.345, \(P = 0.044\)).
dDifference between inhibitor-positive and -negative in severe group: OR = 3.5 (95% CI = 1.112–11.017, \(P = 0.028\)).

Table 4. Genotype, allele, and phenotype frequencies of CTLA-4-318 C/T in Kurdish hemophilia A patients with and without inhibitors

| CTLA-4 polymorphisms | Mild/Moderate (n = 66) | Severe (n = 60) |
|----------------------|------------------------|-----------------|
|                      | Inhibitor-positive (n = 15) | Inhibitor-negative (n = 51) | Inhibitor-positive (n = 20) | Inhibitor-negative (n = 40) |
| –318 C/T             |                        |                              |                            |                             |
| Genotype frequencies |                        |                              |                            |                             |
| CC                   | 10                     | 66.6                         | 37                          | 72.6                        |
| TT                   | 3                      | 20                           | 10                          | 19.6                        |
| CT                   | 2                      | 13.4                         | 4                           | 7.8                         |
| Allele frequencies   |                        |                              |                            |                             |
| C                    | 22                     | 73.3                         | 78                          | 76.5                        |
| T                    | 8                      | 26.7                         | 24                          | 23.5                        |
| Phenotype frequencies|                        |                              |                            |                             |
| C-positive           | 12                     | 80                           | 41                          | 80.4                        |
| T-positive           | 5                      | 33.3                         | 14                          | 27.5                        |

aDifference between inhibitor-positive and -negative in mild/moderate group.
bDifference between inhibitor-positive and -negative in severe group.
cDifference between inhibitor-positive and -negative in mild/moderate group: OR = 1.2 (95% CI = 0.466–2.994).
dDifference between inhibitor-positive and -negative in severe group: OR = 0.5 (95% CI = 0.238–1.296).
eDifference between inhibitor-positive and -negative in mild/moderate group: OR = 1.2 (95% CI = 0.365–4.079).
fDifference between inhibitor-positive and -negative in severe group: OR = 0.6 (95% CI = 0.217–1.759).
positive patients were homozygous for the G allele (GG), nine (45%) were heterozygous (AG), and six (30%) were homozygous for the A allele (AA) compared with two (5%), 14 (35%), and 24 (60%), respectively, of inhibitor-negative patients ($P = 0.026$) (Table 5). Although not significant, the frequency of the G-positive phenotype was higher in patients with inhibitors (70%) than in those without inhibitors (40%), with an OR of 2.2 (95% CI = 0.871–5.639) (Table 5). A similar trend in the correlation between CTLA-4 +49 A/G and inhibitor development was observed among patients with mild/moderate HA as shown in Table 3 and Table 5.

**Discussion**

The ability to anticipate which HA patients have the potential to develop inhibitors and to recognize factors that lead to inhibitor formation would allow the application of appropriate therapies to avoid the inhibitor response. In the current study, we focused on patients with severe and mild/moderate HA, each presenting with and without inhibitors to assess the pertinence of two CTLA-4 polymorphisms in the risk of developing FVIII inhibitors.

CTLA-4 is a surface molecule expressed on activated T cells that plays a crucial role as a negative regulator of T cell activation.\(^{15,16,27}\) The CTLA-4-318 C/T SNP in the promoter region –318 bp from the ATG start codon is associated with increased promoter activity, increased protein expression, a negative effect on the immune response, and hence a lower risk of inhibitor formation.\(^{28,29}\) In the present study, we found no significant protective correlation between inhibitor formation

### Table 5. Genotype, allele, and phenotype frequencies of CTLA-4 +49 A/G in Kurdish hemophilia A patients with and without inhibitors

|             | Mild/Moderate (n=66) | Severe (n=60) |
|-------------|---------------------|---------------|
|             | Inhibitor-positive  | Inhibitor-negative |
|             | (n=15)            | (n=51)        |
|             | (n=20)            | (n=40)        |
| N           | %                  | N             | %            | N            | %    |
| AA          | 5                  | 33.3          | 32           | 62.7         | 6    | 30   | 24  | 60  |
| GG          | 7                  | 46.7          | 6            | 11.8         | 5    | 25   | 2   | 5   |
| AG          | 3                  | 20            | 13           | 25.5         | 9    | 45   | 14  | 35  |
| A           | 13                 | 43.3          | 77           | 75.5         | 21   | 52.5 | 62  | 77.5|
| G           | 17                 | 56.7          | 25           | 24.5         | 19   | 47.5 | 18  | 22.5|
| A-positive  | 8                  | 53.3          | 45           | 88.2         | 15   | 75   | 38  | 95  |
| G-positive  | 10                 | 66.7          | 19           | 37.3         | 14   | 70   | 16  | 40  |

\(^a\)Difference between inhibitor-positive and -negative in mild/moderate group: $P = 0.011$.

\(^b\)Difference between inhibitor-positive and -negative in severe group: $P = 0.026$.

\(^c\)Difference between inhibitor-positive and -negative in mild/moderate group: OR = 4 (95% CI = 1.719–9.437, $P = 0.001$).

\(^d\)Difference between inhibitor-positive and -negative in severe group: OR = 3.1 (95% CI = 1.383–7.024, $P = 0.005$).

\(^e\)Difference between inhibitor-positive and -negative in mild/moderate group: OR = 3 (95% CI = 1.012–8.659, $P = 0.043$).

\(^f\)Difference between inhibitor-positive and -negative in severe group: OR = 2.2 (95% CI = 0.871–5.639).
and the –318 T allele in patients with either severe or mild/moderate HA, similar to the findings reported in an Indian study, a Chinese cohort, and a Brazilian study, but in contrast to MIBS and Argentinean cohorts that identified a substantial protective correlation between CTLA-4-318 C/T and inhibitor formation. It is noteworthy that most previous reports included only patients with severe HA. The current study further investigated the correlation between CTLA-4 and inhibitor development in a new cohort of patients with mild/moderate HA and, to our knowledge, represents the first such study on this group of patients.

Previous *in vitro* studies showed that CTLA-4 + 49 A > G at position +49 in coding sequence 1 produces a missense variant that results in a threonine to alanine exchange (p.Thr17Ala) in the leader peptide. This causes incomplete glycosylation in the endoplasmic reticulum, eventually leading to a decreased surface/total ratio of the protein that might affect its function. The inhibitory effect of the CTLA-4 protein on activated T cells is less potent in subjects carrying the G allele than in those carrying the A allele. Moreover, this A > G SNP has been linked to susceptibility to a number of antibody-mediated autoimmune diseases. Additionally, an increased frequency of the CTLA-4 + 49 G allele was observed in patients with acquired HA compared with healthy controls. In this series, a significantly higher inhibitor risk was observed for patients carrying the +49 G allele in patients with both severe and mild/moderate HA, which is consistent with the Argentinian cohort study. A similar but non-significant trend was obtained in a study of North European patients with HA, showing an OR of 2.2 (95% CI = 0.6–7.8) for the presence of the G allele among patients with severe HA and inhibitors. However, this association was not established in other populations, including a Brazilian study, a Chinese cohort, and a group of Indian patients with HA, and a series of Italian patients. Pavlova *et al.* also reported the lack of a significant difference with regard to analysis of the +49 A > G SNP between patients with severe HA presenting with and without inhibitors.

Currently endorsed treatments for HA patients presenting with inhibitors are substandard, so these patients have worse outcomes than those without inhibitors. Therefore, a major focus of HA research has been to determine risk factors for inhibitor development and optimal management strategies to decrease inhibitor risk. Because of the low level of resources and economic issues in our developing country, the prevalence of mutations established as major determinants of inhibitor development in HA patients were not analyzed in the current cohort. Nevertheless, our study is important because no published report has yet defined the unmodifiable risk factors associated with inhibitor formation in the Iraqi Kurdish population. Moreover, secondary genetic factors inducing inhibitor development in HA patients (e.g., immune gene polymorphisms) from diverse regions and ethnicities show significant differences worldwide. These divergent findings were clarified in massive international studies, such as the Hemophilia Inhibitor Genetics study and other regional studies.

**Conclusion**

This study highlighted the association between CTLA-4 + 49 A > G and inhibitor formation in HA patients, justifying the need for an analysis of modifying risk factors for inhibitor development. Improved knowledge about such risk factors would allow researchers to develop regionally relevant inhibitor risk scores that consider all non-modifiable factors to calculate the genetic predisposition of each patient to develop inhibitors.
Acknowledgements

We greatly thank the staff of Mahan Laboratory for their enormous participation and cooperation. Very special thanks and appreciation to Dr. Payman Ghoraishizadeh for his tremendous role, fruitful ideas, advices, and collaboration. We also greatly appreciate the staff at the Hemophilia Center in Hewa Haemato-oncology Hospital, Sulaymaniyah; Nanakali Hospital, Arbil; and Karkuk Haemato-oncology Hospital for their contribution to this study. Many thanks to Dr. Nareen Tawfeeq for her collaboration and contribution in patients recruitment and data collection. Special thanks to Dr. Sarwar Noori Mahmood for his contribution in conducting this research.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ORCID iD

Aveen M. Raouf Abdulqader https://orcid.org/0000-0002-4301-6470

References

1. Laffan MA and Pasi KJ. Inherited bleeding disorders, Chapter 41. In: AV Hoffbrand, D Catovsky, EGD Tuddenham and AR Green (eds). Postgraduate Hematology. 6th Edition. UK: Blackwell publishing Ltd., 2011, pp.793–812.
2. Wight J and Paisley S. The epidemiology of inhibitors in hemophilia A: a systematic review. Haemophilia 2003; 9: 418–435.
3. Ananyeva NM, Lacroix-Desmazes S, Hauser CA, et al. Inhibitors in hemophilia A: mechanisms of inhibition, management and perspectives. Blood Coagul Fibrinolysis 2004; 15: 109–124.
4. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. Haemophilia 2012; 19: e1–e47.
5. Astermark J. Why do inhibitors develop? Principles of and factors influencing the risk for inhibitor development in haemophilia. Haemophilia 2006; 12: 52–60.
6. Ghosh K and Shetty S. Immune Response to FVIII in Hemophilia A: An Overview of Risk Factors. Clinic Rev Allerg Immunol 2009; 37: 58–66.
7. Chambost H. Assessing risk factors: prevention of inhibitors in haemophilia. Haemophilia 2010; 16: 10–15.
8. Gouw SC, van den Berg HM, Oldenburg J, et al. F8 gene mutation type and inhibitor development in patients with severe hemophilia A: systematic review and meta-analysis. Blood 2012; 119: 2922–2934.
9. Astermark J, Wang X, Oldenburg J, et al. MIBS Study Group. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. J Thromb Haemost 2007; 5: 263–265.
10. Astermark J. FVIII inhibitors: pathogenesis and avoidance. Blood 2015; 125: 2045–2051.
11. Pavlova A, Delev D, Lacroix-Desmazes S, et al. Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-a and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. J Thromb Haemost 2009; 7: 2006–2015.
12. Marchione VD, Zuccoli JR, Abelleyro MM, et al. A prevalent CTLA4 missense variant significantly associates with inhibitor development in Argentine patients with severe haemophilia A. Haemophilia 2014; 20: 150–156.
13. AlFadhli S and Nizam R. Violating the theory of single gene-single disorder: inhibitor development in hemophilia. Indian J Hematol Blood Transfus 2014; 31: 162–168.
14. Jayandharan GR, Srivastava A and Srivastava A. Role of molecular genetics in hemophilia: from diagnosis to therapy. Semin Thromb Hemost 2012; 38: 64–78.
15. Kearney ER, Walunas TL, Karr RW, et al. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28
costimulation and inhibited by CTLA-4. J Immunol 1995; 155: 1032–1036.

16. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctl-a. Science 1995; 270: 985–988.

17. Braun J, Donner H, Siegmund T, et al. CTLA-4 promoter variants in patients with Graves’ disease and Hashimoto’s thyroiditis. Tissue Antigens 1998; 51: 563–566.

18. Ligers A, Xu C, Saarinen S, et al. The CTLA-4 gene is associated with multiple sclerosis. J Neuroimmunol 1999; 97: 182–190.

19. Giscombe R, Wang X, Huang D, et al. Coding sequence 1 and promoter single nucleotide polymorphisms in the CTLA-4 gene in Wegener’s granulomatosis. J Rheumatol 2002; 29: 950–953.

20. Kouki T, Sawai Y, Gardine CA, et al. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves’ disease. J Immunol 2000; 165: 6606–6611.

21. Ahmed S, Ihara K, Kanemitsu S, et al. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. Rheumatology 2001; 40: 662–667.

22. Blanchette VS, Key NS, Ljung LR, et al. Definitions in hemophilia: communication from the SSC of the ISTH. J Thromb Haemost 2014; 12: 1935–1939.

23. White GC II, Rosendaal F, Aledort LM, et al. Factor VIII and Factor IX Subcommittee. Definitions in hemophilia: recommendation of the Scientific Subcommittee on Factor VIII and Factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. J Thromb Haemost 2001; 85: 560–574.

24. Verbruggen B, Novakova I, Wessels H, et al. The Nijmegen modification of the Bethesda assay for factor VIII: C inhibitors: improved specificity and reliability. J Thromb Haemost 1995; 73: 247–251.

25. Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16: 1215.

26. Deichmann K, Heinzmann A, Bruggenolte E, et al. An Mse I RFLP in the human CTLA4 promoter. Biochem Biophys Res Commun 1996; 225: 817–818.

27. Qian J, Collins M, Sharpe AH, et al. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. Blood 2000; 95: 1324–1329.

28. Ligers A, Teleshova N, Masterman T, et al. CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. Genes Immun 2001; 2: 145–152.

29. Wang XB, Zhao X, Giscombe R, et al. A CTLA-4 gene polymorphism at position -318 in the promoter region affects the expression of protein. Genes Immun 2002; 3: 233–234.

30. Pinto P, Ghosh K and Shetty S. Immune regulatory gene polymorphisms as predisposing risk factors for the development of factor VIII inhibitors in Indian severe haemophilia A patients. Haemophilia 2012; 18: 794–797.

31. Lu Y, Ding Q, Dai J, et al. Impact of polymorphisms in genes involved in autoimmune disease on inhibitor development in Chinese patients with haemophilia A. J Thromb Haemost 2011; 107: 30–36.

32. Agostini D, Rosset C, Botton MR, et al. Immune system polymorphisms and factor VIII inhibitor formation in Brazilian haemophilia A severe patients. Haemophilia 2012; 18: e416–e418.

33. Anjos S, Nguyen A, Ounissi-Benkalha H, et al. A common autoimmunity predisposing signal peptide variant of the cytotoxic T-lymphocyte antigen 4 results in inefficient glycosylation of the susceptibility allele. J Biol Chem 2002; 277: 46478–46486.

34. Maurer M, Loserth S, Kolb-Maurer A, et al. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (exon 1 + 49) alters T-cell activation. Immunogenetics 2002; 54: 1–8.

35. Christiakov D and Turakulov R. CTLA4 and its role in autoimmune thyroid disease. J Mol Endocrinol 2003; 31: 21–36.

36. Ueda H, Howson JM and Esposito L. Association of the T cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 2003; 423: 506–511.
37. Pavlova A, Diaz-Lacava A, Zeitler H, et al. Increased frequency of the CTLA4 + 49 A/G polymorphism in patients with acquired haemophilia A compared to healthy controls. *Haemophilia* 2008; 14: 355–360.

38. Bafunno V, Santacroce R, Chetta M, et al. Polymorphisms in genes involved in autoimmune disease and the risk of FVIII inhibitor development in Italian patients with haemophilia A. *Haemophilia* 2010; 16: 469–473.

39. Walsh CE, Soucie JM and Miller CH; United States Hemophilia Treatment Center Network. Impact of inhibitors on hemophilia A mortality in the United States. *Am J Hematol* 2015; 90: 400–405.

40. Astermark J, Donfield SM, Gomperts ED, et al. The polygenic nature of inhibitors in hemophilia A: results from the Hemophilia Inhibitor Genetics Study (HIGS) Combined Cohort. *Blood* 2013; 121: 1446–1454.