Polymorphisms in the TGF-β1 (rs1982037) and IL-2 (rs2069762, rs4833248) genes are not associated with inhibitor development in Iranian patients with hemophilia A

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ABSTRACT

Objectives: Development of neutralizing antibodies against factor VIII is the major complication in hemophilia care which makes replacement therapies ineffective. The reports showed that the relationship between inhibitor development and the polymorphisms of two cytokine genes was studied in severe hemophilic patients from Iran. Methods: In this case-control study, three polymorphisms of immune regulatory genes (TGF-β (rs1982037) and IL-2 (rs2069762, rs4833248)) were analyzed in 100 Iranian hemophilia A patients divided into 55 inhibitor positive and 45 inhibitor negative patients using Tetra primer ARMS PCR, and DNA sequencing. Results: The analysis of polymorphisms in the TGF-β and IL-2 genes showed no association between the genotypes and the production of inhibitors (p > 0.05). Also, comparison of allele frequencies for TGF-β and IL-2 genes between two groups indicated no significant differences associated with the development of FVIII inhibitors (p > 0.05). Discussion: In contrast with some reports involving the correlation between polymorphisms of TGF-β1 and IL-2 genes and inhibitor development in the world, no statistically significant differences in analysis of the alleles and genotypes for TGF-β and IL-2 genes were found between the inhibitor and non-inhibitor Iranian patients. Thus, other genetic markers influencing the immune response to replacement therapy in patients with hemophilia should be identified. Conclusions: Regarding our results in molecular predisposition for inhibitor development, further studies of effective genetic markers are required as a prerequisite for the development of novel immunogenic therapeutic approaches in the future.

Introduction

Hemophilia A (HA) is a hemorrhagic disease resulting from the deficiency of clotting Factor VIII (FVIII) [1-5]. Nowadays, development of alloantibodies against infused FVIII is the most significant complication of hemophilia care [2,3]. Inhibitors are present in about 15% of the hemophilia population and develop in about 30% of previously untreated patients on exposure to FVIII concentrates [2,4,6,7]. Several patient-related factors have been considered for the risk of inhibitor development such as ethnicity, mutation type of F8 gene, family history of inhibitors, HLA haplotypes, single nucleotide polymorphisms (SNP) of cytokine genes [8-11]. In a recent study by Astermark (2013), 53 SNPs were reported to be the predictors of inhibitor status indicating the complexity of immune response and immune modifier genes in the development of inhibitors [11]. In addition to the underlying mutations that cause hemophilia A, inhibitor risk appears to be modified by polymorphisms in various cytokines and immunomodulators. However, HLA haplotypes have not been strong determinants of inhibitor risk [12]. Nijmegen modification of Bethesda assay was performed to identify the level of inhibitors that assess the neutralizing capacity of FVIII specific antibodies [13–15]. Some studies indicated that inflammatory cytokines have a significant role in inhibitor development [2,9]. Interleukin-2 (IL-2) and its receptor are important players in autoimmune disease [16]. IL-2 cytokine is primarily produced by activated CD4+ T cells, and exerts pleiotropic effects in the immune system as well as displays immune regulatory functions [17,18]. The IL-2 (rs2069762) gene is located in the 4q27 chromosomal region, and comprises five exons (www.ncbi.nlm.nih.gov/gene). A study showed that three SNPs in the IL2 gene had significant association with inhibitor development, two of which (rs2069762 and rs4833248) remained significant after correction for multiple hypothesis-testing. One haplotype with 28% prevalence among controls conferred protection against inhibitor development [12].

KEYWORDS

FVIII inhibitors; hemophilia A; cytokine; TGF-β; IL-2

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Moreover, transforming growth factor β (TGF-β) is a multifunctional cytokine that involves in several biological processes such as cell replication, differentiation, migration, apoptosis, healing, bone formation, angiogenesis, and immune system regulation [19–21]. TGF-β is an important regulator of inflammatory responses and immunological homeostasis [22]. The TGF-β gene is located in the 19q13.2 chromosomal region, and comprises 7 exons separated by six large introns (www.ncbi.nlm.nih.gov/gene, 23, 24). TGF-β and IL-2 cytokines were associated with susceptibility to various diseases including cancers, cardiac diseases, inflammatory diseases, bleeding disorders and others. To date, several single nucleotide polymorphisms (SNPs) have been shown to affect TGF-β (rs1982037) and IL-2 (rs2069762, rs4833248) expression and could therefore be used as susceptibility biomarkers [16–26]. The aim of this study was to investigate the association between polymorphic variants of the IL-2 and TGF-β genes, and development of FVIII inhibitors.

**Methods**

**Sample collection**

The peripheral blood samples were collected from 100 un-related severe HA patients (the FVIII level <1%) with and without inhibitors (i.e. among ∼600 HA patients with different FVIII levels) in Iranian Hemophilia Care Center during two years ago. The Nijmegen-modified Bethesda assay was performed for quantification of the FVIII inhibitor titer. The level of inhibitors was measured as Bethesda units (BU/mL). Titer of 0.6 BU/mL or greater were used as inhibitor positive [15]. Low responders had antibody titers <5 BU/mL indicating a lack of anamnestic response on exposure to FVIII, while high responders had antibody titers ≥5 BU/mL demonstrating a brisk anamnestic response to FVIII [25]. The HA was classified based on the FVIII level as severe (<1%), moderate (1–5%), and mild (5–40%). Fifty-five patients were diagnosed with inhibitors, and forty-five patients were without inhibitors and matched with inhibitor patients for mutation type. Patients with limited data on the presence of an inhibitor, as well as patients with transient inhibitors, were also excluded. All non-inhibitor patients had more than 150 exposure days. This study did not contain sibling patients with Hemophilia A. All the patients were multi-transfused with FVIII replacement therapy (plasma-derived FVIII such as Haemoctin, Bioteq) as a standard treatment for bleeding episodes; some of them received it as a prophylactic therapy and others on demand. Moreover, all included patients with and without inhibitors gave written informed consent before entering the study and the Iranian Comprehensive Hemophilia Care Center Ethics Committees cleared and approved the study protocol.

**Detection of polymorphisms**

Genomic DNA was extracted from leukocytes using GF-1 Nucleic Acid Extraction Kits (Vivantis, Malaysia) according to the manufacturer’s instructions. The used DNA concentration was ∼50 ng/μL. For detection of single nucleotide polymorphisms (SNPs) in the TGF-β (rs1982037) and IL-2 (rs2069762, rs4833248) genes, the primers were designed by http://primer1.soton.ac.uk/ database, and sequence validity was investigated using BLAST database and SnapGene software (Table 1). Next, the genotyping of the TGF-β and IL-2 genes was performed by Tetra primer ARMS PCR amplification. The amplified products were subjected to agarose gel electrophoresis and then, visualized through UV trans-illuminator. The validation of SNPs in all genes was determined by DNA sequencing. Finally, statistical analysis was carried out using Chi-square and Logistic regression tests.

**Statistical analysis**

The allele and genotype frequencies were calculated by SPSS version 22 (SPSS Inc., Chicago, IL, U.S.A.) software using Chi-square and Logistic regression tests. The data in two groups with or without inhibitors were in accordance with the Hardy–Weinberg equilibrium. The observed risk (OR) and confidence interval (CI, 95%) were calculated and the p-value less than 0.05 was considered to be significant.

**Results**

**Patients**

From 100 un-related patients with severe HA, 55 patients developed inhibitors. Among this sub-group, approximately 75% of the 55 subjects were high responder; whereas, ∼25% of them were low responder. Patients with inhibitor aged 3–55 years old (median = 26 years old) and the patients without inhibitor aged 6–62 years old (median = 22 years old).

**SNP genotyping in IL-2 and TGF-β genes**

Genotyping of three SNPs in HA patients with and without inhibitors was performed. The statistical data showed that there was no deviation from Hardy-Weinberg equilibrium in both patient groups. As indicated in Table 2, there was no statistically significant difference between the genotypic and allelic frequencies for IL-2 and TGF-β genes in patients with inhibitors compared to patients without inhibitors. Our study represented no correlation between IL-2 genotypes and inhibitor development in Iranian HA patients (p = 0.321 for rs2069762, and p = 0.380 for rs4833248). The lack of correlation was also obtained between TGF-β genotype.
(rs1982037) and inhibitor development \((p = 0.810)\). Comparison of allele frequencies for TGF-β and IL-2 genes \((p = 0.217\) for rs2069762 SNP of IL-2, \(p = 0.410\) for rs4833248 SNP of IL-2, and \(p = 0.462\) for rs1982037 SNP of TGF-β) between two groups showed no significant differences associated with the development of FVIII inhibitors.

**Discussion**

As known, cytokines are more or less directly involved in the antibody-mediated immune responses [26,27]. In patients with autoimmune disease, polymorphisms in the immune response genes were found to be associated with antibody formation. Moreover, point mutations and single nucleotide substitutions (SNPs) in the regulatory regions of cytokine genes are key components in the pathogenesis of many diseases such as cancer, metabolic disorders, infectious diseases, autoimmune diseases, and inflammatory conditions [28,29]. Individual immune response traits may also affect a patient’s reaction to exogenous factor VIII [2]. The development of FVIII inhibitor is the main complication of replacement therapy in patients with haemophilia A. Indeed, the immunomodulatory cytokine genes have been related to the risk of development of alloantibodies in several studies, mainly in HA with severe form [26]. For instance, polymorphic variants at IL-2 gene was associated with several autoimmune conditions including type 1 diabetes, rheumatoid arthritis, multiple sclerosis and bleeding disorders [16]. Fichna et al. indicated that there is no difference between Autoimmune Addison’s disease (AAD) with IL-2 (rs2069762) polymorphism [16]. Also, Lozier et al. showed that two SNPs in the IL-2 gene had significant association with inhibitor development [12]. Our data showed that IL-2 (rs2069762, rs4833248) polymorphisms have no correlation with the risk of inhibitor development \((p > 0.05)\). On the other hand, the TGF-β gene is polymorphic at several sites and these polymorphisms are probably related to differences in the rate of TGF-β synthesis. TGF-β is the main cytokine involved in liver fibrogenesis. It was reported that TGF-β mRNA was increased in the liver of patients with chronic HCV infection [30]. Oliver et al. showed that polymorphisms at the TGF-β gene were not probably related to the risk of advanced alcoholic liver disease (ALD) [31]. On the other hand, Wang et al. showed that TGF-β allelic variations were associated with variability in developing more severe fibrosis during chronic hepatitis C infection in Caucasians [32]. Genotyping of a total of 9 SNPs in genes IL-4, IL-5, IL-10, TGF-β1, and IFN-γ was performed by Fidanci et al. in 103 HA patients (42 positive inhibitor and 61 negative inhibitor patients), and 100 healthy individuals. Among these genes, only a significant association with the T allele of rs2069812 in the IL-5 gene promoter and patients

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**Table 1.** The designed primers for tetra primer ARMS PCR.

| Gene   | Primer sequence Product size (bp) Annealing temp. (°C) |
|--------|------------------------------------------------------|
| IL-2 (2069762) | Forward inner primer AAATCTGAATGAGCTCGGACATAGCGTG 191 61.02  |
|         | Reverse inner primer AAAGFAACTGAGAAATTTCTTGGCCC 289  |
|         | Forward outer primer TAGCGTTAAACAGTACCTCAAGCTCAAT 409  |
|         | Reverse outer primer GATGTAGGTGAAATCCCTCTTTGTTACA  |
| IL-2 (4833248) | Forward inner primer AAATCTGAATGAGCTCGGACATAGCGTG 191 61.02  |
|         | Reverse inner primer AAAGFAACTGAGAAATTTCTTGGCCC 289  |
|         | Forward outer primer TAGCGTTAAACAGTACCTCAAGCTCAAT 409  |
| TGF-β  | Forward inner primer AAATCTGAATGAGCTCGGACATAGCGTG 191 61.02  |
|         | Reverse inner primer AAAGFAACTGAGAAATTTCTTGGCCC 289  |
|         | Forward outer primer TAGCGTTAAACAGTACCTCAAGCTCAAT 409  |

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**Table 2.** Genotype frequencies of 3 SNPs in Iranian hemophilia A patients with and without inhibitors.

| Gene | SNP name | Genotype | Inhibitor negative \(n\) % N = 45 | Inhibitor positive \(n\) % N = 55 | p-Value | CI (95%) | OR | p-Value |
|------|----------|----------|-----------------------------------|-----------------------------------|---------|---------|------|---------|
| IL-2 | (rs2069762) | GT       | 17 (37.8)                         | 25 (45.5)                         | 0.321   | 0.165-1.345 | 0.680 | 0.160   |
|      |          | GG       | 15 (33.3)                         | 21 (38.2)                         | 0.168-1.453 | 0.714 | 0.200   |
|      |          | TT       | 13 (28.9)                         | 9 (16.4)                          | 1.00 (reference) |       |         |
|      |          | G        | 47 (52.2)                         | 67 (60.9)                         | 0.217   | 0.399-1.233 | 0.701 | 0.218   |
|      |          | T        | 43 (47.8)                         | 43 (39.1)                         |         |         |       |         |
| IL-2 | (rs4833248) | AG       | 17 (37.8)                         | 27 (49.1)                         | 0.380   | 0.191-1.331 | 0.629 | 0.167   |
|      |          | AA       | 13 (28.9)                         | 16 (29.1)                         | 0.226-1.866 | 0.812 | 0.423   |
|      |          | GG       | 15 (33.3)                         | 12 (21.8)                         | 1.00 (reference) |       |         |
|      |          | A        | 43 (47.8)                         | 59 (53.6)                         | 0.410   | 0.453-1.382 | 0.728 | 0.410   |
|      |          | G        | 47 (52.2)                         | 51 (46.4)                         |         |         |       |         |
| TGF-β | (rs1982037) | AG       | 12 (26.7)                         | 16 (29.1)                         | 0.810   | 0.335-2.055 | 0.750 | 0.688   |
|      |          | AA       | 5 (11.1)                          | 8 (14.5)                          | 0.203-2.364 | 0.625 | 0.557   |
|      |          | GG       | 28 (62.2)                         | 31 (56.4)                         | 1.00 (reference) |       |         |
|      |          | A        | 22 (47.8)                         | 32 (29.1)                         | 0.462   | 0.419-1.485 | 0.687 | 0.462   |
|      |          | G        | 68 (75.6)                         | 78 (70.9)                         |         |         |       |         |

\(N:\) total number of patients; \(OR:\) odds ratio; \(CI:\) confidence intervals.
with inhibitors was observed [33]. In 2015, de Alencar et al. demonstrated that polymorphisms in IFN-γ and in TGF-β genes but not in IL-2 gene were related to risk of developing inhibitor, and could contribute to a genetic profile of the individual HA for the risk of inhibitors development to FVIII. In their study, from the cohort of 117 patients with severe HA, 35 developed inhibitors [26]. In our study, 55 patients developed inhibitors among 100 patients with severe HA indicating their high number in the collected samples. Moreover, our data showed that polymorphisms not only in IL-2 gene but also in TGF-β gene did not show any significant difference between Iranian patients with and without inhibitors (p > 0.05). Ding et al. also studied the clinical significance of CD4+CD25high T regulatory (Treg) cells in HA patients. The data showed that there were significant differences between the inhibitor and non-inhibitor patients in levels of IFN-γ, IL-2, IL-10, and TGF-β. Indeed, the proportions of Treg cells and the concentrations of T cell cytokines in inhibitor patients were higher than those in non-inhibitor patients. In their study, only 6 severe HA patients with factor VIII inhibitors and 6 HA patients without inhibition of factor VIII were included [34]. Our study did not represent significant differences between the inhibitor and non-inhibitor patients in levels of IL-2 and TGF-β. Generally, inhibitor development in Iranian hemophilia A patients was not restricted to polymorphisms in IL-2 and TGF-β cytokines.

Conclusions

According to our study, there is no significant association between the risk of inhibitors in severe hemophilia A patients and the polymorphisms in the TGF-β and IL-2 genes. This study was performed in the small number of patients and it is needed to obtain the results more exactly in a cohort study with large population. However, further studies of genetic markers are required as a prerequisite for the development of novel immunogenic therapeutic approaches in the future.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] Zhang AH, Skupsky J, Scott DW. Factor VIII inhibitors: risk factors and methods for prevention and immune modulation. Clin Rev Allergy Immunol. 2009;37(2):114–124.
[2] Witmer C, Young G. Factor VIII inhibitors in hemophilia A: rationale and latest evidence. Ther Adv Hematol. 2013;4(1):59–72.
[3] Di Minno MN, Di Minno G, Di Capua M, et al. Cost of care of haemophilia with inhibitors. Haemophilia. 2010;16(1):e190–e201.
[4] Mannucci PM, Shi Q, Bonanad S, et al. Novel investigations on the protective role of the FVIII/VWF complex in inhibitor development. Haemophilia. 2014;20(6):2–16.
[5] Lieuw K. Many factor VIII products available in the treatment of hemophilia A: An embarrassment of riches? J Blood Med. 2017;8:67–73.
[6] Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia. 2003;9:418–435.
[7] Peyvandi F, Mannucci PM, Palla R, et al. SIPPET: methodology, analysis and generalizability. Haemophilia. 2017;23:353–361.
[8] Kruse-Jarres R. Inhibitors: our greatest challenge. Can we minimize the incidence? Haemophilia. 2013;19(1):2–7.
[9] Ghosh K, Shetty S. Immune response to FVIII in hemophilia A: An overview of risk factors. Clin Rev Allergy Immunol. 2009;37(2):58–66.
[10] Pavlova A, Delev D, Lacroix-Desmazes S, et al. Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. J Thromb Haemost. 2009;7(12):2006–2015.
[11] Astemark 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(8):1446–1454.
[12] Lozier J, Rosenberg PS, Goedert JJ, et al. A case-control study reveals immunoregulatory gene haplotypes that influence inhibitor risk in severe hemophilia A. Haemophilia. 2011;17(4):641–649.
[13] Reipert BM. Risky business of inhibitors: HLA haplotypes, gene polymorphisms, and immune responses. Hematology Am Soc Hematol Educ Program. 2014;2014(1):372–378.
[14] Verbruggen B. Diagnosis and quantification of factor VIII inhibitors. Haemophilia. 2010;16(102):20–24.
[15] Krudyisz-Amblo J, Parhami-Seren B, Butenas S, et al. Quantitation of anti-factor VIII antibodies in human plasma. Blood. 2009;113(11):2587–2594.
[16] Fichna M, Zurawek M, Bratland E, et al. Interleukin-2 and subunit alpha of its soluble receptor in autoimmune Addison’s disease – an association study and expression analysis. Autoimmunity. 2015;48(2):100–107.
[17] Bouzid D, Fournati H, Amouri A, et al. Autoimmune diseases association study with the KIAA1109-IL2-IL21 region in a Tunisian population. Mol Biol Rep. 2014;41(11):7133–7139.
[18] Hoyer KK, Dooms H, Barron L, et al. Interleukin-2 and LPS and IFNγ. Biosci. 2006;26(4):281–289.
[19] Kubiczkova L, Sedlarikova L, Hajek R, et al. TGF-beta: An excellent servant but a bad master. J Transl Med. 2015;13:28.
[20] Liao N, Zhao H, Chen ML, et al. Association between the TGF-β1 polymorphisms and chronic obstructive pulmonary disease: a meta-analysis. Biosci Rep. 2017;37(4).
[23] Martelossi Cebinelli GC, Paiva Trugilo K, Badaró Garcia S, et al. TGF-β1 functional polymorphisms: a review. Eur Cytokine Netw. 2016;27(4):81–89.

[24] Poniatowski LA, Wojdasiewicz P, Gasik R, et al. Transforming growth factor beta family: insight into the role of growth factors in regulation of fracture healing biology and potential clinical applications. Mediators Inflamm. 2015;2015:1–17.

[25] Acharya SS, DiMichele DM. Management of factor VIII inhibitors. Best Pract Res Clin Haematol. 2006;19:51–66.

[26] de Alencar JB, Macedo LC, de Barros MF, et al. New associations: INFγ and TGFβ1 genes and the inhibitor development in severe haemophilia A. Haemophilia. 2015;21(4):e312–e316.

[27] Astermark J, Oldenburg J, Pavlova A, et al. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. Blood. 2006;107(8):3167–3172.

[28] Visentainer JE, Sell AM, da Silva GC, et al. TNF, IFNG, IL6, IL10 and TGFβ1 gene polymorphisms in South and Southeast Brazil. Int J Immunogenet. 2008;35(4-5):287–293.

[29] Scheller J, Ohnesorge N, Rose-John S. Interleukin-6 trans-signalling in chronic inflammation and cancer. Eur J Immunol. 2006;36(5):321–329.

[30] Castilla A, Prieto J, Fausto N. Transforming growth factors beta 1 and alpha in chronic liver disease. effects of interferon alfa therapy. N Engl J Med. 1991;324(14):933–940.

[31] Oliver J, Agúndez JA, Morales S, et al. Polymorphisms in the transforming growth factor-beta1 gene (TGF-beta1) and the risk of advanced alcoholic liver disease. Liver Int. 2005;25(5):935–939.

[32] Wang H, Mengstebab S, Tag CG, et al. Transforming growth factor-beta1 gene polymorphisms are associated with progression of liver fibrosis in Caucasians with chronic hepatitis C infection. World J Gastroenterol. 2005;11(13):1929–1936.

[33] Fidancı ID, Zülfikar B, Kavaklı K, et al. A polymorphism in the IL-5 gene is associated with inhibitor development in severe hemophilia A patients. Turk J Hematol. 2014;31:17–24.

[34] Ding KY, Ji WC, Wu JS, et al. Higher frequency of CD4+CD25high Treg cells in hemophilia patients with factor VIII inhibitor. Genet Mol Res. 2014;13(1):1774–1781.