Violating the Theory of Single Gene-Single Disorder: Inhibitor Development in Hemophilia

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Abstract Hemophilia is clinically and genetically heterogeneous blood disorder with several known gene defects accounting for the diversity of disease phenotype and inhibitor production. Although increasing number of causative mutations have been reported, not much is known regarding the root cause of inhibitor development against infused blood clotting factors, which represents a major challenge in the treatment of disease. The variations in the severity and frequency of bleeding in hemophiliacs with same molecular defect, indicates the role of modifier genes in the pathogenesis of disease. Herein, we aim to review and summarise the literature over the past decade, to gain insight into what is critical for the development of inhibitors in hemophilia. Aside from potential mutations in factor VIII and IX, polymorphisms in various genes such as human leukocyte antigen-I (HLA-I), HLA-II, tumor necrosis factor-alpha, interleukin-10 and cytotoxic T-lymphocyte associated antigen-4, also tends to contribute to the development of inhibitors. Violating the theory of single gene-single disorder, new research indicates that inhibitor arise from a complex interplay of multiple genetic, immunological and environmental factors. With the revolutionary advances in whole genome sequencing, we propose a detailed genome wide study to identify the spectrum of genetic markers involved in the development of inhibitors for better diagnostics and therapeutics.

Keywords Hemophilia · Inhibitors · Mutations · Polymorphisms

Abbreviations

TNF- α	Tumor necrosis factor- α
IL	Interleukin
CTLA-4	Cytotoxic T-lymphocyte antigen-4
HLA	Human leukocyte antigen
TGFB1	Transforming growth factor beta 1
DOCK2	Dedicator of cytokinesis 2
MAPK2	Mitogen-activated protein kinase 9
PTPRR	Protein tyrosine phosphatase receptor type R
CD	Cluster of differentiation
IQGAP2	IQ motif containing GTPase activating protein 2
CSF1R	Colony stimulating factor 1 receptor

Introduction

Hemophilia is an inherited blood disorder where blood does not clot properly due to a defect or deficiency in one of the blood clotting factor genes that exist on the X chromosome. It often occurs in male, while females are carriers of the disease. Hemophilia A (HA) also known as classic hemophilia, is characterized by a lack of clotting factor VIII and accounts for about 90 % of hemophilia cases; while Hemophilia B (HB) characterized by a lack of clotting factor IX is found to be very rare [1, 2]. An estimated worldwide frequency of HA is one per 5,000–7,000 male birth and HB is one per 25,000 male births [3]. The third variant of hemophilia known as acquired hemophilia (AHA) is characterized by the development of inhibitors against blood clotting factors. It occurs either spontaneously or as secondary to other autoimmune diseases, hematological malignancies, inflammatory disorders or infectious triggers [4]. Inhibitors are also produced upon replacement therapy of defective clotting factors, representing a major challenge in the treatment of hemophilia.

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Inhibitors are polyclonal alloantibodies that bind to the epitope of clotting blood factor causing it to be recognized as non-self by the immune system. It also tends to destroy the natural clotting factor inside the body, exposing the patients to an increased risk of morbidity and mortality. Exogenous administration of factor VIII leads to inhibitor production in approximately 15–30 % of HA and 2–5 % of HB patients [5].

Although altered gene sequence of candidate genes are believed to be responsible for the development of the disease, the factors that regulate the complexity and the inhibitor development remains elusive. Recent studies indicate that a combination of genetic, immunologic and environmental factors tend to contribute to inhibitor production. Identification of such factors and mechanisms involved is of enormous significance as such attempts would pave the way for better diagnostic parameters and therapeutics. This review intends to evaluate the role of modifier genes towards the susceptibility and complexity of hemophilia development observed in previous years.

Mutations and Hemophilia Development

Molecular genetics in the pathogenesis of Hemophilia is known ever since the detection of mutations underlying the deficiency of clotting factors. Over the last decades, several point mutations have been reported in different ethnic groups that specifically leads to HA or HB [6–11]. These mainly include missense, nonsense, splice mutations and rarely gene rearrangements such as inversions covering a total of 2,107 known mutations for HA and 1,094 mutations for HB [12, 13].

Factor VIII is the only gene found to be associated with HA. It maps to the distal end of the X-chromosome (Xq28) and consists of 26 exons that encode a 2,351 amino acid precursor polypeptide arranged into six domains A1-A2-B-A3-C1-C2 [14, 15]. Each of these domains contains specific binding sites for various components of the clotting cascade. One of the probable reasons for the production of inhibitors is the type of gene defect and its location within the determinant gene [16]. Increased risk of inhibitor production has been associated with mutations that lead to complete destruction of protein or relatively high dysfunctional protein. It has been postulated that the different degrees of bleeding severity and inhibitor development are the outcomes of genetic defect they carry, with dysfunctional proteins resulting in continued production of detectable range of clotting factors, while the null proteins leading to severe deficiencies, each contributing to specific phenotype. A fourfold increased risk has been reported in hemophilia patients with mutations involving A2 and C2 domain of factor VIII gene [17]. The larger the deletion or inversion, the

higher is the risk of inhibitor development as any change in the three dimensional structure of A2 and C2 domain may inactivate the pro-coagulant activity of factor VIII [18]. Subjects with inversion involving intron 22 are more prone to inhibitor development as they lead to truncated mRNA by an intra-chromosomal homologous recombination separating factor VIII exons 1–22 from exons 23–26 [19]. Similarly, intron 1 inversion [20, 21] leading to complete destruction of the protein and mutations in exon 14 leading to partial destruction of the open reading frame [22] are some of the common variants of factor VIII gene that may have a probable role in inhibitor development. Nevertheless, the exact genetic defect that leads inhibitor development remains obscure. Polymorphisms within factor VIII gene have been associated with inhibitor development in Caucasian hemophiliacs [23], indicating their significance in the pathophysiology of disease.

Several mutations contributing to defective Factor IX have also been reported in HB. Factor IX is located on chromosome Xq27.1 and consist of 8 exons that encode a 462 amino acid protein. One third of the mutations responsible for HB results in dysfunctional proteins that generally affects post-translational modifications, Factor IX activation, ligand binding and substrate recognition [24]. In HB, gene deletions or rearrangements are associated with 50 % increased risk of inhibitor development compared to 20 % for those with frameshift, premature stop or splice-site mutations [19]. The onset of inhibitor development leads to persistent allergic reaction that can be life threatening and could no longer be treated by immune tolerance therapy.

HLA and Immune Related Genes in Inhibitor Production

Independent of the causative mutation, the phenotype of major histocompatibility complex tends to influence the development of inhibitors in hemophilia patients [25]. Upon exogenous administration of factor VIII, inhibitors develop in hemophilia as transfused FVIII is considered as a foreign protein and HLA class II molecules mediates the processing of antigenic peptides from antigen presenting cells to CD4+ T lymphocytes and antibody-producing B lymphocytes. Several studies have attempted to explore the association of HLA class I and II alleles with inhibitor production. HLA class II alleles such as *DR4.1*(*DRB1*04:01*), *DQB1*04*, *DQA1*03:01*, *DQB1*06:02*, *DQA1*01:02*, *DRB1*15* and *DRB1*15*; associated with the increased risk of inhibitor development [26–29] and to its contrast, HLA class I *A*24* and *C*05* and HLA class II *DQB1*05:02* and *DRB1*16* are associated with decreased risk of inhibitor production [26, 27, 30].

Table 1 Candidate genes and their polymorphisms shown to be associated with inhibitor development in hemophilia

Study	Gene	Polymorphisms	Allele/haplotype	Association
Chaves et al. (2010) (n = 85)	IL-10	(rs 1800896, rs 1800872, rs 1800871)	GCC/ATA haplotype	P = 0.043, OR = 3.55; 95 % CI = 1.01–12.57
			GCC/ACC haplotype	P = 0.0267, OR = 5.82; 95 % CI = 1.11–30.56
Lozier J et al. (2011) (n = 915)	IL10	(rs6667202, rs407226, rs4072227)	CTT haplotype	P = 0.04, OR = 1.23, 95 % CI = 1.01–1.50
	IL2	(rs10027390, rs2069772, rs2069779, rs2069778, rs2069762, rs4833248)	TGCCTG haplotype	P = 0.008, OR = 0.69, 95 % CI = 0.53–0.19
	IL12A	(rs2243115, rs583911, rs2243131, rs568408, rs2243148, rs2243154, rs2133310)	TAAGCGA haplotype	P = 0.041, OR = 1.31, 95 % CI = 1.02–1.68
	IL1 α	(rs3783557, rs2071373, rs6746923, rs17597976, rs11687624, rs11898680, rs6716046, rs6716046, rs7567619, rs7585707, rs11680809, rs12711742, rs12469600)	GTAGTTTTTCGT haplotype	P = 0.034, OR = 2.2, 95 % CI = 1.1–4.3
	IL1 β	(rs1143627, rs16944, rs1143623, rs1261220, rs13032029, rs13008855, rs6735739, rs12053091)	TAGCCTCCG haplotype	P = 0.02, OR = 0.75, 95 % CI = 0.58–0.96
	Factor VIII	(rs5945250, rs17281377, rs4898399, rs6643622, rs7053448, rs1936645, rs5945269, rs5945270, rs17281398, rs6649625)	TCTGCGGCAT haplotype	P = 0.004, OR = 3.8, 95 % CI = 1.50–9.51
Astermark et al. (2006) (n = 164)	TNF- α	rs 1800629	AA genotype	P = 0.008, OR = 4.0, 95 % CI = 1.4–11.5
Astermark et al. (2006) (n = 164)	IL-10	[CA] <i>n</i> in promoter	Allele 134	P < 0.001, OR = 4.4, 95 % CI = 2.1–9.5
Astermark et al. (2007) (n = 124)	CTLA4	C–318T	Allele T	P = 0.012, OR = 0.3, 95 % CI = 0.1–0.8
	TNF- α	rs 1800629	Allele A	P = 0.114, OR = 1.81, 95 % CI = 1.13–2.86
Pavlova et al. (2006) (n = 260)	TNF- α	(rs1800629, rs361525, rs3093662)	AGA haplotype	P = 0.0002, OR = 2.48, 1.51–4.19
Astermark et al. (2013) (n = 833)	MAPK9	rs4147385		P = 9.57×10^{-6} , OR = 2.03, 95 % CI = 1.48–2.78
	PDGFRB	rs10072056		P = 3.87×10^{-5} , OR = 0.61, 95 % CI = 0.48–0.77
	PCGF2	rs2879097		P = 4.69×10^{-5} , OR = 0.58, 95 % CI = 0.45–0.76
	DOCK2	rs1863993		P = 7.37×10^{-5} , OR = 4.29, 95 % CI = 2.09–8.80
	CD44	rs927335		P = 1.86×10^{-4} , OR = 1.67, 95 % CI = 1.28–2.19
	IQGAP2	rs17652304		P = 3.19×10^{-4} , OR = 3.65, 95 % CI = 1.80–7.40

Table 1 continued

Study	Gene	Polymorphisms	Allele/haplotype	Association
	CSF1R	rs17725712		P = 3.72×10^{-04} , OR = 2.39, 95 % CI = 1.48–3.86
	HSP90B1	rs1882019		P = 6.04×10^{-04} , OR = 0.56, 95 % CI = 0.40–0.78
	F13A1	rs13206518		P = 6.75×10^{-04} , OR = 0.44, 95 % CI = 0.28–0.71
	IGSF2	rs2296449		P = 7.16×10^{-04} , OR = 0.34, 95 % CI = 0.18–0.63
	ALOX5AP	rs4075131		P = 8.40×10^{-04} , OR = 0.63, 95 % CI = 0.48–0.82
	MAP2K4	rs3826392		P = 9.74×10^{-04} , OR = 0.67, 95 % CI = 0.52–0.85
	PTPRN2	rs12667537		P = 9.95×10^{-04} , OR = 0.66, 95 % CI = 0.51–0.84

Likewise, immune cells and inflammatory cytokines has prominent role in inhibitor production. CD4+ T cells are considered as a promising target for inhibitor production [31]. Activation of CD4+ T-cells occurs either by Th1 or Th2 mediated response by the secretion of cytokines involved in cellular response and humoral response. Th1 mediated activation involves secretion of interferon gamma (INFG), tumor necrosis factor alpha (TNFA) and IL-1, while that of Th2 includes IL-4, IL-5, IL-6 and IL-10 [32]. Hemophilia A patients are generally characterized by an overall increase in the frequency of IL-6 and IL-10 with decrease of IL-8 and IL-12 [33]. In a previous study by Hu G et al. (2007), FVIII exposure was reported to significantly increase IFNG-producing CD4(+) T cells in patients and controls, whereas an increase in IL-4- producing CD4(+) T blasts was exclusively limited to patients with inhibitors, indicating the role of Th1 cells in initiating the immune response to FVIII and Th2 cells in the development of inhibitor production [31]. Additionally, increased IL-5/TNF- α ratio in neutrophils and IL-10/TNF- α ratio in monocytes were also reported in hemophilia subjects with inhibitors [34]. A T-cell cytokine deficient environment together with the presence of IL-5 and IL-10 derived from neutrophils and monocytes has been suggested to initiate the activation of B cells towards the synthesis of IgG4. The pleiotrophic role of IL-10 in humoral response tends to regulate T-/B-cell interplay, determining the subsequent immune response and IgG4 induction [35]. A combined action of T-/B-regulatory cells has been proposed to induce the production of inhibitors against infused factor.

Upregulation of co-stimulatory signals such as CD-40 and other regulatory cytokines such as *TGFB1* have also been implicated in inhibitor development [36].

Likewise, several single nucleotide polymorphisms (SNPs) within cytokine and immune genes tend to influence their transcription rate and protein production (Table 1). Recently, genetic markers within IL-10 gene were associated with antibody production in hemophiliacs [37, 38]. Haplotypes GCC, ACC and ATA resulting from IL-10 (−1082, −819 T/C, −592 A/C) were shown to be associated with higher, intermediate and low production of cytokines respectively. A combination of high/intermediate IL-10 haplotype was specifically associated with susceptibility to inhibitor development in hemophilia [37]. IL-10 haplotypes were also found to be associated with inhibitor development in Caucasian and Chinese population [23, 39]. Similarly, individual polymorphisms and haplotypes from IL1 α , IL1 β , IL-2, IL-10, IL-12A and *TNFA* were also found to be significantly associated with inhibitor development in hemophilia subjects [23, 27, 40].

Polymorphisms in other immune related gene such as *CTLA-4*, *DOCK2*, *MAPK9*, *PTPRR* and *CD36* gene have also been linked to inhibitor development [41, 42]. In a recent study by Astermark (2013), 53 SNPs were reported to be the predictors of inhibitor status, further indicating the complexity of immune response and immune modifier genes in the development inhibitors [43]. Five candidate genes *MAPK9*, *DOCK2*, *CD44*, *IQGAP2* and *CSF1R* were most strongly associated with inhibitor development, while 13 other genes were associated with protectivity to

Fig. 1 Represents network of predicted associations for immune genes from the literature that have been speculated to be associated with inhibitor development in hemophilia. The network nodes are proteins and the edges represent the predicted functional associations. Differently colored lines, represent their mode of action. A blue line indicates—Binding; a violet line—Catalysis; a pink line—Post

translational modification; and a yellow line—Expression. Arrow at the end of the edge next indicates the directionality of the action. Red bar indicates down-regulation and green arrow indicates up-regulation. The circle indicates that the directionality of the interaction is known, but the end result is unknown. This figure was constructed using STRING 8 protein network program [47]. (Color figure online)

inhibitor development. Association of aforementioned genes with inhibitor status may vary in their degree; nevertheless, together they may contribute to the development of inhibitor.

Several other immune related genes have also been tested for its association with inhibitor development

(Fig. 1). Insilco analysis of immune genes from the literature indicates that majority of these genes are interrelated and has similar functional significance indicating their vast possibility in disease progression and development. Violating the theory of single gene-single disorder, hemophilia involves multifaceted hemato-immunological interactions

defining complex interplay of multiple genes related to blood coagulation, immune response, defense response, homeostasis, isotype switching, cytokine secretion, cellular metabolic process, signal transduction and intracellular protein transport and localization. Although the root cause remains elusive, a delicate balance between these genetic variants directs the development of inhibitor against infused factor. Hence, a deeper insight into pathophysiology of the disease is mandatory to identify the risk factors underlying inhibitor development.

Other Factors Predisposing to Hemophilia

The role of non-genetic factors in the development of inhibitors was indicated by discordant inhibitor status of monozygotic hemophiliac twins [44]. Racial factors and family histories also tend to influence the development of inhibitors. The risk of inhibitor development was reported to be higher in the subgroup of African descent when compared to Caucasians (55.6 % vs. 27.4 %) [45, 46]. Similarly, higher risk was observed in patients with a family history of inhibitor development [46]. Various other intriguing factors such as infections, vaccinations, tissue damage upon FVIII exposure and therapeutical parameters such product type, duration of exposure and mode of delivery also tend to contribute to the development of inhibitors [44].

Conclusion

Hemophilia is a complex X-linked blood disorder with heterogeneous mutations accounting for its pathophysiology. Replacement of deficient blood clotting factors being the only affordable treatment for this disorder, the development of inhibitors against infused factors represents a major challenge in disease treatment with high risk of morbidity and mortality. Although the types of mutation in determinant gene is considered to be the initial factor responsible for the development of inhibitor, increasing number of publications also hint towards the involvement of HLA molecules, immune related genes and cytokines. Nevertheless, the mechanisms that regulate the complexity and bleeding frequency in hemophiliacs and the exact cause and triggers of inhibitor development remain unknown. Differential response to factor replacement indicates the role of other modifying genes involved in the process; identification of these genes before the start of the treatment would increase the survival rate of patients. To date, limited research has been carried out to detect the polymorphisms of selected genes and resulting haplotypes that increase/decrease inhibitor production. With the revolutionary advances in next generation sequencing, a whole genome sequencing of

selected hemophilia cases would reveal a better insight to what is critical for the development of inhibitors and the complexity of the disease. Such investigation is of paramount significance as it would lead to the development of potential diagnostic markers and better therapeutics.

Conflict of interest The authors declare that they have no conflict of interest.

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