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Risk factors associated with inhibitor development in Chinese patients with haemophilia B

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Introduction: Inhibitor development is a severe complication of factor IX substitution treatment for haemophilia B (HB). Current research examined the association between inhibitor development and *F9* genotypes and polymorphisms in immune response genes in Chinese HB patients. **Materials and Methods:** 11 inhibitor-positive HB patients and 41 inhibitor-negative HB patients were enrolled. Direct sequencing, copy number variation (CNV) detection and fragment length analysis were applied to identify *F9* genotypes and 15 polymorphisms in immune response genes. **Results:** 7 patients developed high titer inhibitors, with 5 of them having histories of consecutive exposure to FIX products on demand for at least 5 days. Allergic reactions/anaphylaxis to prothrombin complex concentrates (PCC) occurred in 3 patients before inhibitors were detected. Five nonsense mutations (E54X, R75X, Q185X, R298X and R379X), two large deletions (E1–6del and E1–8del) and one missense mutation (S411G) were identified in patients with inhibitors. Missense mutations had a low odds ratio for FIX inhibitors development (IOR) of 0.078 ($P = 0.02$), while nonsense mutation presented a high IOR of 8.500 ($P = 0.0044$). The frequency of allele T in *CD44*⁹⁵¹⁰² (A/T) was significantly higher in inhibitor-negative patients, with OR of 0.324 ($P = 0.04$). **Conclusions:** Nonsense mutations conferred a higher risk for while allele T in *CD44*⁹⁵¹⁰² (A/T) might play a protective role against inhibitor development in Chinese HB patients.

Keywords: anaphylaxis, *F9* gene mutation, haemophilia B, immune response genes, inhibitor development

Introduction

Haemophilia B (HB) is an X-linked, recessive disorder characterized by reduced activity of coagulation factor IX (FIX). Based on the residual FIX activity (FIX: C), this disease can be classified as mild (5–40% of normal), moderate (1–5% of normal) or severe (<1% of normal) [1]. So far, replacement therapy is the only effective option to prevent or control the bleeding epi-

sodes in HB patients. However, the development of inhibitors can compromise the management of acute haemorrhage and is a severe and challenging complication of FIX substitution treatment, with an incidence of 1.5–3% in HB patients [2]. Allergic episodes are a unique phenomenon occurring in 60% of HB patients with inhibitors upon treatment with plasma-derived or recombinant FIX concentrates (rFIX), especially in patients with large deletions in *F9* gene [3]. Nephrotic syndrome has been reported to occur in patients with inhibitors and previous history of allergic reaction/anaphylaxis on immune tolerance (IT) regimen [4]. Allergic reactions/anaphylaxis and nephrotic syndrome may prevent inhibitor eradication by an IT approach, making the treatment of HB patients with inhibitors more difficult.

Both environmental and genetic risk factors play decisive roles in inhibitor development in haemophilia patients. Environmental factors include age at first exposure [5,6], prophylaxis [7] and intensity of coagulation factor infusion [8], while the genetic factors include disease severity, ethnicity, type of causative

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mutations and immune-related genetic polymorphisms [9]. In the recent years, numerous studies have been focused on the genetic risk factors for inhibitor development in haemophilia A (HA). HA patients with large deletions, nonsense mutations or intron-22 inversion are more prone to developing inhibitors [10,11]. Since inhibitor formation is a polygenic process, human leucocyte antigen class II molecules (DRB1*15:01 and DQB1*06:02) [12] and single nucleotide polymorphisms (SNPs) both in human immune response genes, such as interleukin-10 (*IL-10*) [13,14], tumour necrosis factor- α (*TNF- α*) [15] and cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) [16], and in genes related to intracellular signalling pathways [17] were reported to be either risk factors for or having protective roles against inhibitor development in HA. In contrast, information regarding the risk of inhibitor development in HB patients is scarce and mostly focused on the type of *F9* gene mutations. Partial/complete deletions and nonsense mutations in the *F9* gene confer a higher risk of FIX inhibitor development [2,18]. Besides, severe disease, ethnicity and age <11 were reported to be significantly associated with inhibitor development in HB patients [19]. Few studies focused on the role of immune response genes in FIX inhibitor development.

This paper presents data collected from 11 HB patients with inhibitors from three haemophilia centres in China. The inhibitor development associated with *F9* genotypes and polymorphisms in the immune response genes of these patients were analysed.

Materials and methods

Subjects

A total of 420 HB patients from unrelated families have been registered in Shanghai Ruijin Hospital, Beijing Children's Hospital and Hunan Xiangya Hospital since 2008 in China. Informed consent was obtained from each patient. Eleven HB patients developing inhibitors against therapeutic FIX products for prophylaxis or on demand were enrolled in our study. Forty-one HB patients with FIX activity around 1% and without inhibitors under FIX products replacement therapy [exposure days (ED) ranging from 4 to 300 days] were also involved in our study. They served as a control group to evaluate the impact of *F9* gene defects and polymorphisms in immune response genes on inhibitor formation.

Characterization of FIX inhibitors in HB patients

Nijmegen modification of Bethesda assay was performed to identify the titre of inhibitors [20]. The immunoglobulin subclass of FIX inhibitors were identified by enzyme-linked immunosorbent assay (ELISA) as previously described [21] using peroxidase-conjugated mouse monoclonal IgG to human IgG1, IgG2, IgG3 and IgG4

(Invitrogen, Carlsbad, CA, USA) respectively. Samples were assayed in duplicate. The normal absorbance range (average $\pm$ 3 SD) for each IgG subclass was obtained by analysing plasma samples from 50 healthy volunteers with 10-fold dilution. Samples with an average absorbance value higher than the value of normal average absorbance plus 3 SD were regarded as positive.

Genetic analysis of the *F9* gene

Genomic DNA was isolated from peripheral blood leucocytes using the QIAamp DNA blood purification kit (Qiagen, Venlo, The Netherlands). Genetic detection of the *F9* gene was performed by direct sequencing [22]. For patients whose partial or entire exons of *F9* gene failed to be amplified by PCR repeatedly, the AccuCopy technique was applied to detect the copy number variation (CNV) of the *F9* gene as described previously [23]. All genetic alterations were reported according to the standard international nomenclature guidelines as recommended by the Human Genome Variation Society [24].

Polymorphisms detection in *IL-4*, *IL-10*, *IL-13*, *IL-16*, *CTLA-4*, *IFN- γ* , *TNF- α* , *CD44* and *CSF1R* genes

In order to investigate the influence of SNPs in the immune response genes to the inhibitor development of Chinese HB patients, polymorphisms proved to be associated with the inhibitor developments in HA patients and SNPs on immune response genes associated with autoimmune disease with minor allele frequency more than 0.1 in Chinese population were involved in our study [13–17]. Briefly, SNPs on *IL-4*, *IL-10*, *IL-13*, *IL-16*, *CTLA-4*, interferon- γ (*IFN- γ*), *TNF- α* , *CD44* and colony-stimulating factor-1 receptor (*CSF1R*) genes were detected using PCR amplification and direct sequencing, including -590C/T on *IL-4*, -592C/A, -819C/T and -1082A/G on *IL-10*, -1112C/T and 2044G/A on *IL-13*, -295T/C on *IL-16*, -318C/T and 49A/G on *CTLA-4*, 874A/T on *IFN- γ* , -308G/A and -827C/T on *TNF- α* , 95102A/T on *CD44* and 10657586C/T on *CSF1R*. PCR primers for each reaction are listed in Table S1. The multiallelic *IL-10* microsatellite *IL-10G* was genotyped by fragment length analysis with the methods of fluorescence PCR (primers listed in Table S1) and capillary electrophoresis, and alleles were named according to the numbers of CA repeats.

Statistical analysis

Statistical analysis was performed on GraphPad Prism v5.0 (GraphPad software Inc., San Diego, CA, USA). Chi-squared tests were applied to evaluate the genotype frequencies in HB patients with and without

inhibitors. Fisher's exact test was used if theoretical frequencies of samples were <5 . All P values were two-sided, and a P value of less than 0.05 was accepted as indicating statistical significance. Odds ratio (OR) were calculated and considered to be significant if the lower value of the 95% CI was larger than 1.0.

Results

Clinical manifestations and treatment information of HB patients with FIX inhibitors

The information of HB patients with and without FIX inhibitors can be found in Table 1 and Table S2 respectively. Eleven out of 420 HB patients developed FIX inhibitors when they were exposed to prothrombin complex concentrates (PCC) or rFIX for prophylaxis (patient 10) or on demand (the other 10 patients), with an incidence of 2.6%. The immunoglobulin subclass of FIX inhibitors was identified as IgG4 in all patients. Patients 1, 2, 7, 9 and 10 received inhibitors surveillance beginning with their initial exposure to FIX products, while the other patients had their inhibitors detected only when they had a reduced response to replacement treatments. The EDs among them ranged from 6 to 1656 days. The exact time when inhibitors developed in some patients are unknown due to the lack of inhibitor surveillance. Seven patients developed high titre inhibitors (>5 BU), with five of them (patients 1, 3–6) having consecutive exposure to FIX products on demand for at least 5 days before due to urgent bleeding episodes as shown in Table 1. Patient 10 developed inhibitors during prophylactic treatment. At one point, inhibitors in patient 11 became undetectable during withdrawal time, but rebounded significantly after exposure to PCC again on demand. The other four patients developed low titre inhibitors. One of them (patient 9) had no inhibitor detected during prophylactic treatment until he was consecutively exposed to PCC for 10 days due to iliopsoas haemorrhage, leading to the development of low titre inhibitors. Patients 1, 6 and 7 suffered from allergic reactions/anaphylaxis responses to the infusion of PCC. Patient 1 developed infusion-related urticaria in the first 10 EDs to PCC and inhibitors were detected on the 15th ED. Patient 6 has had infusion-related urticaria since the age of 16 and inhibitors were detected at the age of 21 when the outcome of PCC treatment on demand was poor. Anaphylactic shock occurred in patient 7 when he received PCC treatment on demand on the 5th ED. Antishock treatments including systemic corticosteroids and antihistamines were applied and the FIX product was switched to rFIX. No anaphylactic response occurred again but the inhibitor was detected on the 15th ED.

Genotypes of the F9 gene in HB patients with and without inhibitors

A total of eight *F9* gene defects were identified in 11 HB patients with inhibitors (Table 1 and Figure S1). Five nonsense mutations (E54X, R75X, Q185X, R298X and R379X) were detected in patients 1–7, and both E54X and R75X were associated with recurrent inhibitor development in two unrelated pedigrees respectively. Large deletions in the *F9* gene were identified in patients 8–10, with complete gene deletions in patients 8 and 9 and partial gene deletions (E1–E6) in patient 10. A missense mutation S411G was found in patient 11. E54X and Q185X were novel mutations. The genotypes of the *F9* gene in 41 HB patients without inhibitors are shown in Table S2.

The impact of F9 mutation type on inhibitor development in HB

Based on the identification of the causative mutations in HB patients both with and without inhibitors, we estimated the relative risk of inhibitor development by the relative risk of each mutation type (present/absent) vs. inhibitor status (yes/no) applying a case-control analysis. Table 2 shows the estimations of the *F9* mutational odds ratio (IOR) for FIX inhibitor development. Missense mutations showed a significantly low IOR of 0.078 (CI: 0.009–0.669), while nonsense mutations presented a significantly high IOR of 8.500 (CI: 1.948–37.094). Three HB patients with large deletions developed inhibitors in our study, although non-significant difference for inhibitor development was found in the group of large deletions ($P = 0.2305$, Table 2) and in entire *F9* deletion as a single group ($P = 0.1690$, Table 2). Splice site mutations and frameshift mutations revealed a non-significant difference for inhibitor development in our study ($P = 0.6339$).

The influence of polymorphisms in immune response genes on FIX inhibitor development

Data shown in Tables 3 and 4 revealed the results of polymorphisms in immune response genes detected in HB patients both with and without inhibitors, represented as genotype and allele frequency. Within each study group the genotype distribution was consistent with those predicted by the Hardy–Weinberg equilibrium. The frequency of allele T in *CD44*⁹⁵¹⁰² (A/T) was significantly higher in patients without inhibitors (47.6%) than in patients with inhibitors (22.7%), with OR of 0.324 (CI: 0.109–0.962; $P = 0.04$). No significant difference in the distribution of the *IL10G* was observed (Table 4), but a trend for higher frequency of the CA26 repeat unit polymorphism was

Table 1. Clinical information and FXI replacement therapy regimen of 11 haemophilia B patients with FIX inhibitors.

PN	FIX: C (%)	Age of ID (year)	EDs (day)	PIT (BU)	Protein mutation HGVS (p.)	Age and treatment for first exposure	Previous treatment at inhibitor onset when inhibitor was detected	Allergic reaction	Regular regimen	
									On demand	Prophylaxis
1	<1.0	4	15	54.4	Y E54X*	4 year, PCC, 20–30 IU kg ⁻¹ qd ⁻¹ for 7 days, then 20 IU kg ⁻¹ qw ⁻¹ cephalhaematoma	See left	Y urticaria	See left	N
2	<1.0	1.5	6	4.2	Y E54X*	1.5 year, PCC, 10–20 IU kg ⁻¹ qd ⁻¹ , 2–3 day, right knee joint bleeding	1.5 year, PCC, 10–20 IU kg ⁻¹ qd ⁻¹ , 2–3 day (2rd) joint bleeding	N	PCC, 10–20 IU kg ⁻¹ qd ⁻¹ , 2–3 day, joint bleeding	N
3	<1.0	32	196	59.2	N R75X	15 year, FFP, 10 IU kg ⁻¹ qd ⁻¹ , 3d, joint bleeding	32 year, rFIX, 20 IU kg ⁻¹ qd ⁻¹ , 2 day, elbow joint bleeding	N	15–24 year, FFP, 15–20 IU kg ⁻¹ qd ⁻¹ , 3–8 day 25–29 year, PCC, 15–25 IU kg ⁻¹ qd ⁻¹ , 3–8 day 30–32 year, rFIX, 15–20 IU kg ⁻¹ qd ⁻¹ , 2–3 day 3–4 timesper year, haemorrhage of knee joint, muscle, teeth and gastrointestinal tract	N
4	1.2	2	23	6.6	N R75X	1 year, PCC, 20 IU kg ⁻¹ qd ⁻¹ , 6 day, incised wound	2 year, PCC, 20 IU kg ⁻¹ qd ⁻¹ , 3 day, right hip haematoma	N	PCC, 20 IU kg ⁻¹ qd ⁻¹ , 3–7 day, once every 2 months, knee joint bleeding, right hip haematoma	N
5	<1.0	23	48	32.0	N Q185X*	10 year, PCC, 10 IU kg ⁻¹ qd ⁻¹ , 7 day, peritoneal cavity haemorrhage	23 year, PCC/rFIX, 15 IU kg ⁻¹ qd ⁻¹ , 12 day, haematuria	N	PCC, 10 IU kg ⁻¹ qd ⁻¹ , 3 day, once per year haematuria, joint bleeding	N
6	1.0	22	250	128.0	N R298X	0.5 year, FFP, 80 IU kg ⁻¹ qd ⁻¹ , 7 day, tongue mucosa bleeding	22 year, PCC, 10 IU kg ⁻¹ qd ⁻¹ , 5 day, haematuria	Y urticaria	PCC, 10 IU kg ⁻¹ qd ⁻¹ , 2–3 day, once per every 2 month, knee joint bleeding	N
7	<1.0	3	15	4.4	Y R379X	3 year, PCC and rFIX, 20 IU kg ⁻¹ qd ⁻¹ for 5 days, then Benefix 20 IU kg ⁻¹ qd ⁻¹ for 7 days, left gastrocnemius muscle trauma haemorrhage	See left	Y anaphyl-actic shock allergic to PCC	See left	N
8	<1.0	7	1656	4.3	N E1-8del	1 year, PCC, 40 IU kg ⁻¹ qd ⁻¹ , 3 day, haemorrhage of muscle	7 year, PCC, 30 IU kg ⁻¹ qd ⁻¹ , 3 day, right thigh haematoma	N	PCC, 30 IU kg ⁻¹ qd ⁻¹ , 2–3 day, 5 times per month, haemorrhage of muscle and ankle, oral bleeding	N
9	<1.0	12	150	1.6	Y E1-8del	9 year, prophylactic treatment, PCC, 20 IU kg ⁻¹ qw ⁻¹	12 year, PCC, 20 IU kg ⁻¹ qd ⁻¹ , 10 day, iliopsoas haemorrhage	N	NO	PCC, 20 IU kg ⁻¹ qw ⁻¹
10	<1.0	4.3	16	20.0	Y E1-6del	4 year, prophylactic treatment, 20 IU kg ⁻¹ qw ⁻¹	See left	N	PCC, 20 IU kg ⁻¹ qd ⁻¹ , 2–3 day joint bleeding	PCC 20 IU kg ⁻¹ qw ⁻¹
11	<1.0	18	>50	50.0	N S411G	Not available	18 year, PCC, 15 IU kg ⁻¹ qd ⁻¹ , 3 months, knee joint synovial haemorrhage	N	PCC, 15 IU kg ⁻¹ qd ⁻¹ , 3–7 day, joint bleeding	N

FIX, factor IX; PN, patient number; FIX:C, the activity level of FIX; Age of ID, age of inhibitor detected; EDs, exposure days; PIT, peak inhibitor titre; IS, inhibitor surveillance; Y, yes; N, no; PCC, prothrombin complex concentrates; rFIX, recombinant FIX concentrates; FFP, fresh frozen plasma.

*Novel mutation.

Table 2. FIX inhibitor risks of the type of *F9* gene mutations in Chinese patients with HB.

Mutation type	HB (%)	Cases N = 11	Controls N = 41	IOR (95% CI) (%)	P-value
Missense	62.7	1	23	0.078 (0.009–0.669)	0.0200
Splicing defect	5.9	0	3	0.478 (0.023–9.953)	0.6339
Frameshift indel	7.8	0	3	0.478 (0.023–9.953)	0.6339
Nonsense	15.7	7	7	8.500 (1.948–37.094)	0.0044
All-large deletions	9.8	3	5	2.700 (0.532–13.691)	0.2305
Entire F9 deletions	3.9	2	2	4.333 (0.536–35.024)	0.1690

FIX, factor IX; HB, haemophilia B; HB (%), the mutation type frequencies in HB patients in our centres; Cases, inhibitor-positive cases; Controls, inhibitor-negative cases; IOR (95% CI), inhibitor likelihood odds ratio (mutation positive/negative), 95% confidence interval; P-value, Fisher exact test P value. Statistically significant associations are shown in bold.

observed in patients with inhibitors (0.18 vs. 0.06; $P = 0.103$; OR = 3.44). No statistically significant differences in the genotype analysis for the other tested polymorphisms were found in this study (Table 3).

Discussion

FIX inhibitor development is a severe and challenging complication of FIX substitution treatment for HB that may increase morbidity and mortality, resulting in higher therapy costs. The incidence of inhibitor development in HB patients is 2.6% in our study, consistent with literature reported [2]. Four out of five patients with inhibitor surveillance had inhibitors detected within 20 EDs, with an exception of patient 9. The other six patients without inhibitor surveillance were supposed to miss the low titre inhibitor detection and the exact time when inhibitors were developed was unknown. This may be a reason that most of them (5/6) developed high titre inhibitors, as re-exposure to FIX concentrates may elicit an anamnestic response, which is common in HA [25]. Allergic reaction/anaphylaxis was temporarily associated with FIX inhibitor development in three patients, with an incidence of 27% (3/11). Anaphylaxis may result from the extracellular distribution of the small FIX protein with potential mast cell activation and an IgE-mediated hypersensitivity response due to high FIX concentration after infusion, or the complement activation by transient IgG1 antibody formation [2]. However, only IgG4 antibody was identified in our study, and the levels of serum IgE, with the half-life less than 2 days, were all within normal reference range in our patients (data not shown) as the blood samples on the day of onset were not available. All three patients had anaphylaxis before inhibitors were detected, which may indicate that patients with anaphylaxis are more likely to develop inhibitors under replacement therapy. Anaphylactic shock occurred in patient 7 on the 5th ED, emphasizing the need for a physician or haemophilia nurse with first-aid equipment to be present during the first 20 FIX products exposures [9]. The anaphylaxis disappeared upon antishock treatments and did not re-occur when switching PCC to rFIX, indicating that HB patients may be

allergic to particular types of FIX products instead of all.

In this study, no statistical significance was found in large deletions as the risk factors for inhibitor development in HB patients. Nonsense mutations conferred a significantly high risk, while missense mutations produced the lowest risk for inhibitor development. About 71.4% (5/7) of patients with nonsense mutations also had high titre inhibitors. The data for large deletions (33.3%) and missense mutations (100%) could not be taken into consideration because of the relatively small number of patients. According to FIX mutation database, a total of 57 nonsense mutations were identified, summarized in Table S3 (www.factor-ix.org). Six nonsense mutations (S49X, R75X, Q237X, W240X, R294X and R298X) are related to inhibitor development and three of them (R75X, R294X and R298X) are located in CpG-rich region. In our study, R379X (located in CpG-rich region) and two novel mutations (E54X and Q185X) were also associated with inhibitor development, in addition to R75X and R298X. So far, only two missense mutations (Q237K and S411G) were reported to be linked to inhibitor development in HB [26,27]. S411 is one of the catalytic triad (His267-Asp315-Ser411) of activated FIX (FIXa) and plays an important role in coagulation FX activation [28]. The S411G mutation accounted for severe HB in the reported case with FIX antigen (FIX:Ag) level of approximately 20% ($0.86 \mu\text{g mL}^{-1}$) and inhibitor titre of 0.8 BU [26]. The inhibitor titre of less than 0.5 BU and FIX:Ag level of 15% (data not shown) during withdrawal time in our patient indicated that inhibitor development was not due to the complete absence of the FIX protein. The S411G mutation leads to an amino acid change from hydrophilic to hydrophobic, which may result in the conformational change in the FIX protein, thus changing the immunogenicity of this molecule and leading to inhibitor formation following FIX replacement therapy. This suggests that S411G mutation produces inhibitors specific for an epitope found on normal FIX, which is similar to the FVIII inhibitors caused by specific F8 missense mutations [29–32]. Since FIX is a less immunogenic protein than FVIII for its considerable conservation of amino acid

Table 3. Genotype and allele frequencies of 14 single nucleotide polymorphisms in Chinese haemophilia B patients with and without inhibitors.

Variant	Genotype	Inhibitor negative <i>n</i> (%)	Inhibitor positive <i>n</i> (%)	<i>P</i> -value (2 df)	Logistic regression OR (95% CI)	<i>P</i> -value
<i>CTLA-4</i> (-318C/T) rs5742909	C/C	30 (73.2)	7 (63.6)	0.566	1.00 (reference)	
	C/T	10 (24.4)	3 (27.3)		1.286 (0.278–5.939)	0.75
	T/T	1 (2.4)	1 (9.1)		4.286 (0.238–77.216)	0.32
	C	70 (85.4)	17 (77.3)	0.348*		
<i>CTLA-4</i> (49A/G) rs231775	T	12 (14.6)	5 (22.7)		1.716 (0.532–5.529)	0.37
	G/G	16 (39.0)	7 (63.6)	0.343	1.00 (reference)	
	A/G	18 (43.9)	3 (27.3)		0.211 (0.084–1.726)	0.38
	A/A	7 (17.1)	1 (9.1)		0.327 (0.034–3.179)	0.33
<i>IL-4</i> (-590C/T) rs2243250	G	50 (60.9)	17 (77.3)	0.156		
	A	32 (39.1)	5 (22.7)		0.460 (0.154–1.369)	0.16
	T/T	23 (56.1)	7 (63.6)	0.898	1.00 (reference)	
	C/T	14 (34.1)	3 (27.3)		0.704 (0.156–3.177)	0.65
<i>IL-10</i> (-592C/A) rs1800872	C/C	4 (9.8)	1 (9.1)		0.821 (0.078–8.604)	0.87
	T	60 (73.2)	17 (77.3)	0.697		
	C	22 (26.8)	5 (22.7)		0.802 (0.264–2.435)	0.70
	A/A	22 (53.7)	5 (45.5)	0.551	1.00 (reference)	
<i>IL-10</i> (-819C/T) rs1800871	C/A	16 (39.0)	4 (36.4)		1.100 (0.254–4.756)	0.90
	C/C	3 (7.3)	2 (18.2)		2.933 (0.383–22.463)	0.30
	A	60 (73.2)	14 (63.6)	0.381		
	C	22 (26.8)	8 (36.4)		1.558 (0.575–4.222)	0.38
<i>TNF-α</i> (-827C/T) rs1799724	T/T	22 (53.7)	5 (45.5)	0.339	1.00 (reference)	
	C/T	17 (41.4)	4 (36.4)		1.035 (0.241–4.454)	0.96
	C/C	2 (4.9)	2 (18.2)		4.400 (0.494–39.210)	0.18
	T	61 (74.4)	14 (63.6)	0.318		
<i>IL-13</i> (-1112C/T) rs1800925	C	21 (25.6)	8 (36.4)		1.660 (0.610–4.513)	0.32
	C/C	31 (75.6)	10 (90.9)	0.512	1.00 (reference)	
	C/T	8 (19.5)	1 (9.1)		0.388 (0.043–3.489)	0.40
	T/T	2 (4.9)	0 (0)		0.600 (0.027–13.529)	0.75
<i>IL-13</i> (-1112C/T) rs1800925	C	70 (85.4)	21 (95.5)	0.291*		
	T	12 (14.6)	1 (4.5)		0.278 (0.034–2.263)	0.23
	C/C	29 (70.7)	10 (90.9)	0.383	1.00 (reference)	
	C/T	11 (26.8)	1 (9.1)		0.264 (0.030–2.308)	0.23
<i>IL-13</i> (2044G/A) rs20541	T/T	1 (2.5)	0 (0)		0.937 (0.035–24.819)	0.97
	C	69 (84.1)	21 (95.5)	0.168*		
	T	13 (15.9)	1 (4.5)		0.253 (0.031–2.047)	0.20
	C/C	18 (43.9)	3 (27.3)	0.313	1.00 (reference)	
<i>IFN-γ</i> (874A/T) rs2430561	C/T	20 (48.8)	8 (72.7)		2.400 (0.551–10.457)	0.24
	T/T	3 (7.3)	0 (0)		0.755 (0.032–18.082)	0.86
	C	56 (68.3)	14 (63.6)	0.679		
	T	26 (31.7)	8 (36.4)		1.231 (0.459–3.297)	0.68
<i>IL-16</i> (-295T/C) rs4778889	T/T	35 (85.4)	7 (63.6)	0.157	1.00 (reference)	
	A/T	5 (12.2)	4 (36.4)		4.000 (0.853–18.753)	0.08
	A/A	1 (2.4)	0 (0)		1.578 (0.058–42.611)	0.79
	T	75 (91.5)	18 (81.8)	0.239*		
<i>IL-16</i> (-295T/C) rs4778889	A	7 (8.5)	4 (18.2)		2.381 (0.629–9.019)	0.20
	T/T	27 (65.9)	6 (54.5)	0.275	1.00 (reference)	
	C/T	10 (24.4)	5 (45.5)		2.250 (0.560–9.043)	0.25
	C/C	4 (9.7)	0 (0)		0.470 (0.022–9.869)	0.63
<i>IL-10</i> (-1082A/G) rs1800896	T	64 (78.0)	17 (77.3)	1.000*		
	C	18 (22.0)	5 (22.7)		1.046 (0.339–3.224)	0.94
	T/T	34 (82.9)	8 (72.7)	0.424*	1.00 (reference)	
	C/T	7 (17.1)	3 (27.3)		1.821 (0.384–8.637)	0.45
<i>TNF-α</i> (-308G/A) rs1800629	T	75 (91.5)	19 (86.4)	0.438*		
	C	7 (8.5)	3 (13.6)		1.692 (0.400–7.163)	0.48
	G/G	35 (85.4)	8 (72.7)	0.616	1.00 (reference)	
	A/G	4 (9.8)	2 (18.2)		2.187 (0.339–14.095)	0.41
<i>CD44</i> (95102A/T) rs927335	A/A	2 (4.8)	1 (9.1)		2.187 (0.176–27.197)	0.54
	G	74 (90.2)	18 (81.8)	0.275*		
	A	8 (9.8)	4 (18.2)		2.056 (0.557–7.589)	0.28
	A/A	13 (31.7)	6 (54.5)	0.121	1.00 (reference)	
<i>CSF1R</i> (10657586C/T)	A/T	17 (41.5)	5 (45.5)		0.637 (0.159–2.557)	0.52
	T/T	11 (26.8)	0 (0)		0.090 (0.005–1.781)	0.11
	A	43 (52.4)	17 (77.3)	0.036		
	0054	39 (47.6)	5 (22.7)		0.324 (0.109–0.962)	0.04
<i>CSF1R</i> (10657586C/T)	C/C	25 (61.0)	6 (54.5)	0.844	1.00 (reference)	
	C/T	14 (34.1)	4 (36.4)		1.190 (0.287–4.946)	0.81

Table 3. (continued)

Variant	Genotype	Inhibitor negative <i>n</i> (%)	Inhibitor positive <i>n</i> (%)	<i>P</i> -value (2 df)	Logistic regression OR (95% CI)	<i>P</i> -value
rs17725712	T/T	2 (4.9)	1 (9.1)	0.580	2.083 (0.161–26.963)	0.57
	C	64 (78.0)	16 (72.7)			
	T	18 (22.0)	6 (27.3)		1.333 (0.455–3.903)	0.60

CTLA-4, cytotoxic T-lymphocyte antigen-4; IFN- γ , interferon- γ ; IL-10, interleukin-10; TNF- α , tumour necrosis factor- α ; CSF1R, colony-stimulating factor-1 receptor.

Statistically significant associations are shown in bold.

*Fisher's exact test was used as theoretical frequencies of sample in chi-square test were <5.

Table 4. The allele frequencies of IL-10.G in Chinese haemophilia B patients with and without inhibitors.

Variant	Allele	Inhibitor negative <i>n</i> (%)	Inhibitor positive <i>n</i> (%)	<i>P</i> -value (2 df)	Logistic regression OR (95% CI)	<i>P</i> -value
IL10.G	21	43 (52.4)	10 (45.5)	0.725		
	22	13 (16.0)	3 (13.6)		0.992 (0.237–4.153)	0.992
	25	7 (8.5)	2 (9.1)		1.229 (0.221–6.830)	0.814
	26	5 (6.1)	4 (18.2)		3.440 (0.780–15.172)	0.103
	17	4 (4.9)	2 (9.1)		2.150 (0.344–13.424)	0.413
	20	4 (4.9)	1 (4.5)		1.075 (0.108–10.688)	0.951
	24	2 (2.4)	0 (0.0)		0.829 (0.037–18.582)	0.906
	27	2 (2.4)	0 (0.0)		0.829 (0.037–18.582)	0.906
	23	2 (2.4)	0 (0.0)		0.829 (0.037–18.582)	0.906

CI, confidence interval; OR, odds ratio.

sequence with other vitamin K-dependent clotting factors [9,33], the mechanism of inhibitor development associated with missense mutations of the *F9* gene needs to be further explored.

Allele T in *CD44*⁹⁵¹⁰² (A/T) played a protective role against inhibitor development for HB patients in the current study, however, this allele was also identified to be associated with increased risk of inhibitor development in HA patients [17]. This may be due to the different mechanisms of inhibitor development between HA and HB. Higher frequencies of -1082G, -819T and -592A and an allele with 134 bp in one of the CA repeat microsatellites (*IL-10G*) in *IL-10* have been confirmed in the earlier published data in inhibitor-positive HA patients [12–14]. However, no significant differences were found in these alleles in our study.

Our study is limited to a relatively small study cohort, due to the low prevalence of HB and the low incidence of FIX inhibitors [2]. The small study group may lead to some bias in the statistical analysis. Further studies encompassing a larger number of HB patients will be needed to increase the statistical power of these potential associations.

In conclusion, this study provides an in depth analysis of 11 HB patients with inhibitors. Nonsense mutations confer a higher risk for the development of inhibitors, while allele T in *CD44*⁹⁵¹⁰² (A/T) might play a protective role against inhibitor development in

Chinese HB patients. The association of inhibitor development to the missense mutations of the *F9* gene implies that inhibitor development is not, as suggested before, exclusively restricted to large deletions and nonsense mutations.

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Authorship contributions

JY Zhou, QL Ding, ZP Chen, HB Yang, L Lin, HL Wang, XF Wang and RH Wu designed the experiments. JY Zhou, ZP Chen, HB Yang and L Lin performed the experiments and analysed the data. JY Zhou, QL Ding and RH Wu wrote the manuscript. XF Wang, QL Ding, RH Wu and HL Wang directed the study. All authors discussed the results and reviewed the manuscript.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The results of direct sequencing and copy number variation (CNV) detection of F9 gene in HB patients with inhibitors. (a–e) Five nonsense mutations identified in seven HB patients with inhibitors (patient 1–7). (f) Missense mutation identified in patient 11. (g) CNV results of F9 gene in patient 8–10.

Table S1. PCR primers for SNPs and IL-10.G genotyping in immune response genes.

Table S2. Forty-one HB patients with no inhibitors enrolled in our study.

Table S3. Inhibitor status in nonsense mutations in HB patients according to the HB database.

180 190

A T T G G A A G T T T C T

Chromatogram showing a DNA sequence. The sequence is G A G G A C G A G A G T T. The peaks are color-coded: green for A, blue for C, black for G, and red for T. The sequence is divided into two segments by a vertical line, with positions 240 and 250 marked above the sequence.

A Sanger sequencing chromatogram showing a DNA sequence. The sequence is A T G T G A T T C A A T T T A T T. A black arrow points to the 'C' at position 110, which is labeled '110 G'.

Chromatogram showing a DNA sequence. The sequence is T C A A G G A G T A G T G G. The peaks are color-coded: red for T, blue for C, green for A, and black for G. An arrow points to the 10th position, which is an 'A' peak.

[illegible]

Table S1 PCR primers for SNPs and IL-10.G genotyping in immune response genes

No.	dbSNP	SNP	5' Flanking reference seq	SNP	3' Flanking reference seq
1	rs5742909	<i>CTLA-4</i> (-318C/T)	CACTTAGTTATCCAGATCCT	C/T	AAAGTGAACATGAAGCTTCA
2	rs231775	<i>CTLA-4</i> (49A/G)	AGGCTCAGCTGAACCTGGCT	A/G	CCAGGACCTGGCCCTGCACT
3	rs2243250	<i>IL-4</i> (-590C/T)	TAAACTTGGGAGAACATTGT	C/T	CCCCAGTGCTGGGGTAGGAG
4	rs1800872	<i>IL-10</i> (-592C/A)	CATCCTGTGACCCGCTGT	C/A	CTGTAGGAAGCCAGTCTCTG
5	rs1800871	<i>IL-10</i> (-819C/T)	CCCTTGACAGGTGATGTAA	C/T	ATCTCTGTGCCTCAGTTTGC
6	rs1799724	<i>TNF-α</i> (-827C/T)	GTATGGGGACCCCCCTTAA	C/T	GAAGACAGGGCCATGTAGAG
7	rs1800925	<i>IL-13</i> (-1112C/T)	CTGGAGGACTTCTAGGAAAA	C/T	GAGGGAAGAGCAGGAAAAGG
8	rs20541	<i>IL-13</i> (2044G/A)	TTTCGAAGTTTCAGTTGAAC	C/T	GTCCCTCGCGAAAAAGTTTC
9	rs2430561	<i>IFN-γ</i> (874A/T)	TGTGTGTGTGTGTGTGTGTG	A/T	GATTTGATTTTGTGTGTAA
10	rs4778889	<i>IL-16</i> (-295T/C)	AAAGCCTTTTGTTCCTATCA	C/T	AAAGAGTCAGGGTCTTCAAG
11	rs1800896	<i>IL-10</i> (-1082A/G)	TTACCTATCCCTACTTCCCC	C/T	TCCCAAAGAAGCCTTAGTAG
12	rs1800629	<i>TNF-α</i> (-308G/A)	AATAGGTTTTGAGGGGCATG	A/G	GGACGGGGTTCAGCCTCCAG
13	rs927335	<i>CD44</i> (95102A/T)	GTTGTTAAGTGCTGGTTGTT	A/T	GTTGTTAAAGTAGAGAAAAC
14	rs17725712	<i>CSF1R</i> (10657586C/T)	CCACTTGCCTAAGCCAGAAT	C/T	AGGTAAAGCACCCACCCAAA
15		<i>IL10.G</i>	FAM-AGGGGAAGGTGAAGGCTCAATC*		GTTTCTTTTCATGGAGGCTGGATAGGAG

* The former primer was 5'-labelled with 6-FAM fluorescent dye (PE Applied Biosystems, Weiterstadt, Germany)

Table S2 41 randomized HB patients with no inhibitors enrolled in our study

No	FIX:C (%)	Severity	EDs (d)	Mutation			
				Exon	Gene level	Protein level	Type
1	<1	S	32	E1	c.77>A	A26D	missense
2	<1	S	29	E2	c.127C>T	R43W	missense
3	<1	S	43	E2	c.172G>C	G58R	missense
4	1	Md	10	E2	c.228A>C	G76D	missense
5	1.6	Md	4	E4	c.349T>C	C117R	missense
6	1	Md	21	E4	c.356G>A	C119Y	missense
7	1	Md	15	E6	c.688G>A	G230R	missense
8	<1	S	28	E7	c.758G>T	G253V	missense
9	<1	S	17	E8	c.942-3TG>AT	HD314-5QY	missense
10	<1	S	32	E8	c.944A>G	D315G	missense
11	1.2	Md	15	E8	c.947T>C	I316T	missense
12	1	Md	8	E8	c.962T>C	L321P	missense
13	<1	S	26	E8	c.1025C>T	T342M	missense
14	1.8	Md	6	E8	c.1105C>T	L369F	missense
15	<1	S	35	E8	c.1219T>G	C407G	missense
16	<1	S	21	E8	c.1231A>G	S411G	missense
17	1	Md	17	E8	c.1238G>A	G413E	missense
18	1.4	Md	8	E8	c.1279G>A	G427R	missense
19	1	Md	13	E8	c.1303>C	C435R	missense
20	1.1	Md	24	E8	c.1306G>A	A436T	missense
21	<1	S	53	E8	c.1322A>G	Y441C	missense
22	1	Md	28	E8	c.1349A>G	Y450C	missense
23	<1	S	41	E8	c.1361T>A	I454N	missense
24	<1	S	68	E2	c.236_239insT	E72X	nonsense
25	<1	S	39	E5	c.484C>T	R162X	nonsense
26	1	Md	27	E8	c.880 C>T	R294X	nonsense
27	<1	S	72	E8	c.880 C>T	R294X	nonsense
28	1.4	Md	50	E8	c.1018G>T	E340X	nonsense
29	1	Md	44	E8	c.1150C>T	R384X	nonsense
30	<1	S	26	E8	c.1358G>A	W453X	nonsense
31	<1	S	37	E2	c.252 +3_+6delGAGT	IVS2 +3_+6delGAGT	Splice-site
32	<1	S	86	E2	c.252 +5G>A	IVS2 +5G>A	Splice site
33	1.3	Md	23	E5	c.519 +1G>C	IVS5 +1G>C	Splice site
34	<1	S	66	E2	c.89-93delT	D33IfsX8	framshift
35	1.5	Md	13	E5	c.488delT	A164QfsX14	framshift
36	<1	S	42	E7	c.813delT	G272VfsX14	framshift
37	1.9	Md	27		E1-3del		large deletion
38	<1	S	89		E2-3del		large deletion
39	<1	S	64		E2-4del		large deletion

40	<1	S	300	E1-8del	large deletion
41	<1	S	154	E1-8del	large deletion

FIX:C, activity level of FIX; S, severe; Md, moderate; EDs, exposure days.

Table S3 Inhibitor status in nonsense mutations in HB patients according to the HB database (www.factorix.org)

Protein mutation	Gene mutation	Severity	Domain	Exon	CpG	TN	IPN	INN	ND/NR
HGVS (p.)	HGVS (c.)								
L20X	c.59T>A	S	SiP	1	N	1	0	0	1
G21X	c.61G>T	S	SiP	1	N	1	0	0	1
C28X	c.84T>A	S	SiP	1	N	1	0	0	1
K45X	c.133A>T	S	ProP	1	N	1	0	0	1
S49X	c.146C>G	S	Gla	2	N	1	1	0	0
Q57X	c.169C>T	S	Gla	2	N	2	0	0	2
E72X	c.214G>T	S	Gla	2	N	3	0	0	3
E73X	c.217G>T	S	Gla	2	N	1	0	0	1
R75X	c.223C>T	S/Md	Gla	2	Y	73	8	5	60
E76X	c.226G>T	S	Gla	2	N	1	0	0	1
E79X	c.235G>T	S	Gla	2	N	1	0	0	1
W88X	c.263G>A	S/md	Gla	3	N	3	0	0	3
K89X	c.265A>T	-	Gla	3	N	1	0	0	1
Q96X	c.286C>T	S	EGF-1	4	N	3	0	0	3
C108X	c.324C>A	S	EGF-1	4	N	1	0	0	1
C117X	c.351T>A	Mi	EGF-1	4	N	1	0	0	1
W118X	c.353G>A	S/Md	EGF-1	4	N	6	0	0	6
C119X	c.357T>A	S	EGF-1	4	N	1	0	0	1
C141X	c.423C>A	S/Md	EGF-2	5	N	8	0	0	8
E142X	c.424G>T	S	EGF-2	5	N	1	0	0	1
Q143X	c.427C>T	S	EGF-2	5	N	1	0	0	1
C157X	c.471T>A	S	EGF-2	5	N	1	0	0	1
G160X	c.478G>T	S	EGF-2	5	N	1	0	0	1
Y161X	c.483T>A	S	EGF-2	5	N	1	0	0	1
Q167X	c.499C>T	S	EGF-2	5	N	1	0	0	1
G179X	c.535G>T	S	Linker	6	N	1	0	1	0
Q237X	c.709C>T	S	SP	6	N	4	1	0	3
W240X	c.719G>A	S/Md	SP	6	N	7	1	0	6
Q241X	c.721C>T	S	SP	6	N	1	0	0	1
G253X	c.757G>T	S/-	SP	7	N	2	0	0	2
E291X	c.871G>T	-	SP	8	N	1	0	0	1
R294X	c.880C>T	S/Md	SP	8	Y	70	4	8	58
R298X	c.892C>T	S/Md	SP	8	Y	63	1	11	51
E320X	c.958G>T	S	SP	8	N	1	0	0	1

E323X	c.967G>T	S	SP	8	N	1	0	1	0
L325X	c.974T>G	S	SP	8	N	1	0	0	1
Y330X	c.990C>A	S/Md	SP	8	N	4	0	0	4
Y341X	c.1023C>A	S	SP	8	N	2	0	0	2
G349X	c.1045G>T	Mi/S	SP	8	N	3	0	0	3
W356X	c.1067G>A	S	SP	8	N	4	0	1	3
G357X	c.1069G>T	S	SP	8	N	2	0	0	2
Q370X	c.1108C>T	S/Md	SP	8	N	3	0	0	3
R379X	c.1135C>T	S/Md/Mi	SP	8	Y	65	0	58	7
C382X	c.1146T>A	S	SP	8	N	1	0	0	1
R384X	c.1150C>T	S/Md	SP	8	Y	5	0	3	2
Y391X	c.1173T>A	S	SP	8	N	1	0	0	1
C396X	c.1188T>A	S	SP	8	N	1	0	0	1
S406X	c.1217C>G	S/Md	SP	8	N	3	0	0	3
G409X	c.1225G>A	S	SP	8	N	1	0	0	1
G413X	c.1237G>T	-	SP	8	N	1	0	0	1
E420X	c.1258G>T	S/Md	SP	8	N	3	0	0	3
L425X	c.1274T>G	S/-	SP	8	N	2	0	0	2
W431X	c.1293G>A	S/Md/-	SP	8	N	9	0	0	9
E434X	c.1300G>T	S/-	SP	8	N	2	0	0	2
Y441X	c.1323T>G	S	SP	8	N	1	0	0	1
Y450X	c.1350T>G	S	SP	8	N	1	0	1	0
W453X	c.1358G>A	S/-	SP	8	N	2	0	1	1

S, severe; Md, moderate; Mi, mild; -, not recorded; Sip, signal peptide; Prop, propeptide; SP, serine protease; TN, total number; IPN, inhibitor positive number; INN, inhibitor negative number; ND/NR, not detected/ not reported; Inhibitor related nonsense mutations are shown in bold.