Inhibitor development in nonsevere hemophilia A
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Chapter 3

The Fc gamma receptor Ila R131H polymorphism is associated with inhibitor development in severe hemophilia A


ABSTRACT

Background
The development of factor VIII neutralizing alloantibodies (inhibitors) is a major complication of the treatment with factor VIII concentrates in hemophilia A and the etiology is still poorly understood. The low-affinity Fc gamma receptors (FcγR), which are expressed on immune cells, provide an important link between cellular and humoral immunity by interacting with IgG subtypes. Genetic variations of the genes encoding FcγRs (FCGR genes) have been associated with susceptibility for infectious and autoimmune diseases.

Objectives
The aim of this study was to investigate the association between genetic variation of FCGR and inhibitor development in severe hemophilia A.

Patients/Methods
In this case-control study samples of 85 severe hemophilia A patients (siblings from 44 families) were included. Single nucleotide polymorphisms and copy number variation of the FCGR2 and FCGR3 gene cluster were studied in an FCGR-specific multiplex ligation-dependent probe amplification assay. Frequencies were compared in a generalized estimating equation regression model.

Results
Thirty-six patients (42%) had a positive history of inhibitor development. The polymorphism 131R>H in the FCGR2A gene was associated with an increased inhibitor risk (Odds Ratio [OR] per H-allele, 1.8; 95% confidence interval [CI], 1.1-2.9). This association persisted in 29 patients with high titer inhibitors (OR per H-allele, 1.9; CI, 1.2-3.2) and in 44 patients with the F8 intron 22 inversion (OR per H-allele, 2.6; CI, 1.1-6.6).

Conclusions
Hemophilia A patients with the HH genotype of the FCGR2A polymorphism 131R>H have a more than threefold increased risk for inhibitor development compared to patients with the RR genotype.
INTRODUCTION

Bleedings that occur in patients with the congenital bleeding disorder hemophilia A can be treated by administration of the deficient coagulation factor: factor VIII. However, in about 25-35% of the patients with severe disease treatment is complicated by the development of factor VIII neutralizing alloantibodies (inhibitors).1,2 Treatment of bleedings in patients with inhibitors remains a great challenge resulting in increased morbidity.3 Eradication of inhibitors is classically attempted by repeated administration of high dose therapeutic factor VIII (immune tolerance induction), which is very expensive and only successful in about 70% of the patients.4 So far, it is unclear why some patients develop inhibitors whereas others become tolerant towards therapeutic factor VIII. Better understanding of the pathophysiology of inhibitor development may help to identify targets for preventive strategies in high risk patients.

The development of inhibitors involves a CD4+ T cell dependent polyclonal IgG response, with IgG1 and IgG4 as dominant subclasses.5,6 The interaction between CD4+ T cell and B cells is fundamental for the immune response to occur and initiates expansion and differentiation of B cells and also triggers isotype switching and affinity maturation of antibodies.7

The immune response elicited by factor VIII administration is believed to result from an interplay of genetic predisposition and the immunological challenge that specific treatment regimens form, such as type of factor VIII products and intensity of treatment.8 Genetic predisposition is primarily determined by the type of F8 mutation, with the highest risk for those patients carrying null-mutations – mutations that cause complete absence of the protein.9 Other genetic risk factors outside the F8 gene are located in immunoregulatory genes, such as HLA class II complex variations and single nucleotide polymorphisms (SNPs) in the genes encoding for IL-1, IL-2, IL-10, TNF-α, and CTLA-4.10-16

This study is the first analysis of the association between inhibitor development in hemophilia A and the genes encoding for Fc receptors for IgG (Fcγ receptors). Fcγ receptors (FcγR) are glycoproteins that are widely expressed throughout the hematopoietic system. They provide an important link between cellular and humoral immunity by binding to the Fc portion of the IgG subtypes (IgG1-4). The low-affinity Fcγ receptors – FcγRIla, FcγRIlb, FcγRIlc, FcγRIllla and FcγRIllb – modulate both pro- and anti-inflammatory responses and differ in their affinity for antibody Fc-fragment and in the signalling pathways they induce (activating of inhibitory). They are encoded by the FCGR genes – FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B – clustered on chromosome 1q23-24.17 Allelic variants of FcγRs are common. However, in certain environmental and genetic contexts they may lead to a more susceptible phenotype. Copy number variation (CNV) and SNPs within the FcγR gene alter the balance between activating and inhibitory
receptor signalling – influencing the susceptibility to, or outcome of, autoimmune/inflammatory diseases.\textsuperscript{18}

The aim of this study was to evaluate whether CNV and polymorphisms in the FCGR2 and FCGR3 gene confer susceptibility to inhibitor development in a unique cohort of siblings with severe hemophilia A.

\section*{MATERIALS AND METHODS}

\subsection*{Patients}

Blood samples from a previously described cohort of hemophilia A patients (Malmö International Brother Study [MIBS]) were available for analysis.\textsuperscript{19} Approval for the study was obtained from the Lund University Institutional Review Board. Written informed consent was obtained from all participants or parents/guardians of participating children according to the Declaration of Helsinki. To obtain a clinical homogenous group of patients, we restricted our analysis to severe hemophilia A patients, defined as baseline plasma factor VIII activity (FVIII:C) < 0.01 IU mL\textsuperscript{-1}.

\subsection*{Data collection and definitions}

The following data were available from the MIBS database: date of birth, ethnicity, severity, F8 gene mutation, inhibitor history including titer at inhibitor detection and peak titer in Bethesda Inhibitor Assay Unit per mL (BU mL\textsuperscript{-1}). A positive inhibitor was defined as having at least two positive Bethesda inhibitor assay titers of ≥ 1.0 BU mL\textsuperscript{-1}. A high titer inhibitor was defined as a historical peak titer of > 5.0 BU mL\textsuperscript{-1}.

\subsection*{DNA extraction}

Genomic DNA was isolated from blood samples with the QIAamp DNA Blood Kit (QIAGEN N.V., Venlo, the Netherlands). DNA was eluted in distilled water to a final concentration of 20 ng μL\textsuperscript{-1}.

\subsection*{Multiplex ligation-dependent probe amplification}

The multiplex ligation-dependent probe amplification (MLPA) method was designed as an alternative for the Southern blot (RLFP) method to detect abnormal copy numbers of the FCGR2A, -2B, -2C, -3A and -3B genes and to detect the presence of known (functional) SNPs in one multiplex assay with 50 different targets (J. Schouten, MRC-Holland, Amsterdam, The Netherlands).\textsuperscript{20,21}

MLPA probes were designed for the FCGR2A, FCGR2B, FCGR2C, FCGR3A, and FCGR3B genes. At least 3 probes per gene were designed to study CNV and partial insertions.
or deletions. Separate probes were designed to study the following polymorphisms and haplotypes: FCGR2A (131H/R), FCGR2B (232I/T), FCGR2C (exon 3 ORF/STOP), FCGR3A (158W/F), and FCGR3B (HNA1a/HNA1b). For location and the specific target sequences of the probes we refer to the publication of Breunis and colleagues. The MLPA assay was validated by DNA of well-typed individuals with respect to CNV and SNPs. The MLPA assay in this study was performed as reported previously.

In brief, 5 μL of DNA (20 ng μL⁻¹) was denatured at 98°C for five minutes and then cooled to 25°C in a thermocycler with heated lid; 2.0 μL probe mix and 1.5 μL buffer were added to each sample and incubated for one minute at 95°C, followed by 16 hours at 60°C.

Thereafter, 32 μL of ligase-65 mix was added at 54°C, followed by 15 minutes incubation at the same temperature and five minutes at 98°C for heat inactivation of the Ligase-65 enzyme. After four times dilution of the ligation mixture at 4°C, 10 μL polymerase mix was added at 60°C, containing one single primer pair. Directly after adding the polymerase mix, the polymerase chain reaction (PCR) was started with the following PCR conditions: 36 cycles of 30 seconds at 95°C; 30 seconds at 60°C; 60 seconds at 72°C; followed by 20 minutes at 72°C and pause at 15°C. After the PCR, 1 μL of PCR product was mixed with 0.4 μL (GeneScan™ 500 LIZ® Size Standard, Life Technologies, Darmstadt, Germany) internal size standards and 8.6 μL deionized formamide, and incubated for 10 minutes at 90°C. The products were then separated by electrophoresis on an ABI-3130XL (Applied Biosystems, Foster City, CA).

The program Genemarker (version 1.40) was used to analyze the samples (Soft Genetics, State College, PA). Given that MLPA is a relative quantification assay, one sample was assigned as reference sample. The normalized height (or the area) of the probe amplification product of the patients sample was divided by the normalized height of the probe amplification product of the reference sample. A ratio between 0.8 and 1.2 was considered normal, below 0.8 was considered as loss in copy number, and above 1.2 as gain of copy number. For information concerning the control probes used in the standard MLPA, we refer to MRC-Holland (Amsterdam, The Netherlands).

**Statistical analyses**

Differences between the proportions of CNV and genotype/allele frequencies of SNPs were compared between patients who did and did not develop inhibitors using logistic regression with Generalized Estimating Equations (GEE) to correct for the relatedness of the observations among siblings (IBM SPSS, version 20.0). For bi-allelic SNPs an additive genetic model was assumed. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. All P values were 2-sided and a P value less than 0.05 was considered statistically significant. High titer inhibitors were evaluated separately and another subgroup analysis was performed in patients with the F8 intron 22 inversion.
RESULTS

In total, 85 Caucasian patients with severe hemophilia A were included in the study. Patients were born between 1934 and 1999. In 86% of the patients (n = 73) the F8 genotype was known and 44 patients (52%) had an F8 intron 22 inversion. Thirty-six patients (42%) had a positive history of inhibitor development, with 29 patients having a high titer inhibitor. The inhibitor patients had a median peak titer of 14.3 BU mL\(^{-1}\) (IQR, 8.0-92.8).

The patients belonged to 44 unrelated families including 38 families with two affected siblings participating in the study and one family with four siblings with hemophilia A. In the other five families no siblings were available. Inhibitor patients belonged to 27 families, of which 16 were discordant and eight were concordant for inhibitor development (three patients with high titer inhibitors had no sibling participating in the study). Inhibitor characteristics of the patients and families are summarized in Figure 1.

**FCGR gene copy number variation and inhibitor development**

The CNV for the FCGR3A gene was observed in five patients (one inhibitor positive) with three gene copies. Twelve individuals had CNV for the FCGR3B gene, five (two inhibitor positive) with three gene copies and seven (four inhibitor positive) with only a single copy. Variation in the copy number in the FCGR2C gene was observed in 16 individuals. Eight patients (five inhibitor positive) had one single copy and eight patients (two inhibitor positive) had three gene copies of the FCGR2C gene. Variation of copy number in the FCGR3A, FCGR3B or FCGR2C was not associated with inhibitor development in our cohort. Variation of copy number of the FCGR3A, FCGR3B or FCGR2C gene was not associated with inhibitor development. As expected, we did not find CNV in the FCGR2A and FCGR2B genes.

**FCGR gene SNPs and inhibitor development**

The distribution of known functional SNPs in FCGR2A, -2B, -2C, -3A and -3B was compared between inhibitor positive and inhibitor negative patients (Table 1). In inhibitor negative patients the observed distribution was comparable to the frequencies reported in the Caucasian population.

The allele frequencies of the FCGR2A for the FcγRIIa-R131 and FcγRIIa-H131 variants were equally distributed in inhibitor negative patients (both 50%) and differed significantly from the distribution in inhibitor positive patients in whom FcγRIIa-H131 was overrepresented (67%; OR per H-allele, 1.8; CI, 1.1-2.9). Thus based on the additive model, patients with the 131 RH or 131 HH genotype have a predicted 1.8 (CI, 1.1-2.9) and 3.3 fold (CI, 1.2-8.7) increase in risk for inhibitor development compared to patients with the 131 RR genotype, respectively. The overrepresentation of the FcγRIIa-H131 variant persisted when analysis was performed in the subgroup of patients with high titer inhibitors (69%; OR per H-allele, 1.9; CI,
**Figure 1.** Overview of the families included in the study
Concordant; both siblings within one family have or do not have an inhibitor; Discordant, one of the two siblings within one family has an inhibitor; No, no inhibitor; HT, high titer inhibitor; LT, low titer inhibitor; Sibs, siblings affected with hemophilia A.

**Table 1.** Genotyping results of SNPs in **FCGR2** and **FCGR3** genes in inhibitor positive and inhibitor negative patients

<table>
<thead>
<tr>
<th></th>
<th>No. of patients with severe hemophilia A</th>
<th>Inhibitor positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibitor negative (n = 49)</td>
<td>All inhibitors (n = 36)</td>
</tr>
<tr>
<td><strong>FCGR2A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>131RR</td>
<td>15 (31)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>131HR</td>
<td>19 (39)</td>
<td>14 (39)*</td>
</tr>
<tr>
<td>131HH</td>
<td>15 (31)</td>
<td>17 (47)</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>131R</td>
<td>49 (50)</td>
<td>24 (33)</td>
</tr>
<tr>
<td>131H</td>
<td>49 (50)</td>
<td>48 (67)</td>
</tr>
<tr>
<td><strong>FCGR2B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>232II</td>
<td>36 (73)</td>
<td>26 (72)</td>
</tr>
<tr>
<td>232IT</td>
<td>12 (25)</td>
<td>7 (19)</td>
</tr>
<tr>
<td>232TT</td>
<td>1 (2)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>232I</td>
<td>84 (86)</td>
<td>59 (82)</td>
</tr>
<tr>
<td>232T</td>
<td>14 (14)</td>
<td>13 (18)</td>
</tr>
</tbody>
</table>
No. of patients with severe hemophilia A

<table>
<thead>
<tr>
<th></th>
<th>Inhibitor negative (n = 49)</th>
<th>Inhibitor positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All inhibitors (n = 36)</td>
<td>High titer (n = 29)</td>
</tr>
<tr>
<td><strong>Inhibitor negative</strong></td>
<td>20 (41)</td>
<td>16 (44)</td>
</tr>
<tr>
<td></td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7 (14)</td>
<td>7 (19)</td>
</tr>
<tr>
<td></td>
<td>18 (37)</td>
<td>12 (33)</td>
</tr>
<tr>
<td></td>
<td>3 (6)</td>
<td>1 (3)</td>
</tr>
<tr>
<td><strong>Inhibitor positive</strong></td>
<td>158F (67)</td>
<td>46 (63)</td>
</tr>
<tr>
<td></td>
<td>35 (33)</td>
<td>27 (37)</td>
</tr>
</tbody>
</table>

| **FCGR3A**            | 158FF                      | 158FFF              |
| Genotype frequency    | 158VV                      | 158VF               |
|                      | 158VFF                     | 158VF               |
|                      | 158F                       | 158V                |

| **FCGR3B**            | HNA1a                      | HNA1b                |
| Genotype frequency    | HNA1aHNA1a                 | HNA1bHNA1b           |
|                      | HNA1bHNA1b                 | HNA1bHNA1b           |
|                      | HNA1bHNA1b                 | HNA1bHNA1b           |
|                      | HNA1aHNA1a                 | HNA1bHNA1b           |
|                      | HNA1aHNA1a                 | HNA1bHNA1b           |
|                      | HNA1aHNA1b                 | HNA1bHNA1b           |
| Allele frequency      | HNA1a                      | HNA1b                |

| **2C exon 3**         | Unknown                    | STOP                |
| Genotype frequency    | STOP/STOP                  | STOP/STOP/STOP      |
|                      | ORF                        | ORF/STOP/STOP       |
| Allele frequency      | ORF                        | STOP                |

Data are no. (%). Due to copy number variation, the 3-allelic variation was observed for FCGR3A-158VF and the 1-allelic and 3-allelic variation was observed for FCGR3B-HNA1aHNA1b and FCGR2C exon 3. Two Patients had nonclassical ORF haplotypes of FCGR2C, which were classified as STOP.

* P = 0.017  † P = 0.011
Figure 2. Observed odds ratios per FCGR2A -131R>H genotype for inhibitor development in patients with severe hemophilia A.

Subgroup analysis was performed within patients with high titer inhibitors and patients with the F8 intron 22 inversion.

1.2-3.2) and in patients with the F8 intron 22 inversion (81%; OR per H-allele, 2.6; CI, 1.1-6.6) (Table 1 and Figure 2).

Genotypes and allele frequencies of SNPs in the FCGR2B (232 I/T), FCGR2C (exon 3 ORF/STOP), FCGR3A (158 V/F), FCGR3B (HNA1a/HNA1b) demonstrated no significant differences between inhibitor positive and negative patients (Table 1).

DISCUSSION

This is the first study investigating the association between FCGR genes and inhibitor development in severe hemophilia A. We found that the FCGR2A polymorphism 131R>H was associated with inhibitor development, conferring a more than threefold increased risk for hemophilia A patients with the 131HH genotype. This association persisted in subgroups of high titer inhibitor patients and patients with the F8 intron 22 inversion. These findings suggest that FcyRIIa may play a role in the complex pathophysiology of immunogenicity against therapeutic factor VIII.
FCγRIIa (CD32a) is a single-chain, low-affinity receptor that includes an extracellular ligand-binding domain and an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain. The activating FCγRIIa is expressed on mononuclear phagocytes, platelets, neutrophils, macrophages and dendritic cells, but not on lymphocytes. It has several cell-type specific functions including initiation of the respiratory burst, degranulation, cytokine production, and phagocytosis.

FCγRIIa has two codominantly expressed alleles, R131 and H131, which differ at amino acid position 131 in the extracellular domain. These allelic variants vary substantially in their ability to bind IgG. FCγRIIa-H131 has a higher binding affinity than FCγRIIa-R131 for IgG1 and IgG2. The genotype distribution of FCγRIIa in white populations follows the Hardy-Weinberg equilibrium: ~25% homozygous R131, ~50% heterozygous, and ~25% homozygous H131. Several investigations have shown the clinical importance of allelic variants of FCγRIIa in various infectious and autoimmune diseases; but it has not previously been associated with development of alloimmunity. The overrepresentation of FCγRIIa-H131 allele in inhibitor-positive patients raises the question how this SNP may be involved in inhibitor development.

A possible pathophysiologic mechanism by which FCγRIIa influences the development and affinity maturation of inhibitors could be by altering the costimulatory signals that drive T cell and sequential B cell responses to factor VIII. Concomitant immune responses involving immune complexes that are present at the time factor VIII neutralizing antibodies are formed can be such a co-stimulatory signal. Alternatively, non-inhibiting antibodies against factor VIII could play a role in shaping this immune response. It is known that inhibitor development is a complex polyclonal response that, apart from neutralizing antibodies, also involves non-inhibiting antibodies against factor VIII, which are widespread and are found in the healthy population at a high prevalence of 19%. Because FCγRIIa is not expressed on either T or B cells, a role for this receptor on antigen-presenting cells seems most logical. Ligation of FCγRIIa has previously been shown to increase the production of pro-inflammatory cytokines such as IL-1β by dendritic cells stimulated with TLR ligands. Current hypothesis on inhibitor pathogenesis suggests that exposure to factor VIII concentrates in the presence of inflammation - caused by injured tissue from surgery or trauma - may provoke the development of inhibitors, suggesting that co-stimulatory signals indeed have an impact on the development of neutralizing antibodies. Still, the precise mechanism by which FCγRIIa-H131 influences this multifactorial process is yet unclear and needs further investigation.

Limitations
Genetic susceptibility for inhibitor development may result from a combination of SNPs and other allelic variants in genes involved in immune regulation. For this study we used blood
samples of the MIBS cohort; this cohort was previously used to demonstrate other genetic associations between allelic variants of other genes and inhibitor development (e.g. TNFA and IL10). As these genetic variants are not located near the FCGR locus we do not expect linkage disequilibrium with these polymorphisms to have confounded the association between FcγRIIa-H131 and inhibitor development.

Recent findings have demonstrated linkage disequilibrium of FcγRIIa-H131 with other genetic variations in the FCGR gene locus, i.e. FcγRIIa-V158 and FcγRIIc-ORF (Nagelkerke & Kuijpers; data submitted). Although the association with FcγRIIa-H131 was the only one that reached statistical significance in our analysis, the effects of other FCGR variants or specific combinations (haplotypes) may also have contributed to the increased risk of inhibitor development. Due to the limited sample size, our analysis was underpowered to detect weak associations of genetic variations and polymorphisms that are less common. Given the present sample size and the observed minor allele frequencies (14-50%), we could detect odds ratios ranging from 2.5-2.9 with 80% power assuming an additive genetic model and a two-sided significance threshold of 0.05. However, given the exploratory character of the present study, we chose to include all known (functional) polymorphisms in the FCGR locus. Because of the exploratory character of the study, a (too liberal) nominal p-value of 0.05 was chosen as threshold for statistical significance. However, a (Bonferroni) correction for the number of SNPS tested would have been too conservative since the actual number of independent tests is lower due to the presence of linkage disequilibrium between these SNPs.

The study was based on concordant and discordant brother pairs. As the relation between family members was the same for all families (i.e. brothers) the GEE seemed to be the most appropriate model to correct for the correlatedness among observations. Moreover, the same associations were found when only one hemophilia A patient from each family was included in the analyses (data not shown).

Our study results only cover a Caucasian population and can therefore not provide further insights in the increased inhibitor risk among individuals from African-American descent. As genetic variations of FCGR genes vary substantially between races, it would be interesting to investigate our findings in a multiethnic population.

**CONCLUSION**

We found an association between FcγRIIa-H131 and inhibitor development in severe hemophilia A. Investigations are definitely needed to understand the immunological mechanism underlying the association between FcγRIIa-H131 and inhibitor risk. This will improve our understanding on the immune response to therapeutic factor VIII and may further help to develop preventive strategies.
REFERENCES


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APPENDIX: The MIBS study group consists of the following centers and investigators: J. Astermark, E. Berntorp, E. Mattson, Malmö, Sweden (coordinator); J. M. Tusell, Barcelona, Spain; N. Ciavarella, Bari, Italy; G. C. White, Chapel Hill, USA; N. P. Ewing, City of Hope, USA; J. Lusher, Detroit, USA; M. Morfini, Florence, Italy; L. Tengborn, Gothenburg, Sweden; F. Ebeling, Helsinki, Finland; J. Coudevandel, Lille, France; M. J. Inwood, London, Canada; D. DiMichele, New York, Cornell, USA; G. Tjønnfjord, Oslo, Norway; G. Mariani, Palermo, Italy; Y. Sultan, Poitiers, France; B. L. Krones, Rockville, USA; P. Petrini, S. Schulman, Stockholm, Sweden; A. Kallas, Tartu, Estonia; D. Brettler, Worcester, USA; and J. Oldenburg/H. Brackmann, Bonn/Würzburg, Deutschland.