Study of common functional genetic polymorphisms of FCGR2A, 3A and 3B genes and the risk for cryptococcosis in HIV-uninfected patients

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A pilot candidate gene association study was conducted to investigate the role of three common functional genetic polymorphisms of the low-affinity Fc gamma receptors, FCGR2A (131H/R), FCGR3A (158F/V) and FCGR3B (NA1/NA2) in Cryptococcus neoformans infections in individuals not infected with HIV. The FCGR2A 131RR and FCGR3A 158VV genotypes were over-represented [OR: 1.67 (1.05–2.63) and 2.04 (1.06–4.00), respectively] whereas the FCGR3B NA2NA2 was under-represented in patients with cryptococcosis (28% vs. 40% in controls). An analysis of haplotypes showed a significant difference in distribution between cases and controls overall and in Caucasians.

Keywords cryptococcosis, candidate gene association studies, genetic polymorphisms, haplotype analysis, Cryptococcus neoformans

Introduction

Cryptococcus neoformans is a ubiquitous pathogenic yeast that causes life-threatening infections in immunocompromised hosts, including those with AIDS and other underlying disorders such as corticosteroid treatment, transplantation, cancer and sarcoidosis [1]. In a substantial percentage of HIV-negative patients, no identifiable risk factors are observed [2]. The role of innate immunity against C. neoformans is critical, especially since alveolar macrophages first encounter inhaled pathogens and are activated to ingest and kill, partly by producing proinflammatory cytokines [3]. T-cells, macrophages, neutrophils and other cells of immune system express low affinity Fc gamma receptors, which couple humoral and cellular immunity [4].

Low affinity Fc gamma receptors are divided in three classes, I, II and III that differ by structure, IgG affinity, distribution on the hematopoietic cells and biologic functions [4]. Three low affinity receptors, FCGRIIA, FCGRIIIA and FCGRIIIB have well-characterized functional polymorphisms which have been associated with infection or auto-immunity [5]. These include two nonsynonymous single nucleotide polymorphisms (SNPs), in FCGR2A arginine (R) to histidine (H) at position 131 located in the ligand binding domain and in FCGR3A, a valine (V) to phenylalamine (F) substitution at position 158 [6]. In FCGR3B, there is a complex polymorphism of four linked amino acid substitutions in this neutrophil antigen, known as NA1 and NA2 alleles [4].

A pilot study was conducted to investigate functional polymorphisms in FCGR2A, FCGR3A and FCGR3B as possible risk factors for C. neoformans infection in HIV-negative patients compared to historical healthy controls collected from blood donors [7].

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Materials and methods

Subjects

A total of 103 incident cases of cryptococcosis were collected between 1999 and 2004 from the University of Alabama at Birmingham, Birmingham, AL; University of Southern California and Los Angeles County Hospital, Los Angeles, CA; Baylor College of Medicine, Houston, TX. Diagnosis was confirmed by either microbiological isolation of Cryptococcus neoformans or histopathology. All patients were seronegative for HIV infection. In 15 cases, underlying conditions were identified which included diabetes, fibromyalgia, leukaemia, lupus erythematosus, polycythemia vera, pulmonary emphysema, severe rheumatological disease, sarcoidosis, sarcoma, and solid organ transplantation. All but 6 patients had central nervous system and/or lung fungal infections. Sixty-seven subjects were male (64%) and the median age was 52.5 years. Seventy-eight were Caucasians, 18 African Americans, 6 Hispanic and 1 American Indian. Cases were collected under a protocol approved by the institutional review board of NCI. Healthy controls comprised 172 African Americans and 223 Caucasians residing in North America as previously published [7].

DNA extraction

Genomic DNA was isolated from peripheral blood using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Determination of the genotype for each subject was performed in duplicate with previously described methods [7–9].

FCGR2A

The previously reported polymorphism 131 H/R in the coding region of FCGR2A was determined by allele-specific restriction digestion according to methods described by Jiang et al. [10]. A mutant oligonucleotide-directed restriction site was created in the 5‘ end using the primer: GGAAAATCCCAGAAATTCT (an introduced G compared with the wild-type A). The antisense primer, CAACAGCCTGACTACCTATTACGCQG, corresponding to a sequence in the next intron, capitalizes on gene specific amplification and introduces a second BstUI restriction site that serves as an internal control for the restriction digestion. PCR amplification was performed in a 20 μl reaction with 10 ng genomic DNA, 100 ng of each primer, 200 μmol/l each dNTP, 0.5 U AmpliTaq Gold DNA polymerase (Roche, Molecular Biochemicals Rahway, NJ), and the manufacturer’s buffer. A denaturation step of 95°C for 10 minutes was followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. After BstUI digestion, samples were analyzed on a 3% agarose gel.

FCGR3A

The FCGR3A 158V/F polymorphism was determined using a nested PCR amplification of genomic DNA followed by direct sequencing [7]. A gene-specific, 1.2-kb fragment was amplified using the following sense and antisense primers, ATATTTACAGAAATGGCAGG and GACTTGGTACCCAGGTAGG, respectively, in a 25 μl reaction with 20 ng of genomic DNA, 75 ng of each primer, 200 μmol/l dNTP, and 0.25 U Taq polymerase. PCR conditions were as follows: 10 min of initiation denaturation at 95°C, followed by 35 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min. One microliter was used as a template for a nested PCR reaction with the primer pair, TCATCATAATTTGACTTCT and CTTGAGTATGGTGATGTTC, and conditions of 30 cycles of 1 min each at 95°C, 62°C, and 72°C. PCR products were purified by a 60 min incubation at 37°C with 5 U shrimp alkaline phosphatase and 1 U of exonuclease I (USB, Cleveland, OH). Enzymes were inactivated by incubation at 75°C for 15 min. Sequence analysis was performed by use of dideoxy chain termination with BigDye terminator reagent, M13 universal primers*, and run on a 3700 Genetic Analyzer (Applied Biosystems, Inc.)

FCGR3B

Two well known, functionally important polymorphic forms were determined by allele-specific PCR with the following primer pairs, NA1-sense, CTCAATGGTACAGGGTGCTC and NA1-antisense, GGCCGCTGGTGAAGATGAGGT or NA2-sense, CTCAATGGTACAGGGTGCTC and NA2-antisense, CACCTGACTTCCACTGTGTC, using a modified protocol according to Hessner et al. [11]. Fifty nanograms of genomic DNA was amplified in 50 μl reaction containing 200 μmol/l dNTP, 10 ng of each NA1 or NA20 primer pair, 5 U Taq polymerase. The PCR conditions were as follows: 30 cycles of 94°C for 1 min, 67°C for 1 min, and 72°C for 1 min. PCR products were visualized on a 3% agarose gel in duplicate.

Genotype and haplotype analysis

Testing for association was performed using Fisher’s exact test for alleles and genotypes (GraphPad Prism4.0)
Software, San Diego, CA). $P$-values, odds ratio (OR) and 95% confidence interval (CI) were calculated. Logistic regression analysis was performed for additive, dominant and recessive models. Multiple logistic regression analysis was performed in an analysis of genotypes and cryptococcosis stratified by gender and race. Association between the three polymorphisms was tested with $\chi^2$ test with 4 degrees of freedom ($3 \times 3$ table).

Haplotype frequencies were estimated using the PHASE 2.1 program [12]. The program PHASE implements a Bayesian statistical method for reconstruction of haplotypes from unphased genotype data using a Markov chain-Monte Carlo algorithm to obtain an approximate sample from the posterior distribution of the (unknown) corresponding haplotype pairs given the (known) genotypes for the individuals [12]. After entering the genotype for the three FCGR genes of each individual belonging to either cases or controls, the program reports the most probable haplotype for each individual, population haplotype frequencies and a $P$ value testing the hypothesis whether haplotype frequencies differ between cases and controls.

**Results**

The overall and Caucasian specific frequencies of $FCGR2A$, $FCGR3A$ and $FCGR3B$ genotypes in cryptococcosis cases and healthy historical controls are summarized in Table 1. Overall, both the $FCGR2A$ R allele and the $FCGR3A$ V allele were associated with an increased risk for cryptococcal infection. The finding for the $FCGR3A$ was also apparent in an analysis restricted to Caucasians only.

A non-random allelic distribution of $FCGR2A$ 131H/R polymorphism was observed with R allele being overrepresented in patients with cryptococcosis (58% vs. 49% in healthy controls; $P=0.022$). The $FCGR2A$ 131RR genotypes were overrepresented among patients with cryptococcosis (39% vs. 27% in healthy controls). An association was found between $FCGR2A$ genotypes and cryptococcosis based on the additive model $[(OR = 2.04 (1.06–4.00)]$ for the mutant (less frequent in healthy controls) V allele.

$FCGR3B$ NA2NA2 genotypes were underrepresented in patients with cryptococcosis (28% vs. 40% in healthy controls). An association was found between $FCGR3B$ genotypes based on the dominant model $[(OR = 1.64 (1.02–2.63)]$ for the mutant (less frequent in healthy controls) NA1 allele.

Similar results were observed in a multivariate logistic regression analysis (data not shown). Analysis of haplotype frequencies resulted in significant differences of their distribution between controls and patients with cryptococcosis overall ($P=0.03$) and Caucasians ($P=0.04$) individuals (Table 2). The most frequent haplotype was 131R 158F NA2 in both healthy controls and patients with cryptococcosis. The haplotypes 131R 158F NA1 and 131R 158V NA2 were overrepresented in patients with cryptococcosis (17.8% and 12.4% vs. 13% and 8.3% in healthy controls, respectively). The genotypes of $FCGR3A$ 176F/V polymorphism and alleles were associated with the genotype of $FCGR3B$ NA1/NA2 polymorphism among patients with cryptococcosis (data not shown).

**Discussion**

The results of this pilot study indicate that common genetic polymorphisms in the low affinity Fc gamma receptors could be associated with risk for $C. neoformans$ infection in individuals not infected with HIV. Our findings are notable because the common polymorphisms in $FCGR$ genes analyzed in the present study have been shown to be functionally important and clinically significant in auto-immunity and other infectious diseases [7]. We observed that $FCGR2A$ 131RR and $FCGR3A$ 158VV genotypes were associated with increased risk for cryptococcosis whereas the $FCGR3B$ NA2NA2 was associated with decreased risk for cryptococcosis. Moreover, an analysis in Caucasians only revealed a possible effect for the V allele of $FCGR3A$.

Previous association studies of HLA phenotype and susceptibility to cryptococcosis in a group of Papua New Guinean population failed to reach statistical significance although there was a trend for susceptibility linked to HLA B*5601 [13]. Thus, it is plausible that common genetic variation in immune function could be an important risk factor in susceptibility to $C. neoformans$ in individuals with alterations in immune function. It is also notable that a substantial number of subjects in our study did not have an identifiable risk factor, suggesting that there could be a complex set of factors, including variants of low
Table 1  Distribution of FCGR2A, 3A and 3B genotypes in patients with cryptococcosis and healthy controls.

<table>
<thead>
<tr>
<th>Populations</th>
<th>FCGR2A gene</th>
<th></th>
<th>FCGR3A gene</th>
<th></th>
<th>FCGR3B gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymorphism</td>
<td>Cases</td>
<td>Controls</td>
<td>Polymorphism</td>
<td>Cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n (%)</td>
<td>(%)</td>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Caucasians</td>
<td>R allele a</td>
<td>80 (56)</td>
<td>205 (47)</td>
<td>V allele a</td>
<td>56 (40)</td>
</tr>
<tr>
<td></td>
<td>HH</td>
<td>16 (22)</td>
<td>69 (31)</td>
<td>FF</td>
<td>27 (39)</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>32 (44)</td>
<td>97 (44)</td>
<td>FV</td>
<td>30 (43)</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>24 (33)</td>
<td>54 (25)</td>
<td>VV</td>
<td>13 (19)</td>
</tr>
<tr>
<td></td>
<td>X², df (P value)</td>
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<td>(0.209)</td>
<td>X², df (P value)</td>
<td>4.218, 2</td>
</tr>
<tr>
<td>Model b</td>
<td>P value</td>
<td>OR (95% CI)</td>
<td></td>
<td>Model b</td>
<td>P value</td>
</tr>
<tr>
<td>Additive</td>
<td>0.08</td>
<td>1.92 (0.93–4.00)</td>
<td></td>
<td>Additive</td>
<td>0.06</td>
</tr>
<tr>
<td>Dominant</td>
<td>0.18</td>
<td>1.58 (0.85–3.03)</td>
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<td>Dominant</td>
<td>0.12</td>
</tr>
<tr>
<td>recessive</td>
<td>0.17</td>
<td>1.54 (0.86–2.78)</td>
<td></td>
<td>recessive</td>
<td>0.09</td>
</tr>
<tr>
<td>allelic</td>
<td>0.07</td>
<td>1.45 (0.98–2.13)</td>
<td></td>
<td>allelic</td>
<td>0.04</td>
</tr>
<tr>
<td>All</td>
<td>R allele a</td>
<td>118 (58)</td>
<td>384 (49)</td>
<td>V allele a</td>
<td>70 (38)</td>
</tr>
<tr>
<td></td>
<td>HH</td>
<td>22 (22)</td>
<td>113 (29)</td>
<td>FF</td>
<td>39 (42)</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>40 (40)</td>
<td>170 (44)</td>
<td>FV</td>
<td>38 (41)</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>39 (39)</td>
<td>107 (27)</td>
<td>VV</td>
<td>16 (17)</td>
</tr>
<tr>
<td></td>
<td>X², df (P value)</td>
<td>5.178, 2</td>
<td>(0.075)</td>
<td>X², df (P value)</td>
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<tr>
<td>Model b</td>
<td>P value</td>
<td>OR (95% CI)</td>
<td></td>
<td>Model b</td>
<td>P value</td>
</tr>
<tr>
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<td>1.92 (1.06–3.57)</td>
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<td>Additive</td>
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<tr>
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<td>0.48</td>
</tr>
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<td>1.67 (1.05–2.63)</td>
<td></td>
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<td>0.04</td>
</tr>
<tr>
<td>allelic</td>
<td>0.02</td>
<td>1.44 (1.05–1.98)</td>
<td></td>
<td>allelic</td>
<td>0.11</td>
</tr>
</tbody>
</table>

aMutant (less frequent in healthy controls) alleles: R allele, V allele, and NA1 allele.
bAdditive; homozygotes (Hm) for wild allele vs. heterozygotes (Ht) vs. Hm for mutant allele, Dominant; Hm and Ht for mutant allele vs. Hm for wild allele, recessive; Hm for mutant allele vs. Ht and Hm for wild allele, allelic; Mutant alleles vs. Wild alleles.
affinity Fc gamma receptors that contribute to *C. neoformans* infection.

There is a strong rationale to investigate functionally important polymorphisms in low affinity Fc gamma receptors. Functionally important *FCGR* polymorphisms could alter *C. neoformans* phagocytosis. Capsular material of *C. neoformans* can bind directly or indirectly after opsonization to multiple cellular receptors including Fc gamma receptors [14]. Fc gamma RI and III may not be essential for natural resistance to cryptococcal infection in mice but are instrumental in IgG1- and IgG2-mediated but not in IgG3-mediated phagocytosis [15]. IgG2 antibodies are the predominant subclass of naturally occurring anti-glucuronoxylomannan (GXM) IgG antibodies in humans and mediate phagocytosis of *C. neoformans* by human peripheral blood monocytes through FCGRIIa [16]. Furthermore, the GXM-mediated immunosuppression could be ascribed to GXM recognition by Fc gamma RI [17,18]. The increased susceptibility to cryptococcosis of individuals with *FCGR2A* 131RR and *FCGR3A* 158VV genotypes could be explained by inefficient phagocytosis of IgG2 and IgG1 opsonized *C. neoformans* [15]. The protective effect of *FCGR3B* NA2NA2 genotype could be explained by inefficient binding to IgG3 opsonized *C. neoformans* which otherwise enhance pathogenicity by providing an intracellular sanctuary for fungus [15].

Published data have shown that the three common polymorphisms alter function and thus provide corroborative evidence for the observed associations. The *FCGRIIa* 131H allotype displays higher binding efficiency for human IgG2 and IgG3 compared to *FCGRIIa* 131R [6]. Since *FCGRIIa* represents the major receptor for IgG2, the capacity to effectively bind IgG2 is associated with the *FCGR2A* genotype [19]. For the *FCGR3A* 158F/V polymorphism, IgG induced NK cell activity was increased in VV donors compared to FF donors due to higher affinity of the former allotype for IgG1, IgG3 and IgG4 [9,20]. Finally, the *FCGRIIIb* NA1 allotype induces phagocytosis of IgG1 and IgG3 opsonized particles and binds immune complex IgG3 more efficiently than *FCGRIIIb* NA2 allotype [21].

A preliminary haplotype analysis showed that the haplotype frequencies were different between the controls and patients with cryptococcosis. In our healthy population the 131R 158F NA2 was the most frequent haplotype whereas it has been reported that in a Japanese population, the most frequent haplotype is 131H 158F NA1 [22].

The results of our pilot study require confirmation in a larger, prospective study. It is possible that our finding could be due to chance based on the small size of the study and the design, which used historical controls. Still, our findings provide the first evidence that alterations in the function of low affinity Fc gamma receptors could contribute to the risk for cryptococcal infection, especially in individuals with an underlying immune deficiency. Since multiple genetic risk factors are expected to be responsible for conferring susceptibility or protection to infectious diseases interacting with environmental and iatrogenic factors, further studies are required to confirm these results and to assess other genetic and non-genetic factors.

## Acknowledgements

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## References


20 Koene HR, Kleijer M, Algra I, et al. Fc gammaRIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIa, independently of the Fc gammaRIIa-48L/R/H phenotype. *Blood* 1997; 90: 1109–1114.
