Role of autophagy genetic variants for the risk of Candida infections

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Received 17 September 2013; Revised 10 December 2013; Accepted 24 December 2013

Abstract

Candida albicans can cause candidemia in neutropenic and critically ill patients and oropharyngeal candidiasis in human immunodeficiency virus (HIV)–positive patients with low CD4⁺ counts. Because all patients at risk do not develop Candida infections, it is possible that a patient’s genetic background might play a role in his or her susceptibility to infection. Autophagy mediates pathogen clearance and modulation of inflammation. Our aim was to assess the effect of genetic variations in the ATG16L1 and IRGM autophagy genes on the susceptibility of patients with candidemia and oropharyngeal candidiasis. We assessed genetic variations in the ATG16L1 and IRGM genes in a cohort of candidemia patients of both African and European origin. In addition, we evaluated the effect of these polymorphisms on the susceptibility to oropharyngeal candidiasis of an HIV-positive cohort from Tanzania. Functional studies have been performed to assess the effect of the ATG16L1 and IRGM genetic variants on both in vitro and in vivo cytokine production. The results indicate that ATG16L1 variants modulate production of tumor
necrosis factor-alpha, but not other cytokines, while no effects were seen in the presence of IRGM polymorphisms. In addition, no significant associations between the single-nucleotide polymorphisms in the ATG16L1 and IRGM genetic variants and the incidence of candidemia or oropharyngeal candidiasis were identified.

Despite moderate effects on the modulation of proinflammatory cytokine production, genetic variation in the autophagy genes ATG16L1 and IRGM has a minor impact on the susceptibility to both mucosal and systemic Candida infections.

**Key words:** HIV, Candida albicans, candidemia, autophagy, oropharyngeal candidiasis, genetic association study.

**Introduction**

*Candida albicans* is a dimorphic fungus that has a commensal relationship in immunocompetent individuals; it colonizes the skin, gastrointestinal tract, and oral and genital mucosa. Under certain host immune defense conditions, *C. albicans* can become pathogenic, being the most common human fungal pathogen [1], and it is involved in both mucosal and disseminated infections. Candidiasis is a systemic infection caused by *Candida* spp., predominantly but not limited to *C. albicans*. The infection occurs primarily in patients who are immunocompromised due to neutropenia, in critically ill patients who use broad-spectrum antibiotics, in patients who have undergone invasive surgery, and in patients who receive total parenteral nutrition [2,3]. In addition, other risk factors, such as ethnic background, might also play a role based on the genetic differences between individuals of European and African ancestry [4]. Oropharyngeal candidiasis (OPC) is a *Candida* mucosal infection that frequently occurs as an opportunistic infection in human immunodeficiency virus (HIV)-positive patients who have low CD4+ counts (<200 cells/μl) [5–7]. In the absence of proper treatment, there is a chance that the infection will spread to the bloodstream [8].

However, not all at-risk patients develop *Candida* infections, and part of the differential susceptibility may be explained by the patient’s genetic makeup, as shown by several previously published reports [9–12]. Autophagy is a highly conserved housekeeping molecular process that is important for cellular development and maintenance, energy turnover, and antigen presentation. The autophagy machinery enables the formation of double membrane vesicles, which leads to the sequestration of components of the cytoplasm into double membrane vesicles that ultimately fuse with the lysosome, leading to degradation of the autophagosomal content [13]. Furthermore, autophagy enables lysosomal digestion and antigen presentation of phagocytosed materials such as fungal and bacterial pathogens, including *Candida* spp. [14].

Previous studies have demonstrated that genetic variation in autophagy genes affects the modulation of inflam-
support the genetic analysis, functional assays were performed with peripheral blood mononuclear cells bearing different ATG16L1 and IRGM genotypes. Serum cytokine was measured by comparing the genotypes for their ability to produce cytokines when stimulated, either in vitro or in vivo, by C. albicans. Finally, a bioinformatic analysis was performed with the aim of further explaining the possible functional consequences of the genetic variants in ATG16L1 and IRGM.

**Patients and methods**

### HIV-positive patients

The effect of the polymorphisms associated with autophagy predisposition to OPC was assessed in a group of 155 HIV-seropositive patients recruited between April 2007 and August 2008 at the Muhimbili National Hospital HIV clinic in Dar-es-Salaam, Tanzania. An independent physician examined the patients according to the World Health Organization (WHO) clinical staging criteria [27]; oral examinations were routinely performed at each visit in accord with WHO standards [28]. Variations in color, size, and shape of intraoral tissue were taken as clinical signs of OPC, which was manifested as pseudomembranous candidiasis or a combination of pseudomembranous and erythematous, hyperplastic, or angular cheilitis. This cohort has been previously included in genetic association studies by our group [29]; the hospital’s ethical committee approved the study. Sampling was performed by firmly swabbing the lesion site with a sterile cotton wool swab. Immediate microbiological confirmation was performed, and the samples were sent immersed in 10% potassium hydroxide. Within this cohort, 82 patients developed OPC, while 73 did not.

### Candidemia patients

Patients were enrolled between January 2003 and January 2009 after informed consent was given (or waiver as approved by the institutional review board) at the Duke University Hospital (DUMC; Durham, NC, USA) and the Radboud University Medical Center (RUMC, Nijmegen, the Netherlands) and after the review boards from these institutions approved the investigations. To be included in the analysis of susceptibility to infection, patients had to have at least one positive Candida blood culture while hospitalized at the participating center. Noninfected controls had to have no history or evidence of candidemia/invasive candidiasis or any invasive fungal infection during their hospitalization. These individuals were recruited from the same hospital wards as infected patients such that comorbidities and clinical risk factors for infection would be similar in both groups. The clinical characteristics of the patients have been previously reported [9,24,30].

### Genetic analysis

Genomic DNA was isolated from whole blood using standard procedures. Genotyping for the ATG16L1 T300A (rs2241880) and the IRGM (rs13361189 and rs4958847) SNPs was performed using the TaqMan single-nucleotide assays C_909577_20, C_31986315_10, and C_1398968_10, respectively, in the 7300 ABI real-time polymerase chain reaction system (all from Applied Biosystems, Carlsbad, CA, USA). Two of these polymorphisms (rs2241880 and rs4958847) were also part of the Sequenom analysis (Sequenom MassARRAY®, Sequenom, San Diego, CA) used in the study by Smeekens et al. [24]. However, we chose to assess the DNA of the patient cohort in the present study using TaqMan technology due to the fact that the quality of DNA necessary for Sequenom analysis was not sufficient to evaluate all patients. The assessment of all three polymorphisms in the studies on mucosal forms of infections has not been presented elsewhere.

### Cytokine stimulation assays

Peripheral blood mononuclear cells (PBMCs) were isolated from 73 healthy volunteers by Ficoll-Paque gradient. Subsequently, the cells were stimulated with heat-killed C. albicans blastoconidia at 24 h (for IL-1β, IL-6, IL-8, and tumor necrosis factor-alpha [TNF-α]), 48 h (for interferon-gamma [IFN-γ] and IL-10), or 7 d (for IL-17). At the end of the incubation period, the above-mentioned cytokines were measured in the supernatants using enzyme-linked immunosorbent assay (purchased from R&D Systems, Minneapolis, MN, USA, or Sanquin Research, Amsterdam, the Netherlands). Cytokine production among patients bearing the different ATG16L1 or IRGM genotypes was compared.

### Serum cytokine measurements

Cytokine concentrations of IL-6, IL-8, and IFN-γ in plasma and serum samples obtained from infected patients were measured by multiplex fluorescent bead immunoassays (xMAP Technology; Bio-Rad, Veenendaal, the Netherlands) and a bio-plex microbead analyzer (Luminex, Austin, TX, USA) according to the manufacturers’ protocols at day 0 up to day 5 after initial positive blood culture.

### Bioinformatic analysis

In order to predict the effect on the conformation of the ATG16L1 protein given the T300A amino acid change,
we used HOPE (http://www.cmbi.ru.nl/hope/), a next-generation web server that performs automatic mutant analysis. No homology model was produced because no template with enough identity to the ATG16L1 sequence exists [31]. The HOPE server assessment was only possible for the ATG16L1 SNP since it is the only SNP from this study that alters the amino acid sequence and the HOPE server works exclusively in case of nonsynonymous mutations and their effect on protein function.

Statistical analysis
For genetic analysis, statistical comparisons of frequencies were made between infected (with either OPC or candidemia) and noninfected patients by performing the \( \chi^2 \) test with SPSS, version 20 (IBM SPSS Statistics for Windows, IBM Corporation, Armonk, NY, USA). For analysis of the in vitro PBMC stimulation and in vivo serum cytokine measurements, a Kruskal-Wallis one-way analysis of variance was performed using GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA, USA). Corrections for multiple testing were not performed due to the absence of significant differences; throughout the article, only uncorrected \( P \) values are given.

Results

Functional analysis of cytokine profiles
The cytokines measured in the in vitro assays were selected based on their role in the anti-Candida immune response, that is, TNF-\( \alpha \), IL-1\( \beta \), IL-6, IL-8, and IL-10 to evaluate the response of monocytes and IFN-\( \gamma \) and IL-17 as T-cell–derived cytokines to monitor the activation of the Th1 and Th17 responses, respectively. A significant difference in C. albicans–induced cytokine production was observed between cells isolated from individuals with different TNF-\( \alpha \) production ATG16L1 genotypes (\( P = 0.0039 \); Fig. 1). However, no differences were observed in the production of IL-1\( \beta \), IL-6, IL-8, IL-10, IFN-\( \gamma \), and IL-17. In addition, results of the effect of the IRGM gene genotype on the in vitro cytokine production revealed that the only statistically significant effect was associated with the rs13361189 genotype, which affected production of IL-8. Furthermore, TNF-\( \alpha \), IL-1\( \beta \), IL-6, IL-10, IFN-\( \gamma \), and IL-17 production was not affected by either of the two investigated SNPs in IRGM.

Bioinformatic analysis using the HOPE server
We also performed a bioinformatic analysis using HOPE [31]. The output indicates that the change from a threo-nine to an alanine at position 300 of the ATG16L1 protein introduces changes in amino acid size and hydrophobicity. The mutant residue is smaller and more hydrophobic than the wild type. Moreover, the server predicted that the polymorphism would cause an empty space in the protein core (or protein complex). The change in hydrophobicity between the wild-type and mutant-type amino acids would cause a loss of hydrogen bonds in the protein core (or protein complex). As a result, it would affect correct folding (Supplementary Fig. 1).

Oropharyngeal candidiasis
Distribution of the ATG16L1 and IRGM polymorphisms in the HIV-positive patient groups with or without OPC is shown in Table 1; as can be noted, no statistically significant differences were observed. Since the CD4\( ^{+} \) count is considered to be a confounder, we stratified the study subjects based on their CD4\( ^{+} \) counts. No significant association was observed in either CD4\( ^{+} \) count group (Table 2).

Disseminated candidiasis
The intergroup comparison between the Dutch RUMC and North American white DUMC controls and patients revealed a similar genetic distribution of the genotyped SNPs. This allowed us to merge the groups into a single group of subjects of European descent (data not shown). Genetic distribution of the ATG16L1 and IRGM polymorphisms in the studied patient groups is shown in Table 3. No significant associations of polymorphisms in ATG16L1 and IRGM with susceptibility to candidemia were revealed (\( P > 0.05 \)).

Serum samples collected from patients with bloodstream Candida spp. infections during the first 5 days after initial positive blood culture were measured for concentrations of IL-6, IL-8, and IFN-\( \gamma \). The results are shown in Figure 2; IL-6, IL-8, and IFN-\( \gamma \) circulating concentrations were not affected by any of the SNPs in ATG16L1 and IRGM. Other cytokines such as TNF-\( \alpha \), IL-1\( \beta \), IL-10, and IL-17 were not detectable in the serum samples (data not shown).

Discussion
Here, we evaluated the effect of polymorphisms in the autophagy genes ATG16L1 and IRGM [15,23] on host susceptibility to OPC and candidemia, respectively. Upon stimulation with Candida blastoconidia, the in vitro cytokine production capacity of primary immune cells with different ATG16L1 genotypes demonstrated a lower production of TNF-\( \alpha \) by cells bearing the ATG16L1 300A allele. On the other hand, the variant allele of IRGM rs13361189
Figure 1. Functional analysis of the stimulation of peripheral blood mononuclear cells with heat-killed *Candida albicans* blastoconidia. Cytokine production capacity of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and interferon-gamma (IFN-γ) was compared between the cells obtained from healthy volunteers bearing wild-type or variant *ATG16L1* or *IRGM* SNPs. Data are presented as means ± standard error of the mean; *P* < 0.05. For the *ATG16L1* rs2241880 polymorphism, the antisense nucleotides are depicted.
Table 1. Frequencies of the genotype of the \textit{ATG16L1} single-nucleotide polymorphism (SNP) rs2241880 and the \textit{IRGM} SNPs rs13361189 and rs4958847 and incidence of oropharyngeal candidiasis in human immunodeficiency virus–positive patients from Tanzania.

<table>
<thead>
<tr>
<th>SNP</th>
<th>OPC</th>
<th>TT* (%)</th>
<th>TC* (%)</th>
<th>CC* (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ATG16L1} rs2241880</td>
<td>Noninfected (N = 73)</td>
<td>40 (54.79)</td>
<td>29 (39.72)</td>
<td>4 (5.48)</td>
<td>0.287</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 81)</td>
<td>56 (69.14)</td>
<td>21 (25.93)</td>
<td>4 (4.94)</td>
<td></td>
</tr>
<tr>
<td>\textit{IRGM} rs13361189</td>
<td>Noninfected (N = 68)</td>
<td>14 (20.59)</td>
<td>36 (52.94)</td>
<td>18 (26.47)</td>
<td>0.818</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 77)</td>
<td>17 (22.08)</td>
<td>35 (45.46)</td>
<td>25 (32.47)</td>
<td></td>
</tr>
<tr>
<td>\textit{IRGM} rs4958847</td>
<td>Noninfected (N = 71)</td>
<td>14 (19.72)</td>
<td>39 (54.93)</td>
<td>18 (25.35)</td>
<td>0.497</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 82)</td>
<td>22 (26.83)</td>
<td>42 (51.22)</td>
<td>18 (21.95)</td>
<td></td>
</tr>
</tbody>
</table>

OPC, oropharyngeal candidiasis.
* For the \textit{ATG16L1} rs2241880 polymorphism, the antisense nucleotides are depicted.

Table 2. Minor allele frequencies of the \textit{ATG16L1} single-nucleotide polymorphism (SNP) rs2241880 and the \textit{IRGM} SNPs rs13361189 and rs4958847 stratified by CD4+ count in a cohort of human immunodeficiency virus–positive patients from Tanzania.

<table>
<thead>
<tr>
<th>Single-nucleotide polymorphism</th>
<th>&lt;100 (N = 40)</th>
<th>100–200 (N = 81)</th>
<th>200–300 (N = 26)</th>
<th>&gt;300 (N = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ATG16L1} rs2241880</td>
<td>16.67%–14.29%</td>
<td>33.33%–28.89%</td>
<td>20.00%–18.75%</td>
<td>0%–30%</td>
</tr>
<tr>
<td>\textit{IRGM} rs13361189</td>
<td>64.52%–50%</td>
<td>50%–51.22%</td>
<td>40%–53.33%</td>
<td>75%–80%</td>
</tr>
<tr>
<td>\textit{IRGM} rs4958847</td>
<td>59.09%–50%</td>
<td>40.28%–46.67%</td>
<td>35%–63.33%</td>
<td>75%–87.5%</td>
</tr>
</tbody>
</table>

\textit{CD4+} T-cell count (minor allele frequency [%] OPC positive–OPC negative). OPC, oropharyngeal candidiasis.

moderately increased IL-8 production under similar conditions. Despite these differences, the genotype of the \textit{ATG16L1} and \textit{IRGM} SNPs did not affect the susceptibility of patients to either OPC or systemic candidiasis.

Genetic variation in genes coding for autophagy regulatory proteins are known to alter the clearance of intracellular bacteria [23]. The SNP rs2241880 of \textit{ATG16L1} causes a partial loss of function in the \textit{ATG16L1} protein, inhibiting the \textit{Salmonella}-induced autophagy process, which in turn results in reduced clearance of the microorganisms in human epithelial cells [15]. In addition, a polymorphism in the promoter region of the \textit{IRGM} gene conferred an increased susceptibility to tuberculosis [32]. Twenty-one mouse IRG genes are located on chromosomes 11 and 18. These genes have been demonstrated to have a very powerful effect on the clearance of intracellular pathogens such as \textit{Listeria monocytogenes}, \textit{Toxoplasma gondii} [33,34], \textit{Mycobacterium tuberculosis} [35], \textit{Salmonella typhimurium}, and \textit{Chlamydia trachomatis} [34]. Furthermore, genetic variation in both \textit{ATG16L1} and \textit{IRGM} genes is associated with a higher susceptibility to Crohn’s disease [16–18,22]. Conversely, the role of these genetic polymorphisms in the susceptibility to fungal pathogens has yet to be studied. In fact, this is what led us to investigate whether SNPs in \textit{ATG16L1} and \textit{IRGM} are related to the propensity to develop oropharyngeal and/or systemic \textit{Candida} infections.

The effect of the T300A polymorphism on \textit{ATG16L1} function is the best characterized among the three SNPs evaluated in this study. The loss of function predicted with the HOPE server was evident in TNF-\textit{α} production, with a decrease paralleling the allelic dosage. This polymorphism is known to reduce autophagy [15]; this is supported by a previous study [36]. Nevertheless, genetic variation in the \textit{ATG16L1} gene did not influence the susceptibility to either oropharyngeal or systemic candidiasis.

In contrast to the \textit{ATG16L1} SNP, the \textit{IRGM} polymorphisms were found not to affect the \textit{IRGM} protein sequence or structure; however, they did modulate \textit{IRGM} gene expression. McCarroll and colleagues demonstrated that \textit{IRGM} haplotypes differentially affected gene expression in different cell types and that the gene expression levels affected the efficiency of autophagy for eradicating intracellular bacteria [23]. Despite the possible consequences of the variation in the two \textit{IRGM} SNPs, the genotype of
Table 3. Frequencies of the genotype of the \textit{ATG16L1} single-nucleotide polymorphism (SNP) rs2241880 and the \textit{IRGM} SNPs rs13361189 and rs4958847 and the incidence of candidemia in a cohort of hospitalized patients of African-American and European descent.

<table>
<thead>
<tr>
<th></th>
<th>African-American</th>
<th>European</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Candidemia</td>
<td>Noninfected (N = 52)</td>
</tr>
<tr>
<td></td>
<td>TT* (%)</td>
<td>28 (53.85)</td>
</tr>
<tr>
<td></td>
<td>TC* (%)</td>
<td>18 (34.62)</td>
</tr>
<tr>
<td></td>
<td>CC* (%)</td>
<td>6 (11.54)</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.303</td>
</tr>
<tr>
<td></td>
<td>Candemia</td>
<td>Infected (N = 62)</td>
</tr>
<tr>
<td></td>
<td>CC (%)</td>
<td>26 (41.94)</td>
</tr>
<tr>
<td></td>
<td>CT (%)</td>
<td>22 (35.48)</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.456</td>
</tr>
<tr>
<td></td>
<td>Candemia</td>
<td>Infected (N = 60)</td>
</tr>
<tr>
<td></td>
<td>GG (%)</td>
<td>25 (48.08)</td>
</tr>
<tr>
<td></td>
<td>AG (%)</td>
<td>7 (13.46)</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.392</td>
</tr>
<tr>
<td></td>
<td>Candemia</td>
<td>Infected (N = 149)</td>
</tr>
<tr>
<td></td>
<td>CC (%)</td>
<td>31 (20.81)</td>
</tr>
<tr>
<td></td>
<td>CT (%)</td>
<td>71 (47.65)</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.090</td>
</tr>
</tbody>
</table>

OPC, oropharyngeal candidiasis.

* For the \textit{ATG16L1} rs2241880 polymorphism, the antisense nucleotides are depicted.

Acknowledgments

D.C.R. received funding from the European Commission through the FINSysMarie Curie Initial Training Network (PITN-GA-2008-214004). T.S.P. was supported by a Veni grant from the Netherlands Organization for Scientific Research. This study was partially supported by an ATG16L1 and IRGM SNPs were generated using the National Institutes of Health grants AI51537 to M.G.N. and by the European Research Council Consolidator Grant 7396 to J.R.P. All authors had full access to all study data and take responsibility for the integrity of the data analysis.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

The observation that the investigated SNPs in \textit{ATG16L1} and \textit{IRGM} are not associated with increased susceptibility to \textit{Candida} infections but are involved in the pathogenesis of Crohn's disease may suggest that the link between \textit{Candida} colonization and Crohn's disease, through the presence of ASCA antibodies, is not mediated by autophagy. However, one must realize that the mechanisms that underlie \textit{Candida} colonization vs. \textit{Candida} infection are largely different, as recently shown by our group [32]. Therefore, although autophagy appears not to be involved in susceptibility to \textit{Candida} infection, as demonstrated in the present study, it still could be associated with \textit{Candida} colonization. Future studies are warranted to assess this aspect.

Autophagy-mediated pathogen clearance is an important process mainly for host defense against intracellular bacteria such as mycobacteria or \textit{S. typhimurium}. Whereas the role of autophagy in the clearance of extracellular bacteria such as \textit{C. albicans} remains unclear. The lack of association of the SNPs in \textit{ATG16L1} and \textit{IRGM} with susceptibility to \textit{Candida} infections implies that host defense against \textit{Candida} infections does not depend on these two genetic variants in autophagy genes. The results of these investigations strongly argue that autophagy is a redundant mechanism for host defense against both systemic and mucosal \textit{Candida} infections.
Figure 2. Cytokine concentrations of interleukin (IL)-6, IL-8, and interferon-gamma (IFN-γ) in plasma and serum samples from infected patients from day 0 to day 5 after initial positive blood culture correlated with the ATG16L1 or IRGM genotype. Data are presented as means ± standard error of the mean; * P < 0.05. HET, heterozygous; HOM, homozygous; WT, wild type.

Supplementary material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References


