

Chapter 7

Host-Derived Biomarkers for Risk Assessment of Invasive Fungal Diseases

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Abstract

Invasive fungal diseases are major complications associated with the treatment of hematologic malignancies. The integration of host-derived biomarkers into clinical processes to predict the risk and progression of fungal disease is a promising approach in immunocompromised patients. Recent insights into human antifungal immunity have highlighted the remarkable influence of host genetics in modulating susceptibility to infection. In this chapter, we describe protocols to examine human genetic variation and to assess its functional consequences using the pattern recognition receptor PTX3 as an example.

Key words Invasive fungal disease, Single nucleotide polymorphism (SNP), Host biomarkers, Long pentraxin 3 (PTX3), Antifungal immunity, Immunocompromised patients, Risk stratification, Personalized medicine

1 Introduction

Invasive fungal diseases, particularly invasive aspergillosis (IA), are major complications associated with the treatment of hematologic malignancies [1]. Vaccines are not available, and despite noteworthy recent developments in diagnostic and therapeutic approaches, these diseases remain associated with unacceptable mortality rates [2, 3]. Concerns over excessive prescription of antifungal drugs and the remarkable burden these diseases convey to the healthcare systems are inspiring a shift from universal prophylaxis to risk stratification and preemptive approaches. Recent evidence continues to highlight the complexity of the multiple fungal-sensing immune systems and the remarkable influence of the host genetics in the ability to control infection risk and its progression. As a result of our improved understanding of the host–fungus interaction, several relevant target genes (and associated genetic variants) with the potential to be exploited in future personalized medical interventions in high-risk settings based on individual genetic risk have been identified.

Chronic granulomatous disease and autosomal-dominant hyper-IgE syndrome (AD-HIES) are the most common examples of primary immunodeficiencies typically associated with susceptibility to IA [4]. These severe immune deficiencies with monogenic inheritance are usually limited to a very small number of individuals or families, but the identification of genetic defects is very informative on immune defense mechanisms. For most individuals however, genetic propensity to fungal disease has a polygenic inheritance, acting in concert with other clinical predisposing variants such as chemotherapy-induced neutropenia. Our increasing ability to analyze human variability at the DNA level has made possible the identification of several host genetic variants amenable to use in the categorization of patients with a high risk of infection and to target antifungal therapy (reviewed in [5, 6]). However, the clinical translation of this active field of research is still limited, mostly due to the heterogeneity of cohorts, sample size, case and control selection bias, and statistical misconceptions.

Among the most encouraging examples reported to date, a donor haplotype in Toll-like receptor 4 (*TLR4*) underlying a delayed T cell and natural killer T cell immune reconstitution [7] has been disclosed as an important risk factor for developing IA in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) [8]. In addition, *TLR4* variants have been previously linked with chronic aspergillosis in immunocompetent individuals [9] and fungal colonization in HSCT recipients [10]. However, and since the fungal ligand (or the host-derived molecule released in response to fungal infection) for *TLR4* remains debated, uncovering the exact mechanism(s) through which *TLR4* deficiency impacts antifungal immunity is required to strengthen the prognostic significance of its genetic variation.

It is noteworthy that, in addition to *TLR4*, genetic variation in other TLRs has also been proposed to influence the risk of IA. For example, a regulatory variant decreasing the expression of *TLR3* in dendritic cells was found to impair the recognition of fungal nucleic acids and to compromise the efficient priming of protective memory CD8⁺ T cell responses, thereby rendering HSCT recipients more prone to develop IA [11]. Ultimately, the evaluation of regulatory variation impacting adaptive immunity might help to enhance the discriminatory potential of recent immunodiagnostic strategies based on the evaluation of fungal-specific adaptive immune responses [12]. Of interest, damage perception is coupled with pathogen-sensing pathways (especially intracellular TLRs) to restrain inflammation in experimental aspergillosis [13]. Therefore, it is not surprising that genetic variants determining a hyperactivation in danger signaling axes, and presumably underlying exuberant inflammatory responses, were also found to increase the risk for IA [14]. Finally, other examples include genetic variants in *TLR1* and *TLR6* [15], and *TLR5* [16], but further studies are warranted to definitely assure their predictive potential for fungal disease.

Dectin-1 deficiency has consistently been reported to contribute to susceptibility to IA [17–19]. In particular, a stop codon polymorphism in Dectin-1 (*CLEC7A*) compromising the surface expression of the receptor in myeloid cells and downstream cytokine production in response to fungal infection [20] significantly increased the risk of IA in HSCT recipients [18]. The fact that Dectin-1 deficiency in both transplant counterparts synergized towards risk of IA highlighted for the first time the pivotal contribution of non-hematopoietic Dectin-1 in antifungal immunity. The prominent biological plausibility of this association suggests Dectin-1 as an attractive candidate not only in risk stratification measures but also in immunotherapeutic strategies aiming at countering the defective Dectin-1 function.

Numerous positive associations between genetic variants in cytokine and chemokine genes and risk of IA have also been reported [21–26]. Most of these initial reports were however largely underpowered and performed in poorly characterized cohorts, thereby precluding definite conclusions about genetic variants affecting cytokine production in the context of IA. There are however some exceptions that assume particular relevance given the robust study design and functional validation [22, 27]. Among the exceptions, a haplotype in C-X-C motif ligand 10 (*CXCL10*) was mechanistically correlated to a deficient expression of this chemokine in dendritic cells and, accordingly, high levels of CXCL10 were also more frequently observed among patients surviving IA compared to unaffected controls [22]. More recently, variants in the genes encoding for IL-1 β and beta-defensin 1 (*DEFB1*) were reported to increase the risk of IA in solid organ transplant recipients, supposedly by impairing fungal-induced pro-inflammatory cytokine secretion by mononuclear cells [27].

Next-generation sequencing technologies now provide exciting possibilities to pin down essential steps in the host-fungus interaction at a level of complexity previously unanticipated. The first genome-wide association studies (GWAS) exploring host susceptibility to IA are underway and are expected to provide unbiased insights into the genetic defects contributing to development of IA. The plausibility of these approaches has been recently demonstrated in invasive fungal diseases, in which functional genomics analyses have allowed the identification of new important players controlling susceptibility to candidemia [28, 29]. Finally, genetic analysis of gene expression represents another powerful approach enabling insights into the human genomic landscape by generating expression maps that might be revealed extremely useful for the functional interpretation of noncoding variants likely to arise from ongoing genome-wide initiatives [30].

A number of alternative strategies using mouse models of infection as a starting point have also been employed to define candidate genes involved in susceptibility to IA [31, 32]. Genetic mapping analysis of survival data of animals subjected to experi-

mental infection led to the identification of a non-synonymous polymorphism in human plasminogen (*PLG*), a regulatory molecule with opsonic properties, as an important modulator of susceptibility to IA in humans [32]. Genetic deficiency of additional molecules with opsonic activity—e.g., mannose-binding lectin (MBL) [33] and the long pentraxin 3 (PTX3) [34]—has also been disclosed as a major determinant of susceptibility to IA, pointing to a key contribution of the innate humoral arm to an adequate activation of protective antifungal immune responses. This is corroborated by the validation of the association of a *PTX3* haplotype and increased risk of IA in two independent, high-powered genetic association studies [34, 35]. The involved haplotype was found to compromise the alveolar availability of PTX3 and, at a cellular level, its expression during the developmental programming of neutrophil precursors in the bone marrow, leading to defective antifungal effector mechanisms of newly reconstituted neutrophils [34]. Importantly, this association was also recently replicated in recipients of lung transplant [36], highlighting a potential applicability of these markers in predicting infection across patients with intrinsically different predisposing conditions. Of interest, the alveolar levels of PTX3 have been demonstrated to discriminate microbiologically confirmed pneumonia in mechanically ventilated patients [37]. Given that these levels vary individually according to host genotypes [34], we can envisage the quantification of PTX3 in bronchoalveolar lavage fluids as a complementary surveillance measure in addition to the currently available diagnostic approaches. Finally, the fact that exogenous administration of PTX3 is able to revert the genetic defect *in vitro* [34] further highlights the potential of PTX3-based immunotherapies to treat (or prevent) IA [38].

Although the overall weight of the antifungal immune response is certainly driven by adding effects of single genetic factors and their complex interactions with clinical immune dysfunctions, PTX3 represents the most robust genetic marker identified to date. These consistent findings are expected to lay the foundations for well-designed prospective trials ultimately endorsing PTX3-based genetic testing in risk stratification approaches for IA. In this chapter, we will use PTX3 as an example to describe protocols to assess genetic variation in *PTX3* associated with susceptibility to IA, and to evaluate the associated functional consequences in circulating neutrophils from patients at-risk.

2 Materials

Prepare all solutions using ultrapure water and cell-culture grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Carefully follow all waste disposal regulations when disposing of human waste materials.

- 2.1 DNA Isolation** 1. Quick-gDNA™ MiniPrep Kit (Zymo Research).
- 2.2 Single Nucleotide Polymorphism (SNP) Genotyping** 1. KASP™ Assay Mix for the rs2305619, rs3816527, and rs1840680 SNPs in *PTX3* and KASP™ Master Mix (LGC Genomics).
- 2.3 Neutrophil Isolation** 1. Histopaque 1077 (Sigma-Aldrich).
2. NaCl 0.2, 0.9, and 1.6 %.
3. Dextran T-500 from Alfa Aesar (cat #: J63702) 3% in 0.9% NaCl (dextran/saline solution).
4. PBS: NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM, pH 7.2–7.4, 0.2 μm filtered.
- 2.4 Neutrophil Lysis** 1. Neutrophil lysis buffer: Tris–HCl 10 mM, EDTA 5 mM, NP40 1%, and 1× protease inhibitors.
- 2.5 ELISA for *PTX3*** 1. ELISA to detect human *PTX3*, for example the Pentraxin 3/TSG-14 DuoSet ELISA (R&D systems).
2. PBS (prepared as above).
3. Wash buffer: Tween 20 0.05% in PBS, pH 7.2–7.4.
4. Reagent diluent: bovine serum albumin (BSA) 1% in PBS, pH 7.2–7.4 (stored at 4 °C).
5. Substrate solution: 1:1 mixture of color reagent A (H₂O₂) and color reagent B (tetramethylbenzidine).
6. Stop solution: H₂SO₄ 2 N.
- 2.6 Assessment of Neutrophil Effector Functions** 1. Live conidia from *Aspergillus fumigatus*.
2. Fluorescein isothiocyanate (FITC) (Sigma-Aldrich).
3. RPMI Medium 1640 (Thermo Fisher Scientific).
4. Sabouraud Dextrose agar (BD Difco™).

3 Methods

For the purpose of this chapter, genetic variants in *PTX3* are given as examples of common variants that have been described to influence the risk of IA; however, these can be replaced with other known and novel genetic markers. The methods addressing the functional consequences of these variants and described below have been verified in human circulating neutrophils.

3.1 Isolation of Genomic DNA from Peripheral Blood

Reagents used to isolate genomic DNA are part of the Quick-gDNA™ MiniPrep Kit. By using the innovative Zymo-Spin™ Column technology, this kit yields highly purified RNA-free DNA, bypassing the need for RNase A treatment and excluding the use

of proteinase K and organic denaturants, thereby ensuring high quality for sensitive downstream applications, including SNP analysis. In addition, all reagents are compatible with commonly used anticoagulants (i.e., EDTA, heparin, citrate) in blood drawing procedures. Additional equipment required includes a microcentrifuge and a vortex.

1. Add 400 μL of lysis buffer to 100 μL of whole blood (4:1). Mix thoroughly by vortexing and allow the mixture to rest for 5–10 min at room temperature (*see Note 1*).
2. Transfer the mixture to a Zymo-Spin™ column in a collection tube and centrifuge at $10,000\times g$ for 1 min.
3. Wash the column with 200 μL of DNA pre-wash buffer and centrifuge as before.
4. Add 500 μL of DNA wash buffer to the column and centrifuge as before.
5. Elute the DNA in 50 μL of elution buffer and collect the purified DNA in a clean microcentrifuge tube (*see Note 2*).
6. Determine the concentration of DNA in each sample by light spectrophotometry (e.g., NanoDrop) using the elution buffer as blank.

3.2 Genotyping of PTX3 SNPs

The Kompetitive Allele-Specific PCR (KASPar) chemistry (LGC Genomics) is hereafter used as an example of a commercial on-demand assay to perform genotyping. This kit contains a KASP™ Assay Mix that is specific to the SNPs under analysis and consists of two competitive, allele-specific forward primers and one common reverse primer. Each forward primer incorporates an additional tail sequence that corresponds with one of two universal fluorescent resonance energy transfer (FRET) cassettes (FAM and HEX) present in the KASP™ Master Mix. This mix also includes the ROX™ passive reference dye, Taq polymerase, free nucleotides, and MgCl_2 in an optimized buffer solution.

1. Vortex thoroughly all the components of the KASPar assay prior to use.
2. Dispense 5 μL of each DNA sample onto a qPCR-compatible 96-well plate (*see Note 3*). Make sure to include at least three non-template controls (NTC) containing ultrapure water.
3. Prepare a master mix containing 5 μL of $2\times$ reaction mix and 0.14 μL of the primer mix for each sample to be analyzed.
4. Add 5 μL of the master mix to each DNA and NTC well.
5. Seal the 96-well plate carefully and run the qPCR in a compatible instrument according to the following thermal profile (*see Notes 4 and 5*).

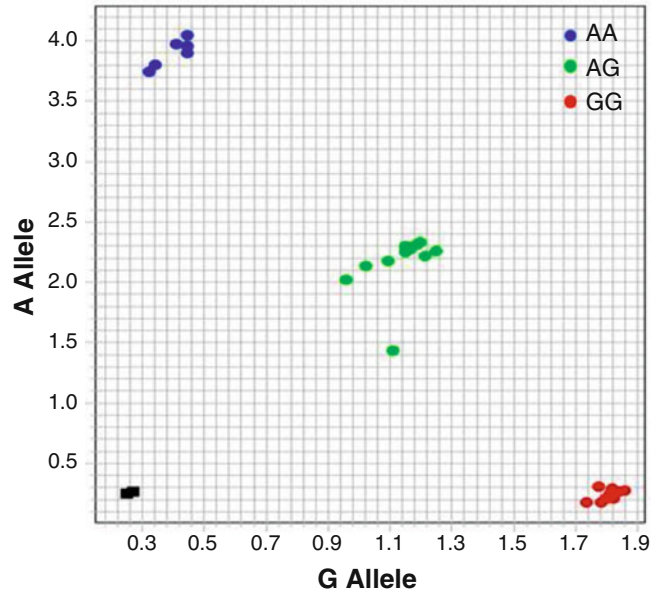


Fig. 1 Typical allelic discrimination plot obtained for the rs2305619 SNP in the *PTX3* gene. Genotyping was performed using KASPar chemistry in a 7500 Fast qPCR system (Applied Biosystems)

Holding stage	Cycling stage (10 cycles)		Cycling stage (32 cycles)		Post-PCR read (holding stage)
94 °C 15 min	94 °C 20 s	61 °C 60 s (-0.6 °C/cycle)	96 °C 20 s	55 °C 60 s	25 °C 60 s

6. Perform the allelic discrimination by reading fluorescence at 25 °C (Fig. 1).
7. In the event clear genotyping clusters are not observed, the plate should be thermally cycled further (three additional cycles of 20 s at 94 °C and 60 s at 57 °C) and read again at 25 °C.

3.3 Isolation of Circulating Neutrophils

1. Dilute the collected whole blood at a 1:4 proportion with 0.9% NaCl.
2. Layer 10 mL of Histopaque 1077 beneath the diluted whole blood using a pipette or a syringe (*see Note 6*).
3. Centrifuge at $400\times g$ for 40 min at 20 °C without brake (*see Note 7*).
4. Aspirate and discard the supernatant and resuspend the granulocyte/red blood cell (RBC) pellet in 20 mL of PBS.
5. Add an equal volume of dextran/saline solution, mix and incubate in the upright position for approximately 30 min at room temperature.

6. Aspirate and save the leukocyte-rich plasma (upper layer) using a pipette.
7. Pellet cells from the plasma by centrifuging at $250\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ (*see Note 8*).
8. To remove residual RBC, subject cells to hypotonic lysis by resuspending the pellet in 20 mL of cold 0.2% NaCl for exactly 30 s.
9. Restore isotonicity by adding 20 mL of ice-cold 1.6% NaCl.
10. Centrifuge at $250\times g$ for 6 min at $4\text{ }^{\circ}\text{C}$ and discard the supernatant. Repeat steps 8 and 9 until the cell pellet appears free of RBC.
11. Resuspend cells in ice-cold PBS and determine cell concentration by counting in a Neubauer chamber.

3.4 Preparation of Neutrophil Lysates

1. Transfer 10^6 neutrophils into an Eppendorf tube and remove the supernatant by centrifuging at $250\times g$ for 6 min at $4\text{ }^{\circ}\text{C}$.
2. Add 200 μL of lysis buffer and incubate 5 min on ice.
3. Collect the cell lysate by centrifugation at $12,000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$.
4. Store at $-80\text{ }^{\circ}\text{C}$ until the determination of intracellular PTX3 by ELISA.

3.5 Measurement of Intracellular PTX3 in Neutrophils by ELISA

All reagents from the ELISA kit for PTX3 should be brought to room temperature before use and the reconstituted components should be allowed to sit for at least 15 min with gentle agitation. Working dilutions should be prepared according to the manufacturer's instructions and used immediately.

1. Dilute the capture antibody to the working concentration using PBS and coat a 96-well microplate with 100 μL per well.
2. Seal the plate and incubate overnight at room temperature.
3. Wash each well three times with 400 μL of wash buffer (*see Note 9*).
4. Block the plate by adding 300 μL of reagent diluent to each well and incubate for 1 h at room temperature. Repeat the washing step as above.
5. Add 100 μL of sample (it may be necessary to dilute the samples prior use) or standards in reagent diluent per well. Cover the plate with an adhesive strip and incubate overnight at $4\text{ }^{\circ}\text{C}$.
6. Repeat the washing step as above.
7. Add 100 μL of the diluted detection antibody to each well and incubate for 2 h at room temperature.
8. Repeat the washing step as above.

9. Add 100 μL of the working dilution of streptavidin-horseradish peroxidase (HRP) to each well.
10. Seal the plate and incubate at room temperature for 20 min (*see Note 10*).
11. Repeat the washing step as above.
12. Add 100 μL of substrate solution to each well and incubate at room temperature for 20 min (*see Note 11*).
13. Add 50 μL of stop solution to each well and gently tap the plate to ensure thorough mixing.
14. Determine the optical density of each well immediately using a microplate reader set to 450 nm (*see Note 12*).

3.6 Measurement of Phagocytosis in Neutrophils

1. To estimate phagocytic activity, plate 5×10^5 freshly isolated neutrophils in 12 mm glass coverslips coated with serum proteins or purified fibrinogen in a 24-well plate.
2. Add live FITC-labeled conidia of *A. fumigatus* at an effector to fungal cell ratio of 1:5 in a final volume of 500 μL (*see Note 13*).
3. Incubate at 37 °C for 1 h in a humidified CO₂ culture chamber.
4. After incubation, remove the non-adherent conidia by washing carefully twice with PBS.
5. Add 300 μL of trypan blue (1 mg/mL in PBS) to each well for 15 min at 25 °C to quench the fluorescence of bound but uningested conidia.
6. Wash twice with PBS and fix the cells in 1% paraformaldehyde for 15 min.
7. Mount coverslips with the cell side down onto microscope slides in 90% glycerol in PBS, and seal with clear nail polish.
8. Quantify phagocytosis via phase contrast and fluorescence microscopy by counting at least 200 neutrophils per coverslip (*see Note 14*).

3.7 Evaluation of Neutrophil Fungicidal Activity

1. Incubate neutrophils with conidia of *A. fumigatus* at an effector to fungal cell ratio of 10:1 for 120 min in a final volume of 100 μL in 96-well flat-bottomed microtiter plates.
2. After incubation, freeze plates at -80 °C and rapidly thaw at 37 °C to lyse cells and harvest conidia.
3. Prepare serial dilutions (1:10) from each well in PBS (900 μL) and plate 100 μL onto Sabouraud dextrose agar plates.
4. Incubate plates at 28 °C for 48 h.
5. Count the number of colony-forming units (CFU) and calculate the percentage of CFU inhibition (referred to as the fungicidal activity) (*see Note 15*).

4 Notes

1. It is recommended to add β -mercaptoethanol to the genomic lysis buffer at a final dilution of 0.5% (v/v) to improve lysis performance.
2. Ensure that the elution process occurs for at least 1 min to increase the DNA yield.
3. The use of at least 20 ng of DNA in each genotyping reaction is recommended. Use an identical DNA concentration for all samples in order to adequately normalize the fluorescence intensity.
4. The thermal profile presented is a typical example that has been specifically provided by the manufacturer with some minor modifications. Because of the underlying chemistry mechanisms of KASPar, the post-PCR read should always be performed below 40 °C.
5. An optimal cluster visualization is typically obtained using a 7500 Fast qPCR system (Applied Biosystems) and a post-PCR read at 25 °C.
6. Take care to preserve the interface between the Histopaque and the diluted blood solution, thereby avoiding contamination between both phases.
7. The temperature of 20 °C is crucial to achieve the best cell separation performance.
8. After separation, cells should be kept on ice at all times and handled gently to prevent damage or undesired activation of neutrophils.
9. Complete removal of liquid from the wells by inverting the plate and blotting it against clean paper towels is essential for an optimal performance of the ELISA test.
10. Avoid exposing the plate to light.
11. The length of incubation may vary depending on the concentration of PTX3. For that reason, check the plate every 5 min and stop the reaction earlier, if necessary.
12. If wavelength correction is available, set it to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm in order to correct for optical imperfections in the plate.
13. For the staining of live conidia of *A. fumigatus*, incubate 2×10^7 conidia in 2 mL of Na_2CO_3 50 mM buffer, pH 10.2 with FITC at a final concentration of 0.1 mg/mL (prepared in the same buffer) at 37 °C for 4 h. Wash the conidia thoroughly to eliminate residual FITC.

14. Enumerate the number of ingested conidia, or bound but not ingested. The data is typically presented as the percentage of neutrophils ingesting one or more conidia.
15. Calculate the inhibition of CFU—also known as fungicidal activity—using the following formula: % fungicidal activity = $(1 - X/C) \times 100$ where X is the number of CFU obtained after infection of neutrophils, and C is the number of CFU in the absence of neutrophils. Perform three biological replicates for each condition and at least two technical replicates.

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