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Host genetics affect susceptibility to invasive aspergillosis

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Invasive aspergillosis is a common and life-threatening infection in immunocompromised individuals. Epidemiologic risk factors do not fully explain susceptibility to disease amongst at-risk persons. Recently, the contribution of host genetics and genetic polymorphisms to disease susceptibility has begun to be explored. This paper highlights methodologies for evaluating the role of host genetics in aspergillosis susceptibility.

Keywords aspergillosis, genetic, polymorphism

Introduction

Invasive aspergillosis (IA), typically caused by *Aspergillus fumigatus* (AF), is the most common filamentous fungal infection following hematopoietic stem cell transplantation (HSCT), occurring in approximately 10% of allogeneic HSCT recepients. Other high risk groups include solid-organ transplant recipients and patients receiving chronic corticosteroid therapy [1–5]. Outcome from such infections is dismal, with an overall one-year survival rate of approximately 20% [1]. Despite growing knowledge regarding epidemiologic risk factors for IA, these factors only explain a portion of disease risk. Investigators are beginning to consider the contribution of the host's genetic background to risk of IA [6,7]. Polymorphisms in genes regulating immune function are likely to be important determinants of host susceptibility to fungal infections and may become critically important during times of immunosuppression. Elucidation of the host's genetic contribution to IA risk and/or progression is facilitated by complementary use of animal models and human cohorts for discovery of genetic polymorphisms regulating susceptibility in the at-risk host.

Both animal models and human cohorts can be used to elucidate the contribution of host genetics to risk of IA. Animal models may focus on candidate genes, utilize positional cloning or gene expression studies, whereas genetic association studies are appropriate methodology for use in human cohorts. Identification of genes/gene variants responsible for increased risk of IA in at-risk populations may serve to individualize prophylactic regimens or to spearhead the development of targeted therapeutics. At present, there are many candidate gene studies evaluating the role of certain genes in the pathogenesis of invasive aspergillosis. Increasing availability of both murine and human genetic polymorphism data should facilitate the development of genetic association studies related to host susceptibility to invasive aspergillosis.

It is well-established that invasive aspergillosis arises from a failure of pulmonary defenses. *A. fumigatus* conidia are continuously inhaled by humans, but rarely have any adverse effects as they are eliminated efficiently by the immune response. Pulmonary alveolar macrophages can ingest inhaled *A. fumigatus* conidia and inhibit germination. If the macrophages are unable to clear the inhaled conidia, the conidia germinate into hyphae. The host must then rely on polymorphonuclear leukocytes (PMNs) to defend against continued fungal growth. Numerous cytokines and chemokines are involved in host defense against aspergillosis, with increasing appreciation for the role of adaptive immunity in response to this disease as well [8,9]. Once germinated, *A. fumigatus* hyphae grow and invade along vascular planes, causing tissue destruction, pulmonary hemorrhage and microvascular obstruction. It is clear that despite similar degrees of exogenous immunosuppression amongst at risk patients impairing the immune clearance of *A. fumigatus* conidia and hyphae, only a subset of at risk patients will develop IA. Functional genetic polymorphisms in any component

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of host defenses may be capable of altering the balance towards establishment of IA.

It is also established that gene variants affect the risk to many types of infections, both in model systems and in human disease. Polymorphisms in genes regulating immune function are likely important determinants of host susceptibility to fungal infections. These polymorphisms may become critically important during times of immunosuppression, a concept known as environmentally determined genetic expression [10]. Studies in *Drosophila* and murine models underscore the importance of genetic variants in susceptibility to fungal infection [11–13]. Variations in susceptibility to fungal infection have been demonstrated in humans as polymorphisms in the mannose-binding lectin gene are associated with both recurrent vulvovaginal candidiasis [14] and chronic necrotizing pulmonary aspergillosis [15]. There are likely numerous as yet unidentified polymorphisms in immune regulating genes that affect host susceptibility to opportunistic fungal pathogens.

Genes controlling effectors of innate immunity are outstanding candidate genes for study of *A. fumigatus* susceptibility. Numerous biologically plausible candidate genes for study exist, including those in the toll-like receptor superfamily, collectins, as well as those governing direct cytokine and chemokine response. Toll-like receptor (TLR) biology in relation to susceptibility to invasive aspergillosis has been studied extensively. Host defenses against invasive aspergillosis in mice and humans involve signaling through the innate immune receptors TLR-2 and TLR-4 [16–23]. This signaling leads to TNFα, IL-1β, and IL-6 production. In humans, the TLR-4 co-receptor CD14 is necessary for TNFα production in response to stimulation with fungal hyphae and conidia [21]. AF antigens induce the activation and maturation of immature dendritic cells, enhance the phagocytosis and production of IL-8 in PMNs and IL-12 production in bone-marrow derived dendritic cells through TLR-2 [18]. Additionally, a low level activation of NFκB and subsequent innate immune response occurs through an alternative pathway not dependent on TLRs. Human studies, however, have not implicated TLR4 polymorphisms in susceptibility to aspergillosis [24], but may show a role of polymorphic variants of TLR1 and 6 that require a larger sample size and testing in another cohort to validate [6]. The interaction of AF with host defenses is complex and incompletely defined. In aggregate, these studies indicate the complexity of the immune response to fungal infection, and the potential for polymorphisms at multiple levels to contribute to disease susceptibility.

Candidate gene studies

Knowledge regarding the pathogenesis of invasive aspergillosis has fostered numerous candidate gene studies regarding aspergillosis susceptibility. Although the immune effects of the Toll system in *Drosophila* were initially discovered through fungal susceptibility studies, the bulk of candidate gene studies in invasive aspergillosis are murine transgenic ('knock-out') studies. While discussion of all murine transgenic studies in *Aspergillus* susceptibility is beyond the scope of this review, the evaluation of IL-10 and its role in susceptibility to invasive aspergillosis provides an example of using a biologically plausible candidate gene approach from *in vitro* studies through human association study cohorts. For more illustrative examples, the reader is encouraged to explore the literature concerning Toll-like receptors and aspergillosis susceptibility through multiple important articles [11,12,16–20,23–27].

The role of interleukin-10 (IL-10) in the pathogenesis of invasive aspergillosis has also been explored through a series of *in vitro* and *in vivo* studies, as well as one human association study in hematopoietic stem cell transplant recipients. The cytokine IL-10 functions to regulate both lymphoid and myeloid cells, with the ultimate outcome of generating a Th2-biased phenotype. IL-10 is mainly produced by the T_H2 subset of $CD4^+$ helper cells. However, it is also produced by some activated B cells, some T_H1 cells (in humans), activated macrophages, and some nonhematopoietic sources (e.g., keratinocytes, colon carcinoma, melanoma cells) [28]. Kinetics studies demonstrate that IL-10 is synthesized later than other immunoregulatory cytokines by activated T cells or monocytes. These data may reveal the regulatory role of IL-10 in later phases of the immune response. Like other cytokines interleukin-10 has many effects upon the functions of cells such as lymphocytes, monocytes, natural killer cells, and dendritic cells. Specifically, IL-10 is a cytokine that regulates immune-mediated inflammation. It appears to have two major functions: (1) to inhibit cytokine (i.e., TNF, IL-1, chemokine, and IL-12) production by macrophages, and (2) to inhibit the accessory functions of macrophages in T cell activation. IL-10 accomplishes the latter function through the reduced expression of MHC class II molecules and certain co-stimulators (e.g., B7). The cumulative effect of these functions acts to inhibit T cell-mediated immune inflammation. IL-10 also has stimulatory actions on B cells and may function as a switching factor for the production of IgG4 in humans (homologous to IgG1 in mice) [29]. *In vitro*, it has been shown that IL-10 suppresses the mononuclear cell oxidative burst in response to *Asper-*

gillus hyphae with a concomitant increase in phagocytic activity [30]. Follow-up studies confirmed a deleterious role of IL-10 in the setting of *Aspergillus* exposure as previously susceptible (median survival 4 days) mice were rendered resistant (median survival 25 days) to disease when administered anti-IL-10 monoclonal antibodies [31]. Finally, IL-10 knockout mice have significantly greater survival in an intravenous model of aspergillosis as compared to wild-type controls [32].

Based on knowledge of how IL-10 relates to the pathophysiology of invasive aspergillosis in *in vitro* and murine systems, the role of IL-10 in human invasive aspergillosis was explored. In a small evaluation, IL-10 levels were measured during invasive aspergillosis in eight non-neutropenic patients who developed IA. Of interest, the six patients who had a favorable outcome from infection had low IL-10 levels throughout the course of infection, whereas the two individuals who had poor outcome demonstrated persistently elevated levels of IL-10 until time of death [33]. This pilot study does not demonstrate cause or effect of the elevated IL-10 levels, but provides further support towards the relationship of IL-10 and unfavorable outcome in invasive aspergillosis. Futhermore, a small association study of 105 HSCT recipients evaluated the relationship of IL-10 promoter haplotypes with the development of invasive aspergillosis over a median follow-up period of 292 days (range 9–2076 days) [7]. In this study, an IL-10 promoter haplotype ACC/ACC (-1082*A/-819*C/-592*C) appeared protective from invasive aspergillosis at two-year follow-up as compared to the ATA/ATA (-1082*A/-819*T/-592*A) or ACC/ATA haplotypes (3/61 patients with ACC/ACC haplotype developed invasive aspergillosis as compared to 6/44 patients with non-ACC haplotypes, $p = 0.0307$). While this study generates interesting hypotheses and provides preliminary data for future studies, its small size and the lack of correlation of IL-10 promoter haplotypes with IL-10 levels in this population hamper the conclusions. Additionally, this study evaluated haplotypes from the HSCT recipient with blood samples taken prior to transplant. As IL-10 is predominately produced by cells of hematopoietic lineage, it is unclear if donor or recipient haplotype is the haplotype of interest in an HSCT population.

Positional cloning

Positional cloning provides a solid framework for identifying the genetic determinants of complex traits. Positional cloning involves identifying a gene responsible for a particular disease based on its location in the genome, when no information about a biochemical basis of the disease is known. The first step in positional cloning is identifying quantitative trait loci (QTLs) associated with a phenotype of interest. QTLs are chromosomal loci statistically associated with quantitative variation in a particular complex trait [34], i.e., an area on the genome that may contain genes that are involved in the phenotype of interest. The choice of candidate genes from a mapped QTL ('positional candidates') combines the strength of positional cloning and candidate gene studies to uncover the genetic determinants of complex diseases.

The mouse is an outstanding model organism for positional candidate discovery to uncover the genetic determinants of AF susceptibility. The availability of inbred mice in combination with the recent revolution in molecular genetics has accelerated the study of the genetic basis of complex traits. The tools available include chromosome substitution and recombinant inbred strains, the nearly complete genomic sequence of four inbred strains, the haplotype maps of common inbred strains and the availability of strains that have undergone genetic engineering to alter genes and chromosomes [35]. Interested readers can refer to the following outstanding review on positional cloning techniques [36]. Using positional cloning for QTL discovery is PCR-based and quite time-consuming, as it involves breeding several generations of mice. However, it remains a time-tested and rigorous method of gene discovery. Using a robust phenotype of host response to AF, the above-mentioned powerful gene discovery techniques can help decode the genetic determinants of IA susceptibility.

Murine single nucleotide polymorphism (SNP) databases

Murine single nucleotide polymorphism (SNP) databases provide the framework for dissecting complex phenotypes [37]. The recent revolution in the identification and cataloging of murine SNPs into functional databases has made it easier to find positional candidate genes [38]. SNPs are the most abundant class of markers in the genome. As SNPs have been shown to be associated with certain phenotypes, they can be used as both diagnostic and mapping tools. Murine SNP databases are currently available, providing both low SNP densities over a great number of strains to high SNP density over a more limited number of strains [39,40]. These databases can initially provide broad mapping of complex traits, with later mapping projects focused on strains exhibiting extreme phenotypes. Whereas the traditional breeding experiments

described above take years to uncover putative QTLs, the availability of murine SNP databases has simplified the initial QTL discovery considerably. There are numerous publicly-available murine SNP and haplotype databases (Table 1). These databases are continuously being updated with new genotyping and new mouse strain information. Each database has strengths and weaknesses, and it is important to be knowledgeable about how the data were derived in order to use the databases effectively. Bioinformatics algorithms used to query these databases are known as '*in silico* haplotype mapping' or 'haplotype based genetic analysis' and serve to analyze murine phenotypic data for differences in SNP patterns that correspond to phenotypic variations. The power of this type of analysis lies in its speed, as chromosomal intervals related to the phenotype of interest can be identified in weeks rather than years. Additionally, defining a chromosomal region of interest may effectively exclude large portions of the murine genome from consideration, simplifying future positional cloning and candidate gene selection. The weakness of this method lies in its false positive rate, therefore validation experiments remain essential. However investigators can use data generated via these algorithms to focus murine breeding experiments and transgenic mouse experiments.

Differential susceptibility

Differential susceptibility to fungal infection between inbred murine strains has been explored through many models, using a variety of inbred strains [9,39]. Broad screening of inbred strains of mice for differential susceptibility to *Aspergillus fumigatus* is a means of evaluating the genetic basis of susceptibility and provides a framework for using computational haplotype mapping to define QTLs or to select parental strains for intercross experimentation. Focus on precise phenotypes such as bronchoalveolar lavage cytokine levels following inhalational challenge with *Aspergillus* conidia would allow for such methodology to locate single genes that relate to the complex phenotype of aspergillosis susceptibility. Biologically plausible positional candidate genes discovered from murine studies can then be further evaluated for polymorphic variants associated with outcomes in both murine strains and human cohorts. Ultimately, proof of functional differences between polymorphic

Table 1 Public domain murine single nucleotide polymorphism databases

http://www.broad.mit.edu/snp/mouse	This database contains a high quality draft of mouse genome produced and analyzed by the Mouse Genome Sequencing Consortium. The Mouse SNP map contains approximately 340,000 SNPs, with a goal of a 20 kb resolution haplotype map of 48 inbred strains.
http://mousesnp.roche.com	The pattern of genetic variation among 18 commonly used inbred mouse strains was analyzed and is available in this SNP database. Using this information, a high-resolution haplotypic map covering 75 Mb of the mouse genome was produced for 16 *Mus Musculus* strains. Two strains were excluded because the authors were unable to produce a stable haplotypic structure when the Cast/Ei and Spret/Ei strains were included (*Mus spretus)*. This haplotype map was used to develop a computational method for genetic analysis of phenotypic traits in mice as described in the following paper: G. Liao, J. Wang *et al. In silico* genetics: identification of a novel functional element regulating H2-Ea gene expression, *Science* 2004; **306** (5696): 690–695. For more detailed methods of constructing mouse haplotype map, please see J. Wang, G. Liao *et al*., In: *Computational Genetics and Genomics: New Tools for Understanding Disease*, (G. Peltz, Ed.), Humana, Totowa, NJ, 2005.
The Mouse Genetic Variation Mapping (vMap) Initiative	Initiative by the National Institute of Environmental Health Sciences (NIEHS) to develop complete DNA variation maps of mouse strain genomes and display of the information in a public database. Initially, 15 commonly used laboratory strains will be assessed. These maps will be constructed within approximately two years using high-throughput DNA sequencing on oligonucleotide microwafer arrays as described recently by Perlegen [*Science* **294**, 1719 (2001)]. The proposed list of 15 mouse strains to be assessed is as follows: B10D2H22, Cast/Ei, Balb/CJ Combined, C57BL/6J, DBA/2J, 129X1/svJ, A/J, C3H/HeJ, Spret/Ei, Akr/J, NZB/BINJ, MRL/MpJ, A/HeJ, BALB/cByJ, NZW/LacJ.
http://www.ncbi.nlm.nih.gov/projects/SNP/MouseSNP	Murine SNP database containing SNPs from 49 inbred strains. Currently in build 34.1. Contains 5,805,387 submitted SNPs with 1,252,186 referenced SNPs and 3,596 validated SNPs. Tools available for comparisons between mouse strains and integration with other NCBI databases.

variants of key susceptibility genes remains the goal for linking a gene of interest with disease susceptibility.

Conclusion

The hypothesis that host genetic polymorphisms affect susceptibility to invasive aspergillosis may hold the key to elucidation of new risk factors for this lethal disease. As validation of *in vitro* and murine experiments with human cohorts is essential, pro-active planning to establish reliable DNA banks from at-risk populations is necessary to ensure appropriate resources are in place for future studies.

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