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Genetic Predictors of Susceptibility to Cutaneous Fungal Infections: a pilot Genome Wide Association Study to Refine a Candidate Gene Search

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Abstract

Background—*Trichophyton tonsurans* is the foremost fungal pathogen of minority children in the U.S. Despite overwhelming infection rates, it does not appear that this fungus infects children in a non-specific manner.

Objective—This study was designed to identify genes that may predispose or protect a child from *T. tonsurans* infection.

Methods—Children participating in an earlier longitudinal study wherein infection rates could be reliably determined were eligible for inclusion. DNA from a subset (n=40) of these children at the population extremes underwent whole genome genotyping (WGG). Allele frequencies between cases and controls were examined and significant SNPs were used to develop a candidate gene list for which the remainder of the cohort (n=115) were genotyped. Cumulative infection rate was examined by genotype and the ability of selected genotypes to predict the likelihood of infection explored by multivariable analysis.

Results—23 genes with a putative mechanistic role in cutaneous infection were selected for evaluation. Of these, 21 demonstrated significant differences in infection rate between genotypes. A risk index assigned to genotypes in the 21 genes accounted for over 60% of the variability observed in infection rate (adjusted $r^2=0.665$, $p<0.001$). Among these, 8 appeared to account for the majority of variability that was observed ($r^2=0.603$, $p<0.001$). These included genes involved in: leukocyte activation and migration, extracellular matrix integrity and remodeling, epidermal maintenance and wound repair, and cutaneous permeability.

Conclusions—Applying WGG to individuals at the extremes of phenotype can help to guide the selection of candidate genes in populations of small cohorts where disease etiology is likely polygenic in nature.

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Introduction

Trichophyton tonsurans is the foremost fungal pathogen of children in the United States. Approximately 1 in 8 African American (AA) children in elementary school and as many as 1 in 2 AA preschoolers bear the fungus on their scalp.[1,2] While such overwhelming infection rates might suggest that *T. tonsurans* has the ability to broadly and non-specifically infect children, this does not appear to be the case. In the U.S., infections are almost exclusively restricted to AA children, yet every child of African ancestry does not appear to be equally susceptible to infection. In a recent 2-year longitudinal study of daycare center attendees we observed that infections did not occur haphazardly. Approximately 1/3rd of children were chronic carriers of *T. tonsurans*, as many as 25% were only transiently infected, and another 1/3rd never demonstrated evidence of infection despite the shared environment.[1] In fact, there were even cases of sibling pairs sharing both the home and school environment that demonstrated different infection patterns.[1]

The data from this earlier study offered compelling evidence that unique host factors mitigate the interaction between the host and the infecting pathogen. This theory is consistent with the reports of others which offer limited evidence that inherited traits influence susceptibility to dermatophyte infections.[3–8] Given the complexity of the human immune system and the involvement of different elements of innate and acquired immunity at various stages of infection, a complement of sequence variations in several genes may well account for differences in susceptibility between children. The current study was designed to identify genes which could predispose a child to, or protect him/her from, infection. Application of a preliminary “extreme phenotype” whole genome genotyping approach was used to inform subsequent candidate gene selection and offer an unbiased look at potential host factors that might influence infection.

Materials and Methods

Study Design

This was a single-center cohort study designed to generate preliminary data on genes or gene families that could influence predisposition to cutaneous fungal infections. Children were eligible for inclusion if they participated in the earlier longitudinal investigation that afforded determination of their infection frequency.[1] Infection frequency was defined by a continuous variable representing the percent of total daycare center visits during the 2 year period in which the participant’s scalp culture was positive for *T. tonsurans* irrespective of whether the child was clinically symptomatic. Only children with visits on 6 or more occasions over 2 years were included in the analysis to ensure the most reliable and conservative estimate of infection rate. Children were excluded from participation if they no longer attended the daycare center at the time of follow-up and/or if their parents did not consent to their participation in the study. All children were enrolled with informed parental permission under a protocol that was reviewed and approved by the Institutional Review Board of Children’s Mercy Hospital’s and Clinics.

Samples

Saliva (200–500 μ L) was obtained from each child using Dacron swabs that were placed under the tongue for 20–30 seconds. The swabs were immediately placed into 200 μ L of stabilizing solution and transported to the laboratory on ice. The composition of the stabilizing solution included; 93.75 μ L PBS, 93.75 μ L AL buffer (Qiagen, Valencia, CA), 5 μ L proteinase K, 5 μ L solution S (0.25 mM EDTA, 2% SDS) and 2.5 μ L RNase. Additionally, 2 buccal swabs were obtained from each child at the time of collection and similarly kept on ice during transport. All specimens were processed within 2 hr of

collection. Genomic DNA (gDNA) was extracted and purified using the column-based QIAamp DNA Blood Mini Kit (Qiagen) with slight modification. DNA was quantitated using the Quant-it double stranded assay kit (Invitrogen, Carlsbad, CA). DNA from the buccal specimens used to supplement the salivary DNA as needed to ensure enough nucleic acid for genotyping.

Whole Genome Genotyping

Cases and controls (n=20 each) were selected from among the children at the population extremes. Cases were required to demonstrate an infection frequency of 90% (i.e. always or almost always infected). Controls were required to demonstrate an infection rate of 10% (i.e. never or almost never infected). Samples were genotyped using the Sentrix® HumanHap650 genotyping bead chip (Illumina, San Diego, CA). In total, 750 ng of gDNA was subjected to whole genome amplification, fragmented, precipitated with isopropanol and subsequently resuspended in formamide-containing hybridization buffer. Reconstituted samples were denatured and allowed to hybridize to the capture probes overnight (48°C) after which the chips were washed, single-base extension performed, the chip stained and subsequently imaged on the Illumina BeadArray Reader. Image intensities were extracted with the manufacturers BeadScan software and normalized according to algorithms nested in the program.

Candidate Gene Selection

Allele and genotype frequencies generated by WGG were examined between the cases and controls using a chi-square test. Independent SNPs demonstrating an unadjusted p-value of <0.001 were eligible for consideration of evaluation in the remaining children. SNPbrowser software (v. 4.0, Applied Biosystems, Carlsbad, CA) was used to examine the proximity of each SNP to a functional gene region. Synonymous and nonsynonymous sequence variations nested within functional gene regions were prioritized further to identify candidate genes with a putative mechanistic rationale for their contribution to the cutaneous infection process.

PCR based assays with allele specific fluorescent probes (TaqMan, Applied Biosystems, Carlsbad, CA) were identified or designed for SNP analysis of the DNA from the remaining children who did not undergo WGG. All reactions were performed in 8 µL and cycled on a Dyad DNA engine (Bio-Rad, Hercules, CA). An end point assay was used to measure fluorescence on an ABI Prism 7900HT (Applied Biosystems). Infection rate was compared across genotypes by ANOVA with a Tukey post-hoc test. The influence of demographic covariates on infection phenotype was also assessed in the same manner. All statistical analyses were performed with SPSS v. 12 (SPSS, Chicago, IL).

Risk Index Assignment

Individual genotypes were assigned a risk index score if the mean infection rate differed significantly from the mean infection rate for the other genotypes at that locus (e.g. infection rate among children with an AA genotype significantly different from children with an AG genotype). Genotypes for which the mean infection rate was less than 20% were ascribed a value of 0. Genotypes for which the mean infection rate ranged from 20–40% were assigned a 1. A value of 2 was used to designate genotypes for which the mean infection rate lay between 40–60%. Finally, genotypes for which the mean infection rate was greater than 60% were assigned a 3. By way of example; mean infection rates were 19.9%, 33.3% and 57.8% among children with an AA, AG and GG, respectively at rs154576 in the semaphorin 6A gene. Consequently, risk values of 0, 1 and 2 were assigned to the AA, AG and GG genotypes, respectively. Multivariable analysis of variance was used to build the initial regression model using all genotypes as independent variables and stepwise linear

regression (backward elimination) was used to identify the combination of genes whose combined risk index demonstrated the greatest association with infection rate.

To explore the validity of a risk-index based strategy for prioritizing gene candidates, a separate set of analyses were performed using the raw genotypes as independent variables. Standardized regression (i.e. beta) coefficients were calculated according to the following: $\text{beta} = b * s_x/s_y$, where b represents the raw regression coefficient, s_x represents the standard deviation of the independent variable (x), and s_y represents the standard deviation of the dependent variable (y). These beta coefficients were used to identify the genotypes that were incorporated into the final model. As above, all statistical analyses were performed in SPSS and the significance limit accepted for all analyses was $\alpha=0.05$.

Results

Of the 446 children in the original longitudinal investigation, 163 remained in attendance at the participating daycare center and 161 were given parental permission to participate. Of these, 155 qualified for inclusion based on the number of visit dates for which data were available (i.e. 6). Participating children were evenly distributed by gender (male-50.3%) and the majority were African-American (90.7%) with the remainder Caucasian (6.8%), mixed African-European ancestry (2.5%) or other (<1%). Among the subset of children selected as cases and controls, 100% were African-American with an even split between males and females (19 vs. 21, respectively). In all participants, there was a sufficient quantity of high quality DNA to permit genotyping.

WGG revealed 1,013 unique sequence variations (693 when analyzed by allele, 320 when analyzed by genotype) that met the *a priori* threshold cutoff defined in the methods. Approximately 40% of these SNPs ($n=410$) were distributed among 275 known or predicted genes. After reviewing existing data on the functionality of each the 275 genes, 23 were prioritized for subsequent analysis in the remaining study cohort based on a putative mechanistic rationale for involvement with cutaneous infection processes (Table 1). When the remaining 115 participants were genotyped at these loci, 21 of the 23 genes originally significant by WGG retained statistical significance for their association between genotype and infection frequency.

The cumulative risk index assigned to the genotypes of these 21 genes accounted for more than 60% of the variability observed in infection rate (Figure 1). Stepwise regression revealed that 8 genes; CsmD1, FibCD1, Fibulin 5, LASS4, MAPK8, SEMA6A, SLIT3 and SMOC2 appeared to account for the majority of variability that was observed in infection rate (Table 2, Figure 1). The inclusion of gender and race in the regression model offered a slight but significant increase in correlation coefficient between infection rate and risk index (Table 2). Slightly less variability (58%) was accounted for when using the β -coefficient based approach (Table 2, Figure 1). Notably, 6 of the genes identified by risk index were also identified by β -coefficient. SLIT3 and SMOC2 were not significant using the latter approach; however, GALP emerged as significant in this model.

Discussion

Identifying individuals for inclusion in a genetic association study of cutaneous infection risk can be a challenge. Adopting a cross-sectional, case-control study design introduces the problems of selection and information bias and necessitate the need for very large sample sizes. Cohort-designs are more powerful; however, they are resource-intensive, and some might argue impractical, as they require large population-based cohorts to be followed for long periods of time while tracking the appearance and disappearance of infections.[9]

The pilot study described herein attempted to capitalize on the advantages of both study designs. The longitudinal assessment of a high-risk cohort deriving from a singular environment allowed us to define infection rates over time in children with presumably similar non-genetic risk factors. Using a subset of the population for a case-control WGG analysis permitted the identification of unique gene candidates that could be further refined for subsequent evaluation in the remainder of the population. With this approach we identified an association between *T. tonsurans* infection rate and genotype in 21 genes for which a plausible mechanism in the development of cutaneous infections could be constructed.

Several of the genes for which a significant association was demonstrated are involved in the recruitment, activation, and migration of leukocytes. Though not detected in normal skin epithelium, semaphorin 6A (SEMA6A) is strongly expressed on Langerhan's cells (LC) residing in the dermis and draining lymphnodes of patients with LC histiocytosis and dermatopathic lymphadenopathy. High levels of expression under conditions of inflammation suggest that SEMA6A may be involved in guiding the migration of these antigen-presenting cells out of the skin.[10,11] Roundabout receptor 1 (ROBO1) and the corresponding SLIT gene products function cooperatively to influence the directional migration of numerous cell types including monocytes and lymphocytes.[12–14]. Though SLIT3 is present in skin, most of the data on ROBO/SLIT guided migration of dendritic cells is described for a related gene family member (SLIT2).[15,16] CD99-Like 2 (cd99L2) was also identified as a candidate and the product of this gene is involved with the extravasation of neutrophils and monocytes into inflamed tissue including skin.[17,18] The Cub and Sushi Multiple Domains-1 (CSMD1) gene has been implicated in inflammatory processes via the activation of complement and was recently identified as candidate gene associated with psoriasis.[19,20] Finally, Grb2-associated binder 2 (GAB2) demonstrates a polyfunctional role in the process of inflammation. This gene appears to be a major effector for IgE-dependent allergic reactions, it participates in the activation of T- and B-cells via cytokine- and growth factor-mediated signaling and it is involved in mast cell signaling and function in response to pathogenic stimuli. Importantly, Gab2 knockout mice demonstrate reduced mast cell recruitment into the skin.[21,22]

A large proportion of the genes in our dataset are involved in the extracellular matrix formation, integrity and remodeling. Fibulin 5 (FBLN5), fibrillin 2 (FBN2) and microfibrillar-associated protein 4 (MFAP4) are all essential components of the extracellular matrix (ECM) and elastin fiber network under normal homeostatic conditions and during periods remodeling and wound healing.[23–25] Further, FBN2 expression is markedly upregulated in sclerotic skin diseases,[26,27]and mutations in FBLN5 disrupt the elastic properties of the skin.[28,29] Secreted modular calcium-binding protein-2 (SMOC2) appears to be involved with melanocyte function and genome wide studies have identified an association between SMOC2 and the autoimmune disorder vitiligo in selected populations.[30,31] Importantly, vitiligo is also associated with lower hydration and altered permeability in the stratum corneum.[32] BH-protocadherin (PCDH7), a member of the cadherin superfamily of genes, is involved with cell-cell recognition and is upregulated during keratinocyte differentiation.[33,34] Finally, the enzymes encoded by matrix metalinoproteinase 3 (MMP3) and a disintegrin and metalloprotease 12 (ADAM 12) are involved with remodeling of the ECM under normal and pathological conditions.[35–39]

A smaller subset of the genes identified in this study are involved with epidermal development, maintenance, and wound repair the most obvious of which is fibroblast growth factor 1 (FGF1). Others include mitogen-activated protein kinase 8 (MAPK8) which is involved in toll-like receptor signaling in the skin and implicated in the pathogenesis of skin diseases (e.g. psoriasis);[40] and insulin-like Growth Factor 1 Receptor (IGF1R) which is

expressed in keratinocytes and responds to IGF1 secreted from dermal fibroblasts.[41] Notably, dermatologic changes stimulated by *Propionibacterium acnes* appear to be mediated through the IGF/IGF1R system [42,43] Other genes in our dataset that can be grouped in this functional class include PCDH7 and ADAM-12.

Among the genes with a more unique role in skin homeostasis and host-pathogen interactions is longevity assurance gene 4 (LASS4). With its involvement in the ceramide synthesis pathway (i.e. the formation of n-acetylsphinganine from sphinganine), LASS4 is involved in maintaining the water permeability and barrier function of the skin. [44–46] In addition, the substrate on which LASS4 acts (sphinganine) is capable of inhibiting mycelial development and growth in a number of cutaneous pathogens including *T. tonsurans* which appears to be highly sensitive to this lipid [47] Another unique gene is galanin-like peptide (GALP), a neuropeptide that is expressed in dermal microvasculature. Although there is no direct evidence for GALP, related members of this gene family regulate cutaneous blood flow and demonstrate the ability to inhibit the budding and growth of the cutaneous yeast *Candida albicans*. [48–50] The Kallmann syndrome 1 (KAL1) gene innervates itch perception and is differentially regulated in atopic dermatitis.[51] Interestingly, it is orthologous to a canine antimicrobial peptide that is active on skin.[52] Finally, Fibrinogen C domain containing 1 (FibCD1) functions primarily to bind chitin from invading fungi and parasites. While interesting with respect to a putative role in cutaneous infections, FibCD1 is highly expressed in the gastrointestinal tract and it is unclear whether it is expressed in the skin. [53,54].

This pilot analysis offers some intriguing gene candidates that may be associated with a child's susceptibility to superficial dermatophyte infections. Interestingly, genes that appear to play a role in susceptibility to chronic mucocutaneous candidiasis (e.g. STAT1 and 3, CARD9, AIRE, CLEC4E and 7A, CD209, IL-17) were not represented among the preliminary 275 genes identified by WGG in this study [55–60]. The recessive nature of the implicated sequence variations for some genes (AIRE, CARD9, IL-17RA) and the low allele frequency of other sequence variations in individuals of African ancestry (CLEC7A) may explain why they were not detected in our population. However, the role of other gene families (e.g. STAT) in the susceptibility to dermatophyte infections remains unknown.

Though the genes reported herein appear to have reasonable predictive power to discriminate children at risk for chronic *T. tonsurans* infection, it is important to clarify that we were simply able to detect an association and cannot infer a causal relationship. It is also important to emphasize that the study is limited by a small sample. It is possible that some of these associations we observed reflect false positive results and equally possible that we failed to identify associations with genes that play a role in disease susceptibility. Equally important is the fact that we restricted our analyses to functional gene regions. Intergenic sequence variations that could influence the regulation and/or expression of relevant genes would have been overlooked unless they exist in linkage disequilibrium with the SNPs that were evaluated.

Given the extent to which infections are observed in the urban pediatric community, numerous genes likely contribute to a child's overall risk of acquiring and sustaining dermatophyte infections following contact with the pathogen. Multiple pathways, rather than a singular common biological pathway likely contribute to the shared phenotype of infection risk. It is conceivable that pathogen, in addition to host, genetics also play a role in the long-term relationship between host and organism; however, we observed too many genetic strain types in our community to identify a relationship between fungal genotype and pathogenicity. Undoubtedly, it is the combination of host and pathogen traits that permit a long-term relationship between the two species.

When demographics and host genetics were considered together, we were able to account for approximately 55 to 65% of the variability observed in infection rate. Though other genetic factors may play a role, it is also important to consider host-environment interactions that may influence infection status. By enrolling children who shared a common daytime environment, we attempted to minimize the contribution of environment. However, the influence of the environment in which the children spend the remainder of their time cannot be discounted. Notably, there were several sibling sets in our earlier investigation wherein the children shared both a home and school environment yet demonstrated different infection patterns. This was the first cue that a unique host-pathogen relationship may exist.

Evidence for the contribution of any of the genes described herein will require replication in an independent cohort of children. Despite the intensive nature of the longitudinal study design that would be required, there is no shortage of populations (U.S. and international) in whom dermatophyte infection rates equal or exceed those observed in our community. Our small cohort is unlikely to provide all of the answers as to what factors predispose or prevent infection but our initial observations suggest a much larger effect on total heritability of the trait than we initially anticipated. Subsequent studies with dermatophytes, or other chronic cutaneous pathogens, that clarify the role of selected gene products in the infection process may facilitate the identification of therapeutic targets that restrict host-pathogen interactions in high-risk populations.

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Figure 1.

Box plots of risk index vs. infection phenotype with all genes considered (left), with the subset of genes demonstrating the greatest impact on phenotype (center) and with the subset of genes demonstrating the greatest impact on phenotype based on β -coefficient (right).

Table 1

gene name	gene abbreviation	rs	ANOVA p-value	Putative Mechanistic Role
Semaphorin 6A	SEMA6A	rs154576	2.03E-07	LRAM
microfibril-associated protein 4	MFAP4	rs6587082	9.18E-05	ECM
Fibrinogen C domain containing 1	FibCD1	rs12343454	3.27E-04	PB
Kallmann syndrome 1	KAL1	rs12007434	7.50E-04	APG
galanin-like peptide	GALP	rs16987032	1.02E-03	CHP
BH-protocadherin	PCDH7	rs11935515	1.41E-03	ECM, EDMW
Fibulin5	FBLN5	rs7411198	1.42E-03	ECM
Slit homolog 3	SLIT3	rs11134534	1.55E-03	LRAM
Marapsin-2	MPN2	rs12079887	1.76E-03	EDMW
Cub and Sushi Multiple Domains-1	Csmd1	rs6986644	1.93E-03	LRAM
Roundabout receptor 1	ROBO1	rs498805	2.24E-03	LRAM
Mitogen-activated protein kinase	MAPK8	rs7075976	5.13E-03	EDMW
Secreted modular calcium-binding protein-2	SMOC2	rs747994	5.20E-03	ECM, MF
Longevity assurance gene 4	Lass4	rs11666866	5.64E-03	APG, CHP
CD99-Like 2	cd99L2	rs2142003	9.04E-03	LRAM
Insulin-like Growth Factor 1 Receptor	IGF1R	rs11633294	1.31E-02	EDMW
Grb2-associated binder 2	GAB2	rs2450129	2.37E-02	LRAM
Matrix metallopeptidase 3	MMP3	rs522616	2.78E-02	ECM
Fibroblast growth factor 1	FGF1	rs2299020	2.93E-02	EDMW
Fibrillin-2	FBN2	rs27855	3.38E-02	ECM
A disintegrin and metalloprotease 12	ADAM12	rs17154587	4.11E-02	ECM, EDMW
Matrilin-2	MTR2	rs4360265	6.76E-02	ECM
Tribbles homolog 1	Trib1	rs2235108	1.53E-01	APG, EDMW

APG- antimicrobial peptides/glycopeptides, CHP- cutaneous hydration and permeability, ECM- extracellular matrix formation/integrity/remodeling, EDMW- epidermal development/maintenance/wound repair, LRAM- leukocyte recruitment/activation/migration, MF- melanocyte function, PB- parasite binding

Table 2

Statistical parameters for multivariable regression

Model	df	r ²	adjusted r ²	SE of the estimate	F	p-value
Risk-index based regression						
21 genes	21	0.704	0.635	17.930	10.178	<0.0001
21 genes + gender	22	0.724	0.656	17.406	10.604	<0.0001
21 genes + gender + race	23	0.758	0.695	16.390	11.979	<0.0001
8 significant genes ^a	8	0.631	0.603	18.696	22.454	<0.0001
8 genes + gender	9	0.661	0.632	18.011	22.523	<0.0001
8 genes + gender + race	10	0.698	0.669	17.081	23.800	<0.0001
β -coefficient based regression						
21 genes	21	0.656	0.577	19.214	8.269	<0.0001
21 genes + gender	22	0.683	0.605	18.565	8.795	<0.0001
21 genes + gender + race	23	0.707	0.631	17.945	9.322	<0.0001
7 significant genes ^b	7	0.561	0.533	20.289	19.570	<0.0001
7 genes + gender	8	0.588	0.557	19.759	18.909	<0.0001
7 genes + gender + race	9	0.612	0.579	19.262	18.411	<0.0001

df- degrees of freedom, r- multiple correlation coefficient, SE- standard error, F- F-statistic

^aC_{sm}D1, FibCD1, Fibulin 5, LASS4, MAPK8, SEMA6A, SLIT3 and SMOC2^bC_{sm}D1, FibCD1, Fibulin 5, GALP, LASS4, MAPK8, SEMA6A