Whole-Exome Sequencing Identifies the 6q12-q16 Linkage Region and a Candidate Gene, *TTK*, for Pulmonary Nontuberculous Mycobacterial Disease

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Abstract

Rationale: Pulmonary nontuberculous mycobacterial disease (PNTM) often affects white postmenopausal women, with a tall and lean body habitus and higher rates of scoliosis, pectus excavatum, mitral valve prolapse, and mutations in the *CFTR* gene. These clinical features and the familial clustering of the disease suggest an underlying genetic mechanism.

Objectives: To map the genes associated with PNTM, whole-exome sequencing was conducted in 12 PNTM families and 57 sporadic cases recruited at the National Institutes of Health Clinical Center during 2001–2013.

Methods: We performed a variant-level and a gene-level parametric linkage analysis on nine PNTM families (16 affected and 20 unaffected) as well as a gene-level association analysis on nine PNTM families and 55 sporadic cases.

Measurements and Main Results: The genome-wide variant-level linkage analysis using 4,328 independent common variants identified a 20-cM region on chromosome 6q12-6q16 (heterogeneity logarithm of odds score = 3.9), under a recessive disease model with 100% penetrance and a risk allele frequency of 5%. All genes on chromosome 6 were then tested in the gene-level linkage analysis, using the collapsed haplotype pattern method. The *TTK* protein kinase gene (*TTK*) on chromosome 6q14.1 was the most significant (heterogeneity logarithm of odds score = 3.38). In addition, the genes *MAP2K4*, *RCOR3*, *KRT83*, *IFNLRI*, and *SLC29A1* were associated with PNTM in our gene-level association analysis.

Conclusions: The *TTK* gene encodes a protein kinase that is essential for mitotic checkpoints and the DNA damage response. *TTK* and other genetic loci identified in our study may contribute to the increased susceptibility to NTM infection and its progression to pulmonary disease.

Keywords: genetic linkage; DNA sequence analysis; nontuberculous mycobacteria

Pulmonary nontuberculous mycobacterial (PNTM) disease is caused by infections with nontuberculous mycobacteria, which are ubiquitous in the environment and normal inhabitants of the drinking water system. Although exposure is nearly universal and

infection is common in many geographic areas, only a proportion of infected people develop pulmonary disease (1–4). The disease is usually chronic, indolent, and difficult to diagnose and treat. According to the 2007 American Thoracic

Society/Infectious Diseases Society of America statement (5), the diagnosis of PNTM is made from specific clinical, microbiological, and chest imaging findings. Population-based data from North America (3, 4, 6–8), Europe (9–13),

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At a Glance Commentary

Scientific Knowledge on the

Subject: Pulmonary nontuberculous mycobacterial disease (PNTM) has posed an increasing health burden globally in aging populations. The shared morphologic and clinical features among individuals affected with PNTM suggest a strong genetic influence. Several studies have connected the disease to multiple gene categories and pathways. However, no specific genetic regions or loci have been identified for PNTM.

What This Study Adds to the

Field: We found evidence of genetic linkage on chromosome 6q12-q16 and further identified the gene *TTK* (TTK protein kinase) as a candidate gene for PNTM. *TTK* may contribute to the susceptibility of PNTM through its role in host DNA damage repair and cell survival.

Australia (14), and Asia (15, 16) have consistently shown a continued increase in both the prevalence and incidence of PNTM in the past two decades. Across these regions, PNTM is more common in individuals aged 50 years and older, with *Mycobacterium avium* complex as the dominant species of PNTM. In the United States, the majority of PNTM cases are white females (3, 4, 7, 8), whereas in Europe middle-aged men with chronic lung conditions predominate, possibly due to a historical excess of men smoking (9, 10).

Host and environmental factors interact to influence PNTM disease risk. It is associated with predisposing conditions including lung cancer, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (17). In addition, a low body mass index has been associated with increased risk of PNTM disease (16, 18, 19). Climatic and population factors, such as greater evapotranspiration and greater population density, are thought to promote mycobacterial growth and disease transmission (17, 20). However, given that the organisms are so widespread yet disease is rare, host susceptibility likely plays a key role.

A distinct group of people affected by PNTM in the United States are postmenopausal women who are white, tall, thin, and

nonsmokers, who have high rates of scoliosis, pectus excavatum, mitral valve prolapse, and cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations (18). PNTM often presents among these women as nodular bronchiectatic PNTM, as opposed to the cavitary PNTM typically seen in men with a history of smoking and COPD (20). The shared morphologic features of women with PNTM, along with the observed familial clustering (19), support the hypothesis of genetic predisposition to the development of PNTM. Candidate gene studies (21–23) and pathway/network analyses (24, 25) have implicated certain genetic factors and biological pathways for PNTM.

In this study, we conducted a genome-wide linkage analysis among nine PNTM families to identify genetic regions that may harbor PNTM genes. With whole-exome sequencing data, we performed a gene-level linkage test, using a collapsed haplotype pattern (CHP) method, to further determine the gene(s) driving the detected linkage signals. A gene-based association analysis was also conducted on PNTM families and sporadic cases to identify rare variants associated with PNTM. Some of the results of this study have been previously reported in the form of an abstract (26).

Methods

A detailed description of the methods can be found in the online supplement.

Subjects and Phenotype

Between 2001 and 2013, 12 PNTM families (21 PNTM-affected and 24 unaffected family members) and 57 sporadic PNTM cases were recruited at the National Institutes of Health Clinical Center (Bethesda, MD). PNTM diagnoses were made according to clinical guidelines (see the online supplement). Informed consent was obtained from all subjects, and this study was approved by the National Institutes of Health (protocols 01-I-0202, 07-I-0033, 07-I-0142, and 09-I-0172).

Whole-Exome Sequencing and Quality Control

Whole-exome sequencing and variants calling have been previously reported (25). All variants were annotated by ANNOVAR (March 2015) (27). After quality controls, the final data set contained nine families

(16 PNTM-affected and 20 unaffected individuals) and 55 sporadic cases with 249,843 single-nucleotide variants (SNVs) and 30,665 small insertion/deletion variants (INDELs). Our study population is primarily of European ancestry with a small number of individuals with East Asian ancestry (see the online supplement).

Genetic Linkage Analysis

We conducted a parametric linkage analysis of the PNTM families at both the variant level and the gene level. The linkage analysis was limited to SNVs only, because the genetic distance between insertion/deletion variants in the human genome is not well characterized.

The variant-level parametric linkage analysis was performed in MERLIN (28), using a subset of common (minor allele frequency [MAF] > 0.10) and independent (pairwise $r^2 < 0.10$) variants with call rate greater than 0.95. The genetic map GRCh37, from the HapMap project (<https://www.genome.gov/10001688/international-hapmap-project/>), was used for the analysis. We tested 60 disease models varying by mode of inheritance (dominant, recessive, and additive), penetrance (40%, 60%, 80%, and 100%), and risk allele frequency (0.01, 0.05, 0.10, 0.15, and 0.20). A heterogeneity logarithm of odds (HLOD) score greater than 3.3 was considered significant for genetic linkage (see the online supplement).

For chromosomal regions with the most significant linkage, a gene-level linkage analysis was performed to determine the gene(s) driving the linkage signals. We first applied the CHP method (29) to all sequenced variants to construct haplotypes for each gene and tested these genes for linkage, using MERLIN (see the online supplement). To infer the MAF range of the causal variants, we conducted a separate analysis to include only the rare variants with MAF not greater than 0.05 and the common variants with MAF greater than 0.05.

Association Study

To search for rare variants associated with PNTM, we performed a genome-wide association test in our study cohort of PNTM families and sporadic cases. We applied a gene-based approach that extended the commonly used collapsing and kernel methods in unrelated case-control data to account for known pedigree relationships (30). East Asian individuals were excluded from the analysis, resulting in 66 case

subjects and 15 control subjects in the analyzed cohort. Analysis was restricted to 16,026 genes with at least two rare variants defined as SNVs with call rate greater than 95% and MAF less than 0.05 in the 1000 Genomes Project European populations (*see the online supplement*). The widely accepted genome-wide threshold $P < 5 \times 10^{-8}$ was used to determine the significance.

Results

A set of 4,328 independent SNVs with known genetic location was tested in the variant-level parametric linkage analysis for nine families (*see Figure E1 in the online supplement*). This analysis identified a 20-cM region on chromosome 6q12-q16 with evidence of linkage, and the evidence was strongest (HLOD score = 3.9) under a recessive disease model with 100% penetrance and a risk allele frequency of 0.05 (Figure 1; and *see Figures E3 and E4*). An empirical P value obtained from a permutation analysis under the null hypothesis of no linkage was less than 10^{-6} . Under this disease model, family A (LOD score = 0.963), family B (LOD score = 0.977), and family D (LOD score = 0.711) each contributed the most to this linkage

signal on chromosome 6 (*see Figure E1*). The information content across markers on chromosome 6 was consistently greater than 80%, suggesting an adequate power of the selected markers in extracting inheritance information (*see Figure E3*) (31).

In gene-level linkage analysis, considering all SNVs on chromosome 6, a total of 1,005 genes were collapsed using the CHP method and tested in a single-point parametric linkage analysis under various recessive disease models with 100% penetrance, varying by risk allele frequencies. The *TTK* protein kinase gene (*TTK*; OMIM [Online Mendelian Inheritance in Man] 604092) on chromosome 6q14.1 was identified as significant with an HLOD score of 3.38. The significance of the *TTK* gene was attenuated when common variants (MAF > 0.05) were excluded from the analysis (Table 1), suggesting that the risk alleles in the *TTK* gene associated with PNTM are likely to have a frequency between 0.05 and 0.10 in European populations.

Among 27 SNVs and 4 INDELs sequenced in the *TTK* gene, 22 of them showed variations across the PNTM families (*see Table E1*). As suggested by the linkage results, the disease-causing variants may cosegregate in the PNTM families as either recessive homozygotes or compound

heterozygotes. We applied a filtering strategy to search for the causal variants for PNTM (*see Figure E5*). Eight variants met the criteria of not being homozygotes of the risk allele in any unaffected individuals across all PNTM families. Variants were then considered recessive if they were homozygotes of the risk allele in all cases of a given family, allowing families to be affected by different variants. Variants were considered compound heterozygous if they were (1) heterozygotes in all cases of a given family, and (2) not compound heterozygotes in any control subjects of any PNTM family. However, we found that no variants in the *TTK* gene region met the above-mentioned criteria (data not shown).

Evidence of suggestive linkage was also found in the linkage region on chromosome 6q12-6q16 for the genes trophoblast glycoprotein (*TPBG* [OMIM 190920]; HLOD score = 3.08), origin recognition complex subunit 3 (*ORC3* [OMIM 604972]; HLOD score = 3.10), and ankyrin repeat domain 6 (*ANKRD6* [OMIM 610583]; HLOD = 3.02). For all of these genes, the linkage signals were likely to be driven by variants with common allele frequencies greater than 0.05 (Table 1).

A total of 16,026 genes across the genome were tested in the gene-based association analysis, with a mean coverage

Figure 1. Multipoint parametric linkage analysis of pulmonary nontuberculous mycobacterial disease under a recessive disease model with 100% penetrance and risk allele frequency of 0.05. The x-axis indicates the genetic position in chromosomes, and the y-axis presents the heterogeneity logarithm of odds (HLOD) score. The horizontal dashed line at HLOD score = 3.3 denotes the threshold for evidence of significant linkage.

Table 1. Gene-Level Single-Point Parametric Linkage Analysis Results

Gene	Location [†]	Disease Model [‡]	All* No. of SNVs HLOD [§]		Common* (>0.05) No. of SNVs HLOD [§]		Rare* (≤0.05) No. SNVs HLOD [§]	
			No. of SNVs	HLOD [§]	No. of SNVs	HLOD [§]	No. SNVs	HLOD [§]
<i>TTK</i>	6q14.1	Recessive, 100% penetrance, and risk allele frequency of 0.05	27	3.38	13	3.35	14	1.30
<i>TPBG</i>	6q14-6q15	Recessive, 100% penetrance, and risk allele frequency of 0.05	6	3.08	1	2.49	5	0.41
<i>ORC3</i>	6q14.3-q16.1	Recessive, 100% penetrance, and risk allele frequency of 0.20	18	3.10	10	3.18	8	0.33
<i>ANKRD6</i>	6q15	Recessive, 100% penetrance, and risk allele frequency of 0.05	22	3.02	12	2.52	10	0.44

Definition of abbreviations: *ANKRD6* = ankyrin repeat domain 6; HLOD = heterogeneity logarithm of odds score; *ORC3* = origin recognition complex subunit 3; *TPBG* = trophoblast glycoprotein; *TTK* = TTK protein kinase; SNVs = single-nucleotide variants.

*Allele frequencies of the alternate alleles were adopted from the European populations in the 1000 Genomes Project.

[†]Location reported in GRCh37.

[‡]Disease models under which the reported genes were most significant.

[§]HLOD score under the suggested disease model for each reported gene.

of seven SNVs in each gene region. Five genes reached genome-wide significance at $P < 5 \times 10^{-8}$ (Table 2).

Discussion

Multipoint parametric linkage analysis identified a 20-cM region on chromosome 6q12-q16 under a recessive disease model with 100% penetrance and risk allele frequency of 0.05. The linkage was most significant at the *TTK* protein kinase gene (*TTK*), as suggested in our gene-level linkage analysis using the CHP method. In the same linkage region, suggestive linkage was also found with genes *TPBG*, *ORC3*, and *ANKRD6*.

The *TTK* gene on 6q14.1 encodes a dual-specificity protein kinase (hMps1), a key spindle assembly checkpoint protein that regulates the proper chromosomal alignment and segregation during mitosis (32–35). The expression level of hMps1 in several human tumors is correlated positively with tumor grade and negatively with survival (36–38), making it one of the most promising drug targets for cancer treatment (39). In addition to regulation of the spindle assembly checkpoint, hMps1 also participates in the DNA damage response. Depletion of hMps1 causes a defect in G₂/M arrest, compromising DNA repair and cell survival (40–42). Dysfunction in DNA repair is a common finding in patients with COPD and tuberculosis (43). As studies on *TTK*/hMps1 have been exclusively focused

on its tumorigenesis, its contribution to bronchiectasis; to bacterial, mycobacterial, or fungal infections; or overall progression to pulmonary disease is currently unknown. Impaired DNA damage repair in host cells induced by defects in the *TTK* gene may provide a beneficial environment for bacterial proliferation or may compromise local innate immune responses, allowing infections to cause disease.

Studies on the genes *TPBG*, *ORC3*, and *ANKRD6* are limited and thus their relevance to PNTM is unclear. All three genes are expressed in lung tissues. The *TPBG* gene encodes an oncofetal antigen that is highly expressed in many types of cancer (44, 45). The protein encoded by the *ORC3* gene is a subunit of the origin recognition complex (ORC), which is essential for the initiation of the DNA replication. Diversin, a planar cell polarity protein encoded by the *ANKRD6* gene, is involved in the Wnt signaling pathways and has been implicated in human neural tube defects (46, 47).

Our gene-based association analysis identified five genes whose rare variants are associated with PNTM, of which two genes may affect PNTM through their involvement in host immune defense in bacterial or viral infection. The most significant association was found with the mitogen-activated protein kinase 4 gene (*MAP2K4*) on chromosome 17p12. The encoded protein is a member of the mitogen-activated protein kinase

Table 2. Top Gene-based Association Results: $P < 5 \times 10^{-8}$

Gene	OMIM No.*	Location [†]	No. of SNVs	P Value
<i>MAP2K4</i>	601335	17p12	2	1.63×10^{-10}
<i>RCOR3</i>	—	1q32.2	4	6.44×10^{-9}
<i>KRT83</i>	602765	12q13	5	1.47×10^{-8}
<i>IFNLR1</i>	607404	1p36.11	4	1.64×10^{-8}
<i>SLC29A1</i>	602193	6p21.2	4	1.78×10^{-8}

Definition of abbreviations: *IFNLR1* = IFN- λ receptor 1; *KRT83* = keratin 83; *MAP2K4* = mitogen-activated protein kinase kinase; OMIM = Online Mendelian Inheritance in Man; *RCOR3* = REST corepressor 3; *SLC29A1* = solute carrier family 29 member 1; SNVs = single-nucleotide variants.

*Gene identifier in the OMIM database.

[†]Location reported in GRCh37.

(MAPK) family, and is involved in a number of KEGG pathways including the Toll-like receptor signaling pathway; epithelial cell signaling in *Helicobacter pylori* infection; and hepatitis B, influenza A, and Epstein–Barr virus infection (48). The product of the IFN- λ receptor 1 gene (*IFNLR1*) on chromosome 1p36.11 forms a receptor complex with IL-10 receptor β (IL10RB) and interacts with IL-28A, IL-28B, and IL-29. Genetic variations in *IFNLR1* are correlated with susceptibility to infection with hepatitis C virus and spontaneous viral clearance (49). Differential expression of *IFNLR1* in low-risk and high-risk human papillomavirus-positive women suggests its potential contribution to lesion progression in high-risk human papillomavirus infection (50). In addition, the solute carrier family 29 member 1 gene (*SLC29A1*) encodes the human equilibrative nucleoside transporter 1 (hENT1) that mediates the transport of nucleosides and nucleoside analog drugs across the plasma membrane. Repression of hENT1 dampens pulmonary inflammation and improves lung function in animal models (51), which could potentially be relevant to progression to pulmonary disease in PNTM.

PNTM is an emerging public health issue, posing substantial health and economic

burdens, particularly in aging populations. Our study systematically investigated the host genetics associated with nontuberculous mycobacterial infection and pulmonary disease. We combined traditional linkage analysis of individual variants with a novel gene-level approach based on collapsed haplotypes, and identified the *TTK* gene on chromosome 6q14.1 as a candidate gene for PNTM. However, our data suggest that the disease-causing variants of the *TTK* gene are likely common (MAF, 0.05–0.10) in the general population and may function as recessive homozygotes or compound heterozygotes, which is consistent with the observed low prevalence of the disease. Importantly, the causal variants may be located outside the exonic regions of the *TTK* gene and therefore not be included in this exome-sequencing data set. Furthermore, small insertion/deletion variants may also explain some of the familial aggregation of PNTM, but these currently cannot be analyzed in linkage and gene-based association analysis. Because the gene-based association analysis using pedigree data has slightly inflated type I errors (30), we applied a threshold ($P < 5 \times 10^{-8}$) more stringent than the Bonferroni-corrected P value ($P < 3 \times 10^{-6}$) to determine the

statistical significance of the association results. This led to the identification of several genes that may be biologically relevant to PNTM.

Like other complex diseases, PNTM has a multifactorial etiology involving many genes and environmental exposures (25). Our family-based study identified the *TTK* gene region as linked to the familial form of the disease in our study population. Future studies with larger sample sizes are warranted to replicate our findings. The impact of this gene depends on the frequencies and effect sizes of the putative alleles in the general population, which requires further investigation with population sampling. Functional studies may also help to elucidate the role of *TTK* and the underlying disease mechanism of PNTM, and hence lead to the development of effective treatments of the disease. Findings from these genetic studies will provide valuable insights for the prevention, diagnosis, and treatment of PNTM. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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