Mapping of a Novel Susceptibility Locus Suggests a Role for MC3R and CTSZ in Human Tuberculosis

Graham S. Cooke[1,2,3], Sarah J. Campbell[1], Steve Bennett[4†], Christian Lienhardt[5], Keith P. W. J. McAdam[6], Giorgio Sirugo[6], Oumou Sow[7], Per Gustafson[8], Frank Mwangulu[9†], Paul van Helden[10], Paul Fine[9], Eileen G. Hoal[10], and Adrian V. S. Hill[1]

[1]Wellcome Trust Centre for Human Genetics, University of Oxford, Churchill Hospital, Headington, Oxford, United Kingdom; [2]Imperial College, London, United Kingdom; [3]Africa Centre for Health and Population Studies, University of KwaZulu-Natal, Somkhele, South Africa; [4]London School of Hygiene and Tropical Medicine, London, United Kingdom; [5]National TB/Leprosy Control Programme, Banjul, The Gambia; [6]Medical Research Council Laboratories, Fajara, The Gambia; [7]CHU Ignace Deen, Conakry, Guinea; [8]Danish Epidemiology Science Centre, Bissau, Guinea-Bissau; [9]Karonga Prevention Study, Chilumba, Malawi; and [10]Medical Research Council Centre for Molecular and Cellular Biology, Stellenbosch University, South Africa

***Rationale*: Tuberculosis remains a major cause of morbidity and mortality in the developing world. A better understanding of the mechanisms of disease protection could allow novel strategies to disease management and control.**

***Objectives*: To identify human genomic loci with evidence of linkage to tuberculosis susceptibility and, within these loci, to identify individual genes influencing tuberculosis susceptibility.**

***Methods*: Affected sibling pair analysis in South African and Malawian populations. Independent case-control study in West Africa.**

***Measurements and Main Results*: Two novel putative loci for tuberculosis susceptibility are identified: chromosome 6p21-q23 and chromosome 20q13.31—33—the latter with the strongest evidence for any locus reported to date in human tuberculosis (single point LOD score of 3.1, $P = 10^{-4}$, with a maximum likelihood score [MLS] of 2.8). An independent, multistage genetic association study in West African populations mapped this latter region in detail, finding evidence that variation in the melanocortin 3 receptor (MC3R) and cathepsin Z (CTSZ) genes play a role in the pathogenesis of tuberculosis.**

***Conclusions*: These results demonstrate how a genomewide approach to the complex phenotype of human tuberculosis can identify novel targets for further research.**

Keywords: tuberculosis; host genetics; MC3R; Africa

Interest in human susceptibility to tuberculosis predates the discovery of the causative pathogen. Evidence has accumulated from a number of sources that human genetics influences an individual's susceptibility to disease and in recent years some of the genes responsible have been identified, most notably HLA class II (1–3), natural resistance-associated macrophage protein-1 (NRAMP1) (4–8), interferon gamma (IFNG) (9–11), and MYD88 adaptor-like (MAL) (12). These successes have generally used a candidate gene approach based on knowledge of human immunology gained from bench, animal, and human studies.

Genomewide linkage studies offer the promise of discovering genes influencing disease susceptibility for which a role might not have been previously suspected. To date, despite success in the closely related disease of leprosy (13–16), genomewide linkage studies have failed to identify a major susceptibility locus for tuberculosis. Previous work in a South American study (17) found the strongest evidence for linkage in the genomic regions 20p12.1 and 17q11–21, and work in African populations (18) has implicated the regions 15q11–13 (19) and Xq27 (20).

We performed further affected sibling pair linkage analysis in two populations: "Coloureds" (South Africans of mixed racial origin) from metropolitan Cape Town and Malawians from the Karonga district. One genomic region, 20q13.31–33, was prioritized for further study. With a two-stage design, this region was then mapped in detail in a large, independent West African case-control study, with the aim of identifying novel loci involved in the pathogenesis of disease.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Using prior knowledge of a gene's function, a limited number of genes have shown convincing evidence of influencing susceptibility to tuberculosis. However, new genomewide approaches can identify novel susceptibility genes with no prior knowledge of function.

What This Study Adds to the Field

Using a genomewide approach and positional mapping, this study identifies two novel genes, melanocortin 3 receptor and cathepsin 7, that play a role in susceptibility to tuberculosis in African populations. Both are related to macrophage function and are potential therapeutic targets.

METHODS

Family Recruitment

Families for genomewide analysis were recruited from the Coloured population in metropolitan Cape Town, South Africa. A total of 131 sibling pairs were identified in 81 families with pulmonary tuberculosis, defined by either a positive sputum smear or positive mycobacterial culture. Twenty-four sibling pairs were identified in 24 families recruited from the Karonga Prevention Study in Malawi. Where possible, DNA was also collected from both parents. When this was not possible, DNA from unaffected siblings was collected to allow reconstruction of parental genotypes. All individuals gave informed consent for their samples to be used for genetic analysis. Approvals were obtained from local ethics committees (University of Oxford, UK; Gambian Government/Medical Research Council Ethical Committee; Ministry of Public Health, Guinea-Bissau; National Ethics Committee, Ministry of Health, Conakry, Guinea; and the ethics committee of the Faculty of Health Sciences, Stellenbosch University, South Africa).

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Correspondence and requests for reprints should be addressed to Graham S. Cooke, Ph.D., M.R.C.P., Africa Centre for Health and Population Studies, PO Box 198, Mtubatuba 3935, Somkhele, KwaZulu-Natal, South Africa. E-mail: gcooke@africacentre.ac.za

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Microsatellite Genotyping

A commercially available set of microsatellite markers (LMSv2 MD10; Applied Biosystems, Foster City, CA) was used for genomewide linkage analysis. Individual markers were amplified with a range of annealing temperatures (55°–61°C) and Mg concentrations (1.5–2.5 mM Mg^{2+}) in a 15-μl reaction mix containing 50 ng of DNA, 40 ng of each primer, 2.5 mmol KCl buffer, 100 μM dNTPs, and 0.2 U Taq polymerase. Details of primers and conditions are available from the authors on request. FAM-labeled products were pooled separately from HEX/NED-labeled products to avoid spectral overlap and analyzed with the AB 3700 system. Genotypes were extracted using Genotyper software (Genotyper Version 2; Applied Biosystems).

Statistical Methods

Pedigrees were checked for nonpaternity with GAS (Oxford University, Oxford, UK) and PedCheck (University of Pittsburgh, Pittsburgh, PA) (21). LOD (logarithm of the odds) scores for individual markers were estimated with the Sibpair analysis program (Queensland Institute of Medical Research, Queensland, Australia) (22). Sibpair analysis provides a likelihood-based statistic for linkage that is equivalent to the LOD score calculated assuming recessive disease with the phase unknown. If parents were unavailable, the likelihood is calculated using the sum of terms corresponding to each possible parental genotype combination calculated assuming Hardy-Weinberg equilibrium (HWE). The method is a nonparametric approach, which is preferred because the mode of inheritance for any putative disease susceptibility allele is unknown. Exclusion mapping was performed with a parametric method such that areas of the genome with sufficiently low LOD scores can be excluded from containing a gene of interest with a specified lambda-s.

Maximum likelihood multipoint mapping was performed with the Mapmaker/Sibs program (Whitehead Institute for Biomedical Research, Cambridge, MA) (23) to increase the information content at any given locus. Only fully independent sibling pairs were analyzed and maximum likelihood scores calculated using Holman's constraints (24). Multipoint data were assessed for possible errors in genotyping using PEDwipe, part of the Merlin program (University of Michigan, Ann Arbor, MI) (25).

Analysis

An initial genomewide screen was performed on 71 sibling pairs with 402 polymorphic microsatellite markers spanning the genome. Thirty-nine sibling pairs had been part of a previous study (18), and for these samples, markers in 15q11-13 and Xq27 were excluded from analysis. Markers for which linkage could not be excluded were subsequently analyzed in the remaining 60 sibling pairs. Regions with evidence for linkage were then studied in the Malawi population and data combined to prioritize regions for further study.

Case-Control Study

Detailed single nucleotide polymorphism (SNP) mapping was performed as part of a large, independent case-control study of adult pulmonary tuberculosis from West Africa, described in detail previously (26, 27). The study includes population collections from The Gambia, Guinea-Bissau, and Guinea. All cases had been confirmed by either two consecutive smear-positive samples or a positive mycobacterial culture. Both HIV-positive and HIV-negative cases were included, the prevalence of each differing between countries, and with prevalence ranging from 2 to 8.1% between countries (26). For further testing, samples were included from another Gambian case-control study (4). A test of HWE was applied to the control population for each SNP. Unless noted, SNPs did not deviate from HWE. In view of the large number of SNPs being tested for association, and the potential pitfalls of multiple testing, a multistage strategy was adopted. The Gambian cohort within the TB, Genetics, and Environment study (TBGENENV) study was investigated first, with significant findings followed up in other remaining populations.

SNP Typing

Within a 1-LOD support region around the peak of linkage, a cluster of genes lying between microsatellite markers d20s832 and d20s173 were prioritized for study. Forty SNPs from this region were identified from publicly available databases. All SNPs were typed using the MassArray platform (Sequenom, San Diego, CA) using the manufacturer's recommended protocols. The seven exons and the 3′ untranslated region (3′UTR) of cathepsin Z (CTSZ) were sequenced as separate polymerase chain reaction products and analyzed on the ABI 3700 system using SeqMan software (DNAStar, Madison, WI) (primers and conditions for all reactions available from authors on request). SPSS version 11 (SPSS, Inc., Chicago, IL) was used to perform binary logistic regression to assess the effect of genotype on disease outcome, adjusted for age, sex, ethnicity, and HIV status.

RESULTS

Genomewide Analysis

Using an LOD score of less than or equal to −2 as a threshold for exclusion, over 40% of the genome was found unlikely to contain a putative disease susceptibility locus with a locus-specific λ_s of 2 or more. The first-round genome screen identified 64 markers that were followed up for further analysis.

A novel region, not previously studied in this population, was identified on chromosome 6 (6p21-6q23) with evidence for linkage found in both stages of the genomewide screen. The highest single point of linkage was found at marker d6s434 (single point LOD, 1.9; MLS, 1.82; $P = 0.002$). Evidence for linkage was found within the region of 15q11-13, identified in previous work (18), and including samples previously analyzed, an MLS (LOD) score of 2.1 is found in the Coloured population alone. Further evidence suggesting linkage was identified in regions including 20q31-33 (LOD score on first round, 0.9; overall MLS, 0.6) and 14q31-32 (LOD, 1.1 on first screen; overall MLS, 0.9). There was weak evidence for linkage in the region 17p11.2-17q11.2 (MLS, 0.6).

Twelve markers used in the complete genome screen were then selected for study in the Malawian population. These 12 markers were drawn from the LMSv2 and chosen if they fell within the regions of suggestive linkage on chromosomes 6p21-q23, 15q11-13, 20q13.31—33, and Xq27. A list of these markers is available from the authors. Two of these markers were within the putative region of linkage on chromosome 20 (d20s171 and d20s173); d20s171 showed evidence of linkage (LOD, 2.0; $P = 0.0005$) with some weak evidence for d20s173 (LOD, 0.91; $P = 0.91$).

To prioritize regions for further study, all markers typed with the same primers were analyzed together with families studied previously (18) as a single dataset. The strongest evidence for linkage was within the region 20q13.31-33 (with an MLS of 2.8 on multipoint mapping, $P = 0.00008$; *see* Figure 1).

Detailed Association Mapping of 20q30.31-33

In an initial screen of 40 SNPs, polymorphism within two genes showed evidence of disease association in the Gambia: melanocortin 3 receptor (MC3R; rs3827103 referred to here as

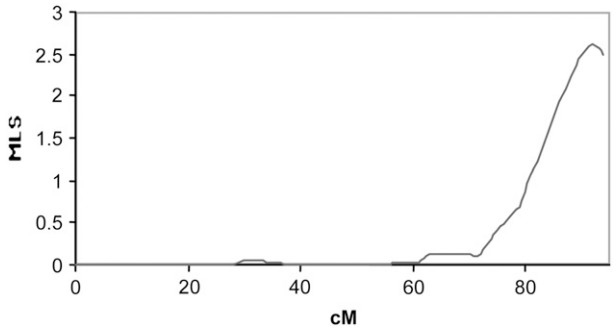

Figure 1. Multipoint mapping of chromosome 20 for all populations studied. Peak of linkage corresponds to marker 20s171.

MC3R241) encoding MC3R (MC3R241 genotype AA protective, $P = 0.04$) and CTSZ (GenBank SNP NM_011362 [refSNIP rs34069356; National Center for Biotechnology Information, Bethesda, MD] referred to here as CTSZ3P) encoding cathepsin Z (CTSZ3P genotype CC susceptible, $P = 0.01$). Associated polymorphisms within these genes were typed in the populations of Guinea-Bissau and Guinea.

CTSZ

The genotype distributions of CTSZ3P were similar in all three populations and combined analysis found significant evidence of disease association among cases (CC genotype 386 [58.0%], CT 255 [38.3%], and TT 24 [3.6%]) versus controls (316 [52.9%], 236 [39.5%], and 45 [7.5%], respectively). These results were significant when regression analysis was performed to correct for age, sex, ethnicity, and HIV status (odds ratio [OR] of TT genotype for disease, 0.49; 95% confidence interval [CI], 0.29–0.84; $P = 0.009$).

Genotyping was repeated to confirm the results and 90 cases and control subjects were sequenced to validate the genotypes. Once strong disease association was confirmed within the 3′UTR SNP, sequencing was extended to the gene's coding exons and other neighboring markers were examined in more detail. Only one nonsynonymous SNP was identified within CTSZ (in exon 5), but this showed no evidence of disease association ($P = 0.31$).

MC3R

Over the three populations studied, the trend to protective effect for the MC3R genotype AA remained between cases (AA genotype 142 [20.4%], AG 357 [51.3%], and GG 197 [28.3%]) versus control subjects (156 [24.1%], 314 [48.5%], and 178 [27.5%], respectively). However, regression analysis was nonsignificant ($P = 0.26$). Genotyping was confirmed by direct sequencing of the single exon of MC3R.

In light of the association seen in the Gambian population within TBGENENV, further genotyping was done in samples from a previous study (4). This independent dataset also found evidence of a protective role for the MC3R241 AA genotype. Genotypes found in cases were AA 63 (22.2%), AG 137 (48.2%), and GG 84 (29.6%), and in control subjects were AA 53 (30.9%), AG 68 (39.7%), and GG 50 (29.2%); OR for the AA genotype, 0.63; 95% CI, 0.40–1.00; $P = 0.04$. Combining the two Gambian populations found a stronger protective effect (OR, 0.67; 95% CI, 0.50–0.88; $P = 0.004$).

DISCUSSION

There are two key aspects to these results: the first is the novel identification of a major locus for susceptibility to human pulmonary tuberculosis on chromosome 20; the second is the identification of two susceptibility genes with a plausible link to tuberculosis, both of which merit further study.

The great attraction of linkage analysis remains that, starting from clinical cases of disease, and with no prior hypothesis of disease immunology, it is possible to identify previously unsuspected molecules playing a role in disease. For tuberculosis, there are three potential susceptibility loci that have been studied in detail to date. Those on 15q11-13 and Xq27 (18) have the most statistically convincing evidence of linkage in published work to date. The former has already been the subject of some preliminary mapping (19) with the gene UBE3A implicated in disease. Key candidates within Xq27 have also been investigated and no disease association was established for either of two candidate genes prioritized on the basis of their position and function, CD40 ligand, and iduronate 2-sulphatase (IDS) (20). More recently, detailed mapping within a locus on chromosome 17q11-q21 has found disease association with several genetic variants within one gene cluster (28), which could not be resolved more finely.

The data presented here suggest two novel putative tuberculosis susceptibility loci in African populations, 6p21-23 and 20q13.31-33. The latter presents the strongest evidence for linkage yet with the complex phenotype of tuberculosis. The challenge of finding disease association beyond the original dataset can often be difficult and confounded by the problems of multiple testing. The multistage strategy described here finds evidence of disease association within the gene encoding cathepsin Z, a member of the cathepsin protease family, and MC3R, a member of the melanocortin receptor (MCR) family.

The cathepsins are a family of cysteine proteases, which form a major component of the lysosomal proteolytic system and thus potentially have a role in immunity to pathogens including *Mycobacterium tuberculosis*. Endosomal proteases have a role in two elements of major histocompatibility class II–restricted antigen presentation: antigen degradation and invariant chain cleavage.

The gene encoding cathepsin Z was cloned after a search for homologs of the then-known cathepsins (29). The enzyme, 303 amino acids in length, has significant similarity to cathepsin B (26%), cathepsin C (34%), and cathepsin L (30). However, the observations that the enzyme differs significantly from other cathepsins with a shorter leader propeptide, a more diverse array of insertions around the active site, and an unusual chromosomal location led the authors to suggest it might be a member of an independent cathepsin subfamily. It is encoded by a gene within the region 20q13.31-33.

To date, evidence supporting a role for cathepsin Z in tuberculosis has been inferred from an appreciation of its role in macrophages and by analogy to other cathepsins. Cathepsin Z is one of only two cathepsins preferentially expressed in murine macrophages (the other is cathepsin F) (31). It is recruited to the murine phagosome and is found there hours after phagocytosis (32). Using murine primary macrophage cell lines (J774), Lennon-Dumenil and colleagues monitored the sequential recruitment and activation of cathepsins to the phagosome. They found that cathepsin Z was one of the four most abundant cysteine proteases present and active in early phagosomal compartments, despite its relatively mild pH, in contrast to other cathepsins that were recruited later (33). That this pattern of recruitment and activation was not observed in a dendritic cell line suggests that the role of cathepsin Z might be specialized to macrophages and, by analogy to other cathepsins, host defense. In contrast to a role in protective immunity, cathepsin Z could mediate tissue damage by degradation of the extracellular matrix. For example, it has been reported that cathepsin Z is strongly overexpressed in malignant melanoma cells and could play a role in allowing tumors to metastasize.

MC3R is part of a wider family of MCRs that have been of key interest in the study of obesity and weight control. They respond to natural melanocortin proteins derived from the larger molecule POMC (pro-opiomelanocortin), and share a single amino acid motif (HFRW sequence). The MC3R protein is 361 amino acids long and encoded by a single exon on 20q13.2. The molecule is a G-protein–coupled membrane receptor with seven transmembrane domains.

The different receptors within the MCR family differ in their specificity for particular POMC derivatives (34), their tissue expression, and their biological effects once activated. MC3R differs from the other melanocortins in that it has no selective specificity for melanocortin-derived products and is the only MCR strongly activated by γ-MSH (gamma melanocyte-stimulating hormone).

Beyond energy homeostasis, it has been realized for some time that derivatives of POMC have antiinflammatory effects in a variety of animal models of inflammation, including mycobacterial arthritis (35, 36). MC1R was originally believed to be the key family member playing a role in inflammatory responses, mediating the proinflammatory effects of γ-MSH in monocytes. However, recent work from Getting and colleagues suggests a more important role for MC3R (37). Getting and coworkers (38, 39) have used a murine model of gouty inflammation to show that inhibition of MC3R reduces the response of peritoneal monocytes to an injection of urate crystals and, more recently, that it reduces the inflammatory reaction in a model of gouty arthritis (40). Although the functional relevance of the associated SNP cannot be inferred from this associated study, it is possible that the MC3R241 AA genotype is associated with a loss of function that might allow a more vigorous inflammatory response to control infection.

The size of the effects seen for both CTSZ and MC3R means it is very unlikely that variation in either gene plays a major role in tuberculosis, at least in the populations studied. However, these results are the first direct suggestion of a role for members of either the cathepsin family or the MCRs in the pathogenesis of tuberculosis, a hypothesis that can now be tested in genetic, cellular, and clinical studies, and that offers potentially important insights into the pathogenesis of this persistent disease.

Conflict of Interest Statement: G.S.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B. is deceased and is unable to provide a conflict of interest statement. C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.P.W.J.M. has been working in Uganda for 3 years at the Infectious Diseases Institute where Pfizer, Inc., Gilead, Exxon Mobil, and Becton Dickinson have been donors. None of these donors supplied work in the Gambia, where they did not have commercial sponsorship. G.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. O.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.M. is deceased and is unable to provide a conflict of interest statement. P.v.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.G.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.V.S.H. is a nonexecutive shareholding director in Oxxon Therapeutics PLC.

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