

## TUBERCULOSIS

No association between interferon- $\gamma$  receptor-1 gene polymorphism and pulmonary tuberculosis in a Gambian population sample

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**Background:** Tuberculosis (TB) is a major global cause of mortality and morbidity, and host genetic factors influence disease susceptibility. Interferon- $\gamma$  mediates immunity to mycobacteria and rare mutations in the interferon- $\gamma$  receptor-1 gene (*IFNGR1*) result in increased susceptibility to mycobacterial infection, including TB, in affected families. The role of genetic variation in *IFNGR1* in susceptibility to common mycobacterial diseases such as pulmonary TB in outbred populations has not previously been investigated.

**Methods:** The association between *IFNGR1* and susceptibility to pulmonary TB was investigated in a Gambian adult population sample using a case-control study design. The coding and promoter regions of *IFNGR1* were sequenced in 32 patients with pulmonary TB, and the frequencies of six common *IFNGR1* polymorphisms were determined using PCR based methods in 320 smear positive TB cases and 320 matched controls. Haplotypes were estimated from the genotype data using the expectation-maximisation algorithm.

**Results:** There was no association between the *IFNGR1* variants studied and TB in this Gambian population sample. Three common haplotypes were identified within the study population, none of which was associated with TB.

**Conclusions:** These data represent an important negative finding and suggest that, while *IFNGR1* is implicated in rare Mendelian susceptibility to mycobacterial disease, the common variants studied here do not have a major influence on susceptibility to pulmonary TB in The Gambian population.

Approximately two billion individuals globally are infected with *Mycobacterium tuberculosis*, yet only 10% develop clinical tuberculosis (TB).<sup>1</sup> Nonetheless, TB, especially in its pulmonary form, remains an important cause of morbidity and mortality worldwide. *M. tuberculosis* is an intracellular parasite and cell mediated immunity is crucial for containment of infection.<sup>2</sup> Activation of infected macrophages by interferon-gamma (IFN- $\gamma$ ) derived from T cells and natural killer cells is the principal antimycobacterial effector mechanism. The importance of IFN- $\gamma$  in human mycobacterial immunity was established by the identification of mutations in the gene encoding the IFN- $\gamma$  receptor ligand binding chain (*IFNGR1*, MIM #107470)<sup>3,4</sup> as a cause of Mendelian susceptibility to mycobacterial infection (MSMI). Mutations in the genes encoding the IFN- $\gamma$  receptor signal transducing chain (MIM #147569),<sup>5</sup> the p40 subunit of interleukin (IL)-12 (MIM #161561),<sup>6</sup> the IL-12 receptor  $\beta$ 1 subunit (MIM #601604),<sup>7,8</sup> and signal transducer and activator of transcription-1 (MIM #600555)<sup>9</sup> also result in increased susceptibility to mycobacterial infection. Several other *IFNGR1* mutations have since been identified in patients with MSMI, the majority of which result either in complete absence of surface expression of the protein or expression of an abnormal protein that does not bind IFN- $\gamma$ .<sup>10</sup> IFN- $\gamma$  signalling is completely abrogated and the clinical phenotype correspondingly severe, often resulting in death from disseminated infection at an early age. Other mutations, however, lead to the expression of a dysfunctional protein and impairment—but not complete abrogation—of IFN- $\gamma$  signalling. In this partial form of IFN- $\gamma$  receptor-1 deficiency the phenotype is less severe and patients respond to IFN- $\gamma$  treatment.<sup>10</sup> Infection with virulent *M. tuberculosis* has been

reported in partial IFN- $\gamma$  receptor-1 deficiency.<sup>11</sup> These observations suggest a spectrum in which disease severity correlates with the degree of functional impairment in the IFN- $\gamma$  receptor.

Host genetic factors have an important role in the development of clinical disease following infection with *M. tuberculosis*, but inheritance of TB susceptibility in the general population is non-Mendelian. The correlation between the molecular pathology, mycobacterial virulence, and clinical phenotype in inherited IFN- $\gamma$  receptor-1 deficiency suggests that more subtle variation in *IFNGR1* could contribute to *M. tuberculosis* disease susceptibility in an outbred population. To test this hypothesis, a case-control association study comparing the frequency of genetic variants of *IFNGR1* in patients with pulmonary TB (the most common clinical phenotype) and healthy control subjects was conducted in The Gambia.

## METHODS

The study was approved by the MRC/Gambia Government ethics committee. The cohort has been described in more detail elsewhere.<sup>12</sup> Briefly, we recruited 320 men with pulmonary TB and 320 healthy male blood donors of similar age and ethnicity. TB is endemic in The Gambia, and the rationale for selecting blood donors as controls is presented elsewhere.<sup>13</sup> All subjects were HIV negative. Assuming a multiplicative model, the study had more than 80% statistical power to detect at  $p = 0.05$  an association with odds ratio of 1.7–2.5 for alleles at 2–10% frequency (the –611, –470 and –270 promoter polymorphisms and the E7+189 single nucleotide polymorphism (SNP) in exon 7) and an odds ratio of 1.4 for alleles at 50% frequency (the –56 promoter SNP and the +95 SNP in exon 1).

10 ml of venous blood was collected into 3.8% sodium citrate and DNA extracted using a standard phenol/chloroform protocol. Sequencing of the *IFNGR1* promoter region between -1400 and +100 relative to the translation start site has been reported elsewhere.<sup>14</sup> Further sequencing between -768 relative to the translation start site and the first exon/intron boundary, the coding regions, and splice sites for exons 2-7 of *IFNGR1* was conducted in 32 patients. These regions were amplified by polymerase chain reaction (PCR) and products sequenced using the ABI Big Dye Terminator v2 kit and an ABI 3700 capillary sequencer (ABI; Foster City, CA, USA). PCR and sequencing primers designed from NCBI sequence data ([http://www.ncbi.nlm.nih.gov/NT\\_025741](http://www.ncbi.nlm.nih.gov/NT_025741)) are shown in table 1.

The polymorphisms at positions -470, -270, and +95 were genotyped using ARMS PCR as described elsewhere.<sup>14</sup> The -611 and -470 promoter polymorphisms and the E7+189 SNP were genotyped using Invader (Third Wave Technologies; Madison, WI, USA) as described elsewhere.<sup>15</sup> Attempts to genotype the -56T>C SNP using ARMS PCR, RFLP and Invader failed, possibly because it lies within a GC rich region. This SNP was successfully genotyped using the multiplex SNaPshot kit (Applied Biosystems; Foster City, CA, USA). The primers 5'-CGGTGACGGAAGTGACGTA and 5'-GGATCCCTCCCTCCCTCT were used to PCR amplify a 238 base pair region of the *IFNGR1* gene which includes the -56 and +95 SNPs. The primers 5'-AGGCCGGGGCTGGAG-GGCAG and 5'-CGGCCGAGCCCTGCCGCGA were used as internal SNaPshot primers to genotype the -56 and +95 SNPs respectively, following the SNaPshot protocol supplied by the manufacturers. SNaPshot products were analysed using the ABI 3100 DNA sequencer (Applied Biosystems). Typing the -470 and the +95 polymorphisms by two different methods allowed us to validate the genotyping performed for this study.

Fisher's exact test was used to compare allele frequencies between cases and controls. The software UNPHASED<sup>16</sup> (<http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/>) was used to construct haplotypes from the genotype data and to compare haplotype frequencies between the case and control groups. This program calculates association statistics for multilocus haplotypes in case-control data using the expectation-maximisation algorithm to estimate haplotype odds ratios across multiple categories, giving a likelihood ratio test of homogeneity.

## RESULTS

The presence of the -470, -270, -56, and +95 polymorphisms identified previously was confirmed. Two additional SNPs were identified in this Gambian sample at positions -611 A>G relative to the transcriptional start codon and at E7+189 G>C (rs1327474 and rs11914, respectively, <http://www.ncbi.nlm.nih.gov/SNP/>). The exon 1 V14M polymorphism previously reported to be associated with systemic lupus erythematosus in a Japanese population<sup>17</sup> and the H318P and L450P polymorphisms associated with anti-*Helicobacter pylori* IgG concentrations in a Northern Senegalese population<sup>18</sup> were not present in this Gambian sample. Allele and genotype frequencies for the -470 and +95 SNPs obtained by two different methods were concordant (correlation 0.99 and 0.98, respectively).

Genotype and allele frequencies at six *IFNGR1* loci are shown in table 2. There was no significant difference in allele or genotype frequencies between cases and controls for any of the polymorphisms. Genotype distribution of all polymorphisms did not deviate significantly from Hardy-Weinberg equilibrium. A total of 11 haplotypes were estimated to be present in this population, of which three major haplotypes with frequency >4% account for 90% of all chromosomes (table 3). There was no significant difference in haplotype frequencies between the two groups, indicating that there was no association between variation in *IFNGR1* and pulmonary TB in this study.

## DISCUSSION

IFN- $\gamma$  is required for host defence against a broad range of pathogens and is especially critical for mycobacterial immunity. Children lacking either chain of the IFN- $\gamma$  receptor are highly susceptible to mycobacterial disease and fail to upregulate in vitro monocyte function in response to IFN- $\gamma$ .<sup>19</sup> Heterozygous carriers have an intermediate phenotype, suggesting that more subtle variation in the IFN- $\gamma$  response pathway may underlie susceptibility to TB in outbred human populations.<sup>19</sup> Since the *IFNGR1* mutations responsible for MSMD are rare and often lethal, it is unlikely they play a role in pulmonary TB. Nonetheless, it is possible that more common genetic variants such as promoter region polymorphisms that influence gene expression are associated with disease. However, we have failed to show an association between six *IFNGR1* variants and pulmonary TB in a Gambian population sample.

**Table 1** Primers used for discovery and genotyping of polymorphisms within the interferon- $\gamma$  receptor-1 (*IFNGR1*) gene

Region	Primer name	Primer sequence (5' to 3')	PCR product (bp)
Promoter	IFNGR1-PPa	CAAGGTGAAAAGTGCCATGA	588
Promoter	IFNGR1-PPb	CACCCATTCTGCAATCTT	
Exon 1	IFNGR1-E1g	GTAGCAGCATGGCTCTCCTC	107
Exon 1	IFNGR1-E1h	GCGAACGACGGTACCTGA	
Exon 2	IFNGR1-E2u	TATCTGGGCAATGTGGCATC	301
Exon 2	IFNGR1-E2d	ACGTGGGAAGGCTGATGAA	
Exon 3	IFNGR1-E3u	TTCTACCGCTTTGTGCTGTG	498
Exon 3	IFNGR1-E3d	ATTTCTTTGAGTGCCATGC	
Exon 4	IFNGR1-E4u	TGTTTATTAAGGACCCGAGA	471
Exon 4	IFNGR1-E4d	TGATCTGTGAGTCTTGCTGAA	
Exon 5	IFNGR1-E5u	TGCATAGTATCGTCTGTGTG	502
Exon 5	IFNGR1-E5d	GGAATGGAACATAATGCAATGA	
Exon 6	IFNGR1-E6u	GCTGGGAAGAACCATTATTC	503
Exon 6	IFNGR1-E6d	TGCTGCACAGCAAAAGACA	
Exon 7	IFNGR1-E7u	GCCATTTGGTGGTCCATTAC	1461
Exon 7	IFNGR1-E7d	TGTTCTGCACACTGGGTGT	
Exon 7	IFNGR1-E7i	CAAATAATAAAGGTGAAATAAA	Internal
Exon 7	IFNGR1-E7i2	CAGTAACGGAACAGTATCC	Internal

**Table 2** Genotype and allele frequencies for interferon- $\gamma$  receptor 1 (*IFNGR1*) gene polymorphisms

Polymorphism	Genotype frequencies, n (%) <sup>*</sup>			Allele frequencies	Odds ratio (95% CI) <sup>†</sup>	p value <sup>‡</sup>
-611	AA	AG	GG	A/G		
Cases	273 (93.2)	19 (6.4)	1 (0.4)	0.96/0.04	1.19 (0.66 to 2.17)	0.34
Controls	260 (91.9)	22 (7.7)	1 (0.4)	0.96/0.04	0.84 (0.46 to 1.53)	
-470	TT/TT	TT/-	-/-	TT/-		
Cases	234 (79.6)	53 (18.0)	7 (2.4)	0.89/0.11	0.83 (0.57 to 1.22)	0.20
Controls	232 (81.7)	49 (17.3)	3 (1)	0.90/0.10	1.20 (0.82 to 1.75)	
-270	TT	TC	CC	T/C		
Cases	244 (95.7)	10 (3.9)	1 (0.4)	0.98/0.02	0.91 (0.39 to 2.13)	0.50
Controls	225 (96.5)	6 (2.6)	2 (0.9)	0.98/0.02	1.10 (0.47 to 2.57)	
-56	TT	TC	CC	T/C		
Cases	62 (27.9)	108 (48.7)	52 (23.4)	0.52/0.48	1.01 (0.78 to 1.30)	0.50
Controls	73 (27.5)	130 (49.1)	62 (23.4)	0.52/0.48	0.99 (0.77 to 1.28)	
+95	TT	TC	CC	T/C		
Cases	74 (27.4)	131 (48.5)	65 (24.1)	0.52/0.48	0.98 (0.77 to 1.25)	0.49
Controls	73 (27.5)	130 (49.1)	62 (23.4)	0.52/0.48	1.02 (0.80 to 1.29)	
E7+189	TT	TG	GG	T/G		
Cases	255 (95.1)	13 (4.9)	0 (0)	0.97/0.03	1.41 (0.68 to 2.91)	0.23
Controls	249 (93.6)	16 (6.0)	1 (0.4)	0.97/0.03	0.71 (0.34 to 1.46)	

<sup>\*</sup>Total number genotyped = 297 cases and 285 controls; discrepancies in numbers arise from genotyping failures.

<sup>†</sup>Odds ratio (OR) and p values shown are for Fisher's exact test comparison of 2  $\times$  2 tables for allele frequencies between cases and controls.

<sup>‡</sup>No multiple testing correction was applied.

Our negative findings have a number of possible interpretations. Firstly, variation in *IFNGR1* may contribute to the susceptibility to TB, but the effect is small and a much larger sample size is required to detect it. Furthermore, we only sequenced gene regions that are most likely to harbour functionally relevant variants—that is, the promoter region, exons, and intron/exon boundaries. We therefore cannot exclude a causal variant elsewhere in the gene. A weak association between the intron 6 *IFNGR1* microsatellite and pulmonary TB has been reported in a Croatian population,<sup>20</sup> but we did not replicate this finding in this Gambian sample.<sup>21</sup>

Secondly, we have investigated pulmonary TB which is caused by reactivation of latent infection. Mycobacterial infections in MSMI are generally disseminated, which suggests that *IFNGR1* may influence the course of primary infection in childhood or the development of disseminated TB (for example, osteomyelitis and meningitis). However, IFN- $\gamma$  is still required for control of pulmonary disease and pulmonary TB occurs in *IFNGR1* deficient individuals. Alternatively, sequence variation in other genes in this pathway may affect susceptibility to pulmonary TB. Two recent studies have reported an association between the +874 A/T SNP in the first intron of the IFN- $\gamma$  gene and pulmonary TB,<sup>22, 23</sup> suggesting that the TT genotype which is associated with lower IFN- $\gamma$  production confers susceptibility to pulmonary TB.

Thirdly, mutations in *IFNGR1* mostly result in infection with avirulent mycobacteria or *M bovis* BCG. This may reflect differences in exposure; environmental mycobacteria are ubiquitous and many affected children received BCG in early life. Alternatively, the role of IFN- $\gamma$  in the control of *M tuberculosis* and environmental mycobacteria may be different. For example, IFN- $\gamma$  has been shown to activate human macrophages to control avirulent mycobacteria in vitro but not *M tuberculosis*.<sup>24</sup> Pathogenic mycobacteria have evolved various mechanisms by which host responses are evaded, including prevention of phagosomal maturation and acidification.<sup>25</sup> Although IFN- $\gamma$  can overcome these phenomena in vitro, *M tuberculosis* can interfere with IFN- $\gamma$  signalling and downregulate the transcription of IFN- $\gamma$  inducible genes.<sup>26</sup> These observations, together with the lack of association between common variants in *IFNGR1* and pulmonary TB, suggest that alternative pathways may be important for immunity to *M tuberculosis* in humans. Further genetic and functional studies are required to find these pathways to identify new drug targets for enhancing critical host immune response pathways against *M tuberculosis*.

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**Table 3** Estimated haplotype probabilities for six polymorphisms in the interferon- $\gamma$  receptor-1 (*IFNGR1*) gene

Haplotype number	Haplotype	Estimated haplotype frequencies <sup>*</sup>	
		Controls, n (%)	Cases, n (%)
1	A, insTT, C, T, T, T	189 (47.6)	188 (46.0)
2	A, insTT, C, C, C, T	124 (31.1)	145 (35.3)
3	A, delTT, C, C, C, T	50 (12.6)	36 (8.7)
	Others	35 (8.8)	41 (10.0)

Markers are shown in chromosomal order (i.e. *IFNGR1*-611, *IFNGR1*-470, *IFNGR1*-270, *IFNGR1*-56, *IFNGR1*+95, and *IFNGR1* E7+189).

<sup>\*</sup>Only haplotypes with an estimated probability of >4% are shown. p value=0.13 (NS).

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