Variants in the IFNγ transcription factor genes TBET, STAT1, STAT4, and HLX and the risk of pulmonary tuberculosis in a Colombian population: a case-control study

Dulfary Sánchez1,2,3, Céline Lefebvre4, Luis F. García1,2, Luis F. Barrera1,2

1 Grupo de Inmunología Celular e Inmunogenética (GICIG), Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia
2 Centro Colombiano de Investigación en Tuberculosis (CCITB), Medellín, Colombia
3 Stanley S. Scott Cancer Center, Health Science Center, Louisiana State University, New Orleans, LA, United States
4 ECOGENE-21 Clinical Trial Center, Centre de Médicine Génique Communautaire de l’Université de Montréal, Centre Hospitalier Affilié Universitaire Régional de Chicoutimi, Pavillon Notre-Dame, Chicoutimi, Canada

Instituciones donde se llevó a cabo la investigación:
Universidad de Antioquia, Medellín, Colombia
Laboratory for Genetics and Genomic Medicine of Inflammation, Montreal, Canada

Introduction: Interferon gamma (IFNγ) is the most potent cytokine involved in the control of Mycobacterium tuberculosis (Mtb), the etiological agent of human tuberculosis (TB). Patients with active TB present reduced levels of IFNγ, which may explain the lack of effective immunity against Mtb in these patients. The diminished expression of or functional alterations in trans-acting factors that regulate IFNγ gene expression may explain the reduced levels of IFNγ in TB patients.

Objective: To investigate the relationships of genetic variants in the transcription factors TBET, STAT1, STAT4, and HLX to susceptibility/resistance to pulmonary TB.

Materials and methods: Eight candidate single-nucleotide polymorphisms (SNPs) were selected, and genotyped in 466 unrelated pulmonary TB patients and 300 healthy controls from Colombia, and the allelic and genetic associations with TB were analyzed.

Results: The results indicate that no SNP in the transcription factors studied is associated with TB. However, polymorphism rs11650354 in the TBET gene may be associated with a decreased risk of TB; the TT genotype was significantly associated with TB protection in a recessive genetic model (OR=0.089, 95% CI: 0.01-0.73, p=0.0069), although this association was not maintained after multiple test correction (EMP2= 0.61).

Conclusion: In this study, the rs11650354 variant of TBET was suggested to promote resistance to TB in a Colombian population. A future replication case-control study using additional samples will be necessary to confirm this suggestive association.

Keywords: Mycobacterium tuberculosis, interferon gamma, transcription factor, STAT1, STAT4, case-control studies.

doi: http://dx.doi.org/10.7705/biomedica.v33i2.790

Variantes en los factores de transcripción para IFNγ, TBET, STAT1, STAT4 y HLX, y el riesgo de desarrollar tuberculosis pulmonar en un estudio de casos y controles de una población colombiana

Introducción. El interferón gama (IFNγ) es la citocina más potente para controlar la infección por Mycobacterium tuberculosis, el agente etiológico de la tuberculosis humana. Los pacientes con tuberculosis activa presentan reducción de los niveles de IFNγ, lo cual parece explicar la inmunidad poco efectiva contra el bacilo. La disminución de su expresión o alteraciones funcionales de los factores transactivadores del promotor del gen de IFNγ, podrían explicar la reducción de los niveles de IFNγ en los pacientes con tuberculosis.

Objetivo. Determinar la asociación de variantes genéticas en los factores de transcripción TBET, STAT1, STAT4 y HLX con sensibilidad o resistencia a tuberculosis pulmonar.

Contribución de los autores:
Luis Fernando Barrera y Luis Fernando García fueron los responsables del diseño del proyecto y obtuvieron su financiación. María Dulfary Sánchez y Céline Lefebvre procesaron y analizaron las muestras de ADN para el estudio de los polimorfismos mediante espectrometría de masas.
Todos los autores participaron en el análisis de datos, redacción y edición del artículo.
It is estimated that one-third of the human population is infected with *Mycobacterium tuberculosis* (*Mtb*), the etiological agent of tuberculosis (TB). TB is a complex disease, and its occurrence is influenced by environmental, immunologic and genetic factors. It is estimated that 10% of *Mtb*-infected individuals may develop active TB during their lifetimes (1), suggesting that the majority of the infected individuals are endowed with a protective immune response.

IFNγ is a potent cytokine with pleiotropic activities in different cell types, and it is considered the major activator of macrophages, a cell type that is critical to the control of *Mtb* replication and dissemination. In addition, IFNγ acts on professional antigen presenting cells, increasing MHC class II expression and thus favoring the adaptive T cell response. IFNγ is produced by different cell populations, including Natural Killer (NK) cells (2), T lymphocytes (LT) αβ (CD4+ and CD8+ T cells), LTγδ cells (3), dendritic cells (DCs) (4), and monocytes/macrophages (5).

IFNγ activity is crucial for mycobacterial control in *Mtb*-infected cells. It stimulates the production of inducible nitric oxide synthase (NOS-2), which is known to be responsible for high levels of nitric oxide, and other reactive nitrogen intermediates that are bactericidal to *Mtb* in mice (6), although the role of NOS-2 in human TB remains controversial. IFNγ also induces the expression of LRG-47, a p47 GTPase that controls *Mtb* by allowing the autophagy of infected macrophages (7). The importance of IFNγ during *Mtb* infection has been demonstrated in both mice and humans. Mice that are deficient in IFNγ or the IFNγ receptor (IFNγR) rapidly succumb to infection with *Mtb* or *M. bovis* BCG (8,9). Individuals with mutations in the *IFNGR1* or *IFNGR2* genes (encoding the IFNγ-R1 and R2 receptors, respectively) or IFNγ-intracellular pathway components such as the STAT1 protein may experience fatal disseminated BCG or non-tuberculous mycobacterial infection during childhood (10). Polymorphic variants in the *IFNG* and *IFNG receptor* genes have been associated with variable levels of IFNγ production (11,12).

Different reports indicate that peripheral blood mononuclear cells (PBMCs) from patients with severe active TB produce lower levels of IFNγ protein and mRNA in response to mycobacterial antigens than do healthy tuberculin reactor subjects (13,14) or with household healthy contacts of TB patients (15). This impaired IFNγ production could be associated with an inability to clear the bacterial load and the consequent progress of the disease and its pathology. This assumption is consistent with evidence suggesting that IFNγ expression correlates with protective immunity against TB (6,8,9); however, according to other studies, high levels of IFNγ protein post-infection represents a risk factor for developing active TB (16-20). These data suggest that the mechanisms that control the excessive inflammatory responses during *Mtb* infection may have a critical role in the immunopathology of TB.

The molecular regulation of IFNγ is a complex process because multiple signals converge to govern its expression (21,22). Different regulatory regions of the IFNγ locus have been identified, and...
multiple transcription factors have been implicated in the regulation of IFNγ gene expression (23). Transcription factors such as TBET (T-box 21) (24), GATA3 (25), STAT1 (Signal Transducers and Activators of Transcription 1) (26), STAT4 (27), HLX (H2.0-like homeobox) (28), CREB (29), ATF-2, c-Jun (30), and NF-κB (22) have been implicated in the regulation of IFNγ gene expression. Few studies have tested whether the reduced IFNγ mRNA and protein production in human TB is due to functional mutations in any of these transcription factors. Reduced CREB protein expression or the absence of phosphorylated nuclear CREB in TB patients resulted in decreased IFNγ promoter activity and reduced IFNγ production (29). However, it is presently unknown whether genetic variants of this and other transcription factors may be associated with susceptibility/resistance to TB.

IFNγ plays a critical role during Mtb infection, and there is evidence indicating the crucial role of regulation of IFNγ production. Therefore, we performed a case-control gene association study in a Colombian population of pulmonary TB cases and healthy controls, using transcription factors that positively or negatively regulate IFNG gene expression as the candidate associated genes. A trend for association with a decreased risk for TB (OR±95%CI=0.089±0.01-0.73; \(\chi^2=7.99; p=0.0069\)) was found for the TT genotype of TBET (rs11650354 SNP) assuming a recessive model of inheritance; however, this significance was lost after multiple test correction via permutation (EMP=0.61). The populations were also genotyped for additional single nucleotide polymorphisms in the STAT1, STAT4, and HLX genes; however, none of these SNPs was found to be associated with susceptibility/resistance to TB.

Materials and methods

Subjects

Pulmonary TB patients (n=499) and healthy controls (n=320) of Mestizo ethnicity were recruited from the metropolitan area of Medellin, the capital city of the Antioquia Department, Colombia. The incidence of tuberculosis in Colombia was last reported as 34 per 100,000 inhabitants in 2010, according to a World Bank report released in 2011 (31). In Medellin, the records of the incidence of TB show rates ranging from 40.4 to 60.7 cases per 100,000 inhabitants (32). The cases included in this study were newly diagnosed TB patients with disease confirmed by sputum smear examinations for acid-fast bacilli and/or culture for Mtb. The cases were enrolled from February 2002 to September 2004 (from a cross-sectional study; n=119) and from March 2005 to November 2006 (from a cohort study (20); n=380). The control subjects were healthy (as defined by Interferon Gamma Release Assays, IGRA), non-related household contacts, enrolled (collected from March 2005 to December 2006; n=105), and non-contact individuals living in the same neighborhood as the TB patients (source population), randomly sampled and age-matched to the TB patients (n=215), enrolled from September 2007 to December 2008. The exclusion criteria for patients included positive serologic tests for Human Immunodeficiency Virus (HIV), a history of organ transplantation, primary immunodeficiency, cancer, treatment with immunosuppressive drugs, endocrine disorders such as diabetes, autoimmune and chronic renal disease, or the development of pleural, miliary or meningeal TB. The exclusion criteria for controls included persistent productive cough (for more than 2 weeks) and a previous history of TB. No household contact participating in this study showed clinical symptoms of TB during the 2 years of clinical follow-up. All subjects signed an informed consent document that was approved by the Ethics Committee of the Facultad de Medicina, Universidad de Antioquia. Table 1 shows the demographic characteristics of the 466 TB patients and 300 healthy controls that were included in the association analyses. Samples from 33 TB patients and 20 healthy controls were eliminated during the genotype quality control analyses, as described below.

Blood samples and DNA isolation

Venous blood samples (3-8 ml in EDTA; BD Vacutainers) were collected. Genomic DNA was extracted using the DNAzol reagent (ES Extra Strength MCR, Cincinnati, OH, USA) according to the manufacturer’s instructions. The isolated DNA was suspended in TE 1X (pH 7.9) and stored at 4°C. DNA integrity was visualized by ethidium bromide staining of 0.7% agarose (Amresco; Solon, OH, USA) gels. DNA samples were quantified using the Quant-iT PicoGreen dsDNA reagent (Invitrogen Molecular Probes, Eugene, OR, USA) as recommended by the manufacturer.

SNP selection and genotyping

Eight SNPs in the TBET, HLX, GATA3, STAT1, and STAT4 genes were evaluated. The SNPs were strategically selected according to their locations in areas of potential importance for gene regulation or protein function and/or regions that
are in LD with other SNPs previously reported to be associated with different diseases. The SNPs rs11578466 and rs11588625 in the \textit{HLX} gene were used as proxies for an \textit{HLX} SNP that lies within the 3' untranslated region (\textit{HLX1-C/T}, 3' UTR; NCBI dbSNP: rs2738756). SNP genotype information for the CEU population was downloaded from HapMap database (phase II release 21a from January 2007). Primer design software (MassARRAY Assay Design) was used to design PCR and hME primers for the high-plexed MassEXTEND (hME) assays to avoid overlapping mass signals in the available spectrum range for each target SNP. Samples were genotyped at the Laboratory for Genetics and Genomic Medicine of Inflammation (http://www.inflammgen.org), Université de Montreal, Montreal, Canada, by MassARRAY (Sequenom, San Diego, CA, USA); this technique uses chip-based Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry, as previously described (33).

**Statistical analysis**

Association testing was performed with PLINK software v1.04. A single marker basic allelic association test (--assoc --Fisher option) and genotypic association test (--model --Fisher option) were performed for each of the post-QC SNPs. To test for the mode of inheritance, 2×3 tables, Fisher’s exact test and ORs with 95% confidence intervals (CI) were calculated. For SNPs that showed a significant genotypic association (p<0.05), a PLINK’s max(T) permutation procedure (--mperm option) with 100,000 iterations was performed to limit type II error. PLINK generates both uncorrected (EMP1) and corrected (EMP2) empirical P-values. In both cases, p-values of <0.05 were considered significant.

The Mann-Whitney statistical test was used for age comparisons, and the $\chi^2$ test was used to test for gender differences between cases and controls. To adjust for potential confounding, gender was included in a forward stepwise logistic regression analysis for SNPs with significant genotypic association using the Statistical Package for the Social Sciences (SPSS v.11.5 for Windows; Chicago, IL). Statistically significant associations were defined by a p≤0.05.

**Results**

The demographic and clinical characteristics of the population analyzed in our study are shown in Table 1. Of the initial 499 TB patients and 320 controls, the DNA samples from 33 TB patients and 20 healthy controls were removed from the analysis due to low genotype call rates. There was no difference in age distribution between cases and controls (p=0.46), but men were more frequent in the TB patient group than in the control group (p<0.001). Two SNPs in \textit{GATA3} (rs4143094 and rs1058240) showed deviations from Hardy-Weinberg Equilibrium (HWE) in the control group and were removed for further
analysis. The remaining 8 SNPs tested passed QC analysis (genotyping success <80%) and were included in the subsequent analyses.

The results of the allelic association analyses did not reveal any differences in the frequencies of TBET, HLX, STAT1, and STAT4 polymorphisms between TB cases and controls (table 2). Genotype frequency analysis was also performed (table 3), and a significant difference between TB patients and controls was found in TBET rs11650354 SNP, with genotypic frequencies significantly decreased in cases compared with controls ($\chi^2=8.06; P=0.019$).

To analyze the genotypic effect, we performed a comparison of genotypic models in which the risk allele was dominant or recessive. The frequency of the TT genotype of TBET (recessive model of inheritance) was significantly lower in TB patients (OR=95%CI= 0.089±0.01-0.73; $\chi^2=7.99; p=0.0069$) than in controls. This difference persisted even after adjusting for the gender distribution between groups (p=0.02), as shown using a logistic regression analysis controlling for gender as a covariate. Genotypic significance was ascertained through a permutation analysis of phenotypes (n=100,000 permutations) over all 81 SNPs (the total number of SNPs included in the complete genetic study using the same Colombian samples ((35) and unpublished data) in the analysis. After these stringently corrected permuted p-values were applied, the rs11650354 SNP did not remain significantly associated with TB protection (EMP2 p=0.61; table 3).

**Discussion**

Studies in the genetics of TB resistance/susceptibility have identified several important candidate genes (discussed in (36)) and genetic regions (37). IFN$\gamma$ has been identified as a key cytokine in the control of mycobacterial infections, as clearly demonstrated in mice models of infection (38,39) as well as in human genetic studies evaluating polymorphisms in the IFNG gene (40-42) and other genes involved in the modulation of IFN$\gamma$ production or signaling (43,44).

Under the hypothesis that IFN$\gamma$ production is critical for the successful control of $Mtb$ infection and multiplication, we studied SNPs from five specific

---

**Table 2. Allele frequencies**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Gene</th>
<th>Chr</th>
<th>SNP position</th>
<th>SNP Alleles</th>
<th>MAF in cases (%)</th>
<th>MAF in Controls (%)</th>
<th>P value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3771300</td>
<td>STAT1</td>
<td>2q32.2</td>
<td>Intron</td>
<td>C/A</td>
<td>0.45</td>
<td>0.43</td>
<td>0.42</td>
</tr>
<tr>
<td>rs7574865</td>
<td>STAT4</td>
<td>2q32.3</td>
<td>Intron</td>
<td>G/T</td>
<td>0.28</td>
<td>0.31</td>
<td>0.25</td>
</tr>
<tr>
<td>rs11650354</td>
<td>TBET</td>
<td>17q21.32</td>
<td>Intron</td>
<td>C/T</td>
<td>0.1</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>rs16947078</td>
<td>TBET</td>
<td></td>
<td></td>
<td>A/G</td>
<td>0.12</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>rs11588625</td>
<td>HLX</td>
<td>1q41</td>
<td>Exon (Ala387Gly)</td>
<td>T/C</td>
<td>0.14</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>rs11578466</td>
<td>HLX</td>
<td>3' UTR</td>
<td>C/G</td>
<td>0.13</td>
<td>0.15</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Fisher’s exact test for 2×2 comparisons of alleles in the different groups calculated using PLINK.

**Table 3. TBET genotype frequencies and inheritance model analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>Status</th>
<th>Number of individuals in each genotype (%)</th>
<th>$\chi^2$ (2 d.f.)</th>
<th>$\chi^2$ (1 d.f.)</th>
<th>2×2 p value$^3$</th>
<th>OR (95% CI)</th>
<th>EMP1</th>
<th>EMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBET</td>
<td>rs11650354</td>
<td>Healthy</td>
<td>233 (80.7) (19.1) 1 (0.2)</td>
<td>8.06 0.019 7.99 0.0069 0.089 0.011 0.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>controls</td>
<td>58 (78.2) (19.5) 7 (2.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Fisher’s exact test for 3×2 comparisons of genotypes in the different populations

$^b$ Fisher’s exact test for 2×2 comparisons of genotypes in the recessive model of inheritance (TT vs CC+CT) between groups

$^3$ Logistic regression for confounding factors in the gender phenotype. The Hosmer-Lemeshow goodness of fit tests indicated no significance (p>0.05). Correction for the effects of gender using logistic regression did not affect the observed associations for TBET.

The results of the permutation testing are displayed as uncorrected P-values (EMP1) and permuted P-values corrected for the number of tests performed (EMP2).

Abbreviations: SNP ID, single nucleotide polymorphism database identification number; OR, odds ratio; CI, confidence interval; d.f., degrees of freedom
genes that control the transcription of the IFNγ gene, namely, TBET (24), GATA3 (25), STAT1 (26), STAT4 (27), and HLX (28), and assessed whether their variants were associated with susceptibility/resistance to TB. We found that the TT genotype of TBET is associated with a decreased risk of TB in the Colombian population studied, although this association was lost after multiple test correction. A study with larger numbers of samples will be necessary to confirm this association.

TBET is a member of the T-box family of transcription factors that regulate the lineage commitment of T helper (Th)-0 lymphocytes toward a predominant Th1 phenotype. Different studies have highlighted the importance of TBET in the diverse subsets of cells that have been implicated in a Th1 immune response, such as dendritic cells (45), NK cells (46), and CD8+ LT cells (47). The important regulatory role of TBET in the course of Mtb infection was established in a murine model. Mice that lack TBET due to a targeted mutation of this gene are more susceptible to Mtb infection than their wild-type (WT) counterparts. These mice exhibited a shorter mean survival time, less efficient control of bacterial replication, and severe lung inflammation following both i.v. and respiratory infection (48). The lack of TBET expression resulted in a weakening of the systemic immune response to M. tuberculosis, evidenced by higher bacterial burdens in the lung, liver, and spleen. In addition, IFNγ production in response to a mycobacterial purified protein derivative (PPD) was significantly reduced in T cells isolated from TB lung lesions from TBET-/- mice (48).

However, it has been suggested that a disruption of the immune response profile balance during active TB facilitates an overregulation of the effector response, characterized by decreased IFNγ production (49), increased IL-4 production (50) and the development of regulatory T cells (Tregs) that inhibit protective Th1 responses (51). A lack of TBET expression generates conditions that strongly favor the development of the Th2 compartment (47). Heritable Th2-associated conditions might be caused by genetic variation in Th1 cytokine regulation through TBET (52). TBET polymorphisms have recently been associated with susceptibility to asthma (53,54), diabetes mellitus (55), autoimmune diseases (56,57), cancer (58,59), and viral infections (60,61). However, to date, no polymorphism in TBET has been associated with mycobacterial disease, including TB. The present report is the first to suggest an association between a TBET variant (rs11650354) and tuberculosis in a Colombian population.

The findings of recent genetic studies suggest an association between the rs11650354 SNP in TBET and systemic sclerosis (56). These authors showed that patients with the TT genotype had a more prominent Th2 cytokine profile, while patients in the wild-type group (CC) had a more prominent proinflammatory cytokine profile. They thus hypothesized that CD4+ T cells fail to differentiate into the Th1 lineage in the presence of the TBET TT polymorphism, leading to a Th2-predominant environment. This finding supports the potential importance of Th1/Th2 cytokine balance in autoimmune disease and asthma; this Th1/Th2 dysregulation could also be expected to affect the response to TB and potentially explains our finding of the TT genotype as a protective factor against TB development.

There is not enough evidence regarding whether specific variants of the TBET gene are associated with TBET expression/function. Interestingly, the levels of TBET messenger RNA (mRNA) in peripheral blood cells have been reported to be lower in asthma patients than in controls (62), and two TBET variants (rs11650354 and rs17699436) have been associated with susceptibility to asthma (63,64), suggesting that TBET SNPs could affect TBET transcription. To better understand the immunologic significance of the TBET variant rs11650354 in TB, further studies need to be undertaken to demonstrate the effects of these polymorphisms on mRNA and protein levels and TBET function, which in turn could affect IFNγ transcription and disturb the immune response.

In summary, this study found evidence of a weak association between the TBET TT genotype (rs11650354) and resistance to pulmonary TB. However, the lack of evidence for this association after multiple test correction via permutation casts doubts on the robustness of this finding. A further replication case-control study will be needed to confirm this result.

Acknowledgements

This study would not be possible without the generous participation of the patients, their families and healthy controls.

Conflicts of interest

The authors have declared that no competing interest exists.
Funding
This work was funded by grants 11150412950, RC-431-2004, 1115408204820, and 11150416335-2004 from Colciencias, Bogotá, Colombia.

References
25. Usui T, Nishikomori R, Kitani A, Strober W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet.


32. Secretaria de Salud de Medellín. Boletín Número 5 de 2011. Fecha de consulta: 9 de diciembre de 2012. Disponible en: http://www.medellin.gov.co/irj/go/km/docs/wpcontent/Sites/Subportal%20del%20Ciudadano/Salud/Secciones/Indicadores%20y%20Estad%C3%A1sticas/Documentos/2011/Bolet%C3%ADn%20%20Epidemi%C3%B3log%C3%ADco%20Sivigila/Boletines%202011/Bolet%C3%ADn%20Epidemi%C3%B3log%C3%ADco%20-%202011.pdf.


34. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81:559-75. http://dx.doi.org/10.1086/519795


50. van Crevel R, Karyadi E, Preyers F, Leenders M, Kullberg BJ, Nelwan RH, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is


267