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ARTÍCULO ORIGINAL

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

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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.

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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.

Materiales y métodos. Se seleccionaron ocho polimorfismos de un solo nucleótido (*Single-Nucleotide Polymorphism*, SNP) y se estableció su genotipo, en 466 pacientes con tuberculosis pulmonar y 300 controles sanos en Colombia; además, se hizo un análisis de asociación alélica y genética.

Resultados. Los resultados indican que los SNP de los factores de transcripción estudiados no están asociados con tuberculosis; sin embargo, el polimorfismo rs11650354 en *TBET* puede estar implicado en la disminución de riesgo de tuberculosis. El genotipo TT de *TBET* se asoció significativamente con protección contra tuberculosis usando un modelo genético recesivo (OR=0,089; $CI_{95\%}$: 0,01-0,73; p=0,0069); sin embargo, la corrección mediante pruebas múltiples de ajuste abolió esta asociación (*Empirical P Value,* EMP2=0,61).

Conclusión. En este estudio se sugiere un efecto de la variante rs11650354 de *TBET* sobre la resistencia a la tuberculosis en la población colombiana. Es necesario desarrollar un estudio de replicación usando muestras adicionales para confirmar esta asociación sugestiva.

Palabras clave: *Mycobacterium tuberculosis*, interferón-gamma, factor de transcripción STAT1, factor de transcripción STAT4, estudios de casos y controles.

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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

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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; χ^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 Medellín, 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

Table 1. Demographic and clinical characteristics of the study population

Demographic characteristic	TB patients (466)[a]		Healthy controls (300)[a]		P value
	Cohort study (350)	Cross-sectional study (116)	Household contacts (96)	Source population (204)	
Female	155	58	56	120	<0.001[b]
Male	195	58	40	84	<0.001[b]
Median age[c]	39 (26-51)		42 (25-54)		0.46[d]

[a]A total of 499 TB patients and 320 controls were enrolled in the study, but 33 samples from patients with TB and 20 from healthy controls were removed for low genotype call rates. The final data set post-quality control analyses gave an average genotype call rate of 98.1%. [b]Pearson's chi-square comparing sex between TB and healthy controls. [c]Years (interquartile range); [d]Mann-Whitney statistical test comparing TB patients with healthy controls.
Abbreviation: TB, tuberculosis

are in LD with other SNPs previously reported to be associated with different diseases. The SNPs rs11578466 and rs11588625 in the *HLX* gene were used as proxies for an *HLX* SNP that lies within the 3' untranslated region (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).

Data processing

To optimize the quality of the data (quality control, QC) while keeping as many SNPs and samples as possible, we selected different parameter thresholds using PLINK v1.04 (34). The PLINK QC options were set to the following thresholds: maximum missing genotypes per person (--mind option) ≤0.20, maximum missing genotypes per SNP (--geno option) ≤0.10 and Hardy–Weinberg Equilibrium (HWE; exact) P-value <0.05 in controls (--hwe option). These QC steps produced a final high-quality dataset with an average call rate of more than 80% per individual and per SNP. Of the initial DNA samples from 499 TB patients and 320 controls, 33 samples from TB patients and 20 from healthy controls were removed due to low genotype call rates. The final post-QC analysis dataset yielded an average genotype call rate of 98.1%.

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 χ^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 *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 (χ^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; χ^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 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). IFNγ 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 IFNγ production or signaling (43,44).

Under the hypothesis that IFNγ production is critical for the successful control of *Mtb* infection and multiplication, we studied SNPs from five specific

Table 2. Allele frequencies

SNP ID	Gene	Chr	SNP position	Alleles (A1/A2)	MAF in cases	MAF in Controls	P value[a]
rs3771300	STAT1	2q32.2	Intron	C/A	0.45	0.43	0.42
rs7574865	STAT4	2q32.3	Intron	G/T	0.28	0.31	0.25
rs11650354	TBET	17q21.32	Intron	C/T	0.1	0.12	0.15
rs16947078	TBET			A/G	0.12	0.14	0.21
rs11588625	HLX	1q41	Exon (Ala387Gly)	T/C	0.14	0.17	0.12
rs11578466	*HLX*		3' UTR	C/G	0.13	0.15	0.33

[a]Fisher's exact test for 2×2 comparisons of alleles in the different groups calculated using PLINK.
Abbreviations: SNP ID, single nucleotide polymorphism identification in rs#; A1, major allele; MAF, minor allele frequency; UTR, untranslated region

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

Gene	SNP ID	Status	Number of individuals in each genotype (%) CC n (%)	CT n (%)	TT n (%)	χ^2 (2 d.f.)	p-value[a]	Recessive mode of inheritance: χ^2 (1 d.f.)	p value[b] (adjusted p value)[c]	OR	(95% CI)	EMP1	EMP2
TBET	rs11650354	TB cases	376 (80.7)	89 (19.1)	1 (0.2)	8.06	0.019	7.99	0.0069 (0.02)	0.089	(0.01-0.73)	0.011	0.61
		Healthy controls	233 (78.2)	58 (19.5)	7 (2.3)								

[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
[c] 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.

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Conflicts of interest

The authors have declared that no competing interest exists.

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