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## -592 and -1082 interleukin-10 polymorphisms in pulmonary tuberculosis with type 2 diabetes

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### ABSTRACT

**Objective:** To determine the polymorphisms of Interleukin-10 (IL-10) (-592, -1082) in pulmonary tuberculosis (PTB) with and without type 2 diabetes (T2D). **Methods:** We studied a Mexican mestizo population of 37 patients with TB in remission (TBr) and 40 with active pulmonary TB (PTB), 21 patients with TB + T2D, 47 blood donors accepted, and 13 healthy health-care workers with tuberculin skin test positive. Determination of IL-10 polymorphisms was performed by real-time Polymerase chain reaction. **Results:** IL-10-592C/A presented in a greater proportion in healthy individuals than in patients with type 2 diabetes and TB in a not quite significant statistically manner. IL-10-1082A/A presented more frequently in the group of patients with both diseases, not being statistically significant in comparison with the group of healthy subjects. **Conclusions:** This study describes two important new findings. First, it reveals that the IL-10 (-592 A/A and -592 C/C) polymorphisms were found in a greater proportion in a group of patients with T2D and TB than in healthy subjects. Second, the study provides evidence that the (-1082 G/G) polymorphism presented with greater frequency in healthy individuals than in patients with both of these diseases.

## 1. Introduction

There is evidence that host genetic factors are important determinants of mycobacterial susceptibility. Different techniques have been employed to identify the genes implicated[1]. Some genes that codify for different cytokines may play crucial roles in host susceptibility to tuberculosis (TB), because the production capacity of these cytokines varies among individuals and depends on the single nucleotide polymorphisms (SNPs) of these[2]. Recently we reported that TB patients not responding to treatment, occurred more frequently genotype LTA +252G/A[3]. Interleukin-10 (IL-10) converts human dendritic cells into macrophage-like cells with increased antimycobacterial activity[4]. IL-10 is an important immunoregulatory cytokine

mainly produced by activated T cells, monocytes, B cells and thymocytes. As an immune response modulator, IL-10 can both stimulate and suppress the immune response[5]. There are three bi-allelic polymorphisms genetically regulating *in vivo* production of IL-10 in promoter gene. In the -1082 position of the transcription initiation site, the polymorphism constitutes a substitution by G of A, and in the -592 position, is the substitution by C of A. The presence of G and C is associated with greater production of IL-10, and A with less in Peripheral blood mononuclear cell (PBMC) cultures[6]. The low-expression IL-10 -1082 AA genotype is found increased in patients with cutaneous malignant melanoma[7].

The IL-10 -1082 G allele can be relevant for susceptibility to celiac disease, mainly in selective IgA deficiency[8]. The G allele of the -1082 single nucleotide polymorphism (SNP) is associated with low liberation of IL-10 after cardiopulmonary bypass. High levels of IL-10 secretion are related with organ dysfunction 24 h post-surgery[9]. The -1082 SNP G→A SNP exerted an influence on the achievement of longevity in males[10]. The A allele of

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the -592 SNP was associated with less stimulation of IL-10 liberation and increased liberation in critically ill patients<sup>[11]</sup>. The IL-10 genotype was related with the frequency of Respiratory syncytial virus (RSV)-associated pneumonia<sup>[12]</sup>. An increased frequency has been demonstrated of the -1082 GG genotype (implicated in high production of IL-10) in patients recovered from hepatitis C virus (HCV) infection<sup>[13]</sup>. Patients homozygous for the G allele of the -1082 SNP had a greater risk for septic shock<sup>[14]</sup>. This was associated more frequently with the GG genotype of the -1082 SNP, and with increased severity of the systemic inflammatory response syndrome (SIRS) associated with Community-acquired pneumonia (CAP)<sup>[15]</sup>.

The IL-10 gene also can be implicated in genetic susceptibility to TB. It has been suggested that -1082 IL-10 AA low-producer polymorphism is associated with pleural TB<sup>[16]</sup>. The heterozygous for the -1082 polymorphism has been associated with susceptibility to TB in Cambodian patients<sup>[17]</sup>. A reduction in the -1082 A allele is related with long-lived Sicilian individuals with chronic TB<sup>[18]</sup>. It has also been demonstrated that the frequency of the -1082 G allele was more common in Turkish patients with TB than in healthy controls<sup>[2]</sup>.

In Uganda, some authors reported that the IL-10 gene is associated with TB<sup>[19]</sup>. A direct inter-relationship was shown between IL-10 and the severity of both pulmonary as well as extra-pulmonary TB<sup>[20]</sup>. The -1082 A allele of the IL-10 gene can be important in susceptibility to TB<sup>[21]</sup>. IL-10 is important for effective control of inflammation in the host with TB<sup>[22]</sup>.

On the other hand, there is an alarming prevalence of T2D in Mexican population; the majority of previously diagnosed are in poor control. The prevalence for T2D was 14.42% (7.3 million diabetics)<sup>[23]</sup>. Because type 2 diabetes (T2D) is very prevalent among cases of TB in Mexico, the objective of this work was to determine the IL-10 polymorphisms in pulmonary TB (PTB) that are complicated by T2D and in TB. We carried out a study in Mexican mestizo population, PTB complicated by T2D (TB + T2D), cured TB, non-cured TB, Blood bank donors, and in positive tuberculin (PPD+) health workers. The real contribution of IL-10 polymorphisms in terms of the risk for TB is unknown. To our knowledge, there are no studies that have investigated the contribution of these T2D-complicated TB. Thus, this study is of the greatest importance.

## 2. Materials and methods

In a study conducted 2 years ago, we collected by simple random sampling, peripheral blood samples from 37 adult patients with TB in remission (TBr), from 40 patients with active pulmonary TB (PTB), from 21 patients with T2D-complicated TB, from 47 Blood Bank donors, and from 13 healthy, tuberculin skin test positive (TST+) health-care workers. We excluded from the study individuals with an acute or chronic infection different from TB, those infected with Human immunodeficiency virus (HIV) and/or hepatitis B or C, and those having autoimmune diseases or any other immune suppression-associate condition. Patients were

treated with a regime recommended by the World Health Organization (WHO).

Criteria for the screening of TBr:

1. We considered patients with TBr as those who presented an adequate microbiological and clinical response to the first treatment scheme.

Criteria for the screening of PTB:

1. Signs and symptoms compatible with TB. History of interpersonal contact with a case of smear-positive pulmonary tuberculosis.
2. Isolation of the bacillus in sputum.
3. Abnormal chest radiograph, showing mediastinal lymphadenopathy and/or hilar or other lesions consistent with TB.

Criteria for the screening of T2D:

1. A casual blood glucose greater than or equal to 200 mg/dL (11.1 mmol/L).
2. Fasting plasma glucose equal to or greater than 126 mg/dL (7 mmol/L).
3. Plasma glucose at 2 h longer or equal to 200 mg/dL (11.1 mmol/L) during the test, oral glucose tolerance.
4. All patients had adequate metabolic control.

We also studied a sample of unrelated blood donors and of health-care personnel with TST+ who were asymptomatic and with a chest X-ray without evidence of pulmonary disease. All participants were of Mexican mestizo origin and were followed-up for 18 months to confirm their diagnosis or control. The study was approved by the local Ethics Committee, and all participants provided written informed consent.

### 2.1. Subjects genotyping

Genomic DNA was extracted with guanidine isothiocyanate and phenol according to the procedure described by Chomczynski<sup>[24]</sup>. IL-10 gene (-592 C/A and -1082 G/A) polymorphisms were typed utilizing ABI PRISM 7300 (Applied Biosystems) Polymerase chain reaction (PCR). We use accessory software to analyze the distribution of each pair of alleles. The TaqMan allelic discrimination assay used 5' nuclease activity of the Taq polymerase to detect a reporter signal generated during or after the PCR reactions. For SNP genotyping, we employed a pair of TaqMan probes and a pair of primers. The assays utilize two TaqMan probes that differ in the polymorphic site, with a complementary probe to the wild-type allele and the remaining probe, to the variant allele. A 5' reporter colorant and a 3' color quencher bind covalently with the probes with the allele or with variant. When the probes are intact, fluorescence is quenched by the physical proximity of the reporter and quencher colorants. During the PCR alignment step, the TaqMan probe hybridize with the target polymorphic target site. During the PCR extension phase, the 5' reporter colorant breaks due to the 5' nuclease activity of the Taq polymerase and leads to an increase in fluorescence, which is characteristic of the reporter colorant. Specific genotyping determined by measuring the signal intensity of the two different reporter colorants after the PCR reaction. The volume of the reaction was 25  $\mu$ L, containing 2 $\times$ TaqMan

Buffer A, 3.5 mmol/L MgCl<sub>2</sub>, 200 μmol dATP, dCTP, and dGTP, respectively, 400 μmol dUTP, 0.025 U/μL Ampli Taq Gold DNA, 900 nmol of forward and reverse primers, 250 nmol/L of each Minor groove binder (MGB) TaqMan probe, and from 1 to 20 ng of DNA substrate. We employed TaqMan MGB probes (assay number C\_1747360\_10), which detects the (–592 C/A SNP) localized in the rs1800896 region, and assay number (C\_1747363\_10), which detects the (SNP –1082 G/A SNP) localized in the rs1800872 region of the IL–10 gene. Probe sequences were the following:

T C C T C T T A C C T A T C C C T A C T T C C C C [ A / C ]  
 T C C C A A A G A A G C C T T A G T A G T G T T G a n d  
 C T T T C C A G A G A C T G G C T T C C T A C A G [ A / G ]  
 ACAGGCGGGCTCACAGGATGTTT, respectively. Each probe contains one reporter colorant in the extreme 5'; the VIC® colorant binds with the catheter with allele 1, and the FAM colorant binds to the catheter with allele 2. The increase of fluorescence in VIC® and FAM followed in real time with each cycle by means of emission filters optimized for the use of fluorophores in the yellow channel (530 nm excitation and 555 nm emission), and in the green channel (470 nm excitation and 510 emission), respectively. The thermal profile was as follows: initial denaturalization, 1 cycle at 95 °C for 10 min; 40 denaturalization cycles at 92 °C for 15 s, and alignment/extension at 60 °C for 1 min. Each sample was mounted and analyzed by duplicate and we included three negative controls without template in each run.

2.2. Statistical analysis

To calculate sample size, we used the overall frequency of alleles in healthy controls reported in previous study[11], the estimated difference for the different alleles of the polymorphisms of the IL–10 in patients with pulmonary TB (PTB) is approximately 25%, we use the method of comparison of proportions taking a confidence level of 5% and 80% power.

Table 2 Allele frequency in the study population.

| Study population              | –592 Allele frequency Allele A/Allele C | –1082 Allele frequency Allele A/Allele G |
|-------------------------------|---|--|
| Healthy subjects (n=60)       | 0.36/0.64                               | 0.28/0.72                                |
| Patients with TB (n=77)       | 0.38/0.62                               | 0.16/0.84                                |
| Patients with TB + T2D (n=21) | 0.26/0.74                               | 0.50/0.50                                |

TB = Tuberculosis; T2D = Type 2 diabetes.

Table 3 Genotype distribution in the study population.

| Genotype                       | Healthy(n=47) | TST+(n=13) | PTB(n=40) | TBr(n=37) | TB + T2D(n=21) | OR (95% CI)      | P value  |
|--------------------------------|---------------|------------|-----------|-----------|----------------|------------------|----------|
| Genotypes of IL–10 (–592 C/A)  |               |            |           |           |                |                  |          |
| AA (%)                         | 0 (0)         | 1 (8)      | 1 (2)     | 0 (0)     | 1 (5)          | 10.8 (3.8–30.7)  | <0.0001* |
| CA (%)                         | 34 (72)       | 8 (61)     | 28 (70)   | 29 (78)   | 9 (43)         | 0.31 (0.10–0.92) | 0.05     |
| CC (%)                         | 13 (28)       | 4 (31)     | 11 (28)   | 8 (22)    | 11 (52)        | 3.5 (1.2–9.9)    | 0.018*   |
| Genotypes of IL–10 (–1082 G/A) |               |            |           |           |                |                  |          |
| AA (%)                         | 4 (10)        | 0 (0)      | 2 (5)     | 1 (3)     | 6 (29)         | 3.0 (0.67–13.4)  | NS       |
| GA (%)                         | 18 (29)       | 7 (54)     | 11 (28)   | 9 (22)    | 9 (42)         | 2.0 (0.62–6.9)   | NS       |
| GG(%)                          | 25 (61)       | 6 (46)     | 27(67)    | 27 (75)   | 6 (29)         | 6.2 (1.32–29.38) | 0.04*    |

Values are the number (%) of subjects with each genotype. Statistical analysis of genotype frequencies was carried out by χ<sup>2</sup> test with the continuity correction. 95% CI = 95% Confidence interval; NS = Not significant; OR = Odds ratio; PTB = Pulmonary tuberculosis; TBr = Tuberculosis in remission; T2D = Type 2 diabetes; TST+ = Tuberculin test positive. \*Comparison between TB + T2D and healthy donor

The study groups were examined by means of descriptive statistics utilizing central tendency and dispersion measurements as quantitative variables, while we employed frequency tables and/or percentages for nominal variables. To determine the possible associations of the variables in the study groups, we used contingency tables and independent χ<sup>2</sup> test or statistical analysis. For continuous variables, we applied the Wilcoxon sum of ranges (the Mann–Whitney U test). Statistical significance was considered with P ≤ 0.05. Odds ratio (OR) was calculated with the 95% Confidence interval (95% CI).

3. Results

The demographic data of patients with TBr and PTB, TB + T2D, normal control subjects, and health–care workers who were TST+ are represented in Table 1.

Table 1 Demographic data of the study population.

| Study population                | Age (years) | Gender(M/F) |
|---------------------------------|-------------|-------------|
| Healthy subjects (n=47)         | 34.0±6.2    | 31/16       |
| Health–care workers TST+ (n=13) | 31.6±5.0    | 8/5         |
| Patients with PTB (n=40)        | 54.9±9.8    | 15/25       |
| Patients with TBr (n=37)        | 44.7±6.5    | 15/22       |
| Patients with TB + T2D (n=21)   | 55.0±10.6   | 9/12        |

F, feminine; M, masculine; PTB, Pulmonary tuberculosis; TBr, Tuberculosis in remission ; T2D, Type 2 diabetes ; TST+, tuberculin test positive.

3.1. Allele frequency IL–10

To analyze the frequency of alleles in the studied population, we brought together patients with TBr and with PTB, and healthy blood donors with health workers who were TST+. The C/A allele distribution of the IL–10 –592

polymorphism and G/A of the –1082 were similar in healthy subjects, with the two alleles occurring at an approximate ratio of 2:1 and all did not show significant differences (Table 2).

### 3.2. Genotype frequency IL–10

The results of genotyping of the population under study may be observed in Table 3. The five groups fell within Hardy–Weinberg equilibrium, with non–significant values by  $\chi^2$  test for the genotype observed and expected for each of the polymorphisms tested<sup>[25]</sup>. The IL–10 genotype did not differ significantly between healthy subjects and cases of cured TB and non–cured TB. In contrast, the –592 CA genotype presented in a greater proportion in healthy subjects than in patients with TB + T2D with statistical significance. Similarly, the –1082 AA genotype was found more frequently in patients with both diseases as compared with healthy individuals, also with statistical significance.

## 4. Discussion

Distribution of IL–10 gene polymorphisms appears to have an ethnic distribution, because its frequency varies depending on the population studied, in addition to its known association with the major histocompatibility complex (MHC)<sup>[11,18,25–28]</sup>. Our study has limitations, the healthy individuals are not a well defined group, may be TST positive or negative, they were much younger than patients, and limitations of groups sizes.

This study describes two important new findings. First, it reveals that the IL–10 (–592 A/A and –592 C/C) polymorphisms were found in a greater proportion in a group of patients with T2D and TB than in healthy subjects. Second, the study provides evidence that the (–1082 G/G) polymorphism presented with greater frequency in healthy individuals than in patients with both of these diseases.

The IL–10 (–592 C/A) allele frequency in healthy subjects correlated with other report, differing for the (–1082 G/A) allele frequency<sup>[28]</sup>. With regard to TB, our findings coincide with those reported in African population<sup>[29]</sup>, and diverging from what was reported in China where they find, the genotype frequencies of A/A of IL–10 in the PTB patient group were higher than that of control group<sup>[30]</sup>. With respect to the G/A genotype of the –1082 SNP, its frequency was greater in healthy subjects, although without statistical significance, diverging from that reported in Turkey, where it was found associated with a risk for TB<sup>[31]</sup>.

Regarding the G/G genotype of the –1082 SNP, this was found to be more frequent in Mexican healthy population compared with patients with both diseases, coinciding with that reported in Brazilian population<sup>[32]</sup>, as well as with those reported in Egypt, where IL–10–1082 G/G genotype did not exhibit significant association except for increased GG frequency in PTB<sup>[33–36]</sup>.

Although the protective mechanisms against clinical TB disease by increased IL10 are not fully understood, several clues were reported. Much higher serum IL10 was detected

in patients with clinical TB suggesting that IL10 plays a role in susceptibility to tuberculosis<sup>[37,38,39]</sup>. The clinical characteristics of TB and maybe, the treatment failure are due to host–mycobacteria interaction, where is not well defined the role of the host’s genetics factors. Although the high failure rate for TB therapy suggests factors like non–compliance or drug resistance, neither we found association between any genotype of the IL–10 SNPs studied with an anti–TB treatment response.

We think that there are many more polymorphic regulator genes that can potentially exert an influence and that although these have not yet been studied in patients with TB, additional studies are necessary in this regard. A larger sample size is needed to study the association between gene polymorphisms and diseases. This was low in our study, and using cohorts such as this can lead to false positive and/or negative associations, which is probably the case here. The results must be confirmed by further studies with larger sample sizes.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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