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MOLECULAR ASPECTS

Possible association of rare polymorphism in the ABCB1 gene with rifampin and ethambutol drug-resistant tuberculosis

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SUMMARY

Human P-glycoprotein (P-gp) is a membrane transporter encoded by *ABCB1* (also known as *MDR1*) that plays a critical role in pharmacokinetics of many unrelated drugs. Rifampin (RMP) and ethambutol (ETB), two anti-tubercular agents, are substrates of P-gp. Single nucleotide polymorphisms (SNPs) in *ABCB1* have been associated with resistance to several drugs; however, their association with RMP and ETB resistance in tuberculosis patients has not yet been studied. Genotype/allele frequencies in C1236T, G2677T/A and C3435T SNPs of *ABCB1* were obtained from 99 tuberculosis patients susceptible or resistant to RMP and ETB (NoRER or RER). 2677G>A allele prevalence was found to be significantly higher in the RER group compared to NoRER (5 resistant vs 2 non-resistant patients, $P < 0.01$; OR, 11.0; 95% CI, 2.00–56.00). No differences were found in genotype/allele frequencies in C1236T and C3435T SNPs of *ABCB1* and resistance to RMP and ETB in tuberculosis patients ($P > 0.05$). The present study suggests the 2677G>A allele of *ABCB1* could be associated with simultaneous resistance to RMP and ETB in pulmonary tuberculosis patients. Further studies with larger sample sizes are needed to confirm this association and explore its nature.

1. Introduction

Pulmonary tuberculosis (TB) is the most frequent form of infection caused by *Mycobacterium tuberculosis (Mtb)*. Nine million new TB cases are reported worldwide and two million people die each year due to this disease [1].

Combination treatment with rifampin (RMP), isoniazid (INH), ethambutol (ETB) and pyrazinamide (PZA) is the standard and generally effective therapy for new TB cases. Nevertheless, in last decades *Mtb* strains resistant to anti-tubercular agents have been emerging [2–4]. Multidrug-resistant TB (MDR-TB) is caused by *Mtb* strains resistant to at least INH and RMP simultaneously [5]. MDR-TB represents a world health problem. Its burden differs among countries, however, rates as high as 28.9% for new TB cases and 51.5% for previously treated cases have been reported [6].

Mutations in several genes of *Mtb* have been documented to be associated to drug-resistant phenotypes in bacterial cultures. Additionally, accumulation of such mutations may lead the bacteria to acquire a multidrug-resistant phenotype [3]. Positive selection of resistant strains is known to occur as consequence of environmental factors, which are related to repeated exposition to antibiotic therapy, and inadequate or insufficient treatment [5]. Although the involvement of bacterial genetics in the genesis of MDR-TB has been relatively well explored, and several authors have investigated the role of host genetics on *Mtb* infection [7,8], much less is known about host genetic factors on the development of resistant forms of TB. In this regard, Takahashi et al. reported a possible association between a variant of the gen *SLC11A1* and MDR-TB [9].

Human P-glycoprotein (P-gp) is an extensively studied ATP-dependent pump that actively transports several different molecules out of the cell [10]. Its most evident function is protection of cells from the toxic effect of some endogenous compounds and xenobiotics [11,12]. P-gp is expressed on apical surface of some

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epithelia, kidney, blood–brain barrier and phagocytic cells as macrophages (main target of *Mtb)* [9,13,14]. In addition, P-gp plays a critical role in pharmacokinetics of some drugs [15]. *ABCB1/MDR1* polymorphisms affect the expression of P-gp and/or its activity against some cardiovascular, anti-cancer, central nervous system drugs, etc. [16–20]. A study demonstrated that two of the first line drugs for tuberculosis treatment, RMP and ETB, are substrates of P-gp [21], setting up the rationale for considering P-gp as an interesting candidate for human genetics' involvement in the generation of a fraction of the MDR-TB cases. Up to date, there are no studies that explore whether P-gp polymorphisms are associated with drug-resistant TB. In this study, we investigated whether polymorphisms in *ABCB1* may be associated with higher risk to develop resistant forms of TB. We explored for associations between alleles, genotypes and haplotypes of C1236T, G2677T/A and G3435T polymorphisms and resistance to any of four first line drugs INH, RMP, ETB and streptomycin (SM), focusing on MDR-TB and simultaneous resistance to RMP and ETB.

2. Materials and methods

2.1. Participants

From 2010 to 2013, a total of 122 consecutive patients who had culture proven TB and attended the TB Clinic of the University Hospital of Monterrey (A referral center for MDR-TB cases from north-east Mexico) were evaluated. The ethics committee approved this study and written informed consent was obtained from all participants. Only individuals with positive *Mtb* cultures and results of drug sensitivity tests (DST) were recruited. DST performed to samples from all patients tested for resistance to INH, RMP, ETB and SM. Subjects with extra-pulmonary infection and those co-infected with HIV were excluded. Demographic data was recorded and a peripheral blood sample was obtained from each patient. Finally, samples that presented genotyping problems were discarded, ending up with 99 participants for analysis (Figure 1). Patients were allocated in 4 non-mutually exclusive study groups according to their DST results: MDR, NoMDR, RMP-ETB resistant (RER) and NoRER. Information about patients' resistance status, risk factors and co-morbidities was obtained from their medical records.

2.2. Genotyping of C1236T, C3435T and G2677T/A polymorphisms

Genomic DNA isolation was performed from 0.5 ml of EDTA-anticoagulated blood using the TSNT technique. DNA samples were stored at −20 °C until their use. Real time PCR was performed using Taqman® technology for C1236T [rs1128503] and C3435T [rs1045642] genotyping (Assay IDs: C___7586662_10 and C___7586657_20 respectively). PCR conditions for C1236T were as follows: Initial denaturation step by 10 min at 95°, 40 cycles of 15 s at 95 °C and annealing-extension step at 60 °C for 60 s. PCR conditions for C3435T were 10 min at 95°, 40 cycles of 30 s at 95 °C and annealing-extension step at 60 °C for 90 s. Both reactions contained 10 μl of IQ Super Mix (2x, Bio-Rad, Hercules, CA), 8 μl miliQ water, 1 μl of isolated genomic DNA and 1 μl of the corresponding Taqman probes (20x, Applied Biosystems, Carlsbad, CA), real time PCR reactions were performed in a CFX96 thermocycler (Bio-Rad, Hercules, CA).

G2677T/A [rs2032582] genotyping was tested using the PCR-RFLP method (Table 1). Forward and reverse primers reported previously by Levran et al. (2008) were used (F: 5′-TCT CAT GAA GGT GAG TTT TCA GA-3′, R: 5′-AAA CAC ATT CTT AGA GCA TAG TAA GCA-3′). Primers were synthesized by Alpha DNA (Montreal, QC). G2677T/A PCR (PTC-200 thermocycler, MJ Research, St.-Bruno, QC) was performed under the following conditions: Initial denaturation step for 5 min at 94 °C, 40 cycles of denaturation at 94.5° for 45 s, annealing at 63 °C for 30 s and extension at 72 °C for 30 s. All restriction enzymes were from New England Biolabs (Ipswich, MA). Each PCR product was separately digested with RsaI or BseYI. Fragments were run in 3% agarose gel and stained with ethidium-bromide or RedGel™ (Biotium, Hayward, CA). Products with restriction patterns matching TA or AA genotypes were also double-digested with NlaIV and RsaI in order to discriminate between them.

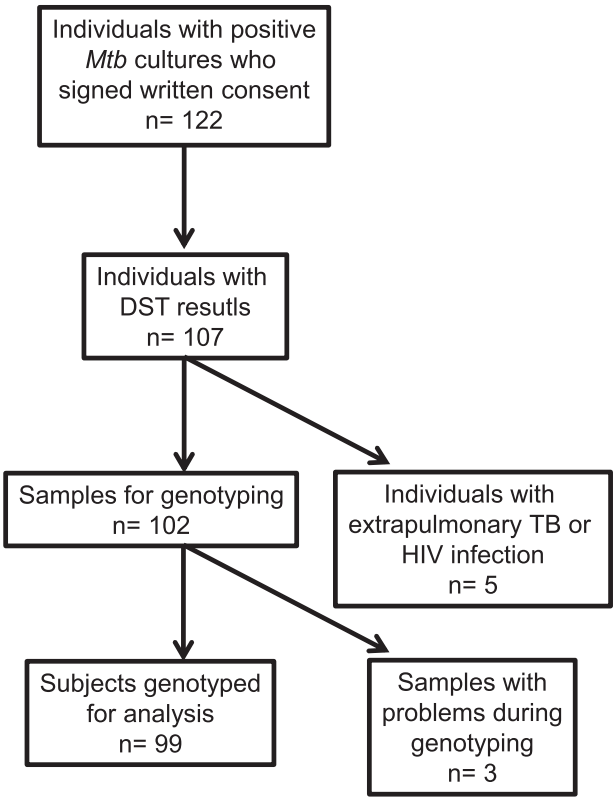

Figure 1. Flow diagram of patient selection.

2.3. Statistical analysis

Power calculations to detect a relative risk of 1.5 assuming a dominant model were between 7% for the A allele of G2677T/A (2677G>A), and 14% for the C allele of C1236T (1236C). Concordance of genotype distribution with the Hardy–Weinberg equilibrium was assessed for individual alleles at each locus using Arlequin software v3.5.1.3 (Bern University, Switzerland), which makes use of a test analogous to Fisher exact test [22]. Hardy–Weinberg test was performed on the NoMDR patients, as genotype frequencies of this group are expected to be more representative of the general population than those of MDR patients. Polymorphisms that were in equilibrium were further analyzed with a Fisher bilateral test comparing among patients with MDR, NoMDR, RER and NoRER using Prism v5.0 (Graphpad, La Jolla, CA). *P*-values below 0.05 were considered statistically significant.

Haplotype inference was performed from G2677T/A and C3435T genotypes using the ELB algorithm found in Arlequin. The ELB algorithm makes use of a Bayesian method to “reconstruct” the gametic phase of multi-locus genotypes. Frequencies of inferred haplotypes were then analyzed using the software WHAP 2.09,

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