Infection, Genetics and Evolution 23 (2014) 42–48

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Association of genetic variants with anti-tuberculosis drug induced hepatotoxicity: A high resolution melting analysis

Meenakshi Singh [a,1], Vinod H. Gupta [b,1], Deepak N. Amarapurkar [c], Jyotsna M. Joshi [d], Rajiv Baijal [e], Praveenkumar H. Ramegowda [e], Anjali D. Amarapurkar [f], Pramod P. Wangikar [a,*]

[a] Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India
[b] Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India
[c] Department of Gastroenterology and Hepatology, Bombay Hospital and Medical Research Centre, Mumbai 400 020, India
[d] Department of Pulmonary Medicine, BYL Nair Hospital, Mumbai 400 008, India
[e] Department of Gastroenterology and Hepatology, Jagjivanram Western Railway Hospital, Mumbai 400 008, India
[f] Department of Pathology, TN Medical College, Mumbai 400 008, India

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 15 January 2014
Accepted 24 January 2014
Available online 31 January 2014

Keywords:
Tuberculosis
Drug induced hepatotoxicity
High resolution melting
Mutation screening

ABSTRACT

Background: Tuberculosis (TB) treatment remains a challenge owing to the high incidence of drug induced hepatotoxicity (DIH). Apart from environmental factors, single nucleotide polymorphisms (SNPs) in drug metabolizing enzymes (DMEs), nuclear receptors (NRs) and transporter proteins (TPs) contribute to DIH. In the present study, we report known and novel SNPs in a total of seven genes of DMEs, NRs and TPs with high resolution melting (HRM) technique.
Methods: DNA samples of 185 TB patients of Western Indian population, of which 50 showed DIH, were analyzed. Grouping of the temperature-shifted difference plots obtained from the DNA melt curves enables identification of known and novel SNPs. Representative samples of each group were sequenced.
Results: We report 18 novel SNPs, of which 3 are in 5′-UTR, 14 in exonic and 1 in intronic region. Of the SNPs in exons, 7 non-synonymous, 3 synonymous and 4 deletion mutations were observed. Among the known SNPs, *CYP2E1* wild-type, *NAT2*5* mutant and *NAT2*6* heterozygous genotypes were associated with DIH ($p < 0.05$). Among the novel SNPs, group 2 of *SLCO1B1* showed a significant association ($p < 0.05$).
Conclusions: While several SNPs showed borderline p values between 0.05 and 0.15, the confidence in association can be improved further by using larger data sets.

1. Introduction

Despite intense efforts worldwide, rates of mortality and morbidity remain significantly high for tuberculosis (TB). Furthermore, incidence of hepatotoxicity towards the first line anti-TB drugs namely, isoniazid (INH), rifampicin (RIF), pyrazinamide (PYZ), and ethambutol (ETB) and the associated non-compliance to treatment has resulted in the emergence of multi-drug resistant strains (Joshi, 2012). We have recently reported the hepatotoxic potential of each of these anti-TB drugs individually and in combination via *in vitro* assays (Singh et al., 2011). Apart from environmental factors, polymorphisms in genes contribute towards development of drug induced hepatotoxicity (DIH). Evidence is now accumulating which strongly suggests that polymorphisms in drug metabolizing enzymes (DMEs), transporter proteins (TP) and nuclear receptor (NR) play a significant role in altered disposition of xenobiotics (Xu et al., 2005) To cite a few examples, polymorphisms in 5′-UTR (un-translated region) of Phase I gene, Cytochrome P450 2E1 (*CYP2E1*) (Vuilleumier et al., 2006), exon of phase II gene, N-acetyltransferase 2 (*NAT2*) DMEs (Gupta et al., 2013), constitutive androstane receptor, *NR1I3* (Hor et al., 2008), TP encoded by multidrug resistance (*MDR1*) (Chen et al., 2011), organic anion transporters

Abbreviations: ALT, alanine transferase; AST, aspartate transaminase; BMI, body mass index; CYP2E1, cytochrome P450 2E1; DIH, drug induced hepatotoxicity; DMEs, drug metabolizing enzymes; ETB, ethambutol; HRM, high resolution melting; INH, isoniazid; MDR1, multidrug resistance; NAT2, N-acetyl transferase 2; NR1I3, nuclear receptor 1I3; NRs, nuclear receptors; OATP1B1, organic anion transporters polypeptide 1B1; OCT1 & OCT3, organic cation transporter 1 & 3; PYZ, pyrazinamide; RFLP, restriction fragment length polymorphism; RIF, rifampicin; SNPs, single nucleotide polymorphism; SLC22A1, solute carrier 22A1; SLC22A3, solute carrier 22A3; SLCO1B1, solute carrier O1B1; TPs, transporter proteins; ULN, upper limit of normal.

* Corresponding author. Address: Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, Maharashtra, India. Tel.: +91 22 2576 7232; fax: +91 22 2572 6895.
E-mail address: wangikar@iitb.ac.in (P.P. Wangikar).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.meegid.2014.01.027
1567-1348/

polypeptide 1B1, *solute carrier* (*SLC*) *O1B1* (Kim et al., 2012), organic cation transporter 1, *SLC22A1* (Umamaheswaran et al., 2011), and organic cation transporter 3, *SLC22A3* (Chen et al., 2010) genes are reported with regards to various clinical conditions including those associated with drug toxicity. Therefore, there is a need to develop a technique that rapidly and economically detects the polymorphisms in DMEs, TP and NR.

Several methods have been employed to detect polymorphism in the genes that metabolize drugs. These include allele specific primer (Newton et al., 1989), PCR followed by restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), gel electrophoresis (Orita et al., 1989), probe real-time PCR (Reuter et al., 2005) and sequencing (Davis et al., 2007). However, low sensitivity, high cost and time requirements or a combination thereof limit the application of these techniques. High resolution melt (HRM) analysis is a non-gel based automated method for mutation scanning, which does not require gene specific probes (Wittwer et al., 2003). By comparing fluorescence as a function of temperature in a melt curve, HRM detects single nucleotide polymorphism (SNPs) and small deletions in amplified DNA. Depending on the alleles present, distinct melting curves are observed. In this study, we employ HRM analysis to study the association of polymorphism in DMEs, TPs and NRs with anti-TB drug induced hepatotoxicity. Using HRM technique, three novel SNPs in the *CYP2E1*5*, four deletions in *NAT2*6*, eleven novel SNPs in *SLCO1B1*, and one novel SNP in *SLC22A3* gene were observed in TB patients. The present study provides insight into the role of genetic variants of DMEs, NR and TP using HRM analysis; that will help in effective management of anti-tuberculosis drug induced hepatotoxicity and the resultant non-compliance.

2. Materials and methods

2.1. Patient samples and DNA extraction

The patient population, inclusion and exclusion criteria and definition of hepatotoxicity has been described in our earlier study (Gupta et al., 2013). Briefly, a total of 185 patients undergoing anti-tuberculosis treatment were recruited from Bombay Hospital, BYL Nair Hospital & TN Medical College and Jagjivanram Western Railway Hospital. Patients with incidence of hepatotoxicity during treatment were categorized as cases ($n = 50$) and those not exhibiting hepatotoxicity as controls ($n = 135$). The patients between age group of 18 and 73 years with normal levels of alanine transferase (ALT), aspartate transaminase (AST) and total bilirubin were included. The patients showing the following symptoms were excluded; (a) chronic liver diseases, (b) hepatitis B and/or C or HIV, (c) alcoholic liver diseases, (d) treatment with other potentially hepatotoxic drugs, (e) ALT, AST and total bilirubin levels above two times upper limit of normal (ULN). Hepatotoxicity is defined as increase in two times ALT levels to ULN and/or a combined increase in AST and total bilirubin provided one of them to be over 2 times the ULN according to International Consensus Meeting (Benichou, 1990). Collection of clinical data and biological materials were subject to informed consent by the patients. Genomic DNA from whole blood of all patients were extracted using salting out method (Miller et al., 1988). Quantity and quality of DNA samples were assessed using Nanophotometer 1265 (Implen, Munchen, Germany). The study was conducted in accordance with principles of the Declaration of Helsinki (Helsinki, 2001). The study protocol was approved by Institutional Ethic Review Board of all participating hospitals mentioned above including ethical committee of Indian Institute of Technology Bombay, Mumbai.

2.2. Primer design

Primers were designed using Primer 3 online software (version 0.2, www-genome.wi.mit.edu). The amplicon length was kept in the range of 66–286 bp with majority being shorter than 160 bp in order to distinguish the HRM curves for alleles with SNPs (Gundry et al., 2003). HPLC purified primers were sourced from Sigma Aldrich (Sigma Aldrich, Bangalore, India). We first optimized the annealing temperature for each primer set on Master Cycler (Eppendroff, Hamburg, Germany) to avoid primer-dimers and non-specific amplification in HRM analysis. Primer sequences, annealing temperature and location of Phase I and II, NR and TP genes are listed in Table 1. Verification of the specific amplicon obtained post PCR using HRM primers was done on ethidium bromide stained gels.

2.3. PCR conditions

The real time PCR was performed on Light cycler® 480 (Roche Diagnostics, Indianapolis, IN) in 96 well micro titre plate filled with 10 μl of mineral oil. The assay was performed in a total volume of 10 μl using 4 μl of 1× Light Scanner® master mix (Idaho Technology Inc., Salt Lake city, Utah, USA), oligonucleotide primers at a concentration of 0.2 μM and 30 ng of DNA according to manufacturer's recommendation. PCR was initiated by 10 min of denaturation-activation at 95 °C, followed by 45 cycles of 95 °C for 10 s (denaturation), 60 °C for 10 s (annealing and elongation). The melting program included three steps: denaturation at 95 °C for 1 min followed by renaturation at 40 °C for 1 min and subsequent melting that consisted of a continuous reading of fluorescence from 65 to 95 °C at the rate of 25 acquisitions per °C with ramping rate of 0.02 °C/s. Reproducibility of each duplicate sample was assessed by checking the shape and peak height.

2.4. HRM techniques and gene scanning

The HRM analysis and clustering were performed using Gene Scanning Software v.1.5.0 (Roche Diagnostics, Indianapolis, IN). After the completion of PCR cycle, melting curve analysis was performed. The software first normalizes raw melting curve data by setting pre-melt and post-melt signals of all the samples to a uniform value. Secondly, it shifts the normalized curves along the temperature axis so that samples are comparable. Finally, the difference plot is obtained by subtracting the shifted normalized curves from a reference curve which allows clustering the samples into groups according to similarities in the difference plots. It has been hypothesized that each group corresponds to a given genotype (de Juan et al., 2009). The novel variants were identified based on the distinct melting peaks obtained from the derivative plot as compared to that obtained for the normal genotypes.

2.5. Sequencing

At least two representative DNA samples from each group were sequenced. The samples were first amplified using conventional PCR. Sample (20 μl reaction mixture) were prepared in duplicates, with each set containing 1× PCR buffer (ABI, CA), 2 mM of $Mgcl_2$, 0.4 mM of dNTPs, 0.2 μM of each forward and reverse primer, 1.5U of AmpliTaq Gold (ABI, CA) and 100 ng of gDNA. Primers for sequencing were different from the ones used for HRM in order to produce larger amplicon. The cycling conditions consisted of an initial denaturation of 95 °C for 10 min, followed by 35 cycles of each for 1 min (denaturation: 95 °C, annealing: 60 °C and elongation: 72 °C) and final extension of 72 °C for 7 min. After amplification, PCR products were checked on 2% agarose gel and 15 μl was