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Ybhg. Prof.,

LAPORAN AKHIR PENYELIDIKAN TACROLIMUS PHARMACOGENETICS: THE INFLUENCE OF GENETIC POLYMORPHISMS OF CYP3A5, MDR1 AND PREGNANE X RECEPTOR (PXR) IN TRANSPLANT PATIENTS.

Merujuk kepada perkara di atas, bersama-sama ini disertakan 3 (tiga) naskah Laporan Akhir Penyelidikan bertajuk: "TACROLIMUS PHARMACOGENETICS: THE INFLUENCE OF GENETIC POLYMORPHISMS OF CYP3A5, MDR1 AND PREGNANE X RECEPTOR (PXR) IN TRANSPLANT PATIENTS".

Sekian, terima kasih.

Yang benar,

PROF DR MOHD ZAKI SALLEH
Ketua
Projek Penyelidikan

PENGHARGAAN

Setinggi-tinggi penghargaan dan ribuan terima kasih diucapkan kepada semua pihak yang terlibat secara langsung dan tidak langsung bagi membolehkan penyelidikan ini disiapkan dengan sempurna. Setinggi ucapan terima kasih juga kepada semua para pesakit serta para sukarelawan yang terlibat yang menyumbang kepada kejayaan projek ini.

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Abstract

Title: Tacrolimus pharmacogenetics: the influence of genetic polymorphisms of CYP3A5, MDR1 and Pregnane X receptor (PXR) in transplant patients.

Introduction: Tacrolimus is an immunosuppressant commonly used for long term in transplant patients to prevent organ rejection. Its use faces clinical challenges in achieving optimum dose for all patients. There are wide variation in doses of tacrolimus required for patients resulting in different drug levels and unpredictable drug responses. Certain patients are predisposed to an increased susceptibility to acute rejection while many are at risk of adverse effects of tacrolimus. Tacrolimus is a substrate for both P-glycoprotein (P-gp, encoded by the gene MDR1) and CYP3A's. Differences in the intestinal P-glycoprotein and hepatic and intestinal cytochrome P4503A activities have been postulated as contributing to the wide inter-patients variations in drug levels and responses. We therefore aim to investigate the impact of the genetic polymorphism as well as expression level of *MDR1* in transplant patients receiving tacrolimus.

Materials and methods: Genotyping method for *MDR1 C1236T, G2677T/A & C3435T* was developed using allele specific primers that were designed to specifically amplify the variants of *C3435T*. The method was then validated by direct sequencing. DNA samples from healthy volunteers comprising of three major ethnic groups in Malaysia as well as kidney-transplant patients prescribed with tacrolimus were screened for the three variants. Next, expression level of *MDR1* gene among patients was quantified by quantitative real-time PCR (qPCR) using specific hydrolysis probe.

Result: The correlation of dose and trough level of tacrolimus was moderate with R^2 =0.32. The initial dose of tacrolimus at 3 months and 6 months were found to be significantly different between the different genotypes of *MDR1* for SNPs C1236T (Post Hoc LSD; $p<0.05$), C3435T (Post Hoc LSD; $p<0.05$), G2677T/A (Post Hoc LSD; $p<0.05$). The dose of tacrolimus was higher for patients with homozygous wild type of *MDR1* despite the lower trough levels achieved. Percentage change of HDL, serum creatinine, total cholesterol and fasting blood sugar were found significantly different between different genotype groups of *MDR1* (Post Hoc LSD; $p<0.05$). The dose of tacrolimus at 12 months were predictable by 51.7% using pharmacogenotyping of *CYP3A5*3* and **6*.

Discussion and Conclusion: This AS-PCR was found to be sensitive and specific in distinguishing the variants and it is also more suitable and cost-effective to be used in large studies compared to genotyping using restriction enzymes. It has been shown in this study that there are correlations between polymorphisms in *MDR1* and expression level. This study also evidently underlines that improved dosing regimen can be achieved using pharmacogenotyping of *MDR1* and *CYP3A5* with the methods that have been developed. However, prospective clinical trials are recommended to assess the dosing algorithm for personalising tacrolimus dose.

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CHAPTER ONE

LITERATURE REVIEW AND INTRODUCTION

1.0 Introduction

The most important criteria to measure the efficacy of a drug are the ability of the drug to reach its target and its lifetime in the body. However, the development of multidrug resistance towards a wide variety of structurally similar drugs which was first observed in cancer cells have become the main obstacle in clinical therapy. Many studies have shown that the resistance is associated with the over-expression of a membrane-bound transporter protein known as P-glycoprotein (P-gp), which is a product of *MDR1* gene (Juranka *et al.,* 1989; Gottesman *et al.,* 1993). This protein has the ability to transport out and reduces the intracellular concentrations of a wide range of drugs and xenobiotics. P-gp exhibits wide substrate specificities for structurally different drugs and consequently mediates drug resistance to a variety of drugs, including Vinca alkaloids, anthracyclines, epipodophyllotoxins, taxols, actinomycin D, cardiac glycosides, immunosuppressive agents, glucocorticoids, and anti-HIV protease inhibitors (Ueda *et al.*, 1992; Gottesman *et al.,* 1993; Saeki *et al.*, 1993). The multidrug resistance transporter belongs to a family of ATP-binding cassette (ABC) protein with a well conserved membrane topology, structure and mechanism of action; is found in almost all living organism from bacterial cells to human. In order to minimize the failure of drug therapy, the understanding of this defense mechanism and factors that influence it will be of major importance.

1.1 *MDR1* Gene

P-gp is encoded by one of the MDR gene(s) that are well conserved in nature. Humans have two types of *MDR* gene namely *MDR1* and *MDR3* (also known as *MDR2*) (Chin *et al.*, 1989) while rodent have three types of *MDR* genes known as *MDR1a*, *MDR1b* and *MDR2* (Gros *et al.*, 1986). In human, only MDR1 gene is responsible for P-gp synthesis while *MDR1a* and *MDR1b* in rodents are involved in the transportation of drugs and development of drug resistance (Fardel, 1996). The *MDR1* gene is located on the long arm of chromosome 7, region 21 (7.q.21) and consists of a core promoter region and 29 exons (Project Ensembl [database online] Hinxton, 2004). From DNA derived human cell lines, it was characterized that the genomic sequence of *MDR1* gene is 209 kb in size (Bodor *et al.*, 2005).

1.2 Structure of P-gp

P-glycoprotein (P-gp) is a 170 kDa phosphorylated and glycosylated protein with N-terminal glycosylation (Juliano, 1976). It was termed P-glycoprotein after it was found that this protein has the ability to change the permeation of drugs in cell (Juliano and Ling, 1976). This protein consists of 1280 amino acid with two homologous halves of 610 amino acids joined by a flexible linker region of 60 amino acids. Each of these homologous halves contains six transmembrane domains with an N-terminal hydrophobic and a hydrophilic domain containing a

nucleotide-binding site. The nucleotide-binding site is crucial for the binding of ATP and its analogs and inactivation of either site inhibits substrate-stimulated ATPase activity. However, the 2 sites are functionally independent and cleavage probably occurs only at one site at a time (Hrycyna, 1998; Loo, 1999). Each nucleotide binding site consists of 3 conserved regions called Walker A, B, C motifs (Gottesman, 1996).

P-gp has a donut shape with 6-fold symmetry, a diameter of about 10 nm and a large pore of about 5 nm in diameter. About half of this molecule is within the plasma membrane because this protein has a thickness in the plane of the plasma membrane of about 8 nm and the plasma membrane lipid bilayer is only about 4 nm. There is an aqueous chamber within the membrane that is exposed to the extracellular medium, which is formed by the closing of the central pore at the inner end. There is an opening from this chamber to the lipid phase. The two-nucleotide binding domains (NBDs) are exposed at the cytoplasmic end as lobes of 3 nm each (Rosenberg, 1992).

1.3 Function of P-glycoprotein

P-gp is known to play a very important role in transporting drugs and other chemicals out of the body. P-gp is also able to transport a wide variety of structurally dissimilar drugs and overexpression of this protein contributes to the development of multi-drug resistance in cancer cells. However, in the absence of

therapeutics or toxins, the normal physiological function of P-gp is unclear that studies were carried out using the knockout (KO) mice. Knockout mice are genetically engineered mice that have one or more of its genes made inactive through a gene knockout and in this case the *MDR1* gene is being inactivated. From the studies carried out on these *MDR1a* knockout mice, it was shown that these mice have normal viability, fertility and a range of biochemical and immunological parameters. However, as predicted, they do have delayed kinetics and clearance of vinblastine and they accumulate high levels of certain drugs (vinblastine, ivermectin, cyclosporin A, dexamethasone, ondansetron, loperamide and digoxn) in their brains (Schinkel *et al.,* 1996; 1997). Apart from transporting drugs and toxins out of the cells, P-gp also function in steroid metabolism, cholesterol metabolism, immune system, cell death and cell differentiation, chloride channel and modulating cytochromes expression. P-gp plays a role in small and large intestine, biliary ductules, and proximal tubules of the kidney by decreasing the absorption from the gut and/or the excretion of endogenous and exogenous hydrophobic amphipathic toxins.

1.4 Cloning and Expression of P-gp

Gene cloning is defined as the production of exact copies of particular DNA or gene of interest using recombinant DNA technology (Hine & Martin, 2004). Gene cloning enables the determination of the structure and function of individual genes and it also led to rapid and efficient DNA sequencing techniques (Brown,

1993). The basic steps in gene cloning include the insertion of a DNA fragment containing the gene to be cloned into appropriate expression vector to produce a recombinant DNA. Vectors or also known as plasmids are circular molecules of DNA that are capable of replicating independently in bacterial cells. Next the recombinant DNA will be propagated into a host cell with the vector the acting as a transport vehicle. The introduced vector will multiplies within the host cell to produce a large number of identical copies of the recombinant DNA molecule. These identical copies of recombinant DNA molecule will be passed on to the progeny when the host cell divides. Lastly, after numerous number of cell division, a colony of identical host cell, which carries one or more copies of the recombinant DNA molecule, will be produced (Brown, 1993).

It has been shown that the *MDR1* gene is 209 kb in length but the coding sequence for this gene is 3843 bp (Acession Number: M14758, http://www.ncbi.nlm.nih.gov/Genbank/). Thus, an appropriate vector must be used to clone this large fragment of DNA. Raymond et al. (1994) have successfully expressed functional P-glycoprotein encoded by the mouse multidrug resistance *MDR3* gene in *Saccharomyces cerevisiae*. This expressed *MDR3* in yeast can confers cellular resistance to the immunosuppressive and antifungal agent FK520 that suggests that this compound is a substrate for P-gp in yeast cells. *MDR3* behaves as a fully functional drug transporter in this heterologous expression system (Raymond *et al.,* 1992). It was also reported that human mdr1 cDNA has been successfully cloned into a high copy number of yeast expression vector under the control of the constitutive promoter of the yeast plasma

membrane H^+-*ATPase*. Yeast cells transformed with the P-gp expression plasmid acquired increased resistance to valinomycin, suggesting that the expressed P-gp is properly folded and functional (Mao & Scarborough, 1997). During this study, the expressed P-gp can be solubilized from the yeast membranes with lysophosphatidylcholine, and when tagged with ten histidines at its C-terminus, can be readily purified to about 90% homogeneity by $Ni2^+$ affinity chromatography. Since the protein expressed from this host is expected to be similar as protein expressed in human, an *in-vitro* model of this protein would be of much importance in studying the property of this protein.

1.5 Polymorphism in *MDR1* Gene

To date, there are more than 50 single nucleotide polymorphisms (SNPs) identified on *MDR1* gene and some of these polymorphisms are thought to have an impact on the expression and function of P-gp. Hoffmeyer *et al.* (2000) had performed a systematic screening of the entire *MDR1* gene for polymorphisms where all the 28 exons and the core promoter region as well as exon-intron boundaries were sequenced from genomic DNA of healthy volunteers. In that screen, 15 single-nucleotide polymorphisms (SNPs) were detected. Some of these polymorphisms change the protein but do not affect the expression levels of *MDR1*. However, a SNP known as *C3435T* shows pharmacological consequences where it correlates with intestinal P-gp levels and influences the uptake of orally administered P-gp substrates (Hoffmeyer *et al.*, 2000). Individuals homozygous

for T/T alleles exhibit lower P-gp expression by fourfold compared with homozygous wild-type (C/C) individuals. This SNP was also shown to play a role as a significant predictor for treatment outcome of children with acute lymphoblastic leukemia although this finding has not been confirmed in adults (Jamroziak *et al.,* 2005) while in breast cancer patients, a study showed that patients homozygous for the *C3435T* genotype have a greater response towards preoperative chemotherapy (Kafka *et al*., 2003).

There is significant variability between different ethnic groups reported previously. A significant variability has been reported for *C3435T* polymorphism between African and Asian/Caucasian population where Africans showed higher C/C genotype (Ameyaw *et al,* 2001; Balram *et al.,* 2003). Although correlations between dispositions of other P-gp substrates with these polymorphisms have been established, the impact of these polymorphisms on the bioavailability of tacrolimus showed contradictory results. A study by Yamauchi *et al.* (2002) showed that only mutation at position 2677 in exon 21 is correlated with tacrolimus-induced neurotoxicity and the C3435T did not. On the other hand, a study in renal transplant patients found that *MDR1* haplotypes derived from the SNPs 2677G > T (exon 21) and 3435C > T (exon 26) have no significant effect on tacrolimus trough concentrations (Mai *et al*., 2004). The correlation of tacrolimus-induced toxicity and the C3435T is yet to be confirmed as studies on these showed contradictory results and therefore further studies need to be carried out.

1.6 Tacrolimus

Tacrolimus (FK506) is a calcineurin inhibitor which acts as an immunosuppressant whose main use is to reduce the risk of organ rejection in transplant patients (Fig 1.1). This 23-membered macrolide lactone was first discovered in 1984 from the fermentation broth sample of *Streptomyces tsukubaensis* in Japanese soil (Tanaka & Marusawa, 1987; Mierke & Karuso, 1992). Tacrolimus shares the same cellular mechanism of action as cyclosporine A although there are some structural differences between the two compounds and tacrolimus was found to be 10-100 times more potent at molecular level (Almawi & Melemedjian, 2000). Upon entering the cell, tacrolimus will bind to the cytosolic immunophilins; FK506-binding proteins (FKBP-12 and FKBP-52), which are the components of the glucocorticoid receptor complex reduce the peptidyl-prolyl isomerase activity. This drug- immunophilin complex will then binds to calcineurin, a calcium/calmodulin-dependent protein phosphatase and inhibits the activity of this enzyme. The complex will then result in the interruption of calcium-dependent signal transduction pathway in T-cells by preventing the dephosphorylation and translocation of nuclear factor of activated T-cells (NF-AT). NF-AT is closely associated with the initiation of gene transcription for formation of lymphokines such as interleukin-2 and gamma interferon and it is the inhibition of NF-AT that results in immunosuppression (Spencer *et al.,* 1995; Gewirtz, A. T., & Sitaraman, S. V, 2002; Drug Facts and Comparisons, 2003).

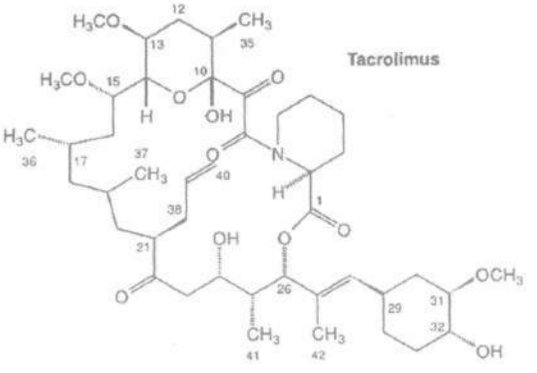

Figure 1.1 Structure of Tacrolimus. (Adapted from LC Paul)

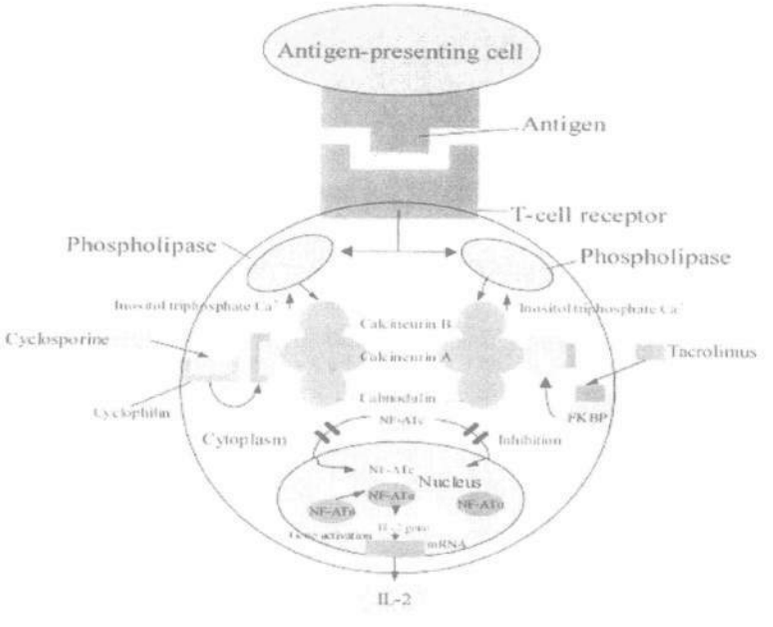

Figure 1.2 Intracellular mechanism of action of Tacrolimus. (Adapted from LC Paul)

Tacrolimus is orally administered and its rate of absorption and bioavailability shows a vast variability with the mean bioavailability of approximately 25% but

can range from 5% to 95% (Jain *et al.,* 1992; Gruber *et al.,* 1994). In order to compare drug exposure after oral and intravenous administration, the oral doses should be 3-4 times higher than intravenous doses. Poor drug uptake among patients is associated with low aqueous solubility of tacrolimus and change of gut motility in transplant patients. This drug is extensively distributed in the body and binds strongly to red blood cells resulting in a whole blood/plasma concentration distribution ratio of approximately 20:1 (Beysens *et al.*, 1991). CYP3A4 and CYP3A5 in the liver and intestinal wall are principle enzymes for the metabolism of tacrolimus with <0.5% remains as parent drug in faeces and urine (Sattler *et al.*, 1992; Karanam *et al.,* 1994). However, a study has shown that CYP3A5 is a more efficient enzyme for the metabolism of tacrolimus as compared to CYP3A4 (Yang *et al.,* 2005). The limited bioavailability of tacrolimus is associated with presystemic metabolism by gastrointestinal CYP3A. Previous studies have shown that intracellular concentration of tacrolimus is being lowered by the action of P-glycoprotein which pumps this drug back into the intestinal lumen (Benet, 1998; Tuteja *et al.,* 2001; Zhang & Benet, 2001). This protein is also believed to regulate the access of this drug to CYP450 3A enzymes by preventing this enzyme from being overwhelmed to the high concentration of tacrolimus in the intestine (Wacher *et al.,* 1998; Benet *et al.,* 1999). To date, eight metabolites have been reported from the studies carried out on *in-vitro* preparation of ^{14}C-labeled tacrolimus but only 3 I-O-demethylated tacrolimus exhibits similar activity to parent compound (Iwasaki *et al.,* 1993, Iwasaki *et al.*, 1995). More than 95% of

the tacrolimus metabolites are eliminated by the biliary route while 2.4 % are eliminated through urinary excretion (Moller *et al.,* 1999).

1.7 Statement of problems/ Hypothesis

Even though studies had shown large interethnic variation in the frequency of alleles for drug metabolizing enzymes, data reported for *MDR11* in local population especially among patients group are lacking. Data available are limited to healthy volunteers. In a previous study, we have genotyped 763 healthy Malaysians of 3 ethnic groups recently; the Malay and Chinese showed a higher frequency of allele C (50–60%) with an increased expression of P-gp. The Indian however exhibits a lower frequency (40%), similar to other Indian populations (15).

There are enough data to suggest that CYP3A5 and MDR1 polymorphisms alter levels of enzyme expression and function. As a result, they appear to be one of the key determinants of tacrolimus bioavailability and may also impact on other immunosuppressive agents. However, the effects of the polymorphisms of PXR gene on tacrolimus have not been reported. Thus, the interaction between these multiple genes with respect to tacrolimus pharmacokinetic variability requires further analysis. What is clear is that there is a scope for genetic analysis to be used in clinical practice, with pre-transplant testing of genes allowing a more scientific dosing in the first few days of post-transplant. The initial aim of this is to identify those patients with increased drug requirements to avoid under-

immunosuppression. It is also possible to identify groups of patients who are more likely to develop drug-related side effects at therapeutic doses. The other objective is to achieve targeted drug level as fast as possible and a prospective genetic test could reduce the time needed to attain a stable and effective blood concentration, resulting in a favorable cost/benefit ratio. With clearer understanding of the interactions involved, it may be possible to predict individual dose requirements and responses. We therefore aim to investigate the impact of the genetic polymorphism of *MDR1* in transplant patients receiving tacrolimus.

1.8 Study Design

The study methodology would be explained in 2 parts; Part A for Genotyping and Part B for genotyping and quantification of *MDR1* gene among kidney-transplant patients. The following flow chats describe the overall study design.

1.8.1 PART A: Study Design and Methodology for Genotyping on healthy volunteers

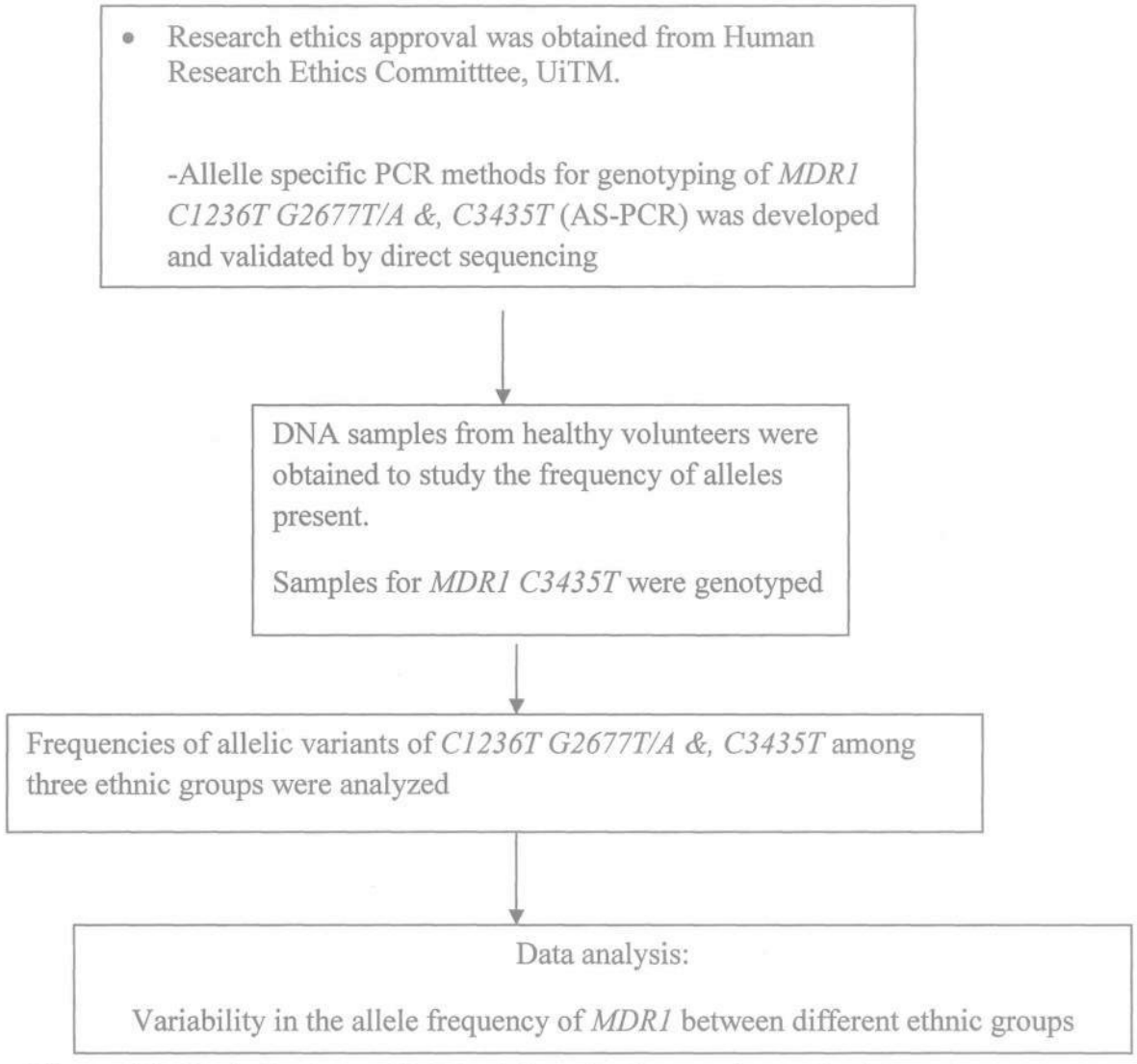

Figure 1.3: Study Design and Methodology for Genotyping on healthy volunteers