SYNOPSIS

Title: Genetic Polymorphism in Indian Population

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1. Introduction

Although the whole sequence of human genome is available, functions of only 15-20% genes are known. A major goal in human genetics is to understand the role of common genetic variants in susceptibility to common diseases. This will require characterizing the nature of gene variation in human populations, assembling an extensive data of candidate genes and polymorphic markers and performing association studies for particular diseases [Cargill M et al., 1999].

The original Mendelian view of genome classified alleles as either wild type or mutant. Subsequently, coexistence of multiple alleles at a locus called genetic polymorphism was recognized each with different effect on the phenotype. The present challenge is polygenic or complex disease phenotypes and multifactorial diseases those, which require an environmental stimulus or cofactor for gene influence. For such diseases and other multifactorial phenotypes, population association analysis is employed to discover genetic effects [Lewontin C, 1985; Chakravarti A 1999]. Population association analysis involving genes or random genome scans could be used for genetic analysis of disease proneness and/ or pharmacogenomic studies related to drug efficacy and drug response analysis.

Over the years there have been major improvements in the length and quality of life. While the infectious diseases are on a decline, the non-communicable and chronic diseases like coronary heart disease, cancers, diabetes, rheumatoid arthritis and hypertension are increasing. There have been number of drugs developed for these non-communicable diseases. But unfortunately variable response to these drugs by patients is a major hindrance in the cure rates. Inter-individual variability in drug response can be attributed to genetic polymorphism in genes encoding different drug metabolizing enzymes, drug transporters and enzymes involved in DNA biosynthesis and repair [Evans WE, 2003]. Mutation in a gene coding for a drug-metabolizing enzyme can cause enzyme variants with high, low or no activity [Meyer UA, 1994]. As a result of inter-individual variation some patients develop adverse drug reactions while some patients respond positively to the same drug administered in same dose. It has a known influence in clinical medicine and also in the development of new drugs. Hence determining an optimal dosage of the drug is a very important factor in therapeutics. There exists a clear evidence of correlation between genetic polymorphisms and efficacy and toxicity of drugs. This has led to the genesis of a concept of “Personalized Medicine” in the field of modern medicine.
The developments in the field of pharmacogenomics and the concept of “Personalized Medicine” are new to the field of modern medical science. However, there is a long-standing tradition regarding this concept of “personalized medicine” in the Indian Medical System of Ayurveda. According to the principles of Ayurvedic medicine, every person has a unique trait, which is called as “Prakriti”. Prakriti is defined by specific and permanent composition of Doshas at conception. It is one of the important factors for management of health and diseases. Each Prakriti has certain physical - psychosomatic characteristics, defined proneness to diseases and specific response to treatment [Charaka Samhita, 2000]. These concepts can be correlated with principles of immunogenetics and pharmacogenetics.

This Ph.D. work was planned in this background, to study the genetic polymorphism of drug metabolizing enzymes (DME) in Maharashtrian population. In the same cohort, this research work explored genetic basis for concept of Prakriti in Ayurveda. By considering Rheumatoid Arthritis (RA) as an example of a complex, chronic disease, this research work studied the pharmacogenetics of Methotrexate (MTX) response (efficacy, toxicity) in Indian patients suffering from RA.

2. Aim
To study genetic polymorphisms in Indian population and effect of these polymorphisms on drug response (efficacy and toxicity) in diseased population.

3. Objectives

3.1 To study Phase I DME (CYP2C19, CYP2C9) gene polymorphisms in Maharashtrian population.

3.1.1. To compare the allele and genotype frequency of Maharashtrian population with other populations.

3.2 To study inter-individual variability in drug metabolism and its association with metabolically polymorphic Prakriti if any.

3.3 To study pharmacogenetics of Methotrexate (MTX) response (efficacy, toxicity) in Indian patients suffering from RA.

3.3.1. Single nucleotide polymorphisms (SNP) in genes coding for MTX metabolism in Indian RA patients

3.3.2. The association of gene polymorphism in MTX metabolic pathway with efficacy and toxicity related to MTX therapy in Indian RA patients
3.3.3. The effect of gene polymorphism on plasma levels of MTX and 70H-MTX metabolite at different time points (pharmacokinetics) in RA patients.

4. Literature Review

Detailed literature search was carried out for:

4.1. Pharmacogenetics
4.2. DMEs: Cytochrome P450 enzyme polymorphism
4.3. CYP2C19 and CYP2C9 (Phase I DME) gene polymorphism
4.4. Genetic basis to concept of Prakriti
4.5. Pharmacogenetics of RA
4.6. Pharmacogenetics of MTX in RA

A brief overview of DMEs: Cytochrome P450 enzyme polymorphism, CYP2C19 and CYP2C9 (Phase I DME) gene polymorphism, genetic basis to concept of Prakriti and pharmacogenetics of MTX in RA has been provided.

- **DMEs: Cytochrome P450 enzyme polymorphism**

Cytochrome P450 enzymes (CYP450) are the phase I enzymes that metabolize number of chemically diverse, endogenous and exogenous compounds, including drugs, environmental chemicals, and other xenobiotics. Genetic variation has been seen in the CYP450 enzymes [Goodman-Gilman, 2001]. Inter-individual variation in CYP expression can lead to marked variability in drug response, drug activity or detoxification and therefore it is important to understand the genetic factors that influence CYP levels and activities. CYP2D6, CYP2C9, CYP2C19 and CYP3A4 are studied widely because of their greater clinical importance.

- **CYP2C19 and CYP2C9 (Phase I DME) gene polymorphism**

CYP2C19, one of the members of cytochrome P-450 (CYP) super family enzymes is involved in metabolism of a number of clinically important drugs such as diazepam, certain barbiturates, tricyclic antidepressants, omeprazole and proguanil [Goldstein JA et al., 1994; Daly AK, 1995]. Among the twenty-five variant of CYP2C19, two principle alleles CYP2C19*2 (rs4244285) and CYP2C19*3 (rs4986893) have been reported with Poor Metabolizer (PM) phenotype in Caucasians and Asian populations [de Morais SMF et al., 1994]. Individuals with homozygous (*1/*1) or heterozygous (*1/*2, *1/*3) wild-type
CYP2C19*1 genotype have efficient enzyme to metabolize CYP2C19 substrates and are Extensive metabolizer (EM).

**CYP2C9:**

CYP2C9 metabolizes range of clinically significant drugs like tolbutamide, phenytoin, S-warfarin, losartan, cyclophosphamide and non steroidal anti inflammatory drugs (NSAIDS) most of which have narrow therapeutic indices. Thus enzyme activity of CYP2C9 is important determinant of clearance and response of a drug within an individual [Minors JO et al., 1998]. Currently 34 allelic variants for CYP2C9 polymorphism have been reported. CYP92C*1 is the most commonly found allele; while CYP2C9*2 and CYP2C9*3 are most common variant allele.

CYP2C19 and CYP2C9 gene polymorphism is widely studied in Caucasians, African and Oriental populations; however, far less is known about other ethnic groups such as Indians.

- **Genetic basis to concept of Prakriti**

Traditional Indian medicine - Ayurveda classifies the human population into three major constituents or Prakriti known as V, P and K types. Earlier, we have demonstrated a proof of concept that supports a genetic basis for Prakriti [Patwardhan B et al. 2005, 2006]. According to Ayurveda texts persons with Pitta Prakriti are fast metabolizers while those of Kapha Prakriti are slow metabolizers.

CYP2C19 gene polymorphism is chosen as case examples to study inter individual variability in drug metabolism. PM and EM condition is similar to metabolic variants in major Prakriti classes

The research question we posed was whether the difference in metabolic rate in Prakriti gets reflected in the differences in drug metabolism. We further hypothesized that different Prakriti may have different drug metabolism rates associated with Drug Metabolizing Enzyme (DME) polymorphism.

- **Pharmacogenetics of MTX in RA**

MTX is the most widely used disease-modifying antirheumatic drug (DMARD) for the treatment of RA. Although MTX is among the best-tolerated DMARDs, major drawbacks of MTX therapy are the large interpatient variability in clinical response and the unpredictable appearance of a large spectrum of side-effects [O'Dell JR, 2005]. Several studies have demonstrated polymorphism of the genes regulating enzymes in the intracellular MTX metabolic pathway [Ulrich CM et al., 2002]. Many of these gene polymorphisms are being linked to drug efficacy and safety but the evidence available is not yet conclusive
[Ranganathan P et al., 2006]. Pharmacogenetic testing [Dervieux T et al., 2005] may help to optimize therapy. Much data has emerged on MTX from the West. Little is known about incidence of SNPs in genes coding for MTX metabolism and its relevance to the MTX response in the Indian scenario.

5. Material and Methods:

5.1 Study Population:

5.1.1 139 unrelated healthy subjects of either sex, residing in Maharashtra (Pune) were recruited for the CYP2C19 and CYP2C9 genotyping study.

5.1.2 The same cohort was studied to explore genetic basis for concept of Prakriti. Prakriti of each subject was determined by a validated questionnaire. Each subject was clinically assessed by senior Ayurvedic physician independently classifying all the subjects in three groups of Vata, Pitta or Kapha.

5.1.3 Pharmacogenetics of MTX in RA:

- **Pilot study**
  1. Thirty six naïve patients completing at least 6 months of supervised MTX, willing and consenting, were selected from Centre for Rheumatic Diseases (CRD), Pune.

- **Study**
  1. A total of 336 naïve RA patients completing at least 1 year of supervised MTX, willing and consenting, selected (according to clinic attendance) from a busy community rheumatology referral clinic.
  2. Period of obtaining patient data and blood samples- May 2007 to January 2008 (36 weeks)

For both the studies:

3. Retrospective analysis of patient clinical and Lab data (CRD database); recall used in several instances to confirm data.

4. ACR (American College of Rheumatology) 20 improvement index response used to evaluate efficacy.

5. MTX toxicity/AE defined as one or combination of the nausea/ vomiting, abnormal LFT (> than twice the upper limit of normal values), aggravated skin...
nODULES, ORAL ULCERS, CYTOPENIA, ANY OTHER KNOWN MTX RELATED AE DOCUMENTED IN DRUG LITERATURE.

6. BOTH OVERALL AE AND INDIVIDUAL AE WERE ANALYZED.

7. 144 UNRELATED HEALTHY CONTROLS (HC) WERE INCLUDED.

THE STUDY PROTOCOLS FOR ALL THE ABOVE STUDIES WERE APPROVED BY THE INSTITUTIONAL ETHICS COMMITTEE AND WRITTEN INFORMED CONSENT WAS OBTAINED FROM ALL SUBJECTS PARTICIPATING IN THE STUDY.

5.2 DNA EXTRACTION:
About 5ml of venous blood was drawn from each subject in a vacutainer containing EDTA as an anticoagulant. DNA was extracted using Miller’s protocol [Miller SA et al., 1988].

5.3 GENOTYPE ANALYSIS:
Genotyping of extracted DNA for CYP2C19 (CYP2C19*1, CYP2C19*2, CYP2C19*3), CYP2C9 (CYP2C9*1, CYP2C9*2, CYP2C9*3), A1298C and C677T of MTHFR, 5’UTR repeat and 3’UTR deletion of TYMS, G80A of RFC1, A2756G of MS, C3435T and C1236T of MDR1, C401TGH of RFC1 and A66G MTRR was done using PCR- RFLP technique [Goldstein JA et al., 1996; Kumagai K et al., 2003; van Ede AE et al., 2001; Urano W et al., 2002]. While genotyping of SHMT1 and ATIC was performed using by Real-time Taqman allelic discrimination assay (Applied Biosystems) [Weisman MH et al., 2006].

5.4 PHARMACOKINETIC STUDY:
Pharmacokinetics of MTX, 7OH MTX (metabolite) and Homocystiene (Hcy) estimation was carried out in these RA patients.

1. 100 RA patients on supervised MTX with known genotypes, willing and consenting, were selected from the main study.

2. Subjects were given weekly dose of MTX (ranging from 3.75 mg-17.5mg) after an overnight fast.

3. 2-3 ml blood sample was collected at following time points:
   1st interval: 0 hr before MTX administration for baseline values
   2nd interval: at 2 hrs after MTX administration.
   3rd interval: at 8 hrs after MTX administration

4. Plasma samples & RBCs were separated within 30 min of collection & stored at -70 until analyzed.
5. Plasma MTX and its metabolite 7-OH MTX levels were determined using High Performance Liquid Chromatography (HPLC) with post column photoxidation-fluorescence detection.

6. Plasma Hcy was estimated at 0 hr in all these patients by HPLC.

5.5 Statistical analysis:
Odd’s ratio (OR) and χ2 at p< 0.05 was performed using the Graph Pad Prism statistical software (San Diego CA. USA). In case of CYP2C19 and CYP2C9 study differences in allele frequencies and Poor metabolizer genotype frequencies between Maharashtrians and population from various geographical regions were measured by Fisher exact test.

6. Results:
6.1 Genetic polymorphism of CYP2C19 and CYP2C9 in Maharashtrian population

**CYP2C19**

CYP2C19*1 (57.1%) was the most frequently identified allele in the Maharashtrian population (95% CI 0.602, 0.714). Forty-seven subjects (33.8%) were homozygous for wild type allele having CYP2C19*1/*1 genotype (95% CI, 0.258, 0.419) and 65 subjects (46.8%) were heterozygous with CYP2C19*1/*2 or CYP2C19*1/*3 genotype. Out of the two mutant alleles CYP2C19*2 (41.7%) was the most frequently observed mutant allele (95% CI, 0.359, 0.476). Twenty-six subjects (18.7%) were homozygous poor metabolizer (PM) having CYP2C19*2/*2 genotype (95% CI, 0.121, 0.253). CYP2C19*3 allele was found in three subjects (1.2%) (95% CI, 0.062, 0.132), of which two (1.4%) were heterozygous for CYP2C19*1 and one (0.7%) for CYP2C19*2. CYP2C19*3 homozygous mutant was absent in this population. The observed frequencies of CYP2C19 genotypes in Maharashtrian population were found to be in Hardy-Weinberg equilibrium.

**CYP2C9**

One hundred and five (87.5%) subjects were homozygous for wild type allele carrying CYP2C9*1/*1 genotype. Seven subjects (5%) carried CYP2C9*1/*2 genotype, while twenty one (15.1%) carried CYP2C9*1/*3 genotype. CYP2C9*1 (87.5%) was the most frequently identified allele in the Maharashtrian population. Amongst the two mutant alleles CYP2C9*3 allele was observed with higher frequency (9.6%) than CYP2C9*2 allele (1.1%). Out of the total population three subjects (2.2%) carried PM genotype of which two were homozygous for CYP2C9* 3 allele, while one was heterozygous for CYP2C9*2. Homozygous for
CYP2C9*2 allele was absent in the population. The observed frequencies of CYP2C9 genotypes in Maharashtrian population were found to be in Hardy-Weinberg equilibrium.

6.2 Genetic basis for concept of Prakriti
One hundred and thirty two subjects were classified in three major categories of Prakriti: 63 (47.7%) of K, 43 (32.6%) of P and 26 subjects (19.7%) of V. The genotypic repertoire in this population represents predominance of CYP2C19 *1/*2 genotype as compared to other genotypes. This genotype was well distributed in all the three dominant Prakriti types. Extensive Metabolizer (EM) genotype (*1/*1, *1/*2, *1/*3) was found to be predominant in P (91%). *1/*3 genotype specific for extensive metabolizer group was present only in P with \( \chi^2 = 4.2, p \text{ value} = 0.04 \) giving an Odds ratio (similar to Relative Risk) of 10.8. PM genotype (*2/*2, *2/*3, *3/*3) was highest (31%) in K when compared with V (12%) and P (9%). CYP2C19 *2/*2 genotype frequency was significantly higher with \( \chi^2 = 7.28, p \text{ value} = 0.007 \) in K type as compared to V + P types yielding an Odds Ratio of 3.5. *2/*3 genotype typical for poor metabolizer group was observed only in K Prakriti. V Prakriti did not show any significant association with any of the genotypes. Despite small sample size our results indicate a reasonable correlation between CYP2C19 genotype and Prakriti types. Some of the genotypes are common to two or more Prakriti classes.

6.3 Pharmacogenetics of MTX response (efficacy, toxicity) in Indian patients suffering from RA.

6.3.1 Pilot Study
The MTHFR A1298C ‘C’ allele incidence among RA patients (46%) was significantly higher. The C allele was associated with RA (\( \chi^2 = 4.24, P < 0.05, OR = 1.68 \)). None of the other allele tested showed any association. We also could not find any association between ACR 20 response and AE and tested alleles.

6.3.2 SNPs in genes coding for MTX metabolism in Indian RA patients.
MTHFR A1298C CC genotype (OR 7.6, CI 3.6-16.1, p=0.0001), RFC1 G80A GG (OR 1.6, CI 1.0-2.5, p=0.02) and GA (OR 1.8, CI 1.2-2.7, p=0.004) genotype distribution among RA patients was significantly higher from that of HC population.

Occurrence of MTHFR A1298C AA genotype (p<0.0001), TS 3 UTR 6bp/6bp (p=0.01), RFC1 G80A AA (p<0.0001), MS A2756G AG (p=0.003) and SHMT1 C1420T CT genotype (p=0.04) was significantly lower among RA patients when compared with HC population.
No association of MTHFR C677T, TYMS 5 UTR, MDR1 C3435T and C1236T, GGH C401T, MTRR A66G, MDR1 and ATIC C347G genotypes with RA.

6.3.3 The Association of Gene Polymorphism in MTX metabolic pathway with toxicity related to MTX therapy in RA.
Among 322 patients, 180 (56%) patients had AE. *TS 3 UTR 6bp/6bp* (OR 2.7, CI 1.5-4.9, p=0.001), *GGH-401TT* (OR 1.8, CI 1.1-2.8, p=0.01) and *SHMT1-1420 CT* (OR 1.8, CI 1.0-2.9, p=0.03) were associated with overall toxicity. While *TS 5 UTR 3R/3R* (OR 0.6, CI 0.4-0.9, p=0.02), *TS 3 UTR 0bp/0bp* (OR 0.6, CI 0.3-0.9, p=0.02), *MS-2756 AA* (OR 0.6, CI 0.4-0.9, p=0.03), *ATIC-347 GG* (OR 0.5, CI 0.4-0.9, p=0.03) reduces the risk of overall toxicity. *MS-2756 AG* (OR 1.7, CI 1.0-2.9, p=0.02), *MTRR-66 AA* (OR 1.8, CI 1.0-3.0, p=0.04) and *SHMT1-1420 CC* (OR 3.9, CI 1.0-16.9, p=0.04) are risk genotypes associated with gastrointestinal toxicity (n=95). *GGH-401 TT* (OR 1.8, CI 1.0-1.3, p=0.01) and *MDR1-1236 CT* (OR 2.0, CI 1.0-3.0, p=0.006) are risk genotypes for hepatic toxicity. *TS5UTR-5R/2R* (OR 11.8, CI 1.0-138.4, p=0.01) associated with alopecia.

6.3.4 Pharmacokinetics of MTX, 7OH MTX (metabolite) and Homocystiene (Hcy) estimation in RA patients.
Statistical analysis of the data generated using HPLC and genotyping to examine association if any with the following parameters is ongoing
- Genetic polymorphism and MTX efficacy
- AE & MTX dosage
- MTX, 7 OH MTX plasma levels & AE
- MTX, 7 OH MTX plasma levels & MTX dosage
- Hcy levels in different MTX dosage
- Hcy levels & MTHFR genotypes

7. Conclusions:
7.1 Genetic polymorphism of CYP2C19 and CYP2C9 in Maharashtrian population
The study takes a systematic review of the population distribution of CYP2C19 and CYP2C 9 allele and genotypes in various populations residing in different geographic areas. Meta-analysis of the studies in various populations confirms [Ghadke Y et al., 2007]

1. The existence of interethnic differences in *CYP2C19* allele/genotype frequencies and *CYP2C9*2 mutant allele.
2. Significant heterogeneity exists among Indian subpopulations in occurrence of CYP2C19 allele/genotype but not for CYP2C9 allele/genotype.

3. 19.4% PM genotype makes it essential to evaluate the effect of various CYP2C19 substrates in Maharashtrians.

4. No significant differences observed in the occurrence of CYP2C9*3 variant allele & PM genotype of CYP2C9 in Maharashtrians when compared with the other populations.

5. Further studies to assess the clinical significance of genotypic differences is needed.

6. It is of potential clinical importance to be able to identify individuals who have altered pharmacokinetics for CYP2C19/ CYP2C9 substrates so that appropriate dosage strategies for these drugs can be adopted, and adverse drug reactions can be avoided.

7.2 Genetic basis for concept of Prakriti

Our study demonstrated:

1. CYP2C19 gene polymorphisms do associate with major Prakriti phenotypes.

2. Although a small sample size; our study for the first time demonstrates genomic basis for metabolic differences attributed by Prakriti in humans.

3. The results allows us to predict that Kapha and Pitta being slow and fast metabolizer groups respectively are likely to require low and high doses of CYP2C19 substrates respectively.

4. Further studies are required to confirm Prakriti Pharmacogenomics relationship in terms of genotype, Prakriti and drug metabolism.

5. An integration of Prakriti concept of Ayurveda with genomics nomenclatured as Ayugenomics is worth exploring for future customized medicine.

7.3 Pharmacogenetics of MTX response (efficacy, toxicity) in Indian patients suffering from RA.

7.3.1 Pilot study data revealed [Ghodke Y et al., 2007 and 2008]

1. MTHFR A1298C ‘C’ allele (46%) and CC genotype (27%) incidence among RA patients was significantly higher than in HC (p<0.05).

2. Though a small sample study, our findings do not suggest a significant association of MTHFR and TS allele & genotype with MTX response in our ethnically distinct Indian (Asian) RA patients.

7.3.2 SNPs in genes coding for MTX metabolism [Ghodke Y et al., 2008]
1. Significant associations in MTX metabolism genes is observed in our cohort of RA.
2. These associations can be further explored to examine MTX response (efficacy & toxicity).

7.3.3 Association of SNPs in genes coding for MTX metabolism with toxicity related to MTX therapy in RA [Ghodke Y et al., 2008]

1. Genes regulating MTX metabolism do contribute to toxicity in Indian RA patients.
2. MS-2756 AA, ATIC-347 GG are reported as risk associations in previous studies however; our study these genotypes illustrates reduced risk of AE.
3. TS 3 UTR 6bp/6bp, GGH-401 TT, SHMT1-1420 CT are newer risk associations that are not reported earlier.
4. Ongoing MTX pharmacokinetic analysis will help to confirm associations reported in the present study.

By using genetic polymorphism study we demonstrate that the study population is distinct from Caucasians, Africans and some of the Asian populations and significant heterogeneity exists among Indian subpopulations as far as drug metabolizing enzymes are considered. Further we also evaluate the role of gene polymorphisms in efficacy and toxicity of Methotrexate – a drug used to treat patients with rheumatoid arthritis. Significant associations in genes coding MTX metabolism is observed in our cohort of RA. Genes regulating MTX metabolism contribute to toxicity in Indian RA patients. Association between genotype and pharmacokinetics in these patients will help us in understanding MTX response in RA. This in turn will help to predict the toxicity and increase the efficacy of MTX in clinical practice.

We observed interesting correlations between CYP2C19 genotypes and Prakriti with fast and slow metabolism being one of the major distinguishing and differentiating characteristics. These observations are likely to have significant impact on phenotype – genotype correlation, drug discovery, pharmacogenomics and personalized medicine.
References

29. Urano W, Tanaguchi A et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002; 12(3): 183-90.