CHAPTER 5

GENOTYPE AND PHENOTYPE CORRELATION OF CYTOCHROME P450 2C9 POLYMORPHISM IN NATIONAL CAPITAL REGION DELHI POPULATION
Chapter 5

Genotype Phenotype Correlation of Cytochrome P450 2C9 in Healthy Human Volunteers Residing in Indian National Capital Region Delhi

5.1 Introduction

Cytochrome P450 2C9 (CYP2C9) is a clinically significant enzyme that demonstrates multiple genetic variants with a potentially functional impact on the efficacy and adverse effects of several drugs including phenytoin, tolbutamide, losartan, and number of anti-inflammatory drugs such as ibuprofen, diclofenac, mefenamic and flubiprofen (Harumi, 2001; Zhou et al., 2009; Yoon et al., 2001; Goldstein, 2001). Polymorphism of CYP2C9 has been reported with number of putative poor metabolizer (PM) alleles of CYP2C9 found in different racial groups. Among the 30 variant alleles of CYP2C9 that have been characterized till date (Bozina et al., 2003; Joyce et al., 2004), CYP2C9*2 and CYP2C9*3 are the most common alleles. These are the two common defective alleles studied both in vitro and in clinical studies (Crespi and Miller, 1997; Sullivan et al., 1996; Lee et al., 2002). The CYP2C9*3 allele has lower affinity and markedly lower intrinsic clearance for numerous drugs. The CYP2C9*2 allele is more moderately defective. CYP2C9*2 mutation results in an Arg144Cys amino acid substitution, which alters the interaction between cytochrome P450 and NADPH: Cytochrome P450 oxido-reductase (Crespi and Miller, 1997). CYP2C9*3 mutation, on the other hand, results in Ile359Leu amino acid substitution in the substrate-binding site. Such single nucleotide polymorphism (SNP) in CYP2C9 results in reduced enzyme activity as compared to wild type allele (CYP2C9*1) (Aithal et al., 1999).

Racial and ethnic differences in both the frequency and types of polymorphisms of CYP2C9 gene are common (Xie et al., 2001; Kidd et al., 2001; Xie et al., 2002). The
identification of polymorphisms in different racial and ethnic populations is vital for understanding differences in clinical responses to drugs (Johansson and Ingelman-Sundberg, 2011). Ethnic or racial inter-individual $\text{CYP2C9}$ polymorphism have been reported in Caucasians (Lee et al., 2002), Japanese (Miyuki et al., 1998), African-Americans (Xie et al., 2001; Kidd et al., 2001), Koreans (Yoon et al., 2001), Spanish (Dorado et al., 2003), Bolivian (Bravo et al., 2005), Egyptian (Hamdy et al., 2002) East Asian (Xie et al., 2002), Turkish (Aynacioglu et al., 1999), Russian (Gaikovitch et al., 2003), French (Yang et al., 2003), Belgian (Allabi et al., 2003), Brazilian (Vianna Jorge et al., 2004), Mexican-American (Lerena et al., 2004), Vietnamese (Lee et al., 2005), Chinese (Hong et al., 2005), Saudi Arabians (Mirghani et al., 2011), Chinese and Malay residing in Singapore (Ngow et al., 2009), and South Indian (Ramasamy et al., 2007; Jose et al., 2005) populations. However, the $\text{CYP2C9}$ polymorphism has not been studied in NCR Delhi. In this chapter, we studied the genetic polymorphism of $\text{CYP2C9}$ gene in NCR Delhi using losartan as probe drug. Drugs including losartan (Yasar et al., 2002b; Yasar et al., 2002c) and diclofenac (Yasar et al., 2001) have earlier been used as probe drug for phenotyping $\text{CYP2C9}$. Losartan is particularly used for good correlation of its oxidation with different genotypes, easily availability and safety. We performed the genotyping of the most common $\text{CYP2C9}$ variants, $\text{CYP2C9}^*2$ and $\text{CYP2C9}^*3$, and correlate it with the phenotype. In the presence of correlation, established by the identification of known SNPs, an individual’s status as rapid or poor metabolizers can be ascertained by genotyping alone, which is a relatively easier technique as compared to the more elaborate process of phenotype profiling. In the absence of correlation between the two, phenotyping would be mandatory to accurately identify the drug metabolizing status.
5.2 Materials and methods

5.2.1 Clinical study

The study protocol and corresponding informed consent form (ICF) were reviewed by the Institutional Review Board. Subjects were informed before initiation of the study through an oral presentation regarding the purpose, procedures to be carried out, potential hazards and rights of the subjects. Subjects (170) were selected randomly from the volunteer bank of clinical pharmacology unit of Ranbaxy Laboratories Limited. The volunteer bank comprised of healthy volunteers from NCR, which encompasses the entire national capital territory of Delhi as well as urban areas of neighboring states of Haryana, Uttar Pradesh and Rajasthan. Volunteers were recruited on the basis of inclusion and exclusion criteria after obtaining written informed consent. Medical histories and demographic data were recorded (described in chapter 4). Each subject underwent physical examination and laboratory tests of hematology, hepatic and renal function. Hematological parameters were analyzed on a fully automated five-part differential count auto analyzer (Sysmex XT 1800xi), and biochemical parameters (plasma glucose, blood urea nitrogen, serum creatinine, serum total bilirubin, serum alkaline phosphatase, serum alanine and aspartate aminotransferases, serum cholesterol, and urine drug of abuse) were analyzed on fully automated biochemistry analyzer (Siemens Diagnostics USA). Urinalysis (routine and microscopic examination) was done by manual dipstick method (Multistick from Siemens Diagnostics). Subjects were housed in the clinical pharmacology unit of Ranbaxy Laboratories Limited, Delhi. Losartan (COVANCE 25 mg tablet manufactured by STANCARE India) was administered orally to 107 volunteers along with 240 ml of water under the supervision of a trained Medical Officer. The blood (6 ml in the presence of EDTA) was collected at 0, 1, 3, 6, and 10 hour after drug administration. Number of volunteers was calculated statistically based on the prevalence of percentage poor metabolizers present worldwide. The sample size of 100 volunteers was calculated statistically. Based on the simulation with
higher sample size (200 or 300 subjects), it was noted that there was not much benefit in precision. Hence the study was done taken into account at least 100 subjects.

5.2.2 Phenotyping using losartan as probe drug

5.2.2.1 Estimation of losartan and losartan carboxylic acid

The activity of CYP2C9 was assessed by the ratio of losartan and its metabolite losartan carboxylic acid in the blood plasma by LC-MS/MS method on Waters Quattro premier mass spectrometer. Samples from each subject were analyzed using a set of calibration standards spiked in human plasma. Three levels of quality control samples were distributed through each batch of study samples assayed to monitor the performance of testing. Experiments were carried out using C18 reverse phase column (Supelco Discovery C18 (5cm) purchased from Sigma Aldrich). The mobile phase consisted of acetonitrile, ammonium acetate (2mM), and formic acid (80:20:0.05). Solid phase extraction method with HLB Oasis cartridges (purchased from Waters, Ireland), methanol as conditioning solvent and ethanol as elution solvent were selected. Acetonitrile was purchased from Spectrochem Pvt. Ltd. (Mumbai, India), and methanol from Qualigens fine chemicals (A division of GSK Ltd, Mumbai, India). Human plasma was collected in-house in the presence of ethylenediaminetetraacetate (EDTA) as anticoagulant. Plasma was free from HIV and hepatitis. All other solvents and reagents of analytical grade were purchased from S.d. fine chem. Ltd (Mumbai, India). Irbesartan was used as an internal standard. It was procured from Ranbaxy, Toansa India. Six replicates of aqueous dilutions of losartan, irbesartan and carboxylosartan were injected and their peak response ratios were recorded to check the interference or specificity. Long-term stability of analytes was evaluated using low and high QC samples stored below \(-50\)°C in deep freezer for a period of 91 days. Six replicates of low and high quality control samples were used for each stability exercise. Stored QC samples were analyzed against freshly spiked calibration curve. The calibration range for losartan (6.81-1063.35 ng/ml) and losartan carboxylic acid (7.24-1131.62 ng/ml) were selected.
Losartan potassium was procured from IPCA Laboratory Limited, India, and losartan carboxylic acid from analytical division of Ranbaxy, India.

5.2.2.2 Phenotyping data analysis

The population was categorized as poor and rapid metabolizers for the group of drugs metabolized by CYP2C9, on the basis of the assessment of enzymatic activity. The enzyme activity was measured by evaluating the ratio of losartan and losartan carboxylic acid. Concentration of losartan and losartan carboxylic acid was initially quantified from the samples drawn at pre dose, 1 hour post dose, 3 hour post dose, 6 hour post dose and 10 hour post dose for 10 volunteers. Based upon the concentration of parent drug at the above mentioned time points, $T_{max}$ (time to reach the drug at the highest concentration) of 3 hour post dose was selected for evaluating the activity of CYP2C9. Frequency histogram was constructed with log (drug/metabolite) versus number of volunteers. The presence of different categories of individuals indicated in frequency histogram is different from the normal distribution. On visual inspection of frequency histogram, approximate antimode position is established as the point on graph where two different modes are separated. However the exact antimode is derived by probit plot analysis. This is a graphical method in which the standard deviates of a normal distribution are plotted against the log drug/metabolite ratio. Deviations from linearity in probit plots have been interpreted as existence of a polymorphism. Scatter type chart was prepared with log (drug/metabolite) ratio on x-scale and probit on y-scale. Trendlines were added to the plot to get the best linear fit. Based on the selected trendline, a polynomial equation of regression is obtained. Intercept at X is the antimode. The individuals having log drug/metabolite ratio more than the antimode were classified as poor metabolizers. The mean of the ratios (drug/metabolite) of poor and rapid metabolizers were analyzed by student $t$-test to evaluate the significance of difference of mean drug/metabolite ratio between poor and rapid metabolizers.
5.2.3 Genotyping

5.2.3.1 Identification of known SNP

After the completion of phenotyping, phenotyped volunteers (poor or rapid metabolizers) were contacted for genotyping. Only 37 volunteers agreed for genotype evaluation. Among the 37 volunteers, 9 were poor and 28 rapid metabolizers. The blood (2 ml each) was collected in K3 EDTA vacutainer. DNA was extracted by salting-out method. After isolation, 1.0μl of extracted DNA was loaded on a 0.8% Agarose gel along with 1Kb DNA ladder. Picogreen estimation is done for further determination of concentration, yield and purity. DNA standards (50 pg/μl – 800 pg/μl) were prepared as per the details provided in Chapter 3. Electrophoresis of isolated DNA is shown in the Figure 5.1. Single base primer extension technology was used by iPLEX procedure. With this technology, a single base mutation can be identified. The portion or site on the DNA where single nucleotide polymorphism is present was identified. CYP2C9*2 and CYP2C9*3 variants were identified in the study population. These variants involved following SNP, rs1799853 (430C>T) and rs1057910 (1075A>C). The detail of the primers used for amplification of the site on the DNA where single nucleotide polymorphism was present is provided in Chapter 3.

After the PCR, the PCR product was treated with the shrimp alkaline phosphatase (SAP) treatment to de-phosphorylate unincorporated dNTPs. The SAP treated product undergoes single base extension reaction with specific primers designed corresponding to the base which is mutated. Followed by iPLEX reaction, resin treatment was given to desalt the iPLEX reaction products. The resin treated extended product was then spotted onto a spectrochip using nanodispenser and the chip was then used to acquire data from the Sequenom’s caller software. The data was retrieved in the form of .xml file and analyzed using the Mass Array Typer software.
Figure 5.1 Gel electrophoresis showing isolated DNA. M indicates DNA ladder and the bands on line a) and b) are genomic DNA of human volunteers labeled as S1-S36. Lanes S1-S36 shows intact genomic DNA band above the 1 kb DNA ladder which indicates good Genomic DNA.
5.2.3.2 Interpretation of genotyping data

The presence of any one of the CYP2C9 variant- CYP2C9*2 and CYP2C9*3 is an indication of poor metabolizer phenotype. CYP2C9*2 was identified as a base change from C (normal) to T (mutant) at position 430 on the gene and the variant CYP2C9*3 was identified as a base change from A (normal) to C (mutant) at position 1075 on the gene coding for CYP2C9. The numbers of genotype data of rapid or poor metabolizers were correlated with phenotype data.

5.3 Results

5.3.1 Clinical study and phenotyping

None of the volunteers reported any undesirable effect or adverse reaction during the study. The LC-MS/MS method developed for the simultaneous estimation of losartan, losartan carboxylic acid and irbesartan had no interference observed in the retention time of any of the above analytes. The retention time of 1.08, 1.13, 0.88 minutes for losartan, losartan carboxylic acid and irbesartan, respectively, was tuned. The sensitivity of the estimation of losartan and losartan carboxylic acid was 95.49% and 91.8%, respectively. The estimation procedure was specific as no interfering peak was observed in six different batches of biological matrix. The coefficient of correlation of linear regression (r) was 0.9997 for losartan and 0.9986 for losartan carboxylic acid calibration. Percent accuracy of the calibrators and quality control samples were between 85-115%. The precision for the estimation of losartan was estimated as %CV (coefficient of variation). It was between 4.5 and 8.28% for all three levels of quality control samples, and was well within FDA defined acceptable limit <15%. This confirms the suitability of the method for the estimation of losartan and its metabolite losartan carboxylic acid from the plasma sample. The probit plot analysis and frequency histogram analysis described the bimodality of the population studied with respect to log (drug/metabolite) ratio (Figure 5.2 and 5.3). Regression analysis done on the probit plot yielded a best linear fit at $R^2 = 0.991$. The trendline equation $y = -4.451 x^4 + 13.7 x^3 - \ldots$
14.60 x^2 + 6.832 x 1.229) was obtained. On solving the equation, intercept at x-axis, which is actually an antimode was found to be 0.73 [log (losartan/losartan carboxylic acid)]. The individuals having log ratio of losartan/losartan carboxylic acid more than 0.73 were categorized as poor metabolizers. Based on the antimode value, 14.28% of population was categorized as poor metabolizers for the category of drugs metabolized by CYP2C9. Significant difference was observed between the mean ratio of drug/metabolite of poor metabolizers (Mean: 11.38) and rapid metabolizers (Mean: 1.18) with p = 0.00032 using student t-test (Table 5.1).

5.3.2 Genotyping

Following genotypes were obtained CYP2C9*1/*1, CYP2C9*1/*2, CYP2C9*2/*2 on genotyping the study population (Table 5.2). CYP2C9*3 allele was not found in the study group. CYP2C9*2 mutant was observed in homozygous and heterozygous form based on which the individuals were categorized as rapid, intermediate and poor metabolizers. The rapid metabolizers identified by phenotyping technique correlated 100% with the rapid metabolizers obtained by genotyping technique. Three out of 9 poor metabolizers (by phenotyping) were found to be heterozygous mutants CYP2C9*1/*2). Six of the nine poor metabolizers were homozygous mutants having genotype (CYP2C9*2/*2).

5.4 Discussion

The present study elucidates the genetic polymorphism of CYP2C9 in NCR population group. Most of the current literature related to pharmacogenetics of CYP2C9 has studied polymorphisms in various populations (Yoon et al., 2001; Lee et al., 2002; Xie et al., 2001; Xie et al., 2002; Dorado et al., 2003; Aynacioglu et al., 1999; Gaikovitch et al., 2003; Yang et al., 2003; Allabi et al., 2003; Vianna et al., 2004; Hong et al., 2005; Jose et al., 2005). However, phenotyping data for the North Indian population, which is distinct from the South Indian population, is scanty. The present study is probably the first attempt to establish the phenotype-genotype correlation of CYP2C9 polymorphism in
Losartan used in the present study for the phenotyping of CYP2C9 is safe, suitable and tolerable, and we observed no adverse event during the entire clinical trial, which is in agreement with earlier reported trial (Yasar et al., 2002). Numbers of studies were done on phenotyping of CYP2C9 using losartan as probe drug; however information on the cutoff to distinguish poor metabolizers from rapid metabolizers is scanty. Recently Liu et al., 2012 distinguished poor, intermediate and rapid metabolizer based on a cutoff, as ratio of Area under curve (AUC) losartan and losartan carboxylic acid while in our study a cut off is defined as ratio of log (losartan and carboxylic acid) hence the cut off value cannot be compared. However metabolic ratio (MR) of losartan and losartan carboxylic acid for rapid and poor metabolizers of our study is comparable to earlier reported values. Mean metabolic ratio of poor metabolizers of our study was 11.38 that is comparable to earlier reported metabolic ratio (MR) [4.0-12.8 (Michaud et al., 2004); 12.4 (Dorado et al., 2012); 4.2-188 (Allabi et al., 2004); 3.4-200 (Sandberg et al., 2004)]. Similarly, MR for rapid metabolizers is comparable to earlier reported values (0.71-4.0) (Allabi et al., 2004; Sandberg et al., 2004, Michaud et al., 2004). Based on the cut off, 14.28% of the study population was found to be poor metabolizer, which is higher than reported in African-American (3.5%; Kidd et al., 2001), East Asian (1.6%; Xie et al., 2002), Chinese and Malays (7.2%, 7.7%; Ngow et al., 2009), Japanese (1.1%; Miyuki et al., 1998), and Korean (1.1%; Yoon et al., 2001) population. However, prevalence of poor metabolizers in this population was lower than Caucasians (19%; Lee et al., 2002); Spanish (26%; Dorado et al., 2003), Turkish (20.6%; Aynacioglu et al., 1999), Russian (17.2%; Gaikovitch et al., 2003), French (23%; Yang et al., 2003), Belgian (17.4%; Allabi et al., 2003), and Brazilian (15.1%; Vianna et al., 2004). The prevalence of poor metabolizers in our study group was comparable to South Indians (12%; Ramasamy et al., 2007; Jose et al., 2005).

The fact that 14.28% of the study population belonging to INCR is poor metabolizer for the categories of drugs metabolized by CYP2C9 make it necessary to identify the status of individuals for clinical management of drug dose for any long duration therapy or in clinical trials to prevent adverse reactions of the drug (Johansson
et al., 2011; He et al., 2011). CYP2C9 is an important drug-metabolizing enzyme that catalyzes the biotransformation of many clinically useful drugs. Of special interest are those drugs with narrow therapeutic index, such as S-warfarin, tolbutamide and phenytoin, where impairment in CYP2C9 metabolic activity might cause difficulties in dose adjustment as well as toxicity. The utility of genotyping for gene variants, $CYP2C9^*2$ and $CYP2C9^*3$ alleles, was recommended to alter clinical management in patients commencing warfarin, who are at a greater risk of bleeding (Higashi et al., 2002). Similarly, phenytoin, a commonly used drug for the treatment of epileptic patient has a narrow therapeutic index and genotyping of CYP2C9 is suggested to lower the risk of toxicity in case of mutants (Van der et al., 2001). The significance of genotyping of CYP2C9 is also recommended in patients with diabetes mellitus for deciding the dose of tolbutamide to regulate blood glucose level (Becker et al., 2008). Significant association of $CYP2C9$ polymorphism with head and neck squamous cell carcinoma has been reported in a study that underlined the importance of pretherapeutic genotyping of $CYP2C9$ in determining the treatment schedule (Paul et al., 2011).

In this study, 37 volunteers were genotyped for the identification of single nucleotide polymorphism ($430C>T$); ($1075A>C$) for the identification of $CYP2C9^*2$ and $CYP2C9^*3$ variants. In the study population, the poor metabolizers phenotype were due to the presence of $CYP2C9^*2$ variants. $CYP2C9^*3$ variant was not observed in the current population, while it has been reported to be 8% in South Indian population. This could be due to a relatively small sample size in our study group. The rapid metabolizers identified by phenotyping technique using losartan as probe drug were 100% correlated with the rapid metabolizers identified by the genotyping technique. However 3 of the 9 poor metabolizers (by phenotyping technique) were found to be heterozygous mutants $CYP2C9^*1/*2$. Six out of nine poor metabolizers were homozygous mutants having genotype $CYP2C9^*2/*2$. The genotype-phenotype correlation has been earlier reported in other populations with various probe drugs including losartan, tolbutamide, warfarin and phenytoin (Sullivan Klose et al., 1996; Yasar et al., 2002b, 2002c; Ramasamy et al.,
2010; Vander et al., 2001). However, studies have been reported where genotype-phenotype correlation was not observed, and hence the usefulness of genotyping was opposed in the management of phenytoin dose regimen in the treatment of epileptic patients (Tauchchi et al., 2005). Similarly no genotype-phenotype correlation was observed using diclofenac as probe drug in various genotypes. However, same study subjects’ genotype and phenotype were well correlated using losartan as probe drug (Yasar et al., 2001). Losartan used to establish genotype-phenotype correlation was given low significance in a comparative study done for three probe drugs, losartan, tolbutamide and flurbiprofen (Lee et al., 2003). However, the usefulness of losartan as probe drug was well established in a number of studies and a good genotype-phenotype correlation has been observed (Yasar et al., 2002b, 2002c; Bae et al., 2011). This study also demonstrates a good genotype-phenotype correlation using losartan as a probe drug, and proposes that CYP2C9 phenotyping is desirable before the enrollment of INCR population for clinical trials or for deciding drug dose regimen as 14.28% of the study population was poor metabolizer for the categories of drugs metabolized by CYP2C9 enzyme.

5.5 Conclusion

Results of this study establish the phenotype-genotype correlation of CYP2C9 and the study concludes that genotyping or phenotyping may be used to evaluate the status of drug metabolizing capacity of CYP2C9 as a primary screening procedure before enrolling subjects for clinical trials or in clinical practice. Genotyping, being a relatively easier technique as compared to elaborate process of phenotyping, is proposed to evaluate the status of drug metabolizing capacity of CYP2C9.
Probit plot of Log (Losartan/losartan carboxylic acid) vs probit

\[ y = -4.4519x^4 + 13.703x^3 - 14.609x^2 + 6.8325x + 1.2291 \]

\[ R^2 = 0.9918 \]

Figure 5.2 Probit plot of log (losartan/losartan carboxylic acid) vs probit
Figure 5.3 Frequency histogram plotted as log ratio of losartan/ losartan carboxylic acid vs. number of individual. Arrow indicating an antimode at 0.73.
Table 5.1 Evaluation of mean ratio of poor and rapid metabolizers of CYP2C9 enzyme using t-test

<table>
<thead>
<tr>
<th>Variables</th>
<th>Rapid metabolizers</th>
<th>Poor metabolizers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.1801304</td>
<td>11.38129</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.116</td>
<td>5.988</td>
</tr>
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<td>Variance</td>
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<td>39.84903</td>
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<td></td>
</tr>
<tr>
<td>Degree of freedom</td>
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<td></td>
</tr>
<tr>
<td>t Statistics</td>
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<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
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<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.8331129</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference in mean ratio of losartan and losartan carboxylic acid for rapid and poor metabolizers was found using t-test.
Table 5.2 Categorization of individuals as rapid, intermediate and poor metabolizer on the basis of \textit{CYP2C9}\textsuperscript{*2} and \textit{CYP2C9}\textsuperscript{*3} mutant genotypes

<table>
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<tr>
<th>Subjects</th>
<th>Genotype</th>
<th>Genotype</th>
<th>Genotype</th>
<th>Phenotype based on genotyping</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CYP2C9\textsuperscript{*2} rs1799853 (430C&gt;T)</td>
<td>CYP2C9\textsuperscript{*3} rs1057910 (1075A&gt;C)</td>
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<tr>
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<tr>
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<td>TT</td>
<td>AA</td>
<td>CYP2C9\textsuperscript{*2/*2}</td>
<td>Poor</td>
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