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ROLE OF CYTOCHROME P450 2C8 AND 2J2 GENOTYPE IN CALCINEURIN INHIBITOR INDUCED CHRONIC KIDNEY DISEASE

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Abstract

Objectives—The calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus (Tac) help prevent allograft rejection but are associated with nephrotoxicity. Cytochrome P450 2C8 (CYP2C8) and CYP2J2 are polymorphic enzymes expressed in the kidney that metabolize arachidonic acid (AA) to epoxyeicosatrienoic acids, promoting kidney homeostasis. This study examined the association between CNI-induced nephrotoxicity in liver transplant patients and *CYP2C8 and CYP2J2* polymorphisms.

Methods—Liver transplantation patients receiving CNIs for at least three years were genotyped for *CYP2C8*3, CYP2C8*4, CYP2C8 Haplotypes B and C*, and *CYP2J2*7* and evaluated for nephrotoxicity (serum creatinine ≥ 1.6 mg/dL) three-years post-transplantation. CYP2C8 proteins were also engineered in *E*. coli and their activity towards AA and inhibition by CNIs was investigated *in vitro*.

Results—The risk of kidney dysfunction post-transplantation was positively associated with *CYP2C8*3* genotype. Odds ratios for all subjects carrying at least one *CYP2C8*3* allele were significant (OR= 2.38 (1.19 – 4.78)). Stratification by CNI indicated a significant association between *CYP2C8*3* and nephrotoxicity among patients receiving Tac but not CsA. The risk of renal dysfunction was not significantly influenced by *CYP2C8*4*, *CYP2J2*7*, or *CYP2C8* haplotype B genotype although inheritance of haplotype C seems to be protective. *In vitro*, the gene products of *CYP2C8*3* and *CYP2C8*4* were deficient in AA epoxidation, retaining 26 and 18% of wild type activity respectively. *In vivo* plasma concentrations of CsA and Tac inhibited CYP2C8 wild type *in vitro* activity by 17% and 35%, respectively.

Conclusions—Inheritance of *CYP2C8*3* is associated with a higher risk of developing renal toxicity in patients treated chronically with CNIs, and especially Tac, possibly by reducing formation of kidney protecting vasodilatory epoxyeicosatrienoic acids.

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Keywords

Arachidonic Acid; Calcineurin Inhibitor; Cyclosporine A; *CYP2C8*; *CYP2J2*; Liver Transplantation; Nephrotoxicity; Polymorphism; Tacrolimus

INTRODUCTION

The calcineurin inhibitors (CNIs), cyclosporine A (CsA) and tacrolimus (Tac), are commonly used to prevent allograft rejection in solid organ transplantation recipients. Both drugs suppress the activation of calcineurin and block T-lymphocyte proliferation through down regulation of interleukin-2 [1]. In a large retrospective review of liver transplantation recipients throughout the US, patient survival rates (censored for graft loss) three years post-transplantation were 88.9% for patients receiving Tac and 87.8% for patients receiving CsA [2]. Unfortunately, despite this remarkable clinical success, both Tac and CsA have been associated with a variety of toxic effects including nephrotoxicity [3-5]. Importantly, CNI therapeutic blood concentration monitoring does not prevent a large number of patients (30-40%) from eventually developing chronic kidney disease and many patients ultimately exhibit chronic kidney disease Stage V [6].

CNIs cause both acute and chronic nephrotoxicity. The acute toxicity is manifested by reduced renal blood flow and glomerular filtration rate (GFR), effects that are dose-dependent and reversible. These direct effects of CNIs on the renal vasculature and activation of systemic responses may involve activation of the sympathetic nervous system, the rennin-angiotensin system, and the creation of an imbalance between endogenous vasoconstrictive and vasodilatory substances. This imbalance includes an increase in thromboxane A2 and a decrease in prostaglandin E2, as well as an increase in endothelin-1 release and a decrease in the production of nitric oxide [7]. In contrast, chronic nephrotoxicity from CNI therapy is not dose-dependent, and is not reversible. It appears to be the result of structural changes in the kidney caused by chronic changes in renal hemodynamics. The rennin-angiotensin-aldosterone system may also be involved in this toxic response [7,8].

Because dose is not predictive of chronic CNI-induced nephrotoxicity, its development in some but not all patients has been evaluated for identification of contributory risk factors, including genetic variation in renal CNI metabolism and transport genes and genes involved in the rennin-angiotensin system [9-11]. In addition to their effect on T-lymphocyte function, CNIs may also influence the production of endogenous vaso-protective arachidonic acid (AA) metabolites, the epoxyeicosatrienoic acids (EETs) in the kidney. EETs are produced via cytochrome P450 (CYP)-mediated epoxidation of arachidonic acid and are hydrolyzed by soluble epoxide hydrolase to the dihydroxyeicosatrienoic acids (DHETs). EETs have several significant physiological roles that help maintain blood pressure homeostasis, including tubular reabsorption of water and Na^+ transport, protection against inflammation, and maintenance of vascular smooth muscle tone [12-16]. Several studies suggest that the EETs are vasodilatory and thus provide protection against hypertension. In particular, it was shown that the 11,12-EET was a vasodilator in rat juxtamedullary nephron preparations, possibly due to its ability to open endogenous potassium channels. The EETs are also inhibitors of Na^+-K^+-ATPase and angiotensin II-induced natriuresis, contributing to their antihypertensive properties [17,18]. Endogenous pools of EETs, and DHETs have been found in mouse, rat, and human kidney, highlighting the importance of these agents in maintaining normal renal function [19].

Cytochromes P450 2C8 and 2J2 (CYP2C8 and CYP2J2) are important members of the P450 superfamily that are expressed in the kidney and are involved in the metabolism of AA to biologically active EETs [20,21]. CYP2C8 forms mainly 14,15- and 11,12-EET while CYP2J2

forms all four isomers — the 5,6- 8,9- 11,12- and 14,15-EETs [21]. Metabolites formed by CYP2C8 and CYP2J2 have been implicated in preventing hypertension and inhibition of these enzymes has been shown to reduce the concentration of EETs found in the kidney tissue. In addition, both CYP2C8 and CYP2J2 are polymorphically expressed and several variants with compromised activity have been identified (http://www.cypalleles.ki.se/).

The purpose of this study was to test for an association between known *CYP2C*8 and *CYP2J2* polymorphisms and the risk of developing CNI-associated nephrotoxicity in liver transplantation recipients. We hypothesize that patients carrying one or more variant CYP2C8 or CYP2J2 allele will have reduced production of the protective EETs and, therefore, will be at a higher risk of developing kidney toxicity when challenged with CNIs. A retrospective evaluation of a case-control study of kidney function in liver transplantation recipients receiving CNIs for at least three years was performed. In addition, *in vitro* experiments were carried out to evaluate the activity of different CYP2C8 variants with respect to EET formation and the ability of steady state circulating plasma concentrations of CNIs to inhibit CYP2C8 and CYP2J2 metabolism of arachidonic acid.

METHODS

Materials

The following chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO): IPTG, δ-ALA, thiamine, imidazole hydrochloride, DTT, sodium cholate, NADPH, Coomassie brilliant blue R, DLPC, TFA, Protease inhibitors cocktail. *E coli* C41(DE3) cells and pGRO7 plasmid were purchased from Fisher Scientific (Pittsburgh, PA)Restriction enzymes and other DNA-modifying enzymes were obtained from New England BioLabs (Beverly, MA). Platinum *Pfx* DNA polymerase, T4 DNA ligase, and *E. coli* DH5αF'IQ Max Efficiency Competent cells were purchased from Invitrogen (Carlsbad, CA). Bactotryptone, bactopeptone, and bactoyeast extract were obtained from BD Biosciences Clontech (Palo Alto, CA). Ni-NTA Superflow was from Qiagen (Valencia, CA), Emulgen 911 was from Kao Corporation (Tokyo, Japan). Baculovirus-insect cell microsomes (Supersomes®) containing expressed CYP2J2 or CYP2C8, co-expressed human cytochrome P450 reductase and human cytochrome b_5 were purchased from BD Gentest Corporation (Woburn, MA). All standards for the analysis of arachidonic acid metabolism (14,15-EET, 11,12-EET, 5,6-EET, 8,9-EET, d8-14,15-EET and 20-HETE) were available from Cayman Chemicals (Ann Arbor, Michigan).

Study Subjects

Subjects for this study were consented for an evaluation of the association between genes directly or indirectly involved in CNI disposition and effect, and chronic kidney disease. The first association was found with *ABCB1* genotype and post-liver transplantation CNI-induced chronic kidney disease [11]. A total of 200 liver transplantation patients were consented. From this pool, final eligibility for inclusion was defined as any non-Hispanic, Caucasian adult patient who underwent a liver-only transplantation at the University of Washington Medical Center (UWMC) between 1989 and 1999, received continuous CsA or Tac immunosuppressive therapy post-transplantation, and were at least three years from the time of transplantation. A total of 163 subjects were found to be eligible and included in the present association study. Consent for using this population for the current study was approved by the Institutional Review Board at the University of Washington (current HSRC approval number 01-19746-D, approved 8/13/2004). The original study reported on data from 120 subjects; the present study includes an additional group of subjects not in the original study that met all of the current study eligibility criteria.

DNA Analysis

DNA was obtained from the study subjects and genotyped for the absence or presence of *CYP2C8* *3 (associated with haplotype D) and *CYP2C8**4 variants (rs10509681, rs11572080 and rs1058930 respectively), the *CYP2C8* haplotypes B and C (characterized by rs7909236 and rs1113129, respectively),[22] and the *CYP2J2**7 and N124S (rs890293 and rs11572243 respectively) variants of *CYP2J2*. These are the most common variant alleles for these two genes in a Caucasian population.

All single nucleotide polymorphism (SNP) detection assays were designed and performed in the Functional Genomics Laboratory, Center for Ecogenetics and Environmental Health at the University of Washington, Seattle, WA. Samples were genotyped using 5′-nuclease assays which employ specific fluorogenic probes and uses probes and primers designed and optimized by the Assay-by-Design service offered by Applied Biosystems (Foster City, CA).

The sequences for all primers and fluorogenic allele-specific oligonucleotide probes are listed in Supplemental Table 1. All probes were 3′-labeled with the TAMRA quencher dye. In addition, the specific wild type and variant probes were 5′-labeled with the 6-FAM reporter dye and the VIC reporter dye, respectively. All probes were designed on the “sense” strand, unless otherwise indicated as “anti”. The fluorescent 5′-nuclease assays were performed and analyzed on an Applied Biosystems 7900HT Fast Real-Time PCR System. The specific PCR reaction conditions were based on the general guidelines provided by the manufacturer and incorporated 25-50 ng of genomic DNA template. Thermocycling parameters are also listed in Supplemental Table 1.

Demographic and Clinical Information

Relevant clinical data for all subjects was abstracted from the written and electronic medical records. This included lab values associated with kidney (serum creatinine (SCr), and blood urea nitrogen concentrations) and liver (alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transferase, albumin, bilirubin and prothrombin time) function. It also included a record of all concomitant medications thought to be nephrotoxic, kidney protective or that can influence affect the clearance of CNIs, as well as CNI dosage and trough blood concentrations over the first three years post-transplantation. Other conditions that might affect post-transplantation kidney function were ascertained, including pre-transplantation kidney disease, pre- and post-transplantation hypertension (subjects with pre-transplantation hypertension were defined as those patients requiring medication for hypertension for at least six months prior to transplantation while post-transplantation hypertension was defined as hypertension that required medication for at least six months) and pre- and post-transplantation hyperglycemia as previously described [11].

Clinical Endpoints

Kidney function, as measured by serum creatinine (SCr) three years post-transplantation, was correlated with *CYP2C8* and *CYP2J2* genotype. A chronic kidney disease case was defined a priori as a patient with a SCr ≥ 1.6mg/dL while a control case was defined as having a SCr < 1.6mg/dL. An evaluation of the frequency distribution of SCr concentrations at three years post transplantation in the study subjects by commingly analysis has been described [11]. It was determined that SCr values were trimodally distributed and that the SCr value of 1.6 mg/dl fell between the means of the first two distributions [11]. This supports the use of a SCr value of 1.6 mg/dl to differentiate between cases and controls [11]. Data on subject pre-transplantation kidney function was also obtained. The same SCr values defining cases and controls were used to define pre-operative kidney function. Pre-transplantation kidney function was considered normal if the SCr was <1.6mg/dL, while pre-transplantation kidney disease was defined as SCr ≥ 1.6mg/dL. Other means of defining kidney dysfunction were either not

available for this analysis or were considered inappropriate for this study population. A measured glomerular filtration rate (GFR) would have been an ideal means of defining kidney dysfunction, but this data was not available. Data was available for defining kidney dysfunction using several equations to calculate GFR, but none of these estimates of kidney dysfunction are appropriate in this post liver-transplantation population [23].

Determining the of Risk of Kidney Disease

Hardy-Weinberg equilibrium was determined for all SNPs. Homozygous variants were grouped with heterozygotes for phenotype comparisons against homozygous wild types, because of the low frequency of the variant alleles and because the mutations confer a decrease in function and/or expression levels. Odds ratios (95% confidence intervals) were calculated to evaluate the association between each genotype and kidney disease for all study subjects and also for only those with normal, pre-transplantation renal function. The analysis was further stratified by the use of CsA or Tac and by gender.

Cloning of CYP2C8 WT and Variants

The CYP2C8 cDNA, a gift from Dr. Frank Gonzales at NIEHS, was inserted into the pCWori + expression vector [24] using the *NdeI* and *Sal1* restriction sites. The modified sequence of Barnes *et al*. [25], MALLLAVF, was used to replace the first eight amino acids of native CYP2C8.1 and a 6-His tag was inserted at the C-terminus to facilitate purification using metal affinity columns as previously described [26].

CYP2C8 variant expression vectors were constructed using the Quickchange II XL site directed mutagenesis kit and using pCWori+-*2C8*1* as a template with the following primers: (for pCWori+-*2C8*4* (I264M)),
5′:CAATCCTCGGGACTTTATGGATTGCTTCCTGATCAAAATGG, and
5′:CCATTTTGATCAGGAAGCAATCCATAAAGTCCCGAGGATTG, 1Unit of *Pfu* Turbo polymerase, and cycling conditions of 95° for 3 minutes followed by 16 cycles of 94° for 35 seconds, 52° for 35 seconds, 72° for 10.5 minutes followed by a 4 hour digestion with Dpn1 to remove template DNA. The *CYP2C8*3* allele product contains a double mutation (R139K and K399R) that was sequentially introduced with the first PCR reaction containing the primers, (for K399R),
5′:CCGTGCTACATGATGACAGAGAATTTCCTAATCCAAATATC and
5′:GATATTTGGATTAGGAAATTCTCTGTCATCATGTAGCACGG and pCWori+*2C8*1* as a template 1Unit of *Pfu* Turbo polymerase, and cycling conditions of 95° for 3 minutes followed by 16 cycles at 94° for 35 seconds, 52° for 35 seconds, 72° for 10.5 minutes followed by a 4 hour digestion with Dpn1 to remove template DNA. An individual clone was sequenced to confirm the presence of the mutation (pCWori+2C8 (K399R) and used as a template with the primers (for R139K), 5′:TTGGGATGGGGAAGAAGAGCATTGAGGACCG and
5′:CGGTCCTCAATGCTCTTCTTCCCCATCCCAA, and amplified with the same cycling conditions mentioned above. All genes were sequenced entirely to confirm the introduction of the desired mutations and the absence of other undesired mutations as a result of PCR.

Protein Expression and Purification

LB/ampicillin/chloramphenicol plates were used to select for *E. coli* C41(DE3) cells freshly co-transformed with each expression plasmid and pGRO7 following the manufacturer's protocol. Single isolated colonies were used to inoculate 5 ml of LB/ampicillin/ chloramphenicol media incubated at 37 °C in a rotary shaker (at 225 rpm) overnight. The following day this starter culture was used to inoculate 500 ml TB media (12 g bactotryptone, 24 g yeast extract, 2 g bactopeptone and 4 ml glycerol per liter), 1 mM thiamine, 100 μg/ml ampicillin, and chloramphenicol (final 40 μg/ml) and incubated at 37 °C in a rotary shaker (at 225 rpm). At one hour post-inoculation, L-arabinose (final 4 mg/ml) was used for induction

of chaperone proteins, and at five hours post inoculation, final concentrations of 1 mM IPTG and 0.5 mM δ-aminolevulinic acid were added to the cultures which were then incubated at 28 °C for 48 hours. Cells were harvested by centrifugation at 5000 × *g* for 10 min, resuspended in storage buffer (100 mM potassium phosphate buffer, (KPi), pH 7.4, containing 20% glycerol), pooled into 50 ml Falcon tubes, and re-centrifuged (4000 × *g*, 30 min). The bacterial cell pellets were stored at −80 °C until further use.

Frozen pellets were thawed on ice and resuspended in 100 mM KPi (pH 7.4) containing 20% glycerol and protease inhibitors cocktail. Emulgen 911 was added to a final concentration of 1% and the cells were lysed with two passes through the French Press. The resulting mixture was centrifuged for 60 min. (100,000 × *g*) and the brown-orange supernatant was loaded directly on a Ni-NTA agarose column that had been pre-equilibrated with 10 column volumes of equilibration buffer containing 100 mM KPi pH 7.4, 0.5 M KCl, 5 mM imidazole, 0.05% sodium cholate and protease inhibitors cocktail. A dark orange band was visible at the top of the column following loading of the sample. The Ni-NTA agarose column was washed with five volumes of wash buffer containing (100 mM KPi, pH 7.4, containing 20% glycerol, 40 mM imidazole, 0.05% sodium cholate, 0.1 mM DTT, 100 mM glycine, 0.3 M NaCl and protease inhibitors). The enzyme was eluted with elution buffer containing (100 mM KPi, pH 7.4, 20% glycerol, 250 mM imidazole, and 0.02% sodium cholate) and the dark orange/red fractions were collected, pooled, and dialyzed twice against 100 volumes of resuspension buffer (100 mM KPi, pH 7.4, 20% glycerol). The enzymes were stored as 15-20 μM aliquots at −80 °C.

General Enzyme Reconstitution and Metabolic Assay Procedure

Purified CYP2C8 enzymes (20 pmols) were reconstituted with P450 reductase and cytochrome b5 (ratio of 1:2:1) in DLPC vesicles which have been extruded through a 200 nm pore size membrane. Reconstituted enzymes were diluted to a final volume of 0.5 mL in potassium phosphate buffer (100 mM, pH 7.4 containing 0.1 mM EDTA) after which substrate was added. The mixture was equilibrated at 37 °C for 5 min, and reactions were initiated with NADPH (1.0 mM final volume). After 20 minutes at 37 °C, reactions were terminated with 200 μL cold acetonitrile and internal standard (d8 14,15-EET Cayman Chemicals) was added. After centrifugation the supernatant was removed and analyzed by LC-MS/MS as described below.

Inhibition of CYP2C8 and CYP2J2 by CsA and Tac

Baculovirus-insect cell microsomes (Supersomes®) containing expressed CYP2J2 or CYP2C8, co-expressed human cytochrome P450 reductase, and human cytochrome b_5 (20 pmol), were reacted with arachidonic acid (5 μM) in the absence and presence of CsA (0.2 μM) or Tac (0.02 μM) and NADPH (1 mM) in potassium phosphate buffer (0.2 mL). The reactions was allowed to proceed for 20 minutes after which they were quenched with the addition of cold acetonitrile (0.2 mL) and internal standard (d8 11,12-EET 50 ng/mL). After centrifugation, 200 μL of the incubation mixture was analyzed directly by LC-MS/MS.

Arachidonic LC-MS/MS Assay

The quantification of arachidonic acid epoxides and 20-hydroxide was performed on a Waters Aquity Ultra Performance Liquid Chromatography (UPLC) coupled with a Micromass Premier-XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA). The system was operated using atmospheric pressure electrospray ionization (API-ES) in the negative ion mode. Chromatographic separation of analytes was achieved on a 2.1 × 50mm, 1.7μm, phenyl BEH column (Waters Corp., Milford, MA) using a gradient elution. The initial mobile phase was 80% 10 mM formic acid/20% acetonitrile. There was a linear increase to 60% organic at 11.0 minutes followed by an increase to 100% acetonitrile from 11.2 to 12.5 minutes. Mobile phase components were then returned to initial conditions at 13 minutes. The flow was kept at

0.4 mL/min. throughout and the sample chamber and column were maintained at 5 °C and 40 °C respectively. MS conditions were as follows: capillary voltage, 3.6 kV; the source and desolvation temperatures were 120 °C and 400 °C respectively; the desolvation gas (Nitrogen) flow, 1000 L/Hr; and collision gas (Argon) flow, 0.2 mL/min. The selective reaction monitoring (SRM) analysis used the optimized cone voltages of 10 +/- 1 V and collision voltages of 13 +/-1 volt for all of the epoxides. For the 20-HETE, the optimal cone and collision voltages were 20 and 15 V respectively. The transitions monitored for the individual compounds were: 14,15-EET (m/z 319>219), 11,12-EET (m/z 319>208), 5,6-EET (m/z 319>191), 8,9 and 11,12-EET (m/z 319>167), 8,9-EET (m/z 319>127), d8-14,15-EET (m/z 327>219), 20-HETE (m/z 319>245).

Data Analysis

All odds ratios calculations were performed in Excel (Microsoft Office, 2003). Velocity verses substrate concentration data were analyzed by non-weighted non-linear regression analysis using a single enzyme Michaelis-Menten model in SigmaPlot (SigmaPlot 9.01, Systat, Point Richmond, CA).

RESULTS

Demographics and Incidence of Renal Dysfunction

The characteristics of the study subjects are listed in Table 1. One hundred sixty-three subjects from the original pool of 200 consented patients were eligible for the current analysis, had DNA of adequate quality for genotyping, and had clinical data available for analyzing the relationship between *CYP* genotype and kidney disease three years post liver transplantation. Of the consented subjects, only adult Caucasians that received a liver-only transplant were eligible. Sixty-four percent of the subjects were men while 36% were women. The average age of the subjects was 48 years.

At the three year post-transplantation time point for evaluating the kidney status of these patients, 39.2% had SCr ≥1.6 mg/dl, defining them as cases. Of the 141 subjects whose pre-transplantation kidney status was known, 15.0% had kidney disease just prior to transplantation. Eleven out of 55 (20%) cases had kidney disease prior to transplantation while 10 out of 86 (12%) controls had kidney disease prior to transplantation. These frequencies for the 163 subjects in this study are very similar to those obtained for the smaller cohort of 120 subjects previously described [11].

Association between CNI-Associated Nephrotoxicity and CYP Genotype

All 163 study subjects were successfully genotyped for the *CYP2C8*3* and ****4*** variants, 155 were successfully genotyped for *CYP2C8* haplotype B, 152 were able to be genotyped for *CYP2C8* haplotype C, and 162 samples were successfully genotyped for *CYP2J2*7* and CYP2J2 N124S. Genotype frequencies in the sample population were as expected and found to be in Hardy-Weinberg equilibrium. The subjects were stratified for analysis by CNI, gender, and kidney status at time of transplantation. One hundred forty-one subjects had pre-transplantation kidney status information available. When subjects were removed from the risk analysis because they showed signs of pre-existing kidney disease at the time of transplantation, there were 120 subjects remaining that qualified for analysis. It is notable that two cases were *CYP2C8*3* homozygous whereas all the controls were heterozygotes for the *CYP2C8*3* allele or homozygous *CYP2C8*1*. There were no homozygous mutants for *CYP2C8*4* or *CYP2J2*7*. Three control subjects were homozygous for *CYP2C8 Haplotype B*. Two control subjects and one case subject were homozygous for *CYP2C8 Haplotype C*.

The relative risks for developing kidney disease by three years post-transplantation as a function of genotype are presented as odds ratios in Tables 2 and 3. Both tables present the odds ratios first for all subjects, then only for those subjects in good kidney health at the time of transplantation. The data is also stratified by gender. The odds ratios in Table 2 are those calculated when considering the use of any CNI. Table 3 presents the odds ratios for subjects on CsA or Tac separately. Odds ratios are presented with their respective 95% confidence interval in parenthesis. The number of subjects included in each calculation is presented as the "n" for each value. As indicated above, homozygous variants were grouped with heterozygotes for phenotype comparisons against homozygous wild types. All samples were wild type for CYP2J2 N124S SNP, therefore no odds ratios were calculated for that specific CYP2J2 variant. When no odds ratio is presented, there were no subjects in that category carrying a variant allele and odds ratios could not be calculated.

As shown in Table 2, when evaluating the risk of developing CNI-associated kidney disease post-transplantation regardless of pre-transplantation kidney status or the CNI used, the presence of the *CYP2C8*4* allele or the *CYP2J2*7* allele was associated with a trend towards the development of CNI-induced kidney disease although the odds ratios were not significant for all eligible men and women considered together, and for all eligible men. However, the odds ratios determined for male and female subjects with one or more *CYP2C8*3* allele evaluated together was significant (OR=2.38 (1.19-4.78)), indicating that the inheritance of this allele is associated with increased risk of developing CNI-induced nephrotoxicity. When all eligible subjects were stratified by gender, the odds ratio for men with a *CYP2C8*3 allele was still significant (OR=2.82 (1.15-6.93)); while the odds ratio for women approached but did not reach significance (OR=2.19 (0.66-7.30)).

Table 2 also illustrates that the risk of developing CNI-associated kidney disease post-transplantation for subjects with normal kidney function before their transplantation operation was not significantly influenced by the presence of the *CYP2C8*4* allele or the *CYP2J2*7* allele, although the risk trended towards significance for men and women evaluated together and for men alone. Similar to the analysis using all eligible study subjects, there was significant association between the presence of the *CYP2C8*3* allele and post-transplantation kidney disease for subjects with normal kidney function prior to transplantation: (OR=3.38 (1.39 – 8.19)) for men and women, and for men only the (OR=7.33 (1.85 – 29.15)). The same analysis was not significant for women only.

Table 3 presents the odds ratios calculated as above, except that the odds ratios were calculated separately for CsA and for Tac. Subjects were stratified by pre-transplantation kidney status and gender, as above. For either immunosuppressant, the same trends in risk were seen for *CYP2C8*4* and *CYP2J2*7* compared to what was seen when the analysis was combined for the two medications. The risk differs between the two drugs when evaluating the presence of *CYP2C8*3*. Inheritance of the *CYP2C8*3* allele was not a significant risk factor for the development of CNI-induced kidney disease for patients of either gender taking CsA, when all eligible subjects were evaluated. In contrast, there was a significant increased risk of kidney disease in patients with the *CYP2C8*3* allele who were taking Tac, as seen by the odds ratio of 16.67 (2.8 – 99.6). Only 41 study subjects were on Tac at the time of evaluation. The spread of the confidence interval was large, but the magnitude of the odds ratio was substantial. The risk for developing Tac-induced kidney disease in drug- and gender-stratified groups could not be evaluated in men only. However, the odds ratio for kidney disease in women with the *CYP2C8*3* allele and on Tac was not significant.

For subjects with normal kidney health at transplantation, men carrying the *CYP2C8*3* allele and receiving CsA had a significant risk of developing kidney disease (OR: 4.91 (1.2 – 20.2)).

For subjects carrying the *CYP2C8*3* allele and taking Tac, the combination of men and women analyzed together again proved to be significant (OR: 15.33 (1.9 – 122)).

Analysis of the risk of developing CNI-associated kidney disease as a function of *CYP2C8* Haplotype B and C showed that Haplotype C may be protective. Significant protective odds ratios were determined for men and women evaluated together and for men evaluated alone regardless of their pre-transplantation renal status when on CsA (OR: 0.36 (0.2 – 0.8) and (OR: 0.33 (0.1 – 0.9)), respectively.

Other Clinical Covariates

Potential clinical covariates listed in Table 1 were evaluated for their effect modifying and confounding contributions *for CYP2C8*3, CYP2C8*4* and *CYP2J2*. These covariates were not found to be effect modifiers but several were found to be significant confounders when evaluated for all eligible men and women grouped together and for men and women grouped together with normal kidney function at transplantation. Due to the limited sample size, confounding was evaluated one covariate at a time. If the inclusion of the covariate in a logistic regression model changed the genotype coefficient by more than 10%, it was deemed a confounder. The ranges of the odds ratios that resulted when adjusting for each covariate individually per genotype are presented in Table 4. The covariates were not consistently confounding across the different genotypes. Liver function laboratory values that were above the normal range did not uniformly affect the odds ratios. Gender and weight also did not uniformly affect the risk of developing kidney disease three years post-transplantation. Except for pre-transplantation kidney status, concomitant diseases also did not uniformly affect the unadjusted odds ratios. Pre-transplantation SCr values ≥ 1.6 mg/dL did increase the risk of having three-year post-transplantation kidney disease for subjects with the *CYP2C8*3* and the *CYP2J2*7* alleles. Of note, the concomitant administration of other nephrotoxic medications did not affect the risk of developing kidney disease three years post-transplantation. The concomitant administration of medications whose mechanism of action may protect the kidney from CNI-induced nephrotoxicity only lowered the adjusted odds ratio for the *CYP2J2*7* allele for all eligible subjects. Interestingly, CsA dose and level each lowered the adjusted odds ratios for subjects with the *CYP2C8*3* and *CYP2C8*4* genotype. However, consideration of Tac dose and level significantly elevated adjusted odds ratios for subjects with *CYP2C8*3* and *CYP2C8*4* alleles. These covariates influenced the adjusted odds ratios to the greatest degree.

In vitro Metabolism of Arachidonic acid by CYP2C8 and CYP2J2

Expressed human wild-type CYP2C8.1 and CYP2J2.1 were found to be active in epoxygenating arachidonic acid. Both enzymes metabolized arachidonic acid to the 14,15-, and 11,12-EET, but only CYP2J2 formed 8,9-EET, 5,6-EET and 20-HETE, as previously described [21]. The relative production of the 14,15-EET by CYP2C8.1, the CYP2C8.3 and CYP2C8.4 variants, and CYPP2J2.1 are presented in Figure 1 as the percentage of the CYP2C8.1 activity. CYP2J2.1 had less than 20% of the activity towards AA compared to CYP2C8.1. CYP2C8.3 and CYP2C8.4 also exhibited reduced activity towards AA, compared to CYP2C8.1, having approximately 25% and 18%, respectively, of the relative activity of the wild type enzyme. Figure 2 illustrates a comparison of the product formation kinetics of CYP2C8.1 with that of CYP2C8.3 and CYP2C8.4 variants. Assuming simple Michaelis-Menten kinetic behavior, K_m and V_{max} values were calculated and are presented in Table 5. The intrinsic clearance of 14,15-EET formation of CYP2C8.3 and CYP2C8.4 was 32 and 30% that of the wild-type enzyme respectively. In contrast, the metabolism of AA by CYP2J2 displayed strong substrate inhibition kinetics and kinetic parameters were not estimated at this time.

Inhibition of EET Formation by CNIs using Expressed Enzymes

Circulating concentrations between the trough and peak concentrations of CsA and Tac (0.2 μM and 0.02 μM respectively) were found to inhibit arachidonic acid metabolism by CYP2C8.1 and CYP2J2.1 (Figure 3). Tac appeared to be a more potent inhibitor than CsA for both enzymes.

DISCUSSION

The key finding of this study was the positive association between inheritance of the *CYP2C8*3* allele and the risk of developing CNI-induced kidney disease post-liver transplantation. Significant odds ratios were calculated for men and women together, and for men alone. There was a trend towards significance for women, which constituted only 36% of the total study population. The gender difference could be due to the lower baseline SCr for women undergoing liver transplantation, which may result in a miss-classification of case-control status for women. Also in our sample we had more men than women and SCr levels at three years post transplantation were also significantly higher in men compared to women [11]. It is also likely that these findings of increased risk of kidney disease in men compared to women in our sample are not spuriously caused by miss-classification since the literature indicates a greater incidence in general of kidney disease in men compared to women [27, 28].

There was a more striking risk of developing kidney disease in patients carrying *CYP2C8*3* allele that had good kidney health before their transplantation operation, compared to all patients evaluated together, including those that underwent liver transplantation with existing kidney disease. This signifies that, in patients with good kidney health at the time of transplantation, expressing the *CYP2C8*3* allele is a major risk factor for developing kidney disease and that pre-existing kidney disease from various causes (e.g., hepato-renal syndrome) masked the genetic association. It is worth noting that the allele frequency of *CYP2C8*3* is only 15-18% of the general white (Caucasian) population. However, inheritance of one or more variant allele compounded with drugs that affect CYP2C8 activity posed a risk that may follow a gene-dose effect.

The data indicates that the use of Tac may cause a greater risk for developing CNI-induced kidney disease than CsA in subjects carrying the *CYP2C8*3* allele. Far fewer subjects were administered Tac compared to CsA, making the significant odds ratios calculated for that particular medication appear to be of even greater importance. This finding should be investigated with future studies. However, the present *in vitro* studies show that Tac is a more potent inhibitor of CYP2C8, which may explain its stronger association with the mutant *CYP2C8*3* allele and kidney disease.

The odds ratios calculated for *CYP2J2*7* and *CYP2C8*4* were not significant for all subjects regardless of pre-transplantation kidney status or gender, although some groupings of subjects trended towards significance (see Tables 2 and 3). No patients carrying the CYP2J2 N124S variant were found in the study population and therefore no odds ratios related to that genotype could be calculated. The lack of statistical significance for the *CYP2J2* variants suggests no causality, but this result may also be due to the low frequency of the *CYP2J2* genetic polymorphism among the study population. Additionally, it may be explained by the fact that CYP2J2 protein expression in the kidney is low compared to CYP2C8. Using immunohistochemistry, Enayetallah *et al.* found a higher signal intensity for CYP2C8 protein expression in proximal tubule cells than CYP2J2 in human kidney tissue samples [29]. The apparent protective affect of the CYP2C8 haplotype C is not clear at this time and the functional importance of this haplotype in metabolizing arachidonic acid has not been established. Further

studies are needed to address the importance of inheriting this haplotype and risk for developing kidney disease.

The *in vitro* data described in this paper supports the risk analysis presented above. Both CYP2C8.3 and CYP2C8.4 variant enzymes exhibited markedly reduced activity compared to CYP2C8.1. Reduced activity of CYP2C8.3 towards AA compared to CYP2C8.1 observed has also been demonstrated by Dai *et al* [30]. In that study, CYP2C8.3 produced only 35-40% of the 11,12- and 14,15-EETs compared to CYP2C8.1. These data, coupled with our clinical observation, indicate that reduced production of EETs by the less active CYP2C8.3 enzyme may contribute to the risk of CNI-induced nephrotoxicity. A possible explanation for why significant odds ratios were not seen for the *CYP2C8*4* allele, even though the gene product also has significantly reduced activity towards AA is again attributed to the low numbers of subjects expressing this allele in this study population. A larger population sample size may result in significant odds ratios that would reflect the CYP2C8.4 *in vitro* activity.

Our data suggest that carrying one or more *CY2C8*3* variant allele confers an increased risk for the development of kidney dysfunction in patients receiving chronic CNI therapy. EETs are known to exhibit vasodilatory effects, particularly in the kidney where they have been measured [19]. Decreased production of these molecules in patients with the variant *CYP2C8*3* allele may reduce the capacity of the organ to counter the vasoconstrictive effects of the CNIs or may simply confer an independent risk factor that accentuates the kidney toxicity of CNIs. However, our data also offers the intriguing possibility that a drug-induced decrement in CYP2C8-catalyzed EET formation is an initiating, causal factor in the pathogenic process.

Several studies have shown that CsA and Tac inhibit CYP2C8 activity *in vivo*. For example, CsA was found to increase the AUC of cerivastatin by ~ 300% [31], paclitaxel by 750% [32] and repaglinide by 143% [33]. All of these drugs are known substrates for CYP2C8 [34]. In addition, one study showed that Tac increased the AUC of cerivastatin by 51% [35]. Inhibition of CYP2C8 by both CsA and Tac *in vivo* may reduce the formation of the protective EETs in the kidney and precipitate acute toxicity. Several studies support the hypothesis that a decrease in the presence of kidney EETs may contribute to kidney afferent hypertension, a potential mechanism for CNI nephrotoxicity. Renal cortical microsomes from spontaneously hypertensive rats produced more 14,15- and 11,12-EET than microsomes from normotensive rats [19]. Increased urinary excretion of the same EETs was higher in the spontaneously hypertensive compared to the normotensive rats. Although this seems to indicate that the increased EET production is a cause for the hypertension seen in this rat model, the authors suggest that the DHET metabolites of the EETs may be responsible for the increased blood pressure or that the increased EETs may be a response to the elevated blood pressure in these animals. The latter conclusion is supported by further findings by Yu et al [36] showing that reduction of EET hydrolysis by soluble epoxide hydrolase (sEH) inhibition resulted in a drop in blood pressure in the spontaneously hypertensive rat model. Administration of an sEH inhibitor in angiotensin hypertensive rats also decreased blood pressure and caused a decrease in urinary albumin excretion (a marker of renal damage) [37].

There may be other factors that influence the risk of nephrotoxicity in our study subjects. One factor may be the presence of the *CYP2C9*2* variant. Although these subjects were not genotyped for this allele, there is strong linkage between *CYP2C9*2* and *CYP2C8*3*. Approximately 95% of subjects carrying *CYP2C8*3* also carry *CYP2C9*2 genotype* [38]. However, CYP2C9 has not been detected in the kidney at the protein or functional level. Accordingly, we did not genotype subjects for this allele because it seems unlikely that it will contribute to the metabolism of arachidonic acid in the kidney.

Besides gaining a better understanding of the risk of developing kidney disease from CNI therapy, and understanding the underlying mechanism of these drugs' kidney toxic effects, the findings in this study may have additional clinical relevance. The occurrence of some adverse drug-drug interactions may be explained in part by these results. We demonstrated that the CNIs can inhibit CYP2C8 at concentrations that are clinically relevant. The clearance of other drugs that are metabolized by this enzyme may be affected by co-administration with a CNI, resulting in elevated, potentially toxic systemic concentrations of the inhibited drug. This has been shown to be the case for repaglinide [39], and CNIs may also affect the antimalarials amodiaquine and chloroquine [40].

In summary, *2C8*3* appears to be predictive of risk of CNI-induced kidney disease, especially when patients are taking Tac. The findings presented in this paper have significant clinical relevance to care of transplantation recipients that receive CNI based immunosuppression, and those taking other medications that may have drug interactions with the CNIs. It may also have relevance to other conditions of kidney dysfunction. A prospective clinical study is currently underway at the University of Washington to confirm these findings as well as the effect of other genetic polymorphisms on CNI-induced renal toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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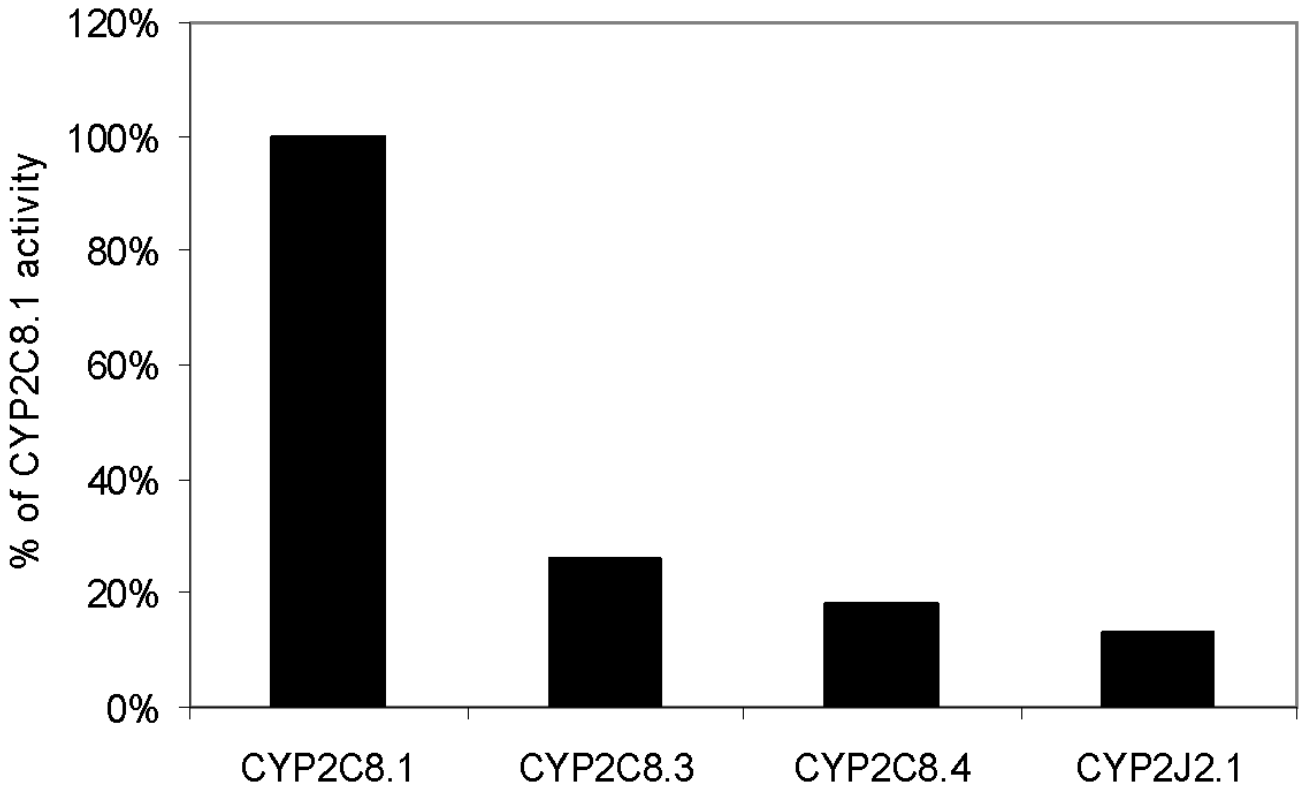

Figure 1.
14,15-EET Formation by CYP2J2 and CYP2C8. Values are reported as the average of two separate experiments

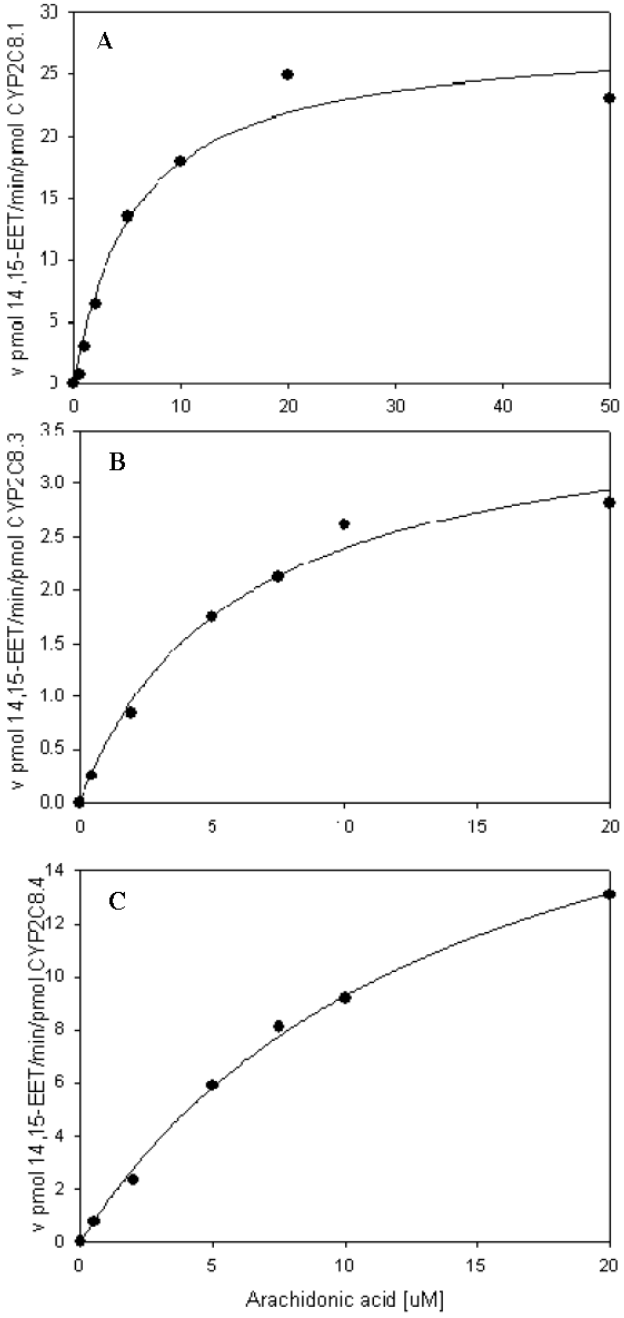

Figure 2.
Steady state kinetics for 14,15-EET formation rate by CYP2C8.1 (panel A), CYP2C8.3 (panel B) and CYP2C8.4 (panel C). Data was fitted to a single enzyme Michealis-Menten model. Values are the average of duplicates.

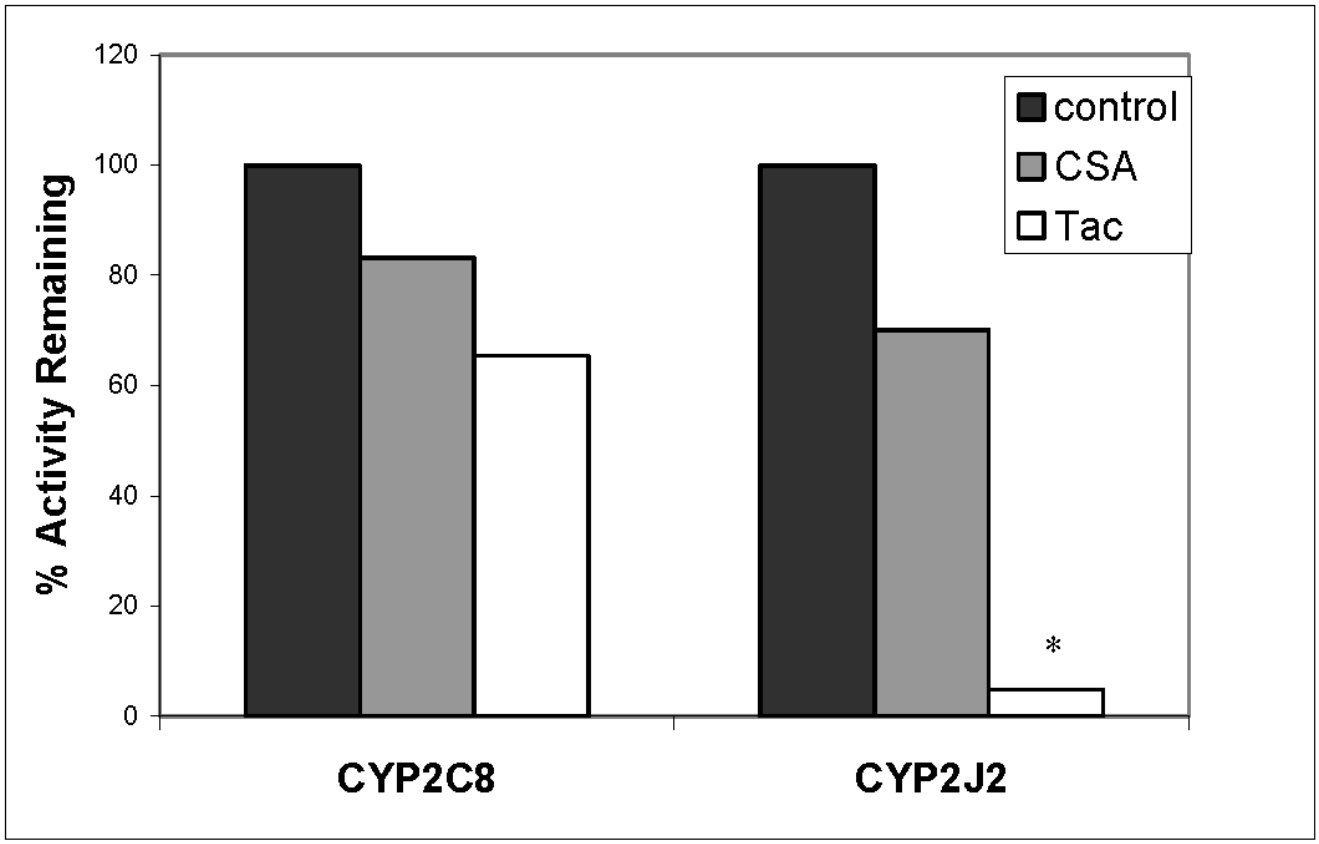

Figure 3.
Inhibition of 14, 15-EET Formation by CSA and Tac. The concentration of AA was 5 μM for CYP28 and 10 μM for CYP2J2. The concentrations of CSA and Tac were 0.2 μM and 0.02 μM, respectively. Experiments were run in duplicates and reported as the average.

Table 1

Subject Characteristics at Three Years Post Transplantation

Variable	SCr ≥ 1.6 mg/dl (cases)	SCr < 1.6 mg/dl (controls)	All Subjects
Demographics; N (percent of total)			
Total	64	99	163
Male	48 (75)	57 (58)	105 (64)
Female	16 (25)	42 (42)	58 (36)
Age at transplantation (years; Mean ± SD)	50 ± 9	47 ± 11	48 ± 10
Relevant concomitant illnesses; N (percent of total)			
Pre-Tx[1] SCr ≥ 1.6 mg/dl[2]	11/56 (20)	10/86 (12)	21/142 (15)
Pre-Tx diabetes	8 (12)	11 (11)	19 (12)
With end-organ damage	5 (8)	2 (2)	7 (4)
New onset post-Tx diabetes	16 (25)	17 (17)	33 (20)
With end-organ damage	10 (16)	7 (7)	17 (10)
Pre-Tx hypertension	4 (6)	5 (5)	9 (6)
New onset post-Tx hypertension	46 (7)	69 (70)	115 (71)
Hepatitis C	30 (47)	46 (46)	76 (47)
CNI therapy; mean ± SD			
CsA dose (mg/day)	299 ± 130	288 ± 106	292 ± 116
CsA blood concentration (ng/ml)	150 ± 65	131 ± 35	138 ± 50
Tac dose (mg/day)	7.8 ± 3.9	6.7 ± 3.9	7.1 ± 3.9
Tac blood concentration (ng/ml)	11.8 ± 3.5	10.2 ± 3.2	10.7 ± 3.3
Lab Values; mean ± SD			
SCr (mg/dl)	2.0 ± 0.4	1.2 ± 0.2	1.5 ± 0.5
Creatinine clearance (ml/min)	54 ± 20	76 ± 22	66 ± 24
BUN (mg/dl)	37 ± 11	24 ± 7	29 ± 11
BUN:SCr (mg SCr/mg BUN)	55 ± 275	21 ± 5	34 ± 171
Serum albumin (gm/dl)	3.9 ± 0.5	4.9 ± 6	4.5 ± 5.0
Aspartate aminotransferase (IU/L)	57 ± 95	53 ± 45	55 ± 68
Alanine aminotransferase (IU/L)	57 ± 59	60 ± 57	58 ± 58
Alkaline phosphatase (IU/L)	129 ± 109	124 ± 114	126 ± 112
Total bilirubin (mg/dl)	1.4 ±2.5	1.2 ± 0.9	1.3 ± 1.7
Gamma glutamyltransferase (IU/L)	188 ± 256	181 ± 248	183 ± 250
Prothrombin time (sec)	12.6 ± 3.8	13.4 ± 2.2	12.9 ± 2.9
Relevant concomitant medications; N (percent of total)			
NSAID/COX-2 Inhibitors	0	0	0
Aspirin (low dose)	13 (20)	17 (17)	30 (18)
Aminoglycosides	1 (2)	1 (1)	2 (1)

Variable	SCr ≥ 1.6 mg/dl (cases)	SCr < 1.6 mg/dl (controls)	All Subjects
Vancomycin	9 (14)	3 (3)	12 (7)
Amphotericin B	1 (2)	2 (2)	3 (2)
ACE/ARB inhibitors	11 (17)	10 (10)	21 (13)
P-glycoprotein/CYP3A inhibitors	8 (13)	6 (6)	14 (6)

[1] Tx: transplantation

[2] Only 142 of the 163 subjects had pre-transplant kidney function status available.

Table 2

Relative Risk of Kidney Disease Three Years Post-Transplantation for CNIs Combined[1]

All Eligible Subjects			
Genotype	**All Subjects**	**Women**	**Men**
2C8*3[2]	2.38 (1.19 – 4.78) n=163	2.19 (0.66 – 7.30) n=58	2.82 (1.15 -6.93) n=105
2C8*4[3]	1.31 (0.38 - 4.50) n=163	-- n=58	1.21 (0.33 – 3.68) n=105
2J2*7[4]	1.40 (0.58 - 3.35) n=162	0.68 (0.07 – 6.60) n=57	1.40 (0.52 – 3.80) n=105
Haplotype B	0.87 (0.5 – 1.7) n=155	1.52 (0.5 – 5) n=57	0.75 (0.32 – 1.73) n=98
Haplotype C	0.36 (0.2 – 0.7) n=152	0.42 (0.1 – 1.5) n=56	0.33 (0.1 – 0.8) n=96
2C8*3[2]	3.32 (1.37 – 8.07) n=119	2.46 (0.64 – 9.55) n=44	7.33 (1.85 – 29.15) n=77
2C8*4[3]	1.02 (0.23 – 4.51) n=119	-- n=44	0.83 (0.22 – 4.52) n=77
2J2*7[4]	1.33 (0.49 – 3.61) n=118	0.85 (0.08 – 9.06) n=43	1.30 (0.42 – 4.03) n=77
Haplotype B	0.70 (0.3 – 1.5) n=114	0.69 (0.2 – 2.9) n=42	0.70 (0.3 – 1.8) n=72
Haplotype C	0.28 (0.1 – 0.7) n=112	0.21 (0.07 – 0.7) n=71	0.49 (0.1 – 2.2) n=41

[1] Relative risk is presented as the odds ratio (95% CI)

[2] The wild type allele for 2C8*3 is G; the variant allele is A.

[3] The wild type allele for 2C8*4 is C; the variant allele is G.

[4] The wild type allele for 2J2*7 is G; the variant allele is T.

Table 3

Relative Risk of Kidney Disease Three Years Post-Transplantation Stratified by CNI[1]

All Eligible Subjects						
	CsA			**Tac**		
Genotype	**All Subjects**	**Women**	**Men**	**All Subjects**	**Women**	**Men**
2C8*3[2]	1.44 (0.7 – 3.2) n=122	1.27 (0.3 – 5.5) n=40	1.65 (0.6 – 4.4) n=82	**16.67 (2.8 – 99.6) n=41**	8.25 (0.8 – 85.6) n=18	-- n=23
2C8*4[3]	1.09 (0.2 – 5.1) n=122	-- n=40	1.11 (0.2 – 5.9) n=82	2.08 (0.3 – 16.6) n=41	-- n=18	1.71 (0.1 – 8.8) n=23
2J2*7[4]	1.76 (0.6 – 4.9) n=122	0.87 (0.1 – 9.3) n=40	1.96 (0.6 – 6.6) n=82	0.8 (0.1 – 4.8) n=40	-- n=17	0.71 (0.1 – 5.0) n=23
Haplotype B	0.97 (0.5 – 2.1) n=116	1.61 (0.4 – 6.9) n=39	1.00 (0.4 – 2.6) N=77	0.61 (0.2 – 2.5) n=39	1.50 (0.2 – 12.8) n=18	0.29 (0.04 – 2.0) n=21
Haplotype C	**0.36 (0.2 – 0.8) n=113**	0.49 (0.1 – 2.3) n=38	**0.33 (0.1 – 0.9) n=75**	0.38 (0.1 – 1.6) n=39	0.29 (0.03 – 3.4) n=18	0.38 (0.1 – 2.3) n=21

Subjects with Normal Kidney Function at Transplantation						
	CsA			**Tac**		
Genotype	**All Subjects**	**Women**	**Men**	**All Subjects**	**Women**	**Men**
	2.43	1.17	**4.91**	**15.33**	13.50	--

Subjects with Normal Kidney Function at Transplantation

	CsA			Tac		
Genotype	**All Subjects**	**Women**	**Men**	**All Subjects**	**Women**	**Men**
2C8*3[2]	(0.9 – 6.5) n=89	(0.2 – 6.3) n=29	**(1.2 – 20.2)** **n=60**	**(1.9 – 122)** **n=32**	(0.9 – 207) n=15	 n=17
2C8*4[3]	1.01 (0.2 – 4.8) n=89	-- n=29	1.08 (0.2 – 5.8) n=60	-- n=32	-- n=15	-- n=17
2J2*7[4]	1.69 (0.5 – 5.5) n=89	1.13 (0.1 – 14.3) n=29	1.76 (0.4 – 7.0) n=60	0.80 (0.1 – 8.5) n=31	-- n=14	1.25 (0.1 – 18.0) n=17
Haplotype B	0.75 (0.3 – 1.8) n=84	0.82 (0.2 – 4.2) n=28	0.86 (0.3 – 2.5) n=56	0.70 (0.1 – 4.6) n=30	0.89 (0.1 – 12.3) n=15	0.86 (0.04 – 16.9) n=15
Haplotype C	0.44 (0.2 – 1.1) n=84	0.75 (0.1 – 3.9) n=29	0.36 (0.1 – 1.2) n=55	0.17 (0.02 – 1.7) n=28	0.33 (0.03 – 4.4) n=14	-- n=14

[1] Relative risk is presented as the odds ratio (95% CI)

[2] The wild type allele for 2C8*3 is G; the variant allele is A.

[3] The wild type allele for 2C8*4 is C; the variant allele is G.

[4] The wild type allele for 2J2*7 is G; the variant allele is T.

Table 4

Range of Adjusted Relative Risks for Kidney Disease Three Years Post Transplantation Stratified by Genotype

Genotype		Odds Ratios Range
All Eligible Subjects		
2C8 *3	GG + AG vs AA	1.62 – 11.76
2C8*4	GG + CG vs CC	0.95 – 1.97
2J2*7	TT + GT vs GG	0.71 – 1.99
Subjects with Normal Kidney Function Prior to Transplantation		
2C8 *3	GG + AG vs AA	2.05 – 12.99
2C8*4	GG + CG vs CC	0.66 – 1.27
2J2*7	TT + GT vs GG	0.46 – 1.96

Table 5

Steady State Kinetic Values for 14,15-EET Formation by CYP2C8 and Variants

Enzyme	Km (μM)	Vmax (pmol/min/pmol enzyme)	Vmax/Km
CYP2C8.1	5.7	28.1	4.9
CYP2C8.3	14.1	22.6	1.6
CYP2C8.4	3.8	5.8	1.5