Short Communication

CYP3A5 genotype - phenotype analysis in the human kidney reveals a strong site-specific expression of CYP3A5 in the proximal tubule in carriers of the CYP3A5*1 allele

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Running title: CYP3A5 genotype - phenotype correlation in the human kidney

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Abbreviations:
ABCB1, ATP-binding cassette, sub-family B, member 1; RT-PCR, real-time polymerase chain reaction; HMBS, hydroxymethylbilane synthase
Abstract

Inter-individual variability in the drug-metabolizing activity of the cytochrome P450 3A5 enzyme (CYP3A5) is mainly due to a single nucleotide polymorphism in CYP3A5 leading to low expression in homozygous CYP3A5*3/*3 individuals compared to CYP3A5*1 allele carriers. In the human kidney, expression of CYP3A5 has been implicated in blood pressure regulation and calcineurin inhibitor-associated nephrotoxicity. The effect of the CYP3A5*1/*3 polymorphism on the expression level and protein distribution within the human kidney is not well characterized. We therefore performed a genotype-phenotype analysis of CYP3A5 mRNA and protein expression in the human kidney. To this end we analysed sections of normal kidney tissue obtained from 93 Caucasian individuals undergoing nephrectomy by quantitative mRNA expression analysis. Qualitative protein expression analysis of CYP3A5 was performed by immunohistochemistry. Mean renal mRNA expression of carriers of the CYP3A5*1 (n = 12) allele was more than 18-fold higher compared to CYP3A5*3/*3 carriers (n = 81, p < 0.001). Immunohistochemical analysis demonstrated CYP3A5 protein in all epithelia of the nephron in kidney sections with the CYP3A5*3/*3 genotype. In carriers of the CYP3A5*1 allele a strong increase in protein expression of CYP3A5 was detected and this was confined to the proximal tubule. This study confirms a significant effect of the CYP3A5*1/*3 polymorphism on CYP3A5 expression in the normal human kidney and reveals a strong nephron segment-specific difference in the CYP3A5 protein expression limited to the proximal tubule.
Introduction

A striking inter-individual difference in the cytochrome P450 3A5 enzyme (CYP3A5) expression is linked to a single nucleotide polymorphism in CYP3A5 leading to only low expression in homozygous CYP3A5*3/*3 individuals compared to CYP3A5*1 allele carriers (Kuehl et al., 2001; Wojnowski, 2004). Recent data suggested a role for CYP3A5 in salt-sensitive blood pressure regulation and indicated an association with hypertension (Bochud et al., 2006; Eap et al., 2007; Fromm et al., 2005; Givens et al., 2003; Ho et al., 2005; Kivisto et al., 2005; Kreutz et al., 2004; Kreutz et al., 2005; Langaee et al., 2007; Lieb et al., 2006; Zhang et al., 2009). Although controversial results regarding this association have been obtained, it has been hypothesized that CYP3A5 modifies renal tubular sodium reabsorption via protection of the mineralocorticoid receptor from glucocorticoid binding in the distal tubular cells (Fromm et al., 2005; Kreutz et al., 2005). Another hypothesis states a gene-gene-interaction between CYP3A5 and ATP-binding cassette, sub-family B, member 1 (ABCB1), which codes for the multi-drug transporter P-glycoprotein, affecting blood pressure regulation in humans (Eap et al., 2007; Bochud et al., 2009).

So far, a detailed analysis of the genotype-dependent CYP3A5 mRNA expression has been described mainly for liver (Kuehl et al., 2001; Lin et al., 2002) whereas data on genotype-dependent CYP3A5 levels in the human kidney are scarce. Nevertheless, renal CYP3A5 expression has been implicated in calcineurin inhibitor-associated nephrotoxicity (Kuypers et al., 2010; Joy et al., 2007). We therefore performed a genotype-phenotype analysis of CYP3A5 mRNA and protein expression in kidneys from Caucasian individuals.
Materials and Methods

Tissue samples. Kidney samples from 93 patients were obtained during surgical interventions conducted at the Department of Urology, Campus Benjamin Franklin, Charité – Universitätsmedizin Berlin. The kidney samples originated from normal tissue surrounding different kidney tumors (see Table 1 for detailed characteristics of the study cohort). All patients gave written informed consent and the local ethics committee approved the use of patient samples. DNA and RNA of all tissue samples were isolated simultaneously using the QIAGEN® AllPrep DNA/RNA Mini Kit including a DNase digestion step (Qiagen GmbH, Düsseldorf, Germany).

Genotyping and mRNA quantification. Genotyping of CYP3A5 (rs776746) was performed as previously described (Kreutz et al., 2005). Correctness of genotyping results was confirmed by direct sequencing using the ABI Prism 3130 Genetic Analyzer. After reverse transcription of total RNA (n = 93 samples) using the First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany), mRNA expression levels of genes of interest, i.e. CYP3A4 and CYP3A5, were quantified as previously reported (Galvez-Prieto et al., 2008). Details of the primers and probes used for quantitative RT-PCR are summarized in the Supplemental Data (Table 1). To normalize our expression data, we used hydroxymethylbilane synthase (HMBS; also known as porphobilinogen deaminase) as a housekeeping gene. All primers and fluorogenic probes were designed using the Primer Express software and synthesized by TIB Molbiol (Berlin, Germany). Quantification was done using the standard curve method with calculation of the transcript number in each sample and dividing it through the respective HMBS transcript number. Specificity of the products was confirmed by direct sequencing.

Immunohistochemistry. Immunostaining for CYP3A5 protein was performed on paraffin sections using a polyclonal primary antibody directed towards human CYP3A5 (#ab22692, Abcam, Cambridge, UK) and second step amplification by Envision and DAB-plus from DAKO (Glostrup, Denmark), as previously described (Mooyaart et al., 2009). For this purpose 20 different renal biopsies were screened as well as a panel of seven normal human kidneys from Eurotransplant, which had not been transplanted for technical reasons. Further analysis was confined to the kidneys from Eurotransplant. Sections were counterstained with hematoxylin and mounted. Only the second step conjugates were used as negative controls.

Statistical analysis. Data are presented as mean ± S.E.M. Regarding CYP3A5*1 allele carriers (CYP3A5*1/*1 and CYP3A5*1/*3) and CYP3A5*3/*3 individuals, differences in categorical measures were compared using Fisher’s Exact test and differences in continuous measures were analysed using the Mann-Whitney-U test or Student’s t test. All reported p values are two-sided; a p value of less than 0.05 was considered to indicate statistical significance.
Results

No significant differences in gender proportion, mean age, and frequency of hypertension were found between *CYP3A5*/*1* allele carriers and *CYP3A5*/*3/*3* individuals (Table 1). In the hypertensive and normotensive groups of patients we observed allele frequencies of 10.9% and 15.8% for the *CYP3A5*/*1* allele, respectively (p = 0.54). The genotype distribution of all polymorphisms investigated was in Hardy-Weinberg equilibrium. The allele frequencies of the *CYP3A5*/*1* and *CYP3A5*/*3* allele were 7.5% and 92.5%, respectively, and thus similar to those reported previously in a large cohort of Caucasians (Kreutz et al., 2005).

The highest levels of CYP3A5 mRNA were found in *CYP3A5*/*1/*1* individuals (n = 2), intermediate levels in heterozygotes (n = 10) and lowest levels in *CYP3A5*/*3/*3* individuals (n = 81). The comparison between genotype groups indicated a significant effect of the *CYP3A5*/*1* allele on CYP3A5 mRNA levels expressed as CYP3A5 transcript number/HMBS transcript number (*CYP3A5*/*1/*1* individuals: 5.18 ± n.a., *CYP3A5*/*1/*3* individuals: 1.74 ± 0.31 and *CYP3A5*/*3/*3* individuals: 0.28 ± 0.03, p < 0.05 and p < 0.001, respectively; Fig. 1a). Mean CYP3A4 mRNA content was almost five-fold lower than the mean CYP3A5 mRNA level of *CYP3A5*/*3/*3* individuals (0.06 ± 0.02 versus 0.28 ± 0.03 CYP3A4 or CYP3A5 transcript number/HMBS transcript number, p < 0.001).

Immunohistochemistry revealed staining for CYP3A5 protein in all renal epithelia. Compared to kidney samples with a *CYP3A5*/*3/*3* genotype (n = 5), higher expression of CYP3A5 was detected in kidneys of *CYP3A5*/*1* allele carriers (n = 2). This genotype-dependent differential expression was exclusively observed in epithelial cells of the proximal tubule, while no influence of the genotype was observed in distal epithelium, collecting ducts, or glomeruli (Fig. 1b).
So far, a detailed analysis of the genotype-dependent CYP3A5 mRNA expression has been described mainly for liver (Kuehl et al., 2001; Lin et al., 2002) or only a small number of kidney samples (Koch et al., 2002). In this respect, this is the first study showing the strong genotype – phenotype correlation between CYP3A5 genotype and its renal mRNA expression levels on a large set of normal human kidney samples from Caucasian individuals. Regarding the genotype-dependent effects on the CYP3A5 protein content in the human kidney, a significant difference between heterozygous CYP3A5*1 allele carriers and CYP3A5*3/*3 individuals was previously reported in microsomal preparations from 21 human kidneys (Givens et al., 2003). In the current study, immunohistochemical analysis in normal kidneys confirmed the genetically determined variability in renal CYP3A5 expression with an increased staining intensity in CYP3A5*1 allele carriers. Of note, the increased expression observed in CYP3A5*1 allele carriers is restricted to the proximal tubule. Although immunohistochemical localisation of renal CYP3A5 protein has been previously reported in biopsies of transplanted kidneys (Joy et al., 2007; Metalidis et al., 2011), no genotype-dependent differences in the CYP3A5 staining pattern have been detected so far (Metalidis et al., 2011). This discrepancy might result from technical differences including antigen retrieval and subsequent amplification of the first antibody as well as the use of a different primary antibody. Moreover, it should be noticed that the aforementioned studies were performed on transplanted kidneys with and without signs of calcineurin inhibitor-associated nephrotoxicity (Joy et al., 2007; Metalidis et al., 2011). In contrast, we analyzed well preserved normal kidneys prior to transplantation. We cannot exclude the possibility that ischemia reperfusion injury after transplantation may affect the expression level of CYP3A5 in proximal epithelium.

In accordance with other reports (Koch et al., 2002; Givens et al., 2003), we found either no or very low CYP3A4 mRNA expression levels compared to CYP3A5, which consolidates the presumption of CYP3A5 being the predominant CYP3A isoenzyme in the human kidney.

Hepatic CYP3A4 mRNA expression has been shown to be influenced by the ABCB1 2677 polymorphism (Lamba et al., 2006) and an interaction between CYP3A5 and ABCB1 with effects on blood pressure has been reported in individuals of East African descent (Eap et al., 2007; Bochud et al., 2011).
2009). For CYP3A5 mRNA levels in carriers of the CYP3A5*1 allele, we did not observe a significant influence of the ABCB1 TTT-haplotype, which is derived from the three ABCB1 genotypes 1236C>T (rs1128503), 2677G>T (rs2032582), and 3435C>T (rs1045642) (Eap et al., 2007; Bochud et al., 2009) (data not shown). But we realize that the analysed subcohort of 12 individuals is too small to draw negative conclusions; this association necessitates further investigation. The same applies to the observation that no significant association between presence of CYP3A5*1 allele and the diagnosis of hypertension was observed, although numerically the CYP3A5*1 allele frequency was higher in the normotensive group.

In summary, we confirmed a genotype-dependent variation in renal CYP3A5 mRNA content as has been described mainly for liver so far. Furthermore, we were able to show that the morphologic correlative of this relationship is a strong nephron segment-specific difference in the CYP3A5 protein expression limited to the proximal tubule.
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Authorship contributions

Participated in research design: Bolbrinker, Kreutz

Conducted experiments: Bolbrinker, Seeberg, Baelde, and de Heer

Contributed new reagents or analytic tools: Schostak, Kempkensteffen, and de Heer

Performed data analysis: Bolbrinker, Seeberg, de Heer, and Kreutz

Wrote or contributed to the writing of the manuscript: Bolbrinker, Seeberg, Schostak, Kempkensteffen, Baelde, de Heer, and Kreutz
References


Footnotes

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The authors declare that they have no competing financial interests.

Juliane Bolbrinker and Stefanie Seeberg contributed equally to this work.
Legends for Figures

FIG. 1. CYP3A5 genotype-dependent expression analyses. (a) CYP3A5 mRNA according to the respective CYP3A5 genotype group, CYP3A5 mRNA levels are given as total transcript number divided through the respective HMBS transcript number (* $p < 0.05$, *** $p < 0.001$). (b) CYP3A5 protein in the human kidney, immunohistochemical stainings detecting CYP3A5 protein are shown for a sample with heterozygous *1/*3 and homozygous *3/*3 allele status; original magnification 100×. For CYP3A5*1/*3 in comparison to CYP3A5*3/*3, a stronger protein expression can be observed in proximal epithelia.
TABLE 1

*Characteristics of nephrectomy patients as one cohort and according to the presence or absence of the CYP3A5*1 allele*

Data are presented as mean ± S.E.M. RCC = renal cell cancer. *p* values are two-sided.

<table>
<thead>
<tr>
<th>CYP3A5*1 allele status</th>
<th>Total (n = 93)</th>
<th>CYP3A5*1 allele carriers (n = 12)</th>
<th>CYP3A5*3/*3 (n = 81)</th>
<th><em>p</em> value</th>
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<tbody>
<tr>
<td>Male / female (n)</td>
<td>58 / 35</td>
<td>8 / 4</td>
<td>50 / 31</td>
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<tr>
<td>Age (years)</td>
<td>64.3 ± 1.0</td>
<td>61.5 ± 2.4</td>
<td>64.7 ± 1.1</td>
<td>0.271</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>55 (59.1)</td>
<td>6 (50.0)</td>
<td>49 (60.5)</td>
<td>0.540</td>
</tr>
<tr>
<td>Disease</td>
<td>82.8% RCC</td>
<td>83.3% RCC</td>
<td>82.7% RCC</td>
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SUPPLEMENTAL DATA
CYP3A5 genotype - phenotype analysis in the human kidney reveals a strong site-specific expression of CYP3A5 in the proximal tubule in carriers of the CYP3A5*1 allele
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Drug Metabolism and Disposition

Supplemental Table 1: Primers and probes used for quantitative RT-PCR analysis

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer forward (F), reverse (R)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A5*1</td>
<td>F: TCGTCAGGGTCTCTGGAAATTT</td>
<td>AAGTATGGAADAATGTGGGGAACGTATGAAGGT</td>
</tr>
<tr>
<td></td>
<td>R: TCTGATCACGTGGGATCTGG</td>
<td></td>
</tr>
<tr>
<td>CYP3A4 exon primers</td>
<td>F: GAACTGAATGAGAAACCAACAAAGTAAA</td>
<td>TTTGGTCATTGTAATCACTGTGCGTGG</td>
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<tr>
<td></td>
<td>R: CAATGGGCAAAGTCACAGTGGAT</td>
<td></td>
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<tr>
<td>CYP3A4 intron primers</td>
<td>F: TCCTCCTAGTCCAGGGC</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>R: AACTCAATGATTTAATGGGAAA</td>
<td></td>
</tr>
<tr>
<td>HMBD (PBGD)</td>
<td>F: CCACACACACAGCTACTTTCCAA</td>
<td>CCATGCTCTGTAACGGCAATGCGG</td>
</tr>
<tr>
<td></td>
<td>R: ACGCGAATCACTCTCATCTTTTG</td>
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</tr>
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</table>

*a* No specific probe was designed for this assay; instead, we used SYBR Green for unspecific DNA intercalation.