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ORIGINAL ARTICLE

The recipient CYP2D6 allele 4-associated poor metabolizer status correlates with an early fibrosis development after liver transplantation

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Conflicts of Interest
The authors declare that there is no conflict of interest.

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Summary

CYP2D6 is part of the cytochrome P450 system, which catalyzes biotransformation of endogenous substrates and xenobiotics. Approximately 10% of the Caucasian population has two null alleles, resulting in a poor metabolizer (PM) status. Mostly, allele four (CYP2D6*4) is responsible for the PM status, which is suspected to be associated with an accelerated fibrosis progression (FP). The aim of the present study was to analyze the role of the CYP2D6*4 genotype for FP after liver transplantation (LT). Genotypes were determined in liver biopsies (donor) and peripheral blood (recipient) by fluorescence resonance energy transfer. Data were correlated with clinical variables and risk factors for fibrosis. We analyzed 517 LTs performed between 1997 and 2009. Overall donor and recipient allele frequencies were comparable (18.0%, 20.5%; $P = 0.43$). The donor genotype did not correlate with FP. In contrast, recipients carrying CYP2D6*4, showed a significant higher risk for an accelerated FP ($P = 0.011$) in HCV positive ($P = 0.038$) and HCV negative patients ($P = 0.033$). Results were confirmed by multivariate analysis (Hazard ratio 1.65; $P = 0.001$). The CYP2D6*4-associated PM status of the donor liver seems to have no influence on FP after LT. Recipients, carrying the allele, have an elevated risk for an accelerated FP.

Introduction

Recurrence of the underlying disease is a frequent complication after liver transplantation (LT). Recurrent cirrhosis (RC) occurs from 20% up to 40% of patients with hepatitis C within 5 years after LT [1,2]. Despite a high number of clinical risk factors including donor-/recipient-variables, complications and interventional factors, mainly immunosuppression and the increasing donor age, are held responsible for the accelerated fibrosis progression after LT [1]. Why some patients develop a recurrent cirrhosis within the first years after LT and others show no significant fibrosis in the five-year protocol biopsy cannot only be explained by these clinical risk factors [3]. To

date, no pretransplant molecular markers exist, which are predictive of fibrosis progression after LT and could be considered during organ allocation.

CYP2D6 is part of the cytochrome P450 system, which catalyzes biotransformation of endogenous substrates and up to 20% of xenobiotics [4]. Genetic polymorphisms of P450s with modified activities have been identified, explaining the differences in the metabolism of various compounds among the population [5]. CYP450s polymorphisms have already been shown to be associated with various liver diseases, e.g. the association between the genetic polymorphism of CYP2E1 and the progression of alcoholic liver disease [6].

CYP2D6 enzymes are located in the endoplasmic reticulum of liver cells and have been associated with the metabolism of arachidonic acid (AA) [7]. It was shown that AA is involved in the regulation of collagen production by hepatic stellate cells [8]. Furthermore, molecular mimicry has been described: CYP2D6 has epitopes for ''liver kidney mikrosome type 1'' (LKM-1) autoantibodies not only in patients with autoimmune hepatitis (AIH) type 2 but also in 10% of HCV positive patients, whereas these LKM-1 detect other epitopes as LKM-1 from AIH type 2 patients [9–11]. Neutralization of these antibodies is possible by incubation with epitopes of HCV-components (RNA-dependant DNA-polymerase NS5) and CMV (alkaline exonuclease) [12]. A further sequence homology has been reported between epitopes of CYP2D6 and the infected cell protein 4 (ICP4) of HSV-1 [13]. Therefore, CYP2D6 presents a link between endogenous and xenobiotic metabolism, autoimmunity (AIH) and viruses (HCV) as inducers of human liver disease.

The CYP2D6 gene displays a high inter-individual variability: more than 80 different alleles have been identified [4]. Approximately 10% of the Caucasian population has two null alleles, resulting in a poor metabolizer (PM) status [4]. The major variant allele, which lacks the enzyme activity in the Caucasian population is allele 4 (CYP2D6*4), which occurs with an allele frequency of about 20–25% and is responsible for 70–90% of all PMs [4,5,14]. The frequency of the other two common PM alleles, *3 and *5, together is much lower (2%) [14]. In previous studies, CYP2D6 polymorphism has been shown to be associated with various carcinogenic processes, such as cancer of the lung or larynx [15], and with hepatocellular carcinoma (HCC), particularly in HCV patients [16,17]. Moreover, in chronic hepatitis patients, different frequencies of the PM alleles were found in cirrhotic patients versus non-cirrhotic patients [16,17]. Fishman *et al.* found CYP2D6*4 to be associated with an accelerated fibrosis progression in the nontransplant hepatitis C population [18]. Therefore, the question whether the CYP2D6*4 allele has any effect on the fibrosis after LT emerged.

The aim of our study was to elucidate the impact of the major PM allele (CYP2D6*4) on fibrosis after LT.

Patients and methods

Study population

Between 1997 and 2009, 517 transplantations in 465 caucasian patients who underwent deceased donor LT were performed at the University of Mainz, Germany. Samples used for DNA analysis and clinical data on these patients were entered prospectively and reviewed retrospectively. Clinical data were collected from electronic medical records and patient charts: recipient- and donor-specific variables, patient history, physical examination and biochemical blood tests were obtained monthly during the first six months after LT and at least every 3–6 months within the first 5 years after LT depending on the clinical status.

We included recipient characteristics, donor characteristics, underlying diseases, immunosuppression-related variables, virological variables, treatment parameters and the CYP2D6*4 genotype. No hepatitis C positive organ has been transplanted. In hepatitis C patients, interferon (IFN)-therapy was initiated within 2 weeks after histological confirmation of diagnosis of a recurrent hepatitis C and administered for 48 weeks. No patient was treated preemptively. Pegylated interferon alpha 2a (PEG-IFNα-2a) was given as 135–180 μg/week in combination with ribavirin (800–1200 mg/day) according to individual tolerability. The ribavirin dosage was then modified according to the hemoglobin levels. Patients were followed up for 24 weeks after the completion of the therapy.

Acute rejections were treated with steroids (500 mg methylprednisolone for 3 days).

Informed consent was given by each patient. The study followed the ethical guidelines of the Declaration of Helsinki and was approved by the local ethics committee.

Histopathology

Liver biopsies in our transplant center are routinely performed before implantation, 1 and 5 years after LT. Additional liver biopsies were performed whenever a biochemical or clinical abnormality with signs of graft dysfunction was detected to diagnose recurrence of underlying disease or to rule out rejection. Hepatic fibrosis (stage) was assessed by the semi-quantitative histological score described by Desmet *et al.* [19]. The degree of fibrosis was staged using a scale of 0–4 (F0: absent; F1: mild portal fibrosis without septa; F2: moderate periportal fibrosis with few septa; F3: numerous septa without cirrhosis; F4: cirrhosis). Significant fibrosis progression was presumed when a difference of at least one fibrosis stage

occurred between the biopsies of a patient (previous biopsy after LT or null biopsy) according to the score published by Desmet *et al.* [19] ($\Delta \geq$ F1). As many patients got additional liver biopsies and all patients got a histology at 1 year after LT according to our follow-up protocol, during this time, no significant fibrosis should be overseen. This allows a relatively exact determination whether an early fibrosis progressed after LT. Overall, 1536 liver biopsies were retrospectively analyzed for fibrosis.

CYP2D6*4 genotyping using FRET analysis

Genomic DNA from the recipient was extracted from peripheral blood by a salting-out procedure (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). Donor DNA was extracted from pre-implantation liver biopsies (QIAamp DNA Mini Kit; Qiagen).

CYP2D6*4 genotype was detected by a real-time PCR method, using Fluorescence energy transfer (FRET) analysis. Probes were measured using a lightCycler instrument (Roche Diagnostics, Basel, Switzerland) as previously described [20]. The fluorogenic adjacent hybridization probes were designed and produced by TIB-MOLBIOL (Berlin, Germany). Sequences for PCR oligonucleotides were 5′-ACCCCGTTCTGTCTGGTGT-3′ (sense) and 5′-CCGAGAGCATACTCGGGAC-3′ (anti-sense) for the CYP2D6*4 allele. The key mutation is a G to A transition, which causes a shift of the consensus acceptor splice site of the third intron by one base, thereby resulting in a spliced mRNA with one additional base that has an altered reading frame and a premature stop codon. Probes were designed that their melting temperatures (T_m) were marginally higher than the T_m of the primers. The anchor probe was labeled with fluorescein (X) at the 3′ end (5′-GAAGGCGACCCCTTACCCGCATCT-X-3′) and the sensor was labeled with the LightCycler Red 705 (LC-Red 705) at the 5′ end (5′-LC-Red 705-ACCCCC **AA**GACGCCCCT phosphorylated; the detected single nucleotide polymorphism is written in bold). The probe recognized adjacent sequences with the shorter probe lying over the mutation site and the probe was separated by three bases. Fluorescein was used as the donor fluorophore and blocked extension from the probe during PCR. LC-Red 705 was used as acceptor of the FRET process with its 3′ end phosphorylated to block extension. The greater stability of the longer anchor probe meant that loss of the fluorescence occurred as the shorter probe melted off the template.

PCR was performed with 400 nM CYP2D6 primers in a standard PCR reaction including a 200 nM anchor and 200 nM sensor hybridization probe, 100 ng DNA, 4.0 mM $MgCl_2$, and 2 μl LightCycler DNA master hybridization mix in a total of 20 μl. The reaction started at 95 °C for 30 s and amplification was done for 50 cycles of denaturation (95 °C, 0 s, ramp rate 20 °C/s), annealing (60 °C, 7 s, ramp rate 20 °C/s), and extension (72 °C, 20 s, ramp rate 20 °C/s).

PCR product identification was performed by analysis of DNA melting curves in the glass capillary (LightCycler Fast Start DNA Master Hybridization Probes; Roche Diagnostics, Basel, Switzerland). DNA was denaturated at 95 °C for 30 s and maximal fluorescence was acquired by holding the reaction at 45 °C for 30 s. Data for the melting curves were generated by heating slowly to 80 °C with a ramp rate of 0.1 °C/s and were collected continuously during that time. When the shorter probe melted off the template, FRET no longer took place and fluorescence was converted to melting peaks using software that plotted the negative derivative of fluorescence with respect to temperature ($-dF/dT$ ν T). The sequence-specific hybridization probes melt off the target sequence at a characteristic temperature: 61 °C (wildtype) and 68 °C (mutation).

Statistical analysis

Data management and all statistical analyses were performed with the SPSS program (SPSS Inc., Chicago, IL, USA). All quantitative variables were expressed as mean ± standard deviation (SD). For categorical variables, between-group differences were analyzed by the chi-square or Fisher's exact test. Between-group differences for quantitative variables were calculated using the Student's *t*-test. All tests were performed using a 5% level of significance (two-sided). Fibrosis free time was calculated using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazard regression models were used to estimate the influence of potential risk factors on fibrosis. Hazard ratios (HR) were computed univariately for each potential risk factor, and accompanied with 95% confidence intervals. A multivariate model was developed using a stepwise process, including terms with $P < 0.05$.

Results

Patient characteristics

In total, 517 LTs in 465 patients were analyzed. Characteristics of the study population are presented in Table 1. Of the 465 patients, 373 (80%) were genotyped. The selection process is shown in Fig. 1. In total, 407 of the 517 transplantations (79%) were eligible for analysis.

The mean age was 54 years, 64% of patients were male and 36% female. HCV was present in 119 of the 465 LT patients (25.6%). The remaining patients were transplanted for hepatitis B virus ($n = 63$; 13.5%), alcoholic

Table 1. Patient characteristics.

Characteristics	Total (n = 517)	Genotyped (n = 407)
Recipient		
CYP2D6 (Wildtype/heterozygous/homozygous) (%)	262/123/22 (64/30/5)	262/123/22 (76/30/5)
Age at LT (years) (range)	54 (16–74)	54 (16–74)
Gender (male/female) (%)	333/184 (64/36)	264/143 (65/35)
Body mass index (BMI) at LT (range)	25 (13–48)	25 (13–47)
UNOS status at LT (before 2007) (HU/T2/T3) (%)	48/177/174 (12/44/44)	30/148/143 (9/46/45)
Lab-MELD score at LT (after 2007) (range)	23 (6–40)	21 (6–40)
CMV-IgG (negative/positive) (%)	152/348 (30/70)	126/271 (32/68)
HCC (yes/no) (%)	177/340 (34/66)	140/267 (34/66)
HCV (yes/no) (%)	133/384 (26/74)	98/309 (24/76)
Donor		
CYP2D6 Donor (Wildtype/heterozygous/homozygous) (%)	99/30/10 (71/22/7)	83/27/7 (71/23/6)
Age (years) (range)	50 (8–81)	50 (8–81)
Gender (male/female) (%)	274/241 (53/47)	211/196 (52/48)
CMV-IgG (negative/positive) (%)	212/294 (42/58)	162/236 (41/59)
Donor–recipient constellation (%)		
Donor–recipient–gender combination (mm, ww, mw, wm)	201/110/131/73 (39/21/25/14)	159/91/105/52 (39/22/26/13)
Gender discongruency (no/yes)	384/131 (75/25)	250/157 (61/39)
CMV high risk constellation at LT	413/82 (83/17)	327/66 (83/17)
(Donor CMV-IgG positive – Empfänger CMV-IgG negative) (no/yes)		
Transplantation		
Cold ischemia time (min) (range)	618 (30–1113)	620 (30–1080)
Initial immunosuppression (%)		
Tacrolimus/Cyclosporin A	434/78 (85/15)	351/56 (86/14)
MMF (no/yes)	342/170 (67/33)	176/131 (68/32)
Methylprednisolon (no/yes)	56/456 (11/89)	51/356 (12/88)
Follow-up		
Steroid bolus after LT (no/yes) (%)	369/148 (71/29)	281/126 (96/31)
Number of steroid boli at rejection (range)	0 (0–6)	0 (0–6)
Steroid bolus after LT at rejection (no/1/≥2) (%)	369/98/50 (71/19/10)	281/84/42 (69/21/10)
Follow-up post-LT (days) (range)	1142 (0–4554)	1381 (0–4554)

HCC, hepatocellular carcinoma; LT, liver transplantation.

cirrhosis (n = 126; 27.1%), PSC (n = 17; 3.7%), primary biliary cirrhosis (PBC, n = 19; 4.1%), AIH (n = 9; 1.9%), cryptogenic cirrhosis (n = 30; 6.5%), familial amyloidosis (n = 20; 4.3%), acute liver failure (n = 12; 2.6%), hemochromatosis (n = 22; 4.7%), Wilson's disease (n = 6; 1.3%); Alpha1-antitrypsin deficiency (n = 8; 1.7%), Budd-Chiari syndrome (n = 4; 0.9%) and other rare causes (n = 10; 2.2%). Thirty-five percent (n = 164) of patients had a history of HCC at the time of transplantation. Sixty-one of the 407 genotyped patients suffered from biliary complications without any difference between genotypes (P = 0.89). In total, 54 of 119 HCV-positive patients underwent interferon therapy. Of these patients, only 13 reached an SVR (24%). Ninety-eight of the 119 HCV-positive patients were genotyped (70 wildtypes, 28 CYP2D6*4 carriers), forty-eight of whom received an IFN-therapy (34 of 70 wildtypes, 14 of 28 CYP2D6*4 carriers).

Mean time of patient follow up (n = 465) after LT was 4.5 ± 3.5 years (range 0–12.9 years) with 1 and 5 year patient survival rates of 79% and 64% and allograft survival rates of 74% and 57%, respectively.

Higher frequency of CMV-high-risk constellation among CYP2D6 WT recipients

Of 465 patients transplanted, DNA was available for genotyping from 373 recipients (80%) and 139 donors (27%) (Table 1). Allele frequency was in Hardy Weinberg equilibrium among donors and recipients. One hundred and twenty-three heterozygous and 22 homozygous recipients for CYP2D6*4 were detected with an overall allele frequency of 20.5%. The allele frequency in HCV positive recipients (n = 98) was 16.8% compared to 21.7% in HCV negative recipients (n = 309; P = 0.19). Donor-allele frequencies were comparable to 18.0% (P = 0.43). Table 2 shows the demographic and clinical characteristics based on the recipient CYP2D6*4 genotype. The only difference between the genotypes was a higher rate of CMV-IgG negative donors and therefore a lower proportion of

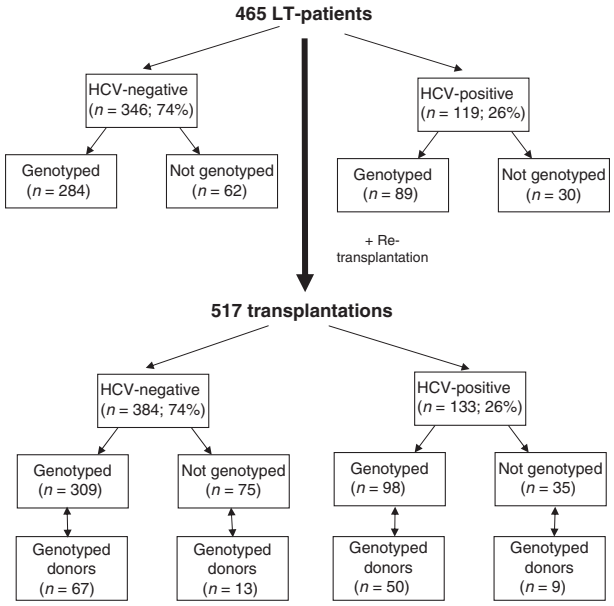

Figure 1 Distribution of genotyped patients and livers in HCV positive and negative patients.

CMV high risk constellation at LT (donor CMV IgG positive – recipient CMV IgG negative) in CYP2D6*4 carriers. There were no differences in allelic distribution between HCV positive and negative patients (Fig. 2).

Accelerated fibrosis development among CYP2D6*4 carriers compared with WT patients within 5 years after LT

Risk factors for fibrosis progression in our patients were identified. We examined liver biopsies and found 49% of transplanted organs ($n = 517$) to develop a fibrosis within 5 years after LT. The main risk factors in our whole study population were HCV-infection ($P < 0.001$) and the CYP2D6*4 genotype of the recipient ($P = 0.016$); Table 3. A donor age >50 years nearly reached significance ($P = 0.057$).

In the hepatitis C subgroup, patients who received multiple steroid boluses, Cyclosporine and immunosuppressive therapy without combination with MMF were at increased risk for fibrosis development. There was no statistically significant difference between IFN-treated and

Table 2. Comparison wildtype – CYP2D6*4 carrier.

	Total ($n = 407$)	Wildtype ($n = 262$)	CYP2D6*4 carrier ($n = 145$)	*P*-value
Recipient				
HCV-infection (no/yes) (%)	309/98 (74/26)	192/70 (73/27)	117/28 (81/19)	NS (0.12)
Age at LT (years) (range)	54 (16–74)	54 (16–74)	55 (18–71)	NS (0.82)
Gender (m/w) (%)	264/143 (65/35)	170/92 (65/35)	94/51 (65/35)	NS (1.00)
Body mass index at LT (range)	25 (13–47)	25 (13–47)	25 (16–35)	NS (0.45)
CMV-IgG (negative/positive) (%)	126/271 (32/68)	85/171 (33/67)	41/100 (29/71)	NS (0.43)
HCC (no/yes) (%)	267/140 (66/34)	174/88 (66/34)	93/52 (64/36)	NS (0.66)
Donor				
CYP2D6 (Wildtype versus CYP2D6*4 carrier) (%)	83/34 (71/29)	54/24 (69/31)	29/10 (74/26)	NS (0.67)
Age (years) (range)	50 (8–81)	50 (13–81)	50 (8–81)	NS (0.61)
Gender (m/w) (%)	211/195 (52/48)	130/131 (50/50)	81/64 (56/44)	NS (0.26)
CMV-IgG (negative/positive) (%)	162/236 (41/59)	96/163 (37/63)	66/73 (48/52)	**0.054**
Donor–recipient constellation				
Donor–recipient–gender combination (mm, ww, mw, wm) (%)	159/90/105/52 (39/22/26/13)	98/59/72/32 (38/23/28/12)	61/31/33/20 (42/21/23/14)	NS (0.67)
Gender discongruency (no/yes) (%)	301/105 (74/26)	189/72 (72/28)	112/33 (77/23)	NS (0.34)
CMV high-risk constellation at LT (no/yes) (%)	327/66 (83/17)	200/55 (78/22)	127/11 (92/8)	**<0.001**
Transplantation				
Cold ischemia time (min) (range)	620 (30–1080)	620 (30–1035)	622 (60–1080)	NS (0.98)
Initial immunosuppression (%)				
Tacrolimus/Cyclosporin A	351/56 (86/14)	227/35 (87/13)	124/21 (85/15)	NS (0.77)
MMF (no/yes)	276/131 (68/32)	180/82 (69/31)	96/49 (66/34)	NS (0.66)
Methylprednisolon (no/yes)	51/356 (13/87)	38/224 (15/85)	13/132 (9/91)	NS (0.12)
Follow-up				
Steroid bolus after LT (no/yes) (%)	281/126 (69/31)	184/78 (67/33)	97/48 (67/33)	NS (0.50)
Steroid bolus after LT at rejection (no/1/≥2) (%)	281/84/42 (69/21/10)	184/53/25 (67/21/12)	97/31/17 (67/21/12)	NS (0.73)
Biliary complications ITBL/IBL (no/yes) (%)	346/61 (85/15)	223/39 (85/15)	123/22 (85/15)	NS (0.89)
Follow-up post-LT (days) (range)	1381 (0–4554)	1419 (0–4554)	1296 (2–4330)	NS (0.65)

HCC, hepatocellular carcinoma; LT, liver transplantation; NS, nonsignificant. Bold value indicates statistically significant ($P < 0.05$).

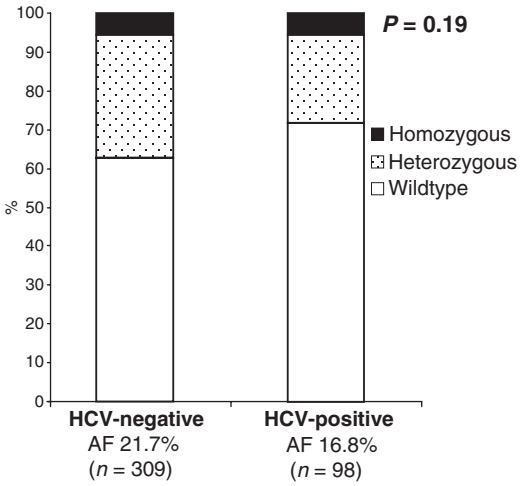

Figure 2 Allele frequencies (AF) of CYP2D6*4 in HCV positive and negative patients.

untreated patients ($P = 0.25$). In the hepatitis C negative subgroup, patients with HCC developed less fibrosis within 5 years after LT (30% vs. 49%; $P = 0.004$). Patients positive for CMV at transplantation (CMV-IgG) developed fibrosis more frequently (54% vs. 39%; $P = 0.035$). The CYP2D6*4 carrier status was the only risk factor associated with fibrosis in the hepatitis C positive and the hepatitis C negative subgroups ($P = 0.032$; $P = 0.021$).

Homozygous CYP2D6*4 individuals showed a much faster recurrence of fibrosis compared with heterozygous patients or persons not carrying the allele (Fig. 3).

In a multivariate analysis of all transplanted patients, HCV-infection is the most important risk factor for fibrosis after LT ($P < 0.001$), followed by the CYP2D6-genotype ($P = 0.001$) and the donor age ($P = 0.027$) (Table 4). In HCV-negative recipients, the CYP2D6-genotype ($P = 0.014$), the CMV status of the recipient

Table 3. Risk factors for fibrosis development within 5 years after liver transplantation (univariate analysis).

	Total	No fibrosis within 5 years after LT (%)	*P*-value	HCV-negative	No fibrosis within 5 years after LT (%)	*P*-value	HCV-positive	No fibrosis within 5 years after LT (%)	*P*-value
Recipient									
Transplantations	517	49		384	56		133	26	
HCV-infection (yes/no)	133/384	26/56	**<0.001**						
Age at LT (<50 years/ >50 years)	168/349	47/50	NS (0.81)	124/260	58/57	NS (0.55)	44/89	21/32	NS (0.19)
Gender (male/female)	333/184	48/50	NS (0.83)	240/144	59/53	NS (0.22)	93/40	21/38	**0.069**
Body mass index at LT (<25/>25)	259/244	46/53	NS (0.77)	183/191	54/61	NS (0.82)	76/53	29/25	NS (0.27)
HCC (no/yes)	340/177	45/56	NS (0.13)	271/113	51/70	**0.004**	69/64	27/25	NS (0.88)
CYP2D6 (Wildtype/ Allele 4 carrier status)	262/145	56/42	**0.016**	192/117	64/47	**0.021**	70/28	35/27	**0.032**
CMV-IgG (positive/negative)	348/152	41/52	NS (0.18)	255/115	61/46	**0.035**	93/37	27/17	NS (0.64)
Donor									
Age (<50 years/>50 years)	255/262	53/45	**0.057**	187/197	62/53	**0.077**	68/65	33/22	NS (0.24)
Gender (male/female)	274/241	47/51	NS (0.52)	202/180	54/58	NS (0.65)	72/61	23/29	NS (0.83)
CMV-IgG (positive/negative)	294/212	52/44	NS (0.44)	219/155	61/49	NS (0.18)	75/57	23/32	NS (0.32)
CYP2D6 (Wildtype/ Allele 4 carrier status)	99/40	41/52	NS (0.26)	54/26	54/69	NS (0.30)	45/14	26/31	NS (0.26)
Donor–recipient constellation									
CMV-high-risk (yes/no)	413/82	45/50	NS (0.40)	61/304	51/58	NS (0.27)	21/109	20/27	NS (0.99)
Gender discongruency (no/yes)	312/205	47/53	NS (0.30)	238/146	55/60	NS (0.41)	74/59	21/35	NS (0.15)
Initial immunosuppression									
Tacrolimus/Cyclosporin A	434/78	51/41	NS (0.35)	320/61	57/52	NS (0.94)	114/17	31/6	**0.011**
MMF (yes/no)	170/342	59/47	NS (0.61)	119/262	61/57	NS (0.45)	51/80	54/18	**0.008**
Methylprednisolon (yes/no)	456/56	49/48	NS (0.28)	334/47	58/51	**0.072**	122/9	25/33	NS (0.92)
Follow-up									
Steroid bolus after LT (no/yes)	369/148	52/43	NS (0.19)	275/109	59/52	NS (0.41)	94/39	32/16	NS (0.13)
Steroid bolus after LT at rejection (<2/≥2)	467/50	50/42	NS (0.35)	345/39	57/53	NS (0.70)	122/11	30/0	**0.041**

The first percentage value corresponds to the first variable, the second percentage value to the second in each table box.
HCC, hepatocellular carcinoma; LT, liver transplantation; NS, nonsignificant. Bold value indicates statistically significant ($P < 0.05$).

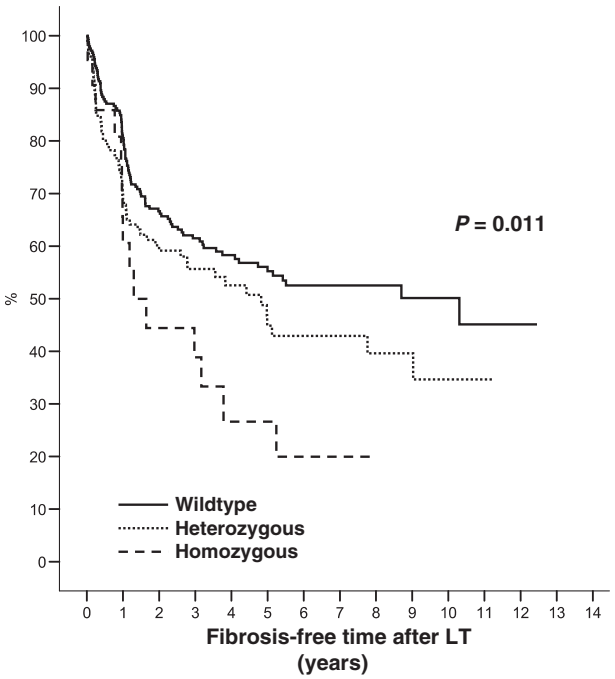

Figure 3 Fibrosis development after liver transplantation (LT) according to CYP2D6*4 recipient genotype.

Table 4. Cox proportional hazard model of variables associated with fibrosis development within 5 years after liver transplantation.

Covariates	Hazard ratio	95% CI	*P*-value
HCV-infection	2.69	1.93–3.76	<0.001
Donor age	1.01	1.00–1.02	0.027
CYP2D6*4 recipient	1.65	1.22–2.25	0.001

before LT ($P = 0.019$) and the absence of a HCC ($P = 0.004$) present the greatest risk factors, whereas in HCV-positive recipients, the CYP2D6-genotype remains the only risk factor ($P = 0.024$).

Results were re-evaluated and confirmed by considering only advanced fibrosis stages ≥F2 ($P = 0.041$; Fig. S1). However, 5 years after LT, heterozygous patients had a similar occurrence of advanced fibrosis compared to wild-type patients.

The CYP2D6*4 recipient genotype is an independent predictor for fibrosis development after LT. Irrespective of their HCV status, recipients of CYP2D6*4-mutated livers showed no differences in fibrosis or graft survival compared with recipients transplanted with WT livers.

However, we found no statistically significant difference for recurrent cirrhosis ($P = 0.88$), in graft or patient survival between CYP2D6*4 mutant (5.7 ± 1.5 year) and WT recipients (6.4 ± 0.5 year). The combination of different risk factors, e.g. the donor age and the CYP2D6*4 genotype, allows a more precise prediction of fibrosis progression (Fig. S2).

Discussion

The results provide evidence that CYP2D6*4, the PM genotype of CYP2D6, is associated with an accelerated rate of fibrosis development after LT. Patients carrying the CYP2D6*4 allele have significantly higher rates of fibrosis within five years after transplantation compared with wild type recipients (Table 3, Fig. 3 and Fig. S1). However, there was no significant difference between the allele frequencies in the HCV positive and negative subgroups (Table 3, Fig. 2). Moreover, it was demonstrated by uni- and multivariate analyses that the CYP2D6*4 carrier state is the only covariate independently from the HCV infection that harbored a higher risk for fibrosis development (Tables 3 and 4). In our study population, the CYP2D6*4 genotype was even more important for fibrosis than the donor age, which nearly reached significance.

Allele frequencies in all recipients were 20.5%. Donors, representing a normal/healthy population had 18.0% allele frequency, whereas it can be argued that it is not to rule out that organs carrying the CYP2D6*4 genotype, which has been shown to be associated with liver diseases and fibrosis progression in hepatitis C patients, could have been more often not allocated or withdrawn, e.g. because of an advanced fibrosis.

Our results are consistent with published data by Sachse *et al.* reporting 20.7% CYP2D6*4 allele frequency in 589 German volunteers [14]. Interestingly, in our HCV-positive and negative patients, we found no significant differences in the allele distribution between the two groups (16.8% and 21.7%; $P = 0.19$; Fig. 2). Previous studies showed an association between the CYP2D6 polymorphism and cirrhosis in HCV patients and HCC in patients with chronic liver disease in the nontransplant setting [16,17]. Silvestri *et al.* examined the prevalence of various genotypes of CYP2D6 in four groups of patients with HCV: asymptomatic carriers, patients with active hepatitis, cirrhotic patients and patients with HCC. The frequency of PM alleles (CYP2D6*4 and CYP2D6*3) was significantly higher in asymptomatic carriers and in patients with hepatitis than in the other two groups. These results in the nontransplant setting are in contrast to our results after LT with a higher rate of fibrosis in patients harboring the CYP2D6*4 carrier state. Another study by Kimura *et al.* examined the association between PBC and polymorphisms of various genes, which are involved in xenobiotic metabolism [21]. They found the presence of CYP2D6*4 allele indicated slightly higher risk for more advanced disease, reflected in a higher

prevalence of ascites ($P = 0.031$). These facts suggested a role for CYP2D6 in hepatic disease severity and its deterioration towards cirrhosis. Fishman *et al.* compared CYP2D6*4 allele frequencies in nontransplanted hepatitis C patients with fast and low fibrosis progression according to Poynard's fibrosis progression curves [18]. Consistent with our results, they found a positive correlation of the CYP2D6*4 carrier state with fast fibrosis progression with an odds ratio of 6.5 ($P = 0.01$).

Interestingly, we found only the recipient CYP2D6*4 carrier state associated with fibrosis. As in our study population, the donor genotype, and therefore the liver genotype, does not play a role in fibrosis, it can be argued that the fibrosis development is not caused by poorly metabolized substrates by the liver itself. This is consistent with the data by Bendriss *et al.*, who found no change in CYP2D6 PM phenotype after LT [22]. Moreover, Carcillo *et al.* showed that the transcriptional regulation of intrahepatic and extrahepatic CYP2D6 mRNA (from peripheral blood mononuclear cells) after LT is coordinated, possibly through a mechanism that is predominantly extrahepatic [23].

Interestingly, the only significant difference between CYP2D6*4 carriers and WT patients in our study population is the CMV state of the donor with a lower proportion of CMV-positive patients in CYP2D6*4 carriers. This is only to explain by chance or immunological phenomena.

Whether molecular mimicry, which is described between epitopes of CYP2D6, HCV, HSV-1 and CMV could be involved in the accelerated fibrosis progression after LT is an open question. The development of LKM1-autoantibodies against CYP2D6 without evidence of autoimmune or viral hepatitis associated with a transplant rejection episode in a patient transplanted for Wilson's disease suggests immunological mechanisms of rejection together with hepatocellular injury as a pathogenetic mechanism [24].

In the hepatitis C subgroup, patients who received multiple steroid boluses, Cyclosporine and immunosuppression without MMF were at increased risk for fibrosis development. This is explained by the fact that most of these patients were transplanted in the period 1990s during a time when fibrosis in hepatitis C patients dramatically increased due to more potent immunosuppression, antibodies, and use of steroids [1]. In the hepatitis C negative subgroup, patients with HCC before LT developed less fibrosis within 5 years post-transplantation (30% vs. 49%), whereas there was no difference between allele frequencies in HCC (Table 2). As HCC is the most frequent indication for LT without presence of an end-stage liver cirrhosis, one can speculate that these patients harbor a less aggressive underlying fibrogenic disease.

We did not examine the other two PM alleles (*3 and *5) in light of their low frequencies in the Caucasian population. In other studies, the frequencies of these two alleles were too low to establish meaningful statistical results [21]. CYP2D6*4 might serve as a genetic non-invasive marker, enabling identification of patients who are prone to develop fibrosis early in the course after LT. In spite of the small sample size of some underlying disease, e.g. PBC, our results should be verified in large study groups in the future. Further efforts should be made to elucidate the pathomechanism by which CYP2D6 is involved in fibrosis development. Our results indicate a pathophysiological role for CYP2D6-associated fibrosis outside the liver, possibly by involvement of de novo molecular mimicry between viral structures (e.g. CMV epitopes), CYP2D6 and/or arachidonic acid metabolism after LT.

In conclusion, our results show, for the first time, an association between the PM allele CYP2D6*4 and fibrosis development after LT. The combination of different markers and risk factors may allow a better prediction of the individual risk for fibrosis progression after LT (Fig. S2). Genome wide association studies of donors and recipients will be necessary to establish such models.

Authorship

TZ: designed research, collected and analyzed data, wrote the paper. MHL: collected and analyzed data, performed statistical analysis. AK and AL: performed DNA extraction and genotyping. MH, DF, and NW: collected liver specimen and blood samples. SB: performed histological evaluation of fibrosis. IS: performed statistical analysis. PRG, GO, and MS: made critical review of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Time until first diagnosis of significant and advanced fibrosis (≥F2) after liver transplantation (LT) according to CYP2D6*4 recipient genotype.

Figure S2 Fibrosis development after liver transplantation (LT) according to CYP2D6*4 recipient genotype and donor age.

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