A functional \textit{TGFB1} polymorphism in the donor associates with long-term graft survival after kidney transplantation

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\section*{ABSTRACT}

\textbf{Background.} Improvement of long-term outcomes in kidney transplantation remains one of the most pressing challenges, yet drug development is stagnating. Human genetics offers an opportunity for much-needed target validation in transplantation. Conflicting data exist about the effect of transforming growth factor-beta 1 (TGF-\(\beta\)1) on kidney transplant survival, since TGF-\(\beta\)1 has pro-fibrotic and protective effects. We investigated the impact of a recently discovered functional \textit{TGFB1} polymorphism on kidney graft survival.

\textbf{Methods.} We performed an observational cohort study analysing recipient and donor DNA in 1271 kidney transplant pairs from the University Medical Centre Groningen in The Netherlands, and associated a low-producing \textit{TGFB1} polymorphism (rs1800472-C \(>\) T) with 5-, 10- and 15-year death-censored kidney graft survival.

\textbf{Results.} Donor genotype frequencies of rs1800472 in \textit{TGFB1} differed significantly between patients with and without graft loss (\(P = 0.014\)). Additionally, the low-producing \textit{TGFB1} polymorphism in the donor was associated with an increased risk of graft loss following kidney transplantation (hazard ratio = 2.12 for the T-allele; 95\% confidence interval 1.18–3.79; \(P = 0.012\)). The incidence of graft loss within 15 years of follow-up was 16.4\% in the CC-genotype group and 31.6\% in the CT-genotype group. After adjustment for transplant-related covariates, the association between the \textit{TGFB1} polymorphism in the donor and graft loss remained significant. In contrast, there was no association between the \textit{TGFB1} polymorphism in the recipient and graft loss.

\textbf{Conclusions.} Kidney allografts possessing a low-producing \textit{TGFB1} polymorphism have a higher risk of late graft loss. Our study adds to a growing body of evidence that TGF-\(\beta\)1 is beneficial, rather than harmful, for kidney transplant survival.

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INTRODUCTION

Short-term outcomes following kidney transplantation have dramatically improved in the past 25 years, adding over one million life-years to patients in the USA alone. Nonetheless, improving the long-term transplant outcomes remains a crucial challenge. The alloimmune response is recognized as a major contributor to late kidney transplant failure. Furthermore, cytokines play a pivotal role in orchestrating the immune response. Understanding the contribution of cytokines to donor-recipient incompatibility in kidney transplantation, therefore, is crucial as it can lead to the development of novel treatment strategies.

Transplantation is a unique situation from a genetic and an immunological perspective, as two genomes are brought together. Genetic differences between the donor and recipient subsequently lead to immunological injury. Although the recipient primarily drives the alloimmune response, the release of inflammatory triggers by the donor kidney is gaining traction as an essential additional mechanism. Genetic differences between the donor and recipient subsequently lead to immunological injury. The recipient primarily drives the alloimmune response, the release of inflammatory triggers by the donor kidney is gaining traction as an essential additional mechanism.

Among all cytokines, transforming growth factor-beta (TGF-β) is a multifaceted and functional cytokine that is synthesized by nearly every cell type. To date, three main isoforms of TGF-β have been identified in humans (i.e. TGF-β1, TGF-β2 and TGF-β3), which are encoded by distinct genes (TGFB1, TGFB2 and TGFB3, respectively). Between these, TGF-β1 is the most common and best-characterized isoform. The functions of TGF-β1 range from regulating cellular processes (such as differentiation, migration and apoptosis) to initiating the production of extracellular matrix proteins. Despite this complexity, the TGF-β signalling pathway relies on a simple ligand-activated receptor complex. More specifically, signalling is initiated when dimerized TGF-β binds surface-tethered TGF-β receptors, namely TGF-βR1 and TGF-βR2 (Figure 1A). This binding activates TGF-βR2, allowing it to phosphorylate TGF-βR1, which then propagates the signal intracytoplasmically by phosphorylating transcription factors of the small mothers against decapentaplegic (SMAD) homolog family, SMAD2 and SMAD3 (Figure 1B).

Among its many biological roles, TGF-β1 is predominantly known for being a critical driver of fibrosis in various diseases and conditions. As a result, modulation of TGF-β1 activation and signalling is currently pursued as a therapeutic strategy to halt cancer progression, as well as to prevent fibrosis after surgery and in chronic diseases. In recent years, however, the protective functions of TGF-β1 have attracted much attention and are now deemed equally important. Evidence from animal models and in vitro experiments demonstrate that the protective effects of TGF-β1 range from inhibiting inflammation to inducing autophagy. Accordingly, the role of TGF-β1 in disease context dependent, and may be protective or harmful.
In kidney transplantation, TGF-β1 has been a topic of interest for many years and TGF-β1 has been suggested to impact allograft survival in different ways [12]. Initially, multiple reports showed upregulation of TGF-β1 expression and signalling in kidney transplants during rejection [13, 14]. Separately, plasma levels of TGF-β1 were shown to be a potential biomarker of progressive chronic kidney disease in certain populations [15]. Animal studies then demonstrated that TGF-β1 overexpression in the kidney induced interstitial proliferation, tubular autophagy and fibrosis [16]. In contrast, genetic deficiency of TGF-β1 in mice leads to multiorgan inflammation (including that of the kidney) [17]. In the context of these findings, Du et al. found that TGF-β1 plasma levels were positively associated with long-term graft survival in kidney transplant recipients [18]. Finally, local TGF-β1 expression in the kidney allograft during rejection has been associated with a favourable outcome [19, 20]. In these studies, however, it often remains unclear whether the association found with TGF-β1 is a cause or consequence of the pathology. Genetic studies have therefore used single-nucleotide polymorphisms (SNPs) in the TGFβ1 gene to dissect the impact of TGF-β1 signalling on kidney transplant outcome. However, these studies have primarily been retrospective and underpowered, and, thus, they are often inconclusive in their analyses.

To elucidate the current conflicting data, we investigated the impact of polymorphism in TGFβ1 on long-term outcomes in kidney transplantation patients as a model for target validation (Figure 1C). We specifically chose to study the TGFβ1 Thr263Ile variant (rs1800472 C > T) since it was identified as a major genetic driver of plasma TGF-β1 levels in a recent study by Höglund et al. using whole-genome sequencing data. In their study, the minor allele (T-allele) of this polymorphism was shown to be significantly associated with lower plasma levels of TGF-β1 [21]. Furthermore, the overall heritability of the differences observed in the plasma TGF-β1 concentration was 22.9%, while the heritability conditioned...
of this variant alone was 16.3%. We evaluated in this study the association between this recently discovered low-producing TGFB1 polymorphism and long-term kidney graft survival. Additionally, our secondary endpoints were delayed graft function (DGF) and biopsy-proven acute rejection (BPAR).

**MATERIALS AND METHODS**

**Patient selection and study endpoint**

The primary endpoint in this study was death-censored graft survival, defined as the need for dialysis or re-transplantation. Secondary endpoints were DGF (defined by the United Network
5-year death-censored graft survival

A. Donor TGFB1 SNP (rs1800472)

10-year death-censored graft survival

B. Donor TGFB1 SNP (rs1800472)

15-year death-censored graft survival

C. Donor TGFB1 SNP (rs1800472)

FIGURE 2: Kaplan-Meier curves for 5, 10, 15-year death-censored graft survival after kidney transplantation according to the presence of the TGFB1 variant in the donor and recipient.

Cumulative 5- (A, D), 10- (B, E), and 15-year (C, F) death-censored kidney graft survival according to the presence of the Thr263Ile variant in the transforming growth factor beta 1 gene (TGFB1, rs1800472 C>T) in the donor (A — C, blue line) and the recipient (D — F, yellow line).

for Organ Sharing as ‘the need for at least one dialysis treatment in the first week after kidney transplantation’ [22] and BPAR (according to the Banff 2007 classification). We enrolled patients who underwent single kidney transplantation at the University Medical Centre Groningen in the Netherlands between 1993 and 2008. From the 1430 kidney transplantations, 1271 recipient and donor pairs were included in the cohort, as previously described [23, 24]. Subjects were excluded due to technical complications during surgery, lack of DNA, re-transplantation or loss of follow-up. This study was performed in accordance with the declaration of Helsinki and all patients provided written informed consent. The medical ethics committee of the University Medical Centre Groningen approved the study under file no. METc 2014/077.

DNA extraction and TGF-β1 genotyping

Peripheral blood mononuclear cells were isolated from blood or splenocytes collected from the recipients and donors. DNA was extracted with a commercial kit as per the manufacturer’s instructions and stored at −80°C. Genotyping of the SNPs was determined via the Illumina VeraCode GoldenGate Assay kit (Illumina, San Diego, CA, USA), according to the manufacturer’s instructions. Genotype clustering and calling were performed using BeadStudio Software (Illumina). The overall genotype success rate was 99.5% and six samples with a high missing call rate were excluded from subsequent analyses.

Statistical analysis

Statistical analyses were performed using SPSS software version 25 (SPSS Inc., Chicago, IL, USA). Data are displayed as mean ± standard deviation (SD) for parametric variables, median [interquartile range (IQR)] for non-parametric variables, and nominal data as the total number of patients and the percentage [n (%)]. Differences between groups were examined with the Student’s t-test for normally distributed variables or the Mann–Whitney U-test for the not normally distributed variables, and χ² test for categorical variables. Log-rank tests were performed between different genotypes to assess the difference in the incidence of graft loss. Univariable analysis was performed to determine the association of genetic, donor, recipient and transplant.
characteristics with graft survival. The factors identified in these analyses were thereafter tested in a multivariable Cox regression. Additionally, multivariable Cox regression with a stepwise forward selection was performed. Tests were two-tailed and regarded as statistically significant when $P < 0.05$.

RESULTS

Patient characteristics and long-term graft survival

Baseline demographics and clinical characteristics of the 1271 kidney transplant donor–recipient pairs are shown in Table 1. The mean follow-up after transplantation was $6.16 \pm 4.21$ years with a maximum follow-up period of 15 years. During follow-up, 215 grafts (16.9%) were lost, and the causes of graft failure included rejection ($n = 126$, including acute rejection, chronic antibody-mediated rejection and transplant glomerulopathy), vascular causes ($n = 12$), recurrence of primary disease ($n = 16$), surgical complications ($n = 33$), other causes ($n = 16$) or unknown ($n = 12$). The following characteristics were significantly associated with graft loss in univariate analysis: donor age, donor blood type (ABO versus others), donor type (living versus cadaveric), recipient age, recipient blood type (ABO versus others), use of cyclosporin, use of corticosteroids, cold ischaemia time (CIT), warm ischaemia time (WIT) and DGF.

Distribution of the TGFB1 genetic variant

The observed genotypic frequencies of the Thr263Ile TGFB1 variant (rs1800472 C>T) did not significantly differ between recipients ($n = 1266$; CC, 96.2%; CT, 3.8%; TT, 0%) and donors ($n = 1270$; CC, 97.0%; CT, 3.0%; TT, 0%) ($P = 0.55$). No homozygosity was observed for this TGFB1 polymorphism, but the distribution of the SNP was in Hardy–Weinberg equilibrium. The genotypic frequencies of the TGFB1 polymorphism in donors and recipients were significantly higher than those reported by the 1000 Genomes Project ($P = 0.013$), but not compared with their European cohort ($P = 0.60$) [25]. The proportion of grafts with DGF significantly differed based on recipient TGFB1 genotype (45.8% in CT versus 32.2% in CC, $P = 0.048$), but not for donor TGFB1 genotype (36.8% in CT versus 32.5% in CC, $P = 0.58$) (Supplementary Data). In logistic regression, recipients carrying the T-allele of the TGFB1 variant showed a trend towards a higher risk of DGF [odds ratio $= 1.78$ compared with C-allele; 95% confidence interval (CI) 1.00–3.19; $P = 0.051$]. There was no difference in the overall BPAR frequency between the TGFB1 genotypes in the donor (33.9% in CT versus 34.2% in CC, $P = 0.97$) and recipient (33.3% in CT versus 32.9% in CC, $P = 0.60$).
or the recipient (25.0% in CT versus 34.4% in CC, \( P = 0.18 \)) (Supplementary Data). In contrast, the distribution of the TGF\( \beta \)1 polymorphism in the donor, but not the recipient, differed significantly between patients with and without graft loss after complete follow-up (Table 1, \( P = 0.014 \)). More specifically, the T-allele of the TGF\( \beta \)1 SNP was more prevalent in kidney grafts that were lost during the follow-up period. These data suggest that TGF-\( \beta \)1 expression by the donor kidney might impact long-term graft survival in kidney transplantation.

Long-term kidney graft survival according to the TGF\( \beta \)1 genotypes

Kaplan–Meier survival analysis showed that the TGF\( \beta \)1 SNP in the donor was associated with an increased risk for graft loss during follow-up (Figure 2). The TGF\( \beta \)1 variant in the donor was significantly associated with 10- and 15-year death-censored kidney graft survival in Kaplan–Meier survival analyses (Figure 2B and C), but not with 5-year graft survival (Figure 2A). After complete follow-up, the incidence of graft loss was 16.4% in the reference CC-genotype group and 31.6% in the CT-genotype group, respectively. The TGF\( \beta \)1 variant in the recipient was not associated with death-censored kidney graft survival in Kaplan–Meier survival analyses (Figure 2D–F). Subgroup analysis for recipient sex and donor type did not change these results (Supplementary Data).

Next, the donor–recipient pairs were divided into four groups based on the presence or absence of the T-allele in the donor and recipient. Kaplan–Meier survival analyses revealed a significant difference in graft survival among the four groups (Figure 3; \( P = 0.034 \)). Moreover, the T-allele of the TGF\( \beta \)1 polymorphism in the donor seemed to have a bigger impact on graft survival than the T-allele in the recipient. Recipients with a CT-genotype receiving a graft with the CT-genotype appeared to have the worst outcome. However, this combined genotype was only identified in five donor–recipient pairs.

Regression analysis for the TGF\( \beta \)1 polymorphism and graft loss

Finally, we explored whether the TGF\( \beta \)1 variant in the donor was an independent risk factor for graft loss. In univariate analysis, the T-allele of the TGF\( \beta \)1 SNP in the donor was associated with a hazard ratio (HR) of 2.12 (95% CI 1.18–3.79; \( P = 0.012 \)) for graft loss after complete follow-up. Next, multivariable analysis was performed to adjust for potential confounders, including donor and recipient characteristics and transplant variables (Table 2). In Cox regression analysis, the TGF\( \beta \)1 SNP in the donor remained significantly associated with graft loss independent of potential confounders. Finally, we performed a multivariable analysis with a stepwise forward selection procedure using all variables that were significantly associated with graft loss in univariable analysis (Table 3). In the final model, the TGF\( \beta \)1 SNP in the donor, donor age, recipient blood type, recipient age and the occurrence of DGF were included, whereas CIT, WIT, donor type, use of corticosteroids, cyclosporin and donor blood type were not.

**DISCUSSION**

New therapeutic strategies to improve long-term allograft survival are urgently needed, but the development of new drugs for kidney transplantation is limited [26]. Studies of human genetics are therefore needed to predict the success of novel drug targets, since genetically supported drug targets are more than twice as likely to be successful in clinical trials and lead to approved therapeutics [27, 28]. Here, we studied a common functional polymorphism in TGF\( \beta \)1 to dissect the role of TGF-\( \beta \)1 signalling in kidney transplant survival. The key finding in our study is that kidney allografts possessing a low-producing TGF\( \beta \)1 polymorphism are associated with a higher risk of graft loss. In contrast, no association was seen between this TGF\( \beta \)1 polymorphism in the recipient and long-term allograft survival. In conclusion, our study provides genetic evidence that the TGF-\( \beta \)1 pathway in the donor could be favourable for long-term graft survival in kidney transplantation.

Whole-genome sequencing of plasma TGF-\( \beta \)1 recently highlighted the TGF\( \beta \)1 polymorphism rs1800472 as the top functional variant in a genome-wide association study for plasma TGF-\( \beta \)1 levels using whole-genome sequencing [21]. Furthermore, the overall heritability of the TGF-\( \beta \)1 concentration in plasma was \( \sim 23\% \), of which 71% of the genetic variance was explained by this polymorphism alone. To our knowledge, our study is the first to demonstrate an association between the TGF\( \beta \)1 Thr263Ile variant in the donor and long-term graft survival after kidney transplantation. In particular, we found that the T-allele in the donor approximately doubled the risk of graft loss. Previously, the minor alleles of two other TGF\( \beta \)1 polymorphisms (rs1800470-C \( \rightarrow \) T and rs1800471-G \( \rightarrow \) C) in the donor have also been associated with worse graft survival after kidney transplantation [29]. In line with our results, the minor alleles of these TGF\( \beta \)1 polymorphisms have also been suggested to lead to lower levels of TGF-\( \beta \)1 [30–32]. Furthermore, Du et al. reported that long-term survival kidney transplant recipients had higher TGF-\( \beta \)1 levels than short-term

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**Table 3. Competitive analysis of the associations with graft loss after kidney transplantation**

<table>
<thead>
<tr>
<th>Variables not in the equation</th>
<th>P-value</th>
<th>Variables in the equation</th>
<th>P-value</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIT (h)</td>
<td>0.054</td>
<td>rs1800472-T in the donor (CT versus CC)</td>
<td>0.017</td>
<td>2.04 (1.14–3.68)</td>
</tr>
<tr>
<td>WIT (min)</td>
<td>0.07</td>
<td>Donor age (in years)</td>
<td>0.003</td>
<td>1.02 (1.01–1.03)</td>
</tr>
<tr>
<td>Donor type (living versus deceased)</td>
<td>0.08</td>
<td>Recipient blood type (ABO versus other)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>0.12</td>
<td>Recipient age (in years)</td>
<td>&lt;0.001</td>
<td>0.98 (0.97–0.99)</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>0.32</td>
<td>DGF (yes versus no)</td>
<td>&lt;0.001</td>
<td>4.01 (3.03–5.31)</td>
</tr>
<tr>
<td>Donor blood type (ABO versus other)</td>
<td>0.98</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multivariable Cox regression was performed for kidney graft survival with a stepwise forward selection. Only variables with a \( P < 0.05 \) in the univariate analysis were included. Data are presented as an HR with a 95% CI and P-value. In the final model, the TGF\( \beta \)1 SNP (rs1800472-T) in the donor, donor age, recipient blood type, recipient age and the occurrence of DGF were included, whereas CIT, WIT, donor type, use of corticosteroids, cyclosporin and donor blood type were not.

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survival kidney transplant recipients [18]. Serum TGF-β1 levels positively correlated with long-term graft survival and function. Altogether, our study adds to a growing body of evidence which indicates that TGF-β1 has protective effects on kidney transplant survival.

The impact of the recipient TGFBI genotype on outcome after kidney transplantation has been investigated by various studies but remains controversial [33–37]. A recent meta-analysis of nine studies including 352 rejection cases and 882 controls concluded that the recipient TGFBI genotype was not significantly associated with acute rejection after kidney transplantation [34]. Similarly, we also found no association between the TGFBI polymorphism rs1800472 in the recipient and BPAR (Supplementary Data). In the past, low-producing genotypes of TGFBI in the recipient have been associated with both superior and worse outcomes in kidney transplantation [33, 36], while others have found no association with kidney allograft survival [35]. In our transplant cohort of 1271 donor–recipient pairs, we did not find an association between a low-producing TGFBI polymorphism in the recipient and graft survival either. We also assessed the relationship between the TGFBI polymorphism rs1800472 and DGF. While we did see a trend for higher risk of DGF in recipients carrying the T-allele of the TGFBI polymorphism, we did not find an association between the donor TGFBI genotype and DGF. Similar to our observations, the study by Israni et al. found no association for the TGFBI polymorphism rs1800472 in the donor and DGF [5]. In conclusion, our results suggest that it is not the circulating TGF-β1 from the recipient, but rather the local TGF-β1 expression by the donor kidney that promotes graft survival in kidney transplantation.

Generally, TGF-β1 is considered to be a critical driver of fibrosis [10]. Given the abundance of evidence from animal models and translational studies, inhibiting the TGF-β1 signalling pathways would hypothetically prevent the development of kidney fibrosis in kidney disease and transplantation [38, 39]. However, contrary to expectations, results from clinical trials have been underwhelming, as therapies targeting TGF-β1 have not translated into approved treatment for patients [8, 40, 41]. Emerging data demonstrate that TGF-β1 is not only capable of inducing fibrosis, but also has protective effects [7]. In conformity with the findings, loss-of-function mutations in the TGFBI gene were recently shown to cause severe inflammatory bowel disease and encephalopathy in humans, demonstrating the anti-inflammatory properties of this cytokine [42]. In preclinical transplantation studies, TGF-β1 was shown to protect against brain death-induced organ damage and ischaemia–reperfusion injury, and to prolong graft survival [43–46]. The mechanisms behind these protective effects include (i) protecting kidney cells against apoptosis, (ii) stimulating tissue regeneration and (iii) diminishing alloimmunity to kidney transplants by inducing tolerance through regulatory T cells [12].

Several limitations of our study warrant consideration. First and foremost, our study is observational in nature and can therefore not prove causality. Further studies are needed to assess whether the observed association is indeed causal. Furthermore, we examined one polymorphism in TGFBI and did not assess TGFBI haplotypes. Lastly, the relationship between genotypes and plasma levels of TGF-β1 was not assessed in our cohort due to the lack of samples. In contrast, crucial strengths of this study include the analysis of a functional polymorphism in both the donor and recipient, the large sample size and the stringent and clinically meaningful endpoint, and the lengthy follow-up time.

In conclusion, we found that patients receiving a donor kidney carrying the T-allele of the TGFBI polymorphism rs1800472 have a higher risk of late graft loss. Considering that this T-allele is a low-producing TGFBI variant, our findings imply a beneficial effect of TGF-β1 signalling on long-term allograft survival in kidney transplantation.

**SUPPLEMENTARY DATA**

Supplementary data are available at ckj online.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**REFERENCES**