The impact of eNOS, MTR and MTHFR polymorphisms on renal graft survival in children and young adults

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Abstract

Background. The main cause of reduced long-term graft survival is chronic allograft injury. Cardiovascular risk factors such as hyperhomocysteinaemia, accumulation of asymmetric dimethylarginine, increased oxidative stress and decreased production of nitric oxide seem to play an important role. Functional polymorphisms of the endothelial isoform of nitric oxide synthase (NOS) gene cause an alteration in nitric oxide production. Nitric oxide levels, and thus oxidative stress, are also influenced by hyperhomocysteinaemia.

Methods. We carried out a genetic analysis of endothelial nitric oxide synthase (eNOS) 894G>T, methionine synthase (MTR) 2756A>G and methylenetetrahydrofolate reductase (MTHFR) 677C>T/1298A>C in 268 renal allograft recipient/donor (D/R) matches, with respect to long-term graft survival.

Results. While MTHFR 677C>T/1298A>C and MTR 2756A>G polymorphism distribution in both recipients (R) and donors (D) showed no significant difference between matches with loss of graft function and those with long-term graft survival, the frequency of the eNOS 894TT genotype of donors was significantly increased (P = 0.040) in matches with better graft survival. The multivariate analysis identified the eNOS 894 genotype and clinically acute rejection episodes as independent risk factors for graft loss (P = 0.0406 and P = 0.0093, respectively).

Conclusions. The association between eNOS 894G>T polymorphism of donors and graft survival seems to suggest a role for this gene in chronic allograft injury; however, further studies are needed to confirm this hypothesis.

Keywords: allograft nephropathy; graft survival; polymorphisms; renal transplantation

Introduction

The main cause of reduced long-term graft survival is chronic allograft injury, which has been so far described as chronic allograft nephropathy (CAN). This is a generic term for all causes of chronic allograft dysfunction with fibrosis, which has been recently replaced with a more specific classification in the 8th Banff Conference on Allograft Pathology [1].

Among non-immunological factors contributing to the development of chronic allograft injury, cardiovascular risk factors seem to play an important role, and it is well known that renal transplant patients are more prone to cardiovascular events [2,3]. The first step is an atherosclerosis-like vasculopathy that leads to ischaemic damage of renal parenchyma, interstitial fibrosis, glomerular sclerosis and tubular atrophy, finally resulting in progressive renal function deterioration. Next to hypertension and dyslipidaemia, several other factors are particular to renal failure, especially hyperhomocysteinaemia, accumulation of asymmetric dimethyl arginine, increased oxidative stress and decreased production of nitric oxide (NO). The development of NO is one of the main pathophysiological processes that precede the appearance of the characteristic atherosclerotic lesions. NO, a multifunctional molecule, contributes to the increment of oxidative stress by increasing the peroxinitrite radicals (ONOO−) [4]. Cellular effects of NO may depend on its concentration, site of release and duration of action, and low levels of NO may be protective but higher levels may be detrimental [5]. In the healthy kidney, NO production is strictly linked to the endothelial isoform of nitric oxide synthase (NOS) gene [6]. Endothelial-derived NO can react with a broad variety of molecules and by different pathways can result in relaxation or vasodilation [7]. Studies have shown that the immunosuppressive agent cyclosporin A (CsA) impairs the release of NO in epicardial coronary...
arteries [8]. CsA might also bind to calmodulin via the formation of the CsA/cyclophilin complex and thus inhibits the activity of the Ca\(^{2+}\)/calmodulin-dependent eNOS. Immunosuppressive therapy with tacrolimus is also significantly associated with improved free-radical metabolism [9].

Clinical and epidemiological and in vitro studies have shown that another important factor that alters NO levels is an increased total homocysteine (tHcy) plasma level [10–13]. A high prevalence of moderate hyperhomocysteinaemia has been shown in adults with chronic renal failure or in renal transplant recipients and is inversely related to glomerular filtration rate (GFR). Few reports have recently demonstrated that hyperhomocysteinaemia may also be present in children with chronic renal failure and in young renal transplant recipients [14–16]. Homocysteine is metabolized to cysteine by transulfuration or to methionine by remethylation, the latter step requiring methionine synthase (MTR) and methylenetetrahydrofolate reductase (MTHFR) enzymes. Impairment of this pathway can lead to hyperhomocysteinaemia [17,18].

Genetic variability due to single nucleotide polymorphisms (SNP) has been associated with kidney transplantation outcomes, such as acute rejection and CAN [19–26].

The aim of this study was to test whether the presence of eNOS, MTR and MTHFR gene functional polymorphisms, in both paediatric kidney recipients and their respective donors, may be associated with long-term graft survival.

Subjects and methods

Subjects

This retrospective study was carried out in a group of 268 renal units that were recruited among transplant events occurring from 1984 to 1998 in three Italian Centers for Paediatric Kidney Transplantation (Genoa, Milan, Padua).

Donor and recipient demographic data (age, gender, source of kidney transplant and cause of death in the case of deceased donor) were collected by each centre. The clinical course was retrospectively analysed with respect to graft loss, and follow-up clinical data were collected until 30 October 2007. Graft loss was defined as an irreversible deterioration in renal function with the need for dialysis (haemodialysis or peritoneal dialysis). Graft survival was defined as the time interval between renal transplantation and the return to dialysis.

We have employed the patient’s main characteristics that can influence graft survival: HLA mismatch, clinical acute rejection episodes, immunosuppressive therapy, duration of pre-transplant dialysis, creatinine clearance at 1 year after transplantation, with a multivariate analysis. Donors’ genomic DNA was provided by NITp (Nord Italia Transplant program), while recipients’ genomic DNA was yielded by the three Italian Centers for Paediatric Kidney Transplantation taking part in the study. Informed consent was obtained from the patient or parents if the patient was a minor.

Genotyping

Identification of eNOS 894G>T, MTR 2756A>G, MTHFR 677C>T and MTHFR 1298A>C SNPs was performed by restriction fragment length polymorphism (RFLP) analysis using restriction enzymes Ban II, Hae III, Hinf I and Mbo II, according to the literature [16,27–29]. Following digestion, restriction fragments were size-fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators who were unaware of the clinical data.

Statistical analyses

Descriptive statistical analyses included mean values ± standard deviations (SD) for continuous data and percentages for categorical data. Allele and genotype frequencies were analysed by the χ\(^2\) or Fisher’s exact test. In all statistical analyses, \(P < 0.05\) was considered statistically significant. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated to express the relative risk of disease with a specific genotype. Statistical analysis was performed by the SAS v.8.2 Statistical Package (SAS Institute, Cary, NC, USA). We built a multivariate analysis (PROC LOGISTIC software SAS® 9.1.3) with some independent variables known to influence graft outcome to evaluate the possible association between long-term graft survival and a determined eNOS genotype without confounders. Graft survival was analysed by use of Kaplan–Meier plots. A statistical difference was examined with a log-rank test (SPPS 15.0).

Results

Genetic analysis of eNOS, MTR and MTHFR SNPs was carried out on 268 renal units, corresponding to 262 recipients (6 patients had received a second transplant) and 218 donors (50 donors had donated both kidneys). The cause of death was accidental head injury in 82% of cases and various other causes (including cerebral vascular malformations, other traumas and tumours) in the remaining 18%. Ten percent of grafts were performed from living donors, mean age 40 years (range 27–56) while deceased donors mean age was 15 years (range 1–51). The mean age of recipients and donors was 14 (range 0–27) and 17 years (range 1–56), respectively. The male/female ratio was 1.4 for recipients and 1.9 for donors. The follow-up time ranged from a minimum of 9 years to a maximum of 23 years.

Approximately in the early 15 years, immunosuppressive regimen included steroids and azathioprine with or without cyclosporin (<5% of recipients). Then, the recipients were treated with steroids and cyclosporin or tacrolimus, with or without mycophenolate mofetil. Induction with ATG or ALG has been employed since 1990.

Genotypic frequencies of all SNPs were in Hardy–Weinberg equilibrium in both recipient (R) and donor (D) groups.

We regarded the D group as a control population, considering that donors and recipients had approximately the same geographic origin and the donation had occurred after an accidental death in 82.2% of cases. Comparing both genotypic and allelic frequencies of all four SNPs, no significant differences between the R and D groups did emerge (Table 1). Furthermore, allelic frequencies of four SNPs were comparable with those reported in the NCBI dbSNP database for Caucasians.

Recipients’ gender and age at transplant and donors’ gender and age were correlated with recipients’ genotype, and no significant differences were observed (Table 2). Each renal unit (i.e. donor’s genome) was matched with its corresponding recipient (i.e. recipient’s genome), so that we obtained 268 R/D matches. Graft loss occurred in 78 units (29% of grafts), whereas four patients died of causes not related to the graft (normal renal function) and were not included in the study.

The 268 matches were then divided into two groups, according to graft loss:

Group 1: 60 matches with graft loss. Twenty-two matches were not included in the group as graft loss was due to surgical complications, low compliance
The impact of eNOS, MTR and MTHFR polymorphisms on renal graft survival

Table 1. Genotype and allele frequencies observed in 262 recipients and 218 donors

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Recipients&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Donors&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Allele frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>eNOS 894</td>
<td>GG</td>
<td>94</td>
<td>(36.3)</td>
<td>78</td>
<td>(36.3)</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>135</td>
<td>(52.1)</td>
<td>108</td>
<td>(50.2)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>30</td>
<td>(11.6)</td>
<td>29</td>
<td>(13.5)</td>
</tr>
<tr>
<td>MTR 2756</td>
<td>AA</td>
<td>176</td>
<td>(67.2)</td>
<td>139</td>
<td>(63.8)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>70</td>
<td>(26.7)</td>
<td>62</td>
<td>(28.4)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>16</td>
<td>(6.1)</td>
<td>17</td>
<td>(7.8)</td>
</tr>
<tr>
<td>MTHFR 677</td>
<td>CC</td>
<td>176</td>
<td>(67.2)</td>
<td>139</td>
<td>(63.8)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>128</td>
<td>(48.8)</td>
<td>102</td>
<td>(46.8)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>56</td>
<td>(21.4)</td>
<td>39</td>
<td>(17.9)</td>
</tr>
<tr>
<td>MTHFR 1298</td>
<td>AA</td>
<td>125</td>
<td>(47.9)</td>
<td>100</td>
<td>(46.1)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>114</td>
<td>(43.7)</td>
<td>95</td>
<td>(43.8)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>22</td>
<td>(8.4)</td>
<td>22</td>
<td>(10.1)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three cases with a missing eNOS 894 genotype, one case with missing MTHFR 1298 genotype.

<sup>b</sup>One case with a missing MTHFR 1298 genotype.

Table 2. Demographic data according to the different polymorphic genotypes of 262 recipients

<table>
<thead>
<tr>
<th>Genotype</th>
<th>eNOS&lt;sup&gt;a&lt;/sup&gt;: GG, 94</th>
<th>MTR: AA, 176</th>
<th>MTHFR: 677 CC, 78</th>
<th>MTHFR: 1298&lt;sup&gt;b&lt;/sup&gt; AA, 125 AC + CC, 136</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GT + TT, 165</td>
<td>AG + GG, 86</td>
<td>CT + TT, 184</td>
<td>AC + CC, 136</td>
</tr>
<tr>
<td>Mean recipient age (years ± SD)</td>
<td>13 ± 6</td>
<td>14 ± 6</td>
<td>14 ± 6</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>Donor age (years ± SD)</td>
<td>18 ± 12</td>
<td>17 ± 13</td>
<td>17 ± 13</td>
<td>16 ± 13</td>
</tr>
<tr>
<td>Recipient gender (male %)</td>
<td>51.7</td>
<td>59.9</td>
<td>57.9</td>
<td>56.9</td>
</tr>
<tr>
<td>Living donor (%)</td>
<td>11.1</td>
<td>88.9</td>
<td>74.1</td>
<td>22.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three cases with a missing eNOS genotype.

<sup>b</sup>One case with a missing MTHFR 1298 genotype.

NE: not evaluated.

or disease relapse. For all patients graft loss was due to CAN and that was proved either clinically or histologically. The mean follow-up time in this group was ∼7 years.

Group 2: 177 matches with long-term graft survival (i.e. renal function was still normal at the latest follow-up). We excluded nine matches that were lost at the follow-up. The mean follow-up in this group was ∼12 years.

SNP frequencies of recipients and donors belonging to Group 1 were compared with those belonging to Group 2 (Tables 3 and 4). For statistical analysis, each recipient’s genotype was counted as a single, even if he/she had received a second graft. Likewise, each donor’s genotype was counted only one time, even if he/she had donated both kidneys. Thus, frequencies were estimated in a total of 59 and 177 recipients of Groups 1 and 2, respectively, and in 57 and 156 donors of Groups 1 and 2, respectively. MTR 2756A>G, MTHFR 677C>T and MTHFR 1298A>G polymorphism distribution both in recipients and in donors showed no significant difference between Groups 1 and 2. The eNOS 894G>T polymorphism frequency was not different for recipients of Group 1 and

Group 2, while its distribution showed a marked difference of allele T in donors of Group 1 compared to those of Group 2. Supposing a dominant model of inheritance, the frequency of eNOS 894TT versus eNOS 894GG + eNOS 894GT genotypes was found to be significantly different
Table 4. Genotype frequencies observed in donors of Groups 1 and 2

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Group 1 (n = 57)</th>
<th>Group 2 (n = 156)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS 894</td>
<td>GG</td>
<td>35.7%</td>
<td>37.7%</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>58.9%</td>
<td>46.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>5.4%</td>
<td>16.2%</td>
<td></td>
</tr>
<tr>
<td>MTR 2756</td>
<td>AA</td>
<td>73.7%</td>
<td>62.8%</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>22.8%</td>
<td>27.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>3.5%</td>
<td>9.6%</td>
<td></td>
</tr>
<tr>
<td>MTHFR 677</td>
<td>CC</td>
<td>35.1%</td>
<td>35.2%</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>47.4%</td>
<td>48.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>17.5%</td>
<td>16.7%</td>
<td></td>
</tr>
<tr>
<td>MTHFR 1298</td>
<td>AA</td>
<td>44.6%</td>
<td>43.6%</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>42.9%</td>
<td>44.9%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>12.5%</td>
<td>11.5%</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Multivariate analysis of risk factors. Predictors of long-term graft survival

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS-894TT versus eNOS-894GG + GT</td>
<td>9.192</td>
<td>1.099–76.879</td>
<td>0.0406</td>
</tr>
<tr>
<td>Clinical acute rejection episodes</td>
<td>9.485</td>
<td>1.741–51.666</td>
<td>0.0093</td>
</tr>
<tr>
<td>HLA mismatch (3 to 4 versus ≤ 2)</td>
<td>1.191</td>
<td>0.312–4.540</td>
<td>0.7468  N.S.</td>
</tr>
<tr>
<td>HLA mismatch (≥ 5 versus ≤ 2)</td>
<td>0.996</td>
<td>0.256–3.876</td>
<td>0.8696  N.S.</td>
</tr>
<tr>
<td>Pre-transplant dialysis</td>
<td>0.986</td>
<td>0.962–1.009</td>
<td>0.2297  N.S.</td>
</tr>
<tr>
<td>Therapy (FK versus CsA)</td>
<td>1.607</td>
<td>0.559–4.614</td>
<td>0.3785  N.S.</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>0.988</td>
<td>0.953–1.023</td>
<td>0.4904  N.S.</td>
</tr>
</tbody>
</table>

N.S., non-significant.

Discussion

The present study analysed a panel of NOS, MTR and MTHFR functional polymorphisms that may influence non-immunological factors implicated in the development of chronic allograft injury and thus in the graft survival. Clinically, chronic allograft injury has been defined as a slow but variable decline in renal function, associated with hypertension (new-onset hypertension or worsening of a pre-existing hypertension) and proteinuria, and can be confirmed by histological assessment of transplant biopsies. The prevalent manifestation of chronic allograft injury is the accelerated development of atherosclerotic lesions within the graft.

It is known that the presence of gene polymorphisms may be associated with an increased susceptibility to the development of chronic allograft injury. Functional eNOS 894G>T polymorphism causes a reduction in endothelial NO synthase activity [30]. Studies on E. coli demonstrated that MTR 2756A>G polymorphism has a lower activity than the wild-type one [23]. MTHFR 677C>T
common MTHFR polymorphism is 1298 A>C, which appears to be functional only if associated with MTHFR 677C>T polymorphism [24].

Recently, some authors analysed a panel of several SNPs of genes encoding enzymes involved in homocysteine metabolism in renal grafts recipients [17,25,26,31–34].

Only two reports studied the frequency of MTHFR 677C>T in both adult recipients and donors. While Llanaos et al. did not find that the polymorphism is an important determinant of renal transplant survival, Föddinger et al. concluded that homozygosity for C677T polymorphism in the MTHFR gene significantly increases total homocysteine concentrations in recipients but not in donors [35,36].

All studies that evaluated the potential influence of eNOS polymorphisms in the development of chronic renal allograft injury have considered only recipients. The eNOS 894G>T functional polymorphism is associated with renal function deterioration, atherosclerosis and cardiovascular mortality in patients with end-stage renal disease [37–40] and on dialysis [41], whereas it seems to have no influence on the long-term renal graft function [19,42–45].

To our knowledge, the present study is the first to analyse a polymorphism panel in both recipients and donors of paediatric kidney transplant and particularly the first to analyse eNOS 894G>T and MTR 2756A>G polymorphisms in donors and recipients. Our results demonstrate that the presence of MTR 2756A>G, MTHFR 677C>T and MTHFR 1298A>C polymorphisms in recipients or donors is not associated with graft survival. A significant association was indeed observed between eNOS 894G>T polymorphism in donor and graft long-term survival. Nevertheless, our results are not in agreement with the literature data, as the presence of the eNOS TT genotype in donors appeared to be protective with respect to renal graft survival. Only one study reported that the presence of the T allele might be genetically important for long-term kidney graft survival [42].

NO plays an important role in the physiology of blood vessels, as it seems to inhibit the early stages of atherosclerosis [46]. Studies have delineated an association between CAN and oxidative stress; the production of reactive oxygen and reactive nitrogen species is increased [47–49]. NO is an essential molecule, nevertheless its production is not always beneficial, as excess or diminished NO production can have detrimental effects in pathophysiological processes. Furthermore, cellular effects of NO may depend not only on its concentration, but also on its release and duration of action [50]. The discrepant beneficial and detrimental effects that have been ascribed to NO may depend on closely regulated levels of NO in the vessel wall [51].

Some authors, examining the effects of selective eNOS deficiency in aortic allografts in a murine chronic rejection model, demonstrated that eNOS graft activity plays a significant protective role in suppressing the development of allograft arteriosclerosis [52]. However, Akyurek et al. found no altered eNOS expression in aortic grafts of rats with transplant arteriosclerosis [53]. As a compensatory mechanism, the decrement of NO in endothelium may stimulate the inducible NOS (iNOS), wherein augmented NO levels in altered eNOS conditions were associated with up-regulation of iNOS [54].

Moreover, it is now becoming apparent that atherosclerotic endothelial dysfunction, particularly at the early stages of disease, is primarily caused by deregulation of eNOS enzymatic activity and inactivation of NO through oxidative stress, rather than downregulation of the eNOS gene [55].

The association study of genetic variants with complex qualitative trait, such as chronic allograft injury, may demand a larger sample size than that for quantitative traits to arrive at worthwhile conclusions. The results of these studies are certainly complicated by methodological limitations inherent to multifactorial diseases. As reported by some authors [56], inadequacy in the sample size, genetic heterogeneity, environmental background and different definitions of the renal disease phenotype are additional limiting factors. Another possible explanation for the differing results between our study and literature data could lie in the age of the individuals analysed in our study. The contribution of genetic effects would be expected to be stronger at younger ages when fewer other risk factors for multifactorial diseases are present, and this could have influenced our results.

The present study fulfils most of the criteria of a good genetic association study [57]. In addition, it is to take into account that the functional consequences of eNOS 894G>T polymorphism are not yet well established [55,58–60], and little is known about a potential regulatory effect of activity variation of this enzyme on the expression of other genes. Our results could be consistent with the hypothesis that this eNOS polymorphism represents only an indirect marker for genetic association with other disease-related variants in either the eNOS gene or at other loci, according to Andrikopoulos et al. [60].

It is, however, important to underline that our results seem to suggest a relationship between a different intrarenal (i.e. donor) eNOS activity and a reduced renal graft survival. The identification of genetic functional polymorphisms associated with an increased risk of renal graft loss remains an important issue, both from basic research and the clinical point of view, as knowledge about the underlying mechanisms could open up new arenas for therapeutic intervention.

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