Effect of Glutamate Carboxypeptidase II and Reduced Folate Carrier Polymorphisms on Folate and Total Homocysteine Concentrations in Dialysis Patients

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Abstract. This study was designed to examine the effect of two single nucleotide polymorphisms in the reduced folate carrier 1 (RFC1 80G>A) and the glutamate carboxypeptidase 2 (GCP2 1561C>T) gene on total homocysteine (tHcy) plasma level and folate status in 120 chronic dialysis patients. Red blood cell folate concentration was higher in patients with the GCP2 CT or TT genotype (ANOVA, P = 0.04). Among patient groups with different RFC1 genotypes, red blood cell folate level was not significantly different. A multivariate analysis confirmed that the GCP2 1561C>T genotype (P = 0.011) had a significant influence on the red blood cell folate concentration. Overall, serum folate, creatinine, and the GCP2 polymorphism explained nearly 50% of the variance of red blood cell folate. A linear multivariate regression analysis showed that red blood cell folate (P < 0.001), creatinine (P < 0.001), and the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677TT allele (P = 0.013) are independent predictors of tHcy plasma level explaining 49% of the variance of tHcy plasma concentration. GCP2 1561C>T and RFC1 80G>A showed no effect on tHcy and folate plasma level. In conclusion, GCP2 1561C>T, but not RFC1 80G>A, is a predictor of red blood cell folate level in chronic dialysis patients. Both polymorphisms have no major effect on tHcy plasma concentration in end-stage renal disease patients.

In recent years, evidence accumulated indicating that the folate and homocysteine (Hcy) metabolism are partly under genetic control. In particular, the MTHFR 677C>T polymorphism in the gene coding for 5,10-methylenetetrahydrofolate reductase was shown to influence total Hcy (tHcy) plasma and/or folate level in healthy adults (1) and in patients on hemodialysis treatment, peritoneal dialysis treatment, and in renal graft recipients (2–5). This effect can also be associated with riboflavin availability in end-stage renal disease (ESRD) (6). Interestingly, in renal failure patients, MTHFR 677C>T modulates the response to folate therapy (7–9). In addition to MTHFR 677C>T, MTHFR 1298A>C, and methionine synthase (MTR) 2756A>G have some effect on folate and/or Hcy metabolism in individuals with compromised renal function (10,11).

Dietary folates are absorbed after hydrolysis of the terminal glutamate residues of folylpoly-γ-glutamates by the brush-border enzyme folylpoly-γ-glutamate carboxypeptidase (glutamate carboxypeptidase II). The reduced folate carrier then reabsorbs monoglutamylfolate from the enterohepatic cycle. Thus, both proteins regulate the availability of dietary and circulating folates.

Recently, two polymorphisms located in the genes coding for glutamate carboxypeptidase II (GCP2 1561C>T; H475Y) and for the reduced folate carrier (RFC1 80G>A; R27H) have been shown to be associated with alterations in folate and Hcy metabolism in healthy individuals (12,13). Because folate and Hcy metabolism is markedly impaired in renal failure, these mutations could further contribute to hyperhomocysteinemia of ESRD. In the present study, we examined the effect of GCP2 1561C>T and RFC1 80G>A genotypes on folate and Hcy metabolism in dialysis patients.

Materials and Methods

Study Design

The association of GCP2 1561C>T and of RFC1 80G>A with tHcy plasma level and with folate status was assessed in a cross-sectional study of 120 ESRD patients (66 hemodialysis, 54 peritoneal dialysis patients; 53 female patients, 67 male patients; mean age, 55.4 ± 15.7 yr; body mass index, 23.7 ± 3.8 kg/m²; duration of dialysis treatment, 2.2 ± 2.0 yr; albumin, 38.7 ± 5.7 g/L; creatinine, 8.5 ± 2.5 mg/dl) who were previously enrolled in two studies related to hyperhomocysteinemia in renal disease (6,9). None of the peritoneal dial-
ysis patients received routine folic acid or B vitamin supplementation. The hemodialysis patients received a low-dose folic acid containing multivitamin preparation (160 µg of folic acid; Dreisavit-Filmtabletten, GRY-Pharma GmbH, Kirchzarten, Germany) that was shown to have no effect on tHcy plasma levels in these patients (14).

The ethical review board at the University of Vienna approved the study. All patients gave written informed consent according to the Declaration of Helsinki and the Austrian Law on Gene Technology.

Biochemical Methods

Blood was drawn after an overnight fast in peritoneal dialysis patients and before dialysis in hemodialysis patients.

For determination of red blood cell folate, plasma tHcy, plasma folate, plasma vitamin B6, and plasma vitamin B12, blood anticoagulated with EDTA was drawn, immediately placed on crushed ice, and protected from light. Blood aliquots were snap frozen at −70°C for extraction of DNA. Blood aliquots for determination of red blood cell folate concentration were hemolyzed with 0.2% ascorbic acid (1:21) for 2 h and frozen at −70°C. Red blood cell folate was determined using a radioassay (SimulTRAC-SNB; ICN Pharmaceuticals Inc., Costa Mesa, CA) according to the instructions of the manufacturer (normal, >272 nmol/L): red blood cell folate (nmol/L of packed red blood cells) = [(hemolysate folate concentration) × 21]/hematocrit (in decimal notation).

The remaining EDTA-anticoagulated blood samples were centrifuged within 30 min at 2,000 × g at 4°C (20 min). Plasma aliquots were snap frozen and stored at −70°C. The tHcy plasma concentration was determined by a fluorescence polarization immunoassay (IMx analyzer; Abbott Laboratories, Abbott Park, IL). Hyperhomocysteinemia was defined as a tHcy plasma level above 15 μmol/L (15). Folate (normal, >3.4 nmol/L) and vitamin B12 (normal, >118 pmol/L) plasma levels were measured using a radioassay (SimulTRAC-SNB; ICN Pharmaceuticals Inc., Costa Mesa, CA). Vitamin B6 plasma level (normal, >20 nmol/L) was determined with a radioenzymatic assay (Bühlmann Laboratories AG, Allschwil, Switzerland).

RFC1 80G>A was analyzed by restriction fragment length polymorphism (RFLP) analysis of a 230-bp PCR product that had been amplified with the primer pair RFC1 (5'-AGTGTCACCTTCGTCC-3', nucleotide 46713 to 46732, AF007544), producing a 498-bp fragment (5'-GGTGAGAATGATGGACTTTAC-3', nucleotide 46235 to 46253, U19720). In the presence of the G allele, cleavage with CfoI produced two fragments of 162 bp and 68 bp, respectively. The mutant A allele diminished a restriction site resulting in two fragments of 156 bp and 280 bp, respectively. Identification of the 677C>T transition and of the 1298A>C transversion in MTHFR was performed as described previously (16,17).

Statistical Analyses

Continuous data are given as means ± SD. Categorical data are given as absolute counts and percentages. Because tHcy, plasma folate, and red blood cell folate measurements were positively skewed, we used logarithmic transformation to normalize the distribution. Linear associations were assessed by Pearson correlation. The effect of the different genotypes of GCP2 1561C>T, of RFC1 80G>A, of MTHFR 677C>T, and of MTHFR 1298A>C on tHcy plasma levels was assessed in an ANOVA for each polymorphism, with and without red blood cell folate levels, vitamin B12 plasma levels, and creatinine levels as covariates. The effect of the alleles on red blood cell folate and plasma folate was calculated in a multivariate linear regression analysis with covariates being those variables showing significant associations with red blood cell folate in the univariate correlation analysis. All analyses were performed using SPSS for Windows (Version 10.0.7).

Results

Vitamin Status and Genotypes

Table 1 depicts vitamin status and plasma tHcy level of all 120 dialysis patients. The allele frequency of GCP2 1561C>T was 0.046. Seven patients (5.8%) were heterozygotes, and two patients (1.7%) were homozygotes for the mutation. The allele frequency of RFC1 80G>A was 0.404. Fifty-nine patients (49.2%) were heterozygous, and 19 patients (15.8%) were homozygous for this variant.

Effect of Genotype on Red Blood Cell Folate and Plasma Folate Concentration

Red blood cell folate level was higher in patients with the GCP2 1561C>T or TT genotype (ANOVA, P = 0.04) (Table 2). There was no effect of this polymorphism on plasma folate level.

A multivariate analysis disclosed that the GCP2 1561C>T genotype (P = 0.011) had a significant influence on the red blood cell folate concentration, as well as serum folate, creatinine and, weakly, the RFC1 polymorphism. Age and MTHFR genotype had no effect in this analysis. Overall, 38% of the variance in red blood cell folate was explained by serum folate, 5.5% by serum creatinine, 4.8% of the variance of red blood

| Table 1. Vitamin status and total homocysteine (tHcy) plasma levels of 120 dialysis patients (mean ± SD) |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | All             | Hemodialysis (n = 66) | Peritoneal Dialysis (n = 54) |
| Plasma vitamin B6 (nmol/L)      | 251 ± 816       | 64.3 (5.7 to 2070)a  | 23.7 (4.0 to 67.8)a |
| Plasma vitamin B12 (pmol/L)     | 294 ± 184       | 286 ± 187         | 304 ± 181        |
| Red blood cell folate (nmol/L)  | 1391 ± 774      | 1410 ± 688        | 1367 ± 873       |
| Plasma folate (nmol/L)          | 18.1 ± 8.5      | 19.7 ± 9.2        | 16.1 ± 7.2       |
| Plasma tHcy (µmol/L)            | 32.2 ± 24.5     | 31.6 ± 24.7       | 33.0 ± 24.5      |

a Median and full range.

b Significant difference between hemodialysis and peritoneal dialysis; P < 0.01.
cell folate was explained by GCP2 genotype, and 1.8% by the RFC1 genotype.

Red blood cell folate and plasma folate level was different among patient groups with different RFC1 genotypes (Table 3), although the effect of the RFC1 alleles was significant in the multivariate regression analysis.

**Effect of Genotype on Plasma tHcy Concentration**

The tHcy plasma levels according to GCP2 and RFC1 genotypes are indicated in Tables 2 and 3. Neither the GCP2 1561C>T polymorphism nor RFC1 80G>A exhibited a significant effect on plasma tHcy level. This result remained after vitamin B12, red blood cell folate, or creatinine had been significant effect on plasma tHcy level. This result remained after vitamin B12, red blood cell folate, or creatinine had been included as covariates into the ANOVA model. Only MTHFR 677C>T showed a significant effect on tHcy level in both calculations with and without adjustment.

A linear multivariate regression analysis showed that red blood cell folate (P < 0.001), creatinine (P < 0.001), and the presence of the MTHFR 677T allele (P = 0.013) are independent predictors of tHcy plasma level explaining 49% of its variance. The vitamin B12 plasma level (P = 0.087), the GCP2 genotype (P = 0.313), the RFC1 genotype (P = 0.324), and the MTHFR 1298 genotype (P = 0.764) had no significant effect on tHcy plasma concentration.

Among five patients with the highest tHcy plasma level, three were homozygous, two were heterozygous for MTHFR 677C>T, and two were heterozygous for MTHFR 1298A>C.

**Discussion**

We show that the GCP2 1561C>T, but not the RFC1 80G>A sequence variation, is a predictor of red blood cell folate in chronic dialysis patients. Both polymorphisms have no significant effect on tHcy plasma concentrations, although a trend for lower tHcy levels was observed for patients with one or two GCP2 T alleles.

The GCP2 gene (OMIM 600934) is located on chromosome 11p11.2 (18). The GCP2 gene product consists of 750 amino acid residues and is termed folylpoly-γ-glutamate carboxypetidase (FGCP) (19). Folylpoly-γ-glutamate carboxypetidase is an exopeptidase that is anchored to the apical brush border membrane and shows folate hydrolase and N-acetylated α-linked acidic dipeptidase activity. It hydrolyses the terminal glutamate residues of dietary folylpoly-γ-glutamates before absorption. Thereafter, the monoglutamyl folate derivatives are transported through the membrane via the folate transporter. Therefore, FGCP possibly regulates the availability of dietary folates (20).

The GCP2 polymorphism GCP2 1561C>T (H475Y) is located in exon 13 at the putative catalytic domain of the enzyme and is associated with a 53% reduction of enzyme activity (12). Among 75 healthy white patients, the allele frequency was 0.04, with 8% being homozygous and 92% being homozygous for the wild-type alleles (12). This allele frequency corre-

Two patients were heterozygous and one was homozygous for RFC1 80G>A. None of these five patients showed mutated GCP2 alleles.

The association of combined MTHFR and RFC1 genotypes on tHcy and folate level is indicated in Table 4. The MTHFR 677TT genotype was associated with alterations in tHcy concentration, whereas combined MTHFR 677 and RFC1 80 alleles showed no major effect.

**Table 2.** Red blood cell folate, plasma folate, and tHcy levels of 120 dialysis patients according to GCP2 genotypes (mean ± SD)

<table>
<thead>
<tr>
<th>GCP2 1561C&gt;T Genotype</th>
<th>CC</th>
<th>CT and TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>111</td>
<td>9</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>1337 ± 679*</td>
<td>2055 ± 1429</td>
</tr>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>18.0 ± 8.5</td>
<td>18.8 ± 8.4</td>
</tr>
<tr>
<td>Plasma tHcy (μmol/L)</td>
<td>32.5 ± 25.2</td>
<td>28.1 ± 14.1</td>
</tr>
</tbody>
</table>

* P = 0.04 versus CT and TT combined.

**Table 3.** Red blood cell folate, plasma folate, and tHcy levels of 120 dialysis patients according to RFC1 genotypes (mean ± SD)

<table>
<thead>
<tr>
<th>RFC1 80G&gt;A Genotype</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>42</td>
<td>59</td>
<td>19</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>1389 ± 575</td>
<td>1428 ± 773</td>
<td>1278 ± 1125</td>
</tr>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>17.4 ± 8.1</td>
<td>19.2 ± 9.2</td>
<td>16.2 ± 7.0</td>
</tr>
<tr>
<td>Plasma tHcy (μmol/L)</td>
<td>35.2 ± 30.3</td>
<td>29.7 ± 20.1</td>
<td>33.4 ± 23.0</td>
</tr>
</tbody>
</table>

**Table 4.** Folate and tHcy levels according to RFC1 80G>A and MTHFR 677C>T genotypes

<table>
<thead>
<tr>
<th></th>
<th>677CC</th>
<th>677CT</th>
<th>677TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80GG</td>
<td>15</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>80GA</td>
<td>28</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>80AA</td>
<td>6</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>1524 ± 486</td>
<td>1270 ± 585</td>
<td>1671 ± 873</td>
</tr>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>1481 ± 976</td>
<td>1342 ± 482</td>
<td>1505 ± 745</td>
</tr>
<tr>
<td>Plasma tHcy (μmol/L)</td>
<td>1775 ± 1937</td>
<td>1026 ± 297</td>
<td>1084 ± 554</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>20.8 ± 9.4</td>
<td>15.0 ± 6.5</td>
<td>19.8 ± 8.6</td>
</tr>
<tr>
<td>80GG</td>
<td>19.7 ± 11.0</td>
<td>19.4 ± 7.7</td>
<td>16.4 ± 6.2</td>
</tr>
<tr>
<td>80GA</td>
<td>18.1 ± 11.5</td>
<td>15.1 ± 4.2</td>
<td>15.6 ± 4.3</td>
</tr>
<tr>
<td>tHcy (μmol/L)</td>
<td>25.3 ± 8.3</td>
<td>38.8 ± 33.6</td>
<td>56.2 ± 61.8</td>
</tr>
<tr>
<td>80GG</td>
<td>24.0 ± 9.2</td>
<td>31.5 ± 22.6</td>
<td>46.3 ± 33.3</td>
</tr>
<tr>
<td>80GA</td>
<td>27.0 ± 15.3</td>
<td>27.1 ± 3.3</td>
<td>51.1 ± 39.2</td>
</tr>
</tbody>
</table>
sponds to the allele frequency of 0.046 among our dialysis patients.

The RFC1 gene (OMIM 600424) is located on chromosome 21q22.3 (21). The RFC1 gene product consists of 591 amino acid residues and is termed human folate transporter (FOLT), which belongs to the SLC19A family of transporters. The folate transporter is possibly important for the development of embryos, providing the folate transport across the placenta (22). Furthermore, a 58-kd protein has been detected in the brush border membrane of the murine intestine, which mediates intestinal folate transport (23). The RFC1 80G–>A polymorphism is located in exon 2 and changes an arginine into a histidine residue (R27H) (13). Among French healthy adults, homozygosity for the mutant A allele has been detected in 21.9% of individuals (13), which is similar to the percentage of 15.8 detected in our dialysis patients.

Low levels of serum folate and hyperhomocysteinemia were related to the 1561C>T polymorphism of GCP2 in 75 healthy white patients (12). This observation was explained by a diminished activity of polyglycyl-γ-glutamate carboxypeptidase in transfected COS-7 cells. The authors observed no relation of this mutation with red blood cell folate or vitamin B12 levels as well as no interaction of MTHFR 677C>T and GCP2 1561C>T with folate status and tHcy concentrations (12). Thus, this study suggested that the mutated GCP2 allele impairs the intestinal absorption of dietary folates, resulting in relatively low blood folate levels and consequent hyperhomocysteinemia.

In our study, we observed no different plasma folate levels, but higher red blood cell folate and lower tHcy plasma levels in hemodialysis patients carrying the mutated GCP2 1561T allele. Lievers et al. (24) obtained comparable data. In this study of vascular disease patients and healthy controls, the GCP2 1561C>T mutation was associated with elevated red blood cell folate and plasma folate levels and had no effect on vitamin B12 levels. Furthermore, no association of GCP2 1561C>T with cardiovascular disease or with plasma tHcy levels was observed.

In 1324 participants of the fifth examination of the Framingham Offspring Study (25) GCP2 1561C>T showed no association with plasma folate or plasma tHcy concentrations. However, this study did not address red blood cell folate levels, which might have been influenced by GCP2 1561C>T similar to dialysis patients of our study and Dutch individuals (24). Thus, GCP2 appears to have a red blood cell and probably also plasma folate elevating effect that has only minor consequences on plasma tHcy concentrations. It is worthwhile to speculate on the reason why a polymorphism in GCP2 that codes for a protein involved in folate absorption has an effect on red blood cell folate and not on plasma folate of dialysis patients. The absorption of folate or folic acid as well as their conversion to 5-methyltetrahydrofolate is intact in uremia (26). In contrast, methylation reactions involved in formation of methionine from homocysteine are markedly impaired (27). Therefore, the observed increase of red blood cell folate in GCP2 1561C>T-positive dialysis patients may be related to aggravation of cellular folate trapping in uremia. Other potential effects of the GCP2 polymorphism that are related to folate status may include an effect on purine or pyrimidine synthesis, an association with cardiovascular disease risk, or eventually with response to treatment of renal anemia with erythropoietic drugs.

After hydrolysis of dietary folates, the human folate transporter that is encoded by RFC1 facilitates cellular uptake. The RFC1 80G–A variant showed no association with folate status or tHcy plasma concentration among 169 French healthy adults (13). In this study, however, the RFC1 80GG/MTHFR 677TT genotype was associated with moderately higher tHcy plasma concentrations as compared with 80GG/677CC or 80GG/677CT genotype patients. Subjects with 80AA/677CT genotype showed higher plasma folate levels as compared with 80GG/677CT patients. There was no other effect of RFC1 80G–A on plasma or red blood folate levels as measured by a microbiologic assay (13). These findings compare well with the results of our study showing no major effect of RFC1 80G–A on tHcy or folate concentrations in chronic dialysis patients. We also found no association of RFC1 80G–A with tHcy levels among MTHFR 677TT genotype patients.

Concerning the effect of the RFC1 and the GCP2 polymorphism on folate status, it is important to mention that an elegant study conducted by Ghandour et al. (26) showed that daily supplementation with high doses of folic acid or folic acid results in an eightfold to tenfold increase in the levels of total folate in plasma, challenging the assumption that hemodialysis patients have impaired intestinal folate absorption. Furthermore, a substantial portion of this rise in plasma folate was comprised of 5-methyltetrahydrofolate, suggesting that folic and folic acid underwent enzymatic conversion to this form of folate. The finding of a somewhat lower 5-methyltetrahydrofolate plasma concentration in the folic acid group points to the substantially slower conversion of folic acid to folate coenzymes because of the reduction step that is rate limiting (26).

Among Dutch individuals with neural tube defects, no association with RFC1 80G–A was observed (28). However, in some contrast, an Italian study demonstrated a decreased prevalence of the A allele among neural tube defect subjects (29). So far, the association of RFC1 80G–A with cardiovascular disease has not been examined. Therefore, on the basis of the available data including our study, RFC1 80G–A appears to have no major effect on folate status or tHcy plasma concentrations.

In view of our finding of a further genetic influence on folate status in dialysis patients, two recent meta-analyses interestingly underscore that hyperhomocysteinemia (30) and MTHFR 677C>T polymorphism together with folate status (31) are risk factors for cardiovascular disease in the general population. Thus, genetically determined differences in tHcy plasma levels or folate status can play a significant role in cardiovascular disease risk.

In conclusion, red blood cell folate concentrations are increased among dialysis patients carrying one or two GCP2 1561T alleles. Considering important predictors of the tHcy
plasma level, we observed no effect of RFC1 80G>A or GCP2 1561C>T variants on tHcy concentrations of dialysis patients.

References
