Gene polymorphisms in patients with type 2 diabetes and diabetic nephropathy

1. Introduction

Diabetic nephropathy (DN) is a progressive kidney disease caused by angiopathy of capillaries in kidney glomeruli. Microangiopathy is the earliest detectable change in the course of diabetic nephropathy. The kidneys begin to excrete more serum albumin than normal; this stage is called “microalbuminuria”. Another notable phenotype of the early diabetic kidney is nephromegalia. During the early stage, glomerular hyperfiltration can be observed; an increase of filter fraction and a renal plasmatic flow, but the hypertrophic glomeruli exhibits a normal structure. As the diabetic nephropathy progresses, increasing numbers of glomeruli are destroyed by nodular glomerulosclerosis.

Hemodynamic alterations with the glomerulosclerosis lead to a deficit of blood flow, decreased renal function followed by renal insufficiency and the development, especially in the case of poor control of hyperglycaemia, of hyperlipidemia and high blood pressure [1-3].

A considerable ethnic variability exists in the incidence and prevalence of DN and roughly about one third of diabetic patients develop end-stage renal disease [4]. Another aspect of genetic predisposition has been proven in many epidemiologic studies where the cumulative risk is about 50% higher in families of relatives with DN than in families of relatives without DN [4-6].

The variability coheres with an important contribution of multigenetic predisposition in the development of DN. Some genes, which probably participate in the pathogenesis of diabetic nephropathy, also play a role in the regulation of blood pressure, familial hyperlipidemia, familial hypertension and other diseases of the cardiovascular system.

We have examined the association of diabetic nephropathy, nephropathy of non-diabetic origin, hypertension and of type 2 diabetes itself with several genetic polymorphisms (the insertion/deletion polymorphism in the gene for angiotensin-converting enzyme, the G/T polymorphism in the glucose transporter 1 gene, the G/T (894) polymorphism and the T/C (-786) polymorphism in the eNOS gene in three groups of patients with diabetes mellitus: 1) patients without diabetic nephropathy (DM); 2) patients with DN; 3) patients with nephropathy of non-diabetic origin (NDRD). Angiotensin-converting enzyme is an important factor in a development of arterial hypertension, but in our groups of Central European diabetic patients the I/D polymorphism was not associated with diabetic nephropathy. Furthermore, we have confirmed that the T/C (T786C) polymorphism in the eNOS gene is associated with metabolic syndrome including type 2 diabetes.
pathogenesis of DN, have been investigated. Those genes also play a role in the regulation of blood pressure, familial hyperlipidemia, familial hypertension and other diseases of the cardiovascular system. The gene for angiotensin-converting enzyme (ACE) is highly studied, because it contains very well characterized polymorphism I/D - insertion and deletion [7]. Allele D is associated with a higher expression of ACE; increased synthesis of ACE is probably the cause of the initialization and the progression of diabetic nephropathy [8] and the IgA nephropathy [9]. Further fifteen polymorphisms have been described in the gene for angiotensinogen, but only two of them have been associated with mechanisms of hypertension [7,10]. Other described mutations are the G/T polymorphism in the glucose transporter 1 gene [11,12], the mutation in the atrial natriuretic peptide gene [13], the G/T (-106) polymorphism in the gene for aldose reductase [14], the polymorphisms in the nuclear factor kappa B gene [15], the G/T (894) and T/C (-786) polymorphisms in the endothelial nitric oxide synthase (eNOS) gene, variable number of tandem repeats in intron 4 (4a/4b) in the eNOS gene [16] and the polymorphism Ser/Leu in the inducible nitric oxide synthase gene (iNOS) [17].

We have studied polymorphisms (G/T (894) in the exon 7 and T/C (-786) in the promoter) in the eNOS gene, I/D polymorphism in the ACE gene and G/T polymorphism in the GLUT1 gene in three groups of patients with type 2 diabetes: patients without diabetic nephropathy (the DM group), patients with diabetic nephropathy (the DN group) and patients with non-diabetic renal disease (the NDRD group). Our objective was to determine whether the selected polymorphisms, which have been identified as a risk factor for diabetic nephropathy, are associated with the etiology of hypertension, diabetic nephropathy, nephropathy of non-diabetic origin or type 2 diabetes itself.

2. Experimental Procedures

2.1 Database
A database of Czech and German type 2 diabetic patients (342) and control healthy subjects (114) were tested for I/D polymorphism in the ACE gene, G/T polymorphism in the GLUT1 gene, G/T (894) and T/C (-786) polymorphisms in the eNOS gene. The diagnosis of diabetes mellitus was determined according to American Diabetes Association (ADA) criteria [18]. Patients were separated into three groups, the DM group of diabetic patients without diabetic nephropathy, the DN group of diabetic patients with diabetic nephropathy and the NDRD group of diabetic patients with non-diabetic renal disease. The study was approved by the Ethical Committee of the Third Faculty of Medicine, Charles University in Prague, and the Medical Ethical Commission of the Medical Faculty Mannheim. All patients signed appropriate informed consent forms before being allowed to participate.

The group of diabetic patients without nephropathy (DM, n=117) was formed by patients fulfilling following criteria: 1) duration of DM as minimum as 5 years; 2) normoalbuminuria (i.e. <30 mg/24-hrs) or albumin/creatinine <22.8 mg/mmol in morning urine sample; 3) normal values of renal function assessed by serum creatinine level <115 μmol/l for men and <110 μmol/l for women, respectively; 4) normal urinary sediment. The presence of diabetic nephropathy (DN, n=149) was defined according to following criteria: 1) presence of the albumin/creatinine ratio ≥300 mg/g in two of three morning urine samples, or microalbuminuria ≥30 mg/24-hrs or proteinuria ≥500 mg/24-hrs; 2) normal kidney size on ultrasound examination performed recently; 3) duration of DM as minimum as 5 years. The third group (NDRD, n=76) was composed of diabetic patients with the presence of nephropathy of non-diabetic origin. The most prevalent NDRD (>85%) was atherosclerotic renal disease and the others were represented by chronic tubulointerstitial nephritis (12%) or others. The presence of tubulointerstitial nephritis was defined according to following criteria: history of repetitive UTI (urinary tract infection), leukocyteinuria in urinanalysis, low grade proteinuria (proven as tubular origin) and negative signs of any glomerular disease, possibly decreased kidney size in ultrasound examination. The presence of hypertension was defined according to blood pressure above 140/90 mmHg or according to any kind of antihypertensive medication. Healthy individuals without any signs of nephropathy were used as a control group (n=114).

2.2 DNA analyses
DNA was extracted from the whole blood with EDTA by QIAamp DNA Blood Mini kits (Qiagen). The G/T (894) polymorphism in the eNOS gene was identified by PCR-RFLP method. The primers 5’-AAG GCA GGA GAC AGT GGA TGG A-3’ (sense) and 5’-CCC AGT CAA TCC CTT TGG TGC TCA-3’ (anti-sense) amplified the 258-bp fragment encompassing the Glu298Asp variant. The PCR reaction was performed with the annealing temperature at 64°C [15,19]. PCR product was digested with the restriction enzyme Eco24I (Fermentas). The G allele (corresponding to a glutamic acid at position 298), digested, was characterized by two fragments of 163 and 85 bp in length. The T allele, minor (corresponding to an aspartic acid at position 298), non-digested, was
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characterized by the 248 bp fragment [19,20]. The T/C (-786) polymorphism in the eNOS gene was analyzed using PCR and RFLP methods. PCR reaction was performed using the primers 5'-GTG TAC CCC ACC TGC ATT CT-3' (sense) and 5'-CCC AGC AAG GAT GTA GTG AC-3' (antisense). The PCR reaction was performed with the annealing temperature at 60°C [15,19]. PCR product was subjected to digestion with Turbo NaeI (Promega) [21,22]. The T allele was digested. The amplified fragment length polymorphism PCR method (AFLP) was used for the I/D polymorphism (intron 16) in the ACE gene. The primers 5'-GCC CTG CAG GTG TCT GCA GCA TGT-3' (sense) and 5'-GGA TGG CTC TCC CCG CCT TCT CTC-3' (antisense) were designed for detection of the I allele with a length of 597 bp and the D allele with a length of 319 bp. The PCR reaction was performed with the annealing temperature at 50ºC [15,23]. The G/T single nucleotide polymorphism in the GLUT1 gene was identified by PCR and RFLP methods. The primers 5'-TGT GCA ACC CAT GAG GTA GTG AC-3' (sense) and 5'-GGG TGG CTC TCC CCG CCT TCT CTC-3' (antisense) were used to identify the G allele, non-digested, was characterized by the fragment 1.1 kb long and the T allele, minor, digested, by the 0.2 and 0.9 kb long fragments. All the PCR and the restriction digest products were analyzed by electrophoresis in 3% agarose gel stained with ethidium bromide and compared to the molecular marker pUC19 (Fermentas Life Science).

2.3 Statistics
Statistical evaluation was performed using SamplePower 2.0, SPSS SamplePower 2.0 (USA) and SPSS 15.0 version (USA) program. A significant difference was assumed if the P-level was ≤0.05. The verification of genotype comparisons was performed by Chi-Square test for independency in contingency tables and the verification of allele comparisons by Chi-Square test for independency in contingency tables and Fisher’s exact test. Overall, the genotype distributions were consistent with Hardy-Weinberg equilibrium. The logistic regression analysis was used to examine relationship between the polymorphisms and diagnoses.

3. Results
All the characteristics of diabetic patients and healthy individuals such as gender, age, DM duration, laboratory results, occurrence of arterial hypertension and diabetic retinopathy are listed in Table 1. In all three groups, the DM group, DN group and NDRD group, were persons aged 65 and over, and both sexes. The DM group was used as a control group in comparison with the DN and NDRD groups. The group of healthy individuals without any signs of nephropathy was used as a control group in comparison with the diabetic groups.

Significant results (P=0.004, P=0.043, P=0.020, respectively) were found for the T/C eNOS polymorphism in the comparison of the genotype’s frequencies variable patients without diabetic nephropathy patients with diabetic nephropathy patients with non-diabetic renal disease healthy individuals

<table>
<thead>
<tr>
<th>variable</th>
<th>patients without diabetic nephropathy</th>
<th>patients with diabetic nephropathy</th>
<th>patients with non-diabetic renal disease</th>
<th>healthy individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>number in group</td>
<td>117</td>
<td>149</td>
<td>76</td>
<td>114</td>
</tr>
<tr>
<td>age (years)</td>
<td>67.112 (SD=12.44)</td>
<td>70 (SD=13)</td>
<td>80 (SD=7.160)</td>
<td>45 (7.315)</td>
</tr>
<tr>
<td>DM duration (years)</td>
<td>24.649 (SD=13.187)</td>
<td>23 (SD=10.27)</td>
<td>23 (SD=8)</td>
<td>-</td>
</tr>
<tr>
<td>gender</td>
<td>58 males</td>
<td>88 males</td>
<td>44 males</td>
<td>77 males</td>
</tr>
<tr>
<td>arterial hypertension</td>
<td>59 females</td>
<td>61 females</td>
<td>32 females</td>
<td>37 females</td>
</tr>
<tr>
<td>diabetic retinopathy</td>
<td>65 yes (56%)</td>
<td>119 yes (80%)</td>
<td>59 yes (78%)</td>
<td>no</td>
</tr>
<tr>
<td>U-Alb (g/24)</td>
<td>29 yes (25%)</td>
<td>116 yes (78%)</td>
<td>15 yes (20%)</td>
<td>no</td>
</tr>
<tr>
<td>DU-prot (g/24)</td>
<td>&lt; 0.0706 g/24</td>
<td>1.007 g/l (Med)</td>
<td>0.060 g/l (Med)</td>
<td>&lt; 0.15 g/24</td>
</tr>
<tr>
<td>U-Alb (g/24)</td>
<td>&lt; 0.03 g/24</td>
<td>3.000 g/l (Med)</td>
<td>0.380 g/l (Med)</td>
<td>&lt; 0.03 g/24</td>
</tr>
<tr>
<td>S-cr (μmol/l)</td>
<td>106.24 (SD=39.988)</td>
<td>265.96 (SD=192.06)</td>
<td>163.268 (SD=54.977)</td>
<td>males 44-110</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6 (SD=0.905)</td>
<td>7.5 (SD=1.65)</td>
<td>8.013 (SD=2.19)</td>
<td>males 44-110</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of the Czech and German patients and control subjects. The measured lab values are in means, standard deviations and medians. DM = diabetes mellitus, U-Alb = Albuninuria, DU-Prot = amount of urinary protein in 24 h, S-Cr = Serum Creatinine, HbA1C = glycated haemoglobin, Med = median
between the healthy individuals and the groups (DM, DN, NDRD, respectively) of diabetic patients (Table 2). Patients suffered from arterial hypertension in the both DN and NDRD groups. The comparison between arterial hypertensive patients and patients without hypertension embodied significantly (P=0.021) different frequencies of genotypes in the I/D ACE polymorphism (Table 3).

Borderline significant results (P=0.055) were demonstrated for the minor C allele in regression analyses of diabetic nephropathy, T/C eNOS polymorphism (both genotype and allele) and arterial hypertension. Similar borderline results (P=0.057) were proven in the case of non-diabetic nephropathy patients. The C allele may be associated with diabetic nephropathy and other diabetic complications. Borderline significant frequencies of alleles (P=0.048) were found in the I/D ACE polymorphism in the comparison of the DM group and the DN group (Table 4).

4. Discussion

We studied the prevalence of four gene polymorphisms, which had previously been published in the context of diabetic nephropathy progression. We examined the I/D polymorphism in the ACE gene [25-32], the G/T polymorphism in the GLUT1 gene [24,33], the G/T (894) polymorphism and the T/C (-786) polymorphisms in the eNOS gene [17,20,34-36]. We studied type 2 diabetic patients which were stratified into the DM group, the DN group and the NDRD group.

The NO pathway is an important factor in hypertension, renal disease, inflammation and atherosclerosis [37]. It was demonstrated in previous studies that the T/C (-786) eNOS polymorphism is associated with diabetic nephropathy and end stage renal disease [16,36], diabetic retinopathy and diabetic macula edema [38,39], albuminuria [40], rheumatoid arthritis [41], acute coronary syndrome [42,43], coronary artery disease [44] essential and arterial hypertension [45,46], inflammation and atherosclerosis [37,47]. In our study, significant results appeared for the T/C eNOS polymorphism in the comparison with genotype’s frequencies between the healthy individuals and the groups (DM, DN, NDRD respectively) of diabetic patients. Additionally, borderline significant results appeared for the minor C allele in both nominal regression analyses of diabetic nephropathy, T/C eNOS polymorphism and arterial hypertension and in nominal regression analyses of non-diabetic renal disease, T/C eNOS polymorphism and arterial hypertension. Type 2 diabetes is a part of metabolic syndrome (Ray syndrome) that directly or indirectly initiates the process of atherogenesis. The metabolic syndrome includes hyperinsulinism with insulin intolerance, hypertension, hyperglycemia, visceral adipose tissue and atherogenic dyslipidemia [48-50]. The majority of diabetic patients in our cohort suffered from arterial hypertension and obesity. We demonstrated the importance of the T/C eNOS polymorphism as a risk factor for metabolic syndrome (which includes also T2D). We have confirmed previously published association studies with insulin resistance [51,52], the features of metabolic syndrome [53] and with type 2 diabetes [54].

The genotypic frequency of the I/D ACE polymorphism was found to be significantly different between arterial hypertensive patients and patients without hypertension. Rigat et al. [8] found that the I/D polymorphism correlated with the level of ACE in the plasma. The quantity of ACE in
the plasma is two-fold higher for the DD genotype than for the II genotype while the ID genotype produces a level of ACE which is intermediate to the DD and II genotypes [8]. Our results are consistent with the conclusions of other scientific groups [55-57] which confirmed that the DD genotype (also ID+DD genotype) and the D allele is associated with an increased risk of hypertension [55-57] which confirmed that the DD genotype (also ID+DD genotype) and the D allele is associated with an increased risk of hypertension [55-57].

Borderline different frequencies of alleles were found for the I/D ACE polymorphism in the comparison of the DM group and the DN group. The conclusions of individual association studies of the I/D ACE polymorphism and nephropathy syndrome differ due to different ethnic backgrounds [4] and different dietary habits. The minor D allele was not associated with type 2 diabetic nephropathy in a European population [7, 58-62] and the results from our study confirmed this fact. On the contrary, the association was proven for type 2 diabetic patients from an Asian population [31,63-70] with the exception of four studies [71-74].

We found no association between diabetic nephropathy and the G/T polymorphism in the GLUT1 gene and the G/T (894) polymorphism in the eNOS gene. The conclusions of other association studies on this issue differ. Some studies suggest an increased incidence of minor T allele in the GLUT1 gene in the group of patients with diabetic nephropathy [75-77]. While, other studies have not found an association between G/T polymorphism in the GLUT1 gene and the progression of DN [78,79]. In Japanese and Korean populations the G/T (894) polymorphism in the eNOS gene has been demonstrated as a predisposing factor for End-Stage Renal Disease and for type 2 DN [80,81]. In contrast, for a cohort of West Africans and Mexican Americans in the USA this association was not demonstrated [82,83]. The conclusions of the studies were variable probably due to different ethnic backgrounds [4]. Further detailed research is needed to clarify all the issues concerning associations of polymorphisms mentioned above and diabetic nephropathy.

In conclusion, we found no association between diabetic nephropathy and the G/T polymorphism in the GLUT1 gene and the G/T (894) polymorphism in the eNOS gene. We confirmed no association between the minor D allele in the ACE gene and diabetic nephropathy. We also confirmed the effect of I/D polymorphism in the ACE gene on the development of hypertension. Finally, we demonstrated the importance of the T/C eNOS polymorphism as a risk factor for metabolic syndrome (which includes also T2D).

Acknowledgements

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