Original Article

Genetic and Functional Analyses of MRAS and HNF1A Genes in Diabetes and Diabetic Nephropathy

(MRAS / HNF1A / diabetic nephropathy / type 1 and 2 diabetes)

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Abstract. Evidence has recently indicated that the MRAS and HNF1A genetic polymorphisms are associated with coronary artery disease. The MRAS and HNF1A genes are located on chromosomes 3q and 1q2 within the regions where associations with diabetes and diabetic nephropathy occur. We thus performed genetic and functional analyses of these two genes to evaluate their impacts on diabetes and diabetic nephropathy. MRAS and HNF1A genetic polymorphisms were genotyped in 1399 Czech subjects including non-diabetic controls (339), type 1 (243) and type 2 (817) diabetic patients with and without diabetic nephropathy using TaqMan allelic discrimination. Gene expression levels in the kidneys of diabetic Goto-Kakizaki and Wistar rats were detected with real-time RT-PCR. Despite no significance in genetic analysis of diabetic subjects, SNP rs2259816 in the HNF1A gene tended to associate with diabetic nephropathy in type 1 diabetic patients. The hnf1a gene expression was significantly decreased in kidney tissues of Goto-Kakizaki rats compared to Wistar and insulin-treated Goto-Kakizaki rats. There was neither significant association in the MRAS genetic polymorphism with diabetic nephropathy nor variation of mras gene expression in the kidneys of Goto-Kakizaki and Wistar rats. Data from the present study have not proved any significant association of the MRAS and HNF1A genetic polymorphisms with diabetes and diabetic nephropathy in a cohort of Czech population. However, the functional analysis and the trend in genetic analysis suggest that the HNF1A gene may have primary genetic impact on the development of diabetic nephropathy.

Introduction

Microvascular lesions and accelerated atherosclerosis are the major causes of morbidity and early mortality in diabetic patients. Diabetic nephropathy (DN) affects ~30–40 % of all diabetic patients and represents a high-risk factor for cardiovascular mortality. DN is also the most common cause of end-stage renal disease (ESRD) (US Renal Data System, 2007). The prevalence and course of DN are similar in patients with type 1 diabetes (T1DM) and type 2 diabetes (T2DM) with the same disease duration. The stages of DN are similar as well, while the routes they take may differ (White et al., 2007; Kanwar et al., 2008). Epidemiological and familial studies suggest that genetic factors influence the risk of developing both micro- and macrovascular complications in patients who have T1DM and T2DM (Seaquist et al., 1989; Quinn et al., 1996; Osterholm et al., 2007). Therefore, genetic and functional analyses of the susceptibility genes could reveal more details about pathogenesis of these diseases.

We have currently searched for the susceptibility genes in diabetes and DN with the positional candidate gene genotyping approach. One of the chromosomal re-
gions that we have focused on in our study is chromosome 3q, because this chromosomal arm is associated with diabetes and DN (Chistiakov et al., 2004; Takeuchi et al., 2008; Zhang et al., 2010). Similarly, hepatocyte nuclear factor 1a gene (HNF1A, OMIM code 142410) on chromosome 12q was recently proved to have strong association with monogenic and multifactorial forms of T2DM (Voight et al., 2010). Concerning these two chromosomal arms, Erdmann et al. have recently reported the new coronary artery disease risk loci on chromosomes 3q22 and muscle RAS oncogene (MRAS, OMIM code 608435) and 12q24 in HNF1A (Erdmann et al., 2009). Whether the coronary artery disease risk SNPs in MRAS and HNF1A are also associated with diabetes and diabetic nephropathy is unknown.

The M-Ras protein belongs to the Ras superfamily of guanosine triphosphate-binding proteins and is widely expressed in all tissues. Previous work has shown that M-Ras is involved in tumour necrosis factor α-stimulated lymphocyte-function-associated antigen 1 activation in splenocytes (Yoshikawa et al., 2007). HNF1A (also known as TCF1 or MIM142410) encodes transcription factor HNF-1α that binds to promoters of a variety of genes. This gene is expressed in the pancreas, liver, intestine and kidneys and regulates a number of genes involved in innate immunity, blood coagulation, lipid and glucose metabolism and cellular detoxication (Skupien et al., 2008). Mutations in the HNF1A gene cause maturity onset diabetes of the young (HNF1A-MODY, formerly MODY3) (Fajans et al., 2001; Vaxillaire and Froguel, 2006) and affect plasma concentrations of CRP (Reiner et al., 2008; Ridker et al., 2008; Kleber et al., 2010), fibrinogen (Soria et al., 2005) and γ-glutamyl transferase (Yuan et al., 2008). Moreover, a risk allele in the HNF1A locus has been linked to higher plasma levels of low-density lipoprotein cholesterol (Kathiresan et al., 2009).

However, it is unknown whether the MRAS and HNF1A genetic polymorphisms are associated with DN. In the present study, we carried out a genetic association study in a cohort of Czech subjects including T1DM and T2DM patients with and without DN. We also performed a functional analysis of these two genes in diabetic Goto-Kakizaki (GK) rats with and without insulin treatment. The aim was to evaluate the genetic impact of the MRAS and HNF1A genes in the development of diabetes and DN.

Material and Methods

Genetic Association Study

Subjects

Clinical material in this study was collected through the years 2002-2010 at the 3rd Department of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic. A total of 1,399 unrelated subjects of European descent (661 males and 738 females) were included. Among them, 339 were non-diabetic healthy controls, while 243 were T1DM patients and 817 T2DM patients with and without DN. Genomic DNA was isolated from human leukocytes using standard methods.

T1DM was considered present if the age at onset of disease was ≤ 35 years and the time to definitive insulin therapy was ≤ 1 year. The group of patients with latent autoimmune diabetes in adults (LADA) was defined by the presence of positive antibodies test (islet cell antibodies, insulin antibodies or glutamic acid decarboxylase antibodies) and uninterrupted insulin treatment started within one year of diagnosis. Patients with LADA were included in the group of T1DM patients. The T2DM patients were not limited by the age of diagnosis and were treated with oral antidiabetic drugs, insulin treatment or both. Non-diabetic controls did not differ from diabetic patients in sex, age, BMI, blood pressure and lipid parameters significantly. All control subjects had normal renal functions.

Subjects were divided into seven groups according to diabetes type and renal function (T1DM without DN, T1DM with microalbuminuria, T1DM with proteinuria or ESRD, T2DM without DN, T2DM with microalbuminuria, T2DM with proteinuria or ESRD and non-diabetic controls). Absence of DN was considered as persistent normoalbuminuria < 30 mg/24 h or < 20 μg/min or < 20 mg/l or albumin/creatinine ratio (ACR) < 2.5 mg/mmol. Presence of microalbuminuria (MA) was defined by urinary albumin excretion rate (AER) 30–300 mg/24 h or 20–200 μg/min or 20–200 mg/l or ACR 2.5–25 mg/mmol. Presence of DN was defined either by persistent proteinuria (> 300 mg/24 h or > 200 μg/min or > 200 μg/min or ACR >25 mg/mmol) or chronic kidney disease (glomerular filtration rate GFR < 60 ml/min) or ESRD (all of them not due to the condition other than diabetes). The clinical characteristics of the subjects are presented in Table 1. Sample collection of Czech diabetic patients was approved by the local ethics committees and all patients gave their written informed consent.

Genotyping experiments

SNPs rs9818870 and rs2259816 in the MRAS and HNF1A genes, respectively, were selected based upon information from a recent report (Erdmann et al., 2009) and the databases of International HapMap Project (HapMap data release 21a/phasell Jan 07) and dbSNP. A standard protocol of TaqMan allelic discrimination was used for genotyping experiments (ABI 7300 Real Time PCR System, Applied Biosystems, Foster City, CA) (Livak, 1999). For quality control, the subjects were distributed randomly across the plates with numbers of cases and controls approximately in the rate of 2:1 on each PCR plate. Negative controls (Universal-mixture blanks) were included onto each plate. Genotype call rate was 99% in MRAS rs9818870 and 89% in HNF1A rs2259816.
Gene Expression Study

Animals

A total of 22 male spontaneously diabetic GK rats, approximately 2.5 months of age, were obtained from a colony at Karolinska University Hospital (Stockholm, Sweden), and 14 male Wistar rats from a local breeder colony at Karolinska University Hospital (Stockholm, Sweden), and 14 male Wistar rats from a local breeder (B&K Universal, Sollentuna, Sweden) were used as controls. In addition, 11 male GK rats were implanted with a sustained release insulin chip containing 26 μg insulin per 10 days. The blood glucose concentrations and body weight were determined prior to pancreas isolation, tissue preparation and RNA extraction. All procedures were approved by the North Stockholm’s Ethical Committee for Care and Use of Laboratory Animals.

Real-time RT-PCR

Kidney tissues from both group GK rats and Wistar rats were harvested and quickly submerged in RNA/ later solution (Ambion, Austin, TX). Tissue homogenization was prepared according to a protocol developed in our laboratory. Briefly, 30 mg of whole kidney tissue was placed into a 2-ml microcentrifuge tube containing 0.5 ml of 1-mm diameter glass beads and 0.6 ml of RLT buffer (Qiagen, Hilden, Germany). The tube was then placed in the Mini Beadbeater (BioSpec, Bartlesville, OK) and shaken twice at 2,000 g for 60 s. The supernatant was collected. Total RNAs were extracted by using an RNAsasy Mini Kit (Qiagen). The integrity of the RNA was assessed by electrophoresis of 10 μl of each sample through 1.2% agarose gel. RNA concentration was determined spectrophotometrically by measuring the A_{260}/A_{280} ratio. First-strand cDNA was synthesized from 1 μg total RNA from the kidney of each rat employing random hexamer oligonucleotide in a final volume of 20 μl using TaqMan reverse transcription reagents (Applied Biosystems), according to the manufacturer’s protocol. The gadph (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a housekeeping control. To perform the real-time RT-PCR, the specific TaqMan assays for mras assay ID No. Rn01445058_m1, for hnf1a assay ID No. Rn00562020_m1, for gadph assay ID No. Rn99999916_s1) were designed (Applied Biosystems) according to the mRNA sequences. The probe was labelled with 6-carboxy-fluorescein (FAM) as reporter dye and TAMRA as quencher dye. Amplification was performed using the 5'-nucleotide TaqMan method with a two-step PCR protocol (95 °C for 10 min, followed by 36 cycles of 95 °C for 15 s and 60 °C for 1 min) in an ABI 7300 real-time PCR system (Applied Biosystems). All studied samples were normalized with standard curve (slope > 3.3, r^2 > 0.99). Expression values were then calculated by test gene Ct/control gene Ct.

Statistical Analyses

Clinical data are expressed as mean ± SD. Basic descriptive statistics was calculated for the presented parameters. ANOVA, Student’s t-test or Wilcoxon’s and Mann-Whitney test were used for comparing data between groups. Tests were selected depending on the normality of data distribution. Consistency of the observed genotype frequencies was assessed with Hardy-Weinberg equilibrium within each group. Analyses were performed using SPSS Statistics (ver. 17.0 SPSS Inc., Chicago, IL). In the animal study, a non-parametric Kruskal-Wallis comparison analysis and/or test for equality of means was done using BMDP (ver. 1.12, Los Angeles, CA). P < 0.05 was considered significant.

Results

We conducted single marker association analysis of the genotype distribution and allele frequency in the Czech population. Information on the examined SNPs, genotype distribution and minor allele frequencies (MAFs) is summarized in Table 2A and B. The minor alleles of both SNPs were assessed according to the information from HapMap Project and dbSNP. Minor allele T frequency in MRAS rs9818870 (0.159) and A in HNF1A rs2259816 (0.348) in the Czech population were consistent with the information of European Caucasians recorded in the databases.

<table>
<thead>
<tr>
<th>T1D</th>
<th>T1D</th>
<th>T1D</th>
<th>T2D</th>
<th>T2D</th>
<th>T2D</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>147</td>
<td>46</td>
<td>50</td>
<td>331</td>
<td>204</td>
<td>282</td>
</tr>
<tr>
<td>age (years)</td>
<td>43.0 ± 13.9</td>
<td>49.0 ± 16.3</td>
<td>49.0 ± 15.9</td>
<td>63.0 ± 10.6</td>
<td>65.0 ± 11.0</td>
<td>68.0 ± 10.2</td>
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<tr>
<td>duration (years)</td>
<td>16.1 ± 11.7</td>
<td>18.2 ± 11.2</td>
<td>23.5 ± 11.2</td>
<td>9.8 ± 8.2</td>
<td>11.3 ± 7.4</td>
<td>15.3 ± 9.0</td>
</tr>
<tr>
<td>HbA1c (% FCCI)</td>
<td>6.9 ± 1.3</td>
<td>8.0 ± 1.8</td>
<td>8.1 ± 2.2</td>
<td>6.1 ± 1.9</td>
<td>6.9 ± 2.3</td>
<td>7.3 ± 2.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1 ± 3.1</td>
<td>25.4 ± 4.9</td>
<td>26.2 ± 4.4</td>
<td>29.6 ± 5.1</td>
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<td>creatinine (μmol/l)</td>
<td>81.8 ± 16.3</td>
<td>95.5 ± 47.6</td>
<td>219.5 ± 229.8</td>
<td>83.3 ± 17.6</td>
<td>110.8 ± 48.1</td>
<td>242.6 ± 190.1</td>
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<td>SBP (mmHg)</td>
<td>132.0 ± 13</td>
<td>130.0 ± 14</td>
<td>145.0 ± 18</td>
<td>139.0 ± 19</td>
<td>142.0 ± 20</td>
<td>148.0 ± 21</td>
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<tr>
<td>DBP (mmHg)</td>
<td>81.0 ± 8</td>
<td>78.0 ± 12</td>
<td>85.0 ± 13</td>
<td>82.0 ± 9</td>
<td>82.0 ± 12</td>
<td>84.0 ± 12</td>
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<td>TC (mmol/l)</td>
<td>4.7 ± 0.8</td>
<td>5.0 ± 1.1</td>
<td>5.2 ± 2.5</td>
<td>4.8 ± 1.0</td>
<td>4.9 ± 1.2</td>
<td>5.0 ± 1.4</td>
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<tr>
<td>LDL-C (mmol/l)</td>
<td>2.5 ± 0.6</td>
<td>2.9 ± 0.8</td>
<td>2.9 ± 1.0</td>
<td>2.7 ± 0.9</td>
<td>2.5 ± 0.9</td>
<td>2.7 ± 1.1</td>
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<tr>
<td>HDL-C (mmol/l)</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.9</td>
<td>2.2 ± 2.0</td>
<td>2.4 ± 7.9</td>
<td>2.3 ± 1.6</td>
<td>3.4 ± 12.7</td>
</tr>
</tbody>
</table>

Data are means ± SD. N = number of subjects, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, LDL-C = LDL-cholesterol, HDL-C = HDL-cholesterol, TG = triglycerides.
To evaluate the association of SNP rs9818870 from *MRAS* and rs2259816 from *HNF1A* with diabetes, we compared non-diabetic controls vs. all T1DM or T2DM (in rs9818870, \( P = 0.897 \) and \( P = 0.940 \); in rs2259816, \( P = 0.176 \) and \( P = 0.077 \), respectively). To avoid the influence of DN, we also evaluated the control group vs. T1DM without DN or vs. T2DM without DN (in rs9818870, \( P = 0.609 \) and \( P = 0.846 \); in rs2259816, \( P = 0.171 \) and \( P = 0.347 \), respectively). No significant association of the examined SNPs with diabetes in the Czech cohort was found.

To test the association of the examined SNPs with DN, we compared T1DM patients without DN vs. the patients with DN (rs9818870, \( P = 0.861 \); rs2259816, \( P = 0.637 \)) as well as in T2DM (rs9818870, \( P = 0.783 \); rs2259816, \( P = 0.105 \)). Despite no significance we have observed a possible trend of SNP rs2259816 to associate with diabetic nephropathy among T1DM Czech patients. The minor allele A frequency of *HNF1A* was higher in T1DM with DN (0.426) compared to T1DM with microalbuminuria (0.330) and T1DM without DN (0.398). These results imply that for DN in T1DM, minor allele A seems to be a risk allele.

No gender differences in genotype distribution of the studied polymorphisms in T1DM and T2DM patients with and without DN were observed. We also analysed the association between *HNF1A* and *MRAS* SNPs and lipid parameters, but no significant association was found.

We further investigated *mras* and *hnf1a* gene expression in kidney tissue of GK rats. The *mras* gene expression levels in Wistar rats compared to GK rats with and without insulin treatment, respectively, have a tendency to decrease, but with no statistically significant difference (\( P = 0.569 \) and \( P = 0.123 \), respectively), data are shown in Fig. 1A. In the *hnf1a* gene, mRNA expression in GK rats with insulin treatment was significantly higher in comparison to Wistar rats (\( P = 0.029 \)) and significantly lower in GK rats without insulin treatment (\( P = 0.001 \)), Fig. 1B.

**Discussion**

We performed a genetic association study of the SNPs rs9818870 and rs2259816 in the *MRAS* and *HNF1A* genes in a cohort of Czech population with diabetes and DN. Our data have not proved any significant association of these genetic polymorphisms either with diabetes or with DN. Nevertheless, we have found that *HNF1A* genetic polymorphism tends to associate with DN in T1DM, because the A allele frequency of rs2259816 in T1DM with DN or ESRD (0.426) is higher than in patients with microalbuminuria (0.330) and in T1DM without DN (0.398). Therefore, SNP rs2259816 in the *HNF1A* gene may contribute to the risk of development of DN in T1DM.

We have not expected to find that the A allele frequency of rs2259816 in T1DM without DN (0.398) is higher than in T1DM with microalbuminuria (0.330). This could be caused by the range of diabetic duration, which is from five to 27 years. We have considered that some patients in this group may develop DN or ESRD later. Furthermore, the sample size of each group in the present study is limited and statistical analysis may have had less power. For confirmation, further investigation in a larger cohort of patients should be done.

The present study also provides evidence that although the *MRAS* gene is located in the region of chromosome 3q where associations with diabetes and DN have been proved (Chistiakov et al., 2004; Takeuchi et al., 2008; Zhang et al., 2010), SNP rs9818870 in *MRAS* is probably not involved in the pathogenesis of diabetes and DN.

We have also performed a functional analysis of the *mras* and *hnf1a* genes in GK rats with and without insulin treatment. Louro et al. (2011) have reported that in-
sulin treatment in GK rats fed with atherogenic diet is able to improve advanced glycation end product formation, glycoxidation, fibrosis and inflammation in the kidneys, and thus it plays a key role in the development of DN. After insulin treatment, the blood glucose levels in GK rats are normalized to a similar level as in Wistar rats, and this provided us with an idea to study whether the gene has a primary or a secondary effect in the development of diabetes and DN. Interestingly, hnf1a mRNA expression levels in the kidney of GK rats without insulin treatment were decreased compared to Wistar rats, while the levels were increased in GK rats after insulin treatment. These results may be related to metabolic and oxidative stress improvement in the kidneys after insulin treatment (Louro et al., 2011).

It is known that HNF1A is involved in the lipid and glucose metabolism through direct or indirect regulation of a large number of genes, mainly in the liver, but also in the intestine, pancreatic islets and kidneys (Armendariz and Krauss, 2009). The data from our study based upon the results from functional analysis and upon the tendency in genetic association study suggest that HNF1A may play a role in the development of DN.

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