Microsatellite DNA polymorphism of human adrenomedullin gene in type 2 diabetic patients with renal failure

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Background. Adrenomedullin (AM) is a hypotensive peptide widely produced in the cardiovascular organs and tissues such as the heart, kidney, and the vascular cells. We have previously cloned and sequenced the genomic DNA encoding human AM gene, and determined that the gene is located in the short arm of chromosome 11. The 3'-end of the gene is flanked by the microsatellite marker of cytosine adenine (CA) repeats. In this study, we investigated the association between DNA variations in AM gene and the predisposition to develop nephropathy in type 2 diabetes mellitus.

Methods. Genomic DNA was obtained from the peripheral leukocytes of 233 normal healthy subjects (NH), 139 type 2 diabetic patients on hemodialysis (DM-HD), 106 control patients with type 2 diabetes without nephropathy (DM-C) and 318 hemodialysis patients due to chronic glomerulonephritis (CGN-HD). The genomic DNA was subject to polymerase chain reaction (PCR) using a fluorescence-labeled primer, and the number of CA repeats were determined by polyacrylamide gel electrophoresis (PAGE).

Results. In our Japanese subjects, there existed four types of alleles with different CA-repeat number; 11, 13, 14, and 19. The frequencies of these alleles were 11: 27.7%, 13: 32.8%, 14: 35.6%, and 19: 3.9% in NH. These allele frequencies were not significantly different in DM-C and CGN-HD. However, DM-HD showed significantly different distribution of allele frequency from other groups ($\chi^2 = 18.9, P = 0.026$). Namely, the frequency of 19-repeat allele in DM-HD was higher (9.0%) than NH, DM-C, and CGN-HD ($P = 0.005, 0.041$, and 0.004, respectively).

Conclusion. The microsatellite DNA polymorphism of AM gene may be associated with the genetic predisposition to develop nephropathy in Japanese patients with type 2 diabetes mellitus.

Key words: adrenomedullin, gene polymorphism, diabetes mellitus, diabetic nephropathy, microsatellite repeats, genetic markers.

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Adrenomedullin (AM) is a hypotensive peptide produced in cardiovascular tissues such as heart, lung, kidney, and vascular wall [1, 2]. Besides the potent vasodilator action, AM has also been shown to cause natriuresis such as the heart, kidney, and the vascular cells. We have previously identified in human plasma [1, 4] and we have previously reported that plasma AM levels are increased in patients with renal failure and hypertension [5]. Moreover, it has been recently reported that gene delivery or chronic administration of AM alleviates renal injuries in hypertensive rats [6–10]. These findings suggest that AM has implications in pathophysiology of the renal diseases.

We have cloned and sequenced the genomic DNA encoding human AM gene, and determined that the gene is located in the short arm of chromosome 11 [11]. The gene consists of four exons and whole nucleotide sequence corresponding to the 52 amino acid residues of mature AM is included in the fourth exon. Nucleotide sequencing of genomic DNA adjacent to the AM gene revealed that the 3'-end of the gene is flanked by the microsatellite marker with a variable number of cytosine adenine (CA) repeat [12]. This microsatellite marker is located approximately 4 kb downstream of the AM gene. Considering the possible implications of AM in the cardiovascular system, it seems of interest to elucidate whether this gene variation has any relation to the etiology of renal diseases.

With regard to the genetic background of renal diseases, it has been demonstrated that diabetic nephropathy, not only type 1 but also type 2, occurs in familial clusters, suggesting that genetic factors may be involved in the pathogenesis [13, 14]. In this aspect, numbers of studies have been performed as to the association of various gene polymorphisms with genetic susceptibility to suffer diabetic nephropathy. In the present study, we investigated the relationship between the microsatellite DNA polymorphism adjacent to the AM gene and genetic predisposition to develop nephropathy in Japanese type 2 diabetic patients.
METHODS

Study subjects

A group of 139 hemodialysis patients with type 2 diabetes (DM-HD) were selected from the hemodialysis unit of Dokkyo University School of Medicine Hospital and eight nearby hemodialysis hospitals and clinics. Type 2 diabetes was diagnosed when diabetes was detected at 30 years old or older and insulin therapy was not given during 2 years after the onset [15]. From the same hospitals and clinics, 318 hemodialysis patients due to chronic glomerulonephritis (CGN-HD) were involved in this study. Glomerulonephritis was diagnosed by previous renal biopsy or the presence of 2+ or more proteinuria and hematuria and exclusion of other renal diseases such as diabetic nephropathy, hypertensive nephrosclerosis, lupus nephritis, polycystic kidney, and pyelonephritis. A hundred and six control type 2 diabetes patients without nephropathy (DM-C) were selected from the outpatient clinic of Dokkyo University School of Medicine Hospital. In addition to the criteria for the diagnosis of type 2 diabetes, three more criteria were applied to select DM-C subjects: (1) normal serum creatinine (men <1.5 mg/dL and women <1.3 mg/dL), (2) absence of microalbuminuria, and (3) 5 or more years of known history of diabetes. Microalbuminuria was denied when the albumin in spot urine was less than 30 mg/g creatinine on multiple occasions [16]. The normal healthy group (NH) included 233 healthy subjects age 50 years old or older. They were recruited from participants of the health check program of Dokkyo University School of Medicine Hospital. Diseases were ruled out through comprehensive checkup, including a full medical history taking and physical examinations, urinalysis, a stool examination, blood cell counts, blood chemistry, a glucose tolerance test, serologic tests for hepatitis viruses, a chest x-ray film, an electrocardiogram, a respiratory function, an alimentary examination of the upper gastrointestinal tract, and abdominal ultrasonography. The physical examination was performed by an internist, a surgeon, an ophthalmologist, an otolaryngologist, and a gynecologist for women. All subjects were Japanese and unrelated each other.

The study protocol was approved by the Institutional Ethical Committee, and informed consent was obtained from each subject.

Genotype analysis

The number of CA repeats adjacent to AM gene was determined by fluorescence-labeled polymerase chain reaction and polyacrylamide gel electrophoresis (PCR-PAGE) as described previously [12, 17]. Genomic DNA was extracted from peripheral leukocytes using Easy DNA kit (Invitrogen, Carlsbad, CA, USA). The microsatellite region containing the CA repeats 4 kb downstream of the AM gene was amplified by PCR. The PCR was performed with 0.5 μg of genomic DNA and 25 pmol of each primer (sense 5’-AAGAGGGCTGACTCAAGA GGATGGG-3’ and antisense 5’-GCAACTCATATTTT AATATCCTGCACAG-3’ in a final volume of 50 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 10 μg/mL gelatin, 0.2 mmol/L of each deoxynucleoside triphosphate (dNTP), 1 unit of Taq DNA polymerase (Perkin-Elmer, Branchburg, New Jersey). The 5’ end of sense primer was labeled with the blue fluorescent dye 6-FAM. The DNA was amplified for 30 cycles with denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes in a thermal cycler. Then, an aliquot of the PCR product was mixed with the internal DNA size standard labeled with the red fluorescent dye ROX. The mixture was electrophoresed on a 6% urea-polyacrylamide gel using ABI 373 DNA sequencer (Perkin-Elmer). The length of PCR product was calculated from the calibration curve of internal standard using GENESCAN 672 software (Perkin-Elmer).

Biochemical measurement

Peripheral blood sample was obtained in the morning after overnight fast in NH and DM-C. In DM-HD and CGN-HD, blood sample was taken before hemodialysis. Serum creatinine was measured by automatic analyzer (HITACHI 736-60; Hitachi, Tokyo, Japan) and blood hemoglobin A1c was determined by high-performance liquid chromatography (HPLC) using ADAMS hemoglobin A1c HA-8160 (ARKRAY, Inc., Kyoto, Japan).

Statistical analysis

Data are presented as means ± SD. Statistical analyses were performed using Stat View software (version 5.0; SAS Institute Inc., Cary, NC, USA). Clinical characteristics between the case and control groups were compared by one-way analysis of variance (ANOVA) for parametric data and by chi-square test for categoric data. If the ANOVA showed statistical significance, Scheffe’s test was applied for the post-hoc comparison between groups. The frequencies of alleles and genotypes in the four groups were compared by chi-square test and Fisher’s exact test. Differences of parametric data in two groups were analyzed by unpaired Student t test. A P value less than 0.05 was considered to indicate statistical significance.

RESULTS

Clinical characteristics of study subjects

Table 1 lists the physical and laboratory findings of the study subjects. Average age and the gender ratio were not significantly different among the four groups. Body mass index was greater in DM-C than in NH (P = 0.026). However, the body mass index of DM-HD was...
Table 1. Clinical characteristics of the study subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NH</th>
<th>DM-C</th>
<th>DM-HD</th>
<th>CGN-HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years</td>
<td>57 ± 6</td>
<td>59 ± 16</td>
<td>60 ± 10</td>
<td>57 ± 12</td>
</tr>
<tr>
<td>Sex male/female</td>
<td>154/79</td>
<td>64/42</td>
<td>93/46</td>
<td>184/134</td>
</tr>
<tr>
<td>Body mass index kg/m²</td>
<td>23.7 ± 2.8</td>
<td>24.9 ± 4.2</td>
<td>21.1 ± 2.8</td>
<td>20.7 ± 2.7</td>
</tr>
<tr>
<td>Systolic blood pressure mm Hg</td>
<td>117 ± 11</td>
<td>146 ± 25</td>
<td>164 ± 19</td>
<td>153 ± 19</td>
</tr>
<tr>
<td>Diastolic blood pressure mm Hg</td>
<td>73 ± 8</td>
<td>85 ± 15</td>
<td>81 ± 8</td>
<td>81 ± 9</td>
</tr>
<tr>
<td>Duration of diabetes years (until nephropathy was revealed)</td>
<td>14 ± 6</td>
<td>15 ± 7</td>
<td>(8 ± 6)</td>
<td></td>
</tr>
<tr>
<td>Duration of dialysis years</td>
<td>5 ± 3</td>
<td></td>
<td>9 ± 6</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine mg/dL</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>10.3 ± 2.9</td>
<td>11.9 ± 2.7</td>
</tr>
<tr>
<td>Blood hemoglobin g/dL</td>
<td>9.5 ± 2.3</td>
<td>6.7 ± 1.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are: NH, normal healthy subjects; DM-C, control diabetic patients without nephropathy; DM-HD, diabetic patients on hemodialysis; CGN-HD, hemodialysis patients due to chronic glomerulonephritis. Data are mean ± SD. F values of ANOVA for body mass index, systolic blood pressure, diastolic blood pressure, and serum creatinine are 72.0 (P < 0.001), 250.4 (P < 0.001), 48.7 (P < 0.001), and 1422.8 (P < 0.001), respectively.

Table 2. Genotype frequencies of microsatellite DNA polymorphism adjacent to the human adrenomedullin gene in the study subjects

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NH</th>
<th>DM-C</th>
<th>DM-HD</th>
<th>CGN-HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>N = 233</td>
<td>N = 106</td>
<td>N = 139</td>
<td>N = 318</td>
</tr>
<tr>
<td>11 CA repeats</td>
<td>22 (9.4%)</td>
<td>14 (10.0%)</td>
<td>8 (6.0%)</td>
<td>6 (1.9%)</td>
</tr>
<tr>
<td>13 CA repeats</td>
<td>44 (18.9%)</td>
<td>30 (21.6%)</td>
<td>54 (17.0%)</td>
<td>36 (11.3%)</td>
</tr>
<tr>
<td>14 CA repeats</td>
<td>37 (15.9%)</td>
<td>24 (17.3%)</td>
<td>76 (23.9%)</td>
<td>11 (3.4%)</td>
</tr>
<tr>
<td>19 CA repeats</td>
<td>4 (1.7%)</td>
<td>6 (4.3%)</td>
<td>11 (3.4%)</td>
<td>9 (2.9%)</td>
</tr>
<tr>
<td>13 CA repeats</td>
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<tr>
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<td>11 (3.4%)</td>
</tr>
<tr>
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<td>13 CA repeats</td>
<td>35 (15.0%)</td>
<td>16 (11.5%)</td>
<td>28 (8.6%)</td>
<td>7 (2.2%)</td>
</tr>
<tr>
<td>14 CA repeats</td>
<td>5 (2.1%)</td>
<td>10 (7.2%)</td>
<td>7 (2.2%)</td>
<td>7 (2.2%)</td>
</tr>
<tr>
<td>19 CA repeats</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
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</table>

Abbreviations are: NH, normal healthy subjects; DM-C, control diabetic patients without nephropathy; DM-HD, diabetic patients on hemodialysis; CGN-HD, hemodialysis patients due to chronic glomerulonephritis.

Table 3. Allele frequencies of microsatellite DNA polymorphism adjacent to the human adrenomedullin gene in the study subjects

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<tr>
<th>Allele</th>
<th>NH</th>
<th>DM-C</th>
<th>DM-HD</th>
<th>CGN-HD</th>
</tr>
</thead>
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<td>N = 139</td>
<td>N = 318</td>
</tr>
<tr>
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</tr>
<tr>
<td>19 CA repeats</td>
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<td>0 (0.0%)</td>
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<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

Abbreviations are: NH, normal healthy subjects; DM-C, control diabetic patients without nephropathy; DM-HD, diabetic patients on hemodialysis; CGN-HD, hemodialysis patients due to chronic glomerulonephritis.

smaller than that of NH and comparable to that of CGN-HD. Systolic and diastolic blood pressures were obviously higher in DM-C, DM-HD, and CGN-HD than in NH (P < 0.001, respectively). Especially, DM-HD had even higher systolic blood pressure than DM-C and CGN-HD (P < 0.001, respectively), and DM-C had higher diastolic pressure than DM-HD (P = 0.007) and CGN-HD (P = 0.008). Duration of diabetes was comparable between DM-C and DM-HD, however, the duration of diabetes in DM-C was longer than the duration of diabetes until nephropathy was revealed in DM-HD, P < 0.001. In hemodialysis patients, DM-HD had shorter duration of dialysis than CGN-HD, P < 0.001. Serum creatinine did not significantly differ between NH and DM-C. Of course, DM-HD and CGN-HD had much higher serum creatinine than NH and DM-C (P < 0.001, respectively). In hemodialysis patients, predialysis serum creatinine was lower in DM-HD than in CGN-HD, P < 0.001. Blood hemoglobin A₁c was lower in DM-HD than in NH, P < 0.001.

Genotype and allele frequencies

As we have reported previously [12, 17], there existed four types of alleles with different CA repeat number; 11, 13, 14, and 19. The alleles with 11, 13, 14, and 19 CA repeat yield DNA fragment length of 248, 252, 254, and 264 bp, respectively. Combination of the four alleles yields ten possible genotypes: 11/11, 11/13, 11/14, 11/19, 13/13, 13/14, 13/19, 14/14, 14/19, and 19/19. Table 2 shows observed frequencies of these ten genotypes of this microsatellite gene polymorphism in the study subjects, and Table 3 lists the frequency of each allele calculated from the genotype frequencies. The genotype frequencies were not significantly deviated from those expected from the Hardy-Weinberg’s equilibrium in each group. The frequencies of 11, 13, and 14, repeat alleles were approximately 30%, while the frequency of 19 repeat allele was less than 10% in each group. There were no homozygotes of the least frequent 19 repeat allele in the examined subjects. Distributions of the genotypes were not significantly different among the four groups. On the other hand, the allele frequencies were significantly different between the groups (χ² = 18.9, P = 0.026). Figure 1 depicts the difference in allele frequency more clearly. The frequency of 19 repeat allele was higher in DM-HD than in NH, DM-C, or CGN-HD. The frequencies of 11, 13, and 14 repeat alleles did not show significant group differences.

DISCUSSION

Nowadays, diabetic nephropathy is the leading cause of end-stage renal failure in Japan as well as in United
DNA polymorphism adjacent to the AM gene with the genetic predisposition to suffer diabetic nephropathy. Namely, the existence of 19 CA repeat allele is supposed to be associated with the risk of developing nephropathy in Japanese type 2 diabetic patients. However, because the frequency of this 19 repeat allele was small, this gene variation alone is not thought to reflect the large part of genetic predisposition to diabetic nephropathy. Any single gene polymorphism, which has been previously shown to be associated with diabetic nephropathy, does not represent the large part of genetic risk for diabetic nephropathy. It is supposed that multiple genes contribute to the development of diabetic nephropathy. In the future direction, information as to the relation between gene polymorphisms and the risk of diabetic nephropathy should be gathered and analyzed comprehensively to construct a way to evaluate the genetic risk of diabetic nephropathy precisely. In this sense, The DNA polymorphism examined in this study seems to add information to the prediction of the development of diabetic nephropathy.

Some gene polymorphisms have been shown to affect expression of the genes or activity of the gene products. For instance, the serum ACE activity is known to be increased in individuals carrying the deletion allele of the gene [28], and it has been reported that the methionine to threonine substitution at amino acid residue 235 (M235T) of angiotensinogen is associated with an increase in the gene expression [29]. Because the microsatellite polymorphism examined in this study is located 3’ downstream of the AM gene, it is unlikely that the gene transcription is affected by this polymorphism. Indeed, the plasma AM levels were not significantly different among the genotypes of this polymorphism in normal subjects [12]. Namely, the subjects carrying 19 repeat allele and the homozygotes of 11, 13, and 14 repeat alleles showed comparable plasma AM levels. Therefore, the association of 19 CA repeat allele with diabetic nephropathy is not likely mediated by the gene expression and the action of AM itself. It may be possible that this microsatellite polymorphism is associated with the expression of other genes. Near the location of AM gene in the short arm of chromosome 11, there exist such genes as sphyngomyelinase, parathyroid hormone, and lactate dehydrogenase [30–32]. Among these, so far the literature does not suggest relation of sphyngomyelinase and lactate dehydrogenase genes to diabetic nephropathy. With regard the parathyroid hormone gene, it has been shown that the secretion of parathyroid hormone is impaired in patients with diabetic nephropathy [33, 34], which suggests relation between the development of diabetic nephropathy and the expression of parathyroid hormone gene, although this may be caused by the long duration of poor glycemic control as well. Besides these,
the microsatellite polymorphism downstream of AM gene may be linked to other yet unidentified gene variations.

Microsatellite markers, like the one examined in this study, consist of variable number of repeats of short nucleotides, and more than hundreds of such repeat markers are scattered throughout the genomic DNA. These microsatellite markers can be utilized to locate the genomic region responsible for hereditary diseases or traits. Until now, several diseases have been shown to be associated with such microsatellite DNA polymorphism. For instance, it has been reported that the CA repeat polymorphism lying upstream of aldose reductase gene affects the development of nephropathy and retinopathy in type 1 diabetes mellitus [35, 36]. However, inconsistent results have been reported as to the association of this microsatellite polymorphism with type 2 diabetic nephropathy [37–39]. On the other hand, genetic predisposition to essential hypertension has been shown to have significant linkage to a certain number of TCAT though this DNA polymorphism is not likely to affect the GT repeat in the intron 2 of type B natriuretic peptide allele is supposed to reflect a certain part of the genetic lesion [48]. Therefore, the relations between gene polymorphisms and type 2 diabetic nephropathy may be affected by the criteria used for the diagnosis of diabetic nephropathy.

The results of this study revealed the association of the microsatellite CA repeat polymorphism adjacent to the AM gene with genetic predisposition to type 2 diabetic nephropathy in Japanese population. The frequency of 19 repeat allele was significantly increased in patients with end-stage renal disease due to type 2 diabetes. Although this DNA polymorphism is not likely to affect the AM gene transcription, the existence of 19 CA repeat allele is supposed to reflect a certain part of the genetic predisposition to develop nephropathy in Japanese type 2 diabetic patients.

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REFERENCES

8. Zhang J, Yoshida H, Chao L, Chao J: Human adrenomedullin gene delivery protects against cardiac hypertrophy, fibrosis, and