A Novel Van91 I Polymorphism in the 1st Intron of the Parathyroid Hormone (PTH)/PTH-Related Peptide (PTHrP) Receptor Gene and Its Effect on the Urinary cAMP Response to PTH

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This study was designed to identify a parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor gene polymorphism in a healthy Japanese population. All known 13 introns of this gene were amplified by PCR, except the 1st intron, which was amplified by the long-PCR method. No restriction fragment length polymorphisms (RFLPs) were detected by BsmI or XbaI in any of these introns. Twenty-one other restriction enzymes (HindIII, BglII, SspI, PvuII, EcoRI, Van91I, BstXI, ScaI, BamHI, MboII, Tth1I1, PshAI, Eam1105I, NorI, SrfI, BglII, FokI, SfiI, ApeI, TaqI) were tested on the 1st intron. Furthermore, digestion by Van91I (CCANNNNNTGG) identified a single, two-allele polymorphism with a fragment of approximately 3.5 kb (f allele) or a fragment of 3.1 and 0.4 kb (v allele). The frequency of the Van91I polymorphism in 106 healthy Japanese volunteers was 77.4% for type v and 19.8% for type Vv and 2.8% for type VV. In addition, the urinal cAMP response to exogenous [1–34]PTH was studied in 17 healthy volunteers and found to be significantly greater in persons with type Vv than type vv (p<0.05). In conclusion, the Van91I polymorphism of the PTH/PTHrP receptor gene can be used to study the role of polymorphism in various disorders involving PTH or PTHrP.

Key words parathyroid hormone/PTH-Related Peptide receptor gene; polymorphism; long-PCR; restriction fragment length polymorphism; cAMP

Parathyroid hormone (PTH) plays a central role in controlling calcium homeostasis by preventing hypocalcemia. PTH acts on the kidney and bone through its specific PTH/PTH-related peptide (PTHrP) receptor. This receptor is a G-protein-coupled receptor with seven membrane spanning domains that has been cloned from rat, mouse, opossum, porcine and human cDNA.1–5 This PTH/PTHrP receptor has an equal affinity for PTH and PTHrP6,7 and can be discriminated from PTH2 receptor which only recognizes PTH.8,9 PTH/PTHrP receptor is only expressed in the kidney and osteoblastic cells10 and the PTH/PTHrP receptor gene consists of at least 14 exons and 13 introns within 20 kb on the chromosome 3p21.1–p24.2 region.11 In uremic patients with secondary hyperparathyroidism, a higher PTH concentration is required to maintain normal bone formation than in non-uremic subjects, a phenomenon known as PTH resistance.12,13 Prolonged exposure to PTH has been known to cause desensitization or down-regulation of the PTH/PTHrP receptor.14 Since variations in PTH-resistance exist among patients, regardless of treatment, PTH/PTHrP receptor gene polymorphism might affect the efficiency or efficacy of the receptor which causes a variation in PTH resistance.

There have been two reports on PTH/PTHrP receptor gene polymorphism. One of these studies detected a BsmI I restriction fragment length polymorphism (RFLP) by a genomic Southern technique but it could not localize the polymorphic allele on a gene.5,9 Another study detected a polymorphism (T or A) in exon M7 by using temperature gradient gel electrophoresis.16 In the present study, we designed a PCR method to amplify all 13 introns and employed frequently used restriction enzymes to detect a RFLP in introns of the PTH/PTHrP receptor gene and found a Van91I RFLP in the first intron (10 kb). In order to clarify the clinical relevance of this polymorphism, we studied its effect on the urinary cAMP response to exogenous PTH in healthy volunteers.

MATERIALS AND METHODS

Extraction of Genomic DNA Genomic DNA was prepared from the peripheral blood of 106 healthy Japanese subjects using the Mag Extractor-Genome-Extraction Kit (Toyobo, Osaka, Japan). Informed consent was obtained from every blood donor.

Design of PCR Primers and Amplification of Each Intron All 13 introns of the PTH/PTHrP receptor gene were amplified by PCR or the long-PCR method and the sequences of the primers are listed in Table 1. These oligonucleotide primers were prepared using the Expedit Nucleic Acid Synthesis System (PerSeptive Biosystems, MA, U.S.A.). The target DNA sequence was amplified by PCR in a reaction mixture (50 μl) containing 50 ng genomic DNA, 1.5 mM MgCl2, 100 mM Tris–HCl (pH 8.3 at 25°C), 500 mM KCl, 200 μM of each deoxyribonucleotide (dNTP), 10 pmol of each forward and reverse primer, and 1.2 units of Ampli-Taq DNA Polymerase (Roche, Basel, Switzerland). Then, reaction mixtures were amplified for 40 cycles in a DNA Thermal Cycler PJet2000 (Perkin-Elmer Cetus, CT, U.S.A.) under the following conditions: denaturation at 94°C for 30 s, and annealing and polymerase extension at 65–70°C for 2.5 min. For the 1st intron (10 kb), the long-PCR method17 was employed. The long-PCR amplification reaction was carried out with a 50 μl mixture containing 50 ng genomic DNA, 2.5 mM MgCl2, 25 mM N-Tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS, pH 9.3 at 25°C), 50 mM KCl, 1 mM mercaptoethanol, 350 μM of each dNTP, 10 pmol of each forward and reverse primer, and 2

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units of TaKaRa LA Taq® (Takara, Kyoto, Japan) with a PCR Thermal Cycler 480. After initial denaturation at 94 °C for 1 min, long-PCR was carried out for 33 cycles, each consisting of denaturation at 98 °C for 20 s, and annealing and polymerase extension at 70 °C for 12 min.

**Restriction Enzyme Digestion of PCR Products**

In the search for the PTH/PTHrP receptor gene polymorphism, 15 μl PCR or long-PCR product was digested with 4 units restriction enzyme in a supplemented reaction buffer in a total volume of 25 μl for 3 h at optimal temperature. Restriction enzymes employed for the 1st intron were HindIII (Takara), BglII (Takara), Styl (Takara), PvuII (Takara), EcorI (Takara), Vpn911 (Takara), BstXI (Toyobo), Sea83871 (Takara), EcorI (Takara), BamHI (Toyobo), MboII (Takara), BsmI (Toyobo), Thh1111 (Takara), PsHaI (Takara), EcoRII (Takara), NolI (Takara), SphI (Takara), BglII (Toyobo), BsmI (Toyobo), SphI (Takara), ApaI (Toyobo), TaqI (Toyobo), XbaI (Takara). All introns were digested with BsmI and XbaI. Each digested product was detected by ethidium-bromide-UVB illumination after electrophoresis on 0.8% agarose gel (1st intron product) or 3% agarose gel (other PCR products).

**Urinary cAMP Response to PTH**

Seventeen healthy volunteers gave written informed consent before participating in the present study. The Vpn911 genotype was type VV in 9 subjects (4 males, 5 females) and type Vv in 8 subjects (3 males, 5 females). Mean age did not significantly differ between the two genotype groups (29 ± 7 and 27 ± 4 years old, respectively). The urinary cAMP response to exogenous PTH was examined by a modification of the Ellsworth–Howard test. After a 16 h fast with access to drinking water, the first urine sample was collected between 9 A.M. and 11 A.M. Every participant drank 500 ml water at 9 A.M. and again 11 A.M. At 11 A.M., 100 units human [1—34]PTH (Human PTH Injection, Asahi Kasei, Osaka, Japan) was administered intravenously immediately after the withdrawal of blood. This [1—34]PTH is a PTH fragment which has biological activity, and used for differential diagnosis of hypoparathyroidism. A second urine sample was collected 2 h after the first. Urinary cAMP was measured by RIA. Concentrations of creatinine, calcium and phosphorus in both serum and urine were analyzed by autoanalyzer AU600 (Olympus, Tokyo, Japan) and urine was acidified before measurement of calcium and phosphorus. Ionized calcium (Ca²⁺) in serum was measured by autoanalyzer SERA252 (Horiba, Kyoto, Japan). A cAMP/creatinine clearance (Ccr) was calculated by subtracting the second urinary cAMP concentration from the first and then correcting the value by the Ccr. The fractional excretion of Ca²⁺ (FECa) or phosphorus (FEP) was given as the percentage of these ions excreted in urine divided by the amount of these ions in the ultrafiltrate.

**Statistical Analysis**

Results are expressed as the mean ± S.D. Data were analyzed by a non-paired Student t-test using StatView 4.0 for the Macintosh computer (Abacus Concepts Inc., Berkeley, CA, U.S.A.). A level of p < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

Using both the PCR and long-PCR methods, we successfully amplified every region that contained the full sequence of all introns (Fig. 1).

It was confirmed that these PCR products amplified each intron of the PTH/PTHrP receptor gene by the cycle sequence method (data not shown). We performed RFLP screening in 106 volunteers. Neither BsmI nor XbaI detected RFLP in any of the introns. Since the 1st intron was the longest (10 kb), we concentrating our efforts on this intron. Twenty-three restriction enzymes were used in the 1st intron of 24 volunteers. Of these enzymes, only Vpn911 detected RFLP. Finally, digestion of the 1st intron by Vpn911 (CCANNNNNTGG) revealed two invariant bands at 2.5 and 0.8 kb and variant bands at either 3.5 or 4.3 kb (V), and 3.1 and 0.4 kb (v) indicating a single, two-allele polymorphism. Therefore, samples homozygous for the Vpn911 polymorphism yielded fragments of 3.4, 3.1, 2.5, 0.8 and 0.4 kb, while samples homozygous for the absence of the site yielded 3.5, 2.5 and 0.8 kb bands. All six bands were observed in heterozygotes (3.5 and 3.4 kb fragments were detected as a single band) (Fig. 2). Vpn911 is known to have the 3.5 kb band cleaved either into 3.4 and 0.1 kb bands (undetectable by this gel electrophoresis procedure) or into 3.1 and 0.4 kb bands. From these results, the Vpn911 genotype was classified by the presence (v) or absence (V) of the 3.5 kb-digested fragment. The frequency of Vpn911 genotypes of the PTH/PTHrP receptor gene was examined in the volunteers and revealed 77.4% with type Vv, 19.8% with type VV and 2.8% with type VvV polymorphism.

For clinical application of genetic polymorphism, detection of the cleavage pattern of the PCR product by restriction enzymes is the easiest and most universal method. There have been only two reports on polymorphism of the PTH/PTHrP receptor gene. One study also investigated RFLP using a restriction enzyme BsmI and found a two-allele polymorphism. This study used Southern blotting with a 2 kb cDNA probe, but could not determine the location of...
Fig. 1. Schematic Representation of the Human PTH/PTHrP Receptor Gene (A) and Agarose Gel Electrophoresis of the PCR Product of Each Intron (B).

(A) Exons and introns are indicated by black boxes and bold lines, respectively. Amplification site are designated (1) to (9). (B) Each PCR product was visualized by 0.8% agarose gel electrophoresis for site (1) or 3% agarose gel electrophoresis for sites (2) to (9) and stained with ethidium bromide. M1, $\lambda$/Hind III marker; M2, 6X174/Hinc II marker; A, B, normal subjects.

Table 2. Effect of PTH/PTHrP Receptor Genotype on Response to Exogenous [1–34]PTH

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$n$</th>
<th>$\Delta$Ca$^{2+}$ (mEq/l)</th>
<th>$\Delta$Pi (mg/dl)</th>
<th>$\Delta$FECa (%)</th>
<th>$\Delta$FEP (%)</th>
<th>$\Delta$CAMP/Cer (µmol/mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Vr$</td>
<td>8</td>
<td>0.04 ± 0.03</td>
<td>11.6 ± 9.1</td>
<td>0.57 ± 0.29</td>
<td>8.3 ± 2.9</td>
<td>90.3 ± 39.0</td>
</tr>
<tr>
<td>$Vv$</td>
<td>9</td>
<td>0.05 ± 0.05</td>
<td>16.9 ± 6.1</td>
<td>0.70 ± 0.77</td>
<td>7.4 ± 5.0</td>
<td>46.4 ± 23.8*</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. $Vr$.

*Fig. 2. Determination of PTH/PTHrP Receptor Genotype

The long-PCR product (10 kb) was digested with restriction enzyme $\lambda$/Hind III and was visualized by ethidium bromide staining. The digested allele was designated as $v$, and the undigested allele as $V$. AGE, agarose gel electrophoresis.

the polymorphism. Since our study could not find a Bsm I polymorphism in any intron, this polymorphism might exist in other untranslated regions. The second study detected a polymorphism in exon 7 (T or C) by temperature-gradient gel electrophoresis. However, this variant does not change the encoded amino acids. Since the first intron is the longest, at 10 kb, we suspected that a polymorphism would most likely be found in this intron. The second longest is the 3rd intron, which is only 2 kb in length. We tested 23 restriction enzymes in the 1st intron and found only one variant allele. Most of the Japanese subjects had type $Vv$ and $Vv$. Type $VV$ occurred in very few.

Table 2 shows differences in the results of PTH-loading tests between genotype $Vv$ and $Vv$. The $\Delta$CAMP/Cer was significantly higher in subjects with $Vv$ than in subjects with $Vv$ ($p < 0.05$). There were no significant differences in $\Delta$ Ca$^{2+}$, $\Delta$ phosphorus, $\Delta$ FEa and $\Delta$ FEP, nor was any significant difference seen between male and female subjects. The Ellsworth-Howard test is the only available method for studying the efficacy of the PTH/PTHrP receptor in humans. This test is designed to diagnose pseudohypoparathyroidism (PHP), which is a hereditary disorder of PTH action, despite normal PTH secretion. This test detects urinary cAMP and the phosphaturic response to PTH. Type la PHP is caused by the mutation of Gs $\alpha$ protein. Gs $\alpha$ protein is coupled to the PTH/PTHrP receptor and mediates the signaling that activates adenylyl cyclase for the production of cAMP. Although type Ib PHP has been suspected to be caused by mutation of the PTH/PTHrP receptor gene itself, there has only been a negative report. In this study, the urinary cAMP response to PTH was significantly higher in people with type $Vv$ than type $Vv$. Although not reading statistical significance, there was a trend toward a lower FEa and higher FEP in people with type $Vv$ than type $Vv$. This trend agrees with the
difference in urinary cAMP response between genotypes. Since only a small number of subjects participated in this test, a larger number need to be examined to confirm this preliminary finding.

In conclusion, the Van911 polymorphism of the PTH/PTHrP receptor gene can be used to study the phenotypes of disorders involving PTH or PTHrP.

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REFERENCES