Detection of Point Mutation for Human Growth Hormone in Patients with Anti-pituitary Antibody Positive Type 1 Diabetes Mellitus

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We investigated genetic mutations in the coding region of the human growth hormone (hGH) gene in anti-pituitary antibody (APA)-positive patients with type 1 diabetes mellitus (n = 6) or autoimmune thyroid diseases (n = 10) and in APA-negative, healthy controls (n = 10). A point mutation in the hGH gene was discovered in two patients with type 1 diabetes mellitus. No mutations were found in the hGH gene in control subjects, patients with autoimmune thyroid diseases (Hashimoto’s thyroiditis, Graves’ disease) or in the remaining four patients with type 1 diabetes mellitus. The mutation was located in the coding region for the second amino acid in the N-terminal region of hGH. This point mutation was identified in codon 2 in exon 2 of the hGH gene. We successfully developed an allele-specific amplification method for detecting this mutation using the polymerase chain reaction.

KEY WORDS: ANTI-PITUITARY ANTIBODY-POSITIVE TYPE 1 DIABETES; HUMAN GROWTH HORMONE; POINT MUTATION; POLYMERASE CHAIN REACTION

Introduction

The presence of anti-pituitary antibodies (APAs) has been reported in patients with a group of endocrinological autoimmune diseases classified as multiendocrine autoimmune syndrome.1 APAs have been found in patients with adrenocorticotropic hormone deficiency,2 panhypopituitarism,3 empty sella syndrome,4 – 6 pituitary tumours,5 pituitary disorders,7 Hashimoto’s thyroiditis,8 Graves’ disease9 and type 1 diabetes mellitus,10,11 which suggests a pathophysiological role for APAs in these disorders.

Previously, we used an enzyme-linked immunosorbant assay (ELISA) to detect a 22-kilodalton (kDa), including anti-growth hormone (GH) antibody,12 and demonstrated that APAs are frequently expressed in patients with type 1 diabetes mellitus.11 However, it has not been determined whether or not the mechanism by which anti-GH antibody is produced in APA-positive patients with type 1 diabetes mellitus involves a mutation in the coding region for hGH.

Anti-pituitary antibodies do not originate from a single antibody and polyglandular autoimmune syndromes, for which APAs are
the likely cause, are not monogenic diseases. However, APAs are present in autoimmune diseases that show several common features, and it is believed that there may be a common mutation at the gene level.

Single nucleotide polymorphism (SNP) has been studied in relation to the onset of polygenic diseases, and further investigation on the disease mechanisms, including lifestyle factors, is warranted. Also, more emphasis should be placed on investigating the mechanism by which autoimmune endocrinopathy develops. This will also be vital in the development of functional genomics at an individual level.

With the aim of elucidating the autoimmune mechanisms involved in patients with autoimmune disorders, we evaluated previously the pathological changes in the pituitary gland, thyroid, pancreas and adrenal gland using a murine model of autoimmune thyroid disease. Inflammatory changes and hGH receptors were detected in the pituitary, pancreas and adrenal glands but not in the thyroid. This suggests that hGH is involved in the development of APA-positive diseases, a structural change in the hGH protein due to a mutation in the gene sequence leading to APA formation.

We investigated genetic mutations in the coding region of the hGH gene, using the polymerase chain reaction (PCR), in APA-positive patients with type 1 diabetes mellitus or autoimmune thyroid diseases and in normal, healthy controls. In addition to determining the base-pair sequence of hGH and conducting a comparative study in different types of APA-positive patients and APA-negative individuals, we also developed a PCR method, using specifically designed primers, to detect a point mutation in the gene sequence of hGH.

Subjects and methods

SERUM SAMPLES
Serum samples were obtained from both APA-positive Japanese patients with a diagnosis of type 1 diabetes mellitus or autoimmune thyroid disease (i.e. Graves’ disease and Hashimoto’s thyroiditis) and from APA-negative healthy control subjects. Written informed consent was obtained from each individual. Demographical characteristics are shown in Table 1. Written consent was also obtained from the local ethics committee.

PREPARATION OF RAT PITUITARY ANTIGENS
Three rat pituitary glands, each weighing 60 mg, (RKL, Gilbertsville, PA, USA) were homogenized in 3 ml buffer (0.25 mol/l sucrose, 0.1 mmol/l EDTA, 3 mmol/l TRIS–HCl buffer, pH 7.4) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) and centrifuged at 10 000 g at 4°C for 10 min. The resultant supernatant was used as the source of pituitary antigens for the subsequent procedures. The protein concentration and protein standard were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA).

ENZYME-LINKED IMMUNOSORBANT ASSAY
Microtitre plates (Nunc-Immuno Module, Nunc A/S, Roskilde, Denmark) were coated with 100 µl rat pituitary antigen (supernatant diluted to 25 µg/ml in 0.05 mol/l sodium bicarbonate buffer, pH 9.6) in each well by overnight incubation at 4°C. The plates were washed with 0.05% phosphate-buffered solution (PBS)-Tween 20 (pH 7.2) and blocked with 300 µl 3% bovine serum albumin (BSA) in PBS at 37°C for 30 min. After washing with 0.05% PBS-Tween 20,
### TABLE 1:
Demographic characteristics of (A) anti-pituitary antibody-positive patients with type 1 diabetes mellitus, (B) APA-positive patients with autoimmune thyroiditis and (C) APA-negative normal control subjects

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<th>FPG (mg/dl)</th>
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<th>TSH (µg/ml)</th>
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<td>Control group (n = 10)</td>
<td>39.3 ± 9.8</td>
<td>160.1 ± 9.7</td>
<td>59.6 ± 12.7</td>
<td>22.67 ± 2.09</td>
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*All of the patients with diabetes were receiving insulin therapy.

BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycosylated haemoglobin; GAD, glutamic acid decarboxylase autoantibodies; CPR, C-peptide immunoreactivity; IGF-1, insulin-like growth factor 1; fT3, free tri-iodothyronine, fT4, free thyroxine, TSH, thyroid stimulating hormone; TRAb, TSH receptor antibody; McAb, anti-microsome antibody; TgAb, anti-thyroglobulin antibody; ND, not determined.
each well received 100 µl sample sera or APA-positive control sera and the plate was incubated, with slow shaking at room temperature for 2 h. Plates were then washed five times with PBS. Peroxidase-labelled rabbit anti-human immunoglobulin G (IgG) polyclonal antibody (Sigma, St Louis, MO, USA) was added (100 µl to each well; antibody concentration, < 25 µg/ml), and the plate was left at room temperature for 1 h. After washing with PBS, 100 µl substrate solution (0.1 mol/l sodium acetate-citrate containing 0.006% hydrogen peroxide and 0.2 mg/ml 3,3′,5,5′-tetramethyl-benzidine dihydrochloride, pH 5.5) was added to each well. This was followed by incubation at room temperature for 30 min. The colorimetric reaction was stopped by 100 µl 0.5 M H₂SO₄. The absorbance at 450 nm was measured using a Behring ELISA Processor III (Behring Werke AG, Marburg, Germany), and the cut-off index was calculated. Assays were typically performed in duplicate.

WESTERN BLOT ANALYSIS
Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was performed according to the method of Laemmli.¹⁵ In brief, 50 µl rat pituitary antigen (approximately 200 µg protein) was mixed with 50 µl sample buffer (0.1 M dithiothreitol, 2% SDS, 15% glycerol, 0.006% bromophenol blue, 5% 2-mercaptoethanol, 0.08 mol/l TRIS–HCl, pH 6.8), heated at 96°C for 1 min, and electrophoresed through a gradient of 4 – 20% polyacrylamide gel (TEFCO, Tokyo, Japan) for 1.5 h at 18 mA in running buffer (25 mmol/l TRIS, 192 mmol/l glycine, 0.1% SDS, pH 8.0). Separated proteins were transferred to a polyvinylidene difluoride membrane (TEFCO) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Richmond, CA, USA) at 6 V for 1 h. Non-specific binding sites were blocked by incubation of the membrane overnight at 4°C with Block Ace (Dainippon Inc., Osaka, Japan). The protein-loaded membrane was washed three times, for 5 min each time, in washing buffer (5% non-fat milk, 0.05% Tween 20 in PBS, pH 7.2). The membrane was then incubated in 1:101 diluted patient serum (dilution buffer: 5% non-fat milk, 3% BSA in PBS, pH 7.2) at room temperature for 2 h and washed three times, for 5 min each time, in washing buffer. It was then incubated in 1:500 diluted biotinylated rabbit anti-human IgG polyclonal antibodies (Sigma) in dilution buffer at room temperature for 1 h, and washed in washing buffer three times, for 5 min each. The membrane was subsequently incubated in 1:50 diluted streptavidin–biotin complex peroxidase (Dakopatts, Clostrup, Denmark) in 3% BSA in PBS, pH 7.2, at room temperature for 30 min and washed in washing buffer three times, for 5 min each. The labelled bands were revealed by chemiluminescence, using the POD Immunostein Set (Wako Pure Chemicals, Kyoto, Japan), for 5 min.

POLYMERASE CHAIN REACTION
Genomic DNA was extracted from the subjects’ blood (3 ml). The PCR was carried out in a thermal cycler (Takara PCR Thermal Cycler, Takara Biomedicals, Tokyo, Japan). A Takara PCR Amplification Kit (Takara Co., Tokyo, Japan) was used and the cycles were as follows: 1 cycle at 94°C for 3 min, 38 cycles at 94°C for 1 min, 38 cycles at 60°C for 1 min and 38 cycles at 72°C for 1 min followed by 1 cycle at 72°C for 10 min. Three microlitres of 25 mM MgCl₂ was used in 50 µl total volume. Primer concentration was 0.5 µM; target DNA, 0.05 µg/50 µl; and Taq polymerase, 1.25 U/50 µl.

The code for the primers was based on part of the coding region of hGH: sense, 5′-TATGAATTCCTCTGCCTGCCCTGGCTTCAAGAG-3′; and anti-sense, 5′-CTAACA\nCTAGTCTCTCAAAGT-3′.
The accuracy of the PCR was confirmed by sequencing (ABIPRISM™ 310 Genetic Analyzer, Perkin Elmer Co., Tokyo, Japan).

DETECTION OF THE C → A MUTATION IN THE HUMAN GROWTH HORMONE GENE
The outline for this PCR approach to detect point mutations within the hGH is shown in Fig. 1. In this method, the 3'-terminal sequence of the PCR primer was constructed specifically so that it would bind and amplify the mutated region of the hGH gene as identified using the techniques described in the previous section.

The cycles used in the PCR were carried out as described in the previous section. The concentration of 25 mM MgCl₂ was 2.6 µl/50 µl of total volume. The primers used in the detection of the point mutation within the coding region of the hGh gene were: sense, primer-F (mutant 1) 5'-CTCTGCCCTGGCTTCTCAAGAGGCGAGTGCC TTCCA-3'; and anti-sense, primer-R 5'-AAA CTCTGGTAGGTTGCAAAGGCGAGCCAGCTG-3'.

The sequence of the PCR products was

Patient 1

Primer-F - A G T G C C T T C A A - 3’

Genome DNA - T C A C G G A A G G T T G G T A A G G A A T A G G - 3’

Amplified DNA - A G T G C C T T C A A A C C A T T C C C T - 3’ → Amplified

Genome DNA - T C A C G G A A G G T T G G T A A G G A A T A G G - 3’

Patient 2

Primer-F - A G T G C C T T C A - 3’

Genome DNA - T C A C G G A A A G G T T G G T A A G G A A G T A G G - 3’

Amplified DNA - A G T G C C T T C A A A C C A T T C C C T - 3’ → Amplified

Genome DNA - T C A C G G A A A G G T T G G T A A G G A A T A G G - 3’

Normal

Primer-F - A G T G C C T C C A - 3’

Genome DNA - T C A C G G A A G G G - 3’

Primer-F - A G T G C C T T C C A - 3’ Not amplified

Genome DNA - T C A C G G A A G G G T T G G T A A G G A A T A G G - 3’

**FIGURE 1:** The detection of a point mutation using the polymerase chain reaction. Patient 1 had one mutation in the sequence of the human growth hormone whereas patient 2 had two, but both sequences were amplified using the specifically designed primers. The unmutated sequence was not amplified using these primers.
confirmed by fluorescence sequencing as described above.

Results

DETECTION OF MUTATION BY DNA SEQUENCING

In the patient and control groups, a 22 kDa APA band was detected by ELISA and Western blotting (Fig. 2). A highly hydrophilic region of the hGH gene was amplified in all patient groups (Fig. 3). In patients 1 and 2, the C → A point mutation was detected, by fluorescence sequencing, in the codon for the second amino acid from the N-terminus of hGH, i.e. CCA → CAA (Fig. 4). This point mutation was identified in exon 2 of the hGH gene. Further, codon 1 of patient 2 was not associated with an amino acid mutation, but a silent mutation of C → T, which would result in a mutation of TTC → TTT, was found. The C → A mutation was found in only two of the six APA-positive patients with type 1 diabetes mellitus and in none of the patients with autoimmune thyroid diseases. In addition, no mutation was found in the normal, healthy control group.

Apart from the mutations described above, the base sequence was identical within the range of PCR amplification in all three groups.

DETECTION OF THE MUTATION BY THE ALLELE-SPECIFIC AMPLIFICATION METHOD

The mutated and normal base sequences of hGH DNA found in the present study are

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**FIGURE 2:** Western blot analysis revealing a 22 kilodalton (kDa) band corresponding to anti-pituitary antibodies (APAs). This 22 kDa band is indicated by an arrow. Lanes 1 – 5 and 8 – 10 contain APA-negative sera, whereas lanes 6 and 7 contain APA-positive sera.

**FIGURE 3:** The detection of human growth hormone (hGH) fragments by polymerase chain reaction (PCR). Exons 2 – 4 of the hGH gene were amplified (A). The exact sequence of the forward (sense) and reverse (anti-sense) primers (B) and the products of PCR anti-pituitary antibody-positive patients with type 1 diabetes mellitus (C) are shown.
shown in Fig. 1. Our primers bound to the mutated sequence, resulting in DNA amplification in patients 1 and 2. The hGH DNA sequences that did not carry the C → A mutation were not amplified.

In patients 1 and 2, positive bands corresponding to the mutated region of the hGH gene were detected. Such bands were not found in patients without this mutation (Fig. 5). This method was carried out for all cases under the present study. Bands were found only in patients 1 and 2, while no band was detected in any of the other individuals.
Discussion

In this study, we used a new procedure based on PCR to identify a point mutation in exon 2 of the hGH gene, which would result in a change in the amino acid sequence in two APA-positive patients with type 1 diabetes mellitus. There was no evidence of a growth disorder in any of the patients, and it is thus believed that this particular location within hGH has no direct effect on function. Our results suggest that the mutation described above could be an SNP, which may be involved in the mechanism by which autoimmune endocrinopathy, i.e. polygenic disease, develops. This new detection method may be instrumental in making future advances in genetic studies in the field of autoimmune disease.

Anti-pituitary antibodies have been detected at a high frequency in patients with type 1 diabetes mellitus.\textsuperscript{10,11,16} APAs have also been detected in patients with a group of endocrinological diseases classified as polyglandular autoimmune syndrome.\textsuperscript{17,18} We detected the presence of a 22 kDa band corresponding to APAs using ELISA\textsuperscript{12,18} and Western blotting.\textsuperscript{17} These APAs are known to contain both anti-GH and anti-prolactin antibody.\textsuperscript{5} Citing lymphocytic hypophysitis\textsuperscript{19,20} as an example, Crock \textit{et al.}\textsuperscript{21} have detected APAs in patients with autoimmune diseases.

Anti-pituitary antibodies have been detected in 7\% of patients with lymphocytic hypophysitis\textsuperscript{22} and in 45\% of patients with pituitary dysfunctions, such as pituitary adenoma and empty sella syndrome.\textsuperscript{5} APAs have also been identified as a factor involved in the disease mechanism for virus-induced diabetes mellitus.\textsuperscript{23} In addition, APA has been detected in some cases of idiopathic GH secretory insufficiency,\textsuperscript{12,21,24} where it was demonstrated that the reactivity of GH decreased in response to administration of APA-positive serum.\textsuperscript{12} These results indicate that anti-GH antibody plays an important role in the function of APAs. In this respect, it is necessary to evaluate the relationship between GH and the development of APAs. Based on the mutation of the codon CCA $\rightarrow$ CAA, the second amino acid from the N-terminal of hGH, i.e. proline, would become a glutamine residue (Fig. 6). This may cause an alteration in the protein structure and final protein configuration and, as a result, the mutated product may be recognized as an antigen.

It is plausible that endocrinological autoimmune diseases may be genetically determined. Thus, the presence of auto-antibodies, including anti-GH antibodies, may also have a genetic cause. The results of the present study were limited to type 1 diabetes mellitus patients who were APA-positive, and this suggests the possibility that these individuals are genetically alike. Furthermore, the relationship of type 1 diabetes mellitus with human leukocyte antigen (HLA)-DR has been proposed in the past,\textsuperscript{25} and it appears that there may also be a relationship between APA-positivity and HLA-DR.\textsuperscript{20,22} In this respect, it is considered that, with the involvement of the HLA-DR genes in the background, autoimmune reactions can be induced at various sites in the body, resulting in the production of APAs. At present, it is not exactly known whether or not this concept can explain all the pathological conditions found in cases of APA-positivity. A comparative study of the presence of mutations in the hGH gene in different types of APA-positive patients and APA-negative individuals therefore seems important.

The new point mutation identified in this study was found in a region in the hGH gene that is remote from the location where a mutation associated with APA positivity has
FIGURE 6: The point mutation in codon 2 in the human growth hormone (hGH) gene and the corresponding amino acid substitution in the hGH protein. The mutation (CCA → CAA) occurs in exon 2 of the hGH gene. This mutation would result in the substitution of proline (Pro) to a glutamine (Glu) residue. Exon 2, in conjunction with exon 1, encodes a region that becomes separated in the final protein structure as a signal peptide. The CCA → CAA mutation would be located very close to the N-terminal boundary of this peptide.

been detected previously.\textsuperscript{26,27} The mutation reported herein was located in exon 2 of the hGH gene and would be associated with an amino acid substitution in the hydrophilic region of hGH, a site which may be involved in antibody formation. In conjunction with exon 1, part of exon 2 codes for a protein region that becomes separated as a signal peptide. The C → A point mutation would result in an amino acid substitution very close to the N-terminal boundary of this peptide.
The C → A mutation was found in two patients with type 1 diabetes mellitus, but not in any of the patients with autoimmune thyroid diseases. This suggests that these diseases develop via different mechanisms, even though the presence of APAs is a common factor. The mechanisms are likely to involve different APAs, such as an anticytoplasm antibody or anti-cell membrane antibody. As evidence for this, hGH receptor (hGHR) expression has been confirmed in various types of tissues.\textsuperscript{28 - 32} The regions of the body in which these receptors are expressed reflect the pattern of inflammatory changes that we found in the APA-positive mouse model.\textsuperscript{14} That is, the expression of GHR was confirmed in the pituitary gland, pancreas, and adrenal gland, but not in the thyroid gland. This indicates that GH may be an important mediator in diseases associated with APA-positivity, which was one of the reasons why this study to investigate mutation of the hGH gene sequence was carried out.

Growth hormone-binding protein (GHBP) is known to bind GH,\textsuperscript{33} but its physiological functions have yet to be elucidated. GHBP functionality may be closely related to autoimmunity, as suggested by the fact that GHBP expression increases in the blood of pregnant mice in the late gestation period.\textsuperscript{34} Pharmacodynamic changes in GHBP have been associated with endogenous turnover of GHR\textsuperscript{35} and a mechanism by which GHBP is generated from GHR has also been considered.\textsuperscript{36,37} The various features of GHBP mean that it is not currently considered to be an APA. Circulating levels of hGH are increased in rats with type 1 diabetes mellitus but the level of insulin-like growth factor-1 is low.\textsuperscript{38} The point mutation described herein may be related to one of these disease conditions.

In the present study, only the point mutation in the DNA sequence was investigated, and it was not confirmed whether or not antibodies were actually produced as a result of the mutation. In addition, it is not certain whether or not a change in the protein structure would trigger antibody formation of antibodies because structural changes in GH were not confirmed. For this reason, we will extend our studies to investigate antibody formation, covering also other autoimmune diseases in which APAs are found. In the future, it may be possible to use genetic analysis to diagnose APA-positive type 1 diabetes mellitus at an early stage.

Single nucleotide polymorphism has become increasingly important in the analysis of polygenic diseases in recent years.\textsuperscript{13} SNP is based on the substitution of a single base, which occurs in the general population at a frequency of ≥ 1%. The exact frequency of the C → A mutation in these diseases is not known, and it is not certain whether or not it falls precisely under the category of an SNP. However, it may be identified in future studies as a gene that predisposes individuals to these diseases. We expect that the scope of such studies will be enlarged through the adoption of our method for detecting mutations. It is believed that further details of autoimmune disease will be elucidated and significant developments in this area of research will be made.

Acknowledgement

The present study was supported in part by a grant-in-aid (No. 04454545 to IK) for Scientific Research from the Ministry of Education, Science and Culture, Japan.
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