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Transgenic Mice Expressing A Mutant Human GH Gene Causing Type II IGHD

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Abstract: We identified several mutations in the intron 3 of human growth hormone gene I (hGH-I) in patients with isolated GH deficiency (IGHD) type II characterized by an autosomal dominant trait. The mutations result in exon 3 skipping and generation of 17 Kd mutant GH. To elucidate how the mutation causes dominant trait, transgenic mice expressing a mutant hGH gene (the first guanine to adenine transversion in intron 3: GH-I; IVS3+1: G-A) were produced in C57BL/6 strain. Genotypes of mice were identified by PCR-amplified products of tail snip DNAs. Delivery of the mutant hGH transgene into 76 fertilized eggs resulted in production of two male heterozygous transgenic mice ($hGH^{+/-}$, the zero filial generation, F0). Since the mating of the transgenic mice with the same strain was unsuccessful, they were outcrossed with CD-I (ICR) strain. Only one mouse gave birth, producing 4 male and 7 female (F1) harboring the mutant hGH gene in one allele ($hGH^{+/-}$). F1 mice were mated again with the wild type ICR strain, generating 82 $hGH^{+/-}$ mice (F2 : 51 males and 31 females). To study whether somatotrophs in F2 mice express the mutant hGH gene, RNA extracted from the pituitary was subjected to RT-PCR. It was demonstrated that the F2, $hGH^{+/-}$ mice express the mutant hGH gene, lacking exon 3. Thus, these heterozygous mice were sib-mated to generate homozygous mice (F3). The mating resulted in 27% $hGH^{-/-}$, 64% $hGH^{+/-}$ and 9% $hGH^{+/+}$ mice, indicating that the transgene was carried stably to the descendants and did not interfere with the reproduction. These mice will be a valuable model to study how type II IGHD develops during the course of development.

Key words: transgenic mice, growth hormone (GH), type II IGHD

Human growth hormone (hGH) is essential for normal postnatal growth. It is synthesized and released from the somatotrophs of the anterior pituitary gland. Hereditary isolated growth hormone deficiency (IGHD) is classified into three types by its inheritance. Type I IGHD is inherited in an autosomal recessive manner, type II in an autosomal dominant, and type III in an X-liked recessive manner.[1)] In type I IGHD, the affected patients are either homozygous or compound heterozygous for mutations in the GH-I gene.[1-5)] In type II IGHD, several different mutations have been reported at the donor splice site of intron 3 of the GH-I gene,[6-12)] and the affected patients are heterozygous for the mutations. Genetic defects in type III IGHD have not yet been established.

We identified several point mutations at the donor splice site of intron 3 of the GH-I gene from Japanese patients with type II IGHD.[10-12)] These mutations include a guanine (G) to adenine (A) transition at the first nucleotide of intron 3 of GH-I gene, and a G to A transition and a G to cytosine (C) transversion at the fifth nucleotide. Transcripts of all these mutant GH-I genes were shown to lack exon 3 consisting of 120 bases. Thus, the protein encoded by the mutant mRNA should lacks in- frame 40 amino acid residues. *In vitro* study expressing the wild type and the mutant hGH gene by transfection demonstrated that secretion of the wild type GH is inhibited by the mutant in cell lines derived from somatotroph and corticotroph but not in those derived from kidney and hepatocyte, suggesting that the mutant GH exerts dominant negative effect in the secretory granules.[13)]

To date, three dwarfism mice models were reported. Little (Lit) is caused by abnormal GH releasing hormone gene, Snell dwarf mice (dw) and Jackson dwarf mice (df) are caused by mutations in pit-1 gene.[1)] However, a mice model developing type II IGHD has not been reported. We thus produced transgenic mice expressing the mutant hGH gene to study the pathophysiology of type II IGHD during the course of development.

Materials and Methods

The experimental protocol was approved by the Committee for Animal Experiment of the Research Institute of Environmental Medicine, Nagoya University. Production of

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transgenic mice harboring the mutant hGH gene was carried out in the Kumamoto University and the protocol was approved by the Recombinant DNA Committee.

1. Animals treatment

Production of transgenic mice expressing a mutant hGH gene (GH-I; IVS3+1: G-A) causing type II IGHD was carried out by the Center for Animal Resources Development in the Kumamoto University. Transgene was constructed by inserting a rat GH gene promoter upstream of the mutant hGH gene. The promoter was 310 bp long upstream from the transcription start site of rat GH gene and was shown to direct somatotroph-specific expression in transgenic mice.[14,15] Transgenic mice were produced by micro injection of the mutant hGH gene into fertilized eggs of C57BL/6 strain mice. A total of 76 mice derived from the injection were transported from Kumamoto University, reared and bred in the SPF room of Research Institute of Environmental Medicine, Nagoya University.

Only two male transgenic mice ($hGH^{+/-}$) were identified (F0). They were cross-bred with siblings. However, the sib matings were not successful. They were thus outcrossed with CD-1(ICR) strain. Only one mouse was successful to give birth (F1). The mice in F1 generation were continued to outcross with CD-1(ICR) strain (F2). Finally, $hGH^{+/-}$ mice were cross-bred with sibling of same genotype (F3) to produce $hGH^{+/+}$ mice. In the each filial generation, pups weaned at 4 weeks. Genotyping and determination of mouse GH (mGH) and human mutant GH mRNA will be described below.

2. Genotyping by PCR amplification

DNA was extracted from tail snip as previously described[10] and amplified by PCR. The primers used were sense (5′- TCC CTG GAG GGA TGG AGA GAG -3′ in intorn 3) and antisense (5′- CTC TAA CAC AGC TCT CAA AGT-3′ in exon 5) for the mutant hGH gene and sense (5′-CTT GGA TTC AAA ATG GTC TCA GAG-3′ 238 bp upstream of transcription start site) and antisense (5′-TCC GAG AGT CTA GAG AGA GAC A-3′ in exon 2) for mGH gene. PCR condition was previously described.[10] After the amplification, both mGH and mutant hGH products were electrophoresed on 5.0% acrylamide gel in 1xTBE buffer (90mM Tris base, 90mM Boric acid, 1mM Na_2EDTA, pH 8.0). A DNA molecular weight marker (Nippon Gene Ltd., Tokyo, Japan) was used to estimate the sizes of the products. The amplified fragments were photographed after staining with ethidiumbromide under UV light. Special caution was taken to standardize the staining and photography of the gels. The photographic data were fed to Adobe Photoshop (Adobe Systems Inc., California, USA), and the density of each mGH and mutant hGH band was analyzed by NIH image (National Institutes of Health, Bethesda, USA). A standard PCR product from heterozygous template was used to calibrate the amount of each fragment. The density ratio (hGH/mGH) in the standard was assigned as 1. A mouse with the ratio more than 2 was regarded as homozygous.

3. RT-PCR to identify hGH expression

Total RNA was extracted from the pituitary of the $hGH^{-/-}$ and $hGH^{+/-}$ mice by the acid-phenol-chloroform method as previously described.[16] To analyze the transcripts from the mGH and mutant hGH genes, complementary DNA (cDNA) was synthesized using $oligo(dT)_{15}$ primer and SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD,USA) according to the manufacture's instruction. Human and mouse GH cDNAs were amplified by PCR using a sense primer (5′-GCC CAA CTC CCC GAA CCA CT-3′ in exon 1) and an antisense primer (5′-GAG GCA CTG GGG AGG GGT CAC-3′ in exon 5) for human[13] and a sense primer (5′-CAG GCA AGG AGG AGG CCC AG-3′ in exon 3) and an antisense primer (5′-GAA GGC ACA GCT GCT TTC CAC A-3′ in exon 5) for mouse GH cDNA. PCR and electrophoresis conditions were previously described.[10] After the amplification , both mGH and mutant hGH products were electrophoresed on 1.5% agarose gel in 1xTAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.4). A wild type hGH cDNA clone[13] was used as a template to confirm that the mutant hGH transcript in transgenic mice lacks the exon 3.

Results and Discussions

Rat GH gene promoter has been shown to direct the expression of the gene of interest in somatotrphs of the transgenic mice.[14,15]

Figure 1 shows PCR-amplified products of mGH and hGH

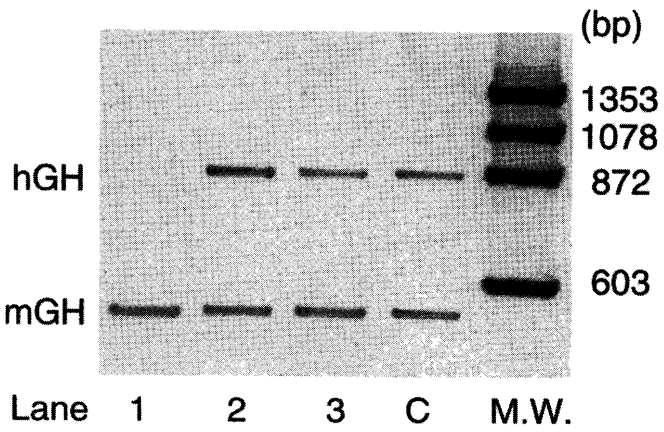

Fig. 1 Determination of genetypes by PCR-amplification of hGH and mGH genes from the tail snip DNAs.
Lane 1: non-transgenic mouse, Lane 2: homozygous mouse ($hGH^{+/+}$), Lane 3: heterozygous mouse ($hGH^{+/-}$), Lane 4: control heterozugous mouse, Lane 5: DNA MW markar. The control heterozygous mouse was selected from F2. The DNA from the mouse was used as control for determination of genotype of each filial generation.

gene using mice tail snip DNAs as templates. The template used in the control lane (C) was obtained from a mouse heterozygous to hGH gene, since it is the product of mating with wild-type and heterozygous F1 mice. In lane 1, only mGH gene product was observed, identifying the mouse to be non-transgenic, $hGH^{-/-}$. In lane 2, the products of both mGH and mutant hGH genes were amplified, identifying the mice to be transgenic. By the calculation of the density of the band of hGH with respect to mouse GH gene, the mouse represented in the lane 2 was homozygous $hGH^{+/+}$ mouse and that in lane 3 to be heterozygous. Homozygousity of the transgenic mice will be confirmed by outcrossing $hGH^{+/+}$ mouse with $hGH^{-/-}$ mouse of CD-1(ICR) strain which should give birth of all heterozygous mice.

Table 1 and 2 show the number and the incidence of mice with different genotypes in each filial generation. In the F0 generation, 76 mice were obtained from the fertilized eggs of C57BL/6 strain injected with the mutant hGH transgene. We identified 2 male transgenic mice (2.6%). The incidence was concordant with a report by Hirabayashi M et al.[17] Sibmating of the two heterozygous mice was unsuccessful, suggesting the low fertility rate of C57BL/6 strain. Therefore, the transgenic mice in the F0 generation were outcrossed with $hGH^{-/-}$ CD-1(ICR) strain. In the F1 generation, the rate of trangenic mice was 18% (11/61). If the transgenic mouse in F0 was heterozygous or homozygous, 50% or 100% of the siblings should be heterozygous, suggesting the mouse was heterozygous and the transgene was unstable in F1 generation. In F2 generation, the proportion of transgenic mice was 45% (82/182), confirming the heterozygosity of the transgenic mice in F1 generation. Since the ratio is in agreement with the Mendelian law, it was suggested that the transgene is stably transmitted from F1 to F2 generation. In the F3 generation in which heterozygous mice were mated, the rate of non-transgenic ($hGH^{-/-}$), heterozygous ($hGH^{+/-}$) and homozygous ($hGH^{+/+}$) mice was 27.0% (39/143), 64.0% (91/143) and 9.0% (13/143), respectively. Since expected ratio of each genotype should be $hGH^{-/-}$; 25%, $hGH^{+/-}$; 50% and $hGH^{+/+}$; 25%, it is possible that the number of homozygous mice was underestimated.

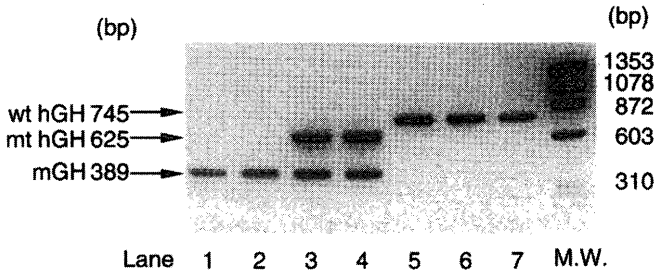

Fig. 2 Expression of mGH and mutant hGH mRNA in the pituitary. RT-PCR was carried out to analyze the expression mGH and the mutant GH mRNA. The expected size of the RT-PCR product for mGH cDNA was 389 bp and for the mutant GH cDNA lacking exon 3 was 625 bp and wild-type hGH cDNA was 745bp. To confirm the exon 3 skipping from the mutant hGH gene, wild type hGH cDNA was amplified with the same primers, yielding a band of 745 bp. Lane 1,2: non-transgenic mice, Lane 3, 4: heterozygous mice ($hGH^{+/-}$), Lane 5, 6 and 7: wild type hGH cDNA. Lane 8: DNA MW markaer.

Table 1 The genotypes of the mice in each filial generation

Genotypes / Generation	Male			Female			
	Wild-type (hGH-/-)	Heterozygote (hGH+/-)	Homozygote (hGH+/+)	Wild-type (hGH-/-)	Heterozygote (hGH+/-)	Homozygote (hGH+/+)	Total
F0	45	2	0	29	0	0	76
F1	24	4	0	26	7	0	61
F2	56	51	0	44	31	0	182
F3	21	56	9	18	35	4	143

Number of mice having genotype was determined by PCR analysis of tail snip DNAs.

Table 2 The proportion of genotypes of mice in each filial generation

Genotypes / Generation	Wild-type (hGH-/-)	Heterozygote (hGH+/-)	Homozygote (hGH+/+)	Mothod of crossing
F0	97.4	2.6	0	C57BL/6
F1	82.0	18.0	0	C57BL6 × CD-1
F2	55.0	45.0	0	Hetero × CD-1
F3	27.0	64.0	9.0	Hetero × Hetero

Data were expressed as % of total mice genotype determined by PCR analysis of tail snip DNAs.

Figure 2 shows expression of mGH and hGH mRNA in the pituitary of $hGH^{+/-}$ and $hGH^{-/-}$ mice in F2 generation. The primers for RT-PCR were designed to contain more than two introns, to avoid possible artifact due to DNA contamination. The RT-PCR products from non-transgenic mice (lane 1 and 2) demonstrates only mGH mRNA expression while heterozygous $hGH^{+/-}$ mice (lane 3 and 4) clearly demonstrates the expression of the mutant hGH mRNA. Wild-type hGH transcript is shown in lane 5–7. Note that the mutant human GH transcript is 120 base shorter than that of wild-type hGH transcript.

Since the stable transmission of the transgene was observed from F2 generation, we are starting to analyze the phenotype in the heterozygous and homozygous mice.

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