A novel mutation of exon 7 in *growth hormone receptor* mRNA in a patient with growth hormone insensitivity syndrome and neurofibromatosis type I

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Abstract. Growth hormone insensitivity syndrome (GHIS), a genetic disease characterized by growth retardation combined with high serum concentration of growth hormone (GH) and low insulin-like growth factor 1 (IGF-1) levels, can be caused by mutations in the *GH receptor* (GHR) gene. We investigated the molecular defects in the *GHR* gene in a patient with neurofibromatosis type 1 (NF-1). The patient, a 2-year-old boy with NF-1, was assessed on his short stature by auxological, biochemical and molecular studies. Height of the patient and his family members were measured and compared to normal control. Serum concentrations of GH, IGF-1 and IGF-binding protein 3 (IGFBP3) in the patient were measured during a GH stimulation test. We examined the *GHR* gene in the patient and his parents. Genomic DNA and mRNA of the *GHR* gene were extracted from peripheral lymphocytes. All the exons and the flanking regions of the *GHR* gene were amplified by PCR, and directly sequenced. The patient's height was 75 cm (-2.89 SDS) with gradually reducing growth velocity, while the heights of the other family members were within the normal range. The GH stimulation test revealed that serum GH concentrations in the patient were much higher than those in the control group, and serum IGF-1 and IGFBP3 levels were extremely low. There was no germline mutation in the exons or the flanking regions of the patient's *GHR* gene. Interestingly, a deletion of 166 bases of exon 7 in the *GHR* mRNA was found, and it was suggested that the novel mutation resulted in premature termination (M207 fs. X8). This mutation decreases GH binding affinity to the GHR, and, thus, would be responsible for growth retardation.

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Introduction

Neurofibromatosis type 1 (NF-1) is a common genetic disorder that is transmitted by autosomal dominant inheritance, and is diagnosed when two of the following signs are found in individuals: six or more cafe-au-lait spots over 5 mm in greatest diameter during prepubertal period, freckling in the axillary or inguinal region 2-3 mm in diameter, two or more Lisch nodules in the iris, optic gliomas, two or more neurofibromas or one plexiform neurofibroma, skeletal abnormalities such as a distinctive osseous lesion like sphenoid dysplasia or pseudo-arthritis, or family history of a first degree relative with NF-1 (1).

Short stature, defined as a height that is less than 2 standard deviations to the population mean, has been known to be one of the most common complications in NF-1. Although it is known to be associated with several clinical risk factors related to NF-1, the exact mechanism for short stature is not yet defined (2).

Growth hormone insensitivity syndrome (GHIS) is known to be a very rare genetic disorder with an autosomal recessive inheritance, and is characterized by normal or elevated GH concentration, very low insulin-like growth factor 1 (IGF-1) and IGF-binding protein 3 (IGFBP3) levels in serum. GH binds to its receptor and then activates an intracellular signal transduction pathway, leading to the production of IGF-1. IGF-1 mediates the actions of GH and performs a negative feedback of GH secretion at the pituitary level. Any defect in this pathway results in GHIS. Consequently, patients with this syndrome have postnatal growth failure, leading to short adult stature, and abnormal facial morphologies such as frontal bossing of the forehead, hypoplasia of the midfacies and depressed nasal bridge (3,4).

GHIS was first reported by Z. Laron in two siblings from an Oriental Jewish family, since then many cases have been described and most of them revealed gene defects of various proteins involved in the GH - IGF-1 signaling cascade (5,6).

We examined a patient with GHIS accompanied by NF-1, the first reported case with biochemical and molecular analysis.

Materials and methods

Patient. A pediatric patient with growth retardation was brought to the department of pediatric endocrinology in our

Table I. Oligonucleotide sequences of the *GHR* primer sets used in this study.

Sense primer (5'→3')		Antisense primer (5'→3')		Amplified region
Exon 2 F	GTCTGCTTTTAATTGCTGGGC	Exon 2 R	ACACTGAGGGTGGAAATGGA	Exon 2
Exon 3 F	CCTCTTTCTGTTTCAGCCAC	Exon 3 R	GGATAGTAGCTTAATTACACTA	Exon 3
Exon 4 F	AGGATCACATATGACTCACCT	Exon 4 R	AGTGTACTTTAGTAGGTACATC	Exon 4
Exon 5 F	TAAGCTACAACATGATTTTTGG	Exon 5 R	TTAGTCTAAAACTATGTCAAAG	Exon 5
Exon 6 F	GTGTCTGTCTGTGTACTAATG	Exon 6 R	AGAAAAGTCAAAGTGTAAGGTG	Exon 6
Exon 7 F	TAGTGTTCATTGGCATTGAG	Exon 7 R	ACAAAAGCCAGGTTAGCTAC	Exon 7
Exon 8 F	AAACTGTGCTTCAACTAGTCG	Exon 8 R	GGTCTAACACAACTGGTACA	Exon 8
Exon 9 F	GAATATGTAGCTTTTAAGATGTC	Exon 9 R	CATATGACAGGAGTCTTCAGGTG	Exon 9
Exon 10 F	GAGTTTCTTTTCATAGATCTTC	Exon 10 R	GGTTTAAACATTGTTTTGGC	Exon 10
Exon 10-1 F	GATCTTCATTTTCTTTCTAT	Exon 10-1 R	CTACCTGCTGGTGTAATGTC	Exon 10-1
Exon 10-2 F	CATCGACTTTTATGCCCAGG	Exon 10-2 R	ATGAATGGAGGTATAGTCTGG	Exon 10-2
Exon 10-3 F	CATGTTCCAGGTTCTGAGAT	Exon 10-3 R	GGTTTAAACATTGTTTTGGC	Exon 10-3

hospital. The patient's birth, medical, nutritional and family history were reviewed. His height, weight and head circumference were measured and assessed by standard deviation score (SDS) as well as the assessment of growth velocity since birth. Informed consent was obtained from three family members who participated in the study.

Biochemical and imaging study. GH stimulation was performed. The basal serum concentrations of GH, IGF-1, IGFBP3, thyroid stimulating hormone (TSH) with free T4, luteinizing hormone (LH), follicle stimulating hormone (FSH) and cortisol were measured. Serum concentrations of GH and cortisol were measured every 30 min during 120 min after stimulation of insulin (0.1 IU/kg, intravenously). Data of serum GH concentrations was also obtained from age-matched control group (n=10) and compared to those of the patient. This test was planned to be discontinued if hypoglycemic symptoms occurred or when serum glucose level after stimulation with insulin was 45 mg/dl or <50% of basal level. Brain magnetic resonance imaging (MRI) scanning was also performed in order to investigate abnormal brain lesions associated with growth retardation.

Extraction of genomic DNA and total-RNA. Genomic DNA (gDNA) was isolated from the patient and his family members using Accuprep Genomic DNA extraction kit (Bioneer, Seoul, Korea), according to the manufacturer's instructions. Total-RNA extraction was carried out in two steps; leukocytes from whole blood were obtained in step I, RNA from the leukocytes was extracted in step II subsequently. Leukocytes were isolated from the patient, his family members and healthy control using Ficoll-Paque Plus (GE Healthcare, USA), according to the manufacturer's instructions.

Reverse-transcription polymerase chain reaction (RT-PCR). cDNA was synthesized from 4 μg of total-RNA in a 20 μl reaction mixture containing ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). First strand cDNA was amplified using PCR. This mixture was placed at 42˚C for 60 min. The synthesized cDNA was incubated at 70˚C for 10 min, and then stored at -80˚C. To verify the amplification, the PCR products were subsequently examined by 1.2% agarose gel electrophoresis.

Direct DNA sequencing. PCR amplification of all growth hormone receptor (GHR) exons, including the flanking intron regions, was performed on extracted DNA and RNA using previously described amplification primers and cycling conditions, as described by Vidal *et al* (7) with some modifications. Primer sequences used for amplification of human *GHR* gene fragments are listed in Table I.

The PCR amplifications were performed and the products were purified with a QIA-quick-PCR-purification kit (Qiagen, Germantown, MD, USA), and then directly sequenced with an ABI-3700 automated DNA sequencer (Applied-Biosystems, Foster City, CA, USA).

Results

Patient. The patient was a 2-year-old boy, who was born by normal vaginal spontaneous delivery with normal birth length and weight. No abnormal findings were observed on the neonatal screening test. The patient's mother and maternal grandfather were diagnosed with NF-1 (Fig. 1). Also, the patient was diagnosed with NF-1 at the age of 6 months when the patient had 6 or more cafe-au-lait spots with >5 mm in greatest diameter on the body. The patient showed normal hair, frontal bossing and a depressed nasal bridge. On admission, 75 cm of height (<3 percentile), 8.3 kg of weight (<3 percentile), and 48 cm of head circumference (25-50 percentile) were recorded. Since birth, the patient had showed reduced growth velocity, resulting in postnatal growth failure (Fig. 2). The heights of the parents and maternal grandfather were within normal range.

Biochemical and imaging study. The GH stimulation showed that GH concentration was 4.13 ng/ml at 0 min but 94 ng/ml at 60 min. The rapid increase in GH serum level following

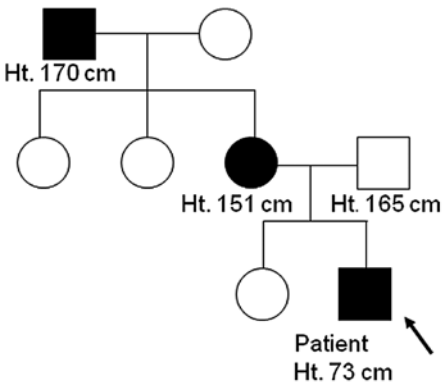

Figure 1. Pedigree of the family with neurofibromatosis type 1 (NF-1). Affected individuals indicated in solid symbols. The proband (arrow) shows a male who has a short stature.

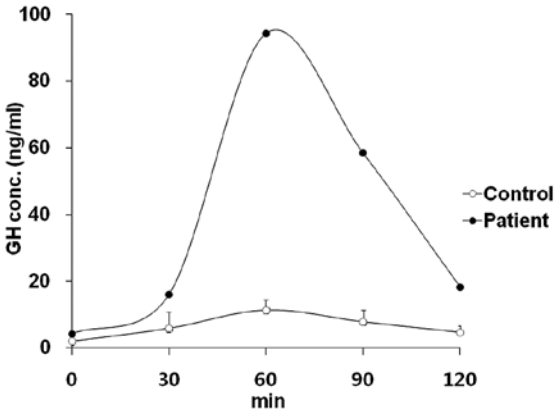

Figure 3. GH secretions in response to stimulation with insulin. Serum GH concentrations in the patient and the control group (mean ± SE, n=10) are shown.

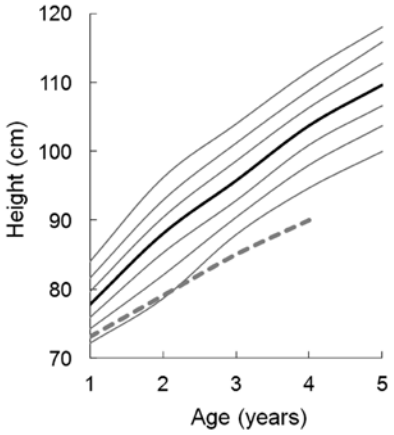

Figure 2. The growth chart of the patient is shown. Dotted lines represent the patient's growth pattern, which reveals a gradual decrease in growth velocity as the age increase.

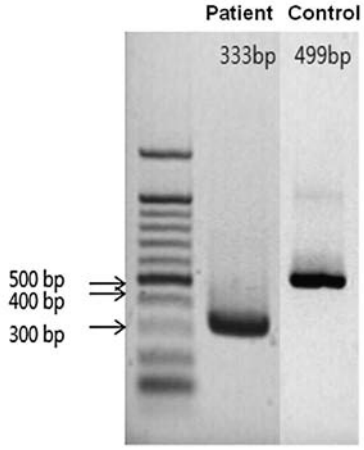

Figure 4. Agarose gel electrophoregram displaying a 166 bases deletion in exon 7 in the *GHR* mRNA in the patient.

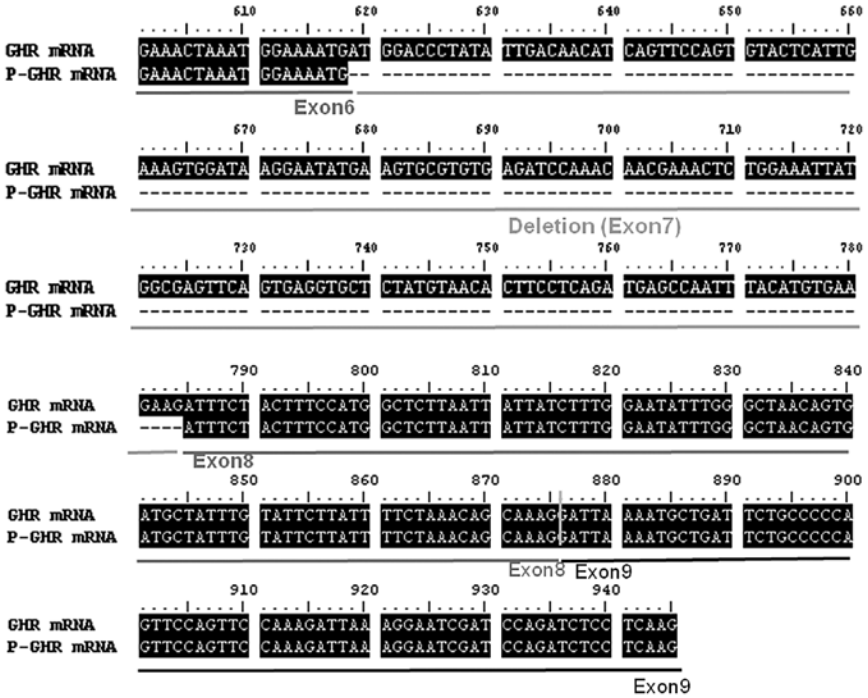

Figure 5. Sequence alignment demonstrating deletion mutation of exon 7 in the *GHR* mRNA in the patient.

insulin stimulation revealed the significant difference from the general pattern of normal control (Fig. 3). The patient's serum IGF-1 and IGFBP-3 levels were low, <25 ng/ml and <1.2 mg/l, respectively. On the brain MRI scanning, there was no evidence of other abnormalities including brain tumor.

Molecular studies. No germline mutation of the *GHR* gene including intron-exon boundaries was found in the patient and his family members. A complete deletion of exon 7 (c.619_784 del) of the *GHR* mRNA was observed. By analysis of agarose gel electrophoresis, a deletion of 166 base pairs in exon 7 was found (Fig. 4). It was verified via direct DNA sequencing analysis that 166 bases in exon 7 were completely deleted (Fig. 5). The deletion results in an 8-base-frameshift from the 207th codon and a premature termination at the 214th codon of the *GHR* mRNA. It was considered to be a frame shifting change from methionine in codon 207, with the new reading frame ending in a stop codon, M207 fs. X8.

Discussion

We report a 2-year-old patient with NF-1 who presented postnatal growth failure with decreased growth velocity. This patient exhibited very high GH concentrations after stimulation with insulin and very low baseline levels of serum IGF-1 and IGFBP3. The patient was ultimately diagnosed as having GHIS through molecular analysis.

GHIS have been attributed to genetic defects along the GH-IGF-1 axis. GH-IGF-1 axis plays a key role for body growth. Therefore, GHIS shows the pathologic conditions associated with low serum IGF-1 and IGFBP3 concentrations, as well as, normal or elevated serum GH concentrations, leading to postnatal growth failure including various clinical facial findings (8). An analysis that investigated growth patterns in untreated patients with GHIS has revealed that most patients showed reduced growth velocity and their growth charts were similar to those with GH deficiency or IGF-1 gene deletion (9).

A GH secretory problem was highly suspected as the etiology of growth failure in our patient because the patient demonstrated postnatal growth retardation and decreased height growth velocity. In the present study, other hormonal secretory functions were normal but serum GH concentrations remained very high after stimulation with insulin. Also, the basal serum IGFBP3 and IGF-1 levels were too low to measure quantitatively. These results reveal that the patient has GHIS and the growth failure would be associated with it, not NF-1.

NF-1 related factors causing postnatal growth failure were not found in the patient. The exact mechanism of growth failure in NF-1 remains unclear, but it is known to be associated with NF-1 itself or its complications (10). These complications include central nervous system tumors, especially suprasellar lesions such as an optic glioma, true precocious puberty, GH deficiency, hypothyroidism, and abnormal skeletal development (11). In particular, GH deficiency and true precocious puberty are the major risk factors of short stature in NF-1 that are highly associated with central nervous system pathology (2). No abnormal findings were observed on MRI scanning of the neuro-musculo-skeletal system in our patient. In addition, the possibility of familial short stature as the etiology was excluded because the heights of the patient's family members were all within normal limits.

It has been reported that in NF-1 patients during puberty, growth spurt was slightly decreased and height according to age gradually reduced, and eventually adult final heights in NF-1 patients were less than those in normal individuals, whereas growth velocity remained within normal range in prepubertal stage (9,12). Because our patient showed gradually reduced growth velocity since birth, the growth curve was different from that of other NF-1 patients. Considering the reduced growth velocity, low serum IGF-1 and IGFBP3 levels and abnormal serum GH secretory pattern in the GH stimulation test, we strongly suggested that the short stature of this patient is due to GHIS, not NF-1.

Molecular studies were performed in order to investigate whether the growth retardation in our patient is associated with molecules involved in the intracellular signaling cascade of the GH-IGF-1 axis. We found a novel mutation of *GHR* mRNA, a complete deletion of exon 7, in the patient. Because of the deletion and resulting frameshift, the mutation results in a premature termination at the 214th codon (M207 fs. X8) of the *GHR* gene. Exon 7 encodes a part of the extracellular domain of the GHR protein (13,14). Therefore, the deletion mutation of exon 7 is considered to make a truncated receptor protein, and then the GH signal can not be transmitted into the cell. Reportedly, almost all mutations causing Laron syndrome and GHIS encode the extracellular domains of the *GHR* gene, while other mutations encode the cytoplasmic domain (15,16).

In the reports regarding *GHR* mutation, mRNA mutations of the *GHR* are uncommon, with splicing errors by mutations of the flanking region of exon being the majority (17). Rarely, mutations due to addition of a pseudoexon in an intron were reported (18-20). The patient in the present study is the first reported case having a GHIS with NF-1, who showed extensive deletion of the *GHR* gene mRNA. To clarify the mechanism of the disease-causing mutation, further studies are required.

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