differences in sensitivity to FC and changes in sensitivity with age.

6. Treatment of NC 21-OHD

Recommendation

1. We suggest that NC 21-OHD associated with excessive adrenal androgen symptoms including enhanced growth rate and bone age and virilization of the external genitalia in females be treated with maintenance therapy similar to that for classical 21-OHD. 2 (●○○)
2. We recommend against treatment of subclinical NC 21-OHD. 1 (●○○)
3. We recommend that the dose of glucocorticoids for NC 21-OHD under glucocorticoid treatment be increased for febrile illness (>38.5°C), gastroenteritis with dehydration, surgery under general anesthesia; and major trauma. 1 (●○○)

Explanation

NC 21-OHD associated with characteristics of 21-OHD in endocrinological tests, but without symptoms of glucocorticoid or mineralocorticoid deficiency; requires routine evaluation of physical findings, height, body weight and bone age to determine the timing of treatment (7, 9, 80, 81). If excessive adrenal androgen symptoms including enhanced growth rate and bone age and virilization of the external genitalia are found in females, maintenance therapy similar to treatment of classical 21-OHD should be started. There are no large-scale studies showing benefits of treatment of subclinical NC 21-OHD. Female NC 21-OHD patients who were diagnosed in the child stage in Japan showed enhanced bone age and virilization of the external genitalia (80, 81); but no early pubic hair development, hirsutism, acne or menstrual irregularity, which have been found in Europe and the US. Some patients developed adrenal crisis after starting treatment, suggesting the importance of stress dosing in maintenance therapy (78). As in classical 21-OHD, the appropriate dose should be determined individually in each patient with NC 21-OHD to adjust the progression rates of height and bone age such that they correspond with age.

7. Treatment of Adult Classical CAH

Recommendation

1. We suggest that patients with classical CAH be treated with short- or long-acting glucocorticoids. 2 (●○○)
2. We suggest that a patient under treatment with glucocorticoids and mineralocorticoids have a consultation at least twice a year and monitoring based on hormone tests. 2 (●○○)
3. We recommend that comprehensive evaluation of test findings and clinical symptoms are required for follow-up in the adult stage. 1 (●○○)
4. We suggest that measurement of serum 17-OHP before administration of glucocorticoids early in the morning be used for monitoring and that the appropriate range is 400–1200 ng/dL. 2 (●○○)
5. We suggest that determination of bone density should be considered if glucocorticoids exceed the recommended dose and Cushing's syndrome is suspected. 2 (●○○)

Explanation

Treatment for adult CAH patients requires that (1) no corticosteroid deficiency symptom develops, (2) a female patient has no virilization or menstrual irregularity, (3) both male and female patients have no gonadotropin suppression and (4) the risk of adrenal rest tumor in the testis is reduced. However, an overdose of glucocorticoids causes iatrogenic Cushing's syndrome, whereas an underdose causes chronic adrenal insufficiency symptoms, including general fatigue. Overdose of mineralocorticoids causes hypertension. A large-scale randomized controlled study to determine the optimal dose of glucocorticoids and mineralocorticoids in the adult stage has not been performed.

The results of a survey in Japanese adults

showed that HC was administered in 47% of male patients (mean 22.5 mg/d) and 61% of female patients (mean 20.0 mg/d). Other patients were treated with a single administration of synthetic glucocorticoids or combined administration with HC. Monotherapy with dexamethasone was administered in 29% of males (mean 0.5 mg/d) and 15% of females (0.5 mg/d). Monotherapy with prednisolone was used in 5% of males (5 mg/d) and 5% of females (7.5 mg/d) (116). In Europe, CAH patients in the adult stage receive replacement therapy with HC (mean 13.75 mg/m^2) in 36% of patients, prednisolone in 14% of patients (4.74 mg/d), and dexamethasone in 33% of patients (0.5 mg/d) (117).

In a nationwide study of 203 patients in the United Kingdom (UK), HC was administered to 26% of patients (mean daily dose 25 mg, range 10–60 mg), prednisolone was administered to 43% of patients (7.6 mg, 2.5–10 mg), dexamethasone was administered to 19% of patients (0.5 mg, 0.25–0.75 mg), and a combination of HC with prednisolone or dexamethasone was administered to 10% of patients (118). In a study in 224 CAH patients in the US, about 80% of pediatric patients received HC (daily dose 15.0±5.9 mg/m^2), and about 30% each of adult patients received HC (daily dose 17.9 ± 7.6 mg/m^2), prednisolone, and dexamethasone (119). Therefore, a patient with no negative reaction to HC in the child stage can receive similar treatment in the adult stage. HC can be replaced with prednisolone or dexamethasone, but the dose should be carefully determined. Long-acting glucocorticoids were more likely to be used in adult patients than in pediatric patients in all studies.

Several studies have examined methods to find the optimal dose of glucocorticoids (9, 120). A study in the US proposed a target range of serum 17-OHP of 100–1200 ng/dL before administration of glucocorticoid early in the morning for monitoring of glucocorticoid treatment, similarly to that in the child stage; however, the dose and range should be decided individually. Another study suggested a target 17-OHP level of < 800 ng/dL before administration of glucocorticoids early in the morning in females of childbearing age; and < 2500 ng/dL for males without adrenal rest tumor in the testis (120). However, these were not large-scale clinical studies. In a study in adult patients in the US, about 30% had a serum 17-OHP level of 100–1200 ng/dL before administration of glucocorticoid early in the morning (119). In a UK study, the target 17-OHP level was achieved in 10% of patients (118). Therefore, appropriate treatment with glucocorticoids is difficult in the adult stage.

A recent study in the UK showed that the quality of life (QOL) of adult patients given prednisolone or dexamethasone was lower than that of patients treated with HC (121). However, it is uncertain if prednisolone and dexamethasone reduce QOL or are used for patients in which CAH is difficult to control. Testosterone in males reflects reproductive function more than adrenal function and is not useful for monitoring of treatment. If a male patient with a large adrenal rest tumor in the testis has a low level of testosterone early in the morning, Leydig cell dysfunction is suspected (9).

The optimal dose of FC in adult patients has not been examined. The results of a nationwide study in the UK showed that FC was administered to 72% of patients at a mean dose of 0.125 mg (0.01–0.5 mg/day) (118). The requirement for FC decreases with age (5), and patients given FC in the child stage often do not need FC in the adult stage. Therefore, adjustment of administration of FC while monitoring the patient's blood pressure and plasma renin activity or concentration is required. If renin activity increases after discontinuation, readministration of FC should be considered. However, prednisolone has less effect on mineralocorticoids in comparison with HC, and dexamethasone has no effect. Therefore, if HC is replaced with these drugs, the dose of FC may require adjustment.

In the adult stage, 21-OHD can be accompanied by metabolic abnormalities

(118–127). Bone mineral density (BMD) may decrease, and fracture and osteoporosis may occur, but there are conflicting studies (123, 124). BMD decreased in adults ≥ 30 yr old and in postmenopausal females with 21-OHD (123), but BMD did not decrease in comparison with controls in pubertal children and young adults with 21-OHD (124). There are reciprocal studies of the correlation of the total dose of glucocorticoids with BMD (123–125, 127). Thus, there is currently no evidence to support routine monitoring of BMD; however, if the dose of glucocorticoids is higher than the recommended dose and obesity and Cushing's syndrome are present, a BMD test should be assessed

A study of metabolic syndrome in 203 adult patients with 21-OHD in the UK found obesity in 41% of the patients, complication with hypercholesterolemia in 46% of the patients, insulin resistance in 29% of the patients, osteopenia in 40% of the patients, and osteoporosis in 7% of the patients (118). The same study group showed that insulin resistance and increased fat were related to decreased QOL (121). The results of a study in American adult patients with 21-OHD showed obesity in 30% of the patients, hypertension in 60% the patients, metabolic syndrome in 18% the patients, and decreased BMD in 50% the patients (119). In a study by the adrenal group in Japan, the rates of high-grade obesity (BMI ≥ 30) were 23% in males with 21-OHD and 16% in females with 21-OHD, with no difference between the types of glucocorticoids; however, the HC dose in the high obesity group was significantly lower (116). No definite relationship of obesity with administration of glucocorticoids can be established from these results due to the small number of subjects.

These results suggest that metabolic abnormalities may be associated with 21-OHD, but it is unclear if these abnormalities are related to overdose of glucocorticoids. No definite monitoring method to find the optimal dose of glucocorticoids is available in adults, which is similar to the pediatric field. Therefore, a large-scale systematic clinical study is required to examine administration of glucocorticoids and mineralocorticoids, monitoring methods, and metabolic abnormalities in the adult stage of 21-OHD.

8. Prenatal Diagnosis and Treatment

Recommendation

1. Prenatal diagnosis and treatment have yet to be established. We suggest that experienced physicians conduct diagnosis and treatment in institutions with genetic counseling after approval of the ethics committees of their institutions. 2 (●○○)

Explanation

8-1. Prenatal diagnosis and treatment

Administration of dexamethasone through placental transfer from the maternal body can reduce adrenal androgen production in a fetus with 21-OHD (128–130). The purposes of prenatal treatment are to block virilization of the external genitalia in female patients and avoid the need for surgery, and to relieve the emotional distress and anxiety of parents that may be caused by an external genitalia anomaly in their child (128–132). However, the disease cannot be completely remitted by prenatal treatment, and routine and careful follow-up and treatment are required. The risk of adrenal crisis also does not disappear.

Prenatal treatment is currently conducted based on previous reports (128, 131), but the dose and administration period for dexamethasone have not been fully established. The fetal cortisol concentration is usually very low in the first trimester (133) and increases from weeks 8 to 12 of gestation, but it is still about 10% of the maternal cortisol concentration in the second trimester (134, 135). Therefore, administration of dexamethasone from the first trimester can result in a 60-fold fetal physiological concentration in the second trimester (136). A method to decrease

dexamethasone in fetal treatment after the completion of formation of the external genitalia has been proposed (136, 137).

21-OHD is an autosomal recessive disease, and the probability of the disease in a fetus of a woman who has already delivered a child with 21-OHD is 25% with the same partner. Virilization of the external genitalia in girls with 21-OHD occurs until 6 wk after conception; therefore, treatment should be performed as soon as possible if the woman recognizes pregnancy. Dexamethasone does not inactivate the placenta and can be used as a therapeutic drug (138). Treatment should start from weeks 6–7 of gestation, but genetic diagnosis using chorionic villus sampling cannot be conducted until weeks 10–12 of gestation. Therefore, the probability of CAH in the fetus is 25%, but dexamethasone should be administered to the pregnant woman in the first trimester. A new test method to determine sex using maternal blood within wk 6 of gestation has been applied to prenatal treatment of 21-OHD to shorten the administration period of dexamethasone (139, 140). Detection of a Y chromosome allows earlier discontinuation of unnecessary treatment. Only girls with 21-OHD receive the benefit of treatment, and only one-eighth of all pregnancies really require treatment. This results in unnecessary dexamethasone administration, and the related ethical issues are under discussion in Japan and other countries (103, 132, 141, 142).

8-2. Fetal safety and long-term prognosis

A survey of children given dexamethasone for prenatal treatment of 21-OHD showed that the children were significantly more introverted in comparison with the general population (143). However, in another survey that compared 174 patients who received prenatal treatment and 313 untreated controls, there were no differences in nine social and developmental indicators (144). In a study of Swedish children treated before birth with a constant therapy protocol and control children of matching sex and age, a survey and a standard neuropsychological test conducted by a clinical psychologist (145) indicated no differences in intelligence, proficiency and long-term memory. However, children who were given dexamethasone for a short period, but did not have 21-OHD; had a reduced verbal working memory, less self-recognition in academic achievement; and increased subjective social phobia (145, 146). Simultaneously, no difference in behavioral and adaptive performance was found, but 7 boys who did not have 21-OHD but were treated with dexamethasone for a short period had greater androgynous behavior in comparison with control boys (147). The authors concluded that it cannot be completely ruled out that the differences in children without disease were due to the small number of patients with 21-OHD who were continuously treated. In contrast, a survey and neuropsychological tests performed by Meyer-Bahlburg *et al.* showed that children who were given dexamethasone for a short period but did not have 21-OHD did not have a reduced verbal working memory, but 21-OHD female patients who were treated with dexamethasone for a long time had slightly reduced recognition (148).

8-3. Studies in Japan

Prenatal diagnosis and treatment of 21-OHD have also been examined in Japan (141, 142). In a survey of members of the Japanese Society for Pediatric Endocrinology, Kinoshita *et al.* identified 13 children who underwent prenatal treatment from 1995 to 2002, of whom 2 girls had 21-OHD and external and internal genitalia that were completely female. Nine of these patients discontinued treatment, including 8 boys and one fetus that was aborted (141). None of the 8 boys and their mothers had adverse reactions, but the results of follow-up tests are not available. In a survey from 2002 to 2007, detailed responses were obtained for only 7 patients (142). Four girls with 21-OHD were treated until birth, of whom 3 had completely female external genitalia and one had slight clitoromegaly, despite being treated

with dexamethasone from wk 8 of gestation. Of the patients who discontinued treatment, 2 were determined to be normal girls by genetic diagnosis, and 1 was diagnosed as a boy in a sex check. Two of the 7 cases were spontaneous abortions, and 1 was an induced abortion, but the details were unknown. Three cases underwent genetic analysis and a sex check using chorionic villus sampling, and 2 underwent amniocentesis. Mild symptoms of Cushing's syndrome were found in 2 mothers, and dyspepsia was present in 1 mother. There is no Japanese study in which children who were given dexamethasone for a short time or until birth were subsequently evaluated by neuropsychological tests. In the survey, 15% of respondents considered that this treatment had ethical problems.

8-4. Current status

The results of a meta-analysis of prenatal diagnosis of 21-OHD were reported in 2010 (149). In this analysis, treatment effects and fetal and maternal adverse reactions were examined in 4 studies and a total of 323 pregnancies. Dexamethasone was effective for prevention of virilization and had no adverse effects on the fetus, but it significantly increased stria and edema in mothers, but with no serious adverse effects. However, the authors concluded that there were too few studies for analysis and that the evidence was insufficient.

The American Endocrine Society has recently published clinical practice guidelines for CAH that indicate that unnecessary maternal and fetal exposure to dexamethasone should be avoided in prenatal treatment because the potential adverse events caused by dexamethasone are more important than the mental distress of parents and patients caused by virilization of the external genitalia (9).

There are no data on long-term prognosis in Japan. Therefore, the Mass Screening Committee of the Japanese Society for Pediatric Endocrinology concluded that the appropriateness of prenatal diagnosis and treatment based on the Guidelines has not been established and should be considered further.

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Conflict of interest of the working committee members: None of the committee members have a conflict of interest regarding development of the Guidelines, based on the criteria for conflict of interest of the Japan Pediatric Society, in accordance with the rules of the Japanese Society for Pediatric Endocrinology.

Appendix

1. Development process

1-1. Understanding current conditions

A survey was conducted via email sent to 156 councilors of the Japanese Society for Pediatric Endocrinology. Initial treatment was examined from August 28 to September 28, 2012, and maintenance therapy and treatment in the pubertal stage were examined from January 10 to February 28, 2013. The response rates were 25.6% (40/156) and 19.8% (31/156), respectively. For the initial treatment in neonatal cases without SW symptoms, the HC dose was indicated to be 100–200, 50–100; and 20–50 mg/m^2/d by 20, 13; and 6 of the 40 respondents, respectively.

1-2. External evaluation

The draft guidelines were published on a website for members of the Japanese Society for Pediatric Endocrinology from March 11 to April 10, 2013, to solicit opinions, and a revised draft was developed on June 7, 2013, with consideration of the received opinions. The validity of the guidelines was discussed in the Guidelines Committee, including external members, and revisions were made based on the proposal of the Guidelines Committee (April 7, 2014). This revision was approved by the

Board of the Society on April 26, 2014, and published.

1-3. Consultation with relevant societies

The draft guidelines were sent to the councilors of the Japanese Society for Mass Screening by email from March 11 to April 10, 2013 to request opinions. A revised draft was developed on June 7, 2013, based on replies to this request.

1-4. Hearing of opinions of patient groups

There is no relevant patient group; therefore, no hearing was conducted.

2. Revision schedule

The Guidelines are planned to be revised within 4 yr after disclosure. The committee for revision will be organized by the board of the Japanese Society for Pediatric Endocrinology. If new developments occur that may have critical effects on the Guidelines, the board of the Japanese Society for Pediatric Endocrinology may decide to revise the Guidelines immediately as "Recommendations."

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Spondyloepiphyseal Dysplasia Congenita Caused by Double Heterozygous Mutations in *COL2A1*

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Spondyloepiphyseal dysplasia congenita (SEDC) is a group of rare inherited chondrodysplasias characterized by short stature, abnormal epiphyses, and flattened vertebral bodies. SEDC is usually caused by substitution of glycine residue with another amino acid in the triple helical domains of alpha 1 chains, which consist of type II collagen (*COL2A1*). Herein, we describe a unique case of SEDC with mild coxa vara (SEDC-M) caused by double de novo *COL2A1* mutations located on the same allele. One mutation, p.G504S, was previously described in patients with SEDC, whereas the other, p.G612A, was a novel mutation; both were located in the triple helical domain. Neither mutation was identified in the parents and appeared to be de novo. To the best of our knowledge, this is the first study involving a patient with a type II collagenopathy with two *COL2A1* mutations on the same allele. The case was characterized by a more severe phenotype compared with previously reported cases involving a single p.G504S mutation, which may have been the result of the double mutation.

Key words: type II collagenopathies; spondyloepiphyseal dysplasia; *COL2A1*; double mutations

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INTRODUCTION

Mutations in the *COL2A1* gene that codes for type II collagen result in a spectrum of autosomal dominant conditions. These phenotypes range from prenatal lethality or short stature to relatively mild entities that may become apparent only during adulthood, the so-called type II collagenopathies. Type II collagenopathies include Stickler dysplasia, spondyloepiphyseal dysplasia (SED), Kniest dysplasia, spondyloperipheral dysplasia, and Strudwick type spondyloepimetaphyseal dysplasia [Spranger et al., 1994; Kannu et al., 2012]. SED exhibits a wide range of phenotypic variation, from lethal achondrogenesis type II and hypochondrogenesis to spondyloepiphyseal dysplasia congenita (SEDC) and late-onset SED (SED tarda: SEDT). Moreover, SEDC is subdivided into the following two groups: SEDC with severe coxa vara (SEDC-S) and SEDC with mild coxa vara (SEDC-M) [Wynne-Davies and Hall 1982; Nishimura et al., 2005].

The most common type of *COL2A1* mutation in the setting of SEDC is a single base substitution in the triple-helical glycine residue. Some glycine-to-serine substitutions, such as p.G504S, usually result in milder skeletal dysplasia. However, glycine-to-nonserine residue substitutions cause varying phenotypic abnormalities [Nishimura et al., 2005].

Herein, we describe a unique case of the SEDC-M phenotype characterized by two de novo *COL2A1* mutations, including the previously described p.G504S mutation and the novel p.G612A mutation, which were located on the same allele. To the best of our knowledge, this is the first reported patient with two *COL2A1* mutations on the same allele, and our study further delineates the genotype-phenotype correlation that characterizes type II collagenopathies.

CLINICAL REPORT

The patient was a girl born to healthy, nonconsanguineous Japanese parents. Her older brother was healthy. There was no family history of skeletal dysplasia. Short limbs were noted during pregnancy. The girl was delivered vaginally at 40 weeks gestation. Her birth length

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was 42.6 cm (−3.5 SD); her weight was 2,582 g (−1.5 SD), and her head circumference was 33.4 cm (± 0.0 SD). Although her psychomotor development was normal, her short stature became more evident as she grew.

At age 2, she was referred to us because of severe short stature. Her height was 72.6 cm (−5.0 SD), and her weight was 10.6 kg (−1.1 SD). Her head circumference was 47 cm and was large compared with her height. Her wing span was 77 cm and was proportional to her height. Her face was unremarkable. Genu valgum was not evident. Ophthalmologic examination demonstrated myopia and intermittent exotropia. Her hearing was not impaired. Routine blood and urine tests were normal.

Radiological examination demonstrated a normal skull. Mild odontoid hypoplasia was observed. The spine was characterized by platyspondyly with dorsal vertebral flattening (Fig. 1A, B). The ilia were broad and short with narrow greater sciatic notches. The iliac crests and acetabula appeared irregular. The proximal femoral epiphyses were mottled together with horizontal clefts. Coxa vara and short femoral necks were noted (Fig. 1C). The metaphyses of the long bones demonstrated irregular radiolucencies, which were intermingled with radiodensities, and were partially fragmented (Fig. 1D).

Based on these clinical and radiological findings, the patient was believed to have type II collagenopathy, or SEDC-M.

Mutation Analysis

The patient's parents provided written informed consent. The ethics committee of our university approved the genetic diagnosis. Genomic DNA was extracted from peripheral blood leukocytes, and the *COL2A1* exon was amplified via polymerase chain reaction (PCR) as reported previously [Williams et al., 1992]. PCR products were purified and sequenced directly using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). To determine whether two mutations were on the same allele, exons 22 to 27 of *COL2A1* were amplified from the patient's genomic DNA with a set of primers located in introns 21 and 27. Thereafter, PCR products were cloned into a pCR 2.1 TA cloning vector (Invitrogen, Carlsbad, CA). We randomly selected several clones and verified the DNA sequence of each allele.

RESULTS

Sequence analysis of *COL2A1* revealed two heterozygous missense mutations in exons 22 and 27 (Fig. 2A). One was a G to A transition at position 1510, resulting in a substitution of glycine for serine at amino acid position 504 (c.1510G>A, p.G504S). The other mutation was a G to A transition at position 1835, also resulting in a

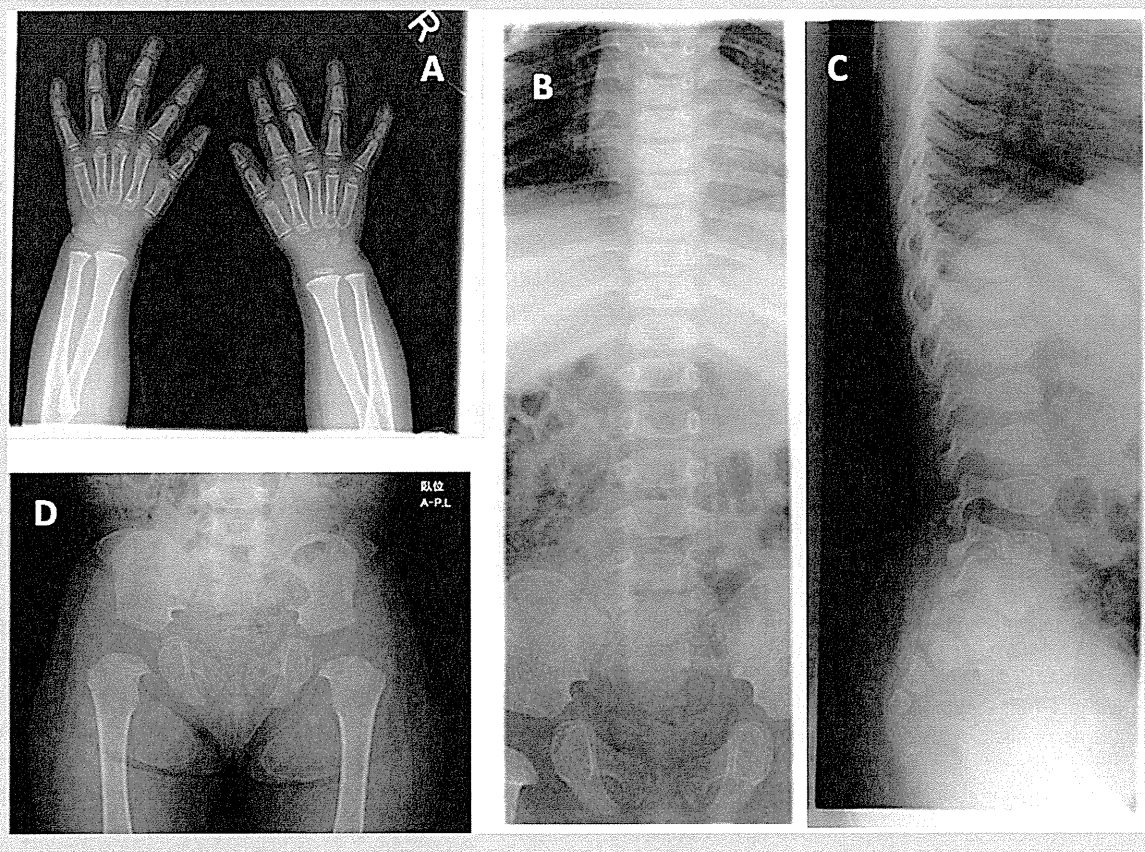

FIG. 1. Radiographs of the patient at 3 years and 1 month of age. A: Hand A–P. Short tubular bones are minimally affected. B, C: Spine A–P and lateral. Note moderate platyspondyly, minimal scoliosis and hyperlordosis. D: Pelvis A-P. Ilia are broad and short with narrow sciatic notches. Iliac crests and acetabula are irregular. Note coxa vara and short femoral necks.

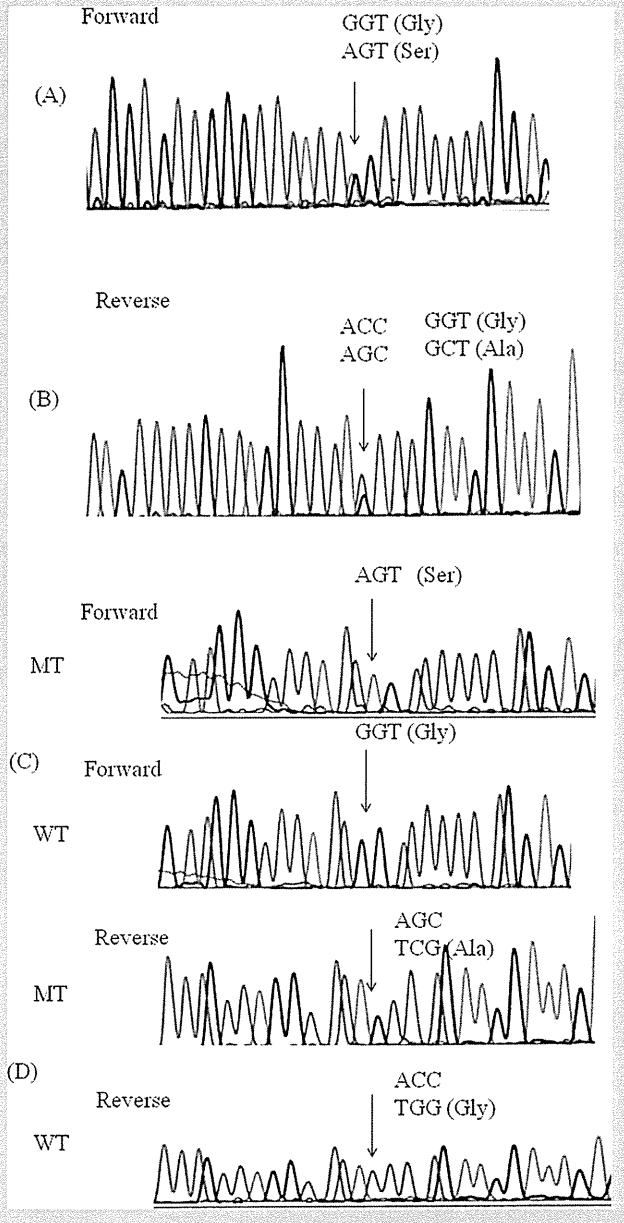

FIG. 2. DNA sequences of exons 22 and 27 of *COL2A1*. Panel A illustrates the presence of the p.G504S mutation (c.1510G>A). Panel B illustrates the presence of the p.G612A mutation (c. G1835>C). The upper row of Panel C demonstrates the DNA sequence obtained with the cloning sample. In this sequence, an A was detected in position 1,510. The upper row of Panel D demonstrates the DNA sequence obtained with the same cloning sample. In this sequence, a C was detected in position 1,815. These results indicate that the mutations of p.G504S and p. G612A are on the same allele. WT, wild-type allele; MT, mutant allele.

substitution of glycine for serine at amino acid position 612 (c.1835G>C, p.G612A) (Fig. 2B). Both mutations were located in the triple helical region, and neither mutation was identified in the child's parents. Using an online software program, PolyPhen2 [Adzhubei et al., 2010], [http://genetics.bwh.harvard.edu/pph2/dokuwiki/about], a computational tool used to predict the functional effects of an amino acid substitution, we determined that the p.G612A mutation was "probably damaging," with a position-specific independent count (PSIC) of 0.998. Using another program, SIFT [Kumar et al., 2009], [http://sift.jcvi.org/], which also predicts functional effects of an amino acid substitution, we predicted p.G612A to be damaging with SIFT score of 0.00 and Median Info of 2.63.

After sequencing 30 clones of PCR product, we found that twelve of the clones had two mutations (c.1510G>A and c.1835G>C), indicating that two mutations were on the same allele (Fig. 2C, D).

DISCUSSION

This report describes a patient with an SECD-M phenotype with double de novo *COL2A1* mutations. To the best of our knowledge, this is the first report of a case with double *COL2A1* mutations. The p.G504S mutation was previously reported in patients with SEDC, SEDC-M, and Strudwick type spondyloepimetaphyseal dysplasia, or SED-T [Nishimura et al., 2005; Xia et al., 2007; Cao et al., 2012]. In contrast, the p.G612A mutation was not reported previously and was not identified in a sample of two hundred normal Japanese males. However, both PolyPhen2 and SIFT analysis indicated that this mutation was likely damaging, and we therefore concluded that the p.G612A mutation was pathogenic.

The occurrence of double mutations on the same allele is very rare, as such a phenomenon occurs once out of every 10^{-11} cases [Kondrashov, 2003]. Four cases of double mutations have been reported in *FGFR3* [Rump et al., 2006; Santos et al., 2007; Pannier et al., 2009; Marquis-Nicholson et al., 2013]. Double *FGFR2* mutations in the setting of Apert syndrome and double *CFTR* mutations in the setting of cystic fibrosis have also been described [Savov et al., 1995; Goriely et al., 2005]. Most double mutations are characterized by more severe clinical phenotypes than may be expected for each single mutation. According to previous reports, p.G405S mutations cause milder SEDC phenotypes. Indeed, p.G504S was identified in two Japanese patients with mild type II collagenopathies [Nishimura et al., 2005]. One patient was diagnosed with SEDC-M, and his adult height was 137.2 cm (−5.2 SD for normal Japanese male); he did not have myopia or suffer from hearing impairment. The other patient with a p.G504S mutation was diagnosed with SEDT and late-onset SED, and his adult height was 151 cm (−3.4 SD for normal Japanese male); this patient did not have eye or hearing impairments. Other reports of p.G405S mutations have also noted intact visual and auditory function [Tiller et al., 1995; Xia et al., 2007; Cao et al., 2012]. By contrast, our patient demonstrated severe early onset myopia, and this visual impairment may have been the result of double mutations.

In conclusion, this report described rare double mutations in *COL2A1* as the cause of SEDC-M in a Japanese patient. The existence of two mutations is likely to affect the disease's phenotypic severity.

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IGSF1 遺伝子変異により先天性中枢性甲状腺機能低下症を引き起こす病態の解明

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はじめに

我々のグループは日本で初めてX連鎖性中枢性先天性甲状腺機能低下症の原因がImmunoglobulin super family gene 1（*IGSF1*）遺伝子異常によることを次世代シークエンサーによる解析で明らかにし、臨床的特徴も含めこれまで報告を行ってきた（1, 2)。過去の報告ではIGSF1がinhibinのco-receptorであり、activin、inhibin、TGFβなどの作用に関与する可能性も示唆されているが（3)、生体内におけるIGSF1の正確な役割や、先天性中枢性甲状腺機能低下症（CH-C）の発症機序に関してはほとんど不明である。

今回我々は、更に日本人における*IGSF1*変異の探索を行うとともに、IGSF1の*in vitro*での機能を明らかにする目的で以下の実験を行ったので報告する。

方法

1. 遺伝子解析

患児の白血球よりgenomic DNAを抽出ののち、*IGSF1*をPCR法によって増幅した。増幅条件と使用したプライマーは既報の通りである（3)。Applied Biosystems 3130 Genetic Analyzer（Applied Biosystems, Foster City, CA, USA）を用いたPCR direct sequencing法により*IGSF*の解析を行った。遺伝子解析に際しては、両親より書面での同意を得て施行した。

2. *In vitro* 解析

2-1　Igsf1のPRL産生に対する効果の検討

ラット下垂体腺腫由来GH3細胞には、ラットIgsf1が発現していないことをRT-PCR法により予備的に確認した。

（A）Activin刺激によるPRL promoter活性化の検討

GH3細胞にPRL promoter-luciferaseベクターをリポフェクション法でトランスフェクトし、24時間後に無血清培地へ交換。無血清培地で24時間培養ののち、activin 1.0nMで6時間刺激を行った。補正には、同時にトランスフェクションしたB-galactosidaseを用いた。

（B）Igsf1がactivin刺激によるPRL promoter活性化に与える影響の検討

GH3細胞にPRL promoter-luciferaseベクターと、*Igsf1*野生型、既報の変異型（p.Q645X, p.Q1189X）発現vectorのいずれかをリポフェクション法でトランスフェクトし、24時間後に無血清培地へ交換。無血清培地で24時間培養ののち、activin 1.0nMで6時間刺激を行った。補正には、同時にトランスフェクションしたB-galactosidaseを用いた。

2-2　TGFβ-betaglycan シグナル伝達における IGSF1 の効果についての検討

アフリカミドリザル腎細胞由来 COS7 細胞において、activin receptor I, Ic, IIa, IIb, TGFβR-II は発現し、Igsf1, activin receptor Ib, TGFβR-I, TGFβR-III（=betaglycan）は発現していないことを RT-PCR 法により予備的に確認した。

Activin/TGFβ は activin receptor, TGFβ receptor, betaglycan を介して、Smad 2, 3 をリン酸化により活性化したのち細胞膜より離れ、common mediator Smad である Smad 4 と複合体をつくり、核内に移行することが知られている（4）。

（A）TGFβ による smad 活性化に Igsf1 がどのような作用を示すかの検討

COS7 細胞に Smad 結合エレメントを持つルシフェラーゼベクター pGL4.48［luc2P/SBE/Hygro］（Promega 社）と、Igsf1 野生型、既報の変異型（p.Q645X, p.V1082E, p.Q1189X）発現ベクターのいずれかをリポフェクション法でトランスフェクトし、24 時間後に無血清培地へ交換。無血清培地で 24 時間培養ののち、TGFβ 10g/ml で 6 時間刺激を行った。同時にトランスフェクションした *Renilla reniformis*-luciferace を用いて補正を行った。

（B）Activin/inhibin による smad 活性化に Igsf1 がどのような作用を示すかの検討

COS7 細胞に Smad 結合エレメントを持つルシフェラーゼベクター pGL4.48［luc2P/SBE/Hygro］（Promega 社）と、betaglycan 発現ベクター、Igsf1 野生型もしくは既報の変異型（p.Q645X, p.V1082E）発現ベクターのいずれかをリポフェクション法でトランスフェクトし、24 時間後に無血清培地へ交換。無血清培地で 5 時間培養ののち、activin、inhibin 単独もしくは両者による刺激を 24 時間行った。同時にトランスフェクションした *Renilla reniformis*-luciferace を用いて補正を行った。

結果

1. 遺伝子解析

3 つの新規 *IGSF1* 変異（p.G1085Wfs39X, c.2335+1G>A, 124kb deletion）を同定した。

今回の検討により、新生児マススクリーニング検査、もしくは低身長を契機に発見された CH－C の男児 3 例において、3 つの新規 *IGSF1* 変異（p.G1085Wfs39X, c.2335+1G>A, 124kb deletion）を同定した。いずれも過去の報告（1-3）と同様、*IGSF1* の C 末端に存在する変異であった。

2. *In vitro* 解析

2-1　Igsf1 の PRL 産生に対する効果の検討

（A）Activin 刺激により PRL promoter 活性は抑制される。（Fig.1）

Activin 刺激により PRL promoter 活性は抑制され、これは既報の報告（5）に矛盾しない結果であった。