natal thyroid status according to Kliegman *et al.* [8]]. The neonatal hyperthyroidism manifested with tachycardia (140-180 bpm), prompting administration of 2 mg of MMI immediately after birth. In addition, on Day 17, 4 mg of atenolol was also started for persistent tachycardia. Thereafter, MMI and atenolol were discontinued at 8 weeks of age. LT4 replacement was implemented on Day 36 because of central hypothyroidism and the infant received LT4 replacement therapy until 2 years of age, when the hypothyroidic state resolved [9]. Follow-up examination at the age of two showed the failure to thrive. He weighted 8.2 kg (< 5th percentile) and was 77 cm tall (<5th percentile).

Two years after the first delivery, she became pregnant again. When first seen at 10 weeks of gestation, she was euthyroid under the LT4 replacement therapy, as follows: TRAb 230.0 IU/L, TSH 0.09 μU/mL, fT4 1.7 ng/dL, fT3 2.7 pg/mL (Table 1a). Obstetric ultrasound showed appropriate-for-date fetus with no abnormalities and the pregnancy course was uneventful. At 21 weeks of gestation, however, the fetus developed baseline tachycardia over 170 bpm. Fetal ultrasound examination revealed an enlarged thyroid gland (15 × 12 mm), pericardial effusion and cardiomegaly (Fig. 2a). Additionally, at 22 weeks of gestation, CTAR and PLI of the inferior vena cava were 53 % and 0.94, respectively. Taken together, the fetus was diagnosed to have *in utero* cardiac failure associated with hyperthyroidism. Based on the clinical course in the first pregnancy, oral maternal potassium iodide (50mg/day) and PTU (300 mg/day) were initiated (Fig. 3). At 24 weeks of gestation, pericardial effusion resolved. In addition, the CTAR as well as the PLI gradually improved to 46% and 0.72 after the initiation of fetal treatment. Since the PLI was still elevated and persistent fetal tachycardia (160 bpm) was seen at 30 weeks of gestation, oral maternal potassium iodide increased to 100 mg/day. Finally, fetal tachycardia and cardiomegaly resolved at the 34 weeks gestation (Fig. 2b), however, the enlarged thyroid gland was still present (Fig. 4) and the circumference of the thyroid was 9.8cm [normal: 3.7~6.0 cm] [reference ranges for fetal thyroid size according to Ranzini AC *et al.* [10]]. Subsequently, because preterm premature rapture of the membrane occurred at 35 weeks gestation, the mother delivered a 2034-g female neonate by repeated cesarean, with Apgar scores of 8 and 9 at 1 and 5 min, respectively. Laboratory examination on Day 0 showed neonatal subclinical hyperthyroidism (TRAb 120.0 IU/L, TSH 0.01 μU/mL, fT4 1.4 ng/dL, fT3 4.9 pg/mL) (Table 1b). Moreover, because the electrocardiogram of the baby after birth showed persistent tachycardia and accelerated maturation of the femoral ossification center was seen, MMI was initiated immediately after birth. Similar to her sibling, LT4 replacement was performed for possible central hypothyroidism. At 7 months of age, the neonate showed growth and gross motor developmental delays. She weighted 5.7 kg (< 5th percentile) and was 62 cm tall (< 5th percentile), and she wasn't able to sit without support and to crawl.

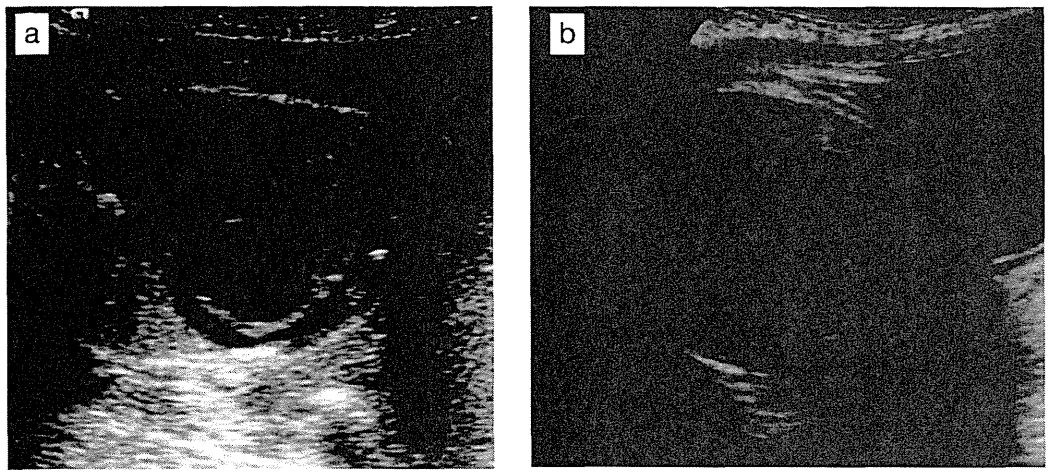

Fig. 2 Sonographic cross-sectional view of fetal thorax
a. Note fetal cardiomegaly with pericardial effusion at 21 weeks gestation.
b. Note cardiomegaly resolved at 34 weeks gestation by *in utero* treatment.

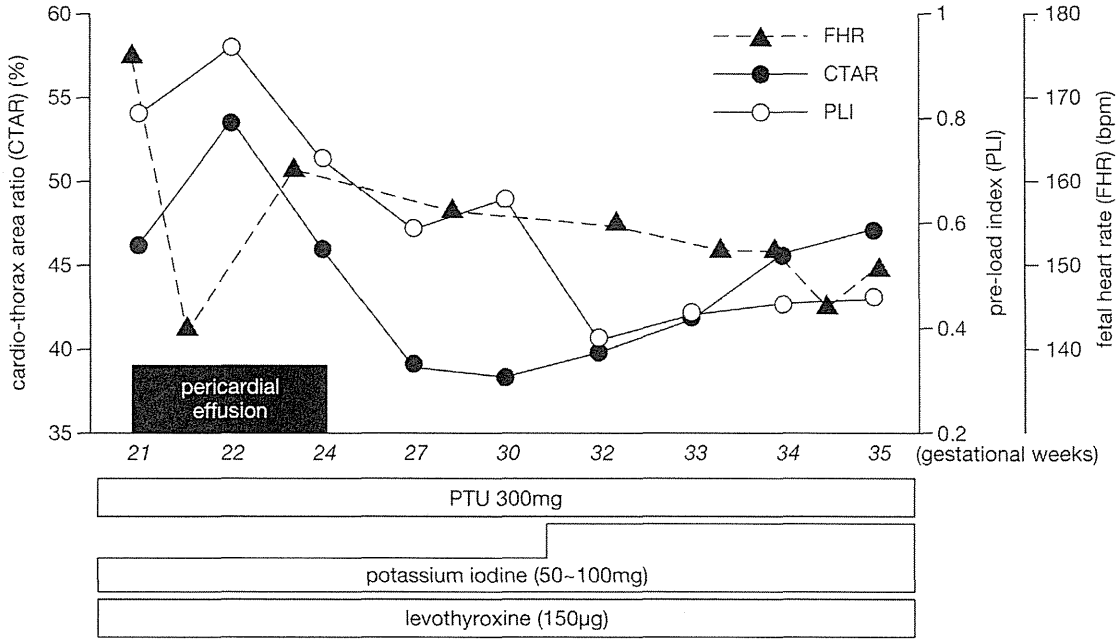

Fig. 3 Changes in sonographic measurements for fetal cardiac function
The cardio-thorax area ratio (CTAR) and pre-load index (PLI) of the inferior vena cava improved after the initiation of *in utero* treatment. Potassium iodide was increased up to 100 mg/day because of persistent fetal tachycardia.

Fig. 4 Fetal magnetic resonance imaging
Note the enlarged thyroid gland showing heterogeneous intensity on the T2-weighted imaging (arrow).

Discussion

In our case, the mother with a history of Graves' disease showed persistent high levels of maternal TRAb, although she was in a hypothyroidic state being treated with LT4 replacement therapy after subtotal thyroidectomy and radioiodine therapy. Our case was significant in that 1) fetal hyperthyroidism was diagnosed based on the clinical features, 2) fetal treatment using oral maternal anti-thyroid medicines was effective for improving cardiac failure, 3) maternal TRAb contributed to the development of fetal and neonatal hyperthyroidism.

Cordocentesis allows direct and accurate evaluation of the fetal endocrine status, however, most concern is the procedure-related complications including transient bleeding at puncture site, transient fetal bradycardia, chorioamnionitis, and cord hematoma [11]. In this case, high serum levels of maternal TRAb values as well as fetal sonographic features led us to the diagnosis of fetal hyperthyroidism, which was confirmed after birth. Therefore, fetal hyperthyroidism could be diagnosed without the invasive procedures (i.e. cordocentesis). In particular, the key feature for the suspicion of fetal hyperthyroidism might be persistent tachycardia in women with Graves' disease, as was shown in previous reports [12].

Fetal treatment using oral maternal potassium iodide and PTU was performed in our patient. Especially, PTU is preferred to MMI during the first trimester because the latter has been associated with aplasia cutis congenital and other malformation [12, 13]. In our case, the patient received PTU because of a history of allergic symptoms to MMI. Potassium iodide acutely inhibits its hormonal secretion within hours in hyperthyroid

patients [14]. The reason for our choice of potassium iodide in combination with PTU was that 1) iodine has few side effects, 2) iodine has prompt therapeutic effects, and 3) the urgent treatment was needed for fetus in hyperthyroid status [15, 16].

Our report demonstrated changes in fetal cardiac function in response to oral maternal anti-thyroid medicine. Based on the indices (*i.e.* PLI and CTAR), we have adjusted the dosage of potassium iodide and PTU during treatment. In pregnant patients with active Graves' hyperthyroidism, it is well established that fetuses can be maintained euthyroid by keeping maternal FT4 in the upper normal to mildly thyrotoxic range for pregnant women [17]. However, there has been no safe and reliable method of determining fetal thyroid function due to the lack of monitoring maternal thyroid function. Morine *et al.* have shown the usefulness of sonographic measurements to gain insights into the pathophysiology of cardiac failure associated with fetal goitrous hypothyroidism [18]. Our results suggest both PLI and CTAR could contribute to the appropriate follow-up in fetal hyperthyroidism. In this case, although fetal cardiac functions were well controlled in both fetuses, the both neonates showed developmental delay at the age of two years and 7 months, respectively. The reason for this discrepancy remained unknown, but could be due to the association with fetal hyperthyroidism or other abnormalities, since both fetuses already showed growth restriction *in utero*. The clinical features of fetal hyperthyroidism were less severe in the first fetus than in the second, but higher levels of maternal and neonatal TRAb were noted in the first pregnancy. Reports of outcomes in siblings born to a mother with Graves' disease after total thyroidectomy or radioiodine treatment are limited. Hamada *et al.* demonstrated that the occurrence of neonatal hyperthyroidism is not only due to high levels of TRAb before pregnancy, but also to TRAb not decreasing during pregnancy [19]. Additionally, fetal thyroid function might depend on the balance between the transplacental passage of maternal TRAb and thyroid inhibiting antithyroid drugs [20]. Thus, it seems difficult to predict fetal and/or neonatal outcome in Graves' disease with high levels of TRAb.

In conclusion, when managing pregnant women with complicated Graves' disease, it should be remembered that two patients are being treated: the mother and the fetus. Especially, women with history of surgery or radioiodine therapy are at risk for fetal hyperthyroidism. To the best of our knowledge, this is the first report on the changes of fetal cardiac function in response to the fetal treatment in two siblings showing *in utero* hyperthyroid status. The fetal heart rate and sonographic parameters for cardiac function could be useful in indicating the onset of hyperthyroidism and monitoring a response to therapy.

Disclosure Statement

The authors declare that no competing financial interests exist.

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Masanori Adachi*

Assessment of user-friendliness of the Norditropin FlexPro for pediatric patients treated with recombinant human growth hormone: results of an open-label user survey

Abstract

Objective: To assess the user-friendliness of the Norditropin FlexPro (Novo Nordisk A/S, Bagsvaerd, Denmark), a newly developed growth hormone (GH) injection pen.
Methods: This study consisted of a single-center, single-arm, open-label, questionnaire based survey of patients undergoing GH treatment for diverse indications who were scheduled to switch from the formerly used device Norditropin NordiFlex (Novo Nordisk A/S) to the FlexPro and evaluate each device's usability.
Results: A total of 82 patients participated in the study. Compared to the NordiFlex, the FlexPro was regarded as easier to grip, easier to hold during injection, and more stable during injection. The degree of pain at insertion perceived by the patient and the patient's fear of injection were significantly decreased when using the FlexPro, and 81% of the respondents selected the FlexPro as the more preferable device.
Conclusions: The FlexPro was more user-friendly than the NordiFlex. Perception of pain at insertion and fear of injection were lessened by using the FlexPro.

Keywords: adherence; growth hormone; injection device; pain.

***Corresponding author: Masanori Adachi,** Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan, Phone: +81 45 711 2351, Fax: +81 45 742 7821, E-mail: madachi@mars.sannet.ne.jp

Introduction

Recombinant human growth hormone (GH) is used in the treatment of a wide range of causes of growth retardation in children, including GH deficiency (GHD), Turner syndrome (TS), and short children born small for gestational age (1–3). In Japan, achondroplasia and hypochondroplasia are also the targets of GH treatment (4). Regardless of etiology, earlier initiation of GH treatment is recommended to obtain a better height outcome (5–8). However, a significant association between insufficient adherence to the treatment regimen and poor treatment outcome has been demonstrated by several studies (9–14). In fact, we often witness insufficient adherence, or even discontinuation of treatment, on either a short- or long-term basis. In addition to poor height outcome, insufficient adherence may result in the waste of health care resources (12). The reason for the high incidence of non-adherence to GH therapy may be multifactorial (9, 11–13). First, GH treatment requires daily subcutaneous self-injections, which may be cumbersome and/or painful, and may lead to a fear of injections. Second, GH therapy requires a long treatment period. Third, skipping GH injections does not necessarily immediately lead to serious health problems.

To promote patients' adherence, it has been suggested that an injection device should be user-friendly (12, 14–16). Accordingly, features enhancing simple handling and an easier injection procedure have been pursued by the manufacturers of GH injection devices.

In this study, we assessed the user-friendliness aspects for pediatric patients of the Norditropin FlexPro (Novo Nordisk A/S, Bagsvaerd, Denmark), one of the newly developed GH injection pens (17), by comparing it to the preceding product, Norditropin NordiFlex (Novo Nordisk A/S) (18).

Patients and methods

Study design

This study was a single-center, single-arm, open-label, questionnaire based survey conducted between 2010 and 2011 at the 419 bed Kanagawa Children's Medical Center, which is one of the largest children's hospitals in Japan, neighboring the Tokyo Metropolitan area.

Pediatric patients who satisfied both of the following criteria were consecutively invited to participate in this survey: i) were currently using the NordiFlex for GH treatment of any indication; and ii) were scheduled to switch to the FlexPro. Originally, at the introduction of GH treatment, the NordiFlex was chosen among all the available GH injection devises either by an attending physician's recommendation or by the patient's own preference. Participants were asked to complete a 16-item questionnaire twice to assess the NordiFlex and the FlexPro (Figure 1). The questionnaire regarding the NordiFlex was provided at the time of switching from NordiFlex to FlexPro, and the FlexPro questionnaire was distributed 12 weeks after the switch. The questionnaire was completed at home by whoever was actually injecting the GH, either the patient or by a caregiver. The questionnaire was then collected at the next outpatient visit. No additional clinical interventions, examinations, or follow-up visits were required for this study.

The questionnaire consisted of four sections: i) evaluation of the design of the pen (three items: pen's weight, pen's size, and the degree of easiness to grip); ii) evaluation of the pen's handling ease (nine items regarding ease of the following: attaching needle, reading the scale on the pen, setting and correcting the dose, dialing up the dose, reverse-dialing, holding the pen during injection, pushing the button, finishing delivery (confidence of full delivery), and stability during injection; iii) questions about pain and fear of injection (three items: pain at insertion perceived by the patient, pain during injection perceived by the patient, and the patient's fear of injection); iv) overall satisfaction with the pen (one item). In addition, in the last part of the second questionnaire, the respondent was asked which pen was preferable. In most question items, respondents were instructed to rate their assessment using a five category ordinal response scale (5=best, 3=unsure, 1=worst) (Figure 1). There were no rewards given to the patients for participating in the study and they were guaranteed that they would be at no disadvantage if they decided not to take part.

This study was reviewed and approved by an ethics committee of the Kanagawa Children's Medical Center. Written informed consent was obtained from all participating patients or their caregivers.

Norditropin FlexPro

The Norditropin FlexPro is a pre-filled, multi-dose, disposable pen containing a liquid GH preparation (17). It is an evolution model of the Norditropin NordiFlex (18), which was one of the most widely used pre-filled GH pens. Compared with the NordiFlex, the FlexPro is 17 mm shorter in length and 4 mm thicker in diameter. It was anticipated that this shape would improve ease of handling the pen and stability during injection, especially for pediatric patients, who have smaller hands. In addition, with its new crafted spring, the FlexPro needs a 4-fold lower injection trigger force than the NordiFlex (19). The FlexPro has been marketed in Denmark, the Netherlands, the USA, Germany, Switzerland, Austria, Australia, and Japan since 2010.

Data analysis

Standard descriptive statistics were used to summarize the characteristics of the patients. Responses rated as 4 and 5 were treated as positive responses, whereas those rated as 1 and 2 were treated as

Please provide your impression about Norditropin FlexPro.	
Evaluation of the pen's design (should be scored by the person who is actually injecting GH)	☺ ····· 😐 ····· ☹ very good ·····average ····· very bad
Is pen's weight appropriate?	5 · 4 · 3 · 2 · 1
Is pen's size appropriate?	5 · 4 · 3 · 2 · 1
How did you feel about the easiness to grip?	5 · 4 · 3 · 2 · 1
Evaluation of the handling ease of the pen (should be scored by the person who is actually injecting GH)	☺ ····· 😐 ····· ☹ very good ·····average ····· very bad
Is it easy to attach the needle?	5 · 4 · 3 · 2 · 1
Is it easy to read the scale on the pen?	5 · 4 · 3 · 2 · 1
Is it easy to set and correct the GH dose?	5 · 4 · 3 · 2 · 1
Questions about pain and fear of injection (should be scored by the patient himself or herself)	☺ ··· 😐 ··· ☹ no pain/no fear···painful/fearful··much painful/much fearful
How do you feel in insertion?	5 · 4 · 3 · 2 · 1
How do you feel during injection?	5 · 4 · 3 · 2 · 1
Is injection fearful for you?	5 · 4 · 3 · 2 · 1

Figure 1 A sample portion of the questionnaire.

negative. The response distribution between the two pens was compared using the Wilcoxon signed-rank test. Responses to the questions about pain and fear of injection were compared according to the difference of needle brand [BD Micro-Fine plus 31G 5 mm (Nippon Becton Dickinson Co., Ltd, Tokyo, Japan) vs. NanoPass 33G 5 mm (Terumo Corp., Tokyo, Japan)] using the Wilcoxon rank sum test.

Statistical tests were set as two-tailed, with a level of significance of 0.05. The above calculations were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Results

Of the 108 patients who were consecutively invited to participate in this survey, 82 completed the questionnaires twice, that is, both before and after switching from the NordiFlex to the FlexPro. The clinical characteristics of the patients are summarized in Table 1. The mean age of the patients was 11.1 years, and 30.5% of patients were younger than 10 years. GHD was the most prevalent indication (42.7%) for GH treatment. When the patients with TS were excluded, the sex distribution of the participants was almost even. The rate of self-injection reported in the first questionnaire (when using the NordiFlex) was 34.1%, and 43.9% in the second questionnaire (after switching to the FlexPro). Regarding self-reported adherence, the proportions of 'complete adherence' responses were 53.7% on the first, and 63.0% on the second questionnaire. In contrast, 'lower than 70% adherence' was reported in 11.0% and 11.1% of the first and second questionnaires, respectively.

Table 1 Patient characteristics (n=82).

Characteristic	Number (%)
Age group	
1–4 years	3 (3.7)
5–9 years	22 (26.8)
10–14 years	43 (52.4)
15 or older	14 (17.1)
Sex	
Male	38 (46.3)
Female	44 (53.7)
Indication	
GHD	35 (42.7) (M16, F19)
SGA	24 (29.3) (M15, F9)
ACH/HCH	15 (18.3) (M7, F8)
TS	8 (9.8)
Administrator of GH injections	
NordiFlex	
Patients (self-injection)	28 (34.1)
Caregivers	54 (65.9)
FlexPro	
Patients (self-injection)	36 (43.9)
Caregivers	46 (56.1)
Needle used in injections[a]	
BD Micro-Fine plus 31G 5 mm	53 (64.6)
NanoPass 33G 5 mm	29 (35.4)

[a]In a given patient, the same brand of needle was used during the switch of the pen. GHD, growth hormone deficiency; SGA, small for gestational age; ACH, achondroplasia; HCH, hypochondroplasia; TS, Turner syndrome; M, male; F, female.

Evaluations of the design of the pen

Positive responses (rated as 4 and 5) to questions about the pen's weight, pen's size, and the degree of easiness to grip all significantly increased after the switch to the FlexPro. Regarding 'pen's weight' and 'pen's size', an approximately 20% increase in positive responses occurred [for 'weight', from 47.6% to 64.6% (p=0.0195); and for 'size' from 35.4% to 58.5% (p<0.0001)]. Positive responses to 'easiness to grip' doubled after switching to the FlexPro [from 28.0% to 56.1% (p=0.0024)], whereas negative responses decreased only slightly (from 12.2% to 11.0%).

Evaluation of the ease of handling of the pen (Figure 2)

For the FlexPro, approximately 70% of respondents expressed positive evaluations in 'reading the scale on the pen' (74.4%), 'setting and correcting the dose' (69.5%), 'dialing up the dose' (69.1%), and 'pushing the button' (67.1%). The positive responses for the FlexPro was about 60% for 'attaching needle' (61.0%), 'holding the pen during injection' (56.3%), 'finishing delivery' (56.3%), and 'stability during injecting' (56.8%).

In comparison to the responses regarding the NordiFlex, an approximately 20% increase in positive responses occurred, with statistical significance, for all items except 'dialing up the dose' and 'reverse-dialing'. In particular, positive responses almost doubled for 'holding the pen during injection' (from 28.8% to 56.3%) and 'stability during injection' (from 25.9% to 56.8%). Positive responses more than tripled for 'pushing the button' (from 22.0% to 67.1%). 'Reverse-dialing' was the only item for which positive responses decreased for the FlexPro compared to the NordiFlex (from 65.4% to 34.6%).

Questions about pain and fear of injection

For the FlexPro, more than two-thirds of patients (whether self-injecting or not) responded positively regarding 'pain

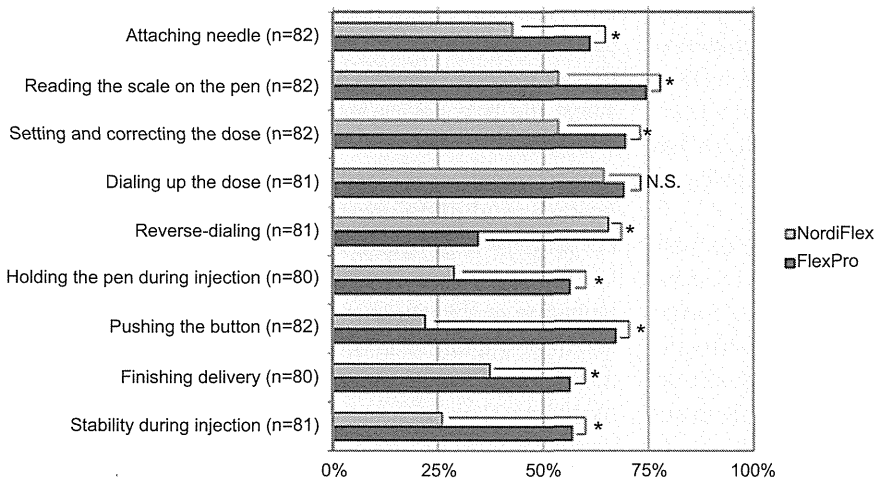

Figure 2 Cumulative positive answers (%) for evaluation of the ease of handling the pens. An asterisk indicates statistically significant differences in patients' ratings between NordiFlex and FlexPro. N.S., not significant.

at insertion', 'pain during injection', and 'patient's fear of injection' (70.2%, 66.7%, and 70.8%, respectively). Furthermore, after switching to the FlexPro, positive responses significantly increased for 'pain at insertion' and 'patient's fear of injection' (Figure 3).

The difference of needle brand did not influence the responses to the above questions. The incidence of positive response to 'pain at insertion' was not statistically significantly different between users of BD Micro-Fine plus 31G 5 mm and those of NanoPass 33G 5 mm, when using either the NordiFlex (58.0% vs. 51.2%; p=0.5926) or the FlexPro (74.0% vs. 63%; p=0.6841). No statistically significant differences were found regarding 'pain during injection' or 'fear of injection' (data not shown).

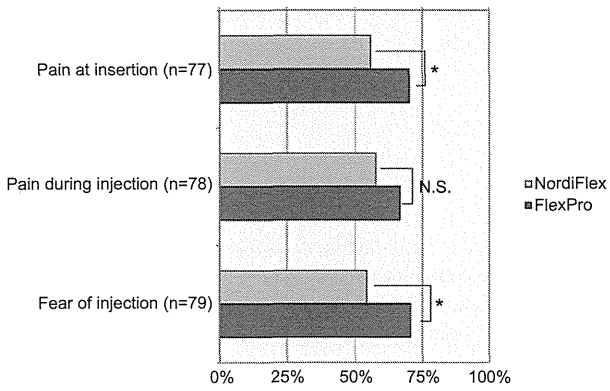

Figure 3 Cumulative positive answers (which indicate presence of little or no pain or fear) (%). An asterisk indicates statistically significant differences in patients' ratings between NordiFlex and FlexPro. N.S., not significant.

Overall satisfaction and device preference

Regarding overall device satisfaction, a significantly higher number of respondents expressed satisfaction with the FlexPro than the NordiFlex (50.0% vs. 82.7%, respectively; p=0.0001). To the question of which pen was preferable, the majority of respondents selected the FlexPro compared to the NordiFlex (81% vs. 19%, respectively).

Discussion

This study demonstrated that the FlexPro was rated better than the NordiFlex in nearly all aspects concerning user-friendliness (Figure 2).

One of the reasons for this improvement may be the pen's shape. The shorter and thicker design seemed to improve the ease of grip, which may be directly related to the significant increase of positive responses to 'holding the pen during injection' and to 'stability during injection'.

The reduced injection trigger force achieved by the new crafted spring may be another reason for improved user-friendliness, considering the 3-fold increase of positive responses to the question in 'pushing the button'. Another simple study assessing usability of the FlexPro vs. the NordiFlex by multinational patients (n=50) demonstrated that 80% of the patients preferred the FlexPro over the NordiFlex (20).

In addition to improved ease in handling, responses to 'pain at insertion perceived by the patient' and those to 'the patient's fear of injection' showed a favorable

trend, irrespective of the needle thickness used. Although the precise reason for this improvement is unknown, increased ease of grip may be relevant to the reduced insertion pain, and thus, to the reduction of fear of injections. In addition, the shorter length of the FlexPro may be less intimidating, resulting in a positive psychological impact.

In contrast, improvement was not evident in 'pain during injection perceived by the patient'. It is possible that an increased injection speed caused by the crafted spring was detrimental in this respect. The problems in 'dialing up the dose' and 'reverse-dialing' may also be as a result of the crafted spring system, which demands increased effort to go against the force of the spring. In addition, an unpleasant mechanical sound is generated by reverse-dialing. A sustained and vigorous effort to develop an even better device is needed.

From this study, it is impossible to estimate the contribution of use of the FlexPro to the improved treatment adherence. However, it was reported that the ease of use of a drug-delivery device was associated with greater patient adherence to treatment of GHD and diabetes (13–16, 21, 22). In addition, pain and fear of injections have been shown to be associated with poor adherence (9, 14, 21, 22). In these respects, the FlexPro may have the potential to improve adherence; assessing this topic is an objective of a planned future study.

In addition to progress in the design and function of pens, a needle insertion accessory has been also used, which was expected to reduce needle fear (18, 20). Additionally, an electrically controlled auto-injection device is an alternative method of improving user-friendliness (13, 23, 24). Although such devices present some difficulties for learning to operate, and may not be preferable for some patients (25), they could be attractive for others (26). In addition, the fact that the personal injection history is recorded automatically by the device may be beneficial for increasing adherence (13). Currently there are a variety of methods for GH delivery. It is important to provide complete information to patients, and to select the best device/method of GH injection for each individual.

An important limitation of this study is a potential bias as a result of the non-cross-over design. First, because all patients were asked to change pens, they may have felt that the newer FlexPro must be better. Second, participation in this study may be relevant to the increased self-injection rate seen (from 34% to 44%), which in turn may have led to a positive impression of the FlexPro. Aside from these biases, the question remains as to which device features had more important roles in determining patient preference. It should also be noted that certain important aspects influencing patient preference might not have been captured by our questionnaire.

In conclusion, the FlexPro was found to be more user-friendly than the NordiFlex. In addition, perception of pain at insertion and fear of injection were decreased in patients using the FlexPro.

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Conflict of interest statement

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Case Report

Association Between Graves' Disease and Renal Coloboma Syndrome: A Case Report

Takeshi Sato[1, 5], Koji Muroya[1], Junko Hanakawa[1], Yumi Asakura[1], Eihiko Takahashi[2], Yoshiyuki Shiroyanagi[3], Yuichiro Yamazaki[3], Yukichi Tanaka[4], Tomonobu Hasegawa[5], and Masanori Adachi[1]

[1]*Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Kanagawa, Japan*

[2]*Department of Nephrology, Kanagawa Children's Medical Center, Kanagawa, Japan*

[3]*Department of Urology, Kanagawa Children's Medical Center, Kanagawa, Japan*

[4]*Department of Pathology, Kanagawa Children's Medical Center, Kanagawa, Japan*

[5]*Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan*

Abstract. Renal coloboma syndrome is an autosomal dominant condition characterized by renal lesions and optic nerve abnormalities. We report an 11-yr-old Japanese girl with familial renal coloboma syndrome, who also had Graves' disease. Four affected family members had a previously reported heterozygous mutation (c.76dupG, p.Val26Glyfs*28) in the *PAX2* gene. We hypothesized that *PAX2* mutations may increase the risk of autoimmune diseases through alterations of human ß-defensin 1 expression.

Key words: renal coloboma syndrome, *PAX2,* mutation, Graves' disease

Introduction

Renal coloboma syndrome (OMIM#120330) is an autosomal dominant condition characterized by renal lesions and optic nerve abnormalities (1). Renal manifestations include renal hypoplasia, enuresis due to urinary concentrating defect, multicystic dysplastic kidney, and renal insufficiency. Ocular manifestations include optic nerve dysplasia, scleral staphyloma, optic nerve cyst and retinal detachment. More than 170 affected individuals and 80 families have been reported to date (2).

PAX2 is the only gene known to cause renal coloboma syndrome. Genotype-phenotype correlation is not evident among *PAX2* mutation carriers. Optic nerve abnormalities and renal lesions are variably manifested by patients with *PAX2* mutations, even within the same family (2, 3). While several nonrenal or non-ophthalmological features, such as hearing impairment, central nervous system anomalies, developmental delay, cardiac defects, abnormality of the hand, and ligamentous laxity, have been reported (4), there is no report documenting autoimmune diseases or thyroid diseases.

Here, we report a patient with Graves' disease and a familial *PAX2* mutation, and discuss the potential relationship between

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Corresponding: Dr. Koji Muroya, Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, 2-138-4 Mutsukawa, Minami-ku, Yokohama-shi, Kanagawa 232-8555, Japan
E-mail address: kmuroya@kcmc.jp

Table 1 Renal and ocular manifestations of the patients

Patient, gender and age	Renal manifestations					Ocular manifestations	Associated findings	Other diseases
	Renal size	Enuresis	Onset of proteinuria	Initiation of hemodialysis	Current Scr (mg/dL)	Fundus		
I-2 F/53 yr[a]	Not available	Not available	29 yr	32 yr	9.64[b]	Bilateral retinal detachment	None	Breast cancer
II-3 M/43 yr	Not available	(+)	10 yr	18 yr[c]	1.40	Bilateral optic nerve dysplasia, left retinal detachment	None	None
II-4 F/37 yr	Bilateral hypoplasia	(+)	12 yr	(–)	1.27	Normal	None	Obesity, hypertension, hyperlipidemia
III-3 Unknown/ abortus[d]	Bilateral hypoplasia	ND	ND	(–)	ND	ND	Oligohydram-nios	ND
III-4 F/11 yr	Normal	(+)	9 yr	(–)	0.60	Bilateral optic nerve dysplasia	Oligohydramni-os, strabismus, nystagmus	Graves' disease, bronchial asthma
III-7 M/2 yr	Bilateral hypoplasia	ND	(–)	(–)	0.41	Normal	Oligohydram-nios	None

F, female; M, male; Scr, serum creatinine concentration. [a] She died at 53 yr of age. [b] At the initiation of hemodialysis. [c] He had a kidney transplant at 19 yr of age. [d] The family elected to terminate the pregnancy at 20 wk of gestation.

Graves' disease and *PAX2* mutations.

Case Report

Family pedigree is shown in Fig. 1A. Clinical information of the family members is summarized in Table 1. We describe clinical pictures of three affected family members.

III-4

The proband is an 11-yr-old girl. She is the second child of non-consanguineous Japanese parents. Serial prenatal ultrasound examinations showed no renal anomalies, although oligohydramnios was noted. She was delivered vaginally at 39 wk of gestation. Her birth weight was 2,752 g (−0.6 SD), length was, 47.5 cm (−0.6 SD), and head circumference was, 31.0 cm (−1.6 SD).

At 4 mo of age, internal strabismus and horizontal nystagmus were noticed, and she was referred to our hospital. Ocular fundus examination showed bilateral optic nerve dysplasia (Fig. 2A, B). She has received patching treatment and worn glasses for myopia.

Nocturnal enuresis continued from birth. At 6 yr of age, diurnal enuresis developed. Lifestyle guidance, water restriction and alarm treatment did not result in a favorable response. At 9 yr of age, her kidneys were normal in size and structure according to an ultrasound examination. The Fishberg concentration test revealed that the maximal urine osmolality was 752 mOsm/kg with elevated antidiuretic hormone concentrations (antidiuretic hormone concentration, 8.7 pg/mL, at a serum sodium concentration of 136 mmol/L), suggestive of a mild urinary concentrating defect. A 24-h specimen of urine contained 0.33 g of protein, suggestive of mild proteinuria. Her blood pressure was within normal limits. Renal biopsy

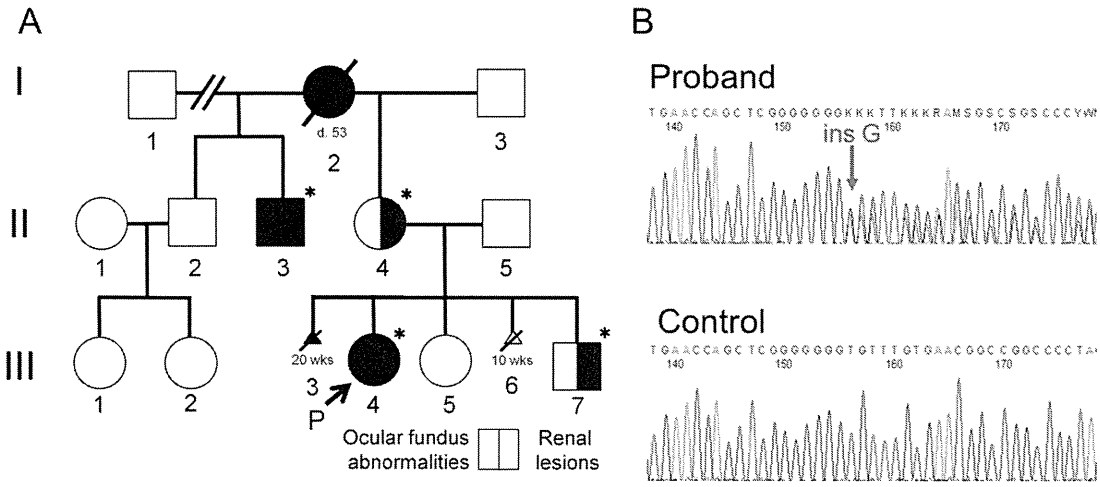

Fig. 1. A: Pedigree of the family. Family members who underwent the genetic testing are marked with an asterisk. B: Sequencing of exon 2 of the *PAX2* gene.

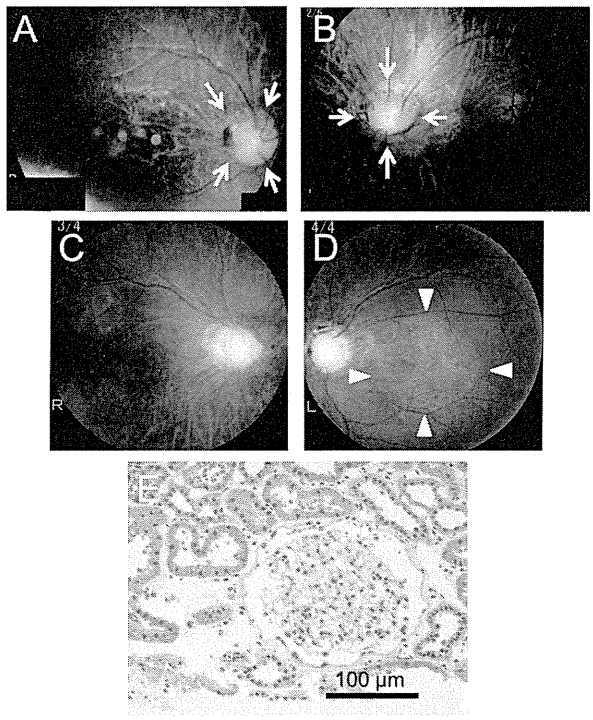

Fig. 2. A–D: Ophthalmological examination of the patients. Right (A) and left (B) eye of the proband (III-4). Bilateral optic discs were wide and deeply excavated (*surrounded by arrows*), suggestive of optic nerve dysplasia, so called "morning glory" variant. Right (C) and left (D) eye of patient II-3. Macular degeneration of his left eye (*surrounded by arrowheads*) and bilateral optic disc dysplasia were shown. E: Renal biopsy from patient II-4 at 26 yr of age. The glomerulus exhibits mesangial cell proliferation slightly. Hematoxylin and eosin stain.

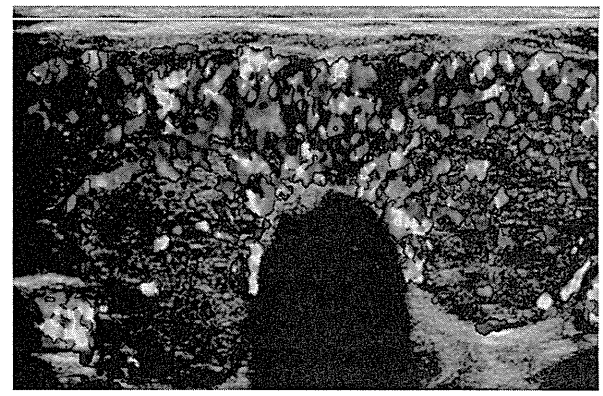

Fig. 3. Color Doppler ultrasound imaging of the thyroid in the proband (III-4). Ultrasonography revealed an enlarged thyroid with hypervascularity and heterogeneously low echogenicity.

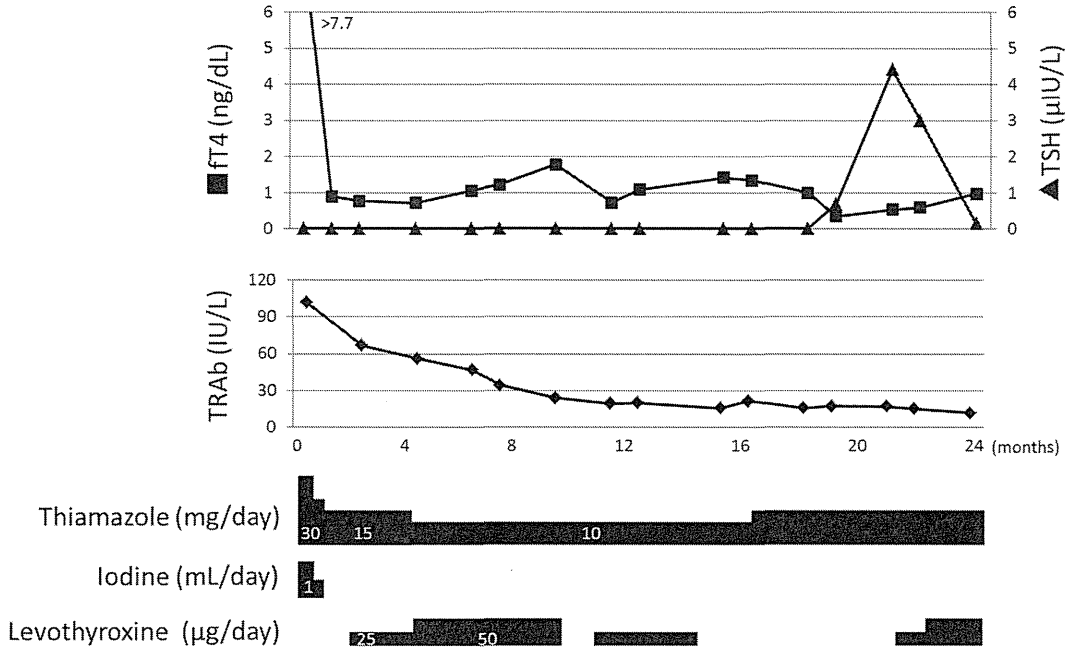

Fig. 4. Clinical course of the proband (III-4). Free thyroxine (fT4) was normalized within a month. Thyroid-stimulating hormone (TSH) levels had been suppressed. Anti-thyroid stimulating hormone receptor antibody (TRAb) titers were reduced.

was not performed.

She was referred to the pediatric endocrinology division at 10 yr of age because of weight loss. On examination, hand tremor and tachycardia (139 bpm) were apparent. Her thyroid was soft and large by palpation. She also had exophthalmos. Blood tests revealed marked hyperthyroidism with a suppressed thyroid-stimulating hormone level (<0.01 μIU/L, reference 0.5–5) and elevated free triiodothyronine (>32.55 pg/mL, reference 2.3–4.0) and free thyroxine levels (>7.77 ng/dL, reference 0.90–1.70). Ultrasonography revealed an enlarged thyroid (estimated size, >34.4 mL, reference 2.9–6.3 (5, 6)), which had heterogeneously low echogenicity. Marked hypervascularity was shown by color Doppler imaging (Fig. 3). The diagnosis of Graves' disease was confirmed by elevated anti-thyroid stimulating hormone receptor antibody (102 IU/L, reference <1.0). Treatment with 30 mg of thiamazole and 1 mL of 2.5% aqueous iodine solution improved clinical symptoms within a month. Then, her anti-thyroid stimulating hormone receptor antibody titers were found to be reduced. Her serum thyroid-stimulating hormone levels had been suppressed. She needed 15 mg of thiamazole two years after diagnosis (Fig. 4).

She has suffered from bronchial asthma with a high immunoglobulin E (IgE) level (511 IU/mL, reference <173) for five years. Her bronchial asthma has been well controlled with montelukast and inhaled beclomethasone. Other immunological tests, including serum levels of immunoglobulins (IgG, IgA, IgM) and blastoid transformation of lymphocytes in response to concanavalin-A and phytohemagglutinin, were within normal limits.

II-3

The proband's uncle is 43 yr old. A periodic medical examination at school revealed proteinuria at 10 yr of age. He presented with enuresis until 11 yr of age. The results of renal

biopsy at 14 yr of age and information about medical treatment was not available. His renal function continued to deteriorate. At 18 yr of age, he was placed on chronic hemodialysis. At 19 yr of age, he had a kidney transplant from his elder brother (II-2). He had complained visual impairment since his late thirties. At 40 yr of age, an ocular fundus examination revealed macular degeneration of his left eye and bilateral optic nerve dysplasia (Fig. 2C, D). At 42 yr of age, retinal detachment of his left eye occurred.

II-4

The proband's mother is 37 yr old. She presented with enuresis until 12 yr old. At 12 yr of age, a periodic medical examination at school revealed proteinuria for the first time. Ultrasonography revealed bilateral renal hypoplasia. At 14 yr of age, left renal biopsy revealed no specific findings. At 26 yr of age, she became pregnant with the proband (III-4), and her renal function became worse. Repeated renal biopsy revealed no specific findings (Fig. 2E). When she became pregnant with a fetus (III-6) at 32 yr of age, her renal function further deteriorated and hypertension developed. The family elected to interrupt the pregnancy at 10 wk of gestation. She was obese, and her body mass index was 32.8 at her last visit.

Mutation analysis of the *PAX2* gene

After obtaining written informed consent, we extracted genomic DNA from peripheral blood samples or nails of four family members (II-3, II-4, III-4 and III-7) using standard protocols. Genomic DNA samples were PCR-amplified for the coding 11 exons and their splice sites of the *PAX2* gene (7), and the PCR products were subjected to direct sequencing from both directions on an autosequencer. All of them had a heterozygous mutation (c.76dupG, p.Val26Glyfs*28) that inserts an extra guanine nucleotide in a stretch of seven guanine nucleotides (Fig. 1B).

Discussion

We described a Japanese family with renal coloboma syndrome and a heterozygous mutation (c.76dupG, p.Val26Glyfs*28) in exon 2 of the *PAX2* gene. Clinical manifestations varied within the family. Ocular fundus manifestations included normal fundus, optic nerve dysplasia, and retinal detachment. Renal manifestations included renal hypoplasia, urinary concentrating defects, proteinuria and end-stage renal insufficiency. One of the two patients with renal failure had a kidney transplant.

The c.76dupG mutation is one of the most frequent *PAX2* mutations (8). It is predicted to have a premature termination codon located in 54–56 nucleotides upstream of the subsequent exon-intron junction. The c.76dupG mutation may trigger nonsense-mediated decay (NMD), although whether the mutation triggers NMD remains to be clarified. Even if the mutant RNA is not destroyed by NMD, the c.76dupG mutation would lead to a truncated protein lacking any functional domains. Clinical presentation of the c.76dupG carriers is known to be highly variable between individuals and even within a family (3, 7). Genotype-phenotype correlation is not evident among *PAX2* mutation carriers. It has been hypothesized that genetic, epigenetic or environmental factors may modulate the clinical manifestations in humans and mice with *PAX2* mutations (9, 10).

Interestingly, the proband had an atypical complication, Graves' disease. Graves' disease is an autoimmune disorder mediated by agonistic antibodies to the thyroid stimulating hormone receptor, which is the commonest cause of hyperthyroidism in childhood and adolescence. *PAX2* mutations or renal coloboma syndrome associated with Graves' disease has not been reported so far. One previous study showed that the increased risk of Henoch-Schönlein purpura nephritis, a putative immune-mediated glomerular disease, was associated with *PAX2* gene polymorphisms (11). Although coexistence

of renal coloboma syndrome and Graves' disease in the proband could be only coincidental, we think it probable that *PAX2* mutations may be associated with development of Graves' disease. PAX2 has been known to be expressed in a subset of lymphocytes (12), and thus would have a physiological role in the immune system. PAX2 represses human ß-defensin 1 expression through binding to the promoter (13). Functional single nucleotide polymorphisms of ß-defensin 1 were reported to be risk factors for development of systemic lupus erythematosus (14) and autoimmune thyroid diseases in type 1 diabetes patients (15). We speculate that *PAX2* mutations may increase the risk of autoimmune diseases, including Graves' disease, through alterations of human ß-defensin 1 expression.

In summary, we reported a patient with a familial *PAX2* mutation, who also had Graves' disease. Further study will clarify whether *PAX2* mutations can be a risk factor for development of autoimmune diseases.

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Novel compound heterozygous mutations of the growth hormone-releasing hormone receptor gene in a case of isolated growth hormone deficiency

Akiko Soneda [a], Masanori Adachi [a,*], Koji Muroya [a], Yumi Asakura [a], Masaki Takagi [b], Tomonobu Hasegawa [b], Hiroshi Inoue [c], Mitsuo Itakura [c]

[a] Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Japan
[b] Department of Pediatrics, Keio University School of Medicine, Japan
[c] Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Japan

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ABSTRACT

Objective: To elucidate the pathogenesis of isolated growth hormone (GH) deficiency in a Japanese girl without consanguinity.
Design: A 2-year-old girl of height 77.2 cm (−3.0 SD for Japanese girls) was found to have an insulin-like growth factor (IGF)-1 level of 7 ng/mL and IGF binding protein-3 (IGFBP-3) level of 0.41 μg/mL. GH responded modestly to a series of pharmacological stimulants, increasing to 2.81 ng/mL with insulin-induced hypoglycemia, 3.78 ng/mL with arginine, and 3.93 with GH-releasing hormone (GHRH). Following direct sequencing of the GHRH receptor (*GHRHR*) gene, evaluation by the luciferase reporter assay, immunofluorescence study, and in vitro splicing assay with minigene constructs was conducted.
Results: Novel compound heterozygous *GHRHR* gene mutations were identified in the patient. A p.G136V substitution elicited no luciferase activity increment in response to GHRH stimulation, with normal membranous expression. Splicing assay demonstrated that the IVS2 + 3a > g mutation would lead to aberrant splicing.
Conclusions: A case of isolated GH deficiency due to novel *GHRHR* gene mutations was identified.

1. Introduction

Growth hormone (GH) deficiency (GHD) refers to inadequate GH secretion from the anterior pituitary gland. When GHD occurs in combination with other pituitary hormone deficiencies, it is defined as multiple pituitary hormone deficiency; when GHD presents without other pituitary involvement, it is called isolated GHD (IGHD). The reported incidence of IGHD has varied widely between 1 in 3500 and 1 in 10,000 live births [1–5]. This great variation may be partly due to the different cut-off thresholds for defining IGHD when interpreting pharmacological stimulation tests for GH.

Although IGHD is usually idiopathic, 5–30% of IGHD patients have genetic backgrounds, including mutations involving the *GH1* gene and GH-releasing hormone receptor (*GHRHR*) gene. Of these, *GHRHR* gene mutations are extremely rare, with most cases described in consanguineous families [6–17]. Genetic screening for IGHD patients in the UK [8] and Netherlands [18] failed to identify any sporadic cases with *GHRHR* mutation. Furthermore, a Japanese study examining the effects of *GHRHR* mutations in 127 short children revealed 1 IGHD family with a homozygous mutation [19]. We herein report the case of a Japanese girl found to have IGHD due to novel heterozygous *GHRHR* gene mutations.

2. Patient report

A 2-year-old Japanese girl was referred for the evaluation of short stature (Fig. 1a). Her non-consanguineous parents were in good health. Her father was 169.5 cm tall (−0.2 SD for Japanese men according to the national reference data [20] and her mother 156.3 cm tall (−0.3 SD for Japanese women). Her 4-year-old brother showed normal growth and development (Fig. 1b). The patient was born at term following a normal pregnancy and uncomplicated vaginal delivery in cephalic presentation. Her birth weight was 3236 g (+0.6 SD) and height 50.0 cm (+0.8 SD). There were no episodes suggestive of hypoglycemia. As depicted in Fig. 1a, her growth curve was quite worrisome throughout her infancy. When she presented at the hospital, her height was 77.2 cm (−3.0 SD), weight 8.9 kg (body mass index: −0.4 SD [20]), and head circumference 44.0 cm (−2.1 SD). Mild frontal bossing was observed. Her developmental milestones were appropriate for her age. According to the Greulich and Pyle method, bone age was 1 year and 8 months. Laboratory findings showed normal results, including the complete blood count, serum electrolytes, and liver and renal function tests. Thyroid function was also normal: TSH, 3.73 μIU/mL (reference range 0.50–5.00); free T4, 1.16 ng/dL [14.9 pmol/L] (reference range 0.90–1.70 ng/dL); and

* Corresponding author at: Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan. Tel.: +81 45 711 2351; fax: +81 45 742 7821.
E-mail address: madachi@mars.sannet.ne.jp (M. Adachi).

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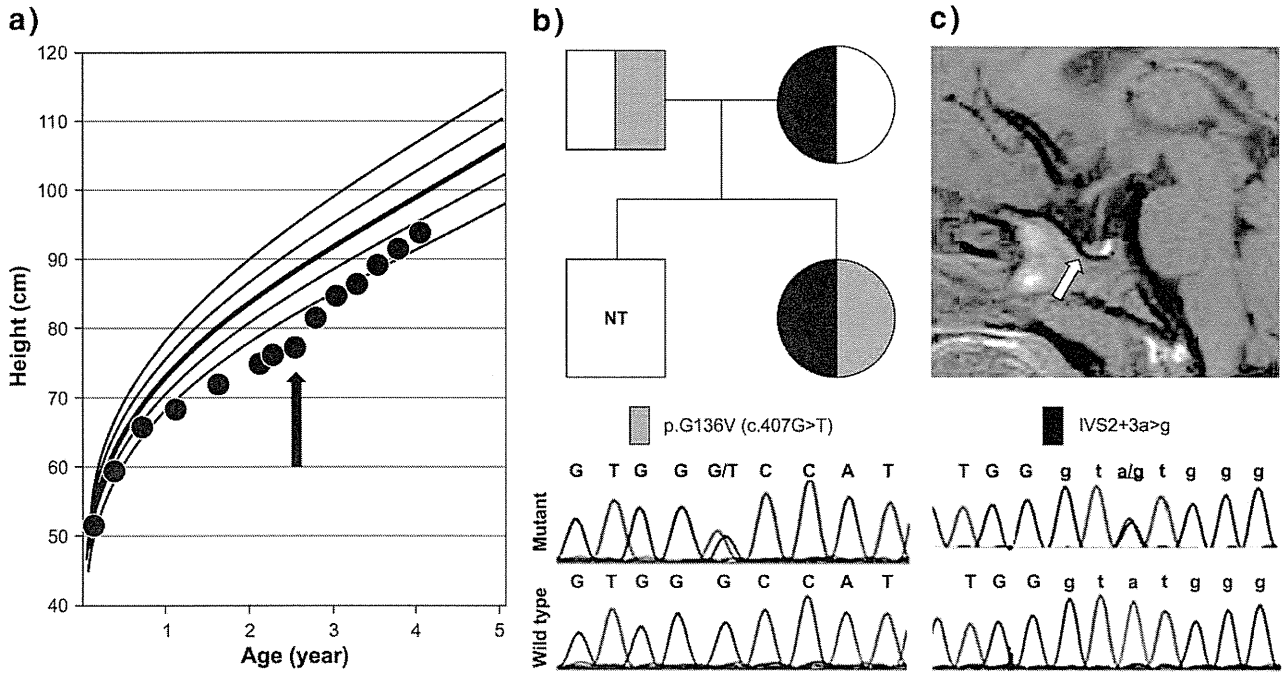

Fig. 1. Clinical characteristics of the patient. a) Height curves. The introduction of GH treatment is depicted by an arrow. b) Family tree. The gray symbol denotes G136V mutation in the *GHRHR* gene, whereas the black symbol indicates IVS2 + 3a > g mutation. Electropherogram of each mutation is shown under the family tree. NT: not tested. c) MR image taken at age 2 years 3 months. The arrow indicates the pituitary gland.

free T3, 3.75 pg/mL [5.76 pmol/L] (reference range 2.30–4.30 pg/mL). Gonadotropins (FSH, 5.11 IU/L; LH, 0.10 IU/L) as well as PRL (23.3 ng/mL) were also appropriate for the patient's age. Because of low levels of both IGF-1 (7 ng/mL; reference range for female infants, 37–229 ng/mL) and IGFBP-3 (0.41 μg/mL; reference range for female infants, 1.33–2.19 μg/mL), a series of GH stimulation tests was conducted. An MRI of the sellar region revealed a pituitary gland of normal size and shape (Fig. 1c). At the age of 2.5 years, GH treatment was initiated at the dose of 0.20 mg/kg/week, divided into 6 injections per week. The patient showed an excellent response to this treatment (Fig. 1a).

3. Materials and methods

3.1. Hormonal evaluations

Serum GH level was determined by a specific RIA kit (GH KIT Daiichi®; TFB Inc., Tokyo, Japan) with recombinant GH (WHO 98/574) set as the standard [21]. IGF-1 concentration was measured by IRMA (IGF-1 IRMA Daiichi®; TFB INC.), and IGFBP-3 concentration was measured by RIA (IGFBP-3 COSMIC®; Cosmic Corporation, Tokyo, Japan). GH stimulation tests were carried out in an overnight fasting state using 4 pharmacological stimuli: insulin-induced hypoglycemia (0.1 U/kg weight); arginine (0.5 g/kg); GHRP-2 (GHRP KAKEN 100®; Kaken Pharmaceutical Co. Ltd., Tokyo, Japan, 2 μg/kg); and GHRH (GRF Sumitomo®, Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan, 1 μg/kg). GHRP-2 is a ghrelin receptor ligand, more likely acting through the enhancement of hypothalamic GHRH release [22,23].

3.2. Mutation detection

Genomic DNA was extracted from peripheral blood leukocytes using standard techniques. The entire coding sequence of *GH-1* comprising 7 exons and of the *GHRHR* gene comprising 13 exons, including intron–exon boundaries and promoter regions, were amplified by PCR and then directly sequenced by the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). PCR primers and conditions for analysis of the *GHRHR* gene were according to those reported in a previous study [6]. In addition, the Multiplex Ligation-dependent Probe Amplification assay (MLPA) was performed using the MLPA kit for GHD (Salsa® MLPA® kit for GHD; MRC-Holland, Amsterdam, Netherlands), which can analyze the following genes involved in genetic GHD: *GH1*, *GHRHR*, *PIT1*, *PROP1*, *LHX3*, *LHX4*, and *HESX1*.

3.3. Construction of expression vectors and site-directed mutagenesis

The wild-type (WT)-GHRHR expression vector was constructed by generating cDNA encoding human *GHRHR* gene by PCR from human pituitary cDNA (Gene Pool Human cDNA; Invitrogen, Carlsbad, CA) and then cloning it into pEGFP-N1 (Clontech Laboratories Inc., Palo Alto, CA) via the introduced *Hin*dIII/*Bam*HI restriction sites.

The p.G136V substitution was introduced by site-directed mutagenesis using the primer set comprising GHRHR-Ex5-Up_ (5′-ACACC GTGGTCCATAGCATCTCTATTGTAG-3′) and GHRHR-Ex5-Lo_(5′-TGCT ATGGACCACGGTGTAGATAATG-3′ (mutated nucleotide underlined). The resulting purified amplicons were digested and then subcloned into the *Hin*dIII/*Bam*HI site of pEGFP-N1. The 3′ end of the vector contained a GFP sequence, thus yielding the following constructs: GFP-GHRHR-Ex5-WT or -mutant. Mutagenesis was confirmed by direct DNA sequencing.

3.4. Cell culture and GHRH-induced luciferase reporter assay

Human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 1% penicillin (10,000 U/mL), streptomycin (10,000 μg/mL), and 10% fetal bovine serum (FBS). Activation of G protein-coupled signal transduction by GHRH (WT or mutant) was studied using luciferase assays. Gs-coupled signaling was analyzed with a reporter vector containing the cAMP response element (CRE-luc; pGL4.29, Promega, Fitchburg, WI). We seeded HEK-293

cells in a 12-well plate and transfected the cells with 400 ng of each GHRHR construct (empty vector or WT or p.G136V) along with 800 ng of CRE-luc reporter vector and 2 ng pRL-CMV internal control vector (Promega) using Lipofectamine 2000. Twenty-four hours after transfection, the medium was removed, and the cells were incubated with 0 or 10^{-8} mol GHRH molecules at 37 °C. Cells were harvested and analyzed sequentially for firefly and Renilla luciferase activities (Dual-Luciferase Reporter Assay System, Promega) according to the manufacturer's protocol. Experiments were conducted in quadruple and repeated at least 3 times.

3.5. Immunofluorescence study

We created an N-terminal hemagglutinin (HA)-tagged GHRHR (HA-GHRHR) expression vector by inserting the HA sequence (TACCCATACGATGTTCCAGATTACGCT) between c.66C and c.67C of the *GHRHR* gene. G136V was introduced by site-directed mutagenesis. HeLa cells grown on sterile glass coverslips were co-transfected with each HA-GHRHR construct (WT or G136V) and a membrane-targeting vector (fusion construct of membrane targeting signal and enhanced red fluorescent protein). Transfected cells were fixed for 10 min in 4% paraformaldehyde/PBS and were incubated with 1:200 anti-HA Alexa Fluor® 488 conjugate antibody (clone 16B12, Invitrogen) at room temperature for 60 min. The fixed cells were permeabilized for 10 min in 0.1% Triton X-100/PBS for staining intracellular antigens. The coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA), and were observed under a confocal microscope (Leica TCS SP5; Leica Microsystems, Mannheim, Germany).

3.6. Cell culture and in vitro splicing assay

HEK 293T cells were maintained in high glucose-DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. The *GHRHR* exons 2–3 and their flanking intronic sequences (929-bp) were amplified by PCR from the DNA of the patient and a control subject using the following set of primers: GHRHR-Ex2-3-Up_*Xho*I, 5′-ggctcgagtttTCGGTCAACCCTAACCTCTG-3′, and GHRHR-Ex2-3-Lo_BamHI, 5′-ccggatcctttGGCTTCTGCTAACACCTGGA-3′ (the lowercase letters indicate a linker sequence containing *Xho*I and *Bam*HI sites [underlined], respectively). The resulting purified amplicons were digested and subcloned into the *Xho*I/*Bam*HI site of the exon-trapping vector pSPL3 (Invitrogen), and designated as pSPL3-GHRHR-Ex2-3-WT or -mutant (IVS2 + 3a > g). The integrity of the constructed plasmid was confirmed by direct sequencing. Splicing assays were performed in HEK 293T cells transfected with 1 μg of the plasmid. Duplicate independent transfections were carried out. Forty-eight hours later, total RNA was isolated and first-strand cDNA was synthesized from 500 ng of total RNA. The resulting 2.5 ng of cDNA (assuming 100% conversion of RNA to cDNA during the reverse transcription step) was amplified, with the primer combination SD6 and SA2 (vector-specific primer set). Amplification of β-actin mRNA (101-bp) (ACTB_ex3F: 5′-AAGGCCAACCGCGAGAAG-3′; ACTB_ex4R: 5′-CCAGAGGCGTACAGGGATAG-3′) was used to assess the reaction efficiency. In addition, RT-PCR products were purified and subcloned into a pCR4-TOPO TA vector (Invitrogen). Approximately 50 individual plasmid clones were randomly selected, and the purified plasmid DNAs were analyzed by *Eco*RI digestion and sequencing using universal M13 primers and a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI

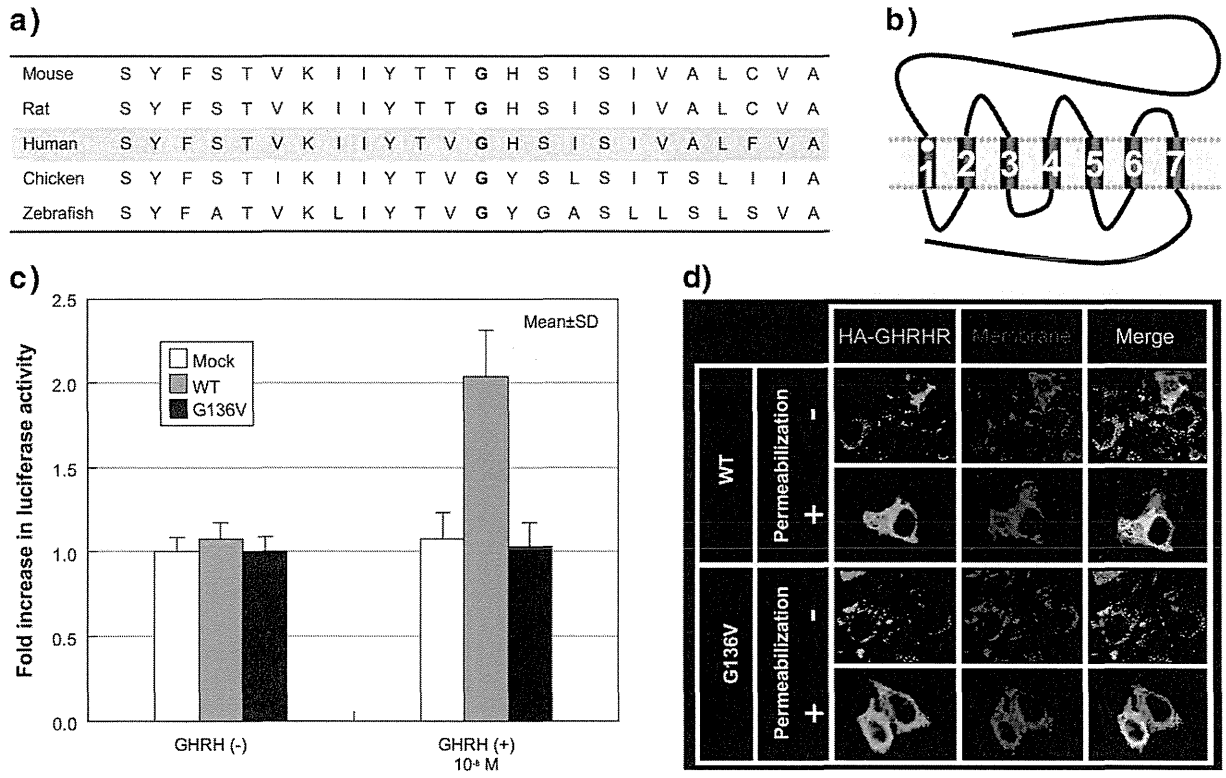

Fig. 2. Delineation of the *G136V* mutation. a) Evolutionary conservation of the 136th glycine among diverse species. b) Schematic representation of the GHRHR protein; the 136th glycine resides in the first transmembrane domain of the receptor. c) Basal and GHRH-stimulated intracellular luciferase activity levels in HEK293 cells transiently transfected with GHRHR. The bars represent the negative control (left, lipofectamine alone), wild-type GHRHR (middle), and G136V mutant GHRHR (right). The assay was conducted in quadruple. d) Subcellular localization analyses using hemagglutinin (HA)-tagged GHRHR constructs. Wild type (WT) and G136V receptors showed comparable surface distribution of fluorescence, indicating equivalent membrane receptor expression.