fasting state, due to the presence of factors stimulating insulin secretion such as postprandial glucose excursion and incretin.

The association between postprandial CPR index and future glycemic control remained significant after adjustment for other confounders including the use of insulin. Insulin is the most potent therapy to improve glycemic control [17], and improvement of beta cell function after introducing insulin therapy has been reported [18, 19, 20]. However, in UKPDS, deterioration of glycemic control was similarly observed among patients treated with an SU, BG or insulin [2, 3]. Consistent with this, the association between baseline CPR value and future glycemic control observed in this study does not seem to be flawed by anti-diabetic medication, which was confirmed in multivariate logistic regression analysis. A negative correlation between postprandial C-peptide and plasma glucose levels has been reported [21, 22], which may result in poor glycemic control in subjects with a low CPR index. Incretin-based therapy has been shown to improve beta cell function [23]; however, it was not available in Japan during this study period.

Multivariate logistic regression analysis showed that, in addition to postprandial CPR index, male sex, shorter duration of diabetes, absence of family history of diabetes, lower BMI, lower HbA1c, administration of a lower number of oral hypoglycemic agents (OHA) and no insulin use before admission were significantly related to good glycemic control, which is largely in line with the results of previous studies [14, 15]. Accordingly, these results indicate that the predictive value of postprandial CPR index is relatively small. Indeed, ROC analysis of postprandial CPR index for predicting good glycemic control revealed a significant but small AUC. Although in this study we were not able to assess insulin sensitivity, it would be interesting to determine whether CPR index adjusted by insulin sensitivity (*i.e.*, disposition index) improves the predictive value for future glycemic control.

There are limitations of this study. Since our study had a retrospective design, unknown confounders might exist. Medication including insulin at the time of CPR measurements might affect CPR levels, but this should affect both groups. Since the wash-out period for previous medication was short in this study, the use of SU, which stimulates insulin secretion, before admission might affect CPR levels. However, the postprandial CPR level was rather lower in SU users than in non-SU users (4.04 ± 2.17 *vs.* 4.58 ± 2.80 ng/mL, $P = 0.015$), suggesting that the effect of previous medication on the CPR value was relatively small. On the other hand, although we carefully adjusted for the effects of medication on future glycemic control in the current study, the dose and duration of medication before admission were not available. Thus, these factors might affect future glycemic control. Also, we did not consider changes in medication after discharge in this study; however, more than 95% of the patients had started medication and most patients had already initiated insulin therapy at discharge. At 2 years after the discharge, proportion of the patients treated with insulin was significantly higher in the inadequate glycemic control group compared with the good glycemic control group (70.2% *vs.* 46.7%, $P < 0.001$). In our previous report using the same cohort, we also have noted that insulin users at the final visit rather showed poorer glycemic control compared with non-insulin users [8], indicating that poor glycemic control in our study was not only due to insufficient intensification of the therapy. Finally, the patients in this study showed poor glycemic control and had been hospitalized and treated with basal-bolus insulin therapy during admission. Therefore, it may not be possible to generalize the results of this study to outpatients with good to moderate glycemic control, and future studies are needed to answer this question. The strengths of this study include 1) the use of a cohort with detailed clinical information including medication, 2) the large sample size and long follow-up duration, 3) that the study was conducted in a single institution, and 4) that all the physicians were specialists in diabetes and treated the patients according to the JDS guidelines for treatment of diabetes.

In conclusion, higher beta cell function assessed by CPR indices was associated with better glycemic control thereafter, confirming the importance of beta cell function for maintaining adequate glycemic control in patients with type 2 diabetes. Postprandial CPR index may add predictive utility to other predictors of future glycemic control and help in the selection of optimal treatment for individual patients with type 2 diabetes. To maintain adequate glycemic control in patients with type 2 diabetes, new therapeutic strategies aiming to foster the recovery of beta cell function are warranted.

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LETTER TO THE EDITOR

Postprandial serum C-peptide to plasma glucose ratio predicts future insulin therapy in Japanese patients with type 2 diabetes

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Type 2 diabetes is a progressive disease, and most patients with type 2 diabetes eventually need insulin therapy to maintain glycemic control. Recent studies have shown that early insulin treatment improved beta cell function as well as insulin resistance in patients with type 2 diabetes[1], suggesting that early introduction of insulin treatment may delay disease progression and be able to maintain good glycemic control thereafter. However, since predictive biomarkers for future insulin therapy have not been established, the introduction of insulin treatment is often delayed in clinical settings [2].

We have recently reported that lower 2 h postprandial C-peptide immunoreactivity (CPR) to plasma glucose ratio (postprandial CPR index; PCPRI) was associated with subsequent need for insulin treatment in Japanese subjects with type 2 diabetes who were admitted to our hospital between 2000 and 2007 [3]. In that study, however, since most patients were started on insulin treatment during the baseline admission, the time-to-event relationship of insulin initiation could not be accounted for in the analysis. Here, we report a sub analysis of this cohort in whom insulin therapy had not been introduced at baseline ($N = 190$), to establish the predictive value of PCPRI for future insulin therapy using univariate and multivariate Cox proportional hazard models. In addition, the prognostic performance of PCPRI was estimated using receiver operating characteristic (ROC) curve analysis.

Baseline characteristics of the subjects were as follows: male 57.9 %, age 65 ± 13 years (mean ± SD), duration of diabetes 10 ± 9 years, BMI 25.3 ± 4.7, HbA1c 9.5 ± 1.8 % (80 ± 20 mmol/mol), fasting CPR 2.14 ± 0.95 ng/ml, fasting CPRI 1.56 ± 0.74, PCPR 5.62 ± 2.59 ng/ml, PCPRI 2.72 ± 1.62 and urinary CPR 78.4 ± 54.7 μg/day. Of the subjects, 52.1 % were treated with a sulfonylurea at baseline.

During the observation period (mean 4.2 ± 2.3 years, median 3.0 years), insulin therapy was introduced in 34 subjects (18 %). Higher PCPRI at baseline was associated with a lower risk of future insulin therapy (hazard ratio (HR) for 1 unit increase in PCPRI was 0.48, 95 % CI: 0.32 to 0.73, $P < 0.001$). However, neither fasting CPRI nor urinary CPR predicted future insulin therapy ($P = 0.30$ and $P = 0.72$, respectively). After adjustment for age, sex, duration of diabetes, family history of diabetes, BMI, HbA1c and sulfonylurea use at baseline, PCPRI remained an independent predictor of future insulin therapy (HR = 0.42, 95 % CI: 0.25 to 0.70, $P = 0.001$). The optimal cutoff value of PCPRI for predicting insulin therapy was 2.16, with 70.6 % sensitivity and 64.1 % specificity (area under the curve (AUC) of ROC curve = 0.698, 95 % CI: 0.609 to 0.788, $P < 0.001$).

In the present study, PCPRI predicted future insulin therapy in patients with type 2 diabetes. Interestingly, neither fasting CPR index nor urinary CPR was significantly associated with future insulin therapy, suggesting that PCPRI is the superior marker for future insulin therapy

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among CPR indices. Because of the small sample size of this study, our findings should be confirmed by future studies including a comparison with other indices such as homeostasis model assessment (HOMA)-β. Nonetheless, PCPRI can be easily measured in clinical practice [4] and may reflect beta cell mass [5]. Our findings emphasize the importance of CPR measurement in clinical settings of diabetes care.

Conflict of interest The authors have no conflict of interest.

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DIABETOLOGY & METABOLIC SYNDROME

RESEARCH Open Access

The efficacy of incretin therapy in patients with type 2 diabetes undergoing hemodialysis

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Abstract

Background: Although incretin therapy is clinically available in patients with type 2 diabetes undergoing hemodialysis, no study has yet examined whether incretin therapy is capable of maintaining glycemic control in this group of patients when switched from insulin therapy. In this study, we examined the efficacy of incretin therapy in patients with insulin-treated type 2 diabetes undergoing hemodialysis.

Methods: Ten type 2 diabetic patients undergoing hemodialysis received daily 0.3 mg liraglutide, 50 mg vildagliptin, and 6.25 mg alogliptin switched from insulin therapy on both the day of hemodialysis and the non-hemodialysis day. Blood glucose level was monitored by continuous glucose monitoring. After blood glucose control by insulin, patients were treated with three types of incretin therapy in a randomized crossover manner, with continuous glucose monitoring performed for each treatment.

Results: During treatment with incretin therapies, severe hyperglycemia and ketosis were not observed in any patients. Maximum blood glucose and mean blood glucose on the day of hemodialysis were significantly lower after treatment with liraglutide compared with treatment with alogliptin ($p < 0.05$), but not with vildagliptin. The standard deviation value, a marker of glucose fluctuation, on the non-hemodialysis day was significantly lower after treatment with liraglutide compared with treatment with insulin and alogliptin ($p < 0.05$), but not with vildagliptin. Furthermore, the duration of hyperglycemia was significantly shorter after treatment with liraglutide on both the hemodialysis and non-hemodialysis days compared with treatment with alogliptin ($p < 0.05$), but not with vildagliptin.

Conclusions: The data presented here suggest that patients with type 2 diabetes undergoing hemodialysis and insulin therapy could be treated with incretin therapy in some cases.

Keywords: Type 2 diabetes, Hemodialysis, Incretin therapy, CGM, Insulin therapy

Introduction

Diabetes is a multifactorial progressive disease accompanied by subsequent systematic vascular complications. Diabetic nephropathy is one of the most critical complications for diabetic patients because it can lead to severe renal failure, which requires treatment with hemodialysis (HD). Indeed, the major cause of the need for HD is diabetic nephropathy, and this has been the case in Japan since 1998. Controlling the blood glucose level of diabetic patients on HD is difficult, because of frequent hypoglycemia, restriction of the use of anti-diabetic agents, and instability of glucose, insulin, and drug metabolites between the day of HD and the non-HD day. However, strict glycemic control is important for the prognosis of diabetic patients with or without renal impairment [1]. Thus, additional effective and tolerable medications are urgently required for diabetic patients with renal impairment.

In recent years, medications that mimic or enhance incretin activity, such as glucagon-like peptide (GLP)-1 receptor agonists and dipeptidyl peptidase (DPP)-4 inhibitors, have emerged as important new treatments

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for type 2 diabetes [2]. Incretins, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are secreted after meals and act directly on pancreatic β cells to stimulate glucose-dependent insulin secretion. GLP-1 has multiple roles in the regulation of glucose metabolism, because it acts on pancreatic β cells and on other organs, including the brain, stomach, and vasculature [3]. However, intrinsic incretins are rapidly inactivated by DPP-4 [4], and as a result GLP-1 receptor agonists and DPP-4 inhibitors have been developed for the treatment of type 2 diabetes.

Incretin therapy is associated with additional benefits compared with other anti-diabetic agents. Firstly, incretin therapy does not induce hypoglycemia, because it controls blood glucose regulation by both insulin and glucagon secretion depending on the blood glucose level [5]. Additionally, incretin therapy may be able to protect pancreatic β cell function and volume, in contrast to sulfonylureas [6,7], and decrease blood glucose level without weight gain [8]. Recently, DPP-4 inhibitors have become one of the most frequently prescribed medications for type 2 diabetes in Japan, and we have previously reported the efficacy of a DPP-4 inhibitor, sitagliptin, in Japanese patients with type 2 diabetes [9]. Furthermore, some incretin therapies could potentially be used for blood glucose control in patients with type 2 diabetes who also have severe renal impairment [10].

According to the guidebook for chronic kidney disease (CKD) in Japan [11], DPP-4 inhibitors including 50 mg oral vildagliptin once-daily, 6.25 mg alogliptin, and the GLP-1 receptor agonist, liraglutide 0.3 mg injected once-daily, are available for treatment of patients with type 2 diabetes and end-stage renal disease (ESRD). However, there are no reports comparing the efficacy and safety of incretin therapies in diabetic patients on HD. In the present study, we examined the efficacy and safety of incretin therapy in patients with insulin-treated type 2 diabetes undergoing HD, using continuous glucose monitoring (CGM).

Subjects and methods

Subjects

Ten Japanese insulin-treated type 2 diabetic patients aged 34–83 years old and on HD were recruited for this study. Patients with a history of type 1 diabetes and diabetic ketoacidosis, severe impairment of intrinsic insulin secretion (serum C-peptide <2.0 ng/dL), requirement of high dose insulin injections (≥20 U/day), severe cardiac disease (New York Heart Association grade ≥III), or severe liver disease were excluded.

Baseline characteristics of the 10 patients are shown in Table 1. In this study, we measured glycated albumin (GA) as a marker of glycemic control, because GA is a more reliable glycemic control marker than glycated hemoglobin in patients with renal failure [12]. Baseline GA was 24.1 ± 1.5% and the mean duration of diabetes was 25.4 ± 2.3 years. Duration of HD was 4.1 ± 1.1 years. Serum-C peptide (S-CPR) was 6.7 ± 1.3 ng/mL, suggesting that patients still had intrinsic insulin secretion. The total dose of daily insulin injection was 11.6 ± 1.9 U/day. No patient had the anti-glutamic acid dehydrogenase (GAD) antibody or history of ketoacidosis. During hospitalization undergoing insulin or incretin therapy, all patients were treated with HD, thrice weekly for 4–5 hours with a bicarbonate dialysate containing 100 mg/dL of glucose. The calculated single-pool Kt/V [13] was 1.44 ± 0.09, suggesting sufficient efficacy of hemodialysis.

Table 1 Patient characteristics at baseline

Parameter	Value
n	10
Sex (male : female)	7:3
Age (years old)	62.9±4.3
Duration of diabetes (years)	25.4 ± 2.3
Duration of HD (years)	4.1±1.1
BMI (kg/m2)	23.0±1.5
Dose of insulin (U/day)	11.6±1.9
Glycated albumin (%) *	24.1 ± 1.5
S-CPR (ng/ml)	6.7 ± 1.3
spKTV	1.44 ± 0.09
Cre (mg/dl)	9.72±0.91
PTH (pg/ml)	88.0 ± 31.4
AST (U/l)	9.3 ± 1.1
ALT (U/l)	9.7 ± 1.3
gGTP (U/l)	16.9 ± 2.2
Total choresterol (mg/dl)	162.1 ± 8.1
Triglyceride (mg/dl)	120.1 ± 20.6
Blood glucose (mg/dl)	162.1 ± 8.1
Complications	
Macroangiopathy (– / +)	(6/4)
Neuropathy (– / +)	(0/10)
Retinopathy (– / +)	(0/10)

Values are means ± SEM or n. *n=9.

All patients provided written informed consent to participate. The study protocol was approved by the ethics committees at Fukuoka University Hospital. The study was performed in accordance with the ethical principles stated in the Declaration of Helsinki, 1964, amended in Edinburgh in 2000.

Methods

The present study design is shown in Figure 1. All patients were hospitalized and switched from insulin

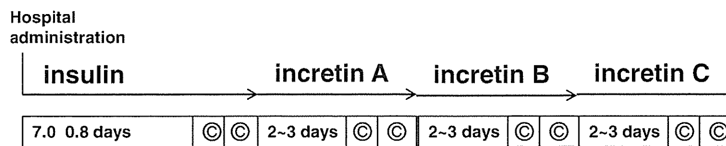

Figure 1 Study protocol. Incretin A-C indicates 0.3 mg liraglutide, 50 mg vildagliptin, or 6.25 mg alogliptin. ©: CGM monitoring day.

therapy to incretin therapy. After patients were admitted to hospital, blood glucose was controlled by insulin therapy targeting a fasting blood glucose level of <130 mg/dL and a 2 h post-prandial glucose level of <180 mg/dL without hypoglycemia for 7.0 ± 0.8 days (3–13 days). After insulin therapy, patients were treated with 0.3 mg liraglutide, 50 mg vildagliptin, and 6.25 mg alogliptin, in a randomized crossover design without a wash-out period.

Continuous glucose monitoring

CGM was performed during the last 2 days of insulin therapy as a baseline evaluation, and then patients were treated with incretin therapy. After at least 2 days of treatment with 0.3 mg liraglutide, 50 mg vildagliptin, or 6.25 mg alogliptin, CGM was performed on both the day of HD and the non-HD day for each incretin therapy. CGM was performed using a CGMS System GOLD system monitor (Medtronic MiniMed Inc., Northridge, CA, USA). Changes in glucose were monitored by CGM for 2 successive days, and injection of liraglutide and administration of vildagliptin or alogliptin once-daily began at least 36 h before CGM. Based on CGM data on the last 2 days, corresponding to the day of HD or non-HD day, the maximum glucose level, minimum glucose level, average and SD of 24 hours glucose, and duration of hyperglycemia (glucose level ≥200 mg/dL) and hypoglycemia (glucose level <70 mg/dL) were determined and compared the baseline on insulin with all therapies.

Statistical analysis

Summary statistics for continuous variables are presented as mean ± standard error. One-way analysis of variance and paired t-tests were performed to analyze differences between incretin therapies. A value of $p < 0.05$ was considered significant for all statistical tests.

Results

No severe hyperglycemia, ketosis, severe nausea, or other adverse effects were observed in patients at any time during incretin therapy. As shown in Figure 2A, maximum blood glucose level was approximately 200 mg/dL for all therapies (insulin; 213.9 ± 11.5 mg/dL (HD), 217.9 ± 15.5 mg/dL (non-HD), liraglutide; 198.2 ± 9.0 mg/dL (HD), 185.9 ± 131.1 mg/dL (non-HD), vildagliptin; 218.8 ± 17.1 mg/dL (HD), 213.2 ± 14.1 mg/dL (non-HD), alogliptin; 240.7 ± 18.2 mg/dL (HD), 233.0 ± 20.1 mg/dL (non-HD)). For incretin therapy, the maximum blood glucose level associated with liraglutide was significantly lower compared with treatment with alogliptin on both the day of HD and the non-HD day ($p < 0.05$), whereas there was no significant difference between liraglutide and vildagliptin. Conversely, there was no significant difference in minimum blood glucose level between the therapies (insulin; 81.8 ± 7.2 mg/dL (HD), 88.5 ± 6.2 mg/dL (non-HD), liraglutide; 81.9 ± 5.4 mg/dL (HD), 89.6 ± 9 mg/dL (non-HD), vildagliptin; 88.1 ± 6.3 mg/dL (HD), 95.6 ± 6.0 mg/dL (non-HD), alogliptin; 89.4 ± 7.1 mg/dL (HD), 92.4 ± 8.2 mg/dL (non-HD)).

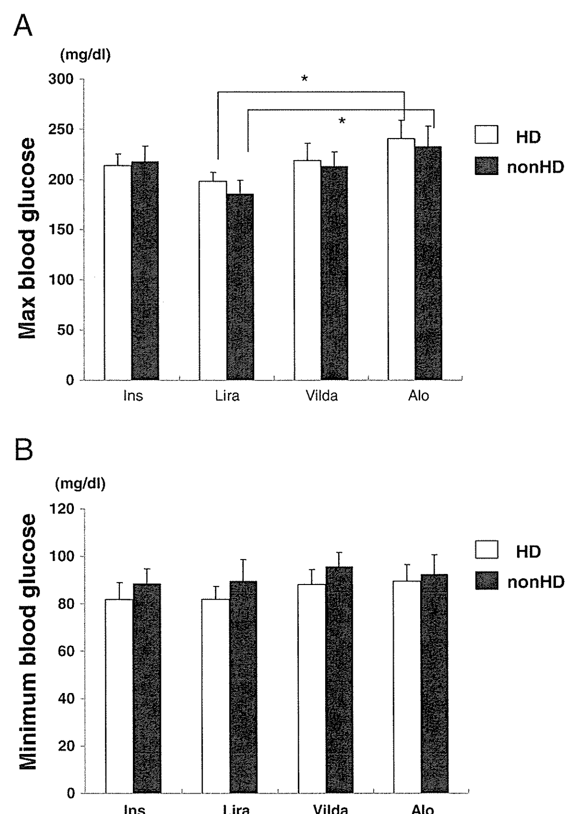

Figure 2 Maximum blood glucose level (A) and minimum blood glucose level (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

We next analyzed the average and standard deviation (SD), a magnitude of glucose fluctuation, measured using CGM. As shown in Figure 2A, the average blood glucose level associated with the therapies was 120–160 mg/dL (insulin; 134.3 ± 6.7 mg/dL (HD), 148.4 ± 10.2 mg/dL (non-HD), liraglutide; 129.0 ± 6.2 mg/dL (HD), 135.9 ± 6.8 mg/dL (non-HD), vildagliptin; 144.3 ± 7.7 mg/dL (HD), 149.6 ± 7.6 mg/dL (non-HD), alogliptin; 147.4 ± 7.3 mg/dL (HD), 149.7 ± 9.1 mg/dL (non-HD)). Compared with alogliptin, liraglutide significantly decreased the average blood glucose level on the day of HD, ($p < 0.05$). Furthermore, we compared the SD of insulin and incretin therapies. As shown in Figure 2B, the SD of liraglutide was lower in comparison to other treatments (insulin; 33.4 ± 4.2 mg/dL (HD), 33.3 ± 4.7 mg/dL (non-HD), liraglutide; 27.3 ± 1.9 mg/dL (HD), 21.5 ± 1.9 mg/dL (non-HD), vildagliptin; 34.7 ± 6.1 mg/dL (HD), 30.2 ± 4.5 mg/dL (non-HD), alogliptin; 38.0 ± 6.5 mg/dL (HD), 32.0 ± 5.1 mg/dL (non-HD)). On the day of HD, the SD of liraglutide was significantly lower compared with insulin and alogliptin treatment ($p < 0.05$), but not with vildagliptin (p=0.14), suggesting that liraglutide controlled blood glucose in patients undergoing HD with smaller glucose fluctuations.

Finally, we measured hyper- (blood glucose ≥200 mg/dL) and hypo-glycemic (blood glucose <70 mg/dL) periods associated with insulin and incretin therapy. As shown in Figure 3A, liraglutide was associated with a decreased hyperglycemic period compared with other treatments (insulin; 40.0 ± 15.0 min/day (HD), 117.9 ± 42.4 min/day (non-HD), liraglutide; 22.9 ± 23.9 min/day (HD), 33.3 ± 34.4 min/day (non-HD), vildagliptin; 87.1 ± 54.6 min/day (HD), 178.7 ± 95.0 min/day (non-HD), alogliptin; 104.1 ± 38.0 min/day (HD), 77.8 ± 26.9 min/day (non-HD). Both on the day of HD and the non-HD day, the hyperglycemic period associated with liraglutide treatment was significantly shorter compared with insulin and alogliptin ($p < 0.05$), but not with vildagliptin. Conversely, there was no significant difference in the hypoglycemic period between the therapies (Figure 3B, insulin; 16.3 ± 9.6 min/day (HD), 21.7 ± 28.6 min/day (non-HD), liraglutide; 49.5 ± 70.7 min/day (HD), 23.9 ± 23.5 min/day (non-HD), vildagliptin; 1.0 ± 1.4 min/day (HD), 1.9 ± 0.0 min/day (non-HD), alogliptin; 6.8 ± 5.7 min/day (HD), 8.0 ± 7.5 min/day (non-HD)). The frequencies of hypoglycemic periods were independent from the duration of hemodialysis.

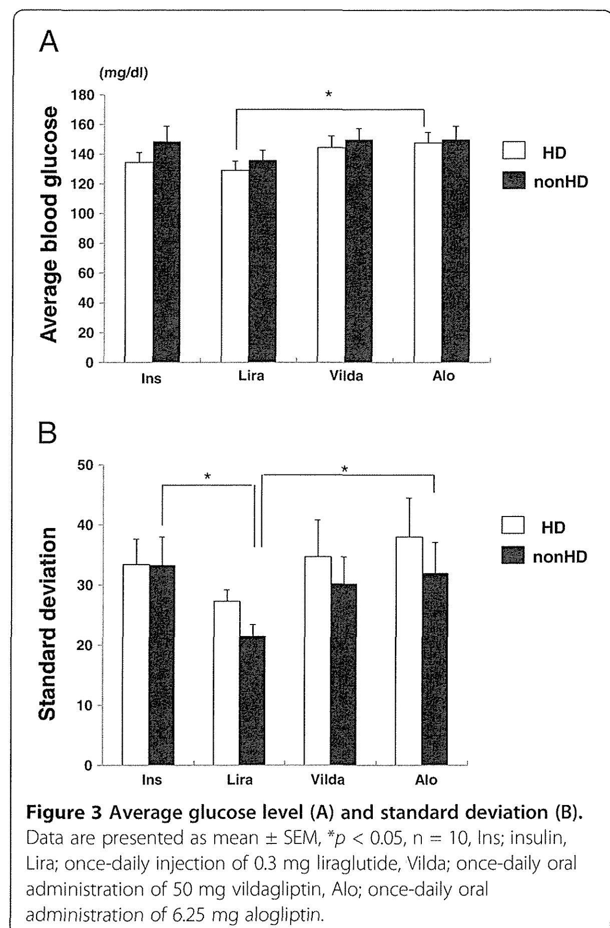

Figure 3 Average glucose level (A) and standard deviation (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

Discussion

In the present study, all 10 type 2 diabetic patients undergoing HD were able to terminate insulin therapy permanently, and were subsequently treated with incretin therapy, including a once-daily injection of 0.3 mg liraglutide, once-daily oral 50 mg vildagliptin, and 6.25 mg alogliptin until at least 3 months after the end of this study. Blood glucose data obtained from CGM suggested that switching from insulin therapy to incretin therapy was effective and well tolerated. After CGM monitoring with incretin therapies, no patient required insulin therapy, and subsequently, six patients continued to be treated with liraglutide, two patients with vildagliptin, and two patients with alogliptin. Their GA 3 months later with incretin therapies was 22.9 ± 0.3%, which was lower compared with the baseline GA, as shown in Table 1, suggesting long term tolerability of incretin therapy in diabetic patients undergoing HD. However, this study has some limitations, because of the small sample size and short-term nature of the study. In the present study, we have shown that it may be possible to use incretin therapy in type 2 diabetes patients undergoing HD, but further study with larger sample sizes over longer terms and including multiple regression analysis of contributing factors to glycemic control by incretin therapy are required to confirm the findings here.

Blood glucose control of diabetic patients undergoing HD is difficult, because of the risk of hypoglycemia. Additionally, therapeutic options for diabetic patients with renal impairments are limited, because reduced glomerular filtration leads to an accumulation of drugs and their metabolites [14]. Before the availability of incretin therapy, standard treatment of diabetic patients with ESRD was with insulin, some glinides, or α-glucosidase inhibitors. However, incretin therapy has emerged as another option for the treatment of diabetic patients with ESRD. Incretin therapy may be an ideal treatment for patients with diabetes and ESRD, because of the low risk of hypoglycemic events. Furthermore, as previously reported by us in an animal model, incretin may also have a vasoprotective effect [15,16]. Indeed, as renal impairment is one of the risk factors that can accelerate coronary artery disease [17]. In addition, incretin has received much attention for its effects on fatty liver [18] and bone [19]. In the present study, we measured liver enzymes and parathormone (PTH) level; however, no changes were observed in these markers after incretin therapy. The lipid lowering effect, as we previously reported [9], was not observed in the present study, probably because baseline lipid level was low in the present study.

In the present study, 0.3 mg liraglutide decreased blood glucose levels and fluctuations of blood glucose more compared with 50 mg vildagliptin and 6.25 mg alogliptin in diabetic patients undergoing HD. Liraglutide is a GLP-1 receptor agonist, which is available for diabetic patients with renal impairment, whereas exenatide, another GLP-1 receptor agonist, is not recommended for use by diabetic patients with severe renal impairment [20]. In a previous report, the safety and pharmacokinetics of liraglutide in subjects with varying stages of renal impairment was examined [21]. In this report, there was no significant difference in the pharmacokinetics and onset of adverse effects depending on the grade of renal impairment. However, there are no reports examining the safety of liraglutide at higher doses and over a longer term in patients with ESRD. In the present study, one patient was treated with liraglutide for over 9 months without adverse effects. However, further studies are required to confirm the safety and efficacy of liraglutide in diabetic patients undergoing HD. Theoretically, incretin therapy should not cause hypoglycemia. However, we observed hypoglycemic periods, and 0.3 mg liraglutide was associated with the highest frequency of hypoglycemia (Figure 4B). These data suggest that both hyperglycemia and hypoglycemia should be monitored when treating diabetic patients undergoing HD with incretin therapy. In the present study, the lowest hypoglycemic period, which was not significantly different from other therapies, was observed with vildagliptin.

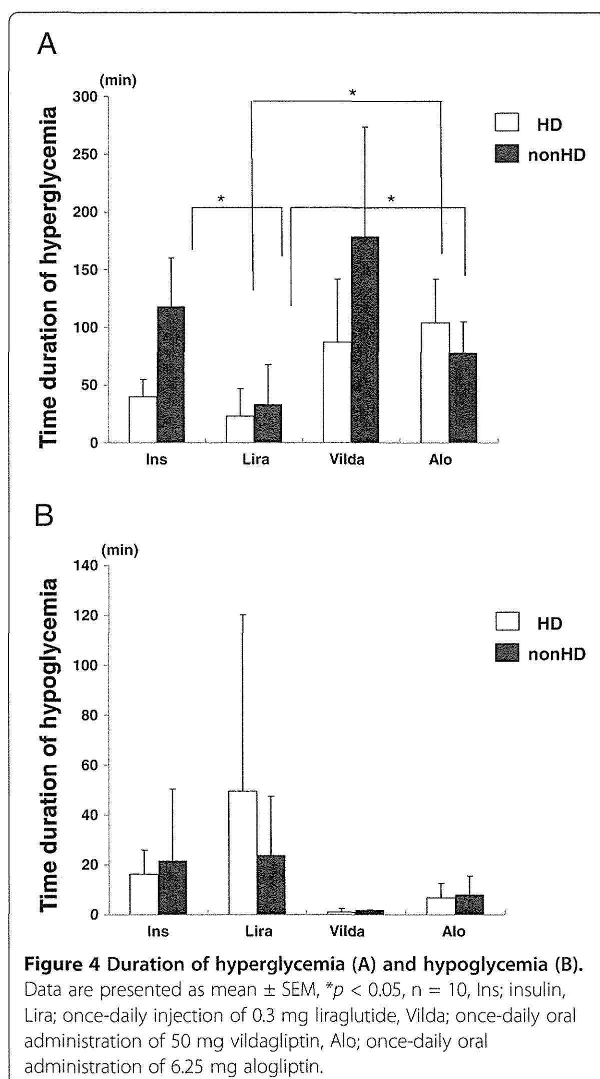

Figure 4 Duration of hyperglycemia (A) and hypoglycemia (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

Similar to our present study, other groups have previously demonstrated the efficacy and tolerability of the DPP-4 inhibitor, vildagliptin, in type 2 diabetic patients undergoing HD [22,23]. Kume et al. treated drug naïve type 2 diabetic patients undergoing HD with 50 mg vildagliptin once-daily for 24 weeks, and observed a significant reduction in postprandial glucose and GA from the baseline data [20]. Ito et al. treated type 2 diabetic patients undergoing HD with once-daily 50 mg or 100 mg vildagliptin for 24 weeks, and observed a significant reduction in HbA1c, GA, and postprandial glycemia [23]. Additionally, the efficacy of alogliptin in type 2 diabetic patients undergoing HD was also reported by another group [24]. Although these reports suggest efficacy and safety of DPP-4 inhibitors for the treatment of patients with type 2 diabetes undergoing HD, caution should still be exercised when treating patients undergoing HD.

Because 85% vildagliptin, 76% alogliptin, and 87% sitagliptin are excreted via the kidney [25], there is a risk that these compounds may accumulate during long-term use. Very recently, linagliptin, which is primarily excreted via bile acid, has become available [26]. According to CKD guidelines [11], linagliptin does not require dose reduction even in patients with ESRD, because of the stable pharmacokinetics of this compound in such patients. Unfortunately, we were not able to include linagliptin in the present study. However, it could potentially become a treatment option for diabetic patients undergoing HD in the future.

As described above, several reports have examined the efficacy of incretin therapy in type 2 diabetic patients undergoing HD. However, there have been no reports demonstrating a switch from insulin to incretin therapy in the treatment of diabetic patients undergoing HD. In the present study, we recruited patients who had S-CPR ≥2.0 ng/dL. Because the glucose lowering effect of incretin therapy depends on intrinsic insulin secretion, S-CPR needs to be high for incretin therapy to be effective. Baseline S-CPR of patients who completed the current study was 6.7 ± 1.3 ng/mL (2.25-16.3 ng/mL), suggesting that 2.0 ng/mL might be the borderline S-CPR concentration with which insulin therapy could be switched to incretin therapy in type 2 diabetic patients undergoing HD. In addition, we recruited patients who did not require insulin injections of >20 U/day to achieve good glycemic control, based on our preliminary experience. In the present study, baseline insulin injection dose was 11.6 ± 1.9 U/day (4–19 U/day).

Furthermore, this is the first report demonstrating CGM of incretin therapy in type 2 diabetic patients undergoing HD. CGM can monitor blood glucose levels for 24 hours, and detect the average and fluctuation range of blood glucose. Because incretin therapy can decrease not only the average blood glucose, but also fluctuations of glucose level, CGM is ideal for the evaluation of glycemic control in incretin therapy [27]. In the present study, incretin therapy, especially liraglutide, controlled both the average and fluctuation of glucose level in type 2 diabetic patients undergoing HD.

Conclusions

This study has shown that it is possible that insulin-treated type 2 diabetic patients undergoing HD might be able to switch from insulin to incretin therapy, if they have a serum C-peptide ≥2.0 ng/dL and an insulin injection dose <20 U/day. However, further studies with larger patient groups and over longer study periods are required to confirm the findings of the present study.

Abbreviations

HD: Hemodialysis; GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase-4; GIP: Glucose-dependent insulinotropic polypeptide; CKD: Chronic kidney disease; CGM: Continuous glucose monitoring; GA: Glycated albumin; S-CPR: Serum-C peptide; GAD: Glutamic acid dehydrogenase; ESRD: End-stage renal disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YU collected data and performed statistical analyses; TN wrote the manuscript and conceived of the research hypothesis; YA, HT, RN, YT, KM, HN, NH, KS, AT, KI, YA, YS, SO, HN, and TS reviewed and edited the manuscript and assisted in patient recruitment; TN assisted in conception of the research hypothesis and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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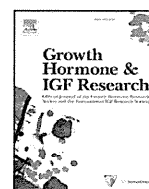

Novel compound heterozygous mutations of the growth hormone-releasing hormone receptor gene in a case of isolated growth hormone deficiency

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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.
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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

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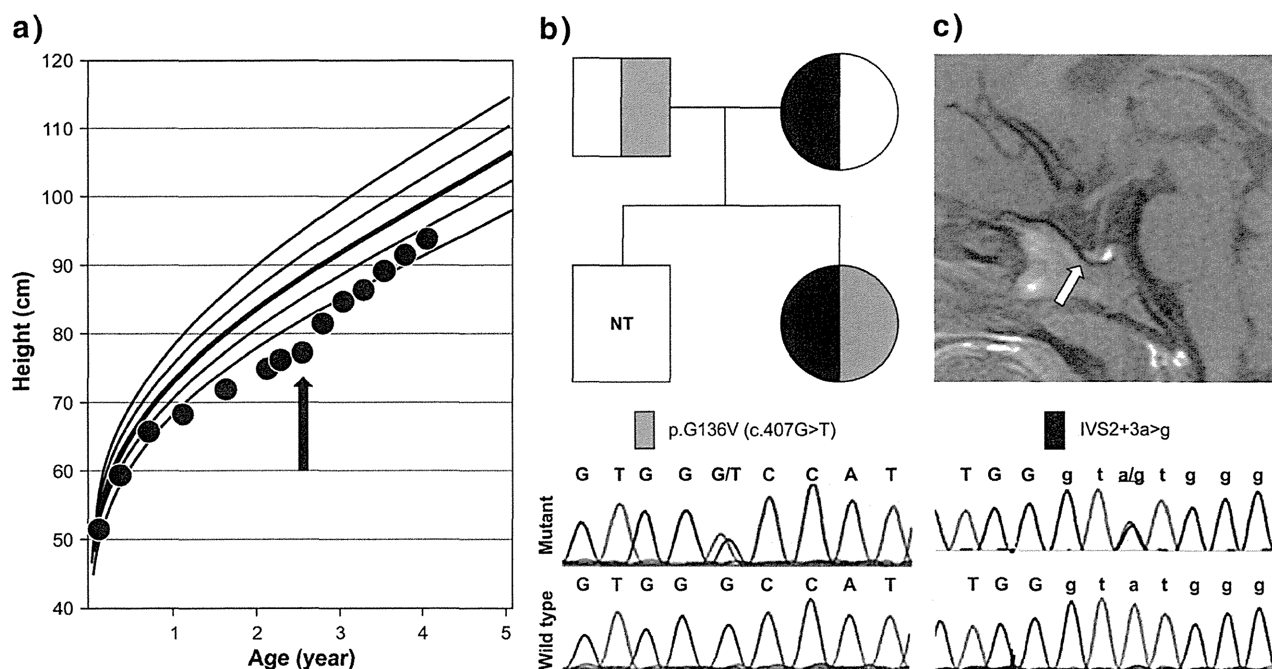

Fig. 1. Clinical characteristics of the patient. a) Height curves. The introduction of GH treatment is depicted by an arrow. b) Family tree. The gray symbol denotes G136V mutation in the *GHRHR* gene, whereas the black symbol indicates IVS2 + 3a > g mutation. Electropherogram of each mutation is shown 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

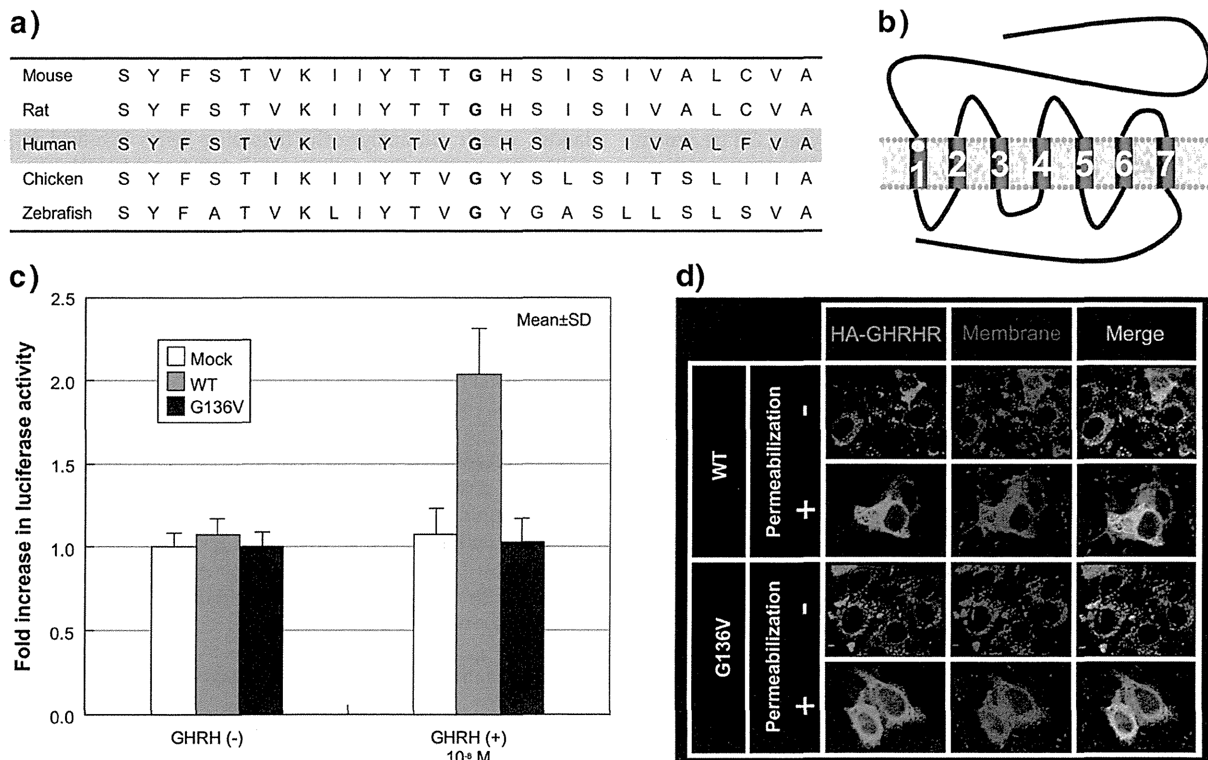

Fig. 2. Delineation of the *G136V* mutation. a) Evolutionary conservation of the 136th glycine among diverse species. b) Schematic representation of the GHRHR protein; the 136th glycine resides in the first transmembrane domain of the receptor. c) Basal and GHRH-stimulated intracellular luciferase activity 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.

Prism 3130xl genetic analyzer. Sequence data were assembled into contigs using the SeqMan II program (DNASTAR®, Madison, WI).

3.7. Bioinformatics

To evaluate the effects of *GHRHR* IVS2 + 3a > g substitution on 5′-splice donor site strength, we used 5 web-based tools for splice-site analysis: the Splice Site Prediction by Neural Network (NN; http://www.fruitfly.org/seq_tools/splice.html), the Splice-Site Analyzer Tool (SS; http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm), the SD-Score Algorithm (SD, http://www.med.nagoya-u.ac.jp/neurogenetics/SD_Score/sd_score.html), the MaxEntScan score5ss program (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), and the HBond (Hydrogen Bond) Score Web-Interface (HBond, http://www.uni-duesseldorf.de/rna/html/hbond_score.php).

3.8. Ethical considerations

The patient's parents provided written informed consent for the genetic studies described above. The Ethical Committee of Kanagawa Children's Medical Center reviewed and approved the study procedures.

4. Results

4.1. Pharmacological GH stimulation tests

The patient showed impaired GH responses. GH peaked at 2.81 ng/mL in response to insulin-induced hypoglycemia, 3.78 ng/mL in response to arginine, 3.93 ng/mL in response to GHRH, and 1.35 ng/mL in response to GH-releasing peptide-2 (GHRP-2; [22,23]). The normal response to the above pharmacological stimulants is defined as above 6 ng/mL, except for GHRP-2, where normal response is defined as above 16 ng/mL [22].

4.2. Mutation detection

In the *GH-1* gene, no pathological mutations were identified. Sequencing of the *GHRHR* gene revealed compound heterozygous mutations consisting of a G to T transition in the 407th nucleotide in the 5th exon, substituting glycine (GGC) with valine (GTC) [p.G136V], and an A to G transition at position 3 in IVS 2 [IVS2 + 3a > g]. Her father was found to be heterozygous for p.G136V, while her mother was heterozygous for IVS2 + 3a > g (Fig. 1b). Neither mutation has been previously reported, nor were they found in 150 Japanese control individuals. The MLPA analysis did not detect any deletion or duplication at the exon level in each gene examined.

4.3. Functional characterization of the missense mutation in GHRHR (p.G136V)

The glycine to valine mutation at position 136 of the *GHRHR* gene is located in the first transmembrane-spanning domain, and is conserved in disparate species such as mouse, rat, chicken, and zebrafish (Fig. 2a,b). The effect of p.G136V was evaluated by GHRH-induced luciferase reporter assay. Fig. 2c shows that GHRH-stimulated luciferase activity was significantly impaired in p.G136V-GHRHR than in WT cells. HA-tagged WT and G136V receptors showed comparable surface distribution of fluorescence, indicating equivalent membrane receptor expression (Fig. 2d).

4.4. Bioinformatics

To predict the effects of the *GHRHR* IVS2 + 3a > g on mRNA splicing, we first used 5 in silico programs (Table 1). In general, these assessments predicted the intrinsic strength of the 5′-splice site of the WT sequence to be relatively low (NN = 0.31 and SS = 71.6,

Table 1
Comparison of 5′-splice donor sites.

	Position									Splice Site Prediction by Neural Network	Splice-site analyzer tool			SD-score algorithm		MaxEntScan::score5ss[a]				HBond score web-interface
	−3	−2	−1	+1	+2	+3	+4	+5	+6	Score	Score	No. of H-Bond between U1 and 5′ splice-site	Free energy of U1/5′ splice-site pairing	SD-score	Coefficient of variation	ME model	MDD Model	FM model	WM model	H-Bond score
Wild	T	G	G	g	t	a	t	g	g	0.31	71.63	7	−4.7	−3.46	0.72	6.23	8.68	5.39	4.88	11.7
IVS2 + 3a > g	T	G	G	g	t	g	t	g	g	0.01	66.67	7	−4.4	−4.80	0.68	1.27	7.28	2.07	3.50	8.3
Cryptic donor site1[b]	C	A	G	g	t	g	g	t	g	0.01	67.69	6	−5.8	−4.10	0.69	1.16	8.08	3.35	4.48	11.8
Cryptic donor site2[b]	G	A	G	g	c	a	g	g	t	0.01	69.73	7	−4.7	na	na	1.90	5.42	2.00	1.74	na
U1 snRNA 3′-	G	U	C	C	A	U	U	C	A	−5′										

na: not applicable. U1 snRNA: U(uridine-rich)1 small nuclear ribonucleic acid. Website of each tool used for splice-site analysis is given in the Materials and Method section.
[a] ME, maximum entropy; MDD, maximum dependence decomposition; FM, first-order Markov; WM, weight matrix.
[b] For the location of each cryptic donor site, see Fig. 3d.

calculated using the Neural Network approach and the Shapiro–Senapathy algorithm, respectively), and that IVS2 + 3a > g further reduces the strength (NN = 0.01 and SS = 66.7), potentially leading to aberrant splicing. The latter hypothesis was further corroborated by other methods. For example, based on the SD-score algorithm, the ΔSD-Score of this mutation was calculated as −1.336, and thus was predicted to cause aberrant splicing. A decrease in the HBond score (11.7 to 8.3) also suggested a reduced sequence complementarity to U1 snRNA (small nuclear RNA).

4.5. In vitro splicing assay

The effect of IVS2 + 3a > g on in vitro splicing efficiency was evaluated using minigene constructs, containing the *GHRHR* exons 2 and 3 with either the WT or mutant sequence (Fig. 3a). RT-PCR of the minigenes revealed that the WT construct generated 2 major splicing products of approximately 470- and 600-bp in size, with the former species being dominant (Fig. 3b). Subcloning and subsequent sequencing showed that the predominant lower band consisted of the expected normal splicing product of 471-bp containing exons 2 and 3 as well as the 474-bp product generated by alternative usage of a CAGCAG tandem acceptor of IVS1, and that the upper band consisted of spliced products but retained the 126-bp sequence of IVS2 (Fig. 3c).

The IVS2 + 3a > g construct also produced 2 bands (Fig. 3b, lane 4), with the 600-bp fragment being the predominant band (Fig. 3b,c). The smaller band (Fig 3b, lane 4) contained either an intronic 20- or 16-bp insertion, consistent with aberrant splicing events due to utilization of cryptic donor splice sites within intron 2 (Fig. 3d, Table 1). The 471/474-bp clones, reflective of normal splicing, were not detected.

Notably, comparison with reference human genomic sequences indicated that the 20-bp-inserted clones used the canonical (gt–ag) splice–site pair, whereas the 16-bp-inserted clones used a non-canonical (gc–ag) splice–site pair (Fig. 3d). In addition, several splicing prediction algorithms (e.g., SS and SD) showed higher 5′-splice site strength for these cryptic splice sites, compared to the mutated counterpart (Table 1). Taken together, our data suggested that IVS2 + 3a > g results in a significant decrease in normal splicing efficiency as well as the activation of cryptic donor splice sites.

5. Discussion

The phenotype of human *GHRHR* gene mutation is IGHD with autosomal recessive trait, classified as IGHD type 1B. First described in 1996 [17], more than 20 mutations have been reported, including a mutation in a POU1F1-binding site of the promoter region [24], missense mutations [8–11,24,25], nonsense mutations [7,8,12,26–28], microdeletions [13,25,29], and splice site mutations [6,14–16,19,26,30–33] (Table 2). Most cases were reported to be severely GH deficient, with extremely low IGF-1 levels and profoundly diminished GH responses to the pharmacological stimulants. Anterior pituitary hypoplasia was a common finding.

This report describes novel compound heterozygous mutations in the *GHRHR* gene in an IGHD patient without consanguinity. The first mutation was a G to T transition in the 407th nucleotide located in

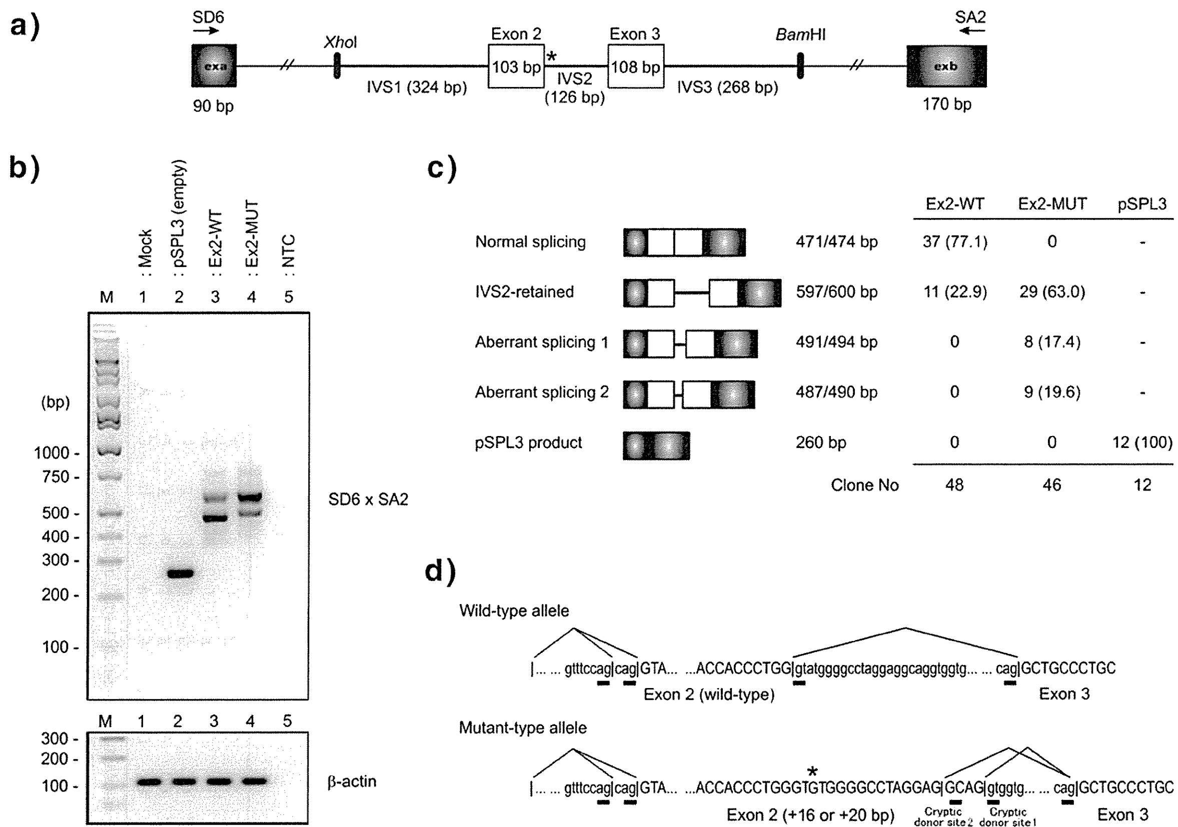

Fig. 3. In vitro splicing assay to verify the effect of the *IVS2 + 3a > g* mutation. a) Schematic diagrams of the *GHRHR* gene locus around the *IVS2 + 3a > g* mutation and the minigene constructs for in vitro splicing assay. SD6 and SA2 are exon-trapping vector pSPL3-specific primers. b) RT-PCR amplification of the GHRHR transcripts obtained from cells transfected with the plasmids pSPL-3-empty (lane 2), pSPL-3-GHRHR exons 2 and 3 wild type (WT; lane 3), pSPL-GHRHR exons 2 and 3 mutant (lane 4), and non-template control (NTC; lane 5). c) Schematic diagrams of WT, various aberrant splicing products, and the appearance frequency. Depending on the alternative usage of CAGCAG tandem in IVS1 acceptor site, the length of normal splice product was either 471 or 474 bp. d) Schematic representation of normal splicing and aberrant splicing with the cryptic 5′ splice sites usage. Underlined nucleotides indicate that both the authentic and cryptic splice sites are involved.

Table 2
Summary of the hitherto reported human mutations in the *GHRHR* gene.

Mutation (site)	Domain	Ethnicity	Consanguinity	Gender	Age at investigation	Height (SDS)	Peak GH response to pharmacological stimuli (ng/mL)					IGF-1 (ng/mL)	IGFBP3 (μg/mL)	Hypoplastic pituitary on MR imaging (age)	Ref.
							GHRH	insulin	arginine	L-DOPA	clonidine				
−124A > C (promotor) K329F (exon 11)	promoter 3rd IC loop	U	−	M	4y	−4.6	1.6	1.2						+ (4 y)	24
IVS1 + 1G > A/IVS1 + 1G > A		Brazil	+	M9, F13	5y − 20y (n = 22)	−2.7 - −8.4	0.63 ± 0.61	flat			flat	6.7 ± 2.8	0.43 ± 0.17	ND	6
IVS1 + 1G > A/IVS4-2A > G		Brazil	−	F	9.5 y	−6.2		1.5			1.9			+ (9 y)	16,30
IVS1 + 1G > A/ E382E (exon 12)		Brazil	−	F	7.1 y	−3.3		0.1						+ (16 y)	16
IVS1 + 2 T > G/IVS1 + 2 T > G		Morroco	+	M F	11y6m 9y4m	−6.6 −4.4		0.1 4.2		0.1 1.2		21 17.4		+ (14 y) + (9 y)	15
Q43X (exon 2) IVS3 + 1G > A	1st EC domain	U	−	M M	11y 8y9m	−5.5 −5.5	<0.05 <0.05		<0.05 <0.05		<0.05 <0.05	20 16		+ (20 y) + (18 y)	26
E72X/E72X (exon 3)	1st EC domain	Sri Lanka	−	M M	7.7 y 6.9 y	−4 −5	<1.5 <1.5					<38.5 <38.5		+ (10 y) + (9 y)	27
		Pakistan	+	M14, F3	3 y - 28 y (n = 17)	−7.2 ± 1.18	0.107 ± 0.053			0.255 ± 0.193	0.374 ± 0.406	5.2 ± 2.0 (n = 14)	0.42 ± 0.13 (n = 14)	ND	7
		U	U	F F	13.8 y 14.8 y	−8.6 −6.74		0.1 0.0		0.1 0.2		7.5 4.2	0.626 0.612	+ (13 y) + (14 y)	28
		India	+ in 2/5 families	M8, F5	4y - 17 y	−4.1 - −8.6	<0.96 (n = 7)			0.9, 1.0	<3.2 (n = 10)	10 - 15 (n = 4)	0.38, 0.42	+ in 4/4 + (4 y - 13 y)	12
		Asia/ Somalia	+	M4, F4	3 y - 15 y (n = 6)	−2.33 - −6.64		<0.1 - 2.2[a]				<−2.5SDS		+ (n = 6) − (n = 2; 4 y, 6 y)	8
E72X/R161W (exon6)	EC/1st IC loop	Asia Asia	−	M F	5.2 y 4.9 y	−4.81 −4.48		0.7[a] 0.6[a]				<−3.5SD <−2.5SD		+ (5 y) − (4 y)	
R94L/R94L (exon4)	1st EC domain	Asia	U	F	5.8 y	−5.6		0.9[a]				−3.0SD		+ (5 y)	

391delG/391delG (exon4)	1st EC domain	Egypt	+	F	5.9 y	−5.5			0.3		0.3	9		+ (5 y)	13
				F	5.9 y	−5.74						<6		− (4 y)	
				F	5.9 y	−6.01						<6		+ (5 y)	
H137L (exon 5)	1st TM	U	−	F	14 y 2m	−7.0		1.0						ND	25
del 1140-1144 (exon11)	7th TM			M	11 y 6m	−5.9		1.0						ND	
L144H/L144H (exon 5)	1st TM	Spain	−	M	5 y	−4.0	0.41					22		+ (5 y)	9
				M	3 y	−3.3	2.0					40		ND	
		Brazil	+	F	26 y	−7.4	<0.3					4.6		+ (26 y)	10
				F	19 y	−7.1	<0.3					2.5		+ (19 y)	
L144H/F242C (exon 7)	1st TM/ 4th TM	US	−	M	17 y 6m	−5.2		3.8						ND	9
				M	16 y 2m	−4.5		2.3						ND	
A176V/A176V (exon6)	2nd TM	Pakistan	+	M	8.5 y	−4.5	<1 mU/L		1.2 mU/L					+ (8 y)	11
				M	8.4 y	−3.5			2.0 mU/L					+ (8 y)	
A222E/A222E (exon 7)	3rd TM	Pakistan	+	M	11 y 2m	−6.8								ND	9
				M	2 y10m	−5.2								ND	
		Asia	+	M	3.2 y	−2.8			0.4[a]			<−3.5SD		+ (3y)	8
				F	2.0 y	−2.98			0.4[a]			<−3.5SD		ND	
IVS7 + 1G > C/IVS7 + 1G > C		Morroco	−	M	16 y	−5.1	absent		5.0 mU/L			14.5		− (25 y)	31,32
				F	14.9 y	−7.3	absent		1.3 mU/L			26.8		− (23 y)	
IVS7-1G > A/IVS7-1G > A		Brazil	+	M	10.1 y	−3.9	2.6				1.3	18		ND	16
				F	3.5 y	−2.5	1.76				3.49			ND	
IVS8 + 1G > A/IVS8 + 1G		China	−	F	56 y	121.2 cm								ND	33
				F	42 y	119.5 cm								+ (42 y)	
				F	36 y	120.2 cm			0.15			31.5	<0.5	+ (36 y)	
W273S/W273S (exon9)	2nd EC loop	Asian	U	F	6.0 y	−4.92						−3.5SD		+ (6 y)	8
		Asian		F	2.6 y	−2.8			0.9[a]			−1.9SD		− (2− y)	
E382E/E382E (exon12)	intracellular tail	Japan	−	F	7 y 2m	−5.2	2.0		2.1					− (7 y)	19
				F	5 y9m	−5.6								− (5 y)	
del 1121-1124/ del 1121-1124 (exon12)	7th TM	Japan	−	M	3 y	−6.0	0.2		0.6	0.8	0.6	67.9		+ (3 y)	29
IVS12 + 2 T > A/IVS12 + 2 T > A		Pakistan	+	M	8 y	−3.6	0.6		0.4			49		− (8 y)	14
				F	3 y	−2.7	0.7		1.7					− (4 y)	
				F	2 y	−2.5	1.0		0.5			41		+ (3 y)	

EC, extracellular; TM, transmembrane; U, unavailable.
[a] The stimulant was either insulin or glucagon.

exon 5, which led to p.G136V substitution. Functional analysis showed that the G136V mutant failed to elicit any discernible luciferase activity increment in response to GHRH stimulation, albeit normal membranous localization in the immunofluorescence study. Salvatori et al. reported a H137L mutation also located in the first GHRHR transmembrane domain and showed that this mutant receptor was normally expressed [25]. In addition, the first transmembrane domain has been shown to be important for ligand binding, both in GHRHR [34] and in the V2 vasopressin receptor [35]. These findings suggest that impairment of receptor function due to p.G136V must occur in the process from ligand binding to cAMP generation.

The second mutation, IVS2 + 3a > g, is an uncommon splice site mutation, considering that both adenosine (A) and guanidine (G) nucleotides are similarly conserved at the + 3 position of the 5′-splice donor site in humans. However, several lines of evidence support our assumption that IVS2 + 3a > g is a disease-causing mutation. First, several examples of disease-associated a > g mutations at this position have been previously described [36,37]. In particular, Ohno et al. reported 10 cases of human disease associated with a > g substitutions at the + 3 site [37]. Second, all the in-silico programs we utilized predicted that IVS2 + 3a > g would have resulted in aberrant splicing. Lastly, a splicing assay using a mini-gene construct and containing the mutant genomic DNA spanning exons 2 to 3 of the *GHRHR* gene demonstrated that aberrant splicing products, with a 20-bp or 16-bp insertion, were mainly generated from the allele containing the IVS2 + 3a > g mutation. The 20- and 16-bp insertions would cause a frameshift and result in truncated GHRHR with 98 and 107 amino acids, respectively. These truncated GHRHRs would lose biological activity, assuming the protein structures were profoundly altered.

The minigene constructs with both WT and the IVS2 + 3a > g mutant also generated the 600-bp product, which retained the 126-bp sequence of IVS2. According to the prediction tool for transmembrane helices in proteins (TMHMM v2.0: http://www.cbs.dtu.dk/services/TMHMM-2.0), the 600-bp products would produce GHRHR with an extended 42-amino acid sequence on its N-terminal (results not shown). Although the biological activity of the 600-bp (+42 amino acid) GHRHR is uncertain, and it is unknown whether the longer GHRHR would be generated in vivo, it may have some residual function, considering that it is normal structure except for the longer N-terminal.

In the majority of patients described with *GHRHR* gene mutations, GH responses to pharmacological stimulants had been completely or nearly absent (Table 2). However, our patient, despite severe growth failure and low IGF-1 levels, showed significant GH responses to the various stimulants. In particular, exogenous GHRH induced a GH response up to 3.93 ng/mL, which seemed paradoxical. For measuring circulating GH levels, we utilized the RIA kit with recombinant GH molecule as the standard. Therefore, the absolute values of serum GH level tended to be lower compared to the values determined by RIA/IRMA with older standards [21]. Thus, methodological differences in GH measurement cannot explain the significant GH response observed in our patient.

Another possible explanation for the GH response may be the younger age of our patient (2 years). As noted in Table 2, our patient was one of the youngest among the reported cases. In the setting of diminished GHRH stimulation, GH response to pharmacological stimulants may gradually decrease with advancing age. In addition, as suggested previously [38], age-dependent changes may also affect pituitary size in patients with *GHRHR* mutations. Whereas our patient was found to have normal pituitary size, previous morphological studies described hypoplastic pituitaries in patients older than 8 years of age [38]. Further research involving serial GH stimulation tests and MR imaging studies may help clarify the presence of age-dependent decline in GH response and pituitary size.

It is also possible that, unlike the in vitro results, splicing inefficiencies at the cryptic donor sites may result in normal splicing in vivo, albeit at very low frequency, and could account for low detection level of stimulated GH.

Yet another possible explanation for the GH response in our patient may be explained by the presence of the 600-bp GHRHR isoform discussed earlier. The increased expression of this form of GHRHR may result in preservation of some receptor function. In addition, different GHRHR isoforms originating from the 4th exon (359 and 337 amino acids) have been reported [39]. Given that these isoforms must also be generated in our patient, it may be the case that they exert some receptor functions.

The parents of our patient harboring heterozygous *GHRHR* mutation had normal stature. This finding is in accordance with other reports arguing against any effects of heterozygosity, although gene–dosage effect [7] or dominant negative effect [29,40] of the mutant *GHRHR* had been suggested previously.

In conclusion, we identified novel compound heterozygous *GHRHR* gene mutations in a Japanese IGHD patient without consanguinity. Although the clinical manifestation was compatible with severe GH deficiency, GH reactivity against pharmacological stimuli including GHRH was partially preserved. To elucidate this mechanism, re-testing after a significant time interval may be helpful.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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