serum vitamin concentrations with Hcy and BMD among subjects in the same study.

In the present study, to evaluate nutritional risk factors for osteoporosis in patients with type 2 diabetes, BMD, Hcy level, and intakes and levels of Hcy-related vitamins including folate, vitamin B_6 and vitamin B_{12} were analyzed.

MATERIALS AND METHODS

Study Population

A total of 125 Japanese patients with type 2 diabetes admitted between December 2008 and June 2009 to Kyoto University Hospital were sequentially enrolled in the study. Lateral lumbar X-ray was carried out to exclude those with scoliosis, compression fractures and ectopic calcifications. Subjects with bilateral hip fractures or prosthesis and other diseases that might influence bone metabolism including liver disease, renal dysfunction (serum creatinine above 2 mg/dL), hyperthyroidism, hyperparathyroidism, hypercorticoidism, and hypogonadism were excluded. All subjects were free of drugs that influence bone and calcium metabolism including glucocorticoids, bisphosphonates, calcitonin injection, estrogens, selective estrogen receptor modulators, vitamin D, vitamin K, thiazide diuretics, heparin and anticonvulsants. The number of patients treated with thiazolidinedione and metformin was 7 and 28, respectively. The present study was cross-sectional in design, and was approved by The Ethical Committee of Kyoto University Hospital and complies with the Helsinki Declaration. Written informed consent was obtained from all participants.

Measurement of Bone Mineral Density

BMD was measured by dual-energy X-ray absorptiometry (DXA; Discovery; Hologic, Waltham, MA, USA) at the lumbar spine (L1-L4) and femoral neck. The coefficient of variation of the measurements of BMD was 0.39%. BMD (g/cm^2) was expressed as *Z*-score calculated on the basis of the normal reference values of the age- and sex-matched Japanese group provided by the DXA system manufacturer. Because male and female patients of different ages were included in the study, comparison of BMD was made based on *Z*-scores. Fat mass and lean body mass (without bone mineral content) were measured by DXA (Hologic Discovery; Hologic) using whole-body absorptiometry software, and each value was expressed in kilograms.

Biochemical Measurements

Blood samples were obtained after overnight fasting immediately after admission. Glycosylated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography (HPLC). The value for HbA_{1c} (%) is estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} [Japan Diabetes Society (JDS); %] +0.4%, considering the relational expression of HbA_{1c} (JDS; %) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[22]. Fasting serum C-peptide was measured by ELISA (ST AIA-PACK C-Peptide; Toso Corporation, Tokyo, Japan). Bone-specific alkaline phosphatase (BAP) was measured by enzyme immunoassay (Osteolinks BAP; DS Pharma Biomedical, Suita, Japan), and urine N-terminal cross-linked telopeptide of type-I collagen (uNTx) was measured by ELISA (Osteomark NTx ELISA Urine; Inverness Medical, Waltham, MA, USA). Plasma Hcy levels were determined by HPLC using a thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfate[23], and the upper limit of Hcy was 13.5 nmol/L. As pyridoxal 5′-phosphate (PLP) is the predominant circulating form of vitamin B_6, serum PLP concentrations were measured by HPLC[24,25] for evaluation of vitamin B_6 status. For vitamin B_{12} measurement, 0.2 mmol/L acetate buffer (pH 4.8) was added to the serum samples, and the vitamin B_{12} was converted to cyanocobalamin by boiling with 0.0006% potassium cyanide at acidic pH. Cyanocobalamin was determined by the microbioassay method using *Lactobacillus leichmanii*, ATCC 7830[24,25]. Serum folate was determined by the microbioassay method using *Lactobacillus casei* ATCC 2733[24,25].

Evaluation of Dietary Nutrient Intake

A food frequency questionnaire (FFQ) validated by Takahashi *et al.*[26,27] was used to calculate nutrient intakes. The FFQ used in the present study included questions on the consumption of various food items over the previous 1 or 2 months. Daily nutrient intake was calculated by multiplying the frequency of consumption of each food by the nutrient content of the portion size and summing the products for all food items. The FFQ is validated against 7-day dietary records and the FFQ-estimated nutrient intake values are 72–121% of those of 7-day dietary records[26]. The reproducibility of the FFQ at intervals of 1–2 months is 93–119% for each nutrient[26]. Correlations of dietary folate intake, serum folate concentration, and plasma Hcy level with intakes of various food groups including grain/rice, potato, green vegetables, other vegetables, fruits, seaweeds, beans/soy products, seafood, meats, egg, milk products and oil/fat were evaluated.

Statistical Analysis

Data were expressed as mean ± SD. SPSS statistical software (version 13.0; SPSS, Chicago, IL, USA) was used for all statistical analyses. Pearson's correlation coefficient was calculated as a measure of association by adjusting for age and sex where appropriate. Stepwise multiple linear regression analyses were carried out to determine independent factors for plasma Hcy levels including (i) dietary vitamin B_6, vitamin B_{12} and folate intake values; and (ii) serum PLP, vitamin B_{12} and folate concentrations as independent variables. The relationship between BMD with Hcy and Hcy-related vitamins was further explored using a quartile-based analysis. Statistical differences among the groups were evaluated using analysis of covariance (ANCOVA) adjusted for age and sex, and Dunnett's multiple comparison tests by comparison with the highest Hcy group. $P < 0.05$ was considered significant.

RESULTS

Clinical characteristics, laboratory data and nutrient intake of subjects are shown in Table 1. The average serum vitamin B_{12} concentration was 1.45 ± 0.45 pmol/mL (Table 1) and there was no difference between patients taking metformin (1.52 ± 0.49 pmol/mL, n = 97) and those without (1.43 ± 0.49 pmol/mL, n = 28). Nutrient intake values were significantly positively correlated with total energy intake (Table 2). Dietary vitamin B_6, vitamin B_{12} and folate intake values were positively correlated with serum vitamin B_6, vitamin B_{12} and folate levels, respectively (Table 2). Plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate (Table 2). Only vitamin B_6 intake and not vitamin B_6 concentration showed a weak negative correlation with Hcy; the influence of vitamin B_{12} on Hcy elevation was unclear (Table 2). Stepwise multiple linear regression analyses were carried out to determine independent factors for plasma Hcy levels. Dietary folate intake was a significant predictor of Hcy when dietary vitamin B_6, vitamin B_{12} and folate intake values were included as independent variables (R^2 = 0.088, β-coefficient = −0.297, P < 0.001), and serum folate concentration also was a significant predictor of Hcy when serum PLP, vitamin B_{12} and folate concentrations were included as independent variables (R^2 = 0.121, β-coefficient = −0.347, P < 0.001). We then evaluated the correlations of folate intake and the concentrations of folate and Hcy with intake of the various food groups determined by FFQ. Dietary folate intake and serum folate concentration were significantly associated with intakes of certain food groups including potato, green vegetables, other vegetables and fruits. Only intake of green vegetables was significantly correlated with the plasma Hcy level (Table 3).

Bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD) were positively correlated with body mass index (BMI) and fat mass, although no significant correlations were found in diabetes-related parameters including fasting plasma glucose, HbA_{1c} and diabetes duration (Table 4). Both SP-BMD and FN-BMD were positively correlated with fasting serum C-peptide, but these correlations were cancelled when adjusted for BMI. Urinary NTx, a marker of bone resorption, was negatively correlated with FN-BMD. As nutrient intake significantly increases with energy intake, nutrition intakes were also evaluated by adjusting for calories. As a result, calorie-adjusted folate intake was positively correlated with SP-BMD, although the association between calorie-adjusted folate and FN-BMD did not reach statistical significance. There were no significant associations between BMD of both sites and serum concentrations of vitamin B_6, vitamin B_{12} and folate. The plasma Hcy concentration was negatively correlated with both

Table 1 | Background characteristics of the study subjects

Characteristic	
No. subjects	125
Male/female	79 (63.2%)/46 (36.8%)
Age (years)	61.2 ± 12.4
Duration of diabetes (years)	11.2 ± 9.4
Diabetes treatment (diet/OHA/Ins/Ins + OHA)	27 (21.6%)/62 (49.6%)/28 (22.4%)/8 (6.4%)
BMI (kg/m^2)	24.9 ± 4.9
Fat mass (kg)	16.5 ± 9.8
Lean body mass (kg)	45.9 ± 9.3
Fasting plasma glucose (mg/dL)	160.2 ± 48.6
HbA_{1c} (%)	9.6 ± 2.2
Fasting serum C-peptide (ng/mL)	1.71 ± 0.89
Serum BAP (U/L)	23.5 ± 8.7
uNTx (nMBCE/mmol Cr)	35.6 ± 19.8
Energy intake (kcal/day)	2073.2 ± 582.5
Protein/fat/carbohydrate intake (g/day)	73.6 ± 19.7/64.4 ± 23.7/278.7 ± 80.2
Calcium intake (mg/day)	596.0 ± 213.6
Vitamin D intake (μg/day)	9.21 ± 4.48
Vitamin B_6 intake (mg/day)	1.22 ± 0.34
Vitamin B_{12} intake (μg/day)	8.81 ± 4.65
Folate intake (μg/day)	287.4 ± 100.5
Serum PLP concentration (pmol/mL)	61.3 ± 29.1
Serum vitamin B_{12} concentration (pmol/mL)	1.45 ± 0.45
Serum folate concentration (pmol/mL)	27.5 ± 10.3
Plasma homocysteine concentration (nmol/mL)	11.2 ± 5.1

Data are number of patients (categorized data) or mean ± SD (quantitative data).
BAP, bone-specific alkaline phosphatase; BMI, body mass index; Ins, insulin; OHA, oral hypoglycemic agents; PLP, pyridoxal 5′-phosphate; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

Table 2 | Correlations among dietary nutrient intake values, serum concentrations and plasma homocysteine levels adjusted for age and sex

	r	P
Correlations of total energy intake with various nutrient intakes		
Vitamin B_6 (mg)	0.521	<0.001
Vitamin B_{12} (μg)	0.253	0.005
Folate (μg)	0.331	<0.001
Correlations of intake values with serum concentrations		
Vitamin B_6	0.192	0.034
Vitamin B_{12}	0.336	<0.001
Folate	0.400	<0.001
Correlations of plasma Hcy levels with B vitamins		
Vitamin B_6 intake (mg)	−0.207	0.022
Vitamin B_{12} intake (μg)	−0.001	0.988
Folate intake (μg)	−0.328	<0.001
Serum PLP concentration (pmol/mL)	0.002	0.982
Serum B_{12} concentration (pmol/mL)	0.001	0.993
Serum folate concentration (pmol/mL)	−0.369	<0.001

Hcy, homocysteine; PLP, pyridoxal 5′-phosphate.

Table 3 | Correlations of dietary folate intake, serum folate concentration and plasma homocysteine level with various food groups

	Dietary folate intake		Serum folate concentration		Plasma Hcy level	
	r	*P*	*r*	*P*	*r*	*P*
Grain/rice	−0.076	0.399	−0.086	0.341	−0.056	0.538
Potato	0.470	<0.001	0.220	0.014	0.012	0.895
Green vegetables	0.843	<0.001	0.361	<0.001	−0.207	0.020
Other vegetables	0.620	<0.001	0.197	0.027	0.077	0.390
Fruits	0.338	<0.001	0.206	0.021	0.018	0.839
Seaweeds	0.322	<0.001	0.072	0.426	0.071	0.435
Beans/soy products	0.390	<0.001	0.156	0.083	0.016	0.856
Seafood	0.313	<0.001	0.075	0.407	−0.017	0.848
Meats	0.065	0.474	0.042	0.643	−0.070	0.435
Egg	0.278	0.002	0.068	0.450	−0.056	0.538
Milk products	0.108	0.230	0.113	0.208	−0.035	0.698
Oil/fat	0.145	0.107	0.161	0.073	−0.112	0.214

Hcy, homocysteine.

Table 4 | Correlations of bone mineral density of lumbar spine and femoral neck with diabetes-related parameters, bone turnover markers and B vitamin status

	SP-BMD		FN-BMD	
	r	*P*	*r*	*P*
BMI (kg/m^2)	0.288	0.001	0.463	<0.001
Fasting plasma glucose (mg/dL)	−0.149	0.098	−0.113	0.210
HbA_{1c} (%)	0.098	0.194	0.053	0.556
Diabetes duration (years)	0.082	0.366	0.057	0.528
Fasting serum C-peptide (ng/mL)	0.182	0.045	0.285	0.001
BAP (U/L)	0.112	0.218	−0.061	0.499
uNTx (nMBCE/mmol Cr)	−0.138	0.084	−0.183	0.042
Vitamin B_6 intake (mg)	−0.032	0.727	−0.053	0.559
Vitamin B_6 intake (mg/100 kcal)	0.113	0.211	0.005	0.959
Vitamin B_{12} intake (μg)	0.012	0.899	0.166	0.065
Vitamin B_{12} intake (μg/1000 kcal)	0.054	0.554	0.148	0.102
Folate intake (μg)	0.103	0.256	0.112	0.216
Folate intake (μg/1000 kcal)	0.198	0.027	0.153	0.090
Serum PLP concentration (pmol/mL)	−0.062	0.497	−0.007	0.936
Serum B_{12} concentration (pmol/mL)	0.023	0.799	0.058	0.524
Serum folate concentration (pmol/mL)	0.104	0.248	0.114	0.205
Plasma Hcy concentration (nmol/mL)	−0.278	0.002	−0.201	0.025

BAP, bone-specific alkaline phosphatase; BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5′-phosphate; SP-BMD, bone mineral density of lumbar spine; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

SP-BMD and FN-BMD, showing that hyperhomocysteinemia is clearly associated with low BMD in patients with type 2 diabetes (Figure 1).

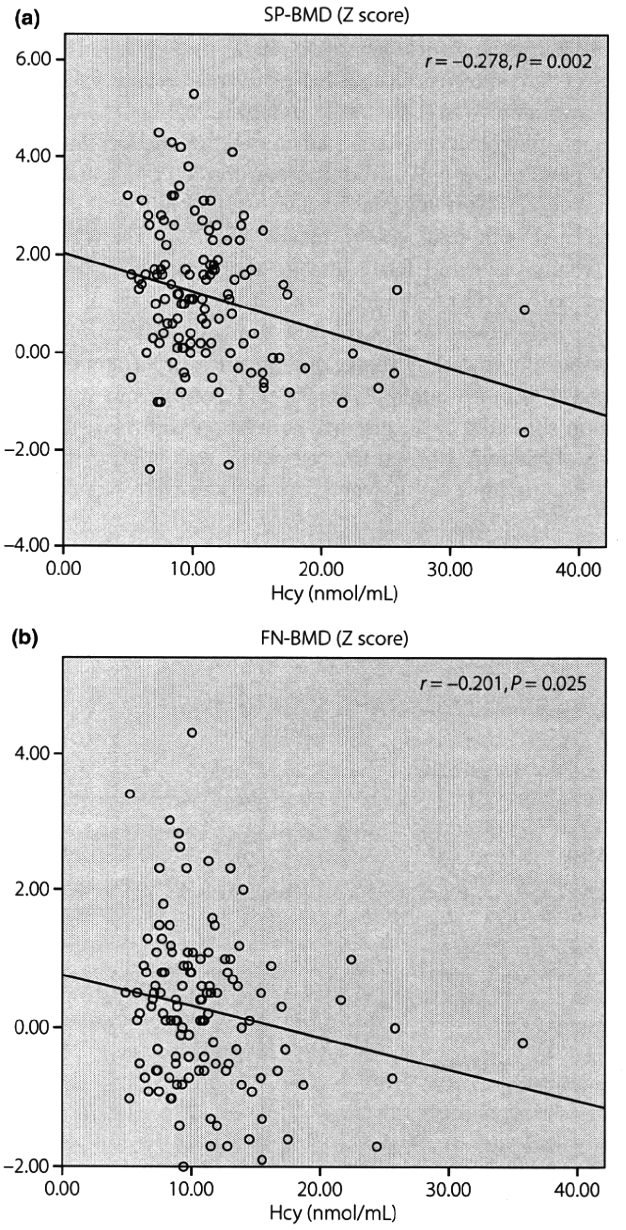

Figure 1 | The relationship between homocysteine (Hcy) and bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD).

As hyperhomocysteinemia derived from folate insufficiency has been suggested to be involved in low BMD, we compared clinical characteristics of the study population across the quartiles of Hcy (quartile 1, n = 31, Hcy < 8.3 nmol/mL; quartile 2, n = 32, Hcy 8.3 to <9.9 nmol/mL; quartile 3, n = 32, Hcy 9.9 to <12.8 nmol/mL; quartile 4, n = 30, Hcy > 12.8 nmol/mL). There were no significant differences across the quartiles in general clinical characteristics including age, BMI, diabetes-related parameters, energy intake, and vitamin B_6 and vitamin B_{12} status (Table 5). However, SP-BMD and FN-BMD were significantly lower in patients in the highest quartile of Hcy than

Table 5 | Comparison of clinical characteristics according to homocysteine quartiles adjusted for age and sex

Hcy concentration (nmol/mL)	Quartile 1 (4.9–8.0)	Quartile 2 (8.1–9.9)	Quartile 3 (10.0–12.8)	Quartile 4 (12.8–35.7)	ANCOVA *P*
Male/female	17/14	21/11	23/9	18/12	
Age (years)	59.3 ± 13.8	58.1 ± 12.6	63.9 ± 8.7	64.0 ± 13.4	0.212
BMI (kg/m^2)	25.0 ± 4.4	25.8 ± 5.0	25.0 ± 5.6	23.8 ± 4.5	0.461
Fasting plasma glucose (mg/dL)	158.8 ± 44.8	162.0 ± 45.8	155.6 ± 50.0	164.6 ± 55.4	0.721
HbA_{1c} (%)	10.1 ± 2.3	9.9 ± 2.5	9.1 ± 1.8	9.4 ± 2.1	0.378
Diabetes duration (years)	9.5 ± 8.4	10.2 ± 9.7	12.6 ± 8.6	12.4 ± 9.0	0.183
SP-BMD (Z score)	1.34 ± 1.43*	1.24 ± 1.38*	1.39 ± 1.24*	0.50 ± 1.18	0.037
FN-BMD (Z score)	0.45 ± 0.99**	0.32 ± 1.23*	0.26 ± 0.96*	−0.27 ± 1.03	<0.001
Energy intake (kcal/day)	2161 ± 543	2145 ± 565	2069 ± 563	1910 ± 650	0.260
Vitamin B_6 intake (mg)	1.31 ± 0.35	1.26 ± 0.36	1.21 ± 0.32	1.09 ± 0.29	0.136
Vitamin B_{12} intake (μg)	8.59 ± 3.44	8.86 ± 4.64	9.49 ± 5.24	8.27 ± 5.21	0.798
Folate intake (μg)	323.5 ± 92.2**	287.2 ± 108.0*	305.0 ± 91.8**	231.7 ± 89.1	0.001
Intake of green vegetables (g/day)	101.9 ± 65.3*	86.1 ± 60.6	89.3 ± 47.5	68.9 ± 49.2	0.043
Serum PLP concentration (pmol/mL)	65.0 ± 33.1	60.0 ± 24.6	58.9 ± 32.9	61.4 ± 26.2	0.943
Serum B_{12} concentration (pmol/mL)	2.39 ± 0.88	2.90 ± 1.61	2.50 ± 0.73	2.53 ± 0.92	0.419
Serum folate concentration (pmol/mL)	33.6 ± 11.5**	26.9 ± 7.6*	26.9 ± 9.0*	21.7 ± 8.7	<0.001
Plasma Hcy concentration (nmol/mL)	6.9 ± 0.9**	9.1 ± 0.5**	11.3 ± 0.8**	17.8 ± 6.1	<0.001

BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5'-phosphate; SP-BMD, bone mineral density of lumbar spine. Mean ± SD, *$P < 0.05$, **$P < 0.01$ relative to the highest homocysteine quartile group.

those in patients in the other quartiles. Furthermore, patients in the highest Hcy quartile showed significantly decreased dietary folate intake, serum folate concentration and intake of green vegetables compared with those in the lower Hcy quartiles. Because the caloric intake was similar across the quartiles, the quality of the diet might be poor in the highest Hcy group. Quartile analysis revealed that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables.

DISCUSSION

In the present study, hyperhomocysteinemia was found to be clearly associated with low BMD in type 2 diabetes patients, as it has been reported to be in non-diabetic subjects[6–14]. Furthermore, folate insufficiency might be one of the important factors in hyperhomocysteinemia, as plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate.

Osteoporosis is a multifactorial disease, a major health problem characterized by low BMD, deterioration of bone microarchitecture and increased risk of fracture. Elevation of Hcy is one of the important risk factors for osteoporosis[28,29], and can be caused by insufficiency of Hcy-related vitamins, such as folate, vitamin B_6 and vitamin B_{12}[6–14]. Because dietary risk factors can be improved when recognized, sufficiency of Hcy-related vitamins and its relationship to osteoporosis in patients with type 2 diabetes is of primary concern.

Elevation of Hcy can be caused by insufficiency of folate, vitamin B_6 or vitamin B_{12}, and the plasma Hcy level is considered to be a fairly sensitive index of folate metabolic status compared with that of the other factors in non-diabetic subjects. Previous studies reported hyperhomocysteinemia was observed in 86% of subjects with clinically expressed folate deficiency[30]; folate is a major determinant of Hcy levels in healthy people[31,32] and vitamin B_{12} influences Hcy levels less than folate does[33,34]. Folate, vitamin B_6 and vitamin B_{12} are water-soluble vitamins, which are in general not readily stored and consistent daily intake is important. Usually, folate and vitamin B_6 deficiency develops within a month of insufficient intake. In contrast, it is known that patients with complete loss of intrinsic factor require 3–5 years to become overtly vitamin B_{12} deficient[35]. Vitamin B_{12} is a unique water-soluble vitamin, and because 80% of the 2.5 mg average whole body stock of vitamin B_{12} is reserved in the liver and vitamin B_{12} excreted in the bile and is effectively reabsorbed in the intestine, clinical signs of vitamin B_{12} deficiency take a long time to appear and progress slowly[36]. Some patients in the present study were taking metformin, which is known to inhibit absorption of vitamin B_{12}[37], but there was no difference between the patients taking metformin and those not taking metformin. As to vitamin B_6, only a weak negative correlation between vitamin B_6 intake and Hcy was not enough to conclude that vitamin B_6 is a nutritional risk factor for osteoporosis, and there have been no other studies showing the effect of vitamin B_6 on BMD.

Leafy green vegetables, such as spinach and broccoli, are rich sources of folate. Folate is also contained in a variety of foods including fruits, beans, seaweeds, liver and egg yolk. To investigate the cause of folate insufficiency, we focused particularly on dietary sources of folate. We evaluated the association of dietary folate intake, serum folate concentration, and plasma Hcy level

with various food groups, and found that intake of green vegetables correlated well with folate status and Hcy levels. Furthermore, it was revealed by the quartile analysis that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables. This analysis suggests that insufficient intake of green vegetables, but not insufficient caloric intake, causes folate insufficiency in the group with the highest Hcy.

The strength of the present study is that it is the first study to show that nutritional status of folate might affect the homocysteine level, a putative risk factor for osteoporosis, in Japanese patients with type 2 diabetes. The present study is also meaningful in promoting awareness of the importance of diet quality, because patients with diabetes are at high risk of developing osteoporosis. In contrast, the present study has some limitations. First, the sample size was not large enough for conclusions regarding marginal insignificant *P*-values. We estimated sample size using a correlation coefficient obtained from a previous cross-sectional study assessing the relationship between BMD and plasma Hcy[8]. The correlation coefficient of femoral BMD with Hcy was −0.18 and the sample size was estimated to be $n = 153$ (two-sided $\alpha = 0.1$, $\beta = 0.2$), while we analyzed 125 patients. Second, we only analyzed patients with type 2 diabetes and comparison with non-diabetic subjects is necessary. An unanswered question is whether diabetes modulates the effects of nutritional state of folate on Hcy metabolism, and the effects of Hcy levels on BMD. Finally, a longitudinal study is required to examine the effects of Hcy on rate of BMD loss and risk of fracture for a longer duration in patients with type 2 diabetes. It is also necessary to determine whether encouraging patients with higher Hcy levels to eat more green vegetables is useful as a dietary intervention to improve Hcy levels and BMD.

In conclusion, the present study shows that BMD inversely correlates to plasma Hcy levels in Japanese patients with type 2 diabetes, and that dietary intake and the serum concentration of folate are determinant factors of Hcy levels. When our group was analyzed across quartiles, BMD, serum folate concentration, folate intake and intake of green vegetables were lowest in the highest Hcy group. Taken together, in Japanese patients with type 2 diabetes, a diet low in green vegetables rather than a calorie-restricted diet might be the more important factor in the declining nutritional status of folate that increases the Hcy level, a putative risk factor for osteoporosis.

ACKNOWLEDGEMENTS

This study was supported in part by Diabetes Masters Conference and Japan Diabetes Foundation. The authors declare no conflicts of interest.

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Hindawi Publishing Corporation
Ulcers
Volume 2011, Article ID 594532, 5 pages
doi:10.1155/2011/594532

Clinical Study

Fat Restriction Is Associated with Impaired Quality of Life in Patients with Ulcerative Colitis and Crohn's Disease

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Received 9 August 2010; Accepted 21 September 2010

Academic Editor: Gyula Mozsik

Inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease, is reported to be associated with impaired health-related quality of life (QOL). Although decreased QOL in these subjects has been reported to be associated with various factors, the effect of nutritional therapy, especially nutrients intake on QOL has received less attention. In this study, we evaluated the various factors including nutrients intake on QOL using SF-8 in 64 patients with IBD. Patients with IBD seem to have decreased QOL especially in the mental aspects. The percentage energy intake from fat of total energy fat intake (% energy) of the whole subjects, was lower than those of the annual National Nutrition Survey in Japan. Multiple regression analyses revealed that fat intake (% energy) was a significant predictor for mental component summary. In conclusion, fat restriction contributes to impaired QOL especially in the mental aspects in IBD patients.

1. Introduction

Inflammatory bowel disease (IBD); ulcerative colitis (UC) and Crohn's disease, is reported to be associated with impaired health-related quality of life (HR-QOL). In this paper, HR-QOL will be simply designated as QOL. Decreased QOL in these subjects has been reported to be related to various factors such as age, gender [1, 2], treatment effects [3], disease activity, and social environment [4]. However, the effect of nutritional therapy on the QOL of IBD patients has received less attention, most of which is devoted to the parenteral nutrition therapy, not the nutritional therapy in general [5, 6].

Since excessive fat intake is considered to worsen the inflammation in the intestine, its restriction has traditionally been employed in Japan as the oral nutritional therapy for IBD patients, especially for those with CD, which, however, has its own pros and cons.

Recently, we have studied the possible involvement of hypovitaminosis D and K in the development of osteoporosis in IBD patients [7]. In face of apparently sufficient intake of these vitamins, their plasma levels were quite low in these patients. Paradoxically, plasma concentrations of vitamin D and K were correlated with the fat intake but not with their intake of these vitamins. These results were more prominent in patients with CD than those with UC. Then it was concluded that fat-soluble substances such as vitamin D and K were not effectively absorbed from the intestine without concomitant intake of enough fat.

Through this paper, we were interested in what fat restriction means from the patients' perspectives and studied

the effect of fat restriction on the QOL of IBD subjects in this paper.

2. Subjects and Methods

2.1. Subjects. Study subjects were 64 patients with IBD attending the gastroenterology clinic at the Kyoto University Hospital; 33 with CD (19 men/14 women) and 31 with UC (20 men/11 women). Detailed information was given and written consent was obtained. The study protocol was approved by the ethical committee of the Kyoto Women's University. Almost all patients (27/33 in CD and 28/33 in UC) were receiving 5-aminosalicylic acid. Glucocorticoid therapy was given to four and two patients with CD and UC, respectively. Immunosuppressive drug therapy was performed in 25 and 4 patients with CD and UC, respectively. Eight patients with CD, but none with UC, were on combined therapy of infliximab, synthetic glucocorticoid, and immunosuppressive drug. Fifteen patients with CD and one with UC were on enteral or total parenteral nutrition therapy, respectively.

2.2. Methods

2.2.1. Dietary Information. Dietary information was obtained from food intake records in 2 weekdays by the patients. By calculating these records, their energy and nutrients intakes were obtained by computer software program (Healthy Maker Pro 501, Mushroom soft Corp.).

2.2.2. QOL Measurement. QOL was assessed using the Japanese Short Form Health Survey (SF-8), a widely used generic questionnaire [8]. Eight subscales are obtained; physical function (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social function (SF), role emotional (RE), and mental health (MH). RP and RE refer to the limitations due to physical or emotional reasons, respectively. They are also summarized into two summary scores: physical component summary (PCS) and mental component summary (MCS). Data are transformed to deviation scores based on Japanese norms [8]. Higher scores indicate better QOL, with 50 corresponding to the national norms.

2.2.3. Statistical Analyses. Statistical analyses were performed using SPSS 17.0J for Windows (SPSS. Japan Inc., Tokyo, Japan). Comparison of data from IBD patients with Japanese norms was done by one-sample t test. The difference between two independent groups was analyzed by unpaired t test or Mann-Whitney test depending on normality. Correlations between two independent variables were analyzed by Pearson's or Spearman's correlations. Multiple regression analysis was performed to determine independent factors for QOL scores in IBD patients.

3. Result

3.1. Background Profiles and Biochemical Indices. The baseline characteristics of the patients are shown in Table 1.

Table 1: Background profiles and results from blood tests in patients with CD and UC.

	CD	UC	P value
Age (y)	35.6 ± 7.3	41.7 ± 17.3	.343[a]
Sex (F/M)	19/14	20/11	—
Disease duration (y)	13.7 ± 7.4	6.8 ± 4.8	<.001[b]
Body mass index (kg/m^2)	19.5 ± 2.3	21.1 ± 3.3	.025[b]
Disease location (involving small bowel/not involving small bowel)	30/2	0/31	—
Glucocorticoid therpy	4	2	—
Immunosuppressive therapy	25	4	—
Immunopotentiating therapy (TNF-α)	8	0	—
Enteral or total parenteral nutrition therapy	15	1	—
C-reactive protein (g/dl)	0.6 ± 1.0	0.3 ± 0.6	.135[b]
Albumin (g/dl)	3.9 ± 0.4	4.3 ± 0.3	<.001[b]
Total cholesterol (mg/dl)	126.9 ± 25.0	177.1 ± 40.3	<.001[b]

Values represent mean ± SD. Comparison of indices between patients with CD and those with UC was done by unpaired t test[a] or Mann-Whitney test[b] depending on normality.

CD patients had significantly longer disease duration and lower BMI than UC patients. While nutritional indices such as serum albumin and total cholesterol were lower in CD subjects, there was no significant difference in C-reactive protein which is an inflammatory parameter between these groups. Most of patients were in remission.

3.2. Energy and Nutrients Intake in CD and UC Patients. Food intake could be evaluated in 62 patients (31 with CD and 31 with UC). Energy and nutrients intake in these patients is shown in Table 2. Fourteen patients with CD were on enteral nutrition, and each one of subjects with CD and UC was on total parental nutrition. Although the energy intake was not significantly different between the two groups, fat intake was significantly lower in CD patients than UC subjects. The annual National Nutrition Survey in Japan (NNS-J) in 2008 showed that in subjects of 30–39 or 40–49, years of age including both genders [9], the daily fat intake (% energy) was 26.5% or 25.6%, respectively. These were significantly higher than those of IBD subjects in this study (P = .001; data not shown). Subjects with enteral or parental nutrition had fat intake only approximately half of that in subjects with oral intake (data not shown). The percentage energy intake from protein, fat, and carbohydrates was significantly different between CD and UC subjects.

TABLE 2: Comparison of nutrient intakes in CD and UC patients.

		IBD (n = 62)	CD (n = 31)	UC (n = 31)	P value
Energy	Intake (kcal)	1816 ± 465 (1804)	1847 ± 392 (1842)	1785 ± 533 (1764)	NS
Protein	Intake (g)	66.0 ± 21.8 (63.5)	71.0 ± 20.6 (67.2)	60.9 ± 22.0 (61.6)	NS
Fat	Intake (g)	44.7 ± 21.6 (43.0)	38.7 ± 17.6 (37.4)	50.6 ± 23.6 (48.1)	$P < .05$
Carbohydrates	Intake (g)	275.4 ± 91.6 (268.6)	298.3 ± 93.1 (275.7)	252.4 ± 85.4 (254.9)	$P < .05$
Protein (% energy)		14.4 ± 2.7 (14.2)	15.0 ± 2.2 (15.6)	13.5 ± 2.9 (13.6)	$P < .001$
Fat (% energy)		22.4 ± 9.6 (24.6)	19.5 ± 8.9 (18.9)	25.2 ± 9.5 (26.8)	$P < .001$
Carbohydrates (% energy)		63.2 ± 9.6 (62.4)	65.2 ± 8.6 (64.0)	56.5±9.5 (60.5)	$P < .001$

Data are expressed as mean ± SD with the values in parentheses showing the median. Comparison of indices between patients with CD and those with UC was done by unpaired t test

TABLE 3: Dimensional SF-8 scores in patients with CD and UC.

	IBD (n = 64)	CD (n = 33)	UC (n = 31)
PF	50.1 ± 4.7 (53.6)	50.1 ± 4.5 (53.6)	50.0 ± 5.0 (53.6)
RP	*48.2 ± 6.8 (48.5)	48.7 ± 5.3 (48.5)	47.7 ± 8.1 (48.5)
BP	50.8 ± 7.6 (51.8)	50.5 ± 6.8 (51.8)	51.2 ± 8.5 (51.8)
GH	*47.8 ± 7.5 (50.7)	*47.7 ± 6.5 (50.7)	47.8 ± 8.5 (50.7)
VT	49.6 ± 6.5 (54.5)	48.4 ± 5.7 (45.3)	51.0 ± 7.1 (54.5)
SF	**46.2 ± 8.3 (45.2)	*46.9 ± 7.2 (45.2)	*45.5 ± 9.4 (45.2)
RE	*48.3 ± 6.4 (49.1)	48.0 ± 6.5 (49.1)	48.6 ± 6.5 (49.1)
MH	**47.3 ± 6.5 (45.0)	*46.8 ± 7.5 (45.0)	*47.8 ± 5.4 (50.3)
PCS	49.0 ± 6.7 (49.1)	49.2 ± 5.4 (49.0)	48.9 ± 7.9 (50.0)
MCS	***46.1 ± 6.6 (46.5)	**45.7 ± 7.1 (46.6)	**46.6 ± 6.0 (46.5)

Data are expressed as mean ±SD with median in the parentheses. One-sample t test was used for comparison between Japanese norms and scores of CD or UC patients. The asterisk denotes the significant difference (*P > .05; **P > .01; ***P > .001).

3.3. QOL Assessment. In Table 3 is shown the eight subscales and two summary scores of SF-8 in subjects with IBD patients. Since data are expressed as the deviation values normalized by the Japanese normative values, the value "50" correponds to Japanese norm. Subscales such as RP, GH, SF, MH, and MCS were significantly lower than the Japanese norms.

Table 3 shows the comparison between CD and UC subjects. There were no significant differences in the eight subscales and two summary scores except for lower VT in CD patients than in those with UC.

3.4. Correlations between PCS/MCS Scores and Clinical Characteristics, Biochemical Markers, and Nutritients Intakes. We analyzed the correlation between these summary scores and biochemical indices, fat intake expressed as the percentage energy intake from fat of total energy, fat intake (% energy) (Table 4). Fat intake (% energy) was significantly correlated with MCS in CD patients. There was significant but weak, correlation between PCS and serum albumin and MCS and BMI in UC patients. In the whole subjects, BMI was significantly correlated with PCS, and fat intake (% energy) was associated with MCS.

3.5. Multiple Regression Analysis for Variable Associated with PCS/MCS Scores. Then multiple regression analyses were done to study the determinant(s) of the subjects' PCS and MCS (Table 5). Variables included in the analysis were types of disease (CD/UC), BMI, serum concentrations of Alb, and fat intake (% energy). BMI was the significant predictor of PCS score (βcoefficient 0.29, P = .023) whereas fat intake was the only significant determinant of MCS score (βcoefficient 0.29, P = .027).

4. Discussion

Recently, various questionnaires have been developed for QOL evaluation, both generic and disease targeted [10]. Generic ones, by their definition, only consist of questions related to the subjects' general status and do not include the questions related to the features which are specific to a certain disease. Therefore, they are applicable to such studies as comparing the impact on QOL by various diseases or even to the evaluation of healthy subjects. In contrast, disease-targeted ones include items specific to a certain disease. They can be more sensitive than the generic ones in detecting the QOL impairment closely related to a certain disease state but are not applicable to the evaluation of patients with other diseases. Various disease-targeted questionnaires have been developed for IBD subjects; the most well known of which would be IBDQ (inflammatory bowel disease questionnaire) including many items related to the patients' gastroenterological problems [11]. Since the purpose of our current work was to study the effects of nutritional therapy on the patients' QOL, we considered it more appropriate to evaluate the patients' QOL using the generic questionnaire.

SF-36 is one of the most commonly used generic questionnaires, and SF-8, used in this study, is the shortened one. Eight subscales, two summary scores are obtained, and expressed as the deviation values, which are normalized by the nations' normative value. Many previous papers on the QOL of IBD patients using SF-36 seem to have handled the data improperly [2, 4]. For example, Bernklev and Andersson expressed their data as the 0–100 scale scores [2, 4], which

TABLE 4: Correlations between PCS/MCS scale scores and clinical characteristics, biochemical markers, and fat intake as proportion of total energy intake.

		IBD (n = 64)		CD (n = 33)		UC (n = 31)	
		PCS	MCS	PCS	MCS	PCS	MCS
Diseaae duration (y)	r	0.012	−0.175	0.070	−0.221	−0.085	−0.066
Body mass index (kg/m^2)	r	0.261*	0.088	0.144	−0.075	0.248	0.415*
C-reactive protein (g/dl)	r	−0.083	0.075	−0.058	0.196	−0.116	−0.045
Albumin (g/dl)	r	0.235	0.082	0.092	0.064	0.424*	0.059
Total cholesterol (mg/dl)	r	0.033	0.196	−0.132	0.169	0.174	0.249
Fat intake(% energy)	r	0.175	0.287*	0.146	0.458***	0.238	0.109

The asterisk denotes the value is significant correlation (*P < .05, **P < .01, ***P < .001) by Pearson's correlation or Spearman's correlation.

TABLE 5: Multiple regression analyses for the predictor(s) of PCS and MCS scores in IBD patients.

	PCS score		MCS score	
	r^2 = 0.086	P = .023	r^2 = 0.081	P = .027
	β	P	β	P
CD/UC (1;CD, 2;UC)	−0.141	.283	−0.059	.657
BMI	0.293	.023	0.069	.594
Alb	0.141	.309	0.024	.855
Fat intake (% total energy)	0.121	.347	0.285	.027

Abbreviations are as follow: β for β coefficient and P for P value. Determinants of independent predictors for PCS/MCS scores were analyzed by multivariate analysis with stepwise method. Variables included were CD/UC, BMI, serum albumin concentration, and fat intake (% total energy)

can be misleading [12]. In the present paper, data were analyzed according to the authorized instruction.

In this study, subscales such as RP, GH, SF, RE, MH, and MCS were significantly lower than the Japanese norms. Decreased RP in face of normal PF is conceivable considering that the patients do not have severe physical impairment but have some limitation in their daily activities by reasons such as the bowel habit problem. Impaired SF would be also conceivable from the similar viewpoint. As a whole, patients with IBD seem to have decreased QOL especially in the mental aspects.

Then, we have analyzed variables associated with PCS and MCS. There were substantial differences in the objective clinical features of patients with CD and UC. For example, CD patients had longer disease duration and lower nutritional status than those of UC subjects. Nevertheless, there were no significant differences in 7 out of 8 dimensions between the two conditions. Namely, QOL which represents the patients' subjective evaluation of their health states seems to be impaired in both CD and UC patients.

Then, we have studied the determinants for PCS and MCS. PCS score was correlated with indices representing their nutritional status such as BMI (r = 0.261, P < .05) and albumin with marginal significance (r = 0.235, P = .066). In contrast, none of these factors were significantly correlated with MCS. Thus, it was considered unlikely that disease activities or other clinical features alone could account for the impaired mental aspects of QOL in these subjects. The association of QOL with mental aspects of the subjects has been previously reported. Boye et al. reported that neuroticism was a significant predictor for mental and vitality subscales of SF-36 in IBD patients using multiple regression analyses controlled for gender, age, and clinical disease activity [13]. Martin also reported that QOL was not closely correlated with the clinical features in CD patients [14]. These results, together with our current findings, suggest that mental aspects can more strongly affect QOL than clinical ones in IBD patients.

Theoretically, it is well known that the QOL scores in subjects with disabilities are higher than those anticipated from their objective physical impairment (disability paradox) [15]. This phenomenon is because subjects with long-term disabilities change their internal standard and make the adaptation to their actual status (response shift) [16].

Next, we have made a hypothesis that nutrients intake such as fat restriction may contribute to the impairment of mental aspects of QOL in these subjects. Although CD patients had lower fat intake than UC subjects, fat intake (% energy) of the whole subjects was significantly lower than those of the NNS-J.

Then, we have analyzed the association between these summary scores and their fat intake (% energy). Fat intake (% energy) was significantly associated with MCS, but not with PCS in patients with IBD. When CD and UC patients were separately analyzed, the correlation coefficients was almost the same, but not statistically significant anymore, probably due to the smaller number of study subjects. We then have performed the multivariate analysis. Of the various factors included for analysis types of disease (CD/UC), BMI, serum albumin, fat intake (% energy), BMI, and fat intake (% energy) were the only significant determinants of PCS and MCS, respectively. Since many IBD patients are young, they are quite likely to favor foods rich in fat. Nevertheless, fat

restriction is the common practice in the nutritional therapy for IBD. It is quite conceivable that fat restriction impairs the mental and social aspects of QOL, and enteral nutrition will make the matter even worse. Of interest, but not apparently compatible with our findings, is the report by Kuriyama et al. They reported that enteral nutrition improved the health-related quality of life of Crohn's disease patients with long-term disease duration, and enteral nutrition was an independent factor for bowel symptoms and systemic symptoms [17]. In their study, IBDQ was employed for the assessment of QOL, which is an IBD-targeted questionnaire with many items related to the patients' gastroenterological problems. Thus it is likely that only the physical aspects of QOL were detected, and mental aspects were overlooked in their study.

Two additional considerations might be added to the current finding: decreased QOL in IBD patients and its association with fat restriction. First, considering the response shift, actual detrimental effect of fat restriction on the mental aspects of QOL might be even greater. Second, the adaptation process seems to be only partial. Chronic pain is known to be associated with response shift [18]. However, the association of fat restriction with impaired mental aspects of QOL was obvious in the current study. Since food intake is one of the most fundamental requirements, it is likely that subjects with fat restriction cannot easily adapt to a situation with long-term fat-restricted diet.

In conclusion, fat restriction exerts undesirable effects on IBD patients in two different ways: decreased intestinal absorption of fat-soluble substances such as vitamin D and K and impaired QOL especially in the mental aspects.

Conflict of interests

None of the authors have any conflict of interests.

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Molecular Genetics and Metabolism 102 (2011) 453–460

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

GCKR mutations in Japanese families with clustered type 2 diabetes

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ARTICLE INFO

Article history:
Received 15 December 2010
Accepted 15 December 2010
Available online 21 December 2010

Keywords:
Genetic susceptibility
Linkage analysis
MODY
HNF1A
GCKR

ABSTRACT

Objective: The aim was to investigate the genetic background of familial clustering of type 2 diabetes.
Subjects and methods: We recruited Japanese families with a 3-generation history of diabetes. Genome-wide linkage analysis was performed assuming an autosomal dominant model. Genes in the linkage region were computationally prioritized using Endeavour. We sequenced the candidate genes, and the frequencies of detected nucleotide changes were then examined in normoglycemic controls.
Results: To exclude known genetic factors, we sequenced 6 maturity onset diabetes of the young (MODY) genes in 10 familial cases. Because we detected a MODY3 mutation *HNF1A* R583G in one case, we excluded this case from further investigation. Linkage analysis revealed a significant linkage region on 2p25-22 (LOD score = 3.47) for 4 families. The 23.6-Mb linkage region contained 106 genes. Those genes were scored by computational prioritization. Eleven genes, i.e., top 10% of 106 genes, were selected and considered primary candidates. Considering their functions, we eliminated 3 well characterized genes and finally sequenced 8 genes. *GCKR* ranked highly in the computational prioritization. Mutations (minor allele frequency less than 1%) in exons and the promoter of *GCKR* were found in index cases of the families (3 of 18 alleles) more frequently than in controls (0 of 36 alleles, P = 0.033). In one pedigree with 9 affected members, the mutation *GCKR* g.6859C>G was concordant with affection status. No mutation in other 7 genes that ranked highly in the prioritization was concordant with affection status in families.
Conclusions: We propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes. The family based approach seems to be complementary with a large population study.

1. Introduction

The national survey in 2007 reported that 8.9 million people suffer from diabetes in Japan [1]. Most of these have type 2 diabetes, and the number of such patients has increased continuously. Both genetic and environmental factors play important roles in the pathogenesis of type 2 diabetes [2].

To elucidate the genetic factors underlying the pathogenesis of type 2 diabetes in the Japanese population, several genome-wide linkage analyses in Japanese sib-pairs have been performed [3–5]. Linkage to 11p13–p12 is consistently implicated in these studies [5]. Recent successes with genome-wide association analyses in the Japanese population have revealed a susceptibility variant in *KCNQ1* located at 11p15.5 [6,7], a locus not far from the region suggested in linkage analyses. The association of susceptibility loci including *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8*, and *HHEX* with diabetes has been established in Caucasian populations and replicated in the Japanese population [8]. However, the loci identified in association studies have uniformly small effect sizes, and can explain only a small portion of the genetic background of diabetes in the Japanese population. Approaches other than sib-pair linkage analyses and association analyses may therefore be required to elucidate a greater aspect of the genetic background of type 2 diabetes.

In the present study, we used a family-based approach, because high degrees of familial clustering can raise the relative risk and provide better insight to novel loci of larger effect size [9]. Familial clustering of diabetes is well known, the typical example being MODY [10]. On the other hand, in most families in Japan, familial clustering cannot be attributed to mutations of the 6 known MODY genes [10], and genetic predisposition in such families has not been ascertained.

We recruited families having a 3-generation history of diabetes and performed genome-wide linkage analysis. We selected candidate genes in the linked chromosomal region and searched for rare and

Abbreviations: GAD, glutamic acid decarboxylase; GCKR, glucokinase regulator; HLOD, heterogeneity logarithm of the odds; HNF4α, Hepatocyte Nuclear Factor 4α; LOD, logarithm of the odds; MAF, minor allele frequency; MODY, maturity onset diabetes of the young; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

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doi:10.1016/j.ymgme.2010.12.009

common nucleotide changes in the genes in familial cases and unaffected controls.

2. Material and methods

2.1. Families and additional index cases

We recruited patients from collaborating hospitals in Japan who had diabetes with a 3-generation family history, which is suggestive of autosomal dominant mode of inheritance [11]. If ≥2 family members with diabetes were alive and donated DNA, the families were regarded as suitable subjects for the present study. Families including members with positive GAD (glutamic acid decarboxylase) antibody were excluded from the study. Four families met these criteria and were included in the linkage analysis (Fig. 1). Affected status of the participants was determined in two ways. First, if participants had been diagnosed with diabetes and treated with oral hypoglycemic agents or insulin injection, they were regarded as affected. Second, if participants had not been treated with oral hypoglycemic agents or insulin injection, they underwent HbA1c (Hemoglobin A_{1c}) measurement for screening of impaired glucose tolerance. The value for HbA1c is estimated as an NGSP (US National Glycohemoglobin Standardization Program) equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS, Japanese Diabetes Society) (%) + 0.4%, considering the relational expression of HbA1c (JDS)(%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [12]. If their HbA1c levels were ≥6.0%, they were also regarded as affected. HbA1c ≥6.0% is the level defined as possible diabetes mellitus in the 2007 survey of the Ministry of Labor, Health and Welfare of Japan [1]. In addition to these subjects, 6 index cases from other families with a 3-generation history of diabetes were included in the study (Supplementary Fig. 1). In these families, although we confirmed the affected status of some of the family members, DNA samples were available only for the index cases but not for other family members. Together with the 4 index cases from the families included in the linkage analysis, a total of 10 unrelated cases with a 3-generation history of diabetes were available for DNA sequencing. The clinical features of family members and additional index cases are shown in Table 1.

2.2. Normoglycemic controls

An annual medical check-up program was performed in Nyukawa district of Takayama City, Japan. Nine-hundred ninety local residents (430 men, 560 women) were recruited in the program and consented to donate their DNA. From 2002 to 2007, participants underwent physical examination and blood tests including fasting plasma glucose and HbA1c every year. We selected normoglycemic controls from the

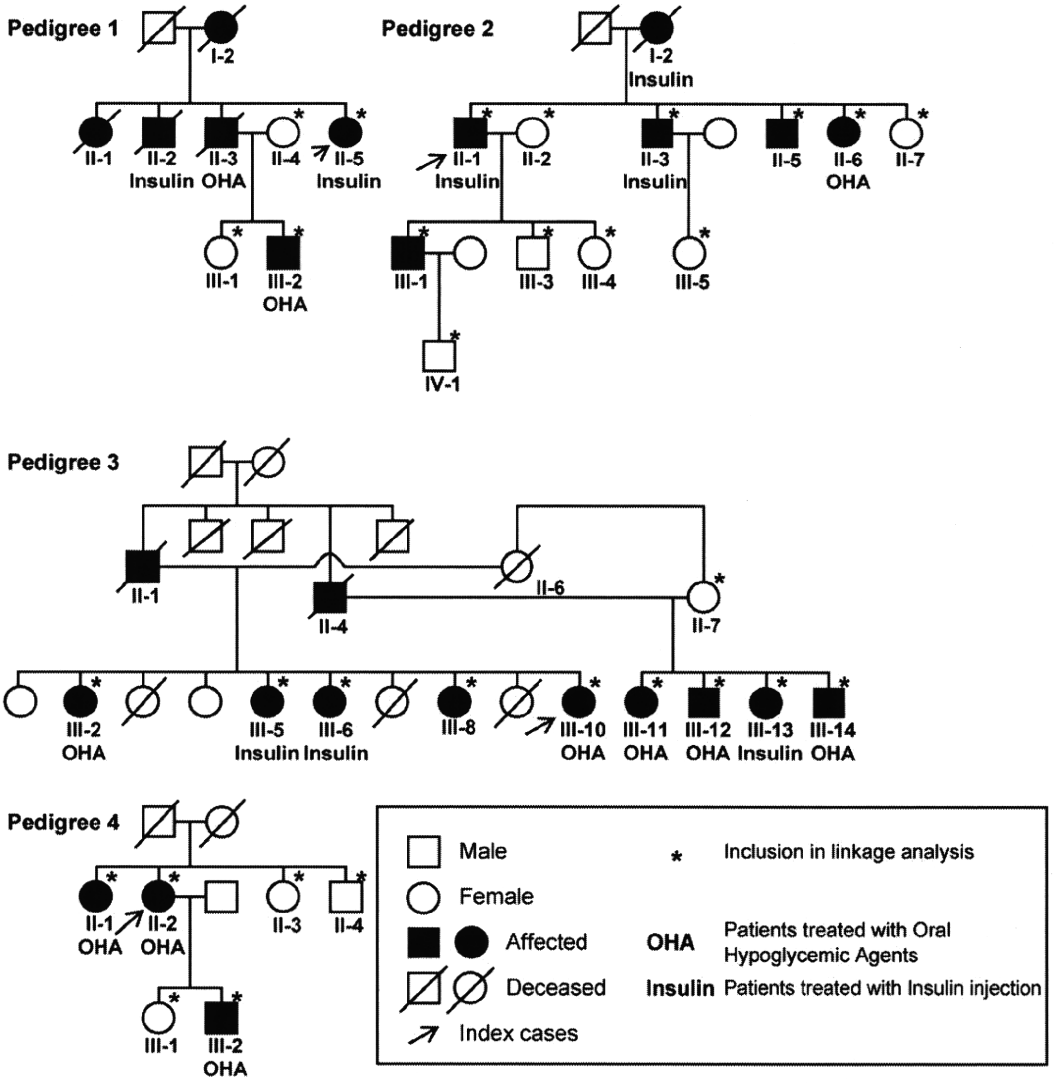

Fig. 1. Four pedigrees with familial aggregated diabetes mellitus.

Table 1
Characteristics of family members and additional index cases.

	ID	Current age	Sex	BMI	HbA1c (%)	Age when diagnosed (diagnosis)	Current therapy
Pedigree 1	II-4	70	F	16.2	5.0		
	II-5	71	F	22.5	10.6	60 (DM)	Insulin 66 U/d
	III-1	40	F	21.9	5.4		
	III-2	37	M	26.0	6.9	20 (DM)	Insulin
Pedigree 2	II-1	79	M	19.2	7.5	50 (DM)	Insulin 25 U/d
	II-2	77	F	18.6	5.6		
	II-3	76	M	17.9	7.2	45 (DM)	Insulin
	II-5	74	M	18.2	6.0	64 (IGT)	Diet
	II-6	71	F	18.4	6.6	N/A (DM)	Oral drug
	II-7	68	F	19.9	5.9		
	III-1	53	M	24.2	6.0	53 (IGT)	Diet
	III-3	51	M	20.4	5.6		
	III-4	47	F	19.3	5.2		
	III-5	46	F	19.6	4.9		
	IV-1	23	M	19.9	5.6		
Pedigree 3	II-7	92	F	22.3	5.9		
	III-2	77	F	23.9	9.3	30 (DM)	Oral drug
	III-5	72	F	22.0	8.1	60 (DM)	Insulin 16 U/d
	III-6	69	F	19.8	8.0	65 (DM)	Insulin 16 U/d
	III-8	66	F	19.1	6.5	64 (IGT)	Diet
	III-10	59	F	19.3	10.2	57 (DM)	Oral drug
	III-11	67	F	20.4	6.9	62 (DM)	Oral drug
	III-12	66	M	21.1	N/A	57 (DM)	Oral drug
	III-13	64	F	20.0	6.6	25 (DM)	Insulin
	III-14	62	M	20.2	10.3	50 (DM)	Oral drug
Pedigree 4	II-1	76	F	28.2	6.7	60 (DM)	Oral drug
	II-2	73	F	25.1	6.4	50 (DM)	Oral drug
	II-3	67	F	19.0	5.5		
	II-4	64	M	N/A	5.4		
	III-1	52	F	20.4	5.3		
	III-2	50	M	20.8	6.2	35 (DM)	Oral drug
Additional index cases	1	57	M	25.7	7.1	30 (DM)	Oral drug
	2	47	F	22.9	10.0	36 (DM)	Insulin 20 U/d
	3	68	F	19.7	7.1	45 (DM)	Insulin 19 U/d
	4	60	F	24.7	10.4	40 (DM)	Insulin 51 U/d
	5	60	F	28.0	9.7	50 (DM)	Insulin 8 U/d
	6	54	F	34.5	9.1	40 (DM)	Insulin

BMI: body mass index, DM: diabetes mellitus, IGT: impaired glucose tolerance.

participants in the cohort. Subjects defined as normoglycemic controls had the following characteristics: HbA1c <6.0% and fasting plasma glucose <5.5 mmol/l during 5-year follow-up span, and age ≥55. The number of subjects that satisfied the definition was 206 (81 men, 125 women).

2.3. Genotyping family members

Genomic DNA was extracted from blood samples with a QIAamp DNA Blood Mini Kit (Qiagen Inc). PCR amplification from genomic DNA was performed with fluorescence-labeled (6-FAM, HEX, NED) and tailed primers. PCR primers to analyze microsatellite markers comprised an approximately 10 cM human index map (ABI Prism Linkage Mapping Set Version 2.5: 382 markers for 22 autosomes), and other microsatellite fine markers were designed according to information from the UniSTS map. PCR reactions were carried out in 7.5 μl with 50 ng genomic DNA, using AmpliTaq Gold DNA Polymerase (Applied Biosystems) in a 2-step amplification program. DNA fragments were analyzed on an Applied Biosystems 3130 Genetic Analyzer. Genotyping errors and inconsistent relationships were checked with the use of GENEHUNTER (version 2.1) software [13]. If the results of genotyping were missed or ambiguous, we treated them as an unknown genotype in the linkage analysis. The rate of genotyping failure was 0.057% (7/11842).

2.4. Linkage and haplotype analyses

Both affected and unaffected family members were included in the linkage analysis. Participants with HbA1c level <6.0% were considered unaffected if the age was ≥55 and unknown if the age was <55, considering the assumed age-dependent penetrance of diabetes. The purpose of including members assigned as unknown was to increase the accuracy of haplotype estimation in affected members, although inclusion did not increase the statistical power. Multipoint parametric analyses for autosomes were run using GENEHUNTER assuming an autosomal dominant model [13]. Because locus heterogeneity could be associated with diabetes, LOD (log of the odds) score and HLOD (heterogeneity LOD) score were calculated. The disease allele frequency was set at 0.00001 and a phenocopy frequency of 0.00001 was assumed. Population allele frequencies for each microsatellite marker were assigned equal portions for individual alleles. We used a 2-stage design: first, all chromosomal regions were screened by genotyping at an approximately 10 cM density (screening), and the regions where LOD scores were highest were considered potentially interesting. Second, these regions were further finely mapped at approximately 1- to 2-cM densities (fine mapping). Regions where LOD scores were above 3.3, a level corresponding to genome-wide significance [9], were considered linkage regions. Haplotypes were constructed with the GENEHUNTER program.

2.5. Prioritization of candidate genes

The 23.6-Mb linkage region on chromosome 2p25-22 contained 106 genes annotated in Ensemble genome browser (http://www.ensembl.org). The genes were computationally prioritized using Endeavour (http://www.esat.kuleuven.be/endeavour/) [14]. We selected 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) as training genes because a dominant mode of inheritance

was assumed in the highly clustered families in linkage analysis. We adopted all databases available in Endeavour, which prioritized glucokinase regulator (*GCKR*) at the first rank.

2.6. Sequencing

We directly sequenced the coding exons of 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) in the 10 index cases. We sequenced *GCKR* including all exons found in the National Center for Biotechnology Information (NCBI) Evidence Viewer (http://www.ncbi.nlm.nih.gov) and the 2-kb promoter region in the index cases from families and in control subjects. We also selected other 7 genes that are highly prioritized within the 11th rank (10.3%) in the linkage region using Endeavour excluding 3 genes with known metabolic functions unrelated to glucose metabolism (Supplementary Table 1). We sequenced the entire coding exons of the 7 genes in the index cases from families included in the linkage analysis. Forward and reverse PCR primers for each exon were selected in an intronic sequence 50 bp away from the intron/exon boundaries and primers to amplify the *GCKR* promoter region were also selected. Sequencing primer data for *GCKR* is shown in Supplementary Table 2. PCR products were run on 2% agarose gel, and the appropriate bands were excised and then purified with the use of the QIAquick Gel Extraction Kit (Qiagen). Sequencing results were analyzed on an ABI Prism 3130 Avant DNA sequencer (Applied Biosystems). Any nucleotide changes identified in sequencing were searched for SNPs (single nucleotide polymorphisms) in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

2.7. Genotyping SNPs

If minor allele frequencies (MAF) of nucleotide changes identified in sequencing were unregistered in the HapMap JPT database on dbSNP as of April 2010 and the minor allele appeared in <2 of all subjects, MAF was determined in the expanded population. We defined mutation as MAF <1% [15]. To determine whether each nucleotide change was a mutation or not, we genotyped 105 normoglycemic controls randomly selected from the cohort (Supplementary Table 3), because genotyping of 210 normal chromosomes is necessary to achieve 80% power to detect a polymorphism present in 1% of the population [16]. The PCR-RFLP (restriction fragment length polymorphism) method for *HNF1A* R583G, *GCKR* g.-689G>A, *GCKR* g.-299G>A, *GCKR* E252K and *FOSL2* R198H and Taqman method for *GCKR* g.6859C>G were used.

2.8. Statistical analysis

Frequencies of mutations (MAF<1%) and common nucleotide changes (MAF≥1%) identified in *GCKR* sequencing in the index cases and in normoglycemic controls were compared by the Fisher exact test with SAS software (version 8.2).

2.9. Ethics

The methods used in this study were approved by the Ethics Committee of the Kyoto University Institutional Review Board, and approved written informed consent was obtained from each participant.

3. Results

3.1. Characteristics of family members

Four families with a 3-generation history of diabetes were enrolled in this study (Fig. 1, Table 1). Every family included no less than 1 member that had been diagnosed with diabetes before the age of 50. Sixteen members (6 men, 10 women) had previously been diagnosed with diabetes. Thirteen out of the 16 members with diabetes were lean (BMI<25). Six members were treated with insulin and another 10 members were treated with oral hypoglycemic agents. Twelve family members who had not been diagnosed with diabetes underwent HbA1c measurement and 3 of them had HbA1c level ≥6.0%. These 3 members had already been diagnosed with impaired glucose tolerance before this study and were included as affected members in the study.

3.2. Exclusion of MODY gene mutations in the index cases

For the 10 index cases, we performed direct sequencing in entire coding exons of the MODY genes. The detected missense SNPs were *HNF1A* I27L (rs1169288), *HNF1A* S487N (rs2464196), *HNF1A* R583G, and *HNF4A* T117I (rs1800961) (Supplementary Table 4). *HNF1A* R583G is a mutation that is reported to cause MODY [17], thus we excluded the carrier of the mutation (additional index case #6, Table 1) from further investigation. *HNF1A* I27L and *HNF1A* S487N are common in the general population (MAF=0.386 and 0.341, respectively in HapMap-JPT). *HNF4A* T117I was associated with late-onset type 2 diabetes but it was not the cause of MODY in a previous report [18].

3.3. Linkage analysis

A total of 30 members (19 affected members) from 4 families were included in the linkage analysis, assuming an autosomal dominant model. The genome-wide linkage results in the screening are shown in Fig. 2. Regions of potential interest by multipoint LOD and HLOD scores were observed on chromosomes 2p24 and 7q34. After fine mapping, 2p25-22 was revealed to be a significant linkage region (Fig. 3, LOD and HLOD=3.47) while the region on 7q34 was discarded. The size of the region with positive HLOD score was 23.6 Mb (D2S2199–D2S2230). In the region, a haplotype segregated in affected and unaffected members in the pedigrees 1, 2, and 3, but not in the pedigree 4.

3.4. Candidate genes

We searched candidate genes in the implicated linkage region by applying a gene prioritization approach implemented in Endeavour software. We selected 6 MODY genes as training genes. The 2 top-ranked genes were glucokinase regulatory protein (*GCKR*) and nuclear receptor coactivator 1 (*NCOA1*). *GCKR* ranked high in prioritization using gene–gene interaction databases (first rank in 5 out of 7 interaction databases), mainly because the interaction of glucokinase and glucokinase regulatory protein has been demonstrated in previous studies [19,20]. *NCOA1* also ranked high in prioritization using gene–gene interaction databases (second rank in 2 out of 7 interaction databases), because nuclear receptor coactivator 1 has been reported to interact with HNF4α (Hepatocyte Nuclear Factor 4α) as a coactivator [21]. Together with *GCKR* and *NCOA1*, genes that are highly prioritized within the 11th rank (10.3% of annotated genes) were considered candidate genes except 3 genes with well-characterized metabolic functions unrelated to glucose metabolism (Supplementary Table 1).

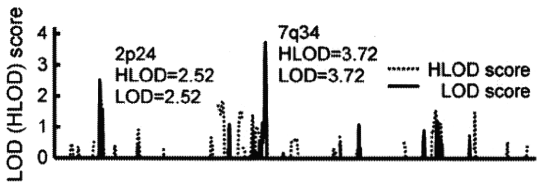

Fig. 2. Multipoint HLOD and LOD scores in genome-wide linkage analysis for 4 pedigrees.

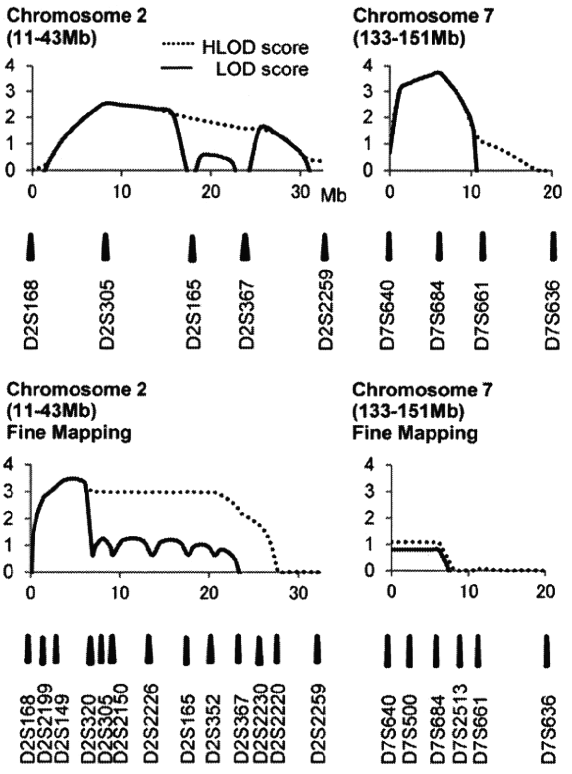

Fig. 3. Multipoint HLOD and LOD scores in fine mapping of D2S168–D2S2259 and D7S640–D7S636.

3.5. Direct sequencing in GCKR and other candidate genes

We performed direct sequencing in exons and the 2-kb promoter region of *GCKR*. Sequencing was performed in 9 index cases from families and in 18 normoglycemic controls in parallel. The 18 control subjects were randomly selected from 206 normoglycemic controls (Supplementary Table 3). Detected sequence changes in the 9 index cases and 18 controls are shown in Table 2. Five nucleotide changes (g.-959 Insertion AATGTTG, E66E, E77G, g.9709G>A, and L446P) were considered to be common variants, because the minor allele was found in not less than 2 subjects out of a total of 27 case and control subjects. To determine whether or not each of the other nucleotide changes (g.-689G>A, g.-299G>A, E252K and g.6859C>G) was a mutation (MAF<1%), genotyping was performed in a total of 105 normoglycemic controls. g.-689G>A, g.-299G>A and g.6859C>G were not detected in the 105 controls, and were regarded as mutations, while E252K was detected in 4 controls out of 105 (MAF = 1.9%) and was regarded as a common change. The number of alleles having mutations was thus significantly larger in the index cases from families than in the controls (3/18 alleles vs. 0/36 alleles, P = 0.033, Fisher exact test).

We performed direct sequencing in the entire coding exons of other 7 candidate genes in index cases from 4 families. One misssense mutation *FOSL2* R198H (MAF = 0.004 in normoglycemic controls) was detected. No other mutations were detected in other 6 genes (Supplementary Table 5).

3.6. Segregation of the mutations with the phenotype in pedigrees

In index cases from the 4 families included in the linkage analysis, 3 sequence changes of *GCKR* were detected (g.-959 Insertion AATGTTG, g.6859C>G and L446P). We tested the segregation of *GCKR* g.6859C>G, a mutation detected in pedigree 3, with the phenotype in the pedigree. Another 2 changes (*GCKR* g.-959 Insertion AATGTG and *GCKR* L446P) were commonly detected in controls (3/36 alleles and 11/36 alleles respectively). In pedigree 3, *GCKR* g.6859C>G was detected in all 9 affected members, but was not detected in the unaffected member (II-7). We performed linkage analysis and haplotype construction in 2p25-22 using the *GCKR* g.6859 genotype together with the microsatellite markers. The parametric multipoint LOD score for pedigree 3 was 2.67 at the *GCKR* g.6859 locus. Haplotype analysis revealed that all affected individuals in pedigree 3 shared a disease haplotype within D2S2199–D2S2230, which includes *GCKR* g.6859G (Fig. 4). In pedigree 3, another sequence change, *GCKR* L446P, was detected, but *GCKR* L446P did not co-segregate with the disease. Haplotype analysis revealed that the minor allele of *GCKR* L446P (g.11169C) resided on a different haplotype than *GCKR* g.6859G in affected subjects III-11, 12, 13, 14 (Fig. 4).

We tested the segregation of *FOSL2* R198H, a mutation detected in pedigree 4, with the phenotype. *FOSL2* R198H was detected in 2 affected subjects (II-2, II-22) but not detected in one subject (II-1).

4. Discussion and conclusions

Recent progress in genome-wide association studies has identified tens of type 2 diabetes susceptibility genes. Even so, only a small

Table 2
Mutations and common nucleotide changes in exons and the promoter of *GCKR* in 9 index cases in families and in 18 controls.

Position	Change	Description	Effect	Detected number of alleles				p^a	Minor allele frequency [MAF]
				Index cases from families (n = 9)		Controls (n = 18)			
				Major	Minor	Major	Minor		
Mutations (MAF<1%)									
Promoter	g.-689G>A			17	1	36	0	0.33	0.000[b]
Promoter	g.-299G>A			17	1	36	0	0.33	0.000[b]
Exon 9	g.6859C>G	Noncoding exon		17	1	36	0	0.33	0.000[b]
Total				15	3	36	0	0.033	
Common changes									
Promoter	g.-959 insAATGTTG			16	2	33	3	1.00	N/D
Exon 2	g.468G>A	Synonymous	E66E	17	1	35	1	1.00	N/D
Exon 3	g.671A>G	Missense	E77G	17	1	33	3	1.00	0.024[c]
Exon 10	g.8817G>A	Missense	E252K	18	0	35	1	1.00	0.019[b]
Exon 11	g.9709G>A	Noncoding exon		17	1	33	3	1.00	0.123[c]
Exon 14	g.11169T>C	Missense	L446P	8	10	25	11	0.087	0.467[c]

GenBank accession no. NT_022184.15.
[a] Fisher exact test.
[b] Frequency in 105 normoglycemic controls.
[c] Frequency in HapMap-JPT.

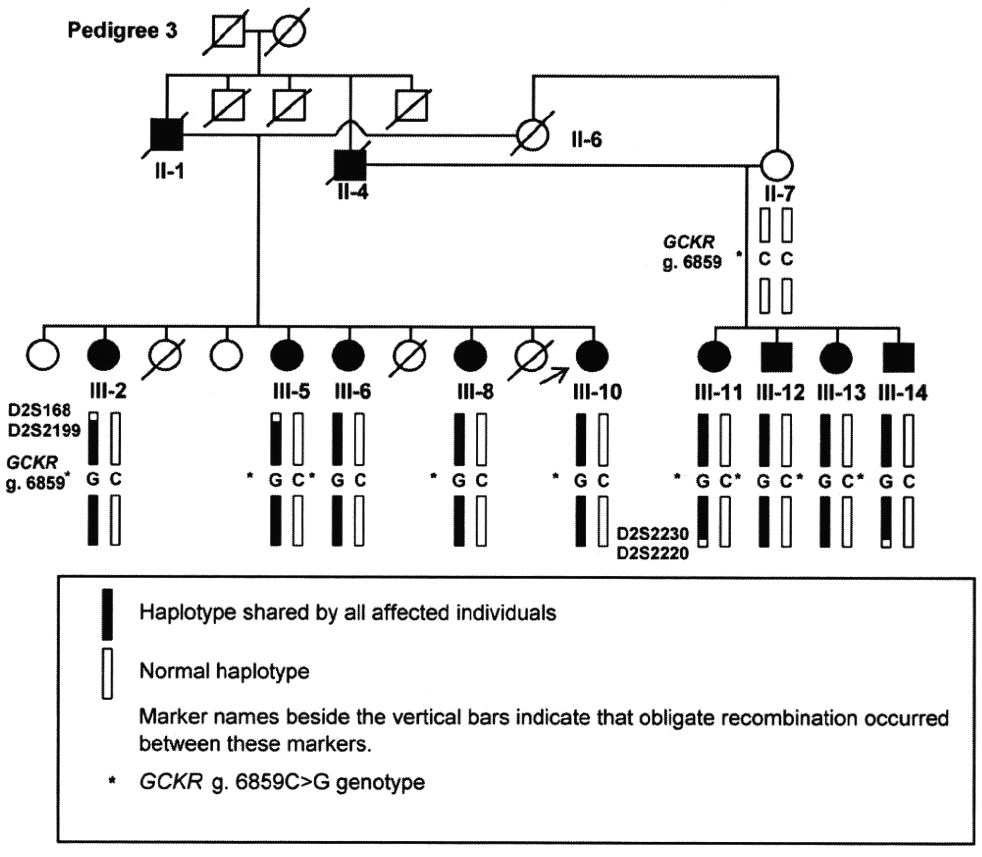

Fig. 4. Haplotype analysis in the D2S168–D2S2259 region and the *GCKR* g.6859C>G genotype for pedigree 3.

portion of the genetic background of diabetes has been explained in the Japanese population. The loci identified in association studies have only very small effect sizes. We hypothesized that rare disease variants with larger effect sizes remain to be discovered that may explain a greater part of the genetic background. Family-based linkage study is an important alternative for the identification of rare disease variants. Indeed, studies with large families with highly clustered diabetes have revealed important mutations involved in MODY and other dominantly inherited diabetes, including a *KCNJ11* mutation [22]. We therefore recruited families with a 3-generation history of diabetes. The validity of our strategy was strengthened by the fact that one case out of the 10 index cases recruited in our study carried a previously reported rare disease variant *HNF1A* R583G.

Our family analysis revealed a significant linkage region on chromosome 2p25-22 that has not been reported in previous Japanese sib-pair analyses [3–5]. Because our approach was based on a higher degree of familial clustering than sib-pair analyses, the linkage region suggested in the present study might well go undetected in sib-pair analyses that include an admixture of sib-pairs with both low and high degrees of familial clustering. In the present study, we conducted a computational approach targeting the linkage region on chromosome 2p25-22. One hundred and six known genes were present in this linkage region. Prioritization of the candidate gene was possible by integrating the information available from multiple publicly available databases [14]. *GCKR* and other 7 gens ranked high in the prioritization, and were selected as candidate genes.

GCKR regulates glucokinase (GCK), the first glycolytic enzyme, in liver. *GCKR*-null mice exhibit elevated postprandial glucose [19]. Adenoviral-mediated overexpression of *GCKR* in mouse liver increases GCK activity and lowers fasting blood glucose. It was suggested that GCKR, a competitive inhibitor of GCK activity, also has a paradoxical role in extending GCK half-life by stabilizing the enzyme [20]. If so, diminished expression of GCKR in human might cause decreased GCK activity in liver and lead to impaired liver glucose uptake, which suggests the *GCKR* mutation as a possible cause of the disease in linked families.

We sequenced entire exons and the 2-kb promoter region of *GCKR* in 9 index 3-generation cases and in 18 control subjects. The rare variants were significantly more frequent in index cases from families than in control subjects. In addition, exonic rare variant g.6859C>G in pedigree 3, which was not detected in 105 control subjects, was clearly segregated in all 9 affected members in pedigree 3. Previous reports have shown the association of common *GCKR* variants with fasting plasma glucose, glucose level after glucose challenge, and diabetes risk in various ethnic groups [23–30]. In Japanese population, a common variant *GCKR* rs780094 is associated with fasting glucose and diabetes risk [27,30]. Our family study suggests the effect of rare *GCKR* variants on diabetes susceptibility that has not been revealed by previous association studies. A recent study has shown the excess of rare *GCKR* variants in individuals with hypertriglyceridemia [31], which supports our idea that rare *GCKR* mutations also affect the diabetes susceptibility.

On the other hand, the only one mutation in other 7 highly prioritized genes was *FOSL2* R198H and it did not co-segregate with the phenotype in the pedigree. Therefore, we tentatively eliminate the possibility that these genes are involved in familial clustering of diabetes patients in the current pedigrees.

Our study has several limitations. First is the large size (23.6 Mb) of the linkage region. Only 4 families could be included in the linkage analysis because we limited the cohort to 3-generation families with ≥2 affected members who donated DNA. Further efforts to recruit large families are needed to narrow down the linkage region. Second, because the *GCKR* g.6859C>G mutation was in a non-coding exon, confirming the relevance of the mutation as the cause of the disease is difficult. Investigation of the effect of the mutation in human liver, where GCKR is predominantly expressed [32], is required, but liver specimens of family

members are currently unavailable. Although we tried to determine the mRNA level in peripheral blood of family members, GCKR mRNA was only barely detectable with the RT-PCR method (data not shown), so comparison of the GCKR mRNA level between affected and unaffected members was not possible. We speculate that the g.6859C>G mutation might affect GCKR function in liver through mRNA transcription or splicing processes [33]. *GCKR* g.-689G>A and g.-299G>A mutations located in the promoter also might affect the expression of GCKR, but TRANSFAC database [34] expected no binding sites of transcription factors at the two promoter mutations.

In conclusion, with systematic investigation we propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes. A family-based approach may be a promising strategy to elucidate the complex genetic background of common diseases including type 2 diabetes.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2010.12.009.

Acknowledgments

This work was supported by a grant for Research on Human Genome Tailor-Made from the Ministry of Health, Labor, and Welfare of Japan, a grant for Intractable Disease Research Program from the Ministry of Health, Labor, and Welfare of Japan, Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Cooperation, and by Kyoto University Global COE Program "Center for Frontier Medicine".

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Biochemical and Biophysical Research Communications 404 (2011) 115–120

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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The effect of gastric inhibitory polypeptide on intestinal glucose absorption and intestinal motility in mice

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ARTICLE INFO

Article history:
Received 21 October 2010
Available online 21 November 2010

Keywords:
GIP
Glucose absorption
Intestine

ABSTRACT

Gastric inhibitory polypeptide (GIP) is released from the small intestine upon meal ingestion and increases insulin secretion from pancreatic β cells. Although the GIP receptor is known to be expressed in small intestine, the effects of GIP in small intestine are not fully understood. This study was designed to clarify the effect of GIP on intestinal glucose absorption and intestinal motility. Intestinal glucose absorption *in vivo* was measured by single-pass perfusion method. Incorporation of [^{14}C]-glucose into everted jejunal rings *in vitro* was used to evaluate the effect of GIP on sodium-glucose co-transporter (SGLT). Motility of small intestine was measured by intestinal transit after oral administration of a non-absorbed marker. Intraperitoneal administration of GIP inhibited glucose absorption in wild-type mice in a concentration-dependent manner, showing maximum decrease at the dosage of 50 nmol/kg body weight. In glucagon-like-peptide-1 (GLP-1) receptor-deficient mice, GIP inhibited glucose absorption as in wild-type mice. *In vitro* examination of [^{14}C]-glucose uptake revealed that 100 nM GIP did not change SGLT-dependent glucose uptake in wild-type mice. After intraperitoneal administration of GIP (50 nmol/kg body weight), small intestinal transit was inhibited to 40% in both wild-type and GLP-1 receptor-deficient mice. Furthermore, a somatostatin receptor antagonist, cyclosomatostatin, reduced the inhibitory effect of GIP on both intestinal transit and glucose absorption in wild-type mice. These results demonstrate that exogenous GIP inhibits intestinal glucose absorption by reducing intestinal motility through a somatostatin-mediated pathway rather than through a GLP-1-mediated pathway.

1. Introduction

Gastric inhibitory polypeptide (GIP), also called glucose-dependent insulinotropic polypeptide, is an incretin of 42-amino-acid polypeptide synthesized by K cells of the duodenum and small intestine [1]. We previously generated GIP receptor-deficient mice (GIPR$^{-/-}$ mice) and showed that GIPR$^{-/-}$ mice have higher blood glucose levels as well as impaired initial insulin response after oral glucose load [2]. Thus, early insulin secretion stimulated by GIP plays an important role in glucose tolerance after oral glucose load.

While GIP receptor mRNA was reported to be present in rat gut [3], the role of the GIP receptor in the gut has not been fully clarified. In this *in vivo* study, we investigated the effect of exogenous GIP on intestinal glucose absorption in mice using the intestinal perfusion method. We investigated the effect of exogenous GIP on SGLT-dependent glucose uptake *in vitro* by using the everted jejunal ring method. Because intestinal motility and absorption are positively related [4,5], we investigated the effect of exogenous GIP on gastrointestinal motility by non-absorbed marker method. Since SST secretion has been reported to be stimulated by GIP and to prolong intestinal motility, we also investigated the involvement of SST in the inhibitory effect of exogenous GIP on both intestinal transit and intestinal glucose absorption by using somatostatin receptor antagonist. Our results demonstrate that exogenous GIP inhibits intestinal glucose absorption by reducing intestinal motility through a somatostatin-mediated pathway rather than through a GLP-1-mediated pathway.

Abbreviations: GIP, Gastric inhibitory polypeptide; GLP-1, glucagon-like-peptide-1; SST, somatostatin; SGLT, sodium-glucose co-transporter; CSS, cyclosomatostatin.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.077