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Longer *HSD11B2* CA-repeat in impaired glucose tolerance and type 2 diabetes

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Abstract. Type 2 11β-hydroxysteroid dehydrogenase encoded by the *HSD11B2* gene converts cortisol to inactive cortisone, and alteration in this enzymatic activity might affect glucose homeostasis by affecting circulating levels or tissue availability of glucocorticoids. We investigated the association of *HSD11B2* variant with glucose homeostasis. Subjects with normal glucose tolerance (n=585), impaired glucose tolerance (n=202) and type 2 diabetes (n=355) were genotyped for a highly polymorphic CA-repeat polymorphism in the first intron of *HSD11B2*. Allele and genotype frequencies differed between normal and impaired glucose tolerance (P = 0.0014 and 0.0407, respectively; 4 degree of freedom) or type 2 diabetes (P = 0.0053 and 0.0078), with significant linear trends between the repeat length and the phenotype fraction. In normal subjects, total CA–repeat length was negatively correlated with fasting insulin and HOMA-β. Thus, subjects having more CA repeats are susceptible to developing abnormal glucose tolerance, whereas normal subjects carrying more CA repeats appeared to have frugal characteristics in insulin secretion.

Key words: 11β-hydroxysteroid dehydrogenase type 2, Cortisol, Cortisone, Insulin, Pancreas

TYPE 2 11β-hydroxysteroid dehydrogenase (HSD11B2), encoded by the *HSD11B2* gene, is expressed in mineralocorticoid target tissues and irreversibly converts cortisol to inactive cortisone [1]. Since impaired activity of this enzyme leads to apparent mineralocorticoid excess [2, 3], HSD11B2 is established as a protective mechanism for the mineralocorticoid receptor [4]. Additionally, HSD11B2 is expressed in tissues other than mineralocorticoid target organs [5, 6]. It may play additional roles as a local modulator of glucocorticoid action, comparable to the role of the type 1 isozyme (HSD11B1) as an enhancer of glucocorticoid action. Whereas the total secretion of glucocorticoids from the adrenal cortex is regulated by ACTH, levels of active and inactive glucocorticoids in systemic circulation are regulated by renal HSD11B2 [7], hepatic HSD11B1 and by adrenal HSD11B2 under certain conditions [8]. Thus, alteration in HSD11B2 activity might affect tissue availability or circulating levels of glucocorticoids.

Among a wide range of glucocorticoid actions, the effects of glucocorticoids on glucose homeostasis have been well documented. Glucocorticoids increase hepatic glucose production and decrease glucose uptake and insulin sensitivity in peripheral tissues [9]. Further, they directly inhibit insulin release from pancreatic β cells [10]. Type 2 diabetes mellitus is a complex and heterogeneous disease that may involve dysregulation of any or all of these processes. Thus, genetic heterogeneity in *HSD11B2* gene might affect glucose homeostasis or the development of type 2 diabetes through modulating circulating levels or intracellular availability of active glucocorticoids. We searched unsuc-

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cessfully for common polymorphisms in the proximal promoter and coding region of *HSD11B2* in Japanese subjects. However, CA-repeat length polymorphism in the first intron was reported to be highly polymorphic [11, 12], and the latter study showed that longer CA repeats resulted in lower *HSD11B2* expression based on *in vitro* transfection experiments in human or rabbit kidney cortical collecting duct cells [12]. According to the hypothesis of a possible effect of longer CA-repeat alleles on the metabolic condition, we genotyped normal subjects and subjects with abnormal glucose homeostasis for this CA-repeat length polymorphism and analyzed its association with type 2 diabetes. Further, functional associations of this polymorphism with putative intermediate phenotypes and clinical parameters of glucose metabolism were examined in normal subjects.

Subjects and Methods

Subjects

The study population was recruited from those coming for medical check-ups at the Japan Self-defense Force Gifu Hospital or the Matsunami General Hospital and patients at the endocrine/diabetes clinic of Gifu University Hospital. The diagnosis of diabetes was based on the criteria of American Diabetes Association [13]. We divided the subjects into 3 groups based on glucose tolerance: normal, impaired glucose tolerance (IGT) and diabetes mellitus. Subjects with impaired fasting glucose as well as type 1 diabetes were not included in the present study. Subjects were 585 normal subjects, 202 subjects with IGT and 355 patients with type 2 diabetes. Body height and weight were recorded with subjects wearing light clothing for computation of body mass index (BMI; kg/m^2). Body surface area (BSA) was calculated according to the following formulae; BSA (m^2)=Body weight$^{0.425}$ (kg)×Body height$^{0.725}$ (cm)×0.007184. All subjects gave informed consent, and the ethical committee of Gifu University School of Medicine approved the study protocol.

Biochemical and Hormonal Assessments

Blood samples were drawn after a 12 hr overnight fast, and the fasting plasma glucose (FPG) and HbA1c level were determined by standard laboratory techniques. HbA1c values are presented as adjusted in line with National Glycohemoglobin Standardization Program (NGSP) values by applying the formula HbA1c (%) = HbA1c (JDS) + 0.4% [14]. Fasting plasma immunoreactive insulin (FIRI) was measured with a double antibody radioimmunoassay. Parameters of insulin resistance (HOMA-R) or β-cell function (HOMA-β) were calculated from FPG and FIRI using the following formulae: HOMA-R (expressed in $mol \cdot \mu U \cdot L^{-2}$) = FPG x FIRI / 22.5 and HOMA-β (expressed as a percentage) = FIRI x 20 / (FPG-3.5) [15].

Clinical parameters including putative intermediate phenotypes such as the ratio of plasma or urinary free cortisone (E) to cortisol (F) were evaluated in normal subjects at the Japan Self-defense Force Gifu Hospital. Peripheral blood samples to examine plasma levels of E and F were drawn from 181 fasting normal subjects early in the morning, yielding the calculation of the ratio of plasma E to F (E/F) which should represent the net activity of HSD11B1 and HSD11B2 in the whole body. 24-hour urine samples were collected from 154 normal male subjects in plain plastic containers without preservatives to determine the urinary excretion of free F and E. The ratio of urinary E/F represents renal HSD11B2 activity. Plasma and urinary free F and E were measured by enzyme linked immunosorbent assays as previously described [7, 8]. Since urinary excretion of E and F are strongly correlated with body surface area, 24 hr excretion values of urinary free F and E were corrected for body surface area ($/m^2$).

Microsatellite typing

Genomic DNA was extracted from peripheral leukocytes by use of a standard phenol-chloroform method and stored at 4˚C thereafter. For genotyping the CA-repeat polymorphism in the first intron of *HSD11B2*, segments were amplified from 20-50ng of each DNA sample in 10 μL of a premix buffer (buffer D, Epicentre Technologies, Madison, WI) with 0.5 U of Ex Taq DNA polymerase (TaKaRa Co., Osaka, Japan) and 4% dimethyl sulfoxide by addition of 5 pmoles each of the sense primer 5'-TCA GGT CAG AAC TGG GAG GTC-3' and the antisense primer 5'-TGG AGA GGG AGG CAA GCA TAT-3', which were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase (TaKaRa Co.). After initial denaturation at 96˚C for 2 min, 28 cycles of 96˚C for 20 sec, 63˚C for 20 sec, and 72˚C for 22 sec, were employed. Each reaction was subjected to electrophoresis in 6% polyacrylamide gels containing 30% formamide along with appropriate size standards. Gels were dried and exposed to Hyperfilm MP at -20˚C.

Statistical analysis

Frequency data were analyzed by chi-square tests, and $P < 0.05$ was considered significant. The association between the CA repeat length and diabetes risk were further analyzed by trend chi-squared tests (Cochran-Armitage test for trend). SAS 9.2 for Windows (SAS Institute Inc., Cary, NC) was used in subsequent analysis. Correlations between CA-repeat length and biochemical parameters were tested by a Spearman's rank correlation analysis after adjustment for age, sex and BMI. A General Linear Models Procedure was used to compare anthropometric and biochemical data in the genotype groups of each polymorphism after adjustment for age, sex and BMI, and results were expressed as least squared (LS) means ± LS-SE where appropriate. All *P* values were adjusted for the number of tests performed by Bonferroni's correction.

Results

Association of CA-repeat length with IGT and type 2 diabetes

The characteristics of all studied subjects are summarized in Table 1. We confirmed 11 alleles (15-25 CA repeats), yielding a heterozygosity of 52.3% on the study population. The allele distributions were shown in Table 2. There were significant differences in allele frequencies between normal subjects and IGT (χ^2 = 17.7; P = 0.0014; 4 degrees of freedom when the 5 most frequent alleles were included) or type 2 diabetes (χ^2 = 14.8; P = 0.0053). Regarding the genotype frequencies, Table 3 depicted distribution of 10 frequent genotypes from all 41 genotypes detected. When the 5 most frequent genotypes were included, there were significant differences in genotype frequencies between

Table 1 Clinical characteristics of the study population

	Normal	IGT	Type 2 diabetes
N	585	202	355
Male/female	539 / 46	178 / 24	211 / 144
Age (yr)	51.2 ± 6.7	52.6 ± 7.6	56.6 ± 10.9**
BMI (kg/m^2)	23.1 ± 2.7	24.4 ± 2.8*	24.9 ± 4.7*
HbA1c (%)	5.5 ± 0.4	5.7 ± 0.5	8.3 ± 1.9*
FPG (mmol/L)	5.2 ± 0.4	5.7 ± 0.6*	8.3 ± 2.6*
FIRI (pmol/L)	35 ± 15	45 ± 23*	56 ± 49*
HOMA-R	1.35 ± 0.62	1.90 ± 1.05*	3.45 ± 3.87*
HOMA-β	69.1 ± 30.9	70.7 ± 36.6	52.0 ± 58.4*

Data are presented as mean ± SD. IGT, impaired glucose tolerance; BMI, body mass index; FPG, fasting plasma glucose; FIRI, fasting plasma immunoreactive insulin; Asterisk means significant *P* value after Bonferroni's correction (**, $P < 0.0034$; *, $P < 0.0167$ *vs*. normal).

Table 2 Allele distributions in normal subjects and subjects with abnormal glucose homeostasis

	Shorter Allele						Longer Allele					
Number of	Normal subjects		IGT		Type 2 diabetes		Normal subjects		IGT		Type 2 diabetes	
CA-repeat	n = 585	%	n = 202	%	n = 355	%	n = 585	%	n = 202	%	n = 355	%
15	6	1.0	2	1.0	1	0.3	0		0		0	
16	3	0.5	4	2.0	4	1.1	0		0		0	
17	96	16.4	27	13.4	46	13.0	11	1.9	3	1.5	6	1.7
18	25	4.3	6	3.0	17	4.8	4	0.7	1	0.5	4	1.1
19	89	15.2	20	9.9	34	9.6	15	2.6	0		4	1.1
20	318	54.4	123	60.9	213	60.0	386	66.0	112	55.5	211	59.4
21	41	7.0	20	9.9	33	9.3	115	19.7	62	30.7	90	25.4
22	6	1.0	0		7	2.0	36	6.2	19	9.4	25	7.0
23	0		0		0		13	2.2	4	2.0	11	3.1
24	1	0.2	0		0		4	0.7	1	0.5	2	0.6
25	0		0		0		1	0.2	0		2	0.6

IGT, impaired glucose tolerance. Frequencies are presented as percentage of total case number.

Table 3 Distribution of 10 frequent genotypes in normal subjects and subjects with abnormal glucose homeostasis

CA-repeat	Normal subjects		IGT		Type 2 diabetes	
Shorter-longer	n = 585	%	n = 202	%	n = 355	%
17-17	10	1.7	3	1.5	5	1.4
17-20*	68	11.6	17	8.4	22	6.2
17-21	8	1.4	6	3.0	9	2.5
18-20	18	3.1	2	1.0	11	3.1
19-20*	71	12.1	15	7.4	29	8.2
20-20*	225	38.5	75	37.1	146	41.1
20-21*	69	11.8	35	17.3	58	16.3
20-22	15	2.6	10	4.9	3	0.9
21-21*	28	4.8	15	7.5	18	5.1
21-22	9	1.5	4	2.0	9	2.5

IGT; impaired glucose tolerance. Frequencies are presented as percentage of total case number.
The asterisk denote the 5 most frequent genotypes.

Table 4 Frequency comparison of the number of alleles more than 21 CA repeats

	Normal subjects n=585	IGT n=202			Type 2 diabetes n=355		
	%	%	OR (95% CI)[a]	*P* value[a]	%	OR (95% CI)[a]	*P* value[a]
Number of alleles with length > 21 CA repeats							
0	71.1	57.4			63.4		
1	20.7	32.7	1.56 (1.20 ~ 2.04)	0.00104	25.4	1.38 (1.10 ~ 1.73)	0.00502
2	8.2	9.9			11.3		
Dominant model							
0	71.1	57.4	1.82 (1.31 ~ 2.54)	0.00034	63.4	1.42 (1.07 ~ 1.88)	0.01362
1+2	28.9	42.6			36.7		
Recessive model							
0+1	91.8	90.1	1.23 (0.71 ~ 2.13)	0.45953	88.7	1.42 (0.91 ~ 2.21)	0.1181
2	8.2	9.9			11.3		

Frequencies are presented as percentage. [a], *versus* normal subjects

normal subjects and IGT (χ^2 = 10.0; P =0.0407) or type 2 diabetes (χ^2 = 13.9; P = 0.0078). Specifically, there were more long alleles among IGT or type 2 diabetes compared with normal subjects. Significant linear trends between the repeat length and the phenotype fraction (against sum with normal subjects) were observed in IGT (χ^2 trend = 11.2; P = 0.0008; 4 degree of freedom) or in type 2 diabetes (χ^2 trend = 10.4; P = 0.0013) by allele. Similar trends were observed also by genotype in IGT (χ^2 trend = 8.2; P = 0.0043) or in type 2 diabetes (χ^2 trend = 13.9; P = 0.0078).

Based on the hypothesis that longer alleles were associated with metabolic condition, we analyzed the association with clinical phenotypes according to the number of alleles more than 21 CA repeats (the median length). As shown in Table 4, there were significant differences for this CA repeat length-related genotype distribution between normal subjects and IGT (P = 0.0010) or type 2 diabetes (P = 0.0050). There were also significant differences noted for the distribution of the dominant model (0 *versus* 1+2) between normal subjects and IGT (P = 0.0003) or type 2 diabetes (P = 0.0136). Thus, subjects with IGT and type 2 diabetes had longer CA-repeat length, compared with normal subjects, suggesting the association of CA-repeat length *per se* with genetic predisposition.

CA-repeat length and putative intermediate phenotypes or clinical parameters

In order to explore the effect of CA-repeat length on putative intermediate phenotypes and clinical indexes, we employed correlation testings. However, in normal

Table 5 Correlation between total CA-repeat length (sum of two alleles) and possible intermediate phenotypes or indexes of glucose metabolism

		Sum of two alleles	
Parameter	n	*r*	*P* value
Urinary E and F	154		
urinary E		0.090	n.s.
urinary F		0.082	n.s.
urinary E / F ratio		-0.057	n.s.
Plasma E and F	181		
plasma E		0.063	n.s.
plasma F		-0.037	n.s.
plasma E / F ratio		0.084	n.s.
Glucose homeostasis	553		
FPG		-0.006	n.s.
FIRI		-0.087	0.042
HOMA-R		-0.082	n.s.
HOMA-β		-0.097	0.022

Data were derived from normal subjects without medication and evaluated by a Spearman's rank correlation analysis after adjustment for age and BMI. *P* values greater than 0.05 were presented as n.s.

Table 6 Differences in clinical parameters by HSD11B2 CA-repeat length related genotype

Parameter	unit	Number of alleles with length ≥ 21 CA repeats		
		0	1	2
Urinary E and F		n = 101	n = 31	n = 22
urinary E	nmol/day/m^2	68.4 ± 3.3	70.4 ± 5.9	64.1 ± 7.0
urinary F	nmol/day/m^2	73.9 ± 4.8	68.1 ± 8.7	63.1 ± 10.3
urinary E / F ratio		1.07 ± 0.04	1.22 ± 0.08	1.11 ± 0.09
Plasma E and F		n = 128	n = 34	n = 19
plasma E	nmol/L	63.6 ± 1.3	67.9 ± 2.5	69.9 ± 3.4
plasma F	nmol/L	252.2 ± 7.1	265.5 ± 13.9	233.6 ± 18.7
plasma E / F ratio		0.268 ± 0.007	0.279 ± 0.015	0.314 ± 0.019
Glucose homeostasis		n = 392	n = 114	n = 47
FPG	mmol/L	5.23 ± 0.02	5.17 ± 0.04	5.19 ± 0.06
FIRI	pmol/L	35.6 ± 0.7	33.1 ± 1.3	29.4 ± 2.0**
HOMA-R		1.39 ± 0.03	1.27 ± 0.05	1.14 ± 0.08**
HOMA-β		70.3 ± 1.5	68.4 ± 2.7	60.7 ± 4.2

Above data were derived from normal subjects without medication. Data were presented as LSmeans ± LS-SE. Asterisk means significant *P* value after Bonferroni's correction (**, $P < 0.0034$ *vs*. 0).
FPG; fasting plasma glucose, FIRI; fasting plasma immunoreactive insulin, E; cortisone, F; cortisol

subjects, no significant linear correlations were detected between total CA-repeat length and urinary or plasma E, F, and E/F (Table 5). As to clinical parameters reflecting glucose metabolism, total CA-repeat length was negatively correlated with FIRI (Spearman's $r = -0.087$, $P = 0.0042$, n = 553) and HOMA- β ($r = -0.097$, $P = 0.022$) but not with FPG ($r = -0.006$, $P = 0.882$) or HOMA- R ($r = -0.082$, $P = 0.055$).

In addition, when we divide these normal subjects by the CA-repeat length related genotype (0, 1 or 2 alleles of more than 21 CA repeats), subjects carrying 2 alleles had lower FIRI ($P = 0.0018$) and HOMA-R ($P = 0.0022$) compared with subjects carrying 0 (Table 6), but the difference in HOMA-β between subjects carrying 0 or 2 did not reach significance ($P = 0.0228$; greater than 0.01695, corresponding for $P = 0.05$).

Discussion

We confirmed the *HSD11B2* intronic CA-repeat to be highly polymorphic with 11 alleles in our population. A significant association was observed between the CA-repeat length and risk of impaired glucose tolerance or type 2 diabetes. Further, in normal subjects without medication, total CA-repeat length was negatively correlated with fasting plasma insulin levels and parameters of insulin secretion (HOMA-β), in other words β-cell function, and insulin levels and parameters of insulin resistance (HOMA-R) were lower in subjects carrying more CA repeats.

HSD11B2 gene has been implicated as a plausible candidate locus for sodium sensitive essential hypertension, but polymorphisms in the gene have not been consistently associated with hypertension [11, 12, 16-18]. A previous report described that longer CA repeat length was associated with lower *in vitro* HSD11B2 expression [12], and decreased renal HSD11B2 activity and/or expression should result in a longer half-life of cortisol. However, the present analysis in normal subjects without medication revealed no linear correlation between *HSD11B2* CA repeats/genotypes and 24hr urinary excretion of cortisol/cortisone or the ratio of urinary cortisone to cortisol, a more reliable marker of renal HSD11B2 activity. Such a discrepancy was noted in the same report [12] describing the association of rather shorter CA repeats with higher salt sensitivity in mean arterial pressure, although salt sensitive subjects did have lower *in vivo* HSD11B2 activity. Together with the present result, no conclusions can be drawn at this point about the associations between more CA repeats and decreased renal HSD11B2 activity/expression suggested by an *in vitro* mini-gene study [12].

In contrast to apparently negative results on the assumed role of HSD11B2 in hypertension, our present results suggest the unexpected connection of the *HSD11B2* intronic CA-repeat to the metabolic condition. Allele and genotype frequencies differed between normal subjects and impaired glucose torelance or type 2 diabetes. The association between the CA-repeat length and diabetes risk was also suggested by trend chi-squared analysis. The number of alleles more than 21 CA repeats was associated with impaired glucose tolerance and, to a lessor extent, with type 2 diabetes in a dominant fashion. A stronger association of longer CA-repeat with impaired glucose tolerance compared with type 2 diabetes may imply the role of HSD11B2 in the induction of abnormal glucose tolerance but not in the development of relative impairment of insulin action. Where is the site of the action other than the renal tissues? Considring the correlation of CA-repeat length with insulin levels in normal subjects, HSD11B2 may play a role in pancreatic islets as well as in peripheral insulin-target tissues.

In general, the HSD11B2 expression was known to be abundant in mineralocorticoid target tissues but not in liver, adipose tissue or muscles, except for a limited time during the fetal stage [6]. Glucocorticoids cause insulin resistance by antagonizing the insulin action as a representative of counter-regulatory hormones. Clinical features in Cushing's syndrome or during the administration of synthetic glucocorticoids are glucose intolerance, central obesity, hypertension and hypertriglyceridaemia, mimicking the metabolic syndrome [19]. In contrast to such a general understanding, different aspects of glucocorticoid action were described. *In vitro*, mouse beta cells secrete less insulin in the presence of glucocorticoids [10], and glucocorticoid receptor overexpression impairs insulin secretion *in vivo* [20]. The initial cloning of human *HSD11B2* described HSD11B2 transcripts in the pancreas [21], but HSD11B2 is not detected immunohistochemically in human islets or acinar cells [22]. However, we have detected HSD11B2 transcript and protein in rodent islets (Mune T, unpublished). In our normal subjects, fasting plasma insulin levels and a parameter of β-cell function (HOMA-β) were negatively correlated with total CA-repeat length, and plasma insulin levels and a measure of insulin resistance (HOMA-R) were lower in subjects carrying more CA repeats. One can hypothesize that pancreatic HSD11B2 locally regulates synthesis and secretion of insulin and/or glucagon within beta and/or alpha cells in an autocrine or a paracrine fashion under some circumstances. If longer CA-repeat length results in decreased expression as in the *in vitro* mini-gene study, the corresponding tissues should be exposed to higher cortisol, and insulin secretion will go down in pancreatic islet. Albeit hypothetical, the significance of CA-repeat length may differ between in normal subjects and in abnormal glucose tolerance. Whether such an association is involved in the etiology of insulin secretion failure in type 2 diabetes should await further studies.

Our study has some limitations. Essentially, circulating cortisol is just one of the intermediate metabolites and regulated by a fine-tuned hypothalamo-pitu-

itary-adrenal feedback mechanism. The clinical and endocrine data including putative intermediate phenotype were not enough for impaired glucose tolerance and type 2 diabetes to proceed to a detailed analysis. The observed association of the more or less repeat with phenotype may reflect a linkage-disequilibrium of an undetected functional mutation in an adjacent chromosome area with the more (or less) repeat polymorphism, and the more CA repeats may represent an ancient haplotype, in which the putative mutation was carried. Further, the other nearby linked genes or combinations of other components in the glucocorticoid pathway may be involved.

In conclusion, our data suggest that *HSD11B2* genotype influences diabetes risk. Functional associations of this polymorphism with putative intermediate phenotypes and clinical parameters of glucose metabolism should be elucidated in greater number of patients with type 2 diabetes.

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