Hypertension Research (2013) 36, 614–619

www.nature.com/hr

ORIGINAL ARTICLE

HSD11B2 CA-repeat and sodium balance

Tomoatsu Mune[1], Hiroyuki Morita[2], Nobuki Takada[3], Yoritsuna Yamamoto[3], Yukinori Isomura[4], Tetsuya Suwa[4], Jun Takeda[4], Perrin C White[5] and Kohei Kaku[1]

Type 2 11β-hydroxysteroid dehydrogenase encoded by the *HSD11B2* gene converts cortisol to inactive cortisone and thus protects the mineralocorticoid receptor from cortisol exposure. Impaired activity of this enzyme leads to mineralocorticoid excess, suggesting *HSD11B2* as a candidate locus for patients at risk of developing low renin or salt-sensitive essential hypertension. In the present study, we searched for frequent polymorphisms in 155 Japanese subjects but detected none in the proximal promoter or coding regions of *HSD11B2*. Following this result, we genotyped a highly polymorphic CA-repeat polymorphism within the first intron in 848 normotensive and 430 hypertensive Japanese patients, and we then analyzed its association with disease and clinical parameters. We confirmed 12 alleles (12, 15–25 CA repeats) in the population and found no significant difference in the distribution of the allele length between normotensive and hypertensive patients. In 174 normal subjects without medication, urinary cortisol excretion was higher in subjects with more CA repeats in the shorter allele, but the ratio of urinary cortisone to cortisol, a reliable marker of renal *HSD11B2* activity, did not differ. However, longer CA-repeat length was positively correlated with 24-h urinary sodium excretion, fractional sodium excretion and potassium clearance, and this observation was confirmed when the longer CA-repeat length was dichotomized. Thus, *HSD11B2* CA-repeat genotype is not associated with hypertension itself, but with renal sodium excretion, probably through salt intake/appetite.
Hypertension Research (2013) **36,** 614–619; doi:10.1038/hr.2013.13; published online 28 February 2013

Keywords: cortisol; human genetics; hypertension; metabolism; sodium appetite

INTRODUCTION

All of the monogenic hypertensions so far identified have been found in genes related to the mineralocorticoid pathway, suggesting its involvement in the etiology of essential hypertension. Encoded by the *HSD11B2* gene, 11β-hydroxysteroid dehydrogenase (11βHSD) type 2 converts cortisol to inactive cortisone, and thus protects the mineralocorticoid receptors in the distal nephron from saturation by physiological concentrations of cortisol.[1] Impaired activity of this enzyme causes sodium retention and potassium excretion, leading to congenital[2,3] or acquired[4] apparent mineralocorticoid excess. Recent experimental studies have revealed the link between reduced 11βHSD type 2 activities and salt sensitivity in the blood pressure (BP) of *hsd11b2* heterozygote mice[5,6] or hypertension associated with abnormal renal function in rats.[7,8] Thus, *HSD11B2* is a plausible candidate locus for the risk of developing essential hypertension, especially of the low renin or salt-sensitive type. A previous report described an association between a nearby microsatellite locus and essential hypertension in Afro-Americans.[9] Another Alu I (Glu178Glu, G534A) polymorphism within exon 3 was associated with end-stage renal disease but not with variations in BP.[10] Furthermore, a highly polymorphic CA-repeat polymorphism in the first intron was reported to be associated with salt sensitivity in human subjects,[11,12] and the latter report also showed a low *in vitro HSD11B2* expression in cultured cells transfected with mini-genes containing longer CA repeats.[12] Theoretically, lower expression should result in reduced renal 11βHSD type 2 activity and a longer cortisol half-life. However, higher salt sensitivity in mean arterial pressure was associated with shorter CA repeats, while salt-sensitive subjects presented with a lower *in vivo* enzymatic activity measured by urinary precursor/product ratios.[12] Thus, there was a discrepancy between *in vitro HSD11B2* expression and *in vivo* renal 11βHSD type 2 activities.

In the present study, we searched for frequent polymorphisms in Japanese subjects but detected none in the proximal promoter or coding regions of *HSD11B2*. Next, we genotyped an intronic CA-repeat polymorphism and analyzed its association with the origin of hypertension and the regulation of salt balance. The functional significance of the polymorphism with regard to clinical parameters, including the ratios of plasma and urinary free cortisone to cortisol and renal electrolyte handling, was evaluated in normal subjects.

METHODS

Subjects

The study population was randomly recruited from patients at our endocrine/hypertension clinic and those coming for a medical check-up at the Japan Self-defense Force Gifu Hospital. Subjects screened for possible *HSD11B2* polymorphisms included 5 patients with pseudoaldosteronism, 10 patients

[1]Division of Diabetes, Endocrinology and Metabolism, Kawasaki Medical School, Kurashiki, Japan; [2]Department of General Medicine, Graduate School of Medicine, Gifu University, Gifu, Japan; [3]Department of Medicine, Japan Self-defence Force Gifu Hospital, Kakamigahara, Japan; [4]Department of Diabetes and Endocrinology, Gifu University, Gifu, Japan and [5]Division of Pediatric Endocrinology, University of Texas Southwestern Medical Center, Dallas, TX, USA
Correspondence: Dr T Mune, Division of Diabetes, Endocrinology and Metabolism, Kawasaki Medical School, 577 Matsushima (Room 7346), Kurashiki, Okayama 701-0192, Japan.
E-mail: mune@med.kawasaki-m.ac.jp
Received 8 October 2012; revised 24 November 2012; accepted 13 December 2012; published online 28 February 2013

Table 1 Clinical characteristics of the study population

Characteristics	*Normotensives*	*Hypertensives*
Number	848	430
Age, years	49.5 ± 9.0	56.3 ± 10.3
BMI, kg m^{-2}	23.2 ± 2.8	24.6 ± 3.4
Systolic BP, mm Hg	117.7 ± 12.2	152.2 ± 16.4
Diastolic BP, mm Hg	73.3 ± 9.0	88.8 ± 11.5
Serum Na, mmol l^{-1}	141.9 ± 2.0	140.7 ± 2.6
Serum K, mmol l^{-1}	4.17 ± 0.30	4.13 ± 0.42
IGT/DM, %	14.9/15.2	14.4/50.7

Abbreviations: BMI, body mass index; BP, blood pressure; DM, diabetes mellitus; IGT, impaired glucose tolerance.
Data are presented as mean ± s.d.

with essential hypertension, 20 patients undergoing hemodialysis resulting from various pathological origins and 120 normal subjects.

The subjects for CA-repeat genotyping included a total of 848 normotensive and 430 hypertensive Japanese. Resting BP was measured using a mercury sphygmomanometer with the subject in a sitting position, and the mean of three readings taken 5 min apart was used. The criteria for normotension included BP $<$140/90 mm Hg without the presence of cardiovascular disease or the use of any antihypertensive therapy. Hypertension was defined as systolic BP $>$140 mm Hg and/or diastolic BP $>$90 mm Hg without treatment or taking any antihypertensive agents after essential hypertension diagnosis. Secondary hypertension was excluded by physical examination and biochemical and radiological investigation where appropriate. Body height and weight were recorded with subjects wearing light clothing to compute body mass index (kg m^{-2}). All subjects provided informed consent, and the ethical committee of the Gifu University School of Medicine approved the study protocol.

The clinical parameters of the subjects are shown in Table 1. Blood was taken into EDTA-2Na or heparin-Na containing tubes, and genomic DNA was extracted from peripheral leukocytes using a standard phenol–chloroform method and stored at 4 °C. Some DNA samples derived from heparinized blood were pretreated at 30 °C for 30 min with 1 U μg^{-1} DNA of heparinase I (Sigma Chemical, St Louis, MO, USA) to exclude heparin inhibition.[13]

Search for polymorphism in *HSD11B2*

To search for frequent polymorphisms in *HSD11B2*, we employed the non-radioisotopic PCR followed by single-strand conformational polymorphism (SSCP) and the RNase cleavage assay. In PCR-SSCP for each exon 2–5, 50 ng of genomic DNA was subjected to PCR as described previously,[2] and the reaction was electrophoresed in 0.5 × MDE gels (FMC Bio Products, Rockland, ME, USA) and visualized with silver staining. To screen polymorphisms in the region from the proximal promoter (nt −679) to 5′-intron 1 (PV) and the region from 3′-intron 1 to 3′-UTR (WZ), we employed the non-isotopic RNase cleavage assay. The 961-bp-long PV segments were amplified using a sense primer located in the promoter region (5′-TGT CCC AGG CAG GTT TTG TGG-3′; nucleotides (nt) −730 to −710) and an antisense primer in intron 1 (5′-CCC TCG AGC CTG GAG TCC-3′), followed by nestic PCR using a sense T7-PV primer (5′-TAA TAC GAC TCA CTA TAG GGG TGA GCG CGC CTT-3′; to nt −679) and an antisense SP6-PV primer (5′-ATT TAG GTG ACA CTA TAG GAG TCC CGC TCC-3′; from 5′-intron 1 + 18 nt). The supplied buffer was used at a concentration of 1.5 mM Mg^{2+} with 2.5 U ml^{-1} of PLATINUM pfx DNA polymerase (Gibco-BRL, Gaithersburg, MD, USA). After initial denaturation at 96 °C for 2 min, a manual hot-start at 80 °C was employed, followed by 24 cycles (1st PCR) and 26 cycles (nestic PCR) of 96 °C for 25 s and 68 °C annealing/extension for 90 s. The 1438-bp-long WZ segments were amplified using a sense primer in intron 1 (5′-CTG CTG GTG GCT TGG TTT G-3′) and an antisense primer in the 3′-untranslated region (5′-ATC GTA ATG CTG GGG GTT TTC-3′), followed by nestic PCR using a sense T7-WZ primer (5′-TAA TAC GAC TCA CTA TAG GGT GAT TCT GGG GTT GTC-3′; to 3′-intron 1 −54 nt) and an antisense SP6-WZ primer (5′-ATT TAG GTG ACA CTA TAG AAT GGC TGG GCC ATA GGT G-3′; from +9 nt from stop codon). The supplied buffer was used at a concentration of 1 mM Mg^{2+} with 5 U ml^{-1} of Pyrobest DNA polymerase (TaKaRa, Osaka, Japan). After initial denaturation at 96 °C for 2 min, a manual hot-start at 80 °C was employed, followed by 24 cycles (1st PCR) and 26 cycles (nestic PCR) of 96 °C for 15 s, 65 °C annealing for 20 s, and 72 °C extension for 90 s. The *in vitro* transcription of the PCR products, the subsequent hybridization and the RNases digestion were completed following the manufacturer's protocol for a MisMatch Detect II kit (Ambion, Austin, TX, USA). The screenings were examined in 40 normal subjects, 10 patients with essential hypertension and 5 patients with pseudoaldosteronism. In addition, several reported polymorphisms or mutations were explored in an additional 80 normal subjects and 20 patients under hemodialysis resulting from various pathological origins by PCR-RFLP. The restriction enzymes used were *Alu*I (TaKaRa) for Glu178Glu (reference SNP no. rs45483293),[10,14] *Bss*HII (TaKaRa) for Ala196Ala (rs5480),[15] *Bsm*AI (New England BioLabs, Beverly, MA, USA) for Ser180Phe (found in a Japanese patient with apparent mineralocorticoid excess)[16] and *Hha*I (TaKaRa) for Arg208His (rs28934592) within exon 3 and *Hha*I for Arg279Cys (rs28934594)[17] within exon 5. Among these four polymorphisms, the HapMap frequency for JPT is available only with Ala196Ala and is reported as 0.

Microsatellite typing

For genotyping the CA-repeat polymorphism in the first intron of *HSD11B2*, segments were amplified from 50 ng of each DNA sample by addition of 5 pmoles of a sense (5′-TCA GGT CAG AAC TGG GAG GTC-3′) and an antisense (5′-TGG AGA GGG AGG CAA GCA TAT-3′) primer end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase (TaKaRa) in 10 μl of a premix buffer D (Epicenter Technologies, Madison, WI, USA) with 0.5 U of Ex Taq DNA polymerase (TaKaRa) and 4% dimethyl sulfoxide. After initial denaturation at 96 °C for 2 min, 28 cycles of 96 °C for 20 s, 63 °C for 20 s, and 72 °C for 22 s were employed. Each reaction was subjected to electrophoresis in a 6% polyacrylamide gel containing 30% formamide along with appropriately sized standards. Gels were dried and exposed to hyperfilm MP at −20 °C.

Biochemical and hormonal assessments

A total of 174 normal male subjects from the Japan Self-Defense Force Gifu Hospital who were not taking medications were asked to collect 24-h urine samples. The samples were collected in plain plastic containers without preservatives to determine cortisol (F), cortisone (E), sodium, potassium and creatinine excretion. Peripheral blood samples were drawn from these subjects on the morning the urine collection began to examine serum electrolyte and creatinine concentrations. Early-morning peripheral blood samples were also obtained from 52 of the 174 above referenced subjects and from the other 153 normal fasting subjects to determine their plasma levels of F and E. Electrolytes and creatinine concentrations in urine and sera were measured by ion selective electrode and the Jaffe method, respectively. Potassium clearance (C_K) was calculated using the formula $C_K(\mathrm{ml\,min^{-1}}) = (U_K \times V)/S_K$, where U_K is the urine potassium concentration, S_K is the serum potassium concentration and V is the urine volume per minute. The fractional excretion of sodium (FENa) was calculated from the formula $\mathrm{FENa}\ (\%) = 100 \times (S_{Cr} \times U_{Na})/(S_{Na} \times U_{Cr})$, where S_{Cr} and U_{Cr} are the serum and urine creatinine concentrations, respectively, and S_{Na} and U_{Na} are the serum and urine sodium concentrations, respectively.

Plasma and urinary free F and E were measured by enzyme-linked immunosorbent assays as previously described,[18,19] yielding the ratio of urinary and plasma E to F (E/F), which reflects renal 11βHSD type 2 activity[20] and gross whole-body activity of 11βHSD type 1 and type 2, respectively. Briefly, 100 μl of plasma or 50 μl of urine was extracted with 4 ml of dichloromethane (the mean recovery rates were 85–90% for ^{3}H-F or ^{3}H-E), reconstituted in 50 μl of phosphate-buffered saline, and subjected to enzyme-linked immunosorbent assays. Sheep polyclonal anti-F and anti-E sera, kindly provided by Dr Celso Gomez-Sanchez (University of Mississippi), were used at final dilutions of 1: 30 000 and 1: 100 000, respectively, and the avidin–biotin–peroxidase method was employed. The cross-reactivities of anti-F and anti-E sera were 4.2% and 3.4% against E and F, respectively. The intra-assay and inter-assay coefficients of variation were as follows: F: 14.9 and 6.9%, E: 12.5

and 8.7%. All assays were completed in triplicate, and the mean determinations were used in all calculations.

Statistical analysis

Allele frequency data were analyzed using the χ^2-test, and SAS 9.2 for Windows (SAS Institute, Cary, NC, USA) was used in the subsequent analysis. Correlations between the CA-repeat length and anthropometric and biochemical phenotypes were tested using Spearman's rank correlation analysis. In addition, diastolic BP was included in the analysis of the urinary data after adjustment for age and body weight. The General Linear Models Procedure was used to compare anthropometric and biochemical data in the dichotomized genotype groups after similar adjustments, and the results are expressed as least squared (LS) means ± LS-s.e. where appropriate. A *P*-value of <0.05, corrected for multiple comparisons, was considered significant.

RESULTS

Screenings with the non-radioisotopic PCR-SSCP and RNase cleavage assay detected no polymorphisms in the coding regions or the proximal promoter region of *HSD11B2* in a total of 55 subjects examined (40 normal, 10 hypertensive and 5 pseudoaldosteronism). Detection of reported polymorphisms and mutations by PCR-RFLP was negative in an additional 80 normal and 20 hemodialytic Japanese subjects.

No association of CA-repeat length with hypertension

We confirmed 12 alleles (12, 15–25 CA repeats) in the population. As shown in Table 2, the distributions of the shorter alleles carried by each subject were bimodal. When the number of CA repeats in the shorter allele was dichotomized by the median length (20 CA repeats), the frequency of the shorter alleles with $\geqslant$20 CA repeats was 66.4% in normotensives and 66.3% in hypertensives. Similarly, when the number of CA repeats in the longer alleles carried by each subject was dichotomized by the median length for this allele (21 CA repeats), the frequency of longer alleles with $\geqslant$21 CA repeats was 32.5% in normotensives and 33.5% in hypertensives. When we divided the subjects based on the 7th JNC report, no differences were observed among normotension, prehypertension and hypertension stages 1 and 2 (data not shown). Thus, the distribution of allele length did not differ between normotensives and hypertensives.

CA-repeat length and putative intermediate phenotypes or clinical parameters

As there were significant negative linear correlations between diastolic BP and urinary excretion of E and F (Figure 1) in normal male subjects not taking medication, diastolic BP adjustment was included in the following analysis.

Considering a previous *in vitro* expression study,[12] the CA-repeat length in the shorter or longer allele might correlate negatively with renal 11βHSD type 2 activity. However, no linear correlation was detected between CA-repeat length and BP, serum sodium, serum potassium, plasma E, plasma F or plasma E/F (upper portion of Table 3, some data not shown). When the number of CA repeats in the shorter or the longer allele carried by each subject was dichotomized, no differences were observed in these variables (upper portion of Table 4).

In contrast to the results on circulating biochemical markers, significant correlations were observed between CA-repeat length and urinary measures of hormones or electrolytes. Regarding a putative intermediate phenotype, there was no linear correlation between CA-repeat length and urinary hormones (lower portion of Table 3). When the subjects were dichotomized by the median length, the urinary excretion of F ($P=0.003$) and, to a lesser extent, E ($P=0.044$) was higher in subjects with more CA repeats in their shorter alleles (lower portion of Table 4), but the urinary E/F ratio did not differ significantly between the groups. Notably, longer CA-repeat lengths had a positive linear correlation with urinary sodium excretion and FENa and 24 h potassium clearance (Table 3 and Figure 2). This observation was confirmed when the subjects were dichotomized according to the median CA-repeat length in the shorter or the longer allele (lower portion of Table 4).

Table 2 Allele distributions in 848 normotensives and 430 hypertensives

	Shorter allele				*Longer allele*			
Number of CA-repeat	*Normotensives*		*Hypertensives*		*Normotensives*		*Hypertensives*	
12	1	**0.0012**	0		0		0	
13	0		0		0		0	
14	0		0		0		0	
15	6	**0.0071**	4	**0.0093**	0		0	
16	7	**0.0083**	4	**0.0093**	0		0	
17	124	**0.1462**	71	**0.1651**	14	**0.0165**	7	**0.0163**
18	31	**0.0366**	18	**0.0419**	5	**0.0059**	5	**0.0116**
19	116	**0.1368**	48	**0.1116**	18	**0.0212**	6	**0.0140**
20	485	**0.5719**	240	**0.5581**	535	**0.6309**	268	**0.6233**
21	68	**0.0802**	37	**0.0860**	193	**0.2276**	94	**0.2186**
22	9	**0.0106**	8	**0.0186**	56	**0.0660**	37	**0.0860**
23	0		0		19	**0.0224**	10	**0.0233**
24	1	**0.0012**	0		6	**0.0071**	1	**0.0023**
25	0		0		2	**0.0024**	2	**0.0047**
χ^2 and *P*-values	**$\chi^2=0.02$, $P=0.968$**				**$\chi^2=0.12$, $P=0.735$**			

Frequencies are in boldface.

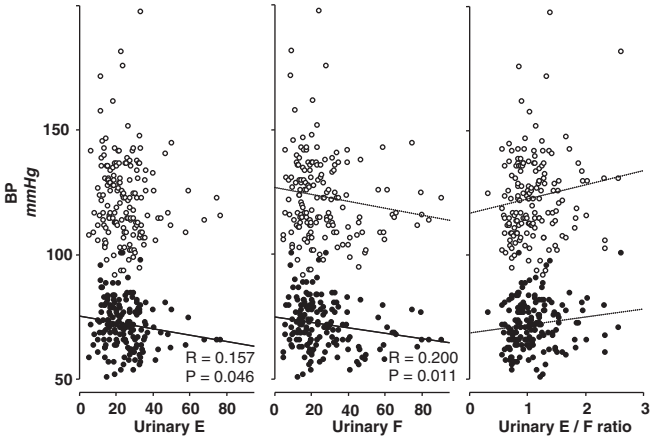

Figure 1 Correlation between blood pressures and 24-h urinary excretion of cortisone (E) and cortisol (F) or its ratio in subjects without medication. Open and closed circles denote systolic and diastolic blood pressures, respectively.

DISCUSSION

In the present study, none of the reported polymorphisms in the coding region of *HSD11B2* or any new polymorphisms were detected, consistent with a comprehensive study from Japan.[21] We confirmed that the *HSD11B2* intronic CA-repeat is highly polymorphic, with 12 alleles (12, 15–25 CA repeats) in the population. However, there were no significant differences in the distribution of the allele length

between normotensives and hypertensives, which supports previous linkage studies.[14,22]

No direct correlations were found between the CA-repeat length and the ratio of urinary free or plasma cortisone to cortisol (parameters of 11βHSD type 2 or type 1 and 2 activity). A lack of effect of this polymorphism on urinary steroid markers was also found by measuring free cortisol and cortisone[12] or tetrahydrometabolites of cortisol.[11] Upon dichotomizing the groups in our study, urinary cortisol excretion was higher in subjects with more CA repeats in their shorter alleles, which is consistent with lower *in vitro HSD11B2* expression in mini-gene constructs carrying more CA repeats, as has been previously reported.[12] However, the ratio of urinary free cortisone to cortisol, a better marker of *in vivo* renal 11βHSD type 2 activity,[20] did not differ upon dichotomization. As mentioned in the Introduction, the discrepancy between *in vitro* expression and *in vivo* renal activity was notable. The salt-sensitive group had a higher ratio of urinary cortisol metabolites ((THF + allo-THF)/THE)[11] or urinary free cortisol to cortisone,[12] whereas salt-sensitive subjects frequently showed a decreased length of the CA repeat that should have higher gene expression based on *in vitro* transfection studies using human or rabbit kidney cortical collecting duct cells.[12]

In our analysis of normal male subjects without medication, the CA-repeat length in the longer allele had positive correlations with 24 h urinary sodium excretion and FENa and potassium clearance. Such a positive correlation with potassium clearance suggests that the CA-repeat length in the longer allele is one of the genetic factors controlling individual renal mineralocorticoid action, but this significant correlation was lost upon inclusion of the urinary sodium excretion for adjustment (data not shown), indicating that this correlation was just secondary to the effect of the changes in urinary sodium excretion. Stronger positive correlations of CA-repeat length in the longer allele with urinary sodium excretion and FENa also suggest a predominant effect on renal sodium handling in this genotype. As urinary sodium excretion essentially depends on sodium intake, our results suggest that this CA-repeat polymorphism might be associated with mechanism(s) controlling habitual salt intake or, in other words, salt appetite. Recent studies in rodents[23,24] have revealed the coexpression of the mineralocorticoid receptor and 11βHSD type 2 in some neurons in the nucleus tractus solitarius. Moreover, aldosterone (and corticosterone) or sodium depletion activated these neurons to affect behavior and to drive sodium appetite.[23] The positive correlation of CA-repeat length with urinary sodium excretion in the present study could be explained by changes in *HSD11B2* expression within these aldosterone-sensitive nucleus tractus solitarius neurons. Thus, in addition to its classical role as an epithelial tissue-specific protector of the mineralocorticoid receptor mainly in the kidneys, 11βHSD type 2 can be involved in the regulation of sodium appetite through the non-epithelial mineralocorticoid action in the central nervous system, such as the nucleus tractus solitarius.

The present results might explain previously mentioned discrepancies. Subjects with short CA repeats are accustomed to low sodium intake, so that they might become sensitive to higher sodium

Table 3 Correlation between CA-repeat length and possible intermediate phenotypes

	Shorter allele		*Longer allele*	
Parameter	r	P-*value*	r	P-*value*
Plasma	***n*=60**	***n*=145**	***n*=142**	***n*=63**
E	−0.038	NS	0.026	NS
F	−0.069	NS	−0.038	NS
E/F ratio	0.071	NS	0.070	NS
Urine	***n*=68**	***n*=106**	***n*=113**	***n*=61**
Free E	0.094	NS	0.062	NS
Free F	0.118	NS	0.002	NS
E/F ratio	−0.094	NS	0.093	NS
Na	0.071	NS	0.188	0.014
K	0.062	NS	0.135	NS
FENa	0.061	NS	0.190	0.013
C_K	0.107	NS	0.179	0.019

Abbrevitions: C_K, potassium clearance; E, cortisone; F, cortisol; FENa, fractional excretion of sodium; NS, not significant.
Data were derived from normal male subjects without medication and evaluated by a Spearman's rank correlation analysis after adjustment for age, body weight and diastolic BP. *P*-values >0.05 were presented as NS.

Table 4 Differences in possible intermediate phenotypes by dichotomized groups by the median length

	Shorter allele			*Longer allele*		
Parameter, unit	*⩽19 repeats*	*20 repeats ⩾*	P-*value*	*⩽20 repeats*	*21 repeats ⩾*	P-*value*
Plasma	***n*=60**	***n*=145**		***n*=142**	***n*=63**	
E, nmol l^{-1}	68.9±2.1	67.5±1.3	NS	67.0±1.4	69.9±2.0	NS
F, nmol l^{-1}	271±12	259±8	NS	265±8	257±12	NS
E/F ratio	0.268±0.011	0.284±0.007	NS	0.273±0.007	0.292±0.011	NS
Urine	***n*=68**	***n*=106**		***n*=113**	***n*=61**	
Free E, nmol per day	106.8±6.5	123.6±5.2	0.044	116.9±5.1	117.2±6.9	NS
Free F, nmol per day	101.1±9.4	137.3±7.5	0.003	126.4±7.5	122.6±10.2	NS
E/F ratio	1.16±0.05	1.06±0.04	NS	1.06±0.04	1.17±0.06	NS
Na, mmol per day	118.3±5.5	132.1±4.4	NS	120.3±4.3	138.4±5.8	0.013
K, mmol per day	36.5±1.6	40.4±1.3	NS	37.6±1.2	41.1±1.7	NS
FENa, %	0.66±0.06	0.75±0.05	NS	0.65±0.05	0.83±0.06	0.017
C_k, ml min^{-1}	6.53±0.59	8.29±0.48	0.022	6.93±0.46	8.85±0.63	0.014

Abbreviations: E, cortisone; F, cortisol; FENa, fractional excretion of sodium; C_K, potassium clearance; NS, not significant.
Samples were derived from normal male subjects without medication. Data were evaluated by a general linear models procedure after adjustment for age, body weight and diastolic BP and presented as LS means±LS-s.e. *P*-values >0.05 were presented as NS.

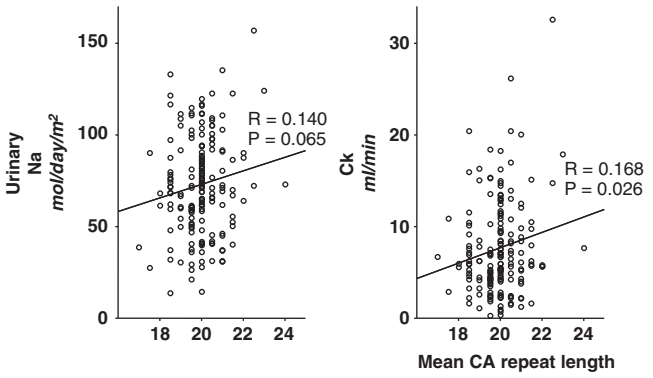

Figure 2 Representative linear correlations between mean CA-repeat length and 24-h urinary sodium excretion (left) or potassium clearance (C_K; right).

intake. Alternatively, considering that salt sensitivity in BP is greater in subjects with high BP and high renal sodium reabsorption,[25] one can even hypothesize that salt sensitivity merely reflects adaptability to individual salt habits. Whether the reported association of salt sensitivity of BP to longer alleles (which is contrary to the relative loss of this enzyme expression in the kidney) is related to the above effects on central mineralocorticoid action deserves elucidation.

As shown in Figure 1, urinary excretion of free cortisone and cortisol was negatively correlated with diastolic BP. In addition, the ratio of urinary free cortisone to cortisol, which is thought to be a reliable marker of renal type 2 11βHSD activity[20] and a putative intermediate phenotype, correlated positively with diastolic BP, suggesting negative regulation of *HSD11B2* expression via a pressor mechanism. There may be a physiological compensatory mechanism by which lower BP enhance the mineralocorticoid action of cortisol, at least in normal subjects.

Lengthening of cortisol half-life and increased urinary cortisol metabolites excretion were reported in some patients with essential hypertension,[26,27] and in one study that was based on the 'four-corners' approach, the impaired conversion of cortisol to inactive metabolites was reported in young men who were predisposed to having higher BP.[28] However, several reports cast doubt on the relationship of impaired renal 11βHSD type 2 activity to salt sensitivity, especially in normotensive subjects.[29,30] As the present analysis is based mainly on data from normal subjects, further study using a greater number of hypertensive patients is required to clarify whether the dysregulation of *HSD11B2* expression or a failure of the compensatory mechanism in renal 11βHSD type 2 activity is involved in the pathophysiology of essential hypertension.

Our results suggest possible ethnic differences in CA-repeat polymorphisms. The allelic distribution in our Japanese population appears to be an intermediate result between those of Caucasians and Afro-Americans.[12,22] In our separate studies on genes related to the mineralocorticoid pathway, the promoter polymorphism of the aldosterone synthase gene[31] and polymorphisms in the gene encoding the alpha-subunit of epithelial sodium channels (unpublished observation) showed similar intermediate distributions. Although Afro-Americans are prone to develop low renin, salt-sensitive hypertension,[32] they usually have lower aldosterone levels than Caucasians,[33] which suggests a mild form of mineralocorticoid excess. An association of longer alleles in CA-repeat polymorphisms with hypertension has been described in people of Afro-Caribbean descent,[22] but Afro-Americans have a higher frequency of short CA repeats.[12,22] Considering that there is no direct linear correlation of this CA-repeat with *in vivo* renal 11βHSD type 2 activity, the involvement of other nearby linked genes or of a combination of other components in the mineralocorticoid pathway should also be investigated.

In conclusion, the *HSD11B2* CA-repeat genotype is not associated with hypertension itself, but is associated with renal sodium excretion, probably through salt intake/appetite.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Celso Gomez-Sanchez (University of Mississippi) for providing the antibody for cortisol and cortisone. This work was supported by grants for 'Disorders of the Adrenal Gland (1998-2006)' from the Ministry of Health, Labor and Welfare, Japan, and National Institutes of Health grant DK42169 (PCW).

1 Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* 1988; **242**: 583–585.
2 Mune T, Rogerson FM, Nikkilä H, Agarwal AK, White PC. Human hypertension caused by mutations in the kidney isozyme of 11β-hydroxysteroid dehydrogenase. *Nat Genet* 1995; **10**: 394–399.
3 White PC, Mune T, Agarwal AK. 11beta-hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. *Endocr Rev* 1997; **18**: 135–156.
4 Stewart PM, Wallace AM, Valentino R, Burt D, Schackleton CHL, Edwards CRW. Mineralocorticoid activity of liquorice: 11β-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* 1987; **2**: 821–824.
5 Bailey MA, Craigie E, Livingstone DEW, Kotelevtsev YV, Al-Dujaili EAS, Kenyon CJ, Mullins JJ. *Hsd11b2* haploinsufficiency in mice causes salt sensitivity of blood pressure. *Hypertension* 2011; **57**: 515–520.
6 Craigie E, Evans LC, Mullins JJ, Bailey MA. Failure to downregulate the epithelial sodium channel causes salt sensitivity in *hsd11b2* heterozygote mice. *Hypertension* 2012; **60**: 684–690.
7 Tang JI, Kenyon CJ, Seckl JR, Nyirenda MJ. Prenatal overexposure to glucocorticoids programs renal 11β-hydroxysteroid dehydrogenase type 2 expression and salt-sensitive hypertension in the rat. *J Hypertens* 2011; **29**: 282–289.
8 Lauterburg M, Escher G, Dick B, Ackermann D, Frey FJ. Uninephrectomy reduces 11β-hydroxysteroid dehydrogenase type 1 and type 2 concomitantly with an increase in blood pressure in rats. *J Endocrinol* 2012; **214**: 373–380.
9 Watson B, Bergman SM, Myracle A, Callen DF, Acton RT, Warnock DG. Genetic association of flanking microsatellites with essential hypertension in blacks. *Hypertension* 1996; **28**: 478–482.
10 Smolenicka Z, Bach E, Schaer A, Liechti-Gallati S, Frey BM, Frey FJ, Ferrari P. A new polymorphic restriction site in the human 11β-hydroxysteroid dehydrogenase gene. *J Clin Endocrinol Metab* 1998; **83**: 1814–1817.
11 Lovati E, Ferrari P, Dick B, Jostarndt K, Frey BM, Frey FJ, Schorr U, Sharma AM. Molecular basis of human salt sensitivity: The role of the 11β-hydroxysteroid dehydrogenase type 2. *J Clin Endocrinol Metab* 1999; **84**: 3745–3749.
12 Agarwal AK, Giacchetti G, Lavery G, Nikkila H, Palermo M, Ricketts M, McTernan C, Bianchi G, Manunta P, Strazzullo P, Mantero F, White PC, Stewart PM. CA-repeat polymorphism in intron 1 of HSD11B2: effects on gene expression and salt sensitivity. *Hypertension* 2000; **36**: 187–194.
13 Taylor AC. Titration of heparinase for removal of the PCR-inhibitory effect of heparin in DNA samples. *Mol Ecol* 1997; **6**: 383–385.
14 Brand E, Kato N, Chatelain N, Krozowski ZS, Jeunemaitre X, Corvol P, Plouin PF, Cambien F, Pascoe L, Soubrier F. Structural analysis and evaluation of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) gene in human essential hypertension. *J Hypertens* 1998; **16**: 1627–1633.
15 Halushka MK, Fan JB, Bentley K, Hsie L, Shen N, Weder A, Cooper R, Lipshutz R, Chakravarti A. Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* 1999; **22**: 239–247.
16 Nunez S, Rogerson FM, Mune T, Igarashi Y, Nakagawa Y, Phillipov G, Moudgil A, Travis LB, Palermo M, Schackleton C, White PC. Mutants of 11β-hydroxysteroid dehydrogenase (11-HSD2) with partial activity. Improved correlations between genotype and biochemical phenotype in apparent mineralocorticoid excess. *Hypertension* 1999; **34**: 638–642.
17 Li A, Tedde R, Krozowski ZS, Pala A, Li KXZ, Schackleton CHL, Mantero F, Palermo M, Stewart PM. Molecular basis for hypertension in the 'type II variant' of apparent mineralocorticoid excess. *Am J Hum Genet* 1998; **63**: 370–379.
18 Morita H, Isomura Y, Mune T, Daido H, Takami R, Yamakita N, Ishizuka T, Takeda N, Yasuda K, Gomez-Sanchez CE. Plasma cortisol and cortisone concentrations in normal subjects and patients with adrenocortical disorders. *Metabolism* 2004; **53**: 89–94.
19 Isomura Y, Mune T, Morita H, Suwa T, Takada N, Yamamoto Y, Takeda J. Physiological roles of 11β-hydroxysteroid dehydrogenase type 2 in kidney. *Metabolism* 2006; **55**: 1352–1357.

20 Palermo M, Schackleton CH, Mantero F, Stewart PM. Urinary free cortisone and the assessment of 11β-hydroxysteroid dehydrogenase activity in man. *Clin Endocrinol* 1996; **45**: 605–611.
21 Kamide K, Kokubo Y, Hanada H, Nagura J, Yang J, Takiuchi S, Tanaka C, Banno M, Miwa Y, Yoshii M, Matayoshi T, Yasuda H, Horio T, Okayama A, Tomoike H, Kawano Y, Miyata T. Genetic variations of HSD11B2 in hypertensive patients and in the general population, six rare missense/frameshift mutations. *Hypertens Res* 2006; **29**: 243–252.
22 White PC, Agarwal AK, Li A, Nikkila H, Pratt JH, Caufield M, Clark A, McTernan C, Stewart PM. Possible association but no linkage of the HSD11B2 gene encoding the kidney isozyme of 11β-hydroxysteroid dehydrogenase to hypertension in Black people. *Clin Endocrinol* 2001; **55**: 249–252.
23 Geerling JC, Engeland WC, Kawata M, Loewy AD. Aldosterone target neurons in the nucleus tractus solitarius drive sodium appetite. *J Neurosci* 2006; **26**: 411–417.
24 Geerling JC, Loewy AD. Aldosterone in the brain. *Am J Physiol Renal Physiol* 2009; **297**: F559–F576.
25 Barba G, Cappuccio FP, Russo L, Stinga F, Iacone R, Strazzullo P. Renal function and blood pressure response to dietary salt restriction in normotensive men. *Hypertension* 1996; **27**: 1160–1164.
26 Walker BR, Stewart PM, Schackleton C, Padfield PL, Edwards C. Deficient inactivation of cortisol by 11β-hydroxysteroid dehydrogenase in essential hypertension. *Clin Endocrinol* 1993; **39**: 221–227.
27 Soro A, Ingram MC, Tonolo G, Glorioso N, Fraser R. Evidence of coexisting changes in 11β-hydroxysteroid dehydrogenase and 5β-reductase activity in subjects with untreated essential hypertension. *Hypertension* 1995; **25**: 67–70.
28 Walker BR, Phillips DIW, Noon JP, Panareli M, Andrew R, Edwards HV, Holton DW, Seckl JR, Webb DJ, Watt GCM. Increased glucocorticoid activity in men with cardiovascular risk factors. *Hypertension* 1998; **31**: 891–895.
29 Kerstens MN, van der Kleij FGH, Boonstra AH, Sluiter WJ, Koerts J, Navis G, Dullaart RPF. Salt loading affects cortisol metabolism in normotensive subjects: relationships with salt sensitivity. *J Clin Endocrinol Metab* 2003; **88**: 4180–4185.
30 Melander O, Frandsen E, Groop L, Hulthen UL. No evidence of a relation between 11β-hydroxysteroid dehydrogenase type 2 activity and salt sensitivity. *Am J Hypertens* 2003; **16**: 729–733.
31 Isaji M, Mune T, Takada N, Yamamoto Y, Suwa T, Morita H, Takeda J, White PC. Correlation between left ventricular mass and urinary sodium excretion in specific genotypes of CYP11B2. *J Hypertens* 2005; **23**: 1149–1157.
32 Weinberger MH. Salt sensitivity of blood pressure in humans. *Hypertension* 1996; **27**: 481–490.
33 Pratt JH, Jones J, Miller JZ, Wagner M, Fineberg NS. Racial differences in aldosterone excretion and plasma aldosterone concentrations in children. *N Engl J Med* 1989; **321**: 1152–1157.