Regulatory SNP in the *RBP4* Gene Modified the Expression in Adipocytes and Associated With BMI

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Retinol-binding protein 4 (RBP4) is a recently identified adipokine that was involved in insulin resistance. RBP4 is predominantly expressed from the liver in normal metabolic state to transport retinoids throughout the body, but the exact physiological function and the regulatory mechanisms of adipocyte-derived RBP4 have not been revealed. We conducted the genetic analysis about metabolic parameters in Japanese and Mongolian; the minor allele carriers of regulatory single-nucleotide polymorphism (SNP –803G>A) showed significantly higher BMI in Japanese men (*P* = 0.009) and women (*P* = 0.017), and in Mongolian women (*P* = 0.009). Relative quantification of RBP4 transcripts in –803GA heterozygotes showed that the minor allele–linked haplotype-derived mRNA was significantly more abundant than the transcript from major allele. RBP4 promoter assay in 3T3L1 adipocytes revealed that the minor allele increased the promoter activity double to triple and the administration of 9-*cis*-retinoic acid (RA) and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) enhanced the activity. Multiple alignment analysis of human, mouse, rat, and cattle RBP4 promoter suggested conserved seven transcription factor binding motifs. Electrophoretic mobility shift assay showed the –803G>A SNP modulate the affinity against unidentified DNA-binding factor, which was assumed to be a suppressive factor. These results collectively suggested that the minor allele of RBP4 regulatory SNP enhanced the expression in adipocytes, which may be associated with the adipogenesis.

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INTRODUCTION

Retinol-binding protein 4 (RBP4) is a recently identified adipokine that was shown to be involved in insulin resistance in a study of adipocyte-specific glucose transporter 4 (GLUT4) knockout mice ($G4A^{-/-}$) (1). The $G4A^{-/-}$ mice developed diabetes, and the adipocytes upregulated both plasma levels and expression of RBP4 mRNA. Overexpression of RBP4 in transgenic mice, as well as recombinant protein injection into wild-type mice, downregulated insulin-induced activation of phosphatidylinositol-3-OH kinase and insulin receptor substrate 1 in muscle and upregulated gluconeogenesis in the liver (1). Downregulation of GLUT4 expression in adipose tissue is an universal feature in patients with insulin resistance and type 2 diabetes (2). RBP4 plasma levels (3–7) and adipocyte RBP4 mRNA levels (8–10) in patients have been controversial, but recent large scale cohort study supports the correlation of RBP4 plasma level (11) and adipose tissue RNA level (8) with metabolic parameters. Administration of thiazolidinedione derivative (TZD) into $G4A^{-/-}$ mice downregulated RBP4 expression in adipocytes toward normal levels as well as improved insulin resistance and glucose intolerance (1). This phenomenon was observed in several clinical studies (12,13); however, other studies have shown that TZD increases RBP4 expression in insulin-resistant patients (14).

The liver is the principal organ for the storage of vitamin A derivatives, and RBP4, which transports retinoids throughout the body, is predominantly expressed from the liver in a normal metabolic state. Adipocytes are the secondary organ for vitamin A storage and secretion of RBP4, which increases in $G4A^{-/-}$ mice to the same level as that seen in the liver (1).

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However, the exact physiological function of adipocyte-derived RBP4 has not been revealed. A recent study showed that vitamin A and its metabolite regulated adipogenesis (15,16). Genetic impairment of the synthesis of retinoic acid (RA) was shown to repress diet-induced obesity in mice (16). We previously carried out a single-nucleotide polymorphism (SNP)–based genetic study in Mongolian subjects with diabetes (17). That case–control study of 511 controls and 281 subjects with type 2 diabetes showed that rare SNP alleles in a tight linkage disequilibrium block were associated with an increased risk of diabetes (P = 0.0015–0.0054) (17). A regulatory SNP (rs3758539) was identified from the linkage disequilibrium block, which increased the transcription efficiency in HepG2 cells based on an increased affinity with a transcription factor, hepatocyte nuclear factor 1α (HNF1α), and the correlation of the SNP with RBP4 plasma level was observed in diabetic patients (17). A recent genetic study in white subjects also suggested an association of RBP4 minor haplotype including the regulatory SNP, with higher fasting plasma insulin levels, BMI, and waist-to-hip ratio (18). Furthermore, several studies have shown an association between plasma RBP4 levels and obesity (5,19). It is thought that RBP4 expression may also be associated with obesity through its adipogenic function. In this study, we re-analyzed the rs3758539 SNP using metabolic parameters of Mongolian and Japanese subjects. In addition, the function of the RBP4 promoter was assessed in 3T3L1 adipocytes.

METHODS AND PROCEDURES

Subjects

Japanese subjects (n = 1,216) admitting for a health check were enrolled in this study under informed consent. The Mongolian nondiabetic population (n = 666) (ref. 17) was also studied under informed consent. The participants with history of diagnosis with diabetes and those with high fasting blood glucose level (>126) were excluded from the population SNP study to minimize the BMI variance based on the illness.

Subcutaneous (SAT) and visceral adipose tissue (VAT) were collected from 36 Japanese men and 29 Japanese women admitted to the Jichi Medical School Hospital for gastrointestinal surgery or gynecological surgery. Under informed consent, small pieces of SAT were collected from the abdominal skin incision and VAT was collected from the extirpated organ, omentum, or mesenterium. Total RNA was extracted from the freshly excised SAT or VAT using RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD). Clinical testing and genome DNA extraction were carried out from the fasting blood sample. This study protocol was approved by the ethics committees of Jichi Medical University, Japan and the Health Sciences University of Mongolia.

Genotyping of RBP4 –803G>A SNP and measurement of molecular ratio of allele-specific RBP4 transcripts

SNP typing of RBP4 −803G>A (rs3758539) was performed with a TaqMan genotyping system using an ABI PRISM 7900HT unit (Applied Biosystems, Foster City, CA). The studied population was stratified based on the ethnicity, gender, and genotype of −803G>A SNP, and the anthropometric and the clinical data were assessed statistically.

Molecular ratio of RBP4 transcripts in adipose tissue of −803GA heterozygotes was determined using the linked SNP marker in the transcript, −179T>G (rs10882283), which generates a restriction fragment length polymorphism of *Hin*fI restriction enzyme. Genomic DNA and complementary DNA from −803G/A heterozygotes were amplified by a primer pair (CGCCTCCCTCGCTCCACG; corresponding to nt −208 to −191 and TGACTCGGAAGCTGCTCACT; complementary to nt +69 to +88) and the PCR products were digested with *Hin*fI. The fully digested PCR products were electrophoresed and the genotype-specific DNA fragments were quantified using a capillary electrophoresis system, HAD-GT12 (eGene, Irvine, CA).

3T3L1 cell culture, mice adipocytes preparation, and RBP4 expression profile

3T3L1 adipocytes were differentiated following standard procedure, briefly, supplementation with 1 µg/ml insulin, 1 µmol/l dexamethasone, and 0.5 mmol/l isobutylmethylxanthine (Sigma-Aldrich, St Louis, MO) for 3 days; after the initial induction, the culture media were changed to fresh Dulbecco's modified Eagle's medium (glucose 4.5 g/l) supplemented with 1 µg/ml insulin for every 2 days except for the insulin withdrawal study. The cultured cells were studied 9 days after induction of differentiation unless otherwise stated, when >90% of the cells was differentiated on the basis of lipid accumulation. Fully differentiated cells were exposed to 10 µmol/l pioglitazone (ALEXIS Biochemicals, Lausen, Switzerland), 30 µmol/l GW9662 (Sigma-Aldrich), 1 µmol/l 9-*cis*-RA (Sigma-Aldrich), or 1 mmol/l 8-bromocyclic adenosine monophosphate (8-Br-cAMP; Sigma-Aldrich). The solvent, dimethyl sulfoxide, was used for the vehicle study. Before the induction of differentiation (day 0), and days 1–9 after induction, and 24 h after stimulation by pioglitazone, GW9662 or 9-*cis*-RA, and 16 h after stimulation by 8-Br-cAMP, total RNA was extracted and the RBP4 mRNA level was measured by real-time PCR system using SYBR Green PCR Master Mix (Applied Biosystems), murine-specific rbp4 primer pair (forward: ACTGGGGTGTAGCCTCCTTTCT and reverse: GCAGGCGGCAGGAGTACTGCA), and 36b4 primer pair (forward: GACCTGGAAGTCCAACTACT and reverse: CTGCTGCATCTGCTTGGAGC). Epididymal fat pads were prepared from 12-week-old C57BL6 male mice fed with standard chow (n = 4). Adipocytes and stroma cells were separated from the fat pad by the method using collagenase type I (GIBCO-Invitrogen, Carlsbad, CA) (20). Total RNA was extracted from the adipocytes, stroma cells, and 11 organs (inguinal fat pad, brain, heart, lung, liver, spleen, kidney, pancreas, small intestine, skeletal muscle, and bone marrow). The specific amplification of rbp4 and 36b4 sequences were verified by fragment size in electrophoresis or by the melting curve profile. The relative amount of rbp4 mRNA in each sample was normalized to 36b4 ribosomal RNA using the comparative ($2^{\Delta C_t}$) method.

Adenoviral induction of reporter constructs of RBP4 promoter into 3T3L1 adipocytes

The DNA fragments from RBP4 promoter (from –1,192 to –1) via luciferase to polyA signal were excised by *Cla*I from previously reported vectors, pGL3-803G-179T, pGL3-803G-179G, pGL3-803A-179T, and pGL3-803A-179G (17), and were inserted in pAxcwit vector at *Smi*I site (Takara, Kyoto, Japan). To standardize the infection efficiency, control vector was generated by insertion of the renilla luciferase (pRL) under CAGGS promoter into pAxCAwit vector. The recombinant adenoviruses were individually isolated by COS-TPC method (21). The cloned adenoviruses were expanded, purified, and titrated by $TCID_{50}$ method (22). Ten multiplicity of infection RBP4 reporter adenovirus and 0.1 multiplicity of infection RL adenovirus were coinfected to 3T3L1 or HepG2 cells. Twenty-four hours after infection, luciferase activity was measured in a Turner Designs Luminometer TD20/20 using the dual luciferase reporter assay system (Promega, Madison, WI). For all infection assays, at least three independent experiments were performed and the relative light units (firefly/renilla light units) were measured and compared among the experiments.

Gel mobility shift analysis (EMSA)

Human (AL356214), mouse (NT_039687), rat (NW_047565), and cattle (NW_001494355) RBP4 genes were aligned by multiple alignment analysis using GENETYX-Mac software (Software Development, Tokyo, Japan) and the conserved binding motifs for transcription factors

were estimated using MatInspector software (Genomatix; http://www.genomatix.de/index.html). Based on these analysis, four ^{32}PαCTP-labeled double-stranded probes were prepared to match the sequences as shown in Figure 4 and 50 fmol–labeled probes were incubated with 10 µg of nuclear extract of day 9 3T3L1 adipocytes or HepG2 cells. Protein concentration of the nuclear extracts was determined by Bradford method using bovine serum albumin as the standard. The competition assays were performed by adding 50 mol/l excess of cold probe against the labeled probes. For supershift assays, 1 µg of anti-HNF1α (Santa Cruz Biotechnology, Santa Cruz, CA) was added. Samples were incubated for 30 min at room temperature and resolved on 5% acrylamide gels in 0.5× Tris–borate–EDTA buffer at room temperature. The gels were dried and exposed for 24 h to BioMax MS film (Kodak, Rochester, NY).

Statistical analysis

Population data were analyzed using Student's *t*-test. Hardy–Weinberg equilibrium was assessed using the χ^2-test. The *in vitro* experimental studies were at least triplicated and the data were evaluated by Student's *t*-test. Statistical analysis was performed using the SNPAlyze (Dynacom, Mobara, Japan) and SPSS (SPSS Japan, Tokyo, Japan) statistical packages. Data are shown as mean ± s.d. Statistical significance was set at $P < 0.05$ level.

RESULTS

Correlation analysis of RBP4 –803G>A SNP with metabolic parameters

The Japanese population genome panel was stratified by gender and the genotype of −803G>A SNP, and metabolic parameters among genotypes were assessed by Student's *t*-test. The mean BMI of −803A carriers was higher than in the G homozygotes in both gender of Japanese ($P = 0.009$ men, $P = 0.017$ women, $P = 0.001$ combined), whereas there were no significant differences in other parameters (Table 1). The allele frequencies in the studied Japanese population were G: 0.917 and A: 0.083, respectively. The observed genotype frequencies were in accordance with the Hardy–Weinberg's law. Due to the lower minor allele frequency, the genotype stratification was carried out about dominant model (−803GG vs. −803GA and AA). In Mongolian women, a similar trend was observed in that the average BMI of −803A carriers was 1.44 higher than −803G homozygotes ($P = 0.009$) (Table 2). In Mongolian men, the BMI of −803A carriers was 0.25 higher than −803G homozygotes, although difference was not significant ($P = 0.692$, Table 2). Although the simple application of Bonferroni's correction against multiple comparison (6×) indicated a lack of statistical significance in all of the factors in the combined analysis other than BMI in the Japanese, the trend of higher BMI of −803T carriers in two Asian ethnic groups suggests a possibility that the −803G>A SNP may be functional in adipocytes, and the promoter efficiency of adipocytes may lead to differences in adipogenesis.

RBP4 expression in 3T3L1 adipocytes

To understand the profile of RBP4 expression along with adipocyte maturation, 3T3L1 adipocytes were induced to differentiation and the relative amount of rbp4 vs. 36b4 internal control mRNA was measured. Rbp4 expression was increased after induction until day 3 but after the peak expression, it decreased gradually (Figure 1a). In the fully matured 3T3L1 adipocytes (day 9), a peroxisome proliferator–activated receptor-γ (PPARγ) agonist, pioglitazone, the antagonist, GW9662, and withdrawal of insulin did not show significant influence on the

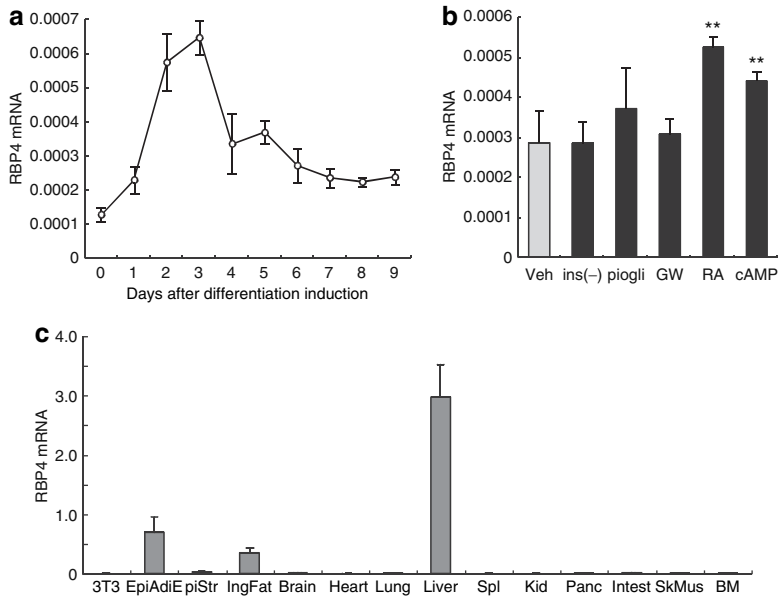

Figure 1 RBP4 expression in 3T3L1 adipocytes. (**a**) Relative amount of murine rbp4 mRNA against 36b4 mRNA in 3T3L1 adipocytes was measured during the differentiation time course; before (day 0) and after differentiation induction (day 1–9). (**b**) Modification of RBP4 mRNA in 3T3L1 adipocytes (day 9) by 48-h withdrawal of insulin (ins(–)), supplementation of pioglitazone (piogli), GW9662 (GW), 9-*cis*-RA (RA), or 8-Br-cAMP (cAMP). The solvent of these chemicals, DMSO, was used as a vehicle study (veh). **Statistical significance against vehicle study ($P < 0.001$). (**c**) Relative amount of murine rbp4 mRNA against 36b4 in day 9 3T3L1 adipocytes (3T3), adipocytes (EpiAdi), and stromal cells (EpiStr) from epididymal fat pad, inguinal fat pad (IngFat), brain, heart, lung, liver, spleen (Spl), kidney (Kid), pancreas (Panc), small intestine (Intest), skeletal muscle (SkMus), and bone marrow (BM).

Table 1 Correlation analysis of RBP4 –803G>A SNP in Japanese

	RBP4 –803 G>A SNP type		Student's *t*-test
	GG	GA+AA	*P* value
All Japanese			
N	1,018	198	
Age	57.2 ± 10.2	56.9 ± 10.6	0.691
BMI	23.9 ± 3.1	24.6 ± 2.9	0.001
HbA_{1c}	5.2 ± 0.9	5.2 ± 0.6	0.885
Total cholesterol	210.0 ± 34.8	209.6 ± 34.3	0.900
HDL cholesterol	61.4 ± 16.0	60.8 ± 15.0	0.664
log TG	2.0 ± 0.2	2.0 ± 0.2	0.060
Men			
N	555	101	
Age	56.0 ± 9.5	55.2 ± 9.7	0.402
BMI	23.9 ± 2.7	24.7 ± 2.8	0.009
HbA_{1c}	5.3 ± 1.3	5.2 ± 0.6	0.408
Total cholesterol	202.8 ± 33.6	205.0 ± 34.5	0.557
HDL cholesterol	57.3 ± 15.8	56.3 ± 13.9	0.562
log TG	2.0 ± 0.2	2.1 ± 0.2	0.280
Women			
N	463	97	
Age	58.5 ± 10.8	58.8 ± 11.1	0.831
BMI	23.7 ± 3.3	24.6 ± 3.0	0.017
HbA_{1c}	5.3 ± 0.7	5.3 ± 0.7	0.553
Total cholesterol	217.6 ± 34.4	214.3 ± 33.2	0.407
HDL cholesterol	65.9 ± 15.0	65.5 ± 14.7	0.821
log TG	1.9 ± 0.2	2.0 ± 0.2	0.078

Data are mean ± s.d.
HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; TG, triglyceride.

Table 2 Correlation analysis of RBP4 –803G>A SNP in Mongolian

	RBP4 –803 G>A SNP type		Student's *t*-test
	GG	GA+AA	*P* value
All Mongolian			
N	496	170	
Age	49.4 ± 10.7	50.3 ± 9.9	0.357
BMI	26.5 ± 4.6	27.5 ± 5.0	0.022
Fructosamine	234.6 ± 19.9	234.3 ± 18.1	0.860
Total cholesterol	179.3 ± 36.5	179.6 ± 36.5	0.928
HDL	54.8 ± 11.4	54.1 ± 11.9	0.485
log TG	2.0 ± 0.3	2.0 ± 0.3	0.210
Men			
N	208	69	
Age	51.1 ± 10.4	53.0 ± 9.2	0.172
BMI	26.5 ± 4.7	26.8 ± 4.5	0.692
Fructosamine	236.0 ± 20.7	235.7 ± 18.5	0.915
Total cholesterol	182.0 ± 41.3	182.7 ± 42.0	0.907
HDL	51.5 ± 11.1	51.0 ± 11.5	0.716
log TG	2.1 ± 0.3	2.1 ± 0.3	0.175
Women			
N	288	101	
Age	48.2 ± 10.8	48.4 ± 10.0	0.872
BMI	26.5 ± 4.5	27.9 ± 5.3	0.009
Fructosamine	233.5 ± 19.2	233.3 ± 17.9	0.908
Total cholesterol	177.3 ± 32.5	177.5 ± 32.4	0.970
HDL	57.2 ± 11.0	56.3 ± 11.7	0.466
log TG	2.0 ± 0.2	2.0 ± 0.2	0.606

Data are mean ± s.d.
HDL, high-density lipoprotein; TG, triglyceride.

RBP4 expression levels. The retinoic acid receptor and retinoid X receptor (RXR) ligand, 9-*cis*-RA, and plasma membrane permeable cAMP, 8-Br-cAMP, significantly increased the expression level of rbp4 toward 1.84-fold and 1.60-fold (**Figure 1b**), respectively. Comparing to 3T3L1, however, adipocytes from mice epididymal adipose tissue expressed extremely high levels of rbp4 transcripts. Liver was the principal organ expressing RBP4 transcripts and the adipose tissue was the secondary organ. Other organs studied expressed rbp4 mRNA 1/170 to 1/5,800 of the amount of liver (**Figure 1c**).

Reporter gene assay of RBP4 promoter in 3T3L1 cells

The influence of the human −803G>A SNP in adipocytes was assessed by induction of reporter constructs using an adenovirus gene delivery system. The promoter function of human RBP4 in the differentiation course of adipocytes was almost identical to that of the intrinsic *rbp4* gene in 3T3L1 cells; it peaked at day 3 and gradually decreased thereafter (**Figure 2a**). The activity of the −803A construct was double to triple that of the −803G constructs, whereas the −179T>G substitution did not show significant alteration of the promoter activity. From day 1 to 9, the difference in luciferase activity between the −803G and −803A constructs was statistically significant (day 1, $P = 0.02$; day 3, $P = 0.01$; day 5, $P = 0.02$; and day 9, $P = 0.003$). In fully differentiated 3T3L1 adipocytes, pioglitazone (pAx-803G-173T, $P = 0.088$; pAx-803A-173T, $P = 0.070$) and GW9662 (pAx-803G-173T, $P = 0.439$; pAx-803A-173T, $P = 0.275$) did not influence this activity. In accordance with the intrinsic promoter, 9-*cis*-RA significantly increased the promoter activity of both −803G and A allele constructs by 2.71-fold ($P = 0.013$) and 1.87-fold ($P = 0.031$), respectively (**Figure 2b**). Furthermore, 8-Br-cAMP significantly enhanced the promoter activity of both −803G and A constructs by 12.7-fold ($P = 0.002$) and 14.2-fold ($P = 0.001$). These differences of fold induction between −803G and −803A constructs were not statistically significant.

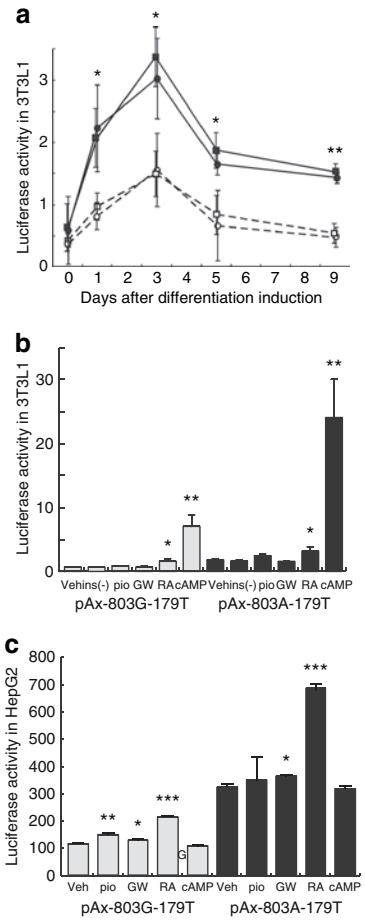

Figure 2 Promoter assay of human RBP4 in 3T3L1 adipocytes. (**a**) Twenty-four hours before the measurement, 10 multiplicity of infection (MOI) reporter constructs of RBP4 promoter-firefly luciferase (–803G or –803A) were delivered into 3T3L1 adipocytes using adenovirus vector system simultaneously with 0.1 MOI of renilla luciferase control vector. The relative luciferase activity (firefly/renilla) of –803G-179T (open circles, dashed line), –803G-179G (open boxes, dashed line), –803A-179T (closed circles, solid line) and –803A-179G (closed boxes, solid line) were measured before (day 0) and after differentiation induction (day 1, 3, 5, and 9). Asterisks indicate the statistical significance between –803G and –803A vectors ($P < 0.05$). (**b**) Modulation of RBP4 promoter activity in matured (day 9) 3T3L1 adipocytes by 48-h withdrawal of insulin (ins(–)), supplementation of pioglitazone (pio), GW9662 (GW), 9-*cis*-RA (RA), or 8-Br-cAMP (cAMP). Veh indicates the experiment using the solvent. Gray and black bars show the results of –803G-179T and –803A-179T constructs, respectively. (**c**) Modulation of RBP4 promoter activity in HepG2 cells by supplementation of pioglitazone (pio), GW9662 (GW), 9-*cis*-RA (RA) , or 8-Br-cAMP (cAMP). Statistically significant results in Student's *t*-test against vehicle study were shown by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$).

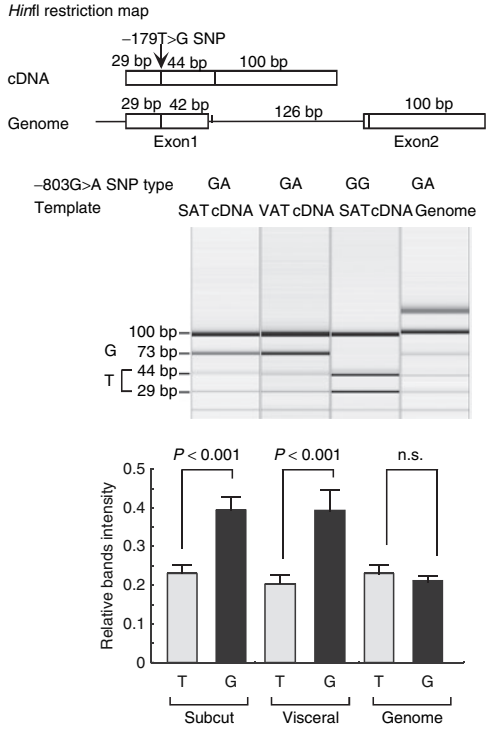

Figure 3 Molecular ratio of RBP4 transcripts in adipose tissue from –803GA heterozygotes. RBP4 complementary DNA and genomic DNA from exon 1 to 2 were amplified and the amplicons were digested by *Hin*fI restriction enzyme. Restriction map illustrates the fragment length. *Hin*fI RFLP in exon 1 was resulted from –179T>G (rs10882283) SNP. The fully digested DNA fragments were resolved in capillary electrophoresis system and the genotype-specific bands in the electropherogram were quantified. The bar graph shows the average value of relative band intensity (–803G-179T haplotype: 44+29 bp bands, –803A-179G haplotype: 73 bp band) against 100 bp band from SAT and VAT complementary DNA and genomic DNA of seven –803GA heterozygotes.

The adenovirus vectors were simultaneously induced in HepG2 cells and the influences of the four chemicals were assessed. Pioglitazone slightly increased the promoter activity of pAx-803G-173T ($P = 0.0002$), whereas a significant effect was not observed on pAx-803A-173T construct ($P = 0.663$). Although GW9662 increased the activity of both constructs (pAx-803G-173T, $P = 0.025$; pAx-803A-173T, $P = 0.016$), the effects were subtle. 8-Br-cAMP did not alter promoter activity in HepG2 cells, but 9-*cis*-RA significantly increased the activity of –803G and –803A constructs by 1.87-fold ($P < 0.000001$) and 2.13-fold ($P < 0.0001$), respectively (**Figure 2c**). These results suggest that the RBP4 –803G>A SNP and RA enhanced the transcription efficiency in both hepatocytes and adipocytes but enhancement by 8-Br-cAMP was observed just in 3T3L1 adipocyte, and TZD had a little effect on promoter activity.

Molecular ratio of RBP4 transcripts in adipose tissue of –803GA heterozygotes

In the 65 studied adipose tissue donors, 7 donors were –803GA heterozygotes and the remaining 58 were GG homozygotes. In order to show the difference of transcription efficiency between the two alleles in adipocytes, the haplotype SNP in the non-coding region of exon 1 (–179T>G, *Hin*fI restriction fragment length polymorphism) was used as a marker. PCR cloning and sequencing analysis showed the molecular linkage of –803G and –179T and of –803A and –179G in the studied –803GA heterozygotes. The –179G reverse transcription–PCR products were significantly more abundant than the –179T products,

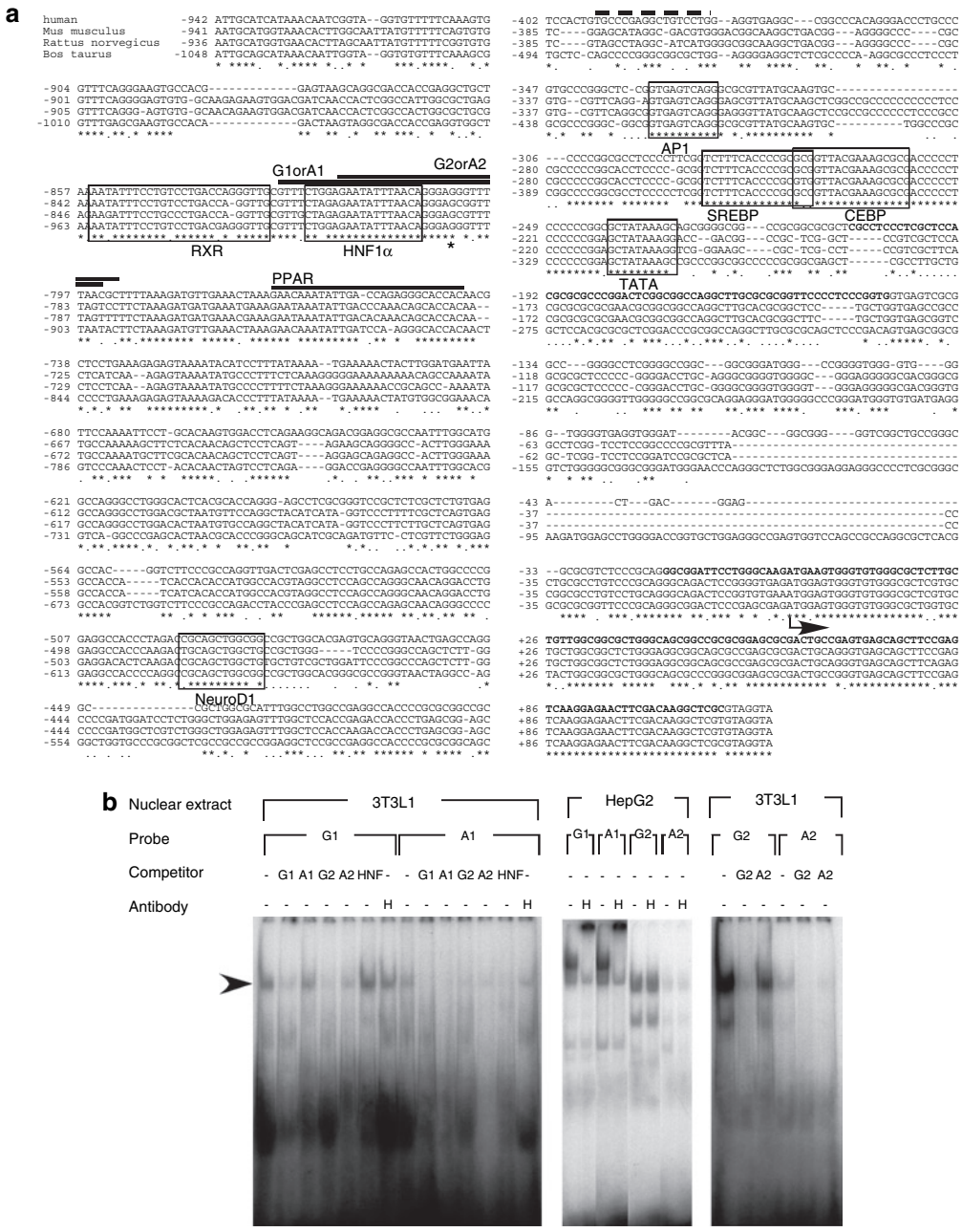

Figure 4 Phylogenetic analysis and electrophoretic mobility shift assay (EMSA). (**a**) RBP promoter sequences of human, mouse, rat and cattle were aligned based on the maximal matching study. The conserved nucleotides were indicated by small asterisks. The big asterisk indicates –803G>A SNP position. The boxes indicate the conserved motifs estimated by MatInspector software. The bold lines indicate the DNA sequences used for EMSA. The bold nucleotide sequences indicate the exon 1 and 2 in human. The bended arrow indicate the translation start position. (**b**) Ten micrograms of nuclear protein from day 9 3T3L1 adipocytes or HepG2 cells were incubated with 50 fmol 32P labeled probes indicated. The competition assays were carried out using the 50-fold molar excess cold competitors indicated above (G1, A1, G2, A2, HNF (hnf1)). For the supershift assay, anti-HNF1α was added to the lane represented by H.

1.71-fold ($P < 0.001$) and 1.93-fold ($P < 0.001$) in SAT and VAT, respectively, whereas fragments from the genome PCR of the heterozygotes showed almost equal amounts (**Figure 3**).

Electrophoretic mobility shift assay

The multiple alignment of humans, mice, rats, and cattle RBP4 promoter region revealed conserved sequences, in which seven transcription factor binding motifs were estimated: RXR heteroduplex, HNF1, NeuroD1, AP1, SREBP, CEBP, and TATA box (boxed in **Figure 4a**). Furthermore, it was also shown that the −803 G allele was the ancestral type and the flanking sequences were highly conserved. An inverse sequence similar to the consensus PPAR/RXR motif (AGGTCAnnnAGGTCA) was found in the human sequence (bold overlined with PPAR) but was not conserved in other species. In HepG2 cells, −803G>A SNP affected the affinity of HNF1α binding to the

motif, which localized 5 bp upstream from the SNP (17). To determine whether the SNP influence the binding of transcription factors in adipocytes, four double-stranded oligonucleotide probes (G1, A1, G2, and A2) encoding the SNPs were prepared. The G1 probe, encoding the HNF1α motif and the −803G allele, showed a shift band after incubation with 3T3L1 nuclear proteins. The shift band was competed out by 50-fold molecular excess G1 and G2 cold probes, whereas the competition was insufficient by A1 and A2 probes, and was not observed by HNF1 probe (**Figure 4b**). The G2 probe also showed a shift band, which was competed out by the G2 probe (**Figure 4b**). The A2 probe, however, significantly diminished the affinity to the unknown nuclear protein. The shift bands of these four probes in 3T3L1 adipocytes did not show supershift bands by anti-HNF1α, which clearly showed supershift bands in HepG2 cells on G1 and A1 probe (**Figure 4b**). These data indicate that HNF1α was not involved in the regulation of RBP4 expression in 3T3L1 adipocytes and that −803G>A SNP significantly modified the affinity for a nuclear protein, which appeared a suppressive factor.

DISCUSSION

We report the correlation of the RBP4 regulatory SNP with BMI in two ethnic groups. The minor allele carriers in Japanese men and women, and Mongolian women showed slightly but significantly higher BMI values than homozygotes of the major allele, whereas Mongolian men did not show statistical difference probably due to its small sample size. We speculate that the adipocytes of minor allele carriers efficiently produce RBP4, which might allow for further adipocyte proliferation. The molecular ratio of RBP4 transcripts in heterozygotes' adipocytes showed a significant dominancy of the transcripts from the −803A allele over those from the −803G allele. Furthermore, the reporter gene assay showed that the −803A allele promoter was two- to threefold more effective in both adipocytes and hepatic cell lines. Carrier of the −803A allele might have bound less effectively to a nuclear factor, which was assumed to be a suppressive factor, to the SNP site in the promoter, so that the −803A carrier might be able to express more RBP4 mRNA than −803G homozygotes. Colantuoni *et al.* reported a negative control element in RBP4 promoter sequence (23), but it was 400 bp downstream from the −803G>A SNP position. Recent reports from Germany also showed an association of RBP4 minor alleles with diabetes (18) and a trend of BMI in diabetic patients and the abundance of RBP4 mRNA in adipose tissue of −803A carriers were shown (18). These results suggest that the RBP4 −803A allele has a potential to increase BMI in multiple ethnic groups. In mice model, however, body weight difference was not observed among wild type, muscle-specific RBP4 overexpressing mice, and rbp4$^{-/-}$ mice at 10–14 weeks of age (1). Adipocyte-specific overexpressing and knockout mice should be generated to resolve a matter whether RBP4 from adipocytes actually promote the adiposity.

In addition to the SNP, the metabolic and the adipocyte maturation state influenced the transcript level of RBP4 *in vivo*. Graham *et al.* reported a positive correlation of serum RBP4 with BMI, waist-to-hip ratio, triglycerides, and systolic blood pressure (3). Klöting *et al.* reported that RBP4 mRNA was increased ~60-fold and 12-fold, respectively, in VAT and SAT of obese white subjects vs. lean subjects (8). Serum RBP4 in that subjects was positively correlated with adipose RBP4 mRNA in both VAT and SAT. They also claimed that the VAT RBP4 expression was approximately fivefold higher in VAT than in SAT adipose tissue.

The physiological function of retinoid metabolism in adipocytes in noninsulin resistant states has been investigated (15,16,24). Retinol transported by RBP4 is imported into adipose tissue and oxidized first into retinaldehyde by alcohol dehydrogenase. Retinaldehyde is then converted into RA by retinaldehyde dehydrogenase. All-*trans*-RA is the ligand for retinoic acid nuclear receptors. RXR is activated by a much less abundant retinoid derivative, 9-*cis*-RA (15). The previous promoter study showed that retinoids significantly enhanced human RBP4 promoter activity in hepatocytes (25,26), whereas retinoid responsive element suggested by Panariello *et al.* (26) was not conserved in other species (dashed overlined in **Figure 4**). Furthermore, Mercader *et al.* showed recently that all-*trans*-RA decreased RBP4 production in 3T3L1 adipocytes and murine adipose tissue (27). These results indicate that adipocyte-derived RBP4 is controlled finely by retinoid metabolites.

Expression levels of RBP4 were higher in VAT than SAT in European subjects (8), although, our 65 Japanese subjects showed an opposite trend (not shown). These distinct differences could have resulted from the physiological characteristics of the two organs (28). RBP4 promoter may provide a tool to resolve molecular mechanisms of functions that are dependent on the location of the adipocytes.

Adipocyte originated excessive RBP4 may overflow into blood and result in a high serum level of RBP4. The RBP4-receptor signaling pathway has been shown to induce insulin resistance in both the liver and muscles (29). Although we reported a positive association of RBP4 −803G>A SNP with diabetes in Mongolian subjects (17), this association study was not repeatable in Japanese diabetes subjects (data not shown). Furthermore, glucose metabolic parameters, hemoglobin A_{1c}, or fasting plasma glucose levels, did not show significant correlations with the SNP, which might have resulted from the lower prevalence of insulin resistance in the Japanese population studied.

RBP4 production in adipocytes of Glut4$^{-/-}$ mice were decreased by pioglitazone (1), but our promoter assay in 3T3L1 adipocytes showed that pioglitazone did not significantly modulate RBP4 production. In human primary adipocytes, TZD was shown to increase RBP4 production whereas tumor necrosis factor-α decreased RBP4 production (30). Clinical studies have also shown contrasting data regarding how TZD affects the expression of RBP4. For example, Yao-Borengasser *et al.* showed that pioglitazone treatment in patients with impaired glucose tolerance increased adipocyte RBP4 mRNA levels, whereas it did not influence plasma levels (14). Jia *et al.* showed that rosiglitazone treatment in patients with diabetes

mellitus resulted in a lower RBP4 levels, which were accompanied by improved insulin sensitivity and reduction of VAT area (13). The current study shows that the investigated human RBP4 promoter function was not substantially enhanced by pioglitazone. Moreover, promoter activity was not influenced by withdrawal of insulin. FOXOs and ATF2 are two transcription factors known to transduce the insulin signal toward cell proliferation and glucose metabolism (31), but those binding motifs were not shown in RBP4 promoter, suggesting that RBP4 expression is not directly regulated by insulin signaling.

The signal mediated by cAMP has been shown to be a pivotal pathway for adipogenesis (32). RBP4 expression was significantly enhanced by 8-Br-cAMP in 3T3L1 adipocytes but not in HepG2 cells, and human RBP4 promoter was more effectively enhanced than mouse promoter in 3T3L1 adipocytes. Jessen *et al.*, however, reported that cAMP induced the rbp4 gene expression in mouse hepatoma cell line, Hepa 1-6 cells (33). It was speculated that these discrepancies were resulted from the difference of the promoter sequences.

Profiling of adipocytes in insulin-resistant patients showed an impairment of expression of Wnt signaling genes and adipogenic transcription factors, for example, WNT1, FZD1, CEBP, PPARγ and SREBP1 (34). The reduction of these transcription factors suggests a possible impairment in the ability to recruit new adipocytes. RBP4 promoter encoded the motifs for CEBP and SREBP1, which expressed most abundantly at day3 after differentiation induction in 3T3L1 adipocytes (32,35,36). RBP4 promoter activity profile during the differentiation time course resembles the expression pattern of these transcription factors, which appeared to be involved in the expression of RBP4 at the early adipocytes differentiation stage. Insulin resistance is a complex disorder involving metabolic, immunological, and neurological axes. Although RBP4 mRNA level increased after induction of 3T3L1 cells, the transcript level was much lower than in mice adipocytes *in vivo*. 3T3L1 adipocytes appear to be a model with insufficient RBP4 expression for comparative analysis of RBP4 expression in adipocytes *in vivo*. The mechanisms of enhancement of RBP4 expression in adipocytes remain to be resolved in ultimately differentiated and insulin-resistant state.

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DISCLOSURE

The authors declared no conflict of interest.

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