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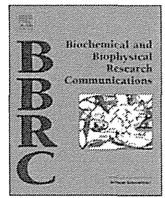

Ablation of *Rnf213* retards progression of diabetes in the Akita mouse

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ABSTRACT

Moyamoya disease (MMD) and moyamoya syndrome are vasculopathies characterized by progressive stenosis in the circle of Willis and its branches. The *RNF213* gene, which encodes a novel class of proteins, characterized by both E3 ligase and AAA + ATPase activities, has been identified as the susceptibility gene for MMD. However, its physiological functions remain unknown. MMD and moyamoya syndrome are often accompanied by diabetes mellitus. In this study, we generated *Rnf213* knockout (KO) C57BL/6 mice ($Rnf213^{-/-}$; $Ins2^{+/+}$), which were mated with Akita (C57BL/6 $Rnf213^{+/+}$; $Ins2^{+/C96Y}$) mice, a strain that develops diabetes spontaneously by 5 weeks of age, to obtain mice lacking *Rnf213* and carrying the Akita mutation (KO/Akita, $Rnf213^{-/-}$; $Ins2^{+/C96Y}$). Body weight and blood glucose concentration were measured from 6 to 20 weeks. Glucose tolerance, insulin resistance, plasma insulin and leptin concentrations, food consumption, pancreatic insulin content and histopathology were evaluated at 18 weeks of age. We found that glucose tolerance, as indicated by AUC, was 20% lower ($p < 0.05$) and insulin contents in pancreas were 150% higher ($p < 0.05$), in KO/Akita than in Akita mice. The number of CHOP positive β-cells assayed by histopathological examination was 30% lower and food consumption was 34% lower in KO/Akita than in Akita mice ($p < 0.05$ each). These findings indicated that the disruption of *Rnf213* improved glucose tolerance by protecting islet β cells.

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1. Introduction

Moyamoya disease (MMD) and moyamoya syndrome are vasculopathies characterized by occlusion at the internal carotid arteries in the circle of Willis and the compensatory formation of an abnormal vascular network, resembling "puffs of smoke", that are called moyamoya vessels [1]. Patients with moyamoya syndrome have a predisposing disease [2], including Down's syndrome [3], neurofibromatosis 1 [4], or microcephalic osteodysplastic primordial dwarfism type Majewski II (MOPDII) [5], whereas patients with MMD have no such predisposing conditions.

Conditions predisposing to moyamoya syndrome are frequently accompanied by diabetes [2,5–7]. Moreover, the prevalence of type 1 diabetes mellitus was shown to be much higher in patients with MMD than in the general population [8], suggesting a pathological link between MMD and diabetes. We recently demonstrated that *RNF213* was the susceptibility gene for MMD, and that the p.R4810K polymorphism (ss179362673: G>A) is a founder variant commonly found in East Asian patients [9]. Although knockdown of *RNF213* in zebrafish caused abnormal vascular development [9], the physiological function of *RNF213* remains largely unknown.

RNF213 encodes a unique, 591-kDa protein with both a ring finger domain and Walker motifs, and *RNF213* mRNA is expressed in various tissues [9]. The E3 ligase activity of the ring finger domain was confirmed by self-ubiquitination, and ATPase in the Walker motifs was confirmed biochemically [9]. Ring-base E3 ligases have been linked to the control of many cellular processes, including proteasome-dependent proteolysis, DNA repair, signal transduction, apoptosis, immunological processes and transcription [10]. *RNF213* is also an AAA + ATPase because it has Walker A and Walker B motifs. AAA + ATPases usually exist and function as oligomers; their cellular functions include vesicular transport, quality control, cargo trafficking and microtubule homeostasis [11].

In this study, we tested whether ablation of *Rnf213* can modify diabetes mellitus in Akita mice (C57BL/6 $Rnf213^{+/+}$; $Ins2^{+/C96Y}$), a model for type 1 diabetes [12], in which β-cell destruction results from endoplasmic reticulum (ER) stress. We found that ablation of *Rnf213* unexpectedly alleviates diabetes by preserving β-cell function through moderating the vicious cycle of hyperphagia and hypoinsulinemia.

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2. Materials and methods

2.1. Generation of *Rnf213* knockout mice

An *Rnf213*-targeting construct was produced using a Multisite Gateway Three-Fragment Vector Construction Kit (Invitrogen). Briefly, a loxP site was cloned into the 5′ site of exon 20, and a fragment containing a loxP site and a neomycin-resistance gene (Neo) was cloned into the 3′ site of exon 20 (Fig. 1A, Supplemental material). The construct was linearized and electroporated into RENKA C57BL/6 ES cells and selected with G418. Integration of the targeting vector into the mouse genome by homologous recombination was verified in targeted ES clones by Southern blotting (data not shown). Correctly targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice with the targeted allele incorporated into the germ lines. The resulting chimeric male mice were mated with female C57BL/6 mice, and germ line transmission of the targeted allele was examined in the offspring. Offspring carrying the target allele were bred with Cre-transgenic C57BL/6 mice to generate mice heterozygous for the *Rnf213* deficiency ($Rnf213^{-/+}$). Heterozygous male and female mice were bred to produce homozygous offspring (KO, $Rnf213^{-/-}$).

2.2. Experimental animals

Akita ($Ins2^{+/C96Y}$) mice on a C57BL/6 background and C57BL/6 (WT) mice were purchased from Japan SLC. To generate mice lacking *Rnf213* and carrying the Akita mutation (KO/Akita, $Rnf213^{-/-}$; $Ins2^{+/C96Y}$), male double-heterozygous ($Rnf213^{+/-}$; $Ins2^{+/C96Y}$) mice were generated and mated with female *Rnf213* KO mice. Experiments were performed on four groups of male mice: (1) KO/Akita ($Rnf213^{-/-}$; $Ins2^{+/C96Y}$), (2) Akita ($Rnf213^{+/+}$; $Ins2^{+/C96Y}$), (3) KO ($Rnf213^{-/-}$; $Ins2^{+/+}$), and (4) WT ($Rnf213^{+/+}$; $Ins2^{+/+}$). Progeny of (1–3), aged 4 weeks, were selected by PCR genotyping for *Rnf213* (Supplemental material) and the *Ins2* locus, as described [13]. Mice were allowed free access to a standard diet (CLEA, Rodent Diet CE-7, 3.4 kcal/g) and tap water. The care of the animals and all experimental procedures were in accordance with the Animal Welfare Guidelines of Kyoto University.

2.3. Culture of Akita and min-6 cell lines and real-time PCR (RT-PCR)

To test *Rnf213* expression in β cells, we used Akita cells and the min-6 cell line [14,15]. Quantitative RT-PCR for *Rnf213* was performed using the specific primers, Rnf213cex29–31F (5′-TAA GGA TGT CCG CTC CTG GTT-3′) and Rnf213cex29–31R (5′-TTG ATG GCA GTA TAC TTG GCA-3′).

2.4. Western blotting

Protein samples from mice pancreas or cultured cells were subjected to immunoblotting using the rabbit polyclonal anti-RNF213 antibody and anti-GAPDH antibody (Santa Cruz Biotechnology). The rabbit polyclonal antibody was produced by inoculation of rabbits with cloned human full-length RNF213 as an antigen. The polyclonal IgG was purified from rabbit serum.

2.5. Measurement of glucose, insulin, proinsulin and leptin

Blood glucose was measured by Glutest Neo Super (Sanwa). All values above 600 mg/dl were treated as 600 mg/dl. Glucose tolerance testing (GTT) was performed by fasting 18-week-old mice for 16 h, followed by an intraperitoneal injection of 1.5 g/kg glucose. Insulin tolerance testing (ITT) was performed by fasting 18-week-old mice for 6 h, followed by an intraperitoneal injection of 1.5 U/kg insulin (Eli Lilly and Company). To measure leptin concentrations, blood was collected from the tail veins of 18-week-old mice after a 16 h fast. Plasma concentrations of insulin, leptin and proinsulin were measured by ELISA (Shibayagi).

2.6. Measurement of pancreatic insulin and proinsulin contents

Mice were sacrificed at 18 weeks of age in the morning after a 6 h fast. Each pancreas was homogenized in acid ethanol (75% ethanol, 1.5% HCl) and extracted at 4 °C overnight. The extracts were centrifuged, and the insulin and proinsulin concentrations of the supernatants were measured.

2.7. Pathological investigations

Mice were sacrificed under sevoflurane at 18 weeks of age after a 6 h fast. Each pancreas was fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The sections were immunostained with guinea pig anti-insulin antibody (Dako) or rabbit anti-C/EBP homologous protein (CHOP)/GADD153 antibody (Santa Cruz Biotechnology). To estimate β-cell mass, consecutive paraffin sections 75 μm apart and spanning the entire pancreas (5–8 sections per pancreas) were prepared, and islet areas and relative abundance of insulin- and CHOP-positive cells were quantified on more than 20 islets per pancreas in three or four mice per genotype using Image-J software (National Institutes of Health). For electron microscopy, pancreases were fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide.

2.8. Statistical analysis

Results are presented as the mean ± standard deviation (SD) or standard error (SE). Differences were analyzed by *t*-test or ANOVA followed by Tukey's honestly significant difference test using STATISTICA software (StatSoft). $p < 0.05$ was considered statistically significant.

3. Results

3.1. General characterization of *Rnf213* KO mice

To determine the physiological function of *Rnf213*, we generated mice with targeted deletion of *Rnf213* exon 20. This targeting strategy, in which a frame shift mutation was introduced into this exon, resulted in the disruption of the Walker motifs and the ring finger domain (Fig. 1A). Complete removal of *Rnf213* exon 20 from genomic DNA (Fig. 1B) and the absence of Rnf213 protein expression (Fig. 1C), were confirmed in KO mice. KO mice were born in the predicted Mendelian ratio and did not show any apparent health problems, including a cerebrovascular phenotype similar to MMD, even at around 80 weeks of age. Both males and females were fertile and produced normal-sized litters (mean, 6–8 pups). The body weight of KO mice was about 13% less than that of WT mice after 25 weeks of age ($p < 0.05$), and GTT results in KO and WT mice did not differ at 50 weeks of age (Supplemental Fig. 1).

3.2. Expression of *Rnf213* in Akita and min-6 cells

Rnf213 protein was expressed in the pancreas (Fig. 1C). To assess the expression of *Rnf213* in β cells, we investigated the expression of *Rnf213* mRNA and protein in Akita and min-6 cell lines by quantitative RT-PCR and western blotting, respectively. We found that *Rnf213* mRNA and protein were expressed in these cells, with no differences between Akita and min-6 cell lines (Fig. 1D and E).

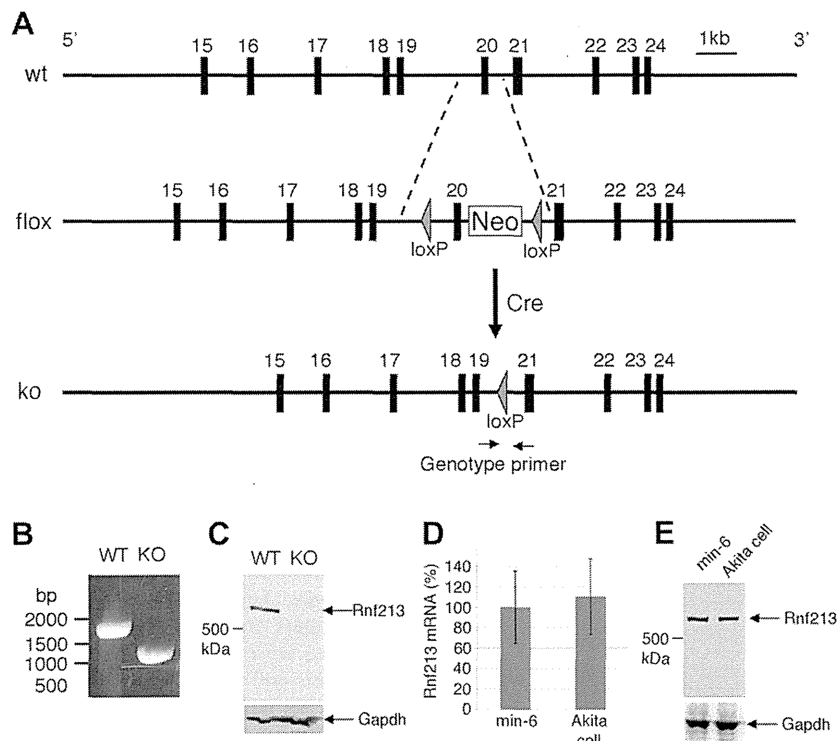

Fig. 1. Generation of *Rnf213* KO mice. (A) Structure of the endogenous mouse *Rnf213* gene, the targeted allele, and the disrupted allele. (B) PCR genotyping of WT and KO mice. (C) *Rnf213* immunoblotting of pancreas extracts from WT and KO mice. (D) Quantitative RT PCR for *Rnf213* in Akita and min-6 cells. Data are shown as mean ± SD. (E) *Rnf213* immunoblotting of extracts from Akita and min-6 cells. Membranes were immunoblotted with antibody to GAPDH as a loading control.

3.3. Body weight over time

The mean body weight of KO/Akita mice was lower than that of Akita mice between 6 and 9 weeks of age, although they did not differ after 10 weeks of age (Fig. 2A). The mean body weights of both KO/Akita and Akita mice were significantly lower than those of KO and WT mice. Between 6 and 20 weeks of age, there were no differences in body weight between KO and WT mice.

3.4. Blood glucose level and glucose tolerance

From 6 to 20 weeks of age, blood glucose concentrations after a 16 h fast were consistently and significantly lower in KO/Akita than in Akita mice (Fig. 2B). Moreover, blood glucose levels after a 6 h fast were significantly lower in 18 week old KO/Akita (348 ± 153 mg/dL) than in Akita (572 ± 42 mg/dL) mice, although both were significantly higher than in KO (140 ± 32 mg/dL) and WT (147 ± 22 mg/dL) mice (Fig. 2C). GTT at 18 weeks showed that glucose tolerance in KO/Akita (Area under the curve [AUC] 49298 ± 8864 mg min/dL) mice was impaired relative to KO (AUC 22179 ± 1516 mg min/dL) and WT (AUC 18284 ± 1170 mg min/dL) mice, but was better than in Akita mice (AUC 62346 ± 9105 mg min/dL) (Fig. 2D and E). These results indicated that deletion of *Rnf213* led to improvements in glucose tolerance in Akita mice. We also investigated the insulin sensitivity of KO/Akita mice. ITT at 18 weeks of age revealed no difference in insulin sensitivity among the KO/Akita, Akita, KO and WT strains (Fig. 2F).

3.5. Plasma insulin and proinsulin concentrations

Plasma insulin concentrations were significantly higher in 18 weeks old KO/Akita (1300 ± 270 pg/mL) than in Akita mice (54 ± 14 pg/mL) after a 6 h fast, but were similar in KO/Akita, KO (1466 ± 323 pg/mL) and WT (783 ± 93 pg/mL) mice (Fig 3A). Plasma insulin concentrations after fasting for 6 h and 16 h showed a significant and positive correlation with blood glucose concentrations in KO/Akita ($R = 0.50$, $p = 0.0009$), but not in Akita ($R = 0.26$, $p = 0.275$), mice (Fig. 3B), indicating that insulin secretion was responsive to increased blood glucose in KO/Akita, but not in Akita, mice. The plasma ratios of proinsulin/insulin concentrations did not differ significantly among KO/Akita, KO and WT mice (Supplemental Fig. 2A). Proinsulin was not detected in the plasma of Akita mice.

3.6. Food intake and plasma leptin concentration

Male Akita mice develop more profound diabetes than female Akita mice. Castration of male Akita mice alleviated such sex differences by reducing hyperphagia [16]. We have shown that castration normalized hyperphagia by acting on plasma leptin and normalizing anorexigenic proopiomelanocortin (POMC) [16]. To examine the regulation of feeding, we measured food consumption and plasma leptin concentration. Food consumption by KO/Akita mice (3.92 ± 0.78 g/day) was similar to that by KO (3.25 ± 0.33 g/day) and WT (3.06 ± 0.23 g/day) mice, but was 34% lower than by Akita mice (5.96 ± 0.68 g/day) (Fig. 3C). Plasma leptin concentrations were similar in KO/Akita (353 ± 226 pg/mL) and Akita (348 ± 43 pg/mL) mice, but lower than in KO (741 ± 156 pg/mL) and WT (744 ± 145 pg/mL) mice (Fig. 3D), suggesting that decreased food consumption in KO/Akita mice was likely attributable to elevated insulin concentration, which stimulates overlapping insulin-leptin signal pathways in the central nervous system to suppress appetite [17].

3.7. Pancreatic insulin and proinsulin concentration

Total pancreatic insulin levels were significantly higher in KO/Akita (2689 ± 746 ng/pancreas) than in Akita (1102 ± 43 ng/pancreas) mice, although they were about one-fifth of those in KO (14,434 ± 3359 ng/pancreas) and WT (13,348 ± 2500 ng/pancreas) mice (Fig. 4A). Pancreatic proinsulin contents were also signifi-

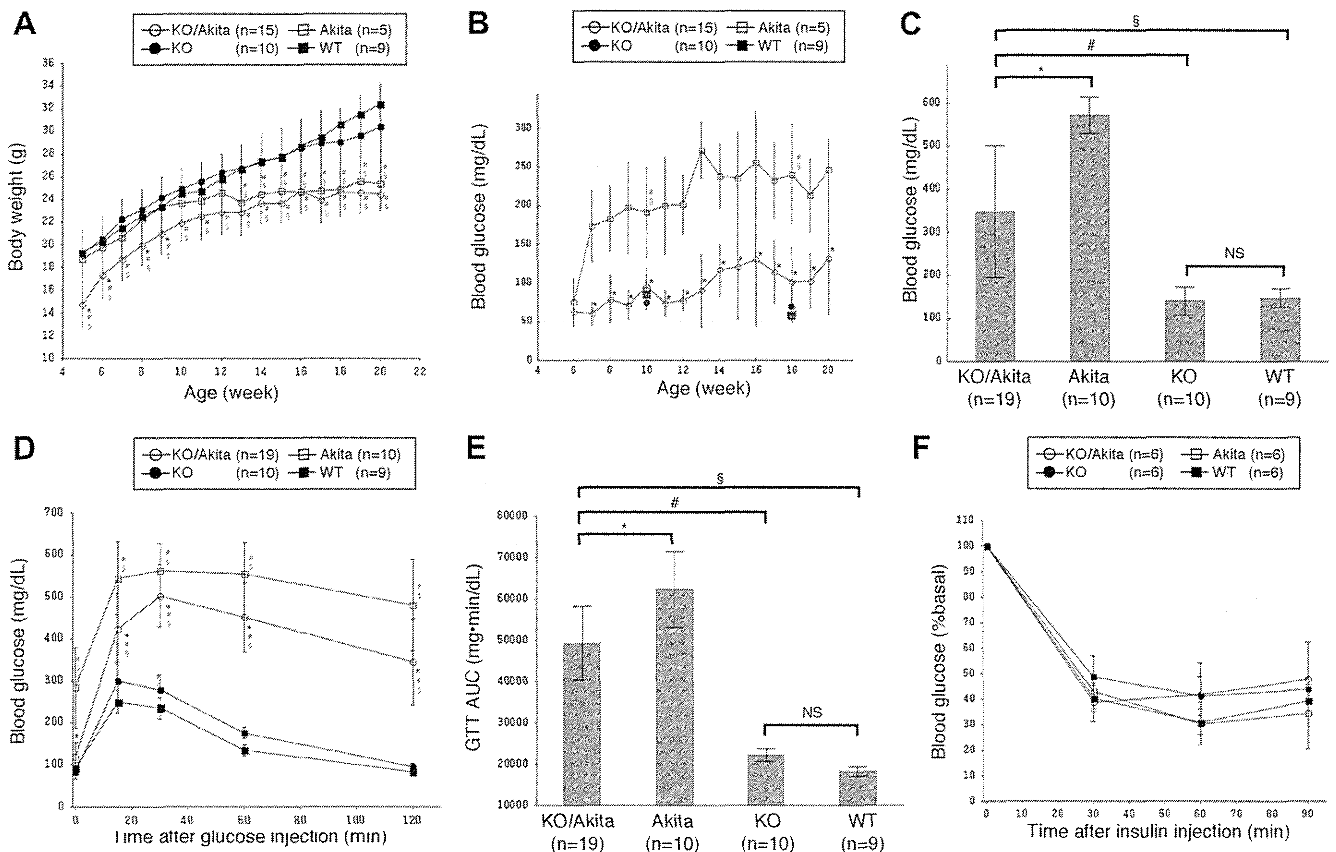

Fig. 2. Mouse growth curves, blood glucose concentrations, GTT and ITT. (A) Time course of body weight of KO/Akita, Akita, KO, and WT mice from 6 to 20 weeks of age. (B) Time course of 16 h fasting blood glucose concentrations in KO/Akita, Akita, KO and WT mice from 6 to 20 weeks of age. Glucose concentrations in KO and WT mice were measured at 10 and 18 weeks of age. (C) Six hours fasting blood glucose concentrations in 18 week old KO/Akita, Akita, KO, and WT mice. (D and E) GTT of 18 week old KO/Akita, Akita, KO, and WT mice. Blood glucose concentrations are shown at indicated times after glucose injections. Area under the curve was calculated for these mice. (F) ITT in 18 week old KO/Akita, Akita, KO, and WT mice. Blood glucose concentrations are shown at indicated times after insulin injections. Data are shown as mean ± SD. *$p < 0.05$ vs Akita, #$p < 0.05$ vs KO, §$p < 0.05$ vs WT, NS, Not significant.

cantly higher in KO/Akita than in Akita mice (Supplemental Fig. 2B). Pancreas weight was similar in these 4 groups (Supplemental Fig. 3A).

3.8. Immunohistochemical assays of insulin and CHOP, and electron microscopy of islets

No morphological abnormalities were observed in the pancreas or islets of KO/Akita and KO mice. Immunohistochemical examination showed that a higher proportion of insulin-positive β cells was preserved in the islets of KO/Akita (0.141 ± 0.046 insulin positive cells/islet) than of Akita (0.088 ± 0.042 insulin positive cells/islet) mice, although both were lower than in KO (0.643 ± 0.080 insulin positive cells/islet) and WT (0.616 ± 0.076 insulin positive cells/islet) mice (Fig. 4B). Mean islet area did not differ among KO/Akita, Akita, KO and WT mice (Supplemental Fig. 3B).

CHOP is an ER stress-inducible transcription factor that promotes apoptosis [18] and that has been used as a marker of ER stress-mediated apoptosis in β cells of Akita mice [19]. To test whether ER stress occurs in the β cells of KO/Akita mice, we assayed for CHOP immunohistochemically. The percentage of CHOP-positive cells in islets was significantly lower in KO/Akita (0.102 ± 0.042 CHOP positive cells/islet) than in Akita (0.135 ± 0.037 CHOP positive cells/islet) mice, but were much lower in KO (0.002 ± 0.000 CHOP positive cells/islet) and WT (0.002 ± 0.000 (CHOP positive cells/islet) mice (Fig. 4C), indicating that ER stress is lower in the β cells of KO/Akita mice.

Electron microscopy of β cells in WT mice revealed abundant mature secretory granules in the cytoplasm, inconspicuous ER, and intact mitochondria with cristae (Fig. 4D, WT). KO mice showed no morphological abnormalities (Fig. 4D, KO). In contrast, examination of Akita mice showed a small number of secretory granules, a tubulovesicular structure comprised of markedly enlarged ER, and swelling or disruption of mitochondria (Fig 4D, Akita), indicators of insulin secretory pathway impairment and ER stress. Unlike Akita mice, KO/Akita mice showed mild ER enlargement and slight swelling of the mitochondria in β cells, although the number of secretory granules was markedly reduced (Fig. 4D, KO/Akita), suggesting less ER stress in the β cells of these mice than in Akita mice. The α cells of KO/Akita, Akita, KO and WT mice were morphologically similar (data not shown).

4. Discussion

We have shown here that targeted disruption of *Rnf213* unexpectedly improved glucose tolerance in Akita mice, although insulin sensitivity was not altered. These findings are consistent with results showing that plasma and pancreatic insulin levels were higher in KO/Akita than in Akita mice. Moreover, disruption of *Rnf213* reduced hyperphagia by elevating plasma insulin concentrations in KO/Akita, but did not alter plasma leptin concentrations in these mice. Taken together, these findings suggest that ablation of *Rnf213* may mitigate the diabetic phenotype by preserving β cell function.

Amelioration by *Rnf213* ablation contradicts a mechanistic link between MMD and diabetes [8], if variants were associated with MMD by loss-of-function or haploinsufficiency of *RNF213*. Alterna-

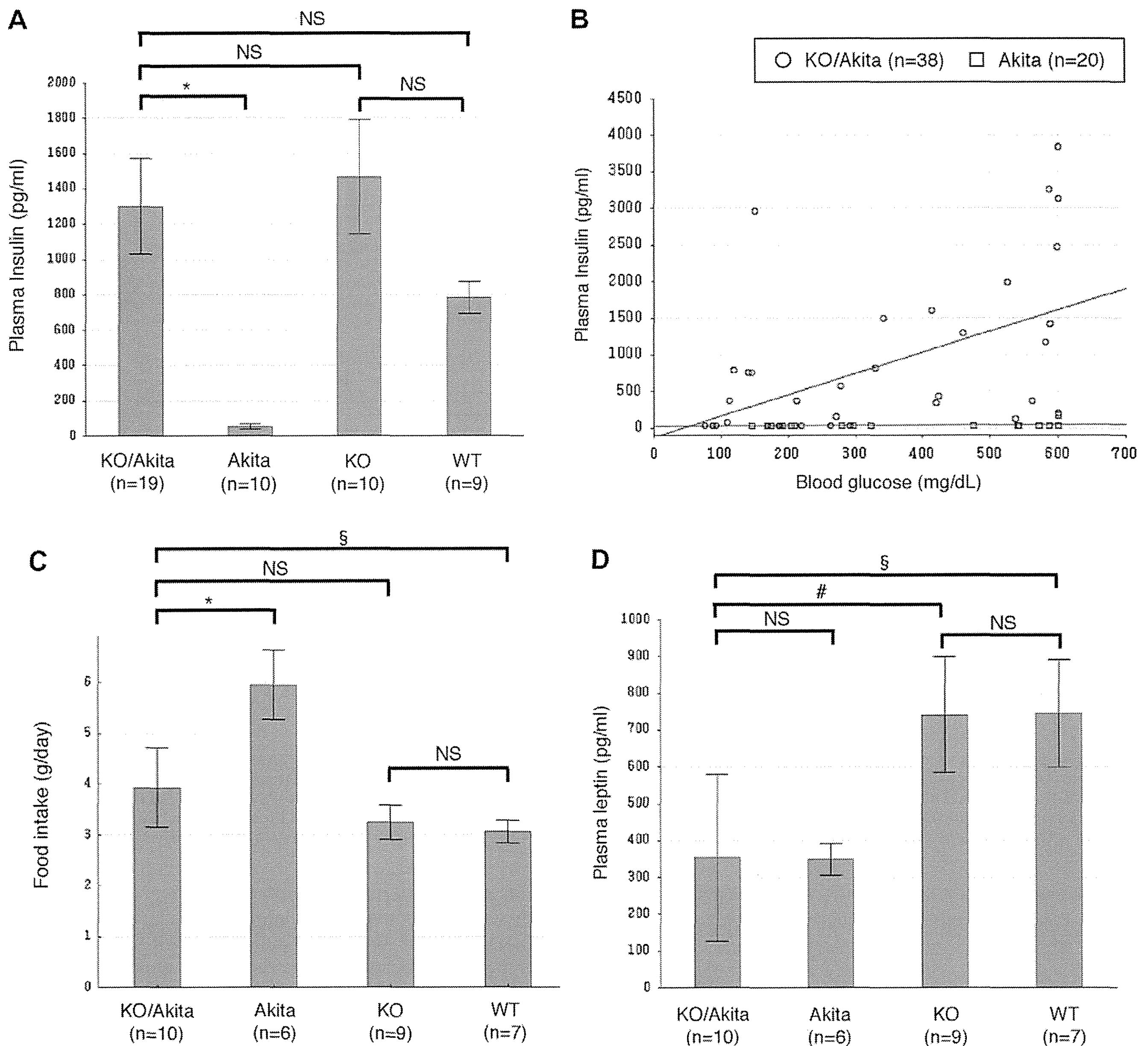

Fig. 3. Plasma insulin and leptin concentrations and food intake at 18 weeks of age. (A) Plasma insulin concentrations in KO/Akita, Akita, KO, and WT mice after a 6 h fast. Data are shown as mean ± SE. (B) Correlation between blood glucose and plasma insulin concentrations of KO/Akita and Akita mice after fasting for 6 h and 16 h (combined). (C) Food intake by KO/Akita, Akita, KO, and WT mice. (D) Plasma leptin concentrations of KO/Akita, Akita, KO, and WT mice after 16 h fasting. Data are shown as mean ± SD except for plasma insulin concentrations. $^*p < 0.05$ vs Akita, $^{\#}p < 0.05$ vs KO, $^{\S}p < 0.05$ vs WT, NS, not significant.

tively, pathological variants including R4810K of *RNF213* may cause MMD and diabetes by a gain-of-function or in a dominant-negative fashion. Among MMD predisposing diseases, diabetogenic mechanisms are well defined in MOPDII, a rare genetic disease characterized by severe growth retardation and early onset diabetes, as well as complication by MMD. Pericentrin, the causative gene for MOPDII, may regulate the intracellular distribution and secretion of insulin, and mutations of pericentrin may result in β-cell dysfunction [20]. The findings presented here indicate that β-cell dysfunction may have a mechanistic link with MMD.

Akita mice carrying a heterozygous C96Y mutation in the *Ins2* gene spontaneously develop hyperglycemia at an early age with reduced pancreatic β cell mass [12,13]. This C96Y mutation causes a conformational change in the insulin molecule, resulting in ER stress. ER stress, in turn, induces an unfolded protein response (UPR), indicating increased degradation of unfolded proteins by ER-associated degradation (ERAD), which is associated with E3 ligase and AAA + ATPase.

Recent studies [21,22] have demonstrated that the $Ins2^{C96Y}$ allele acts dominantly to enhance degradation of both the Akita and wild-type allele proinsulins by the ERAD pathway. We hypothesize that ablation of *Rnf213* may impair ERAD and lead to the sparing of wild-type proinsulin. Then we should explain how such preserved insulin secretion in KO/Akita mice reduced ER stress, as indicated by a reduction in the relative abundance of CHOP positive cells in these mice. Diabetes progresses more rapidly in male than female Akita mice [12]. This gender difference in susceptibility can be reversed by castration of males, thus suppressing hyperphagia [16]. Hyperphagia increases insulin demand due to elevated energy uptake, resulting in enhanced ER-stress with stimulated production of $Ins2^{C96Y}$. Such a vicious cycle may likely accelerate the progression of diabetes in male Akita mice. We found that

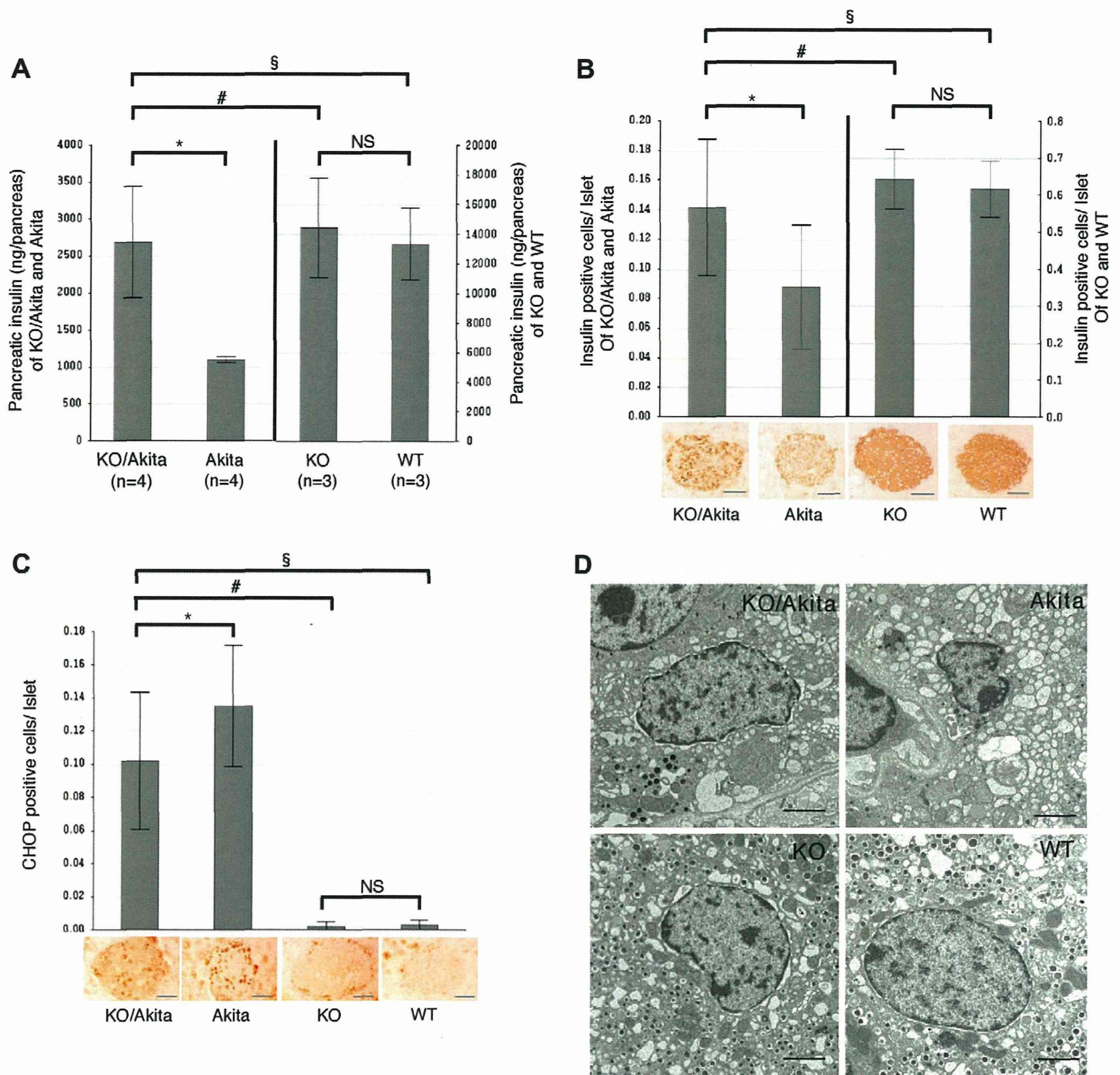

Fig. 4. Pancreatic insulin contents, insulin and CHOP immunohistochemistry, and electron microscopy of 18 week old mice. (A) Pancreatic insulin contents of KO/Akita, Akita, KO, and WT mice. (B) Representative images of islets stained with anti-insulin antibody (lower) and insulin positive cells per islet (upper) of KO/Akita ($n = 4$), Akita ($n = 4$), KO ($n = 3$), and WT ($n = 3$) mice. Quantification was performed on more than 20 islets from each mouse. Scale bar indicates 50 μm. (C) Representative images of islets stained with anti-CHOP antibody (lower) and CHOP positive cells per islet (upper) of KO/Akita ($n = 4$), Akita ($n = 4$), KO ($n = 3$), and WT ($n = 3$) mice. Quantification was performed on more than 20 islets from each mouse. Scale bar indicates 50 μm. (D) Electron micrographs of islets of KO/Akita, Akita, KO, and WT mice. Scale bar indicates 2 μm. Data are shown as mean ± SD. $^{*}p < 0.05$ vs Akita, $^{\#}p < 0.05$ vs KO, $^{\S}p < 0.05$ vs WT, NS, Not significant.

the higher serum insulin levels in KO/Akita mice were sufficient to suppress hyperphagia. Thus, *RNF213* ablation can spare wild-type insulin, thereby ameliorating this viscous cycle. Further study is warranted to test whether *RNF213* is involved in the ERAD pathway.

RNF213 is a single protein with two types of enzymatic activity, E3 ligase and AAA + ATPase [9]. AAA + ATPase is involved in various cellular processes, including vesicular transport, UPR, motor proteins and microtubule severing [11]. The association between *Rnf213* and β cell function is likely mediated by both E3 ligase and AAA + ATPase activities. The core assumption, that the normal allele of *Ins2* is also a target of degradation by ERAD, is intriguing and requires more quantitative assessment in the future. Future studies may help provide clues into a new therapeutic approach for diabetes as well as to gain insight into *RNF213* function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.015.

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Genome-wide association study of the genetic factors related to confectionery intake: potential roles of the *ADIPOQ* gene

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Running head: GWAS for confectionery intake.

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Abstract

Objective: The excessive consumption of confectionery might have adverse effects on human health. To screen genetic factors associated with confectionery-intake frequency, we conducted a genome-wide association study (GWAS) in Japan.

Design and Methods: For the discovery phase (stage 1), we conducted a GWAS of 939 non-cancer patients in a cancer hospital. Additive models were used to test associations between genotypes of approximately 500,000 single-nucleotide polymorphisms (SNPs) and the confectionery-intake score (based on intake frequency). We followed-up association signals with $P < 1 \times 10^{-5}$ and minor allele frequency > 0.01 in stage 1 by genotyping the SNPs of 4,491 participants in a cross-sectional study within a cohort (replication phase [stage 2]).

Results: We identified 12 SNPs in stage 1 that were potentially related to confectionery intake. In stage 2, this association was replicated for one SNP (rs822396; $P = 0.049$ for stage 2 and 4.2×10^{-5} for stage 1+2) in intron 1 of the *ADIPOQ* gene, which encodes the adipokine adiponectin.

Conclusions: Given the biological plausibility and previous relevant findings, the association of an SNP in the *ADIPOQ* gene with a preference for confectionery is worthy of follow-up and provides a good working hypothesis for experimental testing.

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Introduction

Confectionery or snacks are often sweet, fatty, and energy dense, so their excessive consumption might have adverse effects on human health in terms of obesity and the metabolic syndrome (1,2). Genetic as well as environmental factors have recently been implicated as correlates in the consumption of sweet foods. For example, a genetic variation in *TAS1R2*, a sweet taste-receptor subunit, was reported to affect habitual consumption of sugars in overweight and obese individuals (3), whereas a functional polymorphism of the dopamine transporter *SLC6A3* was related to the intake of high-calorie sweet foods among women with high depressive symptoms (4). Furthermore, polymorphisms of the leptin gene (*LEP*) and the leptin receptor gene (*LEPR*) were associated with sweet preference (5). Identification of genetic polymorphisms associated with a preference for sweet foods might therefore help us to understand the physiology and pathophysiology of eating behaviors and addiction to sweet foods (6,7).

However, most genetic findings to date derive from candidate-gene approaches in which biologically possible associations were tested between genetic factors and use of sweet foods. In addition to candidate-gene approaches, a few genome-wide linkage studies have addressed this topic (8,9); however, these studies used only several hundreds of microsatellite markers, so the regions of the genome identified were too broad to locate relevant genes.

Recent genome-wide association studies (GWASs) have utilized several hundreds of thousands of single-nucleotide polymorphisms (SNPs) as a means of finding genes that are potentially related to various phenotypes without prior hypotheses. GWASs have been used to identify novel candidate or target genes regulating obesity (10) and diabetes (11); however, a preference for the consumption of sweet foods has not been examined as a phenotype in recent GWASs. The present study therefore used a GWAS to screen genetic factors associated with the intake frequency of confectionery throughout the human genome, followed by a

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replication study in another independent population.

Methods and Procedures

Study participants

In the discovery phase (stage 1), we conducted a GWAS of 977 participants of the Hospital-based Epidemiological Research Program II at Aichi Cancer Center Hospital (HERPACC-II) between January 2001 and September 2005. All participants were enrolled during their first visit to the Aichi Cancer Center Hospital (ACCH; Nagoya, Japan). The framework of the HERPACC-II has been described elsewhere (12,13). Briefly, all first-visit outpatients to the ACCH aged 20–79 years were asked to fill in a self-administered questionnaire about their lifestyle and medical factors, and trained interviewers checked their responses. The outpatients were also asked to provide a blood sample. In total, 96.7% of contacted patients completed the questionnaire and about 50% of respondents provided a blood sample.

The current analyses were limited to non-cancer participants; approximately 35% of the subjects were diagnosed with cancer within 1 year of their first visit. Our previous study showed that the lifestyle patterns of first-visit outpatients without cancer corresponded well with those of individuals who were randomly selected from the general population of Nagoya city (14).

Association signals selected in the stage 1 GWAS were followed-up by genotyping the SNPs in 4,491 participants aged 35–69 years in a cross-sectional study within the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study (replication phase [stage 2]). We previously reported the detailed design of this cross-sectional study (15) and the J-MICC Study as a whole (16). In brief, participants in the current study completed a questionnaire about lifestyle and medical factors, and donated a blood sample at the time of the J-MICC

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Study baseline survey. J-MICC Study participants were recruited from 10 areas throughout Japan between 2004 and 2008, and included community citizens, first-visit patients to a cancer hospital, and health check-up examinees. The response rates for the baseline survey by study area varied according to the source population, and were recorded as 7.0–24.0% in the community (recruitment by mailing invitation letters or distributing leaflets), 58.4% in first-visit patients to a cancer hospital, and 14.0–65.5% in health check-up examinees. The respondents for the cross-sectional study comprised 400–600 participants who were enrolled consecutively from each area of the J-MICC Study, with the exception of two areas (Kyoto and Tokushima) where fewer participants were recruited.

All participants in the present study gave their written informed consent prior to inclusion. The ethics committees of Kyoto University Graduate School of Medicine (Kyoto, Japan) and Aichi Cancer Center approved the protocol for the stage 1 GWAS. The committee of Aichi Cancer Center also approved the protocols of the HERPACC-II, and the committees of Nagoya University School of Medicine (Nagoya, Japan), Aichi Cancer Center, and all participating research institutions approved the protocols of the J-MICC Study, including the current cross-sectional study. The present study was conducted in accordance with the World Medical Association Declaration of Helsinki and its later amendments.

Confectionery-intake score

We defined the intake score for confectionery as described below, and considered it as a trait in the current GWAS to seek relevant quantitative trait loci (QTLs). Participants in both stage 1 and stage 2 studies were asked to report their usual frequency of consumption of 43 food items in a self-administered questionnaire with the following eight possible responses: 1 = almost never; 2 = 1–3 times per month; 3 = 1–2 times per week; 4 = 3–4 times per week; 5 = 5–6 times per week; 6 = once per day; 7 = twice per day; and 8 = ≥ 3 times per day (17–20).

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The respondents were requested to circle one of the numbers to provide an answer. Western-style and Japanese-style confectionery were included as two separate food items in the questionnaire. The responses were then converted into intake scores of 0, 0.1, 0.2, 0.5, 0.8, 1, 2, and 3, respectively, and the sum of the two intake scores was used for association analysis. Generic or grouped questions about the consumption of confectionery (not those on individual items such as cookies and sponge cakes) are generally used in studies in Japan and have been validated through comparisons with diet records (21,22).

Genotyping

For stage 1, the DNA of each participant was extracted from the buffy-coat fraction using a DNA Blood Mini kit (Qiagen Group, Tokyo, Japan). All 977 samples were genotyped on an Illumina Human610- Quad BeadChip (Illumina, San Diego, CA) with 576,736 SNP markers at the Center for Genomic Medicine of Kyoto University Graduate School of Medicine.

We excluded two participants whose recorded gender was inconsistent with genotyping data. A further sample was excluded because the call rate was below the threshold (0.95), another was excluded because of an extremely high proportion of heterozygotes among the genotyped SNPs, and two were excluded because they were from closely related participants with a pi-hat > 0.4 (estimated using the PLINK whole-genome association-analysis toolset (23)). For each closely related pair, we excluded the member with the lower call rate. Based on principal component analysis, no outlier was identified in terms of ancestry from East-Asian populations. In addition, 32 participants were excluded because of missing data on the intake frequency of confectionery, leaving a total of 939 for the present analysis. After removing SNPs that failed the quality control (QC) criteria (Hardy–Weinberg equilibrium P-value $\geq 1 \times 10^{-6}$ [excluded SNPs: $n = 277$]; SNP call rate > 0.95 [$n = 2,921$]; and minor allele frequency [MAF] ≥ 0.01 [$n = 82,414$]), 491,738 markers were used for the analysis

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(some SNPs were excluded based on two or more criteria). The procedures used to select candidate SNPs for stage 2 are described below.

DNA samples for stage 2 were prepared from the buffy coat or whole blood using a BioRobot M48 Workstation (Qiagen Group) or an automatic nucleic-acid isolation system (NA-3000; Kurabo, Osaka, Japan). We then genotyped the 12 SNPs identified in stage 1 using the multiplex polymerase chain reaction (PCR)-based Invader assay (24) (Third Wave Technologies, Madison, WI) at the Laboratory for Genotyping Development of the Center for Genomic Medicine at RIKEN (Yokohama, Japan). The call rates for all 12 SNPs were 99.6% or higher at stage 2.

Association analysis

In stage 1, PLINK software version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (23) was used to test the association between SNP genotypes and the confectionery-intake score. We utilized standard additive models for assessing associations, and adjusted for gender and age using general linear models. The intake score was regressed on the number of minor alleles for each SNP, and the regression coefficient (β) was estimated, with gender and age included as covariates in the linear model. The SNPs were chosen if the additive model P was $< 1 \times 10^{-5}$. For SNPs within one linkage disequilibrium (LD) block (defined by pairwise $r^2 > 0.8$), we selected the one with the lowest P value. SNPs were excluded from stage 2 analysis when both of the following two conditions were satisfied: the MAF was < 0.05; and they were not within or near to (< 50 kb) a gene. The genome-wide $-\log_{10} P$ value plot (Manhattan plot) from stage 1 was depicted using Haploview version 4.2 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (25). The Q-Q plot was drawn from the PLINK output using Stata version 11.1 (Stata Corporation, College Station,

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TX).

Using stage 2 genotyping data, we repeated the association analyses carried out in stage 1. The combined datasets of stage 1 and 2 studies were analyzed in the same manner to yield pooled *P* values. For the replicated SNPs ($P < 0.05$ in stage 2), the mean confectionery intake scores were computed according to the genotype of the whole, or subgroups of, the pooled population. In this analysis, the heterozygotes were combined with the homozygotes of minor alleles because of the small number of minor homozygotes. The distribution of the intake score was skewed towards lower values compared with the normal distribution. However, the mean scores by genotype would approximate a normal distribution in accordance with the central limit theorem because of the relatively large sample size.

Statistical analyses for stage 2 and the pooled dataset were performed using Statistical Analysis System (SAS) version 9.1 (SAS Institute Inc., Cary, NC). We repeated the analysis for stage 1 using SAS, and reproduced the results obtained using the PLINK software. Accordance with the Hardy–Weinberg equilibrium was assessed by the exact test (26) using Stata software. The background characteristics of participants were compared by the *t* test or the χ^2 test. Body mass index (BMI [kg/m^2]) was calculated on the basis of self-reported height and body weight, because measured values were not available in two study areas. In the remaining eight areas, however, the BMI based on self-reported height and weight was similar to that derived from measured height and weight; the intraclass correlation coefficient between the two indices was 0.98 in both men and women. To consider the potential effect of BMI, we repeated the association analysis of data from stage 1, 2, or 1+2 with further adjustment for BMI (as a continuous variable).

We initially decided to examine the association between SNPs and the sum of the two intake scores for Western- and Japanese-style confectionery, and therefore did not run the analyses separately. However, for SNPs with $P < 0.05$ in the stage 2 study, we did conduct

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separate analyses for Western- and Japanese-style confectionery because the correlation was moderate between the intakes of the two types (correlation coefficient, 0.33 for stage 1+2 data).

Results

The participants of the stage 2 study were older and slightly more likely to be female than those of the stage 1 study, whereas former drinkers and current smokers were more prevalent in stage 1 (Table 1). BMI was slightly higher in the stage 2 study, and the confectionery-intake score was comparable between the two studies. The mean intake score ± standard deviation (SD) was 0.30 ± 0.32 for stage 1 and 0.28 ± 0.27 for stage 2. The distribution of the score was skewed towards lower values compared with the normal distribution: the median was 0.2 (interquartile range, 0.1–0.4) for stage 1 and 0.2 (0.1–0.3) for stage 2.

In stage 1, we found 22 SNPs with P values for the additive model $< 1 \times 10^{-5}$. The selected SNPs are shown as spots above the blue line at $P = 1 \times 10^{-5}$ in the genome-wide $-\log_{10} P$ value plot (Manhattan plot; Figure 1). The Q-Q plot indicates that the observed P values that were less than the cutoff (i.e., 1×10^{-5}) all deviated from the expected P value under the null hypothesis (Figure 2). The genomic inflation factor (λ) was 1.003.

Of the 22 SNPs, five were excluded because they were located within the same LD block of the selected SNPs with the lowest P value in each block. An additional five SNPs were omitted as they were not within or near to a gene (< 50 kb) and their MAF was < 0.05. Eventually, we selected 12 SNPs (Table 2) from HERPACC-II participants for the stage 2 follow-up based on predefined criteria. The genotype distributions of these 12 SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$) in both studies, with the exceptions of rs12351510 in stage 1 ($P = 0.014$) and rs2839519 in stage 2 ($P = 0.020$). The deviation from the expected

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genotype distribution, however, was relatively small, at less than 1% in both cases.

Among the 12 selected SNPs, the association of rs822396 with the confectionery-intake score was replicated in stage 2 of the J-MICC population ($P = 0.049$; Table 2). In the pooled analysis of stage 1 and 2 data, the smallest P was found for rs822396 ($P = 4.2 \times 10^{-5}$), followed by rs17042603, rs13356198, and rs1147522. The polymorphism rs822396 was shown to be an SNP in intron 1 of the *ADIPOQ* gene (IVS1-3971A>G). Further adjustment for BMI did not substantially alter the results. The P values for rs822396 in stage 1, 2, and 1+2 were 4.2×10^{-7}, 0.049, and 4.3×10^{-5}, respectively. The P values for other selected SNPs were also similar to those without BMI adjustment (data not shown), although those in stage 1 for rs10810211 and rs6039211 were 1.1×10^{-5}.

The association of the rs822396 polymorphism with the confectionery-intake score was more dominant for Japanese-style than Western-style confectionery: the respective P values in stage 1, 2, and 1+2 were 1.9×10^{-8}, 0.013, and 4.8×10^{-6} for Japanese-style confectionery, and 5.1×10^{-3}, 0.73, and 0.083 for Western-style confectionary.

We compared the mean confectionery-intake score by the rs822396 genotype (major homozygotes versus heterozygotes + minor homozygotes) and the background characteristics of participants in the pooled dataset (Table 3). The score was higher among participants with at least one minor allele than among those without. Moreover, this difference was consistently observed across the strata of gender, age, smoking and drinking habits, and BMI. It was particularly large (0.083, $P = 5.1 \times 10^{-6}$) in younger participants aged < 55 years.

Discussion

In the present study, we identified 12 candidate SNPs that were potentially related to confectionery intake in a GWAS. Among them, the association was replicated in an independent population for one SNP (rs822396 or IVS1-3971A>G) in intron 1 of the

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ADIPOQ gene.

The *ADIPOQ* gene on chromosome 3q27 encodes adiponectin, which is an adipokine that is extensively expressed in adipose tissue, and is a highly abundant plasma protein with circulating levels that are, in part, genetically controlled (27,28). Adiponectin has excited intense interest because of the robust negative correlations of its circulating levels with indices of insulin resistance and the risk of type 2 diabetes, as well as their consistent inverse associations with fat mass (28). Because central nervous insulin action might be related to inhibition of eating behavior, and might be negatively correlated with peripheral insulin resistance and obesity (29), insulin resistance associated with hypoadiponectinemia could be involved in increases in food intake.

In addition to its potential role as an insulin-sensitizing adipokine, it is hypothesized that adiponectin plays an important role in the regulation of energy homeostasis, including appetite stimulation (27,30), although its effects on food intake show considerable diversity across studies (30). Collectively, however, previous studies show that adiponectin directly and/or indirectly affects eating activities, which might partly explain our current findings.

Physiologically, adiponectin has been shown to stimulate food intake by activating AMP-activated protein kinase (AMPK) in the arcuate hypothalamus via its receptor AdipoR1 (27,31). The putative downstream pathways for food-intake regulation in response to hypothalamic AMPK are acetyl-coenzyme A carboxylase/ malonyl-coenzyme A/carnitine palmitoyltransferase-1/fatty-acid oxidation and mammalian target of rapamycin signaling (31). However, it remains to be investigated whether adiponectin specifically affects consumption of energy-dense foods such as confectionery. Our relatively broad screening approach with a cutoff of $P < 1 \times 10^{-5}$ in stage 1 might have resulted in the observed low replication rate of only one of the 12 candidate SNPs identified in stage 1. Nevertheless, this replicated SNP appears to be biologically plausible as an

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