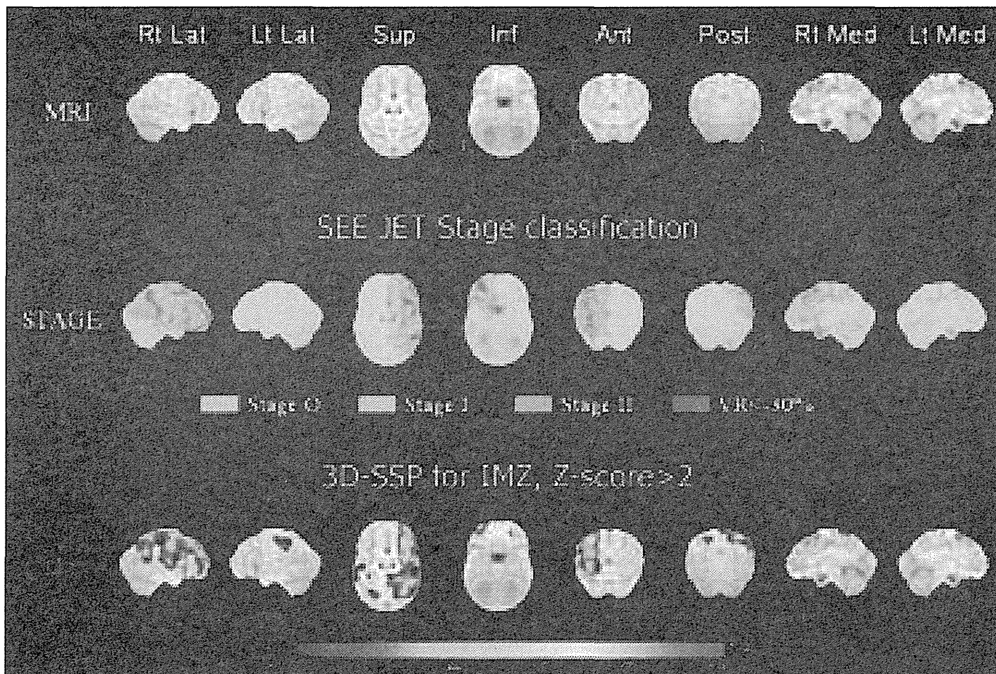

図4 72歳，男性，右頸部内頸動脈狭窄症のIMP-SPECTのSEE-JET解析とIMZ-SPECTの3D-SSP解析（全脳で正規化されたGLB画像での検討，Z-score＞2を有意）の比較

一過性脳虚血発作（左片麻痺）にて発症し来院した．右頸部内頸動脈高度狭窄症（アテローム血栓性）と診断された．IMP-SPECT SEE-JET解析では，右前大脳動脈・中大脳動脈領域に広範な血行力学的脳虚血Stage II（misery perfusion）が認められたが，IMZ-SPECT 3D-SSP（Z-score）解析では主として前大脳動脈と中大脳動脈の境界領域に，皮質神経細胞の脱落領域（不完全脳梗塞）が認められた．

解析を行い，その特異的所見について検討した[10]．

高次脳機能障害と判定された6症例では，正常群18例との群間比較により両側前頭葉内側に皮質神経細胞の脱落領域が認められた（**図5**）．さらに，個々の症例における前頭葉の脳皮質損傷の広がりの程度を脳回のレベルで評価することを目的として，SEE Level 3による脳回レベルでの解析を行い，主として両側前頭葉内側の内側前頭回（medial frontal gyrus: MFG）と前方帯状回（anterior cingulate gyrus: ACG）における脳皮質損傷の広がりの程度を評価したところ，4症例の両側MGF，1症例の一側MGF，2症例の両側ACGに有意な皮質神経細胞の脱落が見られた（**図6**）．一方，高次脳機能障害と判定されなかった12症例では，正常群との群間比較により両側前頭葉内側には皮質神経細胞の脱落領域は認められなかった（**図5**）．SEE Level 3による脳回レベルでの解析では，2症例の両側MGF，4症例の一側MGF，2症例の一側ACGに軽度の皮質神経細胞の脱落が見られた．

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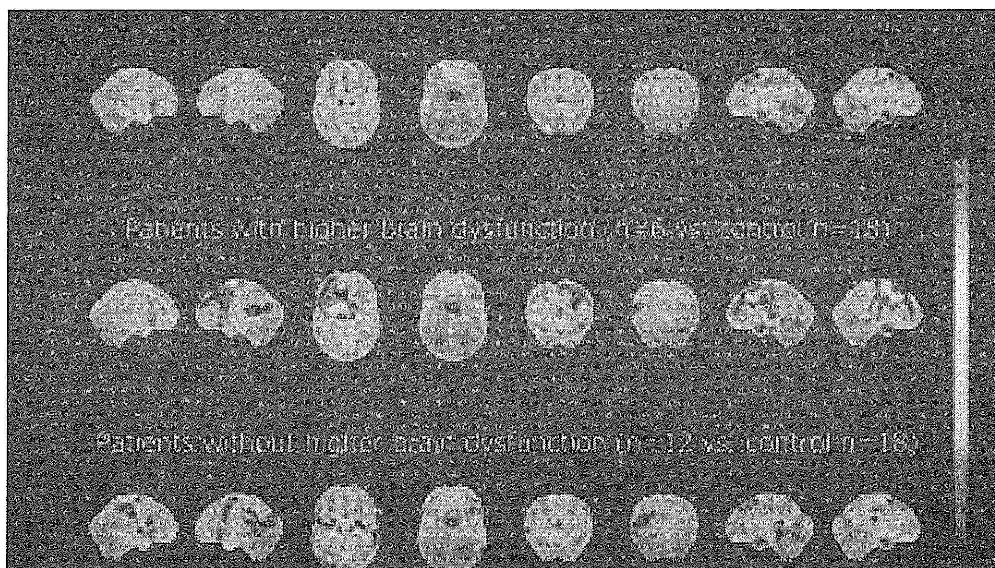

図5 高次脳機能障害と判定されたもやもや病6症例および判定されなかった12症例と正常群（18例）との群間比較（IMZ-SPECT 3D-SSP解析，全脳で正規化されたGLB画像での検討，Z-score＞2を有意）

高次脳機能障害と判定されたもやもや病6症例では，両側前頭葉内側に統計的に有意（Z score＞2）な皮質神経細胞の脱落領域が認められたが，高次脳機能障害と判定されなかった12症例では，両側前頭葉内側には有意な皮質神経細胞の脱落領域は認められなかった．

もやもや病の高次脳機能障害例では，両側前頭葉内側に皮質神経細胞の脱落が認められたが，その機序として，もやもや病の前方循環における血行力学的脳虚血の重症度と長期にわたる持続期間が関与し「不完全脳梗塞」が生じたものと考えられた．現在，IMZ-SPECTの統計画像解析についても，QSPECTパッケージソフトを用いた正常群データベースが作成され，SPECT機器の違いを超えた診断の標準化が進展しつつある．

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Extent	>10%	10%>,>1%	0%
Medial Frontal Gyrus (MFG): bilateral	4	1	0
:unilateral	1	0	0
Anterior Cingulate Gyrus (ACG) :bilateral	2	2	2
:unilateral	0	0	0

図6　高次脳機能障害と判定されたもやもや病6症例のIMZ-SPECTに関するSEE Level 3による脳回レベルでの解析（正常群DBの検討から，各脳回における異常（Z-score＞2）を示すpixelの割合（Extent）が＞10%を有意，10%＞，＞1%以下を軽度と評価）

両側前頭葉内側の内側前頭回（MFG）と前方帯状回（ACG）における脳皮質損傷の広がりの程度を評価したところ，4症例の両側MGF，1症例の一側MGF，2症例の両側ACGに有意な皮質神経細胞の脱落，1症例の両側MGF，2症例の両側ACGに軽度の皮質神経細胞の脱落が見られた．

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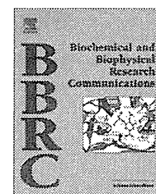

Ablation of *Rnf213* retards progression of diabetes in the Akita mouse

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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).

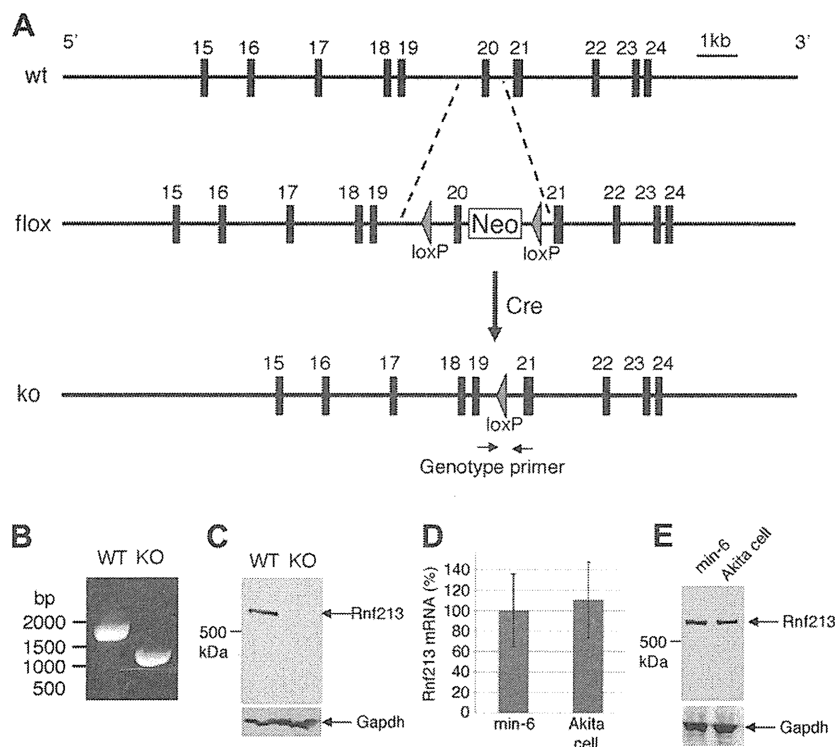

Fig. 1. Generation of *Rnf213* KO mice. (A) Structure of the endogenous mouse *Rnf213* gene, the targeted allele, and the disrupted allele. (B) PCR genotyping of WT and KO mice. (C) *Rnf213* immunoblotting of pancreas extracts from WT and KO mice. (D) Quantitative RT PCR for *Rnf213* in Akita and min-6 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-

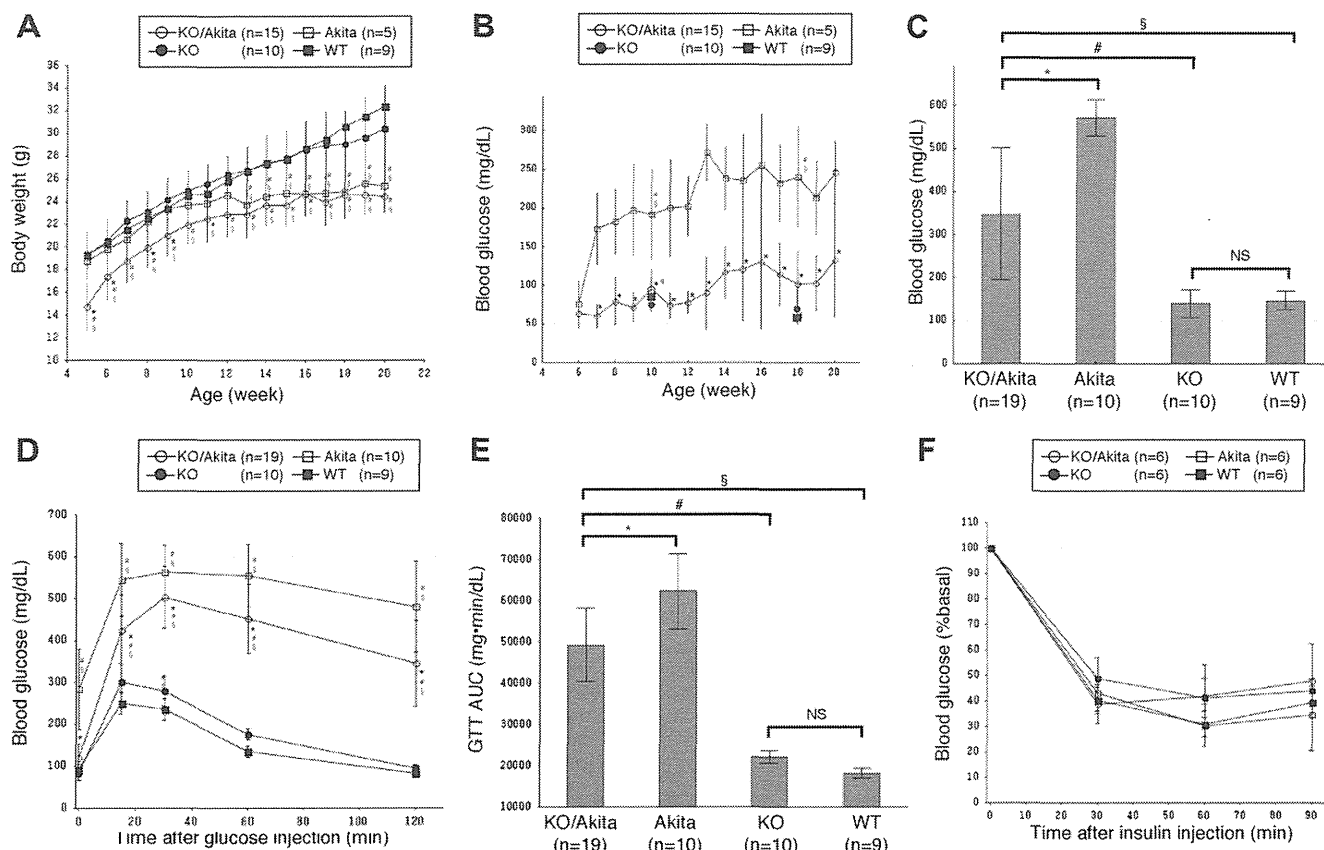

Fig. 2. Mouse growth curves, blood glucose concentrations, GTT and ITT. (A) Time course of body weight of KO/Akita, Akita, KO, and WT mice from 6 to 20 weeks of age. (B) Time course of 16 h fasting blood glucose concentrations in KO/Akita, Akita, KO and WT mice from 6 to 20 weeks of age. Glucose concentrations 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, $^{\S}p < 0.05$ 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-

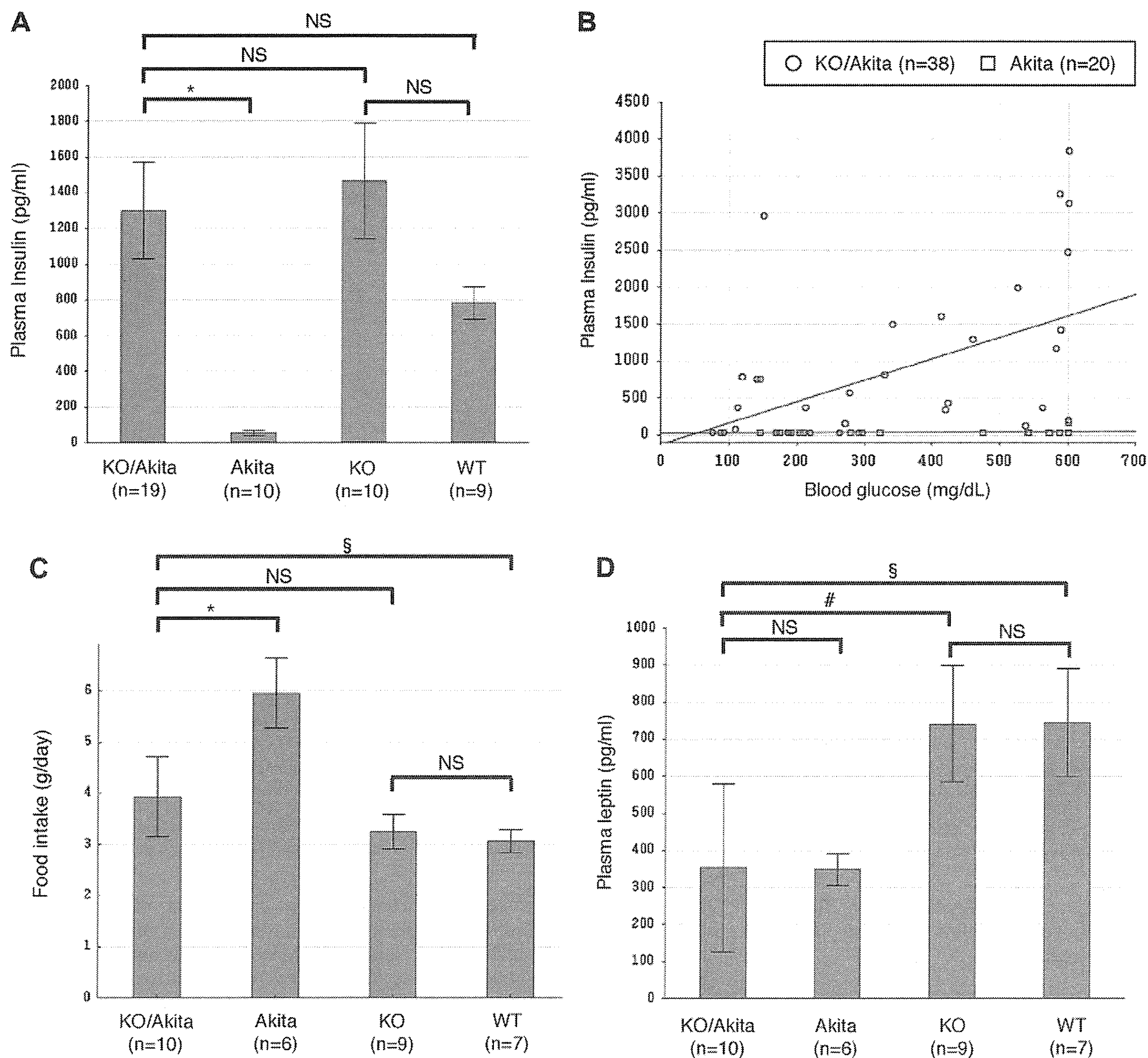

Fig. 3. Plasma insulin and leptin concentrations and food intake at 18 weeks of age. (A) Plasma insulin concentrations in KO/Akita, Akita, KO, and WT mice after a 6 h fast. Data are shown as mean ± SE. (B) Correlation between blood glucose and plasma insulin concentrations of KO/Akita and Akita mice 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

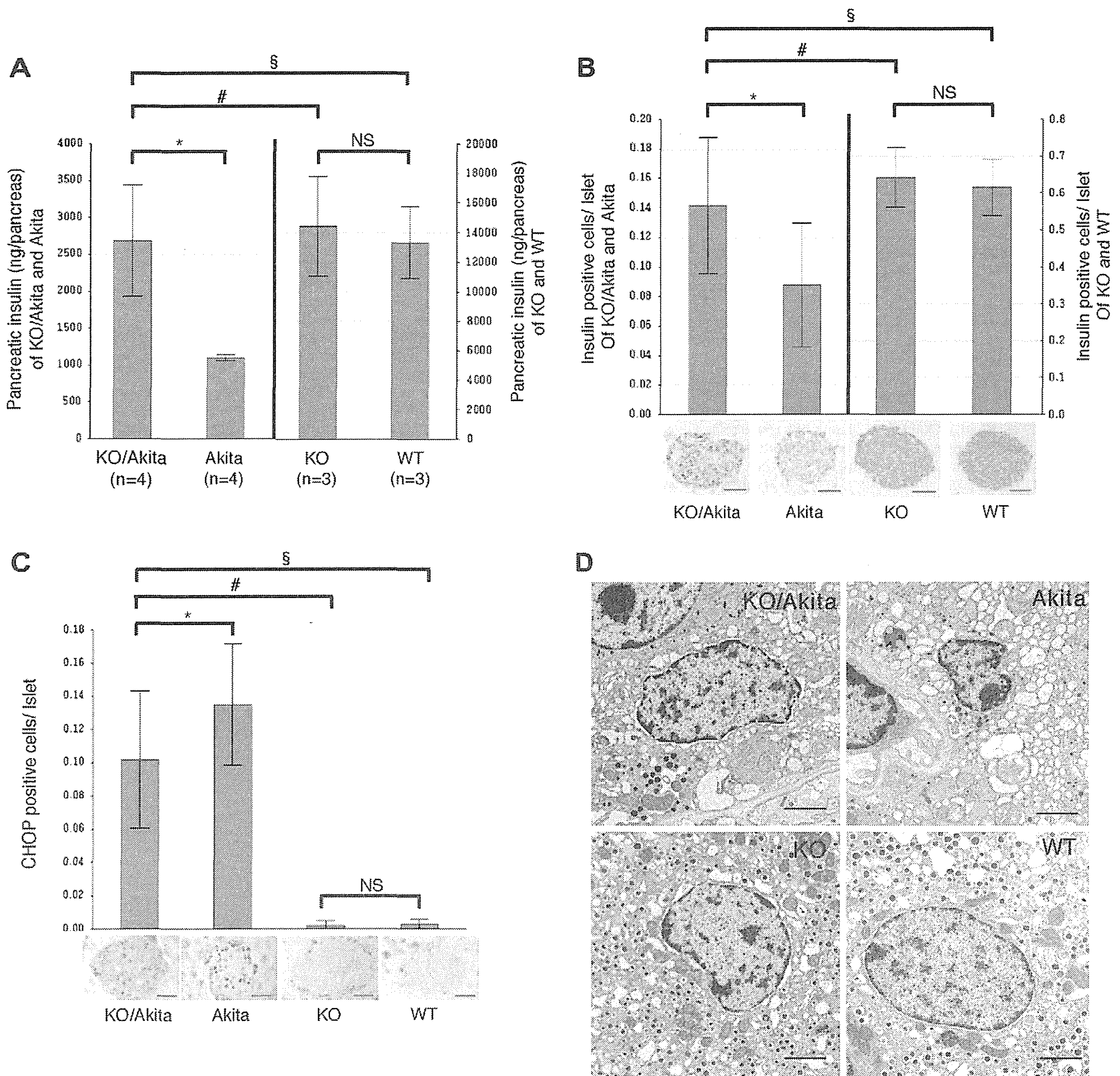

Fig. 4. Pancreatic insulin contents, insulin and CHOP immunohistochemistry, and electron microscopy of 18 week old mice. (A) Pancreatic insulin contents of KO/Akita, Akita, KO, and WT mice. (B) Representative images of islets stained with anti-insulin antibody (lower) and insulin positive cells per islet (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.

Acknowledgments

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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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REGULAR ARTICLE

P.R4810K, a polymorphism of *RNF213*, the susceptibility gene for moyamoya disease, is associated with blood pressure

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Abstract
Background Moyamoya disease—an idiopathic vascular disorder of intracranial arteries—is often accompanied by hypertension. *RNF213* has been identified as a susceptibility gene for moyamoya disease. In the present study, the association of p.R4810K (G>A) with blood pressure (BP) was investigated in a Japanese population.
Methodology/principal findings Three independent study populations, the Nyukawa ($n = 984$), Noshiro ($n = 2{,}443$) and Field ($n = 881$) studies, joined this study. BP, body weight and height were measured. Past and present symptoms and disease and medication histories were assessed by interview. Associations of p.R4810K (rs112735431, ss179362673) of *RNF213* with BP were investigated. Two linkage disequilibrium blocks were constructed for moyamoya patients with p.R4810K ($n = 140$) and the general population ($n = 384$) using 39 single nucleotide polymorphisms (SNPs) spanning 390 kb around *RNF213*. A total of 60 carriers (3 for AA genotype and 57 for GA genotype) were found in these samples, and the minor allele frequencies were 1.4 % in the Nyukawa and Field studies and 0.2 % in the Noshiro study. Regression analyses adjusted for age, sex and body mass index based on an additive model demonstrated significant associations with systolic BP (mmHg/allele): β (standard error) was 8.2 (2.9) in the Nyukawa study ($P = 4.7 \times 10^{-3}$), 18.7 (5.4) in the Noshiro study ($P = 4.6 \times 10^{-4}$) and 8.9 (2.0) ($P = 1.0 \times 10^{-5}$) in the three populations. In contrast, diastolic BP showed significant associations only in the Noshiro study. Linkage disequilibrium blocks contained none of the BP-associated proxy SNPs reported by previous studies.
Conclusions/significance Our study suggests that p.R4810K of *RNF213* is associated strongly with systolic BP.

Keywords *RNF213* · Moyamoya disease · P.R4810K · Systolic blood pressure · Hypertension

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Introduction

Moyamoya disease is an idiopathic occlusive vascular lesion that occurs at the terminal portion of internal carotid arteries in the circle of Willis [1, 2]. Familial clustering has been reported for moyamoya disease, with 15 % of cases being reported to have a family history [3]. This familial clustering led to searches for genetic factors, which identified several loci, including 3p24–p26 [4], 6q25 [5], 8q23 [6] and 17q25 [7, 8]. The locus on 17q25.3 was further refined by a linkage analysis [9] and an association study

[10]. Finally, *RNF213* was identified as the susceptibility gene of the 17q25.3 locus for moyamoya disease [11].

Several polymorphisms specific to Japanese, Korean, Chinese and Caucasian patients with moyamoya disease have been found in *RNF213* [11]. Of these, the p.R4810K (rs 112735431, ss179362673; G>A) polymorphism is a founder variant found commonly in Japanese, Korean and Chinese patients. In particular, it is found at rates of >90 % in Japanese patients and approximately 80 % in Korean patients. More surprisingly, 2–3 % of the Japanese and Korean general populations are carriers of this variant [11]. Given that the prevalence of moyamoya disease in patients is 10.5 per 10^5 people [12] and the rate of carriers of p.R4810K is 3 %, only a minor portion of carriers will develop moyamoya disease.

We conducted molecular cloning of a full-length cDNA of *RNF213* and found that it comprises 5,207 amino acids. *RNF213* has two well-known domains, a RING finger domain and a Walker motif that show ubiquitin ligase activity and ATPase activity, respectively [11]. *RNF213* was shown to be a novel functional E3 ligase [11]. Two splicing variants of *RNF213* were detected ubiquitously in cDNAs isolated from various types of cells, including vascular endothelial cells [11]. After knockdown of *RNF213* in zebrafish embryos, severely abnormal sprouting vessels were seen in the cranial vessels. However, the p.R4810K variant had no apparent detrimental effects on E3 ligase activity.

Moyamoya disease is often accompanied by hypertension [13, 14]. The pathogenesis of hypertension in patients with moyamoya disease has been attributed to stenosis of the renal arteries, i.e., renovascular hypertension. However, in some cases, hypertension was reported to occur without stenosis of the renal arteries [15]. Such observations suggest that hypertension in moyamoya disease may be caused by a novel mechanism associated with *RNF213* variants. If this is the case, it can be hypothesized that hypertension is a phenotype of p.R4810K carriers.

In the present study, we investigated whether p.R4810K elevates blood pressure (BP) in p.R4810K carriers. To examine this possibility, we conducted a genetic epidemiological study in three independent populations.

Methods

Ethical statement

Ethical approval for the three independent studies was given by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (Approval number: G140 approval date 10/18/2004). All the subjects provided written informed consent. All the studies involved the use of humans and adhered to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects.

Study population

Hypertension was defined as systolic blood pressure (SBP) of ≥140 mmHg and/or diastolic blood pressure (DBP) of ≥90 mmHg or treatment with antihypertensive medication. The pulse pressure (PP) was calculated as the difference between SBP and DBP.

Samples were donated by participants in three independent studies: the Nyukawa, Noshiro and Field studies. The Nyukawa and Noshiro studies are cohort studies to investigate common diseases. An annual health check-up is provided to the residents as a subsidy program run by the local autonomy at its public health center under the Industrial Safety and Health Act or Elderly Health law. Weight was measured in light clothes on an electrical balance, and height was measured with a stadiometer. Body mass index (BMI) was computed as weight in kilograms divided by the square of the height in meters. BP was measured on the right arm of seated participants by public health nurses after 5 min of rest at each examination using a mercury sphygmomanometer. BP was measured once or more than once when the readings were much higher than past records, i.e., 10 mmHg for SBP and 5 mmHg for DBP. In cases with more than one reading, the later BP reading was used. Clinical data such as past history of medication, past disease history, past and present symptoms, and demographic data (age and birth date) were collected by interview by public health nurses during the clinical examinations. The Nyukawa study started in 2004 in Nyukawa town in Gifu prefecture and has continued to the present, while the Noshiro study started in 1998 in Noshiro city in Akita prefecture and has continued to the present. All participants who underwent health check-ups at local health centers were recruited, and >95 % of the potential candidates agreed to join the present study. The participants donated blood samples at start of the studies and data collected at the start of the studies were used. Although these two studies are cohort studies, the study design for the present study was a cross-sectional association.

The Field study started in the early 1990s and ended in the mid-1990s as an environmental and nutritional study designed to evaluate exposure to environmental pollutants through dietary routes in nationwide general populations [16, 17]. Participants were recruited from among farmers by local community farming support stations. More than 90 % of subjects agreed to join the study. Owing to its focus on household information, such as dietary habits and

sources of vegetables, we selectively recruited more unrelated housewives than males. The researchers visited local towns and collected blood samples. Weight was measured in light clothes on a scale and height was measured with a stadiometer. BP was measured on the right arm of seated participants by physicians after 5 min of rest at each examination using a mercury sphygmomanometer. BP readings were measured once, and more than once when readings were much higher (i.e., 10 mmHg for SBP and 5 mmHg for DBP) than the values anticipated by the participants. In cases with more than one reading, the later BP reading was used. Clinical data such as past history of medication, past disease history, past and present symptoms, and demographic data (age and birth date) were collected by interview by physicians during the participant's visit. The samples and data were donated to the Kyoto Specimen sample bank [18, 19]. Since the minor allele frequency (MAF) of p.R4810K in the general population was significantly lower for the Noshiro population (0.2 %) than for the Nyukawa population (1.4 %), we selected populations in the western part of Japan in the Field study, i.e., Niigata, Ishikawa, Toyama, Tokyo, Gunma, Nagano, Aichi, Shiga, Kyoto, Kochi, Ehime, Shimane, Yamaguchi and Kagoshima.

We excluded from the analyses subjects with chronic kidney disease, polycystic kidney diseases, diabetic nephropathy and secondary hypertension based on the information obtained by interview. Blood-relatedness of the participants was checked by address, family names and interviews. If there was blood-relatedness among participants, one of the blood-related participants was selected and others were excluded from the analyses. For carriers, interview sheets describing blood-relatedness among carriers within the same communities were re-reviewed. Carriers or related family members were re-interviewed if needed.

Linkage disequilibrium around ss179362673

In the Japanese population, haplotypes carrying p.R4810K in *RNF213* were reported to be derived from a common founder [11]. We investigated linkage disequilibrium (LD) blocks in unrelated Japanese controls ($n = 384$) in the Kyoto and Osaka areas in western Japan, and in Japanese patients with p.R4810K and moyamoya disease ($n = 140$) [11]. Thirty-two single nucleotide polymorphisms (SNPs) (rs6565649, rs7216577, rs7406843, rs8078855, rs7217421, rs9902702, rs11869363, rs12451808, rs55996424, rs7222014, rs35968416, rs4890012, rs12150356, rs8070106, rs4889848, rs6565683, rs9913006, rs6565686, rs8065843, rs4074303, rs4890025, rs11869626, rs9898443, rs12601738, rs12185227, rs7502866, rs9911978, rs12950635 rs4890047, rs4889863, rs11655474, rs8080957) were selected on the basis of information in the Hapmap database (http://hapmap.ncbi.nlm.nih.gov/) to cover a region spanning 390 kb from the 5′ end of *SGSH* to the 5′ end of *Raptor*. These 32 SNPs were selected to illustrate the LD blocks using the Tagger program, with criteria of $r^2 > 0.65$ and a MAF of >0.05 in Japanese people. Typing for these 32 SNPs plus 7 rare SNPs (ss179362670, ss179362671, ss179362672, ss179362673, ss179362674, ss179362675, ss161110142) was selected. Each LD block was constructed using SNP & Variation Suite V7. LDs were evaluated by D'.

Genotyping and quality control

Genomic DNA was extracted from blood samples with a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). Genotyping of the 39 SNPs was conducted using TaqMan probes (TaqMan SNP Genotyping Assays; Applied Biosystems, Tokyo, Japan) using a 7300/7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) as previously reported [11]. Data were cleaned using a quality control process. This process included evaluation of sample and marker call rates, mismatches of sex, age and BP readings, past histories, duplicates and batch effects. Sample relatedness and population stratification were not evaluated genetically. The genotyping results for p.R4810K were confirmed by direct sequencing by the Sanger method in 42 subjects out of 140 patients, as previously reported [11]. The genotype of p.R4810K in two independent genotyping procedures was perfectly matched for control 384 subjects. The call rates for the 39 SNPs were 100 %.

Statistical analysis

Hypertension was analyzed as a binary trait (cases vs controls) using a logistic model under an additive model. Association of p.R4810K with BP was also analyzed by an additive model as a quantitative trait using linear regression models. For subjects taking antihypertensive therapies, BPs were imputed by adding 10 and 5 mmHg to the SBP and DBP values, respectively [20]. Unless otherwise specified, analyses were based on the protocol used in the large studies [21–23]: adjustment for age, sex and BMI: inclusion of subjects taking antihypertensive medications and imputation of their BP. Genetic association analyses were performed using SNP & Variation Suite V7 (Golden Helix, Bozeman, MT). In the analyses, we tested additive and dominant models. Odds ratio was calculated by the SNP & Variation Suite for dichotomous phenotypes. Hardy-Weinberg equilibrium (HWE) was tested in entire populations. The exclusion criteria was HWE $<10^{-3}$.

A Bonferroni correction of $P = 0.05/2 = 0.025$ was applied to the regression analysis. This correction was selected because two parameters, SBP and DBP, were independent, while the other parameters (PP and adjusted

values) were derivatives of these values. Other statistical analyses were conducted using SAS software (SAS Institute, Cary, NC). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Demographic characteristics of three populations

The demographic characteristics of the participants in the three studies are summarized in Table 1. The numbers of participants were 984 for the Nyukawa study, 2,443 for the Noshiro study and 881 for the Field study. The percentages of patients taking antihypertensive agents or diuretics ranged from 10.8 % in the Field study to 15.8 % in the Noshiro study. The percentages of patients with hypertension ranged from 12.8 % in the Field study to 17.6 % in the Noshiro study.

The MAFs of p.R4810K were 1.4 % in the Nyukawa and Field studies, and 0.2 % in the Noshiro study (Table 2). The MAF was significantly lower in the Noshiro study than in the Nyukawa and Field studies (Fisher's exact test, $P < 1 \times 10^{-5}$). Based on interview, none of the carriers had any symptoms related to moyamoya disease or past history of stroke.

Blood-relatedness checked by address and family names did not exclude any subjects in three studies. None of the 60 carriers were confirmed to be blood-related by re-review of the interview sheets.

Table 1 Demographic characteristics of the three samples

Characteristics	Nyukawa study	Noshiro study	Field study
Observation period	2004-present	1998-present	1990s
Number of participants	984	2,443	881
Men:women	425:559	1,229:1,214	188:693
Age (years)	56.1 ± 15.1	49.9 ± 14.0	50.8 ± 10.4
Body mass index (BMI)	22.3 ± 2.9	23.3 ± 3.1	23.2 ± 3.0
SBP (mmHg)	125.2 ± 19.2	126.6 ± 18.2	129.1 ± 13.8
DBP (mmHg)	72.7 ± 12.1	77.5 ± 11.9	76.6 ± 10.3
% Taking antihypertensive or diuretics	10.9	15.8	10.8
% Hypertension	15.5	17.6	12.8

Data are shown as values or mean ± standard deviation (SD)

Table 2 Association of p.R4810K with hypertension by additive model

Study	p.R4810K genotype (G>A)						Dichotomous	
	AA	GA	GG	Total	MAF (%)		HT (+)	HT (−)
Nyukawa	2	23	959	984	1.4	AA	1	1
						AG	6	17
						GG	145	814
						Odds (95 % CI)	2.09 (0.96–4.58)	
	HWE	0.012				*P*	0.08	
Noshiro	0	11	2,432	2,443	0.2	AG	3	8
						GG	428	2004
						Odds (95 % CI)	1.76 (0.46–6.64)	
	HWE	1				*P*	0.368	
Field	1	23	857	881	1.4	AA	0	1
						AG	5	18
						GG	108	749
						Odds (95 % CI)	1.93 (0.70–5.30)	
	HWE	0.159				*P*	0.329	
Combined	3	57	4,248	4,308	0.7	AA	1	2
						AG	14	43
						GG	681	3567
						Odds (95 % CI)	1.71 (0.93–3.13)	
	HWE	1.39×10^{-3}				*P*	0.055	

MAF minor allele frequency, *HT* hypertension, *CI* confidence interval, *HWE* Hardy–Weinberg equilibrium

Table 3 Association of p.R4810K with blood pressures by additive model and dominant model

	SBP			DBP			PP		
	β	SE	p	β	SE	p	β	SE	p
Additive model study									
	Raw data (mm Hg)								
Nyukawa	8.2	2.9	$4.7 \times 10^{-3*}$	−0.4	2.0	0.83	8.5	2.1	$4.6 \times 10^{-5*}$
Noshiro	18.7	5.4	$4.6 \times 10^{-4*}$	11.4	3.4	$6.6 \times 10^{-4*}$	7.3	2.8	$1.0 \times 10^{-2*}$
Field studies	4.7	2.4	5.6×10^{-2}	−0.4	2.0	0.83	5.1	2.2	$1.8 \times 10^{-2*}$
Combined	8.9	2.0	$1.0 \times 10^{-5*}$	0.6	1.9	0.70	8.3	1.3	$5.4 \times 10^{-11*}$
	Adjusted data (mmHg)								
Nyukawa	10.0	3.2	$1.6 \times 10^{-3*}$	0.5	2.1	0.82	9.6	2.2	$1.7 \times 10^{-5*}$
Noshiro	19.1	5.8	$1.0 \times 10^{-3*}$	11.6	3.6	$1.2 \times 10^{-3*}$	7.5	3.0	$1.4 \times 10^{-2*}$
Field studies	6.1	2.6	$2.0 \times 10^{-2*}$	0.3	2.0	0.88	5.8	2.2	$8.6 \times 10^{-3*}$
Combined	10.0	2.2	$5.0 \times 10^{-5*}$	1.2	1.4	0.40	8.9	1.3	$2.3 \times 10^{-11*}$
Dominant model study									
	Raw data (mm Hg)								
Nyukawa	9.2	3.2	$4.4 \times 10^{-3*}$	−0.2	2.2	0.94	9.4	2.4	$7.6 \times 10^{-5*}$
Noshiro	18.7	5.4	$4.6 \times 10^{-4*}$	11.4	3.4	$6.6 \times 10^{-4*}$	7.3	2.8	$1.0 \times 10^{-2*}$
Field studies	6.3	1.7	$3.0 \times 10^{-4*}$	0.04	1.4	0.97	6.2	1.5	$3.1 \times 10^{-5*}$
Combined	9.9	2.2	$5.7 \times 10^{-6*}$	1.1	1.5	0.46	8.8	1.4	$1.0 \times 10^{-10*}$
	Adjusted data (mmHg)								
Nyukawa	10.8	3.5	$2.3 \times 10^{-3*}$	0.6	2.3	0.78	10.2	2.5	$4.0 \times 10^{-5*}$
Noshiro	19.1	5.8	$1.0 \times 10^{-3*}$	11.6	3.6	$1.2 \times 10^{-3*}$	7.5	3.0	$1.4 \times 10^{-2*}$
Field studies	6.7	2.8	$2.0 \times 10^{-2*}$	0.5	2.2	0.82	6.9	2.5	$5.4 \times 10^{-3*}$
Combined	10.8	2.4	$4.9 \times 10^{-6*}$	1.5	1.5	0.92	9.2	1.4	$8.5 \times 10^{-11*}$

* Significant association $p < 0.025$

Association of p.R4810K with hypertension

The associations of p.R4810K by the additive model for dichotomous traits (hypertension vs normal) were not significant in any of the populations (Table 2).

Regression analyses adjusted for age, sex and BMI revealed significant associations of p.R4810K with SBP and PP (Table 3). Specifically, the β [standard error (SE)] values for SBP were 8.2 (2.9) mmHg/allele for the Nyukawa study and 18.7 (5.4) mmHg/allele for the Noshiro study, while those for PP were 8.5 (2.1) mmHg/allele for the Nyukawa study, 7.3 (2.8) mmHg/allele for the Noshiro study and 5.1 (2.2) mmHg/allele for the Field study. The associations of p.R4810K with therapy-adjusted SBP and PP were also confirmed in the three studies. In the three combined populations, p.R4810K was highly associated with SBP [8.9 (2.0) mmHg/allele] and adjusted SBP [10.0 (2.2) mmHg/allele]; with PP [8.3 (1.3) mmHg/allele] and adjusted PP [8.9 (1.3) mmHg/allele]. The results by additive model are essentially the same by dominant model.

The characteristic features of carriers are shown in Table 4. SBP was significantly higher for the AA + GA genotype than for the GG genotype [136.4 (20.3) vs 126.4 (18.2) mmHg/allele, $P < 0.001$], while the male/female ratios, age and BMI did not differ significantly between any two groups. Adjusted SBP (mmHg/allele: mean ± SD) was 155.3 (19.7) for the AA genotype, 137.8 (22.7) for the GA genotype and 138.7 (22.7) for the AA + GA genotype, which is significantly higher than 127.7 (19.9) with the GG genotype ($P < 0.001$). On the other hand, neither DBP nor adjusted DBP differed between any two groups: the adjusted BP was 78.2 (13.2) and 76.9 (12.5) for GG genotypes, respectively. The % hypertension was marginally higher in the AA + AG group than in the GG group (Fisher's exact test, $P = 0.06$).

Effects on associations of protocol changes for statistical analysis

The present statistical analyses were based on the same protocol [21–23]. The protocol included subjects with antihypertensive medications and adjustment for age, sex and BMI. We investigate the effects of modifications of the protocol on the statistical analyses to determine to what degree the current results are dependent on the protocol. First, we eliminated subjects with antihypertensive

Table 4 Characteristics of p.R4810K carriers. Values are mean (standard deviation)

Genotype	*N*	M:F	Age (years)	BMI	Raw blood pressure (mmHg)		% Hypertension
					SBP	DBP	
AA	3	1:2	72.7 (5.1)	23.2 (4.8)	145.3 (19.7)	73.3 (5.8)	66.7
GA	57	14:43	51.9 (21.4)	23.5 (3.9)	135.9 (20.4)	77.3 (13.1)	24.6
AA + GA	60	15:45	52.4 (14.0)	23.5 (3.9)	136.4 (20.3)*	77.1 (12.8)	25.0
GG	4,248	1,827:2,421	51.1 (13.9)	23.0 (3.0)	126.4 (18.2)	76.2 (11.8)	16.0

* Significantly different from the values in GG genotype $P < 0.001$

medications to investigate the effect of genotypes without modifications by medications. Although reduced numbers of subjects decreased statistical power, there were significant associations of p.R4810K with SBP and PP (Table S1). Second, analyses were conducted without adjustment for BMI for the population including subjects with antihypertensive medications. The associations were reproduced without adjustment for BMI (Tables S2 and S3). Changes in the statistical analysis protocol did not alter the results by quantitative regression analyses extensively, suggesting that associations of p.R4810K are robust and reproducible.

LD blocks around p.R4810K of *RNF213*

Analyses of the LD blocks of 39 SNPs spanning 390 kb in patients with p.R4810K and moyamoya disease demonstrated that p.R4810K was in LD with the 3′ half of *RNF213* and *ENDOV* (Fig. 1a). Similar LD blocks were observed in the general control population (Fig. 1b). LD blocks of p.R4810K did not contain any proxy SNPs, which have been reported to be associated with BP in East Asian people or other ethnicities, even when less stringent statistical criteria are applied [21–23].

Discussion

In the present study, quantitative trait locus analyses demonstrated that p.R4810K was associated significantly with SBP and PP in the general population. The p.R4810K allele resulted in consistently elevated SBP from 4.7 to 18.7 mmHg/allele and PP from 5.1 to 8.5 mmHg/allele. Such large and consistent contributions support our hypothesis that p.R4810K increases SBP.

Several large-scale studies have used the genome-wide association study (GWAS) approach [21–23]. However, none of these studies detected a locus on 17q25.3 for hypertension. Investigations of the LD blocks of p.R4810K failed to reveal any SNPs that have been reported to be associated with BP, even with less stringent P values [21–23]. There is no doubt that recent association studies have been successful; however, we consider that there are three major reasons for the failure to detect an association of *RNF213* polymorphisms with hypertension. First, the

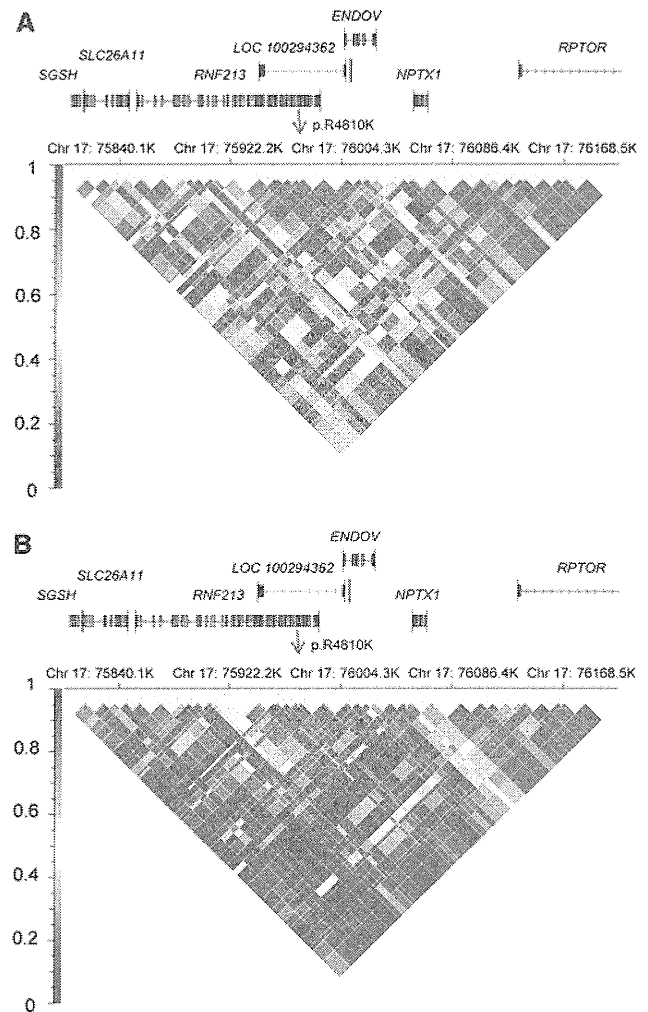

Fig. 1 Linkage disequilibrium (LD) blocks from a region spanning 390 kb from *SGSH* to *RAPTOR* in two independent populations. The LD blocks were constructed with 39 single nucleotide polymorphisms (SNPs). Scales are D'. The physical positions refer to Genome View Build 36 (http://www.ncbi.nlm.nih.gov/mapview/). **a** LD blocks for 140 unrelated Japanese patients with p.R4810K and moyamoya disease. **b** LD blocks for 384 unrelated Japanese subjects

critical assumption for the efficient detection of association through GWAS using LD mapping with TagSNPs $\geq$5 % is that, for the susceptibility locus, there is only low level allelic heterogeneity. In the presence of allelic heterogeneities, GWAS with TagSNPs may have missed potentially significant associations [24, 25]. Second, another GWAS assumption is that variants are common. The GWAS protocol usually discards minor alleles, i.e., with allele frequencies smaller than 10 %. Thus, minor alleles in *RNF213*, such as p.R4810K, might have been eliminated from the analysis. Third, even large studies are not free from sample size limitations to detect small population effects even using TagSNPs. For example, a simple genetic power calculation [26] on the assumption (HT prevalence 0.2; risk of the allele = 2.0; D' between a tag SNP and risk allele 0.9; tag SNP allele frequency = 0.5) reveals that to obtain alpha = 0.05 and power = 0.80, more than 50,000 HT Japanese cases would be needed for the low frequencies of risk allele ~0.01. Given the lower frequencies in the Tohoku areas of Japan, more cases would have been needed. Collectively, although p.R4810K has a discernible influence on BP in individuals, its small contribution to the population BP makes it extremely difficult to detect, even in large scale GWAS studies.

Given that the current observation can be generalized, carriers of p.R4810K are at risk of high SBP, irrespective of whether they have moyamoya disease. An increase of this magnitude overwhelms the population average of BP treatment effects for single antihypertensive agents [27]. Moreover, it has been suggested that differences in SBP of this magnitude elevate total mortality and the incidence of stroke [28]. Therefore, p.R4810K carriers may need special attention to control their SBP to prevent cerebrovascular events, regardless of whether they are affected by moyamoya disease. In this regard, it remains an enigma why p.R4810K has a strong association with SBP while it does not with DBP. Further studies are warranted to explain the preferential association with SBP.

Hypertension in patients with moyamoya disease has been postulated to be caused by renovascular hypertension [13, 14]. Similarly, it may occur in carriers without manifestation of moyamoya disease. There may also be other possibilities for the mechanisms of hypertension. For example, it remains to be addressed why hypertension occurs in some patients with moyamoya disease without stenosis of the renal arteries [15]. The physiological role of the p.R4810K variant of *RNF213* in the pathological process is unknown. *RNF213* comprises a novel class of E3 ligase and is involved in angiogenesis [11]. Therefore, its biochemical and physiological functions need to be elucidated. Further studies are required with a focus on the functional aspects of p.R4810K of *RNF213* in SBP.

Except for Down syndrome [29], various disorders associated with moyamoya syndrome are also known to be associated with BP, including Sickle cell disease [30, 31], neurofibromatosis I [32], Noonan syndrome [33, 34], Seckel syndrome [35], familial thoracic aortic aneurysm/dissection syndrome caused by *ACTA2* mutations [36] and X-linked moyamoya syndrome [37]. The genetic association between BP and p.R4810K of *RNF213* may not be unique to moyamoya disease, but could instead be common to the pathophysiology associated with moyamoya syndrome. The common pathological processes in these diseases are associated with occlusive vascular lesions. The biomedical mechanisms associated with occlusive vascular lesions may be attributable to endothelial dysfunction, as is the case for prehypertension [38]. Alternatively, it is interesting that patients with *ACTA2* mutations have excessive proliferation of vascular smooth muscle cells, and thereby elevated BP [36].

The present study has several limitations, the most major being the population sizes of the carriers of p.R4810K. Although we performed extensive genotyping in the three populations, we could not obtain large numbers of carriers because of the low prevalence of p.R4810K. It is of particular interest that the MAF of p.R4810K is significantly lower in the eastern part of mainland of Japan than in the western part of Japan. It should be investigated in future whether or not the prevalence of moyamoya disease has geographic differences in Japan. Specifically, it may be lower in the eastern part of mainland Japan than in the western part of Japan, as predicted by the low prevalence in Caucasians [11]. In this regard, although we cannot eliminate the effects of population stratification completely, the strength of the present study, namely a single ethnicity, can mitigate such biases. In addition, we did not test genetic relatedness among participants, although we checked it with indirect information. Thus, blood-related samples may be contaminated in associations and lead to inflation of associations. The second limitation of this study is that clinical data were not available for examining renovascular hypertension in carriers. Third, it could be argued p.R4810K is a proxy SNP or that *RNF213* is associated directly with BP, and that p.R4810K of *RNF213* may elevate BP. At present, we cannot say whether *RNF213* is associated with BP, and whether p.R4180K elevates BP directly or not. Further evidence is required to explain the association of p.R4810K with BP biologically. Finally, we cannot exclude a healthy participant effect in the current study. In fact, most of the participants are active both mentally and physically. Handicapped subjects with hypertension-associated cardiovascular diseases or subjects receiving clinical care might have been eliminated in the communities. The present findings may help to determine the pathological consequences of moyamoya disease in

carriers of p.R4810K. Furthermore, our findings suggest that high SBP may be a common phenotype of carriers of p.R4810K. Finally, although the MAF of p.R4810K in the general population is small, antihypertensive measures may be efficient for this high-risk population.

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Conflict of interest None.

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Bypass Surgery for Moyamoya Disease
—Concept and Essence of Surgical Techniques—

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Abstract

This review describes the basic concepts of surgical revascularization for moyamoya disease, including direct and indirect bypass surgery. Direct bypass surgery can improve cerebral hemodynamics and reduce further ischemic events immediately after surgery, but may be technically challenging in some pediatric patients. Indirect bypass surgery is simple and has widely been used. However, its beneficial effects can be achieved 3 to 4 months after surgery, and surgical design is quite important to determine the extent of surgical collateral pathways. Combined bypass procedure, especially superficial temporal artery (STA) to middle cerebral artery anastomosis and indirect bypass, encephalo-duro-myo-arterio-pericranial synangiosis, is a safe and effective option to improve the short- and long-term outcome in patients with moyamoya disease. Alternative techniques are also described for specific cases with profound cerebral ischemia in the anterior cerebral artery or posterior cerebral artery territory. Special techniques to safely complete bypass surgery and avoid perioperative complications are presented, including methods to prevent delayed wound healing, to avoid facial nerve palsy after surgery, and to preserve the STA and middle meningeal artery during skin incision and craniotomy. Finally, the importance of careful management of patients is emphasized to reduce the incidence of perioperative complications, including ischemic stroke and hyperperfusion syndrome.

Key words: moyamoya disease, bypass surgery, surgical technique, direct bypass, indirect bypass

Introduction

Moyamoya disease is an uncommon cerebrovascular disorder that is characterized by progressive occlusion of the supraclinoid internal carotid artery (ICA) and its main branches within the circle of Willis. This occlusion results in the formation of a fine vascular network (moyamoya vessels) at the base of the brain.[46] The predominant feature of the pathology of moyamoya disease is now known to be progressive stenosis of the carotid artery terminations, and the moyamoya vessels are the dilated perforating arteries that function as collateral pathways.[2] Recent studies have rapidly expanded our knowledge of the basic and clinical aspects of moyamoya disease, including the etiology, pathophysiology, surgical treatment, and long-term prognosis of the disorder.[49] In particular, various types of bypass surgery have been developed and are known to improve the long-term outcome in patients with moyamoya disease.[3-5,9,13,14,31,33,38,40,44] However, further understanding of the pathophysiology, diagnosis, and treatments of this disease is needed to improve the long-term outcome for these patients.[22]

In this article, we review recent surgical advances in the treatment of moyamoya disease. Special points of surgical techniques are precisely described. Surgical techniques for specific cases are also presented. Finally, the importance of perioperative management of patients with moyamoya disease is emphasized to reduce the incidence of perioperative complications during and after surgical revascularization.

Superficial Temporal Artery to Middle Cerebral Artery (STA-MCA) Anastomosis and Encephalo-duro-myo-arterio-pericranial Synangiosis (EDMAPS)—Standard, Tips, and Tricks

As we recently reported, STA-MCA anastomosis

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