Impact of *RNF213* founder polymorphism (p.R4810K) on the postoperative development of indirect pial synangiosis after direct/indirect combined revascularization surgery for adult Moyamoya disease

Masaki Ito, Masahito Kawabori, Taku Sugiyama, Kikutaro Tokairin, Ryota Tatezawa, Haruto Uchino, Ken Kazumata, Kiyohiro Houkin, Miki Fujimura

Department of Neurosurgery, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan

Corresponding Author: Masaki Ito, M.D., Ph.D.

Department of Neurosurgery, Hokkaido University Graduate School of Medicine, North 14, West 5, Sapporo 060-8648, Japan

Tel: +81-11-706-5987, FAX: +81-11-708-7737, E-mail: masakiitou-nsu@umin.ac.jp

ORCID: 0000-0002-8032-7198

Abstract

Direct superficial temporal artery (STA) to middle cerebral artery (MCA) anastomosis combined with indirect pial synangi- osis provides favorable surgical collaterals for Moyamoya disease (MMD), especially in adults; however, factors leading to the development of each direct and indirect collateral are not well documented.

We aimed to investigate the association between RNF213 founder polymorphism (p.R4810K) and each direct and indirect collateral development. By qualitative and quantitative evaluations of direct and indirect surgical collaterals using time-of- flight MR angiography, postoperative development of each type of bypass was evaluated independently into two categories. Multivariate logistic regression analysis was performed to study the contributing factors for the development of each surgical collateral.

Excellent development of postoperative direct and indirect bypass was observed in 65 hemispheres (70%) by qualitative evaluation, which was confirmed by quantitative evaluation. Multivariate logistic regression analysis of excellent indirect bypass development revealed a significant positive correlation with the p.R4810K (odds ratio, OR4.0; 95%-confidence interval, CI 1.2–16), advanced MR angiographic stage (OR9.5; 95%CI 1.7–73), and preoperative middle meningeal artery caliber (OR6.8; 95%CI 1.8–35), but a significant negative correlation was found with the excellent direct bypass development (OR0.17; 95%CI 0.03–0.75). No significant correlation was observed between excellent direct bypass development and the p.R4810K (OR0.95; 95%CI 0.37–2.4).

In conclusion, excellent development of indirect collaterals after STA-MCA anastomosis combined with indirect pial synan- giosis occurs more frequently in adult MMD with the RNF213 founder polymorphism, suggesting a role of the p.R4810K variant for marked in-growth of indirect collaterals and the utility of preoperative genetic analysis.

Key Words: Moyamoya; Magnetic resonance imaging; Polymorphism, Revascularization; RNF213; Vascular disorders

Declarations

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Consent to participate: Written informed consent was obtained from all participants (or guardians) to participate in this study.

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Introduction

Moyamoya disease (MMD) is characterized by progressive stenosis of the terminal portion of the internal carotid arteries, accompanied by the formation of abnormally dilated, fragile perforators at the base of the brain. [18, 31, 33] Direct superficial temporal artery (STA) to middle cerebral artery (MCA) anastomosis is accepted worldwide as the primary treatment of choice aiming at improving cerebral blood flow and surgical revascularization for symptomatic ischemic and hemorrhagic presentations of MMD. [17, 26] While direct STA-MCA anastomosis offers the advantage of immediate revascularization, indirect bypass effectively induces the in-growth of new collaterals to the underlying cerebral cortex overtime. [18, 31] In the combined setting using both direct and indirect bypass, the advantages of both bypass procedures are expected, with a perioperative stroke risk of 4.7% per surgery but a favorable long term clinical outcome. [15, 20] Several studies demonstrated a reciprocal relationship between the direct and indirect bypass with a wider extent of surgical revascularization in the context of a combined setting. [1, 2, 36, 37] Very recently, the association between a *RNF213* founder mutation for east Asian MMD (p.R4810K) [14, 23] and postoperative development of surgical collaterals has been demonstrated,[8] however, it is still obscure for which type of surgical collateral (i.e., direct or indirect or both?) the *RNF213* founder mutation is responsible.

The extent of revascularization after Moyamoya bypass surgery is traditionally examined by catheter carotid angiography. [2, 24] In the early 2000s, Yoon et al.[38] and Honda et al.[9] reported the usefulness of magnetic resonance (MR) angiography to evaluate the development of the external carotid artery tributaries, including the STA, middle meningeal artery (MMA), and deep temporal artery (DTA) after direct and/or indirect bypass surgery for MMD. Recent studies using time-of-flight (TOF) MR angiography or its source image provided insights into the discernable and sequential roles in direct and/or indirect bypass in MMD. [1, 37] To comprehensively investigate the clinical and genetic factors associated with the induction of each direct and indirect surgical collaterals, we retrospectively examined our institutional records and MR angiography for adult patients undergoing combined direct/indirect bypass for MMD.

Materials and Methods

Below are the main methods necessary to comprehend the results. Details were provided in the online-only supplementary information.

Study Population

This study included all adult MMD patients (>16 years of age at the surgery) who consented to genetic analysis and underwent repeat MR imaging within 3 years after combined direct/indirect bypass [10, 19] between 2005 and 2019 in our hospital. The diagnosis of MMD was confirmed by the criteria outlined in the Japanese guidelines for the diagnosis and treatment of MMD. [30] The Houkin MR angiographical stage/grading system was used to stratify the angiographical stage of MMD (ranging 1 to 4 [most advanced]). [11] Cerebrovascular reactivity to acetazolamide was evaluated quantitatively as previously reported. [36] According to our surgical protocol, [10, 19] double-barrel STA-MCA anastomosis combined with EDAMS or EDMAPS was performed as the standard combined direct and indirect bypass procedure. Basically, we discontinued antiplatelet(s) before surgery if prescribed, except for 14 operations. Thus, single antiplatelet agent (cilostazol, aspirin, or clopidogrel) was used in fifteen percent (14 out of 93) of the surgery. In accordance with an institutional review board-approved protocol (number14-053), medical records were retrospectively reviewed to gather demographic information, age at the surgery, symptoms at presentation, comorbid conditions before surgery, and results of radiographic studies (Please see supplementary table 1), as well as clinical outcome in terms of global disability and mortality rated by modified Rankin scale (mRS), and recurrence of ischemic or hemorrhagic stroke when evaluating the postoperative development of surgical collaterals by MR angiography as described below.

Evaluation of the Postoperative Development of Direct and Indirect Surgical Collaterals by MR angiography

Evaluation of the postoperative development of direct and indirect surgical collaterals was performed qualitatively and quantitatively using TOF MR angiography and its source images, respectively, by a previously reported protocol with minor modifications [36, 37]. In brief, MR angiography at two time points acquired before and 6 to 36 months after surgery were reviewed and compared by neurosurgeons (M. I. and T.S.) blinded to the results of genetic testing. For qualitative evaluation, [36] postoperative development of each direct and indirect surgical collaterals were dichotomized into excellent or not, respectively. Thus, the development of direct surgical collaterals was evaluated by the development of the STA, while that of indirect surgical collaterals was evaluated by the development of the MMA and DTA. For quantitative evaluation, we reviewed MR angiography source images and measured the calibers of the STA, MMA, and DTA as previously reported.[37] The caliber change ratios (CCRs) of post to preoperative calibers were calculated for each artery.

Genetic Analysis of the *RNF213* founder polymorphism (p.R4810K)

Written-informed consent was received for genetic analysis from MMD patients or their guardians. In accordance with the institutional review board-approved protocol, genetic analysis was conducted at the Department of Neurosurgery of Hokkaido University by K.T. and R.T. who were blinded to clinical data. Taqman single nucleotide polymorphism genotyping assay was employed to determine the allele type for *RNF21*3 founder mutation (p.R4810K).

Data Analysis

Continuous or rank variables were described as the mean or median with standard deviation or range. Dichotomous or categorical variables were expressed as the ratio or frequency. “Continuous (age, donor artery diameter) and rank variables (MR angiographical stage, mRS) were compared between two groups by the unpaired-t test and Mann-Whitney test, respectively. Dichotomous or categorical variables were compared by Fisher’s exact test. For multiple comparisons, two-way repeated measures or ordinary analysis of variance followed by a post-hoc test was used, as appropriate. To assess the correlation between the excellent development of each surgical collateral and

multiple clinical and genetic variables, multivariate logistic regression analysis was performed using the stepwise forward parameter selection that achieved significance levels of P <0.1 in the univariate analysis. All clinical and genetic factors for the multivariate analysis are listed in the main Tables. The level of significance was set at P <0.05. GraphPad Prism (version 9.1.1, San Diego, CA, USA) was used for these analyses.

Results

Study Population

During the study period, 110 adult patients with 160 hemispheres underwent STA-MCA anastomosis combined with indirect pial synangiosis using vascularized tissue, including dura mater, temporal muscle, and pericranium. Of these, 48 were excluded from analysis due to lack of genetic testing, missing follow-up MR imaging within 6 to 36 months after surgery, or the diagnosis of quasi-MMD (n=43, 2, and 3 patients, respectively). Consequently, 62 patients (56%) with 93 operated hemispheres (58%) were included in the analysis: 47 female and 15 male patients with an average age of 42 years. In this study, 79 (84.9%) and five (5.4%) hemispheres underwent double- and single-barrel STA-MCA anastomosis as the direct bypass procedure, respectively. The rest of nine hemispheres (9.7%) underwent STA-MCA and ACA anastomosis. In this series, 99% (92/93 hemispheres) of the direct bypass surgeries were successfully completed. While, in one hemisphere, intraoperative thrombotic occlusion occurred repeatedly at the site of anastomosis where white clot formation was observed. This operation was done by indirect revascularization, since conventional methods were not effective, including a vessel massage with intravenous heparin administration or a takedown and re-anastomosis. *RNF213* founder mutation (p.R4810K) was detected in 40 patients (65%) with 59 hemispheres (63%) in this series (Figure 1A). There was no significant difference in baseline characteristics between *RNF213*-mutant (MT) and -wild type (WT) groups, except in familial occurrence and a comorbid condition of

hypertension (Supplementary Table. 2). A representative patient who was a heterozygote for the *RNF213* founder mutation is shown in Figure 1B-E.

Clinical Outcome

Overall clinical outcome was favorable in terms of global disability and mortality when evaluating the development of surgical collaterals by MRA at 319±140 postoperative days. Thus, fifty-three patients (85.5%) showed favorable outcome (mRS 0-1) and there was no mortality among the 62 patients with 93 operated hemispheres. In terms of the recurrence of ischemic or hemorrhagic stroke or TIA, we observed stroke recurrence in the three operated hemispheres (3.2%) in the three patients (4.8%). There was no significant correlation between the excellent postoperative development of indirect nor direct collaterals and stroke recurrence (Table 1 and 2).

Qualitative and Quantitative MR Angiography Evaluations

Of all 93 hemispheres enrolled in this study, excellent development of postoperative direct and indirect surgical collaterals was observed in 65 (70%), respectively, after the combined bypass with a mean follow-up period of 319±140 days by qualitative evaluation. In terms of the relationship of the postoperative development between direct and indirect bypass, dual/equal development was most frequently observed (62% of the operated hemisphere). Thus, postoperative direct- (STA) or indirect- (MMA and/or DTA) dominant development was observed in 44 and 14 hemispheres, respectively, when evaluated in the above-mentioned follow-up period. By quantitative MR angiography evaluation of all 93 hemispheres, a significant increase was observed in the caliber of the STA after surgery (pre: 1.8±0.35 mm; post: 2.5±0.65 mm, $P<0.0001$). The caliber of the MMA and DTA also significantly increased (pre: 1.6±0.44 mm; post: 1.8±0.50 mm, $P<0.0001$ in MMA; pre: 0.98±0.33 mm; post: 1.6±0.60 mm, $P<0.0001$, respectively). The CCR of post to preoperative-calibers for the STA was significantly higher (1.5±0.42 vs 1.0±0.28, $P<0.0001$) in the excellent direct bypass development group than in the non-excellent group. The CCRs for the DTA (2.0±0.89 vs 1.2±0.53, $P<0.0001$) and MMA (1.3±0.31 vs 1.0±0.29, $P=0.042$) were also significantly higher in the excellent indirect bypass development group.

We further analyzed the association between direct or indirect bypass development and *RNF213* founder polymorphism (p.R4810K). Of note, excellent indirect bypass development was observed more frequently in the *RNF213*-MT group (78%, 46/59 hemispheres) than in the *RNF213*-WT group (56%, 19/34 hemispheres), with a significant difference (P = 0.035) (Figure 2A). Excellent direct bypass development was observed in 40/59 hemispheres (68%) and 24/34 hemispheres (71%) of the *RNF213*-MT and -WT groups, respectively, with no significant difference (P = 0.82). Multiple comparisons of the CCRs demonstrated a significant difference in the DTA, but not in the STA or MMA, between the two groups (Figure 2B). Thus, the CCR for the DTA was significantly higher in the *RNF213*-MT group (1.9±1.0) than in the *RNF213*-WT group (1.4±0.52, P=0.0007).

Factors Correlated with Excellent Development of Indirect and Direct Collaterals After Combined Bypass

To identify clinical and genetic factors that may underlie excellent revascularization after combined bypass in adult MMD, we examined which factors correlated with the excellent development of indirect and direct collaterals (Table 1 and 2). Multivariate logistic regression analysis for excellent indirect bypass development revealed a significant positive correlation with *RNF213* founder mutation (adjusted odds ratio (OR), 4.0), advanced MR angiographic stage (adjusted OR, 13 in stage 3; 9.5 in stage 4), and preoperative caliber of the MMA (adjusted OR, 6.8), whereas a significant negative correlation was noted with excellent direct bypass development (adjusted OR, 0.17). On the other hand, no significant correlation was observed between excellent direct bypass development and *RNF213* founder mutation. Multivariate logistic regression analysis for excellent direct bypass development revealed a significant negative correlation with excellent indirect bypass development (adjusted OR, 0.23) and the comorbid condition of dyslipidemia (adjusted OR, 0.27). Please see supplementary figure 1 and 2 supporting these results.

Discussion

To our knowledge, this is the first study to demonstrate that the *RNF213* gene polymorphism (p.R4810K) plays a role in the postoperative development of indirect, but not direct surgical collaterals after direct STA-MCA anastomosis combined with indirect pial synangiosis for adult patients with MMD. We were able to document this relationship by qualitative and quantitative evaluation of repeated TOF MR angiography during the short to mid-term follow-up period (6 to 36 months after surgery). Growing number of studies demonstrated that indirect bypass for adult MMD is less effective in terms of angiographical outcome (postoperative development of indirect surgical collateral) compared to pediatric or young adult patients. Thus, the successful indirect bypass development were observed in 44-47% of adult MMD patients treated with combined direct and indirect bypass [5, 28] or indirect bypass.[21] Consistent with these past research, our study showed that only 56% of adult MMD showed excellent indirect bypass development in adult patients without *RNF213*-founder polymorphism (*RNF213*-WT group, Please see Figure 2A). On the other hand, most (78%) of patients with *RNF213*-founder polymorphism (*RNF213*-WT group) demonstrated excellent indirect bypass development. Based on these observations, preoperative genetic analysis for the *RNF213* founder mutation might be useful for the clinical management of adult MMD patients, especially when making a decision whether adding indirect bypass on direct bypass procedure for adult MMD.

To further determine key factors responsible for indirect and direct bypass development, respectively, we focused on significant clinical and genetic factors correlated with the excellent postoperative development of surgical collaterals by multivariate logistic regression analyses. We identified a panel of excellent revascularization-related factors for each indirect and direct bypass development after combined bypass for adult MMD (main Tables). Of note, 1) p.R4810K variant, 2) preoperative advanced MR angiographical stage, and 3) preoperative larger caliber of the MMA were positively, and 4) postoperative excellent direct bypass development was negatively correlated with excellent indirect bypass development. This is partly current knowledge. Thus, advanced angiographical stage, preoperative trans-dural collateral vessels (i.e., MMA), and heterozygous p.R4810K variant are known radiographic and genetic biomarkers of the increased capacity of postoperative surgical

collateral production. [8, 32] In addition to the current knowledge, our study suggests significant correlation between p.R4810K and excellent indirect, but not direct bypass development after combined bypass, although its underlying mechanism is unclear based on our study, which is briefly discussed below. Thus, it remains unclear how p.R4810K plays a pathological role in MMD (i.e., why earlier disease onset, higher disease severity, [16, 27] and prolonged/delayed cerebral hyperperfusion after STA-MCA anastomosis [34] occur more frequently in MMD with the *RNF213* founder mutation?). Earlier experimental studies reported that cellular gene expression analysis of *RNF213* in adult human tissues revealed markedly high expression in immune tissues such as the spleen and leukocytes.[14, 25] Genome-wide plasma/serum microRNA profiling [6, 13, 35] revealed a panel of significant MMD-related plasma/serum microRNAs whose target genes were involved in inflammatory or angiogenesis-related molecular pathways. Bidirectional major pathways that are influential in the inflammatory response potentially causing collateral formation in MMD are 1) the anti-inflammatory cytokine pathway and 2) proinflammatory cytokine pathway activating *RNF213*. Fujimura et al. (2018) recently showed increased serum production of soluble CD163 and CXCL5 in MMD patients, suggesting the involvement of intrinsic M2 macrophage-related immune reactions.[7] The immune responses associated with angiogenesis are promoted by M2 macrophages and angiogenic mediators are activated through these anti-inflammatory cytokines.[25] Ohkubo et al. (2015) reported that pro-inflammatory cytokines activated transcription of *RNF213* both *in vitro* and *in vivo.* p.R4810K variant was more likely linked to the functional deficiency of the *RNF213* gene based on markedly high matrix metalloproteinase production upon experimental silencing of *RNF213*.[29] Bang et al. (2016) reported a marked increase in the blood caveolin-1 level in *RNF213* founder mutation carriers.[3] As caveolin-1 negatively regulates proliferation of endothelial cells, but positively regulates endothelial angiogenic function such as tube formation,[22] the increased caveolin-1 levels may accelerate angiogenesis in MMD patients. *In vivo* experimental study demonstrated increased angiogenesis in mice lacking *RNF213* after chronic hind-limb ischemia, [12] suggesting a role of *RNF213* abnormality in the development of pathological vascular networks in chronic ischemia. Taken together, our observation of marked angiogenesis represented by excellent indirect bypass development in adult

MMD patients with P.R4810K variant can be explained bidirectionally by the loss-of-function (i.e., marked angiogenesis by lacking *RNF213* gene function) and gain-of-function (i.e., marked angiogenesis through *RNF213* mutation) mechanisms of the *RNF213* gene, which is the next question to be addressed.

Another novel finding demonstrated in this study is that the comorbid condition of dyslipidemia was negatively correlated with excellent direct bypass development. In this study, all the thirteen patients with dyslipidemia in this series were treated with statin and had well-controlled preoperative blood LDL level before surgery. There was no significant difference in the preoperative blood LDL level between dyslipidemia and non-dyslipidemia group (125.5 ± 32.8 mg/dl and 122.6 ± 27.3 mg/dl, P=0.73, unpaired t-test). Recently, Church and Steinberg et al. reported an association between hyperlipidemia and radiological progression of unilateral type to bilateral type in MMD, with possible explanations including synergistic effects of increased lipids in the underlying moyamoya vasculopathy and the inadvertent inclusion of cerebral atherosclerotic disease in the study population.[4] One possible explanation for our finding may also be the inadvertent involvement of atherosclerosis in our MMD patients, which leads to a poorer condition of both the donor and recipient arterial wall, resulting in poor long-term patency. We should further validate this issue in a larger cohort with optimal stratification by the different (well- or poorly-controlled blood LDL level).

Limitation

Our study is limited by the following several points. First, our study did not include pediatric subgroup of MMD. As we previously reported that almost all (95%) pediatric MMD exhibited effective indirect revascularization after combined bypass,[36] we only investigated adult MMD in this study. Second, catheter angiography follow-up was not available in the most bypass surgeries in this study, although measurement of vessel calibers by catheter angiography is optimal. Last, consistent hemodynamic evaluations were not available for all patients using single-photon emission computed tomography or positron emission tomography. We do not consider these limitations to affect the interpretation of the results, but further studies are warranted.

Conclusions

We found that the excellent development of postoperative indirect pial synangiosis after combined direct and indirect bypass occurs more frequently in adult MMD patients with the *RNF213* founder mutation (p.R4810K) allele. This confirms a novel clinical role of the *RNF213* founder polymorphism in the marked angiogenesis via indirect pial synangiosis in adult patients with MMD, and suggests the utility of preoperative genetic analysis for *RNF213* polymorphism in MMD.

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Figure legends

Figure 1. Flow chart for inclusion of the study subjects and representative MR angiography of a study subject before and after combined direct/indirect bypass

(A) Flow chart shows the breakdown of inclusion and exclusion of the study subjects. **(B-E)** MR angiography (MRA) of a 45-year-old female with Moyamoya disease and the *RNF213* founder polymorphism (G/A genotype) obtained before and 6 months after left combined direct and indirect bypass. **(B, C)** Preoperative axial time-of-flight MRA with maximal intensity projection (MIP) reconstruction **(B)** and its source image **(C)** depicted the left superficial temporal artery (STA, double arrows) and middle meningeal artery (MMA, dotted arrows), whereas the deep temporal artery (DTA) was hardly seen in MIP or its source images preoperatively. **(D, E)** Follow-up MRA obtained 6 months after left STA-MCA anastomosis combined with encephalo-duro-myo-arterio-pericranio-synangiosis demonstrated excellent development of postoperative direct (STA) and indirect (DTA) surgical collaterals. The caliber of the STA and DTA were markedly increased after surgery (STA: pre: 1.54 mm; post: 1.99 mm, DTA: pre: 0.53 mm; post: 2.34 mm, MMA: pre: 1.18 mm; post: 1.23 mm) as shown in panel **(C)** and **(E)**.

Figure 2. Qualitative and quantitative analysis of postoperative direct and indirect collateral development using time-of-flight magnetic resonance angiography

(A) Interleaved bar graph showing the results of qualitative analysis of the axial time-of-flight MR angiography with maximal intensity projection (MIP) reconstruction before and after combined bypass to evaluate the development of postoperative indirect and direct collaterals in adult MMD. ns, not significant; *P <0.05, Fisher's exact test. **(B)** Interleaved scatterplot with bars showing the caliber change ratios for the superficial temporary artery (STA), deep temporal artery (DTA), and middle meningeal artery (MMA) from quantitative analysis of the source images of MR angiography

1 before and after combined bypass in adult MMD. ns, not significant; ***P <0.001,
2 two-way ANOVA followed by Bonferroni multiple comparison.

3

TABLE 1
Correlation of Postoperative Indirect bypass development with Clinical and Genetic Variables

Variables		Indirect bypass development		Unadjusted		Adjusted	
		Excellent (n = 65)	Non-excellent (n = 28)	Odds Ratio (95% CI)	*P* value	Odds Ratio (95% CI)	*P* value
RNF213 founder polymorphism, G/A		46/65 (71%)	13/28 (46%)	2.8 (1.1 to 7.1)	0.028	**4.0 (1.2 to 16)**	**0.034**
Age, y, mean, SD		41 ± 11	43 ± 9.5	0.98 (0.94 to 1.0)	0.46		
Sex, Male		13/65 (20%)	8/28 (29%)	0.63 (0.23 to 1.8)	0.37		
Familial occurrence		23/65 (35%)	7/28 (25%)	1.6 (0.63 to 4.7)	0.33		
Hemisphere, Left		33/65(51%)	12/28 (43%)	1.4 (0.57 to 3.4)	0.48		
Clinical presentation	Ischemia	45/65 (69%)	17/28 (61%)	1.5 (0.57 to 3.7)	0.43		
	Hemorrhage	5/65 (7.7%)	5/28 (18%)	0.38 (0.10 to 1.5)	0.16		
MR angiographical stage	2 (reference)	12/65 (18%)	18/28 (64%)	-	-	-	-
	3	34/65 (52%)	7/28 (25%)	7.3 (2.5 to 23)	0.0004	**13 (3.4 to 62)**	**0.0004**

	4	19/65 (29%)	3/28 (11%)	9.5 (2.6 to 47)	0.0019	**9.5 (1.7 to 73)**	**0.017**
PCA involvement		17/65 (26%)	1/28(3.6%)	9.6 (1.8 to 177)	0.033	4.1 (0.48 to 92)	0.25
Co-morbidities	Hypertension	20/65 (31%)	10/28 (36%)	0.80 (0.32 to 2.1)	0.64		
	Diabetes mellitus	4/65 (6.3%)	2/28 (7.1%)	0.85 (0.16 to 6.4)	0.86		
	Dyslipidemia	12/65 (19%)	1/28 (3.6%)	6.1 (1.1 to 114)	0.09	12 (1.3 to 278)	0.052
Decreased cerebrovascular reserve (10% or less)		24/40 (60%)	9/17 (53%)	1.3 (0.42 to 4.2)	0.62		
Direct bypass development, Excellent		40/65 (62%)	25/28 (89%)	0.19 (0.043 to 0.62)	0.013	**0.17 (0.03 to 0.75)**	**0.029**
Preoperative donor artery diameter mm, mean, SD	STA	1.8 ± 0.36	1.9 ± 0.32	0.83 (0.23 to 3.0)	0.78		
	MMA	1.6 ± 0.45	1.4 ± 0.40	3.9 (1.3 to 14)	0.023	**6.8 (1.8 to 35)**	**0.010**
	DTA	1.0 ± 0.36	0.95 ± 0.34	1.6 (0.41 to 6.7)	0.50		
	BA	3.0 ± 0.47	3.1 ± 0.61	0.67 (0.27 to 1.6)	0.36		
Clinical outcome							
	mRS, median	0	0	NA	NA		
	Stroke recurrence	11/65 (16.9%)	1/28 (3.6%)	4.9 (0.87 to 92)	0.14		
Days after bypass for the assessment		305 ± 127	350 ± 167	1.0 (1.0 to 1.0)	0.17		

Abbreviations: RNF213, ring finger protein 213; CI, confidence interval; SD, standard deviation; PCA, posterior cerebral artery; STA, superficial temporal artery; MMA, middle meningeal artery; DTA, deep temporal artery; BA, basilar artery; NA, not applicable

TABLE 2

Correlation of Postoperative Direct bypass development with Clinical and Genetic Variables

Variables		Direct bypass development		Unadjusted Odds Ratio (95% CI)	*P* value	Adjusted Odds Ratio (95% CI)	*P* value
		Excellent (n = 65)	Non-excellent (n = 28)				
RNF213 founder polymorphism, G/A		41/65 (63%)	18/28 (64%)	0.95 (0.37 to 2.4)	0.91		
Age, y, mean, SD		42 ± 9.9	42 ± 11	1.0 (0.96 to 1.0)	0.93		
Sex, Male		15/65 (23%)	6/28 (21%)	1.1 (0.39 to 3.4)	0.86		
Familial occurrence		21/65 (32%)	9/28 (32%)	1.0 (0.40 to 2.7)	0.99		
Hemisphere, Left		33/65 (51%)	12/28 (43%)	1.4 (0.57 to 3.4)	0.48		
Clinical presentation	Ischemia	42/65 (65%)	20/28 (71%)	0.71 (0.26 to 1.8)	0.52		
	Hemorrhage	7/65 (11%)	3/28 (11%)	1.0 (0.26 to 5.0)	0.99		
MR angiographical stage	2 (reference)	23/65 (35%)	7/28 (25%)	-	-		
	3	28/65 (43%)	13/28 (46%)	0.66 (0.22 to 1.9)	0.44		
	4	14/65 (22%)	8/28 (29%)	0.53 (0.15 to 1.8)	0.31		
PCA involvement		11/65 (17%)	7/28(25%)	0.61 (0.21 to 1.9)	0.37		
Co-morbidities	Hypertension	21/65 (32%)	9/28 (32%)	1.0 (0.40 to 2.7)	0.99		
	Diabetes mellitus	3/65 (4.6%)	3/28 (11%)	0.40 (0.07 to 2.3)	0.29		
	Dyslipidemia	5/65 (7.7%)	8/28 (29%)	0.21 (0.057 to 0.70)	0.012	**0.27 (0.073 to 0.93)**	**0.042**

Decreased cerebrovascular reserve (10% or less)		20/39 (51%)	13/18 (72%)	0.40 (0.11 to 1.3)	0.14		
Indirect bypass development, Excellent		40/65 (62%)	25/28 (89%)	0.19 (0.043 to 0.62)	0.013	**0.23 (0.05 to 0.77)**	**0.029**
Preoperative donor artery diameter mm, mean, SD	STA	1.8 ± 0.32	1.9 ± 0.36	1.2 (0.35 to 4.6)	0.75		
	MMA	1.5 ± 0.32	1.6 ± 0.49	1.9 (0.67 to 5.7)	0.24		
	DTA	0.98 ± 0.30	0.98 ± 0.34	1.1 (0.27 to 4.2)	0.94		
	BA	3.1 ± 0.51	3.0 ± 0.52	0.79 (0.33 to 1.9)	0.60		
Clinical outcome							
	mRS, median	0	0	NA	NA		
	Stroke recurrence	7/65 (10.8%)	4/28 (14.3%)	0.72 (0.20 to 3.0)	0.68		
Days after bypass for the assessment		307 ± 105	323 ± 154	1.0 (1.0 to 1.0)	0.61		

Abbreviations: RNF213, ring finger protein 213; CI, confidence interval; SD, standard deviation; PCA, posterior cerebral artery; STA, superficial temporal artery; MMA, middle meningeal artery; DTA, deep temporal artery; BA, basilar artery; NA, not applicable

Main figures

A

160 hemispheres (110 MMD patients)

Exclusion
- lacking genetic testing
- lacking follow-up MR imaging
- quasi-MMD

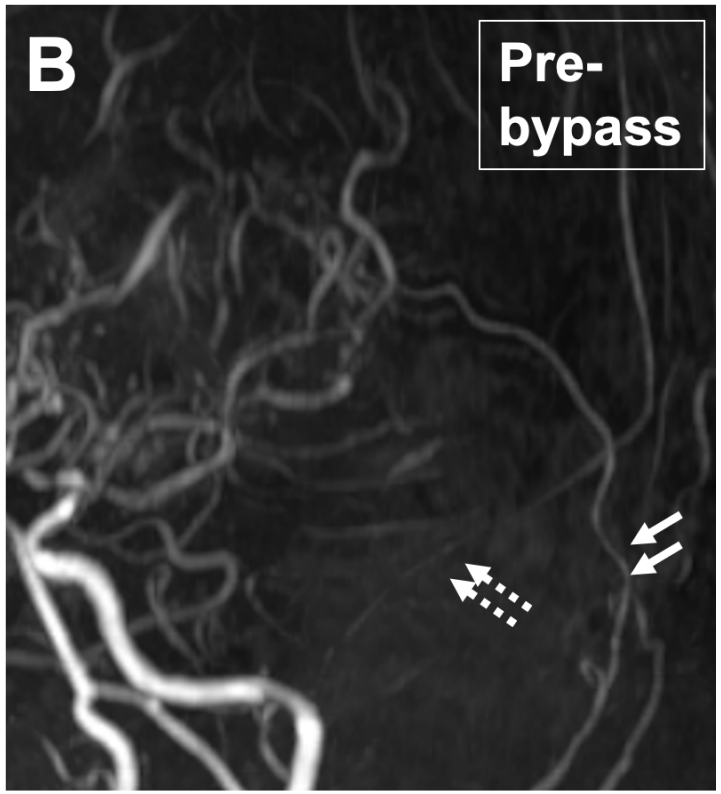
93 hemispheres (62 MMD patients)

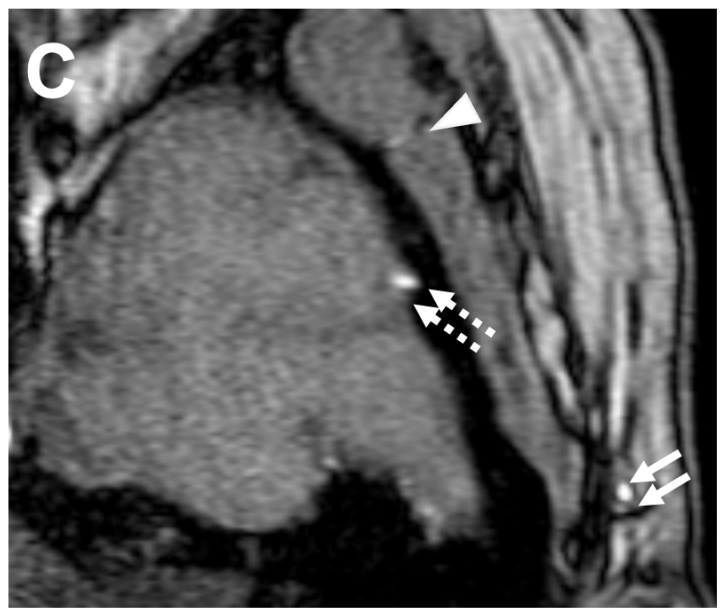
Combined direct STA-MCA anastomosis and indirect pial synangiosis

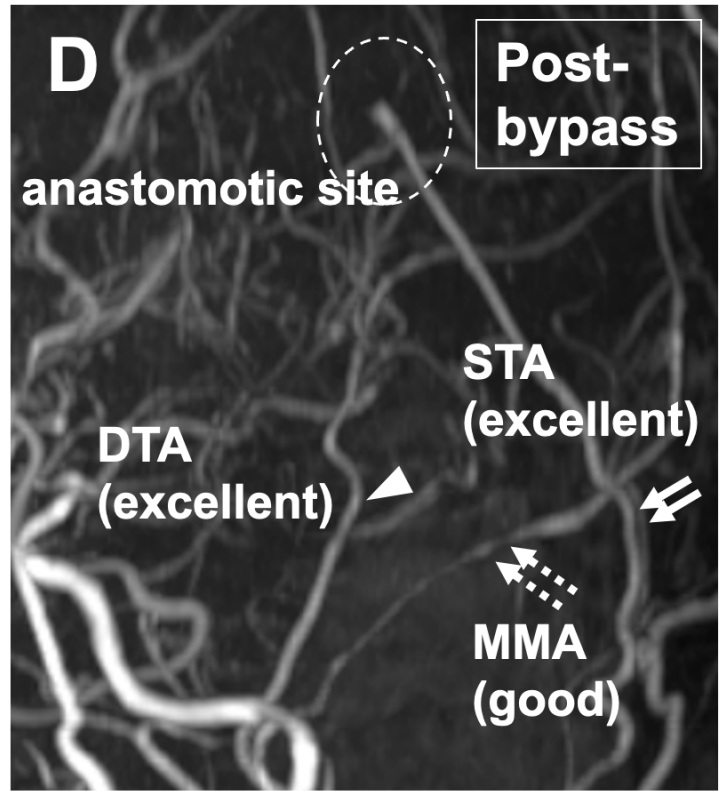
RNF213 founder polymorphism (p.R4810K)

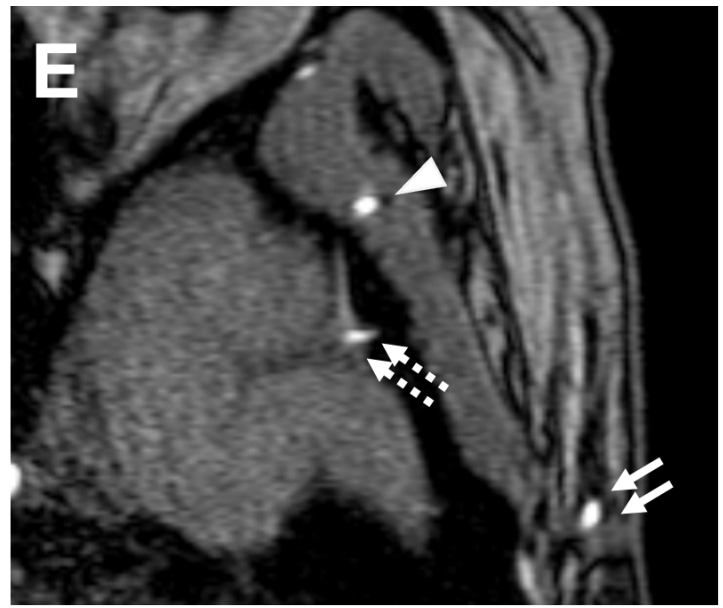
Wild-type: (G/G) 34 hemispheres, 37% (22 patients)	Heterozygous: (G/A) 59 hemispheres, 63% (40 patients)

Postoperative MR angiography evaluation



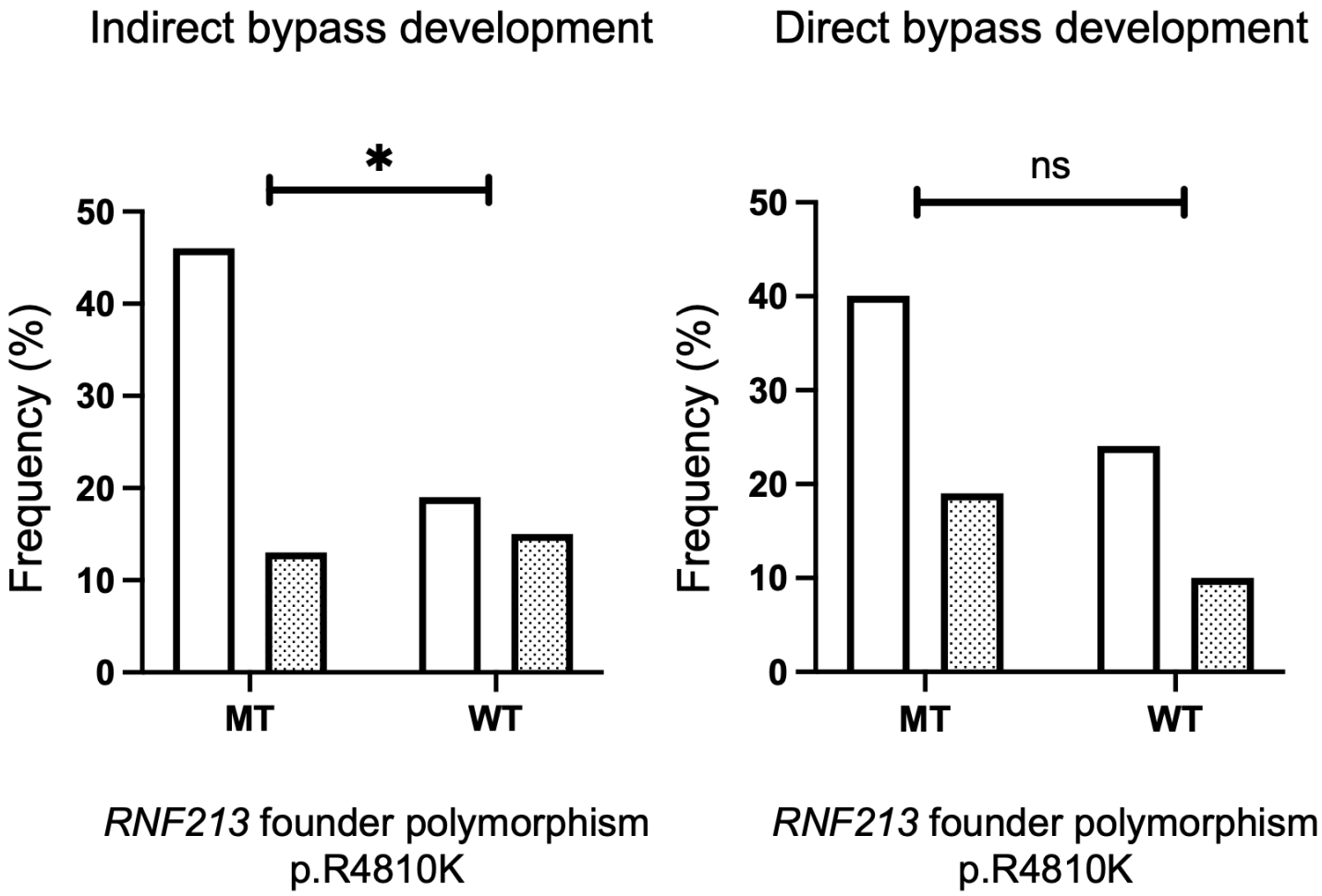






A

Qualitative Analysis



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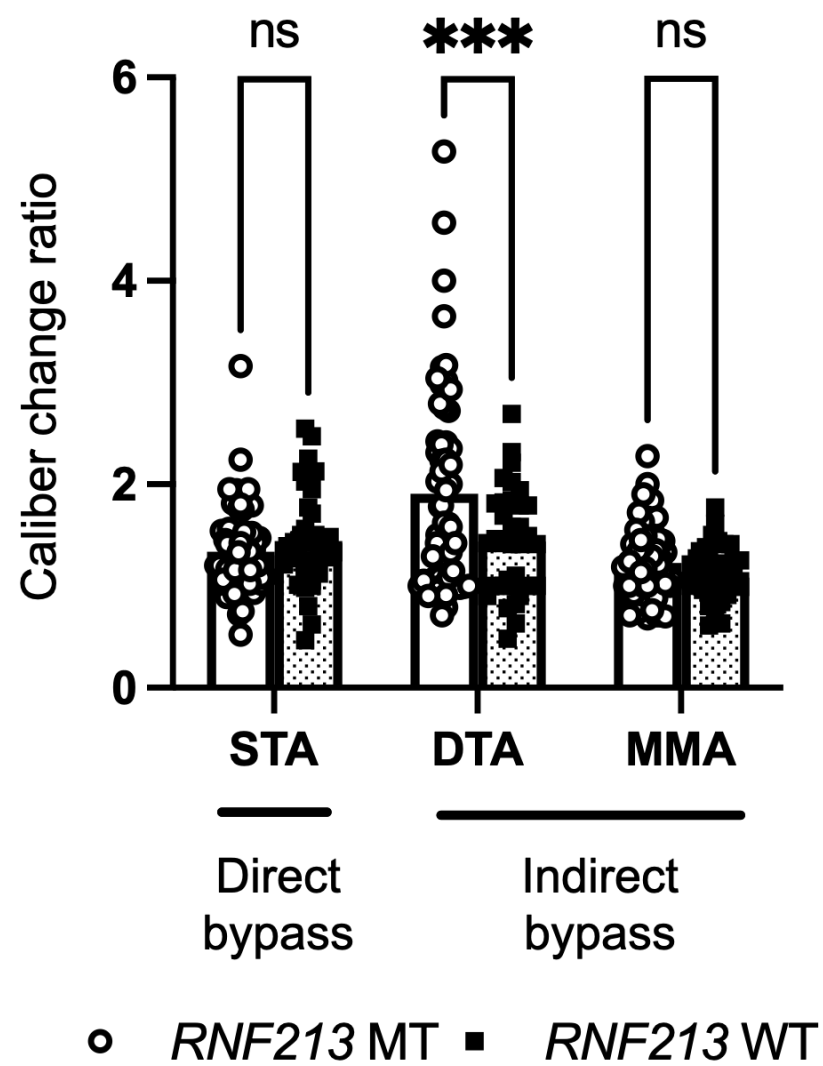
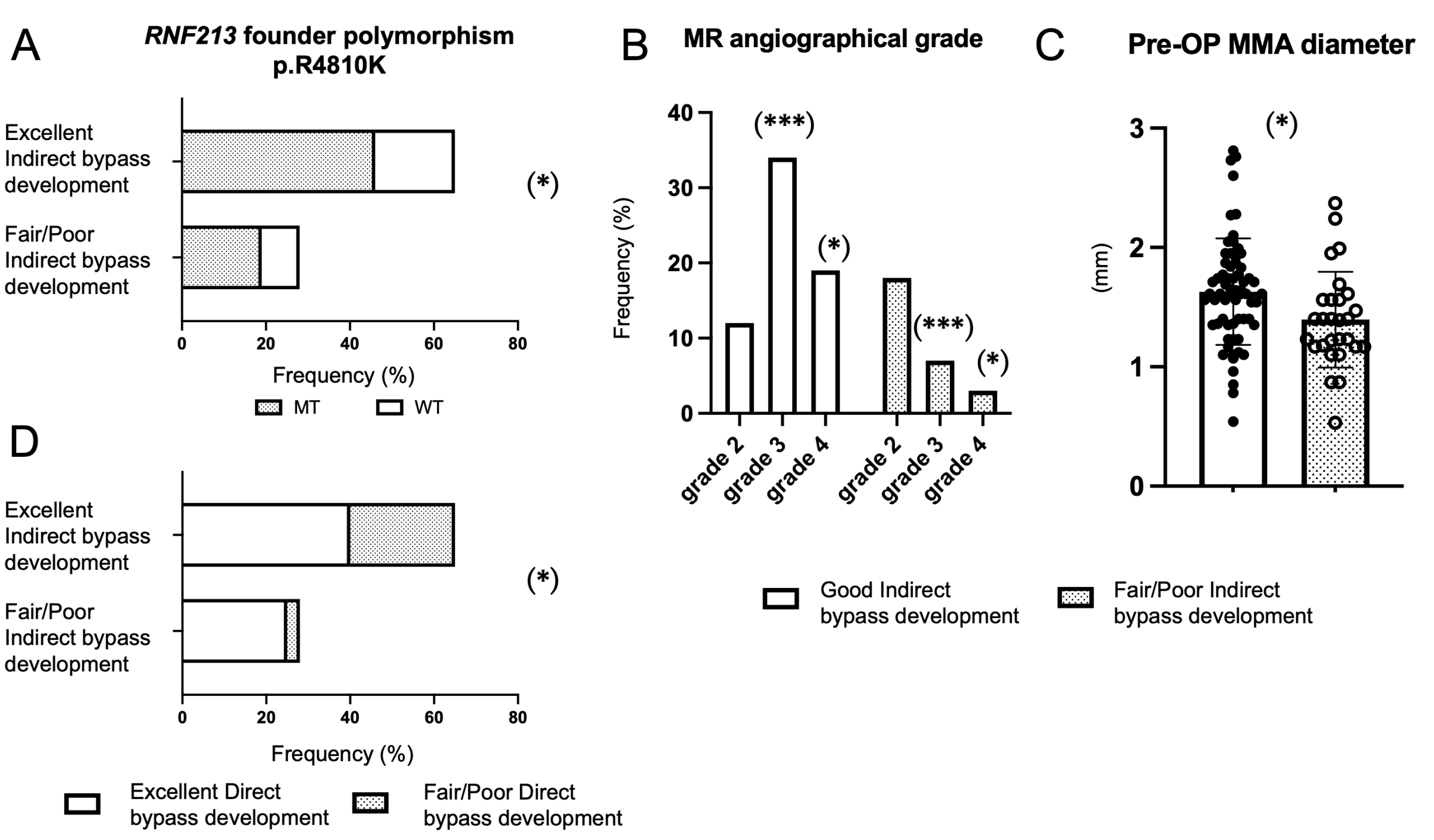
Quantitative Analysis



Supplementary data


A
RNF213 founder polymorphism p.R4810K
Excellent Indirect bypass development
Fair/Poor Indirect bypass development
(*)
0
20
40
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80
Frequency (%)
MT
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MR angiographical grade
Frequency (%)
40
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grade 2
grade 3
grade 4
grade 2
grade 3
grade 4
Good Indirect bypass development
Fair/Poor Indirect bypass development
C
Pre-OP MMA diameter
(mm)
3
2
1
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D
Excellent Indirect bypass development
Fair/Poor Indirect bypass development
(*)
0
20
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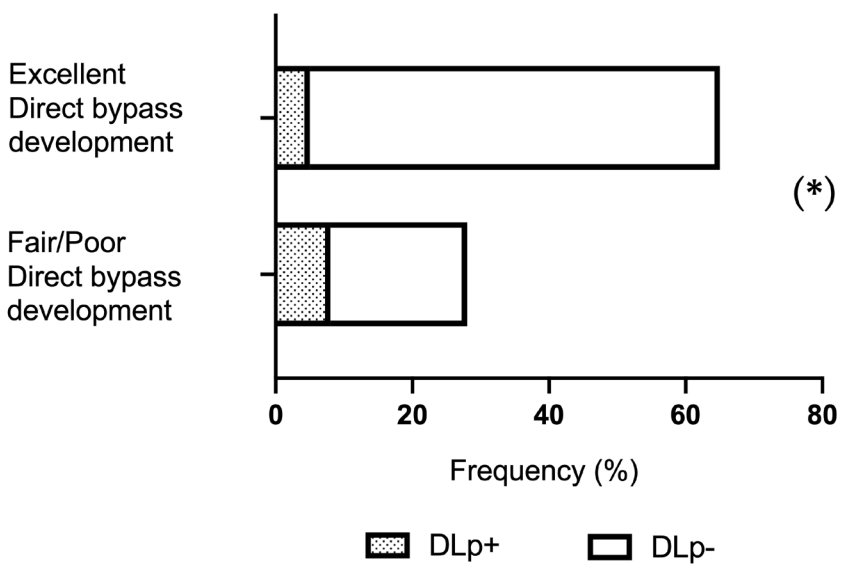
60
80
Frequency (%)
Excellent Direct bypass development
Fair/Poor Direct bypass development

A


Excellent
Direct bypass
development
Fair/Poor
Direct bypass
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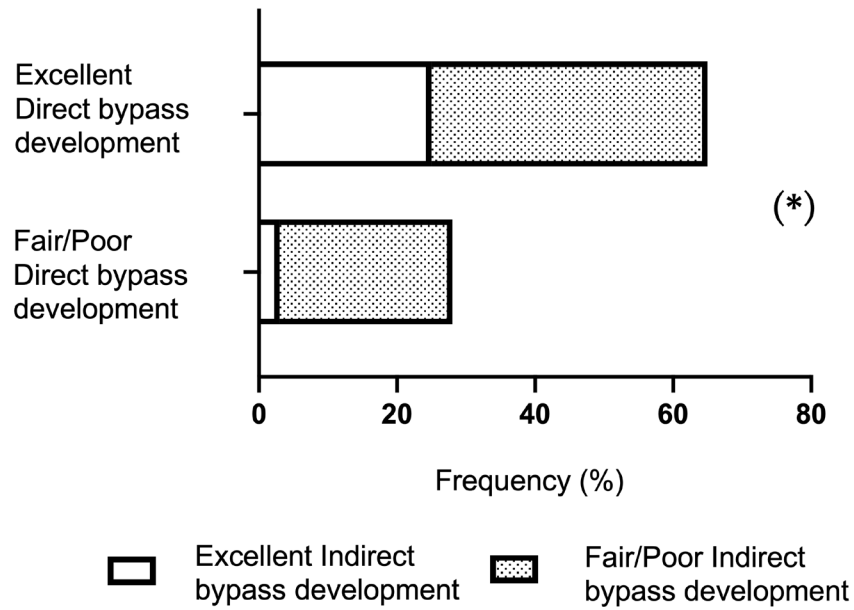
DLp-

B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Frequency (%)
Excellent Indirect
bypass development
Fair/Poor Indirect
bypass development

Supplementary Table 1

Baseline clinical and genetic characteristics of the MMD patients in this study

Variables		**N = 93 hemispheres**
Age, y, mean (range)		41.9 (19 - 67)
Sex, M:F		21:72
Hemisphere, Rt:Lt		48:45
Clinical presentation	Ischemia	62
	Hemorrhage	10
	Others	21
Co-morbidities	Hypertension	30 (32.3%)
	Diabetes mellitus	6 (6.5%)
	Dyslipidemia	13 (14.0%)
	CKD	1 (1.1%)
Familial occurrence		30 (32.2%)
RNF213 founder mutation (p.R4810K)	Homozygous, A/A	0
	Heterozygous, G/A	59 (63.4%)
	Wild-type, G/G	34 (36.6%)
MR angiographical stage, median (range)		3 (2-4)
Preoperative donor artery diameter, mm, mean (range)		
	STA	1.8 (1.2 - 2.7)
	MMA	1.6 (0.53 - 2.8)
	DTA	0.98 (0.37 - 1.8)
	BA	3.1 (1.9 - 4.3)

Supplementary Table 2

Comparison of baseline characteristics among MMD patients with or without *RNF213* founder mutation

Variables		*RNF213* Mutant, N=59	*RNF213* Wild-type, N=34	Odds Ratio (95% Confidence interval)	Significance
Age, y, mean (SD)		43.2 (9.07)	39.6 (12.4)		0.11
Sex, M:F		15:44	6:28	1.6 (0.55 to 4.7)	0.45
Familial occurrence		26/59 (44.1%)	4/34 (11.8%)	5.9 (1.9 to 17)	**0.0013**
Co-morbidities	Hypertension	24/59 (40.7%)	6/34 (17.7%)		
	Diabetes mellitus	3/59 (5.08%)	3/34 (8.82%)	3.2 (1.1 to 8.9)	**0.024**
	Dyslipidemia	8/59 (13.6%)	5/34 (14.7%)	0.55 (0.12 to 2.5)	0.67
	Chronic Kideny Disease	1/59 (1.72%)	0/34 (0%)	0.91 (0.30 to 2.7)	>0.9999
Hemisphere, Rt:Lt		29:30	19:15	Infinity (0.065 to infinity)	>0.9999
Clinical presentation	Ischemia	44	18	0.76 (0.32 to 1.8)	0.67
	Hemorrhage	4	6		0.083
	Others	11	10		
MR angiographical stage, median		3	3		0.36
Preoperative donor artery diameter mm, mean (SD)	STA	1.9 (0.37)	1.7 (0.3)		0.36
	MMA	1.6 (0.48)	1.6 (0.38)		
	DTA	0.95 (0.34)	1.0 (0.30)		0.05
	BA	3.0 (0.54)	3.2 (0.44)		0.88

MMD, Moyamoya disease; RNF213, ring finger protein 213; CI, confidence interval; SD, standard deviation; M, male; F, female Rt, right; Lt, left; STA, superficial temporal artery; MMA, middle meningeal artery; DTA, deep temporal artery; BA, basilar artery

Supplementary materials and methods

Study Population

A consecutive surgical series of patients with MMD or quasi-MMD was reviewed to identify all patients undergoing combined direct/indirect bypass [1, 4] between 2005 and 2019 at our hospital. The diagnosis of MMD was confirmed by the criteria outlined in the Japanese guidelines for the diagnosis and treatment of MMD. [5] In accordance with the guidelines, patients were offered the surgery if they were symptomatic based on a combination of neuropsychological testing, as reported previously,[3] and radiological studies, including MR imaging, TOF MR angiography, or had evidence of hemodynamic compromise by cerebral blood flow (CBF) measurements by [^{123}I] N-isopropyl-iodoamphetamine single photon emission tomography, with acetazolamide challenge if safely applicable. Repeat MR imaging and MR angiography after surgery were recommended at 3, 6, and 12 months, and yearly thereafter, but due to patient preference or willingness, it was not always available at all time points. This study included adult MMD patients (>16 years of age at the surgery) who consented to genetic analysis and underwent repeat MR imaging within 3 years after surgery. quasi-MMD was excluded. The Houkin MR angiographical stage/grading system was used to stratify the angiographical stage of MMD (ranging 1 to 4 [most advanced]).[2] Cerebrovascular reactivity (CVR) to acetazolamide was evaluated quantitatively as previously reported.[6] In accordance with an institutional review board-approved protocol (number14-053), medical records were retrospectively reviewed to gather demographic information, age at the surgery, symptoms at presentation, comorbid conditions before surgery, and results of radiographic studies.

Surgical Procedure

According to our surgical protocol, [1, 4] double-barrel STA to MCA anastomosis combined with EDAMS or EDMAPS was performed as the standard combined direct and indirect bypass procedure. Briefly, after large frontotemporal craniotomy, the dura was opened while preserving the MMA. Both of the parietal and frontal branches of the STA

were anastomosed to the cortical branches of the MCA in an end-to-side manner. The dural flap was inverted and placed on the surface of the brain to increase collateral formation. The temporal muscle was sutured to the edge of the dura. In the EDMAPS procedure, paramedian craniotomy was also performed to improve revascularization in the area of the anterior cerebral artery (ACA). The majority of these patients underwent indirect bypass using the periosteal flap, whereas the remaining patients underwent STA to ACA anastomosis. Basically, we discontinued antiplatelet(s) before surgery if prescribed, except for 14 operations. Thus, single antiplatelet agent (cilostazol, aspirin, or clopidogrel) was used in fifteen percent (14 out of 93) of the surgery.

Evaluation of the Postoperative Development of Direct and Indirect Surgical Collaterals by MR angiography

Evaluation of the postoperative development of direct and indirect surgical collaterals was performed qualitatively and quantitatively using TOF MR angiography and its source images, respectively. For qualitative evaluation,[6] postoperative development of each direct and indirect surgical collaterals were dichotomized into excellent or not, respectively. As defined below, the development of direct surgical collaterals was evaluated by the development of the STA, whereas that of indirect surgical collaterals was evaluated by the development of the MMA and DTA. Thus, we reviewed the caliber and course of the STA towards the site of direct anastomosis on TOF MR angiography with maximal intensity projection (MIP) reconstructions after surgery. When we were able to clearly follow the STA towards the anastomotic site with increased caliber after surgery, postoperative STA development was considered excellent. When we were unable to follow the STA to the anastomotic site with its unchanged or reduced caliber, we considered it to be fair or poor. For the indirect surgical collaterals, we compared the calibers of the MMA and DTA on MR angiography after surgery. When the caliber of both arteries increased, we considered the indirect bypass development to be excellent. When the caliber of either artery increased, we considered the indirect bypass development to be fair. In all other cases, it was considered poor. Lastly, the postoperative development of direct surgical collaterals was defined as excellent for the hemispheres with excellent STA development, and that of indirect collaterals was defined as excellent for the hemispheres with excellent or fair MMA and/or DTA development. We also

evaluated the relationship between direct or indirect bypass and postoperative development based on their dominancy as follows: direct dominant, indirect dominant, and dual/equal development.

For quantitative evaluation, we reviewed MR angiography source images and measured the calibers of the STA, MMA, and DTA as previously reported.[7] For direct bypass, we compared the caliber of the STA after surgery at the proximal portion of the STA bifurcation into frontal/parietal branches. For indirect bypass, the calibers of the DTA at the most proximal portion and MMA near the foramen spinosum were measured after surgery. The caliber change ratios (CCRs) of post to preoperative calibers were calculated for each artery. The calibers of the basilar artery were also measured before and after surgery as an internal control to validate the reliability of the measurement. The mean caliber of the basilar artery was not significantly different after surgery (3.1±0.52 mm vs. 3.0±0.50 mm).

The successful patency of the direct bypass immediately after surgery was further assessed using MR angiography acquired on 2.1±2.5 postoperative days. When we were able to clearly follow the STA towards the anastomotic site, the direct bypass was considered patent. When we were unable to follow the STA to the anastomotic site with its reduced caliber, we considered it was not patent. As a result, the rate of successful patency of direct bypass was 93.5% (86/92 hemispheres with completed direct bypass surgery) right after surgery. While, at the timing of follow-up (319±140 postoperative days), it was 96.7% (89/92 hemispheres), indicating immediate postoperative patency was not significantly different between right after surgery and about a year follow-up. Out of the six direct bypasses poorly visualized by MR angiography immediately after surgery, all (6 hemispheres) showed excellent indirect bypass development in the follow-up MRA. Whereas, 58 out of 86 hemispheres (67%) with good direct bypass immediately after surgery showed excellent indirect bypass development. There was no independent relationship between poor direct bypass immediately after surgery and postoperative development of indirect collaterals (P=0.17, Fisher's exact test). This is probably because dual/equal development was most frequently observed (62% of the operated hemisphere) in this series. Taken together, we could not claim that poor direct bypass immediately after the surgery potentially affect better indirect bypass eventually.

Genetic Analysis of the *RNF213* founder polymorphism (p.R4810K)

In accordance with the institutional review board-approved protocol, written-informed consent was received for genetic analysis from MMD patients or their guardians. Whole blood samples were collected from the study participants with written informed consent, centrifuged immediately, and blood cell pellets were stored at −80 °C until further analysis at Hokkaido University Hospital Clinical Research and Medical Innovation Center. Genetic analysis was conducted at the Department of Neurosurgery of Hokkaido University by K.T. and R.T. who were blinded to clinical data. Thus, genomic DNA was extracted using the QIAsymphony DSP DNA Midi Kit (937255, QIAGEN, Hilden, Germany) from the blood cells. Real-time polymerase chain reaction (PCR) was performed using Light Cycler 96 (Roche, Mannheim, Germany). PCR reaction mixtures were prepared using the Taqman genotyping master mix (4371355, Applied Biosystems, Foster City, CA, USA) and Taqman single nucleotide polymorphism genotyping assay (4351379, Thermo Fisher Scientific, Waltham, MA, USA) to identify *RNF21*3 founder mutation (p.R4810K). According to the manufacturer's instructions, the thermal cycling conditions were as follows: primary enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. After PCR amplification, an endpoint plate read was performed on the real-time PCR instrument to determine the allele type in each sample (p.R4810K, rs112735431, c.14429G>A located at chr 17:80385145 [GRCh38.p12], https://www.ncbi.nlm.nih.gov/snp/rs112735431).

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Legends for supplementary figures

Supplementary figure 1: The independent factors correlated with excellent development of indirect surgical collaterals after combined bypass in adult MMD.

(A-C) Stacked bars indicate significant positive correlations of excellent indirect bypass development with the *RNF213* founder mutation (adjusted odds ratio [OR], 4.0) **(A)**, advanced MR angiographical grade (adjusted OR, 13 in stage 3; 9.5 in stage 4) **(B)**, and preoperative caliber of the middle meningeal artery (MMA, adjusted OR, 6.8) **(C)**. **(D)** Significant negative correlations were noted between excellent indirect bypass development and excellent direct bypass development (adjusted OR, 0.17). *P <0.05, *P <0.001, multivariate logistic regression analysis.

Supplementary figure 2: The independent factors correlated with the excellent development of direct surgical collaterals after combined bypass in adult MMD.

Stacked bars indicate significant negative correlations between excellent direct bypass development and the comorbid condition of dyslipidemia (adjusted OR, 0.27) **(A)** and excellent indirect bypass development (adjusted OR, 0.23) **(B)**. *P <0.05, multivariate logistic regression analysis.