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Downregulation of Securin by the variant RNF213 R4810K (rs112735431, G>A) reduces angiogenic activity of induced pluripotent stem cell-derived vascular endothelial cells from moyamoya patients

Toshiaki Hitomi [a,1], Toshiyuki Habu [b,1], Hatasu Kobayashi [a], Hiroko Okuda [a], Kouji H. Harada [a], Kenji Osafune [c], Daisuke Taura [d], Masakatsu Sone [d], Isao Asaka [c], Tomonaga Ameku [c], Akira Watanabe [c], Tomoko Kasahara [c], Tomomi Sudo [c], Fumihiko Shiota [c], Hirokuni Hashikata [e], Yasushi Takagi [e], Daisuke Morito [f], Susumu Miyamoto [e], Kazuwa Nakao [d], Akio Koizumi [a,*]

[a] Department of Health and Environmental Sciences, Kyoto University, Kyoto, Japan
[b] Radiation Biology Center, Kyoto University, Kyoto, Japan
[c] Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan
[d] Department of Medicine and Clinical Science, Kyoto University, Kyoto, Japan
[e] Department of Neurosurgery, Kyoto University,Kyoto, Japan
[f] Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan

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ABSTRACT

Moyamoya disease (MMD) is a cerebrovascular disease characterized by occlusive lesions in the circle of Willis. The RNF213 R4810K polymorphism increases susceptibility to MMD. Induced pluripotent stem cells (iPSCs) were established from unaffected fibroblast donors with wild-type RNF213 alleles, and from carriers/patients with one or two RNF213 R4810K alleles. Angiogenic activities of iPSC-derived vascular endothelial cells (iPSECs) from patients and carriers were lower (49.0 ± 19.4%) than from wild-type subjects ($p < 0.01$). Gene expression profiles in iPSECs showed that Securin was down-regulated ($p < 0.01$) in carriers and patients. Overexpression of RNF213 R4810K downregulated Securin, inhibited angiogenic activity (36.0 ± 16.9%) and proliferation of humanumbilical vein endothelial cells (HUVECs) while overexpression of RNF213 wild type did not. Securin expression was downregulated using RNA interference techniques, which reduced the level of tube formation in iPSECs and HUVECs without inhibition of proliferation. RNF213 R4810K reduced angiogenic activities of iPSECs from patients with MMD, suggesting that it is a promising *in vitro* model for MMD.

1. Introduction

Moyamoya disease (MMD) is an idiopathic cerebrovascular disease. It is characterized by occlusive lesions at the terminal portion of internal carotid arteries in the circle of Willis, with compensatory development of a fine vascular network that resembles "puffs of smoke" [1,2]. It is now recognized as one of the major causes of stroke in adults and children worldwide [3–6]. We recently identified *RNF213* as the susceptibility gene for MMD, and the p.R4810K (rs112735431, ss179362673: G>A; herein referred to as RNF213 R4810K) polymorphism as a founder variant commonly found in East Asian (Japanese, Korean and Chinese) patients [7]. RNF213 encodes a 591 kDa protein that exhibits ATPase and ubiquitin ligase activities. Although knockdown of RNF213 in zebrafish impaired angiogenesis, the physiological and biochemical functions of RNF213, and pathological consequences of MMD associated with RNF213 R4810K remain unknown [7].

The minor allele frequency of the founder RNF213 R4810K polymorphism in the general population is estimated to be 0.43–1.36% for East Asia, equivalent to a prevalence of 0.86–2.72% for carriers. RNF213 R4810K elevates the risk of MMD by more than 100-fold in carriers [7], with approximately 15 million individuals thought to be at extremely high risk [8]. The prevalence of patients with MMD (0.01%) is much lower than that for RNF213 R4810K carriers (3%) in Japan and Korea [3,4]. We have sought to determine the triggering factors that induce MMD in RNF213 R4810K carriers. Considering the social and economic dimensions of a large high-risk population in East Asia, determination of these MMD triggering

* Corresponding author. Address: Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan. Fax: +81 75 753 4458.
E-mail address: koizumi.akio.5v@kyoto-u.ac.jp (A. Koizumi).
[1] These authors contributed equally to this work.

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factors is a high-priority issue. Such triggers are considered to act through RNF213 R4810K, but elucidation of these triggers has been hampered, mainly because of the lack of knowledge with respect to RNF213 R4810K pathology.

The primary aims of our study were to characterize RNF213 R4810K and the physiological functions of RNF213. To determine the pathological defects attributable to RNF213 R4810K, we tested whether vascular endothelial cells from patients with MMD have lowered angiogenic activities. Our hypothesis was based on reports of defective angiogenic activities for circulating endothelial progenitor cells in MMD patients [9]. We used induced pluripotent stem cell (iPSC) technology with the hope that it might yield useful *in vitro* disease models [10]. This approach is particularly useful for diseases in which the pathological processes have yet to be elucidated. Once an *in vitro* model has been established it is possible to reveal pathological clues about a disease; it can then be employed as a drug-screening tool, paving the way for translational research. We characterized *ex vivo* phenotypes of vascular endothelial cells differentiated from iPSCs (iPSECs) and conducted a series of *in vitro* experiments to understand the underlying mechanisms of MMD.

2. Methods

2.1. Participants

We studied three probands from three unrelated families with MMD. Diagnosis was made based on criteria from the Japanese Research Committee on MMD (Ministry of Health, Labour and Welfare, Japan) [11]. Participants consisted of six affected, or unaffected and unrelated subjects (Table 1 and Supplementary data). Genotyping revealed a AA genotype (homozygous for RNF213 R4810K) for two affected subjects, a GA genotype (heterozygous for RNF213 R4810K) in one affected and one unaffected subject, and a GG genotype (wild-type for RNF213 R4810K) for two unaffected subjects. We obtained written informed consent from all participants in this study. Our study was approved by the Institutional Ethical Review Board of Kyoto University.

2.2. Establishment of iPSECs

Dermal fibroblasts were isolated from arms and cultured. Induction of iPSCs was performed as described previously (Supplementary data) from primary fibroblasts for three MMD patients, an unaffected carrier and two controls (Table 1). We then induced the differentiation of iPSCs into vascular endothelial cells (Supplementary data). Angiogenic activity of iPSECs was assayed by tube formation. Gene expression profiles were determined using a GeneChip microarray (Human Gene 1.0 ST; Supplementary data).

Table 1
Summary of donor fibroblast information.

ID	Diagnosis	Gender	Age at onset	Age at biopsy	R4810K (G>A) of RNF213
Control 1	Healthy control	F	NA	81	GG
Control 2	Healthy control	F	NA	6	GG
Unaffected carrier	Healthy control	M	NA	36	GA
Patient 1	Familial MMD	F	10	43	GA
Patient 2	Familial MMD	F	55	63	AA
Patient 3	Familial MMD	F	50	64	AA

MMD, moyamoya disease.

2.3. Cell culture and transfection

Fibroblasts were maintained in Dulbecco's Minimal Essential Medium (DMEM; Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Japan Bioserum, Hiroshima, Japan). The iPSCs were maintained in Primate ES medium (ReproCELL, Tokyo, Japan) and supplemented with 500 U/ml penicillin/streptomycin (Invitrogen) and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF; WAKO, Tokyo, Japan) as previously reported [10,12]. Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD, USA) were maintained in EGM-2 (Lonza). An mCherry-tagged wild-type RNF213 or an mCherry-tagged RNF213 R4810K was cloned into pcDNA3.1 (Invitrogen) (Supplementary data). The plasmids were introduced with an Amaxa Nucleofector Device (Lonza).

2.4. Assessment of angiogenic activity

Endothelial tube formation was assessed as described previously [13]. The iPSECs (5000 cells/well) or HUVECs (5000 cells/well or 20,000 cells/well) were seeded onto matrigel-coated (BD Biosciences, Bedford, MA, USA) 96-well plates. Cells were incubated for 12 h at 37 °C and digital images of tubes that formed were captured. For quantitation, tube area, total tube length and the number of tube branches were calculated using Image J software (National Institute of Health, USA). Parameters for assessing tube formation function were obtained from three or four independent tube formation assays.

2.5. RNA interference (RNAi)

Transfection of small interfering RNAs (siRNAs) was conducted using Dharmafect (#1 or #3; Dharmacon, Lafayette, CO, USA) following the manufacturer's recommendations. We also used Nucleofector instruments to transfect HUVECs and iPSECs according to the manufacturer's protocols. We purchased and used Securin siRNA (sc-37491; Santa Cruz Biotechnology, Santa Cruz, CA, USA), RNF213 siRNA 1 (sc-94184; Santa Cruz Biotechnology) and RNF213 siRNA 2 (s33568; Ambion, Austin, TX, USA), with control siRNA-A (sc-37007, Santa Cruz Biotechnology) and silencer select negative control #1 siRNA (Ambion) used as controls. To monitor knockdown of gene expression, real-time quantitative polymerase chain reaction (qPCR), immunostaining and/or western blotting assays were conducted.

2.6. Growth curves

Cell proliferation was assessed using colorimetric 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assays, which were carried out as described previously [14] unless otherwise specified. For growth curves for HUVECs in the overexpression experiment of RNF213 wild type and R4810K, at 2 days post-transfection, HUVECs were re-seeded at a density of 8×10^4 -cells/3.5-mm dish. Viable cells were assessed and counted each day using trypan blue (Nacalai Tesque) exclusion.

2.7. Western blotting

We used the CelLytic M (Sigma–Aldrich, St Louis, MO, USA) cell lysis buffer containing a protease inhibitor cocktail (Nacalai Tesque). In certain cases we also used a lysis buffered comprising 50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40 and 150 mM NaCl. Samples were subjected to immunoblotting using the anti-RNF213