Biochemical and Biophysical Research Communications xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The moyamoya disease susceptibility variant RNF213 R4810K induces genomic instability by mitotic abnormality

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ARTICLE INFO

Article history:
Received 16 August 2013
Available online xxxx

Keywords:
Moyamoya disease
iPS cells
Mitotic phase
Genomic instability
RNF213
MAD2

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. In the present study, we characterized phenotypes caused by overexpression of *RNF213* wild type and R4810K variant in the cell cycle, to investigate the mechanism of proliferation inhibition. Overexpression of RNF213 R4810K in HeLa cells inhibited cell proliferation and extended the time of mitosis 4-fold. Ablation of spindle checkpoint by depletion of mitotic arrest deficiency 2 (MAD2) did not shorten the time of mitosis. Mitotic morphology in HeLa cells revealed that MAD2 colocalized with RNF213 R4810K. Immunoprecipitation revealed an RNF213/MAD2 complex: R4810K formed a complex with MAD2 more readily than RNF213 wild-type. Desynchronized localization of MAD2 was observed more frequently during mitosis in fibroblasts from patients ($n = 3$, $61.0 \pm 8.2\%$) compared with wild-type subjects ($n = 6$, $13.1 \pm 7.7\%$; $p < 0.01$). Aneuploidy was observed more frequently in fibroblasts ($p < 0.01$) and induced pluripotent stem cells (iPSCs) ($p < 0.03$) from patients than from wild-type subjects. Vascular endothelial cells differentiated from iPSCs (iPSECs) of patients and an unaffected carrier had a longer time from prometaphase to metaphase than those from controls ($p < 0.05$). iPSECs from the patients and unaffected carrier had significantly increased mitotic failure rates compared with controls ($p < 0.05$). Thus, RNF213 R4810K induced mitotic abnormalities and increased risk of genomic instability.

1. Introduction

Moyamoya disease (MMD: MIM 607151) is characterized by occlusive lesions at the terminal portion of internal carotid arteries in the Circle of Willis [1,2]. It is now recognized as one of the major causes of stroke in adults and children worldwide [3–6]. RNF213 has been recognized as the susceptibility gene for MMD, and the *p.* R4810K polymorphism (rs112735431 or ss179362673: G > A; herein referred to as RNF213 R4810K) as a founder variant commonly found in East Asian (Japanese, Korean and Chinese) MMD patients [7].

We recently found that vascular endothelial cells developed from induced pluripotent stem cells (iPSECs) of patients with MMD, carrying RNF213 R4810K, had reduced angiogenic activity [8]. This was partially mediated by the down-regulation of *Securin* [8]. In addition to *Securin*, various mitosis-associated genes were down regulated in iPSECs from patients [8]. Furthermore, the overexpression of RNF213 R4810K inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) [8]. The primary aim of our study was to characterize the phenotypes associated with RNF213 R4810K in the cell cycle.

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0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.08.067

2. Methods

2.1. Participants

We studied three probands from three unrelated families with MMD, a carrier of RNF213 R4810K and seven controls. Details of the patients were described previously [8] and in Table S1. 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. Cell culture and transfection

Fibroblasts and HeLa cells were maintained in Dulbecco's Minimal Essential Medium (DMEM; Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Japan Bioserum, Hiroshima, Japan). Fibroblasts from passages 3–5 were used for all experiments. Induced pluripotent stem cells (iPSCs) were maintained as previously reported [8,9].

An mCherry-tagged wild-type RNF213 or an mCherry-tagged RNF213 R4810 K was cloned into pcDNA3.1 (Invitrogen) (Fig. S1) [8]. To monitor the localization of MAD2, HeLa cells stably expressing EGFP-MAD2 were used. MAD2 cDNA cloned into pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA) was introduced into HeLa cells, and a G418-resistant clone was verified by western blotting and fluorescent microscopy. The plasmid was introduced into HeLa cells using Lipofectamine 2000 (Invitrogen) and successfully transfected cells selected with 500 μg/ml G418 (Nacalai Tesque, Kyoto, Japan) for 10 days.

Transfection of small interfering RNAs (siRNAs) was conducted using Dharmafect (#1 or #3; Dharmacon, Lafayette, CO, USA) as previously reported [8]. We purchased and used RNF213 siRNA (Santa Cruz Biotechnology) and MAD2 siRNA (Santa Cruz Biotechnology), with control siRNA-A (Santa Cruz Biotechnology) used as controls.

2.3. Karyotyping

For karyotyping, fibroblasts from six controls (Control 1 to Control 6), one carrier, and three patients were treated with nocodazole (100 ng/ml) for 72 h. Well-isolated chromosomes were chosen and counted three times for each chromosome set. For each fibroblast culture, duplicate karyotyping experiments were conducted. For MAD2 staining, fibroblasts were treated with nocodazole (100 ng/ml) for 72 h, fixed with 4% paraformaldehyde and permeabilized in phosphate-buffered saline (PBS) containing 0.2% Triton X-100. An anti-MAD2 antibody (Covance, Berkeley, CA, USA) was used for immunostaining.

To evaluate chromosomal instability, six iPSC clones from controls (Control 1 to Control 7 except Control 4) and four from a carrier and patients were karyotyped (Table S1).

2.4. Colony formation assays

Following transfection, HeLa cells were reseeded at densities of 1×10^3 to 2.7×10^4 cells/100-mm dish and maintained in DMEM with 10% FBS for 5 days. Medium containing G418 (Nacalai Tesque) was exchanged twice a week. After 10 days, resistant colonies were scored using formalin fixation and crystal violet staining.

2.5. Time-lapse imaging using confocal laser scanning microscopy

Transfected HeLa cells and iPSECs were plated on 35-mm glass-bottom culture dishes. Time-lapse 3D imaging was performed using an FV10i confocal microscope (Olympus, Tokyo, Japan) at 37 °C/5% CO_2. The recording interval was 40 min, and Z-stack images were generated with Fluoview (Olympus).

2.6. Western blotting

Samples were subjected to immunoblotting using the anti-RNF213 antibody, which we generated previously [8], anti-MAD2, anti-dsRed (BD Biosciences), or anti-β-actin (Abcam, Cambridge, UK) antibodies. Quantitation was conducted using Image J software.

2.7. Co-immunoprecipitation of MAD2 with RNF213

HeLa cells transiently expressing the wild-type RNF213 mCherry or RNF213 R4810K mCherry or naïve HeLa cells were lysed in RIPA buffer without sodium dodecyl sulfate (SDS) but with protease inhibitors (Nacalai Tesque). Cell lysates from 4×10^6 cells were incubated with protein A agarose (Santa Cruz Biotechnology) for 30 min at 4 °C with normal mouse immunoglobulin G (IgG; MBL, Nagoya, Japan). After magnetic separation, beads were discarded and supernatants incubated for 4 h at 4 °C with a monoclonal anti-dsRed or polyclonal anti-RNF213 antibody [8] followed by magnetic beads for 4 h. Beads were washed three times with lysis buffer, and bound proteins dissolved in SDS sample buffer at 95 °C for 5 min, subjected to SDS polyacrylamide gel electrophoresis (PAGE) and analyzed by western blotting with anti-MAD2 antibody.

2.8. Statistical analysis

Results are presented as the mean ± standard deviation (SD) unless otherwise stated. Differences between groups were analyzed using analysis of variance (ANOVA), followed by Tukey's honestly significant difference test for comparisons involving more than two means (SAS Institute Inc., Cary, NC, USA). The variance in chromosome numbers as determined by karyotyping was compared with controls using an F-test. Subcellular localization of MAD2 was categorized into four groups and compared with controls using Fisher's exact test with Bonferroni correction. A p-value with Bonferroni correction less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of RNF213 R4810K overexpression

mCherry-tagged wild-type RNF213 and/or RNF213 R4810K proteins (Fig. S1) were overexpressed in HeLa cells (Fig. S2A and B). Localization of exogenous RNF213 R4810K was similar to that of exogenous and endogenous wild-type RNF213, where proteins were observed in the cytoplasm around the nucleus (Fig. S2B). Overexpression of RNF213 R4810K highly repressed colony formation units of HeLa cells (Fig. S2C). In contrast, RNAi-mediated depletion of RNF213 in HeLa cells did not repress colony formation units (Fig. S2D).

To understand better the causes underlying inhibition of cell proliferation, cell cycle distribution of HeLa cells expressing wild-type RNF213 and RNF213 R4810K were monitored. Overexpression of RNF213 R4810K caused a G2/M-plus-higher-DNA-content (4N>) accumulation in HeLa cells (Fig. S3), but overexpression of wild-type of RNF213 did not. Live imaging analyses showed that mitotic stages were severely delayed in HeLa cells overexpressing RNF213 R4810K (Fig. 1A–C). In cells overexpressing the control vector, wild-type RNF213, control siRNA or RNF213 siRNA, the mean time from prometaphase to metaphase was 37 ± 10 min. The mean time from anaphase to telophase was 63 ± 24 min (Fig. 1B and C). In

Please cite this article in press as: T. Hitomi et al., The moyamoya disease susceptibility variant RNF213 R4810K induces genomic instability by mitotic abnormality, Biochem. Biophys. Res. Commun. (2013), http://dx.doi.org/10.1016/j.bbrc.2013.08.067