

SHORT COMMUNICATION

FLNA p.V528M substitution is neither associated with bilateral periventricular nodular heterotopia nor with macrothrombocytopenia

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Filamin A is encoded by the *FLNA* gene on chromosome Xq28 and functions in cross-linking actin filaments into orthogonal networks in the cortical cytoplasm. *FLNA* p.V528M was initially detected in a female autopsy case of X-linked bilateral periventricular nodular heterotopia (BPNH), a neuronal migration disorder characterized by subependymal nodules of gray matter. During our mutation analysis of *FLNA* in a boy with apparent X-linked thrombocytopenia, we detected the p.V528M variant. The patient, mother and sister, who were heterozygous for the substitution, did not have BPNH. We observed an allele frequency of 4.8% in healthy control Japanese, but did not observe the variant in Caucasian subjects. Hemizygous controls had a normal platelet count and size. We suggest that p.V528M is neither associated with BPNH nor with thrombocytopenia and giant platelets, and represents a functional polymorphism.

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INTRODUCTION

X-linked dominant bilateral periventricular nodular heterotopia (BPNH; MIM #300049) is a neuronal migration disorder characterized by subependymal nodules of gray matter.^{1,2} Most patients are heterozygous females who suffer from epilepsy and have normal to borderline intelligence.³ Hemizygous males are likely to be embryonic-lethal or severely affected with mental retardation and skeletal dysplasia, in addition to epilepsy. X-linked BPNH is caused by mutations in *FLNA* (MIM #300017) on chromosome Xq28, which encodes the actin-binding protein filamin A.³ This cytoskeletal protein cross-links actin filaments into orthogonal networks in the cortical cytoplasm and participates in the anchoring of membrane proteins for actin cytoskeleton.^{4,5} So far, > 50 *FLNA* mutations have been reported as a cause of BPNH. Most are nonsense, frameshift or splicing mutations, predicted to result in a severe loss of function, whereas some missense mutations also have been identified.^{6–8}

Filamin A is a widely expressed protein^{5,9}. In blood platelets, it interacts with the glycoprotein Ib/IX transmembrane receptor and contributes to the normal platelet morphology by linking the platelet membrane skeleton and cytoskeletal actin filaments.^{10,11} Inherited glycoprotein Ib/IX deficiency leads to Bernard–Soulier syndrome, a rare autosomal recessive bleeding disorder exhibiting abnormally large platelets and thrombocytopenia (macrothrombocytopenia).^{12,13} Defects in *FLNA* could possibly lead to congenital macrothrombocyto-

penia. During our mutation analysis of *FLNA* in a patient with apparent X-linked thrombocytopenia, we detected the p.V528M variant that has been reported as a BPNH mutation.¹⁴ We present evidence that p.V528M is neither associated with BPNH nor with thrombocytopenia and giant platelets.

MATERIALS AND METHODS

Patient

A 6-year-old boy was referred to Kobe University hospital for the evaluation of thrombocytopenia. A laboratory test revealed a platelet count of $30 \times 10^9 \text{ l}^{-1}$. Although the patient exhibited no bleeding tendency, he showed persistent thrombocytopenia ($30\text{--}60 \times 10^9 \text{ l}^{-1}$; Table 1). Peripheral blood smears showed the prominent appearance of giant platelets. Other blood cell counts, blood chemistry and coagulation tests were within the normal ranges. Platelets were normally aggregated in response to ADP, collagen and ristocetin. Flow cytometry showed the normal expression of platelet glycoprotein Ib/IX.¹² There were no granulocyte inclusion bodies on May–Grünwald–Giemsa (MGG)-stained peripheral blood smears, which was confirmed by immunofluorescence analysis of granulocyte myosin IIA localization.¹⁵ These results excluded Bernard–Soulier syndrome and *MYH9* disorders, the most frequent forms of macrothrombocytopenia.¹⁶ The boy's maternal grandfather (I-3) had a previous history of immune thrombocytopenia and received platelet transfusion (Figure 1). Two sons (II-1 and 2) of the maternal grandfather's (I-3) sister (I-2) had thrombocytopenia, one of whom (II-2) underwent splenectomy. Based on the family history, an X-linked dominant thrombocytopenia was

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Table 1 Clinical and laboratory features in individuals of the family

Case	Sex	Age (years)	FLNA mutation	Platelet count ($\times 10^9 l^{-1}$)	Platelet size (μm) ^a	Epilepsy	Cognitive level	Brain MRI
Father (II-3)	M	40		15.2	2.6 ± 0.7			
Mother (II-4)	F	36	p.V528M (hetero)	14.0	2.8 ± 0.8	No	Normal	Normal
Patient (III-1)	M	6	p.V528M (hemi)	3.5	4.8 ± 1.5	No	Normal	Normal
Sister (III-2)	F	4	p.V528M (hetero)	27.6	2.5 ± 0.5	No	Normal	Normal

Abbreviations: F, female; hemi, hemizygous; hetero, heterozygous; M, male; MRI, magnetic resonance imaging.

^aDetermined by microscopic observation of 200 platelets on a stained peripheral blood smear. Controls, 2.5 ± 0.3 μm ($n=31$).

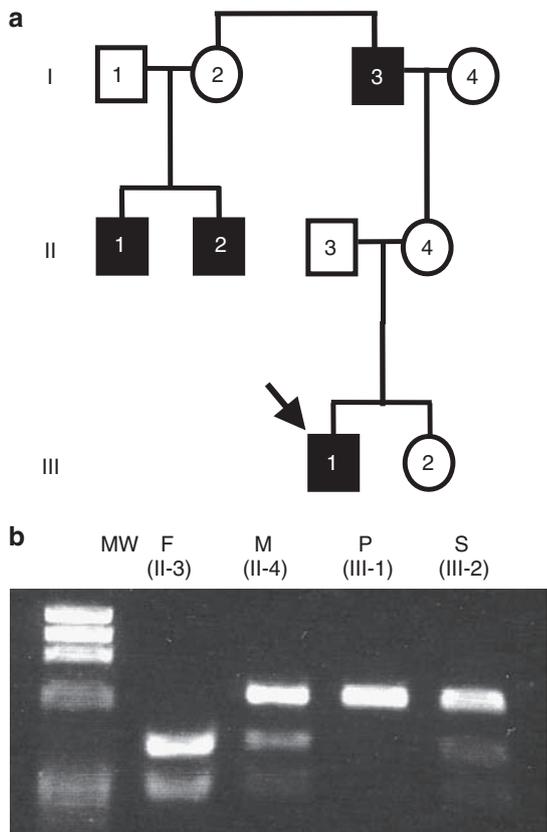


Figure 1 Family tree of the patient with apparent X-linked dominant thrombocytopenia. (a) Circles represent females and squares represent males. Solid symbols denote affected and open symbols denote unaffected individuals. The proband (III-1) is indicated by an arrow. Samples were available and analyzed from II-3, II-4, III-1 and III-2. (b) Allele-specific restriction fragment length polymorphism analysis. The c.1582G>A substitution abolishes a recognition site for *Bst*UI: the hemizygous patient displays a 547-bp band (uncut) and the wild-type father displays 215- and 332-bp bands. The heterozygous mother and sister display three bands. MW, *Hae*III digest of Φ X 174 DNA; F, father (II-3); M, mother (II-4); P, patient (III-1); S, sister (III-2).

suggested. The parents, his sibling and maternal grandfather's sister were negative for a history of a bleeding tendency. All these individuals were of normal intelligence and did not show signs of psychotic behavior, cerebral palsy, epilepsy or other neurological disorders. Brain magnetic resonance imaging scans of the patient, mother and sister revealed a normal architecture of the brain (Figure 2).

Mutation analysis of *FLNA*

The entire coding regions of the patient's *FLNA* gene were amplified from genomic DNA by PCR and amplified DNA fragments were subjected to direct

cycle sequence analysis (Supplementary Table 1). The presence of the c.1582G>A substitution was analyzed in the healthy control DNA of 192 Japanese (114 males, 78 females) and 83 Austrian Caucasians (32 males, 51 females, kind gifts from Dr Christoph Gassner, Blutspende Zuerich, Switzerland¹⁷). The gender of each control was determined by PCR detection of the *SRY* gene (UniSTS: 482099). DNA fragments amplified using primers FLNA105, 5'-ACCCACCAATCCTGACAGC-3', and FLNA113, 5'-AGGCAGGAAGAGCCCATGTG-3', were digested with *Bst*UI (New England Biolabs, Beverly, MA, USA), electrophoresed on 2% agarose gels and stained with ethidium bromide. Expected frequencies of genotypes in the population were calculated using the Hardy-Weinberg equation.¹⁸ The 95% confidence interval of the proportion was calculated. Peripheral blood samples were obtained after obtaining a written consent. The Ethics Committees of Nagoya Medical Center and Kobe University approved this study.

Platelet size determination

Platelet size was determined as the platelet diameter by the microscopic observation of 200 platelets on MGG-stained peripheral blood smears.

RESULTS AND DISCUSSION

We performed mutation analysis of *FLNA* in a patient with apparent X-linked thrombocytopenia (Figure 1). The entire coding sequence of the gene including the intron-exon junctions was investigated. This revealed a hemizygous substitution, c.1582G>A, changing the conserved valine at codon 528 to methionine (p.V528M). The patient's mother and sister were heterozygous for the substitution. The c.1582G>A has been reported in a heterozygous condition in a female autopsy case of BPNH.¹⁴ In that report, the substitution was not found in 100 unrelated control individuals. Although the substitution was not found in the single-nucleotide polymorphism database (<http://www.ncbi.nlm.nih.gov/SNP/>, accessed May 2010), our restriction fragment length polymorphism analysis of control Japanese subjects identified four hemizygotes in 114 males and nine heterozygotes in 78 females. The allele frequency was 4.8% (95% confidence interval (2.8–8.1)). A comparison of the allele frequency between males (3.5%, 95% confidence interval (1.4–8.7)) and females (5.8%, 95% confidence interval (3.1–10.6)) showed no significant difference ($P>0.05$). The exact test for female data showed no significant deviation from the Hardy-Weinberg equilibrium ($P>0.05$). In contrast, c.1582G>A was not detected in a total of 83 control Caucasian subjects, suggesting that c.1582G>A is rare or absent in this population.

The previously described BPNH case with p.V528M reportedly had characteristic heterotopic gray matter lining the walls of the lateral ventricles on a brain magnetic resonance imaging study.¹⁴ However, the present hemizygous patient and heterozygous mother and sister had normal brain magnetic resonance imaging findings (Figure 2). *FLNA* is the major gene currently known to be associated with BPNH.^{6–8} Most *FLNA* mutations are nonsense, frameshift or splicing mutations, predicted to result in a severe loss of function, whereas some missense mutations have been identified. Missense mutations

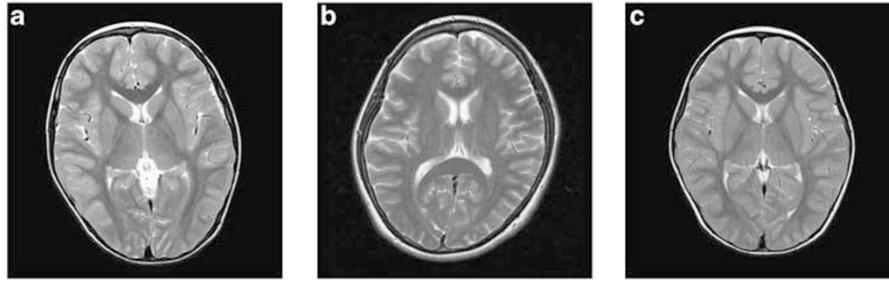


Figure 2 Magnetic resonance imaging of the brain. T2-weighted axial images demonstrate a normal architecture of the brain. (a) Patient (III-1); (b) mother (II-4); (c) sister (III-2).

may result in the production of partially functional proteins and lead to milder phenotypes. A rare autosomal recessive BPNH associated with microcephaly, with mutations of *ARFGEF2*,¹⁹ and BPNH patients with duplication of 5p15.1 or trisomy of 5p15.33²⁰ have also been reported. We suggest that p.V528M is not associated with BPNH and that it represents a functional polymorphism.

Although the present patient hemizygous for p.V528M had macrothrombocytopenia, hemizygous healthy control individuals showed a normal platelet count and size ($21.1 \pm 2.1 \times 10^9 l^{-1}$ and $2.7 \pm 0.4 \mu m$, respectively, $n=4$). The patient's mother and sister, both of whom were heterozygous for the substitution, also had a normal platelet count and size (Table 1). We thus suggest that p.V528M does not affect the platelet count and size.

Which gene was responsible for macrothrombocytopenia in the patient? As clinical information was obtained by interview, it is not known whether thrombocytopenia in other affected males was associated with giant platelets. The most common forms of macrothrombocytopenia, that is, Bernard–Soulier syndrome and *MYH9* disorders, were first excluded by flow cytometry for platelet glycoprotein Ib/IX and immunofluorescence analysis of granulocyte myosin IIA localization, respectively.¹⁶ Mutation screening of the known candidate genes for X-linked thrombocytopenia such as *WAS*^{21,22} and *GATA1*²³ excluded the presence of mutations in the patient (data not shown). We hypothesized that the macrothrombocytopenia in the present patient was caused by the mutation of an unknown gene on chromosome X. It is also possible that thrombocytopenia and giant platelets occurred incidentally and mimicked macrothrombocytopenia.

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- 1 Eksioglu, Y. Z., Scheffer, I. E., Cardenas, P., Knoll, J., DiMario, F., Ramsby, G. *et al*. Periventricular heterotopia: an X-linked dominant epilepsy locus causing aberrant cerebral cortical development. *Neuron* **16**, 77–87 (1996).
- 2 Kamuro, K. & Tenokuchi, Y. Familial periventricular nodular heterotopia. *Brain Dev.* **15**, 237–241 (1993).
- 3 Fox, J. W., Lamperti, E. D., Eksioglu, Y. Z., Hong, S. E., Feng, Y., Graham, D. A. *et al*. Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. *Neuron* **21**, 1315–1325 (1998).

- 4 Gorlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J. *et al*. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J. Cell. Biol.* **111**, 1089–1105 (1990).
- 5 Robertson, S. P. Filamin A: phenotypic diversity. *Curr. Opin. Genet. Dev.* **15**, 301–307 (2005).
- 6 Parrini, E., Ramazzotti, A., Dobyns, W. B., Mei, D., Moro, F., Veggiotti, P. *et al*. Periventricular heterotopia: phenotypic heterogeneity and correlation with Filamin A mutations. *Brain* **129**, 1892–1906 (2006).
- 7 Sheen, V. L., Dixon, P. H., Fox, J. W., Hong, S. E., Kinton, L., Sisodiya, S. M. *et al*. Mutations in the X-linked filamin 1 gene cause periventricular nodular heterotopia in males as well as in females. *Hum. Mol. Genet.* **10**, 1775–1783 (2001).
- 8 Sole, G., Coupry, I., Rooryck, C., Guerineau, E., Martins, F., Deves, S. *et al*. Bilateral periventricular nodular heterotopia in France: frequency of mutations in *FLNA*, phenotypic heterogeneity and spectrum of mutations. *J. Neurol. Neurosurg. Psychiatry* **80**, 1394–1398 (2009).
- 9 Feng, Y. & Walsh, C. A. The many faces of filamin: a versatile molecular scaffold for cell motility and signalling. *Nat. Cell Biol.* **6**, 1034–1038 (2004).
- 10 Cunningham, J. G., Meyer, S. C. & Fox, J. E. The cytoplasmic domain of the α -subunit of glycoprotein (GP) Ib mediates attachment of the entire GP Ib-IX complex to the cytoskeleton and regulates von Willebrand factor-induced changes in cell morphology. *J. Biol. Chem.* **271**, 11581–11587 (1996).
- 11 Hartwig, J. H. & DeSisto, M. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J. Cell. Biol.* **112**, 407–425 (1991).
- 12 Kunishima, S., Kamiya, T. & Saito, H. Genetic abnormalities of Bernard-Soulier syndrome. *Int. J. Hematol.* **76**, 319–327 (2002).
- 13 Lanza, F. Bernard-Soulier syndrome (hemorrhagic thrombocytic dystrophy). *Orphanet. J. Rare Dis.* **1**, 46 (2006).
- 14 Kakita, A., Hayashi, S., Moro, F., Guerrini, R., Ozawa, T., Ono, K. *et al*. Bilateral periventricular nodular heterotopia due to filamin 1 gene mutation: widespread glomeruloid microvascular anomaly and dysplastic cytoarchitecture in the cerebral cortex. *Acta Neuropathol.* **104**, 649–657 (2002).
- 15 Kunishima, S., Matsushita, T., Kojima, T., Sako, M., Kimura, F., Jo, E. K. *et al*. Immunofluorescence analysis of neutrophil nonmuscle myosin heavy chain-A in *MYH9* disorders: association of subcellular localization with *MYH9* mutations. *Lab. Invest.* **83**, 115–122 (2003).
- 16 Kunishima, S. & Saito, H. Congenital macrothrombocytopenias. *Blood Rev.* **20**, 111–121 (2006).
- 17 Kunishima, S., Gassner, C., Inoue, C., Kamiya, T. & Ozawa, K. Expression of low-frequency Ala108Pro substitution in the platelet glycoprotein *Ib β* gene. *Eur. J. Immunogenet.* **30**, 159–161 (2003).
- 18 Guo, S. W. & Thompson, E. A. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48**, 361–372 (1992).
- 19 Sheen, V. L., Ganesh, V. S., Topcu, M., Sebire, G., Bodell, A., Hill, R. S. *et al*. Mutations in *ARFGEF2* implicate vesicle trafficking in neural progenitor proliferation and migration in the human cerebral cortex. *Nat. Genet.* **36**, 69–76 (2004).
- 20 Sheen, V. L., Wheless, J. W., Bodell, A., Braverman, E., Cotter, P. D., Rauen, K. A. *et al*. Periventricular heterotopia associated with chromosome 5p anomalies. *Neurology* **60**, 1033–1036 (2003).
- 21 Derry, J. M., Kerns, J. A., Weinberg, K. I., Ochs, H. D., Volpini, V., Estivill, X. *et al*. *WASP* gene mutations in Wiskott-Aldrich syndrome and X-linked thrombocytopenia. *Hum. Mol. Genet.* **4**, 1127–1135 (1995).
- 22 Villa, A., Notarangelo, L., Macchi, P., Mantuano, E., Cavagni, G., Brugnani, D. *et al*. X-linked thrombocytopenia and Wiskott-Aldrich syndrome are allelic diseases with mutations in the *WASP* gene. *Nat. Genet.* **9**, 414–417 (1995).
- 23 Nichols, K. E., Crispino, J. D., Poncz, M., White, J. G., Orkin, S. H., Maris, J. M. *et al*. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in *GATA1*. *Nat. Genet.* **24**, 266–270 (2000).

Supplementary Information accompanies the paper on Journal of Human Genetics website (<http://www.nature.com/jhg>)