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ORIGINAL ARTICLE

ACTN1 rod domain mutation associated with congenital macrothrombocytopenia

Motoko Yasutomi[1] · Shinji Kunishima[2] · Shintaro Okazaki[1] · Akihiko Tanizawa[1] · Shinya Tsuchida[3] · Yusei Ohshima[1]

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Abstract Mutations in *ACTN1*, the gene encoding the actin-crosslinking protein α-actinin-1, cause autosomal dominant macrothrombocytopenia. α-Actinin-1 exists as antiparallel dimers, composed of an N-terminal actin-binding domain (ABD), four spectrin-like repeats (SLRs), which form the spacer rod, and a C-terminal calmodulin-like (CaM) domain. All of the previously reported *ACTN1* mutations associated with macrothrombocytopenia reside within the ABD and the CaM domain and not within the SLR domain. In this report, we describe a mutation in SLR2 of α-actinin-1 (p.Leu395Gln) associated with familial macrothrombocytopenia. A 3-year-old boy and his mother both had this mutation. They showed a mild form of thrombocytopenia without severe bleeding, accompanied by an elevated mean platelet volume. Consistent with the previous reports of mutations that reside in the ABD or the CaM domain, immunofluorescence examination revealed disorganization of the actin cytoskeleton in Gln395 mutant-transduced Chinese hamster ovary cells. Our findings suggest a novel mechanism for the pathogenesis of *ACTN1*-related macrothrombocytopenia that does not involve functional domain mutations.

Motoko Yasutomi and Shinji Kunishima contributed equally to this work.

✉ Motoko Yasutomi
yasutomi@u-fukui.ac.jp

[1] Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, 23-3 Shimoaizuki, Matsuoka, Eiheiji, Yoshida, Fukui 9101193, Japan

[2] Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 4600001, Japan

[3] Tsuchida Pediatrics Clinic, 11-10-2 Yoshimasa, Maruoka, Sakai, Fukui 9100372, Japan

Keywords *ACTN1* · α-actinin-1 · Macrothrombocytopenia · Spectrin-like repeat · Rod

Abbreviations

ABD	Actin-binding domain
CaM	Calmodulin-like
CHO	Chinese hamster ovary
SLRs	Spectrin-like repeats

Introduction

Congenital macrothrombocytopenia is a heterogeneous group of diseases characterized by a reduced platelet count and abnormally large platelets. Recently, a subgroup of macrothrombocytopenias caused by mutations in *ACTN1*, the gene encoding α-actinin-1, was reported [1–3]. α-Actinin-1 is an actin-crosslinking protein that participates in the formation of the cytoskeleton [4–6]. All of the previously reported *ACTN1* mutations related to macrothrombocytopenia reside within functional domains—either the actin-binding domain (ABD) or the calmodulin-like (CaM) domain; none have been identified within the spacer rod domain, which is composed of spectrin-like repeats (SLRs). In this report, we describe for the first time a mutation in one of the SLRs of α-actinin-1 that is associated with congenital macrothrombocytopenia.

Case report

A 3-year-old boy was referred to our hospital due to thrombocytopenia. Peripheral blood counts revealed the following: the white blood cell count was 5.8×10^9/L;

hemoglobin was 13.5 g/dL; platelets were at 109×10^9/L; and the mean platelet volume was 12.2 fL. Peripheral blood smears revealed macrothrombocytopenia accompanied by anisocytosis (Fig. 1a). In vitro platelet aggregation in response to collagen and ristocetin was normal, but ADP was slightly reduced (37 %). Platelet-associated immunoglobulin G and anti-platelet antibody were negative. The mother was also found to have thrombocytopenia for more than 15 years. Her platelet count was 74×10^9/L with a 13.4 fL mean platelet volume. Peripheral blood smears revealed macrothrombocytopenia with anisocytosis, as with her son (Fig. 1b). For both mother and child, bleeding diathesis was mild: there was occasional epistaxis and difficulty with hemostasis during delivery. The pedigree was constructed through genealogical studies (Fig. 1c). Written informed consent was obtained from the parents in accordance with the Declaration of Helsinki. The study was approved by the institutional review boards of the National Hospital Organization, Nagoya Medical Center, and Fukui University.

Materials and methods

ACTN1 analysis

MYH9 disorders, heterozygous and homozygous Bernard-Soulier syndrome, and GPIIb/IIIa-macrothrombocytopenia were excluded by immunofluorescence analysis for neutrophil myosin IIA and by flow cytometry for platelet GPIb/IX and GPIIb/IIIa expression, respectively [7, 8]. *ACTN1* sequence analysis was performed by PCR amplification of all exons and intron-exon boundaries and direct Sanger sequencing [1].

In vitro expression analysis

Full-length *ACTN1* sequences were amplified from normal platelet complementary DNA (cDNA) and inserted into the mammalian expression vector *pcDNA3.1* (Invitrogen, San Diego, CA) with a 5′Myc tag sequence. The *ACTN1* variant from the patient (p.Leu395Gln) and the reported ABD variant (p.Val105Ile) constructs were also prepared. Plasmid DNA was transfected into Chinese hamster ovary (CHO) cells with

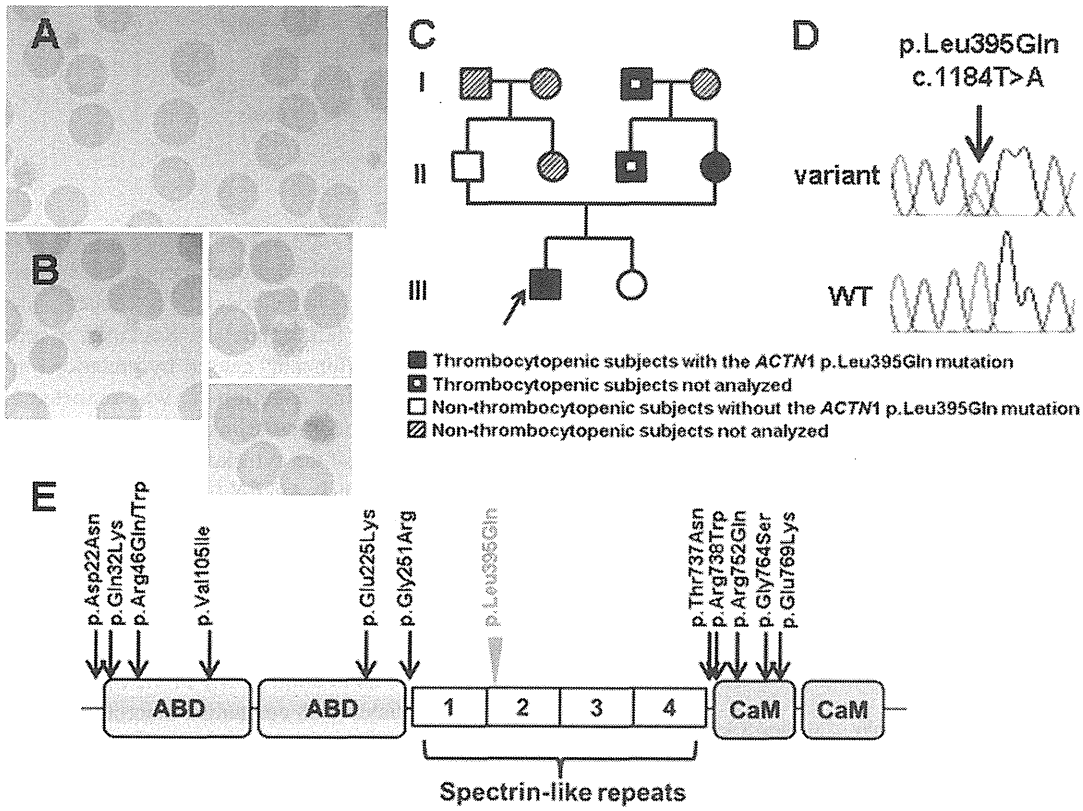

Fig. 1 p.Leu395Gln mutation of *ACTN1* causes macrothrombocytopenia with a form of autosomal dominant inheritance. **a**, **b** Peripheral blood smears from the patient (**a**) and his mother (**b**) were stained with May-Grunwald Giemsa. They showed macrothrombocytopenia accompanied by anisocytosis. **c** The family pedigree. The *arrow* indicates the proband. **d** The sequencing electrogram for the heterozygous p.Leu395Gln mutation. **e** Domain structure of α-actinin-1 and localization of *ACTN1* mutations identified in previous reports and in this report (shown in *red*) [1–3]

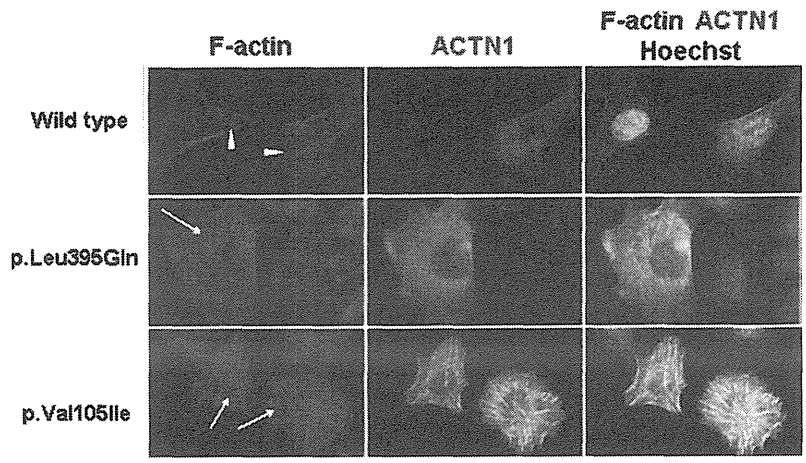

Fig. 2 Expression analysis of the mutant *ACTN1*. CHO cells were transiently transfected with Myc-tagged wild-type or mutant *ACTN1* cDNAs. After the cells were replated on fibronectin-coated slides, they were fixed, permeabilized, and stained with anti-Myc antibody, followed by Alexa-488-labeled goat anti-mouse antibody, Alexa-555-labeled phalloidin, and Hoechst. *Arrows* indicate deformation of actin filaments and *arrowheads* indicate fine actin filaments

the FuGENE HD Transfection Reagent (Promega, Madison, WI). Twenty-four hours after transfection, the *ACTN1*-transduced cells were replated on bovine fibronectin (SIGMA, St. Louis, MO, 20 μg/mL)-coated Millicell EZ slides (Merck Millipore, Billerica, MA) for immunofluorescence analysis. Briefly, the cells were fixed with 4 % paraformaldehyde and permeabilized with 0.5 % Triton X-100. Cells were then fluorescently labeled by incubation with anti-c-Myc antibody (Life Technologies, Carlsbad, CA), followed by Alexa-488-labeled goat anti-mouse IgG, Alexa-555-conjugated phalloidin (Life Technologies, Carlsbad, CA), and Hoechst (Enzo Life Sciences, NY). Images were obtained with an IX-70 fluorescence microscope using a ×40/1.00 numerical aperture oil objective lens (Olympus, Tokyo, Japan).

Results and discussion

ACTN1 sequence analysis revealed a novel c.1184T>A, p.Leu395Gln heterozygous mutation in the SLR2 domain (Fig. 1d, e). To determine the pathogenetic role of the novel variant, we performed in vitro expression analysis and evaluated the organization of actin filaments. As shown in Fig. 2, cells transduced with wild-type *ACTN1* had well-organized, finely stretched actin filaments, where *ACTN1* colocalized with actin filaments, whereas unbound *ACTN1* was finely distributed within the cytoplasm. In contrast, Gln395 variant-transduced CHO cells showed disorganized, thickened, and shortened actin filaments, which were similar to those of Ile105 variant-transduced cells.

Among the four known isoforms of α-actinin, platelets mainly express α-actinin-1 [9]. α-Actinins exist as antiparallel dimers, with each monomer consisting of an N-terminal ABD, a C-terminal CaM domain, and a rod domain composed of four SLRs (Fig. 1e). The actinin rod domain is responsible for dimerization and functions as a spacer between ABDs at both ends [10, 11]. An additional function of the rod domain is to act as the binding site for other proteins. Previously reported *ACTN1* variants in the ABD and CaM domains were found to cause disorganization of actin filaments in mutant-transduced cells [1–3] and abnormal proplatelet formation in mutant-transduced mouse megakaryocytes [1]. Defects in the regulation of terminal platelet production could result in macrothrombocytopenia [12].

Similar to the mutations found in the ABD and the CaM domain, here, we report that a p.Leu395Gln mutation in SLR2 of α-actinin-1 also leads to a disorganized actin cytoskeleton. In contrast, the only previously reported mutation within the rod domain (p.Asp666Val in SLR4) was not found to be related to macrothrombocytopenia [3]. The possible mechanisms underlining different contribution of p.Leu395Gln and p.Asp666Val mutations to pathogenesis are as follows: (1) The leucine mutation at position 395 of SLR2 is located within the dimer interface, whereas the aspartic acid mutation at position 666 of SLR4 is not (Fig. 3) [10, 11]. Therefore, the p.Leu395Gln mutation might affect the formation of antiparallel dimers. (2)

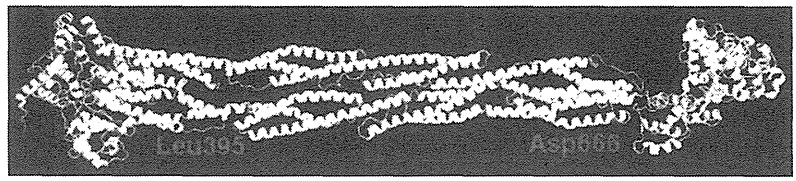

Fig. 3 Structural analysis of α-actinin-1 SLR domain mutations. The figure is constructed according to the coordinates of α-actinin-2 (1SJJ) with MacPyMol software (DeLano Scientific, Palo Alto, CA). Locations of Leu395 and Asp666 are indicated in *red*

SLR1 and SLR4 dimers have diverged further during evolution with respect to SLR2 and SLR3 [10]. Therefore, it might be possible that the mutation within SLR4 affects dimerization to a lesser extent than mutations in SLR2. (3) Whereas the precise location of binding sites is not known, proteins binding to the rod domain might have an effect on the function of α-actinin [5, 13]. Collectively, our results provide new insight into the pathogenesis of *ACTN1*-related macrothrombocytopenia. We propose that mutations outside the ABD and the CaM domains also should be studied for the differential diagnosis of *ACTN1*-related macrothrombocytopenia.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments.

Conflict of interest The authors declare that they have no conflict of interest.

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To the editor:

Diagnostic biomarker for *ACTN1* macrothrombocytopenia

Congenital macrothrombocytopenia is a heterogeneous group of rare disorders characterized by abnormally giant platelets and thrombocytopenia with a variable degree of bleeding tendency.[1,2] A definitive diagnosis is possible in only approximately half of the patients; the rest of the patients remain without a definitive diagnosis and some patients are even misdiagnosed with chronic immune thrombocytopenia and treated accordingly. The most common congenial macrothrombocytopenias are *MYH9* disorders/*MYH9*-related disease and Bernard-Soulier syndrome. These 2 disorders comprise ~40% of all cases and can be definitively diagnosed by an immunofluorescence analysis for neutrophil nonmuscle myosin heavy chain IIA (NMMHCIIA) localization and flow cytometry for platelet glycoprotein Ib/IX expression.[1] Recently, mutations in *ACTN1*, the gene encoding α-actinin-1, have been found to be the next prevalent cause for congenital macrothrombocytopenia and should be considered in the differential diagnosis.[3-5] We herein propose a diagnostic screening test for *ACTN1* macrothrombocytopenia.

ACTN1 macrothrombocytopenia is characterized by mild macrothrombocytopenia with platelet anisocytosis and mild bleeding tendency without nonhematologic complications.[3-5] α-Actinin-1 exists as antiparallel dimers and cross-links actin filaments and participates in the organization of the actin cytoskeleton.[6] The majority of *ACTN1* mutations reside within the functional actin-binding or calmodulin-like domains. *ACTN1* is the major actinin isoform in megakaryocytes/platelets. Mutations exert a dominant-negative effect, causing defective proplatelet formation from megakaryocytes and resulting in the production of large platelets.[5] There are no apparent abnormalities in platelet aggregation, spreading on glass surfaces, or clot retraction in patients. Furthermore, there is no apparent abnormal α-actinin-1 localization in resting as well as

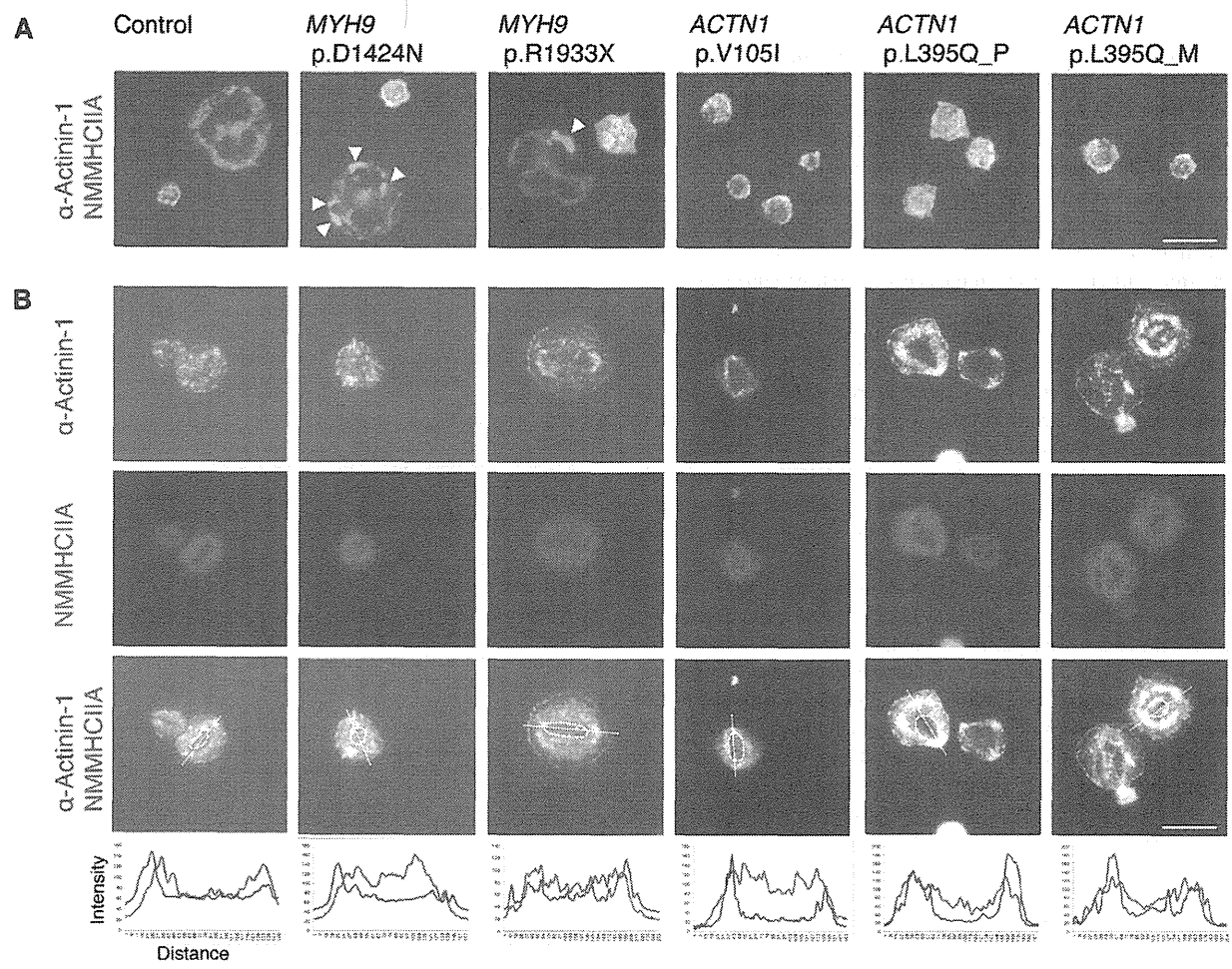

Figure 1. Localization of α-actinin 1 and NMMHCIIA in platelets. (A) Localization of α-actinin 1 and NMMHCIIA in resting platelets. Peripheral blood smears were stained with anti-α-actinin-1 antibody (Alexa Fluor 488, green) and anti-NMMHCIIA antibody (Alexa Fluor 555, red). Merged images are shown. There were abnormal NMMHCIIA aggregations in the neutrophils of patients with *MYH9* disorders (indicated by arrow heads). M, mother; P, patient. Scale bar, 20 μm. (B) Localization of α-actinin 1 and NMMHCIIA in surface-activated spreading platelets. Surface-activated platelets were double-stained with anti-α-actinin-1 antibody (green) and anti-NMMHCIIA antibody (red). The fluorescence intensity of α-actinin-1 (green) and NMMHCIIA (red) along the lines of interest on the merged images was measured using the ImageJ plot profile function (National Institutes of Health, Bethesda, MD) and the data were exported to Microsoft Excel for the construction of graphs. Representative images from 10 spreading platelets in each individual are shown. The granulomere zone is indicated by dotted lines. Scale bar, 20 μm.

surface-activated platelets. This may be partly explained by the fact that only a fraction of α-actinin-1 is associated with the cytoskeleton and large amounts are present as a cytosolic-free form,[7] thereby subtle abnormal localization, if present, may not be readily detected. Accordingly, a molecular genetic analysis is only available for the diagnosis and there is an unmet need for the development of a diagnostic test for clinical diagnostic practice.[3-5]

Peripheral blood smears from controls (n = 4) and patients with *MYH9* disorders (n = 2) and *ACTN1* macrothrombocytopenia (n = 3) were double-stained with anti-α-actinin-1 antibody (sc-17829; Santa Cruz Biotechnology) and anti-NMMHCIIA antibody (BT561; Biomedical Technologies) and examined using a BX50 fluorescence microscope with a UPlanApo 100×/1.35 objective lens (Olympus).[5] α-Actinin-1 was fine-granularly distributed throughout resting platelets from both the controls and patients with *MYH9* disorders and *ACTN1* macrothrombocytopenia (Figure 1A).

Surface-activated platelets undergo dynamic cytoskeletal reorganization, which allow them to change from a discoid to a spherical shape, extend filopodia, and form lamellipodia.[8] We have found in patients with *MYH9* disorders that mutant NMMHCIIA was only distributed in the central granulomere zone, indicating that upon surface activation, mutant NMMHCIIA is unable to translocate to lamellipodia.[9] We therefore hypothesized that during actomyosin cytoskeletal reorganization in surface-activated spreading platelets, α-actinin-1 alteration in *ACTN1* macrothrombocytopenia would influence NMMHCIIA localization. Platelets were seeded onto chamber slides (Millicell EZ Slide; Millipore) for 30 minutes, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then processed for a double immunofluorescence analysis of α-actinin-1 and NMMHCIIA (Figure 1B). In control platelets, α-actinin-1 localized to lamellipodia in a discrete granular pattern and at the circumference of the surface membrane, corresponding to a focal adhesion, indicating that during the process of platelet activation, cytosolic α-actinin-1 was incorporated into the reorganized actin cytoskeleton. In patients with *MYH9* disorders and *ACTN1* macrothrombocytopenia, α-actinin-1 was similarly distributed as in control platelets. The distribution profile of NMMHCIIA was distinct from that of α-actinin-1. In control platelets, NMMHCIIA was diffusely distributed in lamellipodia but not in the central granulomere zone, whereas in patients with *MYH9* disorders and *ACTN1* macrothrombocytopenia, NMMHCIIA was additionally distributed diffusely in the granulomere zone. A density plot analysis of the fluorescence intensity of NMMHCIIA illustrated a clear difference between the controls and patients. In control platelets, the fluorescence intensity was evenly low at the granulomere zone, whereas in patient platelets, it was rather high and uneven. No differences of the distribution profile of α-actinin-1 were found among the controls and patients with *MYH9* disorders and *ACTN1* macrothrombocytopenia. These data suggest that detection of NMMHCIIA in the granulomere zone in surface-activated platelets could be indicative of actomyosin cytoskeletal alterations. Thus, we propose the immunofluorescence analysis of NMMHCIIA in surface-activated platelets as a potential diagnostic screening test for *ACTN1* macrothrombocytopenia.

Shinji Kunishima
Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan

Katsumasa Kitamura
Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan

Motoko Yasutomi
Department of Pediatrics, University of Fukui, Fukui, Japan

Ryoji Kobayashi
Department of Pediatrics, Sapporo Hokuyu Hospital, Sapporo, Japan

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Contribution: S.K. designed and performed experiments, analyzed data, and wrote the manuscript; K.K. helped with the experiments; and M.Y. and R.K. provided the patient samples.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Shinji Kunishima, Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya 4600001, Japan; e-mail: kunishis@nnh.hosp.go.jp.

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