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

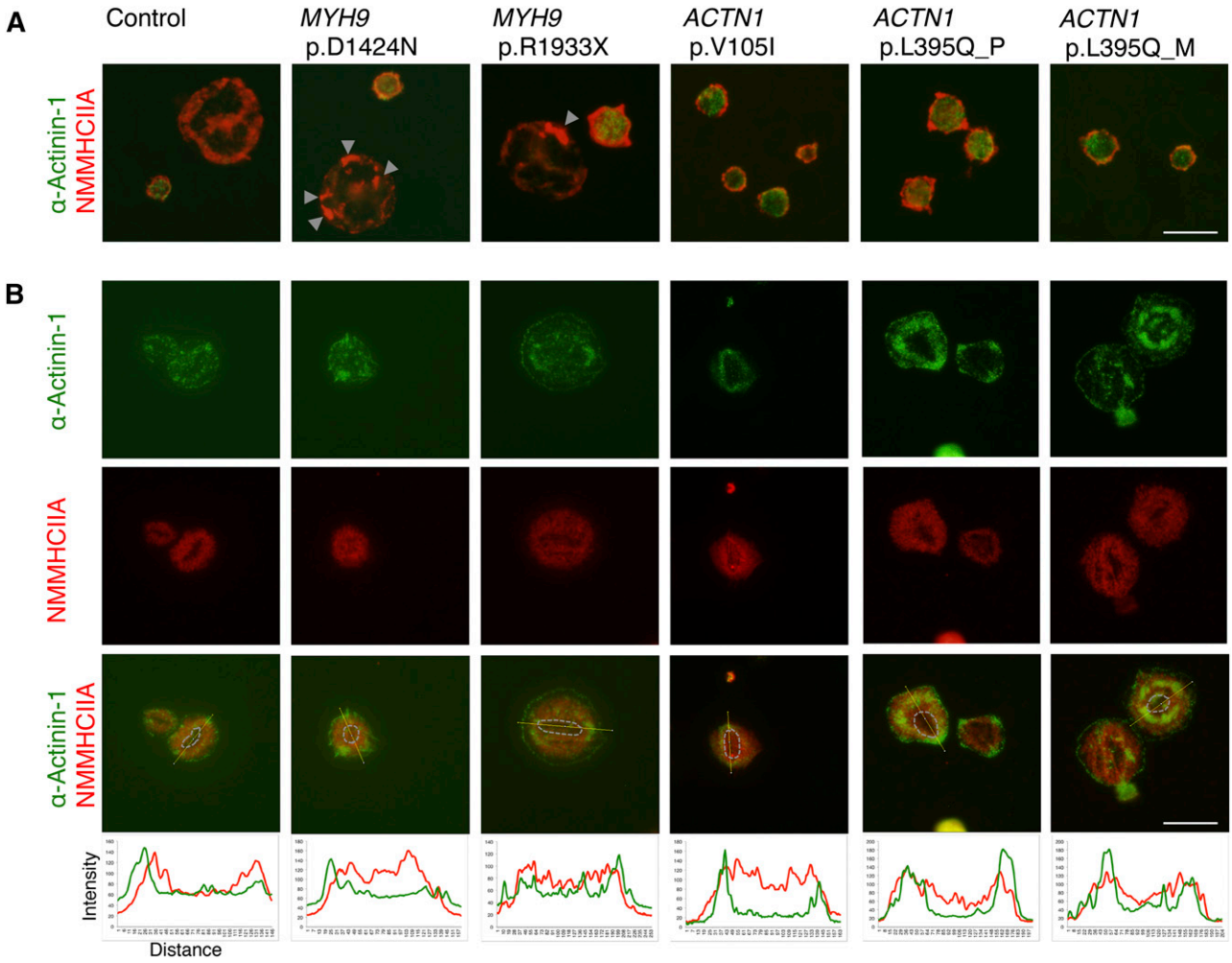

Figure 1. Localization of α-actinin 1 and NMMHCIIA in platelets. (A) Localization of α-actinin 1 and NMMHCIIA in resting platelets. Peripheral blood smears were stained with anti-α-actinin-1 antibody (Alexa Fluor 488, green) and anti-NMMHCIIA antibody (Alexa Fluor 555, red). Merged images are shown. 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.

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