Differential expression of wild-type and mutant NMMHC-IIA polypeptides in blood cells suggests cell-specific regulation mechanisms in MYH9 disorders

Shinji Kunishima,1 Motohiro Hamaguchi,1 and Hidehiko Saito2

1Department of Hemostasis and Thrombosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya; and 2Nagoya Central Hospital, Nagoya, Japan

MYH9 disorders such as May-Hegglin anomaly are characterized by macrothrombocytopenia and cytoplasmic granulocyte inclusion bodies that result from mutations in MYH9, the gene for nonmuscle myosin heavy chain-IIA (NMMHC-IIA). We examined the expression of mutant NMMHC-IIA polypeptide in peripheral blood cells from patients with MYH9 S770delG and 5818delG mutations. A specific antibody to mutant NMMHC-IIA (NT629) was raised against the abnormal carboxyl-terminal residues generated by S818delG. NT629 reacted to recombinant 5818delG NMMHC-IIA but not to wild-type NMMHC-IIA, and did not recognize any cellular components of normal peripheral blood cells. Immunofluorescence and immunoblotting revealed that mutant NMMHC-IIA was present and seques-tered only in inclusion bodies within neutrophils, diffusely distributed throughout lymphocyte cytoplasm, sparsely localized on a diffuse cytoplasmic background in monocytes, and uniformly distributed at diminished levels only in large platelets. Mutant NMMHC-IIA did not translo-cate to lamellipodia in surface activated platelets. Wild-type NMMHC-IIA was homogeneously distributed among megakaryocytes derived from the peripheral blood CD34+ cells of patients, but coarse mutant NMMHC-IIA was heterogeneous scattered without abnormal aggregates in the cytoplasm. We show the differential expression of mutant NMMHC-IIA and postulate that cell-specific regulation mechanisms function in MYH9 disorders. (Blood. 2008;111:3015-3023)

© 2008 by The American Society of Hematology

Methods

Patients

We performed MYH9 mutational analysis on 4 patients with a heterozygous one-base deletion mutation in exon 40 (Table 1) as described.9 All of the patients had moderate-to-severe thrombocytopenia, but none had a bleeding tendency. Patient 2 and the father of patient 1 who also had an MYH9 mutation in exon 38 and 40 are strictly associated with type I localization and those in exons 16, 26, and 30 are associated with type II localization.11 Thus the different MYH9 mutations result in different influences on the assembly of mutant NMMHC-IIA polypeptides. Given that myosin consists of 2 heavy chains and the wild-type NMMHC-IIA molecules are contained in inclusion bodies, the nature of the disease at the molecular level is apparently dominant negative. Although inclusion bodies might be derived as a result of mutant molecules, these conclusions were deduced from indirect observations using antibodies that recognized only wild-type NMMHC-IIA. Recent findings have suggested a haploinsufficiency effect in platelets and megakaryocytes.12,13 Inclusion bodies are undetectable in lymphocytes, and their presence in monocytes remains controversial. Accordingly, whether mutant NMMHC-IIA is expressed in these cells is obscure.1,2,14-16 We demonstrated cell-specific mutant NMMHC-IIA expression in MYH9 disorders for the first time using a specific antibody against mutant NMMHC-IIA.
mutation had been diagnosed with idiopathic thrombocytopenic purpura and treated accordingly. In some experiments, peripheral blood smears from patients with E1841K and a patient with somatic mosaicism for 5818delG were analyzed. Peripheral blood smears were collected after the patients and/or their parents gave informed consent in accordance with the Declaration of Helsinki to participate in the study, which was approved by the ethics review committees at Nagoya Medical Center and at each of the hospitals where the patients were followed up. Immunofluorescence staining, immunoblotting, and quantitative fluorescent polymerase chain reaction (PCR) of peripheral blood samples were analyzed in all of the 4 patients, while analysis of megakaryocytes cultured from CD34+ peripheral blood cells was performed in patients 2, 3, and 4.

**Table 1. Clinical and hematologic characteristics of the 4 patients with MYH9 disorders**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/age, y</th>
<th>Platelet count, 10/L</th>
<th>Platelet size, μm mean ± SD</th>
<th>MYH9 mutations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/1</td>
<td>88</td>
<td>5.6 ± 1.6</td>
<td>5770delG</td>
<td>This study</td>
</tr>
<tr>
<td>2</td>
<td>F/36</td>
<td>68</td>
<td>5.2 ± 1.8</td>
<td>5818delG</td>
<td>Mother of patients 3 and 4</td>
</tr>
<tr>
<td>3</td>
<td>M/10</td>
<td>68</td>
<td>5.7 ± 2.3</td>
<td>5818delG</td>
<td>Kunifuji et al.11</td>
</tr>
<tr>
<td>4</td>
<td>M/4</td>
<td>40</td>
<td>5.1 ± 1.5</td>
<td>5818delG</td>
<td>Brother of patient 3</td>
</tr>
</tbody>
</table>

All 4 patients had leukocyte inclusion bodies and type I NMMHC-IIA localization. None of the patients had nephritis, hearing loss, or cataracts.

### Antibodies

We raised an antigenic NMMHC-IIA antibody (NT629) against 6 abnormal amino acid residues generated by the 5818delG mutation (Figure 1A). Rats were immunized with synthetic peptides (CKGAGMAAPTKR; linker cysteine and the normal sequence are underlined) conjugated with keyhole-limpet hemocyanin as a hapten carrier. Antiserum was collected, absorbed with corresponding wild-type peptides (CKGAGDGSDEE) followed by human platelet myosin purified from outdated platelet concentrates, and then affinity-purified by chromatography on immobilized antigen peptides. Other antibodies included anti-NMMHC-IIA C-terminal peptide (GKADGAEAKPAE) polyclonal antibody PRB440P (BabCO, Richmond, CA), anti-GPlib monoclonal antibody SZ22, fluorescein isothiocyanate (FITC)-conjugated anti-GPlib antibody SZ21, phycoerythrin (PE)-conjugated anti-GPβα antibody ZZ2 (Immunotech SA, Marseille, France), anti-GPβα polyclonal antibody, antiaxin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti–α-tubulin antibody DM1A (Abcam, Cambridge, United Kingdom), anti–myc-tag rabbit polyclonal antibody (Medical and Biological Laboratories, Nagoya, Japan), and anti–myc-tag mouse monoclonal antibody (Invitrogen, San Diego, CA).

### Expression of NMMHC-IIA rod

Lymphocyte mRNA was extracted from patient 2 using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, Little Chalfont, United Kingdom), and first-strand cDNA was synthesized using SensiScript reverse transcriptase and oligo-dt primer (Qiagen, Hilden, Germany). MYH9 rod sequences (nt 4016-6118 of GenBank no. NM002473) corresponding to light meromyosin (wild-type allele, 1278-1960 aa; 5818delG mutant allele, 1278-1946 aa) were amplified from the cDNA containing the wild-type and mutant sequences using LA Taq DNA polymerase (Takara Bio, Otsu, Japan) and cloned into the pCR2.1Topo vector (Invitrogen). We introduced a myc epitope tag fused at the 5' end to MYH9 cDNA using inverse PCR mutagenesis.10 Oligonucleotide primers were designed in inverted tail-to-tail directions to amplify the wild-type and 5818delG MYH9 plasmids. PCR amplification proceeded with a forward primer (5'-CTCGAGGTTGGAGCTGGACAACGTGACC-3') and a mutagenic reverse primer, which included a myc-tag–coding sequence (5'-CAGATCCCTC-TCTGAGATGAGTTTTGTTCTCATACCAAGCTGACG-3') and a mutant gene sequence, which was subsequently amplified with LA Taq DNA polymerase. Amplified DNA fragments were self-ligated with T4 polynucleotide kinase and T4 ligase (Promega, Madison, WI), and used to transform DH5α competent cells. The recovered plasmids were sequenced, and each insert containing myc-MYH9 cDNA was excised with HindIII/NotI and shuttled into the mammalian expression vector, pcDNA3.1 (Invitrogen). All constructs were confirmed by restriction analysis and sequencing.

We transfected the pcDNA3.1 vector containing the normal-type rod MYH9 cDNA or 5818delG rod MYH9 cDNA transiently transfected into 293T cells. Briefly, 106 293T cells were seeded in 35-mm dishes and incubated overnight. Plasmid DNA (1.0 μg) was then transfected into the cells using Peptide transfection reagent (Qiagen) according to the manufacturer’s instructions. Cytosin preparations were analyzed by immunofluorescence staining, and whole cell extracts were immunoblotted 24 hours later.

### Fractionation of peripheral blood cells

Neutrophils, platelets, monocytes, and lymphocytes were isolated from peripheral blood collected into acid-citrate-dextrose. After erythrocyte sedimentation using dextan, supernatants were centrifuged on Ficoll-Paque (GE Healthcare) at 400g for 30 minutes. Platelet-rich plasma prepared from the intermediate mononuclear cell fraction by centrifugation at 100g for 10 minutes was passed through a leukocyte reduction filter (Terumo, Tokyo, Japan). Platelets were washed once, resuspended in Hanks balanced salt solution (Invitrogen) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin, and then incubated with anti-CD61 antibody conjugated to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD61+ cells (platelet fraction) were captured using magnetic separation columns. Monocytes were isolated from the Ficoll-Paque mononuclear cell fraction using anti-CD14 magnetic beads. We isolated lymphocytes by washing the CD14+ monocyte-depleted pass-through fraction 3 times for 10 minutes each at 100g to remove platelets. Erythrocytes in the Ficoll-Paque pellet were lysed in hypotonic saline to obtain CD16+ cells (neutrophil fraction). The cells in each fraction were counted using a hemocytometer and purity was evaluated by May–Grünwald-Giemsa staining and microscopy. The final platelet fraction contained no more than 2 leukocytes/105 platelets, and monocytes, neutrophils, and lymphocytes were more than 98%, 99%, and 98% pure, respectively.

### Analysis of megakaryocytes from CD34+ peripheral blood cells

CD34+ cells (5-10 × 103 cells) were isolated from the Ficoll-Paque mononuclear cell fraction of 10 mL peripheral blood using anti-CD34 magnetic beads (Miltenyi Biotec). Cells were cultured in StemSpan Serum-Free Expansion Medium supplemented with 50 ng/mL TPO, 50 ng/mL SCF, and 10 ng/mL IL3 (Stem Cell Technologies, Vancouver, BC) in a humidified atmosphere of 5% CO2 for 12 days. Culture supernatants were centrifuged at 100g for 8 minutes and analyzed as anti-GPβα antibody antibody S21 and PE-conjugated anti-GPβα antibody S22. Cytosin preparations of cultured megakaryocytes were analyzed by immunofluorescence staining.

### Quantitative fluorescent PCR analysis

Normal and mutant alleles were amplified by quantitative fluorescent PCR that discriminated a one-nucleotide difference in each PCR product.17 Total RNA was extracted from platelets, neutrophils, monocytes, and lymphocytes using a NucleoSpin RNA/Protein kit (Machery-Nagel, Düren, Germany), and first-strand cDNA was synthesized using SensiScript reverse transcriptase and an oligo-dt primer (Qiagen). Primers 395 (5’-ATGCCATGAACCGC-GAAGTC-3’) and 6-FAM-labeled 4035 (5’-6-FAM-GCCTATTCGGCAG-GTTTGC-3’) were included in the PCR mixture and amplification proceeded over 35 cycles of 20 seconds at 95°C, 20 seconds at 58°C, and...
20 seconds at 72°C using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). For comparison, fluorescent PCR was also performed on genomic DNA extracted using QIAamp Tissue Kits (Qiagen), using primers 405 (5'-TGAAGATGTTGCGTGC-3') and 6-FAM–labeled 4035. The PCR products were examined using an ABI 310 Genetic Analyzer and data were analyzed using GeneMapper 3.7 software (Applied Biosystems). Peak areas of normal and mutant alleles were calculated for each sample.

Figure 1. Characterization of NT629 antibody. (A) Site of single nucleotide deletions in MYH9 exon 40 and amino acid sequence of NMMHC-IIA carboxyl-terminal region. Nucleotides encoding amino acids are numbered beginning with initiation codon. Deduced amino acid sequence is written under nucleotide sequence. Deletions of a single guanine nucleotide (bold type) results in frameshift that cause replacement by aberrant amino acids at the carboxyl terminus (bold type) and premature termination. Epitopes of PRB440P and NT629 antibodies are underlined. (B) Total proteins of 293T cells transiently transfected with vectors expressing N-terminal myc-tagged wild-type MYH9 rod (1278-1960 aa) and 5818delG mutant MYH9 rod (1278-1946 aa) were analyzed by immunoblotting. Band corresponding to recombinant myc-tagged NMMHC-IIA rod was detected with antimyc antibody in both wild-type MYH9– and 5818delG MYH9–transfected cells. PRB440P and NT629 detected recombinant NMMHC-IIA rod only in wild-type MYH9–transfected cells and in 5818delG MYH9–transfected cells (arrow head), respectively. Endogenous 220-kDa NMMHC-IIA band was detected in 293T cells with PRB440P and NT629. PRB440P and NT629 intensely stained cytoplasm only in wild-type– and only in 5818delG-transfected cells, respectively. PRB440P weakly stained endogenous NMMHC-IIA in nontransduced 293T cells. (D) Immunofluorescence analysis of peripheral blood smears from a patient with E1841K double stained with PRB440P and NT629. PRB440P showed abnormal type I NMMHC-IIA localization in all neutrophils with diffuse staining background, and diffuse cytoplasmic staining in lymphocytes, monocytes, and platelets. In contrast, cells were not stained with NT629 antibody. (E) Immunofluorescence analysis of peripheral blood smears from a mosaic patient for 5818delG double stained with PRB440P and NT629. PRB440P showed abnormal type I NMMHC-IIA localization in approximately 10% neutrophils, in which inclusion bodies were stained with NT629. Normal neutrophils with diffuse NMMHC-IIA distribution were not stained with NT629. (F) Immunoblots of buffy coat samples from healthy control and from patients 1 (5770delG) and 2 (5818delG). NT629 detected band corresponding to NMMHC-IIA polypeptide and additional small band in blood from patients, but not control.
Immunofluorescence analysis
Peripheral blood smears were analyzed by immunofluorescence staining as described. In brief, EDTA-anticoagulated peripheral blood samples were smeared on glass slides, air-dried, fixed in methanol, permeabilized with acetone, hydrated, and blocked with normal goat serum. The slides were concomitantly incubated with PRB440P and NT629 and then reacted with Alexa 555–labeled anti–rabbit IgG and Alexa 488–labeled anti–rat IgG (Invitrogen). Megakaryocytes derived from CD34+ peripheral blood cells and 293T cells transfected with the myc-tagged NMMHC-IIA polypeptide were simultaneously analyzed. Stained cells were examined using a BX50 fluorescence microscope with a 100×/1.35 numeric aperture oil objective (Olympus, Tokyo, Japan). Images of the slides were acquired using a DP70 digital camera and DP Manager software (Olympus).

Immunoblotting
Protein samples were simultaneously isolated with total RNA using NucleoSpin RNA/Protein kits, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 4 to 12% gradient acrylamide slab gels (Invitrogen) under reduced conditions, and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Labs, Hercules, CA). The blots were cut horizontally in half at the approximate position of the 75-kDa molecular marker. The upper portion of each blot was probed with PRB440P or NT629, and the lower portions were reacted with antiactin antibody followed by horseradish peroxidase–conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescent substrate. Signal ratios of NT629 to PRB440P were determined densitometrically on immunoblot images using National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/, Bethesda, MD). Total expression of the myc-tagged NMMHC-IIA polypeptide in the transfected cells was also analyzed using antimyc antibody PRB440P or NT629.

Results
Mutant MYH9 mRNA is expressed in peripheral blood cells, at lower levels in platelets
Deutsch et al have found normal amounts of mutant MYH9 mRNA in the total peripheral blood cells of patients with MYH9 disorder.

Characterization of antimutant NMMHC-IIA antibody specific for abnormal C-terminal amino acids generated by 5818delG
To specifically detect mutant NMMHC-IIA polypeptide and explore its subcellular localization, we developed the rat polyclonal antibody NT629 that reacts to the unique abnormal C-terminal peptide generated by 5818delG. Figure 1A shows that any exon 40 one-base nucleotide deletion located 5’ to nt 5818 resulted in a frameshift and generated the abnormal C-terminal MAPTKR sequence before premature termination. Accordingly, the antigenic sequence is maintained in the 5770delG mutant. An enzyme-linked immunosorbent assay showed that affinity-purified NT629 reacted to immobilized immunogenic peptides but not to corresponding wild-type peptides (data not shown). Immunofluorescence and immunoblot analyses of 293T cells expressing recombinant NMMHC-IIA rod and peripheral blood samples were performed to validate the specificity of NT629.

We transiently transfected 293T cells with the N-terminal myc-tagged wild-type and 5818delG mutant MYH9 rod constructs. An approximately 80-kDa band, which is consistent with the calculated molecular weight of the recombinant wild-type (79.0 kDa) and of 5818delG (77.7 kDa) NMMHC-IIA rod, was detected in total proteins of 293T cells expressing recombinant NMMHC-IIA rod that were immunoblotted with anti–myc-tag antibody (Figure 1B left panel). Probing the blots with PRB440P revealed the
approximately 80-kDa band only in the wild-type. An endogenous NMMHC-IIA of the 220-kDa band was detected in mock-, wild-type--, and 5818delG-transfected 293T cells (Figure 1B middle panel). NT629 revealed only the approximately 80-kDa band in cells transfected with 5818delG (Figure 1B right panel). Immunofluorescence analysis with PRB440P revealed weak cytoplasmic staining in the mock-transfected cells due to the presence of endogenous NMMHC-IIA. Anti–myc-tag antibodies and NT629 were not reactive in these cells. Cytoplasmic staining with PRB440P was intense only in wild-type–transfected cells, and NT629 was intensely stained only in 5818delG-transfected cells. Positive staining with anti–myc-tag antibody and the negative staining with PRB440P and NT629 in the wild-type– and 5818delG-transfected cells, respectively, confirmed that PRB440P and NT629 did not recognize 5818delG and wild-type NMMHC-IIA rod, respectively (Figure 1C).

We concomitantly stained conventional peripheral blood smears with PRB440P and NT629. PRB440P diffusely stained granulocytes, platelets, lymphocytes, and monocytes, whereas NT629 reactivity was absent in control smears (data not shown). PRB440P detected abnormal type I NMMHC-IIA in all neutrophils with a diffusely stained background from patients with MYH9 5770delG and 5818delG (patients 2, 3, and 4). Immunofluorescence analysis of peripheral blood smears from patients 1 and 2 double stained with PRB440P (red) and NT629 (green). (A) PRB440P stained granular NMMHC-IIA accumulation and diffusely stained cytoplasm of neutrophils, whereas NT629 stained only intense cytoplasmic foci and no background. (B) Lymphocytes were diffusely stained with both PRB440P and NT629. (C) In monocytes, both PRB440P and NT629 detected small dots in addition to diffuse cytoplasmic staining. Platelets in panels A-B are notoriously not stained with NT629. (D) PRB440P diffusely stained platelet cytoplasm and intensely stained cell periphery. In contrast, most platelets are NT629 negative; only extraordinarily large platelets are diffusely stained. (E) Mutant NMMHC-IIA polypeptide cannot translocate to lamellipodia in surface-activated platelets. Platelets were adhered to glass slides for 10 minutes, fixed, and processed for PRB440P and NT629 immunofluorescence analysis. Left column: images stained with May-Grünwald-Giemsa. Mutant NMMHC-IIA was weakly distributed only in granulomere zone of platelets from patients. Platelets from patients 2, 3, and 4 were examined. Original magnification, ×1000.

Mutant NMMHC-IIA polypeptide is sequestrated only in inclusion bodies in neutrophils

We applied the NT629 to investigate the expression and localization of mutant NMMHC-IIA on peripheral blood smears from patients with MYH9 5770delG (patient 1) and 5818delG (patients 2, 3, and 4; Table 1). Figure 3 shows representative immunofluorescence staining of peripheral blood cells. Immunofluorescence analysis of neutrophil NMMHC-IIA with PRB440P disclosed the type I pattern containing 1 or 2 large, intensely stained, oval- to spindle-shaped cytoplasmic NMMHC-IIA–positive granules. In addition to bright foci, PRB440P diffusely stained the cytoplasm, indicating that wild-type NMMHC-IIA accumulates in inclusion bodies and that residual amounts are distributed outside such structures. In contrast, NT629 intensely stained foci, but not the background in the same neutrophils, indicating that mutant NMMHC-IIA is sequestrated and localized only in inclusion bodies (Figure 3A).

Mutant NMMHC-IIA polypeptide is diffusely distributed in lymphocyte cytoplasm, and sparsely localized on a diffuse cytoplasmic background in monocytes

Inclusion bodies are absent in lymphocytes. We previously showed diffuse NMMHC-IIA distribution in lymphocytes from patients with MYH9 disorders. We found that both wild-type and mutant NMMHC-IIA were diffusely distributed in the lymphocyte cytoplasm (Figure 3B). Although the original report of May-Hegglin anomaly described that inclusion bodies are absent in monocytes, this remains controversial. We detected small punctate foci on a diffuse cytoplasmic background using both PRB440P and NT629 in some monocytes, although the foci were smaller than those observed in neutrophils (Figure 3C). This indicated that some mutant NMMHC-IIA accumulated in definite structures and the remainder does not accumulate or aggregate but distributes outside such structures in monocytes.
Mutant NMMHC-IIA polypeptide is uniformly distributed at lower levels only in large platelets

The staining profiles of PRB440P and NT629 in platelets from patients were quite different. NMMHC-IIA is homogeneously distributed in the cytoplasm of normal resting platelets. PRB440P diffusely stained the cytoplasm of platelets from the patients, and intensely stained the cell periphery (Figure 3A,B left panels). In contrast, most platelets were negative for NT629 staining (Figure 3A,B right panels) and only those that were similar to or larger than erythrocytes (~10% of total platelets) were weakly stained in the cytoplasm but not in the cell periphery (Figure 3D). Although mutant NMMHC-IIA expression was remarkably decreased, it did not accumulate or aggregate to form inclusion bodies, and remained uniformly distributed in the cytoplasm.

The distribution profiles of wild-type and mutant NMMHC-IIA were more distinct in surface-activated platelets (Figure 3E). When platelets are activated on glass slides for 10 minutes, they change shape from discoid to spherical, extend filopodia and lamellipodia, and spread with large circumferential lamellae. Immunofluorescence analysis revealed the mutually exclusive localization of wild-type and mutant NMMHC-IIA. NMMHC-IIA was distributed in a discrete granular pattern in the cell body but not in the central granulomere zone of normal platelets. Wild-type NMMHC-IIA was normally distributed in the cell body and weakly in the granulomere zone of platelets from patients 2, 3, and 4. However, mutant NMMHC-IIA detected by NT629 was weakly distributed only in the granulomere (the images are bright, but they were acquired over a long exposure time). These results indicated that upon surface activation, wild-type NMMHC-IIA reorganized, whereas mutant NMMHC-IIA was unable to translocate to lamellipodia. In addition, residual wild-type NMMHC-IIA localization in the granulomere zone, which was undetectable in control platelets, might result from wild-type and mutant NMMHC-IIA association.

The decreased expression of mutant NMMHC-IIA in platelets was consistent with the results of immunoblotting, in which the relative amount of mutant NMMHC-IIA was more decreased than in leukocytes. Densitometric quantitation showed that the signal ratios of NT629 to PRB440P were 0.41 (± 0.10), 0.26 (± 0.06), 0.40 (± 0.17), and 0.04 (± 0.01; n = 3) in neutrophils, monocytes, lymphocytes, and platelets, respectively (Figure 4).

Mutant NMMHC-IIA polypeptide is heterogeneously distributed in megakaryocytes

We examined mutant NMMHC-IIA expression in platelet precursor megakaryocytes. Because bone marrow specimens could not be obtained from the patients, CD34+ cells derived from peripheral blood were cultured in liquid serum-free medium supplemented with a cocktail of cytokines to induce megakaryocyte differentiation. Twelve days later, cultured megakaryocytes were identified by cell morphology (large cells with multilobulated nuclei and abundant cytoplasm) and the expression of GPIIb or von Willebrand factor on cytospin preparations. NMMHC-IIA expression was analyzed by double staining with anti-GPIIb and PRB440P or NT629. NMMHC-IIA was homogeneously distributed in the cytoplasm of normal megakaryocytes (Figure 5A top panels). Although wild-type NMMHC-IIA was normally distributed in megakaryocytes from patients, mutant NMMHC-IIA was coarse and heterogeneously distributed (Figure 5A top and bottom panels). The limited numbers of megakaryocytes derived from peripheral blood CD34+ cells caused difficulties when trying to determine the relative levels of mutant NMMHC-IIA by immunoblotting. The percentage of megakaryocytes was less than 10% of cultured cells.

In this culture system, platelet-like particles were released from megakaryocytes. Although no typical proplatelet-forming megakaryocytes were observed, some platelet-producing megakaryocytes were detected on cytospin preparations (Figure 5B). Platelet-like particles derived from the patients were larger than those of healthy controls according to their mean diameters on cytospin preparations (controls vs patients: 2.6 ± 0.7 vs 6.0 ± 1.3 μm; n = 3 each) or geometric means of forward scatter of GPIbα and GPIIIa double-positive cells determined by flow cytometry (controls vs patients: 233.0 ± 1.7 vs 294.8 ± 9.3; n = 3 each; Figure 5C).

Discussion

We produced a rat polyclonal antibody, NT629, that recognizes the unique abnormal C-terminal peptide generated by 5818delG, and presented the first evidence of mutant NMMHC-IIA polypeptide expression and localization in peripheral blood cells from patients with MYH9 disorders. We extensively validated the specificity of NT629 through studies of 293T cells expressing recombinant NMMHC-IIA, peripheral blood smears from healthy individuals and patients, anduffy coat extracts. NT629 reacted only with mutant NMMHC-IIA polypeptide generated by an MYH9 exon 40 one-base deletion such as 5770delG and 5818delG and it did not recognize any normal cellular components.
Concomitant immunofluorescence staining with NT629 and PRB440P antibodies enabled the differential detection of intracellular mutant and wild-type NMMHC-IIA polypeptides. Mutant NMMHC-IIA detected by NT629 was localized only as punctuate or granular structures in the cytoplasm of neutrophils in doubly stained peripheral blood smears. In contrast, wild-type NMMHC-IIA detected by PRB440P was localized as such structures and also diffusely distributed in the cytoplasm. Nonmuscle myosin II assembly involves the dimerization of 2 α-helices to form a coiled-coil rod structure and lateral associations of the coiled-coils to form a functional filament.22 Since myosin consists of 2 heavy chains, then it should assemble in a cell as a mixture of wild-type homodimers, heterodimers consisting of one mutant and one wild-type molecule, and mutant homodimers. MYH9 disorders are heterozygous conditions in which one allele contains the mutation. If the expression of the wild-type and mutant NMMHC-IIA is equal and the association occurs randomly, 50% of the myosin molecules should be homodimers and the remainder should comprise homodimers of the mutant (25%) or the wild-type (25%), and thus 75% of the myosin molecules will contain at least 1 mutant NMMHC-IIA. Mutant myosin molecules might also copolymerize into filaments with wild-type myosin molecules that could impair the function and localization of normal wild-type myosin. This assumption is consistent with the findings of Franke et al, who demonstrated that recombinant NMMHC-IIA rod mutants form aberrant aggregates and form dominant insoluble aggregates in the presence of wild-type NMMHC-IIA.23 Pecci et al reported that NMMHC-IIA levels in granulocytes from patients with MYH9 disorders were decreased to 32% of controls.13 They might have detected only wild-type NMMHC-IIA by immunoblotting because myosin molecules containing mutant NMMHC-IIA were trapped within inclusion bodies and lost during granulocyte lysate preparation. If so, then 68% would have been myosin-containing mutant NMMHC-IIA instead of the predicted 75%. Taken together, mutant NMMHC-IIA aggregates and accumulates in granulocytes to form cytoplasmic inclusion bodies and thus dominant-negative effects indeed serve as the molecular mechanism underlying the formation of such bodies.

The results of our study provide important insights into the molecular mechanisms underlying the production of giant platelets in MYH9 disorders. Mutant NMMHC-IIA was expressed at obviously decreased levels only in large platelets. Comparative quantitative analysis was not possible because control samples expressing wild-type and mutant NMMHC-IIA polypeptides cannot be obtained. However, the relative amount of mutant to wild-type NMMHC-IIA expression levels in platelets was approximately 10% of the leukocyte levels. The obviously decreased amount of mutant NMMHC-IIA in platelets predicts that the total NMMHC-IIA content would be reduced to approximately 50% of normal levels. This is exactly what Deutsch et al12 and Pecci et al13 found independently in platelets from patients with MYH9 disorders, namely an approximately 50% reduction of NMMHC-IIA compared with the total actin concentration. In addition, we did not detect aggregation or accumulation of mutant NMMHC-IIA in platelets from patients, which is consistent with the fact that overall NMMHC-IIA localization in platelets from patients with MYH9 disorders is normal.11,13 Instead, it was uniformly distributed in the cytoplasm and the localization was distinct from the wild-type molecule. Upon contact with a glass surface, platelets change from a discoid to a spherical shape, expand filopodia, and spread with large sheetlike lamellipodia. Myosin becomes associated with actin and migrates to the front of the lamellipodia, and associates with the granulomere zone.24-26 Biochemical analyses have also shown that activation increases the myosin content in the cytoskeleton.27,28 Canobbio et al recently reported that the increase in cytoskeletal-associated myosin in activated platelets is impaired in patients with MYH9 disorders.29 The most recent findings described by Leon et al demonstrated that megakaryocyte-restricted MYH9 knockout mice lack platelet contractile phenomena, including clot retraction, platelet shape change, and stress-fiber formation on a fibrinogen-coated surface, whereas the heterozygous knockout mice have no abnormalities.30 Our results substantiate previous findings that mutant NMMHC-IIA does not translocate to the lamellipodia in surface-activated platelets. We conclude that the overall defect in platelets with mutant NMMHC-IIA is haplosufficiency. We also propose that only residually expressed mutant myosin has a loss of function and cannot participate in the reorganization of cytoskeletal contractile structures.

The macrothrombocytopenia associated with MYH9 disorders could be caused by impaired platelet release due to abnormal megakaryocyte fragmentation.31,33 Recent findings have shown that NMMHC-IIA attenuates proplatelet formation,33,34 which occurs in terminally mature megakaryocytes where most of the cytoplasm is converted into lengthy beaded extensions.35 Thus, NMMHC-IIA might restrain thrombopoiesis until megakaryocytes accumulate sufficient quantities of the materials required for optimal platelet assembly; loss of myosin-IIA function could trigger precocious proplatelet formation, whereas gain-of-function mutations might limit platelet production. Consistent with recent findings,12,13 we found that wild-type NMMHC-IIA localization was normal in megakaryocytes derived from the peripheral blood CD34+ cells of healthy controls and patients were double-stained with anti-GPlla mouse monoclonal antibody S222 (green) and PRB440P (red, top panels) or with anti-GPlla rabbit polyclonal antibody (green) and NT629 (red, bottom panels). PRB440P diffusely stained cytoplasm of megakaryocytes from control and patients, whereas NT629 coarsely and heterogeneously stained those of patients. (B) Megakaryocytes that produce platelets. Original magnification, ×1000. (C) Flow cytometric analysis of plateletlike particles double stained with anti-GPlla and anti-GPllla antibodies. Geometric mean of forward scatter of GPllla and GPIlla double-positive cells was shown in inset.

Figure 5. NMMHC-IIA localization in megakaryocytes derived from peripheral blood CD34+ cells. (A) Megakaryocytes derived from peripheral blood CD34+ cells of healthy controls and patients were double-stained with anti-GPlla mouse monoclonal antibody S222 (green) and PRB440P (red, top panels) or with anti-GPlla rabbit polyclonal antibody (green) and NT629 (red, bottom panels). PRB440P diffusely stained cytoplasm of megakaryocytes from control and patients, whereas NT629 coarsely and heterogeneously stained those of patients. (B) Megakaryocytes that produce platelets. Original magnification, ×1000. (C) Flow cytometric analysis of plateletlike particles double stained with anti-GPlla and anti-GPllla antibodies. Geometric mean of forward scatter of GPllla and GPIlla double-positive cells was shown in inset.
patients with MYH9 disorders. However, the distribution of mutant NMMHC-IIA was coarse and heterogeneous. Although immunofluorescence staining for the first time revealed that mutant NMMHC-IIA is expressed in megakaryocytes, this finding was not quantitative. From this viewpoint, Pecci et al demonstrated from indirect immunoblot findings that mutant NMMHC-IIA is absent and that the whole or the NMMHC-IIA is wild type in megakaryocytes derived from peripheral blood CD34+ cells, indicating that MYH9 mutations result in haploinsufficiency in megakaryocytes.13 However, simple haploinsufficiency for MYH9 alone cannot explain the pathogenesis of giant platelets and thrombocytopenia in MYH9 disorders. Mice heterozygous for Myh9-null allele do not exhibit giant platelets and thrombocytopenia.36,37 Evidence also indicates that different MYH9 mutations result in different consequences for the platelet phenotype. Patients with MYH9 mutations in the head domain, especially those at the R702 residue, have significantly larger platelets than those with mutations in the tail domain.38 These findings indicate that despite decreased expression levels, mutant NMMHC-IIA affects the production of platelets in some manner.

We found that the expression levels of mutant MYH9 mRNA in leukocytes (neutrophils, lymphocytes, and monocytes) from fractionated peripheral blood were normal. Our results are generally consistent with those of Deutsch et al who reported normal amounts of mutant MYH9 mRNA in total peripheral blood cells of patients with a D1424N mutation.12 However, we found moderately decreased levels of mutant MYH9 mRNA in platelets. Because platelets are anucleated, their mRNA content is a consequence of gene expression in precursor megakaryocytes. Thus, mutant MYH9 mRNA could be slightly more unstable than wild-type mRNA in platelets, and such a subtle difference was undetectable in leukocytes. Furthermore, since leukocytes contain approximately 12 500-fold more mRNA than platelets, residually contaminated leukocytes could affect mRNA levels in platelets and thus they might be overestimated in platelets.39

Inclusion bodies are present in granulocytes but not in lymphocytes in patients with MYH9 disorders.1,2 Accordingly, whether mutant NMMHC-IIA is expressed in lymphocytes has remained obscure. The present study discovered that the distribution of mutant NMMHC-IIA in lymphocytes is diffuse. Meanwhile the original report of the May-Hegglin anomaly, the prototype of macrothrombocytopenia with leukocyte inclusion bodies, described the absence of inclusion bodies in monocytes.1,2 So far, the presence of inclusion bodies in monocytes remains controversial.1,2,14-16 Mutant NMMHC-IIA was sparsely localized on a diffuse cytoplasmic background in monocytes. Transcription of MYH9 is regulated in a cell type–specific manner and by its differentiation stage, and differential translational mechanisms are also suggested.40 Although the mechanisms of how considerable amounts of mutant NMMHC-IIA can be expressed without abnormal aggregation in these cells remain to be elucidated, the differential expression of mutant NMMHC-IIA provides important insights into cell-specific regulation mechanisms in MYH9 disorders. A comprehensive understanding of cell-specific NMMHC-IIA regulation will not only identify the hematologic abnormalities but also help to reveal the mechanisms of nonhematologic manifestations of MYH9 disorders.

**Acknowledgments**

We thank Drs T. Echizenya and M. Higashigawa for providing the patient samples, and Ms Y. Ito for skillful technical assistance.

This work was supported by grants to S.K. from the Japan Society for the Promotion of Science (nos. 16590971 and 18591094), the Ministry of Health, Labor and Welfare (Grant for Child Health and Development 19C-2), the Mother and Child Health Foundation, Charitable Trust Laboratory Medicine Foundation of Japan, and National Hospital Organization Research Fund.

**Authorship**

Contribution: S.K. designed and performed research, analyzed data, and wrote the paper; M.H. analyzed data and wrote the paper; and H.S. supervised the research.

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

Correspondence: Shinji Kunishima, Department of Hemostasis and Thrombosis, Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 4600001, Japan; e-mail: kunishis@nnh.hosp.go.jp.

**References**

Differential expression of wild-type and mutant NMMHC-IIA polypeptides in blood cells suggests cell-specific regulation mechanisms in MYH9 disorders

Shinji Kunishima, Motohiro Hamaguchi and Hidehiko Saito