Macrothrombocytopenia and dense granule deficiency associated with FLI1 variants: ultrastructural and pathogenic features

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

High-throughput gene sequencing

Two high-throughput sequencing methods were used to identify candidate variants. Whole Exome Sequencing was performed at the Centre National de Génotypage platform (CNG, Evry, France) on an Illumina HiSeq2000 instrument using the Agilent SureSelect Human All Exon V5 kit. Sequenced reads data were mapped to the human genome reference sequence hg19 using the BWA software.[1] Sequenced data with a number of reads (i.e. depth) lower than 20 were excluded and duplicates were removed using Picard tools (broadinstitute.github.io/picard/). Variant calling was performed using the GATK suite.[2]9 Detected variants were annotated using the SNPEFF (http://snpeff.sourceforge.net/) and SNPSift 0 (http://snpeff.sourceforge.net/SnpSift.html) programs. For the gene panel sequencing, the DNA has been enriched for the exons of 308 genes of interest (list available upon request) using Haloplex technology (Agilent technologies). It was then sequenced on a NextSeq500 sequencer (Illumina) by the 2-channel SBS technology, using a mid Output kit. The quality of the sequencing was checked with the "Sequencing analysis viewer 1.8.37" (Illumina). The sequences were then aligned with BWA-MEM (Hg19 version of the genome). Variant calling was done with GATK v3.3.0, and annotated with ANNOVAR[3] using VarAFT (http://varaft.eu/). We applied the following filters for variant analysis: SNP with frequency <1% (based on ExAC, 1000G and ESP6500 data), located in coding regions or splice junctions, and called deleterious by diverse prediction algorithms (UMD Predictor,[4] PROVEAN,[5] Polyphen[6]). Sanger sequencing using a 3500 XL sequencer confirmed the variants.

Structural model of FLI1-DNA interactions

The positions of the variant residues were mapped onto the structure of the FLI1 protein (NP_000432.1). Analysis was performed using modular visualization and modelling with Coot[7,8] and PyMOL software (The PyMOL Molecular Graphics System; Version 1.5.0.4; Schrödinger LLC).

Platelet phenotyping

The mean platelet volume was analyzed with an optic method (ADVIA 120, Siemens). Platelet-rich plasma (PRP) was prepared according to standard procedures. The platelet agonists were purchased from Helena Laboratories (ADP and arachidonic acid), Bio/Data Corporation (collagen), American Biochemical & Pharmaceuticals (TRAP-6) or Polypeptide group (TRAP-14) and were used at concentrations given in

Table 1 or Figure 2. Light transmission was recorded on an APACT 4004 optical aggregation system (Labor Bio-Medical Technologies GmbH). For platelet surface glycoprotein expression assay, PRP were incubated in presence or not of the platelet agonist TRAP-14 with the antibodies anti-αIIbβ3-PC5 (clone P2; Beckman Coulter), anti-GPIbα-PC5 (clone HIP1; BD Biosciences), anti-CD63-FITC (clone CLBgran/12; Beckman Coulter) and anti-P-selectin-PC5 (clone CLBThromb/6; Beckman Coulter) for 30 min at 20°C. Scatter signals and fluorescence intensity were analyzed using a FC500 flow cytometer (Beckman Coulter).

PRP serotonin level

For PRP serotonin measurement, the PRP was prepared according standard procedures. For the serotonin measurement, the PRP was frozen at -20 °C and unfrozen, in order to disrupt the platelet membranes. The measurement of serotonin was performed using high performance liquid chromatography with the following parameters: isocratic flow 0.95 ml/min, sensor potential 0.5V, sensitivity 10nA, column temperature 30 ± 1 °C, analysis time 10 min, injection volume 20µl, serotonin retention time 5.2 min, eluant retention time 6.8 min. An automated platelet count of the PRP was performed prior the serotonin measurement in order to normalize the serotonin level to the platelet count. The plasma serotonin level was considered negligible as compared to the intraplatelet serotonin content[9,10]. The platelet serotonin level was calculated using the following formula: *platelet serotonin level (µg/10^9 platelets) = PRP serotonin level (ng/ml) / PRP platelet count (x10^9 platelets)*.

ATP secretion assay

The platelet ATP secretion was assessed in diluted PRP using the ENLITEN ATP Assay System Bioluminescence Detection Kit (FF2000; Promega) according to the manufacturer's instructions. The luminescence was measured using a VICTOR X4 luminometer (Perkin Elmer). The PRP was prepared according to standard procedures and diluted to obtain a platelet concentration of 10^7 platelet / ml. The ATP concentration was measured before and after stimulation with 100 µM TRAP-6 (American Biochemical & Pharmaceuticals).

Mepacrine uptake and release assay

PRP was prepared according to standard procedures and diluted to obtain a platelet concentration of 10^7 platelet / ml. The diluted PRP was incubated with 1.1 or 2.4 µM mepacrine (Q 3251, Sigma-Aldrich) or in absence of mepacrine for 30 min at 37 °C. Then, the platelets were stimulated or not with 40 µM TRAP-14 for one minute and immediately analyzed using a Navios Cytometer (Beckman Coulter). The platelets mepacrine uptake was defined as MFI ratio of platelets incubated with mepacrine to platelets incubated without mepacrine and the platelet mepacrine release was defined as the MFI ratio of resting platelets to stimulated platelets as previously described.[11]

Flow-cytometric quantification of MYH10 expression (whole blood-based assay)

A volume of 50 µL Fresh EDTA whole blood was treated with a modified procedure of the PerFix EXPOSE kit (Beckman Coulter, For Research Use Only (RUO), not for use in diagnostic procedures). Briefly, the blood was fixed in 4% formaldehyde (buffer R1) for 10 min, then permeabilized and red blood cells lysed (500µL buffer R2) for 5 min, centrifuged at 1000G for 5 min. The cell pellet was suspended in 200 µL staining buffer (R3), and a 20 µL fraction was labeled with rabbit anti-MYH10 antibody at 10 µg/mL final concentration (Cell Signaling; #3404 ; RUO) for one hour. After a washing step with 2 mL PBS-BSA 0.2%, the platelets were incubated with goat anti-rabbit PE-labeled secondary antibody 1/200 (Southern Biotech, ref 4050-09S ; RUO) for 30 min in PBS-BSA 0.2%. After another washing step in wash buffer (R4), the cell pellet was suspended in 0.5 mL buffer R4, and data were acquired using a Navios Cytometer (Beckman Coulter), at low speed, and analyzed with FlowJo software (TreeStar).

Western blot assay

To isolate nuclear and cytoplasmic subcellular fractions from protein lysate, GripTite 293 MSR cells (genetically engineered Human Embryonic Kidney 293 cell line, Thermofisher Scientific) were lysed 48h after transfection (PolyJet In Vitro DNA Transfection Reagent, SignaGen Laboratories). Fractionation was performed using the NE-PER Nuclear and Cytoplamsic Extraction Reagent (Pierce). Total platelet proteins (washed platelets) and proteins (30 µg) from the subcellular fractions were separated on NuPAGE gels with MES SDS running buffer (Thermofisher Scientific) and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked and labeled overnight with the following primary antibodies: rabbit anti-FLI1 (Santa-Cruz Biotechnology; sc-356), rabbit anti-HA (Santa-Cruz Biotechnology; sc-805), rabbit anti-MYH10 (Santa-Cruz Biotechnology; sc-376942), rabbit anti-lamin-B1 (Abcam; ab16048), mouse anti-GAPDH (Millipore; MAB374) and rabbit anti-actin (Abcam, ab3280) primary antibodies. The membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Biorad). Chemiluminescence signals were obtained using Novex ECL Chemiluminescent Substrate Reagent, detected Using CCD Imager Image Quant LAS 4000 (GE Healthcare) and quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Site-directed mutagenesis

Directed mutagenesis of the human expression plasmid pCMV3-FLI1-HA (Sino Biological Inc.) was performed using the GeneArt Site-directed Mutagenesis System kit (Thermofischer Scientific) according to the manufacturer instructions. The primer sequences are available upon request. The mutagenesis efficiency was confirmed by sequencing (Beckman Coulter Genomics).

Luciferase reporter assay

Transcriptional regulatory properties of wild-type and variant FLI1 were analyzed using E743tk80Luc plasmid containing the luciferase gene driven by an enhancer / promoter cassette composed of three tandem copies of the Ets Binding Site (E74-binding sites AACCGGAAGTA, found in the Drosophila E74 gene promoter) inserted 5′ of the herpes simplex virus thymidine kinase promoter, as previously described.[12] This plasmid was a gift from J. Ghysdael.[13] GripTite 293 MSR Cells were transfected with the indicated reporter gene constructs (166 ng), the expression plasmid (333 ng) and the pGL473-hRLuc (50 ng) to normalize for transfection efficiency. The luciferase activity was assessed 48h after transfection (Dual-Luciferase Reporter Assay system, Promega).

Epifluorescence microscopy

Forty-eight hours after transfection, H9C2 cells (cell line derived from embryonic BD1X rat heart tissue; ATCC CRL-1446) were fixed in 4% paraformaldehyde for 20 min at room temperature. After washing, the cells were permeabilized with 0.3% Triton X100 in PBS for 5 min, blocked using 3% BSA PBS and incubated overnight with rabbit anti-FLI1 antibody (Santa-Cruz Biotechnology; sc-356). Next, the cells were incubated with anti-rabbit Alexa-488-labeled secondary antibody (Abcam; ab150077) and DAPI staining was performed. Finally, after washing steps, the slides were mounted with Fluoromount and examined using an AXIO Imager M1 microscope (Carl Zeiss, Germany).

***In vitro* megakaryocyte differentiation and proplatelet formation**

After Ficoll separation, circulating $CD34^+$ cells were purified using positive selection on magnetic beads (Miltenyi-Biotec SAS) and cultured as previously reported[12] in serum-free medium supplemented with 20 ng/mL thrombopoietin (TPO) and 25 ng/mL stem cell factor (SCF) (Thermofisher Scientific). For MK differentiation, cells were incubated at day 11 of culture with anti-CD41-APC and anti-CD42a-PE antibodies (BD Biosciences) and Hoechst-33342 (Sigma-Aldrich). The cells were then analyzed using a Navios cytometer (Beckman Coulter). For proplatelet (PPT) formation, cells were seeded on fibrinogen-coated (20µg/ml) culture slides at day 11 and examined on days 12-13 of culture among 300-500 total cells. PPT-forming MKs were identified as exhibiting cytoplasmic extensions with constriction areas.

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SUPPLEMENTARY FIGURES LEGENDS

Supplementary Figure 1: Expression of MYH10 MFI as a function of mean platelet volume

Intraplatelet flow cytometric quantification of MYH10 in the platelets from 13 control individuals (MPV range 7.2-11.4 fl) and two FLI1 variants carriers F1-II2 and F1-III1 (MPV 10.7 and 13 fl) in whole-blood. The MYH10 MFI was expressed as a function of the mean platelet volume. A linear regression analysis showed a significant correlation between the MYH10 MFI and the mean platelet volume among control individuals (black dots). The control individuals were selected so that a large MPV range was represented among individuals explored in the Bleeding and Thrombosis Exploration Center (University Hospital of Marseille) for a hemostatic defect without thrombocytopenia or platelet dysfunction.

Supplementary Figure 2: Transmission electron microscopic quantification of dense granules

Quantification of dense granules per platelet section using transmission electron microscopy in two FLI1 variants carriers (F1-II2 and F2-II4). Values are shown as the mean ± SEM as quantified for ≥100 randomly selected platelets; ****$p<0.0001$ vs. controls (Mann-Whitney test).

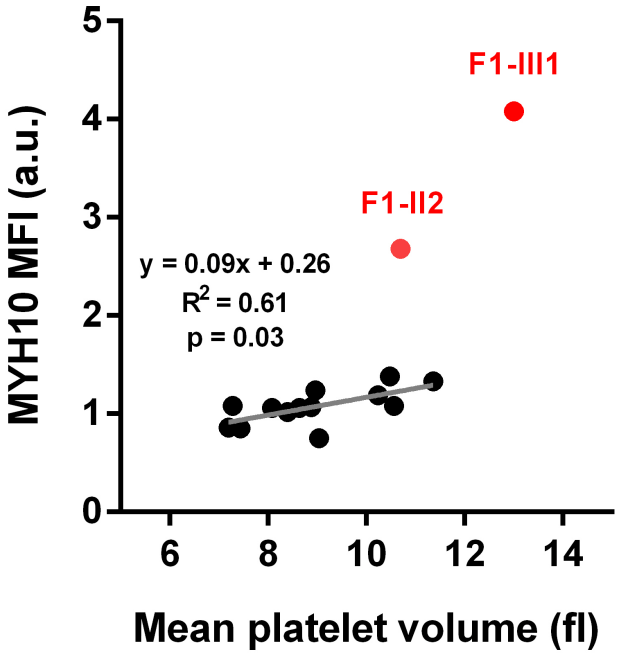
SUPPLEMENTARY VIDEOS LEGENDS

Supplementary Video 1: FIB/SEM acquisition of washed platelets suspension from a healthy control

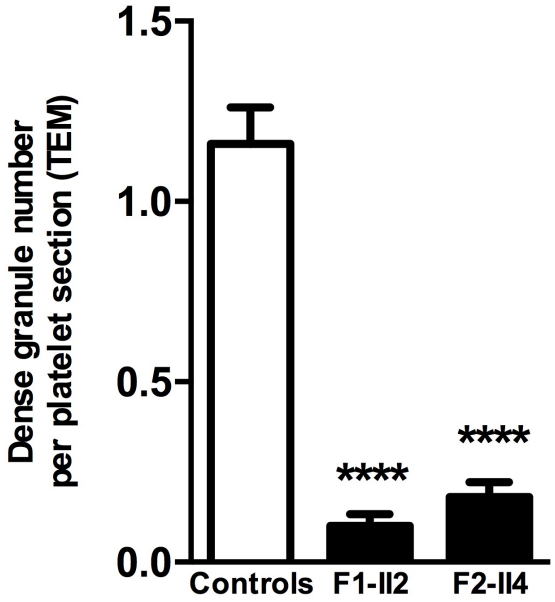
Resin embedded washed platelets from a healthy control were observed using FIB/SEM. The arrows indicate dense granules. The arrowheads indicate empty or abnormal granules.

Supplementary Video 2: FIB/SEM acquisition of washed platelets suspension from F1-II2 (p.R337Q FLI1 variant carrier)

Resin embedded washed platelets from a FLI1 variant carrier (F1-II2; p.R337Q) were observed using FIB/SEM. The arrows indicate dense granules. The arrowheads indicate empty or abnormal granules.



Supplementary Figure 1: Expression of MYH10 MFI as a function of mean platelet volume



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