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ORIGINAL ARTICLE

Mosaicism of an *ELANE* Mutation in an Asymptomatic Mother

Tomonari Shigemura[1] · Norimoto Kobayashi[1] · Kazunaga Agematsu[2] · Osamu Ohara[3] · Yozo Nakazawa[1]

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Abstract

Purpose We report normal neutrophil count in a mother, who carries the same *ELANE* mutation as her daughter with severe congenital neutropenia. We hypothesized that the mother possessed wild- and mutant-type clones and the wild-type clones could generate neutrophils, whereas the mutant clones could not.

Methods We confirmed mutant variant ratio by sequence signals and measured the frequency of the mutant allele by subcloning in various cell types. We established the *ELANE*-mutated and non-mutated induced pluripotent stem cells (iPSCs) from the mother's T cells and compared granulopoiesis between these iPSCs.

Results In the sequence analysis of isolated peripheral blood (PB), nail and hair, the mutant variant was detected in approximately 40–60% of lymphocytes, monocytes, hematopoietic progenitor cells, and hair as well as in a small percentage of nail, but in none of the neutrophils. In the subcloning analysis of extracted DNA from $CD3^+$ and $CD34^+$ cells, the mutant allele was identified in 37.5% and 38.1%, respectively. We reprogrammed the mother's PB cells and established the *ELANE*-mutated and non-mutated iPSCs. Granulopoiesis from mutated iPSCs revealed little sensitivity to granulocyte colony-stimulating factor in comparison with non-mutated iPSCs.

Conclusions These observations strongly suggest that mutant-carrying neutrophils did not appear in the mother's PB because mutated clones could not differentiate into neutrophils. The mother's normal hematological phenotype could be explained by the perseverance of normal, non-mutated granulopoiesis.

Keywords Severe congenital neutropenia · *ELANE* gene · mosaicism · induced pluripotent stem cells · granulopoiesis

Introduction

Severe congenital neutropenia (SCN) is a rare disorder characterized by recurrent bacterial infection due to severe neutropenia and maturation arrest in the bone marrow (BM) at the promyelocyte or myelocyte stage. In SCN patients, the absolute neutrophil counts (ANCs) are consistently lower than 200/μL at birth. [1–3] Heterozygous mutations in the *ELANE* gene encoding the human neutrophil elastase have been identified in 35–69% of SCN [3–5] cases with autosomal dominant inheritance. [6, 7] These mutations also underlie most cases of cyclic neutropenia, which is characterized by regular oscillations of ANC, generally with a 21-day periodicity. [2] In previous analyses, *ELANE* mutations have been shown to cause the misfolding of the encoded protein and subsequent activation of unfolded protein responses, leading to premature apoptosis in myeloid cells. [8, 9]

Here, we present the clinical outcomes of a Japanese girl with SCN due to a heterozygous missense mutation, c.607G>C, in *ELANE* (p.G203R, formerly G174R). The mutated allele was also detected in her asymptomatic mother, who had a normal ANC. We examined the mutant variant ratio by sequence signals in various isolated cell populations. We generated *ELANE*-mutated and non-mutated induced pluripotent stem cells (iPSCs) from the mother's T cells and then examined the occurrence of granulopoiesis.

✉ Yozo Nakazawa
yxnakaza@shinshu-u.ac.jp

[1] Department of Pediatrics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

[2] Department of Infection and Host Defense, Graduate School of Medicine, Shinshu University, Matsumoto, Japan

[3] Department of Technology Development, Kazusa DNA Research Institute, Chiba, Japan

Methods

Detection of Genetic Mutations

Genomic DNA was extracted using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA, USA) from blood samples and using ISOHAIR (Nippon Gene Co., Ltd. Toyama, Japan) from hair and nail samples. PCR amplification of the affected sequence in the *ELANE* gene was performed with primer sets (forward 5′-GGACTTCCCAACCCTGAC; reverse 5′-AGCCAAGGAGCATCAAACAC). The PCR products were subjected to direct sequencing from both directions using the BigDye Terminator v3.1 Cycle Sequencing kit and ABI 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). Maximum peak intensities were extracted manually from raw sequencing traces using Sequence Scanner Software 2 (Allied Biosystems). The fraction of mutant (C) versus wild-type (G) allele representation was calculated using the formula $C/(C+G) \times 100\%$ for the forward and $G/(G+C) \times 100\%$ for the reverse. The values from forward and reverse traces showed semiquantitative data [10].

Cell Purification and TA Cloning

Mononuclear cells and granulated leukocytes were isolated from PB by Ficoll centrifugation and dextran sedimentation, respectively. $CD14^+$ monocytes, $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and $CD34^+$ hematopoietic stem cells/progenitor cells from mononuclear cells were then purified using MicroBeads assays (Miltenyi Biotec, Tokyo, Japan). [11] PCR products from isolated cells were cloned into the pMD20T-vector using the Mighty TA cloning kit (Takara, Kusatsu, Japan), and individual colonies were analyzed by sequence analysis.

Generation of Human iPSCs from PB T Cells

PB T cells were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming kit (MBL Life science, Nagoya, Japan) according to the procedure described previously. [12] All human iPSCs were maintained on mitomycin C-treated mouse embryonic fibroblast feeder cells (ReproCELL, Yokohama, Japan) in human ES/iPS cell maintenance medium [Primate ES Cell Medium (ReproCELL) and 5 ng/mL human basic fibroblast growth factor (ReproCELL)]. iPSCs were passaged every 5 days.

Murine Stromal Cells

AGM-S3 cells (kindly provided by Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) within 10 passages were used throughout the experiments, as previously described. [11] AGM-S3 cells ($1–2 \times 10^5$) were cultured in each gelatin-coated six-well dish (FALCON, Corning, NY, USA) overnight to generate a confluent feeder layer. On the next day, they were exposed to 25 Gy of radiation.

Hematopoietic Differentiation from iPSCs

Hematopoietic differentiation was conducted as described previously. [13, 14] Briefly, undifferentiated iPSCs were transferred into medium containing irradiated AGM-S3 cells. The culture medium was then replaced with hematopoietic differentiation medium [Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 3 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 0.1 mM non-essential amino acids] supplemented with 10 ng/mL vascular endothelial growth factor (Human VEGF165 aa, Miltenyi Biotec) and changed every day. Twelve days later, the co-cultured cells were harvested, and $CD34^+$ cells were separated using positive immunomagnetic selection with CD34 MicroBeads kit (Miltenyi Biotec). [11]

Colony-Forming Cell Assay

Clonal cell cultures were performed in 35-mm Lux suspension culture dishes (#153066; Nunc), as reported previously. [15] Cultured $CD34^+$ cells (2500) were plated in dishes containing methylcellulose medium (Methocult H4034 Optimum, Stemcell Technologies Inc.). On day 14, the colonies of hematopoietic cells were scored in situ using an inverted microscope.

Myeloid Cell Differentiation from iPSC-Derived CD34 Cells

To carry out the differentiation of iPSC-derived CD34 cells into neutrophils, a co-culture system with AGM-S3 and combinations of cytokines was used according to a procedure described previously. [14] Briefly, $CD34^+$ cells (0.5×10^4 cells) were co-cultured with irradiated AGM-S3 cells in hematopoietic differentiation medium supplemented with stem cell factor (Human SCF, Miltenyi Biotec), thrombopoietin (Human TPO, Miltenyi Biotec), interleukin (IL)-3 (Human IL-3, Miltenyi Biotec), granulocyte colony-stimulating factor (Human G-CSF, Miltenyi Biotec), and fusion protein of IL-6 and IL-6 receptor (FP6, kindly provided by Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan). The concentration of each cytokine was as follows: 100 ng/mL SCF, 10 ng/mL TPO, 10 ng/mL IL-3, 10 ng/mL G-CSF, and 100 ng/mL FP6, as described previously. [14] Thereafter, the medium was changed every 4 days. On day 14, non-adherent cells were cytospun and then stained with May-Grünwald-Giemsa. Samples were examined using the NDP.view2 viewer software.

Apoptosis Assay

The annexin V assay (MBL Life Science) was performed according to the manufacturer's instructions on day 7 of myeloid cell differentiation culture. Cell surface markers were analyzed with a FACScan flow cytometer using the BD FACStation™-Data management System software (BD Biosciences, Tokyo, Japan). Fractions of annexin V-positive cells were estimated.

Statistical Analysis

All data are presented as individual data and mean ± SD. A P value of < 0.05 was considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). [16]

Study Approval

This study was performed following approval from the independent ethics committee of Shinshu University School of Medicine, Matsumoto, Japan; written informed consent was obtained from participants.

Results

Proband and Her Asymptomatic Mother

The proband was a 5-year-old Japanese girl with a history of recurrent bacterial infections since infancy. She had been diagnosed with SCN at the age of 1 year based on consistently severe neutropenia (ANC < 200/μL) and maturation arrest in BM. Repeated cytogenetic analysis results were normal. She exhibited no increase in ANC despite daily treatment with G-CSF (10 μg/kg subcutaneously). Sequencing analysis of the *ELANE* gene revealed a heterozygous missense mutation in exon 5 of c.607G>C (p.G203R, formerly G174R). This mutation had been previously reported for SCN [17, 18] and was present in the mother. The mother had no obvious recurrent infections, and repeated PB examinations showed normal white blood cell counts (4170, 4160, and 4130/μL) and near-normal ANCs (1271, 1385, and 1581/μL).

Proof of Maternal Mosaicism

To confirm whether there was a difference in mutant variant ratio among cell lineages, we separated PB and confirmed mutant variant ratios by sequence signals. In the sequence analysis of isolated PB, nail and hair, the mutant variant was observed at approximately 40–60% frequency in $CD14^+$, $CD3^+$, $CD19^+$, $CD56^+$, $CD34^+$ cells, and hair and at low frequency in nail. Conversely, the mutant variant was not detected in neutrophils (Fig. 1, Table 1).

Next, we attempted to establish iPSCs from the maternal PB to assess whether both *ELANE*-mutated and non-mutated clones were present and then determined the occurrence of granulopoiesis in these iPSCs. Although iPSCs derived from $CD34^+$ cells are ideal for estimating hematopoietic capacity into neutrophils, we could neither generate iPSCs from few PB $CD34^+$ cells nor ethically obtain BM from the mother, who had no symptom or low ANCs. Therefore, we attempted to generate iPSCs from maternal T cells. Before that, we evaluated the mutant variant ratio between $CD3^+$ and $CD34^+$ cells with

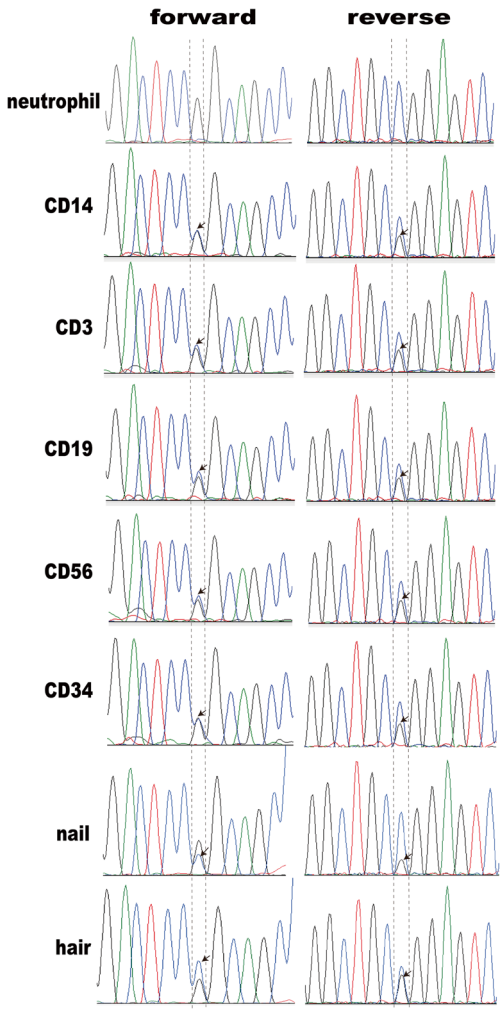

Fig. 1 Direct sequencing of the affected *ELANE* gene in purified maternal cells expressing the CD14, CD3, CD19, CD56, and CD34 antigens, isolated neutrophils, and nail tissue. Sequencing chromatograms showed that maternal lymphocytes, monocytes, and hematopoietic progenitor cells carried approximately half mutant alleles, whereas nail tissue carried only a few (arrow, c.607G>C). However, the mutant allele was not detected in neutrophil cells

Table 1 Mutant allele frequencies

Semiquantitative data from sequencing traces			
Cell type	Forward (%)	Reverse (%)	Average (%)
Neutrophil	0	0	0
CD14	50	34.7	42.35
CD3	53.5	35.9	44.7
CD19	55.2	38.8	47
CD56	54.4	35.2	44.8
CD34	50	33.6	41.8
Nail	37.5	19.8	28.65
Hair	63.8	43	53.4
Subcloning analysis from genomic DNA			
Cell type	No.	%	
CD3	8 of 21	38.1	
CD34	6 of 16	37.5	

another method. PCR products amplified from selected $CD3^+$ and $CD34^+$ cells were subcloned, and the frequency of the mutant allele was analyzed by sequencing. The mutant allele was detected in 8 of 21 clones (38.1%) from $CD3^+$ cells and 6 of 16 clones (37.5%) from $CD34^+$ cells, indicating that mutant variant ratios were similar to the sequence results. Overall, 12 iPSC clones were established from the asymptomatic mother who carried the *ELANE* mutation by infecting PB $CD3^+$ T cells with Sendai virus encoding the reprogramming factors *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*; 10 of the clones were found to contain heterozygous *ELANE* mutation, two of which contained the wild-type. *ELANE*-mutated iPSCs (Mt-1, Mt-2) and non-mutated iPSCs (Wt-1, Wt-2) were established after > 20 passages. We also generated iPSCs derived from her daughter (SCN) and a healthy individual (Cont). These iPSCs had no mutations in the mutation-sensitive region of the G-CSF receptor gene. [19]

Comparison of Granulopoiesis Between $CD34^+$ Cells Derived from *ELANE*-Mutated and Non-mutated iPSCs

For the functional analyses of $CD34^+$ cells derived from *ELANE*-mutated and non-mutated iPSCs, we performed colony-forming cell assays using methylcellulose medium containing cytokine mixtures for myeloid cells. The colonies were picked up, stained with May-Grünwald-Giemsa, and analyzed for the presence of myeloid differentiated cells. Histologic analysis of CFU-G, CFU-GM, and CFU-M confirmed that the colonies contained neutrophils, neutrophils and macrophages, and macrophages, respectively. As shown in Fig. 2, $CD34^+$ cells derived from Mt-1, Mt-2, and SCN generated approximately 75% and 35% reduction in CFU-G and CFU-GM colonies, respectively, compared with $CD34^+$ cells derived from Wt-1, Wt-2, and Cont. However, $CD34^+$ cells derived from Mt-1, Mt-2, and SCN increased the generation of CFU-M by comparison. For the precise quantitation of neutrophil development, $CD34^+$ cells derived from these iPSCs were co-cultured in suspension with AGM-S3 in the presence of neutrophil differentiation medium. The number of cultured cells was not different (data not shown), but a variety of differentiation stages was identified by morphological analysis. As shown in Fig. 3b, Wt-1, Wt-2, and Cont-derived myeloid cells differentiated into increasing frequencies of differentiated neutrophils. Meanwhile, the granulocytic population from *ELANE*-mutated iPSCs (Mt-1, Mt-2, and SCN) was shifted to the left with immature myeloid cells and a largely reduced neutrophil. Scoring for myeloid cell types, the ratio of neutrophils was significantly lower in Mt-1, Mt-2, and SCN than in Wt-1, Wt-2, and Cont (Fig. 3a). The maturation arrest of neutrophil development in SCN is associated with the induction of apoptosis in myeloid cells. [8, 9] Hence, we compared these iPSC-derived myeloid cells by flow cytometric analysis using annexin V. Mt-1-, Mt-2-, and SCN-derived myeloid cells contained a significantly higher proportion of annexin V-positive cells than Wt-1-, Wt-2-, and Cont-

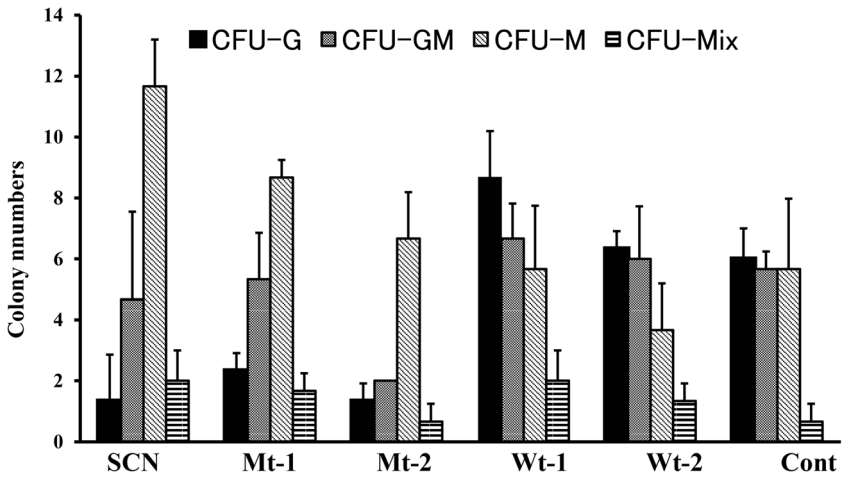

Fig. 2 Colony-forming cell assay of $CD34^+$ cells derived from *ELANE*-mutated iPSCs (Mt-1, Mt-2) and non-mutated iPSCs (Wt-1, Wt-2) and SCN-iPSCs (SCN) and healthy control-iPSCs (Cont). A hematopoietic colony assay was performed using 2500 iPSC-derived $CD34^+$ cells in the presence of a cytokine mixture, and the myeloid (CFU-G, CFU-GM, CFU-M) and mixed colonies were scored on day 14. The data represent mean ± SD ($n = 3$)

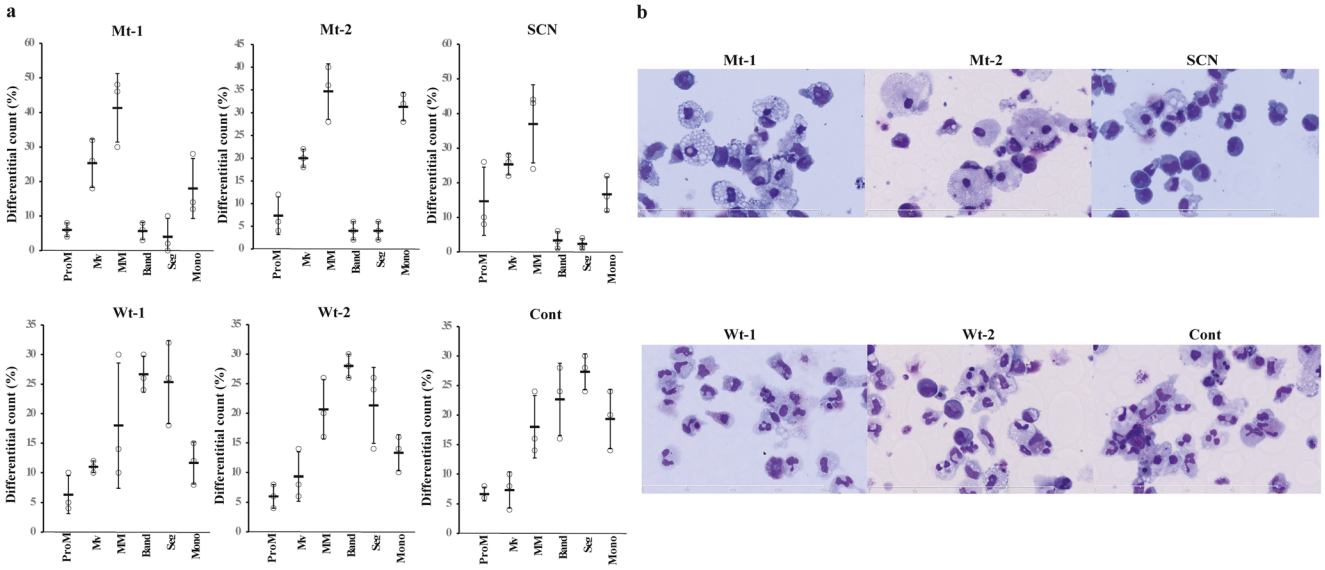

Fig. 3 Granulopoietic differentiation of CD34$^+$ cells derived from Mt-1, Mt-2, Wt-1, Wt-2, SCN, and Cont. iPSCs-derived CD34$^+$ cells differentiated into a variety of granulocyte differentiation, including promyelocyte (ProM), myelocyte (My), meta-myelocyte (MM), n-banded neutrophil (Band), segmented neutrophil (Seg), and monosyte (Mono). (i) The proportion of myeloid cells from iPSC-derived CD34$^+$ cells on day 14. The data represent mean ± SD ($n = 4$). (ii) Photographs of May-Grünwald-Giemsa stained cytospins

derived myeloid cells on day 7 of culture (Fig. 4), suggesting enhanced apoptosis of *ELANE*-mutated iPSCs derived myeloid cells.

Discussion

We present the case of a family of a girl affected with SCN, whose healthy mother was found to carry her daughter's p.G203R *ELANE* mutation. Sequencing chromatograms showed that lymphocytes, monocytes, and hematopoietic progenitor cells carried approximately half of the mutant alleles, whereas in the nail tissue, only a few were present. However, the mutant allele could not be detected in neutrophils (Fig. 1). *ELANE* mutations are known to produce autosomal dominant phenotypes [6, 7] and have extremely high penetrance in SCN. Therefore, we hypothesized that the mother possessed wild- and mutant-type clones; the wild-type clones could generate neutrophils, whereas the mutant clones failed to generate neutrophils.

Somatic and germline mosaicisms have been reported in several genetic disorders, including SCN. Similarly to our case, three reports on SCN and one on cyclic neutropenia have been published previously. [20–23] According to these reports, the same *ELANE* mutation was identified in affected children and in their asymptomatic parent. In three cases, somatic mosaicisms were demonstrated by various molecular methods. Furthermore, Ancliff et al. [21] revealed the presence of homozygous wild-type and heterozygous colonies cultured in vitro from PB. In the current study, another proof of mosaicism was obtained from generated iPSCs from the mother's PB; we could establish both iPSCs with wild-type and heterozygous *ELANE* mutations.

iPSCs have proven to be excellent tools for studying the disease mechanisms. Recently, some groups have demonstrated that *ELANE*-mutation-associated SCN-derived iPSCs could be induced by the arrest of granulopoietic differentiation and apoptosis of myeloid cells. [14, 15] Therefore, we compared the neutrophil differentiation potential between

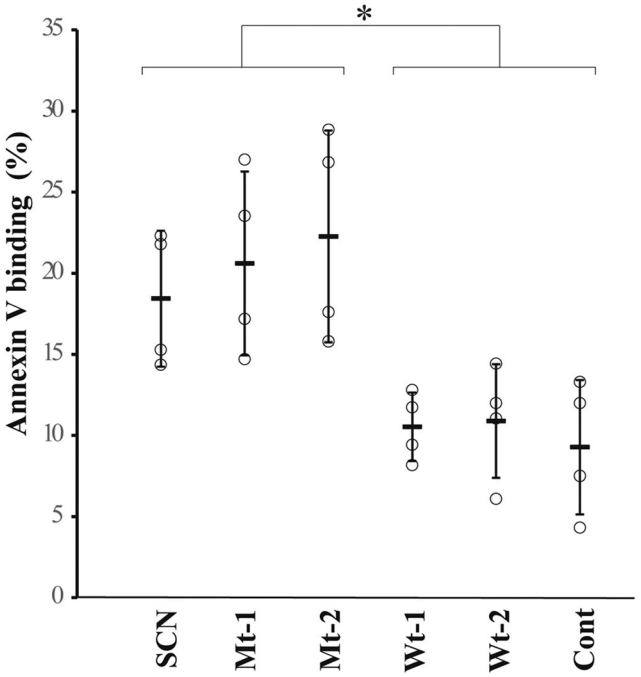

Fig. 4 Quantitation of the annexin V binding % on cultured myeloid cells derived from Mt-1, Mt-2, Wt-1, Wt-2, SCN, and Cont. Annexin V apoptosis assay was performed on day 7. The data represent mean ± SD ($n = 4$). One-way ANOVA was performed, followed by pair-wise comparisons using the Bonferroni criterion. *$P < 0.05$

established *ELANE*-mutated and non-mutated iPSCs. The number of granulocytic colonies and the ratio of neutrophils were significantly lower for mutated iPSCs than for non-mutated iPSCs (Figs. 2 and 3). The granulocytic differentiation potential of non-mutated iPSCs was similar to that observed in iPSCs obtained from a healthy individual. Also, similar to previous reports [14, 15], higher number of apoptotic cells were detected in *ELANE*-mutated iPSCs and SCN than in non-mutated iPSCs and Cont.

Our results demonstrate that the mother's PB harbored somatic mosaicism for the *ELANE* mutation and that her normal hematological phenotype could be explained by the perseverance of normal, non-mutated granulopoiesis. In addition, we conclude that mutant-carrying neutrophils did not appear in her PB because mutated clones could not differentiate into neutrophils due to *ELANE*-mutation-induced apoptosis during myelopoiesis.

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Authors' Contributions T.S. and O.O. performed experiments; T.S., N.K., and K.A. treated the patient; and T.S. and Y.N. designed the study and drafted the manuscript.

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Compliance with Ethical Standards

This study was performed following approval from the independent ethics committee of Shinshu University School of Medicine, Matsumoto, Japan; written informed consent was obtained from participants.

Conflicts of Interest The authors declare that they have no competing interests.

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