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Supplemental Data

De Novo Mutations Activating Germline *TP53* in an Inherited Bone-Marrow-Failure Syndrome

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Supplemental Note: Case Reports

Individual 1 was a male born at term after an uncomplicated pregnancy to healthy, unrelated parents. Birth weight was 2,972g (−0.7 SD), height 48.2 cm (−0.8 SD), and head circumference 31.7 cm (−1.3 SD). There was no family history of mental or hematological disorders. At 1 month of age, he visited a hospital because of intractable diarrhea, at which point hypogammaglobulinemia (IgG 1.53 g/L) was recognized. Anemia was noted at 2 months of age, when complete blood counts (CBC) showed hemoglobin 59 g/L, mean corpuscular volume (MCV) 103.5 fL, a white blood cell (WBC) count of 11.1 × 10^9/L with normal differential counts, platelet count 583 × 10^9/L, and 0.1% reticulocytes. Laboratory tests confirmed hypogammaglobulinemia, IgG 0.2 g/L, IgA 0.05 g/L and IgM 0.09 g/L. Bone marrow aspiration showed severe selective erythroid hypoplasia with otherwise normal cellularity. Chromosomal analysis showed normal karyotype (46XY, 9gh+). At 3 months of age, he had an episode of tonic-clonic seizure. He was diagnosed as having epilepsy and anticonvulsants were initiated with limited efficacy. At 5 months of age, regular IgG replacement and red blood cell transfusions were initiated. At one year of age, DBA was considered the probable diagnosis, and he was treated with methylprednisolone pulse therapy without improvement. At 6 years of age CD19+ B cells and CD3+ T cells were 2% and 97%, respectively. $CD19^+$ B cells were progressively decreased to 0.3% at 12 years of age, whereas CD3+ T cells remain unchanged (98%). He received chronic transfusion therapy to maintain a hemoglobin level above 60 g/L. He developed secondary hemochromatosis due to transfusional iron overload and chelation therapy was initiated from 8 years of age. He had hypothyroidism due to hemochromatosis and thyroid hormone replacement therapy was initiated. At 13 years of age, his anemia showed a spontaneous remission and he became transfusion-independent, although the platelet counts gradually decreased from 12 years of age (51 x 10^9/L). He was suspected of having DBA. However, no mutations were detected in the known DBA genes.[1,2] The activity of erythrocyte adenosine deaminase was normal (1.24 IU/gHb) and the erythrocyte reduced glutathione concentration was not elevated (81.4 mg/dLRBC).[3] Bone marrow analysis revealed hypocellurarity and mild tri-lineage dysplasia, which was consistent with refractory cytopenia of childhood.[4] The growth chart showed severe retardation. He had reticular skin pigmentation, tooth anomalies and hypogonadism with atrophy of the testis. He had severe microcephaly and general psychomotor

retardation: his head circumference as of 16 years of age was 42.0 cm (< −6.0 SD). Although he shared some features of DC, telomeres were not significantly shortened compared to typical DC cases with mutant *TERT*-related genes. He is now 20 years old. However, his bone age is that of a 12–year-old due to hypogonadism and he continues growing slowly. Although he remains transfusion-independent, he needs regular IgG replacement therapy for persistent hypogammaglobulinemia. No cancers were identified in this individual.

Individual 2 was a male born to healthy, unrelated parents. Pregnancy had been full-term and uncomplicated. Birth weight was 2,412 g (−1.4 SD), height 50 cm (+0.5 SD), and head circumference 31 cm (−1.6 SD). Anemia was first noticed at 5 days of age. There was no family history of mental or hematological disorders. He was referred to our hospital at 15 days of age. CBC showed the following: hemoglobin, 51 g/L; MCV, 99.4 fL; WBC, 6.84 × 10^9/L with normal differential counts; platelets, 357 × 10^9/L; and 0.6% reticulocytes. Bone marrow examination showed selective erythroid hypoplasia (5%). Chromosome analysis revealed a normal 46,XY karyotype and chromosome fragility test with mitomycin C was normal. Telomere shortening was not remarkable. From 1 month of age, he became transfusion-dependent and required a regular red cell transfusion every 4 to 5 weeks. At 9 months of age he had an afebrile convulsion, for which an anticonvulsant was initiated. At that time he was found to have hypogammaglobulinemia (IgG 2.92 g/L, IgA 0.01 g/L and IgM 0.05 g/L), for which regular IgG replacement was initiated. CD19+ B cells and CD3+ T cells were 19.2% and 72.7%, respectively. DBA was considered as a probable diagnosis for the cause of pure red cell anemia, and steroid therapy was initiated. However, his anemia did not respond to prednisolone (2 mg/kg/day). No mutations were detected in the known DBA genes.[1,2] Growth was severely retarded. He had severe microcephaly and general psychomotor retardation: as of 5 years of age, his head circumference was 42.7 cm (−4.9 SD). Because of a persistent Norovirus infection with a duration of 2 years, he underwent bone marrow transplantation from an HLA-matched unrelated donor at 7 years of age. However, he died of interstitial pneumonia with chronic graft-versus-host disease 250 days after transplantation. No cancers were identified in this individual.

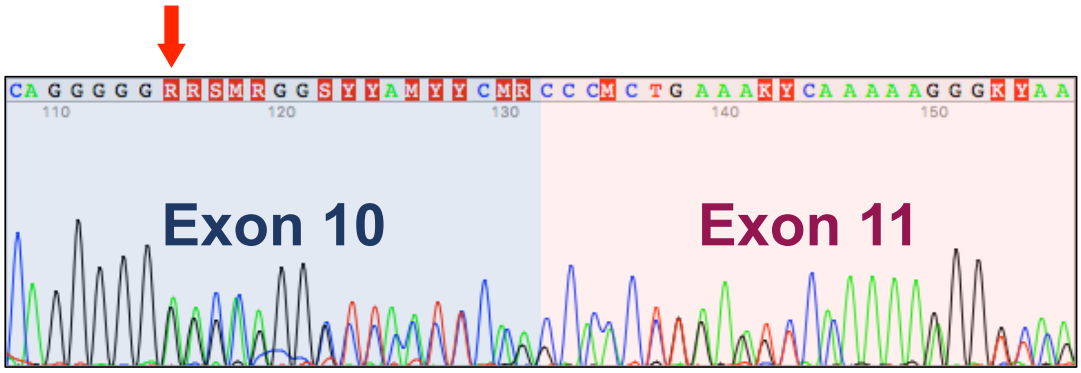

Figure S1. Sanger sequencing detected the mutant transcripts at a comparable level to normal transcripts in peripheral blood mononuclear cells from Individual 1. Arrowhead indicates the position of single nucleotide deletion at c.1083G.

Supplementary Table 3. Erythroid differentiation of human iPS cells carrying a heterozygous *TP53* mutant allele that lacked the C-terminal 32 amino acid.

Experiment 1

Day		Totall cell number (A)	Glycophorin A-positive cells (B)	Red cells (A*B)
0	WT	2.00E+04		2.00E+04
	hetero	2.00E+04		2.00E+04
6	WT	4.56E+05	0.7352	3.35E+05
	hetero	5.28E+05	0.871	4.60E+05
12	WT	3.93E+06	0.9688	3.80E+06
	hetero	2.00E+06	0.9367	1.87E+06
18	WT	2.25E+06	0.924	2.08E+06
	hetero	9.38E+05	0.785	7.36E+05

Experiment 2

Day		Totall cell number (A)	Glycophorin A-positive cells (B)	Red cells (A*B)
0	WT	2.00E+04		2.00E+04
	hetero	2.00E+04		2.00E+04
6	WT	4.64E+05	0.932158	4.33E+05
	hetero	2.50E+05	0.780205	1.95E+05
12	WT	4.55E+06	0.9585	4.36E+06
	hetero	2.30E+06	0.9471	2.18E+06
18	WT	2.00E+06	0.9691	1.94E+06
	hetero	5.00E+05	0.9643	4.82E+05

Experiment 3

Day		Totall cell number (A)	Glycophorin A-positive cells (B)	Red cells (A*B)
0	WT	2.00E+04		2.00E+04
	hetero	2.00E+04		2.00E+04
6	WT	7.10E+05	0.92837	6.59E+05
	hetero	5.00E+05	0.80994	4.05E+05
12	WT	4.90E+06	0.99314	4.87E+06
	hetero	2.37E+06	0.99034	2.35E+06
18	WT	2.87E+06	0.9846	2.83E+06
	hetero	1.00E+06	0.9783	9.78E+05

Supplemental Materials and Methods

Sample preparation

Genomic DNA was extracted from peripheral blood (PB) using a QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

Whole exome-sequencing and detection of germline mutations

For exome sequencing, genomic DNA from each member of the two pedigrees was enriched for protein-coding sequences with a SureSelect Human All Exon V3, V4 or V5 kit (Agilent Technologies, Santa Clara, CA, USA). This isolation was followed by massively parallel sequencing with the HiSeq 2000 platform with 100 bp paired-end reads (Illumina, San Diego, CA, USA). Candidate germline variants were detected through our in-house pipeline for exome-sequencing analysis with minor modifications for the detection of germline variants.[5] The resultant sequences were aligned to hg19 using the Burrows-Wheeler Aligner.[6] After removal of duplicate artifacts caused by PCR, the single nucleotide variants with allele frequencies > 0.25 and insertion-deletions with allele frequencies > 0.2 were called. With a mean coverage of 98.5× (79×–125×), > 92% of the 50 Mb target sequences were analyzed by > 10 independent reads.

Measurements of telomere length

The average relative telomere length (RTL) of peripheral lymphocytes was measured by flow-fluorescence *in situ* hybridization (flow-FISH), using a Telomere PNA kit (Dako Cytomation, Glostrup, Denmark). Details of the technical methods can be found in Baerlocher et al.[7] Briefly, peripheral blood lymphocytes were isolated by Ficoll separation and denatured in formamide at 82°C, hybridized with a fluorescein-conjugated $(CCCTAA)_3$ peptide nucleic acid probe and counterstained with LDS751 DNA dye. The analysis of fluorescence was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA). After gating on diploid cells based on staining with propidium iodine, lymphocytes were isolated on the basis of size and granularity. Relative telomere length (RTL) was calculated as the ratio between the telomere signal of each sample and that of the control (cell line 1301) using the following formula: (mean FL1 of sample cells with probe – mean FL1 of sample cells without probe) × DNA index of control cells / (mean FL1 of control cells with probe – mean FL1 control cells without probe) × DNA index of sample cells.

Construction of plasmids

To introduce the *TP53* mutations derived from Individuals 1 (c.1077delA) and 2 (c.1083delG) into the p53 pcDNA3.1 vector,[8] we performed inverse PCR (Primer STAR: Takara Bio) with the primer pair 45A-F (5' – GAGCCGGGGGGAGCAGGGCTCACTCCAGCCACCTGAAG- 3') and 45R (5' – CCTTCCCAGCCTGGGCATCCTTGAGTTCCAAGGCCTCA- 3') or 124G-F (5' – GAGCCGGGGGAGCAGGGCTCACTCCAGCCACCTGAAG-3') and 45R. PCR products were self-ligated and cloned to TP53 c.1077 pcDNA3.1 or TP53 c.1083 pcDNA3.1 expression vector.

For promoter assays, the luciferase reporter plasmid PICA p21–2.3k was constructed as follows.[8] A fragment containing 2.3 kb of the *CDKN1A* promoter region was amplified from CMK11-5 genomic DNA by PCR using the primers 5'-AGGGTACCAGGAACATGCTTGGGCAGC-3' and 5'-TGAAGCTTCCGGCTCCACAAGGAACTGA-3'. The PCR products were digested with *Kpn*I and *Hind*III, then subcloned into the PICA gene basic vector (Toyo Ink, Tokyo, Japan).

Reporter assay

The human osteosarcoma cell line MG-63 and quail fibroblast cell line QT6 were maintained in Eagle's minimal essential medium (MEM) with non-essential amino acids and 10% fetal bovine serum (FBS) at 37℃ in 5% CO_2. Cells were seeded at a density of 2 x 10^4 cells/well (24 well plate). Following overnight culture, MG63 cells were transfected with 3 μL FuGENE HD transfection reagent (Promega, Fitchburg, WI) whereas QT6 cells received 1.5 μL of reagent according to the manufacturer's protocol. The transfection mixtures contained 400 ng (for MG-63) or 200 ng (for QT6) of firefly luciferase reporter plasmid, 600 ng (for MG-63) or 300 ng (for QT6) of plasmids expressing wild-type or mutant p53. *Renilla* luciferase expressing vector, pEF-Seapansy, was also transfected as a normalization control. Cells were collected 24 h after the transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Functional analysis using zebrafish

A Morpholino antisense oligo (MO) targeting the intron 10/exon 11 boundary of zebrafish *tp53* was obtained from Gene Tools, LLC (Philomath, OR, USA). The sequence was TGTCATGCTGAAAGAAAAAGAATGA. The MO was injected at a concentration of 1.0, 2.5 or 5.0 μg/μL into single-cell stage embryos. The MO-injected embryos were grown at 28.5°C. Hemoglobin staining was performed at 48 h post-fertilization (hpf) using *o*-dianisidine.[9]

Total RNA was isolated from wild-type and the MO-injected embryos at 8 hpf. Reverse transcription (RT)-PCR was used to distinguish normal and intron-containing sizes of the *tp53* transcripts. This was performed by using a primer pair designed at exons 8 and 11. The primer sequences were 5'- ACCACTGGGACCAAACGTAG -3' (exon8) and 5'- AAATGACCCCTGTGACAAGC -3' (exon11).

Gene targeting of human iPSCs

The plasmids expressing CRISPR guide RNA and Cas9 were prepared by ligating oligos into the BbsI site of pX330 (Addgene no. 42230). CRISPR guide sequences were designed using web resources to introduce frame-shift mutations in the C-terminal domain (CTD) of *TP53*. The targeting constructs for genome editing and neomycin resistance gene expression plasmids were transfected into human induced pluripotent stem cells #8 (hiPSCs #8)[10] using FuGENE HD transfection reagent (Promega, Fitchburg, WI) and selected with neomycin (G418). Drug-resistant clones were manually transferred into 96-well plates and expanded for genomic DNA extraction and continued culture. Targeted clones were identified by PCR.

Real-time-quantitative reverse transcription PCR

For analysis of gene expression, the iScript System (Bio-Rad, Hercules, CA, USA) was used to synthesize single strand cDNA from 100 ng of total RNA isolated from hiPSC lines using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each cDNA was used for real-time quantitative PCR. Reactions were performed using iQ SYBR Green Supermix (Bio-Rad) on a CFX system (Bio-Rad). Gene-specific primers for *GAPDH*, *CDKN1A*, *MDM2*, *PMAIP1* and *TIGAR* were selected from the Perfect Real Time Support System (TAKARA Bio, Kusatsu, Japan). The amplification program consisted of 1 cycle of 95°C for 3 min and then 40

cycles of 95°C for 5 s, 60°C for 20 s. Gene expression levels were normalized against the level of the *GAPDH* gene.

Erythroid differentiation via sac-formation from human iPSCs

To differentiate hiPSCs into erythroid cells, we used our previously established protocol.[10] In brief, basic differentiation medium contained IMDM (Sigma-Aldrich, MO, USA) supplemented with 15% FBS, L-glutamine (Thermo, MD, USA), insulin-transferrin-selenium (Thermo), 50 μg/mL ascorbic acid (Sigma-Aldrich), and 450 μM 1-thioglycerol (Sigma-Aldrich). Small clumps of hiPSCs (< 200 cells) were cultivated on C3H10T1/2 stromal cells in basic differentiation medium supplemented with 20 ng/mL human VEGF (R&D) to obtain CD34+ cells at day 14. To further induce erythroid lineage cells, $CD34^+$ cells at day 14 were purified by flow cytometry (FACS Aria II, BD Bioscience, CA). Aliquots of the $CD34^+$ cells (2×10^4) were then transferred onto fresh C3H10T1/2 stromal cells and maintained in basic differentiation medium with 50 ng/mL SCF (R&D), 50 ng/mL TPO (R&D), and 5 U/mL EPO (KYOWA KIRIN, Tokyo, Japan), until Ery phase Day 6 (total of 20 days from the hiPSC stage). From Ery Day 6 to Day 12 (total of 26 days) or Day 18 (32 days), the cells were cultured in the presence of 5 U/mL EPO alone. Non-adherent cells were collected and analyzed at Ery Days 6, 12, and 18. Cell growth was analyzed by flow cytometry (FACS Aria II, BD Bioscience, San Diego, CA).

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