Short report

Congenital anaemia associated with loss-of-function variants in DNA polymerase epsilon 1

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SUMMARY
DNA polymerase epsilon (Pol ε), a component of the core replisome, is involved in DNA replication. Although genetic defects of Pol ε have been reported to cause immunodeficiency syndromes, its role in haematopoiesis remains unknown. Here, we identified compound heterozygous variants (p.[Asp1131fs;[Thr1891del]) in POLE, encoding Pol ε catalytic subunit A (POLE1), in siblings with a syndromic form of severe congenital transfusion-dependent anaemia. In contrast to Diamond-Blackfan anaemia, marked reticulocytopenia or marked erythroid hypoplasia was not found. Their bone marrow aspires during infancy revealed erythroid dysplasia with strongly positive TP53 in immunostaining. Repetitive examinations demonstrated trilineage myelodysplasia within 2 years from birth. They had short stature and facial dysmorphism. HEK293 cell-based expression experiments and analyses of patient-derived induced pluripotent stem cells (iPSCs) disclosed a reduced mRNA level of Asp1131fs-POLE1 and defective nuclear translocation of Thr1891del-POLE1. Analysis of iPSCs showed compensatory mRNA upregulation of the other replisome components and increase of the TP53 protein, both suggesting dysfunction of the replisome. We created Polε-knockout medaka fish and found that heterozygous fishes were viable, but with decreased RBCs. Our observations expand the phenotypic spectrum of the Pol ε defect in humans, additionally providing unique evidence linking Pol ε to haematopoiesis.

INTRODUCTION
DNA replication is an accurate process governed by a multi-protein complex, named a ‘replisome’, which unwind double-stranded DNA and duplicates the strands. DNA polymerase epsilon (Pol ε), a member of the core replisome, is composed of four subunits, namely, catalytic subunit A (POLE1) and three other subunits (POLE2 to POLE4) with distinct structural roles. A mouse model of Pol ε hypomorphy (Pole4−/−) presents systemic manifestations, including leucopenia, anaemia and increased frequency of lymphoma, suggesting the role of Pol ε in haematopoiesis.1 In humans, genetic variants leading to Pol ε deficiency were first described in a large consanguineous French family with FILS (facial dysmorphism, immunodeficiency, levedo and short stature) syndrome (MIM*615139).2 The patients had a homozygous variant (c.4444+3A>G) in POLE (MIM*174762), encoding the POLE1 protein, that resulted in 90% reduction of the mature protein. No major haematological abnormalities other than immunodeficiency were observed in the French family, whereas pancytopenia and myelodysplasia were observed in the second (Palestinian) family.3 More recently, combinations of a seemingly non-functional POL ε variant and deep intronic variant (c.1226+234G>A or c.1686+32C>G) were shown to cause another multisystem disorder named IMAGE-I (intratuderine growth restriction, immunodeficiency, hypoglycaemia, adrenal hypoplasia congenita, genitai anomalies and immunodeficiency) syndrome (MIM*618336).4 Patients with IMAGE-I syndrome were reported to have immunodeficiency and lymphoma, but no major haematological abnormalities. Collectively, although a relationship between Pol ε and haematopoiesis has been speculated in humans, no clear evidence has yet been established.

Here, we describe Japanese siblings with novel compound heterozygous POLE variants (p.[Asp1131fs;[Thr1891del]), both located in the protein-coding exons. Both siblings commonly had congenital anaemia without immunodeficiency.

SUBJECTS AND METHODS
Patient 1 (P1) was a Japanese female patient followed up for IUGR and was born at 29 weeks of gestation with a weight of 726 g (−3.3 SD) and a height of 32 cm (−2.9 SD). At birth, she had severe macrocytic anaemia without reticulocytopenia (red blood cells (RBCs) 0.95×1012/L, haemoglobin (Hgb) level 43 g/L, mean corpuscular volume (MCV) 123 fL, reticulocyte count 158×10 7/L, white blood cells (WBCs) 2.3×10 9/L, and platelets 396×10 9/L). Patient 2 (P2), the younger sibling of P1, also had IUGR and was born at 37 weeks of gestation with a weight of 2124 g (−1.6 SD) and height of 43 cm (−1.9 SD). Her Hgb level was 34 g/L at birth; there were no decreases in reticulocyte counts, WBC...
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Figure 1  Clinical phenotypes of the patients. (A) The pedigree of patients 1 and 2. (B) Myelograms with May-Giemsa staining of tissues of patient 1 and patient 2 in infancy show erythroid dysplasia without marked erythroid hypoplasia. (C) Myelograms with May-Giemsa staining of tissues of patient 1 and patient 2 in toddlerhood show dysplastic changes, such as hypersegmented neutrophils, megaloblastic change, multinucleated erythroblasts and micromegakaryocytes. (D) Immunostaining for TP53 using clot sections of the bone marrow from patients 1 and 2 demonstrate strongly positive signals compared with those of a patient with immune thrombocytopenia (ITP) in early childhood and a patient with autoimmune neutropaenia in infancy.

Exome sequencing of the patients and their parents was performed with SureSelect Human All Exome V6 (Agilent Technologies, Santa Clara, California, USA) and the Illumina HiSeq 2500 system (Illumina, San Diego, California, USA). We filtered the dataset using the Japanese variant database JPN38K (https://jmpr.megabank.tohoku.ac.jp/). To obtain a list of candidate pathogenic variants, the following filtering criteria were applied: (1) non-synonymous or frameshift variants in coding exons or splicing sites, (2) allele frequencies below 0.5% in 38KJPN, and (3) absence of the variant in our in-house exome data to filter pipeline-specific systematic errors. Potential pathogenicity was assumed from inheritance patterns—de novo, homozygous, compound heterozygous or hemizygous—and functional effects were predicted using SIFT, PolyPhen2 and CADD. Direct sequencing was performed to confirm the presence of the identified POLE variants.

Structural data of yeast Pol ε and human replisome were retrieved from the Protein Data Bank (https://www.rcsb.org/). Doxycycline-inducible stable HEK293 cells expressing N-terminal haemagglutinin-tagged POLE1 were established with previously described methods. Induced pluripotent stem cells (iPSCs) were established from a small intestinal tissue biopsy specimen from the proband, P1, and peripheral blood mononuclear cells from P2. Clones were established for P1 (three) and P2 (one). Two iPSC lines, each created from healthy individuals, were used as controls. Total RNA samples prepared from iPSCs were subjected to RNA sequencing (RNA-seq). Antibodies against haemagglutinin, human POLE2 and human TP53 were used for western blotting and immunofluorescence imaging. We generated two lines of Pole-deficient medaka (Oryzias latipes) using CRISPR/Cas9 genome editing. To assess pre-hatching stunting, we observed the embryo at 7 days after fertilisation using hatching enzymes. Peripheral blood was collected from the bulbus arteriosus of fish using glass capillaries, and cells were counted with a haemocytometer.
Details of experimental methods are described in online supplemental data and supplemental figures 3-5.

RESULTS

Our exome sequencing revealed that both P1 and P2 carried compound heterozygous POLE variants: NM_006231.2:c.3392del, p.Asp1131Alafs*24 and c.5672_5674del, p.Thr1891del (ClinVar accession number: SCV003806449 and SCV003806448, respectively). No other candidate variants were found in any genes implicated in congenital anaemia, including 71 causative genes of Fanconi anaemia, Diamond-Blackfan anaemia, Swachmann-Diamond syndrome, congenital dyserythropoietic anaemia and other syndromes, including TP53, SAMD9, GATA1 and GATA2. These two variants were not observed in 38,000 healthy Japanese individuals (https://jmorp.megabank.tohoku.ac.jp/). The p.Asp1131fs variant, if translated, would lose 51% of the amino acid sequence and was considered non-functional.

The p.Thr1891del variant was predicted to have a single residue deletion located in the centre of an α-helix (figure 2A), likely affecting the spatial arrangement of approximately 400 residues after the helix (figure 2A, coloured in blue).

We tested the pathogenicity of the two variants in vitro. Western blotting of the cytoplasmic and nuclear extracts of HEK293 cells expressing haemagglutinin (HA)-tagged POLE1 variants showed markedly decreased amounts of the two variants in the nuclear extracts (figure 2B). Immunofluorescence recapitulated the impaired nuclear localisation of the two variants (figure 2C). Intriguingly, endogenous POLE2 protein failed to localise at the nucleus in cells expressing Thr1891del-POLE1 (figure 2C).

We performed transcriptome analysis with patient-derived iPSCs. The mRNA levels of 12 out of the 17 human core replisome components were increased by more than 15% in the patients’ cells (figure 2D). This putative compensation was restricted to the core replisome, but was not observed in Pol α or Pol δ. POLE1 mRNA level was increased by approximately 50%, variant was predicted to have a single residue deletion located in the centre of an α-helix (figure 2A), likely affecting the spatial arrangement of approximately 400 residues after the helix (figure 2A, coloured in blue).

We tested the pathogenicity of the two variants in vitro. Western blotting of the cytoplasmic and nuclear extracts of HEK293 cells expressing each POLE1 variant showed markedly decreased amounts of the two variants in the nuclear extracts (figure 2B). Immunofluorescence recapitulated the impaired nuclear localisation of the two variants (figure 2C). Intriguingly, endogenous POLE2 protein failed to localise at the nucleus in cells expressing Thr1891del-POLE1 (figure 2C).

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although the transcript from the p.Asp1131fs allele was depleted (figure 2E). This suggests that mRNA with p.Asp1131fs was degraded via nonsense-mediated decay, and the p.Thr1891del allele was expressed at approximately threefold higher level compared with normal. As the loss of Pol ε could lead to replication stress and DNA damage, we addressed whether TP53 was upregulated in the patients’ iPSCs and showed a trend toward higher expression in patients’ cells (figure 2F). Western blotting further displayed a clear upregulation of TP53 protein in patients’ cells (figure 2G).

Finally, we studied the effect of POLE1 deficiency on haematopoiesis using the medaka (O. latipes). Homozygous Pole−/− fish and some heterozygous fish showed early embryonic lethality (figure 2H). In morphologically normal Pole−/− fish, the number of RBCs in the peripheral blood was significantly decreased (p=0.03), whereas a number of other blood cells were comparable to those in the wildtype fish (figure 2I).

**DISCUSSION**

We identified novel compound heterozygous POLE variants (p.[Asp1131fs];[Thr1891del]) in siblings with severe congenital anaemia. This may be the first report to demonstrate that POLE mutations could be a potential cause of congenital anaemia, which constitutes the most severe phenotype of Pol ε dysfunction and the consequential TP53 hyperexpression.

The analyses using HEK293 cells and patient-derived iPSCs confirmed the loss of function of the p.Asp1131fs and p.Thr1891del variants: POLE1 mRNA level was decreased in the p.Asp1131fs variant and nuclear translocation was defective in the p.Thr1891del variant. Thr1891del-POLE1 was predicted to retain most of the binding surface to POLE2 and might be able to sequester POLE2 in the cytoplasm. Previous studies found that patients with FLS syndrome and IMAGE-I syndrome were still able to produce reduced amounts of non-mutated POLE1; however, our patients were unable to produce any non-mutated protein. Indeed, heterozygous POLE variants (p.[Asp1131fs];[Thr1891del]) might result in more severe phenotypes than the replication stress-related phenotypes observed in FLS syndrome and IMAGE-I syndrome. POLE1 is ubiquitously expressed (Human Protein Atlas, proteinatlas.org) and the reported IMAGE-I syndrome had variability in phenotypes. Haematopoietic cells with high DNA synthesis are susceptible to the Pol ε defect, and intrauterine growth retardation and short stature after birth could be attributed to the ubiquitous POLE1 expression. No adrenal insufficiency and only epicanthic folds as facial abnormalities until the present age in our patients could be within the variability of IMAGE-I syndrome.

The pathophysiology of haematological abnormalities in our patients was not confirmed; however, intravascular and extravascular haemolysis was less involved in the congenital anaemia based on the laboratory data (online supplemental table 1). We did not observe burr/helmet cells in the peripheral blood, splenomegaly, increased reticulocyte counts/lactate dehydrogenase/bilirubin/urinary urobilinogen or decreased haptoglobin. Despite the low growth rate of patient-derived iPSCs, we believe the contribution of impaired regenerative anaemia was limited. Marked reticulocytopenia or erythroid hypoplasia was not found, in contrast to that observed in Diamond-Blackfan anaemia. The clinical course in which they had severe anaemia at birth and developed myelodysplasia later indicates that anaemia was not caused by the MDS associated with somatic mutations. The consequent TP53 overexpression leading to the apoptosis suggests an association of ineffective haematopoiesis, as in other disorders related to congenital anaemia or inherited bone marrow failure. In Fanconi anaemia, the hyperactivation of TP53 or TP21 before and after birth by replicative or cellular stress is considered the major factor leading to apoptosis and impaired haematopoietic stem cell expansion. In Diamond-Blackfan anaemia and 5q− syndrome, the myelogram of patients shows TP53 hyperexpression, and animal experiments have revealed the improvement of haematological abnormalities following p53 KO. The associated TP53 activation has been observed in fibroblasts from patients with IMAGE-I, and the phenotypes of size reduction and lymphopenia in KO-POLE4 mice were improved by p53 KO.

This report was based on siblings in one family and a limited functional analysis due to the low growth rate of patient-derived iPSCs and embryonic lethality of Pole-KO fish; nevertheless, our observations not only expand the phenotypic spectrum of the human Pol ε defect but also provide unique evidence linking Pol ε and haematopoiesis.

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**Acknowledgements**

We would like to express our sincere gratitude to the staff of the National Center for Child Health and Development for their aid in treating the patients. We are grateful to NBPR Medaka (https://shigen.nig.ac.jp/medaka/) for providing OK-Cab (Strain ID: MT830) and Hatching Enzyme.

**Contributors**

IT, SN and Alishiguro conceived the study and drafted the manuscript. KT, N and H participated in designing and coordinating the study, carried out molecular genetic studies and drafted the manuscript. AO, KN and TKato participated in designing and coordinating the study, carried out the animal experiments and drafted the manuscript. IT, AIguchi, HS, KA and AIshiguro collected the clinical data of the patients. MKashima, TY, HH, IT, HM and MKobayashi participated in the study design and coordination and carried out the animal experiments. OM, KT and KH participated in the genetic analysis and study design and helped draft the manuscript. TU and TKawai performed immunological studies. VN performed the endocrinological studies. TS, KM, KH and KA supervised the study and added important intellectual content. All authors critically revised the manuscript, commented on the drafts and approved the final draft.

**Funding**

This study was supported by the Initiative on Rare and Undiagnosed Diseases grants (JP21ek0109489, 22bm0104017h0003) (KH and HA, respectively) from the Japanese Agency for Medical Research and Development; MEXT KAKENHI.
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Grant (grant JP21K07787) (IT); and a national Grant-in-Aid from the Japanese Ministry of Health, Labour, and Welfare (grants 2019 A-3, 2020B-3 and 2020B-10) (KA, SN and IT, respectively).

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by the Ethics Committee of the National Center for Child Health and Development (#378). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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