NF-E2 mutation as a novel cause for inherited thrombocytopenia

Inherited thrombocytopenias (IT) are genetic diseases that affect platelet production and function, resulting in thrombocytopenia and a tendency for bleeding. Novel genetic mutations responsible for IT are being reported continuously (Almazni et al., 2019; Lentaigne et al., 2019). We present a case of thrombocytopenia with a homozygous frameshift mutation of nuclear factor, erythroid 2 (NF-E2), which has not been reported before.

The patient was an eight-week-old Filipino boy born at full term of non-consanguineous parents with no family history of bleeding disorders. The mother had gestational diabetes. The patient developed multiple petechiae on day 2 of life and thrombocytopenia was identified. Physical examination revealed a well-developed baby boy with petechiae and purpura on face, trunk and extremities. There was no other active bleeding. Examination of respiratory, cardiovascular, abdominal and lymphatic systems was unremarkable.

Further investigations for viral infections, immunoglobulin levels, lymphocyte subsets, Coombs test and abdominal ultrasound were unrevealing (Table SI). Bone marrow examination revealed cellular marrow with the presence of all cell lines including megakaryocytes. The thrombocytopenia was refractory to platelet transfusion, intravenous immunoglobulin and prednisolone (Fig 1). He was referred to The University of Hong Kong for genetic work-up. Genetic and functional studies on referred patients, data archival in the Asian Primary Immunodeficiency Network (APIN) database (Lee & Lau, 2011), and DNA storage were approved by the Clinical Research Ethics Review Board of the University of Hong Kong and Queen Mary Hospital (Ref. no. UW 08-301) with informed consent obtained from the parents of subjects. DNA from the patient and his mother were sent to Hong Kong together with his peripheral blood and bone marrow slides. A peripheral blood smear revealed thrombocytopenia with normal-sized platelets (Figure S1). A bone marrow smear revealed few normal-looking megakaryocytes without dysplasia, active erythropoiesis and granulopoiesis (Figure S2). Unfortunately the patient was lost to further follow-up in the Philippines.

The patient was initially screened for Wiskott–Aldrich syndrome but PCR-Sanger sequencing of WASP was normal (Lee et al., 2009). Therefore, whole-exome sequencing (WES) was performed on the patient and his mother, which revealed the patient had a homozygous frameshift mutation in NF-E2 (c.952delA, p.T318fsX326), which resulted in a truncated transcription factor NF-E2 45 kDa subunit (p45/NF-E2) (Fig 2). The mother was a carrier of the mutation. The WES was performed as described in Data S1 and Figure S3. Analysis in silico using PROVEAN (Choi et al., 2012) and SIFT Indel (Hu & Ng, 2012) predicted the frameshift mutation in NF-E2 to be deleterious. PROVEAN predicted the mutation as deleterious with a PROVEAN score of $-143 \cdot 071$ (scoring less than $-2 \cdot 5$ is considered deleterious). SIFT Indel predicted the mutation effect as damaging with a confidence score of $0 \cdot 858$.

Mutations in NF-E2 were further searched for in a group of 13 patients in our APIN database with documentation of thrombocytopenia in the first two months of age, or between two and four months of age if without small platelets or organomegaly. None of the 13 patients had a mutation in NF-E2.

NF-E2 is a heterodimeric transcription factor formed by p45/NF-E2 and small MAF proteins through the interaction of their basic leucine zipper bZIP domains (Fig 2) (Levin et al., 1999). NF-E2 is essential for promoting megakaryocytic maturation (Motohashi et al., 2010), proplatelet formation and release (Lecine et al., 2000; Tiwari et al., 2003; Chen et al., 2007) (Figure S4). The bZIP domain facilitates binding of NF-E2 to DNA, allowing the p45/NF-E2 subunit to transactivate platelet gene expression (Fig 2).

p45/NF-E2 is expressed in erythroid cells, megakaryocytes, granulocytes and mast cells (Levin et al., 1999). Although studies demonstrated that mice without either p45/NF-E2 or small MAF proteins had thrombocytopenia, only those without p45/NF-E2 died from lethal haemorrhage, indicating p45/NF-E2 is the critical component (Shivdasani et al., 1995; Catani et al., 2002).

Post-translational modifications of p45/NF-E2 are critical to modulate its functions. Lysine 368 on p45/NF-E2 is the site for sumoylation, the addition of small ubiquitin-like modifier (SUMO), enhancing the DNA binding affinity and transactivation capability of p45/NF-E2, which has been shown to be critical in β-globin expression (Shyu et al., 2005).

The frameshift mutation in our patient (c.952delA, p.T318fsX326) resulted in a truncated p45/NF-E2 protein

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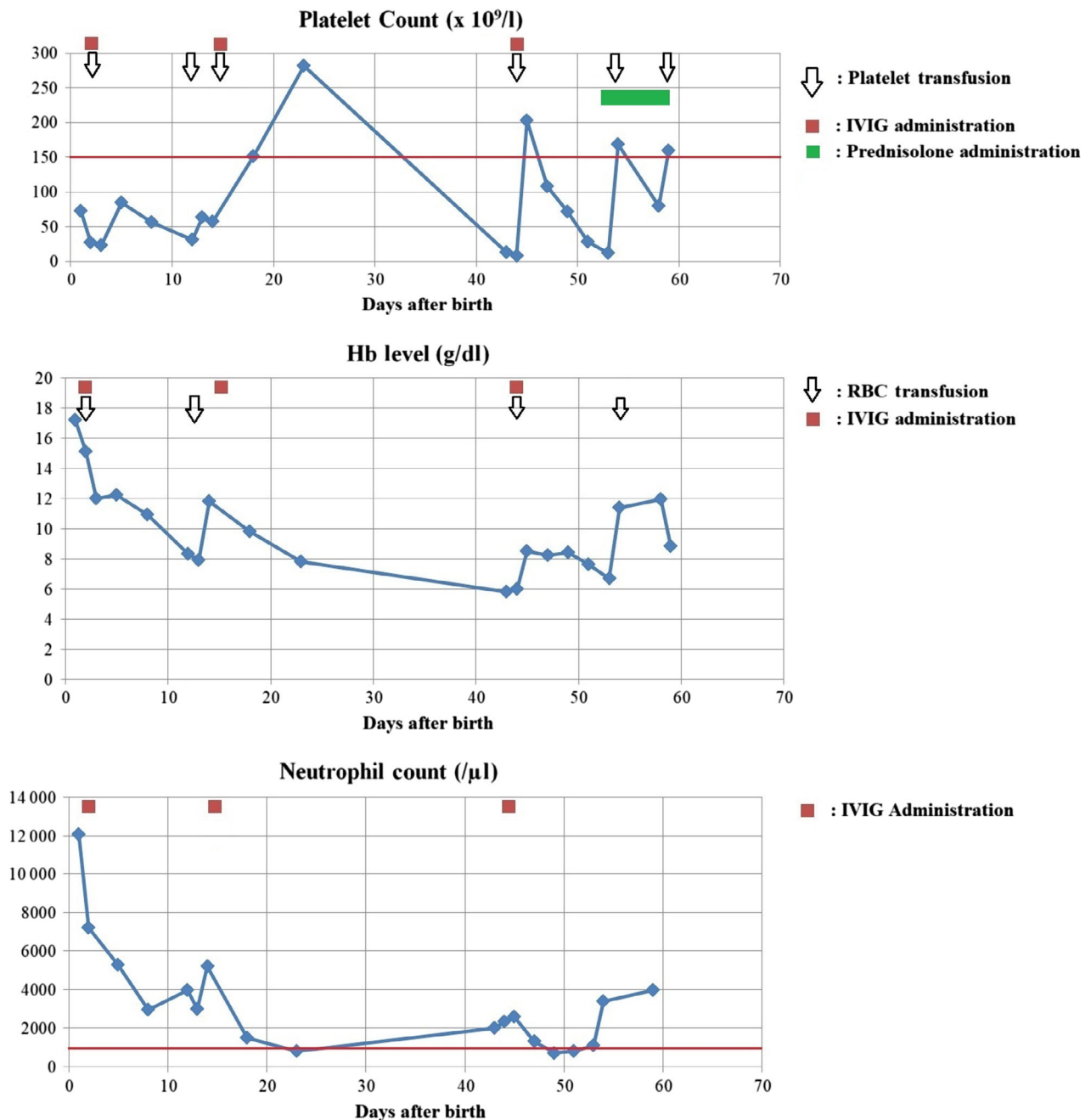
Fig 1. Platelet count, haemoglobin level and neutrophil count of the patient after birth. Red lines represent the lower limit of reference range for platelets (150×10^9/l) and neutrophils (1000/µl). IVIG, intravenous immunoglobulin infusion.

and loss of the sumoylation site, leading to impaired DNA binding, transactivation, megakaryocyte maturation and platelet release.

There are currently at least 41 genes that have been reported to cause IT (Almazni et al., 2019; Lentaigne et al., 2019). We classified them based on inheritance pattern, the presence of megakaryocytes and the size of platelets as well as clinical features, namely dysmorphism, immunodeficiency and bleeding tendency (Figure S5). The majority of them did

not have the matching phenotypes and inheritance pattern to those of our patient. WES did not identify mutations in these 41 genes either (Table SII).

p45/NF-E2 is important for globin production in animal models (Shyu et al., 2005), and loss of p45/NF-E2 function in neonatal mice results in anaemia (Levin et al., 1999). Furthermore, a study in humans has shown that the presence of mixed truncated p45/NF-E2 and normal p45/NF-E2 promotes erythropoiesis through enhancing the action of normal
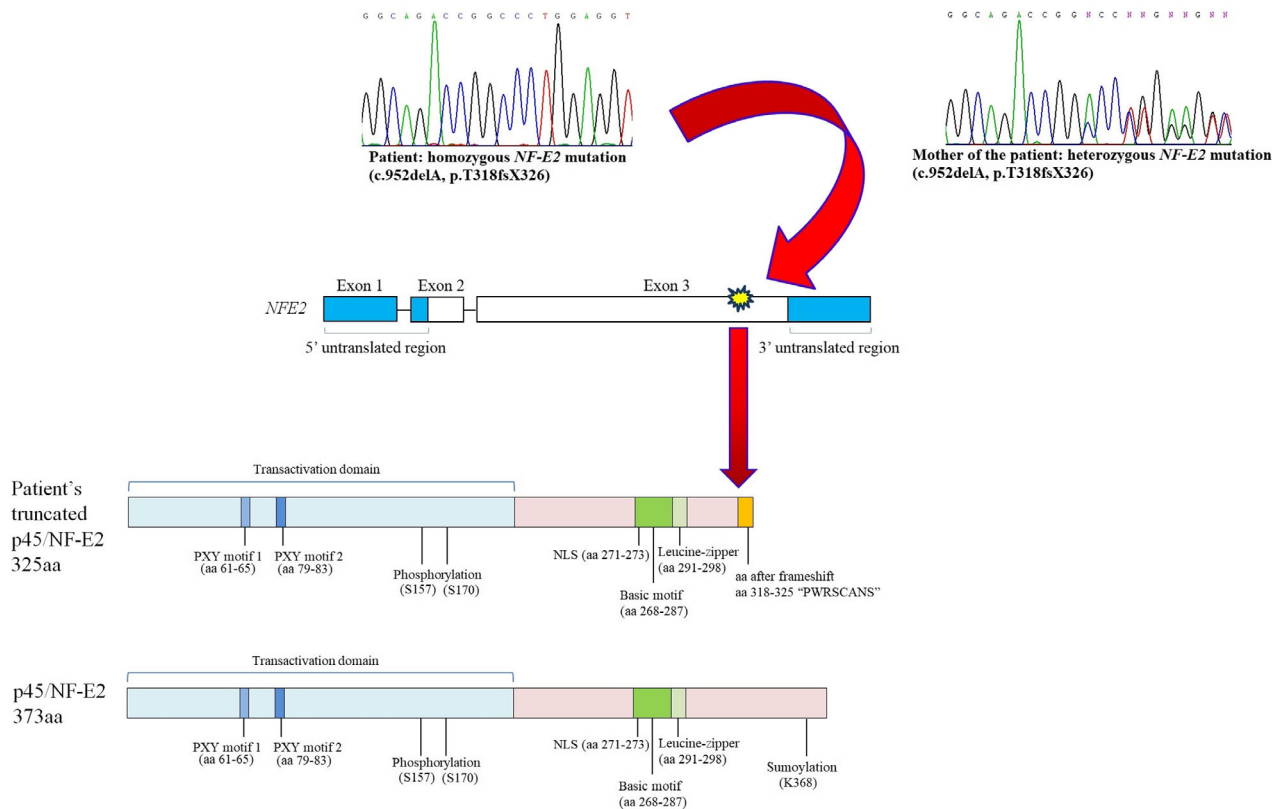
Fig 2. Sanger sequencing result, p45/NF-E2 structure and the truncated form in our patient. Sanger sequencing identified the patient had a homozygous frameshift mutation (*c.952*delA, p.T318fsX326) in exome 3, which produced a truncated p45/NF-E2, with loss of its sumoylation site.

p45/NF-E2 in patients with polycythaemia vera (Jutzi *et al.*, 2013). Since our patient had only truncated p45/NF-E2, he would not have enhanced erythropoiesis. Instead, his haemoglobin level dropped to 8 g/l on day 12 of life (Fig 1). Investigation then revealed a normochromic normocytic anaemia with elevated indirect bilirubin and LDH, but a Coombs test was negative, suggesting a mechanism other than immune-mediated haemolysis. His haemoglobin level dropped again to 6 g/l on day 43 of life. A previous study has suggested lack of p45/NF-E2 function may lead to increased erythrocyte sensitivity towards oxidative stress (Chan *et al.*, 2001), increasing haemolysis susceptibility. However, another study suggested haemolysis does not account for the anaemia observed in p45/NF-E2-deficient mice (Shivdasani & Orkin, 1995). Hence it is still unclear the anaemia observed was due to the loss of p45/NF-E2 function.

To our best knowledge, this is the first report of a homozygous frameshift mutation of *NF-E2* in a patient with thrombocytopenia. As the patient was lost to follow-up, we did not perform mRNA, protein or cellular studies, which is a limitation of our study. Nevertheless, as a guideline for genetic studies in a single patient, it has been proposed to ascribe a novel genetic mutation as the cause of the observed phenotype (Casanova *et al.*, 2014). Our study has fulfilled this guideline with analyses to exclude reported genetic causes of IT in our patient (Almazni *et al.*, 2019; Lentaigne *et al.*, 2019) and identification of a homozygous frameshift mutation of *NF-E2* which would impair the function of the protein, as well as previous reports of relevant animal phenotypes of severe thrombocytopenia due to loss of p45/NF-E2 function (Shivdasani *et al.*, 1995; Levin *et al.*, 1999; Shyu *et al.*, 2005).

In conclusion, mutation in *NF-E2* may be a novel cause of IT in humans.

Author contributions

ADWL and YLL developed the research plan. APA and RCH referred the patient and provided care for the patient. XY, KWC, JY and WY analyzed the whole-exome sequencing. GCFC and JCCS analyzed the bone marrow sample. ADWL, PPL and YLL analyzed clinical information. ADWL and YLL wrote the manuscript. All authors approved the final version.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Peripheral blood smear of the patient. The peripheral blood smear of the patient is on the left, with marked reduction of platelets (marked by red arrows) seen. The peripheral blood smear on the right is the normal control, with blue arrows marking the platelets.

Figure S2. Bone marrow of the patient. Normal megakaryocytes are seen in the bone marrow of the patient.

Figure S3. Analysis of the whole-exome sequencing result for the patient. Following the whole-exome sequencing, variants were analyzed based on the quality of the read, population prevalence, matching of maternal variants, genomic location of variants and predicted functional impact of the variants.

Figure S4. Roles of NF-E2 in platelet formation and function. HSC, haematopoietic stem cell; iMK, immature megakaryocyte; MK, mature megakaryocyte; NRF2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species.

Figure S5. Classification of the genetic causes of inherited thrombocytopenia. BSS, Bernard–Soulier Syndrome.

Table SI. Investigations performed on the patient.

Table SII. In silico analysis of variants of genes causing inherited thrombocytopenia.

Data S1. Whole-exome sequencing for the patient: methods and result.

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