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Distinct molecular abnormalities underlie unique clinical features of essential thrombocythemia in children

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Essential thrombocythemia (ET) is rare in children, with an annual incidence of ~100-fold lower than that in adults.[1] The rarity of the disease in children makes the clinical course and pathogenesis of childhood ET far less clear. It is reported that clonal markers are much less common in children with ET (25.8%) than that in adult cases (80–90%).[2–4] Except for *JAK2* V617F, and *MPL* and calreticulin (*CALR*) mutations, no other mutations have yet been reported in childhood ET.[2,5–8] More molecular markers are needed to distinguish clonal from reactive thrombocytosis in children. The present study investigated the *JAK2* V617F, and *MPL* and *CALR* mutations in a large cohort of children with ET. We conducted the first study to analyze the molecular profiles by targeted next-generation sequencing and to investigate the *JAK2* 46/1 haplotype in childhood ET.

Sixty-three children diagnosed with sporadic ET according to the 2008 World Health Organization criteria were enrolled.[9] Bone marrow histology was consistent with a diagnosis of ET in all cases. The molecular patterns were evaluated before any cytoreductive drug use. The *JAK2* V617F, and *MPL* and *CALR* mutations were investigated as previously reported.[4] The *JAK2* 46/1 haplotype (rs12340895) was assessed by Sanger sequencing. Fifty-five genes (Supplementary Table 1) associated with myeloid malignancies were analyzed by targeted sequencing in 25 children. Polymorphisms in existing database were excluded. Mutations were validated by Sanger sequencing (Supplementary Table 2). Each true-positive mutation was further investigated in 100 normal controls. Germline DNA was used to identify somatic mutations. Detailed information is shown in the Supplementary Methods.

The median age was 11 years (range, 3–14 years). Different from adult ET,[10] a male preponderance was observed among childhood patients, with a male/female ratio of 1.5 (38/25). Compared with the adult patients that we previously reported,[10] childhood ET had higher platelet counts (median 1224×10^9 vs 900×10^9/l; $P<0.001$), lower hemoglobin level (median 127 vs 137 g/l; $P<0.001$) and comparable white blood cell counts (median 10.6×10^9 vs 9.9×10^9/l; $P=0.312$). It indicates a pronounced and isolated megakaryocyte proliferation in child patients. The *JAK2* V617F mutation was found in 14 children (22.2%), with a median allele frequency of 22% (range, 10–31%). In 49 patients among whom *CALR* and *MPL* mutations were investigated, only an 11-year-old girl harbored a *CALR* mutation (52-bp deletion), and none had *MPL* mutations. The molecular markers were much less common than that in adult patients.[3,4] In adult patients, compared with patients with wild-type *JAK2*, V617F-mutated patients display higher white blood cell counts, higher hemoglobin level but lower platelet counts.[4] However, there were no differences in blood cell counts between children with and without the *JAK2* V617F mutation (Supplementary Table 3).

Three children (4.8%) displayed major thrombosis, and two of them were V617F-mutated. Microvascular disturbances were more common ($n=30$, 47.6%) than that in Caucasian children (30.3%).[2] Headache was the most frequent symptom ($n=22$, 34.9%). The incidence of headache was significantly related to platelet counts at diagnosis ($P=0.013$). Headache was relieved by antiplatelet and/or cytoreductive therapy in 17 children (77.3%), providing important evidence for the central role of platelets in the etiology of headache. No major bleeding events were observed. The rate of minor bleeding episodes was 14.3% ($n=9$), similar to that in the Caucasian children (9.0%).[2] The risk of minor bleeding events was not related to platelet counts ($P=0.126$) or *JAK2* V617F mutation ($P=0.194$)). Two children (3.2%) evolved to myelofibrosis after 20 and 7 years of follow-up, respectively. One harbored the *JAK2* V617F mutation and received 1 year of intermittent hydroxyurea treatment before transformation. The other one did not have *JAK2* V617F or *CALR* mutations, and received 2 months of interferon-alpha and then 5 years of hydroxyurea before transformation.

Targeted sequencing was performed in 25 children (Supplementary Table 4). The average depth (median, 349-fold) and coverage (median 99.6%) of the target regions were excellent (Supplementary Figure 1). A total of 135 single-nucleotide variants were identified (Supplementary Table 5). A detailed filtration pipeline was developed to select mutations that might be related to tumorigenesis (Supplementary Figure 2). After filtration, eighteen types of somatic mutations (single nucleotide variants and indels) involving 13 genes and 7 germline mutations involving 7 genes were selected (Supplementary Table 6). Somatic mutations were present in 14 children (56.0%), with a median allele frequency of 33.6% (range, 18.8–60.4%). Other than *JAK2* V617F ($n=6$, 24.0%), the most frequently observed somatic mutations were *ASXL1* mutations ($n=4$, 16.0%) (Figure 1a). In 18 children with wild-type *JAK2* and *CALR*, seven (38.9%) harbored somatic mutations that were previously undocumented in childhood ET. Six (24.0%) children harbored two or more somatic mutations (Figure 1b).

The molecular profiles were different between childhood and adult ET patients. On the one hand, mutations that were commonly involved in adult ET were not found in childhood ET. In adult patients, other than *JAK2*, *CALR* and *MPL*, mutated genes most commonly include *TET2* (4%–11%) and *DNMT3A* (1–5%).[11] However, none of the children had *TET2* or *DNMT3A* mutations. On

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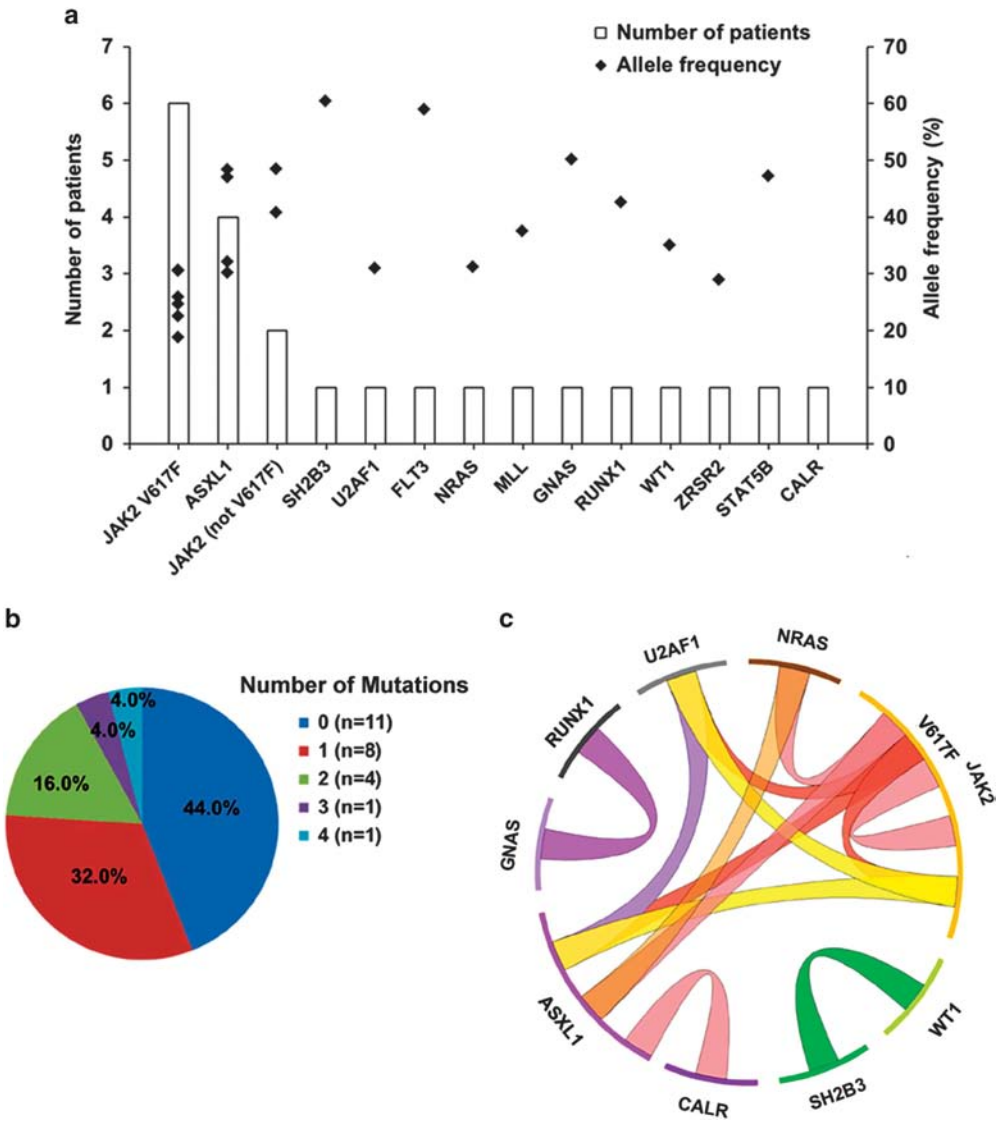

Figure 1. Frequency and distribution of somatic mutations in children with essential thrombocythemia. (**a**) Number of patients and allele frequency of each mutated gene. (**b**) Number of patients with different number of somatic mutations. (**c**) Co-occurrence of the somatic mutations in the same individual.

the other hand, the newly identified mutations in childhood ET (that is, mutations in the gene *NRAS*, *MLL*, *U2AF1*, *ZRSR2*, *GNAS*, *FLT3*, *RUNX1* and *WT1*) were rarely seen in adult ET but were recurrent in myelodysplastic syndrome, primary myelofibrosis or blast phase myeloproliferative neoplasms.[11–13] The rate of the *ASXL1* mutations was much higher (16%) than that in adult ET (2–5%).[11] The discovery of recurrent mutations indicates that targeted sequencing can be used to distinguish clonal from reactive thrombocytosis in children.

By analyzing co-occurrence of the somatic mutations (Figure 1c), we revealed a genetic complexity in childhood ET. Lundberg *et al.*[14] reported that in 60 adults with somatic mutations, 17 (28.3%) had two or more mutations. In our study, about half of the children with somatic mutations (6/14, 42.9%) had more than one mutation. The higher rate of co-occurrence of rare somatic mutations in childhood ET suggests that children with ET might have a more complex and unstable genetic composition than adult patients have. Acquisition and accumulation of the somatic mutations in early life might be the main reason for the early onset of ET in children. Mutual exclusivity was observed in gene pairs with similar biological function, such as genes both involved in the JAK–STAT pathway. However, co-occurrence of mutations in different alleles of *JAK2* (that is, *JAK2* V617F and *JAK2* I354T; *JAK2* V617F and *JAK2* G127D) was observed in two children, indicating that the *JAK2* gene might be more sensitive to the genetic instability. This might be one of the reasons why *JAK2* was most commonly affected in ET. The definite existence of genetic instability needs more evidence.

The *JAK2* 46/1 haplotype was assessed in 49 children. The number of children with CC, CG and GG genotypes was 17 (34.7%), 31 (63.3%) and 1 (2.0%), respectively. The frequency of the *JAK2* 46/1 haplotype (33.7%) was significantly higher than that in normal Chinese population (21.9%; $P = 0.013$).[15] It suggests that inherited predisposition may also exist in childhood patients.

Clusters of mutations based on allele frequencies distinguish founding clones from subclones.[12] Figure 2 shows diverse clonal hierarchies in two representative children. The germline *IDH1* mutation in patient E1 and the *JAK2* 46/1 haplotype in patient E3 reflected the inherited genetic background. In patient E1, three variants (that is, *NRAS*, *ASXL1* and *JAK2* V617F) with similar allele frequencies (~30%) might exist in the same founding clone or in separate founding clones (Figures 2a and b). Different from

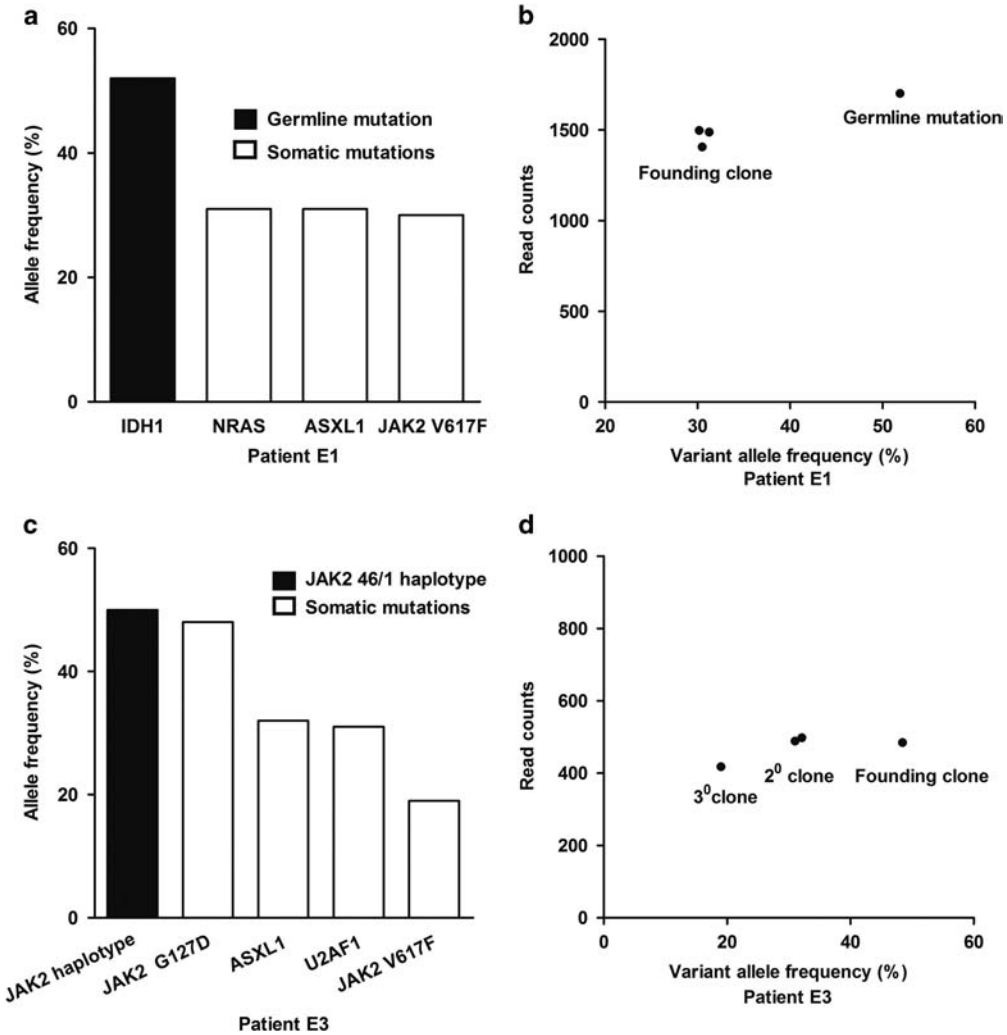

Figure 2. Clonality assessment in two representative cases of essential thrombocythemia. (**a**) Allele frequencies of mutations in patient E1. (**b**) Clusters of variants identify the founding clone in patient E1. (**c**) Allele frequencies of mutations in patient E3. (**d**) Clusters of variants identify the founding clone and subclones in patient E3. In (**b**) and (**d**), the allele frequency is plotted versus the total number of sequencing reads covering the corresponding mutated nucleotide.

patient E1, patient E3 had the founding clone (that is, *JAK2* G127D), first-level subclones (that is, *ASXL1* and *U2AF1*), and second-level subclone (that is, *JAK2* V617F) (Figures 2c and d). Since the heterozygous *JAK2* G127D mutation (allele frequency, 48.5%) was present in almost all cells, *JAK2* V617F (allele frequency, 19.0%) was not the earliest genetic alteration. Similarly, the *CALR* mutation was also not the initial abnormality, which could be speculated from the co-occurrence of the heterozygous *ASXL1* M1096L mutation (allele frequency, 48.4%) and the *CALR* mutation (allele frequency, 21.1%) in the same individual.

Concerning gender, age and blood cell counts, no differences were found between mutated and non-mutated children, except for white blood cell counts ($P = 0.025$). The only child with thrombosis among the 25 children had a single *ASXL1* mutation. The types of antiplatelet and cytoreductive treatment between mutated and non-mutated children were similar (Supplementary Table 4).

In conclusion, the molecular profiles are different between childhood and adult ET patients, and the genetic composition in childhood ET may be more complex than that in adults. The difference in clinical and hematological characteristics between childhood and adult ET may be due to the different molecular profiles underlying the two entities. Additional molecular markers are found by targeted sequencing to identify children with a clonal blood disorder. After *JAK2* V617F, the most frequently observed somatic mutations are *ASXL1* mutations. The *JAK2* V617F or *CALR* mutations may not be the initial abnormalities in some children. Whole genome sequencing would help to reveal initial molecular events.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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R Fu[1], D Liu[2], Z Cao[1], S Zhu[2], H Li[1], H Su[2], L Zhang[1], F Xue[1], X Liu[1], X Zhang[2], T Cheng[1], R Yang[1] and L Zhang[1]

[1]*State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China and* [2]*BGI-Shenzhen, Shenzhen, China*

E-mail: rcyang65@163.com or zlpumc@hotmail.com

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Gene dosage reductions of *Trf1* and/or *Tin2* induce telomere DNA damage and lymphoma formation in aging mice

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Telomeres are essential structures that cap the end of chromosomes, which is required for maintenance of chromosomal stability, cell viability and the capacity of cells to proliferate. A complex of specific telomere-binding proteins (TRF1, TRF2, POT1, TIN2, TPP1 and RAP1), also known as the Shelterin complex, is essential for telomere capping by assisting the formation of tertiary telomeric structures.[1] Gene mutations in components of the Shelterin complex (*hTIN2*, *hPOT1* and *hTPP1*) lead to bone marrow failure and cancer formation in human genetic diseases including dyskeratosis congenita (DC), which is caused by Tin2 mutation in 20% of the cases.[2,3] All known *TIN2* mutations are heterozygous, autosomal-dominant and patients normally show extremely short telomeres. In addition, mutations in the telomere binding protein *POT1* were shown to lead to lymphocytic leukaemia formation.[4] Aside from genetic diseases, a variety of studies reported reduced expression of telomere-binding proteins in human cancers compared with non-cancerous tissue suggesting that downregulation of the expression of telomere-binding proteins may also contribute to carcinogenesis in somatic cells and tissues.[5,6] It was shown that Epstein–Barr virus-encoded LMP1 and Epstein–Barr virus-infection itself induce the downregulation of TRF1, TRF2 and POT1 at the transcriptional and translational level resulting in complex chromosomal aberrations, alternative lengthening of telomeres and the induction of Hodgkin's lymphoma.[7,8]

The causal relation between gene dosage reductions of telomere binding protein and the development of cancer and tissue aging remains elusive. Mouse knockout studies revealed that homozygous deletions of *Tin2* or *Trf1* lead to early embryonic lethality.[9,10] The conditional homozygous deletion of *Trf1* was shown to provoke severe defects in tissue maintenance[11] and in combination with homozygous *p53* deletion led to cancer formation in skin.[12] However, these models did not address the question of whether moderate reductions in the gene dose of telomere-binding proteins contribute to tissue aging and/or carcinogenesis. To address this question we followed aging cohorts of mice carrying heterozygous deletion of *Trf1* and/or *Tin2* in comparison with wild-type mice.[9,10]

Heterozygous $Trf1^{+/-}Tin2^{+/-}$ knockout mice showed a 40–50% reduction in the mRNA expression level of Trf1 and Tin2, but had no effect on the mRNA expression profile of other telomere-binding proteins (Figures 1a and b, Supplementary Figures 1a and d). Protein analysis of whole-spleen extracts revealed an ~50% reduced Tin2 expression in $Tin2^{+/-}$ mice compared with $Tin2^{+/+}$ mice (Figure 1c, Supplementary Figure 1e). Trusty antibodies for detection of endogenous Trf1 protein in tissues are still lacking. To monitor the Trf1 protein amounts in *Trf1* heterozygous mice, a *Trf1* hemagglutinin (HA)-tag knockin mouse line was generated carrying the HA-tag at the N-terminus of the endogenous *Trf1* gene locus. Opposed to the homozygous *Trf1* knockout mouse, homozygous HA-*Trf1* knockin mice ($Trf1^{ki/ki}$) are viable, do not exhibit an overt organismal phenotype and show normal telomere structure indicating that the HA-tag did not interfere with Trf1 function. Trf1 protein from the knockin mice was quantitatively immunoprecipitated with an anti-HA antibody using equally concentrated lysates. Heterozygous *Trf1* deletion led to a reduction in Trf1 protein amounts, whereas the heterozygous deletion of *Tin2* had no significant impact on Trf1 protein levels

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