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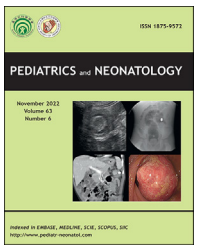

Original Article

Diagnosis and genetic analysis of polycythemia in children and a novel EPAS1 gene mutation

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Background: Unlike in adults, there is no consensus on management and diagnosis of polycythemia in children. This study aims to evaluate the diagnosis and verify the algorithm in children with polycythemia.
Methods: Seventy-nine children with polycythemia were followed-up in our pediatric hematology-oncology clinic between December 15, 2019, and July 15, 2021. After eliminating secondary causes (hypoxia, pulmonary, cardiac diseases), we checked for genetic mutations, including congenital erythrocytosis gene panel (*JAK*, *EPOR*, *EPAS1*, *EGNL1*, *HBB*, *HBA*, *BPGM*, and *VHL*). We also compared parameters for secondary and idiopathic polycythemia groups.
Results: Of the 79 children, thirty-five had secondary polycythemia (hypoxia, pulmonary, cardiac diseases), and one was diagnosed with a novel likely pathogenic mutation c.2089C $>$ G; p.Pro697Ala in exon 13 of *EPAS1* gene. Others (n $=$ 35) had persistent and idiopathic polycythemia. Here, we compared the idiopathic and secondary cases. We found that the ratio of family history of polycythemia (n $=$ 4 (9.5%) vs 0%, respectively) was higher in the second group (p $=$ 0.009). In addition, the mean age (14.7 $\pm$ 3.52 vs 13.4 $\pm$ 4.67 respectively) (p $=$ 0.042) and the ratio of erythroid hyperplasia in bone marrow [n $=$ 3 (8.6%) vs 0% respectively] (p $=$ 0.003) was higher in the idiopathic polycythemia group, compared to secondary polycythemia patients.
Conclusion: Finding the genetic defect in polycythemia is a significant issue. Due to being a rarity in children, the first line JAK mutation analysis should be performed in selected cases. This study is the first description of a Turkish patient with *EPAS1* p.Pro697Ala mutation, thereby expanding our knowledge about the clinical features of the disease. However, new

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investigations are required to confirm its function.
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1. Introduction

Polycythemia (erythrocytosis) is an increase of 2 g/dl in basal hemoglobin (Hb) levels. Another definition is two different hemoglobin and hematocrit levels measured above 99th percentiles of the age and sex. After excluding dehydration and smoking polycythemia, serum erythropoietin (EPO) level is the first parameter to measure.[1] Therefore, it is possible to distinguish EPO secreting tumors (renal, hepatic tumors, pheochromocytoma, cranial hemangioblastoma) and physiological EPO elevations due to pulmonary, cardiac, renal, and hepatic diseases. Undiagnosed patients must be tested for *JAK2* mutations (*JAK2-V617F* and *JAK2* exon 12 mutations).[2] Incidence of polycythemia vera in children is below 0.1%, and it is rarer than congenital erythrocytosis. The difference in bone marrow morphology is seen as a trilineage proliferation in polycythemia vera. In congenital erythrocytosis, erythroid precursors are only increased. Therefore, in selected cases, targeted next-generation sequencing for congenital erythrocytosis should be performed. This panel contains *HBAA, HBB, BPGM, VHL, EGLN1, EPAS1,* and *EPOR* genes.[3] Still however, a group of patients remain with no genetic diagnoses with this comprehensive genetic analysis. Patients with idiopathic erythrocytosis are candidates for whole-genome analysis.[4] Here, in this study, we aimed to report differential diagnoses in children with polycythemia. In addition, we also discussed genetic analysis in idiopathic cases. There are different algorithms for polycythemia in children. Therefore, we aimed to evaluate the effectiveness of these approaches.

2. Material and methods

This study was a retrospective study including 88 patients with polycythemia. These patients were observed at a pediatric hematology-oncology clinic between December 15, 2019, and July 15, 2021. Ethics committee has approved this study (Project number: 2021-333). Inclusion criteria were: having at least two hemoglobin or hematocrit counts above 99th percentile for age and sex and being 1−18 years of age. In addition, hypoxia, erythropoietin secreting tumor, and respiratory and heart diseases were excluded in the first place. Finally, *JAK2-V617F* mutations were investigated for all patients. In addition, we performed *JAK2* Exon 12 mutation analysis and bone marrow analyses in selected patients. Patients with persistent polycythemia had undergone erythrocytosis panel sequencing. The mutations, included in this panel were *HBB, HBA, JAK, EPOR, EGLN1, EPAS1, BPGM,* and *VHL* genes. We compared diagnostic parameters between idiopathic and secondary polycythemia (heart, respiratory diseases, hypoxia) groups. SPSS (Statistical Package for Social Sciences) 21.0 program for Windows was used for data evaluation. We measured continuous variables as mean ± standard deviation and used t-test and chi-square. A p-value of <0.05 was considered significant.

3. Results

Of 79 children, 19 were female, and 60 were male. Four of the males and none of the girls had splenomegaly. There was family history of polycythemia in four participants. Oxygen saturation measurement with a pulse oximeter (n = 50), cranial magnetic resonance imaging (MRI) (n = 28), echocardiography (n = 25) and respiratory function tests (n = 8) were performed. Cranial MRI revealed structural anomalies (n = 4), demyelination (n = 1), venous anomalies (n = 1), cranial cyst (n = 1) and neuroglial tumor (n = 1). Twenty patients had normal echocardiography findings. Pulmonary stenosis (n = 1), coronary arteriovenous fistula (n = 1), left ventricle hypertrophy (n = 1), pulmonary embolism (n = 1) and tricuspid valve failure (n = 1) were reported. Forty-two had secondary polycythemia who had pulmonary, heart diseases, or hypoxia. All patients were analyzed for *JAK2* V617F mutation and erythropoietin gene mutations. Twenty patients had normal *JAK2* exon 12 mutation analysis (n = 20). After excluding these secondary causes, we studied the erythrocytosis gene sequencing panel in 11 of 36 (30.5%) patients. In addition, *CALR* gene mutations were normal in two patients. Genetic studies revealed congenital polycythemia in one patient. The index patient was subjected to targeted exome sequencing (TES) using the Illumina HiSeq platform. TES revealed a heterozygous missense mutation in the *Endothelial PAS domain protein 1 (EPAS1)* gene at position c.2089C > G in exon 13, translating into p. Pro697Ala substitution at the protein level (Fig. 1). His mother also had the same mutation with heterozygosity. The mutation was absent in the father and the brother of the index patient.

In this variant, a C to G substitution occurred at the 2089th base, leading to an amino acid change from proline to alanine at the 697th residue. This variant was not found in several public databases (1000Genomes, ExAC and GnomAD) and also in our internal exome database. The online bioinformatics software, includingPolyPhen-2, SIFT and MutationTaster predict this variant as probably damaging (0.97), damaging (0.01), and disease-causing (0.999), respectively. Our investigations suggested that the heterozygous missense mutation of EPAS1 (c.2089C > G; p.Pro697Ala) may be a potential causal variant in the child with polycythemia.

The remaining (n = 35) were idiopathic. One child had an unknown etiology. We also studied hemoglobin electrophoresis (n = 22) and serum methemoglobin levels (n = 6), all of which were normal. Bone marrow morphology and biopsy revealed erythroid hyperplasia in four (66.6%) patients. None had trilineage hyperplasia consistent with polycythemia

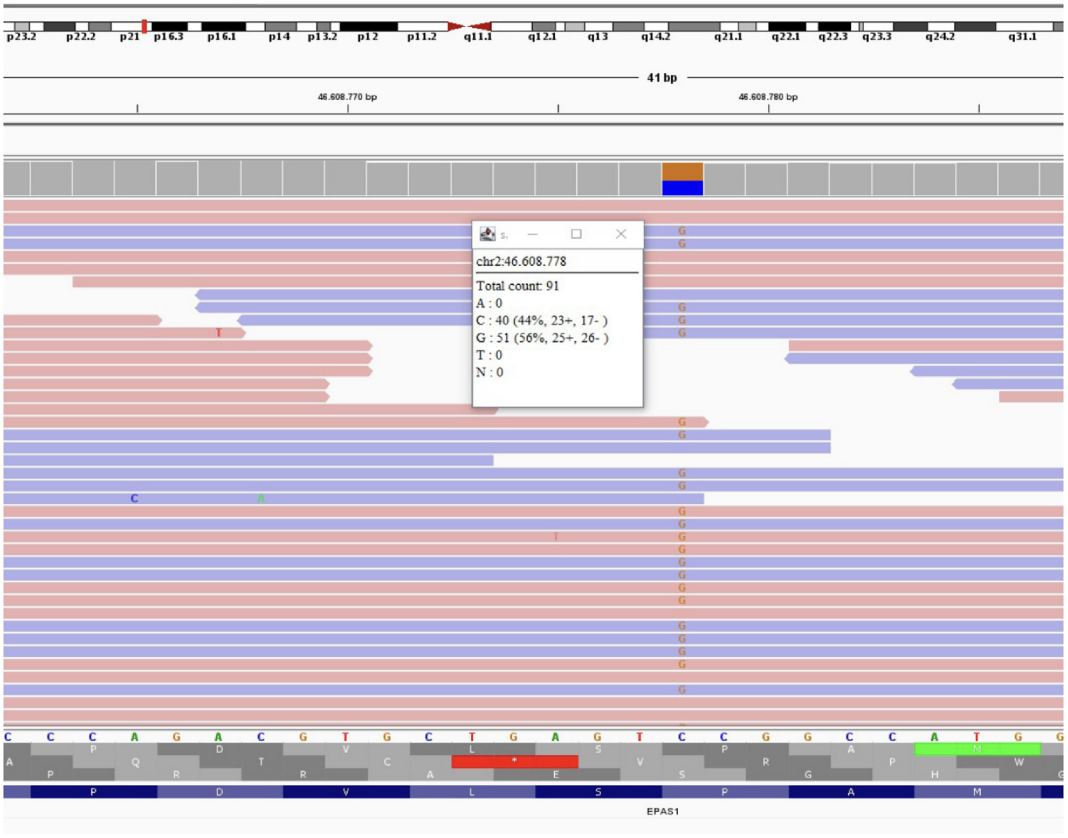

Figure 1 Targeted sequencing detected a novel missense mutation in the EPAS1 gene.

Table 1 Comparing secondary and idiopathic polycythemia groups.

Variables	Polycythemia groups		P-value
	Secondary (n = 42)	Idiopathic (n = 35)	
Age (year)	13,4 ± 4,67	14,7 ± 3,52	**0.042**
Sex (M/F), n	31 (73.8%)/11 (26.2%)	28 (80%)/7 (20%)	0.600
Hemoglobin (g/dl)	146.33 ± 121.92	118.33 ± 102.62	0.591
Hemotocrit (%)	48.42 ± 40.02	50.66 ± 3.36	0.307
Thrombocyte ($10^3/\mu L$)	266,000 ± 57,677	311,735 ± 335,258	0.152
Monocyte ($10^3/\mu L$)	543.57 ± 168.85	563.91 ± 150.41	0.341
Leukocyte ($10^3/\mu L$)	7269.05 ± 2138.43	6754.86 ± 1447.65	0.260
Erythropoietin (Mu/ml)	8.66 ± 4.41	7.93 ± 3.21	0.235
Methemoglobin (%)	0.40 ± 0.03	0.39 ± 0.05	0.169
Splenomegaly (absent/present) (n)	40 (95.2%)/2 (4.8%)	3 3 (%94,3)/2 (%5,7)	0.930
Family polycythemia history (absent/present) (n)	38 (90.5%)/4 (9.5%)	35 (100%)/0 (0%)	**0.009**
Echocardiography (normal/pulmonary stenosis/tricuspid insufficiency) (n)	41 (97.6%)/1 (2.4%)/0 (0%)	34 (97.1%)/0 (0%)/1 (2.9%)	0.710
Bone marrow morphology (normal/erythroid hyperplasia) (n)	42 (100%)/0 (0%)	32 (91.4%)/3 (8.6%)	0.003

vera. No complications of thrombosis and leukemia transformation were observed within two-year term. The cause of pulmonary embolism (n = 1) was post-operative orthopedic immobilization. Compared with the secondary polycythemia cases, patients in the idiopathic group were older (13.4 ± 4.67 vs. 14.7 ± 3.52, respectively) (p = 0.042). In addition, comparing the idiopathic and secondary groups, the ratio of bone marrow erythroid hyperplasia (8.6% vs 0% respectively) was significantly higher in the idiopathic (p = 0.003). However, the percentage of having polycythemia family history (9.5% vs. 0% respectively) was significantly higher in the secondary cases (p = 0.009). Sex, laboratory parameters, splenomegaly, and echocardiographic findings were similar (Table 1). Erythropoietin level was lower than 5 mU/ml in nine of the secondaries and five idiopathic cases.

4. Discussion

Erythrocytosis is divided into primary and secondary erythrocytosis. In primary erythrocytosis, serum erythropoietin is low, and erythroid progenitors are independently proliferating. In addition, congenital erythrocytosis type 1 with EPOR1 mutations and polycythemia vera, exists. Secondary erythrocytosis includes hypoxia, abnormal autonomous erythropoietin secretion, and oxygen sensation defects.[1] There is no consensus on diagnosis of childhood polycythemia, so Lam et al. presented an algorithm for children.[2] *JAK2* mutations are to be studied first. Here, EPO and Ph50 serum levels are determining the algorithm. After exclusion of cardiac and hypoxic conditions, another review disagreed with the priority of *JAK2-V617F* mutations in children.[3,4] Our results support this finding, as we found no *JAK2-V617F* mutations. Filser et al., used Ph50 level in the blood gas first in the erythrocytosis algorithm (n = 270). Other tests were serum erythropoietin, electrolytes, and respiratory function tests. Subsequently, after eliminating secondary causes, *JAK2* mutations and next-generation sequencing tests were performed. *Erythropoietin receptor* (*EPOR*) mutations were only found in four patients. Only one patient with complications received phlebotomies. Only one patient developed thrombotic complications like myocardial infarct and transient ischemic attack.[5] Contrary to the study on adults, mentioned above, we did not detect *EPOR* gene mutations in any patients. The genetic profile is different among different studies of erythrocytosis. Giona et al., have reported a group of eleven sporadic polycythemia (<20 years of age). All patients had normal serum erythropoietin levels. Three patients had *JAK2-V617F* mutations. Their (n = 3) bone marrow examination has included increased endogen erythroid colony growth, erythropoiesis, and myelopoiesis. Among these three patients with polycythemia vera, all received phlebotomies and aspirin; two had been given hydroxyurea. During 113-month follow-up period, no complications had been observed. In two congenital polycythemia cases, the only treatment was phlebotomy. The two patients with congenital polycythemia had *HIF2-alfa* mutations and no complications. They also only had undergone phlebotomy.[6] Mallik et al., have reported eighteen children with idiopathic erythrocytosis. *VHL* homozygotes mutations (n = 11) and high-affinity oxygen hemoglobin variants (n = 3) were present.[7]

Similarly, after elimination of secondary causes and polycythemia vera, we checked gene mutations for congenital erythrocytosis. This targeted gene sequencing panel investigated *HBB, HBA, JAK, EPOR, EGLN1, EPAS1, BPGM*, and *VHL* genes. The benefits of this panel was the elimination of the need for a Ph50 study depending on the EPO level.[8] Only one patient had a novel (c.2089C > G; p.Pro697Ala) *EPAS1* mutation. Moreover*in silico* analysis (Polyphen-2, SIFT, and MutationTaster) indicated that this mutation probably damaged the EPAS1 protein structure. This mutation is a novel variant with uncertain clinical significance (VUS) and predominantly pathogenic evidence. It is linked to the phenotype and probably is pathogenic. Reported VUS depends on phenotype–genotype correlation. Type 3A of VUS is defined as of uncertain clinical significance and predominantly pathogenic evidence.[9] In the literature, it has been reported that sixteen out of 20 variants in the *EPAS1* gene were within exon 12. Others have been reported to be on exon 2, 4, 9, and 16. Among 55 patients with EPAS1 gene variants in the literature, more than 55 *EPAS1* variants are defined within 12 families. All variants are heterozygous, similar to our patients.[10,11] Our investigation suggested thatheterozygous missense mutation of EPAS1 (c.2089C > G; p.Pro697Ala) could be a potential causal variant in the child with polycythemia.

This study compared secondary (hypoxia, cardiac diseases) and idiopathic erythrocytosis. Family history of polycythemia and bone marrow findings were significantly different between the groups. In our study, erythroid hyperplasia was significant in idiopathic erythrocytosis without trilineage proliferation. Laboratory parameters were similar. Here, reaching a precise diagnosis is a considerable problem. Therefore, genetic tests should be employed. Despite extensive diagnostic studies, seventy percent of the patients remain without a genetic diagnosis, and are so-called 'idiopathic' patients. *SH2B3/LNK* exon two mutations or polymorphisms, *LNK* mutations, and *SLC30A10* mutations have been reported. Therefore, whole gene analysis is recommended in idiopathic cases.[11] The limitation of this study is the preliminary genetic results. All idiopathic cases could not be checked by genetic analysis. Therefore, targeted gene sequencing analysis should be performed for the idiopathic and persistent erythrocytosis cases.

What is known

1) We found no polycythemia vera in the population of children with erythrocytosis
2) Forty-three percent of polycythemia remains idiopathic. Therefore, genetic evaluation must progress with the targeted genome analysis.

What is new?

1). We report a novel c.2089C > G (p.Pro697Ala) EPAS1 gene mutation in a polycythemic patient. This mutation is VUS, probably pathogenic. Further studies are essential.
2). The family history of polycythemia is unexpectedly higher in the secondaries than in the idiopathic group.
3). The idiopathic erythrocytosis population is older, and bone marrow erythroid hyperplasia is more frequent than in the second group.

Declaration of competing interest

There are no conflicts of interest between the authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pedneo.2022.06.006.