Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios

Renata Mojzikova,1* Pavla Koralkova,1* Dusan Holub,2 Zuzana Zidova,1 Dagmar Pospisilova,3 Jaroslav Cermak,4 Zuzana Striezencova Laluhova,5 Karel Indrak,6 Martina Sukova,7 Martina Partschova,1 Jana Kucerova,1 Monika Horvathova1 and Vladimir Divoky1,6

1Department of Biology, Faculty of Medicine and Dentistry, Palacky University, 2Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, 3Department of Paediatrics, University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc, 4Institute of Haematology and Blood Transfusion, Prague, Czech Republic, 5Children’s Faculty Hospital with Policlinic, Bratislava, Slovak Republic, 6Department of Haematology-Oncology, University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc, and 7Department of Paediatric Haematology and Oncology, University Hospital Motol, Prague, Czech Republic

Received 25 October 2013; accepted for publication 18 December 2013

Correspondence: Monika Horvathova
Department of Biology, Faculty of Medicine and Dentistry
Palacky University, Hnevotinska 3, 77515 Olomouc, Czech Republic.
E-mail: priwitzer@seznam.cz

*These authors contributed equally to this work.

Summary

Pyruvate kinase (PK) deficiency is an iron-loading anaemia characterized by chronic haemolysis, ineffective erythropoiesis and a requirement for blood transfusion in most cases. We studied 11 patients from 10 unrelated families and found nine different disease-causing PKLR mutations. Two of these mutations - the point mutation c.878A>T (p.Asp293Val) and the frameshift deletion c.1553delG (p.(Arg518Leufs*12)) - have not been previously described in the literature. This frameshift deletion was associated with an unusually severe phenotype involving neonatal hyperferritinaemia that is not typical of PK deficiency. No disease-causing mutations in genes associated with haemochromatosis could be found. Inappropriately low levels of hepcidin with respect to iron loading were detected in all PK-deficient patients with increased ferritin, confirming the predominant effect of accelerated erythropoiesis on hepcidin production. Although the levels of a putative hepcidin suppressor, growth differentiation factor-15, were increased in PK-deficient patients, no negative correlation with hepcidin was found. This result indicates the existence of another as-yet unidentified erythroid regulator of hepcidin synthesis in PK deficiency.

Keywords: red blood cell, pyruvate kinase deficiency, iron overload, hepcidin, ferritin.
neonatal death and non-immune hydrops foetalis have also been reported (Ferreira et al, 2000). Similar to patients with ß-thalassaemia, PK-deficient patients may develop secondary iron overload. Although repeated blood transfusions were considered to be the major cause, iron accumulation also affects non-transfused PK-deficient patients (Zanella et al, 1993). The pathogenesis of iron overload appears to be multifactorial, involving chronic haemolysis, ineffective erythropoiesis, splenectomy and, eventually, coinheritance of hereditary haemochromatosis (Zanella et al, 2001). Importantly, the levels of hepcidin, the negative regulator of iron absorption in the gut and iron recycling from macrophages (Ganz, 2004), are reduced in patients with PK deficiency (Finkenstedt et al, 2008). Based on recent analyses, growth differentiation factor-15 (GDF15) is one of the candidate molecules proposed to suppress hepcidin production and enhance iron loading in the setting of ineffective erythropoiesis (Tanno & Miller, 2010; Tanno et al, 2010). The negative correlation between hepcidin and GDF15 was first reported in patients with ß-thalassaemia (Tanno et al, 2007). Although GDF15 levels are increased in PK deficiency, the magnitude of GDF15 elevation is markedly lower than that in ß-thalassaemia (Tanno et al, 2010). Here, we analysed PKLR mutations and iron status parameters in a cohort of 11 patients with PK deficiency. Our genetic data, together with calculations of the hepclin/ferritin ratio, indicate the involvement of an as-yet unknown erythroid-derived hepcidin suppressor in PK deficiency.

Design and methods

Patients, haematological and biochemical analysis

We investigated 11 patients with congenital nonspherocytic haemolytic anaemia from 10 unrelated Czech and Slovak families. The patient cohort consisted of five adults, three children and three infants. The Ethics Committee of Palacky University Hospital approved the collection and analysis of samples. Informed consent was obtained according to the Declaration of Helsinki. Red blood cell parameters were measured using a Sysmex XE-5000 analyser (Sysmex Corp., Kobe, Japan). Serum parameters of iron metabolism, including iron, ferritin and transferrin saturation as well as bilirubin levels, were determined with standard biochemical methods. PK and hexokinase (HK) activities were measured in erythrocyte lysates that were purified with a QIAquick kit (Qiagen) and sequenced under conditions are available upon request. The PCR products were amplified. PCR primers and conditions are available upon request. The PCR products were purified with a QIAquick kit (Qiagen) and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the ABI Prism 310 Genetic Analyser (Applied Biosystems) with software provided by the manufacturer.

Molecular analysis

Genomic DNA was isolated from peripheral blood drawn in EDTA using a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). The exons of the following were amplified by polymerase chain reaction (PCR): PKLR (accession: NM_000298.5) (Lenzner et al, 1997); genes known to be responsible for hereditary haemochromatosis (Camaschella & Poggiali, 2011), including HFE (accession: NM_000410.3), HFE2 (haemojuevin) (accession: NM_213653.3), HAMP (hepcidin) (accession: NM_021175.2), TFR2 (transferrin receptor 2) (accession: NM_003227.3) and SLC40A1 (ferroportin 1) (accession: NM_014585.3); and a gene encoding the light chain of ferritin (FTL) (accession: NM_000146.3). The iron response element (IRE) regions of the 5'-UTR of SLC40A1 and FTL were also amplified. PCR primers and conditions are available upon request. The PCR products were purified with a QIAquick kit (Qiagen) and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the ABI Prism 310 Genetic Analyser (Applied Biosystems) with software provided by the manufacturer.

Statistical methods

Student’s t-test was used to determine the statistical difference between the patients and controls using Origin 6.1
Patients had increased reticulocyte counts (4.8–558 × 10^9/l) and hyperbilirubinemia (33 to 172 μmol/l). All but one of these patients had increased reticulocyte counts (4.8- to 80.5%). Neonatal jaundice was reported in most of the patients. Seven patients were transfusion-dependent; three were concomitantly treated with chelators and two underwent splenectomy. Two paediatric patients received transfusions during infancy, one adult patient was on intermittent transfusion in his lifetime. In 10 patients, erythrocyte PK activity was below the normal reference values (4.60–6.28 u/g Hb) (Table II). In one case (Patient 7), the activity was false normal (6.58 u/g Hb) due to substantial reticulocytosis (80.5%) and PK deficiency was therefore established based on low PK/HK activity ratio (1.6; normal range 3.6–6.3) (Table II). Reduced PK activity compared with the controls was also detected in the available healthy parents, confirming a congenital defect in the child. All parents tested were later shown to be heterozygous carriers of specific PKLR mutations (Table II).

### Patients' characteristics and PK activity

The clinical findings of nine unrelated patients and two siblings obtained at the time of diagnosis are summarized in Table I. All patients suffered from mild to severe haemolytic anaemia (with Hb levels ranging from 65 to 121 g/l) and hyperbilirubinemia (33 to 172 μmol/l). All but one of these patients had increased reticulocyte counts (4.8- to 80.5%). Neonatal jaundice was reported in most of the patients. Seven patients were transfusion-dependent; three were concomitantly treated with chelators and two underwent splenectomy. Two paediatric patients received transfusions during infancy, one adult patient was on intermittent transfusion in his lifetime. In 10 patients, erythrocyte PK activity was below the normal reference values (4.60–6.28 u/g Hb) (Table II). In one case (Patient 7), the activity was false normal (6.58 u/g Hb) due to substantial reticulocytosis (80.5%) and PK deficiency was therefore established based on low PK/HK activity ratio (1.6; normal range 3.6–6.3) (Table II). Reduced PK activity compared with the controls was also detected in the available healthy parents, confirming a congenital defect in the child. All parents tested were later shown to be heterozygous carriers of specific PKLR mutations. (Table II).

### Mutational analysis of the PKLR gene

Direct sequence analysis of the PKLR gene in the patient cohort revealed nine different mutations in either the homozygous state (four patients) or the compound heterozygous state (seven patients) (Table II). Seven of the mutations are known; five of these (c.1594C>T, c.1529G>A, c.1493G>A, c.1456C>T, and c.994G>A) have previously been identified in Czech and Slovak populations (Lenzner et al., 1997). The c.823G>A substitution and the insertion-deletion (c.347_350delinsAACATTG) were identified in these populations for the first time. The clinical picture and the severity of the disease in our patients bearing the above-mentioned PK mutations were comparable to the phenotype previously reported for patients with identical mutations (Baronciani et al., 1995; Zanella et al., 1997, 2005, 2007). The c.878A>T and c.1553delG mutations are novel and have not been previously described in the literature. The maternally inherited c.878A>T missense mutation was detected in a heterozygous state with the previously reported, paternally inherited c.1529G>A substitution (Fig IA, Table II), which is the most common PKLR mutation in the northern and central European population (Zanella et al., 2005). The c.878A>T mutation involves the alteration of positively charged aspartic acid to hydrophobic valine on the

### Table I. Clinical and haematological data of the patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Neoratal jaundice</th>
<th>ExTx</th>
<th>Spleen</th>
<th>HB, g/l</th>
<th>Ret, %</th>
<th>Bilirubin, μmol/l</th>
<th>Ferritin, μg/l</th>
<th>TFS,</th>
<th>Hepcidin, ng/l</th>
<th>GDF15, 344</th>
<th>EPO,iu/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>9 years</td>
<td>Yes</td>
<td>Yes</td>
<td>Out</td>
<td>79</td>
<td>32</td>
<td>2000§</td>
<td>0.79</td>
<td>n.d.</td>
<td>3052</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>6 months</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>77</td>
<td>7.2</td>
<td>33</td>
<td>4852</td>
<td>0.97</td>
<td>90.7</td>
<td>935</td>
<td>46-2</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>7 years</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>95</td>
<td>10.6</td>
<td>35</td>
<td>16</td>
<td>n.d.</td>
<td>1399</td>
<td>78-1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>10 months</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>75</td>
<td>7.5</td>
<td>59</td>
<td>303</td>
<td>n.d.</td>
<td>15.5</td>
<td>876</td>
<td>123</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>7 years</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>97</td>
<td>8.1</td>
<td>39</td>
<td>205</td>
<td>0.50</td>
<td>24.5</td>
<td>386</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>42 years</td>
<td>No</td>
<td>Yes</td>
<td>In</td>
<td>103</td>
<td>5.9</td>
<td>50</td>
<td>645</td>
<td>0.37</td>
<td>11.4</td>
<td>2581</td>
<td>46-8</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>22 years</td>
<td>Yes</td>
<td>Yes</td>
<td>Out</td>
<td>65</td>
<td>80.5</td>
<td>139</td>
<td>372§</td>
<td>0.88</td>
<td>9.1</td>
<td>514</td>
<td>122</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>31 years</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>95</td>
<td>6.6</td>
<td>34</td>
<td>948</td>
<td>0.54</td>
<td>29.1</td>
<td>805</td>
<td>126</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>3 months</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>67</td>
<td>2.1</td>
<td>172</td>
<td>802</td>
<td>n.d.</td>
<td>2163</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>25 years</td>
<td>Yes</td>
<td>Yes</td>
<td>In</td>
<td>73</td>
<td>25.3</td>
<td>124</td>
<td>29.7</td>
<td>0.20</td>
<td>5.7</td>
<td>1259</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>50 years</td>
<td>No</td>
<td>No</td>
<td>In</td>
<td>121</td>
<td>5.2</td>
<td>49</td>
<td>906</td>
<td>0.34</td>
<td>68.8</td>
<td>854</td>
<td>51-5</td>
</tr>
</tbody>
</table>

Patients 3 and 4 are siblings; ExTx: exchange transfusion; TFS: transferrin saturation; GDF15: growth differentiation factor-15; EPO (serum erythropoietin); n.d.: not determined.

*normal range for ferritin levels, women: <200, men: <300, children: <70 μg/l (Brugnara, 2009). The control range for GDF15 was determined from eight healthy controls (four children and four adults).

†Dependent on blood transfusion during the first year of life due to severe anaemia.

‡Intermittent ExTx.

§Patients on chelation therapy. Two sets of data are shown for Patient 2; the first set represents values at the time of diagnosis, the second corresponds to values after 6 months of chelation therapy.
Table II. Biochemical and molecular data of the patients and their family members.

<table>
<thead>
<tr>
<th>Patient</th>
<th>PK activity, u/g Hb</th>
<th>PK/HK</th>
<th>PKLR mutation</th>
<th>Amino acid alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.64</td>
<td>0.78</td>
<td>c.[347_350delinsAACATTG];</td>
<td>p.[Arg116_Leu117delinsGlnHisCys];</td>
</tr>
<tr>
<td>2</td>
<td>1.63</td>
<td>1.63</td>
<td>c.[1553delG];</td>
<td>p.[Arg518Leufs*12];</td>
</tr>
<tr>
<td>3</td>
<td>1.43</td>
<td>0.51</td>
<td>c.[1529G&gt;A];</td>
<td>p.[Arg510Gln];</td>
</tr>
<tr>
<td>4</td>
<td>1.14</td>
<td>0.46</td>
<td>c.[1529G&gt;A];</td>
<td>p.[Arg510Gln];</td>
</tr>
<tr>
<td>5</td>
<td>1.73</td>
<td>1.75</td>
<td>c.[1493G&gt;A];</td>
<td>p.[Arg498His];</td>
</tr>
<tr>
<td>6</td>
<td>1.16</td>
<td>0.84</td>
<td>c.[1456C&gt;T];</td>
<td>p.[Arg523Trp];</td>
</tr>
<tr>
<td>7</td>
<td>6.58</td>
<td>1.6</td>
<td>c.[1594C&gt;T];</td>
<td>p.[Arg532Trp];</td>
</tr>
<tr>
<td>8</td>
<td>1.56</td>
<td>0.81</td>
<td>c.[878A&gt;T];</td>
<td>p.[Asp293Val];</td>
</tr>
<tr>
<td>9</td>
<td>1.72</td>
<td>2.07</td>
<td>c.[823G&gt;A];</td>
<td>p.[Gly275Arg];</td>
</tr>
<tr>
<td>10</td>
<td>2.32</td>
<td>1.12</td>
<td>c.[1529G&gt;A];</td>
<td>p.[Arg510Gln];</td>
</tr>
<tr>
<td>11</td>
<td>1.54</td>
<td>1.14</td>
<td>c.[994G&gt;A];</td>
<td>p.[Gly332Ser];</td>
</tr>
</tbody>
</table>

PK: pyruvate kinase (normal activity range: 4.60–6.28 u/g Hb); PK/HK: pyruvate kinase and hexokinase activity ratio (normal range: 3.6–3.3). The normal activity range was determined in 10 healthy controls.

![Fig 1](image)

Fig 1. Detection of two novel PKLR mutations by sequencing amplified DNA. (A) In the family of Patient 8, the propositus carried the newly described c.878A>T mutation in exon 7 and the c.1529G>A mutation in exon 11; the mother was heterozygous for the c.878A>T mutation, and the father was heterozygous for the c.1529G>A mutation. (B) In the family of Patient 2, the propositus was homozygous for the second newly described mutation in the PKLR gene, a single nucleotide deletion (c.1553delG) in exon 11 leading to a frameshift (p.[Arg518Leufs*12]) and a premature stop codon. One allele was inherited from his heterozygous mother; his father was unavailable for the analyses.
surface of the A domain of PK. This alteration might increase the hydrophobicity of PK, which is thermodynamically less favourable for protein folding. PK activity was markedly reduced to 1.56 u/g Hb in an affected patient (Patient 8) who presented with a mild phenotype (Hb 95 g/l, reticulocytes 6.6%, and bilirubin 34 µmol/l; Table I).

The novel PKLR frameshift deletion associated with atypical severe phenotype involving neonatal hyperferritinaemia

Patient 2 carried the second previously unidentified frameshift deletion, c.1553delG (p.(Arg518Leufs*12), short p.Arg518fs), in a homozygous state; the mother is a heterozygous carrier of this deletion (Fig 1B, Table II). Because the father is unknown and thus unavailable for analyses, a possible hemizygous genomic deletion (loss of heterozygosity) was excluded by quantitative PCR-based copy number analysis (Supplementary methods; data not shown). The mutation causes premature termination of translation at codon 529, with predicted formation of a truncated protein lacking 46 C-terminal amino acids including the critical activator binding site Arg532 (Valentini et al, 2002). The activity of PK was reduced in both the patient (1.63 u/g Hb) and his mother (2.57 u/g Hb). The patient’s clinical picture was very severe from birth, with a need for repeated blood transfusions. The patient exhibited profound anaemia (Hb, 77 g/l), with signs of hypoxia, neonatal jaundice (bilirubin, 90 µmol/l), thrombocytopenia, prominent hepatosplenomegaly and dramatic hyperferritinaemia (in the range of 3500–5000 µg/l), and transferrin saturation reaching almost 100%. Elevated levels of liver enzymes (not shown) suggested possible liver damage, which led us to perform liver biopsy. Massive iron deposits in both hepatocytes and Kupffer cells corresponding to grade IV haemosiderosis were detected. Taken together, these findings suggested the presence of another co-inherited defect in the child aside from PK deficiency. Sideroblastic anaemia was ruled out by the absence of sideroblasts in the bone marrow. The nucleotide sequence of the IRE region of the FTL gene, which encodes L-ferritin, was not mutated; thus, a possible association with hereditary hyperferritinaemia cataract syndrome (HHCS) was excluded (Brooks et al, 2002). Sequence analysis of all known genes that cause primary haemochromatosis (HFE, HFE2, HAMP, SLC40A1 and TFR2) (Camaschella & Poggiiali, 2011) did not reveal any causative mutations. Only five different previously described SNP variations were identified in TFR2 (c.2213G>A (p.Arg73His) - rs41299542), SLC40A1 (c.44-24G>C - rs1439816, c.-330CGG[8] - rs16836041, c.663T>C (p.Val221 = ) - rs2304704) and FTL (c.1637>C (p.Leu55 = )) (Table SI). Of these polymorphisms, only rs1439816:C>C was previously shown to be associated with the clinical aggressiveness (liver damage) of hereditary haemochromatosis 1 caused by the p.Cys282Tyr HFE mutation; however, this SNP had no effect on ferritin levels in the control group, and it was therefore concluded that the negative effect of the rs1439816:C allele was restricted to pathological conditions (Altès et al, 2009). Because nine of our remaining PK-deficient patients were also homozygous for the rs1439816:C allele (Table SI), this polymorphism probably does not contribute to the severe phenotype of this patient and cannot explain his iron overload. Recently, at an age of two and a half years, the patient’s ferritin levels dramatically decreased (to 145 µg/l), which may be a result of combined chelation therapy lasting 6 months and a prolonged interval between transfusions. Based on these new findings, we conclude that the severe neonatal phenotype of this patient was caused by the PKLR mutation itself.

Iron status in PK-deficient patients

One of the main health complications in PK deficiency is secondary iron overload with multifactorial pathogenesis (Zanella et al, 1993, 2001). In our cohort, nine patients had elevated serum ferritin (median, 724 µg/l), including the patient with no transfusion history (Patient 11). Of nine available cases, five showed a concomitant increase in transferrin saturation (from 0.50 to 0.97) (Table I).

Firstly, we assessed a possible effect of splenectomy on iron loading. One of our two splenectomized patients (Patient 7) showed a progressive increase in ferritin levels after spleen removal. Nevertheless, sustained haemolysis was observed in this patient after spleen surgery, which probably contributed to a further increase in her ferritin levels. On the other hand, no significant difference in ferritin was recorded for the second splenectomized patient (Patient 1); splenectomy improved this patient’s anaemia, nevertheless his pre-splenectomy ferritin levels were already dramatically elevated (2213 µg/l). This patient is a child; thus, the contribution of spleen removal to iron loading may become more apparent over time.

We next searched for the presence of known HFE mutations (p.His63Asp, p.Cys282Tyr), which have been proposed as a condition that may predispose a patient to increased iron absorption (Arruda et al, 2000; Zanella et al, 2001). Abnormal HFE genotypes were identified in four of our patients (Table SI). One of these patients (Patient 3), who was a p.[His63Asp];[Cys282Tyr] HFE compound heterozygote, was the only paediatric patient from the group with normal ferritin levels.

Hepcidin levels in PK deficiency

To address the role of hepcidin in the pathogenesis of iron overload in our patient cohort, we established a proteomics-based method for hepcidin measurement. As expected, the levels of hepcidin were reduced in PK-deficient patients (median, 15.8 µg/l) (Table I) compared with healthy age-matched controls (median, 27.6 µg/l) (Table I), reflecting the impact of ineffective erythropoiesis in PK deficiency. The difference, however, was not statistically significant. We
therefore calculated the hepcidin/ferritin ratio which represents a more accurate estimation of proper hepcidin production with respect to iron loading, and found it to be very low in our PK-deficient patients (median, 0·06) compared with a healthy age-matched group of controls (median, 0·35) (Fig 2). Even the patient with the highest hepcidin level (Patient 2) showed a dramatically reduced ratio, which was detected in repeated measurements (ranging from 0·01 to 0·04) and eventually increased to 0·11 as a response to 6 months of chelation therapy and improved anaemia (Hb, 98 g/l) (Table I and Fig 2). The direct effect of chelation therapy on hepcidin levels and hepcidin/ferritin ratio is difficult to address in our cohort, given the complexity of positive and negative signals regulating hepcidin, the small number of patients receiving chelation therapy, and the lack of hepcidin pre-chelation values for remaining patients.

**Candidate erythroid signals regulating hepcidin**

To clarify the involvement of erythropoiesis in iron metabolism in PK deficiency, serum levels of GDF15 and EPO, two markers of erythropoietic activity known to be associated with hepcidin production, were evaluated. The levels of GDF15, a putative negative regulator of hepcidin production in the setting of ineffective erythropoiesis (Tanno & Miller, 2010), were determined using a commercial enzyme-linked immunosorbent assay. PK-deficient patients showed elevated GDF15 compared with healthy age-matched controls (median, 905·5 μg/l and 223 μg/l, respectively) (Table 1), confirming previously reported results (Zanella et al, 2005). Importantly, no correlation between GDF15 and hepcidin or GDF15 and hepcidin/ferritin ratio was observed. In addition, all available patients from our cohort showed increased levels of EPO (median, 100·1 iu/l) (Table 1), probably reflecting tissue hypoxia. Also, EPO levels did not correlate with hepcidin or hepcidin/ferritin ratio.

Lastly, as inflammatory processes are known to independently influence hepcidin levels (Ganz, 2004), it is important to note that none of our patients had signs of inflammation at the time of the analyses. In concordance, a substantial increase in hepcidin (from 90·7 μg/l to 386 μg/l) was observed in one of our patients (Patient 2) during bacterial infection.

**Discussion**

In this report we characterized PKLR mutations and systemic iron metabolism in eleven patients with PK deficiency from ten unrelated families. Nine different disease-causing PKLR mutations were identified; two of these mutations - the point mutation c.A878T (p.D293V) and the frameshift deletion c.1553delG (p.R518fs) - were novel. The patient affected by the frameshift deletion presented with an unusually severe phenotype involving neonatal hyperferritinaemia, which eventually ameliorated by two and a half years of age, probably in response to combined chelation therapy and a prolonged interval between transfusions.

PK-deficient patients usually develop secondary iron overload over time as a consequence of chronic haemolysis, ineffective erythropoiesis and transfusion therapy; splenectomy and inheritance of HFE mutations have been proposed as additional risk factors (Zanella et al, 2001). Consistently, all our transfusion-dependent patients presented iron overload; one patient has developed hyperferritinaemia independently of blood transfusions. Similar findings, iron overload independent of blood transfusions in PK deficiency, were previously reported by Zanella et al (1993). Although splenectomy reduces the symptoms of haemolysis, it is also known to be a risk factor for iron loading. Due to a low number of splenectomized patients in our cohort, however, the involvement of spleen surgery cannot be adequately addressed. The contribution of co-inherited HFE mutations to excessive iron accumulation is also difficult to assess, especially because three of the HFE mutant patients were children and HFE-related haemochromatosis is an adult-onset disease (Pietrangelo, 2010). Moreover, one paediatric patient with abnormal HFE genotype did not have any signs of iron overload. Based on these observations we can conclude that the HFE genotype does not play a major role in determining iron loading in PK-deficient paediatric patients; however, it may be important to follow up these patients due to a potential higher risk of iron overload in adulthood (Zanella et al, 2001).
Analysis of factors regulating iron homeostasis revealed that hepcidin levels were relatively low for the degree of iron loading in all PK-deficient patients with increased ferritin. Similar results were published for β-thalassaemia intermedia (Kearney et al., 2007; Origa et al., 2007), which clearly confirm the suppression of hepcidin by an erythroid signal that overrides iron loading-induced signalling. Although the levels of the putative hepcidin suppressor, GDF15, were increased in our patient cohort, the levels were considerably lower than those reported for β-thalassaemia (Tanno et al., 2010). No correlation between GDF15 and hepcidin or hepcidin/ferritin ratio indicates that GDF15 can only be used as a marker of accelerated ineffective erythropoiesis in PK deficiency (Tanno & Miller, 2010; Tanno et al., 2010).

Besides elevated GDF15 in the patients’ serum, we also found increased levels of EPO (Ashby et al., 2010; Ganz, 2011) that again did not correlate with hepcidin. This evidence further suggests that EPO by itself is an indirect suppressor of hepcidin as mice with disrupted erythropoiesis are not able to attenuate hepcidin synthesis in response to EPO administration (Pak et al., 2006; Vokurka et al., 2006). In addition to GDF15 and EPO, a suppressive effect of twisted gastrulation protein homolog 1 (TWSG1) (Tanno et al., 2009) and hypoxia (Yoon et al., 2006; Ganz, 2011) should be considered with respect to the regulation of hepcidin production. Nevertheless, the role of TWSG1 has not been clearly confirmed so far (Tanno et al., 2010). Although hypoxia inducible factor (HIF) signalling was reported to directly and also indirectly (through EPO) downregulate hepcidin (Gordeuk et al., 2011), the exact signalling remains elusive. Altogether, our findings indicate that the main erythroid-derived regulator of hepcidin synthesis in PK deficiency remains to be identified.

In conclusion, we have shown that the hepcidin/ferritin ratio is consistently low in PK deficiency, which reflects relatively low hepcidin levels, even in PK deficiency associated with hyperferritinemia.

Acknowledgements
This work was supported by grant NT/11208 (Ministry of Health, Czech Republic) and partially supported by grant LF_2013_010 (Internal Grant Agency of Palacky University). DH was partially supported by CZ.1.05/2.1.00/01.0030 (Ministry of Education, Youth and Sports, Czech Republic), MS was supported by NT/13587 (Ministry of Health, Czech Republic) and MH and VD were partially supported by P305/11/1745 (Czech Science Foundation). We thank Prof. Thomas Ganz (UCLA) for the validation of our HPLC-MS-based hepcidin measurements, Prof. Tomas Adam (Palacky University) for some biochemical measurements, and Lenka Radova (Masaryk University in Brno, Czech Republic) for statistical analyses.

Author contributions
RM and PK designed the study, performed enzyme assays and some molecular analyses, collected and analysed data and contributed to manuscript writing. DH established the hepcidin assay. ZZ determined GDF15 levels. DP, JC, ZSL, KI and MS treated the patients, collected patient material and provided clinical information. MP and JK participated in molecular analyses. MH contributed to the design of the study, interpretation of the results and wrote the manuscript. VD participated in the design of the study, interpretation of the results and revision/editing of the manuscript.

Conflict of interest
The authors declare no competing financial interests.

Supporting Information
Additional Supporting Information may be found in the online version of this article:
Methods S1. qPCR-based copy number analysis.
Table S1. HFE, TFR2 and SLC40A1 SNPs.

References