Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency

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Supplemental Material and Methods

Patients

The DBA cohort consists of twenty-five patients (eleven males and fourteen females) from the Czech National DBA Registry at the age of 1.0-42.9 years (median 23.7 years) with different clinical severity. Most of the patients have been previously described;[1] the group also includes five newly diagnosed patients. The causative RP mutation (RPS7, RPS17, RPS19, RPS26, RPL5 and RPL 11, respectively) was found in twenty patients; five patients are negative for all known RP mutations. Detailed characteristics of the patients are shown in Supplementary Table 1.

The study and the informed consent as per the Declaration of Helsinki were approved by the Ethics Committee of Palacky University Hospital, Olomouc, Czech Republic. A cohort of 12 children examined prior to planned minor surgery (inguinal or umbilical hernia, plastic surgery) and 5 young healthy adults served as controls for hepcidin levels interpretation; the normal range for GDF15 was determined in a group of four children and four adults.[2]

Hematological and biochemical analysis

Blood was taken during routinely performed venous puncture; for transfusion-dependent patients pre-transfusion samples were collected. Blood counts were examined on Sysmex XE-500 analyzer (Sysmex). Biochemical serum parameters of iron metabolism and inflammation: serum iron (Fe), total iron binding capacity (TIBC), transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and CRP levels were determined with standard methods. The patients' serum erythropoietin (EPO) concentrations were measured by a solid-phase chemiluminescent immunochemical reaction.[1] Bone marrow samples were used for cytological and histopathological examination and immunohistochemistry. Liver biopsies were subjected to histopathological examination, determination of liver iron concentration (LIC, mg/g dry weight) and Perl's Prussian blue staining for non-heme iron.

Hepcidin analysis

The serum hepcidin levels were measured by reverse-phase liquid chromatography using the UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to the QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) as we previously described.[2]

GDF15 measurements

The serum levels of growth differentiation factor 15 (GDF15) were quantified according to the manufacturer's instructions using the Human GDF-15 Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA).[2]

Immunohistochemistry

Bone marrow biopsy samples were fixed in neutral-buffered formalin for 24 hours and embedded into paraffin. Immunohistochemical staining was performed as previously described.[3] Glycophorin A staining (H-60, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was followed by analysis of apoptosis using the alkaline phosphatase (AP) In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The slides were analyzed with an Olympus BX 51 light microscope (Olympus, Hamburg, Germany).

HFE mutational analysis

The sequence analysis of the two most common HFE variants (p.C282Y and p.H63D) in exons 4 and 2 (accession: NM_000410.3) was performed as previously described.[2]

Statistical methods

All statistical analyses were done using the Statistica 10 software (StatSoft, Inc.). The Mann-Whitney test was used in comparisons between patients and healthy donors. The Spearman coefficient was used in correlation analyses. The significance level was set at $p < 0.05$ in all analyses.

References

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Supplementary Table S1

Clinical and laboratory parameters

Patient Number	Mutated RP	Sex	Age (years)	Age at Dg.of Anemia	SGA (BW (g))	Anomalies	Short Stature	Current Treatment	Leucine	eADA (nmol hour^{-1} mg Hb^{-1})
CZUH10	RPS19	F	**23.7**	New.	Yes (2500)	CD	No	T, DRX	Yes	90
CZUH14	RPS19	F	**27.9**	2 m	Yes (2450)	No	Yes	T, DRX	Yes	254
CZUH01	RPS26	M	**20.2**	2 m	No (3010)	No	Yes	T, DRX	Yes	96
CZUH03	RPS26	M	**15.5**	New.	Yes (2300)	Vesicoureteral reflux	No	T, DRX	Yes	68
CZUH41	RPL5	M	**9.6**	New.	Yes (1900)	Thenar hypoplasia, CD, HAP, PDA	No	T, DRX	Yes	*ND*
CZUH18	No	F	**17.3**	New.	No (2900)	No	Yes	T, DRX	No	17
CZUH43	RPS19	F	**5.5**	New.	No (3320)	ASD, CD	No	T, DRX	Yes	74
CZUH44	RPS26	F	**5.0**	New.	No (2900)	Ribs malformation, ASD	No	T, DRX	No	*ND*
CZUH46	RPS26	F	**3.0**	New.	No (2950)	Microcephaly	No	T, DRX	No	*ND*
CZUH45	RPS19	M	**4.7**	New.	No (3580)	No	No	T, DRX	Yes	*ND*
CZUH49	No	F	**2.7**	2 m	No	CD, kidney dystopia	No	T	No	*ND*
CZUH50	RPS19	M	**1.0**	4 w	No (2820)	Atypical thumb position	No	T	No	No
CZUH11	RPS19	F	**30.9**	6 w	No (2650)	No	No	R	No	204
CZUH15	RPS19	M	**21.7**	New.	Yes (1650, 35 w)*	CD, astenism	No	R	No	145
CZUH04	RPL5	M	**26.7**	3 m	Yes (2300)	Thenar hypoplasia, CD, HAP	Yes	R	No	487
CZUH25	No	F	**26.9**	6 m	No (3150)	Dermal syndactylia, low hair border, CD	Yes	R	No	183
CZUH51	RPS19	M	**34.0**	6 w	No (3300)	No	Yes	R	No	*ND*
CZUH20	No	F	**35.8**	New.	No (2550)	Kidney aplasia	Yes	R	No	191
CZUH33	No	F	**13.3**	New.	No (3150)	Vesicorenal reflux, CD	No	R	No$^{\#}$	78
CZUH19	No	F	**42.9**	3 m	No (3800)	No	Yes	S-LD	No	387
CZUH07	RPS7	F	**20.8**	5 m	No (3080)	No	No	S	No	429
CZUH12	No	M	**27.0**	10 m	No (3400)	ASD	No	S-LD	No	745
CZUH21	RPS17	M	**38.2**	1 m	No (2600)	Thenar hypoplasia	No	S	Yes	212
CZUH37	RPL11	F	**25.8**	7 m	Yes (2010)	Thenar hypoplasia, triphalangeal thumb, HAP	No	S, T‡, DRX	No	303
CZUH24	RPL5	M	**25.9**	1 m	Yes (2450)	Thenar hypoplasia, CD, HAP	No†	S, T‡	Yes	78

Patients CZUH45, 46, 49, 50 and 51 are newly diagnosed. RPS, small subunit ribosomal protein; RPL, large subunit ribosomal protein; New., newborn age; SGA, small for gestational age (SGA babies are those whose birth weight lie below or are equal to –2 standard deviations for that gestational age. They have usually been the subject of intrauterine growth restriction (IUGR)); BW, birth weight; *, born before week 38 of gestation; CD, craniofacial dysmorphism; HAP, high arched palate; PDA, patent ductus arteriosus; ASD, atrial septal defect; †, after growth hormone treatment; T, transfusions (10-17 transfusions per patient per year); DRX, deferasirox; R, remission; S, steroids (the maintenance dose, 0.5 mg/kg on alternate days); S-LD, low doses of steroids (i.e. less than 0.3 mg/kg twice a week); ‡, occasional transfusions (CZUH37: 2-8 transfusions per year, CZUH24: 6-7 transfusions per year); #, published patient in remission after leucine treatment;[4] eADA, erythrocyte adenosine deaminase, normal values: 24 – 96 nmol hour^{-1} mg Hb^{-1}; *ND*, not determined.

Supplementary Table S2

HFE mutational screening

Patient Number	C282Y	H63D
CZUH10	**wt/wt**	**wt/wt**
CZUH14	**wt/wt**	**wt/wt**
CZUH01	**wt/wt**	**wt/wt**
CZUH03	**wt/wt**	**wt/H63D**
CZUH41	**wt/wt**	**wt/wt**
CZUH18	**wt/wt**	**wt/wt**
CZUH43	**wt/wt**	**wt/wt**
CZUH44	**wt/wt**	**wt/H63D**
CZUH46	**wt/wt**	**wt/wt**
CZUH45	**wt/wt**	*NA*
CZUH49	**wt/wt**	**wt/wt**
CZUH50	**wt/wt**	**wt/wt**
CZUH11	**wt/wt**	**wt/wt**
CZUH15	*NA*	*NA*
CZUH04	**wt/wt**	**wt/wt**
CZUH25	**wt/wt**	**wt/wt**
CZUH51	**wt/wt**	**wt/wt**
CZUH20	**wt/wt**	**wt/wt**
CZUH33	**wt/wt**	**wt/wt**
CZUH19	**wt/wt**	**wt/H63D**
CZUH07	**wt/wt**	**wt/wt**
CZUH12	**wt/wt**	**wt/wt**
CZUH21	**wt/wt**	**wt/wt**
CZUH37	**C282Y/C282Y**	*NA*
CZUH24	**wt/wt**	**wt/wt**

NA, not analyzed

Supplementary Figure legend

Supplementary Figure S1. Apoptosis of bone marrow erythroblasts. TUNEL assay shows significant elevation in the number of erythroid Glycophorin A^+ cells (brown color) undergoing apoptosis ($TUNEL^+$, dark purple color) in the bone marrow of two DBA patients compared to healthy control. The arrows indicate Glycophorin $A^+/TUNEL^+$ cells. Immunohistochemical slides were analyzed with an Olympus BX 51 light microscope (Olympus), original magnification 400×. Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Images were cropped, assembled and labeled using Adobe Photoshop software (Adobe Systems).

Supplementary Figure S1