Mutating heme oxygenase-1 into a peroxidase causes a defect in bilirubin synthesis associated with microcytic anemia and severe hyperinflammation

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disorder characterized by fever, hepatosplenomegaly, cytopenia, hypertriglyceridemia, hypercytokinemia, and hemophagocytosis. The genetically determined forms of HLH, also referred to as familial hemophagocytic lymphohistiocytosis, commonly manifest in infants or in young children and are characterized by genetic abnormalities of natural killer (NK)-cell function. HLH may also develop secondary to inborn errors of metabolism.[1,2] Heme oxygenases are the first and rate-limiting enzymes of heme degradation. Heme oxygenase-1 (HO-1) is ubiquitously expressed at low levels, but is highly inducible in response to stress.[3] The cytoprotective, antioxidant and anti-inflammatory roles of HO-1 are mainly attributed to the elimination of free heme, thus limiting the generation of reactive oxygen species (ROS), but also to the generation of the protective products of heme degradation carbon monoxide, biliverdin, and bilirubin.[4]

We describe a patient carrying a G139V mutation of the active center of HO-1 resulting in loss of normal HO-1 function and gain in pathological peroxidase activity. The patient showed increased urinary excretion of *in vivo* markers for lipid peroxidation, oxidative protein and

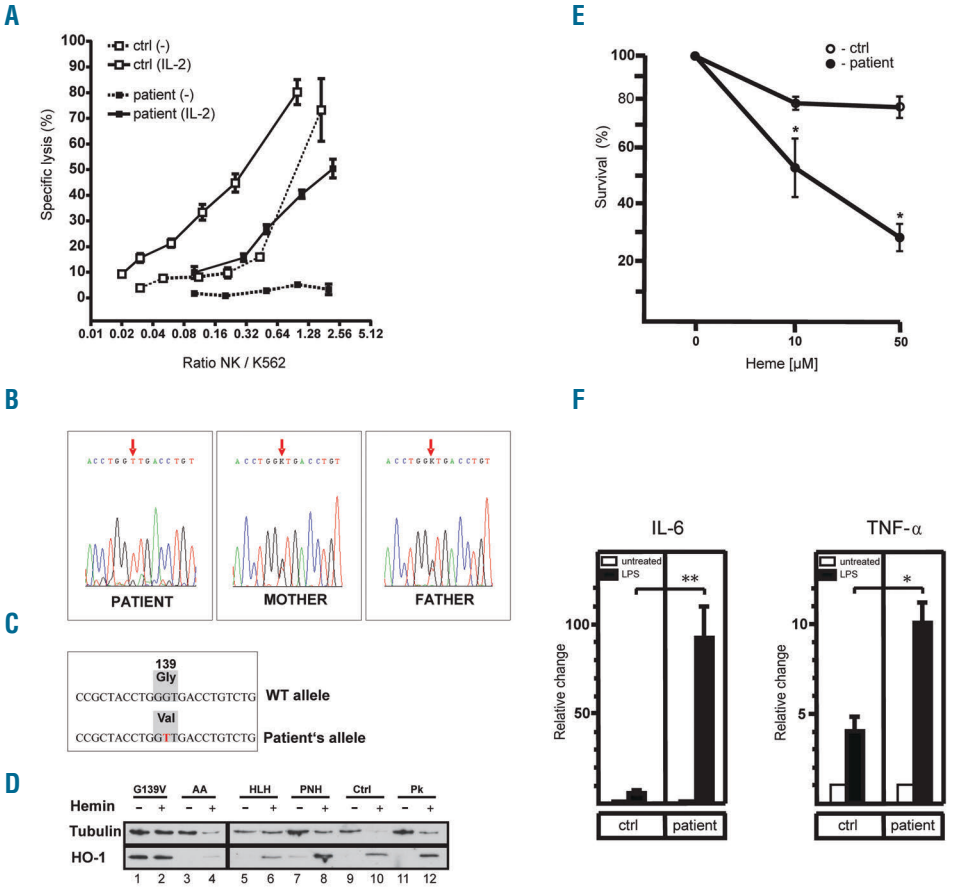

Figure 1. Genetic and functional characterisation of HO-1 G139V. (A) Impaired cytotoxic activity of the patient's NK cells. Whole blood samples of healthy individuals and the patient were treated with or without 100 U / ml IL-2 overnight at 37 °C. NK-cell activity was then determined by a 4 h ^{51}Cr release assay against the NK-cell sensitive target cell K562. All experiments were performed in triplicate. Data are presented as mean ± SD. Ctrl, control; NK, natural killer cell. (B) DNA sequence of nucleotides 405 to 427 of the HO-1 coding sequence including the GGT to GTT mutation of codon 139, which was found homozygously in the patient and heterozygously in his parents. (C) The mutation identified in the patient causes the replacement of a glycine by a valine at position 139 in the HO-1 catalytic center. (D) HO-1 and tubulin immunoblot of protein isolated from PBMC of the G139V mutated patient, a normal control (Ctrl) and control patients with aplastic anemia (AA), genetic HLH with underlying immunodeficiency (HLH), paroxysmal nocturnal hemoglobinuria (PNH), and pyruvate kinase deficiency (Pk). The PBMC were cultured for 4 hours post-isolation, in the absence (lanes 1, 3, 5, 7, 9, 11) and in the presence (lanes 2, 4, 6, 8, 10, 12) of 100μM hemin. (E) PBMC of the patient (closed circles) and healthy controls (open circles) were isolated and cultured with the indicated concentrations of heme (10 μM and 50 μM). Dead or apoptotic cells were identified by annexin V staining. Percent survival was determined and compared to cells cultured in medium without heme. Each point represents the mean ± SD of 3 independent experiments. Statistics, Student's *t*-test for unpaired values: significant differences patient *vs.* control, * =*P*<0.05. (F) PBMC of the patient and healthy controls were isolated and cultured as described in the Methods section. After overnight culture, medium was replaced with medium containing either LPS (1 μg/ml; solid bars) or no LPS (open bars) for another 24 h. Concentrations of IL-6 and TNF-α were measured in the supernatant using an ELISA and were normalized to cell number. Values are means ± SEM of duplicates from three independent experiments; a value of 1 was assigned to the cytokine concentration in untreated cells and the relative change after LPS treatment is expressed as -fold induction. Statistics, Student's *t*-test for unpaired values: significant differences LPS *vs.* control, * =*P*< 0.05; ** = *P*<0.001. ctrl, control; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.

Table 1. Clinical and laboratory features of the patient with HO-1 G139V.

Age/months Phases of treatment	0 to 2 antibiotic treatment for suspected neonatal infection	3 to 7 watch and wait	8 to 19 iron substitution, red cell transfusions	20 to 39 Immunochemo-therapy with dexamethasone, etoposide and cyclosporine A, red cell transfusions	40 to 71 Low dose cylcyclosporine A, phlebotomy	72 to 120 phlebotomy
Persistent fever	absent	absent	present	absent	absent	absent
Hepatosplenomegaly	absent	splenomegaly only	present	present	present	absent
Hemophagocytosis	n.d.	n.d.	present	absent	absent	absent
Hb (g/dl)*	10.4-18.0	8.8-10.9	6.9-12.8	6.5-12.7	5.7-11.6	11.3-14.1
MCV (fl)	79-91	50-72	46-81	52-85	48-67	65-73.5
Platelet count (/nl)	90-233	120-362	70-572	100-478	180-605	319-576
ANC (/nl)	6.5-8.0	4.2-7.6	1.8-10.1	1.5-19.9	4.8-13.6	5.2-12.5
Serum bilirubin (mg/dl)	0.2-1.6	0-0.4	0-0.2	0-0.6	0-1.5	0.2
LDH (U/l)	1,674-1,949	852-1,848	1,457-10,148	891-15,713	812-4,167	1,212-1,632
Free Hb (mg/dl)	n.d.	n.d.	n.d.	47-48	27-260	12-92
Reticulocyte count (‰)	21-29	17-55	1-45	2-130	0-51	8-10
Normoblasts (per100 WBC)	4-95	0-4	0-54	0-143	0-30	0-1
Haptoglobin (g/l)	n.d.	0.1	0.6-1.6	0.8	0.1-1.41	0.1-0.37
Triglycerides (mg/dl)	n.d.	n.d.	308-1,111	252-386	96-323	111-143
Fibrinogen (g/l)	n.d.	0.8-1.4	2.1-5.9	1.7-4.1	3.1-3.9	2.4-3.5
Serum iron (µmol/l)	n.d.	n.d.	<1-7	1.1-4.2	3.2-4.4	1.5-11.2
Transferrin saturation (%)	n.d.	n.d.	7.7-8.4	5-21.3	5	n.d.
Transferrin (g/l)			2.6-2.8	2.0-2.88	3.23	n.d
vWF (%)	n.d.	n.d	n.d.	192-285	110-214	n.d.
CRP (mg/l)	3.2-31	2-32.9	2-240.3	2-240.3	3.2-132.7	5.8-20.9
Ferritin (µg/l)	660	48-395	71-11,044	235-4,855	65-923	135-227
D-dimer (mg/l)	1.4	n.d.	n.d.	0.64	0.17-3.4	
IL-1 (pg/ml)	n.d.	n.d.	8.6-13	14.2-70.2	4-8.5	6.4
IL-6 (pg/ml)	n.d.	n.d.	0.6-88	10.4-410	0.2-152.5	12.8
TNF-α(pg/ml)	n.d.	n.d.	119.4-374	61.8-354	60.8-100.8	100.8
sCD25 (U/ml)	n.d.	n.d.	766-4,192	824-852	668-1,348	668

**includes values before and after transfusion; Hb: hemoglobin; MCV: mean corpuscular volume; ANC: absolute neutrophil count; LDH: lactate dehydrogenase; CRP: C-reactive protein; vWF: von Willebrand Factor; IL: interleukin; TNF: tumor necrosis factor; n.d.: not determined. Patient's sCD25 was only elevated once. Age dependent reference values for: Hb: 31-182 days 9.5 – 13.5 g/dl, 183-365 days 11 - 16 g/dl; 1-6 years 11 – 14.5 g/dl; 7-12 years 11.5 - 15 g/dl; MCV: 31-90 days 77 - 115 fl; 91-182 days 74 - 108 fl; 183-365 days 74 - 102 fl; 1-2 years 73 - 101 fl; 3-12 years 69 - 95 fl; Platelet count: > 150/nl; Bilirubin: 31-365 days 0.1 – 1.2 mg/dl; > 1 year 0.1 – 1.0 mg/dl; LDH: 31-365 days <416 U/l; 1-5 years <364 U/l; 6-14 years <312 U/l; Free Hb: < 20 mg/dl; Reticulocyte count: 31-60 days 4-48 ‰; 61-90 days 3-36 ‰; 91-365 days 2-28 ‰; 1-99 years 5 - 15 ‰; Normoblasts: 0-30 days <50 /100 WBC; 31-365 days <1 /100 WBC; 1-99 years <1 /100 WBC; Haptoglobin: 91-365 days < 1.35 g/l; 1-16 years 0.12 – 1.72 g/l; 17-99 years 0.3 – 2.0 g/l; Triglycerides: 31-365 days 40 - 230 mg/dl; 1-19 years 25 - 180 mg/dl; Fibrinogen: 1.7 – 4.5 g/dl; Serum iron: 0-1 year 7 - 29 mol/l; 2-99 years 14 - 32 mol/l; Transferrin saturation: 16 – 45%; Transferrin: 91-365 days 2.35 – 4.03 g/l; 1-4 years 2.35 – 4.03 g/l; 5-99 years 2.0 – 3.6 g/l; vWF: 70 – 120%; CRP: < 5.0 mg/l; Ferritin: 31-60 days 85 - 430 g/l; 61-120 days 35 - 230 g/l; 121-365 days 7 - 140 g/l; 1-14 years 7 - 140 g/l; D-dimer: < 0.15 mg/l; IL-1: < 5 pg/ml; IL-6: < 6 pg/ml; TNF-α: < 3pg/ml: sCD25: <1,000 U/ml.*

DNA damage, oxidative glycation and oxidative bilirubin metabolites. Functional studies of peripheral blood mononuclear cells revealed exquisite oxidative stress sensitivity and lipopolysaccharide hyper-responsiveness. Clinically, the mutation was associated with hemophagocytic lymphohistiocytosis (HLH), a severe inflammatory disorder hallmarked by excessive macrophage activation and abnormal NK-cell function. This distinguishes the phenotype from two published pediatric cases caused by HO-1 null-mutations with loss of enzyme function, which are not associated with HLH.[5,6] We show that the disease is clinically modulated by limiting heme challenge through calculated iron depletion, thus highlighting the importance of HO-1 function in oxidative stress defense and in the regulation of the macrophage-dependent inflammatory response.

The patient is the son of consanguineous Turkish parents. At the age of three months, he developed microcytic anemia and progressive splenomegaly. At 8 months, oral iron treatment was started, but the anemia did not resolve. On the contrary, the anemia worsened and repeated red cell transfusions were required. The parents and the healthy brother of the patient showed hemoglobin-concentrations and red cell indices at the lower normal limit of the normal range or slightly below (father: Hb 12.7 g/dl, MCV 81 fl; mother Hb 14.4 g/dl, MCV 79 fl; brother age 7 years Hb 11.4 g/dl, MCV 65 fl). At 18 months, the patient developed persistent fever and pro-

Table 2. Quantitative determination of ROS metabolites in urine samples.

	Patient (n=3)	Age matched controls (n=15, mean±SD)	Mother (n=1)	Father (n=1)	Brother (n=1)
Acrolein-lysine (nmol/mg Cr)	391-502	208±84	393	287	728 (+13.37 SD)
Bilirubin oxidative metabolites (U/g Cr)	4.7-10.8	1.7±0.9	1.7	1.0	1.6 (+0.49 SD)
8-hydroxy-2'-deoxyguanosine (ng/mg Cr)	108-135	18.3±4.0	8.1	7.1	13.7 (+0.25 SD)
Pentosidine (pmol/mg Cr)	60.9-105	30.3±9.6	15.4	16.0	20.9 (-0.56 SD)

Cr: creatinine.

gressive hepatosplenomegaly (see Table 1 for results of clinical laboratory assessment). Liver histology showed severe hemophagocytosis and Kupffer cell siderosis plus extramedullary hematopoiesis and slight hemophagocytosis was observed in the bone marrow. At 20 months, immunochemotherapy (HLH 2004 protocol[7]) was started and resulted in sustained remission of the clinical and laboratory signs of HLH. Remarkably, IL-1β, IL-6, TNF-α, ferritin and CRP remained highly elevated indicating ongoing inflammation, whilst the increased sCD25 level normalized rapidly (Table 1). DNA sequence analysis excluded known mutations as potential causes of familial HLH.

In vitro NK-cell function, measured at age 39 and 45 months, showed decreased killing activity in unstimulated cells, but appropriate killing following IL-2 stimulation (Figure 1A). We tapered immunochemotherapy after 14 months, but four weeks later the patient showed inflammatory activity flare-up (CRP 122 mg/l). CSA was therefore re-started. With improved understanding of the disease pathophysiology, iron depletion was started by periodical low volume phlebotomy; this resulted in the improvement of anemia and complete resolution of liver fibrosis, Kupffer cell siderosis, and hemophagocytosis. At age 10 years, the patient is currently off immunosuppression with no clinically apparent hyperinflammation. However, elevation of vWF, CRP and D-dimers indicate an ongoing inflammatory vascular process.

The persistently low bilirubin, despite excessively elevated LDH, led us to sequence the HO-1 gene; this revealed a homozygous G139V mutation in the patient which was heterozygous in both parents (Figure 1B and 1C). The mutation is located directly in the enzyme's catalytic domain.[8] Replacements of glycine at this position with larger aliphatic residues cause loss of oxygenase activity but, importantly, also result in gain in peroxidase activity.[8,9] This mutation was not identified in an additional analysis we performed on 20 HLH patients with unknown genetics, indicating that HO-1 G139V is not a common cause of familial HLH.

We then confirmed the defective enzyme activity by documenting bilirubin synthesis in PBMC. Control cells showed the expected >4-fold heme-induced increase of bilirubin from 18.8 & 23.6 to 95.9 & 84.6 pmol/mg protein/60min following heme treatment. In contrast, patient cells showed a marginal increase from 21.9 & 26.1 to 33.1 & 27.0 pmol/mg protein/60min.

We next assessed HO-1 protein levels in cultured PBMC and found the expected low constitutive and strongly heme-inducible level in healthy controls and in controls with different hemolytic anemias. In contrast, the patient's HO-1 G139V cells showed a strong constitutive HO-1 expression, which did not further increase following hemin treatment (Figure 1D).

The strong constitutive expression of HO-1 G139V with defective normal but gained abnormal peroxidase function was paralleled by increased excretion of peroxidation products in the urine of the patient and, to some degree, in his heterozygous parents and heterozygous brother (Table 2).

We then analysed the effect of HO-1 G139V on immunological function in PBMC and showed that exposure to heme led, in contrast to controls, to increased cell death in cytotoxicity assays (Figure 1E), which implies a confounding effect of cell death on HO-1 expression.

Next we determined inflammatory cytokine levels in cultured PBMC in the presence or absence of LPS. In HO-1 G139V cells, the basal IL-6 and TNF-α levels were elevated and responses to LPS were much stronger than in control cells (Figure 1F); this documents a remarkable inflammatory hyperresponsiveness of the patient's PBMC. Considering potential mechanisms of how the mutation may induce hyperinflammation, it is notable that CO, a stoichiometric by-product of heme degradation and bilirubin biosynthesis, also stimulates the interaction between caveolin-1 (cav-1) and TLR4, thus inhibiting TLR4 function.[10] Furthermore, excess heme is known to stimulate TLR4 function.[11] Deregulated TLR4 function may therefore play a role in the pathogenesis of hyperinflammation in this patient.

With the broader availability of whole exome sequencing, we searched for other mutations which could plausibly explain the phenotype of the patient. *De novo* homozygous mutations in the SPON2 and IBTK genes, which bind LPS and regulate B-cell function, respectively, were detected in the patient and were found to be heterozygous in the parents. These mutations may contribute to the phenotype. Additionally, in the absence of whole genome sequencing, significant non-exonic mutations might have been missed.

It is interesting to compare the features of this patient carrying the G139V mutation with those of two children with absent HO-1 expression.[5,6,12,13] Both null-mutations and G139V cause hemolytic anemia with almost completely abolished bilirubin synthesis. Interestingly, in HO-1$^{-/-}$ mice splenic macrophages are eliminated by the toxic effect of intracellular heme, resulting in splenic fibrosis and intravascular hemolysis.[14] Consistent with this phenotype in mice, asplenia occurred in previously described patients with HO-1 null-mutations. In contrast, the G139V-mutated patient had splenomegaly. Both the null-mutations and G139V resulted in signs of endothelial damage; though this was much more severe in the patients with null-mutations. Abnormal red cell mor-

phology was also observed in both types of mutation; however, the patients with null-mutants suffered the most severe transfusion-dependent hemolytic anemia, whilst the G139V-patient showed only transient severe anemia and mild anemia subsequently. In remarkable contrast to the patient with the deletion mutant, iron supplementation and red-cell transfusion in the G139V-patient were associated with the development of life-threatening inflammation. Additionally, iron depletion by calculated phlebotomy was associated with continuous improvement of anemia and long-term remission of HLH. These important differences can be explained by the gain of abnormal peroxidase function of the strong, constitutive expression of HO-1 G139V, which was predicted by previous biochemical analyses[8,9] and confirmed by the increased excretion of urinary peroxidation products in the patient's urine. Despite the fact that we cannot provide a genetic model for this disease, it is intriguing that this abnormal biochemical function distinguishes this novel clinical entity from that caused by HO-1 null-mutations, although there is overlap caused by loss of HO-1 function in both types of mutants.

These clinical and functional observations confirm the pathogenetic role of the iron and heme inducible mutated HO-1 protein in this disorder. In principle, one may consider complementing this treatment with other antioxidant or iron chelating medication; this has not been done here due to the current stable condition of the patient without further intervention.

The case reported here demonstrates the clinical importance of HO-1 function in oxidative stress defense and in the regulation of macrophage-dependent inflammatory responses.

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