HHS Public Access

Author manuscript

Mol Genet Metab. Author manuscript; available in PMC 2020 July 01.

Published in final edited form as:

Mol Genet Metab. 2019 November ; 128(3): 391–395. doi:10.1016/j.ymgme.2018.10.005.

Strong correlation of ferrochelatase enzymatic activity with Mitoferrin-1 mRNA in lymphoblasts of patients with protoporphyria

John Phillips[a,*], **Collin Farrell**[a], **Yongming Wang**[b], **Ashwani K. Singal**[b], **Karl Anderson**[c], **Manisha Balwani**[d], **Montgomery Bissell**[e], **Herbert Bonkovsky**[f], **Toni Seay**[b], **Barry Paw**[g], **Robert Desnick**[d], **Joseph Bloomer**[b]

[a]Department of Medicine, Division of Hematology, University of Utah School of Medicine, Salt Lake City, UT, United States

[b]Department of Medicine, Division of Gastroenterology and Hepatology, University of Alabama at Birmingham, Birmingham, AL, United States

[c]Department of Medicine, Division of Gastroenterology, University of Texas Medical Branch, Galveston, TX, United States

[d]Department of Genetics, Icahn school of Medicine, New York, NY, United States

[e]Department of Medicine, Division of Gastroenterology, University of California in San Francisco, San Francisco, CA, United States

[f]Department of Medicine, Division of Gastroenterology, Wake Forest University, United States

[g]Department of Medicine, Hematology, Brigham and Women's Hospital, Boston, MA, United States

Abstract

Accumulation of protoporphyrin IX (PPIX) and Zn-PPIX, are the clinical hallmarks of protoporphyria. Phenotypic expression of protoporphyria is due to decreased activity of ferrochelatase (FECH) or to increased activity of aminolevulinic acid synthase (ALAS) in red blood cells. Other genetic defects have been shown to contribute to disease severity including loss of function mutations in the mitochondrial AAA-ATPase, CLPX and mutations in the Iron-responsive element binding protein 2 (IRP2), in mice. It is clear that multiple paths lead to a common phenotype of excess plasma PPIX that causes a phototoxic reaction on sun exposed areas. In this study we examined the association between mitochondrial iron acquisition and utilization with activity of FECH. Our data show that there is a metabolic link between the activity FECH and levels of *MFRN1* mRNA. We examined the correlation between FECH activity and *MFRN1* mRNA in cell lines established from patients with the classical protoporphyria, porphyria due to defects in ALAS2 mutations. Our data confirm *MFRN1* message levels positively correlated with FECH enzymatic activity in all cell types.

[*]Corresponding author at: Medicine, University of Utah School of Medicine, 30 N. 1900 E, Salt Lake City, UT 84132, United States. John.phillips@hsc.utah.edu (J. Phillips).

1. Introduction

The nuclear encoded mitochondrial enzyme ferrochelatase (FECH) catalyzes the insertion of ferrous iron into protoporphyrin IX in the last step of heme biosynthesis. The active form of human FECH is a homodimer, each half containing a 2Fe-2S cluster that is essential for FECH enzymatic activity and stability [1,2]. FECH is part of an oligomeric complex in mitochondria that includes Mitoferrin-1 (MFRN1) and ABCB10 [3,4]. Mitoferrin-1, located in the inner mitochondrial membrane, transports ferrous iron in mitochondria for formation of the 2Fe-2S clusters, and is the source of the ferrous iron used in the final step of heme biosynthesis [5,6]. ABCB10 enhances MFRN1 stability and function through physical interaction, by binding on the *N*-terminus of MFRN1 as a MFRN1-ABCB10 complex [7].

Enzymatic activity of FECH is deficient in patients with Erythropoietic Protoporphyria (EPP) [8,9]. The most common genotype in patients with EPP is a mutation in one *FECH* allele that severely alters enzyme structure and/or function, and an intronic polymorphism in the other *FECH* allele (IVS3-48 T > C) that causes alternative splicing, resulting in low *FECH* expression allele (LEA) and decreased enzymatic activity [10-13]. The diminished FECH activity causes excess accumulation of Protoporphyrin IX (PPIX), primarily in the bone marrow, with an increase of free PPIX in circulating erythrocytes, and increased hepatobiliary excretion of PPIX. The major clinical features that result are lifelong skin photosensitivity from sunlight exposure in nearly all cases [14], and a variable degree of hepatobiliary injury in approximately 20% of patients due to the toxic effects of hepatic PPIX [15,16], which can lead to liver failure necessitating liver transplantation in 1 to 5% of cases [17].

Biochemical and clinical manifestations like those in EPP also occur in patients who have no genetic deficiency of FECH enzymatic activity, but have *C*-terminal truncation mutations of the *ALAS2* gene expressed in the developing erythroid population of the bone marrow [18]. These mutations cause a 2–3 fold increase in enzymatic activity of ALAS2 [19], the rate limiting step of heme biosynthesis, resulting in the excess production and accumulation of PPIX, a condition designated X-Linked Protoporphyria (XLP) [18,20].

A pilot study, published in 2011, examined the relationship between FECH enzymatic activity and the *MFRN1* mRNA level in cultured lymphoblasts of five normal individuals, one individual with classical EPP, and four individuals with XLP (3 members of the same pedigree), and two individuals with positive biochemical studies for an EPP phenotype, but without identifiable mutations of *FECH* or *ALAS2* enzymes [21]. This study showed a positive relationship between FECH activity and *MFRN1* mRNA level, with correlation coefficient of 0.75. However, there were insufficient sample numbers from each well characterized erythropoietic porphyria variant (*FECH nonsense*/LEA, *FECH missense*/LEA, XLP), were not available to draw statistically significant conclusions. The present study was performed on increased sample numbers to determine if this relationship between *MFRN1* message levels positively correlated with FECH enzymatic activity.

2. Methods

2.1. Study population

Studies were done on a) ten patients with confirmed *ALAS2* mutations, b) ten patients with a *FECH C411G* mutation (C411, one of the four cysteine residues coordinating 2Fe-2S cluster binding, located on *C*-terminal domain is substituted with glycine), that prevents the 2Fe-2S complex from binding to FECH, and c) ten patients with *FECH* nonsense or splicing null mutations. All individuals with *FECH* mutations (groups b and c) had the low expression allele (IVS3-48c > t) in trans, which was not present in any of the individuals with *ALAS2* mutations. The sample size of 10 was chosen because it is the smallest number that can provide a correlation coefficient of > 0.6 with $p = 0.05$ and study power of 80%. Studies were also done on 21 healthy individuals with no personal or family history of porphyria. The control population tested negative for *FECH* or *ALAS2* mutations and the low expression allele. The study was approved by the Institutional Review Boards at the University of Alabama at Birmingham, Mount Sinai School of Medicine in New York, University of Utah in Salt Lake City, University of California at San Francisco, University of Texas Medical Branch at Galveston, Wake Forest University at Winston Salem, and Children's Hospital of Boston.

2.2. Lymphoblast generation and culture

After written informed consent from participants, peripheral blood samples were obtained to establish Epstein-Barr virus-transformed lymphoblasts (EBVTL), using methods previously described [22,23]. EBVTL were cultured in RPMI 1640 containing penicillin/streptomycin sulfate (Cellgro) and 20% heat-inactivated fetal bovine serum (Biomedia) until growing well, followed by culture in the same medium containing 15% fetal bovine serum for three to four days to reach the log phase of growth. They were then harvested for measurement of FECH enzymatic activity, MFRN1 mRNA, and mitochondrial iron levels. The iron concentration in fetal bovine serum used in the cultures ranged from 279 to 309 μg/dL.

2.3. Measurements of FECH activity and MFRN1 mRNA in cultured lymphoblasts

FECH enzymatic activity was assessed in sonicates of EBVTL, using a fluorometric assay that measures the formation of zinc-deuteroporphyrin, and is expressed as nmol/mg protein/h [22]. Quantitative levels of *MFRN1* mRNA were measured in total mRNA isolated from EBVTL; qRT-PCR using the Quanti Tect™ Custom Assay (Qiagen) as previously described [21]. Primers and probes for *MFRN1* were designed by Qiagen Quantiprobe Design Software (Qiagen). Real-time PCR was performed on an ABI Prism 7700 instrument (Applied Biosystems).

Mitochondrial isolation from EBVTL was done with a commercial kit (Thermo Scientific Cat # 89874), followed by traditional Dounce homogenization to separate mitochondria from cytosolic components [24]. The iron level in the mitochondria was determined by measurement of bathophenanthroline sulfonate Fe (BPS) 3 formation after incubation with nitric acid at 100 °C overnight [25,26].

2.4. Western blot

Total protein was extracted from cultured cells, 80 μg protein were subjected to SDS-PAGE followed by Western blotting onto PVDF membranes. Western transfers were immunoblotted with anti-FECH (1:1000), anti-MFRN1 (1:1000), anti-GAPDH (1:2000) antibodies. Western blot assay was performed to evaluate levels of FECH protein and MFRN1 protein in mitochondrial fractions extracted from EBVTL [22]. Polyclonal Anti-FECH (SC-99138) obtained from Santa Cruz Biotechnology, polyclonal anti-MFRN1 (SLC25A37/PAS-26720) from Thermo Scientific, and Polyclonal anti-GAPDH (ab9485) purchased from Abcam Inc. In all experiments, membranes were subsequently incubated with a secondary horseradish peroxidase (HRP)-labelled goat anti-rabbit secondary antibody (1:3000) for 1 h at room temperature. The ECL detection reagent (Pierce Chemical) was prepared according to the manufacturer's instructions.

3. Results

3.1. EBVTL measurements

The levels of FECH enzymatic activity in EBVTL classic EPP (*FECH nonsense*/LEA) were significantly reduced compared to the normal controls (Table 1). There was also a reduction in FECH enzymatic activity in the group with *ALAS2* mutations (XLP) compared to normal controls. This was accompanied by decreased formation of normal *MFRN1* mRNA and reduced FECH enzymatic activity compared to normal lines.

The correlation coefficient between FECH enzymatic activity and *MFRN1* mRNA level in this study confirms the presence of a positive relationship between these two mitochondrial entities. The correlation coefficient is 0.94 when the three types of mutant cell lines are combined, and remains high at 0.85 when the normal cell lines are also included (Fig. 1 and 2). Iron levels, in mitochondria isolated from each of the four EBVTL groups, were highest in the normal EBVTL group, and lowest in the EBVTL group with *FECH Nonsense/Splicing* mutations (p-value 0.0015 between the two groups). (Table 2).

3.2. Western blot results

Western Blot evaluations of FECH protein and MFRN1 protein (Fig. 3) levels in mitochondrial fractions of the four EBVTL groups show that the highest protein levels were in normal cells, followed by *ALAS2* cells (XLP), *FECH C411G*/LEA cells, and *FECH Nonsense*/LEA cells. The levels of FECH are normal in the ALAS2 cells and approximately 35% of wild type in cells with the C411G and the non-sense FECH alleles. FECH activity was 25.4 in wild type, 4.2 in C411G 11.7 in ALAS2 and 4.1 nmol/mg protein/h and correlated highest with the levels of mitoferrin 1 protein (Fig. 3).

4. Discussion

The main findings of our study are: a) reduction in FECH enzymatic activity in patients with EPP and XLP, b) reduction in *MFRN1* mRNA levels in both EPP and XLP patients, c) excellent correlation between *MFRN1* mRNA and FECH activity, and d) reduction in mitochondrial iron levels among patients with EPP with *FECH nonsense*/LEA mutations.

Reduction in FECH activity positively correlating with *MFRN1* mRNA levels was previously shown in a pilot study [21]. In the present study, these findings were confirmed with better correlation between FECH activity and *MFRN1* mRNA levels, with a positive correlation coefficient of 0.94. Further, results in the current study extend these findings to all cause of increased PPIX accumulation, mutations in FECH or ALAS2. However, mitochondrial iron level reduction was seen among only one subgroup of EPP patients, those with *FECH nonsense*/LEA alleles, and not in subjects with *FECH C411G*/LEA alleles or in XLP patients. Reasons for these discordant findings on mitochondrial iron levels remain unclear and need further investigation. It would also be interesting to examine the effects of exogenous ferrous iron administration in EPP and XLP patients by measuring the changes in the FECH activity, *MFRN1* mRNA, and mitochondrial iron levels.

In addition to being a substrate for FECH in heme biosynthesis, iron has other important roles in heme metabolism. Experiments with K562 erythroleukemia cells and Cos7 cells expressing human FECH have shown that formation of the 2Fe-2S cluster bound to the *C*-terminal region FECH is regulated by availability of intracellular iron [23]. When intracellular iron levels are low, rapid degradation of FECH protein occurs, indicating that the post-translational stability of FECH depends on iron availability and formation of the structurally important 2Fe-2S cluster. Thus, MFRN1 is an essential component of heme synthesis, as it transports ferrous iron for the final step in heme biosynthesis in the mitochondria, as part of the FECH metabolon [27]. The multi-protein complex is thought to sequester iron, preventing tissue damage that could occur if ferrous iron were free in solution. When MFRN1 expression is low, there is a decrease in the levels of 2Fe-2S clusters being produced by the mitochondrial machinery, leading to decreased FECH activity and potentially disrupting overall iron homeostasis leading to alterations in translation of mRNAs controlled by the Iron Responsive Element/Iron Responsive Element-Binding Protein (IRE/IRE-BP) system [28]. These observations have been made in context to ferritin and other components responsible for iron homeostasis [5]. While the mechanism tying mRNA levels for MFRN1 to activity of FECH are not clear, the evolution of a system to prevent excess free iron and to prevent excess PPIX seems clear [29].

Independent regulatory systems to prevent the toxicity associated with “free iron” and/or “free PPIX” have evolved in multiple cells types. Excess PPIX is pumped out of erythrocytes by ABCG2, a transporter that has a role in regulating PPIX levels during erythroid differentiation, and therefore, could be a genetic determinant of EPP [30]. The plasma membrane bound heme transporter FLVCR has also been shown to export potentially toxic PPIX from the erythron during development [31]. The liver transferrin pathway has a role in the orchestration of iron distribution between peripheral iron stores, the spleen, and the bone marrow [32]. In FECH-deficient protoporphyria patients, ALAS2 expression is enhanced, and the erythrocytic PPIX concentration correlates with iron availability [33].

Other proteins that could also impact phenotypic expression in EPP are the mitochondrial protein that co-localizes with FECH in mitochondria of mouse erythroleukemia cells [34], heme-regulated elF2-kinase (HRI) that is essential for translational regulation of alpha and beta globulins [35], and the iron-regulatory protein 2 (IRP2) that post-transcriptionally regulates iron-responsive proteins [36]. It is notable that patients with EPP often have a mild

anemia, with variable evidence of iron deficiency that includes microcytosis, low serum iron levels, and low serum ferritin levels. Iron availability has been shown to modulate aberrant splicing of *FECH* through the iron-and 2-oxoglutarate dependent dioxygenase Jmjd6 and U2AF^{65} [33]. However, the role of iron replacement in EPP remains controversial, with both clinical improvement and exacerbation of photosensitivity having been reported [37]. The ultimate goal of these studies is to understand how the mitochondria communicates to the nucleus to coordinate proteins levels where a single member of a multi-subunit complex is deficient. In this specific case, tying the ability to import iron, that can be toxic in excess, to the capacity to produce an essential cofactor- heme.

Acknowledgements

This research was supported in part by the Porphyrias Consortium (U54 DK083909), which is a part of the NCATS Rare Diseases Clinical Research Network (RDCRN). RDCRN is an initiative of the Office of Rare Diseases Research (ORDR), NCATS, and is funded through a collaboration between NCATS and the NIDDK. MB is supported in part by the NIH Career Development Award (K23 DK095946). BW is supported in part by the American Porphyria Foundation's "Protect the Future Program".

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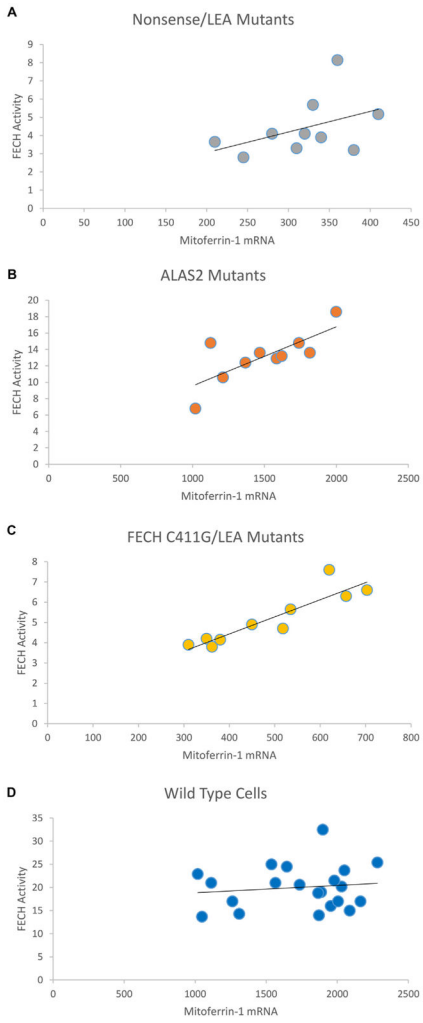

Fig. 1.

Correlation between FECH activity (nmol/mg protein/h) and Mitoferrin-1 mRNA (relative to total mRNA level) in patients with protoporphyria due to A) *FECH Nonsense*/LEA alleles, B) ALAS2 mutants, and C) *FECH C411G*/LES alleles. Panel D is on healthy controls with wild-type lymphoblasts.

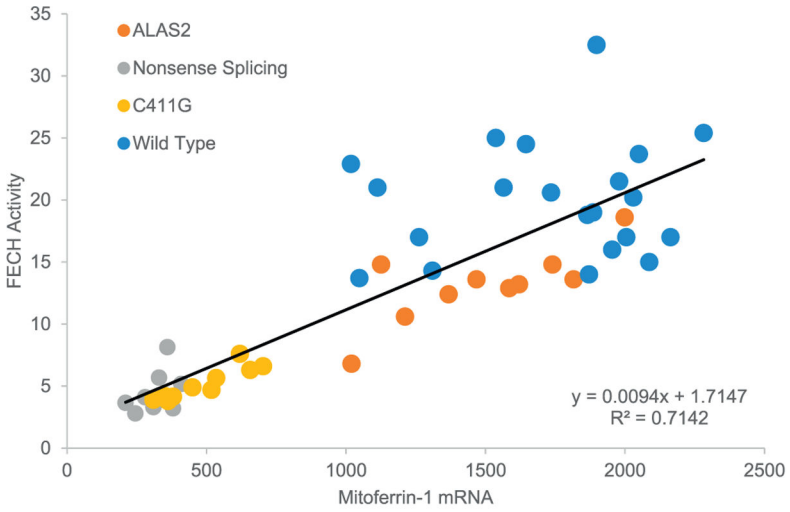

Fig. 2.

Correlation between FECH activity (nmol/mg protein/h) and Mitoferrin-1 mRNA (relative to total mRNA level) in patients with protoporphyria and healthy controls.

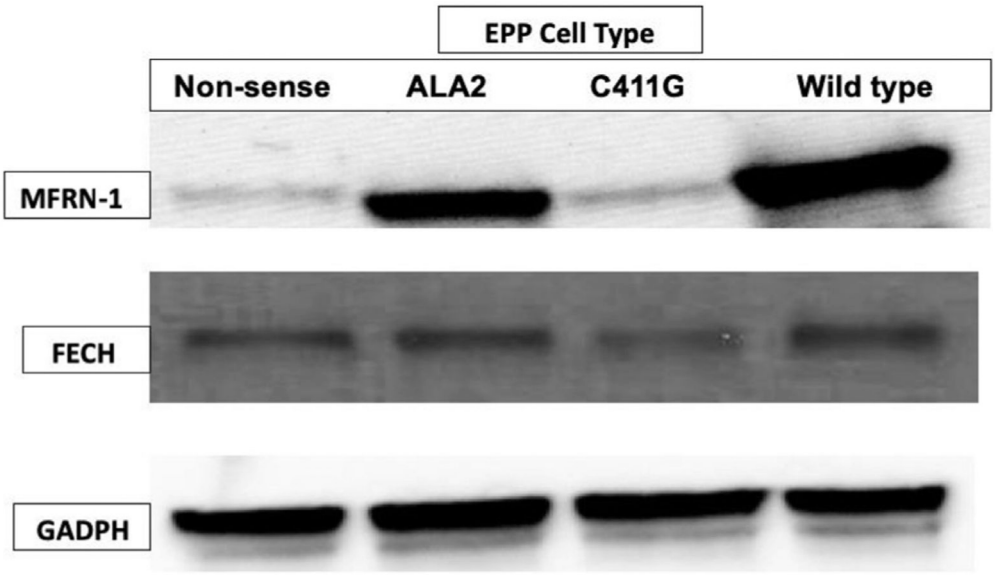

Fig. 3.

Western Blot of MFRN1/FECH/GAPDH in patients with protoporphyria due to mutations in either FECH (non-sense or C411G) or gain-of-function mutations in ALAS2. Total protein, 80 μg, from EBVTL of patients were subjected to SDS-PAGE then transferred to PVDF membranes. Blots were developed with anti-FECH (1:1000), anti-MFRN1 (1:1000), anti-GAPDH (1:2000) antibodies followed by a secondary horseradish peroxidase (HRP)-labelled goat anti-rabbit secondary antibody (1:3000).

Table 1

Ferrochelatase Enzymatic Activity and Mitoferrin-1 mRNA levels in EBVTL.

Genotype	**Wild type N = 21**	***FECH C411G*/ LEA N = 10**	***FECH Nonsense*/ LEA N = 10**	***ALAS2 Mutations* N = 10**
FECH activity[a]				
Mean(SD)	19.7(3.8)	5.2 (1.3) *	4.4(1.6) *	13.1(3.0) *
Mitoferrin-1 mRNA[b]				
Mean(SD)	1728.7(372.3)	488.5(139.5) *	318.5(60.7) *	1495.3(316.1) **

* p value < .001.

** p value = .09.

[a] nmol zinc-deuteroporphyrin/mg protein/h.

[b] copies/μg total RNA.

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Table 2

Mitochondrial Iron in EBVTL (μg/g protein).

Genotype	**Wild type N = 8**	**FECH C411G N = 7**	***FECH Nonsense/ Splicing* N = 10**	***ALAS2 Mutations* N = 8**
Mean(SD)	154(58)	112(84)	66(28)	149(60)
$^{*}p$	–	0.15	0.0015	0.428

*p value – compared with normal group.