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PhD thesis summary

STRUCTURAL-FUNCTIONAL CORRELATIONS OF HYDROXYMETHYLBILANE SYNTHASE

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SOUHRN

Akutní intermitentní porfyrie (AIP, OMIM 176000) je autosomálně dominantní vrozená porucha, která se řadí mezi akutní jaterní porfyrie. Je způsobená částečným snížením aktivity hydroxymetylbilan syntázy (HMBS, EC 4.3.1.8), třetího enzymu biosyntetické dráhy hemu. Klinické příznaky zahrnují gastrointestinální, neurologické a kardiovaskulární symptomy, ale nejčastějším projevem je abdominální bolest neuroviscerálního původu.

Základním cílem této disertační práce bylo provádění molekulární analýzy DNA u pacientů s AIP. V případě, že je u pacienta nalezena mutace, testování DNA na molekulární úrovni je nabídnuto rovněž ostatním příbuzným. Tento individualizovaný přístup umožňuje zachycení asymptomatických jedinců v postižené rodině. Ti jsou pak informováni o vhodnosti požívání léků a o dalších faktorech, které mohou vyvolat nebezpečné akutní ataky.

V ideálním případě je DNA diagnostika složena z robustní a efektivní skenovací metody a potvrzujícího kroku, který využívá DNA sekvenování. Rozhodli jsme se zavést skenovací metodu nové generace, která je vysoce citlivá a relativně časově i finančně nenáročná. Zvolili jsme metodu high-resolution melting (HRM) s využitím přístroje LightScanner.

Dalším důležitým aspektem tohoto projektu bylo studium molekulární heterogenity AIP ve vztahu k struktuře proteinu. Snažili jsme se o popsání vlivu mutace v genu pro HMBS na strukturu a funkci enzymu a dále o předvedení, jak tato informace může přispět k interpretaci klinických, biochemických a genetických dat při určování diagnózy AIP. Pro tento účel jsme použili prokaryotickou expresi a charakterizaci mutantních HMBS enzymů společně s počítačem modelovanou prediktivní strukturálně-funkční korelační analýzou.

SUMMARY

Acute intermittent porphyria (AIP) is an autosomal dominantly inherited disorder, classified as acute hepatic porphyria. It is characterized by a deficiency of hydroxymethylbilane synthase (HMBS, EC 4.3.1.8), the third enzyme in heme biosynthesis. Clinical features include gastrointestinal, neurologic and cardiovascular symptoms, but the most common clinical presentation is abdominal pain caused by neurovisceral crises.

The purpose of this study was first to perform molecular analysis of the AIP patients. Once a mutation is detected in a patient, molecular testing is offered to family members. In each affected family, this becomes an important tool for individualised medicine, allowing for careful drug prescription; in addition, it is very important for the asymptomatic carriers to be warned of precipitating factors, thus avoiding an acute attack.

The proper DNA diagnostics can be achieved by a combination of a robust and effective pre-screening method and a confirmatory DNA sequencing step. We decided to establish a new generation pre-screening method, which will be highly sensitive and relatively time- and cost-effective. Our method of choice was high-resolution melting (HRM) analysis using the LightScanner instrument.

Another important aspect of this project was to study the molecular heterogeneity of AIP in relation to the HMBS protein. We aimed at characterisation of the impact of the HMBS gene mutation on the structure and function of the enzyme, and demonstration of how this aids the interpretation of clinical, biochemical and genetic data in establishing an AIP diagnosis. To demonstrate this, we used expression and characterisation of mutant HMBS enzymes in the prokaryotic system together with the use of predictive computer-assisted structure-function correlation studies.

REVIEW OF THE LITERATURE

Heme and hemoproteins

Heme serves as a prosthetic group for a wide range of proteins, either covalently or non-covalently bound to the protein itself; the biological functions diverge with heme type. The core structure of a heme molecule is tetrapyrrole, a substance significantly called 'pigment of life' (Leeper, 1989) since it gives the specific colour to green leaves and red blood.

Recent protein databank surveys identifies at least several hundred metalloproteins with heme as their subunit, all involved in diverse basic cellular functions.

Heme biosynthesis

Heme biosynthesis is widely distributed process in eukaryotic cells although considerable quantitative variations exist between different cell types. The synthesis takes place in all living cells, but predominantly in the liver and bone marrow.

There are eight enzymatic steps in the heme biosynthesis. The initial enzymatic step take place in the mitochondrion, the following four enzymes operate in the cytosol and the final three steps are directed back into mitochondria.

Porphyrias

The porphyrias are a group of predominantly inherited disorders of heme biosynthesis in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. With the exception of the first enzymatic step, seven deficiencies linked to seven enzymes of the biosynthetic pathway have been described (Anderson, 2001). Each type of porphyria results from a specific decrease in the activity of a corresponding enzyme. The genes coding for the enzymes of this pathway have been characterised at the molecular level (Anderson, 2001) and several mutations have been identified. Inheritance of one copy of an affected allele can cause loss of enzymatic activity, but output from the normal allele appears to be sufficient for normal cellular metabolism in individuals (Gouya, et al., 2004).

Most porphyrias are autosomal dominant conditions, only two of them share an autosomal recessive mechanism of inheritance. Low clinical penetrance is an important feature of all of the autosomal dominant porphyrias (Anderson, 2001; Gouya, et al., 2004).

Porphyrias can be divided into hepatic or erythropoietic types depending on the anatomical origin, and further divided into acute or cutaneous types depending on the clinical

presentation. Two types of clinical expression can occur separately or in combination: acute life-threatening neurovisceral attacks, which are associated with the overproduction of ALA and PBG; and/or cutaneous symptoms that result from photosensitisation caused by porphyrins (Albers and Fink, 2004; Anderson, 2001; Meyer, et al., 1998). All types of porphyria are summarised in **Table 1**.

Table 1 Classification of porphyrias

Porphyria	Deficient enzyme	Classification	Inheritance	Clinical	Mutations (HGMD)	Symptoms
ALAD deficiency	ALAD	acute	autosomal recessive	hepatic	12	neurovisceral
Acute intermittent porphyria	HMBS	acute	autosomal dominant	hepatic	309	neurovisceral
Congenital erythropoietic porphyria	UROS	cutaneous	autosomal recessive	erythro-poietic	38	photosensitivity
Porphyria cutanea tarda	UROD	cutaneous	sporadic / autosomal dominant	hepatic	103	photosensitivity
Hereditary coproporphyria	CPO	acute + cutaneous	autosomal dominant	hepatic	45	photosensitivity/ neurovisceral
Porphyria variegata	PPOX	acute + cutaneous	autosomal dominant	hepatic	149	photosensitivity/ neurovisceral
Erythropoietic protoporphyria	FECH	cutaneous	autosomal dominant	erythro-poietic	120	photosensitivity

Acute intermittent porphyria

With the exception of South Africa and Chile, acute intermittent porphyria (AIP, MIM # 176000) represents the most frequent type of acute porphyria throughout the world (Hift and Meissner, 2005). This predominantly inherited autosomal dominant disorder, classified as acute hepatic porphyria, is characterised by a deficiency of HMBS, the third enzyme in heme biosynthesis. In such cases, the activity of the enzyme is decreased by about 50% in all tissues (Meyer, et al., 1972).

The prevalence of symptomatic disease varies from 1 to 10 per 100 000, but due to frequent misdiagnosis and low penetrance this number may be much higher (Badminton and Elder, 2002; Meyer, et al., 1972; Strand, et al., 1970).

Expression of the disease is highly variable. It is determined in part by environmental, metabolic, and hormonal factors that share the ability to induce hepatic ALAS, the rate limiting enzyme of heme biosynthesis, and thereby to increase the production of porphyrin precursors, ALA and PBG.

Over 300 mutations in HMBS gene are known (http://www.hgmd.cf.ac.uk) (Stenson, et al., 2009). Mutations are equally distributed along the HMBS gene and no particularly sensitive site for mutations has been identified. Regarding the prevalence of AIP in the Czech Republic, no statistical data exist. To date, 14 different mutations have been identified in the Czech and Slovak population. Some of them are described in this thesis.

Clinical manifestation

The manifestation of the disease is associated with an acute neurological syndrome characterised mainly by an acute attack (Albers and Fink, 2004; Meyer, et al., 1998). The porphyric manifestation usually occurs in the third life decade, though there is the rare occurrence before puberty or after menopause (Elder, et al., 1997). Clinical expression, in general, is highly variable and ~90% of AIP heterozygotes remain asymptomatic through life (Elder, et al., 1997; Petrides, 1998). Individual gene carriers differ from each other in both biochemical and clinical manners. Symptoms may be recurrent and last from a few days to a few weeks, or they may occur as a single sporadic incident in a lifetime.

Acute attacks are manifested by a wide variety of clinical features including: autonomic neuropathy, central nervous system impairment, peripheral motor neuropathy, and most commonly a severe pain, usually of abdominal or back origin (Meyer, et al., 1998; Nordmann and Puy, 2002). Among psychiatric disturbances, symptoms widely range from minor behavioural changes to more severe agitation, hysteria, psychosis with hallucinosis or even delirium (Crimlisk, 1997; Millward, et al., 2001; Regan, et al., 1999). An unmistakable symptom of an acute attack, which can often lead to the correct diagnosis, is the occurrence of red or dark-coloured urine resulting from high concentrations of porphyrins and porphyrin precursors.

Acute attacks can be potentially life-threatening, especially when respiratory paralysis occurs (Goldberg, 1959). In patients with severe AIP, hepatocellular cancer may be a potential and serious complication (Kauppinen and Mustajoki, 1988). The chronic complications include the development of renal disease which eventually leads to kidney failure (Andersson, et al., 2000).

Triggering factors of an acute attack

Clinical presentation appears to require additional factors that affect the heme pathway by increasing demand for heme, by causing an additional decrease in enzyme activity, or by

combination of both these factors. These factors include genetic and environmental conditions. The environmental factors can be divided into two main groups: exogenous and endogenous agents. Of the exogenous factors, porphyrinogenic drug are of great importance, since AIP was determined to be pharmacogenetic (Moore and Hift, 1997; Tschudy, et al., 1975). Other exogenous agents include alcohol, infection, major surgery, restricted calorie intake and various kinds of stress (Albers and Fink, 2004; Anderson, et al., 2005; Bonkovsky and Barnard, 2000; Bonkovsky, et al., 1992; Hift and Meissner, 2005; Kauppinen and Mustajoki, 1992; Moore and Hift, 1997; Thunell, et al., 1992). Of the endogenous factors, the use of steroid hormones and the fluctuations in female sex hormones are particularly important, and together explain the higher frequency of disease manifestation in woman (Andersson, et al., 2003). Although it appears that an individual's genetic background can influence susceptibility to acute attacks, the genes involved have not yet been identified.

Diagnosis

Since acute porphyric attack can be potentially life-threatening, prompt and proper diagnosis of AIP heterozygotes is crucial to prevent attacks in both symptomatic and asymptomatic carriers. Traditionally, the diagnosis of porphyria is made on the basis of clinical symptoms, characteristic biochemical findings and enzyme assays.

Of the biochemical findings, the marked increase in urinary PBG accompanied by an increase in ALA levels and some other porphyrins are common features for AIP. These precursors, essential for the diagnosis, are highly elevated during an acute attack and high excretion of these metabolites is often detected in the asymptomatic phase. Nowadays, the biochemical detection of porphyrin precursors have been implemented as the first step in analysis of clinical syndromes and of the severity of the disease, e.g. in detecting acute or latent phase (Sassa, 2006).

Measurement of erythrocyte HMBS activity is another component of laboratory diagnostic tools. In most of the AIP patients, HMBS activity is approximately half of the normal activity (Meyer, et al., 1972).

Several DNA-based screening molecular techniques have been included in the clinical diagnostic process as the final step to confirm the gene carrier status (Frank and Christiano, 1998; Grandchamp, et al., 1996; Kauppinen, 2004; Sassa, 2006). The search for the disease-causing mutation in each affected family is an important tool for individualised medicine, allowing for careful drug prescription and acute attack prevention.

Treatment

An acute porphyric attack requires immediate intervention because it is known to be a life-threatening event and may result in serious neurological damage. Due to modern therapeutic options, the mortality rate nowadays is low.

In management of the porphyric attack, which usually requires hospitalisation, treatment is focused on a specific cure of symptoms and complications. This involves disease-specific therapy, and identifying and removing precipitating factors of an acute attack.

Of the specific therapies, the administration of intravenous heme followed by large amounts of carbohydrates is considered the most powerful treatment (Anderson, et al., 2005; Anderson, 2001; Bonkovsky and Barnard, 2000; Handschin, et al., 2005; Li, 2005; Stein and Tschudy, 1970; Watson, et al., 1978). There are two types of heme derivates available for use to suppress severe acute attacks - hematin (Panhematin®, Abbott Laboratories) and heme-arginate (Normosang, Orphan Europe).

Gene

Human HMBS is determined by a single gene located on chromosome 11 (Meisler, et al., 1980), assigned to the locus to the long arm in the segment 11q24.1-q24.2 (Namba, et al., 1991). HMBS gene is divided into 15 exons ranging from 39 to 438 bp and 14 introns ranging from 87 to 2913 bp in length and, spans approximately 10 kb of DNA (Chretien, et al., 1988; Yoo, et al., 1993).

The HMBS gene was the first gene ever described to be a single gene having dual purpose of being encoded by mRNAs transcribed from two promoters, a housekeeping and an erythroid-specific (Grandchamp, et al., 1987; Chretien, et al., 1988). The housekeeping promoter is in the 5' flanking region and its transcript is encoded by exons 1 and 3 through 15. The erythroid-specific promoter is located 3 kb downstream from the housekeeping promoter in the first intron and its transcript is encoded by exons 2 trough 15 (Grandchamp, et al., 1987; Gubin and Miller, 2001; Chen, et al., 1994).

Protein

Hydroxymethylbilane synthase (also known as porphobilinogen deaminase or uroporphyrinogen I synthase, EC 4.3.1.8 or EC 2.5.1.61), is the third enzyme of the heme biosynthetic pathway. This monomeric protein with a single catalytic active site (Louie, et al., 1992) is organised into three domains approximately equal in size (Louie, et al., 1996). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isoenzymes have

been isolated and characterised (Grandchamp, et al., 1987; Raich, et al., 1986). The housekeeping isoform of the protein consist of 361 amino acids, with an additional 17 amino acid residues at the N-terminus compared to the erythroid variant of 344 amino acids (Grandchamp, et al., 1987; Raich, et al., 1986). Both isoforms catalyse the same reaction.

Relatively high amino acid sequence conservation is found in HMBS and its homologs. It has been reported that the *E. coli* and human HMBS amino acid sequences have 43% identity and more than 60 % similarity (Brownlie, et al., 1994; Jordan and Warren, 1987; Shoolingin-Jordan, et al., 2003)). Several crystallographic structures of *E. coli* HMBS have been determined (PDB 1gtk, 1pda (Helliwell, et al., 2003; Louie, et al., 1996; Louie, et al., 1992)). Recently, the crystallographic structures of human housekeeping HMBS and human mutant protein Arg167Gln HMBS have been determined (PDB 3ecr and 3eq1; (Gill, et al., 2009; Song, et al., 2009)).

Structure-function correlations

To predict the impact of pathological mutations on the protein structure and function of human HMBS, the homology between human HMBS and the *E. coli* enzyme was used to build a structural model (Brownlie, et al., 1994). Now, the discovery of the human structure further facilitates an understanding of the structural correlations. Of these mutations, over 120 of them are missense/nonsense single base changes that result in one amino acid substitution or in the formation of a premature stop codon and subsequent protein truncation (http://www.hgmd.cf.ac.uk) (Stenson, et al., 2009). These mutations are of special interest, since single amino acid change can provide information on the functional or conformational importance of the wild-type residue.

Pathological mutations can be divided into three broad groups according to their molecular basis. The first group represents a change of amino acid that impacts on protein folding and stability. Such residues are usually located in close proximity to the hydrophobic core and in conformational restricted areas. The second group consists of residue changes with an effect on the binding, reaction and assembly of the DPM cofactor. If the DPM cofactor, important not only for the enzyme function, is absent in the enzyme structure, its stabilising ability is missing and the unstable apo-enzyme is therefore rapidly degradated. Finally, the last group consists of mutations that affect the catalytic or substrate binding residues and thereby result in inactive proteins (Gill, et al., 2009; Song, et al., 2009).

AIMS OF THE STUDY

In general, the aim of this study was to enable the proper molecular diagnosis of AIP patients at the DNA level in order to investigate the structural-functional consequences of mutations at the protein level.

The specific aims were as follows:

- The molecular diagnosis of newly diagnosed AIP patients and the molecular diagnosis of affected families using an established method.
- The optimisation of the next generation diagnostic method, High-Resolution Melting, using the LightScanner instrument for detection of DNA variations in the HMBS gene.
- The expression, purification and biochemical characterisation of human mutant HMBS enzymes with introduced pathological mutations of interest in the prokaryotic (*E. coli*) system.
- The structure-function correlation studies - the assessment of detrimental effects of DNA variations on enzyme function.

RESULTS AND DISCUSSION

Publication A - De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study *(Ulbrichova D et al., 2006)*

The patient, a 15-year-old boy, was hospitalised while having his first acute attack, characterised by clinical features typical of AIP.

Using denaturing gradient gel electrophoresis (DGGE) and direct DNA sequencing a novel heterozygous mutation, the c.965_966insA, was identified. The molecular screening in family members failed to reveal any DNA variation. Therefore, we suggest that 966insA is a *de novo* mutation since nonpaternity was excluded by DNA microsatellite analysis.

The mutation c.965_966insA is localised in exon 15 of the HMBS gene. At the protein level, it results in a frameshift and production of a STOP codon after expression of 36 completely different amino acids compared to the original sequence (p.Asn322LysfsX36).

We expressed wild-type and mutant proteins in the prokaryotic system and we performed biochemical testing of the purified mutant enzymes.

The SDS-PAGE analyses of the mutant protein revealed several bands, in contrast to a single homogenous band in the case of wild-type protein. The purified mutant enzyme had a relative activity level of 0.18% of level achieved by wild-type enzyme.

We designed the mutant protein structure using the computer-assisted structure prediction program, using the 3D structure of *E. coli* HMBS as a template. The c.965_966insA mutation is localised in the $\beta 3_3$ sheet of domain 3. Due to the incurred frameshift, part of the third enzyme domain has a different formation. In the wild-type protein, the C-terminal helices protect the beta-strands from being exposed to solvent. This is in agreement with the severely decreased stability of the mutant HMBS.

In summary, the *de novo* mutation c.965_966insA (p.Asn322LysfsX36) was found in a young patient with AIP. Due to a truncated protein sequence with an abnormal C-terminus domain, this small insertion mutation c.965_966insA leads to an almost complete loss of the enzymatic function and decreases the stability of the protein. This case is of particular interest as the identification of a *de novo* mutation is a rare event.

Publication B - A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family *(Flachsova E et al., 2007)*

Our laboratory was contacted by a 50-years-old proband from Nepal who suffered from severe abdominal pain accompanied by dark urine. After considering the possibility of having acute intermittent porphyria, he, as a non-health professional, searched the internet for help. He arranged to send samples to us from himself and from 15 members of his family.

Molecular testing revealed a novel heterozygous mutation c.972_973insG in exon 15 of the HMBS gene. Analysis of the protein sequence indicated that following the insertion mutation, leading to a prematurely truncated protein in which 44 amino acids of the C-terminus of HMBS was missing.

We expressed wild-type and mutant proteins in the prokaryotic system and we performed biochemical testing of the purified mutant enzymes.

The SDS-PAGE analyses of the mutant protein displayed again several bands, suggesting high protein instability. The purified mutant enzyme had an activity level 0.5% of the average wild-type level.

Using the computer-assisted structure prediction, the 3D structure of the mutant protein was designed. The c.972_973insG mutation is located in domain 3 in close proximity to the terminal helix. The mutations in the penultimate helix or its removal destabilise the whole C-terminal domain; in turn, the N-terminal domain cannot fold in a stable unity and therefore the whole protein is destabilised.

After DNA screening in the proband's family members, the same mutation was subsequently found in 12 of them, 7 out of them were asymptomatic.

In summary, a novel mutation c.972_973insG within the HMBS gene was identified in 12 members of an extensive Indian family from Nepal. This mutation results in a truncated and highly unstable protein with loss of enzymatic function. The uniqueness of this case lies in the fact that the proband diagnosed himself based on information from the internet. This highlights the importance of the accessibility of online information especially in the case of rare diseases.

Publication C - Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria *(Xiaoye Schneider-Yin et al., 2008)*

In this study, we report on mutational analysis and *in vitro* characterisation of HMBS variants identified in two individuals who were suspected of having AIP. The first patient, a 17-year-old Ashkenazi Jewish female, had been experiencing menstruation-related recurrent episodes of severe abdominal pains. Biochemical analyses failed to show elevations in urinary PBG and ALA levels. However, erythrocyte HMBS activity showed an average activity that was 60% of normal. The patient was treated with either glucose or heme-arginate (Normosang, Europe) for acute attacks. The second patient, a 30-year-old Ashkenazi Jewish female, was suffering from recurrent acute attacks of abdominal pain for 12 years. Biochemical analyses showed increased urinary ALA and PBG, and reduced erythrocyte HMBS activities about 50% of normal. She was successfully treated with glucose.

After molecular screening of the HMBS gene, two novel heterozygous mutations c.176C>T in exon 5 and c.643G>A in exon 11 was identified separately in each subject. At the protein level, the mutation c.176C>T leads to an amino acid substitution p.Thr59Ile and the mutation c.643G>A leads to an amino acid substitution p.Val215Met.

We expressed both variant proteins in the prokaryotic system and we performed biochemical testing of the purified mutant enzymes. Recombinant p.Thr59Ile and p.Val215Met mutant enzymes had residual activity of 80.6% and 19.4%, respectively, compared to that of the wild-type enzyme.

While the clinical symptoms and *in vitro* and *in vivo* biochemical analyses suggested a causal relationship between p.Val215Met and AIP, the association between the p.Thr59Ile substitution and AIP is less obvious. In view of the results we received, we conclude that p.Thr59Ile might represent a mutation with a weak effect rather than a mere polymorphism. The evidence supporting the association between the p.Thr59Ile mutation and the AIP phenotype was a successful treatment of the clinical symptoms of AIP with glucose and Normosang. Moreover, the patient's erythrocyte HMBS activity was reduced to half-normal value. It may very well be that in this compound case, there is more than one causative factor with clinical relevance, which remains undetected.

In summary, two novel mutations c.176C>T (p.Thr59Ile) and c.643G>A (p.Val215Met) within the HMBS gene were identified. Despite not having full AIP-affirming biochemical evidence in one case, both mutations were associated with AIP. The study demonstrates that

in vitro characterisation of mutations in the HMBS gene can add valuable information to the interpretation of clinical, biochemical and genetic data in establishing a diagnosis of AIP.

Publication D - Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria *(Ulbrichova D et al., 2008)*

In this study, a total of 26 individuals from six unrelated Israeli AIP families of Caucasian origin underwent biochemical and mutational analysis in order to establish an AIP diagnosis. Variability with respect to the ALA/PBG levels and erythrocytic HMBS activity was found among the index patients. Each family carried a unique mutation in the HMBS gene.

Following the molecular screening of the HMBS gene, one novel heterozygous missense mutation c.95G>C (p.Arg32Pro) was shown to exist *de novo* in one family, along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA identified separately in each family.

We expressed p.Arg32Pro and p.Asp178Asn mutant proteins in the prokaryotic system, since the other missense mutations have been expressed and characterised in our previous study. We performed biochemical testing of the purified mutant enzymes. The structure-function consequences of all mutations were studied at the protein level.

Of the four missense mutations, p.Arg32Pro and p.Val215Met had not only detrimental effects on the enzyme *in vitro*, with residual activities of 1% and 19% respectively, but these mutations were also associated with high levels of ALA/PBG comparable with that of frameshift mutations c.730_731delCT and c.982_983delCA in patients. In addition, p.Val215Met was shown to be extremely thermo labile. Therefore we labelled them "strong" mutations. In contrast, the *in vitro* effect of both of the "weak" p.Thr59Ile and p.Asp178Asn mutations was much lower, as demonstrated by the relatively high residual activity of 81%. In accordance with this analysis, a common feature shared by these two patients with "weak" mutations was their normal or borderline levels of ALA/PBG although they presented characteristic clinical symptoms.

All six HMBS mutations were evaluated at the structural level based on the 3D structure of the *E. coli* enzyme. Based on the 3D structure, the two "strong" missense mutations as well as the two frameshift mutations were all predicted to have detrimental effects on the structure and function of the enzyme. The two "weak" mutations on the other hand were located at less critical positions and therefore exerted limited impact on the structure and function of the enzyme.

In summary, one novel heterozygous mutation c.95G>C (p.Arg32Pro), along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA, were identified separately in each family. We performed an extensive *in vitro* characterisation of the proteins with the introduced mutations. The results of the *in vitro* study broaden our understanding of the impact of individual mutations on enzyme activity.

Publication E - Acute intermittent porphyria - impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties *(Ulbrichova D et al., 2009)*

In the present study, six patients who were newly diagnosed with AIP were studied. The diagnosis of AIP was made on the basis of clinical features typical for AIP and the excretion pattern of porphyrin precursors. Overall, 33 individuals from their families were screened.

Denaturing gradient gel electrophoresis (DGGE) revealed six samples with abnormal patterns suggesting mutations were detected. Direct DNA sequencing revealed seven mutations in these samples. Of the identified mutations, three were novel, c.610C>A (p.Gln204Lys), c.750A>T (p.Glu250Asp) and c.675delA (p.Ala226ProfsX28); and four mutations were previously reported c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln) and c.771+1G>T (r.spl?). One patient had two mutations, c.[518G>A; 610C>A], located in the same allele.

To study the impact of the various mutations on the protein structure and subsequent functional consequences, mutated proteins were expressed in *E. coli* and the enzymatic properties were characterised. The residual enzymatic activity measurement of the HMBS proteins with mutant alleles revealed that, with the exception of the p.Gln204Lys mutation (which exhibited ~ 46% of wild-type activity), all mutations lead to little, if any, enzymatic activity. These findings further support the AIP-causality of these mutations in the HMBS gene. In the case of the patient with two combined mutations, both located on the same allele, mutation p.Arg173Gln has a severe effect on enzyme function. From the additional testing of the protein properties, we concluded that the p.Gln204Lys mutation has a milder impact on protein function and structure, but can still be associated with AIP.

To further determine the structure–function relationships for these mutations, the 3D structure of the *E. coli* and newly-determined human proteins, as well as the sequence alignment was used. From the structure and sequence information, it can be inferred that the patient's mutations of Arg26 to Cys or His may lead to the loss of interactions with the cofactor. In the case of the Arg173 to Gln mutation, the change results in an apo form of the

enzyme that is incapable of catalysis. In the case of the p.Gln204Lys mutant, the mutant enzyme exhibited ~ 46% of wild-type activity. This can be explained by the fact that the Gln204 residue is exposed on the surface of the central domain, remote from the active site of the protein. It is likely that the introduction of the positive charge of the lysine amino group changes the configuration of the two surface loops, which may destabilise the enzyme. The small deletion p.Ala226ProfsX28 causes a truncation which leads to an unstable and inactive protein. In mutant p.Glu250Asp, the Glu250 residue is conserved in all sequences with no exception. It creates an interaction that fixes the C-terminal domain to the interdomain hinge whose mobility is important for access of the substrate to the active site. In the case of such mutations as c.771+1G>T, the deletion of the entire exon 12 is expected, and the function of this mutant is expected to be completely abolished.

In summary, we identified four previously reported mutations c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln), c.771+1G>T (r.spl?); and three novel ones c.610C>A (p.Gln204Lys), c.675delA (p.Ala226ProfsX28), c.750A>T (p.Glu250Asp) in Czech AIP patients. Of particular interest, one patient had two mutations (c.518G>A; c.610C>A), both located in the same allele. We performed an extensive *in vitro* characterisation of the mutant proteins. These findings provided further insights into the causal relationship between HMBS mutations and AIP.

Publication F - Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis *(Ulbrichova Douderova D et al., in press in Anal Biochem 2009)*

In this report, we tested the high-resolution melting (HRM) procedure on the LightScanner instrument as a method of screening DNA variations in the polymorphic HMBS gene.

In the selected subjects tested, the diagnosis of porphyria was made based on the clinical features typical for each porphyria type and the related specific porphyrin excretion pattern. The samples were amplified in the presence of the saturating DNA dye LC-Green PLUS dye. Straight afterwards, the PCR plate was transferred to the LightScanner in which the HRM analyses were performed. Collected data were analysed with the commercial LightScanner software. Identified DNA variations were confirmed by sequencing.

To start off, we determined whether the presence of previously detected polymorphisms would adversely interfere with further testing of the gene using this method. In all four cases of polymorphisms consistently detected in our patients, we detected three discrete groups of genotypes (two homozygous and one heterozygous).

The ability of the HRM method to detect DNA variations in the HMBS gene was tested on DNA samples with ten known mutations using a curve shape scan generated with the LightScanner instrument. Each of the ten mutations tested had an altered melting profile compared to the melting profile of the controls. Even if the mutation was localised in the amplicon together with the other known polymorphisms, the mutation was identified correctly.

Finally, we evaluated the HRM method on the group of 97 subjects with suspected acute hepatic porphyria. From the DNA variations identified, three were previously described mutations: c.70G>A (p.Gly24Ser), c.87+5G>T (r.(spl?), c.[518G>A; 610C>A] (p.[Arg173Gln; Gln204Lys]), one was a novel mutation c.899_900delins TGCCTGCATCTG (p.His300LeuFsX10), two were previously described polymorphisms g.3119T/G (rs1006195) and g.7998G/A (rs1799997), and three were novel, rare DNA variations g.2922T>G, g.3059G>A and g.7175A>G (found in one subject). A rare, novel DNA variation, g.2922T>G, found in one subject with porphyria variegata suggests the possibility of a rare case of dual porphyria. This finding requires further investigation, since this variation is localised in the regulatory segment of the erythroid promoter of HMBS.

In summary, screening the group of subjects with suspected porphyria revealed nine different DNA variations, four of which were novel. HRM is a fast, cost-effective pre-screening method for detecting DNA variations in the HMBS gene. Moreover, the screening can be extrapolated to an entire family in the event of possible misdiagnosis or rare dual porphyria. We showed that the HRM method can serve as a useful screening tool to identify DNA variations, even in amplicons with other polymorphisms.

Publication G - Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation *(Ulbrichova Douderova D et al., prior to submission)*

In this case report, we present a latent AIP patient incidentally identified during clarification of a skin disorder that was eventually diagnosed as lichen sclerosus et atrophicus (LS). A cutaneous porphyria disorder was suspected based on skin problems of a 48-year-old female Swiss patient. Biochemical analyses failed to reveal any typical features of cutaneous porphyria. However, repeated measurement of erythrocytic HMBS activity revealed a ~ 50% reduction in enzyme activity and slightly increased urinary ALA and PBG, biochemical findings that are compatible with the latent status of AIP. The skin condition in this patient was subsequently diagnosed as LS by biopsy.

After molecular screening of the HMBS gene, the novel heterozygous substitution mutation c.601C>G in exon 10 was identified and the status of latent AIP was confirmed, since the patient had never experienced an acute attack. At the protein level, this missense mutation leads to a change of Arg201 amino acid residue to glycine residue (p.Arg201Gly).

The mutation p.Arg201Gly was subsequently expressed and the mutant protein was characterised *in vitro*. A residual enzymatic activity of 5.9% of wild-type was measured in the mutant enzyme. Moreover, compared to the wild-type enzyme, the mutant was extremely unstable when faced with heat treatment and exhibited a shift in the optimal pH.

In the 3D structure of human HMBS published recently, two lobes of the central domain are joined by ion-pairing between Arg201 and Asp178. Both of these amino acid residues are highly conserved among prokaryotic as well as eukaryotic HMBS enzyme sequences. Such high degree of conservation suggests that the ion pair plays an important role in the enzyme structure.

In summary, the novel mutation c.601C>G (p.Arg201Gly) was found in a Swiss patient with AIP. The result of the *in vitro* characterisation of the mutant suggested that the p.Arg201Gly mutation has a deleterious effect on the HMBS protein. This result is in accordance with the *in vivo* measurement of decreased erythrocytic HMBS activity. Thus, a latent status, so typical in this disorder, but hardly identifiable without family history, was granted in this patient, suffering simultaneously with lichen sclerosus et atrophicus, taking into account that she has so far not shown any symptoms of AIP.

Report of a novel mutation identification

Publication H - *Gene symbol: HMBS. Disease: Porphyria, acute intermittent* *(Ulbrichova D et al., 2008)*

Turkish AIP patient diagnosis was made based on clinical findings typical for AIP and almost half-normal (57.8%) erythrocytic HMBS activity. After gDNA isolation, we performed molecular screening in the HMBS gene by direct DNA sequencing. The DNA screening revealed a novel heterozygous mutation in exon 4, c.89T>G. At the protein level, this mutation results in the amino acid substitution of leucine to arginine, p.Leu30Arg.

We expressed the mutated gene in the prokaryotic system and performed biochemical testing of the purified enzyme. The purified mutant enzyme had a relative activity 0.03% of the average wild-type level.

In summary, the novel mutation c.89T>G (p.Leu30Arg) was found in a Turkish patient with AIP. This finding further confirmed the diagnosis at the molecular level.

Publication I - ***Gene symbol: HMBS. Disease: Porphyria, acute intermittent*** *(Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

The patient, female, was hospitalised while having her first acute attack characterised by severe abdominal pain and behavioural disturbances. From the biochemical measurements, the highly elevated level of porphyrin precursors was detected, a distinct peak in the fluorimetric plasma scan at 404/622 nm was identified and her erythrocytic HMBS activity was 71% of the normal value.

DNA sequence analyses revealed a small heterozygous insertion c.184_185insT within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Lys62IlefsX3 causes a frameshift and creates a stop codon after three completely different amino acids, resulting in a truncated protein of 64 amino acids. The effect of such a truncation is expected to be detrimental.

In summary, the novel mutation c.184_185insT (p.Lys62IlefsX3) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

Publication J - ***Gene symbol: HMBS. Disease: Porphyria, acute intermittent*** *(Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

The patient, a Ukrainian female, was diagnosed with AIP by a clinician while having clinical manifestations typical for acute hepatic porphyria. During an acute attack, the level of porphyrin precursors PBG and ALA were elevated (16.6 mg/100ml and 18.2 mg/100ml, respectively).

DNA sequence analysis revealed a novel heterozygous small insertion c.384_385insT in exon 8 within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Val130CysfsX80 causes a frameshift and creates a stop codon after eighty completely different amino acids resulting in a truncated protein. The effect of such truncation is expected to be detrimental.

In summary, the novel heterozygous mutation c.384_385insT (p.Val130CysfsX80) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

CONCLUSIONS

In the present study, the patients carrying mutation in the HMBS gene were characterised at the molecular level. The major achievements of the work in this thesis are the following:

- Twenty-eight DNA variations were identified in patients with AIP. Out of them, thirteen were novel mutations, ten were previously reported mutations, two were previously reported polymorphisms, and three were novel rare DNA variations, which require further investigation. Moreover, out of the novel mutations identified, two were *de novo* mutations, which are rare events in this disorder. To the six mutations known to exist in the Slavic population to date, another nine mutations were identified, broadening the molecular heterogeneity of the HMBS gene in our population.

- The comparison of the clinical manifestation of AIP patients disclosed the evidence of the variability with respect to the ALA/PBG levels and erythrocytic HMBS activity among the index patients. This clearly demonstrates that although biochemical measurements should be included as a first diagnostic step, the detection of the causal mutation in the HMBS gene is the ultimate diagnostic criteria for AIP.

- In order to improve molecular testing of the HMBS gene, a fast, cost-effective pre-screening method of high-resolution melting using the LightScanner instrument was established.

- Fourteen different proteins with introduced mutations were expressed in the prokaryotic system. These mutants were characterised at the biochemical level. Even though most of them exhibited a residual activity close to zero, some of them exhibited as high a residual activity as 81% that of wild-type. Only further characterisation allowed the association of these mutations with AIP. This demonstrates that *in vitro* expression of HMBS mutant genes and characterisation of their structure-function consequences can improve the interpretation of clinical, biochemical and genetic data and the diagnosis of AIP. Moreover, the results suggest

that there is more than one causative factor with clinical relevance, which remains undiscovered.

- Based on the identification of the causal mutation in the HMBS gene of the index patient, appropriate genetic counselling based on the DNA diagnostics was applied within the AIP affected families.

Future challenges

One of the unknown aspects in the pathogenesis of AIP is the mechanism of incomplete penetrance and high variability of the clinical manifestation among the porphyria patients. According to the literature, only 10-20% of the mutation carriers will ever develop clinical symptoms. This suggests that the genetic background of individuals may explain differences in susceptibility to an acute attack, though the genes involved have not yet been identified. Future studies may be focused on the identification of such modifier genes which influence the susceptibility of patients with acute hepatic porphyrias to acute attack.

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The thesis is based on the following publications:

Ulbrichova D, Flachsova E, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. 2006. *De Novo* mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study. Physiol Res 55 Suppl 2:S145-54. (IF2006 2.09)

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Report of the novel mutation identification

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Kavan D, Vancurova M, Ulbrichova D, Hladikova I, Pospisil M, and Bezouska K. 2004. Identification of heparin-binding sites in the fibronectin type III domains of the leukocyte common antigen (CD45). Collect Czech Chem Commun 69 (IF2004 1.06)