Univerzita Karlova v Praze

1. lékařská fakulta

Charles University in Prague

First Faculty of Medicine

Autoreferát disertační práce

PhD thesis summary

Cytochrome P450 oxidoreductase: Structurally functional study
Molecular pathology of Antley-Bixler syndrome

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2015

Postgraduate studies in Biomedicine

Charles University in Prague and Academy of Sciences of the Czech Republic

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The full text of the thesis will be available at least five days before the PhD. defense at the Department of Science and Research and International Relations of the 1st Faculty of Medicine, Charles University in Prague.

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ABSTRAKT

NADPH-P450 oxidoreduktáza (POR) je membránový flavoprotein, který transportuje elektrony na široké spektrum hemoproteinů, řada z nich hraje podstatnou úlohu v metabolizmu xenobiotik a steroidů. Vzhledem k nezastupitelné roli POR v metabolismu léků je v posledních letech pozorován zvýšený zájem o analýzu genu *POR,* zejména v oblasti farmakogenomiky. Mutace v genu *POR* způsobují onemocnění, které souborně nazýváme POR deficience. Toto onemocnění se projevuje širokým spektrem fenotypových projevů sahajících od poruch steroidogenéze až po kosterní malformace známé jako Antley-Bixlerův syndrom (ABS). Cílem předložené práce je analýza genu *POR* u pacientů s podezřením na POR deficienci a poprvé v běžné české a židovské populaci. Analyzovali jsme 644 alel nepříbuzných českých jedinců a 1128 alel v populaci židovské, 330 alel Aškenázů, 798 alel Sefardů. Následně byl studován vliv vybraných nových genetických variant na aktivitu POR proteinu. Definovali jsme frekvence alel genu *POR* v obou populacích, popsali jsme 14 nových variant vedoucích k aminokyselinovým záměnám POR proteinu a prozkoumali jsme dvě z nich s cílem určit jejich vliv na aktivitu proteinu. Dostupnost struktury lidské POR umožnila modelování nově popsaných variant a popis defektu na molekulární úrovni. Analýza genu *POR* byla následně v laboratoři zavedena do rutinního vyšetření a jsme v České republice jedinou laboratoří, která v současné době poskytuje diagnostiku POR deficience.

Klíčová slova: CYP oxidoreduktáza; frekvence alel; Česká populace; Židovská populace, POR; farmakogenetika; syndrom Antley-Bixler

ABSTRACT

NADPH–P450 oxidoreductase (POR) is a membrane bound flavoprotein that donates electrons to a wide spectrum of heme-containing proteins, among which are several steroidogenic and many xenobiotics-metabolizing enzymes. Given the important role of POR protein in drug metabolism and pharmacogenomics, there is a particular need to understand the contributions of POR genetic variants to these processes. Mutations in *POR* gene cause a disorder called POR deficiency, which manifests with a wide phenotypic spectrum ranging from disordered steroidogenesis to skeletal malformation, namely, Antley-Bixler syndrome (ABS). The aim of the present work was to investigate the *POR* gene in patients suspected to have POR deficiency syndrome from Czech Republic and to perform genotyping in Czech and Jewish control populations. We analyzed 644 alleles in unrelated individuals from the general Czech population and 1128 alleles in Jewish population, where 330 alleles were of Askhenazi and 798 of Sephardic Jews. We have also studied the impact of selected new genetic variants on POR activity and identified fourteen amino acid variations, two of which we have studied in detail to establish their influence on POR activity. Using the available human POR three-dimensional structure, we then modelled the newly identified variants to describe these defects at the molecular level. Through this study, notably, we have systematically performed analysis of the *POR* gene and are providing POR deficiency diagnostics - the only laboratory in Czech Republic to provide this service. In conclusion, we have, for the first time, defined *POR* allele frequencies in the Czech and Jewish populations that have 14 novel amino acid variations of which two variations studied in detail impinge upon POR activity.

KEYWORDS: CYP oxidoreductase; allele frequencies; Czech Slavic population; Jewish population, *POR*; pharmacogenetics; Antley-Bixler syndrome

INTRODUCTION

NADPH-cytochrome P450 oxidoreductase (POR)

Human NADPH-cytochrome P450 oxidoreductase (POR) is a membrane bound flavoprotein that donates electrons to a wide spectrum of heme-containing enzymes. It belongs to a small group of proteins containing two flavins, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide). It is located on the outer cytosolic side of endoplasmic reticulum. Flavin cofactors are essential for POR function and they serve as a bridge trough which electrons channel from nicotinamide adenine dinucleotide phosphate (NADPH) to metal ion centers of electron acceptors. Electron flow within POR protein starts at NADPH, goes to FAD and FMN and then to heme-containing electron acceptors (Kurzban and Strobel, 1986). The electron flow through the POR electron transport assembly is based on the redox properties of the flavin cofactors (Munro et al., 2001). In metabolism, POR plays an important role in oxidative clearance of endogenous and exogenous compounds. By interacting with microsomally located cytochromes P450 (CYP), POR participates in xenobiotic and drug metabolism and steroidogenesis. By other biochemical pathways, it takes part in metabolism of prostaglandins and fatty acids. Additionally, it was shown that POR also directly activates the anticancer prodrug Mitomycin C (Wang et al., 2007). Mutations in *POR* cause disease called POR deficiency (Miller et al., 2005).

POR genetics

Human POR is encoded by a single gene, which is located on the long arm of the chromosome 7 (Shephard et al., 1989). The *POR* gene contains 15 coding exons. In 2007, a first untranslated exon that lies 38.8 kb upstream the first exon, was described (Scott et al., 2007). Thus the human *POR* gene contains 16 exons spanning 73.2 kb. More than 40 different disease causing *POR* mutations have been identified to date, including missense mutations, frameshift mutations, deletions and splicing errors. First mutations were described in 2004 (Adachi et al., 2004; Arlt et al., 2004; Fluck et al., 2004). The most common mutation in the European population is p.Ala287Pro, while in Japanese population it is p.Arg457His (Adachi et al., 2004; Arlt et al., 2004; Fukami et al., 2005; Huang et al., 2005) and these mutations are by far the most frequent mutations in POR deficiency patients. With a raising number of sequence variants found in the *POR* gene, the question of whether these variants might contribute to inter-individual variations in drug response (both drug safety and efficacy) is being asked more and more often.

POR protein

Human POR is a 78 kDalton, 680 amino acid protein. Sequence of 25 amino acids in the N-terminal part of the protein determined the microsomal localization of the protein and its linkage to the membrane (Wang et al., 1997). The first three dimensional structure of rat POR molecule was published by Wang *et al.* (Wang et al., 1997). Structure revealed that molecule is composed of four structural domains. Atomic structures of the entire soluble human wild-type POR confirmed this findings (Xia et al., 2011). POR protein has a dynamic nature, FMN containing domain motion is required to enable the electron flow from POR to its protein partner (Ellis et al., 2009).

POR studies

Initial studies of *POR* variants focused on catalytic assays with steroid metabolizing CYPs (Fluck et al., 2007; Fluck et al., 2004; Huang et al., 2008; Huang et al., 2005), but lately interest has been on drug metabolizing enzymes (Agrawal et al., 2008; Agrawal et al., 2010; Fluck et al., 2010; Sandee et al., 2010) and several *in vivo* studies have been performed (de Jonge et al., 2011; Oneda et al., 2009; Tomalik-Scharte et al., 2010; Zhang et al., 2011). With a growing number of POR assays becoming available, it has been demonstrated that the catalytic ability of one POR mutant with a particular CYP cannot predict its catalytic ability with another CYP (Agrawal et al., 2010; Sandee et al., 2010), or with another redox partner such as heme oxygenase (Marohnic et al., 2011). POR function may also vary with CYP isoform (Subramanian et al., 2012) and specific substrate metabolized (Agrawal et al., 2010; Sandee et al., 2010). Thus every single mutant must be individually assayed with the specific CYP of interest combined with the unique investigated substrate. Six population genetic studies addressing the distribution of *POR* genetic differences within various populations have been performed (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011; Tomkova et al., 2012; Tomkova et al., 2015).

POR deficiency syndrome

P450 oxidoreductase deficiency (POR deficiency) is a recently described disorder caused by mutations in the *POR* gene (Miller, 2004). It comprises wide spectrum of clinical manifestations including skeletal malformations referred to as Antley-Bixler syndrome (ABS), disorders of sex development, pubertal failure, adrenal dysfunction, and maternal virilization during pregnancy. Phenotype manifestations of different *POR* mutations vary. Genetic changes in POR modulate

activity of its partners, microsomal cytochrome P450s, to different degrees (Sandee et al., 2010), apparently explaining the great variability in the clinical findings of POR deficiency patients. Clinical spectrum of PORD phenotypes ranges from mildly affected individuals with polycystic ovary to severely affected children with ambiguous genitalia and Antley Bixler syndrome (Miller, 2004).

Because of a broad range of clinical manifestations, the diagnosis of POR deficiency is not straightforward and it is assumed that there is large number of under-diagnosed patients. The POR deficiency diagnosis may be considered from clinical and biochemical findings, but always requires confirmation by genetic analysis. Prenatal diagnosis is possible from ultrasound findings and amniotic fluid or urine of a mother (Shackleton et al., 2004). The urine steroid profile of the mother carrying a POR deficiency fetus is characteristic for low estriol, increased epiallopregnanediol disulfate and increased aldosterone (Reisch et al., 2013). Pregnancy complications may include also maternal virilization with an onset in second trimester (Reisch et al., 2013).

AIMS OF THE STUDY

The general aim of the present work was to investigate gene *POR* in selected patients and in Czech and Jewish general population and to study the impact of described polymorphisms on POR protein.

Specific aims of the study:

1. To establish molecular analysis of a *POR* gene in our laboratory and to provide a possibility of a molecular genetic diagnostic for patients suspected to have POR deficiency

2. To look for genetic variations in *POR* gene in general Czech and Jewish population and to establish *POR* allele frequencies in these populations

3. To suggest *POR* genetic variants that could play a role as a bio-markers in pharmacogenetics

4. To investigate *POR* gene in patients suspected to have POR deficiency

5. To address the pathology of found mutations

6. To establish a molecular analysis of a *PGRCM1* gene in our laboratory and to study *PGRMC1* gene in selected patients

MATERIAL AND METHODS

DNA Samples

Patients

Ten patients (7 males and 3 females) with skeletal deformities were investigated for the mutations in the *POR* gene.

Healthy controls

The study enrolled a total of 886 healthy controls, including 564 individuals from Jewish population and 322 individuals from Czech population. DNA samples from Jewish population were obtained from the collection of The National Laboratory for Genetics of Israeli Populations at Tel Aviv University (Tel Aviv, Israel). All samples in the study group were from healthy unrelated adult donors who have self-identified their ethnicity as Ashkenazi (AJ, 35 males and 130 females), Bulgarian (BJ, 59), Ethiopian (EJ, 19), Moroccan (MJ, 57 males and 79 females), Turkey (TJ, 68) and Yemenite Jews (YJ, 117). In BJ, EJ, TJ and YJ groups a gender was not provided. DNA samples from Czech population (CZ 144 males and 178 females) were obtained from both adults and children. 227 DNA samples of adults were acquired during the longitudinal collection of control samples of healthy individuals from Czech population in our laboratory. DNA of 95 healthy neonates was extracted from cord blood.

DNA extraction

Genomic DNA was extracted from peripheral blood sample anticoagulated with EDTA according to a standard protocol. Alternatively, when only a small amount of blood (1-2 ml) was available, QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) was used according to manufacturer´s protocol. Cord blood samples were collected from the placental part of the

umbilical cord immediately after the delivery of the child and cord blood DNA was extracted according to the previously described techniques (Pejznochova et al., 2008).

Ad 1) and 6) DNA analysis

POR gene was analyzed by sequencing and a new generation amplicon high-resolution melting (HRM) analysis. Samples were sequenced on an automatic sequencer ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and an automatic sequencer ABI PRISM 3100-Avant Genetic analyzer (Applied Biosystems). Four *POR* exons were analyzed on the Light Scanner (Idaho Technology). *PGRMC1* gene was analyzed by direct sequencing. All primers used for *POR* and *PGRMC1* analyzes were newly designed.

Long Range PCR and cloning

To identify, whether two mutations (p.Val164Met and p.Glu398Ala) found in the one DNA samples are localized on the one or two different alleles, we amplified a fragment containing these mutations, spanning from exon 4 to exon 10. To do so, we used Expand Long Template (LT) PCR system (Roche, Mannheim, Germany). Fragment with mutations was ligated into the pCR4-TOPO vector from the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA, USA) and then transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Plasmids from ten different colonies were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using the universal T7 and T3 primers.

Purification of PCR products

Post-PCR clean-up for sequencing was performed by ExoSap cleanup method.

Site directed mutagenesis

Vector for mutagenesis and further expression of the full-length POR were kindly provided by prof. Bettie Sue Masters from UT Health Science Centrum at San Antonio (Texas). Site-directed mutagenesis was performed to generate desired mutations using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol with the designed mutagenic primers.

Protein expression

Wild type POR protein and two POR variants (p.Val164Met and p.Pro384Leu) were expressed in *E. coli* strain BL21 (Stratagene). After an overnight incubation at 37°C, cells were

induced by addition of 0.4 isopropyl 1-thio-β-D-galactopyranoside (Fisher HC, Houston, TX, USA) followed by further overnight incubation in TB media containing 100 μM riboflavin and 125 μM ampicillin at 28°C with 160 rpm shaking. Cells were harvested by centrifugation at 4°C for 15 minutes at 6000 g.

Protein purification

Harvested cells were lyzed using lyzozime and by sonification. Soluble and solid fractions were separated by ultracentrifugation (Beckman J25, Brea, CA, USA) for 1 h at 100 000 x g. Pellets acquired after centrifugation were homogenized using dounce homogenizer. The detergent-solubilized protein fraction was cleared from cellular debris by ultracentrifugation for 1 h at 100 000 x g and the supernatant was applied to 2', 5'- ADP – Sepharose 4B (GE Healthcare).

Flavin content analysis

Flavin separation and quantification was performed by high-performance liquid chromatography (HPLC) using a Waters Corporation (Milford, MA, USA) analytical HPLC system, equipped with a 2487 absorbance detector.

Cytochrome c assay

The ability of p.Val164Met and p.Pro384Leu POR variants to reduce cytochrome c was measured by determining the absorption rate at 550 nm using the extinction coefficient, $\varepsilon = 21$ $mM^{-1}cm^{-1}$. All measurements were carried out in triplicate in 96-well plate using VERSA maxmicroplate reader (Molecular Devices, Sunnyvale, CA, USA).

RESULTS

Ad 2) POR genetic variations in Czech and Jewish general population

Czech population

In the Czech population, a total of 25 *POR* SNP genetic variations (table 1) were identified. Four of them were found in 5´flanking region of the gene, 7 in introns and 14 in the exons. From the 14 variations found in exons, 7 were synonymous, 6 were missense SNPs and one substitution

was localized in the first untranslated exon not coding for any amino acid. Of the 25 found variants, 7 were new SNPs, not described previously, including two SNPs in the 5´flanking region (g.4965 C>T and g.4994 G>T), one intronic variant (c.1899 -20C>T), one synonymous SNP (p. 20Ala=) and three nonsynonymous SNPs (p.Thr29Ser, p.Pro384Leu and p.Thr529Met). All 7 SNPs were found as individual heterozygotes at allele frequencies 0. 002. Described SNPs are summarized in table 1.

Jewish population

Analyzing of the *POR* gene in the Jewish population showed a total of 38 different *POR* genetic variations. We described 4 genetic changes in 5´-flanking region, 7 intronic and 27 exonic variants. Sixteen of the 27 exonic genetic variations changed the encoded amino acid. We detected 17 previously uncharacterized genetic changes, two in the 5´-flanking region (g.5003A>C and g.5078G>A), one in the intronic region (c.189 -10T>C), three silent changes (p.143Tyr=, p.248Tyr= and p.Ser400=) and 11 missense genetic changes (p.Thr64Ile, p.Val85Met, p.Ser102Pro, p.Val164Met, p.Val191Met, p.Asp344Asn, p.Glu398Ala, p.Val472Leu, p.Arg550Gln, p.Arg570His and p.Asp648Asn). Variants found in the Jewish cohort are summarized in the table 1.

Comparison of Czech and Jews populations

Twenty-three of the polymorphisms described in the investigated cohorts were population-specific, found in only one ethnic group (for this statement we excluded SNPs found in the proximal promoter and exon 1U since they were not analyzed in all studied groups). Most of them (18) were previously undescribed SNPs. None of the known *POR* amino acid variants causing disordered steroidogenesis or ABS were found in our cohort (Arlt et al., 2004; Fluck et al., 2011; Fluck et al., 2004; Fukami et al., 2005; Huang et al., 2005; Sahakitrungruang et al., 2009).

Described allele frequencies of common SNPs were basically similar to frequencies found in previous studies (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011). Comparison of allele frequencies between Czech and Jewish populations for seven common SNPs (rs1135612, rs4732516, rs2286822, rs2286823, rs2228104, rs1057868, rs1057870) showed statistically significant differences ($p<0.05$) in two cases (rs4732516 and rs1057870). Another relatively frequent SNP, rs10262966, was surprisingly found in 32 samples from Jewish population, but was not described in any sample from Czech population. The statistical comparison

of the allele frequencies of this SNPs between observed populations showed high statistically significant difference ($p<0.001$).

Table 1. **Allele frequencies of the *POR* gene in Czech and Jewish populations.** New *POR* variants in red.

EXON	g.	c.	p.	SNP ID	CZ (644)	AJ (330)	BJ (118)	EJ (38)	MJ (272)	TJ (136)	YJ (234)
5´-Flanking	4849 C>A			rs72553972	0,175	-	-	-	-	-	-
5´-Flanking	4883 C>T			rs139824475	0,005	-	-	-	-	-	-
5´-Flanking	4965 C>T				0,002	-	-	-	-	-	-
5´-Flanking	4994 G>T				0,002	-	-	-	-	-	-
Exon 1U	5003 A>C				0	0,003	-	-	0	-	-
Exon 1U	5036 A>C			rs3823884	0,247	0,224	-	-	0,221	-	-
Exon 1U	5050 G>T			rs72553977	0	0	-	-	0,018	-	-
Exon 1U	5078 G>A				0	0,006	-	-	0	-	-
Intron 1U	5099 C>T			rs72553978	0,008	-	-	-	-	-	-
Exon 1	43906 A>G	15 A>G	Gly5=	rs10262966	0	0,012	0	0,158	0,037	0,044	0,026
Exon 1	43976 A>T	85 A>T	Thr29Ser		0,002	0	0	0	0	0	0
Exon 1	43978 G>A	87 G>A	Thr29=	rs41295381	0	0,003	0	0,026	0,011	0,015	0
Intron 1	62302 T>C	189 -10 T>C			0	0,003	0	0	0,004	0	0
Exon 2	62314 C>T	191 C>T	Thr64Ile		0	0	0	0	0	0	0,004
Exon 3	69365 G>A	253 G>A	Val85Met		0	0	0	0,026	0	0	0
Exon 3	69416 T>C	304 T>C	Ser102Pro		0	0,003	0	0	0	0	0
Exon 4	69456 C>T	344 C>T	Ala115Val		0	0	0,008	0,079	0,004	0	0,004
Exon 4	70240 C>T	369 C>T	Ala123=	rs41299490	0	0	0,008	0,079	0	0	0,004
Exon 4	70258 A>G	387 A>G	Pro129=	rs1135612	0,284	0,318	0,246	0,237	0,360	0,184	0,432
Exon 4	70300 C>T	429 C>T	Tyr143=		0	0	0	0	0,015	0	0
Exon 4	70361 G>A	490 G>A	Val164Met		0	0	0	0	0,004	0	0
EXON	**g.**	**c.**	**p.**	**SNP ID**	**CZ (644)**	**AJ (330)**	**BJ (118)**	**EJ (38)**	**MJ (272)**	**TJ (136)**	**YJ (234)**
Intron 4	70943 G>A	517 -4 G>A		rs41299496	0,002	0	0	0,053	0,000	0	0
Exon 5	71001 G>A	571 G>A	Val191Met		0	0,003	0	0	0	0	0
Intron 5	71411 C>G	642 -5C>G		rs72555509	0	0,009	0	0,026	0,007	0,015	0,013
Exon 6	71457 C>T	683 C>T	Pro228Leu	rs17853284	0	0,003	0	0	0	0,007	0
Exon 6	71461 C>T	687 C>T	Ala229=	rs72557906	0	0,003	0	0	0	0,007	0
Exon 7	72135 C>T	744 C>T	Tyr248=		0	0	0	0	0,004	0	0,004
Exon 8	73486 G>A	898 G>A	Glu300Lys	rs11540674	0	0,006	0,008	0	0	0	0
Exon 9	73673 C>T	984 C>T	Ala328=	rs72557941	0,011	0	0	0	0	0	0
Exon 9	73718 C>T	1029 C>T	Ala343=	rs72557942	0	0	0	0	0,004	0	0
Exon 9	73719 G>A	1030 G>A	Asp344Asn		0	0	0	0	0,004	0	0

Intron 9	74663 C>G	1067 -13C>G		rs4732516	0,984	0,991	0,992	0,737	0,952	0,956	0,970
Exon 10	74721 G>A	1112 G/A	Arg371His		0,002	0	0	0	0	0	0
Exon 10	74760 C>T	1151 C>T	Pro384Leu		0,002	0	0	0	0	0	0
Exon 10	74800 G>A	1191 G>A	Ser397=	rs72557928	0,002	0	0	0	0	0	0
Exon 10	74802 A>C	1193 A>C	Glu398Ala		0	0	0	0	0,004	0	0
Exon 10	74809 G>A	1200 G>A	Ser400=		0,002	0	0,008	0	0,004	0	0,004
Intron 10	74869 C>T	1248 +12C>T		rs2286822	0,345	0,385	0,339	0,421	0,393	0,257	0,483
Intron 10	74877 G>A	1248 +20G>A		rs2286823	0,343	0,382	0,331	0,421	0,393	0,257	0,483
Intron 10	74946 G>A	1249 -11G>A			0	0	0,008	0	0	0	0
Exon 12	75493 G>C	1414 G>C	Val472Leu		0	0	0	0	0	0	0,004
Exon 12	75534 T>C	1455 T>C	Ala485=	rs2228104	0,981	0,991	1,000	0,868	0,952	0,956	0,974
Exon 12	75587 C>T	1508 C>T	Ala503Val	rs1057868	0,269	0,294	0,381	0,132	0,206	0,346	0,286
Exon 12	75665 C>T	1586 C>T	Thr529Met		0,002	0	0	0	0	0	0
Exon 12	75728 G>A	1649 G>A	Arg550Gln		0	0	0	0	0	0	0,004
Exon 13	75861 G>A	1709 G>A	Arg570His		0	0	0	0	0	0,007	0
Exon 13	75868 G>A	1716 G>A	Ser572=	rs1057870	0,345	0,306	0,271	0,079	0,335	0,353	0,188
Exon 14	76133 G>A	1891 G>A	Val631Ile	rs145782750	0,002	0	0,008	0	0,004	0,007	0
Intron 14	76153 G>T	1898 +13G>T		rs72557956	0,002	0	0	0	0	0	0
Intron 14	76216 C>T	1899 -20C>T			0,002	0	0	0	0	0	0
Exon 15	76279 G>A	1942 G>A	Asp648Asn		0	0,003	0	0	0	0	0

Ad 4) Investigation of POR gene in patients suspected of POR deficiency

Direct sequencing of the *POR* gene in patients with skeletal deformities did not reveal any molecular genetic defect.

Ad 5) Addressing the pathology of the found variants

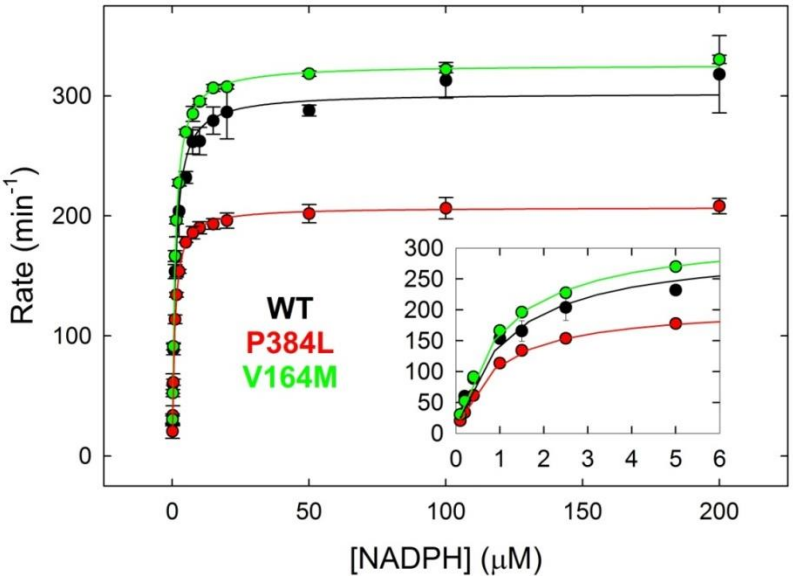
Allele localization

We have identified that two variants (p.Val164Met and p.Pro384Leu) identified in one sample were located on two different alleles, thus the individual is composed heterozygote for the two *POR* SNPs.

Kinetic analysis of purified full length variants

In cytochrome c assay, the p.Pro384Leu variant retained ~65% of WT turnover with no significant effect on K_m^{NADPH} (WT = 1 μM and p.Pro384Leu = 0.9 μM), while the p.Val164Met variant showed modest increase in its activity (~110% of WT turnover) (figure 1).

Figure 1. Cytochrome c assay.



Full length POR flavin content

The HPLC analysis showed that the wild-type enzyme and both new variant proteins had the full complement of FAD and FMN and FAD:FMN:protein ratio is 1:1:1.

Ad 6) Investigation of PGRMC1 gene in patients suspected of POR deficiency

We have analyzed the *PGRMC1* gene in 7 of 10 patients with skeletal deformities. None of them showed to harbor any *PGRMC1* genetic variations.

DISCUSSION

Ad 1), 2) and 3) POR genetic variations in Czech and Jewish general population

POR deficiency is a relatively newly described disorder (Miller et al., 2005). In our laboratory, we introduced the analysis of *POR* gene and currently we are the only laboratory in the Czech Republic that implements POR deficiency diagnostics. Furthermore, by population genetic analysis of *POR* gene, we provided valuable pharmacogenetics data. POR is the unique electron donor to all microsomal CYPs. It is known that CYPs from families 1 to 3 mediate 80-90% of all phase I-dependent metabolism of drugs used clinically (Bertz and Granneman, 1997; Evans and

Relling, 1999), therefore, contribution of common *POR* variants to inter- and intra-individual variability is of great pharmacogenetic interest.

A total of 51 SNPs were identified in 886 individuals. The present work confirmed several of the already reported *POR* SNPs in both, Czech and Jewish populations. Allele frequencies of the observed variants were generally similar to published data where available. We observed the striking difference in allele frequency only in two cases. We have not detected any rs10262966 in Czech population, but it has been described in Caucasian population in all previous studies with frequencies between 0.007-0.045 (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008). Further, present also in Czech population, we have reported relatively high frequency of rs72557941 (more than 1%), which was described in previous studies only by Gomes *et al.* (Gomes et al., 2009) with the frequency of 0.007.

We have identified a total of 20 genetic changes causing amino acid substitution, six of which were known genetic variants and 14 were newly described. All of them were found as individual heterozygotes. By far the most common polymorphism changing amino acid was the variant p.Ala503Val. The other five known amino acid changes (p.Ala115Val, p.Pro228Leu, p.Glu300Lys, p.Arg371His and p.Val631Ile) were found much less frequently. Recombinant expression and analysis of the p.Ala115Val mutant activity showed complete loss of function with CYP1A2 and CYP2C19 drug-metabolizing enzymes (Agrawal et al., 2008), whereas the activities for the steroid-metabolizing CYPs and for drug-metabolizing CYP3A4 were not affected dramatically (Fluck et al., 2010). We found the p.Ala115Val variant in the Jewish population with the frequency distribution of 0.8-7.9%, but not in the Czech population. Likewise the variant p.Ala115Val also the *POR* variant p.Pro228Leu (first described on the BioVentures Website) exhibits significantly decreased support of CYP1A2- and CYP2C19-mediated activities, but do not appear to substantially affect steroid–metabolizing P450s either CYP3A4 (Agrawal et al., 2008; Fluck et al., 2010). In our study we found this genetic change in two Jewish individuals. The third variant p.Glu300Lys was described in three Jewish subjects, but again not in the Czech population. This variant has according to Agrawal *et al.* (Agrawal et al., 2008) practically no effect on CYP activity, but further biochemical essays should be done for better biochemical characterization. Amino acid variant p.Val631Ile, located near NADPH binding site, had in CYP17A1 and CYP3A4 assays relatively mild effect on POR activity (Fluck et al., 2010) and had low but measurable activity (6–41% of wild-type) with both CYP1A2 and CYP2C19 (Agrawal et

al., 2008). All four described variants were not associated with POR deficiency, but as seen three of them (p.Ala115Val, p.Pro228Leu and p.Val631Ile) severely decrease the activity of some drug-metabolizing CYPs (Agrawal et al., 2008), thus we think these *POR* missense variants may be potential biomarkers for future POR pharmacogenetics screening. Impact of the amino acid change p.Arg371His on the POR function has not been studied yet. For further investigation, allele frequency data from a larger population will inevitably be required.

All together, we have identified four genetic variants in the proximal promoter region. None of them lay in the critical SP1 binding sites, but one of the described common polymorphism, rs72553972 (-152), was shown previously to significantly reduce *POR* transcription (Tee et al., 2011).

Ad 4) Investigation of *POR* gene in patients suspected to have POR deficiency

In our cohort of patients with skeletal deformities, we have not identified any of the *POR* genetic defects. Negative results might include misdiagnosis or so called ″false negativity″ when the disease is not determined with available laboratory methods. Since in our study we analyzed only exons and adjacent intronic regions, it is not excluded that *POR* mutations are hidden somewhere in deep intronic regions.

Ad 5) Addressing the pathology of the found mutations

All protein expression and purification steps and also the biochemical assays were carried out in the laboratory of professor Bettie Sue Siler Masters at UT Health Science Centrum at San Antonio (Texas). In time of my staying in Texas, we had discovered only two of the 14 new *POR* SNPs, therefore, up to date we have had expressed only two POR variants. In the future we are planning to expresses and purify all the rest of the new *POR* polymorphisms.

To study the impact of the p.Val164Met and p.Pro384Leu genetic variants on protein structure and functional consequences, mutated proteins were expressed in *E. coli* and enzymatic properties were characterized. The classical assay of POR activity is the measuring of cytochrome c reduction. Cytochrome c assay revealed that the p.Pro384Leu substitution reduced the POR activity to ~65% of WT, while the p.Val164Met slightly increased its activity to ~110% of WT.

Ad 6) Investigation of *PGRMC1* gene in patients suspected of POR deficiency

We have done *PGRMC1* gene analysis in 7 patients with skeletal deformities, but none of them carried any *PGRMC1* genetic change. There can be several reasons for the negative results; some of them I already mentioned. In spite of the negative results I consider it important to further investigate this gene in patients with disordered steroidogenesis.

CONCLUSION

Human NADPH-cytochrome P450 oxidoreductase is a protein of high importance since it affects wide spectrum of hem-containing proteins, including several steroidogenic and most drug metabolizing enzymes. In recent years, especially pharmacogenomics is raising its interest in POR genetic variants studies. Current work provides valuable data that may help to highlight some important questions regarding *POR* sequence differences among various populations, POR function and role of the POR protein in pharmacogenomics. Main achievements of present work are:

- We introduced the analysis of *POR and PGRMC1* genes. Currently we are the only laboratory in the Czech Republic that provides the POR deficiency diagnostics and speeches given at seminars and conferences helped to familiarize Czech doctors with this relatively new described disorder.

- We determined the allele frequencies of a *POR* gene in the, so far unstudied, Czech and Jewish populations.

- We described 14 novel *POR* amino acid variations, five of which are already reported on the official website of *POR* polymorphisms.

- We examined the effect on POR activity of two newly described *POR* variants. Reduction in the activity of p.Pro384Leu change provides evidence that some of the uncommon *POR* variants can potentially alter enzyme activity without causing POR deficiency.

- In the studied populations, we indicated *POR* variants that might play a role in altered drug metabolism.

For further investigation, it is important to investigate *POR* gene deeper intronic sequences, especially in patients suspected to have POR deficiency syndrome, and to augment the general population cohort.

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LIST OF ORIGINAL PUBLICATIONS

Publications *in extenso* related to the thesis:

Tomková M., Panda SP., Šeda O., Baxová A., Hůlková M., Siler Masters BS., Martásek P. *Genetic variations in NADPH-CYP450 oxidoreductase in a Czech Slavic cohort*. Pharmacogenomics, 2015 Mar; 16(3):205-15.

Impact factor: 3.425

Tomková M., Marohnic CC., Gurwitz D., Seda O., Masters BSS., Martásek P. *Identification of six novel P450 oxidoreductase missense variants in Ashkenazi and Moroccan Jewish populations*. Pharmacogenomics**,** 2012 Apr; 13(5): 543-54.

Impact factor: 3.857

Rewiev:

Tomková M., Marohnic C.C., Baxová A., Martásek P. *Antley Bixler syndrome or POR deficiency?* Cas. Lek. Cesk. 147, 2008, 261-265.

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