Molecular Genetic Background of an Autosomal Dominant Hypercholesterolemia in the Czech Republic

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Summary
Autosomal dominant hypercholesterolemia (ADH), more known as familial hypercholesterolemia (FH), is a lipid metabolism disorder characterized by an elevation in low-density lipoprotein cholesterol (LDL-C) and increased risk for cardiovascular disease. In this study, we assessed a spectrum of mutations causing ADH in 3914 unrelated Czech patients with clinical diagnosis of hypercholesterolemia. Samples have been collected within the framework of the MedPed project running in the Czech Republic since 1998. So far we have found 432 patients (11.0 %) with the APOB gene mutation p.(Arg3527Gln) and 864 patients (22.1 %) with the LDLR gene mutation. In 864 probands carrying the LDLR gene mutation, 182 unique allelic variants were detected. We have identified 14 patients homozygous for mutations in the LDLR or APOB genes. We performed function analyses of p.(Leu15Pro) and p.(Gly20Arg) sequence variations.

Key words
Autosomal dominant hypercholesterolemia • LDLR • APOB • LDL cholesterol • MedPed

Introduction
Autosomal dominant hypercholesterolemia (ADH), more known as familial hypercholesterolemia (FH), is a lipid metabolism disorder that results in both total and low-density lipoprotein cholesterol (LDLc) elevation and increased risk for cardiovascular disease. Pathogenic variants in the three genes – LDL receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin/kexin type 9 (PCSK9) have been established as causatives of the disease (Chiu and Charng 2010). Recently, mutations in signal transducing adaptor family member 1 gene (STAP1) (Fouchier et al. 2014) and several other genes (Santos et al. 2016) were described in association with ADH. Recent studies in unselected general populations suggest that the prevalence of heterozygous autosomal dominant hypercholesterolemia may be as high as 1 in 200 (Nordestgaard et al. 2013, Sjouke et al. 2015). Consequently, homozygous autosomal dominant hypercholesterolemia may affect as many as 1 in 160,000-300,000 people (Cuchel et al. 2014).

In 1998, the MedPed (Make Early Diagnosis for prevent Early Deaths in medical pedigrees; http://www.medped.org/) project was established in the Czech Republic. This international humanitarian nonprofit project was founded in the USA. The goal of this project is to prevent premature deaths from ischemic cardiovascular
events by finding high risk individuals with ADH who are either undiagnosed or inadequately treated and by consequent identification of their affected relatives.

Development of molecular diagnostics

Our cohort of unrelated patients referred to molecular diagnostics included 1) patients with untreated total and/or LDL cholesterol serum levels above the 95th percentile of age, sex and population-specific values (Čífková R, personal communication, Šamánek et al. 1997); 2) patients with elevated total and/or LDL cholesterol in serum but untreated levels unavailable or not exceeding the 95th percentile of age, sex and population-specific values, and, in addition, having high clinical suspicion of ADH based on personal history and/or family history of premature coronary heart disease and/or elevated total and LDL cholesterol serum levels in first degree relatives.

In the Czech Republic, ADH molecular diagnostics started in 1997. Large scale of methods has been used for analysis since that time. The first diagnostics scheme for LDLR gene analysis was based on the amplification of all LDLR gene exons and intronic junctions and their analysis by denaturing gradient gel electrophoresis (DGGE). In cases of positive results, direct sequencing was performed in these samples.

In 2002, we published the first spectrum of mutations in Czech hypercholesterolemic patients (Kuhrova et al. 2002). There was evidence of some mutations being more prevalent in our population. Due to this fact, we changed the diagnostics scheme in 2002 and introduced denaturing high performance liquid chromatography (dHPLC) to rapidly screen for mutations in the LDLR gene. DNA analysis of ADH patients was divided into several consecutive steps: 1) PCR-RFLP (Restriction Fragment Length Polymorphism) detection of the most common mutation in the APOB gene [p.(Arg3527Gln)]; 2) PCR-RFLP detection of the most common mutations in the LDLR gene determined on the basis of the pilot study mentioned above [p.(Gly592Glu), p.(Asp266Glu), p.(Cys209Tyr), and p.(Arg416Trp)]; 3) PCR-sequencing of LDLR exon 4 (the exon with the markedly most frequent occurrence of mutations in our patients); 4) PCR-sequencing of the promoter and LDLR exons 1, 5, 6, 9, 10, 12, 14; and 5) PCR-dHPLC of LDLR exons 2, 3, 7, 8, 11, 13, 15, 16, 17, and 18, followed by sequencing of regions which were tested positive.

In 2005, we added multiplex ligation-dependent probe amplification (MLPA). In mutation detection, further DNA analysis continued when 1) a phenotypic manifestation was serious and possibly associated with the presence of two ADH mutations or 2) a detected mutation was new with an effect on the protein structure and function, which was difficult to predict. This diagnostic process was common in ADH/FH molecular genetic testing worldwide in that time (Civiera et al. 2008).

In 2010, we introduced high throughput Arrayed Primer Extension (APEX)-based genotyping DNA microarray (Dušková et al. 2011). In populations such as Ashkenazi (Meiner et al. 1991) and Sephardic Jews (Leitersdorf et al 1993), Icelanders (Gudnason et al. 1997), or Finns (Koivisto et al. 1995), a few mutations predominate due to founder effects. On the contrary, we found over 180 types of mutations in the LDLR gene in Czech patients and on average, 6 novel types of ADH mutations are found per year. We concluded that APEX-based genotyping DNA microarray is not suitable for routine use in diagnostics in our population. Complete direct sequencing of whole coding sequence of LDLR gene in all samples was performed from 2012 to 2014.

The current diagnostic scheme was initiated in 2014 and is based on next generation sequencing for all exons (and adjacent intronic junctions) of the LDLR and the PCSK9 genes, partial sequencing of the APOB gene (part of exon 26 from c.10200 to c.11100) and analysis of 12 functional polymorphisms in other genes (Talmud et al. 2013). We use ADH Master kit manufactured by Multiplicom, Niel, Belgium. In negative samples, LDLR gene MLPA is performed as the second step of the standard diagnostic scheme.

Results and milestones

The group of 3914 unrelated patients diagnosed with ADH, submitted to the database of the MedPed project in the Czech Republic, has been analyzed for the presence of mutations in the APOB and LDLR genes since 1997. Analysis of the PCSK9 gene was completed in 533 patients. We confirmed the FH diagnosis in 1296 patients (33.11 %) – 432 patients carrying a mutation in the APOB gene and 864 patients in the LDLR gene. No ADH causing gain-of-function mutation in the PCSK9 gene was found in this study group of patients. These results are comparable with published studies from other European countries (Chmara et al. 2010, Widhalm et al. 2007, Grenkowitz et al. 2016, Kindt et al. 2014).
et al. 2013, Gabčová et al. 2017, Nauck et al. 2001). It is
notable that the most frequent mutation in Czech ADH
patients p.(Gly592Glu) is also the most common in
Slovakia (Gabčová et al. 2017) and in Poland (Chmara et
al. 2010), the second most common mutation p.(Asp266Glu) is the most common in
Germany (Nauck et al. 2001) and Austria (Widhalm et al. 2007) and the
third most common mutation p.(Arg416Trp) is also very
frequent in Slovakia. In probands carrying an LDLR gene
mutation, 182 unique allelic variants were detected:
72.8% of these variants were DNA substitutions, 15.8% of
classic DNA rearrangements, and (of before each of the
percentages or none) of 11.4% large DNA
rearrangements. Detailed differentiation of the so far
detected variants is shown in Figure 1. In the Czech
MedPed project database there are actually 6929 patients
clinically diagnosed with ADH. Preliminary data suggest
a polygenic base of ADH in part of this cohort
(Fellnerová et al. 2014, Schwarzova et al. 2016). In
comparison with published data (Nordestgaard et al.
2013), the Czech Republic has the third highest
proportion of clinically diagnosed patients from all
assessed countries (Fig. 2). We found 14 patients
homzygous for mutation in causative genes – 10 patients
carry mutations in the LDLR gene (7 of them are
compound heterozygotes, while 3 remaining are true
homozygotes) and 3 patients are carriers of the
p.(Asp3527Gln) mutation in both APOB gene alleles.

Fig. 1. Detailed differentiation of types of mutations in the LDLR gene found in Czech ADH patients. Sequence events short than
100 bp are considered as small duplication/insertion/deletion.

Fig. 2. The proportion of clinically diagnosed ADH patients from all suggested cases of ADH in the Czech Republic compared to other
countries. Data from assessed countries are adopted from publication Nordestgaard et al. (2013). Considering a prevalence 1:500, used
there, the total number of ADH cases in the Czech Republic is 20,000. Recent studies in unselected general populations suggest that
the prevalence of heterozygous familial hypercholesterolemia may be as high as 1 in 200 (Sjouke et al. 2015). Considering this
prevalence, the proportion of ADH cases in all mentioned countries is 2.5x higher.
In 2004, we published our first functional assay for a mutation found in Czech patients (Francová et al. 2004). The described mutation c.-120C>T is located between the TATA box and the sterol-dependent regulatory element repeat 3. Using a luciferase reporter assay system, we analyzed the transcriptional efficiency of the normal and mutant alleles. The mutation reduced promoter activity to a background level. The next three promoter mutations found in Czech patients (c.-153C>T, c.-149C>A and c.-140C>A) are located in the sterol regulatory element (SRE) or the SP1 site. The analyses revealed an 80-95% decrease in activity compared with a wild type promoter (Südhof et al. 1987).

In 2005, we introduced the MLPA reaction into a diagnostic scheme. During the following five years we found 8 different types of large rearrangements, among these 6 were of novel types, not described so far. In all these rearrangements, we characterized their exact extent and breakpoint sequences (Goldmann et al. 2010). The results showed that 6 events were products of NAHR (non-allelic homologous recombination) between Alu repeat sequences. The remaining 2 events apparently originated from NHEJ (non-homologous end joining). While NAHR was described in relation to the LDLR gene earlier, our study was the first describing NHEJ in LDLR genomic rearrangements. Actually, duplication including exons 2-6 is the fifth most common mutation in Czech ADH patients.

In 2012, we published an actual list of mutations found in ADH patients in the Czech Republic up to date (Tichý et al. 2012). The large set of clinical information in the Czech MedPed database allowed us to perform detailed phenotype-genotype correlation in our ADH patients. For lipid profile analyses, ADH probands were divided into 3 groups: i) patients with the LDLR mutation (LDLR+), ii) with the APOB p.(Arg3527Gln) mutation (APOB+) and iii) without a detected mutation (LDLR-/APOB-). Each group was divided into subgroups according to gender. Significant gradients in i) total cholesterol (LDLR+ patients > APOB+ patients = LDLR-/APOB- patients), ii) LDL cholesterol (LDLR+ patients > APOB+ patients = LDLR-/APOB- patients in men and LDLR+ patients > APOB+ patients > LDLR-/APOB- patients in women), iii) triglycerides (LDLR-/APOB- patients > LDLR+ patients > APOB+ patients), and iv) HDL cholesterol (APOB+ patients > LDLR-/APOB- patients = LDLR+ patients) were shown. The occurrence of cardiovascular events and tendon xanthomats was 16.4% and 3.8% respectively, in the set of our ADH patients older than 30 years of age. The highest incidence of these clinical features was recorded in the LDLR+ group (20.8% and 13.1%), in which the highest LDL cholesterol level was also demonstrated (Tichý et al. 2012).

Here we present the list of point mutations, small deletions/insertions (Table 1) and large rearrangements (Table 2) found in our patients, which were not included in our previous publications. Pathogenic impact on the LDL receptor protein structure and function was predicted by in silico approaches (PolyPhen2, SIFT and Mutation Taster). We use aggregated data from ExAC (http://exac.broadinstitute.org/) and 1000 Genomes (http://browser.1000genomes.org) databases for evaluation of sequence variants frequencies in random population.

Most recently, we published our results from the function analysis of controversial sequence variant c.58G>A (p.(Gly20Arg)) in signal peptide (Pavlousková et al. 2016) of the LDL receptor protein. This substitution was described as disease causing on the Leiden Open Variation Database (LOVD), http://www.ucl.ac.uk/ldlr/Current/index.php?select_db=LDLR and also on the Human Gene Mutation Database (HGMD), https://portal.biobase-international.com/hgmd/pro/gene.php?gene=LDLR. Family segregation analysis performed in this variant gave ambiguous results as well as in silico predictions of causality. T-Rex CHO cells were transfected with the wt LDLR plasmid or the plasmid carrying the LDLR change p.(Gly20Arg). Confocal laser scanning microscopy was used to analyze the LDLR expression and binding of the LDL particles to LDLR in stably transfected CHO cells. To see the localization of the wt or mutated LDLR proteins, we studied signal co-localization from fluorescently tagged LDLR proteins and fluorescently labeled endoplasmic reticulum. To see if the receptor proteins are able to bind to the LDLR particles, we incubated transfected CHO cells with fluorescently labeled LDL particles. Functional analyses revealed that the sequence variant p.(Gly20Arg) is able to reach the cell membrane and to bind the LDL particle in a similar way as the wt LDLR protein. We assume that the binding activity of the mutated protein is not affected because p.(Gly20Arg) is localized in signal peptide, which is cleaved in endoplasmic reticulum and is not present in mature protein. Currently, we continue with these analyses in other mutations.
Table 1. List of point mutations and small deletions/insertions found in the LDLR gene, which were not published in our previous study (Tichý et al. 2012). Sequence events shorter than 100 bp are considered as small duplication/insertion/deletion.

<table>
<thead>
<tr>
<th>Mutation at cDNA level</th>
<th>Mutation at protein level</th>
<th>No. of probands</th>
<th>PolyPhen 2</th>
<th>SIFT</th>
<th>Mutation Taster</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-140C&gt;A</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.3G&gt;T</td>
<td>p.(Met1Ile)</td>
<td>1</td>
<td>Possibly damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.300C&gt;A</td>
<td>p.(Asp100Glu)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.383G&gt;C</td>
<td>p.(Cys128Ser)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.502G&gt;A</td>
<td>p.(Asp168Asn)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.529T&gt;C</td>
<td>p.(Ser177Pro)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.683_694del</td>
<td>p.(Glu228_Cys231del)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.940_940+14del</td>
<td>Splicing defect</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.1065C&gt;G</td>
<td>p.(Ile355Met)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1091G&gt;A</td>
<td>p.(Cys364Tyr)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1112T&gt;C</td>
<td>p.(Leu371Pro)</td>
<td>2</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1117G&gt;T</td>
<td>p.(Gly373Cys)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1177A&gt;C</td>
<td>p.(Lys393Gln)</td>
<td>2</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1184_1185delTG</td>
<td>p.(Val395Glyfs*45)</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.1204_1205delTT</td>
<td>p.(Phe402Leufs*38)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.1358+5G&gt;T</td>
<td>Splicing defect</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.1595A&gt;G</td>
<td>p.(Tyr532Cys)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.1741A&gt;T</td>
<td>p.(Lys581*)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.2072C&gt;A</td>
<td>p.(Ser691*)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.2072C&gt;T</td>
<td>p.(Ser691Leu)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.2132G&gt;A</td>
<td>p.(Cys711Tyr)</td>
<td>1</td>
<td>Probably damaging</td>
<td>Not tolerated</td>
<td>Disease causing</td>
</tr>
<tr>
<td>c.2184insTCAGGG</td>
<td>p.(Leu729Serfs*3)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.2256_2257dupGCTG</td>
<td>p.(Pro753Alafs*30)</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c.2407T&gt;C</td>
<td>p.(Cys803Arg)</td>
<td>1</td>
<td>Benign</td>
<td>Not tolerated</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>

In silico approaches, called PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) (Adzhubei et al. 2010), Refined SIFT (http://sift.jcvi.org/) (Kumar et al. 2009) and Mutation Taster (http://www.mutationtaster.org/) (Schwarz et al. 2014), were used to predict the effect of missense mutations or small deletion/insertion on the LDLR protein. Although helpful, it should be remembered that these computer prediction programs provide only an indication that an amino acid substitution may affect the biological activity of the mature protein.

Table 2. List of large rearrangements found in the LDLR gene, which were not published in our previous study (Tichý et al. 2012). Sequence events long than 100 bp are considered as large duplication/insertion/deletion.

<table>
<thead>
<tr>
<th>No.</th>
<th>Deletion/Duplication</th>
<th>Number of proband</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ex1_12dup</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ex2_10del</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Ex4_6del</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Ex13_14del</td>
<td>2</td>
</tr>
</tbody>
</table>

In conclusion, the molecular diagnostic of ADH in the Czech Republic is well organized especially due to the MedPed project. The results achieved in the number of diagnosed patients as well as in the field of translation medicine are comparable with other countries worldwide.

Conflict of Interest
There is no conflict of interest.

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