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REVIEW

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

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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 covertase subtilisin/kexin type 9 (*PCSK9*) have been established as causatives of the disease (Chiou 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 (Cífková 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. 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 small 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 homozygous 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.

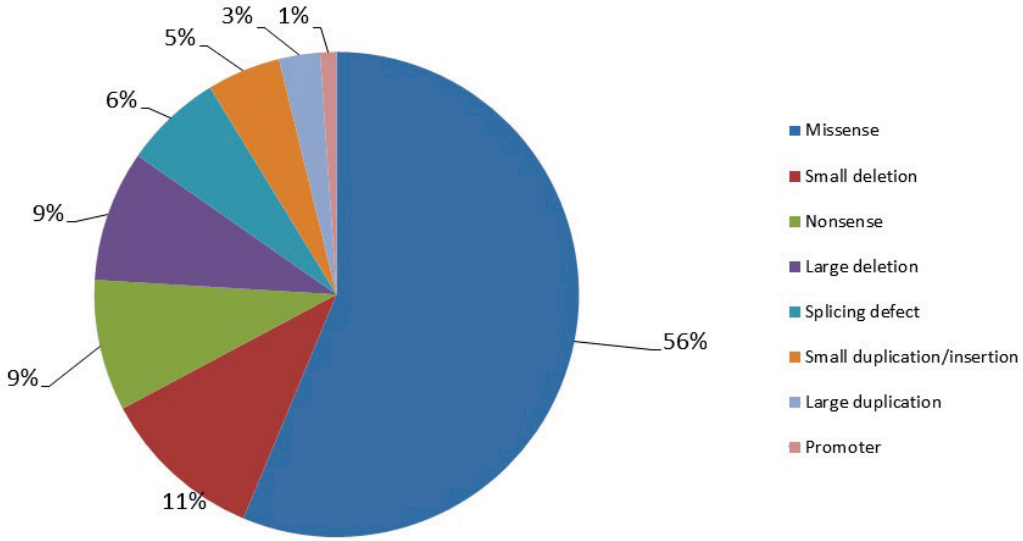

Fig. 1. Detailed differentiation of types of mutations in the *LDLR* gene found in Czech ADH patients. Sequence events short than 100 bp are considered as small duplication/insertion/deletion.

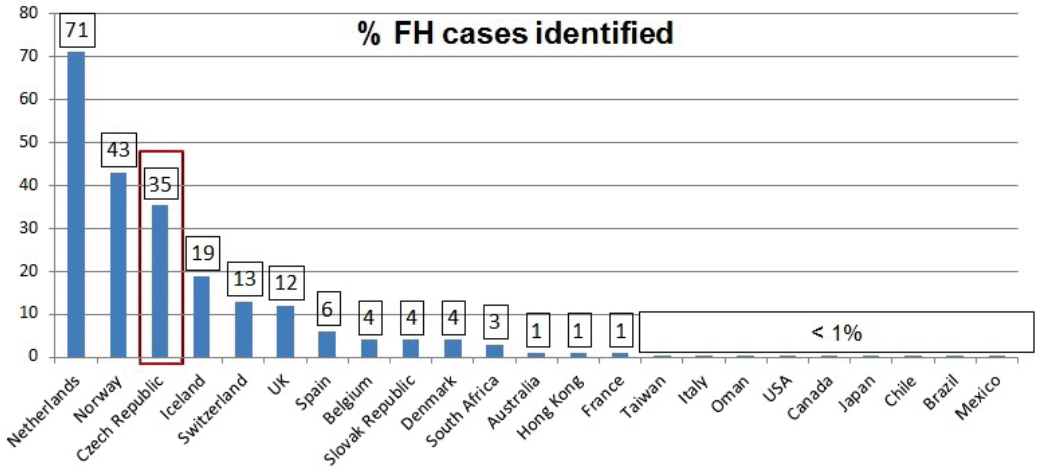

Fig. 2. The proportion of clinically diagnosed ADH patients from all suggested cases of ADH in the Czech Republic compared to other countries. Data from assessed countries are adopted from publication Nordestgaard *et al.* (2013). Considering a prevalence 1:500, used there, the total number of ADH cases 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 sequence variation impact in these regulation sites 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 xanthomas 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 (Pavloušková *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.

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

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.

No.	Deletion/Duplication	Number of proband
1	Ex1_12dup	1
2	Ex2_10del	1
3	Ex4_6del	1
4	Ex13_14del	2

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.

Acknowledgements

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