Review

Polymorphic Cytochromes P450 *CYP2B6* and *CYP2D6*: Recent Advances on Single Nucleotide Polymorphisms Affecting Splicing

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Abstract

Recent work on functional polymorphisms in drug metabolizing cytochromes P450 CYP2B6 and CYP2D6 emphasizes the role of single nucleotide polymorphisms (SNPs) in non-consensus splicing elements such as exonic and intronic splicing enhancers. In the *CYP2D6* gene the intron 6 SNP 2988G > A (allele **41*) shifts the balance of spliced transcripts towards a variant that lacks exon 6. In the *CYP2B6* gene, the 516G > T SNP, a marker of allele *CYP2B6**6, encodes an amino acid change in exon 4 [Q172H] but also leads to increased amounts of a transcript lacking exons 4 to 6. In both cases aberrant splicing results in reduced amounts of functional transcript and reduced amounts of functional protein in the liver. Although expression of functional protein is only partially diminished, reduced activity phenotypes arise in homozygous genotypes or in compound heterozygotes carrying other severely affected alleles. We here describe the elucidation of these genetic polymorphisms and their mechanisms as well as their clinical relevance.

Keywords: Allele, CYP2B6, CYP2D6, cytochrome P450, drug metabolism, genetic polymorphism, splice variant

1. Polymorphisms of Drug Metabolizing Cytochromes P450

About one dozen P450 isozymes of families CYP1, CYP2 and CYP3 are collectively responsible for most phase I biotransformations of drugs and other xenobiotics in human liver. These cytochromes P450 catalyze over 90% of all drug and xenobiotic metabolism pathways in humans.[1,2] Highly variable expression and function of these isozymes, both inter- and intraindividually, constitutes a major determinant for unpredictable drug and drug metabolite plasma concentrations and can lead to unforeseen drug responses including over-reaction, toxicity, or lack of response.[3] Genetic polymorphisms in cytochrome P450 and other drug metabolism enzyme and transporter genes are an important source of variation, but induction/ repression or inhibition by drugs and other xeno- or endobiotics, biological or physiological conditions including sex, age, disease, and many other confounding factors often limit or modulate their penetrance. There are hundreds of single nucleotide polymorphisms (SNPs) and other common or rare sequence variations in the drug metabolizing P450 genes. Most of them are not functional in the sense that they are causative for substantial changes in expression, substrate selectivity or enzymatic activity. In this article we summarize our recent advances on common polymorphisms of CYPs *2B6* and *2D6*. In both genes we identified novel SNPs that are quite common in certain populations and that can explain a considerable part of the expression variability of these genes. We elucidated their mechanisms of action and we found that these SNPs cause partially reduced expression by affecting the correct splicing of pre-mRNA.

2. CYP2D6 Phenotypes and Polymorphisms

The polymorphic expression of CYP2D6 was originally discovered in the 1970s when two research groups in England and in Germany observed that some volunteers participating in pharmacokinetic studies with debrisoquine[4] and sparteine,[5] respectively, developed unexpected ad-

verse reactions. In both cases it was shown that these individuals had a substantially impaired capacity to oxidize the drug and that this metabolic deficiency is inherited as an autosomal recessive trait. The gene for *CYP2D6* is localized together with two pseudogenes *CYP2D7* and *CYP2D8P* on chromosome 22.[6,7] More than 60 distinct alleles which result in CYP2D6 protein with normal, increased, decreased or complete lack of function have been documented on the CYPallele nomenclature homepage (http://www.cypalleles.ki.se).[8] The individual inherited allele combination (genotype) determines the drug oxidation phenotype, which can be assessed *in vivo* by measuring the metabolic ratio (MR), i.e. the ratio between the amount of unchanged drug and drug metabolite of a suitable probe drug (e.g. sparteine, debrisoquine, dextromethorphan) excreted in the urine within a certain time. The enzyme is responsible for oxidative metabolism of numerous clinically used drugs most of which contain a basic nitrogen. Drug substrates of CYP2D6 include antiarrhythmics (e.g. propafenone), antidepressants (e.g. amitriptyline, venlafaxine), antipsychotics (e.g. thioridazine), beta-blockers (e.g. metoprolol), opioids (e.g. codeine) and many more.[9]

Between 5 and 10% of Caucasians carry two null-alleles, of which about 20 are known to date. The most common null-allele of *CYP2D6* is **4*, which occurs at a frequency of 20 to 25% in Caucasians. The null-mutation of **4* (1846G > A) changes the splice acceptor site of intron 3 such that it results in a shift of reading frame and ultimately a premature stop codon.[10] Inheritance of two **4* alleles (or any other combination of two null-alleles) leads to the CYP2D6 poor metabolizer (PM) phenotype (Fig. 1). All other individuals have residual enzyme activity due to the presence of at least one allele encoding a functional protein. However, the quantitative differences among these individuals are extremely large. Most individuals carry either one or two functionally normal alleles (in most cases either *CYP2D6*1* or **2*). Higher than normal activity (ultrarapid metabolizer, UM) results from inheritance of one or two alleles with multiple functional copies, which occur in European populations with allele frequencies of ~ 1 to 10%.[11] A phenotypically distinct subgroup of about 10 to 15% of Caucasians with MR between those of EMs and PMs are commonly termed intermediate metabolizers (IM).[9] In other ethnicities, the frequencies of variant alleles and of the resulting phenotypes are very different. Thus, in Asians and Africans null-alleles are much rarer than among Caucasians whereas alleles with reduced function, i.e. **10* in Asians[12] and **17* in Africans,[13] are much more common. High frequencies of alleles with multiple functional CYP2D6 copies have been observed in certain Arabian and Eastern African populations.[14]

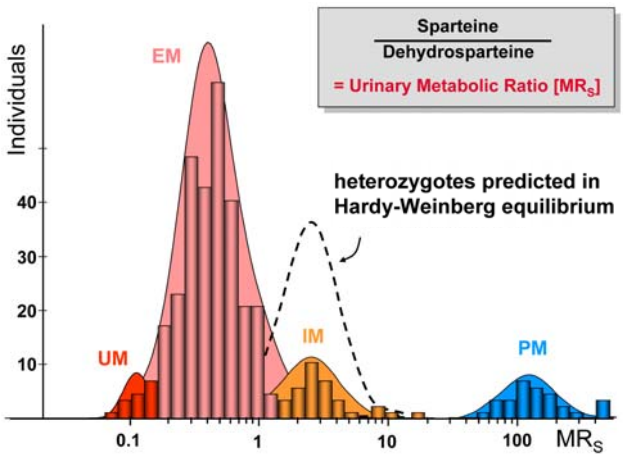

Figure 1. Distribution of sparteine oxidation phenotypes in a Caucasian population. Phenotypes are indicated: UM, ultrarapid metabolizer; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

3. Elucidation of the Genetics Underlying the Intermediate Metabolizer Phenotype

We have recently concentrated on the elucidation of the molecular basis of the IM phenotype in Caucasians.[9] A critical initial observation was that the frequency of this subgroup was much higher than predicted on the basis of already known alleles with reduced function (**9* and **10*, both of which occur in Caucasians at about 1–2%) but at the same time it was much lower than the calculated frequency of ordinary "heterozygotes", i.e. carriers of one null-allele. As indicated in figure 1, this frequency should be around 30 to 40% based on the PM frequency and Hardy-Weinberg equilibrium. This suggested that either the IM phenotype was not genetically determined in a large fraction of these individuals, or that a previously unidentified allele or genotype was responsible.

By genotyping and phenotyping an Italian family we obtained first evidence for a genetic variant of the common functional **2* allele, which harbours two nonsynonymous SNPs [R296C + S486T] that do not alter catalytic activity for most substrates.[15] Interestingly, all members of this family carried one **2* allele combined with the most common null-allele **4* (genotype **2/*4*) but whereas the father and two daughters were phenotypic IMs, the mother and one further daughter were EMs. Segregation of the phenotypes within the family was thus in agreement with the assumption that the paternal **2*-allele coded for an enzyme with lower activity as compared to the maternal one. Sequence analysis revealed no differences between the maternal and paternal coding sequences but several novel polymorphisms were found within ~1600 bp of upstream sequence. Only at one polymorphic position we found a difference between the maternal and the paternal alleles, namely a C > G change at –1584 bp.[15] This SNP was present in the two EMs of the family, whe-

reas all IMs carried the wild type sequence at this position. Remarkably, this SNP could be used to predict the IM phenotype in a subsequently genotyped larger population sample and it was associated with significant changes in CYP2D6 expression and enzyme function in human liver.[16] The *2 allele lacking the –1584C > G SNP was subsequently renamed **41*.

However, the situation was quite complex because the –1584C > G SNP was associated with the EM phenotype, whereas IMs were carriers of a **2* allele lacking this mutation, in combination with one null-allele (Fig. 2). A molecular explanation of these findings was thus difficult, in particular because experimental approaches to determine transcriptional activation failed to reveal functional consequences of the –1584C > G SNP. This suggested that possibly another mutation, either in linkage disequilibrium with the –1584C > G SNP on the maternal allele or located on the paternal one existed. Indeed, a large sequencing effort comprising about 4 kb of upstream sequence and the entire gene including all introns in a number of individuals with different genotypes revealed a single novel SNP that was possibly responsible for the low activity phenotype.[17] This SNP 2988G > A is located at nucleotide +39 of intron 6 of the paternal gene and in other IMs and it could be shown that genotype-phenotype correlations based on this SNP were more accurate than those based on –1584C > G, leading to a re-definition of the **41* allele (Fig. 2).[17]

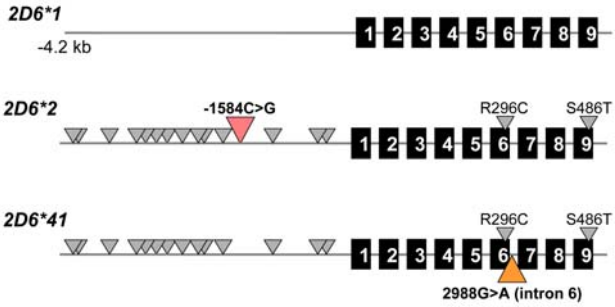

Figure 2. Haplotypes of *CYP2D6* alleles **1, *2,* and **41*. Exons are indicated by boxes. SNPs are indicated below the gene symbols, corresponding amino acid changes are indicated above the gene symbols.

4. An Intronic SNP Affects Splicing

The 2988G > A SNP was then shown to cause decreased protein expression and activity in a recombinant approach by using mammalian expression plasmid that contained the entire *CYP2D6* gene including all eight introns.[18] The constructs including the **2* and **41* alleles differed only by the 2988G > A SNP (Fig. 2) but while the *2 construct expressed at about similar levels than the reference gene, the **41* plasmid resulted in several-fold lower expression in both Huh7 hepatoma cells and COS-1 cells. Using RT-PCR to amplify CYP2D6 transcripts from genotyped liver samples, it could be shown that the 2988G > A SNP affects splicing events. Namely, whereas samples with **1* or **2* alleles expressed almost exclusively the normal transcript, samples genotyped positive for 2988G > A had decreased amounts of the normal transcript and substantially increased amounts of a splice variant lacking the entire exon 6. This variant transcript harbours a stop codon at position 291 of the mature mRNA and can thus not contribute to functional CYP2D6 protein. Thus, the 2988G > A shifts the balance between the functional and a non-functional splice product towards the non-functional variant.[18] We have so far not experimentally analyzed the mechanism by which the 2988G > A SNP affects splicing of CYP2D6 pre-mRNA. It is most likely that this position is located within a splicing enhancer element. Such elements serve as binding sites for specific serine/ arginine (SR)-rich proteins, a growing family of structurally related and highly conserved splicing factors which promote exon definition by directly recruiting the splicing machinery and/or by antagonizing the action of nearby silencer elements.[19] Using the ESE-finder algorithm (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home)[20,21] 2988G > A was shown to change the scores for binding of certain splice factors to known splicing enhancer elements, as illustrated in figure 3. The 2988G > A SNP results in the loss of two binding sites for SF2/ASF (IgM-BRCA1) and a new predicted binding site for SRp40. The 2850C > T SNP (R296C of **2*) in exon 6 leads to the loss of binding sites for SRp55 and for SF2/ASF whereas a new binding site for SC35 is predicted. Although the results of *in silico* prediction of splicing events remain speculative, the 2988G > A SNP is an interesting example for

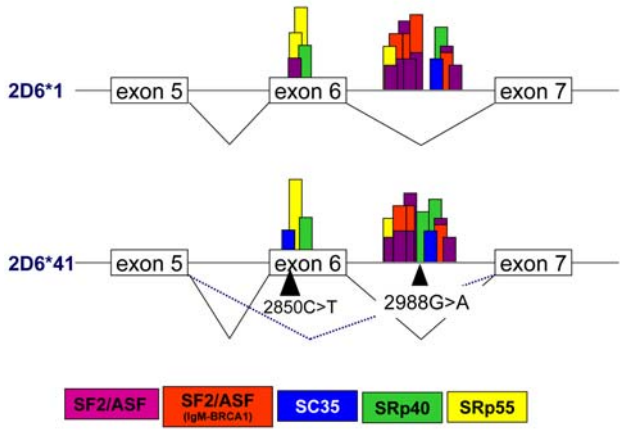

Figure 3. In-silico analysis of CYP2D6 pre-mRNA splicing factor binding sites in exon 6 and intron 6. Sequence windows of 20 nt surrounding the *CYP2D6* SNPs 2850C > T and 2988G > A were analysed for putative exonic splicing enhancer motifs using ESE-finder 3.0. Predicted binding sites are shown schematically for the indicated alleles. The height of the bars indicates the strength of binding sites as calculated by the ESE-finder. ESE, exonic splicing enhancer, SR, serine-arginine rich protein; SC35, SRp40, SRp55 and AF2/ASF.

an intronic polymorphism which does not affect the consensus splice sites at exon/ intron borders but rather changes gene expression quantitatively by affecting intronically located elements. Hopefully, future studies will clarify the involved mechanisms in greater detail.

5. CYP2B6 Phenotypes and Polymorphisms

CYP2B6 is the only functional isozyme of the 2B subfamily in humans and its gene is located, together with the expressed pseudogene *CYP2B7,* within a 350 kb *CYP2ABFGST* gene cluster on chromosome 19 that contains genes and pseudogenes of the *CYP2A, 2B, 2F, 2G, 2S* and *2T* subfamilies.[22] CYP2B6 contributes about 5% to the total liver microsomal P450 content. Clinically used drug substrates include cytostatics (cyclophosphamide)[23], HIV drugs (efavirenz, nevirapine),[24,25] antidepressants (bupropion),[26] antimalarials (artemisinin),[27] anaesthetics (propofol),[28] and synthetic opioids (methadone)[29] and several others.[30] Variability of CYP2B6 expression in liver is high and protein levels can be 100-fold different between individuals.[31,32] Like the rodent phenobarbital-inducible *CYP2B* genes, human CYP2B6 is strongly inducible by numerous drugs and chemicals including rifampicin, barbiturates, cyclophosphamide, artemisinin, carbamazepine, metamizole, efavirenz and nevirapine.[33–36]

Compared to the model polymorphism of *CYP2D6*, which was discovered by clinical observations, *CYP2B6* polymorphisms were discovered following reverse genetics approaches with initial polymorphism screening and subsequent functional and clinical studies.[32] Over 100 DNA variations including numerous nonsynonymous mutations as well as silent, promoter and intronic changes were found in the *CYP2B6* gene, many of them showing extensive linkage disequilibrium giving rise to distinct haplotypes.[37–44] Taking the number of variants and their frequency in different populations into account, *CYP2B6* belongs to the most polymorphic human cytochrome P450 genes. Given the strong inducibility of CYP2B6 as described above, it is rather remarkable that polymorphisms of CYP2B6 are highly predictive for expression and function of the enzyme, at least with certain substrates. However the influence of numerous confounding factors like sex, induction by drugs and other xenobiotics, as well as possible substrate dependent effects contribute to variability and complicate analyses.[36]

6. Elucidation of the Molecular Events Leading to low Expression of the *CYP2B6*6* Allele

The most common variant allele is *CYP2B6*6*, which harbours two nonsynonymous mutations, one in exon 4 (c.516G > T, Q172H) and one in exon 5 (c.785A > G, K262R).[36] The allele occurs with high frequency across different populations, ranging from about 15% in Koreans to ~40% in Africans and over 60% in Papua New Guineans. Following the discovery that CYP2B6 is the major enzyme for oxidative metabolism of efavirenz,[24] a number of pharmacogenetic studies with HIV-infected individuals have convincingly shown that homozygosity for *CYP2B6*6* predicts elevated plasma levels of the drug and increased risk for neurotoxicity.[45–49] Homozygotes for the **6* allele express only about 50% or less CYP2B6 protein in human liver microsomes, compared to homozygotes of the reference **1* allele.[32,50] Microsomal activities towards suitable probe drugs were also found to be decreased to similar extent as shown for S-mephenytoin N-demethylation,[32] as well as bupropion and efavirenz hydroxylation.[50]

Functional analysis of recombinantly expressed variants of the involved amino acid variants (Q172H, K262R, or both) revealed some controversial results. When expressed as N-terminally modified enzyme in *E. coli*, 7-ethoxycoumarin O-deethylase activity of the Q172H mutant (allele **9*) did not follow Michaelis-Menten type kinetics but displayed sigmoidal kinetics with increased turnover.[41] In COS-1 cells the Q172H + K262R double variant expressed at normal levels compared to the wild type but bupropione hydroxylation was catalyzed less efficiently.[48,51]

We used our large human liver bank to investigate the potential role of additional candidate polymorphisms including promoter, intronic and exonic variants in phenotype determination of the *6 allele. The analyses revealed that only the c.516G > T polymorphism was clearly correlated with reduced expression, in contrast to other candidate polymorphisms in the 5'–flanking region (–750T > C) and in introns.[52] It could furthermore be shown that a prominent hepatic splicing variant termed SV1 which lacks exons 4 to 6 is tightly correlated to the 516G > T polymorphism and that mRNA levels for the normal transcript are substantially reduced in homozygous **6* carriers. This was confirmed by a recombinant approach, in which *CYP2B6* "minigenes" were constructed which included all 9 exons as well as most introns. Transfection of several constructs containing single or combined mutations into Huh-7 and COS-1 cells demonstrated that c.516G > T alone results in a phenotype characteristic of the **6* allele, i.e. increased expression of SV1,[37] decreased functional transcript, as well as decreased protein and activity.[52] We do not know yet how the 516G > T SNP leads to altered splicing. Figure 4 shows the results of in-silico analysis of CYP2B6 pre-mRNA splicing factor binding sites in exons 4 and 5, again using the ESE-finder algorithm. The 516G > T SNP in exon 4 leads to the predicted loss of two binding sites for ESE-binding factors SC35 and SRp40. This probably results in the modulation of splicing events in favor of an alternative process that results in the observed splice variant lacking exons 4, 5 and

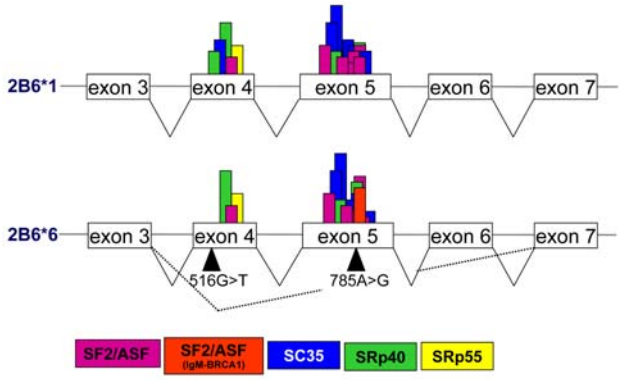

Figure 4. In-silico analysis of CYP2B6 pre-mRNA splicing factor binding sites in exons 4 and 5. Sequence windows of 20 nt surrounding the SNPs 516G > T and 785A > G were analysed for putative exonic splicing enhancer motifs using ESE-finder 3.0. Predicted binding sites are shown schematically for the indicated alleles. The height of the bars indicates the strength of binding sites as calculated by the ESE-finder. ESE, exonic splicing enhancer, SR, serine-arginine rich protein; SC35, SRp40, SRp55 and AF2/ASF.

6. The 785A > G SNP in exon 5 results in only minor changes of binding strength and the creation of one additional SF2/ASF(IgM-BRCA1) binding site.

7. Clinical Implications and Conclusions

Both polymorphisms described here, the 2988G > A variant in intron 6 of *CYP2D6* and the 516G > T variant in exon 4 of *CYP2B6* are impressive examples for SNPs causing erroneous splicing which ultimately lead to pharmacogenetic phenotypes. With an allele frequency of about 8.5%, *CYP2D6*41* is the second-most frequent impaired allele present in Caucasian populations. The genotype **41/*null* accounts for about 50 to 70% of all IMs in Caucasians.[9] There is evidence to suggest that these individuals may behave similar to PMs, especially under conditions of long-term treatment as shown in several clinical studies with metoprolol,[53] amitriptylin,[54] and, more recently, tamoxifen.[55,56] Numerous clinical studies with HIV-infected patients have confirmed a strong association between *CYP2B6*6* homozygosity and efavirenz or nevirapine plasma levels and in some cases neurological side effects.[36,46–49] Recently *CYP2B6* genotyping in Japanese HIV-infected individuals was successfully applied to reduce the therapeutic dose of EFV, which resulted not only in the expected pharmacokinetic changes but also in improvement of CNS-related side effects.[57] Other drug therapies in which *CYP2B6* polymorphisms have been proposed to contribute clinical variability are immunosuppressive and cytostatic therapy with cyclophosphamide,[58] maintenance treatment for opiate addicts with methadone,[59] and smoking cessation treatment with bupropione.[60]

In conclusion, single nucleotide polymorphisms that lead to aberrant splicing are very common among drug metabolizing cytochromes P450. The role of splicing in several important polymorphisms has been discussed earlier.[61,62] Thus, the most frequent null-alleles of *CYP2D6* as well as of *CYP2C19* harbour mutations at splice acceptor sites that prevent the expression of full length functional proteins. Similarly, the polymorphism leading to almost complete lack of expression of CYP3A5 in almost 90% of Caucasians has also recently been elucidated to be mainly due to a splicing defect of the common **3* allele.[63,64] Here we have summarized our recent work on two further common polymorphisms in the *CYP2D6* and *CYP2B6* genes. Both of these polymorphisms do not lead to a total lack of functional gene products but they result in partial reduction of expression. Nevertheless these common polymorphisms are clinically relevant as has been demonstrated in numerous studies. Therefore, aberrant splicing can be regarded as the major mechanism behind polymorphic expression of human drug metabolizing cytochromes P450. It is finally noteworthy that a large number of additional splice variants have been found especially for CYPs 2B6 and 2D6 in liver, brain and other tissues.[37,52,65–67] Whether their occurrence is also linked to the presence of DNA variations is a tempting hypothesis for future research.

8. Acknowledgements

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9. Nonstandard Abbrevations

CNS	central nervous system
CYP	cytochrome
EFV	efavirenz
ESE	exonic splicing enhancer
EM	extensive metabolizer
IM	intermediate metabolizer
HIV	human immunodeficiency virus
MR	metabolic ratio
PM	poor metabolizer
SNP	single nucleotide polymorphism
SV	splicing variant
SR-protein	serine arginine-rich protein
UM	ultrarapid metabolizer

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Povzetek

Zadnje objave s področja funkcijskih polimorfizmov v presnovo zdravil vključenih citokromov P450 CYP2B6 in CYP2D6 so pokazale vlogo eno-nukleotidnih polimorfizmov (SNP) v neohranjenih DNA zaporedjih, kot so eksonski in intronski pospeševalci izrezovanja. V genu *CYP2D6*, SNP polimorfizem v intronu 6, 2988G > A (alel**41*), premakne izrezovanje tako, da nastane oblika prepisa, ki ji manjka ekson 6. V genu *CYP2B6*, SNP 516G > T, ki je označevalec alela *CYP2B6**6, spremeni aminokislino v eksonu 4 [Q172H], privede pa tudi do povečane količine prepisa, ki mu manjkata eksona 4 in 6. Okrnjeno izrezovanje intronov v obeh primerih povzroči zmanjšanje količine funkcionalnih prepisov in posledično zmanjšane količine funckionalnih proteinov v jetrih. Čeprav je izražanje funcknionalnega proteina le delno zmanjšano, pa fenotipi z zmanjšano aktivnostjo proteinov izhajajo iz homozihotnih genotipov ali iz kombinacij z drugimi težko okvarjenimi aleli. V prispevku so opisani omenjeni genetski polimorfizmi in njihovi mehanizmi, kot tudi klinična uporabnost teh dognanj.