Alkaptonuria (AKU) [OMIM 203500] represents a classical example of inborn error of metabolism and is characterised by deficiency of homogentisate dioxygenase (HGD) activity (EC 1.13.11.5) (La Du et al. 1958). Darkening of the urine upon standing is usually the first sign of AKU, caused by excretion of homogentisic acid (HGA). HGA also accumulates in the body and forms a melanin-like polymer that gets deposited in the connective tissues, causing a pathologic pigmentation known as ochronosis. Later on, in the patients’ life, begins a painful degenerative ochronotic arthropathy of intervertebral discs and joints, especially in the shoulders, hips and knees. For now, no cure exists for this disorder. However, since November 2012, the DevelopAKUre project underway is focussed on clinical testing of nitisinone as a possible treatment for AKU. The SONIA1 (Suitability Of Nitisinone In Alkaptonuria) study, the first part of this project, showed that this drug decreases urine HGA in a dose-dependent manner (Ranganath et al. 2014).

HGD gene and mutations

The enzymatic defect in AKU is caused by mutations within the HGD gene (HGNC:4892). This gene was mapped to chromosome 3q13.3 and is composed of 14 exons. In about 400 patients, 142 pathogenic variants were reported that are listed in HGD mutations database (http://hgddatabase.cvtisr.sk/). In this review, we summarise different aspects of AKU genetics and impact of the HGD variants on enzyme function.

Keywords

alkaptonuria – HGD gene – HGD mutations – mutation analysis – inborn error of metabolism

Abstract

Alkaptonuria (AKU) is the first described inborn error of metabolism and a classical example of rare autosomal recessive disease. AKU patients carry homozygous or compound heterozygous mutations of the gene coding for enzyme homogentisate dioxygenase (HGD) involved in metabolism of tyrosine. The metabolic block in AKU causes accumulation of homogentisic acid (HGA) that, with advancing age of the patient, leads to severe and painful ochronotic arthropathy. HGD gene was mapped to chromosome 3q13.3 and is composed of 14 exons. In about 400 patients, 142 pathogenic variants were reported that are listed in HGD mutations database (http://hgddatabase.cvtisr.sk/). In this review, we summarise different aspects of AKU genetics and impact of the HGD variants on enzyme function.

The first human HGD mutations were demonstrated in Spanish AKU families: missense mutations p.P230S in exon 10 and p.V300G affecting exon 12 (Fernández-Cañón et al. 1996). Since then, mutation screening within the HGD gene has been performed in about 400 AKU patients and 173 variants have been reported, 142 of which most likely represent pathogenic mutations (Habbal et al. 2014; Li et al. 2014; Nemethova et al. 2015; Sakhthivel et al. 2014; Usher et al. 2015; Yang et al. 2013; Zatkova et al. 2012). All variants identified so far are summarised in the HGD mutation database (http://hgddatabase.cvtisr.sk/) (Zatkova et al. 2012).

AKU-causing mutations are distributed throughout the entire HGD gene with some prevalence in exons 8, 7, 6, 3 and 13. Missense mutations are the most numerous, followed by splicing, frameshift, nonsense, genomic deletions and finally, one change leading to expansion (Figure 1A). About 73% AKU chromosomes worldwide carry actually one of the 23 most frequent HGD gene mutations (Zatkova 2011).

It can be seen that some HGD mutations are spread throughout the world, such as p.S59fs or one of the first identified AKU mutations, p.V300G (Zatkova 2011). On the other hand, there are mutations rather specific for some countries or regions; for example c.342 + 1G > A [p.(Leu95_Ser114del)] for Slovakia and Czech Republic (Zatkova et al. 2000a), c.87+1G>A [p.(Tyr6_Gln29del)] for the gypsy community Nanikuravar in India (Sakhthivel et al. 2014), p.A122V in Jordan (Nemethova et al. 2015), p.C120W for the Dominican Republic (Goicoechea De Jorge et al. 2002) or 12 variants specific for Italy (Nemethova et al. 2015). Excluding the Slovak population that has its own characteristics, the most common mutations worldwide include p.M368V, p.S59fs, p.P230S and p.V300G (Zatkova 2011).

By re-examination of the position of the mutations and polymorphisms within the HGD gene sequence, several specific hot-spots have been described for HGD gene, such as the ‘CCC’ sequence motif and its inverted complement, ‘GGG’ (Beltrán-Valerio de Bernabé et al. 1999), and c.342 + 1G (Zatkova et al. 2000a). 31.7% (45/142) of the HGD variants identified worldwide affect these sequences and CpG dinucleotides that are known to be highly mutated in humans.

Interestingly, for eight AKU patients reported in the HGD mutation database, no HGD mutations were identified, and in 22 cases, only one mutation was found (Nemethova et al. 2015). Recently, the large genomic deletion of exon 2, including intronic sequences was reported in one case from Lebanon (Habbal et al. 2014) and it was identified also in two siblings from Israel (Nemethova et al. 2015) and several patients from Jordan (unpublished data). It is possible that in addition to deep intronic mutations, also large deletions encompassing one or more exons might be occurring in cases where genomic sequencing does not lead to mutation identification. Therefore, MLPA (Multiplex Ligation-dependent Probe Amplification) analysis or array CGH (Comparative Genomic Hybridization) might be considered as screening methods of choice in such patients.

**HGD protein and effects of the mutations**

The HGD gene codes for the protomer composed of 445 amino acids (NP_000178.2), with the highest expression found in the prostate, small intestine, colon, kidney and liver (http://www.uniprot.org/uniprot/Q93099). Laschi et al. (Laschi et al. 2012) showed the HGD gene expression in osteoarticular compartment cells (chondrocytes, synoviocytes and osteoblasts), which indicates formation of the ochronotic pigment also in loco.

The crystal structure of the HGD protein was defined (PDB code 1EY2 and 1EYB) and it has been shown that the active form of the enzyme is organised as a highly complex and dynamic hexamer comprising two disc-like trimers (Titus et al. 2000). An intricate network of non-covalent interactions is required to maintain the spatial structure of the protomer, the trimer and finally, the hexamer. This delicate structure can be easily disrupted by mutations leading to aberrant effects on enzyme function, which is confirmed by high proportion of missense mutations identified in AKU patients.

There are several possibilities how to evaluate the effect of variants on HGD protein function. PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al. 2010) and SNAP (screening for nonacceptable polymorphisms, http://cubic.bioc.columbia.edu/services/SNAP/) (Bromberg and Rost 2007) programs are frequently used for this purpose and both use 3D protein structures. However, they have their limitation. In the case of AKU, amino acid substitutions, which would be benign if HGD functioned as a monomer, show deleterious effects due to disturbance to the higher organisation of the functional hexamer (Zatkova et al. 2012). Recently, we performed an analysis of the effect of the HGD variants in the context of the molecular interactions of the wild-type residue and mCSM (Pires et al. 2014b) and DUET (Pires et al. 2014a) were used to predict the effects of the variants on protomer and hexamer thermal stability and mCSM-PPi (Pires et al. 2014b) to predict the effects of the variants on the affinity of the protomers to interact with each other. Using these novel effective computational approaches, we showed that the missense mutations are predicted to affect the activity of the enzyme by three mechanisms: decrease of stability of individual protomers, disruption of protomer–protomer interactions or modification of residues in the region of the active site (Nemethova et al. 2015). The subdivision of the variants into these classes might have relevance for a selection of possible treatment strategies in the future.

**Genotype–phenotype correlation**

So far, no information is available on correlation between genotype and phenotype in AKU. These kinds of studies are
complicated due to the complex hexameric structure of the HGD enzyme, especially in patients who carry two different mutations. It has been shown that some mutations lead to different residual HGD enzymatic activities (Rodríguez et al. 2000; Vilboux et al. 2009) but further studies are necessary. In addition, an effective AKU severity scoring system AKUSSI has been developed only recently (Cox and Ranganath 2011). Within the frame of the DevelopAKUre project, detailed clinical characterisations of AKU patients, including mutations, various biochemical parameters as well as AKUSSI will be obtained, which will contribute to better understanding the significance of different HGD variants.

**AKU disease models**

Several human ochronotic cell, tissue and serum models were introduced that contributed to understanding the effect of the HGA on cell viability (Braconi et al. 2010; Tinti et al. 2011), cell protein expression (Braconi et al. 2010; Tinti et al. 2010; Tinti et al. 2011) and joint destruction in AKU (Martin and Batkoff 1987). Genome editing methodologies (TALENs, CRISPR-Cas9) are utilised to develop Xenopus lines carrying specific human AKU mutations (Schmitt et al. 2014). In addition, the murine AKU genotype exists, developed at the Pasteur Institute in 1994 by induced mutagenesis. These mice have a truncated HGD protein, resulting from a splice mutation in the HGD gene (Manning et al. 1999). Originally, it was reported that these animals do not demonstrate ochronotic osteoarthropathy consistent with the human disease despite excreting sufficient HGA to cause darkening of the urine (Manning et al. 1999; Montagutelli et al. 1994). However, Taylor et al. (Taylor et al. 2012) showed ochronosis of tissues in this murine model of AKU. These preliminary histological observations provide a stimulus for further studies focussed on the natural history of the disease to provide a greater understanding of this class of arthropathy.

Based on the studies of AKU mouse model, Galagher et al. (2012) described the model of progression of ochronosis in articular cartilage from initiation in calcified cartilage to eventual destruction of the joint. He postulates that ochronosis begins with the deposition of pigment in individual chondrocytes and their territorial matrix in calcified cartilage. Pigmentation leads to focal increases of stiffness altering the load distribution and inducing stress risers; then it spreads to other chondrons in the calcified matrix and proliferates throughout the hyaline cartilage. The ochronotic cartilage shields the underlying bone from normal mechanical loading leading to aggressive resorption of the subchondral plate, including calcified cartilage and bone. Despite the increased stiffness, the pigmented shell of the remaining articular cartilage fails catastrophically. As a result, pigmented cartilage becomes impacted on the underlying trabecular bone and embedded in the marrow space. However, it has to be considered that also HGA itself as well as free radicals formed during the oxidation of HGA to benzoquinone acetic acid (BQA), can cause degeneration of tissue due to inflammatory processes that they cause.

**Population genetics of AKU**

Alkaptonuria belongs to a large group of rare diseases, it has a very low prevalence (1:1,000,000–250,000) in most ethnic groups but it presents a remarkable allelic heterogeneity (142 pathogenic variants). So far, about 950 AKU patients have been reported in 61 countries worldwide [AKU Society (www.akusociety.org) in: https://www.indiegogo.com/projects/cure-black-bone-disease#/story, 20 October 2013]. Slovakia and the Dominican Republic exhibit prevalence of AKU increased up to 1:19,000 (Milch 1960; Srsen and Varga 1978). Recently, a high number of AKU cases were also found in Jordan (Al-Sbou and Mwafi 2012) and India (Sakthivel et al. 2014), indicating that the overall prevalence of this disease in some countries might be underestimated.
In India, a founder mutation c.87 + 1G > A [p.(Tyr6_Gln29del)] was reported in gypsy community Narikuravar (Sakhivel et al. 2014). Similar situation was observed in Dominican Republic with founder mutation p.C120W (Goicoechea De Jorge et al. 2002). In Jordanian villages, characterised by a high level of consanguineous marriages, five different AKU-causing mutations were reported originally (Al-sbou 2012; Al-Sbou and Mwafi 2012). However, we re-analysed three patients from this study, but we were not able to confirm results reported before. Instead, we show a clear founder effect in the villages of southern Jordan, where most of the patients carried the p.A122V missense mutation in exon 6 (Nemethova et al. 2015).

On the contrary, there are countries where heterogeneity is rather high. One example is Italy, with 34 families reported in the HGD mutation database and published so far (Beltrán-Valero de Bernabé et al. 1998; Gehrig et al. 1997; Mannoni et al. 2004; Muller et al. 1999; Nemethova et al. 2015; Porfirio et al. 2000; Srsen and Varga 1978; Zatkova 2011; Nemethova et al. 2015). In this rather small group, 26 different AKU-causing mutations were described, with 12 mutations that seem to be specific for Italy (Nemethova et al. 2015).

AKU in Slovakia

Very specific is situation in Slovakia where AKU shows increased incidence. In this country, with rather small population of about 5 millions, 208 patients were reported, including 110 children (Srsen et al. 2002). Most of the patients come from a previously genetically isolated region in the north-west of Slovakia, but a classical founder effect is not the only explanation of this evidence since 13 different AKU mutations are reported in 121 Slovak AKU chromosomes (Gehrig et al. 1997; Muller et al. 1999; Zatkova 2011; Zatkova et al. 2000a; Zatkova et al. 2003; Zatkova et al. 2000b; Zatkova et al. 2012). Nevertheless, the most frequent mutation in Slovakia is p.G161R, which is present in 42% of all AKU alleles (Figure 1B, HGD mutation database) (Nemethova et al. 2015; Zatkova 2011; Zatkova et al. 2000a; Zatkova et al. 2000b; Zatkova et al. 2012). The history of the AKU research in Slovakia has been described recently (Rovensky et al. 2014).

In previous studies also, analysis and comparison of AKU haplotypes that were constructed using polymorphisms associated with AKU mutations in the families was performed. Our results showed that HGD mutations in Slovakia can be divided into two groups: five mutations (17%) that are shared by different populations and most likely have been introduced into Slovakia by the founder populations that spread throughout Europe (p.M368V (2%), p.V300G (3%), p.P230S (4%), c.87 + 1G > A [p.(Tyr6_Gln29del)] (6%), p.S59fs (2%); and 8 mutations (83%) that most likely originated in Slovakia (p.G161R (42%), p.D153fs (14%), p.H371fs (13%), p.G270R (9%), c.342 + 1G > A [p.(Leu95_Ser114del)] (2%), p.S47L (1%), p.E178G (1%) and p.T167I (1%) (Nemethova et al. 2015; Zatkova 2011; Zatkova et al. 2000a; Zatkova et al. 2000b; Zatkova et al. 2012).

As the combined sequence and haplotype analysis further shows, 7 of the 13 AKU mutations (53.8%) found in Slovakia are associated with hyper-mutated sequences in the HGD, while worldwide, it is 45/142 (31.7%). Therefore, the high genetic heterogeneity in Slovak AKU might be caused by an increased mutation rate in the HGD gene in a small geographical region (Zatkova et al. 2000a). Alternatively, it can be the result of random accumulation of mutations in the region that might be related to the Valachian colonisation during the 14–17th centuries (Srsen et al. 2002; Zatkova et al. 2000a). The preservation of the most prevalent AKU variants that either arose in Slovakia or were brought there, may be the result of a founder effect and genetic drift due to the geographic isolation.

Patients’ organisations

There is strong collaboration between the AKU patients’ associations and researchers that has contributed highly to the increased scientific interest in this rare disease. There are several support networks for AKU patients, including the AKU Society (www.akusociety.org), French ALCAP (http://www.alcap.fr/), Italian AIMAKU (http://www.aimaku.it/), German DSAKU (http://dsaku.de/), the U.S. AKU society (http://www.akusocietyna.org/index.html), and AKU Society in the Netherlands (no web page available). In Slovakia, in 2013, AKUSSaC (AKU Society Slovakia and Czech Republic, www.akussac.sk) started its activity in collaboration with the National Institute of Rheumatic Disease (NIRD) in Piešťany, which is one of the clinical trial centres or DevelopAKUre project. Shortly after, AKUSSaC became a member of the Slovak Alliance of Rare Diseases (Alliance RD), which was founded with the effort to solve problems in the area of RDs in Slovakia in a complex and systemic way. The objective of the Alliance RD is to keep improving the health and social life conditions of rare disease patients and their families, to improve the quality of their lives, and to support their social integration, which AKUSSaC helps to achieve for AKU patients (Ramljakova 2013).

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