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Review

Alkaptonuria: A Very Rare Metabolic Disorder

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Alkaptonuria (AKU) is a very rare autosomal recessive disorder of tyrosine metabolism in the liver due to deficiency of homogentisate 1,2 dioxygenase (HGD) activity, resulting in the accumulation of homogentisic acid (HGA). Circulating HGA pass into various tissues through-out the body, mainly in cartilage and connective tissues, where its oxidation products polymerize and deposit as a melanin-like pigment. Gram quantities of HGA are excreted in the urine. AKU is a progressive disease and the three main features, according the chronology of appearance, are: darkening of the urine at birth, then ochronosis (blue-dark pigmentation of the connective tissue) clinically visible at around 30 yrs in the ear and eye, and finally a severe ochronotic arthropathy at around 50 yrs with spine and large joints involvements. Cardiovascular and renal complications have been described in numerous case report studies. A treatment now is available in the form of a drug nitisinone, which decreases the production of HGA. The enzymatic defect in AKU is caused by the homozygous or compound heterozygous mutations within the HGD gene. This disease has a very low prevalence (1:100,000-250,000) in most of the ethnic groups, except Slovakia and Dominican Republic, where the incidence has shown increase up to 1:19,000. This review highlights classical and recent findings on this very rare disease.

Keywords: Alkaptonuria, Homogentisic acid, Mutation analysis, Ochronosis, Ochronotic arthropathy

Introduction

Alkaptonuria (AKU, MIM: 203500) is a rare autosomal recessive disorder biologically characterized by the presence of homogentisic acid (HGA) in blood and urine. The urine darkens on standing in air and is characteristic of this disorder[1]. This particular phenotype makes it possible to carry out the diagnosis on the initials days of life and represent the first stage of the disease. The presence of HGA is due to the deficiency of homogentisate 1, 2 dioxygenase (HGD), the third enzyme of the catabolic pathway of tyrosine in the liver and results from mutations in the corresponding gene[2,3]. Clinically, this disorder is characterized by ochronosis, a deposition of a yellow-brown pigment into connective tissues due to an oxidation and a polymerisation product of HGA. Ochronosis represents the second evolutionary stage and appears around the age of 20 and 30 as a bluish colouring of the pinna or/ and a brownish colouring of the sclera of the eyes. The third evolutionary stage is ochronotic arthritis between the age of 40 and 60. It affects mainly the coxofemoral and femorotibial joints and the whole of the rachis. These lead to lower back pain and stiffness, loss of height and serious disability[4]. This review highlights classical and recent findings on this very rare disease.

Historical data

The first clinical description of this disease was reported in the year 1584, when a schoolboy in good health excreted urine as black as ink, and coined the term "black urine disease"[5]. This abnormal black pigment was named alcapton or alkapton (*Arabic* word, "al" = "al(k)cali"; *Greek* word, "kaptein" = "to fix"), i.e. a substance that binds molecular oxygen (O_2) from the air with great affinity and was oxidized very fast in alkaline medium to an insoluble brown black pigment[6]. The disease was called alcaptonuria or alkaptonuria (AKU)[6].

In his famous "Croonian lectures" series in London, Archbild Garrod[7-9] described AKU with the prophetic title of "inborn error of metabolism". He had suggested that this disease was due to an enzyme deficiency. On the advice of Bateson, a geneticist, he also proposed that inheritance of AKU was autosomal recessive based on the very large proportion of AKU individuals who were the children of first cousins.

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Abbreviations: AKU, alkaptonuria; *p*-BQA, para-benzoquinone acetate; HGA, homogentisic acid; HGD, homogentisate 1, 2 dioxygenase.

Mundial repartition

Alcaptonuria is a very rare disease with a low prevalence (1:100,000-250,000) in most of the ethnic groups, except Slovakia[10] and Dominican Republic[11], where the incidence was reported to 1:19,000. The occurrence of AKU is probably more frequent in some countries, such as Jordan, United Arab Emirates (UAE) or India, where consanguineous marriages are more common. Another possibility is that there may be patients with features of AKU, who have not been recognised.

A worldwide census revealed 604 AKU cases in the literature (1584-1962) in 35 countries[1]. The most numerous cases in this report were from Europe (377 cases) and North America (152 cases)[1]. One study from Liverpool reported 626 AKU cases in 2011, based on their website established in 2003, with the most numerous cases in Europe (246 of which 75 in UK) and North-America (86 cases)[12]. A census showed 96 AKU patients from 81 families found in the French literature from 1892 until 2011[4]. Based on personal initiative, 40 cases with AKU were identified in one village in Jordan[13]. Though numerous papers on AKU appeared in India from 2008 to 2012, these were mostly on one case report on clinical aspects[14-16]. Five cases from three unrelated Romani colonies were reported[17].

Biochemical abnormalities: HGA and HGA-melanin pigment formation

AKU results from deficiency of HGD, the third crucial enzyme in the tyrosine degradation pathway in liver[2], resulting in the accumulation of HGA in the liver (Fig. 1). Alcaptone to homogentisic acid (HGA) was identified in 1891[18]. HGA is a highly water-soluble molecule which possesses acidic and *p*-diphenol fractions. HGA pass into the circulation then into various tissues in the body, mainly in cartilage and connective tissues. It is eliminated in great amounts in the urine.

In the urine, the *p*-diphenol fraction of HGA is easily oxidized by molecular oxygen (O_2) present in the air into *p*-benzoquinone acetate (p.BQA) which undergoes a polymerization process to form an insoluble melanin (brown-black pigment) termed "HGA- melanin" by analogy with the natural melanin which is formed from DOPA and was termed DOPA-melanin (Fig. 1). This black-brown coloration of urine, characteristic of the disease, is the first stage of AKU. It appears at birth and persists life-long. Pink staining of the napkin may also reveal AKU with a production of a deep brown colour, when washing both the napkins and baby's buttocks[19].

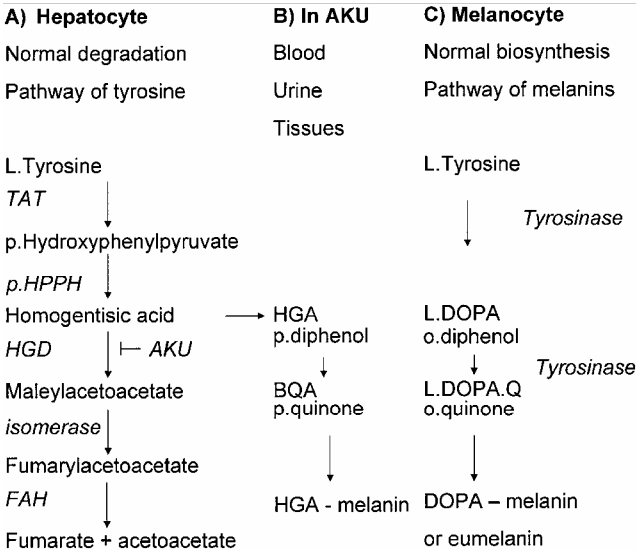

Fig. 1—(A): Normal degradation pathway of L-tyrosine in hepatocytes by five enzymatic steps: TAT, tyrosine amino transferase; p.HPPH, p.hydroxy phenyl pyruvate hydroxylase; HGD, homogentisate dioxygenase, FAH, fumarylacetoacetate hydrolase; (B): In AKU, deficiency of HGD activity leads to accumulation of homogentisic acid (HGA), which pass into the blood and tissues and finally in the urine. HGA is an acidic *p*-diphenol easily oxidized in *p*-benzoquinone acetate (p-BQA), which undergoes a polymerization process to form an insoluble black pigment termed ''HGA-melanin" by analogy with (C) the natural eumelanin or "DOPA-melanin" which is formed from L-DOPA, an *o*-diphenol in melanocytes.

In the blood and tissues, HGA is oxidized by the same mechanism forming HGA-melanin.

i) BQA is a highly labile and reactive compound and appears to undergo addition reactions to form adducts with compounds containing free sulfhydryl (-SH) and amine ($-NH_2$) groups and like free or protein-bound cysteine and lysine residues. These adducts are generated with tissular structural protein like collagen increasing intermolecular cross-linking with a process similar to that found in aging or tanning. The other tissular proteins like elastine and enzymes, where -SH and $-NH_2$ groups are necessary for enzyme activity are also altered[20]. These modified proteins could act as foreign molecules and results in inflammatory process.

ii) HGA-melanin is deposited by adsorption or by chemical binding, particularly to cartilage and connective tissues. Deposit of this insoluble

pigment in connective tissues could be identified macroscopically as a black coloration like in the urine or in a shard (Fig. 2A) and microscopically as an ochre coloration. An unusual necropsy finding in an old man with articular cartilages jet black "as if dipped in ink" was reported in 1866[21]. Microscopically the pigment appears yellow-brown in color and is deposited as granules in the cartilage cells. So, the condition is named "ochronosis" from the Greek words ωχρόσ, yellow and νόσόσ, disease. Due to a quasi steady-state amount of HGA in circulating plasma, this pigment could also deposit into organs like endocardium[22,23] or into the endothelium of vessels, such as coronary arteries and aorta giving a peculiar bluish black tinge[24].

Histochemical abnormalities: HGA-melanin pigment deposit and ochronosis

Microscopic examination of the ochronotic tissue like ligamentous capsule after haematoxylin and eosin staining shows the presence of extracellular pigmentation associated with the collagen fibres and also intracellular pigmentation within fibroblasts[25]. Transmission electron microscopy image reveals three types of collagen fibres in transverse section: numerous fibres with small electron-dense granules located within the fibre body, some fibres appear to have been completely replaced by the pigment and some showing no obvious association with the pigment[25].

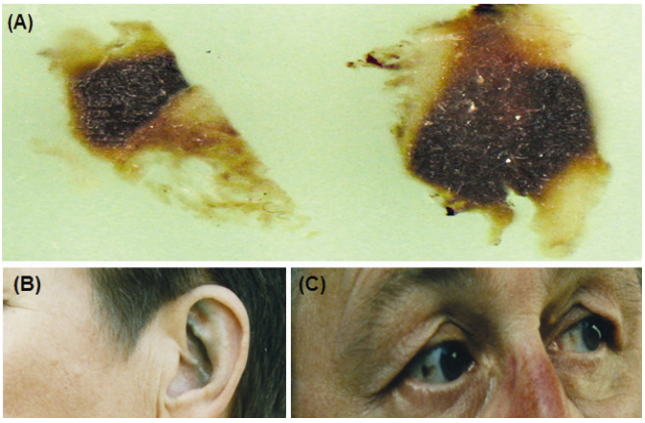

Fig. 2—(A): Shards from the shoulder of the AKU patient. Macroscopically, ochronotic pigment was clearly visible alongside non-pigmented regions; (B): Ear ochronosis in female patient with AKU; and (C): Eyes ochronosis in male patient with AKU

Clinical signs: Ochronosis and ochronotic arthropathy

Clinical ochronosis is the second stage of the disease and appears around 20-30 yrs of age. Ochronosis affects several connective tissues with variable frequencies. The most affected tissues are ears (~70%), which present as a bluish discoloration of the pinna (Fig. 2B). The second one are eyes (~50%) of cases with brown sclera pigmentation, generally oval in shape in temporal or/and nasal part of the limbus (Fig. 2C). The other tissue could be affected with a smaller frequency, 5-10%: hands, nose with bluish discoloration and gum, teeths with a brown tinge[1,17].

Ochronotic arthropathy is the third stage of the disease and usually appears during the fourth decade of life. It begins with dorsolumbar spine involvement: lower back pain and stiffness due to articular cartilage degeneration with bone remodelling, disc calcifications, porotic vertebral bodies and joint spaces narrowing. This condition is quite similar to osteoarthritis[26]. Involvement of large joints like knees, shoulders and hips usually occurs several years later. The course is chronic, progressive and leads to disability and crippling that needs to often undergo surgical replacement. It is frequent to see AKU patients with hip(s), knee(s) and shoulder(s) replacements[27]. Radiographies of the lumbar, dorsal and cervical spine show a narrowing of the intervertebral discs, massive and staged calcifications of the intervertebral discs associated with diffuse osteoporosis[27] (Fig. 3).

Renal and/or prostatic complications

Like liver, kidneys and prostate express HGD and the upstream enzymes of the phenylalanine/tyrosine catabolic pathway. Due to HGD insufficiency, HGA is also produced by these two organs in AKU patients. Kidneys play a critical role for eliminating plasma and renal HGA both by filtration and active secretion. During renal insufficiency, the clearance of HGA decreases, leading to accumulation of HGA and then deposition of HGA-melanin in glomerular cells and destroying connective tissue over the years. In certain cases, renal insufficiency could lead to chronic kidney disease[28]. Renal and/or prostate coloured stones are sometimes found in AKU patients[29]. Brown-black semen has also been reported in two of AKU patients[27].

Cardiovascular complications

Cardiovascular complications result from the deposition of ochronotic pigment within

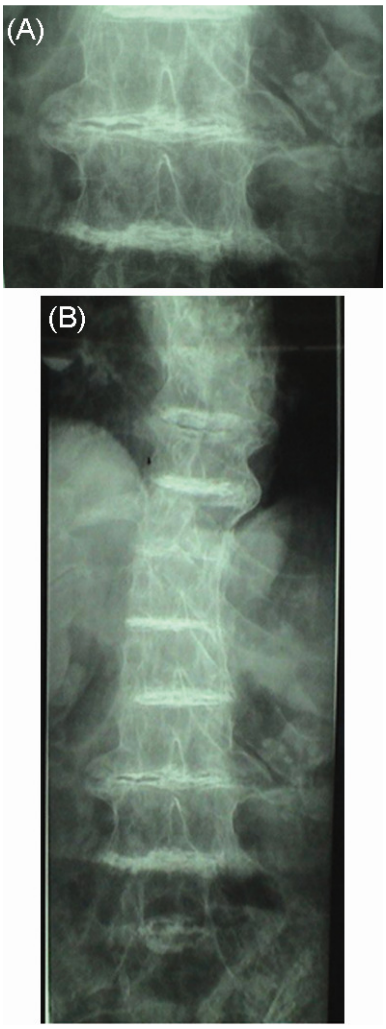

Fig. 3—Radiographies of (A) dorso-lumbar and (B) dorso-lumbar-cervical spine showing a narrowing of joint spaces, calcifications of the intervertebral discs and porotic vertebral bodies

endocardium, aortic intima, heart valves and coronary arteries, giving rise to aortic stenosis, mitral and tricuspid regurgitation, coronary artery disease[22,23]. Deposition of ochronotic pigment within the connective tissue acts as a trigger for dystrophic calcification. In a published case series of 64 patients, 40% have been found to have cardiovascular involvement[29]. Patients with AKU above the age of 40 yrs should undergo routine echocardiographic screening for early diagnosis of cardiac disease[23].

Muskulo-skeletal complications

They are characterized by thickened Achilles tendons, tears of ankle ligaments and ruptures of the patellar and Achilles tendons during the normal activities or with minimal trauma[17,30]. Bilateral spontaneous rupture of the quadriceps tendon has also been described[31]. Quadriceps and hamstring muscle tears due to minimal trauma have also been identified[31].

Genetics

The human HGD gene is mapped to chromosome 3q21-23 and is now completely sequenced[3,32]. The HGD transcript is split into 14 exons and encodes the HGD protein which is composed of 445 amino acids. Northern blot hybridization shows expression of HGD in liver, renal and prostatic tissues[3]. It is also demonstrated that AKU patients are homozygous or compound heterozygous for loss of function mutations in the HGD gene[3]. The first two mutations in the HGD gene have been described in two Spanish families in 1996[3]. These are two missense mutations: P230S (Pro230Ser) in exon 10 and V300G (Val300Gly) in exon 12. To date, more than 100 different mutations of the HGD gene have been identified in patients from many different countries and are described in the new online HGD mutation database (http://hgddatabase.cvtisr.sk/)[33].

The recent establishment of the crystal structure of the human HGD protein provides a framework for understanding the pathogenic effect of AKU mutations[34]. The HGD is a complex structure, which assembles as a functional hexamer arranged as a dimer of trimers. The active site contains a Fe^{2+} atom, close to the interface between the two trimers. This biologically active structure requires many non-covalent bonds (hydrogen, salt, and hydrophobic bonds) between amino acid residues to maintain the spatial structure of the monomer, but also of the dimer and the hexamer. This complex structure can be easily disrupted by mutations of the HGD gene. The effects of some mutations on the HGD enzyme's activity have been studied using mutant HGD proteins in *E. coli*[35]. For other mutations, especially for the missense mutations which are reported to be around 65%, when no functional studies are available, bioinformatics tools (SIFT, POLYPHEN, PANTHER, PNUT, SNAP and FASTSNP) have been generated in order to predict with a 50-80% accuracy the pathogenic effect of these mutations[36,37].

Treatments

Several therapeutically approaches have been used in AKU patients with little success. Current treatments are usually palliative and four types are available:

(I) Reduction of HGA formation i) with a regimen of low tyrosine intake i.e low protein diet but it is difficult to maintain on long term and has no demonstrable efficacy on the symptoms of AKU, or ii) by nitisinone, a triketone herbicide that inhibits p.HPPH, the second enzyme in the tyrosine catabolic pathway (Fig. 1). In a murine model of AKU, oral nitisinone reduce urinary HGA excretion by about 80%, but with a side effect, an increase of plasma tyrosine level. In human, nitisinone has the same biological effect, but during a 3-yr randomized therapeutic trial in 40 alkaptonuria patients, one individual has developed keratopathy classical for tyrosine toxicity[38]. With a 2 mg/day oral administration, nitisinone is well tolerated with a reduced urinary HGA excretion by >95% and a plasma tyrosine levels averaged to 800 µM. Nitisinone treatment should be applied early in life to prevent ochronosis and associated joint arthropathies. Recently in a mouse model of AKU, nitisinone has been shown to completely prevent pigment deposition in the chondrocytes within the articular cartilage of knee[39].

(II) Reduction of pain by classical analgesic drugs, physiotherapy and/or rest, pain control is crucial in the day-life of AKU patients and is tackled by a wide range of analgesic drugs: paracetamol, non-steroidal anti-inflammatory drugs and opioids[27]. Physiotherapy has also been shown to improve activity.

(III) Reduction of physical disabilities by knee, shoulder and/or hip replacements. It is quite frequent in old AKU patients to have 3 to 5 surgical procedures[27]. Hyaluronic acid joint injections may be effective, with a short-term efficacy, as it is demonstrated in a patient with early ochronotic arthropathy[40].

(IV) Enzyme or gene replacement by liver transplant or gene therapy, but enzyme or gene replacements are not yet available[41].

Patients associations

Patients associations are numerous in European countries. One of the most active was the AKU society of the UK, established in 2003 and based in Liverpool. A website for AKU was also established in 2003 (www.alkaptonuria.info). The AKU society used several strategies to help and identify people with AKU in UK[42]: questionnaire based survey for general practitioners, a dedicated website and patient network contact, targeted family screening. By this way, 75 patients with AKU were identified in the UK. Understanding the condition and finding hope for a cure is often a goal for patients with AKU[43]. On the other hand, interactions between patients and doctors/investigators are beneficial, in particular during medical conferences launched by the AKU society (Liverpool, 2008; Sienna, 2009; Cambridge and Liverpool, 2010; Piestany, 2012). ALCAP (www.alcap.fr) is a France based support group for AKU patients, family and care takers. ALCAP was established in 2006 and 34 patients were registered. AKU society, ALCAP and five other European associations from Italy, Sweden, Slovakia, Netherland and Denmark organize a long term (4 yrs) clinical trial with nitisinone. It is a simple blind study, termed SONIA, which begins this year in order to obtain European approval for AKU treatment.

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