Mutational analysis of the xanthine dehydrogenase gene in a Turkish family with autosomal recessive classical xanthinuria

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Abstract
Background. Classical xanthinuria is classified into two categories: type I, deficient only in xanthine dehydrogenase (XDH) activity; and type II, deficient in both XDH and aldehyde oxidase. Both types present mainly with renal stones and lead to renal failure in some cases. We studied the molecular basis of xanthinuria in a Turkish family with two affected siblings.

Methods. We examined two brothers aged 1 and 14 years who presented with histories of passing several urinary stones. We measured their serum and urine levels of uric acid and oxypurine, chemically analysed their stones and performed allopurinol loading tests to diagnose the type of xanthinuria. In addition, we studied the coding regions of the XDH gene in family members.

Results. In the siblings, serum uric acid was undetectable and serum oxypurine was elevated. Laboratory studies showed that the stones that they passed were composed of xanthine, and both were diagnosed as having classical xanthinuria. The allopurinol loading tests indicated their xanthinuria to be type I. Within the entire coding region of the XDH gene, an A to T base change at nucleotide position 2164 was identified in the siblings, indicating a nonsense substitution from AAG (Lys) to TAG (Tyr) at codon 722. Concerning this novel nonsense mutation, restriction fragment length polymorphism (RFLP) analysis showed that the brothers were both homozygous, while the parents were heterozygous, and this confirmed the autosomal recessive inheritance of the XDH gene mutation.

Conclusions. In a Turkish family, we identified a novel point mutation in the XDH gene responsible for classical type I xanthinuria. That both parents had a history of passing renal stones in spite of being heterozygous for that mutation may indicate that individuals with a heterozygous nonsense XDH mutation are more susceptible to nephrolithiasis than healthy individuals. This raises the point that individuals with a heterozygous XDH mutation may also present with renal stones.

Keywords: aldehyde oxidase; hypouricaemia; molybdenum cofactor; urolithiasis

Introduction
Xanthine dehydrogenase (XDH) catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid in the final two steps of purine degradation. Classical xanthinuria, one of the inherited XDH deficiencies, is classified into two categories, classical xanthinuria types I and II. While in classical xanthinuria type I, only XDH activity is lacking, in type II, both XDH and aldehyde oxidase (AO) are absent [1]. Classical xanthinuria is a rare autosomal recessive disorder with an estimated incidence of between 1:6000 and 1:69 000 [1]. Human XDH cDNA was cloned in 1993 and localized to chromosome 2p23 [2,3]. Recently, genes responsible for classical xanthinuria type I and II have been identified. It was reported that the XDH gene was responsible for classical xanthinuria type I, and some mutations in Japanese and Iranian Jewish patients with classical xanthinuria type I have been identified [4–6]. The molybdenum cofactor sulfurase gene, which provides a sulfur atom for the molybdenum cofactor of XDH and AO, has been cloned and is considered to be responsible for xanthinuria type II [7]. However, classical xanthinuria type I and II are not classified according to their clinical symptoms.

In ~40% of individuals with classical xanthinuria, xanthine calculi have been reported, in some cases leading to renal failure [8]. The hallmark of xanthinuria...
is excessive excretion of xanthine in the urine and multiple non-opaque urinary stones. Other occasionally observed manifestations of classical xanthinuria are arthropathy, myopathy and duodenal ulcers. However, some individuals may remain asymptomatic throughout their lives [9].

We report a novel mutation in the XDH genes in two siblings with classical xanthinuria type I in a Turkish family, and we suggest that some individuals who are heterozygous for an XDH mutation could have high risks of developing renal stones.

Subjects and methods

Case 1: A.K.

One of the siblings, A.K., first presented at the age of 8 months with failure to thrive, vomiting, discomfort during urination, gross haematuria and passage of stones. He was the son of consanguineous parents. His older brother, father, paternal grandfather, paternal aunt, mother, maternal grandmother, maternal aunt and maternal uncle had also passed urinary stones (Figure 1). His physical examination was unremarkable. His serum studies revealed the following values: blood urea nitrogen (BUN) 18 mg/dl, creatinine 0.25 mg/dl, uric acid undetectable, sodium 138 mEq/l, potassium 5 mEq/l, chloride 108 mEq/l, calcium 10.4 mg/dl, and phosphorus 5.32 mg/dl; BUN 6 mmol/l (1.78–6.42 mmol/l), creatinine 22.1 mmol/l (26.52–61.88 mmol/l), uric acid undetectable (0.11–0.32 mmol/l), sodium 138 mmol/l (138–145 mmol/l), potassium 5 mmol/l (3.4–4.7 mmol/l), chloride 108 mmol/l (98–107 mmol/l), calcium 2.6 mmol/l (2.2–2.7 mmol/l) and phosphorus 1.71 mmol/l (1.45–1.77 mmol/l). His hepatic enzymes were within their normal ranges. His urine had a specific gravity of 1.013, pH 5.5 and many erythrocytes per h.p.f. The urine culture yielded no growth. Abdominal and renal ultrasonographies were normal after the passage of calculi. The renal stones were analysed using an X-ray diffractometer, and were found to be composed of xanthine.

Case 2: M.K.

M.K., the older brother of case 1, was 14 years old when he was diagnosed to have classical xanthinuria. In the past, he also had had gross haematuria and loin pain (at 4 years of age), and the evaluation at that time had revealed two bladder stones and one left renal stone. The stones were extracted surgically at 5 years of age. X-ray diffractometry revealed the stones to be composed of xanthine. His physical examination was unremarkable. His urine had a pH of 5.0, specific gravity of 1.021, and many erythrocytes per h.p.f. His renal and liver function tests and electrolytes were normal, but serum uric acid was undetectable. Features of the family members with renal stones are presented in Table 1. Serum and urine xanthine and hypoxanthine levels were measured in the siblings and in some members of the family using high-pressure liquid chromatography (HPLC) (Tables 2 and 3).

Analysis of stones

Renal stones were analysed using an X-ray diffractometer (PW3710 BASED PHILIPS, Tube anode: Cu, generator tension 40 kV, generator current 30 mA). Crystal specimens were analysed with X-ray micro-beams at several spots. The X-ray diffraction patterns were recorded with an X-ray goniometer. The diffractograms of the renal stone conformed to that of a specific chemical, xanthine.

Allopurinol loading tests

In order to identify the type of xanthinuria, we performed allopurinol loading tests. Serum was obtained 3 h after allopurinol administration (10 mg/kg) [8,10]. As allopurinol...
is catalysed to oxipurinol by XDH and AO, the serum oxipurinol level in patients with xanthinuria type I is similar to its level in healthy subjects and the serum oxipurinol in patients with the type II is undetectable after allopurinol loading. Serum hypoxanthine, xanthine, allopurinol and oxipurinol were measured using HPLC according to the modified method of Kojima et al. [8,9], who carried out allopurinol loading on four healthy Japanese males to compare their serum oxipurinol levels with those of xanthinuric patients to obtain normal values for the allopurinol loading test [9].

Direct sequencing of DNA

Genomic DNA was isolated from peripheral blood cells by using a QIAGEN blood and cell culture DNA kit (Qiagen, Hilden, Germany). The amplification of the genomic DNA of human XDH is performed by polymerase chain reaction (PCR) as previously described [4]. The PCR products were separated by agarose gel electrophoresis, cut out from the gel, and isolated using a Gene Clean kit (BIO101, Vista, CA). Direct sequencing was performed using a Dye Terminator Cycle Sequencing Kit and an automated sequencer (ABI, Foster City, CA).

Analysis of XDH genomic DNA at nucleotide 2164 (restriction fragment length polymorphism analysis)

For the detection of the genotype of the XDH gene mutation, a part of exon 20 of the XDH gene was amplified by nested PCR using the intronic primer Ex20f (5'-CCCTGCTAAAAGCCACATTTTCTG-3') and exonic primer XDH-2164RsaI (5'-ATTATCTGCTTCGGAAAACCCGT-3'). These primers finally amplified a 131 bp sequence of genomic DNA including the base substitution at nucleotide 2164. The amplified DNA was digested with AfaI under the conditions recommended by the supplier, and subjected to 4% NuSieve GTG agarose gel electrophoresis (BioWhittaker Molecular Applications, Rocklands, ME). By setting G instead of C at nucleotide

Table 1. Features of the family members with renal stones

<table>
<thead>
<tr>
<th>Patients and relatives</th>
<th>Age/ gender</th>
<th>Serum uric acid (µmol/l)</th>
<th>Renal stone (mm)</th>
<th>Mutation</th>
<th>Stone history</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-3 (M.K.)</td>
<td>16/M</td>
<td>0.0</td>
<td>Left 3.6/3.8</td>
<td>Homozygous</td>
<td>Yes</td>
</tr>
<tr>
<td>III-1 (A.K.)</td>
<td>3.5/M</td>
<td>0.0</td>
<td>Left 3.0/3.2</td>
<td>Homozygous</td>
<td>Yes</td>
</tr>
<tr>
<td>III-2 (sibling)</td>
<td>15/M</td>
<td>249.8</td>
<td>–</td>
<td>NS</td>
<td>No</td>
</tr>
<tr>
<td>II-6 (father)</td>
<td>40/M</td>
<td>190.3</td>
<td>Right 2.9</td>
<td>Heterozygous</td>
<td>Yes</td>
</tr>
<tr>
<td>II-7 (mother)</td>
<td>31/F</td>
<td>178.4</td>
<td>–</td>
<td>Heterozygous</td>
<td>Yes</td>
</tr>
<tr>
<td>II-9 (maternal aunt)</td>
<td>22/F</td>
<td>107.0</td>
<td>Left 4.8/4.5/4.6</td>
<td>NS</td>
<td>Yes</td>
</tr>
<tr>
<td>I-1 (paternal grandfather)</td>
<td>65/M</td>
<td>231.9</td>
<td>Right 4.2/Left 3.0</td>
<td>NS</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Normal range serum of serum hypoxanthine and xanthine is from Ichida et al. [10].

Table 2. Serum hypoxanthine and xanthine concentration

<table>
<thead>
<tr>
<th>Patients and relatives</th>
<th>Creatinine (mmol/l)</th>
<th>Hypoxanthine (µmol/l)</th>
<th>Xanthine (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-3 (M.K.)</td>
<td>54.6</td>
<td>10.6</td>
<td>21.0</td>
</tr>
<tr>
<td>III-1 (A.K.)</td>
<td>22.9</td>
<td>14.2</td>
<td>14.2</td>
</tr>
<tr>
<td>II-6 (father)</td>
<td>51.0</td>
<td>17.0</td>
<td>2.9</td>
</tr>
<tr>
<td>II-7 (mother)</td>
<td>51.0</td>
<td>13.4</td>
<td>2.4</td>
</tr>
<tr>
<td>II-9 (maternal aunt)</td>
<td>74.8</td>
<td>20.3</td>
<td>5.5</td>
</tr>
<tr>
<td>I-1 (paternal grandfather)</td>
<td>47.5</td>
<td>13.0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Normal range 4.56–6.61 4.34–10.39

Table 3. Urinary hypoxanthine and xanthine concentration

<table>
<thead>
<tr>
<th>Patients and relatives</th>
<th>Creatinine (mmol/l)</th>
<th>Urate (µmol/l)</th>
<th>Hypoxanthine (µmol/l)</th>
<th>Xanthine (µmol/l)</th>
<th>Urate/Cr (µmol/mmol)</th>
<th>Hypoxanthine/Cr (µmol/mmol)</th>
<th>Xanthine/Cr (µmol/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-3 (M.K.)</td>
<td>4.4</td>
<td>0.0</td>
<td>124.8</td>
<td>281.9</td>
<td>0.0</td>
<td>28.4</td>
<td>64.2</td>
</tr>
<tr>
<td>III-1 (A.K.)</td>
<td>6.0</td>
<td>0.0</td>
<td>171.7</td>
<td>1366.6</td>
<td>0.0</td>
<td>28.5</td>
<td>227.2</td>
</tr>
<tr>
<td>II-6 (father)</td>
<td>14.0</td>
<td>832.0</td>
<td>54.6</td>
<td>20.6</td>
<td>59.4</td>
<td>3.9</td>
<td>1.5</td>
</tr>
<tr>
<td>II-7 (mother)</td>
<td>21.7</td>
<td>2554.7</td>
<td>105.0</td>
<td>60.7</td>
<td>117.4</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>II-9 (maternal aunt)</td>
<td>12.7</td>
<td>938.1</td>
<td>29.1</td>
<td>29.9</td>
<td>74.0</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>I-1 (paternal grandfather)</td>
<td>11.0</td>
<td>2495.7</td>
<td>21.9</td>
<td>25.5</td>
<td>226.9</td>
<td>2.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Normal range 221.7–417.6 3.1–7.9 3.9–8.4

Normal range of urinary hypoxanthine and xanthine is from Kawachi et al. [15].
position 2166 in primer XDH-2164RsaI, a new AfaI digestion site was created in the PCR product. Whether the nucleotide at position 2164 was A or T could be determined by digestion of the amplified genomic DNA with AfaI. When the nucleotide position at 2164 is T, the amplified DNA should be digested with AfaI into two fragments, while at A, the DNA should be resistant to the digestion.

Results

The serum hypoxanthine and xanthine of the patients A.K. and M.K. were 14.2 and 14.2 m mol/l, and 10.6 and 21.0 m mol/l, respectively. The undetectably low uric acid in serum and urine and the increased serum hypoxanthine and xanthine demonstrated that the patients suffered from classical xanthinuria. The serum oxipurinol of patients A.K. and M.K. was 6.5 and 4.1 µg/ml, 42.7 and 27.0 µmol/l, respectively, at 3 h after allopurinol loading. The serum oxipurinol levels of the patients were similar to levels in normal subjects (normal range 19.1–27.6 µmol/l, n = 4), and therefore compatible with classical xanthinuria type I. Urine and serum xanthine and hypoxanthine levels of the siblings and some members of the family are given in Tables 2 and 3.

We amplified and sequenced all the coding regions of the XDH genes of the patients by PCR. Within the entire coding region, an A to T base change at nucleotide position 2164 was identified in the subjects when compared with the sequence reported previously [4]. The nucleotide substitution should generate a nonsense substitution from AAG (Lys) to TAG (Tyr) at codon 722 (Figure 2).

Genomic DNA from the patients, their parents and a control subject was submitted to the PCR and the procedure of digestion with AfaI followed by restriction fragment length polymorphism (RFLP) analysis, as described in Subjects and Methods. The patients turned out to be homozygous with two bands, while their parents were heterozygous with three bands, for the nonsense mutation at codon 722 (Figure 3).

Discussion

Classical xanthinuria is a rare metabolic disorder, and to date just over 100 cases have been reported, one of them from Turkey [8,9,11]. Classical xanthinuria is suspected in the presence of hypouricaemia, reduced urinary uric acid excretion and increased urinary xanthine excretion. An accurate diagnosis is based on the absence of XDH activity in the duodenum or liver [4,8].

Although gene analysis of classical xanthinuria became possible recently, the number of gene-analysed xanthinuric patients is still very small. This is the first report on the gene analysis of a Turkish family with classical xanthinuria type I. We studied two siblings with classical xanthinuria who lacked XDH activity. Their hypouricaemia was the result of underproduction of uric acid. By molecular analysis, we identified an XDH gene mutation in part of exon 20. Within the entire coding region, an A to T base change at nucleotide positions 2164 was identified, indicating a nonsense nucleotide substitution from AAG (Lys) to TAG (Tyr). Whereas the parents were heterozygous, RFLP showed the siblings to be homozygous for this nonsense mutation. According to the Japanese xanthinuria case report, the replacement of C with T at nucleotide position 682 causing a termination codon has been observed in several individuals who are...
unrelated [4]. This mutation may have spread in Japan, whereas in an Iranian Jewish family a different mutation (a 1658insC mutation in exon 16 of the XDH gene) has been reported [5]. The mutation found in the present family is a novel mutation. The mutations of the XDH gene might be different in each population; therefore, further analysis of the XDH mutation is needed in Turkey and in various populations.

The incidence of childhood urolithiasis varies in various geographic regions, and childhood urolithiasis is not rare in Mediterranean countries, such as Turkey [11,12]. The incidence of urolithiasis among primary school children in a metropolitan area in Turkey has been reported to be at least 1.0% [12,13]. In general, underlying metabolic diseases are among the more common causes of childhood urolithiasis. In Turkey, a metabolic disorder was found in 26.1% of 92 children with urolithiasis, and one of them had had a xanthine stone, which suggests the presence of xanthinuria [9,11]. The metabolic reasons for childhood urolithiasis include: hypercalciuric states, cystinuria, hyperoxaluria, distal renal tubular acidosis and disorders of purine metabolism.

Gene analysis of xanthinuria had not been achieved until recently; therefore, identification of the heterozygotes was difficult. Since most heterozygotes do not have symptoms or hypouricaemia, although their urinary oxypurine excretion sometime increases, the rate of urolithiasis in heterozygotes has not been measured reliably. Some studies have tried to identify and characterize xanthinuric heterozygotes from their family trees or their urinary excretion of oxypurine [14,15]. Using the family tree and amounts of urinary oxypurine, Wilson et al. [14] tried to classify xanthinuric family members of one large kindred into homozygotes, obligate heterozygotes, probable heterozygotes, possible heterozygotes and questionable heterozygotes. Although of 29 family members, two had xanthinuria and three individuals who did not suffer from xanthinuria had urolithiasis, their stones were not all composed of xanthine. We too recognized a history of urolithiasis in nine of 16 individuals of our family in spite of the absence of other risk factors of metabolic urolithiasis. However, we were not able to analyse the composition of the stones of all members who passed renal stones, but only the xanthine stones of A.K. and M.K. On the other hand, Kawachi et al. [15] did not report any urolithiasis in a xanthinuric family, in which eight heterozygotes were identified based on their urinary oxypurine excretion. Accordingly, it is difficult to conclude that the individuals with heterozygous XDH mutation have high risks of urolithiasis. Under some conditions, however, heterozygous XDH mutation might have a relationship, directly or indirectly, to urinary stone formation, as there are many individuals with urolithiasis in xanthinuric families. In this study, the urinary xanthine concentrations in both patients with heterozygous XDH mutations were almost normal and did not exceed xanthine solubility. The concentration of xanthine in urine changes easily due to several factors such as dehydration, the purine content of food and catabolic states. Hence, urinary xanthine stones might be formed when urinary xanthine is increased, or urinary xanthine could accelerate stone formation. Alternatively, stone formation could be affected by mutations in another gene near the XDH locus or by factors yet to be identified. XDH mutations may actually influence the frequency of urolithiasis more than we previously have supposed.

In this family, the parents with the heterozygous XDH mutation also had a history of urolithiasis. The rate of urolithiasis in subjects with a heterozygous XDH mutation was not obvious in many previous reports. The subjects with heterozygous XDH mutation may also have recurrent urolithiasis. In general, the number of subjects with a heterozygous mutation is much greater than the number with a homozygous mutation. Moreover, it would be difficult to recognize the subjects with a heterozygous XDH mutation because their serum uric acid might be within the normal range, as in the parents reported here. Since we do not have the stone analysis from the parents, we cannot be sure, but our present study suggests that in such individuals the ability to synthesize XDH may indeed be genetically impaired and some patients with heterozygous mutation may also produce renal stones with additional genetic or non-genetic factors which have yet to be defined. Accordingly, many XDH mutations may actually account much more for the urolithiasis frequency than we previously supposed. We may need to change the diagnostic principles about xanthinuric families including subjects with heterozygous XDH mutations in order to prevent urolithiasis.

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