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Screening for CLCN5 mutation in renal calcium stone formers patients

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ABSTRACT

Thirty-five patients (23 males and 12 females), age 35 ± 13 years old, presenting either idiopathic calcium nephrolithiasis, nephrocalcinosis or mild renal failure with idiopathic calcium nephrolithiasis were selected for the analysis of low molecular weight proteinuria and the possible mutations occurrence in the chloride channel gene CLCN5. The urinary ratio of β2-microglobulin and creatinine (β2M/Cr) was very high in a transplanted woman with nephrocalcinosis (>3.23 mg/mmol) and slightly high in five patients (>0.052 or <1.0 mg/mmol) with multiple urological manipulations. Other studied patients showed β2M/Cr ratio at normal range (0.003-0.052 mg/mmol) without gender difference (p>0.05). Mutation analysis of CLCN5 gene was performed in 26 patients of 35 selected (11 with idiopathic hypercalciuria; 6 men with normal calciuria; 3 with mild renal insufficiency and 6 with nephrocalcinosis) and was normal in all subjects even in those with abnormal molecular weight proteinuria. Conclusion: CLCN5 gene mutation is not a common cause of kidney stone disease or nephrocalcinosis in a group of Brazilian patients studied.

Key words: nephrolithiasis, nephrocalcinosis, low-molecular-weight proteinuria, CLCN5 mutation.

INTRODUCTION

The genetic background of the idiopathic calcium nephrolithiasis is unknown. Some advances have been made in the understanding of disorders that can exhibit nephrolithiasis as a symptom such as primary hyperoxaluria (Danpure et al. 1993), cystinuria (Stoller et al. 1999) and Dent’s disease (X-linked hypercalciuria and nephrolithiasis) (Scheinman et al. 1993), giving some insight into the ethiopathogenesis of calcium idiopathic nephrolithiasis. Dent’s disease, for example, is a rare form of renal tubular disorder and it is characterized by hypercalciuria and low molecular-weight proteinuria besides all features of idiopathic nephrolithiasis, such as calcium stone formation and occasionally nephrocalcinosis and renal failure (Scheinman et al. 1993, Frymoyer et al. 1991, Wrong et al. 1994, Igarashi et al. 1995, Hoopes et al. 1998). The discovery of a...
hypercalciuric man, apparently idiopathic, that was in fact a true case of asymptomatic Dent’s disease (Scheinman et al. 1993), has excited a stone investigators group to look for CLCN5 (gene that encodes for ClC-5 chloride channel) gene mutations. Mutation in CLCN5 is the pathophysiological basis for Dent’s disease that can also present calcium stone formation with idiopathic hypercalciuria, nephrocalcinosis and renal insufficiency. Scheinman et al. in a study that screened 101 patients for low molecular weight proteinuria (LMWP) and presenting idiopathic hypercalciuric found only slight abnormalities in the LMWP in nine patients, none of them had a mutation in CLCN5 (Scheinman et al. 2000). Although the LMWP (β2-microglobuline or retinol-binding protein) study still a useful tool for screening genetic involvement in these patients that usually are male. The same procedure can also be used for the screening of genetic defect carrier female (Scheinman et al. 2000).

In idiopathic lithiasis, which is the main etiologic diagnosis of the renal stone formers, the hypercalciuria is the major urinary risk factor identified. The cellular mechanism of this metabolic disorder is unclear. Nephrocalcinosis is found during imaging studies of renal stone patients and in such occasion idiopathic hypercalciuria, hyperparathyroidism and distal acidification defect must be investigated.

Among all factors involved in the lithogenesis, the present study aims to search for a CLCN5 gene mutation in the context of renal idiopathic calcium lithiasis and/or nephrocalcinosis patients.

MATERIALS AND METHODS

PATIENTS

Recurrent calcium stone formers and/or patients with nephrocalcinosis from Outpatient Clinic of Pedro Ernesto Hospital, Rio de Janeiro, underwent a routine etiologic and metabolic investigation as previously described (Rebele et al. 1996). Thirty-five patients (23 males), age 35 ± 13 (SD) years old, with idiopathic calcium nephrolithiasis, nephrocalcinosis or mild renal failure with idiopathic calci-}

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nary citrate was measured enzymatically with citrate liase (Sigma-Aldrich Corporation, St. Louis, MO, USA) and oxalate by enzymatic-colorimetric assay (Sigma-Aldrich Corporation, St. Louis, MO, USA). Urine pH was measured using a pH meter (Metronic, Minneapolis, MN, USA). The term idiopathic hypercalciuria is applied to hypercalciuria with normocalcemia in the absence of other mineral disorders known to cause hypercalciuria.

β2-MICROGLOBULIN (β2M)

The subjects collected 250 ml of the first morning urine in sodium azide, 200 mg/l final concentration, sent to the laboratory at room temperature. The pH was measured immediately and, if necessary, adjusted to pH > 5.5 with alkali. β2M was measured by fluorimmunoassay (Vidas β2-microglobulina; bioMérieux, MO, USA) within four hours of collection. Creatinine and total proteinuria were also evaluated in the same sample.

The β2M results were expressed in relation to the creatinine in the same sample (β2M/Cr ratio; mg/mmol) and as concentration (mg/l). Normal β2M/Cr ratio is less than 0.052 mg/mmol (Scheinman et al. 2000).

The reference concentration (mg/l) ranges are: from 20 to 39 years old: mean 0.01 and upper limit 0.74; from 40 to 59 years-old: mean: 0.05 and upper limit 1.2.

MUTATION ANALYSIS OF THE CLCN5 GENE

In 26 patients leukocyte DNA was extracted (Miller et al. 1998) and used with CLCN5 specific primers for polymerase chain reaction (PCR) amplification utilizing the conditions described in Table I. The PCR products were purified (QIAquick PCR purification kit; Qiagen, Valencia, CA, USA) and DNA sequence of the PCR products was determined by the use of Taq polymerase cycle sequencing and a semi-automated detection system (Perkin-Elmer, Applied Biosystem, Foster City, CA, USA). The primers were designed based in the CLCN5 gene (genebank accession number 15309448).

RESULTS

The urinary β2-microglobulin (β2M) was evaluated in 35 subjects (23 male) of whom 25 presented idiopathic calcium stone disease (3 with mild renal insufficiency), 6 presented nephrocalcinosis and 4 were asymptomatic offspring of stone and nephrocalcinosis patients. The mean and median of results are shown in Table II. The urine pH varied from 5.53 to 7.60 (median 6.20). The results of β2M expressed as a creatinine ratio, disclosed six patients as having abnormal low molecular weight proteinuria: the transplanted one and 5 cases with multiple urological manipulations for relief of stone obstructions; the total proteinuria was slightly increased, i.e., less than 700 mg protein/g creatinine. Overall, not including the transplanted patient, β2M represented less than 20% of total proteinuria (3% to 16%). In the transplanted patient the β2M corresponded to more than 73% of total protein excretion. (Table II).

The normal β2M/Cr ratio (mg/mmol) ranged from 0.003 to 0.052; median: 0.012; men: from 0.003 to 0.052; median: 0.010, and women: from 0.006 to 0.028; median 0.0125; There was no gender difference, Zcalc = –1.08; p = 0.28; Mann-Whitney test.

DNA ANALYSES FOR CLCN5 MUTATIONS

The CLCN5 gene was analyzed in 26 subjects: 11 with idiopathic hypercalciuria; 6 idiopathic calcium lithiasis men with normal calciuria; 5 calcium stone disease and light to moderate renal insufficiency degrees, 6 nephrocalcinosis (3 without renal stone). Family history of renal stone disease could be obtained in 23 cases and it was positive in about 70%. Some features of these patients are in Table III.

Direct DNA sequencing of the CLCN5 gene did not show any mutation even in those cases with low molecular weight proteinuria.

DISCUSSION

The renal stone is a clinical symptom which has high prevalence (affecting 1 to 12% of the population), significant recurrence rates and high mor-
TABLE I

Polymerase Chain Reaction (PCR) amplification conditions and primers sequences. The temperature, time of each PCR step, number of PCR cycle, and primers used to amplify the different exons of CLCN5 are shown. Denaturation (Denat); Annealing (Anneal); Extension (Ext).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Conditions</th>
<th>No. cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1-2</td>
<td>P1: 5’TGATGTGATATGGCTGCAAG3’ (1-20) P2: 5’CATGACCCTCTAAGAATGAG3’ (738-718)</td>
<td>94°C 45 sec 58°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 3</td>
<td>P3: 5’GCAATCACTACTGATAGT3’ (2784-2803) P4: 5’CAGCATTATTAAATGTGAG3’ (3208-3189)</td>
<td>94°C 45 sec 54°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 4</td>
<td>P5: 5’GCATTTCTCTAGCAAAAG3’ (6219-6238) P6: 5’CATTTTAAAATAAGCGTC3’ (6696-6677)</td>
<td>94°C 45 sec 55°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 5</td>
<td>P7: 5’GAATCCATGGTAAATTTCC3’ (10864-10882) P8: 5’CAACTCCCTTTAATTTCC3’ (11409-11392)</td>
<td>94°C 45 sec 51°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 6</td>
<td>P9: 5’GGAAACATGAAAGCTTAAAG3’ (11946-11964) P10: 5’CTATCCTTATACACCTTTG3’ (12534-12516)</td>
<td>94°C 45 sec 52°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 7</td>
<td>P11: 5’GTATAGTGTGATATGGCTGCAAG3’ (10864-10882) P12: 5’CAACTCCCTTTAATTTCC3’ (11409-11392)</td>
<td>94°C 45 sec 51°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 8</td>
<td>P13: 5’GCAATCACTACTGATAGT3’ (16637-16657) P14: 5’CAGCATTATTAAATGTGAG3’ (17458-17439)</td>
<td>94°C 45 sec 53°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 9</td>
<td>P15: 5’GAATCCATGGTAAATTTCC3’ (19553-19533) P16: 5’GTAATATTTGGAAGCTTTTGG3’ (18979-18999) P17: 5’GGCATATTTTATTGAGTTC3’ (21072-21055)</td>
<td>94°C 45 sec 60°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 10</td>
<td>P18: 5’GCAATCGGTTGAAGCTTTTGG3’ (19553-19533) P19: 5’GTCATTTGTTTACACCG3’ (20437-20454) P20: 5’CTCAACCAACATGTCTTTTGC3’ (21131-21149) P21: 5’CTCAACCAACATGTCTTTTGC3’ (21590-21571)</td>
<td>94°C 45 sec 50°C 45 s 72°C 1 min 37</td>
</tr>
<tr>
<td>Exon 11</td>
<td>P22: 5’CAAAGACAAAGTTGAAAGAAG3’ (22526-22544) P23: 5’TAAATTCATTGAGAGATTT3’ (23366-3346)</td>
<td>94°C 45 sec 50°C 45 s 72°C 1 min 37</td>
</tr>
</tbody>
</table>

In this study, patient selection was based on the presence of either idiopathic calcium nephrolithiasis or hypercalciuria (vitamin D receptor gene, sodium-phosphate co-transporter gene, human homologous with the rat soluble adenylate cyclase gene, renal chloride channel gene and others), so far none has been found to be prevalent (Scheinman et al. 2000, Reed et al. 2002).

In idiopathic calcium stone disease, the high frequency of nephrolithiasis familial history, as in this casuistic, suggest a genetic base. Although there are many possibilities of altered gene to account for idiopathic hypercalciuria (vitamin D receptor gene, sodium-phosphate co-transporter gene, human homologous with the rat soluble adenylate cyclase gene, renal chloride channel gene and others), so far none has been found to be prevalent (Scheinman et al. 2000, Reed et al. 2002).
TABLE II
Results of $\beta^2$-microglobulin ($\beta^2$M), total protein and pH in the first urine in the morning; $n = 35$ (23M/12F).

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>$\beta^2$M (mg/l)</th>
<th>$\beta^2$M/Cr (mg/mmol)</th>
<th>$\beta^2$M/Pr (%)</th>
<th>$\beta^2$M mg/g Cr</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>35,00</td>
<td>0,08</td>
<td>0,01</td>
<td>1,20</td>
<td>101,00</td>
<td>6,20</td>
</tr>
<tr>
<td>Mean</td>
<td>35,20</td>
<td>0,28</td>
<td>0,15</td>
<td>4,36</td>
<td>197,38</td>
<td>6,30</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>13,30</td>
<td>0,70</td>
<td>0,56</td>
<td>12,94</td>
<td>187,71</td>
<td>0,49</td>
</tr>
</tbody>
</table>

TABLE III
Casuistic, etiologic diagnoses and urinary metabolic abnormalities in selected patients for CLCN5 mutation analyses.

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>Familial Antecedent</th>
<th>IPTH* (pg/ml)</th>
<th>Hypo-citraturia</th>
<th>Hyper-calciumia</th>
<th>Hyper-uricosuria</th>
<th>Hyper-phosphaturia</th>
<th>Hyper-oxaluria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic hypercalciuric (n=11)$^1$</td>
<td>7M/4F</td>
<td>8/10</td>
<td>33</td>
<td>9/11</td>
<td>11/11</td>
<td>5/11</td>
<td>3/9</td>
<td>1/11</td>
</tr>
<tr>
<td>Idiopathic calcium nephrolithiasis, normal renal function (n=6)</td>
<td>6 M</td>
<td>3/4</td>
<td>29</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
<td>1/3</td>
<td>0/5</td>
</tr>
<tr>
<td>Idiopathic calcium nephrolithiasis and renal failure (n=6)</td>
<td>3 M</td>
<td>2/3</td>
<td>36</td>
<td>2/3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1/2</td>
</tr>
<tr>
<td>Nephrocalcinosis (n=6)$^2$</td>
<td>2M/4F</td>
<td>3/6</td>
<td>20</td>
<td>4/5</td>
<td>1/4</td>
<td>2/5</td>
<td>0/4</td>
<td>0/5</td>
</tr>
</tbody>
</table>

(X/Y) = positive/analysed; (*) median; (1) one patient had iRTA; (2) including two mild renal failure, two had stones, one had tubular ectasia; intact parathormone (iPTH); Male (M), Female (F).

CLCN5 mutation and calcium nephrolithiasis.

The higher percentage of idiopathic hypercalciuria – 52% – than previously described (Rebelo et al. 1996), is in part due to selection criterion. As it is known, absorptive hypercalciuria type II patient on low calcium diet can reduce urinary calcium excretion to normal range and, in this study, the diet calcium content was not taken into account. This was the reason to include “normal” calciuric idiopathic nephrolithiasis with or without nephrocalcinosis in the search for CLCN5 mutation disease, generally described as a hypercalciuric disease.

Although clinical features of CLCN5 mutation diseases manifest mainly in affected man, female nephrolithiasis and/or nephrocalcinosis have been reported but normally the carrier woman is not symptomatic (Scheinman 1998, Reed et al. 2002).

Based on aforementioned clinical features, the patients were involved in a CLCN5 gene study, whose mutations led to a rare condition that could be misinterpreted as idiopathic nephrolithiasis and nephrocalcinosis, since the affected patients can manifest any feature of metabolic disarrangements seen in idiopathic nephrolithiasis. However, con-
Contrasting to idiopathic lithiasis, in men, not in women, chloride channel disease has a worse prognostic caused by progressive renal failure culminating to end stage renal failure in a young age.

None of our patients had CLCN5 gene mutation, supporting the well known idea that most of calcium stone formers and nephrocalcinosis patients are not phenotypes of the chloride channel disease named Dent’s disease or X-linked calcium nephrolithiasis (Scheinman et al. 2000).

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