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Dent's disease and prevalence of renal stones in dialysis patients in Northeastern Italy

Received: 22 August 2005 / Accepted: 12 September 2005 / Published online: 25 October 2005
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Abstract Dent's disease (DD) involves nephrocalcinosis, urolithiasis, hypercalciuria, LMW proteinuria, and renal failure in various combinations. Males are affected. It is caused by mutations in the chloride channel *CLCN5* gene. It has been suggested that DD is underdiagnosed, occurring in less overt forms, apparently without family history. A possible approach to this problem is to search for *CLCN5* mutations in patients who may have a high prevalence of mutations: end-stage renal disease (ESRD) patients with previous calcium, struvite, or radio-opaque (CSR) stones. We looked for *CLCN5* mutations in 25 males with ESRD–CSR stones selected from all of the patients (1,901 individuals, of which 1,179 were males) of 15 dialysis units in the Veneto region. One DD patient had a new DD mutation (1070 G>T) in exon 7. The new polymorphism IVS11–67 C>T was detected in intron 11 in one patient and one control. We also found 28 females with ESRD and stone history, and seven more

males with ESRD and non-CSR stones. The prevalence of stone formers among dialysis patients in our region was 3.2%, much lower than the prevalence observed in older studies. Struvite stones continue to play a major role in causing stone-associated ESRD.

Keywords *CLCN5* · Dent's disease · End-stage renal disease · Nephrolithiasis · Single strand conformation polymorphism · Struvite stones

Introduction

Dent's disease (OMIM 300009) (DD) is now the generally accepted name for a group of hereditary tubular disorders including X-linked recessive nephrolithiasis (XRN) with renal failure, X-linked recessive hypophosphatemic rickets (XLRH), and idiopathic low molecular

Preliminary data from this paper were presented at the 10th International Symposium on Urolithiasis, Hong Kong, May 25–28, 2004, and published in abstract form in *Urol Res* 32:152–153, 2004

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weight proteinuria (Thakker 2000). This very rare disease tends to manifest itself in childhood or early adult life, being more severe in males than in females. It is characterized by medullary nephrocalcinosis, nephrolithiasis, hypercalciuria, low-molecular-weight proteinuria and other tubular dysfunctions, and renal failure in various combinations. The disease is caused by mutations in the chloride channel *CLCN5* gene (Thakker 2000), which is located on the short arm of the X chromosome (Xp11.22) (1), and encodes a 746-amino acid protein expressed in the renal proximal tubules, the thick ascending loop of Henle, and α -intercalated cells of the collecting ducts (Thakker 2000). However, genetic heterogeneity has been discovered quite recently. Indeed, Hoopes et al (2004) have suggested that a disorder similar to DD may be caused by mutations in other genes: the *CLCN4* gene encoding for the chloride channel ClC-4, and the *OCRL1* gene that encodes for the phosphatidylinositol-4,5-bisphosphate-5-phosphatase and is mutated in the Lowe syndrome (Ludwig and Utsch 2004; Hoopes et al 2005). According to previous studies, mutations in genes others than the *CLCN5* are found in up to 10% of subjects with the DD phenotypes (Hoopes et al 2004).

Different *CLCN5* mutations associated with DD have been reported in unrelated families worldwide. The mutations that have been identified include nonsense, missense, splice site, deletional, and insertional mutations. Approximately 70% of the *CLCN5* mutations are likely to result in truncated or absent ClC-5 channel, and most are scattered over the entire *CLCN5* coding region; genotype to phenotype correlations have not been established.

It has been suggested that the disease may occur more commonly than it is generally believed to, in less overt forms and perhaps without apparent family histories (Reinhart et al 1995). This hypothesis arose from the observation that in the large kindred described by Frymoyer et al (1991), a man was identified with an inactivating mutation in the *CLCN5* chloride channel gene, but he did not have low molecular weight proteinuria (together with the other features of DD) and the only biochemical abnormality he did have was hypercalciuria (Scheinman et al 2000). This raised the question as to whether mutations in *CLCN5* might contribute to the phenotype in patients diagnosed with idiopathic hypercalciuria and/or idiopathic calcium nephrolithiasis, conditions that are twice as common in males as in females, suggesting X-linked transmission in some of the patients. Scheinman et al (2000) looked for *CLCN5* gene mutations in a group of 32 adults and children with idiopathic hypercalciuria, but no *CLCN5* gene mutation was observed. From this data, taking into consideration the sensitivity of DNA analysis methods, it was concluded that DD could account for no more than 3% of patients with idiopathic hypercalciuria (Scheinman et al 2000).

Another way to approach the problem is to look for *CLCN5* gene mutations in another group of patients

who possibly have a high prevalence of mutations: end-stage renal disease (ESRD) patients with previous calcium stones.

We have performed such a study, looking for *CLCN5* mutations in dialysis patients with a personal history of renal stones. Since the prevalence of DD patients without *CLCN5* gene mutations is very low, we did not look for mutations in other candidate genes such as *CLCN4* and *OCRL1*. The survey has also allowed us to determine the prevalence of nephrolithiasis in ESRD patients. The results are reported in the present paper.

Patients and methods

The enrolment of patients among those undergoing chronic hemo- or peritoneal dialysis treatment was carried out during the year 2001 in the Veneto region, which is located in Northeastern Italy. This region has over 4.5 million inhabitants, and according to the Veneto Register of Dialysis and Transplantation, 2,258 patients (1,400 males) underwent chronic dialysis (both peritoneal and hemodialysis) in 21 dialysis units in 2001. Fifteen dialysis units participated in the screening of patients for this study; these cover 84% of the entire population of this region with 1,901 patients (1,179 males) on chronic dialysis.

By interviewing all of the patients, consulting clinical records, and studying the results from X-ray or US evaluations, it was possible to ascertain whether they had passed one or more renal stones, its chemical composition, whether they have or have had a renal stone, the radio-opaque nature of it, and whether they had undergone renal stone surgery or nephrectomy.

For the molecular genetic study, among the subjects who were positive for renal stones, only males with known calcium or struvite stone composition or radio-opaque stones (hereinafter indicated as CSR stone patients) were considered. Blood samples were obtained from all subjects after obtaining written informed consent. Statistics was investigated via χ^2 -test.

Mutational analysis of the *CLCN5* gene

The leukocyte DNA was extracted by NucleoSpin Blood Quick Pure minicolumns (Macherey-Nagel GmbH & Co., KG, Duren, Germany) and used with specific primers for polymerase chain reaction (PCR) amplification of the 12 exons and exon-intron boundaries of the *CLCN5* gene. Primers and PCR conditions have been described by Lloyd et al (1997). Single strand conformation polymorphism (SSCP) analysis was applied to detect DNA mutations as described. PCR products demonstrating mobility shift under specific electrophoretic conditions were sequenced directly.

Sequencing

Direct sequencing of PCR products was performed using the ABI Prism 373 DNA Sequencer and the Big-Dye Terminator Cycle Sequencing ready reaction Kit (Applied Biosystem, Milano, Italy). NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html) was executed for analyzing potential splice sites.

Results

We found 60 (32 males) patients, age range 39–75 years, with a history or current incidence of renal stones. This corresponds to a prevalence of 3.2% renal stone formers among chronic dialysis patients in our region; 48 were positive for CSR stones (prevalence 2.6%); in five it was not possible to ascertain the chemical or radio-opaque nature of the previous stone(s). The prevalence of CSR stone patients was 2.1 and 3.2% in males and females, respectively; these values were not statistically different ($\chi^2=1.475$). All male CSR stone subjects agreed to participate in the molecular study. We screened all the coding sequences (exons 2–12) and the exon–intron boundaries of the *CLCN5* gene with SSCP analysis in 25 male patients. A SSCP mobility shift was found in two samples. By sequencing the PCR products, a nucleotide C–T substitution in intron 11 at position –67 upstream from the 3′-splice site (IVS11–67 C>T) and the nucleotide substitution 1070 G>T in exon 7 were identified.

The first variant was inherited from the mother. The patient was affected by a severe type 1 diabetic disease, and the clinical history leading to ESRD fits with a form secondary to diabetic nephropathy. His family history was negative for renal stones, renal diseases, or ESRD. The IVS11–67 C>T variant was not detected in any of the 47 X chromosomes of the DD patients, but it was found in one of the 49 X chromosomes from a control group comprising 24 male and 6 female patients with idiopathic ESRD or ESRD due to autosomal dominant polycystic kidney disease (ADPKD). According to the NNSPLICE prediction program, this nucleotide substitution should not change the mRNA splicing.

The second nucleotide substitution was also found in the proband's daughter and his teenage male nephew, who had mild proteinuria and hypercalciuria. It causes the nonconservative substitution of glycine to valine G260 V in the CIC-5 protein.

Table 1 Clinical data for patients identified as stone sufferers

	Males (32 patients in total)	Females (28 patients in total)
Struvite/infection stones	3	7
Nephrectomy/polectomy	1	3
Open renal surgery	5	8
ESRD attributed to stones or stone-correlated causes	22	17

Table 1 shows clinical data for the patients identified as stone sufferers. Although the differences were not statistically significant, it is evident that females have an increased prevalence of struvite/infection stones (25% versus 9% in males), of nephrectomy (11% versus 3%) and open renal surgery (29% versus 16%); however, the prevalence of ESRD attributed to known etiologies other than stones or stone-related causes is lower in males (31% versus 39% in females).

Discussion

It is important to investigate the prevalence of DD, to be able to recognize it and to discover *CLCN5* gene mutations in particular groups of patients because of the risk of renal failure associated with this specific condition. As a matter of fact, correct recognition of this disease, which leads to uremia, might help to define the clinical profiles of patients at high risk of being affected, allowing it to be detected (and possibly treated) early.

Unfortunately, at times the clinical picture may be very poor or not specific enough. For instance, isolated hypercalciuria, and/or tubular proteinuria in the early stages might be the unique manifestations of the disease, but regrettably both conditions are usually asymptomatic.

Furthermore, knowing the nature of the *CLCN5* gene mutations alone does not allow us to make assumptions about the clinical phenotype, since there appears to be no genotype–phenotype correlation in DD. Indeed, among the varieties of DD, XLRH and XRN (which share the same pattern of *CLCN5* mutations) have proximal tubular defects in common, but rickets is absent in XRN, while nephrocalcinosis and renal failure are more notable in XLRH.

Finally, inheritance cannot always be clearly recognized, due to the fact that the clinical picture may be very poor or not specific. Thus, relying on the occurrence of familial cases as a pointer toward diagnosis could be misleading.

In terms of the typical manifestations of DD, the fact that patients with low molecular weight proteinuria are sometimes affected by the disease is now well known. In Japan, by governmental mandate, elementary and junior high school students have been screened annually since 1974 for proteinuria, hematuria, and glucosuria to identify children with possible renal disorders. The prevalence of proteinuria among elementary school children is 0.08%, while junior high-school students have a prevalence of 0.37% (Murakami et al 1991). Starting from the first detection of low molecular weight proteinuria up to 1995, over 60 patients were recognized as also having hypercalciuria, nephrocalcinosis (one of the phenotypes belonging to the DD) and *CLCN5* gene mutations (Igarashi et al 1995). However, although the precise prevalence of DD has not been established, this presentation is probably quite rare.

The isolated hypercalciuria phenotype may sometimes also be due to a *CLCN5* gene mutation. Thus, looking for DD within the hypercalciuric patient pool may be a possible way to identify these patients. However, once again, very few DD patients could be identified in the group of subjects with such a phenotype (Scheinman et al 2000).

Renal failure is another interesting and important phenotype in DD, due to its clinical cost. As a matter of fact, ESRD is quite frequent in this condition. Nine out of 15 male cases described by Wrong et al (1994) developed ESRD at the age of 32–65; others have probably not been followed for long enough to establish whether this condition is inevitable, although decline in renal function seems to occur in most patients (Wrong et al 1994). DD patients who received renal transplantation have also been reported (Frymoyer et al 1991; Wrong et al 1994; Scheinman 1998). In fact, it is estimated that 35–100% of male DD patients develop terminal renal failure (Frymoyer et al 1991; Scheinman 1998).

On the other hand, idiopathic ESRD, which in theory might include unrecognized DD cases, constitutes a significant proportion of all ESRD cases. Prevalences vary according to ethnicity and geography, and in the 2001 Veneto region registry it was 16%. Since in this area there are no limitations of any kind on access to health services, the likely explanation for undetermined causes of ESRD is late presentation. In the absence of significant multi-system involvement, hematuria or nephritic and nephrotic range proteinuria, late presentation is likely on clinical grounds alone and can be the corollary of this particular kind of renal pathology. Hence, if a renal disorder proceeds subclinically, or its clinical and laboratory manifestations are not adequately considered or correctly recognized, it can contribute to this category of ESRD. We speculated that one of these renal disorders might be DD.

The scenario behind our working hypothesis was that, since most cases of renal stones are idiopathic in origin, and nephrolithiasis is very frequent in the general population, being as prevalent as 10% (Gambaro et al 2004), the association between renal failure and renal stones, a possible clue for DD, may be overlooked. In our opinion, this hypothesis was made even stronger by considering that the diagnosis of DD may be difficult since, as previously observed, patients with *CLCN5* mutations may have atypical presentations: just hypercalciuria without any other feature of the disease (Scheinman et al 2000), or ESRD without nephrocalcinosis (Wrong et al 1994).

We found 32 male subjects with ESRD and a previous history of, or current, renal stone(s) whatever the composition or the X-ray appearance. We restricted the molecular investigation to the 25 male subjects with CSR stones. The reason for investigating only males was that the disease is an X-linked recessive disorder, and that so far, ten years after the initial description (Wrong et al 1994), only one female, a 59-year-old British lady

who was found to be carrier of a *CLCN5* mutation, has been reported to have developed ESRD (Wrong et al 1994). Furthermore, only CSR stone cases were considered because DD associates with calcium stones only and hypercalciuria, which quite frequently pre-exists struvite stones.

Thus, we screened all of the coding sequences (exons 2–12) and the exon–intron boundaries of the *CLCN5* gene by SSCP analysis in 25 ESRD–CSR males and found one case with a *CLCN5* disease-causing mutation. This patient had a history of recurrent mixed calcium oxalate and phosphate renal stones, and because of nephrocalcinosis was previously diagnosed as a sponge kidney patient. A thorough family enquiry performed after the identification of the *CLCN5* variant in this case led to the discovery of two young male nephews (sons of two proband's daughters) both with proteinuria lower than 1.5 g/24 h mainly of tubular origin. In one of the two sisters, increased urinary excretion of β 2-microglobulin was found, and her son was hypercalciuric. Both revealed the same *CLCN5* gene variant observed in the proband, which can therefore be considered as a disease-causing mutation. The pattern of inheritance of the disorder (only male to male transmission through female healthy carriers), and the clinical phenotypes in the index case's family are indeed typical of DD. The nucleotide substitution 1070 G > T in *CLCN5* exon 7, not reported until now, causes the nonconservative substitution of glycine with valine (G260 V), most likely affecting the function of the CIC-5 carrier.

A further inherited nucleotide substitution IVS11–67 C > T in intron 11 of the *CLCN5* gene not previously reported in the literature was identified in a second ESRD–CSR patient. An accurate examination of his family history failed to reveal any case of DD, renal diseases, ESRD or calcium stones; on the contrary he most likely developed ESRD because of type 1 diabetes mellitus. Due to its position, at the –67 nucleotide of the invariant consensus acceptor splice site of intron 11, the variant could be considered a polymorphism. It is now widely accepted, however, that several bases adjacent to the splice donor and acceptor sites follow consensus sequence rules and, thus could represent crucial sites for the correct mRNA splicing (Lim and Burge 2001). An analysis of single nucleotide polymorphism (SNP) distribution in introns showed that SNPs are under-represented, not only near splice sites at both intron ends, but also in the first and last 150 bp of introns, indicating that those regions probably contain a significant number of elements required for the splicing process (Majewski and Ott 2002). The nucleotide variation detected in our study lies 60–70 bp upstream of the 3'-splice, and could affect regulatory elements of intron 11. RT/PCR analysis of patient's mRNA could reveal whether nucleotide substitution affects functional splice elements. Unfortunately, RNA was unavailable since the patient died in the meantime from myocardial infarction. However, the use of NNSPLICE, a splice site prediction program, allowed us to predict that the variation should not

change mRNA splicing. Indeed, the IVS11–67 C>T variant was not detected in any of the 47 X chromosomes coming from the DD patients, while it was found in one out of 49 X chromosomes from a control group (30 subjects) formed by male and female patients with idiopathic ESRD or ESRD due to ADPKD. The nucleotide substitution was detected in a male patient with ESRD and ADPKD without any history of stones. These findings support the idea that the nucleotide substitution IVS11–67 C>T is not a disease-causing mutation, rather it is a SNP. Indeed, a widespread analysis of the *CLCN5* gene variant in a normal population from our region has shown that individuals carrying IVS11–67 C>T represent 5.5% of the normal population giving an allele frequency of 0.033 (unpublished), thus confirming that the nucleotide substitution is indeed a SNP.

While several techniques have been set up for the efficient detection of point mutations, no “best method” has yet been found. Among PCR-coupled mutation detection techniques, SSCP analysis is probably the most widely used technique, having proved to be a relatively simple and cost-effective method for the direct analysis of genetic variations, particularly when large numbers of PCR samples are required for analysis. Given its technical simplicity and high mutation detection ability, SSCP also provides a useful tool for the molecular diagnosis of genetic diseases with a high rate of nonrecurrent mutations such as DD. The principle of the method is that the electrophoretic mobility of a single-stranded DNA molecule in a nondenaturing gel is dependent on its size and structure (conformations). Since conformations are highly dependent on primary nucleotide sequence and on the applied electrophoretic conditions, a number of studies have shown that the mutation detection rate of SSCP can vary. Usually, 75–100% of point mutations can be resolved by SSCP over fragment lengths of 100–400 bp (Gasser and Zhu 1999). In our experience, SSCP analysis is an efficient and sensitive technique for the detection of mutations in the 12 exons including intronic splice-site sequences of the *CLCN5* gene, and our policy is to reserve DNA sequencing to the exons revealing variant SSCP patterns. As a matter of fact, to validate the use of SSCP in our laboratory, in a “positive control” group of 14 patients from 14 different families with a classical Dent phenotype and *CLCN5* mutations, we succeeded in detecting mutations using SSCP in 11 subjects, giving a sensitivity of 78%.

The finding of one mutated patient among the investigated 25 corresponds to a prevalence of 4% in the population of ESRD and CSR stone patients. We cannot rule out over- or under-estimation of the DD prevalence in this population because of the low number of cases. However, the initial hypothesis that among ESRD patients a subgroup may constitute a “reservoir” of undiagnosed DD is confirmed. It seems that we probably misdiagnose DD rather than missing the case completely because of subclinical manifestations. Actually,

the only patient we found to be misinterpreted was a medullary sponge kidney case. This disorder is also frequently characterized by nephrocalcinosis, although with a very particular location in the papillae rather than in the medulla (outer medulla) as in DD. Anyway, our data suggests once more that DD is a very rare condition, confirming findings in patients with different clinical phenotypes: low molecular weight proteinuria, and hypercalciuria. Even if we were to consider a 78% SSCP sensitivity, the conclusion does not change: a total of six of our ESRD–CSR male subjects might indeed be DD patients; this number would correspond to a prevalence of 0.54% of all male dialysis patients in the Veneto region, a low prevalence indeed!

An interesting finding of this study is the current prevalence of a history of renal stones among dialysis patients. Among this group of subjects, the prevalence of urinary tract lithiasis was reported to range from 3.6 to 8% (Gambaro et al 2001). These estimations tend to originate from the pre-extracorporeal shock wave lithotripsy (ESWL) era and from areas where access to medical and urological treatment may be not completely free. Our data shows that now, more than two decades after the introduction of the ESWL, the prevalence is 3.2%, at least in our region. This area has a number of very efficient urological units, and, as previously noted, no limitations in access to health care. Thus, although we do not have data on the prevalence of urolithiasis in the pre-ESWL era in this region, we believe that our finding indirectly demonstrates that present standards of urological practice, and the current options of urological treatment and medical prophylaxis, have significantly improved the prognosis of renal stone disease. Actually, Jungers et al (2004) have shown that the proportion of patients with nephrolithiasis-related ESRD decreased from 4.7% in the triennial period 1989–1991 to 2.2% in the period 1998–2000.

Of course, among our patients, previous or current renal stones were not responsible for all cases of ESRD; actually, ESRD was ascribed by the referring nephrologist to stones or stone-related conditions (pyelonephritis, obstruction, and so on) for only 65% of the patients identified in this survey (Table 1). This corresponds to 2.1% of the entire ESRD population we investigated, an intermediate value between the 3.2% recently reported by Jungers et al (2004), and the 1.2% observed in the United States (US Renal Data System 1999). The high prevalence of nephrolithiasis in the general population (Gambaro et al 2004) or the occurrence of stones as a complication of ESRD (Viterbo and Mydlo 2002) could be responsible for the casual concurrence of renal failure and nephrolithiasis. In actual fact, in a survey of an unselected series of 2,000 stone formers, less than 2% experienced low-to-moderate grade renal failure, and among these, an overall percentage of 59% had idiopathic calcium stone disease (Gupta et al 1994). Thus, renal stones and their complications quite rarely lead to ESRD, as previously pointed out (Worcester et al 2003). Certainly, among

different kinds of renal stones, struvite calculosis is the form most frequently responsible for ESRD, and while genetic forms (such as cystinuria, primary hyperoxalurias, DD, and 2,8-dihydroxyadenine crystalluria) lead to ESRD in most cases, they are so rare as to account for only a few cases of ESRD secondary to lithiasis (Gambaro et al 2001; Jungers et al 2004). It is well known that struvite/infection stones are more frequently observed in females, contrary to all other forms of nephrolithiasis which are more frequent in males (Worcester et al 2003). Moreover, females have an approximately twofold higher risk of kidney loss compared to males (Worcester et al 2003), a finding which goes parallel to the significant rate of kidney loss in patients with struvite stones. All of these findings are confirmed by our data (Table 1). In idiopathic calcium stone formers, the male:female ratio is 3:1. According to our study 3.3% of female versus 2.2% of male, ESRD patients (male:female ratio 1:1.5) had CSR stones, which confirms a recent report (Jungers et al 2004), and 26% of females versus 8% of males had struvite/infection stones (Table 1); these facts both support the notion that it is probable that struvite stones continue to play a major role in the cause of ESRD for all kinds of nephrolithiasis.

In conclusion, within the limitations of this study (relying in part on patient recalls), we have demonstrated that DD is indeed a very rare disease; that it is not missed at diagnosis in ESRD patients, but that it could be misdiagnosed; that it is a marginal cause of ESRD globally; and that the prevalence of stones in ESRD patients has decreased, suggesting that modern treatment options have significantly improved the prognosis of urolithiasis.

Acknowledgments The Veneto Collaborative Study Group on Dent's Disease comprised the following members: E. De Paoli Vitali, Belluno; G. Bonadonna, Camposampiero; C. Cascone, Castelfranco Veneto, and Treviso; V. Goepel, Chioggia; G. Meneghel, Dolo; J. Nachtigal, F. Antonucci, Feltre; A. Fracasso, Mestre; P. Conz, C. Catalano, Monselice; A. Antonello, A. D'Angelo, L. Citron, F. Nalesso, R. Zagatti, U. Vertolli, Padova (two centers); M. Piva, Rovigo; T. Teodori, S. Donà di Piave; M. Nordio, Venezia; R. Dell'Aquila, Vicenza, and G. Gambaro, A. Lupo, Verona. This study was supported by the Italian Ministry of the University, program Cofin 40%, No. 2002062925-003, year 2002, and by Regione Veneto, Ricerca Sanitaria Finalizzata, Malattie Rare, year 2000 (No. 798/03/98).

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