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Searching for CYP24A1 mutations in cohorts of patients with calcium nephrolithiasis

J Sayers[1]*, AM Hynes[1], SJ Rice[1], P Hogg[2], JA Sayer[1]

Abstract

Introduction

The genetics underlying the idiopathic hypercalciuria leading to calcium-containing renal stones remains elusive. The discovery of rare monogenic tubulopathies, often leading to hypercalciuria, has increased our understanding of tubular physiology and patho-physiology. However, insights into idiopathic calcium stone formation have not been gained from these disorders. The aim of this study is to examine CYP24A1 mutations in cohorts of patients with calcium nephrolithiasis.

Materials and Methods

We examined two cohorts of stone-forming patients for mutations in CYP24A1, which encodes the vitamin D24-hydroxylase enzyme. The first cohort had a biochemical phenotype of suppressed parathyroid hormone and high normal serum calcium, whilst the second cohort had a hypercalciuria phenotype. We did not identify bi-allelic sequence variants in CYP24A1 in our cohorts.

Results

In cohort 1, we identified 9 known sequence variants. In cohort 2 we identified 7 known sequence variants.

Conclusion

CYP24A1 mutations remain a rare cause of calcium nephrolithiasis and hypercalciuria.

* Corresponding author
Email: j.a.sayers@ncl.ac.uk

[1] Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle NE1 3BZ, United Kingdom
[2] Lithotripsy Unit, Freeman Hospital, Newcastle NE7 7DN, United Kingdom

Introduction

Renal stones have afflicted human for many thousands of years. The oldest known stone (from approximately 4800 BCE) was found in an Egyptian grave in 1901[1]. Today, renal stones pose a significant public health problem, with lifetime risk of forming a stone exceeding 5% in women and 12% in men[2]. The direct and indirect costs are substantial, with the annual cost in the United States estimated to be in excess of $5 billion[3].

Genetic factors are suspected to be important in the aetiology of renal stone disease, based on evidence of familial clustering and data from twin studies[4,5], but so far the specific allelic variants contributing to nephrolithiasis susceptibility have been difficult to elucidate[6]. Several rare monogenic disorders associated with hypercalciuria (urinary excretion >6.2 mmol/24 h in females and >7.5 mmol/24 h in males) that cause stones have been identified[7–9]. However, despite initial excitement, the genes involved in these disorders, such as *CLCN5*, have not proved to have a wider role in the more common 'idiopathic' renal stone disease[10]. The genes involved in the majority of nephrolithiasis patients therefore remain unknown[11].

Calcium-containing stones account for over 80% of all renal stones[2], and thus much of the existing research into the genetics of nephrolithiasis has focused on candidate genes believed to be important in calcium regulation and excretion[11].

Genes involved in vitamin D-mediated calcium regulation have caused considerable interest. For example, the vitamin D receptor gene has been identified as a candidate gene for nephrolithiasis through linkage analysis[12]. More recently, a novel cause of infantile hypercalcaemia has been described involving mutations in the gene *CYP24A1*[13].

CYP24A1 encodes the vitamin D24-hydroxylase enzyme, the enzyme responsible for the degradation of vitamin D from its active to inactive form. The enzyme also catalyses the reaction by which 25(OH) vitamin D, a precursor to the active form, is metabolised to an inactive product. It, thereby, acts as a major enzyme in the regulation of vitamin D[14] (Figure 1).

CYP24A1 represents a candidate gene for calcium nephrolithiasis. Indeed, very recently a genetic analysis of two patients (a 9-year-old boy and a 38-year-old man) with elevated 1,25 $(OH)_2$ vitamin D, suppressed parathyroid hormone (PTH) and nephrocalcinosis or nephrolithiasis, respectively, were found to have bi-allelic mutations in *CYP24A1*[15]. Furthermore, the predicted frequency of some potentially pathogenic sequence variants within this gene is high, suggesting these variants may be important genetic risk factors for stone formation[15]. We therefore examined two cohorts of calcium stone formers with phenotypes that might be suggestive of an underlying defect in vitamin D metabolism mutations in *CYP24A1*.

Materials and Methods

Full ethical approval was obtained by the Newcastle and North Tyneside Research Ethics Committee and informed written consent were obtained from participants.

Recruitment of patients

Adult kidney stone formers (age range 18–80 years) attending for lithotripsy at the Newcastle upon Tyne

For citation purposes: *Sayers J, Hynes AM, Rice SJ, Hogg P, Sayer JA*. Searching for CYP24A1 mutations in cohorts of patients with calcium nephrolithiasis. OA Nephrology 2013 May 01;1(1):6.

Competing interests: none declared. Conflict of Interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

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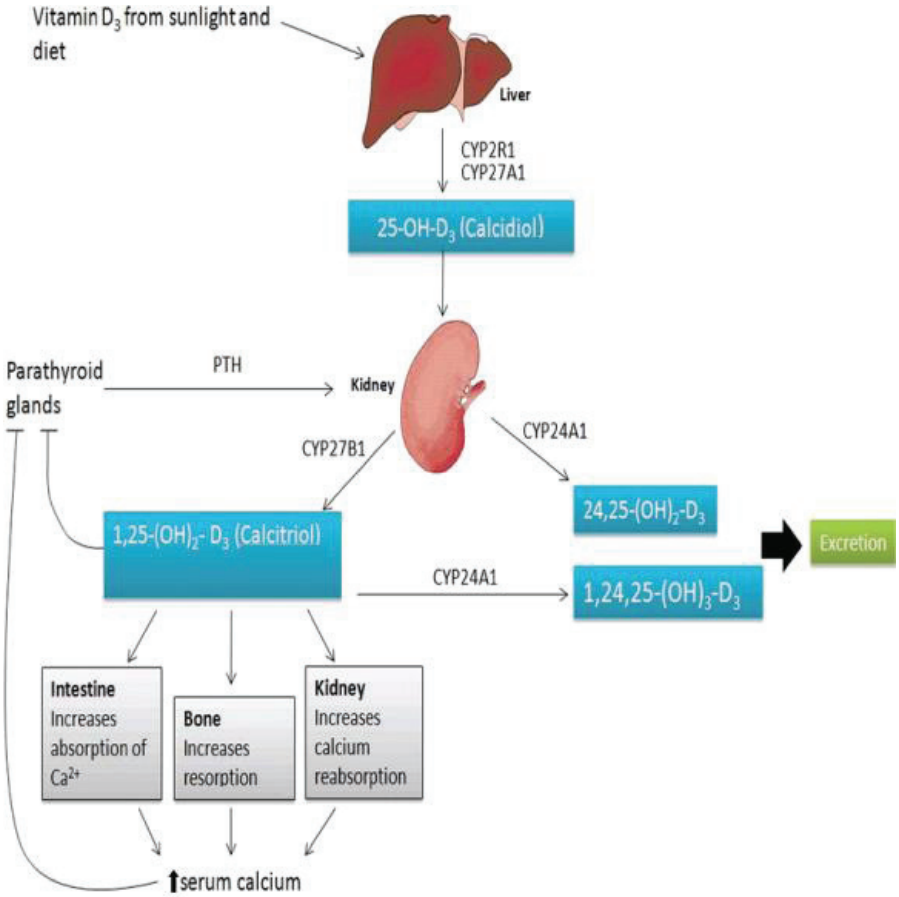

Figure 1: Overview of vitamin D metabolism and role in calcium regulation. Vitamin D is either ingested or is synthesised by the body in response to sunlight exposure. It undergoes a series of hydroxylations in the liver and kidneys to convert it to its biologically active form 1,25$(OH)_2$ vitamin D. 1,25$(OH)_2$ vitamin D regulates serum calcium levels by increasing calcium absorption from the intestine, increasing bone turnover and decreasing calcium excretion from the kidneys. When serum calcium levels are high parathyroid hormone (PTH) is released and increases the conversion of vitamin D to this active form. *CYP24A1* catalyses the hydroxylation of 1,25$(OH)_2$ vitamin D, and its precursor 25(OH) vitamin D, to inactive forms for excretion. This figure is adapted from reference[13].

NHS Foundation Trust Hospital, UK were recruited following informed consent. Patients underwent a biochemical screen (serum biochemistry including calcium and PTH) and a spot urine estimation of urine calcium/creatinine ratio[16].

Two cohorts of patients were selected from 703 renal calcium stone patients based on their biochemical phenotype. Cohort 1 consisted of 72 patients who displayed a high normal (corrected for albumin) serum calcium together with a low/low normal (suppressed) parathyroid hormone (PTH; level <31 pg/ml). Cohort 2 consisted of 94 patients with a tendency towards hypercalciuria as evidenced by a high or high normal calcium/creatinine ratio.

Molecular analysis

Mutational analysis of *CYP24A1* was performed using exon PCR, mutational screening of amplicons by CEL1 digest[17] and direct sequencing. Oligonucleotide primer sequences are listed in Table 1. For the DNA screening of control patients, 92 samples were obtained from blood donor (Caucasian healthy control) panels. Sequence variants were identified using Mutation Surveyor V4.0 software and cross-referenced to dbSNP[18].

***In silico* analysis of mutations**

Online *in silico* analyses were performed when sequence variants were identified. These included MutationTaster (www.mutationtaster.org), SIFT (http://sift.jcvi.org/) and PolyPhen (http://genetics.bwh.harvard.edu/). PyMOL molecular visualisation system was used to model the protein structure of *CYP24A1*[19].

Results

The biochemical phenotypic characteristics of the 72 stone-forming patients in cohort 1 are shown in Table 2. The main features of these adult patients include a high normal serum calcium together with a relatively suppressed PTH. Unfortunately, data are not available for serum vitamin D metabolites in these patients. This cohort did not have prominent hypercalciuria, with a mean urinary calcium creatinine ratio of 0.49 mmol/mmol creatinine). Cohort 2 consisted of an additional 94 patients with evidence of hypercalciuria as determined by a spot urine analysis for calcium/creatinine ratio. The mean urinary calcium creatinine ratio was raised at 0.72 mmol/mmol creatinine). Where available, 24 h urine collections for calcium were performed and confirmed the hypercalciuria evident on the spot urine samples. In this cohort, serum calcium was within normal range (mean 2.3 mmol/L) and PTH was also within normal limits (Table 3).

In cohort 1, we identified nine known sequence variants (Table 4). All variants had a low minor allele frequency (MAF) except

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All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
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Table 1. Oligonucleotide primer sequences.			
Forward primer	**Sequence**	**Reverse primer**	**Sequence**
CYP24A1 Exon1_1	AGGGCATGCTCTGTCTCC	CYP24A1 Exon1_1	AAGGCAGGAGGATGGGG
CYP24A1 Exon1_2	CCCTCTTTGCTTCCTTTTCC	CYP24A1 Exon1_2	ATGTCGGGGAGGGTTTG
CYP24A1 Exon2	GAGGAAGGAGGCGGGAG	CYP24A1 Exon2	CCGTCAGGCTCATCAGGTC
CYP24A1 Exon3	GCTGGAGTATTTCTGCATCTCC	CYP24A1 Exon3	CCACCAATATCCCTATGTCCC
CYP24A1 Exon4	ATGCGATGTAGCAAGACCTG	CYP24A1 Exon4	TGCCTGTTTACAAAAGAGTTGTC
CYP24A1 Exon5	GGCATAGAATTGAGTCTTTAATAACC	CYP24A1 Exon5	TGGGAATCACTGTGAAGTTCTG
CYP24A1 Exon6	CCTCTTCCAGAACGAACATTG	CYP24A1 Exon6	TGAAGCTCCAGACACGGG
CYP24A1 Exon7	TGCAAGAAGGAGTTTGGACTG	CYP24A1 Exon7	TGAATCCCAGTGAAATGAATG
CYP24A1 Exon8	TTGCAGAATAAGGTGGTGGG	CYP24A1 Exon8	TAATTAGCTAGGGGAAGCCG
CYP24A1 Exon9	AATCTGCATTCCCATTGACAC	CYP24A1 Exon9	CAAAGTCTAGGGAGATCTGGTG
CYP24A1 Exon10-11	CAATTTTGCCATTCAAAGGTC	CYP24A1 Exon10-11	GCTCATCCCTCGTCATTCTC
CYP24A1 Exon12_1	CCGGAAAGCAAACTTCAAAC	CYP24A1 Exon12_1	AACAAAATAATGCCCCAGTG
CYP24A1 Exon12_2	GCTGGGAGTAATACTGACAATCC	CYP24A1 Exon12_2	TATTGCATGCATTTCTGTGC
CYP24A1 Exon12_3	TTAGGATCTGTGGTGCAGGG	CYP24A1 Exon12_3	TTTGTGATATAGGGCTTGTAGGC

Table 2. Biochemical Phenotype of cohort 1.			
Number of patients	**Mean serum calcium ± SD (mmol/L) (NR 2.12-2.55)**	**Mean urine calcium creatinine ratio ± SD (NR < 0.60 mmol/mmol creatinine)**	**Mean serum PTH ± SD (pg/mL) (NR 11-54 pg/mL)**
72	2.45 ± 0.12 (Range 2.25-2.95)	0.49 ± 0.3	22 ± 7 (Range 4-30)

NR, Normal range; SD, Standard deviation.

Table 3. Biochemical Phenotype of cohort 2.			
Number of patients	**Mean serum calcium ± SD (mmol/L) (NR 2.12-2.55)**	**Mean urine calcium creatinine ratio ± SD (NR < 0.60 mmol/mmol creatinine)**	**Mean serum PTH ± SD (pg/mL) (NR 11-54 pg/mL)**
94	2.3 ± 0.09 (Range 2.08-2.58)	0.72 ± 0.24	33 ± 14 (Range 18-57)

NR, Normal range; SD, Standard deviation.

for two; a common synonymous single-nucleotide polymorphism (SNP) (rs2296241) and an intronic SNP (rs4809960). In cohort 2, we identified seven known sequence variants (Table 5), all with a low MAF, and a single novel heterozygous non-synonymous sequence change was identified in one patient (c.576G>C, E192D), together with a homozygous synonymous change in the same patient (p.A184A). The E192D variant was not detected in a panel of 96 healthy controls. The glutamic acid residue at position 192 is conserved in chicken and mouse but not in lower organisms. MutationTaster and PolyPhen both suggest that the amino acid change in E192D is benign and the algorithm SIFT also predicted that the substitution would be tolerated. Structural modelling of *CY24A1* protein demonstrates that the E192

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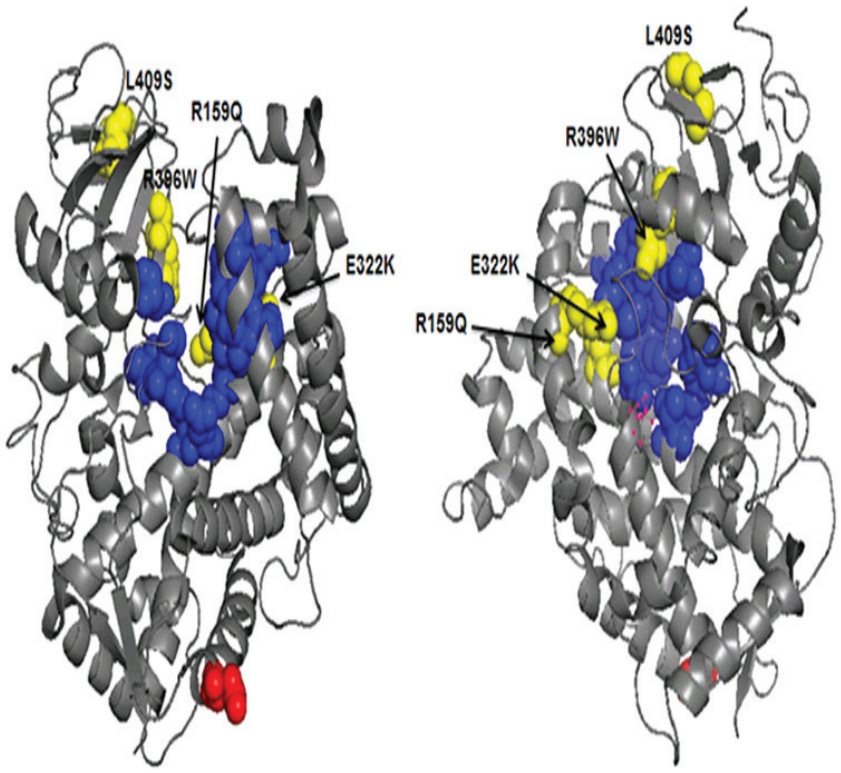

Figure 2: Functionally inactivating *CYP24A1* mutations. The locations of previously identified functionally inactivating mutations in *CYP24A1* are highlighted in yellow in (a) an anterior view and (b) a posterior view of the enzyme structure. They can be seen to cluster around the substrate access channel close to important substrate binding residues (blue). Amino acid 192 is highlighted in red. Image generated using PyMOL molecular visualisation system.

Table 4. Cohort 1 *CYP24A1* sequence variants.

SNP ID	Location of change	MAF in this cohort	Reported MAF
rs6022998	Intronic	0.055	n/a
rs2296241	p.A184A	0.125	0.47
rs4809960	Intronic	0.25	0.184
rs6022998	Intronic	0.007	0.001
rs2274130	Intronic	0.055	0.377
rs1570670	Intronic	0.055	0.377
rs4809957	UTR-3	0.035	0.37
rs11907350	UTR-3	0.007	0.05
rs6022987	UTR-3	0.007	0.211

amino acid is spatially distant from the other reported mutations and the active enzyme site (Figure 2). Unfortunately, additional family samples to look for segregation of this variant were not available.

Discussion

Recent studies have identified mutations in *CYP24A1* to be a novel cause of hypercalcaemia[13,20,21]. Currently, more than 10 loss-of-function mutations in *CYP24A1* have been described with this phenotype, supported by evidence of *in vitro* and *in vivo* loss of enzyme function.

Schlingman et al.[13] identified homozygous and compound heterozygous mutations in *CYP24A1* in eight hypercalcaemic infants with an autosomal recessive pattern of inheritance. Transfection experiments, which provided *in vitro* evidence of loss of enzyme function, were also carried out.

This was confirmed by findings by Dauber et al.[20] who identified a homozygous *CYP24A1* mutation in one child with idiopathic infantile hypercalcaemia and demonstrated decreased enzyme activity *in vivo*. However, *CYP24A1* mutations were not identified in 27 other children with idiopathic infantile hypercalcaemia, implying that *CYP24A1* mutations remain a rare cause of infantile hypercalcaemia.

A third study by Tebben et al.[21] describes a 44-year-old Caucasian male with compound heterozygous splice junction mutations in *CYP24A1* with a clinical phenotype of intermittent hypercalcaemia, hypercalciuria and renal stones. Two children of the proband, each with single heterozygous mutations, also displayed a clinical phenotype, with hypercalcaemia in infancy and nephrocalcinosis. Other family members with heterozygous and compound heterozygous *CYP24A1* mutations also had a biochemical phenotype (of elevated $1,25(OH)_2$ vitamin D concentrations) but no clinical phenotype. This family indicates that considerable phenotypic heterogeneity may exist with *CYP24A1* mutations.

While these fascinating studies have provided new insights into the pathophysiology of idiopathic

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Table 5. Cohort 2 *CYP24A1* sequence variants.			
SNP ID	Location of change	MAF in this cohort	Reported MAF
rs139947227	UTR-5	0.005	0.007
rs2296241	p.A184A	0.03	0.47
n/a	p.E192D -novel	0.005	n/a
rs4809960	Intronic	0.001	0.184
rs6068816	T248T	0.03	0.163
rs2296239	P375P	0.001	0.377
rs6022987	UTR-3	0.012	0.211
rs11907350	UTR-3	0.005	0.05

infantile hypercalcaemia and collectively confirm mutations in *CYP24A1* as a cause of hypercalcaemia, it was unclear, until recently, whether mutations in *CYP24A1* might contribute to the phenotype of patients with 'idiopathic' nephrolithiasis. Several findings suggest that they could.

First, the pedigree described by Tebben et al.[21] includes an adult (detailed above) with active renal stone disease, who was asymptomatic throughout childhood. Second, in response to the initial report describing *CYP24A1* mutations in idiopathic infantile hypercalcaemia[13], Streeten et al. describe an adult patient with hypercalcaemia, nephrolithiasis and bone disease who was found to have a homozygous mutation in *CYP24A1*. His initial presentation was at the age of 19 years, with nephrolithiasis in the context of only mild hypercalcaemia and hypercalciuria[22].

Third, Dauber et al.[20] also reported that the mother of their proband, who had a heterozygous mutation in *CYP24A1*, suffered from nephrolithiasis.

This evidence for *CYP24A1* variants contributing to nephrolithiasis was recently strengthened by a report detailing two patients with bi-allelic mutations in *CYP24A1*[15]. First, a 9-year-old boy with hypercalciuria, increased $1,25(OH)_2$ vitamin D and medullary nephrocalcinosis was found to have compound heterozygous mutations (c.428_430del; p.E143del, previously reported by Schlingman[13] together with c.443T>C; p.L148P). Each heterozygous change segregated from his mother and father, respectively. The second patient was described as a 38-year-old man, with recurrent (calcium phosphate) renal stones since the age of 25 and hyperkalaemia, hypercalciuria, suppressed PTH and elevation of $1,25(OH)_2$ vitamin D. He also had bi-allelic mutations, a c.1226T>C, p.L409S change inherited from his mother and the c.428_430del; p.E143del mutation inherited from his father. Metabolic studies in both patients confirmed reduced enzyme function with undetectable activity of *CYP24A1*[15].

The hypothesis of *CYP24A1* mutations contributing to renal calculi is also supported by observations from a *CYP24A1* knockout mouse model[23]. The *CYP24A1* knockout mice show high perinatal mortality (about 50%), believed to be due to hypercalcaemia. However, the mice that survive the perinatal period develop normally indicating that there is capacity in alternate pathways of vitamin D disposal or down-regulation of vitamin D activation in later life. Despite this, in response to vitamin D supplementation, the mice developed nephrocalcinosis. This raises the possibility that humans with *CYP24A1* variants could also be clinically asymptomatic under normal conditions but develop nephrolithiasis in response to high vitamin D intake.

Considering this evidence, implicating *CYP24A1* variants in calcium stone formation/nephrocalcinosis with specific biochemical phenotypes[15] could *CYP24A1* be contributing to adult onset nephrolithiasis in the 'idiopathic' hypercalciuria population?

We set out to determine whether variants in *CYP24A1* were a common cause of nephrolithiasis by screening a large group of adult stone formers for mutations in the *CYP24A1* gene. This was a reasonable strategy given both the previously reported cases and the noted frequency of potentially pathogenic non-synonymous sequence variants reported in dbSNP[15]. Two cohorts of renal stone-forming patients from the Northeast of England with metabolic phenotypes potentially consistent with loss of *CYP24A1* function were screened. The cohorts included patients with high normal serum calcium levels and low/low normal parathyroid hormone levels and hypercalciuria. We did not identify pathogenic mutations were in these cohorts of calcium stone forming patients. A single novel heterozygous change was observed in one patient with hypercalciuria, but we conclude that this is likely to be a rare variant of unknown significance. We did not find any patients with bi-allelic mutations. Based on these data, *CYP24A1* mutations do not therefore appear to be a common cause of nephrolithiasis in the populations we have studied. *CYP24A1* mutations leading to renal stone formation/nephrocalcinosis in adult patients remain a rare, autosomal recessive disease. We hypothesise that as the clinically relevant effects of *CYP24A1* mutations may be sensitive to vitamin D intake, defects in the enzyme may account for clinical renal stone disease in populations with different dietary and sunlight exposures. Refining the biochemical phenotype by

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measuring 25(OH) vitamin D levels as well as 1,25 and 24,25 vitamin D would help to identify patients with potential *CYP24A1* mutations. A phenotype of normal serum 25(OH) vitamin D combined with elevated 1,25 $(OH)_2$ vitamin D and decreased 24,25 $(OH)_2$ vitamin D would be very suggestive[15]. Osteopenia may also be an important clinical manifestation of this disease[15].

Conclusion

On the basis of our screen, *CYP24A1* mutations are unlikely to be a prominent cause of idiopathic hypercalciuria and nephrolithiasis. However, *CYP24A1* mutations should be considered in stone-forming patients with suppressed PTH, hypercalciuria and hypercalaemia and increased 1,25 vitamin D levels and vigilance is required to identify such patients.

Acknowledgements

This research was funded by the Northern Counties Kidney Research Fund. We are grateful to the patients who helped us to perform this study.

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