Original Article

Genetic Testing of the mucin 1 gene-Variable Number Tandem Repeat Single Cytosine Insertion Mutation in a Chinese Family with Medullary Cystic Kidney Disease

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Abstract

Background: Medullary cystic kidney disease (MCKD) is clinically indistinguishable from several other autosomal-dominant renal diseases; thus, molecular genetic testing is needed to establish a definitive diagnosis. A specific type of single cytosine insertion in the variable number tandem repeat (VNTR) of the mucin 1 (*MUC1*) gene is the only known cause of MCKD1; however, genetic analysis of this mutation is difficult and not yet offered routinely. To identify the causative mutation/s and establish a definitive diagnosis in a Chinese family with chronic kidney disease, clinical assessments and genetic analysis were performed, including using a modified genotyping method to identify the *MUC1*-VNTR single cytosine insertion.

Methods: Clinical data from three patients in a Chinese family with chronic kidney disease were collected and evaluated. Linkage analysis was used to map the causative locus. Mutation analysis of uromodulin (*UMOD*) gene was performed using polymerase chain reaction (PCR) and direct sequencing. For *MUC1* genotyping, the mutant repeat units were enriched by *MwoI* restriction, and then were amplified and introduced into pMD-18T vectors. The 192 clones per transformant were picked up and tested by colony PCR and second round of *MwoI* digestion. Finally, Sanger sequencing was used to confirm the *MUC1* mutation.

Results: Clinical findings and laboratory results were consistent with a tubulointerstitial lesion. Linkage analysis indicated that the family was compatible with the *MCKD1* locus. No mutations were found in *UMOD* gene. Using the modified *MUC1* genotyping method, we detected the *MUC1*-VNTR single cytosine insertion events in three patients of the family; and mutation-containing clones were 12/192, 14/192, and 5/96, respectively, in the three patients.

Conclusions: Clinical and genetic findings could support the MCKD1 diagnosis. The modified strategy has been demonstrated to be a practical way to detect *MUC1* mutation.

Key words: Autosomal Dominant Tubulointerstitial Kidney Diseases; Genotyping; Medullary Cystic Kidney Disease; *MUC1* Gene; Variable Number Tandem Repeat

Introduction

Medullary cystic kidney disease (MCKD) is a progressive tubulointerstitial nephropathy leading to end-stage renal disease (ESRD) and need for dialysis or kidney transplantation.[1] MCKD occurs in adulthood with a variable age of onset, ranging from 20 to 70 years within and between families.[2,3] The manifestations of MCKD are highly variable and nonspecific. Typical manifestations include impaired

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.216408

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Received: 13-06-2017 **Edited by:** Xin Chen
How to cite this article: Si N, Zheng K, Ma J, Meng XL, Li XM, Zhang X. Genetic Testing of the mucin 1 gene-Variable Number Tandem Repeat Single Cytosine Insertion Mutation in a Chinese Family with Medullary Cystic Kidney Disease. Chin Med J 2017;130:2459-64.

renal function, hyperuricemia, and salt wasting. Urinalysis can reveal a bland urinary sediment. The findings of kidney biopsy may show focal tubular atrophy, interstitial fibrosis, or secondary glomerular scarring. Small corticomedullary cysts and slightly reduced kidney size may also be identified by renal ultrasound.[4] However, none of the above findings are specific indicators of this disease; thus, it is very difficult to make a definite diagnosis and counsel families only relying on clinical manifestations.

MCKD belongs to a recently termed group of autosomal-dominant tubulointerstitial kidney diseases (ADTKDs) and is classified into two types according to the responsible genes: mucin 1 (*MUC1*) gene for MCKD type 1 (MCKD1) and uromodulin (*UMOD*) gene for MCKD2.[5-8] These two types of MCKD and other ADTKDs are phenotypically similar, and genetic testing of pathogenic mutation(s) in responsible genes is needed to confirm a diagnosis. ADTKD is mostly caused by mutations of *MUC1* and *UMOD* genes in patients of European ancestry.[9] Several mutations in *UMOD* gene, which encodes the urinary protein uromodulin (Tamm-Horsfall glycoprotein), have been reported as the causative mutation of MCKD2 since this gene was first identified by Hart *et al*.[6] in 2002. Till now, the insertion of a single cytosine in a coding variable number tandem repeat (VNTR) region of the *MUC1* gene, a rare type of mutation, is the only reported causative mutation of MCKD1.[8] This single insertion creates an eight-base stretch of cytosine instead of a seven-base stretch harboring in a coding VNTR region, and thus creates extreme difficulty for genetic testing. To date, genetic analysis for the *MUC1*-VNTR single cytosine insertion is still not offered in routine practice, even some established methods which require a batch of samples in advance can only be done in a limited number of laboratories.[10] Furthermore, no practical genotyping methods are available for genetic laboratories that only test ADTKD patients occasionally.

Here, we presented the case of a Chinese family with chronic kidney disease. To identify the causative mutation/s and establish a definitive diagnosis in this family, clinical assessments and genetic analysis were performed. A modified genotyping method was developed to detect the *MUC1* mutation by *MwoI* restriction, TA cloning, and sequencing in this study.

Methods

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Institutional Review Board of Chinese Academy of Medical Sciences. Informed written consent was obtained from all patients prior to their enrollment in this study.

Subject recruitment

Three patients with tubular interstitial lesions from one family were recruited from Shanxi province in China, and three unrelated individuals without any kidney problems were also recruited as healthy controls. Blood tests, urine tests, and abdominal ultrasounds were performed in three patients, and two of them received renal biopsy. A diagnosis of tubular interstitial lesions was made in each case by two experienced nephrologists based on clinical examinations. Blood samples were collected, and genomic DNA was extracted from whole peripheral blood for further genetic analysis.

Linkage and haplotype analysis

Linkage analysis was performed in three patients using four polymorphic micro-satellite markers that span the critical MCKD1 region in the following order: cen-D1S305, D1S153, D1S1595, and D1S2624-tel. Haplotypes were manually constructed and compared between patients. D16S3054 and D16S3036 around the *UMOD* gene were also genotyped in the three affected individuals to determine linkage to the MCKD2 locus.

Mutation Screening of *UMOD* gene

All exons and intron-exon boundaries of the *UMOD* gene were amplified from genomic DNA, and the polymerase chain reaction (PCR) products were directly sequenced in an ABI Prism 3730xl Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Primer sequences and amplification conditions are available upon request. All sequences were analyzed using CodonCode Aligner software (CodonCode Corp., Dedham, MA, USA).

Detection of the *MUC1*-variable number tandem repeat cytosine insertion mutation

A modified strategy was developed to detect the *MUC1*-VNTR single cytosine insertion based on Kirby *et al*.'s method [Figure 1].[8] To enrich the mutant repeat units containing the cytosine insertion against a reference sequence background, the restriction enzyme *MwoI* was used to completely digest genomic DNA. The *MUC1*-VNTR cytosine insertion disrupts the recognition site of *MwoI* (GCNNNNNNNGC) in the reference repeat unit sequences. After the digestion, reference repeat units (GCCCCCCCAGC) were selectively cleaved, while mutant repeat units containing the cytosine insertion (GCCCCCCCCAGC, where the inserted cytosine is underlined) remained. Then, M13-tailed primers targeting the 60-bp repeat unit were used to amplify the remaining VNTR fragment via PCR (forward primer: 5'-CAGGAAACAGCTATGACACCAGGCCG GCCCCGGGCTCCA-3'; reverse primer: 5'-GTTTTCCCAGTCACGACGTCCG GGGCCGAGGTGACAC-3'; M13 sequences are underlined). The PCR program employed 35 cycles of 98°C for 15 s for denaturation, 60°C for 15 s for annealing, and 72°C for 20 s for extension. The 94-bp PCR products were purified and cloned into the pMD18-T vector. We picked 96–192 clones per transformant from DH5α cells, and then performed colony PCR with BcaBEST sequencing primers for the pMD18-T vector. Colony PCR products were digested with *MwoI* for a second round of mutant

repeat unit enrichment and a prior screening for cytosine insertion. Digestion products were run on 2% agarose gels to select candidate clones containing the mutant repeat unit. Candidate clones were further directly sequenced to confirm the cytosine insertion event and eliminate the sequences similar with reference repeat units.

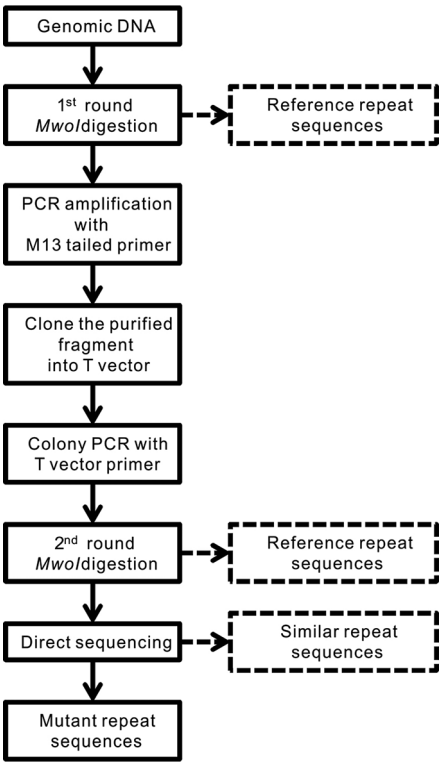

Figure 1: *MUC1*-VNTR single cytosine insertion detection strategy. Background sequences (dashed box), including canonical sequence and other similar sequences, were eliminated after *MwoI* restriction, TA cloning, and Sanger sequencing. VNTR: Variable number tandem repeat.

Results

Clinical findings

We collected a large Chinese Han kindred spanning at least five generations with chronic kidney disease [Figure 2a]. Three individuals, whose blood samples were available and who had histories of abnormal kidney function, were recruited for the present study. Clinical features and laboratory results of these three patients are summarized in Tables 1 and 2. For practical reasons, no detailed clinical information was available from the other family members except that several members had "kidney problems." Three patients recruited for the present study presented with a nephronophthisis-like kidney morphology and a lack of extrarenal manifestations. The patients showed abnormal kidney function at early ages ranging from 29 to 39 years, and one patient (IV-8) was already on hemodialysis. Two patients (III-5, IV-27) presented with polydipsia and had histories of gout and hypertension. None had a history of kidney stones or refractory urinary infection. Laboratory tests showed elevated serum creatinine (sCr) and cystatin C and low urine gravity. Abdominal ultrasound examination showed small kidneys in all the three patients, and a few small cysts located in cortices and/or cortical-medullary boundaries in two patients. Kidney biopsies from these two patients also showed focal tubular atrophy, interstitial fibrosis, and fitted tubulointerstitial lesions [Figure 3a and 3b].

Linkage analysis

There were shared alleles in four polymorphic markers of the *MCKD1* locus, and there was full co-segregation between

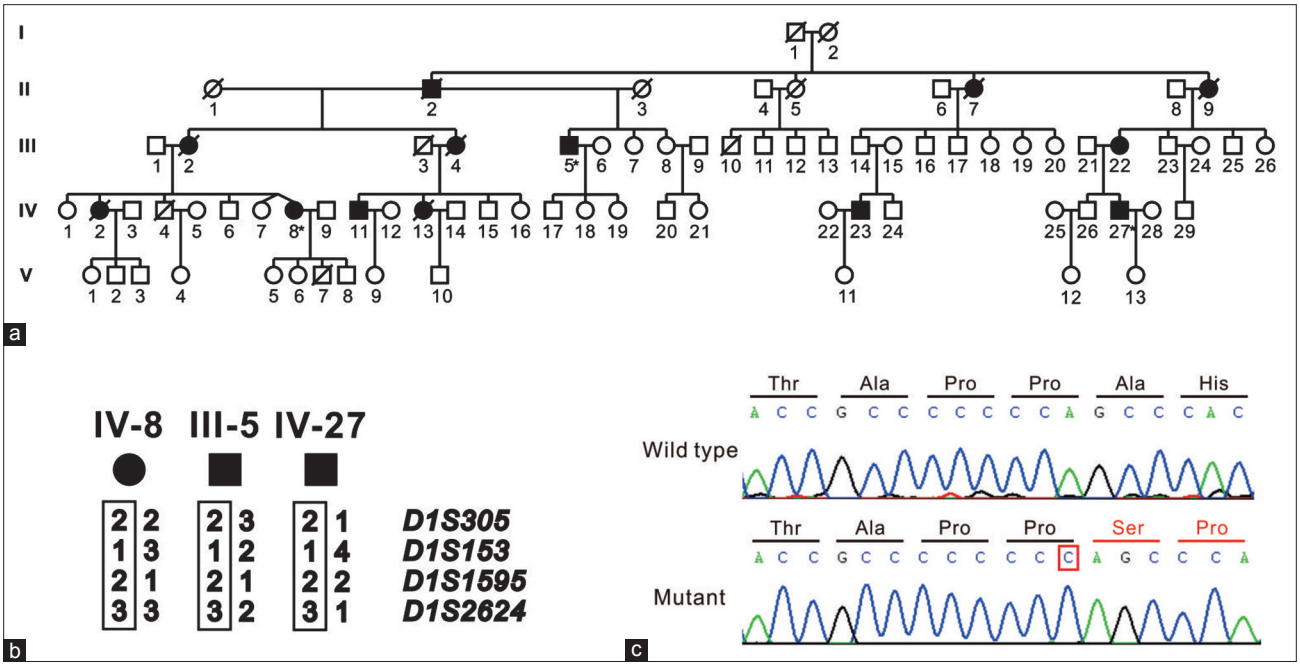

Figure 2: Genetic testing in a MCKD family. (a) Pedigree of the Chinese MCKD family showing autosomal-dominant inheritance. Asterisks indicate the three recruited patients. (b) Disease haplotype segregation at the MCKD1 locus. (c) Sequence chromatograms showing the identified *MUC1*-VNTR single cytosine insertion. MCKD: Medullary cystic kidney disease; VNTR: Variable number tandem repeat.

Table 1: Clinical features and laboratory results of three patients from a Chinese family with medullary cystic kidney disease

Individuals	Age (years)	Gender	Mutation carrier	Age of onset (years)	Polydipsia	Hypertension	Gout	Renal ultrasound
IV-8	40	Female	Yes	39	No	No	No	Hyperecho of renal parenchyma
IV-27	35	Male	Yes	33	No	Yes	Yes	Small kidney, hyperecho of renal parenchyma, 1–2 small cysts in cortex, cyst diameter of 0.4 cm
III-5	51	Male	Yes	29	Yes	Yes	Yes	Small kidney, multiple small cysts in cortex and the boundary area of cortex and medullar cyst diameter of 0.8–1.0 cm

Table 2: Laboratory results of three patients from a Chinese family with medullary cystic kidney disease

Individuals	Hemoglobin (g/L)	Serum uric acid (μmol/L)	Serum creatinine (μmol/L)	Cystatin C (mg/L)	Serum Ca^{2+} (mmol/L)	Serum phosphorus (mmol/L)	Serum osmolality ($mOsm \cdot kg^{-1} \cdot H_2O^{-1}$)	Urine gravity	Urine albumin/ creatinine ratio	Urine α1-microglobulin (mg/L)
IV-8	102	362	136	1.56	2.36	1.24	299	1.005	0.54	<5.7
IV-27	100	756	184	1.76	2.19	1.01	298	1.010	35.43	26.1
III-5	63	469	932	7.01	1.87	1.74	312	1.010	111.1	175.0

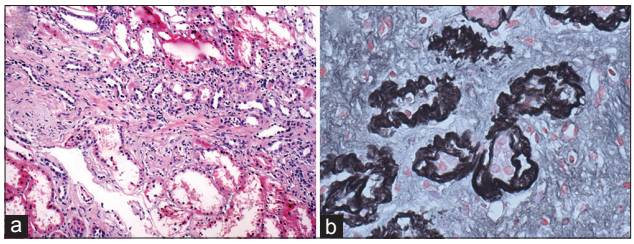

Figure 3: Pathological changes of renal tissue in two patients of a medullary cystic kidney disease family. (a) H & E staining showing interstitial fibrosis (original magnification ×100). (b) Periodic Schiff-Methenamine silver staining showing tubular atrophy with tubular basement membrane shrank and interstitial fibrosis (original magnification ×400).

the shared haplotype and affected status [Figure 2b]. For the *MCKD2* locus, the genotypes for IV-8, III-5, and IV-27 were 1/3, 1/2, and 3/4 at D16S3036 and 3/4, 2/4, and 1/3 at D16SS3054, respectively. Three confirmed patients shared no common alleles in either of the two markers. These results indicated that the family was compatible with linkage to the *MCKD1* locus, but was excluded from linkage to the *MCKD2* locus.

UMOD mutation screening

We found no potential pathogenic mutations in coding sequences, untranslated regions, or intron-exon boundaries of the *UMOD* gene by direct sequencing. Together with the above linkage analysis, *UMOD* mutation screening could rule out a diagnosis of MCKD2.

Detection of *MUC1*-variable number tandem repeat single cytosine insertion

In the index patient (IV-8) of this MCKD family, the *MUC1*-VNTR single cytosine insertion was detected in 7/96 clones in the first screening [Figure 2c]. To further confirm this result, we performed a second screening of 96 clones and detected the mutation in five. As a result, we confirmed the diagnosis of MCKD1, and this established method could be successfully applied to detect the *MUC1*-VNTR single cytosine insertion. With this method, we detected the *MUC1* mutation in the other two patients; mutation-containing clones were 14/192 and 5/96, respectively.

To further confirm the reliability of this established method, we used the same method to genotype *MUC1* gene in three unrelated individuals as negative controls. As expected, we screened 192 clones for each individual, but none had a *MUC1*-VNTR single cytosine insertion.

Discussion

MCKD1 is classified into the recently termed group of ADTKD. Several MCKD families with autosomal-dominant transmission have been reported; however, the specific clinical presentation has not yet reached recognition.[9] It was reported that *MUC1* mutation led to ESRD with a higher incidence and at younger age;[11-13] moreover, families with a clearly autosomal-dominant transmission of kidney disease and ESRD by the age of 60 had a higher chance of carrying a *MUC1* mutation.[14] Consistent with this, we observed an autosomal-dominant inheritance with incomplete penetrance in a large family of five generations, and three patients in this family had been referred to the nephrologists around their third decades; the patient (III5) whose onset age is the youngest among these three patients who had already developed ESRD. For *MUC1* mutation-driven ADTKD, the primary cellular origin of the disease is the renal tubular apparatus, and renal fibrosis is a common feature. In the present study, predominant interstitial fibrosis and tubular atrophy were observed on renal biopsy in two of the three familial patients, thus further confirming a diagnosis of tubulointerstitial renal disease. Renal ultrasound revealed

small kidney size and multiple cysts in 2/3 of patients, but the appearance of renal cysts is neither an early nor a specific feature of the disease. Contrary to the previous understanding that gout is a sequel of kidney failure in MCKD, two patients had a history of gout before ESRD and one (IV-27) had uric acid not associated with declined renal function. Furthermore, no common extrarenal features were observed among the affected patients.

As clinical presentation and pathological findings are nonspecific, it is difficult to make a definitive diagnosis of MCKD or other ADTKDs. However, the responsible genes are different among each ADTKD; thus, a differential diagnosis could be made based on the results of genetic testing results. Ekici *et al*.[9] proposed a diagnostic workflow for clinically suspected ADTKD, in which genotyping the *MUC1*-VNTR single cytosine insertion is especially difficult.[8,15,16] A mass spectrometry-based probe extension assay[8] and SNaPshot minisequencing[9] were established to detect the *MUC1*-VNTR single cytosine insertion; however, these assays are still not routine procedures in genetic laboratories and are only performed in a limited number of research institutes. Here, we established an alternative genotyping method by simply using a restriction assay, cloning, and sequencing to identify *MUC1*-VNTR single cytosine insertion in a MCKD family. As same as previous genotyping methods, we used *MwoI* digestion to reduce the background of the reference repeat unit sequence in the genome, followed by M13-tailed primer-mediated amplification of the remaining VNTR segment. After that, the 94-bp products are cloned into a T vector for further selection because we found it is difficult to obtain enough digestion products for purification after a second round *MwoI* enrichment directly from PCR fragments. Second round *MwoI* digestion of amplification products from constructs is essential because the remaining reference repeat unit sequences are still in the majority. However, digestion results are not indicative of the mutation because there exist other types of 60-mer units similar to the canonical unit but without *MwoI* recognition site in the VNTR region. We performed final sequencing of the positive clones after *MwoI* digestion as a confirmation test, as this directly reveals the single cytosine insertion. In this study, positive clones with mutant sequences could be detected in 96 clones for each affected individual. However, we suggested screening an additional 96 clones for those asymptomatic at-risk relatives in the family to ensure that the mutant is not missed. The two established methods, a mass spectrometry-based probe extension assay and SNaPshot minisequencing, are based on primer extension reactions to detect a single locus, while TA cloning sequencing reveals the whole sequences of repeat units harboring *MUC1*-VNTR single cytosine insertion and shows more directly visible results. Furthermore, mass spectrometry-based probe extension assay and SNaPshot minisequencing require a batch of samples in advance to reduce detection costs and lack flexibility. If an individual sample fails, the cost of additional testing is relatively high. The TA clone sequencing method could be easily performed in any molecular genetics laboratory and requires no additional materials or equipment. For most genetic laboratories that only occasionally test MCKD1 patients, the TA cloning method is an alternative practical detection method with flexibility.

To date, the only mutational mechanism identified in families with MCKD1 is heterozygous single cytosine insertion in the VNTR region of the *MUC1* gene. The *MUC1* gene encodes a transmembrane glycoprotein mucin 1 that is highly expressed at the apical surface of mucosal epithelial cells.[17] The full-length protein contains three domains: short cytoplasmic and transmembrane domains that are highly conserved among species, a large glycosylated extracellular domain containing the VNTR, and a self-cleavage module (SEA) to allow proteolytic cleavage of the extracellular domain.[18,19] Several studies have implicated that *MUC1* gene is an important player during nephrogenesis. Chambers *et al*.[20] first reported high expression level of *MUC1* gene restricted to the collecting ducts during kidney development. Further studies indicated that multiple structures were characterized by *MUC1* expression, including ureteric buds, cap mesenchymal cells undergoing the mesenchymal-to-epithelial transition, renal vesicles, comma bodies, and S-shaped bodies.[21,22] The *MUC1*-VNTR single cytosine insertion is predicted to cause a frameshift mutation and generate a premature stop codon shortly beyond the VNTR terminus, resulting in a truncated protein (termed MUC1-fs) with a new amino acid sequence on the terminal end instead of a downstream SEA module as well as the transmembrane and intracellular domains. MUC1-fs is expressed and retained in the cytoplasm of renal epithelial cells from the Henle's loop, distal convoluted tubule, and collecting ducts in MCKD1 patients.[8] While the specific mechanism of pathogenesis of the MUC1-fs remains unclear, the mutant protein might be improperly processed in the cytoplasm resulting in chronic kidney disease due to dominant-negative and/or gain-of-function mode of action of the mutation, since kidneys of *muc-1* null mice appear to develop normally.[23]

In conclusion, this study identified *MUC1*-VNTR single cytosine insertion mutations in three patients in a Chinese family with chronic kidney disease and made a definite diagnosis of MCKD1. The herein-established method could be a practical *MUC1* mutation genotyping method for genetic laboratories that only occasionally test MCKD1 patients. For practical reasons, this study has only recruited three patients. The reliability of the established method should be further tested in more individuals in the present family and in other unrelated confirmed MCKD1 patients. Genotyping the *MUC1* mutation would be especially important for identifying the asymptomatic carriers in the high-risk families. We recommend that all at-risk relatives should receive *MUC1*-VNTR single cytosine insertion genotyping and all mutation carriers should be involved in CKD management. Genotyping results will also be helpful when considering the potential donors in the family for kidney

transplantation. In the future, a family will possibly benefit from regular sCr monitoring and early CKD treatment, and precise medical approaches may be possible to target the *MUC1*-VNTR single cytosine insertion.

Financial support and sponsorship

This study was supported by a grant from CAMS Innovation Fund for Medical Sciences (CIFMS; No. 2016-I2M-1-002).

Conflicts of interest

There are no conflicts of interest.

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