Pediatr Nephrol (2001) 16:110–112

Tetsuji Morimoto · Akiko Chiba · Yoshiaki Kondo
Shori Takahashi · Takashi Igarashi
Chiyoko N. Inoue · Kazuie Iinuma

A new approach to mRNA in proximal tubule cells of patients with CLCN5 channelopathy

Received: 3 May 2000 / Revised: 16 October 2000 / Accepted: 19 October 2000

Abstract ClC-5 is a chloride channel whose gene mutations have been reported to be associated with X-linked nephrolithiasis (XRN), X-linked recessive hypophosphatemic rickets (XLRH), Dent disease, and idiopathic low-molecular-weight proteinuria (ILMWP) in Japanese children. To establish more efficient screening for CLCN5 abnormalities, we developed a new diagnostic method using reverse transcription and polymerase chain reaction (RT-PCR) of cultured renal tubular cells from the urine of patients. Using this new method, we successfully detected microdeletion of ClC-5 mRNA in a patient and splicing abnormality of the CLCN5 Cl channel.

Keywords CLCN5 · RT-PCR · Cultured renal tubular cells · Splicing · Microdeletion

Introduction

Lloyd et al. recently identified mutations of the CLCN5 chloride channel gene in three disorders that result in kidney stone formation, i.e., Dent's disease, X-linked recessive nephrolithiasis (XRN), and X-linked recessive hypophosphatemic rickets (XLRH) [1]. The human CLCN5 gene located on chromosome Xp 11.22 consists of at least 12 exons spanning 25–35 kb of genomic DNA and encodes a 746-amino-acid protein [2]. CLCN5 belongs to a family of voltage-gated chloride channel genes that have about 12 transmembrane domains. During the following year, Lloyd et al. found four different CLCN5 mutations in Japanese patients with low-molecular-weight proteinuria, hypercalciuria, and nephrocalcinosis [3]. In previous reports, single-stranded conformational polymorphism analysis (SSCP), direct DNA sequence analysis, and Southern blot hybridization have been used for the mutational screening of the CLCN5 gene. However, the mutational diversity within the 2238 basepairs comprising the CLCN5 gene makes it arduous and time consuming to perform mutational screening for CLCN5 channelopathy by these approaches. It has become clear that approximately 10% of CLCN5 abnormalities are due to gene mutations that lead to changes in the sizes of the mRNA products of genomic CLCN5. The identification of these changes in the sizes of mRNA products will provide a quick and easy diagnosis of CLCN5 channelopathy in a certain number of cases. Further, a combination of the conventional technique with this simple identification of the changes in sizes of mRNA products will facilitate and confirm the diagnosis. We have tried to identify a new diagnostic method using reverse transcription and polymerase chain reaction (RT-PCR) for the diagnosis of CLCN5 channelopathy.

We recently succeeded in regenerating a renal tubular epithelial monolayer in an in vitro culture of exfoliated tubular cells into urine. The tubule cells were preserved so well that the in vitro culture even led to tubule formation and branching of the cells in a collagen gel preparation [4]. This method should be suitable to examine whether the ClC-5 mRNA is expressed properly in the targeted organ, the proximal tubule cells.

Therefore, we tried to identify a new diagnostic method using RT-PCR for CLCN5 channelopathy.

Materials and methods

Our initial step was to culture renal tubular cells from the urine of an XLRH patient and an ILMWP patient. The information on these patients and results of CLCN5 gene analysis were previously reported [5, 6]. A genomic deletion from exons 5 to 8 was found in the CLCN5 gene of the former [5], and an acceptor splice site

T. Morimoto (✉) · A. Chiba · Y. Kondo · C N. Inoue · K. Iinuma
Department of Pediatrics, Tohoku University, School of Medicine, Seiryou-machi 1-1, Aoba-ku, Sendai 980-8574, Japan
Tel.: +81-22-7177289, Fax: +81-22-7177290

S. Takahashi
Division of Nephrology, Shizuoka Children's Hospital, Shizuoka, Japan

T. Igarashi
Department of Pediatrics, Faculty of Medicine, The University of Tokyo, Tokyo, Japan

mutation (**a**g-**c**g) in intron 4 was found in the CLCN5 gene of the latter [6]. We collected their mid-stream spot urine specimens after disinfecting their genital areas. For harvesting of voiding cells, urine specimens were gently centrifuged at 1200 rpm for 10 min and the pellets were resuspended in minimum essential medium (MEM; Gibco BRL) containing 20% fetal calf serum (FCS; Summit), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL). The specimens were centrifuged again at 1200 rpm for 10 min and the pellets were resuspended in the same medium. After a third centrifugation at 1200 rpm for 10 min, the pellets were resuspended in the following growth medium: Dulbecco's modified Eagle's medium/Hams F12 (DMEM/F12) containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mmol/l nicotinamide, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenate, 3×10^{-4} M 3, 3, 5-triiodo-*l*-thyronine, and 10^{-5} M dexamethasone. The specimens were centrifuged under the same conditions and the pellets were resuspended in the growth medium. The cell suspension was then dispensed into six-well plates within 6 h after collecting the patient's urine specimens and cultured at 37°C in 5% CO_2–95% O_2 air atmosphere. When the cultured cells grew to 80–90% confluence, they were resuspended with 1 ml TRIzol reagent (Gibco-BRL, Life Technologies) and then 200 µl chloroform was added to separate the mixture into an aqueous and organic phase. The aqueous phase was removed after centrifugation and the total RNA pellet was precipitated with 500 µl isopropanol. The RNA pellet was washed with 75% ethanol and allowed to dry at room temperature, and then the total RNA was resuspended in 15 µl RNase-free water. A first-strand cDNA synthesis kit (Boehringer Mannheim) was used to reverse transcribe RNA extracted from the cultured renal tubular cells. The total RNA (750 ng) was heated at 65°C for 15 min, rapidly chilled on ice, and then mixed with 11.8 µl of the reagents supplied with the kit. The reaction mixture contained 1.6 µg oligo-p$(dT)_{15}$ primer, 10 mmol/l TRIS (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l dNTPs, 50 units RNase inhibitor, and 20 units AMV reverse transcriptase (RT). The reaction mixture was incubated once at 25°C for 10 min, incubated a second time at 42°C for 60 min, and then heated at 95°C for 5 min to inactivate the RT. The PCR reactions were performed in a total volume of 50 µl using an Expand High Fidelity PCR system (Boehringer Mannheim) containing 200 mmol/l dNTPs, 5 µl 10 × conc. Expand HF buffer, 1.5 mmol/l $MgCl_2$, 1 µl RT reaction mixture, 2.6 units enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases, and 50 pmol of the specific primer pairs for CLCN5. The primer sequences were 5'-AGG ACA AGT CGT ACA ATG GTG GAG G-3' (sense, bp 245–269) and 5'-ACA TAT CCA TGG TCT GTA ATG TCC-3' (antisense, bp 2563–2586), and the expected PCR product size was 2342 bp. The 10 µl PCR products were size fractionated by electrophoresis on a 0.8% agarose gel in 1×TRIS-borate-EDTA (TBE) buffer, stained with ethidium bromide, visualized under ultraviolet light, and photographed.

Results

The cultured renal tubular cells were positive for γ-glutamyltransferase (γGTP) staining (data not shown), so it was confirmed that these cells were derived from the proximal tubules. RT-PCR procedures using specific primer pairs for CLCN5 have demonstrated the expression of ClC5 mRNA in cultured renal tubular cells from the urine of the XLRH, ILMWP, and IgA nephropathy (control) patients. Figure 1 shows the PCR products separated on a 0.8% agarose gel and visualized after staining with ethidium bromide. As seen in Fig. 1, when the coding region of the CLCN5 gene was amplified using specific primers in the IgA nephropathy (control) patient, a band of the expected molecular mass (2342 bp) could be visualized. However, in the XLRH and ILMWP patients, abnormal transcripts shorter than the expected size sequencings were identified (954 bp and 123 bp shorter, respectively). The specificities of the three PCR products were confirmed by sequencing (data not shown).

Discussion

In 1991, Racusen et al. succeeded in culturing the renal tubule cells collected from voided urine of patients with cystinosis [7]. They characterized the properties of the primarily cultured renal tubule cells and came to the conclusion that their preparation only cultivated the proximal tubule cells. Quite recently, we also succeeded in culturing the tubule cells in urine and analyzed the normal acid-extruding mechanism of these cells by physiological and molecular biological techniques. The cultured renal tubular cells were positive for γ-glutamyltransferase (γGTP) staining (data not shown), so it was confirmed that these cells were derived from the proximal tubules. We examined the ClC5 mRNA in two unrelated Japanese patients with XLRH and ILMWP using RT-PCR analysis and detected abnormal transcripts. To date, more than 40 disease-associated CLCN5 genetic abnormalities have been reported in Dent's disease,

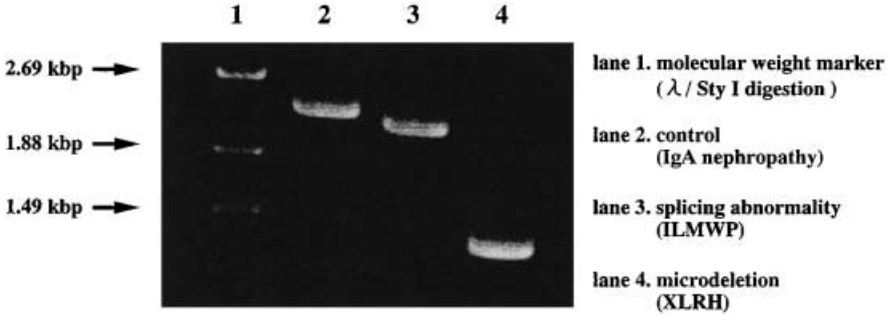

Fig. 1 Detection of ClC5 mRNA in cultured renal tubular cells from the urine of the XLRH, ILMWP, and IgA nephropathy (control) patients: *lane 1* molecular weight marker (λ/*Sty*I digestion), *lane 2* control (2342 bp), *lane 3* acceptor splice consensus sequence mutation in intron 4 (2219 bp), *lane 4* microdeletion from exons 5 to 8 (1388 bp). Abnormal transcripts shorter than the expected size sequencings were identified in the ILMWP (*lane 3*) and XLRH (*lane 4*) patients

XLRH, XRN, and ILMWP patients [1, 3, 5, 6, 8–11]. The CLCN5 mutations were found to be spread throughout the gene, although a clustering of mutations was observed in some regions. The original methods for the screening of the CLCN5 gene using SSCP, direct sequencing, and Southern blot analysis are time consuming. In comparison with the conventional method for diagnosing the abnormalities in the CLCN5 gene, our new method is suitable for the screening of microdeletions and splicing abnormalities. Recently, Cox et al. investigated CLCN5 mRNA processing by the detection of illegitimate transcription of the CLCN5 gene in EBV-transformed lymphoblastoids [12]. We succeeded in detecting a similar type of splicing abnormality of CLCN5 using the voided tubule cell culture system. Before performing the screening of exons 2–12 of the CLCN5 gene for the mutations using SSCP or direct sequencing, our newer, easier diagnostic method will provide us with information about the splicing abnormality and the mutational problem in the CLCN5 promoter region. At the very least, more than 10% of patients with large deletions or splicing abnormalities of the CLCN5 gene will be detected by this new method. This method can also be used to partially check the abnormalities of the promoter gene cascade since it enables direct examination of the expression of the CLCN5 gene in the renal tubule cells.

The present paper is the first to report the applicability of this method to the diagnosis of abnormalities in the expression of the messages for the membrane transport system in the voided urine cells. From our present experience, we conclude that our new method will be successfully applied to patients with CLCN5 abnormalities, as well as various other renal tubular disorders.

References

1. Lloyd SE, Pearce SHS, Fisher SE, Steinmeyer K, Schwappach B, Scheinman SJ, Harding B, Bolino A, Devoto M, Goodyer P, Ridgen SPA, Wrong O, Jentsch TJ, Craig IW, Thakker RV (1996) A common molecular basis for three inherited kidney stone diseases. Nature 379:445–449
2. Fisher SE, Bakel IV, Lloyd SE, Pearce SHS, Thakker RV, Craig IW (1995) Cloning and characterization of CLCN5, the human kidney chloride channel gene implicated in Dent disease (an X-linked hereditary nephrolithiasis). Genomics 29: 598–606
3. Lloyd SE, Pearce SHS, Gunther W, Kawaguchi H, Igarashi T, Jentsch TJ, Thakker RV (1997) Idiopathic low molecular weight proteinuria associated with hypercalciuric nephrocalcinosis in Japanese children is due to mutations of the renal chloride channel (CLCN5). J Clin Invest 99:967–974
4. Inoue CN, Kondo Y, Ohnuma S, Morimoto T, Nishio T, Iinuma K (2000) Use of cultured tubular cells isolated from human urine for investigation of renal transporter. Clin Nephrol 53:90–98
5. Morimoto T, Uchida S, Sakamoto H, Kondo Y, Hanamizu H, Fukui M, Tomino Y, Nagano N, Sasaki S, Marumo F (1998) Mutations in CLCN5 chloride channel in Japanese patients with low molecular weight proteinuria. J Am Soc Nephrol 9:811–818
6. Igarashi T, Gunther W, Sekine T, Inatomi J, Shiraga H, Takahashi S, Suzuki J, Tsuru N, Yanagihara T, Shimazu M, Jentsch TJ, Thakker RV (1998) Functional characterization of renal chloride channel, CLCN5, mutations associated with Dent's Japan disease. Kidney Int 54:1850–1856
7. Racusen LC, Fivush BA, Andersson H, Gahl WA (1991) Culture of renal tubular cells from the urine of patients with nephropathic cystinosis. J Am Soc Nephrol 1:1028–1033
8. Nakazato H, Hattori S, Furuse A, Kawano T, Karashima S, Tsuruta M, Yoshimuta J, Endo F, Matsuda I (1997) Mutations in the CLCN5 gene in Japanese patients with familial idiopathic low-molecular-weight proteinuria. Kidney Int 52:895–900
9. Akuta N, Lloyd SE, Igarashi T, Shiraga H, Matsuyama T, Yokoro S, Cox JP, Thakker RV (1997) Mutations of CLCN5 in Japanese children with idiopathic low molecular weight proteinuria, hypercalciuria and nephrocalcinosis. Kidney Int 52: 911–916
10. Lloyd SE, Gunther W, Pearce SHS, Thomson A, Bianchi ML, Bosio M, Craig IW, Fisher SE, Scheinman SJ, Wrong O, Jentsch TJ, Thakker RV (1997) Characterisation of renal chloride channel. CLCN5, mutations in hypercalciuric nephrolithiasis (kidney stones) disorders. Hum Mol Genet 6:1233–1239
11. Bosio M, Bianchi ML, Lloyd SE, Thakker RV (1999) A familial syndrome due to Arg 648 Stop mutation in the X-linked renal chloride channel gene. Pediatr Nephrol 13:278–283
12. Cox JP, Yamamoto K, Christie PT, Wooding C, Feest T, Flinter F, Goodyer P, Leumann E, Neuhaus T, Reid C, Williams P, Wrong O, Thakker RV (1999) Renal chloride channel, CLCN5, mutations in Dent's disease. J Bone Miner Res 14: 1536–1542