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CLINICAL STUDY

Coexistence of normotensive primary aldosteronism in two patients with Gitelman's syndrome and novel thiazide-sensitive Na–Cl cotransporter mutations

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Abstract

Background: Primary aldosteronism (PA) is the most common form of secondary hypertension, while Gitelman's syndrome (GS) is the most common inherited renal tubular disease. However, coexistence of these two diseases has never been previously reported.
Aim and subjects: The aim of our study was to describe the association of GS and PA in two unrelated patients and compare their clinical presentation with a group of patients with GS.
Methods: Ten subjects suspected to have only GS were assigned to the control group. Saline infusion test was used to confirm the diagnosis of PA. GS was confirmed by sequencing of the causal genes (*SLC12A3* and *CLCNKB*) and functional analyses in *Xenopus laevis* oocytes.
Results: Confirmatory tests, gene analysis, and functional studies demonstrated the coexistence of GS and PA in both patients. In total, nine novel *SLC12A3* gene variants, including seven missense mutations, one splice mutation, and one frameshift deletion, were found in 12 subjects. Four mutations (p.T60M, p.T304M, p.T465P, and p.N611T) harbored by the two patients with both PA and GS were revealed to be loss-of-function variants. Although both patients were normotensive, neither of them had normal nocturnal dip.
Conclusions: Two rare diseases GS and PA may occasionally coexist in one subject. In these patients, salt depletion and volume constriction might explain the absence of hypertension normally seen in PA patients. However, the protective mechanism against hypertension via down-regulation of renal sodium handling was probably not sufficient in those patients, since their normal circadian rhythm of blood pressure was disrupted.

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Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension. Its prevalence among hypertensive patients may exceed 10%, as demonstrated by a large prospective survey (1).

Gitelman's syndrome (GS; OMIM #263800), an autosomal-recessively inherited renal salt-losing disorder, is mainly caused by mutations in the *SLC12A3* gene (MIM: 600968) (2), which encodes the thiazide-sensitive Na–Cl cotransporter (NCC) (3). Additionally, a few patients with GS-like phenotype carry mutations in *CLCNKB* gene (MIM: 602023) (4, 5). GS is the most common primary renal tubular disease, with a prevalence of about 1 per 40 000 (6).

Theoretically, by chance only, the coexistence of PA and GS in one patient should be very rare (~1/2 500 000), given the prevalence of hypertension at about 17%. Indeed, according to our knowledge, the coexistence has never been documented so far. Herein, we report two cases with the coexistence of both diseases and attempt to explore the potential mechanism(s) under their special clinical features.

Subjects and methods

Patients

A 39-year-old married male (patient 1) was admitted for frequent muscle cramps that had occurred in the

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recent 4 months. Twelve years ago, he was once referred to our outpatient clinic for an examination of generalized muscle weakness associated with acral paresthesia. At that time, his blood chemistry showed persistent hypokalemia (1.7–2.6 mmol/l), in association with alkalosis (plasma bicarbonate, 29–32 mmol/l), inappropriate kaliuresis (fractional excretion potassium (FEK): 31%), and salt losing (24 h urinary Na, 261 mmol, normal values, 137–257 mmol). Plasma renin activity (PRA, 11.1 ng/ml per h, normal values, 0.1–2.9 ng/ml per h) and plasma aldosterone concentration (PAC, 17.2 ng/dl, normal values, 2.9–16.1 ng/dl) were elevated. Renal ultrasound findings were normal. Casual blood pressure (BP) averaged 105/70 mmHg. The association of hypokalemic alkalosis, renal salt loss, and hyperreninemic hyperaldosteronism associated with normotension led us to suspect a diagnosis of Bartter's syndrome (BS). This patient did not comply with regular therapy except for intermittent administration of potassium chloride. After hospitalization this time, his physical and biochemical indices were revaluated (Tables 1 and 2). The laboratory findings of hypomagnesemia, with a magnesium level of 0.31–0.44 mmol/l, and hypocalciuria (urine calcium/creatinine (Ca/Cr): 0.06–0.1 mmol/mmol), suggested a diagnosis of GS. Repeated examinations demonstrated his suppressed PRA and elevated PAC. The PAC/PRA ratio was extremely high (Table 2). Thus, the collective evidence of renal salt loss and unexpectedly suppressed PRA led to the suspicion of coexistence of PA and GS in this patient. Computerized tomography showed bilateral enlarged adrenal glands (data not shown). None of his family members had hypokalemia and hypertension.

Patient 2 was a 42-year-old woman who was hospitalized because of persistent muscle weakness for 5 years. She was first diagnosed as GS according to electrolytes analysis of her serum and urine. However, the suppressed PRA, elevated level of PAC, and high PAC/PRA ratio also raised our suspicion of her coexistence of PA (Table 2). No positive renal and adrenal gland image findings were detected. All her family members had normal levels of serum potassium and BP.

To make the diagnosis of these two patients clear, and compare their biochemical features with GS, we selected ten age- and body mass index-matched subjects (six males and four females) with only GS as controls. They came from nine unrelated families, and their age ranged from 34 to 46 years (mean 39 ± 4 years). Their gross clinical features and biochemical data were shown in Tables 1 and 2. Criteria for GS diagnosis were: serum magnesium level <0.65 mmol/l, serum potassium level lower than 3.5 mmol/l, and hypocalciuria (urinary Ca/Cr ratio <0.1 mmol/mmol).

All subjects had normal renal function, and claimed no laxatives or diuretics abuse. Informed consent was obtained from each subject and the study protocol was approved by the ethics committee of the affiliated hospital, Qingdao University School of Medicine.

Table 1 Clinical characteristics of the two patients and control subjects.

Clinical characteristics	Patients suspected of PA and GS ($n=2$)		Control subjects with only GS ($n=10$)
	Patient 1	Patient 2	
Male/female	1/1		6/4
Age (years)	39	42	39 ± 4
Body mass index (kg/m^2)	26.5	21	23.6 ± 2.2
Systolic office BP (mmHg)	135	130	112 ± 10*
Diastolic office BP (mmHg)	85	80	71 ± 9.1
Systolic 24-h BP (mmHg)	129	126	105 ± 9*
Diastolic 24-h BP (mmHg)	79	76	65 ± 6*
Dipper/non-dipper	0/2		9/1*

*$P<0.05$.

Office BP and ambulatory blood pressure monitoring

Office BP was measured with a mercury sphygmomanometer after the patient had rested by sitting for at least 5 min according to AHA guidelines (7) with the mean of two readings used for analysis. All patients also underwent non-invasive ambulatory blood pressure monitoring (ABPM; Spacelabs model 90207; Spacelabs Inc., Richmond, WA, USA). The monitor recorded systolic and diastolic BP every 20 min during the daytime (0600–2200 h) and every 30 min at night (2200–0600 h). Nocturnal decline was defined as the percentage difference in ambulatory day versus night BP levels. Dippers were defined as patients with $\geq10\%$ decline in both systolic and diastolic BP.

Saline infusion test and adrenal venous sampling

Sodium intake was unrestricted. After overnight recumbency, 2 l of 0.9% saline solution were administered intravenously in 4 h between 0800 h and noon. BP and heart rate were monitored closely during the test. PRA and PAC were measured before and after the test. Adrenal venous sampling (AVS) was carried to identify the lateralization of aldosterone secretion.

Mutation analysis

Genomic DNA was extracted from peripheral blood of these two patients, control subjects, their family members, and 200 normal healthy controls by the GenElute blood genomic DNA kit (Sigma, NA2010). *SLC12A3* and *CLCNKB* genes were analyzed as described (8, 9). To analyze transcriptional profiles for

Table 2 Biochemical summary of the two patients and control subjects.

	Patients suspected of PA and GS ($n=2$)			
Biochemical data	Patient 1	Patient 2	**Control subjects with only GS** ($n=10$)	**Normal range**
Serum levels of electrolytes				
Na (mmol/l)	140	142	138±3.3	130–147
K (mmol/l)	2.1	2.2	2.54±0.35*	3.5–5.1
Cl (mmol/l)	97	93	96±2.9	95–108
Mg (mmol/l)	0.42	0.46	0.49±0.08	0.7–1.0
Blood gas analysis				
pH	7.50	7.46	7.45±0.03	7.35–7.45
HCO_3 (mmol/l)	31	29	29.1±1.54	22–27
Urinary electrolytes analysis				
Urinary Na (mmol/24 h)	196	187	237±53	137–257
Urinary Cl (mmol/24 h)	227	204	250±56	170–250
Fractional excretion of K (%)	36	29	24.6±7.4*	8–12%
Urinary Ca/Cr ratio (mmol/mmol)	0.04	0.06	0.043±0.02	0.2–0.6
PRA (ng/ml per h)				
Supine PRA	<0.1 (0.2)[a]	0.3	13.0±8.9†	0.1–2.9
Standing PRA	0.23	1.1	18.7±11.9†	0.6–11.0
PAC (ng/dl)				
Supine PAC	25.6	16.2	18.5±3.9	2.9–16.1
Standing PAC	51.6	39.1	38.8±6.1	3.8–31.3
PAC/PRA ratio				
Supine	128	81	1.76±0.8†	
Standing	224	35.5	2.3±1.2†	
Saline infusion test				
PAC before test (ng/dl)	22.9	21.5	16.9±4.1	
PAC after test (ng/dl)	18.7	20.4	5.1±1.3†	

All biochemical data of each subject were the mean of three examinations except the values of saline infusion test. To convert from metric units to International units, multiply PAC by 28 and PRA by 0.28. PRA, plasma renin activity; PAC, plasma aldosterone concentration. *$0.05<P<0.1$, †$P<0.01$.
[a]When calculating the PAC/PRA ratio, we arbitrarily fix the lowest PRA value at 0.2 ng/ml per h to avoid overinflating the value.

the *SLC12A3*-splicing mutations, we extracted total RNA from blood using TRIzol (Invitrogen/Gibco). After DNAase treatment, RNA was purified with the Total RNA Purification System (Invitrogen) and was reverse transcribed with Superscript III (Invitrogen). After the detection of a substitution at or close to a splice consensus sequence, exonic pairs of primers were designed as described (10) to amplify cDNA regions. One primer of each pair was used to sequence the PCR product. As the pathophysiology of normotensive primary hyperaldosteronism is unknown and has shown wide phenotypic variability among carriers of the chimeric *CYP 11-β/aldosterone synthase* gene (11), we carried out a genetic analysis of these two patients to rule out a peculiar clinical presentation of dexamethasone (DXM)-sensitive hyperaldosteronism. The presence of the *CYP11B1/CYP11B2* chimeric gene was studied using the long PCR technique introduced by Jonsson *et al.* (12).

Functional studies of SLC12A3 mutations

NCC-directed mutagenesis and* in vitro *human NCC cRNA translation Four mutations identified in these two patients were selected for functional analysis (T60M, T304M, T465P, and N611T). Wildtype (WT) human NCC (hNCC) cDNA was cloned into pT7TS vector (kindly provided by Dr Paul Krieg, University of Texas) using the restriction sites of BgIII and SpeI. A FLAG epitope tag was added at the 5′ site of NCC to facilitate detection. Site-directed mutagenesis (QuikChangeII XL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) was performed according to the manufacturer's instructions. Direct sequencing of the full-length cDNA was performed to confirm mutagenesis. The four mutant constructs were linearized with EcoRI, and cRNA transcripts were synthesized *in vitro* using T7 RiboMAX Express System (Promega). Transcription product integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm (Gene Quant α, Pharmacia Biotech).

Expression studies in* Xenopus laevis *oocytes Oocytes at stages V–VI were obtained from *X. laevis* (13). Each oocyte was injected with either 50 nl water or 10 ng WT or mutated hNCC cRNAs. $^{22}Na^+$ uptake assay, western blotting, and immunocytochemistry were performed as described (13) after 48 h of incubation. Assessment of tracer $^{22}Na^+$ uptake (New England Nuclear, Waltham, Massachusetts, USA) was determined in groups of at least 15 oocytes. Oocytes were transferred to Cl^- free medium for 24 h and next transferred to 500 µl uptake medium (containing 1 µCi/ml $^{22}Na^+$, 1 mM ouabain, 100 µM bumetanide, and 100 µM amiloride) for 2 h at 32 °C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without the isotope to remove extracellular fluid tracer. After the oocytes were dissolved in 10% (w/v) SDS, tracer activity was determined for each oocyte by β-scintillation counting. Western blotting was used to compare WT and mutant hNCC protein in cRNA-injected oocytes. Isolation of total membranes was performed in 15 oocytes. Protein samples were immunoblotted onto PVDF membranes and successively incubated with 1:8000 dilution mouse anti-FLAG (Sigma) and 1:2000 diluted sheep HRP conjugated to anti-mouse IgG (Sigma) antibodies, bands were detected by using ECL system (Pierce, Rockford, IL, USA). The subcellular localization of hNCC was determined in immunocytochemical analyses. To the end, the remaining vitelline membrane was removed after 48 h of incubation, and oocytes were fixed at room temperature for 2 h at room temperature in 1% (w/v)

paraformaldehyde fixative, dehydrated, and paraffined. Six micrometer thick sections were cut and incubated overnight at 4 °C with mouse anti-FLAG antibody (Sigma) diluted 1:200 followed by incubation at room temperature for 1 h with Alexa fluor 488 goat anti-mouse IgG (Invitrogen) diluted 1:250.

Statistical analysis

The biochemical data were expressed as mean ± S.D. The Student's unpaired *t*-test and χ^2 test were used to compare the differences between these two patients suspected of having both PA and GS and control subjects with only GS. $P<0.05$ was considered statistically significant, while $0.05<P<0.1$ was borderline significant.

Results

As shown in Table 1, both patients 1 and 2 had normal values of office BP and mean 24-h BP. However, their systolic and 24-h diastolic BP levels were significantly higher than control subjects with only GS (Table 1). In addition, ABPM confirmed that neither patients had normal nocturnal dip (Figs 1 and 2), while nine out of ten subjects had normal circadian rhythm in control group ($P=0.045$; Table 1). The mean ambulatory BP of patient 1 was 129/79 mmHg (mean diurnal pressure 128/78 mmHg; mean nocturnal pressure 131/81 mmHg), and the value of patient 2 was 126/76 mmHg (127/77 mmHg by day and 121/73 mmHg by night).

Laboratory data are shown in Table 2. Both patients presented persistent hypokalemia, hypomagnesemia, hypocalciuria and increased renal salt excretion as seen in subjects with only GS. However, they had borderline significant lower serum potassium level ($P=0.08$) and borderline significant higher FEK ($P=0.07$) than patients with only GS.

The most distinctive feature in these two patients was their suppressed PRA levels as shown in Table 2. Repeated examinations demonstrated that the first patient had severe suppressed PRA and elevated PAC. The PAC/PRA ratio was extremely high. The levels of supine and standing PRA of patient 2 were within normal range, though at the low limit of normal range. The level of her PAC was higher than normal value, and her PAC/PRA ratio was higher than the cut-off value (30 ng/dl per ng/ml per h), which was often used to screen PA. By contrast, in the control group, the PRA of each subject was higher than the upper normal range, and the decline of PAC was significantly higher than these two patients after saline infusion. Additionally, it is worth noting that PAC levels of each subject in the control group were <10 ng/dl, while PAC levels of both patients in the study group were above that value.

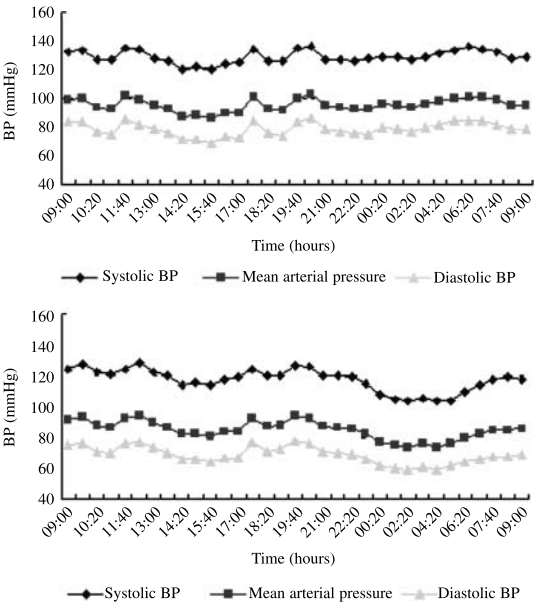

Figure 1 Patient 1: results of ambulatory blood pressure monitoring (ABPM) before and after treatment. Top panel: before treatment by spironolactone. The mean ambulatory BP was 129/79 mmHg; the circadian rhythm was disrupted with a mean diurnal pressure of 128/78 mmHg and a mean nocturnal pressure of 131/81 mmHg. Bottom panel: after treatment by spironolactone. The 24-h BP slightly decreased by ~10 mmHg with the mean 24-h BP of 117/69 mmHg. The normal circadian rhythm characterized with nocturnal dip reappeared with a mean diurnal pressure of 121/71 mmHg and a mean nocturnal pressure of 106/61 mmHg.

AVS was carried to identify the lateralization of aldosterone secretion. The plasma aldosterone levels from the right and left adrenal veins and the inferior vena cava of patient 1 were 592.3, 458.8, and 38.7 ng/dl, and the cortisol levels were 106.0, 126.1, and 16.5 μg/dl respectively (Table 3). Adrenal vein cannulation was considered successful given that the bilateral adrenal vein/inferior vena cava cortisol gradients were 6.4 and 7.6 respectively. The ratio of the higher to lower levels of aldosterone in the right and left adrenals (aldosterone ratio) was 1.3, aldosterone to cortisol ratio (A/C ratio) in the right and left adrenals were 5.6 and 3.6 respectively, and the ratio of higher to lower levels of A/C ratio in the right and left adrenals (lateralization index, LI) was 1.56. The data of patient 2 were also available in Table 3, her LI was 1.19.

After 2 months of potassium and magnesium supplementation together with spironolactone (50 mg/day) treatment, serum potassium levels in both patients could reach the low limit of normal level (~3.5 mmol/l). As shown in Figs 1 and 2, ABPM demonstrated that the mean value of 24-h BP slightly decreased by nearly 10 mmHg, and the normal circadian rhythm characterized with nocturnal dip was restored in both patients. The mean 24-h BP of patient 1 was 117/69 mmHg (diurnal: 121/71 mmHg; nocturnal: 106/61 mmHg), while the value of

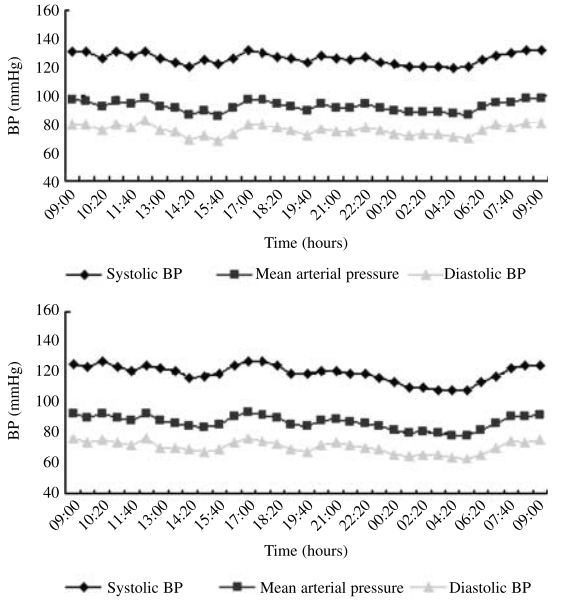

Figure 2 Patient 2: results of ambulatory blood pressure monitoring (ABPM) before and after treatment. Top panel: before treatment by spironolactone. The mean ambulatory BP was 126/76 mmHg; the circadian rhythm without sufficient normal dipper was shown with a mean diurnal pressure of 127/77 mmHg and a mean nocturnal pressure of 121/73 mmHg. Bottom panel: after treatment by spironolactone. The 24-h BP slightly decreased by nearly 10 mmHg with the mean 24-h BP of 120/70 mmHg. The circadian rhythm characterized with normal nocturnal dipper restored with a mean diurnal pressure of 122/72 mmHg and a mean nocturnal pressure of 110/65 mmHg.

patient 2 was 120/70 mmHg (122/72 mmHg by day and 110/65 mmHg by night) by ABPM. The decrease of BP in night time was more predominant than that in day time in both patients.

Sequence analysis of the *SLC12A3* gene of these two patients and ten control subjects revealed 16 different punctual mutations (Supplementary Table, which can be viewed online at http://www.eje-online.org/supplemental/ and Fig. 3A), including nine novel mutations (Supplementary Figure, which can be viewed online at http://www.eje-online.org/supplemental/). The novel variants are seven missense mutations (p.C146F, p.T304M, p.N359D, p.T465P, p.P556L, p.N611T, and p.Y857C), a deletion of a guanine (c.402delG) that caused a frameshift that resulted in a truncated polypeptide with 141 acid residues (p.Arg135AlafsX8), and a splice mutation (c.964+2T>C), which was confirmed to lead to a shorter transcript characterized as skipping exon 7 by cDNA sequencing (p.Ala285 ArgfsX48). Additionally, seven mutations (p.T60M, p.G439S, p.S555L, p.R655C, p.R928C, p.Thr114AlafsX142, and p.Arg959SerfsX11) identified in this study had been reported previously (8, 10, 14–20). To both patients suspected of PA and GS, patient 1 showed compound heterozygosity for mutations of p.T60M and p.T304M, patient 2 was also a compound heterozygote with variants of p.T465P and p.N611T. In the control group, only one mutant allele was identified in two subjects. All mutations co-segregated with the phenotype, and all missense mutations identified in this study are substitute amino acids that are highly conserved in all transporter proteins belonging to the protein superfamily of cotransporters (NCCT, NKCC1, NKCC2). Sequence analysis for the above-mentioned 16 hNCC mutations revealed heterozygous p.T60M in 2, p.R928C in 1, p.Thr114AlafsX142 in 1, and p.Arg959SerfsX11 in 2 out of 200 unrelated healthy subjects respectively. No *CLCNKB* gene mutation was found and gene tests ruled out DXM-sensitive hyperaldosteronism.

Table 3 Adrenal venous sampling in the two patients.

Patient	1	2
Aldosterone (ng/dl)		
Right	592.3	399.4
Left	458.8	423.4
Higher/lower	1.3	1.06
IVC	38.7	23.1
Cortisol (μg/dl)		
Right	106.0	107.8
Left	126.1	96.3
IVC	16.5	13.8
Cortisol ratio (AV/IVC)		
Right	6.4	7.8
Left	7.6	7.0
Aldosterone to cortisol ratio		
Right	5.6	3.7
Left	3.6	4.4
Higher/lower (LI)	1.56	1.19

IVC, inferior vena cava; AV/IVC, adrenal vein/inferior vena cava ratio; LI, lateralization index.

To further determine whether those mutations will affect hNCC's functionality, we used the *Xenopus* oocytes model system. As Fig. 3C shows, we observed in a western blot from oocytes expressing WT and mutant hNCC, which mutants of p.T304M and p.T465P produce proteins that are not fully glycosylated with a single band of 110 kDa, whereas p.T60M and p.N611T generate proteins in which glycosylation patterns appear indistinguishable from WT with bands 110 and 130–140 kDa. The results of $^{22}Na^{+}$ uptake assay were demonstrated in Fig. 3B; the p.T60M, p.T304M, and p.T465P mutants showed the same level of activity as H_2O-injected oocytes, whereas mutant p.N611T showed a rate of $^{22}Na^{+}$ uptake of 54%, compared with oocytes that expressed the WT protein. As displayed in Fig. 3D, in sections of oocytes expressing WT hNCC, clear immunostaining at the plasma membrane was observed, whereas staining was absent in H_2O-injected oocytes. Sections of oocytes expressing the mutant p.T60M exhibited similar pattern with WT, and those expressing p.T304M and p.T465P mutants demonstrated predominant intracellular staining, with only minor staining at the plasma membrane, whereas those expressing mutant p.N611T revealed significant immunopositive staining of both the plasma membrane and the cytoplasm. These results indicate that among

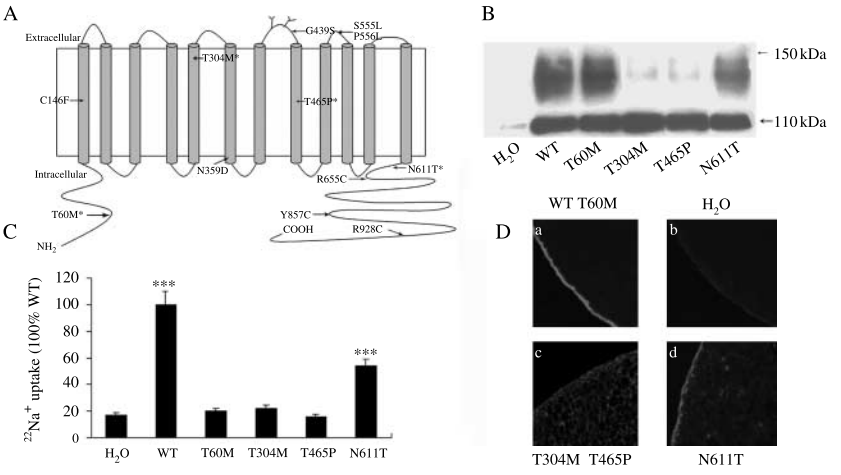

Figure 3 Functional studies of missense mutations in SLC12A3. (A) Schematic of the thiazide-sensitive Na–Cl cotransporter (NCC) and missense mutations identified in this study. The NCC is represented as a 12-transmembrane-domain protein with intracytoplasmic amino and carboxyl termini. The sites of mutations are denoted by arrows, the mutations underlined are novel variants, the remainders are found by others. *Position of the selected mutations for functional study. (B) Immunoblots of total membrane extracts that were isolated from *Xenopus* oocytes, either H_2O-injected (H_2O) or injected with 10 ng WT or mutant hNCC mRNA. (C) $^{22}Na^+$ uptake in oocytes that expressed WT or mutant hNCC. Uptake values are shown as percentage of the WT, where 100% represents an uptake of 2.8 nmol Na^+/oocyte per 2 h. ***$P<0.0001$ in relation to the uptake of H_2O-injected oocytes. (D) Representative immunocytochemistry of oocytes injected with WT or mutant hNCC: plasma membrane staining in an oocyte injected with WT hNCC (a similar pattern was observed for p.T60M; a); H_2O-injected oocyte with only background staining (b); oocyte expressing nonfunctional p.T304M mutant with intracellular staining only (a similar pattern was observed for p.T465P1); c); oocyte injected with functional p.N611T mutation that presented both plasma membrane and intracellular staining (d).

the four mutations studied, p.T304M and p.T465P are not complex glycosylated, absent from the plasma membrane, and nonfunctional. The mutant p.N611T exhibit complex glycosylation, presence in both the plasma membrane and the cytoplasm, and an intermediate level of activity compared with WT. Although the mutant p.T60M show complex glycosylation and similar cell surface expression with WT, its intrinsic activity was abolished.

Discussion

In this report, we described two rare cases presenting with features of GS such as normotensive, hypokalemia, hypomagnesemia, hypocalciuria, and hyperaldosteronism. However, the unexpectedly suppressed PRA made the diagnosis elusive. To confirm the coexistence of GS and 'autonomous' hyperaldosteronism in both patients, we performed a series of studies, such as *SLC123* gene analysis, functional studies, and saline infusion test.

The diagnosis of GS was confirmed by genetic tests and functional studies besides their clinical and biochemical features in both patients. All the four variants of p.T60M, p.T304M, p.T465P, and p.N611T found in them were confirmed to be potentially pathogenic to GS by functional studies in *Xenopus* oocytes. Among the four mutant sites in hNCC, Thr60 has recently been revealed to be the key activating phosphorylation site on NCC, and played an important role in the activation of NCC (21). Our study demonstrated that hNCC harboring mutant p.T60M almost completely lost its intrinsic activity without affecting the surface expression. These results were similar with the expression study by Pacheco-Alvarez *et al.* on rat NCC carrying mutant p.T58A (Thr58 equivalent to Thr60 in hNCC) (22). It is noteworthy that mutant p.T60M has been frequently reported to be correlated with GS (8, 10, 14–17). In fact, it was the most common mutation in Asian populations so far. At codon 611, another mutant, p.N611S, caused by an A-to-G substitution at nucleotide 1832 (c.1832A>G), has also been proven to have decreased activity compared with WT based on an *in vitro* study (23). Therefore, it appears that the substitution of asparagine by threonine at codon 611 has a similar effect on NCC function as the substitution by serine.

Saline infusion test demonstrated that aldosterone secretion was independent, at least partially, from renin–angiotensin system in both patients, whereas the production of aldosterone was significantly suppressed by saline infusion in the control group. Therefore, integration of the results of gene analysis, functional studies, and saline infusion tests suggested concurrence of PA and GS in both patients. The exploitation of the type of hyperaldosteronism in these two patients was interesting. Aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) are two major subtypes of PA. APA, also termed as Conn's adenoma, is a surgically curable disease.

However, IHA could be improved by mineralocorticoid receptor (MR) antagonist. Only APA can be unequivocally diagnosed with strict criteria such as evidence of adenoma at pathological examination besides biochemical evidence of PA and lateralization of aldosterone secretion at AVS. However, no such diagnostic gold standard exists for identifying IHA. Therefore, we could not obtain conclusive diagnosis as neither of them suffered adrenalectomy. Luckily, a recent investigation by Rossi *et al.* (24) has calculated an optimal LI cut-off value of 1.98, which provided the best trade-off between sensitivity (79.5%) and specificity (75%) for exclusion of APA. With decrease in LI cut-offs from 1.98 to 1.125, the possibility (about 20%) of having APA further decreased steadily. From the statistical data provided by Rossi *et al.* the AVS results suggested that both patients (with LI 1.56 and 1.19 respectively) very likely had IHA other than APA.

The classic form of PA is characterized by hypertension and hypokalemia. Recent statistical data have demonstrated that more than one half of patients with PA were normokalemia (1). Although the hypertension is usually mild and may fluctuate, only 27 cases with consistently normotensive, due to APA or IHA, have been reported so far (25–27). The mechanism(s) underlying the maintenance of normal BP levels, despite an overproduction of aldosterone, are unknown. It is a well-acceptable hypothesis that aldosterone-induced salt retention and volume expansion may be the prime factor to hypertension in PA, and meanwhile an important contributor to the development of hypertension in the long term (28, 29). GS is a salt-losing disease due to the loss of function of NCC in the distal convoluted tubule. Salt depletion and volume constriction are the main pathophysiologic characteristics of GS. Coexistence of salt-losing disease might just explain the absence of hypertension in these two patients with PA and give further evidence to the above-mentioned hypothesis. In addition, angiotensin II (Ang II) is one of the most important humoral factors involved in the vascular alterations in hypertension. It is well known that Ang II leads to vasoconstriction and cardiovascular remodeling via its complex intracellular signaling pathways, such as activation of Gq protein signal, along with down-regulation of nitric oxide (NO) system and the up-regulation of RhoA/ROK pathway. Extensive studies of patients with GS performed by Calò *et al.* have shown that cellular pathways induced by Ang II are blunted (30). This explained well the reduced peripheral resistance, vascular hyporeactivity, and normohypotension in GS patients, in spite of high Ang II and activation of the renin–angiotensin–aldosterone system (RAAS). The plasma levels of Ang II in PA patients are suppressed; however, the local RAAS may play a more important role in the development of hypertension. Therefore, the mechanisms maintaining normal BP by blunting Ang II signaling may also exist in these two patients.

However, ABPM proved disrupted circadian rhythm of BP in both patients, with mild nocturnal hypertension in patient 1, and the level of nocturnal BP in patient 2 was at the upper limit of normal ambulatory BP (normal $<120/70$ mmHg). This suggested that the protective mechanism against hypertension via down-regulation of renal sodium handling was probably not sufficient in patients with PA. This situation resembles that of secondary hypertension such as aldosteronism, which is more often than not resistant to diuretics. It is well known that hyperaldosteronism is associated with endothelial dysfunction and impaired vascular reactivity in patients with hypertension (31). When present, endothelial dysfunction is an independent predictor of adverse cardiovascular events. The MR antagonist spironolactone restored both patients' normal BP circadian rhythm and reduced their BP to optimal level. We supposed that this occurs, in part, as a result of improved vascular function. In addition, the diagnosis of PA in both patients was further confirmed by the response to treatment with spironolactone.

It is interesting that the secondary aldosteronism coexisting with GS in patient 1 progressed to PA within 12 years. However, the precise underlying mechanism is difficult to elucidate since the pathogenesis of PA is still unknown. It should also be noted that both patients probably had IHA rather than APA. It is well known that APA and IHA had different physiological aldosterone regulation, as evidenced by the APA's ACTH responsiveness and holding to be functionally autonomous from the renin–angiotensin system, conversely, IHA still maintain the normal regulation of adrenocortical zona glomerulosa. This suggests the existence of an alternative etiology under the descriptive term of 'IHA'. Whether IHA is tertiary hyperaldosteronism remains controversial (28, 32, 33). Theoretically, the underlying mechanism for tertiary hyperaldosteronism is persistent stimulation of the adrenal by excessive Ang II, causing aldosterone overproduction, which eventually evolves into an autonomous phase (28, 34). However, the existence of tertiary hyperaldosteronism has been strongly challenged by Conn *et al.* who reviewed five cases of primary reninism associated with juxtaglomerular tumors. They noted that two patients had been exposed to prolonged hyperreninemia for many years, and that after surgical intervention, both showed a decline in aldosterone excretion. They recommended that the term 'tertiary' hyperaldosteronism be abandoned (33). Recently, Lim *et al.* have again proposed that it is feasible to redefine IHA as a form of tertiary aldosteronism by incorporating new data (28). On the other hand, they considered that polymorphisms of the aldosterone synthase gene may play a part in the development of hyperaldosteronism, and suggested that individuals with certain polymorphisms may be predisposed to the development of tertiary hyperaldosteronism (28, 32). Since both PA and GS are not common diseases, the coexistence of these two diseases may be

due to chance; however, this does not completely rule out the possibility that IHA in these two patients is also acquired under the condition of lasting stimulation factors (salt, Ang II, etc.) that interact with predisposing genes over a long time. Therefore, the evolution from secondary aldosteronism to PA in patient 1 might be well explained by the acquired or tertiary hyperaldosteronism. However, it is worth noting that only two cases were found in nearly 100 GS patients we encountered, and the vast majority of subjects did not evolve to the autonomous phase of aldosteronism under the long-term stimulation of Ang II and therefore, the possibility of existence of 'tertiary' hyperaldosteronism is very low.

In summary, these two cases illustrate that both PA and GS may rarely occur in the same patient. It is noteworthy that either disorder may be initially discovered although GS was discovered initially in our patients. We propose that careful excluding examination should be done to explore the possibility of existence of renal-salt-losing disease such as GS in a patient with normotensive PA, and vice versa, PA should be considered in a patient with renal salt-losing disease and jointly with suppressed PRA.

Declaration of interest

All authors have no conflict of interest to report.

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