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Mutation Analysis of *SCNN1B* in a Family with Liddle's Syndrome

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Liddle's syndrome has been known as a disorder associated with abnormal sodium reabsorption in the distal tubule and transmitted as a rare autosomal dominant trait. It is caused by mutations in the *SCNN1B* or *SCNN1C* gene, which truncate the cytoplasmic carboxyl terminus of the β and γ subunit of the epithelial sodium channel (ENaC). Genetic analysis of ENaC in a Chinese family with Liddle's syndrome revealed P616H of *SCNN1B* coaggregated with the phenotype, while this variant was not detected in 100 unrelated subjects. No mutation at γ ENaC could be detected in all members of the family. P616H is located in the conserved proline-rich PY motif of the βENaC. The PY motif can interact with the WW domain in Nedd4 and affect the activity of ENaC. Structural bioinformatics analysis confirmed that the functional interaction between Nedd4 and ENaC reduces in Liddle-ENaC (P616H) when compared with wild-type ENaC. In summary, P616H may be an underlying mechanism for the signs and symptoms of this family.

Key Words: Liddle's syndrome; epithelial sodium channel; hereditary disease; hypertension.

Introduction

Liddle's syndrome is a monogenic form of hypertension with autosomal dominant inheritance. It is caused by excessive sodium reabsorption in the distal tubule and is characterized by hypertension associated with low plasma renin activity, low aldosterone level, and hypokalemia *(1)*. Genetic analysis has revealed that mutations in the COOH terminus of epithelial sodium channel (ENaC) β or γ subunit can increase Na^+ absorption in the distal nephron of the kidney and thus lead to Liddle's syndrome. These mutations are mainly base substitutions leading to premature stop codons or frameshift mutations that result in the truncation of COOH terminus of the β or γ subunit of ENaC *(2–12)*. Seventeen mutations, consisting of 14 in the βENaC and 3 in the γENaC, have been reported. However, no mutation in the α subunit of the ENaC has yet been detected.

The amiloride-sensitive epithelial sodium channels, which are expressed at the apical membrane of a variety of epithelia, including the kidney, lung, and colon, mediate the entry of sodium across the apical membrane. ENaC is the rate-limiting step of sodium reabsorption in the distal nephron; therefore, its regulation is the key for the maintenance of sodium balance and blood pressure *(13)*. In Liddle's syndrome, hypertension and hypokalemia can be corrected by low salt diet in conjunction with antagonist of the ENaC, e.g., triamterene and amiloride, but no effect by mineralocorticoid antagonist, e.g., spironolactone.

The amiloride-sensitive ENaC complex is composed of three homologous subunits, α (gene symbol *SCNN1A*), β (gene symbol *SCNN1B*), and γ (gene symbol *SCNN1C*), each has two transmembrane-spanning domains, a large extracellular loop, and amino end carboxyl termini within the cytoplasm *(14,15)*. Although with only 30–35% sequence identity, the COOH-terminal region of each subunit shares conserved proline-rich sequences, including the sequence xPPxY, called the PY motif *(2–12)*. The PY motif is important for appropriate regulation of the number of ENaC channels on the cell surface. Systematic mutagenesis studies in *Xenopus* oocytes showed that a highly conserved PY motif of C terminus of the α, β, or γ subunit was critical for regulation of channel activity. Nedd4, the ubiquitin-protein ligase expressed in the neural precursor cell, was identified as a binding partner for the PY motifs in ENaC and can negatively control ENaC cell surface expression *(16)*. The interaction

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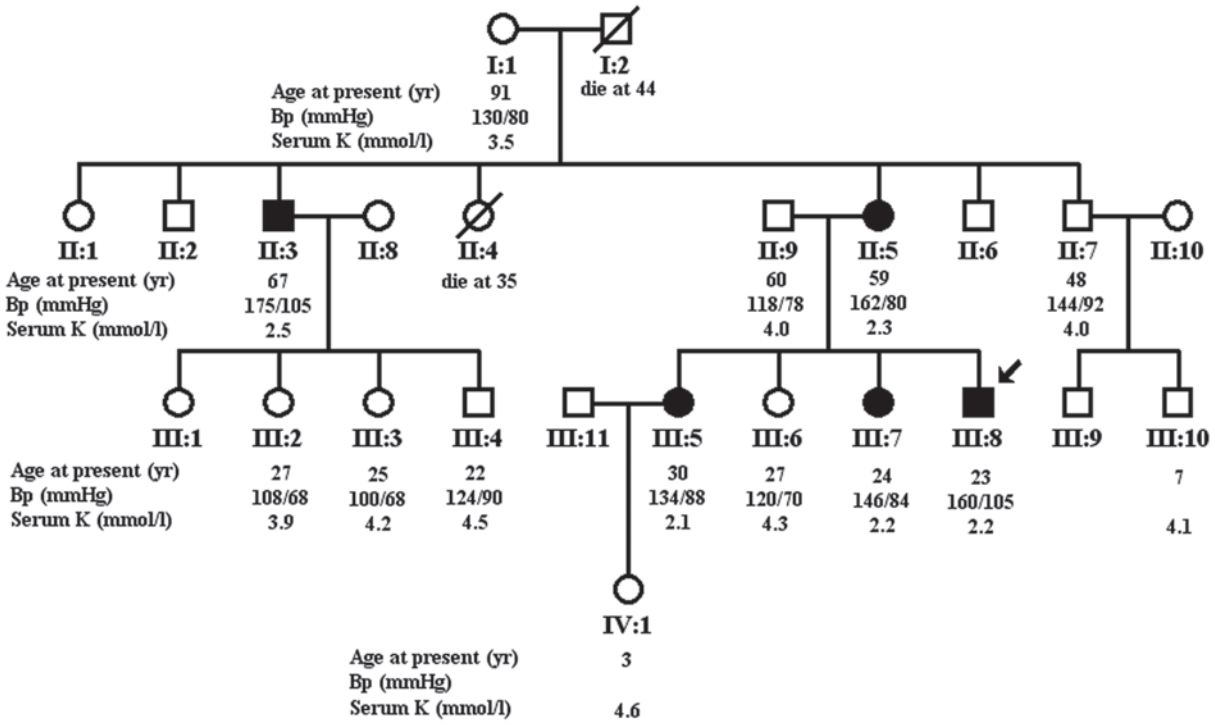

Fig. 1. Pedigree of a family with Liddle's syndrome. The index case is shown by the arrow. Squares indicate males and circles females. Individuals with the P616H mutation are shown as filled symbols. Below the symbols, in descending order, are shown the ages of subjects (in years as of 2005), blood pressure (mmHg), and serum potassium level (mmol/L).

between the WW domain in Nedd4 and the PY motif of βENaC could affect the activity of ENaC.

Here we report a family with Liddle's syndrome carrying a missense mutation in the carboxyl terminus of the β subunit of ENaC. Primary structural bioinformatics analysis suggested abnormal interaction between Nedd4 and PY motif in Liddle-ENaC (P616H) compared with the wild-type ENaC.

Results

Clinical Features of the Liddle's Family

The pedigree of this family is shown in Fig. 1. The proband (shown by the arrow in Fig. 1) presented with severe hypertension and hypokalemia. The laboratory data revealed completely suppressed plasma renin activity and low urinary excretion of aldosterone (Table 1). In response to triamterene (400 mg/d), blood pressure was lowered from 160/110 mmHg to 130/75 mmHg and serum potassium increased from 2.28 mmol/L to 4.52 mmol/L. Plasma renin activity and aldosterone levels were also elevated after administration for 1 mo (Table 2). Thus, the proband was diagnosed as Liddle's syndrome on a clinical standpoint. Extensional analysis demonstrated that severe hypokalemia was found in another four family members who were highly suspected of Liddle's syndrome and three of whom had various degrees of hypertension (Table 3). I-2 had already died of accident.

Detection of the Mutation

As shown in Fig. 2, genetic analysis of the cytoplasmic carboxyl terminus of the β subunit revealed a missense mutation of the βENaC gene in which 616Pro (CCC) was changed to 616His (CAC), P616H, detected in the proband. The same mutation was also identified in his older sisters (III-5, III-7), his mother (II-5), and his uncle (II-3). However, this variant was not detected in 100 unrelated subjects. No mutation was found in γ ENaC gene.

Mutation Analysis

Because human and rat share conserved sequence and function in PY motif and WW domain, we used a rat model to predict the interface between the PY motif and WW domain by using *i*MolTalk. In wild-type ENaC, Pro 616 (PY motif) interacts with Thr 485, Phe 476, and His 470 (Nedd4-WW domain) in the wild-type ENaC. When Pro 616 is substituted with His 616, it can only interact with Thr 485 and Thr 471 through H-bond in addition (Fig. 3). When compared with wild-type ENaC, the functional interaction between Nedd4 and ENaC reduces in Liddle-ENaC (P616H).

Discussion

Liddle's syndrome–associated mutations increase activity of the epithelial sodium channel expressed in the distal nephron, resulting in excessive reabsorption of sodium and hypertension. All the reported mutations in Liddle's syn-

Table 1
Laboratory Data of the Proband with Liddle's Syndrome on Admission

Complete blood counts	Within normal limits
Urinalysis	Within normal limits
Liver function tests	Within normal limits
Renal function	
Blood urea nitrogen	6.8 mmol/L (2.5–7.1)
Creatinine	92 umol/L (53–115)
Uric acid	461 umol/L (160–430)
Serum electrolytes	
Sodium	143.9 mmol/L (130–147)
Potassium	2.51 mmol/L (3.5–5.1)
Chloride	103.7 mmol/L (95–108)
Calcium	2.39 mmol/L (2.00–2.75)
Phosphorus	1.02 mmol/L (0.80–1.60)
Urine electrolytes	
Potassium	68.75 mmol/24 h
Arterial blood gas analysis	
pH	7.434
$PaCO_2$	5.67 kPa
PaO_2	13.50 kPa
HCO_3^-	27.6 mmol/L
BE	3.6 mmol/L
SO_2	98.2%
Hormone levels in serum	
Renin, supine	0.01 ng/mL·h (0.1–5.5)
Renin, upright	0.01 ng/mL·h (0.73–17.4)
Angiotensin II, supine	25 pg/mL (18–103)
Angiotensin II, upright	47 pg/mL (26–208)
Aldosterone, supine	45.8 pg/mL (29.4–161.5)
Hormone levels in urine	
Aldosterone	2.18 μg/24 h (2.25–21.4)
Adrenaline	11.16 μg/24 h (<22.00)
Noradrenaline	24.58 μg/24 h (7.00–65.00)
Dopamine	153.67 μg/24 h (75.00–440.00)

Table 2
Laboratory Data of the Proband Before and After Using Triamterene

Using Triamterene	Before	After 1 mo	Normal Range
BP (mmHg)	160 / 110	130 / 75	
Serum K^+ (mmol/L)	2.28–2.73	4.52	3.5–5.1
Urine K^+ (mmol/24 h)	68.75	43.74	36–90
Serum Aldo (pg/mL)	45.8	59.8	29.4–161.5
Urine Aldo (μg/24 h)	2.18	2.45	2.25–21.47
PRA basal (ng/mL·h)	0.01	0.29	0.1–5.5

Table 3
Clinical Features of Subjects with the P616H Mutations

	II-3	II-5	III-5	III-7	III-8
Age (yr)	67	59	30	27	23
Age at Dx of HTN (yr)	59	56	—	—	21
BP (mmHg)	175 / 105	162 / 80	134 / 88	146 / 84	160 / 105
K^+ (mmol/L)	2.5	2.3	2.1	2.2	2.2

Dx, diagnosis; HTN, hypertension; BP, blood pressure; K^+, serum potassium (normal, 3.5–5.1 mmol/L).

drome are either missense mutations or frameshift mutations altering the conserved PPPxY sequence (PY motif) present in the C-terminal ends of the βENaC or γENaC, pointing to this region as an important site for normal regulation of the channel activity.

In this study, a three-generation family with Liddle's syndrome was diagnosed. All affected subjects in the kindred of Liddle's syndrome show remarkable hypokalemia and various degrees of hypertension (Table 3). We sequenced the C-terminus of the β or γ subunits of the epithelial sodium channel in each available member. As a result, we found P616H coaggregated with the phenotype. The proband (III-8), his older sisters (III-5, III-7), his mother (II-5), and his uncle (II-3) all carried the mutation. This P616H mutation was not detected in 100 unrelated subjects, excluding the variant as a common polymorphism. Because the proband's father (II-9) and grandmother (I-1) did not carry this mutation, it can be speculated that the proband (III-8) inherited this condition from his grandfather (I-2). In Liddle's syndrome, deletion or mutation of conserved amino acids in the PY motif in ENaC increases Na^+ absorption, resulting in hypertension. The symptom of hypertension usually begins at an early age in the affected individuals and worsens through the lifetime. Although the proband's 30-yr-old older sister (III-5) carried P616H and presented hypokalemia, she was not hypertensive at the time of this study. This is consistent with the findings in a Liddle's original pedigree that hypokalemia and hypertension are not universal in affected members *(1,17,18)*. Normotensive family members were also found in the original kindred *(1)*. To date, mutations of the 614PPPxY618 sequence (PY motif) of the βENaC have been reported in five other kindreds *(2–7)*. Pro616His was first reported in an Afro-Haitian sporadic case with early-onset severe hypertension, suppressed plasma renin activity, and reduced serum aldosterone levels *(19)*. Differing from the present proband, the Afro-Haitian girl's serum potassium was within normal range and only her father had a history of hypertension in the whole family. We speculate that the degrees of hypertension and hypokalemia may be influenced not only by gene mutations, low salt intake may explain the lack of high blood pressure, even in the presence of an activating ENaC mutation.

The PY motif of the βENaC is generally involved in protein–protein interactions. Recently, Nedd4, a ubiquitin protein expressed in epithelia containing three or four WW domains and a ubiquitin protein-ligase domain, has been shown to interact specifically with PY motif of β and γ ENaC subunits *(20–24)*. It had been demonstrated that the WW

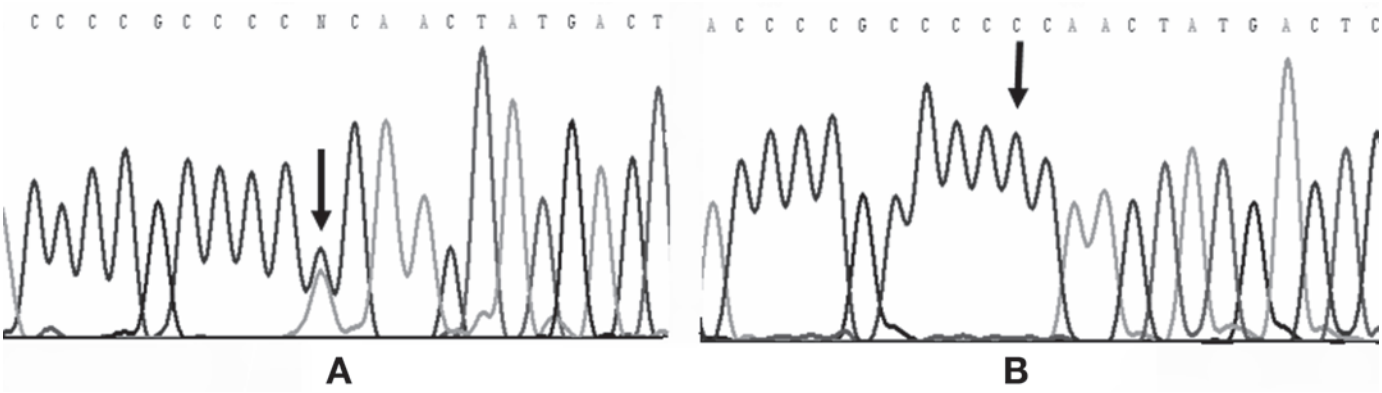

Fig. 2. Identification of mutation by direct sequencing of the *SCNN1B*. The C terminus of βENaC, amplified by PCR, was directly sequenced in the proband and in a normal control subject. As indicated by the arrow, both C and A signals were observed in the second base of codon 616 in the proband (A), while only C signals were observed in a normal subject (B). This point mutation causes an amino acid substitution of His for Pro at codon 616 (P616H).

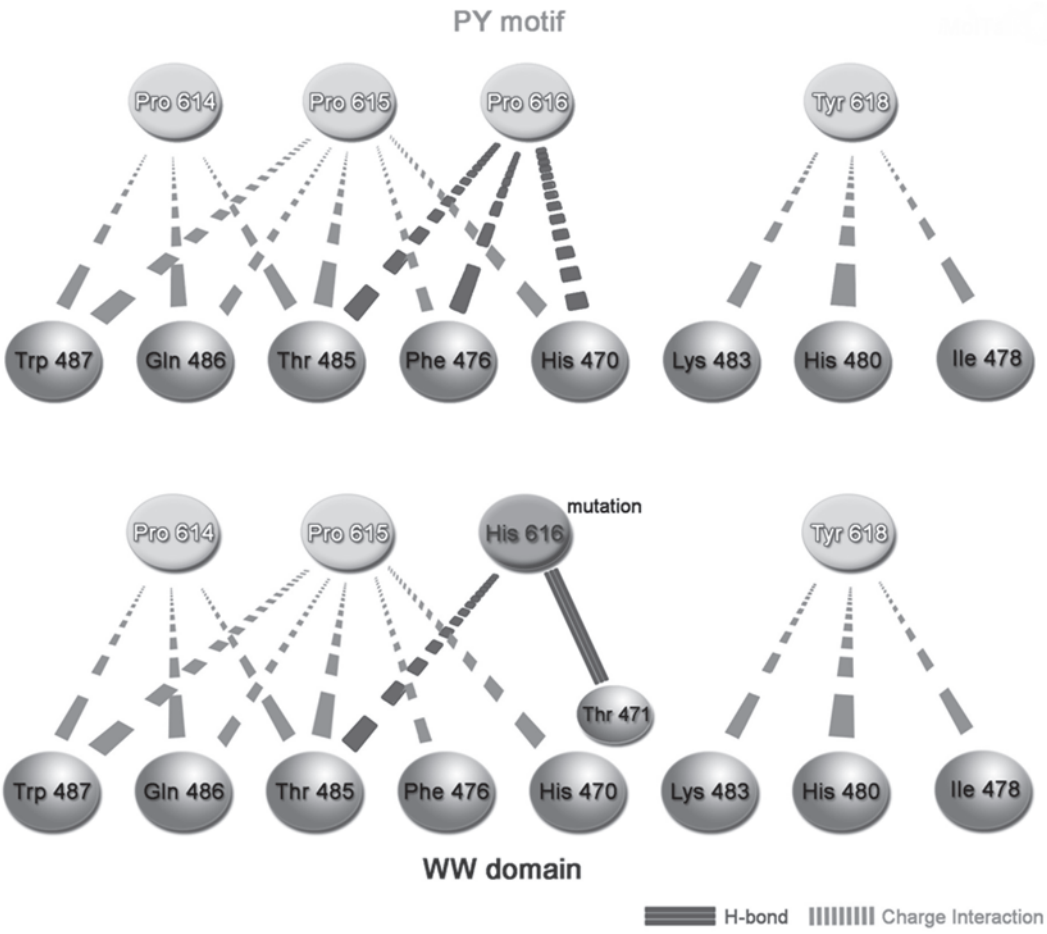

Fig. 3. The interface difference between wild and mutant. The interaction between PY motif and Nedd4-WW domain in wild-type ENaC and Liddle-ENaC (Pro616His) are compared by using *i*MolTalk. Pro 616 (PY motif) interacts with Thr 485, Phe 476, and His 470 (Nedd4-WW domain) in wild-type ENaC. When Pro 616 is substituted with His 616, it can only interact with Thr 485, binding to Thr 471 through H-bond in addition. Pro616His attenuates the interface of PY motif and WW domain.

domain–PY motif interaction could direct the ubiquitin ligase domain to the channel, where it could ubiquitinate one or more subunits to target the complex for degradation *(20)*. Strong evidence had been provided that Nedd4 as a suppressor of ENaC activity could regulate the number of ENaC channels on the plasma membrane and PY motifs were necessary for Nedd4-dependent regulation. The effects that a catalytically inactive Nedd4 stimulated channel activity were

dependent on the PY motifs when using the *Xenopus* oocytes system, for no Nedd4-mediated changes in channel activity were observed in ENaC lacking them *(21)*. Because P616H coaggregated with the phenotype, we wonder how the P616H can cause Liddle's syndrome. *i*MolTalk is an interactive, Internet-based service for computational analysis in structural biology (http://i.moltalk.org/); we used this bioinformation software to find molecular interface between two structural chains *(25,26)*. Because there is no human 3D model for PY motif and Nedd4-WW domain and the sequences between rat and human are conserved, we analyzed the wild-type and mutation interactions between PY motif and Nedd4-WW domain with rat model (PDB:1I5H). We calculate the H-bondings and charge interactions between WW domain in Nedd4 and PY motif in ENaC (Fig. 3). The computational analysis results show that Pro 616 in PY motif interacts with three amino acids, Thr 485, Phe476, and His470 in Nedd4-WW domain in wild-type ENaC. While His 616 changed to interact with only one amino acid Thr485 and a new one Thr 471, which abolished the interface of PY motif and WW domain, the three-dimensional structure would be also changed. This result suggested that a defective PY motif would attenuate the binding and suppression effect of Nedd4 on ENaC, thereby increasing the numbers of sodium channels and resulting in overactivity of the sodium channel. This is the likely mechanism responsible for the excessive sodium reabsorption in the distal nephron and Liddle's syndrome in this family.

In summary, we have described a family with Liddle's syndrome caused by a missense mutation, P616H, in the proline-rich PY motif of the βENaC. The analysis of missense mutation expands our understanding of the mechanisms involved in WW domain–ligand recognition and the molecular basis underlying Liddle's syndrome. These findings provide additional clinical evidence that a conserved PY motif is critically important for the regulation of ENaC activity.

Materials and Methods

Subjects

The proband (Fig. 1; the index case number is subject III-8, representing generation III, subject number 8) was a 23-yr-old Chinese male. He presented with elevated blood pressure at 21 yr for the first time when undergoing a health check-up. Two months later, he became easily fatigued, especially in the lower limbs. A routine examination showed that his serum potassium level was 1.8 mmol/L and blood pressure was 160/100 mmHg. Relief of symptoms followed supplementation with potassium chloride tablets. Because of the continuance of high blood pressure, drug administration was started. However, there was little blood pressure reduction. The patient was treated with a variety of antihypertensive medications with inadequate control of blood pressure, leading to hospital admission at age 22 yr and 2 mo. Blood pressures ranged from 140 to 175 mmHg (systolic) and from 80 to 105 mmHg (diastolic) (1 mmHg = 133 Pa) in the hospital. Physical examination revealed no abnormalities in his heart sounds or respiratory sounds. Serum potassium was 2.28 mmol/L. Other electrolytes, hematocrit, blood urea nitrogen, creatinine, and urinalysis were within normal limits (Table 1). Further evaluation revealed suppressed plasma renin activity (0.01 ng per mL per h, supine; 0.01 ng per mL per h, upright), suppressed plasma aldosterone concentration (45.8 pg/mL, supine) and suppressed urine aldosterone concentration (2.18 μg/24 h) (Table 1). Renal ultrasound was normal. The proband did respond to triamterene (400 mg/d) with normalization of hypertension, hypokalemia, plasma renin activity, and plasma aldosterone levels 1 mo later (Table 2). Based on these findings, he was clinically diagnosed as having Liddle's syndrome.

As shown in Fig. 1, subjects II-3, II-5, II-7, and III-7 also presented with hypertension. The proband's maternal grandfather (I-2) had died from accident at the age of 44. He had no history of hypertension and hypokalemia. His uncle (II-3) was a 67-yr-old man who presented with hypertension 8 yr prior and has a history of hypokalemia since age 59 yr. The proband's mother (II-5) and older sister (III-7) both have a history of hypertension and perioral numbness occurred occasionally in his mother. Although his older sister (III-5) has never developed symptoms of hypokalemia, her serum potassium was only 2.1 mmol/L.

PCR and DNA Sequencing

To identify a mutation in the ENaC gene in this family, venous blood samples for gene analysis were drawn from the proband and his family members. Genomic DNA was extracted from peripheral blood lymphocytes using the classical phenol chloroform protocols. Primers specific for exon 13 of *SCNN1B* and *SCNN1G*, which encode the second transmembrane segment and the COOH termini of β and γ subunits, respectively, were designed and used to generate PCR products. The sequences were as follows: *SCNN1B*-forward: 5'-TGGGCTAGAGGCAAGAATG-3'; *SCNN1B*-reverse: 5'-GTAGGAACCAGGTGAAGATAC AAT-3'. *SCNN1G*-forward: 5'-TAGCCAGGTCTCAGGT CG-3'; *SCNN1G*-reverse: 5'-CCAAGCGCAAGGTATT GT-3'.

Reactions were performed employing 200 ng of genomic DNA in 20 μL volume containing standard PCR buffer 2 μL, 25 m*M* dNTP 0.4 μL, each specific primer 1 μL, 5 u/μL LA-Taq polymerase 0.2 μL (Takara, ostu, Shiga, Japan). The PCR conditions were as followed: an initial denaturing cycle at 94°C for 45 s, followed by 30 cycles of 94°C for 30 s, T°an (T°an for different primers shown in Table 1) for 30 s, and 72°C for 1 min. A final extension step of 72°C for 7 min was used. PCR products were visualized with ethidium bromide by ultraviolet transillumination. The products were purified using QIAGEN PCR purification kits (Qiagen GmbH, Hilden, Germany) and then were subjected to direct

sequencing using ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA).

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