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Functional analysis of a double-point mutation in the *KCNJ2* gene identified in a family with Andersen-Tawil syndrome

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ABSTRACT

Andersen-Tawil syndrome (ATS) is a skeletal muscle channelopathy with autosomal dominant inheritance resulting in periodic paralysis, arrhythmia characterized by QT prolongation, and dysmorphic features. The *KCNJ2* gene has been identified as the causative gene of ATS. Herein, we reported 2 cases of a 21-year-old man and his mother, with episodic paralytic attacks and/or arrhythmia, which are characteristic of ATS. Both G144A, a reported ATS mutation, and V296F, a novel mutation, were identified in the *KCNJ2* gene on the same allele from the proband and his mother, but not from his father. In the present study, we investigated the functional effect of these variants on the potassium channel Kir2.1 and the significance of the double mutation. G144A, V296F, and G144A-V296F mutant channels expressed in cultured cells revealed a loss-of-function effect of these mutations on Kir2.1. The K^+ currents of G144A and G144A-V296F channels were more suppressed than that of V296F channel alone, whereas was no difference between G144A and G144A-V296F. To our knowledge, a double mutation in the *KCNJ2* gene has not been reported previously. While either of 2 mutations potentially causes ATS, the G144A mutation might cause the dominant effect on the patients' clinical presentation.

1. Introduction

Andersen-Tawil syndrome (ATS) is a skeletal muscle channelopathy with autosomal dominant inheritance showing periodic paralysis, arrhythmia characterized by QT prolongation, and dysmorphic features [1,2]. *KCNJ2* and *KCNJ5* genes have been identified as the causative genes of ATS [3,4]. About 60–70% of the patients exhibiting clinical ATS symptoms show genetic abnormalities in the *KCNJ2* gene [5]. The *KCNJ2* gene encodes the inward rectifier potassium channel Kir2.1 which is important to stabilize the resting membrane potential of the sarcolemma [2]. Most causative *KCNJ2* mutations in ATS result in a loss of function indicated by decreased inward rectifying potassium currents and prolonged action potential durations in cardiac myocytes and skeletal muscles [6]. Over 50 different mutations of *KCNJ2* have been reported so far. We identified two missense mutations in the same allele in the *KCNJ2* gene of members of a family exhibiting clinical ATS phenotypes. A double mutation in the *KCNJ2* gene has not been reported before to our knowledge. In the present study, we investigated the functional effect of these variants on the potassium channel Kir2.1 and the significance of the double mutation.

2. Methods

2.1. Case

The proband was a 21-year-old man, born from unrelated parents (Fig. 1A,C). Pregnancy, delivery, and psychomotor development were described as normal. At the age of six, he experienced the first attack of

Abbreviations: ATS, Andersen-Tawil syndrome; CMAP, compound muscle action potential; ECG, Electrocardiography

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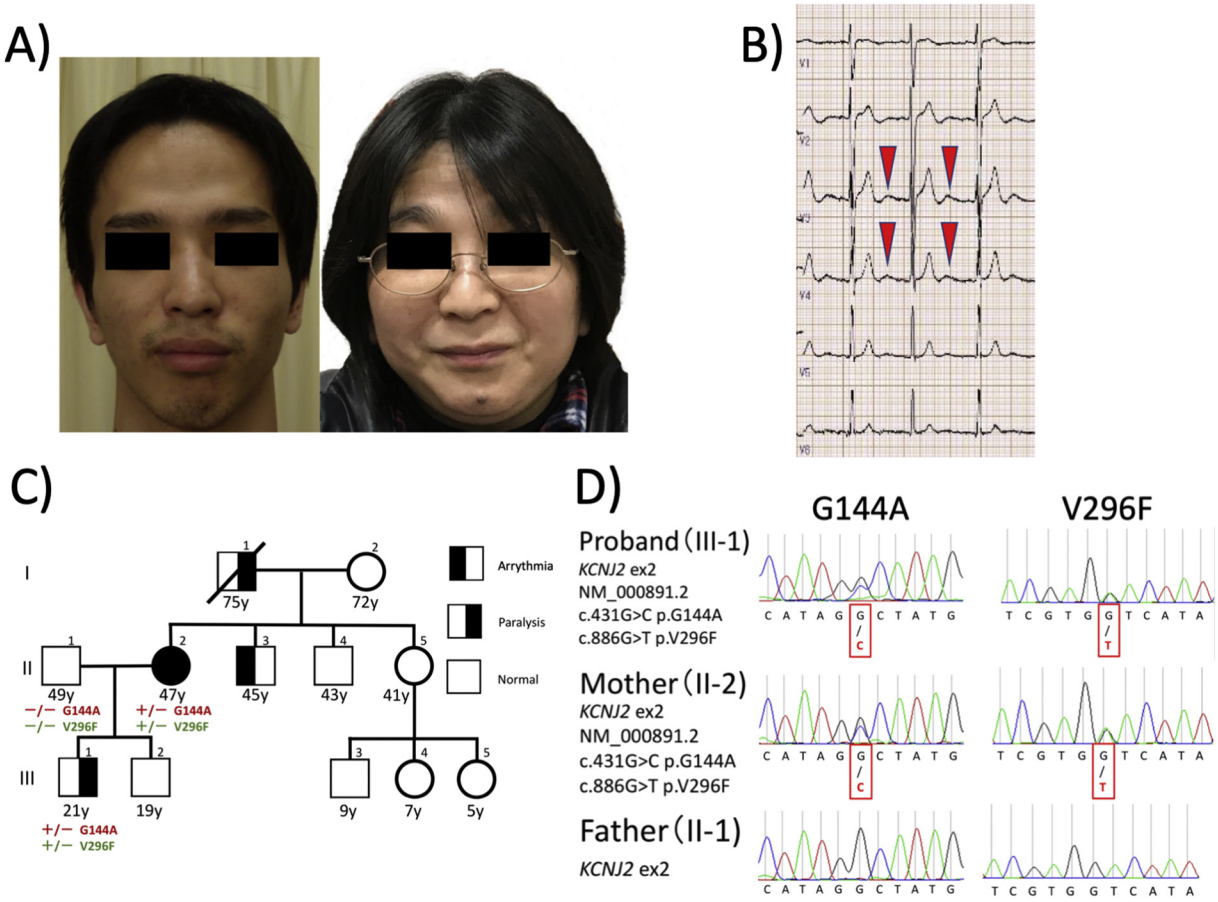

Fig. 1. Clinical features of the family with Andersen-Tawil syndrome (ATS).
A) Facial characteristics of the proband (left) and his mother (right): Broad forehead, hypoplastic mandible, low-set ears.
B) Electro cardiogram (ECG) of the proband: The characteristic U waves (inverted red arrows) are detected.
C) Family tree of the family: The inset in the right upper panel indicates the clinical phenotype of the family members. The proband (III-1) revealed paralysis without arrhythmia though his ECG showed a U-wave. The mother of the proband (II-2) has both paralysis and arrhythmia.
D) Gene analysis for the family with ATS identified two mutations in the same allele, G144A and V296F in the *KCNJ2* gene.

lower limbs weakness. He had presented with recurrent and transitory weakness episodes of upper and lower limbs since then, especially in the winter season. The attacks became more frequent and severe recently. He consulted our institute when he showed severe generalized paralysis.

He reported long-time exercise and consuming extreme amounts of food as a potential trigger. The attacks caused difficulties in walking and climbing steps lasting for approx. One week. During the interictal period, he was asymptomatic.

His clinical manifestation during the episode was tetraplegia (level 2 of the MRC grade in the upper limbs and level 3 in the lower limbs). Grip power was unmeasurable. Deep tendon reflexes were weak but symmetric. No dysarthria and no disturbance of swallowing were observed. Examination revealed low-set ears, broad nose, small mandible, broad forehead, and clinodactyly of the fifth fingers. He also has a peculiarly high-pitched voice. There was no clinical myotonia. He had not experienced palpitations or syncope. Serum potassium levels were reduced to 2.6 mEq/L (normal range 3.5–5 mEq/L). Serum creatine kinase levels during the episode were mildly elevated to 324 IU/L (normal range: 24–190 IU/L). Thyroid function was normal.

On a later day after recovery from the paralysis, a detailed clinical neurophysiological study was performed. Motor conduction velocity, distal latency, and F-waves were normal in the right ulnar nerve. The baseline amplitude of the compound muscle action potential (CMAP) was 6.0 mV, recorded from the *abductor digiti minimi*. With the "prolonged exercise test" [7,8,9], the CMAP amplitude caused a transient increase (8.2 mV) followed by a reduction of CMAP amplitude (4.3 mV) that lasted over 60 min. Needle electromyography showed no myotonic discharge. Sensory nerve action potentials were normal in the radial nerve, and in the sural nerves. Electrocardiography(ECG) showed a U wave (Fig. 1B) and the corrected QT interval was measured as 392 ms, which was normal.

The mother of the proband was a 47-year old woman. At the age of 16, bidirectional ventricular tachydysrhythmia was detected by mass electrocardiographic screening of high-school-aged children and she had been treated with β-blockers. ECG showed a normal QTc interval. She had had no clinical symptoms like palpitation or syncope. At the age of 40, she first noted limb weakness after waking up, which resolved within two hours. Such attacks, usually associated with resting after exercise, resembled the attacks her son was experiencing. She experienced these attacks three times since then. Examination revealed low-set ears, broad nose, clinodactyly of the fifth fingers, and scoliosis. Findings of the neurologic examination were normal. There was no clinical myotonia. The patients provided informed consent for the use of their clinical courses and facial images in publications.

2.2. Gene analysis

After obtaining informed consent from the proband and the mother in written form, we performed gene analysis using the DNA extracted from their lymphocytes. The protocol of the gene analysis was approved by the ethical committee of Osaka University. Sanger sequencing was

performed for hot spots of known pathogenic mutations for periodic paralysis including exons 4, 11, 21, and 30 of *CACNA1S* (NM_000069.3), exons 5, 12, 13, 18, 20, 21, 23, and 24 of *SCN4A* (NM_000334.4), and the entire exon of *KCNJ2* (NM_000891.2). Targeted exons of *CACNA1S*, *SCN4A,* and *KCNJ2* genes were amplified by PCR and the PCR products were sequenced by dideoxynucleotide chain termination with a DNA sequencer (ABI 3100, Applied Biosystems, Foster City, CA, USA).

2.3. Molecular biology

Mouse Kir2.1 (mKir2.1) cDNA cloned into the pBluescript SK(−) (Agilent Technologies) vector was kindly provided by Prof. Yoshihiro Kubo of the National Institute for Physiological Sciences. The amino acid identity between mouse and human Kir2.1 is 98.4%. There are only seven amino acids difference, and the region encompassing the identified mutations between 120th and 387th amino acid is identical. Using a conventional mutagenesis method with PfuUltraII High Fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA), we generated three clones, mKir2.1 with G144A, mKir2.1 with V296F, and mKir2.1 with both G144A and V296F. The successful introduction of mutations was confirmed by Sanger sequencing with Big Dye Terminator Sequencing Kit (Thermo Fisher Scientific) and Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific). The cRNAs of the mKir2.1 clones were synthesized *in vitro* using linearized the cDNA using an mMESSAGEmMACHINE transcription kit (Thermo Fisher Scientific).

2.4. Electrophysiology

Xenopus oocytes were harvested from female *Xenopus laevis* frogs. We injected 50 nL of cRNAs per oocyte in two different conditions, 50 ng/μL or 500 ng/μL. We prepared cells of four different conditions; WT mKir2.1(WT), mKir2.1 with G144A (G144A), mKir2.1 with V296F (V296F) and mKir2.1 with both G144A and V296F (G144A-V296F). After incubation for two days at 18 °C in ND96 solution (5 mM HEPES, 96 mM NaCl, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.5), oocytes expressing mKir2.1 clones were mounted in a two-electrode voltage clamp set-up.

Macroscopic currents were recorded with the two-electrode voltage clamp technique using a bath-clamp amplifier OC-725C (Warner Instruments, Hamden, CT). Stimulation and data acquisition were performed using a Digidata 1440A (Molecular Devices, San Jose, CA) and pClamp 10 software (Molecular Devices). Glass microelectrodes were filled with 3 M KCl whose resistance ranged from 0.5 to 0.7 M Ohm. Recordings were performed at room temperature (25–28 °C) in the external recording solution, ND96 solution. Step pulses ranging from −150 to 100 mV in 10-mV steps were applied from a holding potential of 0 mV for 200 ms (inset shown in Fig. 2B). Data were analyzed using Clampfit 10 software (Molecular Devices).

3. Results

3.1. Gene analysis

To confirm the diagnosis, we performed gene analysis using DNA extracted from the proband's and his parents' lymphocytes. Sanger sequencing revealed that the proband and his mother had two heterozygous mutations in exon 2 of the *KCNJ2* (NM_000891.2), c.431G > C (p.G144A) and c.886G > T (p.V296F), positioned 17: 70175470 and 70,175,925 (GRCh38) respectively (Fig. 1D). Therefore, we concluded these two heterozygous mutations were inherited from his mother to the proband and lay on the same allele. Both mutations were not registered in the database of Tohoku Medical Megabank Organization (ToMMo) which has genotypes of 4.7 thousand Japanese individuals (4.7KJPN) [10]. However, G144A was reported in ClinVar [11] (VCV000067575.1).

3.2. Electrophysiology

cRNA injection of WT mKir2.1 expression into *Xenopus* oocytes (50 nL × 50 ng/μL, “x 1”) revealed robust inward rectifying currents (Fig. 2A and Fig. 2B). By contrast, injecting the same amount of cRNA of the mutants, G144A and V296F, into *Xenopus* oocytes, did not trigger significant inward rectifying currents, indicating that these mutations have a loss-of-function effect. In addition, the double mutation, G144A-V296F, which was found in our patients, did not show significant currents. The observed currents were so tiny that their current properties could not be characterized. Therefore, we prepared oocytes injected with the ten-fold higher amount of cRNA, (“x 10”) per cell, to obtain sufficiently large currents that could be resolved. Compared to the result of “x 1”, WT mKir2.1 showed robust inward rectifying currents and V296F showed small, but distinct currents (Fig. 2C and Fig. 2D). However, G144A and G144A-V296F did not show significant inward rectifying currents even in cells injected with “x 10”. These results suggest that G144A and G144A-V296F have a bigger loss-of-function effect greater than V296F.

4. Discussion

We identified a novel mutation, G144A-V296F, with features characteristic of ATS. Our patients demonstrated periodic paralysis and dysmorphic features, and/or ECG abnormalities consistent with ATS. To our knowledge, this is the first report of ATS harboring two causative mutations in *KCNJ2* gene. In addition, our electrophysiological experiments provided quantitative assessment of each mutation effect, suggesting that G144A, the known causative mutation for ATS, is most likely dominant for the pathogenesis of our cases.

In the clinical aspect, there was some variation of the severity of the symptom between the proband and his mother even though they have the same mutations. According to a previous report, the patient with the G144A mutation in the *KCNJ2* gene showed proximal muscle weakness, hyporeflexia, scoliosis, dysmorphic facial features, and complex ventricular arrhythmia. The mother of the proband also reported a history of ventricular arrhythmia and ECG abnormality consistent with ATS but denied a history of muscle weakness [12].

The prolonged exercise test has been proposed as a useful examination to make a diagnosis of periodic paralysis [8,9]. Our patient showed an interesting feature, the significant CMAP increases after exercise followed by moderate declines in the CMAP peak, indicating that the patients is likely to have periodic paralysis. Songs et al. reported that this pattern was observed in patients with other mutations in the *KCNJ2* gene (T305A and M307I) [13]. More clinical evidence will be needed to pursue whether the significant CMAP increase is the unique findings for specific mutations of ATS or not.

One of the unique features of our cases is that they have two different causative mutation in the causative gene. A few rare cases of skeletal muscle channelopathy, which harbored two pathogenic mutations with additive effects on the clinical phenotype, have been reported [14,15,16]. To clarify how these double mutations in our cases influence the phenotype, we investigated the functional effect of each variant on the potassium channel Kir2.1 using electrophysiological experiments, resulting in that this double mutation revealed the loss of function effect most likely due to G144A predominantly.

G144A mutation has been reported before in a family with ATS [12]. The previous study showed that the channel with the G144A mutation traffics to the plasma membrane normally, but potassium currents were not detected, which is consistent with our result. The glycine at position 144 is a part of the “GYG motif” that plays a crucial role as the selectivity filter of potassium channels, being compatible with the fact that the mutation of this residue would lead to non-functional channels.

To our knowledge, the V296F mutation has never been reported before and is not found in available databases. Our data revealed that

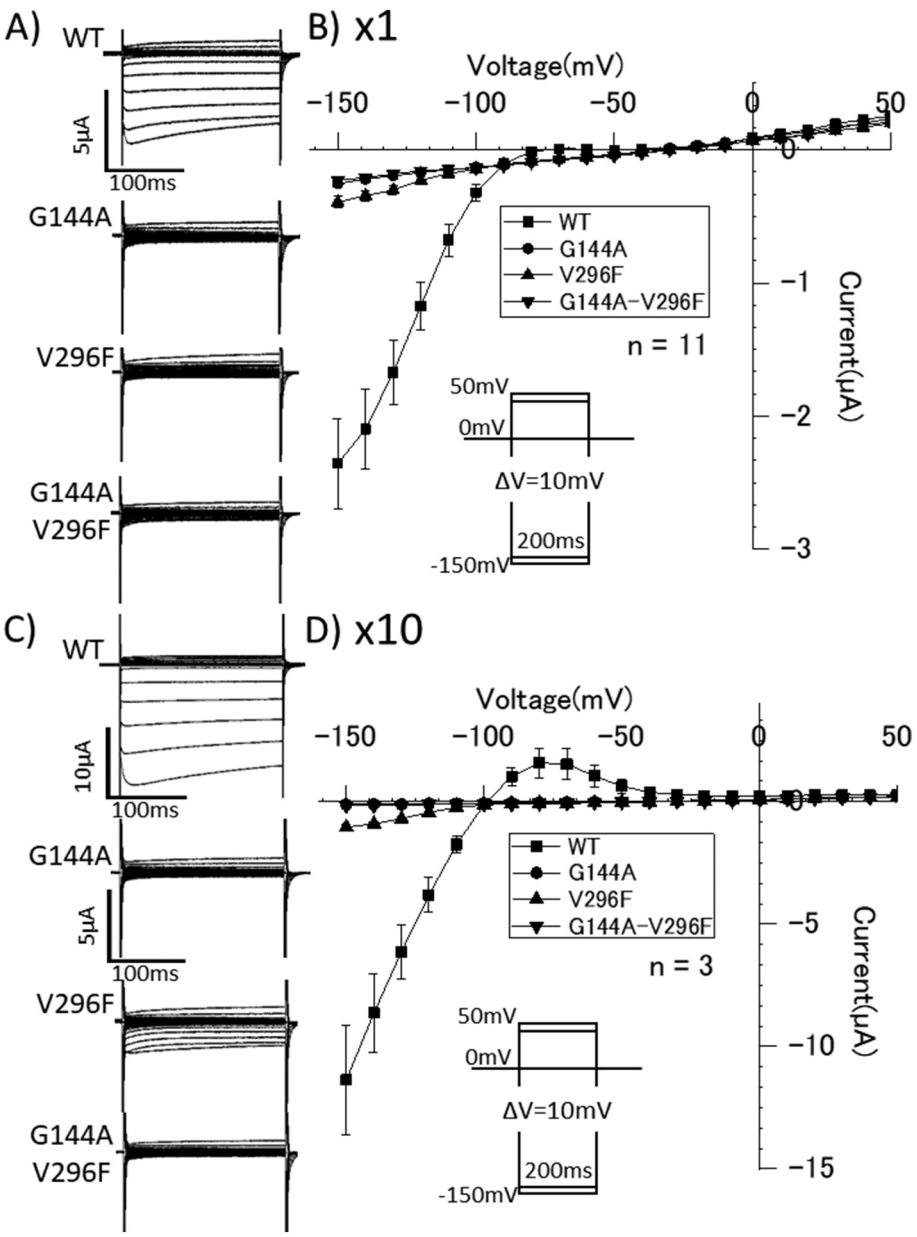

Fig. 2. Functional characterization of mKir2.1 WT, G144A, V296F, and G144A-V296F in *Xenopus* oocytes.
A) Representative current traces recorded from oocytes which were injected with WT mKir2.1 (WT), mKir2.1 with G144A (G144A), mKir2.1 with V296F (V296F) and mKir2.1 with both G144A and V296F (G144A-V296F). The amount of cRNA injected into oocytes was 2.5 ng (50 nL × 50 ng/μL ("x 1")) per cell. Currents were elicited at test potentials ranging from −150 to 50 mV for 200 ms in 10-mV steps from a holding potential of 0 mV. The pulse protocol is shown in inset B. B) Current-voltage relationships of WT (filled squares), G144A (filled circles), V296F (up triangles), and G144A-V296F (down triangles) are shown. Plots represent the current value at 190 ms after the step-wise test pulse. Data are presented as mean ± SEM (n = 11). "x1" indicates that the amount of cRNA injected into oocytes was 2.5 ng. C, D) Representative current traces from oocytes injected with 25 ng (50 nL × 500 ng/μL ("x 10")) of cRNA (C) and current-voltage relationships (D) (n = 3). "x10" indicates that the amount of cRNA injected into oocytes was 25 ng.

V296F causes a loss of function effect in mKir2.1 and presumably associated with ATS. The mutation locates in the intracellular domain, not in the pore domain. Although the structure-function relationship of the intracellular domain has not been fully elucidated, the role of a nearby residue, E299, has previously been investigated [17]. In that study, mKir2.1 with E299S did not show a significant decrease in current amplitude but differed from wild type in terms of the rectification and permeation properties. More experimental evidence will be needed to clarify the molecular function of the intracellular domain including V296F.

The double mutation, G144A-V296F identified in our patients locate in the same allele and lead to a loss of function. Its current was suppressed more than that of V296F alone in our electrophysiological study, whereas there seems to be no difference between G144A and G144A-V296F, indicating that G144A might be the dominant cause of the patients' clinical manifestation. In addition, the current measurements from co-injected oocytes of WT and each mutant revealed that V296F did not exhibit a significant dominant-negative effect, while G144A had a robust dominant-negative effect (data not shown). This result also supports that G144A is the dominant pathogenic factor in our cases although V296F also has a loss-of-function effect.

In conclusion, we have identified ATS patients with a double mutation in the *KCNJ2* gene on the same allele, G144A (reported mutation) and V296F (a novel mutation). Our functional analysis showed that both mutations induced a loss-of-function effect on Kir2.1, but G144A has a more potent effect than V296F, indicating that the pathogenesis of our case is most likely due to G144A. Accumulating the functional analysis data of mutant Kir2.1 channel associated with ATS is essential for the progress of clinical science of ATS including the diagnosis and treatment.

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Declaration of Competing Interest

The author has no declaration of competing interest.

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