Original Research

Effects of SCN5A mutation on intracellular calcium concentration

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Abstract: Objective: To construct the delQKP1507-1509 mutant of SCN5A channel, and observe the effects of this mutation on intracellular calcium concentration. Methods: SCN5A delQKP1507-1509 mutation was engineered using site-directed mutagenesis. Wild-type (WT), mutant (MT) and mixed (the mixture of wild-type and mutant plasmids at a ratio of 1:1) Nav1.5 plasmids were transfected into human embryonic kidney 293 (HEK293) cells, respectively. Intracellular free calcium concentration was determined by laser confocal scanning microscope (LCSM) after the cells were loaded with the Ca2+ indicator dye Fluo-3/AM. Results: Gel electrophoresis and DNA sequencing proved a deletion of nine base pairs in the SCN5A gene at position 1507-1509. Protein expression and localization of wild-type and mutant SCN5A gene in HEK293 cells were detected under the LSCM. Fluorescence intensity in wild-type group, mutant group and co-expression group (1:1 expression of WT and MT sodium channel) mimicking the heterozygous state were 79.3683±3.051, 94.6165±8.383, 90.7463±6.421 respectively, and there was no significant difference among these three groups (P>0.05). Conclusions: SCN5A delQKP1507-1509 mutant is successfully constructed and transfected into HEK293 cells. The mutation does not affect calcium concentration and further study is required to describe the possible pathophysiological mechanisms of dilated cardiomyopathy phenotype in sodium channel overlap syndromes caused by this SCN5A delQKP1507-1509 mutation.

Keywords: SCN5A Mutation; Calcium Concentration; Dilated Cardiomyopathy; Cardiac sodium channel overlap syndromes.

1. INTRODUCTION

Dilated cardiomyopathy (DCM) is a common myocardial diseaseand the leading cause of heart transplantation, characterized by dilated left ventricle/both ventricles and decreased systolic function. The main clinical manifestations of DCM are heart failure, malignant arrhythmias and sudden death associated with high morbidity and mortality [1]. About 20-35% of idiopathic DCM patients have a family history of the condition, which suggests that genetic factor plays a crucial role in the pathogenesis [2]. More than 50 genes have been linked to DCM, most of which were identified in the contractile apparatus and cytoskeletal protein-encoding genes [3]. Recent studies have shown that genetic variations in SCN5A encoding α-subunit of the cardiac voltage-gated sodium channel ($Na_v1.5$) is also associated with DCM and may induce cardiac dilatation through injuring myocardium directly [4]. However the potential mechanism is still unclear. It is well known that the concentration and distribution of intracellular Ca^{2+} is closely related to cardiac excitation-contraction coupling, the electrical stability and Ca^{2+}-dependent signaling. Disequilibrium of calcium homeostasis can result in cardiac structural and functional abnormalities [5]. Accordingly, the altered sodium activities induced by SCN5A mutations are considered as a potential mechanism of DCM. We identified a SCN5A mutation

delQKP1507-1509 in a-three generation Chinese family associated with mixed clinical phenotype: DCM, long QT syndrome type 3 (LQT3) and cardiac conduction disorder (CCD) [6]. The aim of the present study was to explore whether this SCN5A mutation favor the development of DCM through disturbances of calcium homeostasis. A better understanding of the cellular and molecular mechanisms helps to prevent or delay the progression of DCM and provide new and effective strategies for therapies.

2. MATERIALS AND METHODS

2.1. Site-directed Mutagenesis

The SCN5A-delQKP1507-1509 mutation was engineered into WT cDNA cloned in pEGFP-N2 by QuickChange XL site-directed mutagenesis kit using a PCR technique, according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The nucleotide sequences of the mutagenic sense and antisense primers used were as follows:

sense primer5'-GGCTCCAAGAAGCCCATCCCACGGCCCC-3';

antisenseprimer5'CAGGGGCCGTGGGATGGGCTTCTTGGAGC C-3'

The presence of the mutation was confirmed by 0.8% agarosegel electrophoresisand sequence analysis.

2.2. Transfections and Grouping

HEK293 cells were plated into 35mm-glass bottom culture dishes and grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37℃ in a 5% CO_2 humidified atmosphere. Wild-type SCN5A (pEGFP-SCN5A-WT), mutant SCN5A-delQKP1507-1509 (pEGFP-SCN5A-MT) and mixed plasmids (the mixture of WT and MT plasmids in a 1:1 molar ratio) were transiently transfected into HEK293 cells with X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Germany) according to manufacturer's instructions respectively. Each group had three parallel wells. At 48 hour post-transfection the expression of green fluorescent protein (GFP) was detected and the baseline fluorescence intensity (FI) measurement at an excitation wavelength of 488 nm and an emission wavelength of 526 nm was performed by Laser Scanning Confocal Microscope (LSCM, OLYMPUS-FV1000, Japan).

2.3. Cell Loading with Ca^{2+} Indicator

After washing gently three times with PBS, cells were incubated with the Ca^{2+} indicator dye Fluo-3/AM (Biotium, USA). Loading was proceeded for 30 minutes in darkness at room temperature. Then cells were washed three times to remove residual dye adequately.

2.4. Measurement of Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$)

After cells were loaded with Ca^{2+} indicator, the fluorescence intensities were recorded again. Changes in intracellular Ca^{2+} concentration were measured using quantitative data characterizing the increase in Fluo-3/AM fluorescence above the basal level of fluorescence. The experiments were repeated three times independently. During each experiment, each group had three parallel wells. The results from each well consisted of the average data collected from 8 randomly selected sights.

2.5. Statistical Analysis

The statistical analysis was performed with SPSS for windows (version 20.0). Data were presented as mean±SD. All Statistics were analyzed by one-way ANOVA. $P < 0.05$ was considered as statistically significant differences.

3.RESULTS

3.1. Construction and Identification of Eukaryotic Expressing Vector pEGFP-SCN5A-delQKP1507-1509

Agarose gel electrophoresis indicated that the bands of WT (pEGFP-SCN5A) and mutant recombinant plasmid (pEGFP-SCN5A-delQKP1507-1509) had the same size **(Figure 1)**. DNA direct sequencing showed a deletion of nine base pairs (CAGAAGCCC) in the SCN5A gene at position 1507-1509. The results confirmed that the recombinant eukaryotic expression plasmid had been constructed correctly and the remaining sequence did not change **(Figure 2)**.

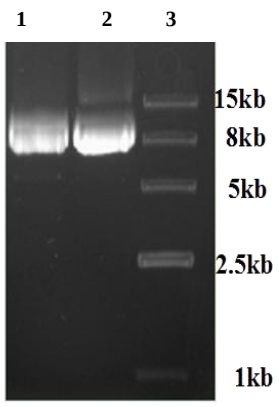

Figure 1. Agarose gel electrophoresis of recombinant plasmids.
1: pEGFP-SCN5A plasmid (WT);
2: pEGFP-SCN5A-delQKP1507-1509 plasmid (MT);
3: Marker.

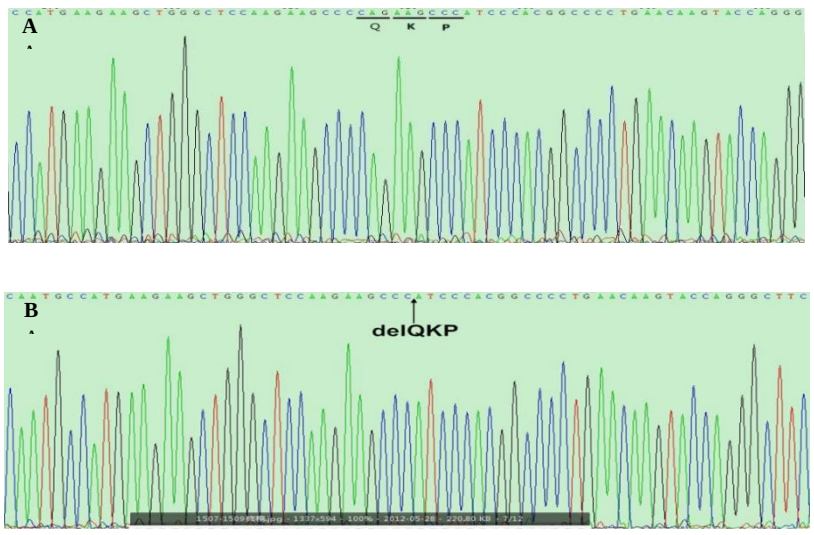

Figure 2. Sequence analysis of recombinant plasmids.
A: DNA sequence of pEGFP-SCN5A plasmid (WT);

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B: DNA sequence of pEGFP-SCN5A-delQKP1507-1509 plasmid (MT).

3.2. Transfection and Localization of wild-type and mutant $Na_v1.5$ channel in HEK293 cells

After transfection with the recombinant plasmids, the green fluorescences were observed at the cell membrane under the LSCM. The results showed that the wild-type and mutant SCN5A gene were transfected into cells successfully and mainly localized at the plasma membrane, which laid a foundation for the following research **(Figure 3).**

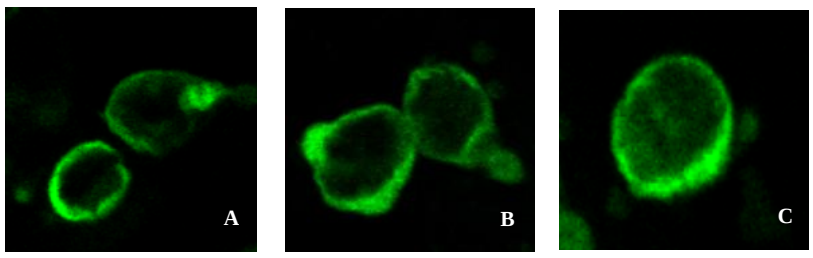

Figure 3. Confocal micrographs of HEK293 cells transfected with the recombinant plasmids.

A: HEK293 cells transfected with WT plasmid;

B: HEK293 cells transfected with MT plasmid;

C: HEK293 cells transfected with mixed plasmid.

3.3. Measurement of Intracellular $[Ca^{2+}]_i$

Fluo-3 fluorescence was excited at wavelengths 488 nm by LSCM and was captured at wavelengths 526 nm. After cells were loaded with Ca^{2+} indicator Fluo-3/AM, the mean fluorescence intensity were significantly enhanced **(Figure 4)**. Fluorescence intensity in wild-type group, mutant group and co-expression group were 79.3683 ± 3.051, 94.6165 ± 8.383, 90.7463 ± 6.421 respectively **(Table 1)**, and there was no significant difference among these three groups **(Figure 5)**.

Table 1. Effect of SCN5A mutation on fluorescence intensity of $[Ca^{2+}]_i$.

	wild-type group	mutant group	mixed group
$[Ca^{2+}]_i$	79.3683±3.051	94.6165±8.383	90.7463±6.421

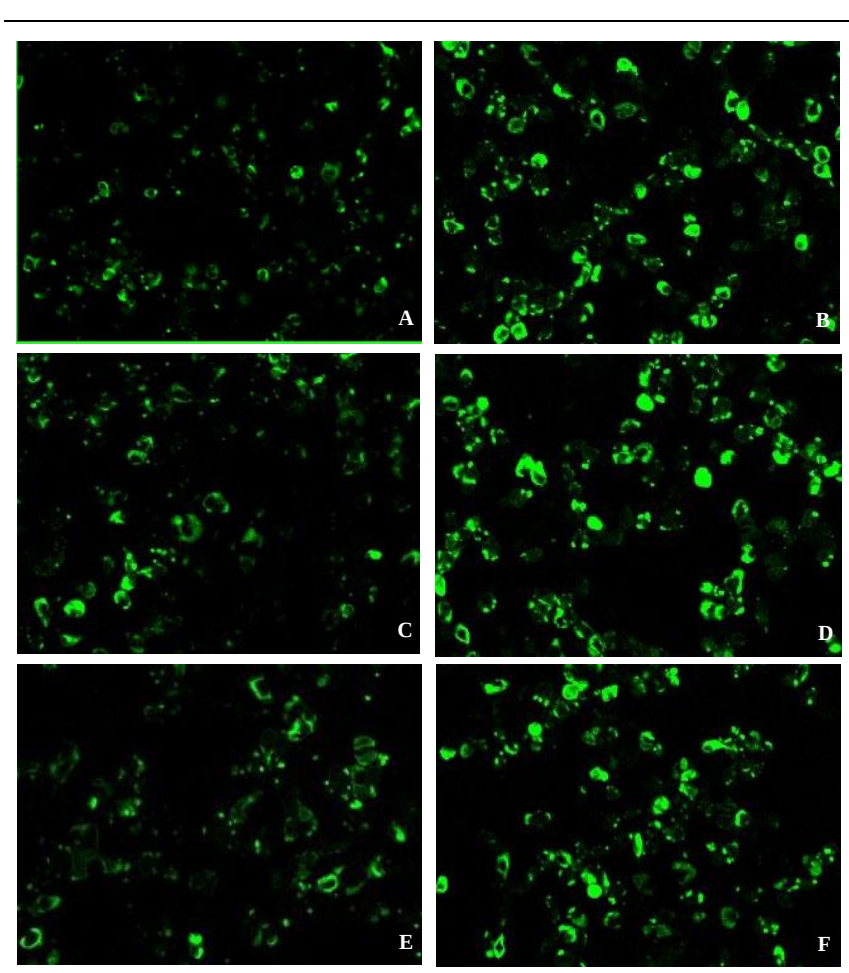

Figure 4. Changes of fluorescence images of $[Ca^{2+}]_i$ in three groups.

A, C, E: The baseline fluorescence of WT, MT and co-expression group, respectively.

B, D, F: The fluorescence of the fluo-3-loaded cells in WT, MT and co-expression group, respectively.

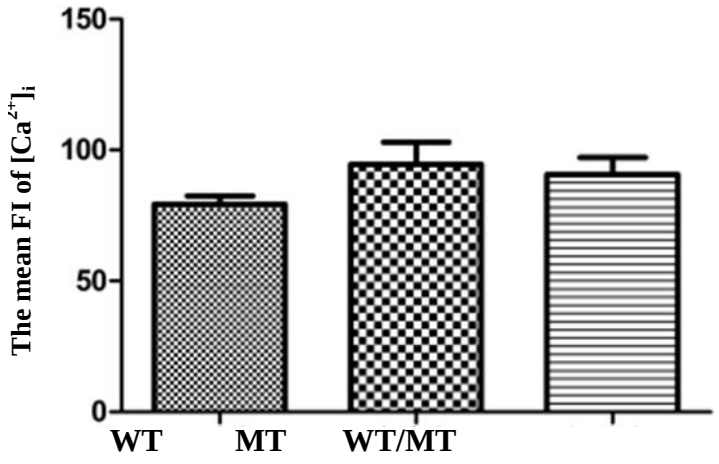

Figure 5. The mean fluorescence intensity of $[Ca^{2+}]_i$ in three groups.

4.DISCUSSION

The SCN5A gene encoding α-subunit of the cardiac voltage-gated sodium channel ($Na_v1.5$) locates on chromosome 3p21 and consists of 28 exons [7]. Mutations in SCN5A gene are associated with inherited arrhythmia syndromes including congenital long QT syndrome type 3 (LQT3), Brugada syndrome (BrS), progressive cardiac conduction disease (PCCD), familial atrial fibrillation (FAF) and sudden infant death syndrome (SIDS) [8-11]. Because of the diversity of clinical presentation and biophysical defects, these hereditary arrhythmias were considered as independent disease entities originally. However accumulating evidence indicates that a single SCN5A mutation can lead to clinical and biophysical overlap of multiple rhythm disturbances, known as an overlap syndrome of cardiac sodium channelopathy [12-14].

Recent studies show that SCN5A mutations are also associated with the development of DCM. In 2004, McNair et al demonstrated that SCN5A-D1275N mutation co-segregated with DCM phenotype in a large kindred with overlap of cardiac conduction disorder, sinus node dysfunction and varied arrhythmia, first proposing that disruption of sodium channel function contribute to the DCM [12-14]. Subsequently, Olson and colleagues discovered the other 4 DCM-related SCN5A mutations (T220I, D1595H, R814W, 2550-2551insTG) [4]. To date, nearly 20 mutations in SCN5A linked to DCM have been identified, including missense, frame shift and deletion mutation. Hesse and colleagues found that MHC-Snail transgenic mice show a progressive DCM through suppression of SCN5A expression in 2007. And this study is the first time to confirm a functional connection between SCN5A function and DCM [4].

It's still a matter of public debate over the years about whether DCM is a direct consequence of SCN5A mutation, or merely a result secondary to long-lasting arrhythmias. Chamber enlargement

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usually occurs simultaneously or is preceded by a wide range of cardiac arrhythmias and conduction disorders [17]. Moreover, reduction of the incidence rate of arrhythmia through antiarrhythmic agents or ablation can ameliorate the cardiac function for some patients [18, 19]. Accordingly, previously DCM is considered as a result of pre-existing electrical arrhythmias.

However, a tremendous amount of knowledge indicates that the alteration of sodium current (I_{Na}) activity can directly impair cardiac structure and function independently of effects of arrhythmia, suggesting that DCM may be the directed result of SCN5A genetic variants [20-22]. A study on transgenic mice without any arrhythmias displayed that the level of the reduction in Na^+ current was closely related to the severity of DCM [16]. Cardiac biopsy or autopsy in SCN5A-positive DCM patients revealed myocardial structural defects, including ventricular hypertrophy, fibrosis and necrosis [4]. The 1-year-old patient of DCM phenotype and conduction disease with W156X/R225W mutations only present tachycardia for a short period before death, implying that ventricular dilation was secondary to sodium channel abnormality rather than arrhythmia [23]. The electrophysiological research and exercise testing in SCN5A-A1180V mutation carriers associated with dilated cardiomyopathy in a Chinese family provided strong support for the hypothesis that structural heart disease can be induced directly by altered sodium currents [24].

The functional properties of mutant sodium channel associated with DCM are highly diverse. Gain of function mutations in the SCN5A gene displayed increased late sodium current or larger sodium window current [25, 26]. Conversely, loss of function mutations of $Na_v1.5$ lead to a decreased I_{Na} peak, or rate-dependent reductions of Na^+ current [25, 26]. Rare mutant Na channels exhibit both loss- and gain-of-function properties, which is relatively common manifestation in sodium channel overlap syndrome by affecting different properties of the sodium channel [24].

However, the concrete mechanism concerning DCM caused by altered $Na_v1.5$ properties is still not elucidated. It is well known that intracellular Na^+ concentration ($[Na^+]_i$) is an essential mediator of calcium handling and myocardial metabolism [28]. Calcium plays a crucial role in triggering excitation-contraction (EC) coupling, maintaining electrical stability and regulating multiple cellular functions as a second messenger. Abnormal handling of intracellular Ca^{2+} can cause the mechanical and electrical dysfunction in various cardiac diseases. Wagner et al. found that $[Na^+]_i$ was significantly increased in cardiomyocytes from CaMKIIδ_C-overexpressing mice developing DCM and supposed that the increased $[Na^+]_i$ lead to DCM through disrupting calcium homeostasis [29]. Several studies have shown that DCM-causing SCN5A mutations may cause cardiac structural and functional damages by disequilibrium of calcium homeostasis [24-26]. It is speculated that the enhanced late sodium current or window current in gain-of-function SCN5A mutation associated with DCM can drive up intracellular calcium concentrations mediated mainly via the Na^+/Ca^{2+} exchanger (NCX). The imbalance of Na^+ and Ca^{2+} homeostasis may promote DCM through diminishing the sarcomere's sensitivity to calcium, increasing myocardial apoptosis and reducing cardiac contraction function.

The mutant $Na_v1.5$ delQKP1507-1509 we reported previously led to a gain of function of the late sodium current [30]. The purpose of this study is to explore the effects of this gain-of-function mutation on intracellular calcium concentration. In order to be convenient for investigating gene transfection efficiency and of Nav1.5, enhanced green fluorescent protein (EGFP) was used as a report gene in our study. After transfected in HEK293 cells, the expression of GFP was detected in the cell membrane and fluorescence intensity of the three groups were roughly the same under the LSCM. The experimental results indicated that SCN5A gene was successfully transfected into HEK293 cells in high transfection efficiency, and both expression and trafficking of wild-type and mutant sodium channel were normal. The calcium concentration in MT group and WT/MT group were higher than wild-type group, suggesting that the quantity of intracellular calcium ion had a growing trend. However, calcium concentrations were not significantly different among groups, demonstrating that the mutant gene did not result in calcium-overload. Due to the modulation of calcium is complicated, it is necessary to have a further investigation on the expression of the key Ca^{2+} handling proteins, calcium current and action potential. In addition, it was noteworthy that SCN5A-delQKP mutation exhibited an overlapping phenotype in our report but only resulted in LQT3 in another family [30]. It is a relatively common phenomenon that the identical SCN5A mutation in different families or among members of the same family can lead to different clinical phenotypes, such as A1180V in SCN5A [24, 31]. The highly clinically heterogeneous suggest that the link between Nav1.5 mutations and DCM is multifactorial, including the complex genetic background, environmental factors, gender and so on. Nowadays induced pluripotent stem cells (iPSCs) technology provides patient-specific disease models for investigating pathogenic mechanism, drug screening and individual treatment [32]. Thus, human induced pluripotent stem cells (hiPSCs) from SCN5A-delQKP patients will be used to recapitulate the phenotypic characteristics, study changes of calcium homeostasis and channel biophysical properties, which help to deeply understand calcium handling, genotype-phenotype correlations of DCM. Currently, drug treatments directed targeting Na^+ channels demonstrated more effective than standard symptomatic supportive therapies for some SCN5A-associated DCM patients. The effects of selective inhibitors of late I_{Na} on sodium channel and calcium homeostasis will be

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further studied using SCN5A-delQKP hiPSCs, which may provide a direction for personalized therapy.

In brief, although it becomes increasingly clear about the relationship between sodium channel function and DCM, the precise mechanism is still unknown yet. We need more knowledge of the available clinical, molecular and biophysical information, which contribute to further improvement of diagnosis, risk stratification and treatment in patients with sodium channelopathies.

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