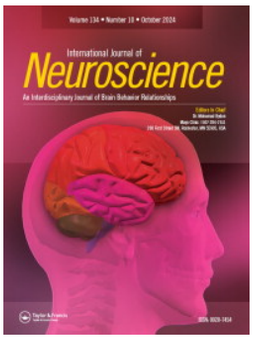

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RESEARCH ARTICLE

Functional characterization of *KCNMA1* mutation associated with dyskinesia, seizure, developmental delay, and cerebellar atrophy

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ABSTRACT

KCNMA1 located on chromosome 10q22.3, encodes the pore-forming α subunit of the 'Big K+' (BK) large conductance calcium and voltage-activated K+channel. Numerous evidence suggests the functional BK channel alterations produced by different *KCNMA1* alleles may associate with different symptoms, such as paroxysmal non kinesigenic dyskinesia with gain of function and ataxia with loss of function. Functional classifications revealed two major patterns, gain of function and loss of function effects on channel properties in different cell lines. In the literature, two mutations have been shown to confer gain of function properties to BK channels: D434G and N995S. In this study, we report the functional characterization of a variant which was previously reported the whole exome sequencing revealed bi-allelic nonsense variation of the cytoplasmic domain of calcium-activated potassium channel subunit alpha-1 protein. To detect functional consequences of the variation, we parallely conducted two independent approaches. One is immunostaining using and the other one is electrophysiological recording using patch-clamp on wild-type and R458X mutant cells to detect the differences between wild-type and the mutant cells. We detected the gain of function effect for the mutation (NM_001161352.1 (ENST00000286628.8):c.1372C>T;Arg458*) using two parallel approaches. According to the result we found, the reported mutation causes the loss of function in the cell. It should be noted that in future studies, it can be thought that the functions of genes associated with channelopathies may have a dual effect such as loss and gain.

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Introduction

The potassium calcium-activated channel subfamily m alpha 1 (*KCNMA1*) gene encodes the alpha subunit of the large conductance, voltage, and calcium-sensitive potassium channel (BK channels) that plays a critical role in neuronal excitability [1]. The *KCNMA1*, located on chromosome 10q22.3, encodes the α-subunit of the BK channel [2]. This α-subunit contains seven transmembrane domains, named as S0-S6 at N-terminus and a large intracellular C-terminus [3]. In the literature, several studies were reported on every segment for N-terminus [4–6] and also for C-terminus [7–9]. The comprehensive structure of BK channels was determined by Yuan et al. [10]. Mutations in the *KCNMA1* gene cause neurological phenotypes such as susceptibility to generalized epilepsy (MIM: 618596), cerebellar atrophy, developmental delay, seizures (MIM: 617643), Liang-Wang syndrome (MIM: 618729), and paroxysmal nonkinesigenic dyskinesia, 3 (MIM: 609446). Among these diseases overlapping phenotypes have been reported and inheritance pattern differences vary, from autosomal dominant to autosomal recessive.

Herein, we present the functional characterization of R458X variation in *KCNMA1* (NM_001161352.1:c.1372C>T,p.Arg458*) that our team had previously identified a patient who presented with a mixed phenotype with nonkinesigenic dyskinesia, cerebellar atrophy, and epilepsy showing both gain and loss of

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function [1]. The variation examined constitutes the entire clinical presentation since it has a pleiotropic effect. To demonstrate the functional effect of the variant a two step-approach was conducted; on the one hand, immunostaining was carried out to show the subcellular effect of the protein level, and on the other hand, patch-clamp technique was accomplished to determine how the membrane conductance differs between mutant vs. wild-type protein. In this way. NM_001161352.1:c.1372C>T,p.Arg458* variation was considered as a causative mutation for the patient's phenotype. To our knowledge, this is the first truncating mutation in *KCNMA1* gene that has a gain of function potential. We have demonstrated the importance of the BK channels and the relationship between changes in their functions, both for gain or loss of function, and the disease.

Materials and methods

Genetic analysis and functional characterization

In this study, we report the functional characterization of a variant which was previously reported by Yesil et al. [1]. The detected variant was evaluated using a proper in-silico prediction tool to confirm previous findings and control update variations. Population cohort databases were also searched in terms of the variant. To detect functional consequences of the variation immunostaining and electrophysiological studies were conducted, respectively. For this purpose, the eGFP-tagged wild type and mutant type *KCNMA1*-expressing plasmids were obtained from Gene Universal (Delaware, USA). Then the transfection process started.

Transfection process

MCF7 (Michigan Cancer Foundation 7) cells (ATCC® HTB-22™) were used for functional assessment. The cells were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Whole cultivation supplies were purchased from PAN-Biotech (Aidenbach, Germany). Cells were cultivated in a humidified atmosphere with 5% CO_2 at 37 °C. In a typical 96-well plate, the transfection efficiency was optimized. In this experiment; 5×10^3 cells/well MCF7 cells were transfected in Opti-MEM by the addition of Lipofectamine 2000 (ThermoFisher Scientific, MA, USA) containing 200 and 400 ng of each eGFP-tagged-pDNA encoding both wild and mutant type separately from *KCNMA1*. The lipofectamine and pDNA ratio was 1:1. After 24 h of cultivation, the medium was removed and the wells were washed with 1X PBS then the efficiency was compared macroscopically between different pDNA groups. Determining concentrations of pDNA were further used for confocal microscopy and patch-clamp experiments, respectively. All experiments were performed in triplicates.

Laser scanning confocal microscopy

The two-well chamber slide was used for confocal microscopy. 10 x 10^5 cells were seeded in each well. Transfection was performed with Lipofectamine 2000 including 400 ng pDNA/well for wild and mutant type eGFP-tagged-KCNMA1. After transfection for 24 h, MCF7 cells were incubated with 0.01 mg/mL DAPI at room temperature for 15 min. After this, the fluorescence signal of cells was assessed by laser scanning confocal microscopy (Leica TCS-SPE, Leica Microsystems, Wetzlar, Germany) at Aziz Sancar Institute of Experimental Medicine of Istanbul University. Three images were analyzed for each wild type and mutant group by ImageJ (National Institutes of Health, Bethesda, MD) [11].

Patch-clamp

Membrane currents were recorded using the whole-cell configuration of the patch-clamp method. For the electrophysiological recordings of the selective potassium channel, 140 mM NaCl extracellular bath solution was prepared as 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 15 mM glucose (pH: 7.3 with NaOH), while the intracellular pipette solution was 130 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA (pH: 7.2 with KOH). Also, 3-5 MΩ pipette resistance was used. The recordings were taken using the voltage-clamp method with a whole cell configuration. Holding potential was fixed at −60 mV and voltage from −100 mV to +50 mV (step by step 10 mV) was applied for 500 ms and current responses were measured (Axon Digidata 1550, Axon Multiclamp 700B). Recordings were made at room temperature.

Results

As previously reported, the whole exome sequencing revealed bi-allelic nonsense variation (NM_001161352.1 (ENST00000286628.8):c.1372C>T; Arg458*) of the cytoplasmic domain of calcium-activated potassium channel subunit alpha-1 protein by Yesil et al. [1].

This variation has not been reported in population and mutation databases and is predicted to have a deleterious effect according to prediction databases

such as mutation taster, DANN, and FATHMM-MKL. According to ACMG classifications, this variant is classified as PVS1 pathogenic since it is a null variant where the gene *KCNMA1* has a high LOF Z score (6.08) and seemed to be located in a highly conserved locus throughout different species with a PhloP score of (phylogenetic p-values) 2.672 [12].

Immunostaining was carried out to compare the expression level between wild-type and mutant cells. For this aim, 400 ng pDNA was transfected and the average transfection efficiency was found as 70% for the wild type and 62% for the mutant type (Figure 1). MCF7 cell growth rate was reduced after mutant type KCNMA1 transfection however this outcome was not evaluated specifically as this was not a primary research question for this study. We have detected the decreased proliferation rate when we compared the mutant type with the wild-type (Figure 1). Later, patch-clamp recordings were taken in triplicate from MCF-7 cells for both wild-type and mutant/variant cells in standard conditions as mentioned above and representative current densities are shown in Figure 2. Outward currents of X channel mutant cells were found significantly higher than wild type groups ($p = 0.0005$).

Discussion

The presence of different currents in various tissues affects the complex role of BK channels in the regulation of excitability [13]. Therefore, defining the mutation's functional characterization seems mandatory.

Numerous phenotypes were associated with *KCNMA1* mutations. Particularly epilepsy, movement disorders, developmental delay, and cerebellar atrophy have been reported [14–17]. In the context of genetic heterogeneity, some of these variations show different BK current properties. BK channels play an important role in neuronal excitability *via* balancing potassium and calcium potential from depolarization to hyperpolarization. However, the exact mechanisms by which KCNMA1 variations effects BK channel activity remain unclear [13]. The understanding mechanistic explanation of the BK channel features and activities are not only crucial for neurological systems, but also muscular and osteoblastic activity [18–20].

As previously accounted, BK channel activity decreases the excitation feature of synaptic activity at neuromuscular junctions [21]. Interestingly BK channels have a dual effect on neurons both for excitation and inhibition. Therefore, finding BK current activity shows different expression patterns for suppression to induction. For instance, Du et al. showed a missense variation, Asp434Gly in the regulator of conductance for K+ (RCK) domain in *KCNMA1*. In their study, Du et al. evaluated a large family with 13 individuals. Among them, one patient had only epilepsy, seven patients had paroxysmal dyskinesia and five patients had epilepsy with paroxysmal dyskinesia. Detected variations exhibited autosomal dominant inheritance patterns [14]. Latterly, the same mutation was detected and analyzed with different materials by other study groups [22–24]. They demonstrate that the variation

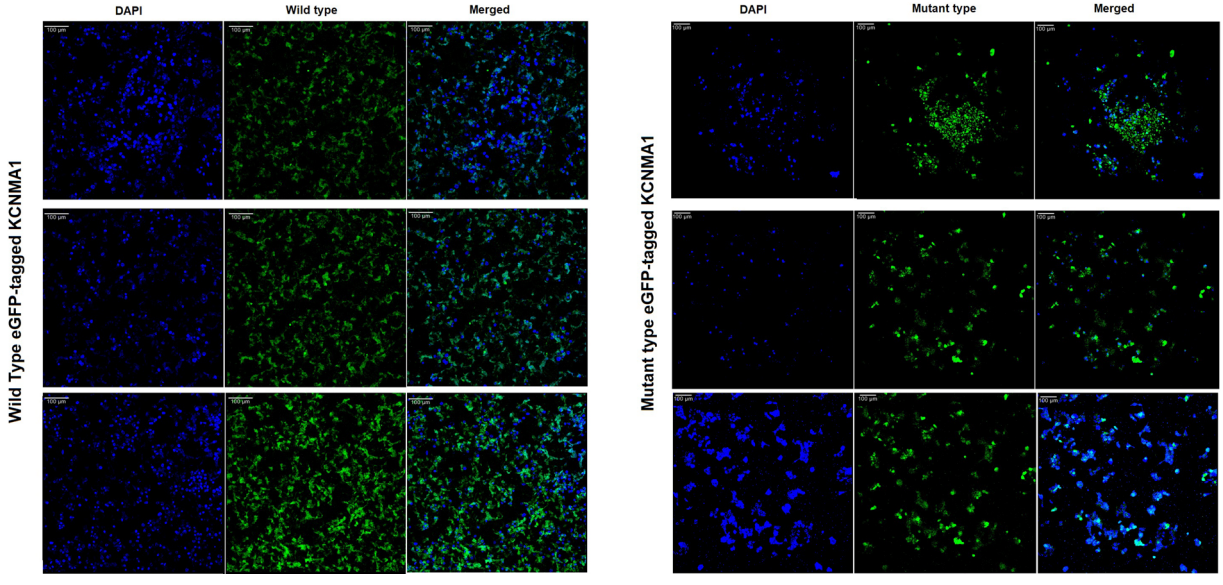

Figure 1. Transfection efficiency results in MCF-7 cells for both wild and mutant type eGFP-tagged-KCNMA1. The nuclei were stained with DAPI in blue and transfected cells are in green. Reduced proliferation were observed cells expressing mutant type KCNMA1 when compared with wild type. Results were demonstrated in triplicates. Images were taken using confocal laser scanning microscopy.

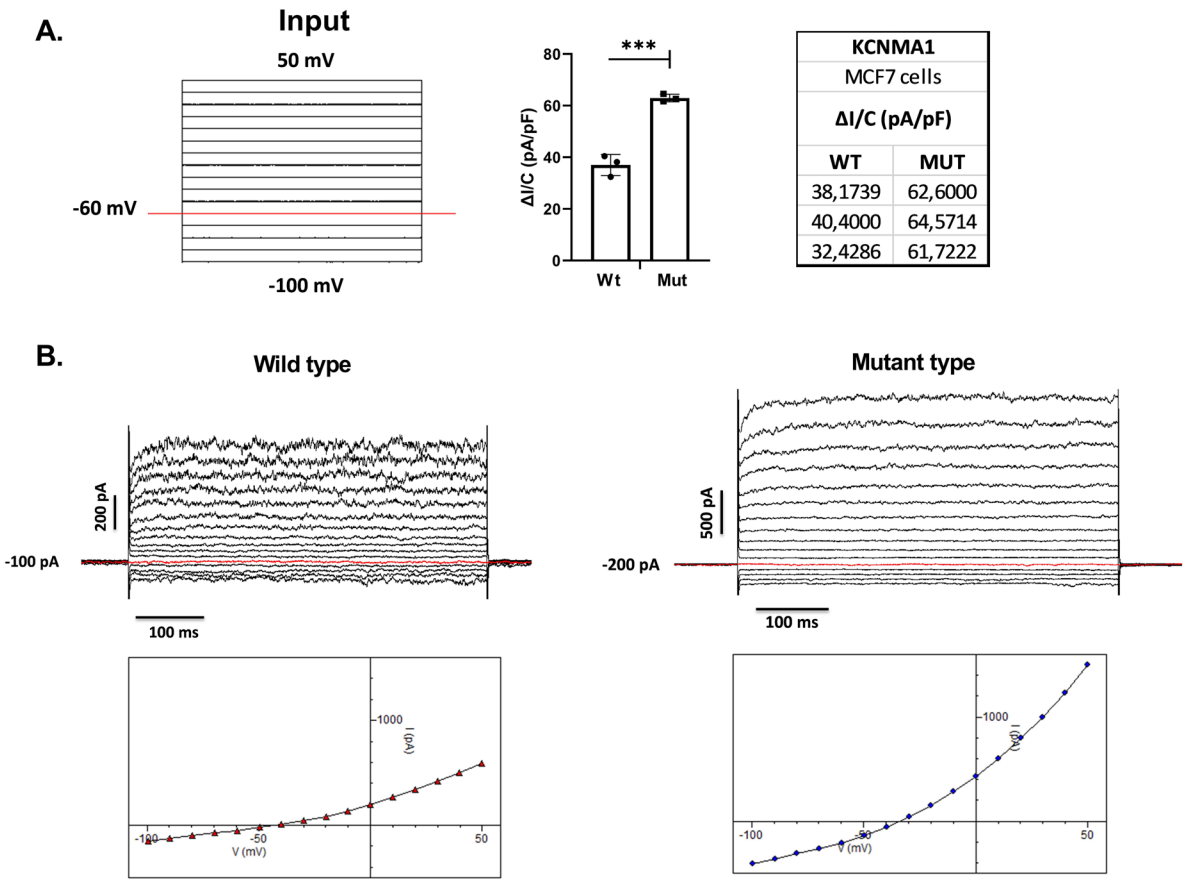

Figure 2. Patch-clamp recordings from MCF7 cells. **A.** Shows voltage protocol, statistical analyze of the pA/pF ratio and raw data of peak current amplitude to the cell membrane capacitance respectively. Statistical analyzis performed with a *t-test* ($p = 0.0005$). **B.** Whole cell patch-clamp recordings of wild and mutant type respectively. pA/pF: picoamperes per picofarad.

causes increased current activity, and they speculated a potential mechanism to account for disease in terms of this very specific variation. Briefly, this mechanism is G-V shift to hyperpolarized potentials, increased open probability, faster activation, slower deactivation, and increased Ca^{2+} sensitivity [13]. In contrast to these findings, Liang et al. reported three unrelated patients who had clinical heterogeneity, i.e. multiple malformation syndromes, such as facial abnormalities, global developmental delay, axial hypotonia, and visceral malformations. Also, one patient had a movement disorder, and two of the three had epileptic seizures [3]. Similar to R458X reported by Yesil et al. the variations C413Y, N449fs, and I663V reported by Liang et al. are placed in the RCK1 domain which is a binding site for Ca^{2+} [3]. The clinical comparison of the patient carrying biallelic mutations C413Y/N449fs presented in and the patient presented by Yesil et al. had developmental/intellectual deficiency and cerebellar ataxia in common however the patient reported by Yesil et al. had also dyskinesia and epilepsy phenotype [1]. Liang et al. observed that C413Y is a LoF variant that inhibits the function of the BK channel substantially and likely reduces the activation and macroscopic current amplitude of the BK channel significantly at calcium concentrations ranging from nominal 0 µM–10 Mm. Moreover, the frameshift variant p.(Asn449fs) did not elicit any potassium current under the voltage stimulus from −160 mV–160 mV at a 10 µM calcium concentration suggesting that it is also a LoF variant [3].

In this study report, we conducted patch-clamp recordings on WT and R458X mutant cells. To understand how a truncating mutation abolishes function and the electrophysiological outcome, a patch-clamp with cell-attached recordings is a direct method. We measured current density from the ratio of peak current amplitude to the cell membrane capacitance (picoamperes per picofarad (pA/pF)) under a stable pH. We found out that under a stable pH the mutant type showed an increasing sequence of actions when compared to the wild type. While the stimulus continued, changes in the potassium channel activation might have altered downstream potential as well. It seems that the increased current and thus the hyperpolarized state -of the channel firstly alters the function but negatively affects the viability of the cell later

on (Figures 1 and 2). The dual phenotype with epilepsy/dyskinesia and cerebellar atrophy, may be explained with this mechanism in such severe gain of function mutations. Although gain-of-function have been generally shown in missense mutations nonsense mutations with a gain of function consequence have also been reported in the literature [25,26].

Taken together, the nature of the BK-channel and its effect on organisms, show a giant heterogeneity, from transcript variation to cellular electrical activity. Both the literature and this study have shown that the, changes in the RCK domain of BK channels directly affect protein function [14,27,28]. This is the first functional study observing an increased current in the *KCNMA1* gene resulting from a truncating mutation. Since the variation we detected is non-sense, it should be considered in possible therapeutic approaches in future studies. In conclusion, due to its complexity of their nature every causative variant in the channel genes should be evaluated in terms of functional characterization, particularly electrophysiological analyzes.

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Disclosure statement

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