Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2020; 32(3): 22-31.

CACNA1C gene mutation in Thai young adult with sudden unexplained death

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KEYWORDS

Molecular autopsy; Sudden unexplained death syndrome; Sudden death; Cardiac ion channelopathies.

ABSTRACT

Sudden unexplained death syndrome (SUDS) is an unexpected natural death in young healthy adults, especially Southeastern Asian populations, wherein no severe diseases can be detected to explain death's cause. Several studies confirmed that SUDS associated with genetically changed cardiac ion channels. However, CACNA1C mutations in SUDS are rarely reported in the Thai population. This study aimed to characterize CACNA1C gene mutation in Thai young adults who died with SUDS. Characteristics data of 32 SUDS cases in the Thai population were collected. Blood samples were collected from 27 males and 5 females of which categorized as an unexplained cause of death in a postmortem examination. Genomic DNA was extracted, amplified, and screened for nine variants mutation of CACNA1C gene, using pyrosequencing assay. Most cases were domiciled in the central region of Thailand. Men aged 19 - 39 years old (mean age 29.44 ± 5.59) is the most common SUDS cases. In a total of 32 cases, we detected 7 cases (21.9 %) with heterozygous A/G, c.720016 G > A within exon 44. The variation was defined as a missense mutation of p. R1880Q at C-terminus of α1C subunit of the L-type cardiac calcium channel. The heterozygous missense mutation of p. R1880Q of CACNA1C gene was identified in Thai SUDS. However, there is no evidence of studies about the effect of function and pathogenicity of CACNA1C-R1880Q. Further study is needed to clarify the role of this variant in the functional effect of pathogenesis in SUDS.

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Received: 21 February 2020/ Revised: 30 April 2020/ Accepted: 9 May 2020

Introduction

Sudden unexplained death syndrome (SUDS) is defined as a sudden unexpected death that a significant cause of death of young adults and healthy. The annual report of SUDS incidence rate showed about 37, 43 and 38 cases per 100,000 populations in Japan, Philippine and Thailand, respectively[1-3]. This syndrome usually occurs within a short period of time after the onset of acute symptoms[4]. The SUDS cases are often due to primary electrical diseases or cardiac channelopathies that are genetically changed in ion channels or regulatory proteins of the heart muscle that lead to an increased risk of arrhythmia and SUDS in individuals with a structurally normal heart[5].

The abnormal ECG pattern of right bundle-branch block and ST-segment elevation in Lead V_1 through V_3, is indicated as Brugada syndrome (BrS) in Thai SUDS patients[6]. In addition, the epidemiological and clinical characteristics like BrS among SUDS cases were related to *SCN5A* gene mutations associated with a sodium ion channel[7]. Recently, several studies from many countries including Thailand have focused on this gene and its phenotype characteristics[8-11]. The *SCN5A* gene mutation associated with BrS was found in 11-28 % of SUDS patients[12-13] followed by *CACNA1C* gene mutation (6-7 %)[14].

CACNA1C gene encodes the pore-forming α1c subunit of the L-type cardiac calcium channel (LTCC). The α1c subunit comprises four transmembrane domains (DI, DII, DIII, and DIV). The α1c subunit pore is known to mediate the influx of calcium ions into the cardiac muscle cell upon membrane polarization. In a normal heart, this plays an important role in excitation-contraction coupling[15]. However, an insufficient amount of calcium inward depolarizing current can trigger the development of potentially fatal ventricular fibrillation, whereas the enhancement of calcium current can prolong cardiac of action potential duration, thereby conferring an increased risk of sudden death.

CACNA1C gene mutation was reported in some mental illnesses. *CACNA1C* gene contributes to the depolarization of neurons through calcium ion influx. Variations in SNPs in *CACNA1C* gene affect both the structure and function of the nervous system, including synaptic plasticity and neuron viability. In 2016, Kim et al. reported that CACNA1C rs723672 and rs1051375 SNPs were associated with bipolar disorder in the Korean population[16]. *CACNA1C* gene also plays an important role in the development of dendrites, neuron survival, synaptic plasticity, and memory formation. Dysfunction of ion channels plays a key role in the pathogenesis of hereditary ataxia[17]. Although *CACNA1C* gene mutation was reported in several diseases other than SUDS. Gene mutation of each disease was found on different exon.

The first implication of mutations in *CACNA1C* was found in cardiac arrhythmias with Timothy syndrome (TS), a rare multisystem disorder associated with long QT syndrome (LQTS8), and childhood sudden death[18]. Since then, several *CACNA1C* mutations have recently been identified in a patient presenting with cardiac ion channelopathies, including the long QT syndrome type 8 (LQTS8)[19-20], the short QT syndrome type 4 (SQTS4), as well as the Brugada syndrome type 3 (BrS3)[14,21]. These different-point mutations of *CACNA1C* lead to different molecular mechanisms, either gain-of-function or loss-of-function of the L-type calcium channel (*LTCC*), resulting in abnormality of calcium ion concentration in the cardiac muscle cells. Then it was found to cause the failure of excitation-contraction coupling until sudden death. A mutation in exon 2, A39V reduced current density in the wake of impaired channel trafficking for the α1C of LTCC[14]. A mutation in exon 8a, G406R revealed a marked reduction in voltage-dependent inactivation (VDI) and an increase in Ca2+ influx is known to prolong the potential of cardiac action[18-20]. However, the data of *CACNA1C* mutation has been limited among the Thai population. This study, therefore, aimed to characterize *CACNA1C* gene mutation in Thai young adults with death from SUDS.

Materials and methods

Subjects of study

This study was approved by the Research Ethics Committee of Thammasat University (COE No.054/2560) and Central Institute of Forensic Science, Ministry of Justice, Thailand (CIFS). Blood samples were collected from 32 SUDS cases submitted to Forensic Pathology of CIFS, Bangkok, Thailand, during April 2017 - December 2018. The information of 32 cases was collected from postmortem inquest reports and postmortem examination reports.

The inclusion criteria of SUDS subjects included factors as follows: (1) Thai nationality, (2) young adult (18 - 39 years of age), and (3) negative comprehensive postmortem investigation such as autopsy, toxicology, histology, and death-scene investigation. Meanwhile, decomposition cases of which the post-mortem changes occur from 12 - 24 hours after death were excluded from this study. The characteristics of 32 SUDS subjects are summarized in Table 1.

Regional classification of SUDS cases

The address of civil registration is used to classify 5 regions of SUDS subjects including Central, Northeastern, Northern, Western, and Southern. Each region was sub-classified into separated provinces

Genomic DNA extraction

Genomic DNA was extracted from 300 µL whole blood using the Genomic DNA Mini Kit (Blood/Cultured Cell) (*Geneaid*, *Taiwan*). The DNA was quantified by measuring the optical density of 260 nm and 280 nm using spectrophotometer (e-spect malcom, Japan).

Primer design

The polymerase chain reaction (PCR) and sequencing primers of *CACNA1C* (GenBank, accession no.NG_008801.2) were designed using PyroMark Assay Design program 2.0 (Qiagen, Singapore) to obtain primer sets that were assigned as 1-7 assays (Table 2). The target genes were amplified using biotin tagged on 5´-end of forward primer in assay 1, 2, 3, 4, and 7, whereas assay 5 and 6 using biotin was tagged on 5´-end of reverse primer.

Polymerase Chain Reaction

PCR mixture consisted of 5 ng/µL of DNA template. Pyromark® PCR kit (Qiagen, Germany), including 12.5 µl of 2x PyroMark PCR Master Mix, 2.5 µl of 10x CoralLoad Concentrate and DNase free water, was used in the reaction. 0.5 µL of 10 µM forward and reverse primers were added for total volume 25 µL/reaction. These reactions were performed with 45 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C (68 °C for assay 6) for 30 s and extension at 72 °C for 30 s. This amplification was achieved using a T100™ Thermal cycler (Bio-RAD Laboratories, USA).

DNA Sequencing by pyrosequencing assay

Based on the sequencing-by-synthesis principle, pyrosequencing is a DNA sequencing method that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. Pyrosequencing analysis was performed using PyroMark® Q48 Advanced Reagents (Qiagen, Hilden, Germany) and analyzed on Pyromark Q48 Autoprep system (Qiagen, Hilden, Germany) with the sequencing primers. The performed steps ensured compliance with the manufacturer's instruction.

Biotinylated PCR products were added into each well of a PyroMark Q48 Autoprep disc. Thereafter, it was immobilized with sepharose magnetic beads. Subsequently, nucleotides, lyophilized enzyme, substrate mixture, and pyromark binding buffer from PyroMark® Q48 Advanced Reagents were automatically added into the cartridge chambers. When nucleotides are incorporated into the analyzed DNA strand, pyrophosphate (PPi) is released and converted to ATP. The generation of ATP drives a detectable light signal through a luciferase reaction and this is proportional to the number of nucleotides incorporated. The pyrograms data were then analyzed using pyrosequencing software (PyroMark Q48 software).

Table 1 Characteristics of each SUDS subject

Domicile/ Province	ID	Gender	Age	Occupation	Month of death (2017-2018)	Time of death (24 hr.)	BMI (kg/m^2)	Heart Weigh (g)	CACNA1C gene Mutation
Central region									
C1	1	F	25	Bl	May 2017	07.30	23.2	305	g.720016 G > A
	2	M	21	Bl	August 2017	01.00	20.8	350	g.720016 G > A
	3	M	19	Bl	August 2017	08.00	22.1	345	g.720016 G > A
	4	M	30	Me	July 2017	06.50	23.9	380	-
	5	M	20	St	May 2018	08.00	20.8	325	-
	6	M	36	Bl	June 2018	05.45	21.5	375	-
C2	7	M	29	Bl	April 2017	08.51	27.7	350	-
C3	8	M	36	Bl	May 2017	08.46	23.9	370	g.720016 G > A
	9	M	29	Bl	September 2018	18.00	27.5	370	-
	10	M	37	Me	May 2017	11.05	26.2	385	-
C4	11	M	30	Bl	June 2018	08.30	26.3	375	-
C5	12	M	32	Bl	August 2017	07.00	23.2	310	-
C6	13	F	34	Bl	September 2017	16.43	22.2	296	-
	14	M	25	Bl	March 2018	8.50	21.2	390	-
C7	15	F	37	Bl	January 2018	6.30	20.7	270	-
	16	M	39	Bl	June 2018	6.50	29.7	425	-
C8	17	M	33	Bl	May 2018	7.00	22.3	420	-
Northeastern region									
NE1	18	M	30	Bl	April 2017	02.30	25.2	380	g.720016 G > A
NE2	19	M	37	Bl	May 2017	22.00	22	355	-
NE3	20	M	27	Bl	May 2017	10.30	21.5	290	-
	21	M	25	Bl	December 2017	09.45	28	335	-
NE4	22	M	31	Bl	May 2017	09.05	22.4	365	-
NE5	23	M	35	Go	June 2017	08.00	25.7	390	g.720016 G > A
	24	M	23	Bl	March 2018	23.30	22.2	425	-
NE6	25	M	26	Bl	August 2017	15.15	24.1	295	g.720016 G > A
NE7	26	M	19	Bl	March 2018	8.30	29.8	420	-
NE8	27	F	28	Bl	June 2018	8.00	21.8	250	-
NE9	28	M	27	Bl	May 2018	16.30	20.8	350	-
	29	M	24	Bl	June 2018	09.00	20.2	305	-
NE10	30	M	32	Bl	October 2017	06.30	30.4	385	-
Northern region									
N1	31	M	26	Bl	March 2018	01.30	21.5	380	-
Western region									
W1	32	F	30	Bl	June 2017	06.00	22.8	315	-
Age (Mean ± SD)			29.44 ± 5.59						
BMI (Mean ± SD)			23.8 ± 2.96						
Heart weight (Mean ± SD)			352.53 ± 45.86						

Note: NE, Northeastern; C, Bangkok and Central; N, Northern; W, Western; M, male; F, female
BMI, Body mass index (normal range) = 18.5 - 24.9 kg/m^2 [26]
Bl, Blue-collar construction workers; Me, Merchant; St, Student; Go, Government official

Table 2 Sequence of oligonucleotide primers used for PCR and pyrosequencing

Assay	Exon/Amino acid change	Primer	Product size (bp)	Ref.
1	2 p. A39V	Forward B 5´-TTG CCA CAG GTT CCA ACT ATG-3´ Reverse 5´-TCT TGG GTT TCC CAT ATT GCT-3´ Sequence 5´-GGG GAT GTG CTC AGG-3´	231	[14]
2	8 p. G406R	Forward B 5´-GGC CCT GGA TCT ATT TTG TTA CAC-3´ Reverse 5´-ATA CAG CCA GGA ATA GCA GAA AGA-3´ Sequence 5´-CCT TGG TCC TGC TTA C-3´	155	[15,16]
3	10 p. G490R	Forward B 5´-GAG CAT GCC CAC CAG TGA GA-3´ Reverse 5´-CTC GCC GTG CCT ACT CAC GC-3´ Sequence 5´-ACG CCA GCC TGG CCC-3´	108	[14]
4	19 p. E850 del	Forward B 5´-TAA GTG GGA GTG CTG GAG TTA TT-3´ Reverse 5´-TGG GCA CTG CCT TTT CCT TA-3´ Sequence 5´- CGA CAG GCA TCT CTG G -3´	138	[18]
5	42/43 (45) p. C1837Y	Forward 5´-GTT CGG CAA CCA CGT CAG CT-3´ Reverse B 5´-TTG GAG CCG GTG GAC GAG TA-3´ Sequence 5´-GCT GCA CAT CAA CAA G-3´	186	[18]
6	44 p. R1880Q 46 p. G1911R 48 p. Q1916R	Forward 5´-AGG ATG ACG AAA ATC GGC AAC T-3´ Reverse B 5´-GCC TGT CCA AAA GTG TGA GCT AC-3´ Sequence 1 5´-GAC AAG AGG GAC ATC C-3´ Sequence 2 5´-CGA CAG AAG GAC CGA-3´	546	[18] [19]
7	47 p. V2014I	Forward B 5´-ATC CAT CCA CTG CGG CTC CT-3´ Reverse 5´-CGA GTC ACC TAC CGC TTC CAC-3´ Sequence 5´-GCA CCA TGA GGG AGA-3´	166	[18]

Note: B is biotinylated on end of primer. Sequence shows primers was used in pyrosequencing.

Results

Characteristics of SUDS cases

The domicile of 32 Thai SUDS cases was reported in different regions of Thailand (Table 1). Among 6 regions, we found SUDS cases in 4 regions: the central region, the northeastern region, and the western region of Thailand. There were 9 of 22, 9 of 20, 1 of 9, and 1 of 5 provinces reported with SUDS cases in the central, the northeastern, the northern, and the western region, respectively. Most cases were found in the central region (18 cases, 56.25 %) and the highest cases were in C1 (7 cases, 38 %), followed by the northeastern region (12 cases, 37.5). The other 2 cases were found in the northern (3.12 %) and the western region (3.12 %). There were 27 males (84.4 %) and 5 females (15.6 %) in the age range of 19-39 years with a mean age of 29.44 ± 5.59 years with 30 years of age being the most common. Twenty-eight of the 32 SUDS cases (87.5 %) were blue-collar construction workers, followed by 2 merchants (6.25 %), 1 government official (3.12 %), and 1 student (3.12 %). In 2017, the monthly incidence of 18 SUDS cases was relatively higher in May (6 cases, 33 %) followed by August (4 cases, 22%), whereas. While, in 2018, the monthly incidence of 14 SUDS cases was relatively higher in June (5 cases, 36 %) followed by March (4 cases, 29 %).

SUDS victims died in 6 periods. Eighteen cases (53.13 %) died from 6 to 9 A.M. followed by 4 cases (12.5 %) died from 9 to 12 A.M. , 4 cases (12.5%) died from 3 to 6 P.M., and 3 cases (9.38%) died

from 0 to 3 A.M. Finally, 2 cases (6.25 %) of the victims died from 21 to 24 P.M. and 03:01 to 06:00 A.M. respectively.For body mass index (BMI), 21cases (65.6 %) and 11 cases (34.4 %) had a normal BMD (18.5 - 24.9 kg/m^2) and high BMD (23.8 ± 2.96 kg/m^2), respectively. The autopsy data showed the mean heart weight of 352.53 ± 45.86 g, ranging from 250 to 425 g. All 32 cases were found a normal structural appearance.

Genetic analysis

This study found a heterozygous missense mutation transition of A→G at position 720016 of *CACNA1C* (GenBank, accession no. NG_008801.2) in the exon 44 that forecasted a substitution of arginine to glutamine at position 1880 (p. R1880Q, rs182208896) located in the intracellular C-terminus of CaV1.2 α1C. It was identified in 7 SUDS cases (21.9 %) (ID: 1,2,3,8,18,23 and 25), including 6 males (22.3 %) and 1 female (20 %).

Discussion

In this study, the domicile of 32 Thai SUDS cases was in different regions of Thailand. Most cases (53.13 %) were found in the central region, especially province C1 (6 cases, 35.3 %), followed by the northeastern region (40.6 %). Contrary to other Thai studies, most SUDS cases were found in the northeastern region[2, 23-25]. This is the novel evidence indicating that the most common SUDS cases were not from the northeastern region. Interestingly, the majority of SUDS cases were found in province C1 in the central region. Field investigations of SUDS families or villages of this province should be further focused on reducing the burden of sudden death at a young age.

Males (27 cases, 84.4 %) were found more than females (5 cases, 15.6 %), which is consistent with previous reports[2,25-29]. The prominent sex difference may be possible pathogenesis of heart-related problems. Valladares reported cardiac vagal tone significantly declined while sympathetic nerve activity markedly increased during the rapid eye movement sleep in the male as compared with the female[30].

The mean age in this study was 29.44 ± 5.59 and was close to that in the previous of Thai SUDS cases[3,24-25]. Simultaneously, the population aged 18 - 39 in our study was is in line with the results of previous studies [3,23-25]. The study of Cheng, 80.56 % of the SUDS cases were related to overwork including psychological and physical stress resulting in high fatigue level[31]. related to overwork including psychological and physical stress resulting in high fatigue level[31]. The high occurrence rates for SUDS were blue-collar construction as several studies reported in Thailand[26, 23-25] and southern China study[31].These workers seem to be susceptible to SUDS because of sympathetic nervous activity increase[30] and blood oxygen decrease[31] after heavy physical labor. Taken together, these factors combined with a genetic predisposition may contribute to the death of SUDS cases.

The incidence of SUDS cases relatively peaked in the summer and monsoon months that was May 2017, followed by August 2017 June 2018, and March 2018 respectively, which are consistent with the findings reported from previous Thai studies[23-24] and a Chinese study[31]. The summer and monsoon climate in the South of China and Southeast Asia including Thailand generally have high temperatures all year round resulting in hot weather and humidity. Cheng reported that these seasons emerge more fever symptoms of SUDS cases[31]. Also, ante-mortem symptoms such as headache and fever in SUDS cases[28] may be a predisposing factor of death.

The mean BMI (23.8 ± 2.96 kg/m^2) of Thai SUDS cases in this study was within normal range which is consistent with previous Thai studies, whereas the mean heart weight (352.53 ± 45.86 g) was in over range compared to that of Thai SUDS cases in other studies[24-25]. However, the mean heart weight was similar to that of southern China study in 148 SUDS cases which found mean heart weight of 352.0 ± 54.4 g[26]. There seems to be the association of heart weight increase and death as described in Steinhaus et al. about sudden arrhythmia death from the increased mean cardiac mass[33].

This study demonstrated *CACNA1C* gene mutation in 32 SUDS cases in the Thai population using nine different exon amino acid changes including *CACNA1C*-A39V, *CACNA1C*-G406R, *CACNA1C*-G490R, *CACNA1C*-E850del, *CACNA1C*-C1837Y, *CACNA1C*-R1880Q, *CACNA1C*-G1911R, *CACNA1C*-Q1916R, and *CACNA1C*-V2014I. The only one mutation *CACNA1C*-R1880Q was detected in 7 cases (21.9 %). It was different from other reports including *CACNA1C*-N547S, *CACNA1C*-R632R, *CACNA1C*-R858H, *CACNA1C*-R1780H, *CACNA1C*-C1855Y, *CACNA1C*-R1910Q[20] *CACNA1C*-A519Y[25] and *CACNA1C*-N2091S[34]. Owing to different exon amino acid changes of *CACNA1C* gene selection for each study, those variants were not identified in our study.

Hereby, we reported a missense mutation of *CACNA1C*-R1880Q, rs182208896 similar to Risgaard et al. (2013). They studied the prevalence of genetic variants associated with Brugada syndrome (BrS) of 6152 persons in the Exome Sequencing Project. The missense mutation of *CACNA1C*-R1880Q was rarely reported in 6 cases of European Americans genotype and 1 case of African Americans genotype[35]. This gene mutation was also reported in 1 case associated with BrS of 205 patients associated with BrS, BrS/SQT, idiopathic ventricular fibrillation (IVF), and early repolarization syndrome (ERS)[21]. Contrary to our study, Suktitipat et al. (2017)[25] detected only 1 female indicated as the missense mutation of *CACNA1C*-A519Y. The most common mutation was *TTN* gene found in 44 % of the cases. From our study, the missense mutation of *CACNA1C*-R1880Q is only located at exon 44 in the C-terminus of the α1 subunit of L-type calcium channel (LTCC). Burashnikov et al. (2010)[21] studied the functional effect in p. E1829_1833dup and p. V2014I-CACNA1C mutations at exon 43 and 46, respectively. They found that these variant mutations lead to loss of function for LTCC. However, functional studies for other mutations including the missense mutation of *CACNA1C*-R1880Q have not been done yet. Besides, previous studies analyzed variants data and classified actionable pathogenic single-nucleotide variants in 500 European and 500 African-ancestry participants[36], in 4300 European and 2203 African-ancestry participants[37]. These reported that *CACNA1C* - R1880Q was classified as likely benign classification of highly penetrant pathogenic variants. In addition, Campuzano et al. (2019)[38] reviewed data of 42 genes associated with BrS and classified *CACNA1C*-R1880Q as a variant of uncertain significance (VUS). To date, there is no evidence of studies about the effect of function and pathogenicity of *CACNA1C*-R1880Q.

Conclusion

From the results, only one missense mutation of *CACNA1C*-R1880Q was found in Thai SUDS; hence it may not a major cause of the disease. Therefore, further study is needed to clarify the role of this variant in the functional effect of pathogenesis in SUDS. In addition, identification of all genes associated with cardiac ion channelopathies in SUDS cases could potentially be used for familial risk screening or surviving relatives and help predict the prognosis of the channelopathies gene mutation along with the diagnostic tool to identify patients who might be at a higher risk of sudden deaths in the future. In addition, identification of all genes associated with cardiac ion channelopathies in SUDS cases could potentially predict the real prognosis of the channelopathies gene mutation.

Take home messages

A heterozygous missense mutation of *CACNA1C*-R1880Q at C-terminus of α1C subunit of the L-type cardiac calcium channel were found in Thai SUDS. Seven cases (21.9 %) with heterozygous A/G, c.720016 G>A within exon 44 were detected. This genetic variant is associated with Brugada syndrome (BrS).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank the Faculty of Allied Health Sciences, Thammasat University, Thailand, and Central Institute of Forensic Science, Thailand, for supporting. The financial was

supported by Capacity Building Program for New Researcher 2018 from National Research Council of Thailand NRCT) Contact Number 8/2561) and Thammasat University Research Fund, Thailand (Contact Number 111/2560).

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