Hirosaki Med. J. 70 : 148—155, 2020

ORIGINAL ARTICLE

Mutational analysis of β-arrestin in Japanese patients with coronary spastic angina

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Abstract Coronary spastic angina (CSA) has become increasingly important for clinical practice. β-arrestin regulates a large network of cellular responses including inflammation and muscle contraction. However, little is known about its role in the pathogenesis of CSA. In this study, we performed mutational analysis of β-arrestin1 and β-arrestin2 genes in CSA patients. The study population included 20 CSA patients and 5 control subjects. Mutational analyses of the coding regions of β-arrestins were performed by direct sequencing. In β-arrestin1, single base heterozygous substitution at nucleotide position 176 in exon 5 was detected in 2 of 20 CSA patients, whereas no substitution was detected in control subjects. We subsequently performed an association study involving 50 CSA patients and 50 control subjects. No difference was found in the incidence of the mutation between the two groups (p=0.44). In β-arrestin2, single base substitution at nucleotide position 61 in exon 11 was detected. This substitution was homozygous in 13 and heterozygous in 7 of 20 CSA patients, whereas all homozygous in 5 control subjects. These substitutions were well-annotated SNPs (rs877711 and rs1045280, respectively) and did not cause amino acid replacement. No significant substitution of the coding regions of β-arrestins was detected in Japanese CSA patients.

Hirosaki Med. J. 70 : 148—155, 2020

Key words: Coronary spastic angina; Prinzmetal variant angina; β-arrestin; Mutational analysis; Direct sequencing.

Introduction

Coronary spastic angina (CSA) causes not only angina attack without atherosclerotic lesion, but myocardial infarction or malignant ventricular arrhythmias, both of which lead to sudden cardiac death[1, 2)]. Although CSA has become increasingly important for clinical practice, the precise mechanism is still largely unknown. We previously identified that phospholipase C (PLC) activity in cultured skin fibroblasts obtained from CSA patients was enhanced, and positively correlated with coronary artery tone[3)]. Furthermore, we found one mutation at position 864 in PLC-δ1 gene sequence obtained from CSA patients, resulted in replacement of amino acid at 257th from arginine to histidine[4)]. This mutation led to enhancement of PLC enzymatic activity and coronary spasm induction in animal model[4, 5)]. These results indicate that genetic factors may contribute to the pathogenesis of CSA.

β-arrestin, originally identified as a mediator to desensitize G protein-coupled receptors (GPCRs), acts as a scaffold in its own signaling and regulates a large network of cellular responses including inflammation, apoptosis, and regulation of muscle contraction[6)]. For instance, β1-adrenergic receptor (β1AR), one of the GPCRs, activates adenylate cyclase via Gs protein and promotes cyclic AMP generation, resulting in inotropic

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Received for publication, December 5, 2019
Accepted for publication, December 13, 2019

and chronotropic effects in the heart. Catecholamine binding to β1AR also activates another signaling pathway known as β-arrestin-dependent signaling, which exerts anti-apoptotic and cardioprotective effects[7]. β-arrestin exists with almost all types of GPCRs including angiotensin II receptor, endothelin receptor, μ-opioid receptor, and acetylcholine (ACh) muscarinic receptor and regulates various physiological and pathological processes[6, 8-11]. There are two subtypes of β-arrestin, β-arrestin1 and β-arrestin2, which independently and differentially regulate downstream signalings[6, 12, 13]. Loss of either β-arrestin1 or β-arrestin2 reduces angiotensin II-induced myosin light chain phosphorylation and contraction force in rat vascular smooth muscle cells (VSMCs)[14]. Furthermore, β-arrestin1 knockdown reduces intracellular calcium increase and contraction force in response to ACh muscarinic receptor agonist methacholine in airway smooth muscle cells. These findings indicate the involvement of β-arrestin in contraction of smooth muscle cells[9]. However, little is known about the role of β-arrestin in the pathogenesis of CSA. In the present study, we performed mutational analysis of β-arrestin genomic DNA in CSA patients to evaluate the possible involvement of β-arrestin in the pathogenesis of CSA.

Materials and methods

Study patients

All procedures were approved by the ethics committee of the Hirosaki University Graduate School of Medicine, and written informed consent was obtained from all patients prior to the study. This study population included 20 CSA patients (15 men and 5 women with an average of 59.2 years) and 5 control subjects without any history of angina pectoris (4 men and 1 woman with an average of 51.8 years). The diagnosis of CSA was made by total or subtotal occlusion or severe vasoconstriction of the coronary artery associated with chest pain and ischemic electrocardiogram (ECG) change induced by intracoronary injection of ACh in 15 patients. After intracoronary injection of isosorbide dinitrate, coronary angiograms revealed normal or almost normal coronary arteries with stenosis ≤50% of the lumen diameter in these patients. In the remaining 5 patients, ST segment elevation on ECG was recorded during a spontaneous attack and no significant stenosis was detected in coronary angiography. We subsequently performed an association study involving a total of 50 CSA patients (40 men and 10 women with an average of 60.2 years) and 50 control subjects (26 men and 24 women with an average of 51.8 years) in the analysis of β-arrestin1 gene. In this population, the diagnosis of CSA was made by intracoronary injection of ACh in 37 patients and ST segment elevation on ECG during a spontaneous attack in 13 patients.

Extraction of genomic DNA and amplification of the coding regions of β-arrestin1 and β-arrestin2

Genomic DNA was extracted from the whole blood using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) as previously described[15]. β-arrestin1 and β-arrestin2 consist of 16 and 15 exons, respectively (Figures 1A and 1B). The coding region was divided into 12 segments in β-arrestin1 and 4 segments in β-arrestin2, and these segments were amplified by polymerase chain reaction (PCR) using corresponding primers (Figures 1A and 1B, Tables 1A and 1B). The PCR products were separated by electrophoresis on 1.5% agarose gel to confirm amplifications of the target regions (Figures 2A and B). Each PCR reaction contained 1 mM Mg^{2+}, 200 μM dNTP mixture, 0.2 μM primers, 1.25 U/μl Tks Gflex DNA Polymerase (TAKARA, Shiga, Japan), and 50-150 ng of genomic DNA in a total reaction volume of 50 μl. The thermal cycling parameters for PCR reaction were as follows: 1 cycle of denaturation (94 °C for 1 min), 30 cy-

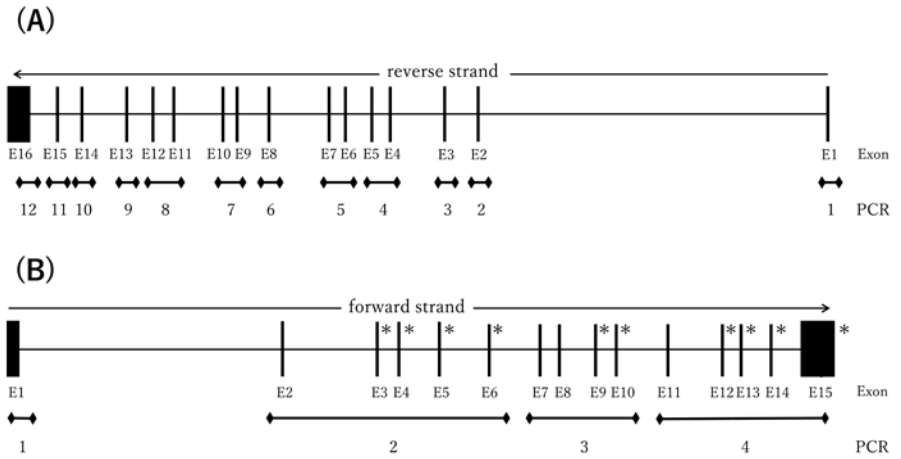

Figure 1. Gene targeting map and the regions of PCR fragments of β-arrestin1 and β-arrestin2. (A) β-arrestin1 coding region consists of 16 exons. The region was divided into 12 segments and amplified by polymerase chain reaction (PCR) using corresponding primers. (B) β-arrestin2 coding region consists of 15 exons. The region was divided into 4 segments and amplified by PCR using corresponding primers. Asterisks indicate exons needed to design oligonucleotide for direct sequencing.

Table 1A. Primer sets used to amplify 12 segments of β-arrestin1 gene.

		Sequence	Amplicon size	Exons included in each PCR amplicon
PCR1	Sense Antisense	5'- TGGGGATTTTCCAGCCTGG 5'-AGAACCAGGACGCAATCGG	166 bps	Exon 1
PCR2	Sense Antisense	5'- TGGTGCCTGTCAGTCACTC 5'-TGTGCATTTCAGGGGACATG	246 bps	Exon 2
PCR3	Sense Antisense	5'-ATGGGTCTGGAGTTTCAGAG 5'-TGAGAGCTATTTCTGGGAGG	238 bps	Exon 3
PCR4	Sense Antisense	5'-AACAGATGGTTGGATCTGGG 5'-TACCAGGTAACACAGGTGAC	1249 bps	Exon 4,5
PCR5	Sense Antisense	5'-TACATGAGACAGGACAGTGG 5'- ATCTCCTCTTGGACTTTGCC	1083 bps	Exon 6,7
PCR6	Sense Antisense	5'-AATGATGTGAGGCTCGCAAG 5'- ATGAGAGGTCTCAGATTCCC	368 bps	Exon 8
PCR7	Sense Antisense	5'- AAGGTCTGCGCAAGGTCTG 5'-AACAGGACACCTAGAGCTTC	799 bps	Exon 9,10
PCR8	Sense Antisense	5'- TAGATATTCTGCTGGGCTGG 5'- ATGGCCAATCCTCAGTGCG	1900 bps	Exon 11,12
PCR9	Sense Antisense	5'- TTCCTCTCTGAACTCTCTCC 5'- AATTGTGTCTCCCAGCTCAC	231 bps	Exon 13
PCR10	Sense Antisense	5'- ATCAAGTCTCACAGCCTGTG 5'-ATAGAGGCTCAAAGCATGGC	540 bps	Exon 14
PCR11	Sense Antisense	5'-TTTCAGTACCAGGAATGAGTC 5'- AAATCCACATCGGTTGGTCC	637 bps	Exon 15
PCR12	Sense Antisense	5'- AGAGCCATACTCTGAGTCAC 5'- TCATCACCGTGATCTGGAAG	356 bps	Exon 16

cles of denaturation (98 °C for 10 sec), annealing (60 °C for 15 sec) and extension (68 °C for 30 sec) followed by a final extension at 72 °C for 10 min on iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

Direct sequencing of β-arrestin1 and β-arrestin2 genes

All PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Since some PCR products in β-arres-

Table 1B. Primer sets used to amplify 4 segments of β-arrestin2 gene.

		Sequence	Amplicon size	Exons included in each PCR amplicon
PCR1	Sense Antisense	5'- TGTCTGCTAGGAGAGGG 5'-TCATTATCCTCGAGGACCTG	406 bps	Exon 1
PCR2	Sense Antisense	5'-AAATAGGAAGGACCTTCCGG 5'-TCTTCTTCCTCTCCAAAGCC	2718 bps	Exon 2,3,4,5,6
PCR3	Sense Antisense	5'-ATGTGGTGGTCATTGTGCAC 5'- TTCTCCTGATCCTGCTCTTC	1340 bps	Exon 7,8,9,10
PCR4	Sense Antisense	5'-AAACAAGGGACTAGTGAGGG 5'-TATGAACACAGCTTGCCACC	2140 bps	Exon 11,12,13,14,15

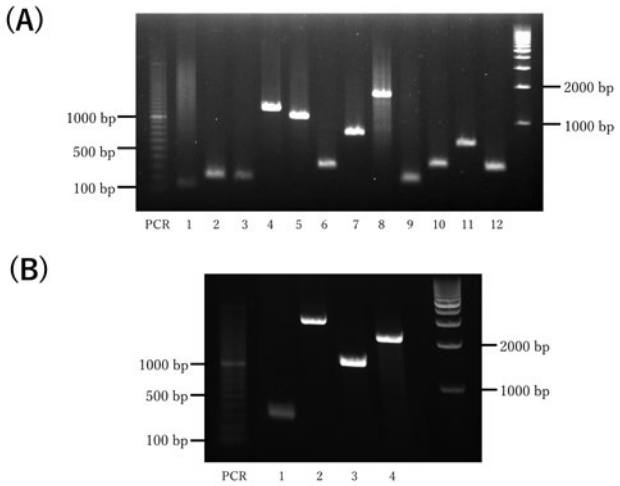

Figure 2. The target amplicons in β-arrestin1 (A) and β-arrestin2 (B) genes were separated by electrophoresis in 1.5% agarose gel. The size of each amplicon is consistent with the designed one. Ladder markers are shown in both sides.

Table 2. Oligonucleotides for direct sequencing of exons with an asterisk in β-arrestin2 shown in Figure 1B.

Fragment		Sequence
PCR2	Exon 3,4	5'- AACCTGGAACAACTCCTTCC
	Exon 5	5'- TAGGGAAGTGAAATGGGCTG
	Exon 6	5'- TCTTAGGTTGAGATTTGGAGG
PCR3	Exon 9	5'- TGATCCGAAAGGTGCAGTTC
	Exon 10	5'- TTACTGCACAGGTGGCTCC
PCR4	Exon 12	5'- TAATGGAGAGCCAGACGGTG
	Exon 13, 14	5'- TCTAGTCCCATGTCGTCGTC
	Exon 15	5'- AGATGCCTTAGCCTTGTGAG

tin2 include plural exons as shown in Figure 1A and 1B, oligonucleotides for sequence of each exon in β-arrestin2 were designed (Table 2). Each exon was sequenced using a fluorescent dye terminator reaction (BigDye Terminator v1.1 cycle sequencing kit, Applied Biosystems, Foster City, CA, USA) on the ABI Prism 3730 genetic analyzer (Applied Biosystems). The sequences of the coding region of β-arrestin1 and β-arrestin2 genes obtained from CSA patients

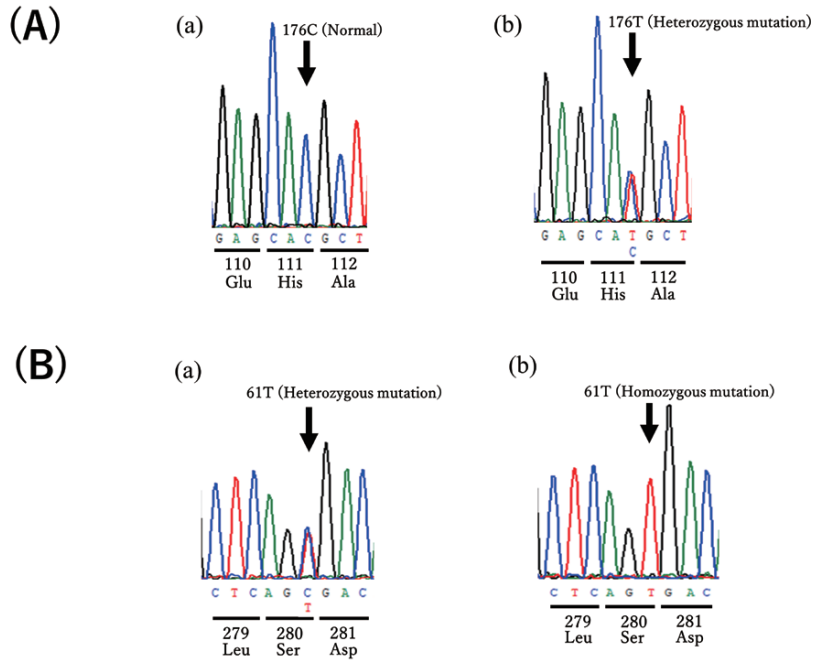

Figure 3. Sequence patterns of human β-arresutin1 and β-arresutin2 genes. (A) Sequence pattern from codons 110 to 112 of human β-arresutin1 gene. Normal (a) and heterozygous mutation (b) at nucleotide position 176 in exon 5. Glu: Glutamine, His: Histidine, Ala: Alanine. (B) Sequence pattern from codons 279 to 281 of human β-arresutin2 gene. Heterozygous (a), and homozygous mutations (b) at nucleotide position 61 in exon 11. Leu: Leucine, Ser: Serine, Asp: Aspartic Acid.

and control subjects were compared with those of β-arrestin1 (ENST00000420843.7) and β-arrestin2 (ENST00000269260.7) genes previously reported.

Statistical analysis

The incidence of gene mutation between CSA patients and control subjects was compared using chi-square test. Statistical analysis was performed with JMP 14.0.0 software (SAS, Cary, NC, USA) and p-value <0.05 was considered as statistically significant.

Results

In β-arrestin1, single base heterozygous substitution (C to T) at nucleotide position 176 in exon 5 was detected in 2 of 20 CSA patients, whereas no substitution was detected in 5 control subjects (Figure 3A). We subsequently performed an association study involving 50 CSA patients and 50 control subjects. This substitution was detected in 11 of 50 CSA patients (22%) and 8 of 50 control subjects (16%), and no difference was found in the incidence of the mutation between the two groups (p=0.44). This substitution was a well-annotated SNP (rs877711) and no amino acid replacement was occurred (His111His, silent mutation). In β-arrestin2, single base substitution (C to T) at nucleotide position 61 in exon 11 was detected. This substitution was homozygous in 13 and heterozygous in 7 of 20 CSA patients, whereas all homozygous in 5 control subjects (Figure 3B). This substitution was also a well-annotated SNP (rs1045280) and did not cause amino acid replacement (Ser280Ser, silent mutation).

Discussion

The prevalence and characteristics of CSA

are different between Japanese population and Caucasians[16], indicating that genetic factors may be involved in the pathogenesis of CSA. CSA is caused by spontaneous hypercontraction of the coronary artery, which is attributed to dysfunctions of endothelial cells (ECs) and/or VSMCs[17, 18]. Nitric oxide (NO) acts as strong vasodilator via relaxing VSMCs and plays a key role in regulating coronary artery tone[19]. NO is synthesized by endothelial NO synthase (eNOS) in ECs[20], therefore, eNOS dysfunction by gene mutation was focused as a critical factor for CSA. In fact, Glu298Asp variant in eNOS gene was reported to be associated with CSA[21]. Furthermore, T786C mutation in the 5'-flanking region reduced promoter activity of eNOS gene, and caused CSA in Japanese patients[22, 23] and in white Americans[24]. Regarding VSMCs, we found Arg257His variant in PLC-δ1 gene obtained from CSA patients, leading to enhancement of PLC enzymatic activity and coronary spasm induction in VSMC-specific variant PLC-δ1 overexpressing mice[4, 5]. These findings indicate that genetic alterations cause dysfunctions of ECs or VSMCs, which potentially lead to coronary spasm.

β-arrestin is reported to regulate muscle contraction, especially in VSMCs and airway smooth muscle cells[9, 14, 25-28]. Furthermore, down-regulation of either β-arrestin1 or β-arrestin2 inhibited shear stress-induced eNOS phosphorylation in human saphenous vein ECs[25]. From these findings, we hypothesized that β-arrestin might be involved in the pathogenesis of CSA. To investigate this hypothesis, we performed mutational analysis of β-arrestin1 and β-arrestin2 genes in CSA patients and control subjects. We found single base substitutions in β-arrestin1 and β-arrestin2 genes with no amino acid replacement and no difference in its ratio between CSA patients and control subjects. Therefore, our results indicate that alternation of primary structure of β-arrestins may not be involved in the pathogenesis of CSA. Since β-arrestins act as a scaffold in the regulation of various intracellular signaling in VSMCs and ECs, further studies including expression and functional assays of β-arrestins are required.

In conclusion, no significant gene mutation in β-arrestin1 and β-arrestin2 was detected in Japanese CSA patients in the present study. Abnormality of the primary structure of β-arrestins may not be involved in the pathogenesis of CSA in humans.

Conflicts of interest

All authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgements

Drs. Hirofumi Tomita and Kenji Hanada have received research grant support from JSPS KAKENHI Grant Numbers JP19K08530 and JP18K15875), and Hirofumi Tomita has received research grant support from The Uehara Memorial Foundation.

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