Mutational analysis of Kir6.1 in Japanese patients with coronary spastic angina

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Abstract. The etiology of coronary spastic angina (CSA) remains uncertain. Mice lacking the gene encoding the inwardly rectifying K^+ channel Kir6.1 were developed as an animal model of CSA. We investigated whether mutation in the coding region of the Kir6.1 gene is detected in Japanese patients with CSA. The study population included 19 Japanese patients with CSA (10 men and 9 women with a mean age of 61±14 years). Mutational analysis of the coding region of Kir6.1 was performed by direct sequencing. We found no missense or nonsense mutations in these samples, but we found in one female CSA patient, a single base substitution (C to T) at nucleotide position 111 in exon 2 of the coding region, which was heterozygous and did not cause amino acid substitution (Ile37Ile, silent mutation). In the remaining 18 patients, no base substitution was detected in the coding region of the Kir6.1 gene. No mutation that alters primary structure of Kir6.1 was detected in Japanese patients with CSA. The results indicate that abnormality in the primary structure of Kir6.1 may not be involved in the genetic pathogenesis of CSA in humans.

Introduction

Coronary artery spasm plays an important role in the pathogenesis of coronary spastic angina (CSA) (1,2) and acute coronary syndromes (3,4). We and other investigators showed that the basal vasomotor tone of the entire coronary artery system of Japanese patients with CSA is enhanced (5-7). Although the underlying mechanisms for its enhancement remain uncertain, racial differences in the coronary artery vasomotor reactivity between Japanese and Caucasian have been pointed out (8). Japanese patients with CSA or myocardial infarction have coronary arteries hyperreactive to constrictive stimuli compared with Caucasian patients despite

no apparent differences in risk factors (9). These findings suggest that genetic factors may be involved in the pathogenesis of the enhanced coronary artery smooth muscle contraction.

A recent study showed that the inwardly rectifying K^+ channel Kir6.1-containing K_{ATP} channels is critical in the regulation of vascular tone, that the disruption of Kir6.1 in mice causes coronary vasospasm, and that Kir6.1-deficient mice were developed as an animal model of CSA (10). However, no mutation in the coding region of the Kir6.1 gene was detected in 18 Italian patients with impaired coronary vasomotility (11). Since CSA is more common in Japanese than in Caucasian, we investigated whether mutation in the coding region of the Kir6.1 gene is detected in Japanese patients with CSA and whether it causes abnormality in the primary structure of Kir6.1.

Materials and methods

Study patients. The ethics committee of our institution approved the study protocol. Written informed consent was obtained from all patients before the study. This study population included 19 Japanese patients with CSA (10 men and 9 women with a mean age of 61±14 years). The coronary arteriographic study was performed in all patients. Coronary spasm, defined as total or subtotal occlusion or severe vasoconstriction of the coronary artery associated with chest pain and ischemic ECG change, was induced with intracoronary injection of acetylcholine in 15 CSA patients. In the remaining 4 patients, ST segment elevation was recorded on the electrocardiogram during a spontaneous attack. After intracoronary injection of isosorbide dinitrate, the coronary arteriograms revealed normal or almost normal coronary arteries with diameter stenosis <50% of the lumen diameter in all patients.

Extraction of genomic DNA and direct sequencing of Kir6.1 gene. Genomic DNA was extracted from the whole blood using the QIAamp DNA blood kit (Qiagen). As shown in Fig. 1, the coding region of the Kir6.1 gene is divided into two exons (exon 2 and 3) located downstream of the first non-coding exon. It was amplified by polymerase chain reaction (PCR) using 3 sets of primers (Table I). Each PCR reaction contained the following: 1X Ex Taq buffer, 200 μM dNTP mixture, 500 nM primers, 0.025 U/μl Takara Ex Taq, and

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Table I. Primers used for direct sequencing of the Kir6.1 gene.

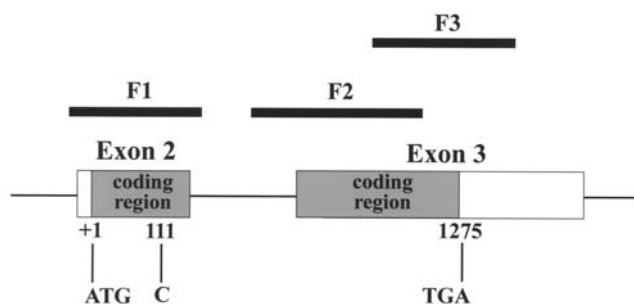| Fragment		Sequence	Amplicon size	Annealing temperature
F1	Sense	5'-AATACAAGGACCCAAGAGG	683 bp	56˚C
	Antisense	5'-ATACTTACTGTGTTAGGTGTTG		
F2	Sense	5'-ACGCAATAAATAATAAACATC	1039 bp	53˚C
	Antisense	5'-ACAATGGACACAAAGCGGT		
F3	Sense	5'-ATCATTAGTGCCTCTGTGC	888 bp	56˚C
	Antisense	5'-TTGAGAGAAACGAGCATAC		

Figure 1. Structure of the coding region of the Kir6.1 gene. Three PCR fragments are shown. The A of ATG translation initiation site is used as nucleotide +1.
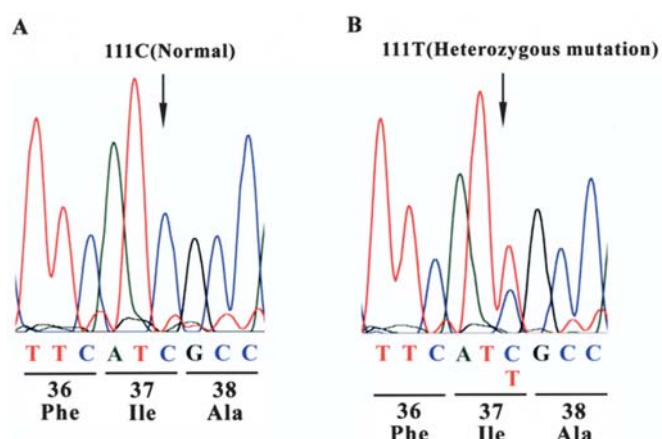

Figure 2. Sequence pattern from codons 36 to 38 of the human Kir6.1 gene. Normal (A) and heterozygous (B) conditions at nucleotide position 111 in exon 2. Phe, Phenylalanine; Ile, Isoleucine; and Ala, Alanine.

40-80 μg 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 5 min), 30 cycles of denaturation (94˚C for 45 sec), annealing (temperature indicated in Table I for 45 sec) and extension (72˚C for 45 sec) followed by a final extension at 72˚C for 7 min on the GeneAmp PCR system 9700 (Perkin-Elmer). All PCR products were purified using Rapid PCR purification system (Marligen, Germany). Both strands of each amplicon were sequenced using a fluorescent dye terminator reaction (BigDye Terminator v1.1 cycle sequencing kit, Perkin-Elmer) on the ABI Prism 310 genetic analyzer (Perkin-Elmer). The sequences of the coding region of the Kir6.1 gene obtained from CSA patients were compared with that of the Kir6.1 gene previously reported (NCBI NM_004982) (Fig. 2A).

Results

Genomic DNA obtained from 19 CSA patients was analyzed. As shown in Fig. 2B, single base substitution (C to T) at nucleotide position 111 in exon 2 of the coding region was detected in one female CSA patient. This substitution was heterozygous and did not cause amino acid substitution (Ile37Ile, silent mutation). In the remaining 18 patients, no base substitution was detected in the coding region of the Kir6.1 gene.

Discussion

K_{ATP} channels are hetero-octameric complexes which consist of four pore-forming inwardly rectifying K+ channel (Kir6.x)

and four regulatory sulfonylurea receptor subunits (SURx), a member of the ATP-binding cassette protein family (12,13). K_{ATP} channel in human coronary artery smooth muscle (HCASM) is composed of Kir6.1 and SUR2B subunits, and is essential in maintaining basal coronary vascular tone (14,15). Kir6.1 and SUR2 knockout mice cause coronary vasospasm leading to sudden cardiac death (10,16). These findings raise the possibility that a loss of function of K_{ATP} channels may play a pivotal role in the pathogenesis of CSA in humans. However, no mutation in the coding region of the Kir6.1 gene was detected in 18 Italian patients with impaired coronary vasomotility (11). Moreover, we could not find any amino acid substitution in the primary structure of Kir6.1 in Japanese patients with CSA. These findings suggest that abnormality in the primary structure of Kir6.1 may not be involved in the genetic pathogenesis of CSA in humans. Genetic analysis of SUR2B subunit is also required to clarify the pathogenesis of CSA associated with K_{ATP} channels.

We found a heterozygous single base substitution in exon 2 of the Kir6.1 coding region in one female Japanese patient with CSA. Although this mutation does not cause abnormality in the primary structure of Kir6.1, it is possible that the substitution is in linkage disequilibrium with the known or unknown genes which are associated with the patho-genesis of CSA. Gender difference in the incidence of CSA is also well known, and CSA occurs much more frequently in

n in females. Although its underlying mechanisms uncertain, several candidate gene polymorphisms associated with CSA differ between Japanese men and women (17). Gender also appears to affect the expression of Kir6.x subunit (18). However, no gender difference in the primary structure of Kir6.1 was shown in the present study. Further large scale association studies compared with control subjects are needed to clarify whether this mutation is associated with the pathogenesis of CSA.

We recently showed that the activity of phospholipase C (PLC) in the cultured skin fibroblast obtained from the Japanese CSA patients is enhanced, and that the $\delta 1$ isoform is the major PLC isozyme detected in the membrane fraction (19). Moreover, the incidence of amino acid substitution from arginine to histidine (Arg257His) in PLC-$\delta 1$ is higher in patients with CSA than in male control subjects, and this variant is associated with the enhancement of PLC-$\delta 1$ activity (20). It should be noted that receptor-mediated activation of PLC inhibits K_{ATP} channel activity (21,22). These observations suggest that gene or protein expression of Kir6.1 might be altered in HCASM obtained from patients with CSA.

In conclusion, the present study provides evidence that no mutation that alters primary structure of Kir6.1 was detected in Japanese patients with CSA. Taken together with a previous study (11), abnormality in the primary structure of Kir6.1 may not be involved in the genetic pathogenesis of CSA in humans. Further expression analysis of Kir6.1 and exploration of genetic variants in additional candidate genes for CSA are required to identify the pathogenesis of CSA.

Acknowledgements

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