GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

MEF2A gene mutations and susceptibility to coronary artery disease in the Chinese population

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Genet. Mol. Res. 13 (4): 8396-8402 (2014)
Received August 14, 2013
Accepted December 16, 2013
Published October 20, 2014
DOI http://dx.doi.org/10.4238/2014.October.20.15

ABSTRACT. We investigated mutations and polymorphisms of the coronary artery disease (CAD)-related myocyte enhancer factor 2A (*MEF2A*) gene in a Chinese population. Polymerase chain reaction-single-strand conformation polymorphism and DNA sequencing were used to detect exon 11 of the *MEF2A* gene in 210 Hubei patients with CAD and 190 healthy controls. The following mutations were identified: a) a synonymous heterozygous mutation (147191G→T) combined with a 6-base deletion (147123-147128); b) a synonymous heterozygous or homozygous mutation (147191G→T) combined with a 9-base deletion (147123-147131); c) a synonymous mutation (147143G→A); d) a synonymous mutation (147191G→T) combined with an 18-base deletion (147111-147128); and e) a 21-base deletion (147108-147128). Mutations (a) and (b) and a 3-base deletion (147126-147128) with

or without the synonymous heterozygous mutation (147191G→T) occurred in more than 1% of controls. However, mutations (c), (d), and (e) were not observed in the control group. The polymorphism in exon 11 of the *MEF2A* gene was observed in the Chinese population. Six or seven amino acid deletions and synonymous mutations (147143G→A) may be correlated with susceptibility to CAD.

Key words: Coronary artery disease; *MEF2A*; Gene mutation; Polymorphism

INTRODUCTION

Coronary artery disease (CAD) has high morbidity and mortality rates worldwide. Thus, the pathogenesis of CAD has long been the focus of medical studies. Myocyte enhancer factor 2A (*MEF2A*) was first discovered as a CAD-related gene by Wang (2005) and Wang et al. (2003, 2005). Three mutation points in exon 7 of *MEF2A* were subsequently identified by Bhagavatula et al. (2004); however, Altshuler and Hirschhorn (2005) and Weng et al. (2005) predicted that the *MEF2A* gene lacked mutations. Zhou et al. (2006a,b) analyzed the mutations and polymorphisms in exons 7 and 11 of the *MEF2A* gene in the Han population in Beijing, and various rare mutations were found in exon 11 rather than in exon 7. The clinical significance of specific 21-bp deletions in *MEF2A* was also explored, and previous studies have shown mixed results. In this study, polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and DNA sequencing were used to detect exon 11 of the *MEF2A* gene in samples collected from 210 CAD patients and 190 healthy controls and to investigate the function of the *MEF2A* gene in CAD pathogenesis and their correlation.

MATERIAL AND METHODS

Subjects

A total of 210 patients, including 160 males and 50 females, 39-77 years old (average, 50.4 ± 10 years), were selected to participate in the study. Another group consisting of 130 healthy elderly males and 60 healthy elderly females with an average age of 60 ± 10 years were recruited as controls. Patients from Hubei, Heilongjiang, Henan, and Hunan Provinces were screened and investigated according to the following diagnostic criteria for CAD and myocardial infarction (MI): presence, frequency, times, inducing factors, relieving factors, durations, and electrocardiogram characteristics of chest stuffiness and pain; family history of CAD; history of alcohol and tobacco use; blood lipid status; and coronary angiography (CAG) results. The final diagnostic indices for positive cases were as follows: 1) MI patients; and 2) coronary stenosis severity ≥70%, as shown by CAG combined with myocardial ischemia (ST-T change and/or stenocardia). The following criterion was used for suspected CAD: coronary stenosis severity ≥50 and <70%, as indicated by CAG. All cases were in accordance with the World Health Organization (WHO) diagnostic criteria of 1979 for ischemic heart disease, and 90% of cases were consistent with the criteria for clinical diagnosis and investigation. This

study was conducted in accordance with the Declaration of Helsinki and was conducted with approval of the Ethics Committee of Wuhan University of Science and Technology. Written informed consent was obtained from all participants.

Primers

The forward and reverse primer sequences for exon 11 of the *MEF2A* gene were 5'-TGCAGAGGTACTTGCAAGCC-3' (forward), 5'-ACTGTCCACAGGGGAGCG-3' (reverse); and 5'-CAGGAAATGGGGCGCTC-3' (forward), 5'-AGATATGTAGGGCAGGTCA-3' (reverse). The following sequencing primers were used: 5'-TGCAGAGGTACTTGCAAGCCAT-3' (forward) and 5'-AGATATGTAGGGCAGGTCACT-3' (reverse). PCR-SSCP product lengths were 257, 241, and 487 bp, respectively. Primers were designed and synthesized by Wuhan Berger Biological Technology Co., Ltd. (Wuhan, China). Sequencing primer sequences were provided by Dr. Wang from the Center for Molecular Genetics in the Department of Molecular Cardiology at Cleveland University and were synthesized by Shanghai Biological Engineering Co., Ltd. (Shanghai, China).

Template preparation

The genomic DNA template was extracted from leukocytes in human femoral arterial blood and peripheral venous blood using Triton-proteinase K (Ameresco, Framingham, MA, USA). The template was stored at -80° or -20°C until further study.

PCR

PCR (Applied Biosystems GeneAmp PCR 9700, USA) was carried out in a 25-μL reaction volume comprising 18.75 μL sterilized double-distilled water, 2.5 μL $MgCl_2$ buffer, 1 μL 10 μM dNTP (Shanghai Jingmei Biological Technology Co., Ltd., Shanghai, China, and Beijing Jiehui Biological Technology Co., Ltd., Beijing, China), 1 μL template, 0.625 μL 10 μM forward and reverse primers, and 1 U *Taq* DNA polymerase (Biostar, Canada and TaKaRa Bio Group; Japan) under the following PCR conditions: 1) 95°C for 5 min; 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; followed by 30 cycles and then elongation at 72°C for 7 min for the first pair of primers; 2) 94°C for 5 min; 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s; followed by 30 cycles and then elongation at 72°C for 7 min for the second pair of primers. The PCR volume of sequencing primers was doubled for all ingredients. The following PCR profile was used: 95°C for 5 min; 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 30 cycles and then elongation at 72°C for 7 min.

SSCP analysis

Vertical neutral 11% polyacrylamide gel electrophoresis was performed (polyacrylamide:methylene acrylamide, 29:1 g). Four microliters of each PCR product and denaturing loading buffer were mixed and denatured at 98°C for 5 min. The mixture was immediately transferred to an ice bath for 8-10 min, and loaded onto the gel. Vertical electrophoresis was performed in a 4°C fridge with a constant voltage of 600 V for 2.5 h.

Silver staining and imaging

The gel was fixed in 10% acetic acid for 30 min, followed by 3 washes in distilled water for 2 min. Next, the gel was stained in 0.1% silver nitrate for 30 min and washed again in distilled water for 20 s. After staining, the gel was placed into a mixed solution of 1.5 μL/mL formaldehyde and 2.8% sodium carbonate until the bands were fully developed. Finally, the gel was fixed in 10% acetic acid for 10 min to terminate the reaction. The gel was washed in distilled water for 1 min and photographed for further analysis. A change in the location of a single band or the band number compared with the control indicates a base pair mutation in a certain patient sample.

Direct DNA sequencing

Purified PCR products of abnormal samples screened by PCR-SSCP analysis were sequenced bidirectionally using DNA sequencing (3730 BigDye Terminator, Shanghai United Cell Biotechnology Co., Ltd., Shanghai, China).

RESULTS

PCR-SSCP analysis

Compared with the negative sequencing results of control samples, abnormal bands were observed following SSCP electrophoresis in the PCR product of exon 11 from healthy controls or patients. The location of a single band or the number of bands had changed, indicating potential *MEF2A* mutations (Figure 1).

Figure 1. PCR-SSCP electrophoresis. *Lanes 1-8* = results from healthy subjects; *lane 9* = negative control; *lanes 10-14* = results from CAD patients; *lanes 1*, *2*, *5-7*, *10*, and *12-14* = mutated samples.

Direct DNA sequencing

The following multiple base changes in CAD patients were observed in partial samples after DNA sequencing: a) a synonymous heterozygous mutation (147191G→T, GGG→GGT, codes for glycine) combined with a 6-base deletion (147123-147128, CAGCAG) that caused a deletion of 2 amino acids (429QQ430); b) a synonymous heterozygous or homozygous mutation (147191G→T) combined with a 9-base deletion (147123-147131, CAGCAGCCG)

resulting in the deletion of 3 amino acids (429QQP431); c) a synonymous mutation (147143G→A, CCG→CCA, codes for proline); d) a synonymous mutation (147191G→T) combined with an 18-base deletion (147111-147128, CAGCAGCAGCAGCAGCAG) that caused the deletion of 6 amino acids (425QQQQQQ430); and e) a 21-base deletion (147108-147128) resulting in the deletion of 7 amino acids (424QQQQQQQ430). Mutations (a) and (b) were observed in more than 1% of subjects. Three-base deletions (147126-147128, CAG) resulting in a 1-amino acid (430Q) deletion combined with or without the synonymous heterozygous mutation (147191G→T, GGG→GGT, codes for glycine) were also observed in more than 1% of healthy controls. However, mutations (c), (d), or (e) were not observed in the control group. Thus, the following changes occurred: 1) codon 451G/T (147191) heterozygous or homozygous synonymous mutation (Figure 2A, B, and C); 2) deletion of 1, 2, 3, 6, and 7 amino acids (147108-147131, 424QQQQQQQP431) (Figure 2D, E, F, G, and H); and 3) codon 435G/A (147143) heterozygous mutation (Figure 2I, J, K, and L). A synonymous heterozygous mutation (147143G→A) and the deletion of 6 or 7 amino acids were only observed in CAD patients (CAG repeat sequence was based on 11 CAG repeats).

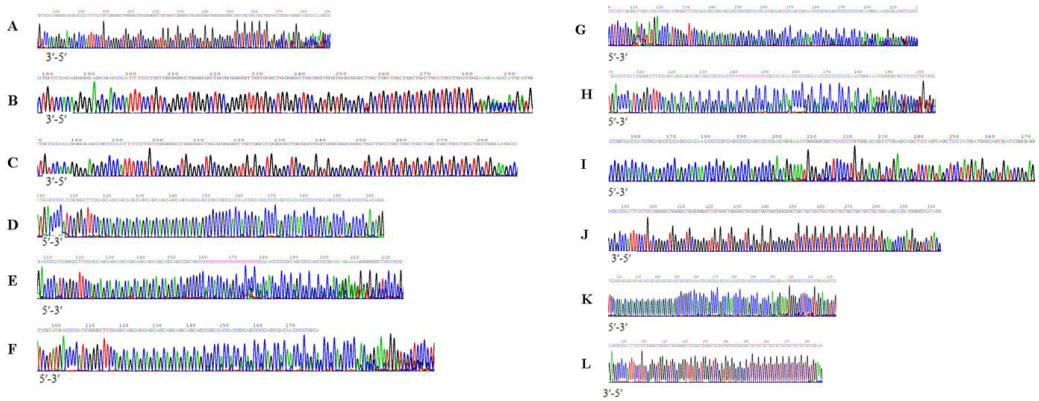

Figure 2. DNA sequencing results. **A.-C.** Codon 451G/T (147191) heterozygous or homozygous mutation; **A.** 451 G/T heterozygous mutation (GGG/GGT); **B.** 451 G/T homozygous mutation (GGG/GGT). **C.** Wild-type codon 451 (GGG/GGG); **D.-H.** 1-, 3-, 6-, and 7-amino acid (147108-147131, 424QQQQQQQP431) deletion; **D.** wild-type (11 CAG repeats); **E.** 3-base deletion (147126-147128, CAG) that resulted in 1-amino acid (430Q) deletion (10 CAG repeats); **F.** 9-base deletion (147123-147131, CAGCAGCCG) that caused 3-amino acid (429QQP431) deletion (9 CAG repeats); **G.** 18-base deletion (147111-147128, CAGCAGCAGCAGCAGCAG) that led to 6-amino acid (425QQQQQQ430) deletion (5 CAG repeats); **H.** 21-base deletion (147108-147128) that resulted in 7-amino acid (424QQQQQQQ430) deletion (4 CAG repeats); **I.-L.** codon 435G/A (147143) heterozygous mutation; **I. J.** codon 435G/A (CCG/CCA) heterozygous mutation; **K. L.** wild-type (CCG/CCG).

DISCUSSION

CAD, a common disease in China, is induced by multiple factors, such as genetics, the environment, and lifestyle. Thus, a multi-faceted approach is necessary in the study of CAD pathogenesis, particularly in molecular biology research, which is important for developing comprehensive treatment of CAD based on gene therapy.

The *MEF2A* gene was first identified as a CAD-related gene through linkage analysis

of a large family with CAD (9 of 13 patients developed MI) in 2003.

In this study, we found the following mutations: 1) codon 451G/T (147191) heterozygous or homozygous mutation; 2) loss of 1 (Q), 2 (QQ), 3 (QQP), 6 (425QQQQQQ430), and 7 (424QQQQQQQ430) amino acids (147108-147131); and 3) codon 435G/A (147143) heterozygous mutation. Among these mutations, the synonymous mutation at locus 147191 was confirmed by reference to the National Center for Biotechnology Information (NCBI) database to be a single nucleotide polymorphism, which was also demonstrated in our study by the extensive presence of this polymorphism in healthy controls. However, the heterozygous mutation at locus 147143 was only found in the genomes of CAD patients, and was therefore identified as a mutation.

Given that *MEF2A* is a CAD-related gene, the results of various studies are controversial among several countries. Weng et al. (2005) screened gene mutations in exon 11 of the *MEF2A* gene from 300 CAD patients and 1500 healthy controls. They hypothesized that the changes in 5-12 CAG repeats are genetic polymorphisms and that the 21-base deletion in exon 11 of the *MEF2A* gene did not induce autosomal dominant genetic CAD. Gonzalez et al. (2006) suggested that the CAG repeat polymorphism was independent of MI susceptibility in Spanish patients. Kajimoto et al. (2005) reported that the CAG repeat sequence was not correlated with MI susceptibility in Japanese patients. Horan et al. (2006) also found that the CAG repeat sequence was not associated with the susceptibility to early-onset familial CAD in an Irish population. Hsu et al. (2010) identified no correlation between the CAG repeat sequence and CAD susceptibility in the Taiwanese population. Dai et al. (2010) found that the structural change in exon 11 was not related to CAD in the Chinese Han population. Lieb et al. (2008) and Guella et al. (2009) hypothesized that *MEF2A* was independent of CAD. However, Yuan et al. (2006) and Han et al. (2007) suggested that the CAG repeat sequence was correlated with CAD because 9 CAG repeats was an independent predictor of CAD. Elhawari et al. (2010) and Maiolino et al. (2011) suggested that *MEF2A* is a susceptibility gene for CAD. Dai et al. (2013) showed that mutations in exon 12 are associated with the early onset of CAD in the Chinese population. Liu et al. (2012) failed to demonstrate a correlation between the CAG repeat sequence and CAD through case-control analysis, systematic review, and meta-analysis, but found that the 21-base deletion in exon 11 was strongly associated with CAD, and that genetic variations in *MEF2A* may be a relatively rare, but specific, pathogenic gene for CAD/MI. Kajimoto et al. (2005) reported 4-15 CAG repeats. However, only 4-11 CAG repeats were observed in our study, possibly because of genetic differences in patients in this study. Eleven CAG repeats were observed in most samples from the control group, and the proportion of 10, 9, and 8 repeats exceeded 1%. The heterozygous mutation at 147143, as well as the 4 and 5 CAG repeats, was only observed in CAD patients. Thus, we speculated that the CAG repeat sequence is correlated with CAD susceptibility, and the presence of 4 or 5 repeats may be a risk factor for CAD, which was inconsistent with the results obtained by Han et al. (2007). The inconsistency in these results may be explained by the differences in subjects and sample sizes among studies. The small sample size in our study may have resulted in the observed deviation. Thus, more samples are required for further confirmation. Whether the degree of change in CAG repeats is related to CAD severity requires further investigation.

CAD is a multi-factorial disease with pathogenic factors that are difficult to identify, which explains the controversy in the reports.

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