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Original Article

Prevalence of calreticulin exon 9 mutation in Iranian population with cardiovascular disease

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Abstract

Introduction: Calreticulin (CALR) is a 46 kDa protein in the endoplasmic reticulum and is one of the major proteins in ca2+ binding; it has a key role in oxidative stress, transcription factor activation, and as a chaperone in newly synthesized protein and glycoprotein folding. The high expression of CALR is pivotal for cardiac development in the embryonic period. It has been showed that mutation in exon 9 of CALR causes loss of C-terminal function and contributes to cardiovascular disease (CVD) development. It could conceivably be hypothesized that in addition to the general risk factors, the specific gene defects which are less considered can contribute to CVD development. In this regard, this study determined the possible existence of CALR mutations in CVD development in patients younger than 40.

Methods: Thirty patients younger than 40 were recruited for this study, 86.7% (26) were male, and just 13.3% (4) were female. The amplification refractory mutation system- polymerase chain reaction (ARMS-PCR) was used to identify mutation in exon 9. The CVD risk factors, including blood pressure, type 2 diabetes, dyslipidemia, history of smoking, alcohol drinking, and familial CVD development were evaluated.

Results: In none of the patients, CALR mutations were detected. Since CALR defect causes accumulation of glycogen in the heart's cells and contributes to CVD development, our results confirm this, so that 76.7% of patients did not have diabetes.

Conclusion: The findings of the current study show there is no significant differences between exon 9 CALR mutation and CVD development.

Introduction

The calreticulin (CALR) was separated for the first time by Michalak et al in 1974.[1] CALR is a 46 kDa protein in the endoplasmic reticulum that is one of the major proteins in ca^{2+} binding. Additionally, it plays a role in oxidative stress, transcription factor activation, and as a chaperone in newly synthesized protein and glycoprotein folding.[2-4] So, the CALR defect is accompanied by a spectrum of the disorder, including heart failure, cancer, brain, and metabolic disorder.[3] In the embryonic period, CALR has a high expression, and a pivotal role in tissue development, including heart, brain, and liver, but its expression suppresses after birth.[3] Additionally, to low CALR expression in the embryonic period, the high CALR expression after birth causes cardiac hypertrophic.[3] Since CALR has an essential role in cardiac development, defect in its expression (decrease or increase) is accompanied by a wide spectrum of cardiovascular disease (CVD). Different kinds of CALR mutations were added to the diagnostic panel in some laboratories. Type 1 deletion in exon 9 (52-bp deletion; c.1092_1143del) and type 2 insertion in exon 9 (5-bp insertion; c.1154_1155insTTGTC) in CALR were considered of pathologic significance.[5]

There is considerable literature about risk factors, including smoking, obesity, diabetes, familial history, and etc. in the incidence trend of CVD development in younger patients. Despite the progression in early diagnosis of CVD and awareness about cardiovascular risk factors, the age of CVD incidence has declined.[6] Hence, it could conceivably be hypothesized that in addition to the general risk factors, the specific gene defects that are less considered can contribute to this condition. In this line, the specific objective of current study is to determine the possible existence of CALR mutations in CVD development in patients younger than 40.

Methods

Study population

With aging, the risk factors for developing CVD increase, so that the presence of gene defects in these subjects has the least likely risk. To address these interfering factors, subjects with younger 40 were included. Since the

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frequency of non-sever stenosis is more prevalent than sever, approximately two-thirds of myocardial infarction occur in non-sever stenosis; and patients with lower 50% vascular stenosis usually are without any significant clinical symptoms at rest (i.e., chest pain), unless the plaque is unstable or be raptured, hence in the current survey, stenosis higher than 50% in CT angiography was considered as one of the main inclusion criteria. Disrupted echocardiography and electrocardiography were not considered because each cardiac pain does not necessarily accompany pathological heart function, like ejection fraction reduction and ischemic mitral regurgitation.

All patients were investigated for underlying disease. The traditional CVD risk factors include hypertension, type 2 diabetes, dyslipidemia, a history of smoking, alcohol, and familial CVD were evaluated. All patients signed an informed consent form before testing.

CALR mutation analysis

Mutation in exon 9 was identified by the amplification refractory mutation system- polymerase chain reaction (ARMS-PCR). The primers used for CALR mutations involved CALR 436 bp F, 5′AAGCAAGGGCTATCGGGTAT3′; CALR436-bp R, 5′ GCCTCTCTACAGCTCGTCCTT 3′.

DNA extraction

The genomic DNA was extracted from the blood based on the FAVORGEN manufacturer's protocol (Biotech Corp, Cat. No.: FABGK 001, Taiwan). The DNA samples were analyzed for concentration and quality by using Thermo NanoDrop One (Thermo Fisher, USA) with a concentration of 100-200 ng/μL and 1.8-2.0 ratio in 260/280 nm.

PCR reaction

The PCR reaction was carried out by FlexCycler Thermocycler as following: 12.5 μL Master Mix 2X (Taq Mix Red, PCR bio, UK), 2 μL DNA (100ng), 1 μL forward primer. 1 μL reverse primer, and H2O up to a final volume of 25 μL. The PCR reaction was performed as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 60 sec, and 72°C for 5 min the final extension. Electrophoresis was done by 2% agarose gel in 1X TBE buffer. The gel was run for 60 minutes in 100 voltage (PAYA PAZHOOHESH, Iran).

Sequencing

Following conformational PCR reactions, it was necessary to sequenced PCR products. For this aim, Sanger sequencing was carried out. All samples were sequenced by ABI-3130 XL (USA). The result of the sequences data was visualized by UGENE software.

Statistical analysis

Chi-square test was used to determine the relationship between qualitative variables between the case and control data. The odds ratio (OR) with a 95% confidence interval (CI) was calculated. A p-value was considered less than 0.05 (P<0.05). Data management and analysis were performed using SPSS software (V24).

Results

Thirty-four patients were included; 4 patients were excluded due to a lower 50% stenosis in CT angiography. 86.7% (26) of patients were male, and just 13.3% (4) were female. 86.7% (26) of participants had coronary artery stenosis more than 50%, and just 13.3% (4) had moderate coronary artery stenosis. In none of the patients, CALR mutations were detected. The clinical and characteristics of patients are shown in Table 1.

Discussion

CALR gene is located on chromosome 19p13.2 and contains 9 exons and has a pivotal role in cellular functions include quality control of newly synthesized proteins, regulation of cell adhesion, chaperone activity, and modulation of steroid-mediated gene expression. CALR is one of the essential members in the Ca^{2+} dependent pathway in the cardiovascular system. For cardiac development, the high expression of CALR is necessary for the embryonic period.[7] In this regard, in vitro studies have shown that mutated CALR is accompanied by cardiac disruption.[8]

The C-terminal of CALR is essential for muscle contraction, signaling, and gene expression.[9] Mutation in exon 9 in CALR causes synthesized mutated protein with novel C-terminal, which loss the function of C-terminal and Ca^{2+} binding capacity. NF-AT and GATA-4 transcription factors have a crucial role in cardiogenesis.[10,11] CALR,

Table 1. Patients' characteristics

Patients' characteristics	No. (%)
Male sex	26 (86.7)
History of smoking	10 (33.3)
History of alcohol consumption	1 (3.3)
History of CVD familial	6 (20)
Type 2 diabetes	7 (23.3)
Significant coronary artery stenosis	26% (86.7)
Hypertension	9 (30)
Dyslipidemia	7 (23.3)
Thyroid disorder	2 (6.7)
Renal disorder	3 (10)
Gastrointestinal disorder	1 (3.3)
Calreticulin exon 9 mutation	0 (0)

CVD; cardiovascular disease.
Stenosis of the coronary arteries was considered when lumen narrowing was 50%, hypertension greater than 140 mm Hg for systolic and greater than 90 mm Hg for diastolic considered for treatment.

by regulating Ca^{2+} release from inositol three phosphate, regulates activation of NF-AT transcription factor.[7,12] It was demonstrated that mutation in CALR with disrupting the NF-AT and GATA-4 signaling pathway contributes to cardiac dysfunction.[4,7,11]

It is possible to hypothesize that CVD development in younger patients is likely due to gene defects. For this purpose, 30 patients younger than 40 with CVD were investigated for CALR mutations. Our finding with no CALR mutations in CVD is in agreement with Jaeger et al that the incidence of CALR mutation is rare in CVD development.[13] It is in regard with Andreasen. C et al. that in an exome sequencing project, found no association between CALR mutation and hypertrophic cardiomyopathy.[14] CALR3 protein is another member of the CALR family, which has a lower Ca^{2+} binding capacity; nevertheless, the mutation in CALR3 is accompanied by cardiac hypertrophic. Verhagen et al. could not show the association between cardiomyopathy and CALR3 variants.[15]

Diabetes is one of the CVD risk factors; it was shown that CALR defect causes accumulation of glycogen in the heart's cells and contributes to the CVD development[16]; Our results match with this that 76.7% of patients did not have diabetes.

These findings may be somewhat limited by the small sample size, and we cannot exclude CALR mutations entirely from gene defect panels in CVD development. Further investigations with larger samples and evaluating other types of CALR mutations are strongly recommended.

Conclusion

CALR is an essential cardiac embryogenic gene for cardiac development. The finding of the current study is regarding the other investigations that show no significant differences between exon 9 CALR mutation and CVD development. However, CALR mutations and alteration in expression of CALR accounts for complete heart block, embryonic lethal, and dilated cardiomyopathy.

Conflict of Interest

The authors declare no conflict of interest. All procedure performed in studies involving human participants were following the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or a compared ethical strand.

Ethical Approval

The study is based on the approval of the Medical Ethics Committee of Jundishapur Ahvaz University (reference number: CVRC-9806).

Authors' Contributions

HH conceived the manuscript and revised it. MT and NS did experimental analysis and wrote the manuscript, and prepared table. All authors have read and approved the manuscript.

Study Highlights

What is current knowledge?

- CALR has an essential role in cardiac development, defect in its expression (decrease or increase) is accompanied by a wide spectrum of cardiovascular disease

What is new here?

- No significant relation between exon 9 CALR mutation and CVD development.

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Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available in the PubMed repository, [https://www.ncbi.nlm.nih.gov/nuccore/]. The sequences for primer design were extracted from the genomic reference sequence of Ensemble (http://www.ensembl.org).

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