MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

EFFICIENCY OF PRIMER DESIGN TOOLS IN EVALUATION OF TWO MOLECULAR METHODS TO DETECT TWO SINGLE NUCLEOTIDE POLYMORPHISMS RELATED WITH ATHEROSCLEROSIS

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History
Received: 1st June 2019
Accepted: 12th September 2019

Keywords:

Allele specific PCR, Tetra ARMS,CAD,SNPs

Abstract

Atherosclerosis is one of the most important coronary artery disease(CAD) caused by lipid accumulation, hypertension, smoking ,and many other factors such as environmental and genetic factors, its recorded that genetic variations in rs10757278, rs1333049correlated with CAD.In present study,100 blood samples were collected(50CAD patients and 50 appeared to be healthy controls), who referred to Ibn-Albytar general hospital/in Bagdad city for heart disease from February to March 2019.Genotyping for two SNPs rs10757278, rs1333049 were done by Allele specific PCR and Tetra ARMS technique.The results revealed that, Tetra ARMS technique is more accurate to detect the mutant and normal cases than allele specific PCR. In conclusion, Tetra ARMS technique was suggested to be more specific, sensitive and accurate method used as a molecular biology tool to detect genetic polymorphisms.

SHORT COMMUNICATIONS

INTRODUCTION

Atherosclerosis is a reformer ailment of the blood vessels as an evolution of heart disease, an incipient incision in the artery endothelial induced by mechanical, environmental and genetic laborer (Rose, 1999). Cardiovascular diseases are the most important cause of death in the developing countries. Many genetic alterations are known to manipulate coronary artery disease (CAD) (Ganesh*et al*., 2013).

Several studies found a strong correlation between many single nucleotide polymorphisms and many heart diseases (Liu*et al*., 2017). Abundant of genome wide association studies (GWAS) have exposed that rs10757278 polymorphism is connected to heart diseases like atherosclerosis, myocardial infarction; it is located on chromosome 9p21 near the tumor suppressor geneCDKN2A and CDKN2B. Many studies on Caucasians, Asian, European ancestry have reported that there is a significant association with heart diseases (Chen *et al*., 2015; Kathiresan*et al*., 2009; Helgadottir*et al*., 2007).

Globally, there are numerous polymorphisms on chromosome 9p21 locus exist, but only rs1333049 is correlated with coronary artery diseases (CAD), as first conveyed by Samani*et al*. (2007), whom first reported in the German people, the C allele of this single nucleotide polymorphism (SNP) transformed to G nucleotide.

There were many methods that had been used for assessment of SNP, and each of these methods was depended on PCR technique that has a specific primer design (Alina*et al*., 2017). Tetra primer amplification refractory mutation system (T-ARMS-PCR) and Allele specific are two methods to detect SNP and widely used in many research (Tanha*et al*., 2015). Allele specific PCR technique is simple and frugal. Specific primers for this method have only one mismatch nucleotide to produce allele specific reaction (Seri and Rus, 2018), while in Tetra ARMS technique it uses four primers to determine the conventional genotype; 2 outer primers and 2 inner primers to produce allele specific fragments, and it depends on the competition between two primers on the SNP position to produce specific fragments. This fragment can be distinguished by its separation in an agarose gel by electrophoresis (Vieira and Andre´a, 2014). This work aimed to evaluate the primer design techniques for two SNPs in Iraqi patients with atherosclerosis in case control study.

MATERIALS AND METHODS

Patients and samples

Peripheral blood specimens were collected from 100 subject50CAD patients who referred to Ibn-Albytar general hospital for heart disease from February to March 2019 and 50 appeared to be healthy controls with no family history of heart disease. Patients were chosen according to the coronary angiography and electrocardiogram (ECG) criteria.

DNA extraction

DNA was extracted from blood samples using a mammalian genomic DNA extraction kit (Geneaid Biotech), it was checked for integrity by agarose electrophoresis and the purity and concentration were checked by Nanodrop. The extracted DNA was stored at -20 °C until use.

Primer Design

Two methods were used to design primers for two SNPs; each one is available online as primer design tool. For allele specific PCR the online program:

1. http://bioinfo.biotec.or.th/WASP/ used to design the primers in table 1 , and
2. for Tetra ARMS technique the online tool was used http://primer1.soton.ac.uk/primer1.html

The primers are listed in **Table 1.**

The PCR protocol for Allele specific PCR was conventional PCR method by two separated reactions using BIONEER primex PCR ready to use tube, while for Tetra ARMS the multiplex master mix was used to prevent dimer formation and the condition mention in **Table 1**.

Table 1. Primer sequence for SNPs detection

SNP	Type of Technique	Sequence 5--3
rs10757278	Allele specific PCR	WT F GGTGTGGTCATTCCGGTTA MT F GGTGTGGTCATTCCGGTTG Common R TAGCTGAGACGACTTCTGGC
	Tetra ARMS	I F AGGGTGTGGTCATTCCGGGAG I R CTACTCTGTCTTGATTCTGCATCGCTTCT O F CTGAGGTCGCAACTAAAAGCCAAGATT O R CGCTGTTCCCAAGTAGCCAGGATA
rs1333049	Allele specific PCR	WT F ATACTAACCATATGATCAACAGTCC MT F ATACTAACCATATGATCAACAGTCG Common R CTAGCGCAATACCACAGTGAA
	Tetra ARMS	I F CCTCATACTAACCATATGATCAACAGATC I R TCTGCGAGTGGCTGCTTATC O F AAGTAAAAAAAGAATGGGCTGCTG O R TGAGCATAGCTGTAAAACAAAGGG

Statistical Analysis

The results obtained from the two techniques were collected from electrophoresis result of PCR reactions and analyzed by Hardy-Weinberg equilibrium. Alleles and genotypes of rs10757278 and rs1333049gene SNPs were given as percentage frequencies and significant differences between patients and controls were assessed by the two-tailed Fisher's exact probability (p), which was corrected for the number of comparisons that were made (Bonferroni correction). In addition, the odds ratio (OR) and its 95% CI (confidence interval) was also estimated for each allele and genotype. The WinPepi software version 11.65 was used to obtain these estimations.

RESULTS AND DISCUSSION

In our present study, 100 samples were collected and divided into 50 patients with CAD, 50 controls. As shown in **Table 2 and Table 3,** there are statistically differences in calculation between the two methods even if applied on the same samples in the same handling process. The result showed that the Tetra ARMS technique gives accuracy and sensitivity more than allele specific PCR method. In Tetra ARMS the result appeared significant while in another method it wasn't significant. The result also showed that the SNP rs10757278 were more distributed in Iraqi patients with atherosclerosis. In comparison with the result of ECG and triglyceride and cholesterol check (data not shown) the results of Tetra ARMS were more accurate than Allele specific PCR.

Atherosclerosis is one of coronary artery diseases(CAD), caused by deposition of cholesterol in the arteries due to the buildup of plaque, the generality of the dissolutions in the developing world's as a result of (CAD) (Heidari*et al*., 2019). In the present study, two SNPs rs10757278 and rs1333049 have an association with CADas proven by the study with two molecular techniques; Allele specific PCR and Tetra ARMS PCR. The Allele specific PCR method was observed to be low-cost, but less accurate because the mismatch locationare located in the 3' terminus of the amplicon which the target DNA can be genotyped. Tetra ARMS PCR method is considered as a simple, more accurate and high sensitivity as compared to Allele specific PCR (Honardoost *et al*., 2014). Based on the Tetra ARMS PCR results, therefore it is more accurate in risk factor allele detection than Allele specific PCR method. GG alleles in SNP rs10757278 were 15 in the first method while it is only 8 were found the other method which indicates the accuracy of detection of risk alleles (Suhda*et al*., 2016). The distribution of rs10757278 is more significant in the patients than rs1333049.

CONCLUSION

We compare the results of two molecular methods for genotyping two SNPs rs10757278; rs1333049 correlated to CAD and outcomes revealed that the sensitivity and precision ofTetra ARMS PCRtechniques is more accurate, sensitive and easier than Allele specific PCR technique.

ACKNOWLEDGEMENT

Authors appreciate College of Science, University of Anbar for the support in this work. The work was carried out under the approval of the Scientific Research Ethics Committee at University of Anbar.

Table 2. Allele specific PCR result according to Hardy-Weinberg equilibrium

SNP	ALLELE/ GENOTYPE	N (%)		ODDS RATIO	95% CI	*P*-VALUE	*PC*-VALUE
		PATIENTS (N = 50)	CONTROL (N = 50)				
RS10757278	*A*	73 (73.0)	88 (88.0)	0.37	0.18 - 0.78	0.012	0.06
	G	27 (27.0)	12 (12.0)	2.71	1.29 - 5.71	0.012	0.06
	AA	31 (62.0)	40 (80.0)	0.41	0.17 - 0.99	0.077	NS
	AG	11 (22.0)	8 (16.0)	1.48	0.55 - 4.02	0.611	NS
	GG	8 (16.0)	2 (4.0)	4.57	0.93 - 22.37	0.092	NS
HWE-P		< 0.01	NS				
RS1333049	G	60 (60.0)	69 (69.0)	0.67	0.38 - 1.20	0.237	NS
	C	40 (40.0)	31 (31.0)	1.48	0.83 - 2.65	0.237	NS
	GG	19 (38.0)	23 (46.0)	0.72	0.33 - 1.58	0.544	NS
	GC	22 (44.0)	23 (46.0)	0.92	0.42 - 2.01	1.000	NS
	CC	9 (18.0)	4(8.0)	2.52	0.73 - 8.71	0.234	NS
HWE-P		NS	NS				

Table 3. Tetra ARMS result according to Hardy-Weinberg equilibrium

SNP	ALLELE/ GENOTYPE	N (%)		ODDS RATIO	95% CI	*P*-VALUE	*PC*-VALUE
		PATIENTS (N = 50)	CONTROL (N = 50)				
RS10757278	*A*	63 (63.0)	88 (88.0)	0.23	0.11 - 0.48	$6.1*10^{-5}$	< 0.001
	G	37 (37.0)	12 (12.0)	4.31	2.09 - 8.88	$6.1*10^{-5}$	< 0.001
	AA	28 (56.0)	41 (82.0)	0.28	0.11 - 0.69	0.009	0.045
	AG	7 (14.0)	6 (12.0)	1.19	0.38 - 3.80	1.000	NS
	GG	15 (30.0)	3 (6.0)	6.71	1.83 - 24.67	0.003	0.015
HWE-P		< 0.001	< 0.01				
RS1333049	G	59 (59.0)	70 (70.0)	0.62	0.34 - 1.10	0.139	NS
	C	41 (41.0)	30 (30.0)	1.62	0.91 - 2.90	0.139	NS
	GG	22 (44.0)	29 (58.0)	0.57	0.26 - 1.25	0.230	NS
	GC	15 (30.0)	12 (24.0)	1.36	0.56 - 3.27	0.653	NS
	CC	13 (26.0)	9 (18.0)	1.60	0.62 - 4.14	0.470	
HWE-P		<0.01	<0.01				

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