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Utility of Amplification-refractory mutation system-PCR [ARMS-PCR] for the detection of clinically significant Sirtuin 1 gene variations in smoker and nonsmoker population of Tabuk -Saudi Arabia

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Abstract:

BACKGROUND: *The sirtuins are a family of highly conserved NAD$^+$-dependent deacetylases that act as cellular sensors to detect energy availability and modulate metabolic processes. Genetic variations such as single-nucleotide polymorphisms in the SIRT1 gene have been associated with several disease-related phenotypes, including cardiovascular disease ,diabetes, body mass index, obesity, cholesterol metabolism, energy expenditure, glucose tolerance .Therefore, the aim of this study was to establish a rapid and sensitive molecular based assay like ARMS-PCR system for the detection of Sirtuin 1 rs7895833A>G gene variation in the Smoker and nonsmoker population of Tabuk -Saudi Arabia.*

METHODOLOGY: *This study was conducted on 100 confirmed subjects of Smoker and nonsmoker population of Tabuk .DNA extraction was done by using Qiagen Kit and ARMS-PCR system was optimized to detect Sirtuin 1 rs7895833A>G gene variation in Smoker and nonsmoker population.*

RESULTS: *All demographic features of the subjects are depicted in table 3. This study was done on 100 subjects among whom 50 were confirmed participants of smokers and 50 were confirmed subjects of nonsmokers. Out of 50 Smokers, 15[30%] were below or equal to 40 years age and 35[70%] were above 40 years of age. Of 50 consecutive smokers, 40[80%] were males and 10 [20%] were females. Out of 50 nonsmokers or healthy controls 45[90%] were males and 05[10%] were females. Out of 50 controls, 20[40%] were below or equal to 40 years age and 30 [60%] were above 40 years of age. ARMS –PCR system was optimized to detect Sirtuin 1 rs7895833A>G gene variation in confirmed subjects of Smoker and nonsmoker population. Gradient PCR was performed for SIRT-1-rs7895833A>G gene variation optimization. The technique was successfully optimized by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known SNPS in SIRT-1-rs7895833A>G gene. This assay does not entail any special equipment other than a thermocycler and gel documentation system.*

CONCLUSION: *ARMS-PCR system for SIRT-1-rs7895833A>G gene variation was successfully optimized. The assay proved to be fast, accurate, simple and economical that does not entail any special equipment other than a thermocycler and gel documentation system. It was indicated that ARMS-PCR system can be used as a potential molecular tool for the detection of SIRT-1-rs7895833A>G gene.*

Key Words: *Sirtuins [SIRTs] , T-ARMS PCR: tetra primer-amplification refractory mutation system-PCR,SD Pol: strand displacement polymerase , AS-PCR: allele specific-PCR.*

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INTRODUCTION:

The sirtuins [SIRTs] are a family of highly conserved histone deacetylases [HDACs] consisting of seven members [SIRT1-SIRT7]. Over the past few decades, SIRT1 has been the most extensively studied and garnered tremendous attention in the scientific community due to its emerging role in cancer biology. Sirtuin proteins are widely expressed in normal tissues and reported to be involved in several biological processes. [1] Sirtuins are a class of NAD+-dependent proteins involved in a wide range of biological processes such as transcription, aging, apoptosis and inflammation. [2] Sirtuin 1 [SIRT1] is located in the nucleus and cytoplasm, and plays an important role in epigenetic regulation by deacetylating a range of transcription factors to control downstream gene expression. [3] Recent studies have demonstrated a protective role of SIRT1 in atherosclerosis, the underlying process of coronary artery disease [CAD]. [4] SIRT1 performs an anti-inflammatory function by downregulating the expression of several pro-inflammatory cytokines by interfering with the NF-κB signalling pathway. Oxidative stress arises from environmental exposure such as cigarette smoking and inflammatory responses to viral and bacterial infections in the lungs. Oxidative stress causes protein oxidation and lipid peroxidation, injuring the lung tissue .[5] Therefore, targeting oxidative stress with antioxidants or increasing the endogenous levels of antioxidants is thought to be beneficial for COPD treatment.Endogenous Sirt-1 I was involved in the cell death/survival process and the pathogenesis of CVD. [6] A heart failure model indicated that Sirt-1 could improve heart function by increasing AMP-activated protein kinase [AMPK] expression.[7] Furthermore, low Sirt-1 expression in aging contributed to the decrease of antioxidants and increase of proapoptotic molecules through oxidative stress pathways .[8] Furthermore, SIRT-1 directly deacetylated ATG5, ATG7, ATG8 and ATG12 proteins and led to autophagy in the heart .[9] Nevertheless, the roles of Sirt-1 in controlling autophagy and apoptosis in the heart were still multifaceted. Determination of single nucleotide polymorphisms [SNPs] has been increasingly utilized in various genetic disciplines, especially in studying genetic determinants of complex diseases. Such studies will be facilitated by rapid, simple, low cost and high throughput methodologies for SNP genotyping. One such method is reported here, named tetra-primer ARMS-PCR, which employs two primer pairs to amplify, respectively, the two different alleles of a SNP in a single PCR reaction. In conventional ARMS-PCR, the amplification of normal and mutant allele is carried out in two separate reactions. Here in T-ARMS-PCR, both normal and mutant alleles can be run altogether with a control fragment in a single reaction. In T-ARMS-PCR, a pair of common [outer] primers produces a non–allele-specific PCR product and in combination with two inner, allele-specific primers [in opposite orientation to each other] produces 2 PCR products. Among the several methods that have been developed to genotype SNPs, the tetra-primer amplification refractory mutation system PCR [T-ARMS-PCR] has proved to be rapid, simple and economical. [10-14] Through combination of two outer primers and two allele-specific inner primers, genotyping requires only a single PCR followed by electrophoresis separation. [15] Genetic variations such as single-nucleotide polymorphisms in the SIRT1 gene have been associated with several disease-related phenotypes, including cardiovascular disease ,diabetes, body mass index, obesity, cholesterol metabolism, energy expenditure, glucose tolerance [16].However, very little is known about the association of polymorphism of SIRT1 and smoking associated diseases development in the Tabuk population. Based on the previous reports, we hypothesized that polymorphisms in SIRT1 might also be associated with susceptibility to smoking associated disorders like cardiovascular disease, lung cancer. Therefore, the aim of this study was to establish a rapid and sensitive molecular based assay like ARMS-PCR system for the detection of Sirtuin 1 rs7895833A>G gene variation in the Smoker and nonsmoker population of Tabuk -Saudi Arabia.

MATERIALS AND METHODS:

Study Population:

In order optimize Sirtuin-1 rs7895833A>G gene variation in Smokers and nonsmokers of Tabuk, we selected 100 specimens among which 50 were Smokers and 50 nonsmokers.

Inclusion criteria:

The study included confirmed subjects Smokers and nonsmokers, All participants were of Saudi origin.
Both male and female subjects.

Exclusion Criteria:
Participants who had already left nonsmokers, Participants of neutralized Saudi, Non-Saudi Arabians were excluded.

Sample collection:
Both Smokers and nonsmokers were interviewed using a structured questionnaire regarding epidemiological/demographic data, past history, family history of subjects. **A** 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from Smokers and nonsmokers in EDTA tubes after obtaining a written informed consent form.

Sample Size:
The study was conducted on 100 specimens among whom 50 were Smokers and 50 nonsmokers.

Genomic DNA extraction:
Genomic DNA was extracted from the peripheral blood samples of Smokers and nonsmokers using DNeasy Blood & Tissue Kit [50] with Cat No-69504 as per the manufacture's instructions. The DNA was extracted from Smokers and nonsmokers was dissolved in nuclease-free water, and stored at 4°C until use. The DNA quality and yield was assessed using Nanodrop [optical density] and 1% agarose gel electrophoresis.

Genotyping for Sirtuin 1 rs7895833A>G by amplification-refractory mutation system:
Sirtuin 1 rs7895833A>G gene variation was detected by ARMS-PCR system approach. ARMS primers were designed by using Primer3 software as shown table 1. The amplification refractory mutation system [ARMS] is an amplification strategy in which a polymerase chain reaction [PCR] primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue. ARMS has also been termed allele-specific PCR or PCR amplification of specific alleles [PASA]. Thus, an ARMS primer can be designed to amplify a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by as little as a single base from the former.

Table 1 : ARMS-PCR primers for SIRT1rs7895833A>G gene polymorphism

Direction	Primer sequence	PCR product	Annealing temperature
FO	5'-CCCAGGGTTCAACAAATCTATGTTG-3'	320 bp	62 °C
RO	5'-GCTTCCTAATCTCCATTACGTTGAC-3'		
FI-A	5'-GGTGGTAAAAGGCCTACAGGAAA-3'	241 bp	
RI-G	5'-CCTCCCAGTCAACGACTTTATC-3'	136 bp	

Fo-outer forward primer :Ro-Reverse outer primer ,FI-Inner forward primer :RI-Inner Reverse primer: AT-annealing temperature

The ARMS primers for SIRT1rs7895833A>G gene polymorphism was performed in a reaction volume of 25uL containing template DNA [50ng]. The cocktail was prepared for 5 samples as depicted in table 2. F1-0.20uL, R -0.20uL, F2-0.30uL, R -0.30uL of 25pmol of each primers and 5uL from DreamTaq green Master Mix [Thermo , USA]. The final volume of 25 uL was adjusted by adding nuclease free ddH_2O .Finally 2ul of DNA was added from each T2D patient.

Table No 2: Preparation of PCR cocktail for SIRT-1-rs7895833A>G

Reagent	1x	5 x
PCR master mix	5ul	25 ul
Forward FO	0.20 ul	1.0 ul
Reverse RO	0.20 ul	1.0 ul
Forward FI	0.30 ul	1.50 ul
Reverse RI	0.30 ul	1.50 ul
Nuclease free water	17.00 ul	85ul
Total volume	23ul	115ul
Add sample	**2ul**	
Total reaction volume	**25ul**	

Thermocycling conditions:
The amplification conditions used were at 95 ºC for 10 minutes followed by 40 cycles of 94ºC for 35sec, 62ºC for 40 sec, 72ºC for 40 sec followed by the final extension at 72 ºC for 10 minutes.

Gel electrophoresis:
The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator.

Statistical analysis:
Deviations from Hardy-Weinberg disequilibrium [HWD] was calculated by Chi-square [χ^2] goodness-of-fit test. All statistical analyses were performed using Graph Pad Prism 6.0 or SPSS 16.0.

RESULTS:

Study Population:
All demographic features of the subjects are depicted in table 3. This study was done on 100 subjects among whom 50 were confirmed participants of smokers and 50 were confirmed subjects of nonsmokers. Out of 50 Smokers, 15[30%] were below or equal to 40 years age and 35[70%] were above 40 years of age. Of 50 consecutive smokers, 40[80%] were males and 10 [20%] were females. Out of 50 nonsmokers or healthy controls 45[90%] were males and 05[10%] were females. Out of 50 controls, 20[40%] were below or equal to 40 years age and 30 [60%] were above 40 years of age.

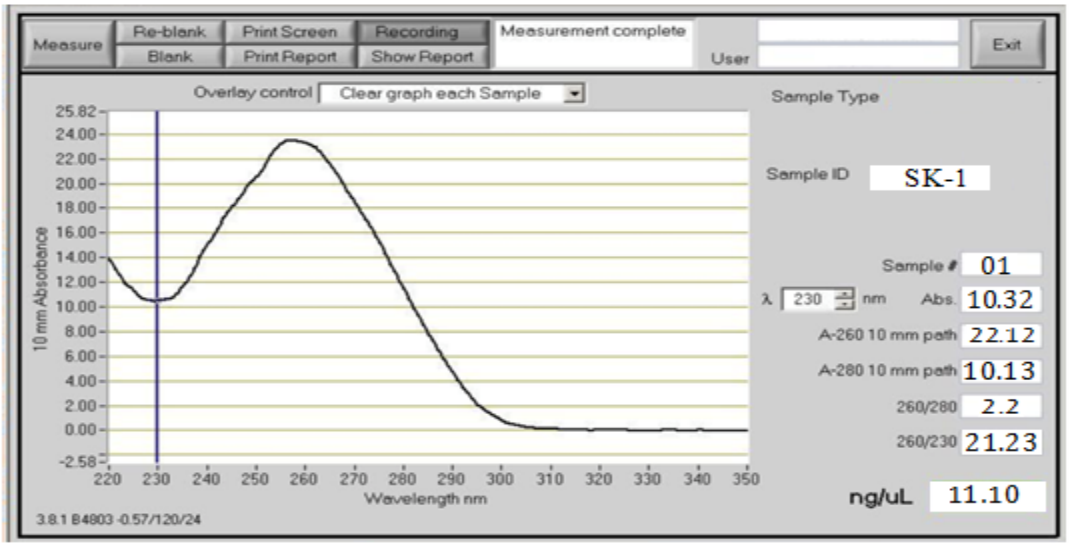
Table.3 :Baseline characteristics of Smokers and nonsmokers

	Smokers	
No of subjects	N=50	Percentage
Males	40	80%
Females	10	20%
Age >40	35	70%
Age <40	15	30%
Baseline characteristics of nonsmokers		
	Nonsmokers	
No of subjects	**N=50**	Percentage
Males	45	90%
Females	05	10%
Age >40	30	60%
Age <40	20	40%

Quality and integrity check of genomic DNA extracted from Smokers and nonsmokers:
The DNA was extracted from Smokers and nonsmokers was dissolved in nuclease-free water, and stored at 4°C until use. The DNA quality and yield was assessed using Nanodrop [optical density] and 1% agarose gel electrophoresis.

Purity of DNA by Nanodrop analysis
The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA . A ratio of ~1.8 is generally accepted as "pure" for DNA. We used NanoDrop™ [Thermo Scientific, USA] Spectrophotometer to measure the purity of DNA. Normally the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. All DNA samples from Smokers and nonsmokers were screened for purity by measuring optical density [OD] at 260nm [OD_{260}] and 280 nm [OD_{280}] as depicted in figure 2. The λ260/λ280 ratio ranged from 1.83-2.05 indicating good quality DNA.

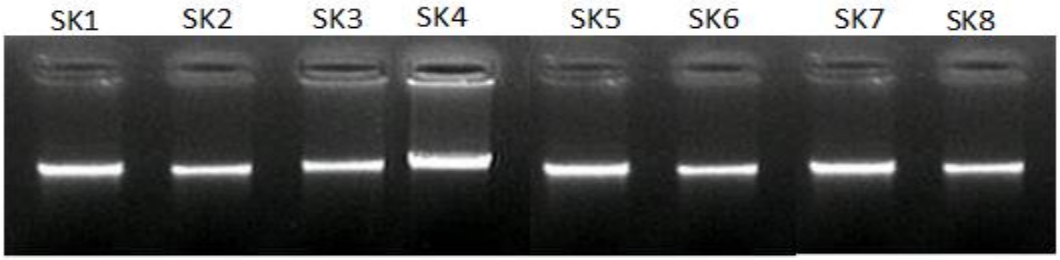
Figure :2 : Nanodrop analysis of genomic DNA extracted from Smokers by NanoDrop



Analysis of genomic DNA from Smokers and nonsmokers by running on 1% gel electrophoresis:

Gel electrophoresis is the standard lab procedure for separating DNA by size for visualization and purification. The 1% gel electrophoresis or gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. It can determine the length of a DNA segment by running it on an agarose gel alongside a DNA ladder. The purity and quality of genomic DNA extracted from Smokers and nonsmokers was checked in 1% gel electrophoresis as depicted in figure 2.

Figure 2: Analysis of genomic DNA from Smokers by running on 1% gel electrophoresis

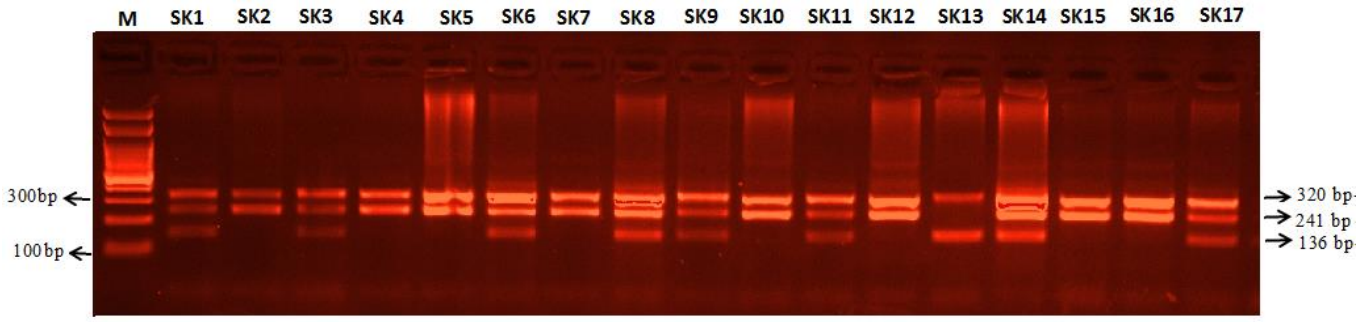


SK-Smokers

Optimization of ARMS primers SIRT-1-rs7895833A>G gene variation:

Sirtuin 1 [silent mating type information regulation 2 homolog 1; EC 3.5.1.-], encoded by the gene SIRT1 [OMIM 604479], belongs to a family of nicotinamide adenine dinucleotide [NAD]-dependent histone deacetylases.The ARMS primers for SIRT-1-rs7895833A>G genotyping were designed using primer3 software. Gradient PCR is mainly the gradient in annealing temperature and is a kind of technique used to optimize a primer and to check the quality control of a new primer. Gradient PCR was performed for SIRT-1-rs7895833A>G gene variation in smokers and nonsmokers.

Figure-3:Optimization of Amplification-refractory mutation –PCR for the detection of SIRT-1 rs7895833A>G gene polymorphism in smokers and non-smokers



LEGEND

M-100 bp DNA ladder
Homozygous for TT allele- SK2, SK4, SK5, SK7, SK10, SK12, SK15 & SK16
Homozygous for GG allele- SK13
Heterozygous for T/G allele- SK1 SK3 SK6 SK8 SK9 SK11 SK14 SK17

320 bp-control band
241 bp –T allale
136 bp- G allele

The best temperature was determined to be at 62°C in the temperature range of 58°C to 66°C tested with a gradient PCR thermocycler [Biorad PCR]. During gradient PCR, the number of cycles was increased from 30 to 35 cycles, which enhanced the yields of all three PCR products. Together, these changes resulted in a more robust amplification of the polymorphic allele and a less competing reaction from the control, as shown by the relative intensities of the corresponding bands on agarose gel electrophoresis.

Primers FO and RO flank the exon of the SIRT-1 gene, resulting a band of 320 bp to act as a control for DNA quality and quantity. Primers FI and RO amplify a wild-type allele [C allele], generating a band of 323 bp, and primers FO and RI [mt] generate a band of 136 bp from the mutant allele [G allele] and primers FI and RO [wild] generate a band of 241 bp from the normal allele [T allele] as depicted in figure 3.

The ARMS-PCR amplification products of SIRT-1-rs7895833A>G genotyping were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Tetra-primer amplification refractory mutation system [ARMS] polymerase chain reaction [PCR] was simple to perform for SIRT-1-rs7895833A>G genotyping. Together, the introduced method could be suggested as a powerful tool for genotyping single-nucleotide mutations and polymorphisms.

DISCUSSION:

Sirtuin 1 [silent mating type information regulation 2 homolog 1], encoded by the gene *SIRT1* , belongs to a family of nicotinamide adenine dinucleotide [NAD]-dependent histone deacetylases. An important thing to know about the *SIRT1* gene is that its homolog *SIR2* makes round worms and fruit flies live longer and protects mice against cancer, dementia, and heart disease . Now, it may be of interest to biologists or exterminators that pests with better *SIR2* live longer and stay on top of their game until a ripe old age, but what does it mean for humans. [17] Recently, description of the SIRT1/AMPK/PGC-1α pathway and its role in mitochondria metabolism presented new research track as depicted in figure 4. SIRT1 encodes sirtuin-1, a NAD+-dependent deacetylase that is activated in response to DNA damage and plays a key role in mitochondrial biogenesis. [18] SIRT1 promotes mitochondrial biogenesis through deacetylation and activation of it subtract PGC-1α [19], a master regulator of mitochondrial biogenesis that co-activates the nuclear respiratory factors [Nrf-1 and Nrf-2], which induce the transcription of genes involved in mitochondrial biogenesis [20].Since sirtuin enzymatic activity is dependent upon the presence of NAD^+, sirtuin activity is directly linked to the metabolic state of the cell. The downstream effects of target deacetylation include changes in cellular metabolism [lipid metabolism, insulin sensitivity, reverse cholesterol transport, and gluconeogenesis] as well as cell survival and senescence effects [cell survival and DNA repair] [21]. Indeed in addition to the activation of Nrf1 and Nrf2, the deacetylation of PGC-1α induces activation of other transcriptional factors such as PPARα and ERRα [22]. All these transcription factors were implicated not only in mitochondrial biogenesis but also in the use of glycolytic and lipidic cell substracts.

Figure 4: SIRT1 cell signaling pathways.

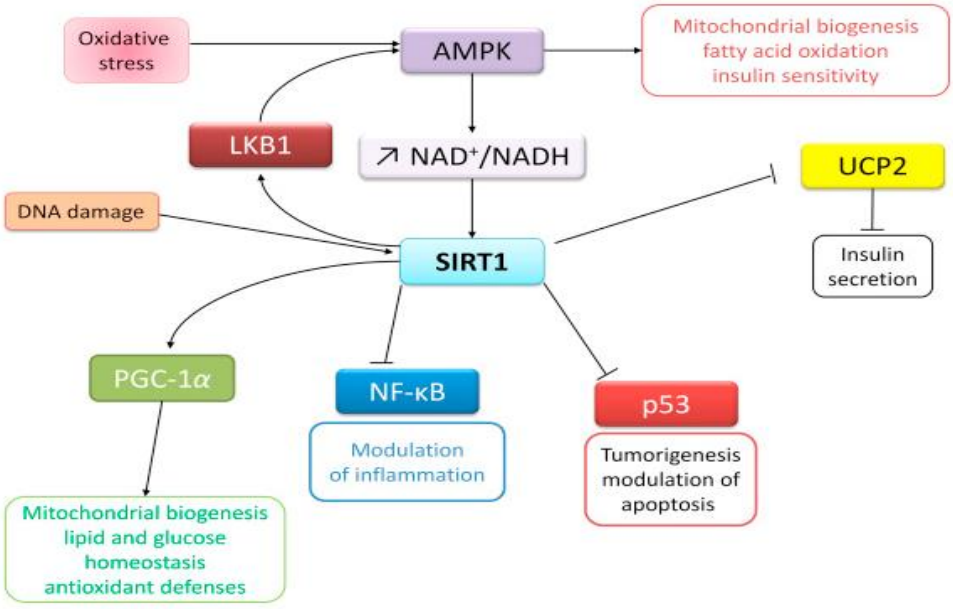


Figure 4: SIRT1 cell signaling pathways. AMP-activated protein kinase (AMPK) can activate SIRT1 through regulating the NAD/NADH ratio. Conversely, SIRT1 activates AMPK by deacetylation of the serine/threonine liver kinase B1 (LKB1), constituting a positive feedback system. Other SIRT1 substrates include PPAR-γ coactivator-1α (PGC-1α), protein tyrosine phosphatase 1B (PTP1B), p53, and NF-κB. Interestingly, although SIRT1 inhibits E2F1 transcriptional activity, E2F1 can upregulate SIRT1 expression. In addition, SIRT1 can stimulate glucose-dependent insulin secretion from pancreatic β cells via repressing the uncoupling protein gene UCP2. Oxidative stress could activate SIRT1 signaling pathways by regulating AMPK and/or directly via SIRT1.

Amplification-refractory mutation system [ARMS-PCR] utilizes four primers viz. outer forward [OF], outer reverse [OR], inner forward [IF] and inner reverse [IR] primers. The OF/OR primer combination generates the outer fragment of the SNP locus and acts as an internal control for the PCR. The IF/OR and OF/IR primer combinations yield allele-specific amplicons depending on the genotype of the sample used. The inner primers are positioned unequally from the corresponding outer primer to generate amplicons with different sizes and hence easily resolvable in a gel and distinction is made accordingly. The potential use of T-ARMS PCR in faster, economical, and precise genotyping, the need for extensive standardization at the initial stages hampered its wide application [23]. Some of sequences and the GC-rich regions are least accessible to this methodology [[24-25]. Recognizing the the potential of T-ARMS technology and recognizing its pitfalls, Mesrian Tanha and co-workers [26] modified the outer primers to equalize the primer strengths and included an additional parameter of equal annealing temperature of specific fragments. The use of chimeric primer-based temperature switch PCR [TSP] strategy with T-ARMS primers enabled multiplexing of T-ARMS PCR . The Taq DNA polymerase, used in the classical T-ARMS PCR, has 5′–3′ polymerase as well as 5′–3′ exonuclease activity. Taq DNA polymerase is commonly used in T-ARMS PCR genotyping. The properties of SD polymerase and Taq polymerase are different and influence the T-ARMS PCR differently. This 5′–3′ exonuclease activity could degrade the annealed IF and IR primers similar to the 5′ Nuclease Assay by the Taq polymerase of the OF/OR while the 3′ end of IF and IR primers are been extended by another Taq polymerase molecule causing the noisy non-specific bands of varying sizes and intensity. [27] The SD polymerase is a variant of Taq polymerase with a 5′–3′ polymerase and 5′–3′ strand displacement activities but devoid of exonuclease activity [28]. T-ARMS PCR is flexible, rapid and economical SNP detection tool compared to contemporary genotyping tools such as allele-specific PCR [AS-PCR], high-resolution melting analysis [HRMA], PCR single stranded conformation polymorphism [PCR-SSCP], PCR-primer introduced restriction analysis [PCR-PIRA], real-time PCR-based genotyping etc.

CONCLUSION:

This study successfully optimized the ARMS-PCR technique for the detection of SIRT-1-rs7895833A>G gene variation in Smoker and nonsmoker population. The assay proved to be fast, accurate, simple and economical that does not entail any special equipment other than a thermocycler and gel documentation system. It was indicated that ARMS-PCR system can be used as a potential molecular tool for the detection of SIRT-1-rs7895833A>G gene variation.

Competing interests:

The authors declare that they have no competing interests.

Authors' contributions:

All authors read and approved the final manuscript.
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