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DETECTION OF CHOLINERGIC RECEPTOR NICOTINIC ALPHA 3 SUBUNIT (CHRNA3 GENE-RS938682 G>A) GENE VARIATION IN NICOTINE DEPENDENT IN SMOKER AND NON-SMOKER POPULATION OF TABUK, SAUDI ARABIA.

[1]BERNARD C. SILVALA , [2]RASHID MIR

[1]Department of Medical Lab Technology, Prince Fahd Bin Sultan Research Chair, Faculty of Applied Medical Sciences, University of Tabuk, Kingdom of Saudi Arabia.

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Abstract:

Background: *Nicotine acetylcholine receptor genes are expressed in the key regions of the brain and play an important role in controlling smoking behavior. Located on chromosome 15q25, they initiate the brain responses to nicotine that binds primarily to these receptors. Tobacco smoking is by far the greatest risk factor for developing lung cancer. Sequence variants in CHRNA SNPs on chromosome 15 have been associated with increased (self-reported) cigarette dose and nicotine dependence and increased risk of carcinogenesis including lung cancer in smokers. Therefore the aim of this study was to establish a rapid and sensitive molecular based assay like ARMS-PCR system for the detection of CHRNA3 gene-rs938682 G>A gene variations in smokers and nonsmokers.* ***Methodology:*** *This study was conducted on 100 specimens among whom 50 were smokers and 50 nonsmokers or healthy controls. DNA was extracted by Qiagen Kit and ARMS PCR system was optimized to detect CHRNA3 gene-rs938682 G>A gene variations in smokers and nonsmokers.* ***Results:*** *The study included 100 specimens among whom 50 were Smokers and 50 nonsmokers or healthy controls. The DNA quality and yield was assessed using Nanodrop (optical density) and 1% agarose gel electrophoresis .Genotyping for CHRNA3 -rs938682 G>A gene was performed on the genomic DNA using a tetra-primer ARMS PCR approach. The ARMS primers were designed by using Primer3 software. ARMS PCR was optimized using gradient PCR to detect CHRNA3-rs938682 G>A gene variations in Smokers and nonsmokers. In this study, it was successfully developed the ARMS technique using the wild-type or mutant-type primers with matched or one-base mismatched to examine the known SNPS in CHRNA3-rs938682 G>A gene. It was indicated that ARMS technique can be used as a potential molecular tool in the diagnosis of potential CHRNA3 gene variations for in smokers and nonsmokers.* ***Conclusion:*** *This study successfully developed the ARMS-PCR technique for the detection of SNPs in cholinergic receptor nicotinic alpha 3 subunit (CHRNA3 gene-rs938682 G>A) gene variation in smoker and nonsmoker population of Taluk. ARMS method could be useful for time-efficient, unbiased, sensitive, accurate, rapid, and reliable for CHRNA3 gene-rs938682 G>A other gene variations.*

Keywords: *ARMS-PCR amplification-refractory mutation system* ,CHRNA3 *(cholinergic receptor nicotinic α3) SNP- Single-nucleotide polymorphism.*

Corresponding author:

DR. BERNARD C. SILVALA,

Department Supervisor, *Department of Medical Laboratory Technology,*
Faculty of Applied Medical Science, university of tabuk,

QR code

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INTRODUCTION:

Tobacco smoking is a complex and multifactorial disease involving both environmental and genetic factors. Tobacco smoking is one of the leading preventable causes of many adverse health outcomes and there are estimated 1.3 billion smokers all over the world. (1). Epidemiological evidence indicates that tobacco smoking can exert its pathogenic effect on almost every organ through direct or indirect tobacco exposure, and the smoking associated mortality still remains at a high level for decades (2). Premature death of smokers dis-possesses families of income, increases healthcare costs and slows down economic advancement (3). Therefore, the prevention of smoking is a crucial factor in work-place health promotion. Saudi Arabia is ranked fourth in the world in terms of tobacco sales and importation (4). The overall prevalence of 12.2% has been reported, with men more likely than women to smoke (21.5% vs. 1.1%). It has been reported that every year, > 4700 individuals die from tobacco-related disease .In Saudi Arabia 23.3% of the Saudi population, 32.3% of men and 13.5% of women, are exposed to secondary smoke for at least 24 hours/week at home, work or school (5).Smoke-free workplace policy has been implemented in Saudi Arabia but it is not strictly enforced , and recently announced VAT on tobacco products that commenced in the second quarter of 2017.World Health Organization (WHO) estimates that if smoking prevention interventions continue at the same rate in Saudi Arabia, ~6 268 400 individuals, about 24% of the population, will be tobacco smokers by 2025 (6). The chromosome region 15q25.1 contains the *CHRNA5* and *CHRNA3* (cholinergic receptor nicotinic α5 and 3) and *AGPHD1* (aminoglycoside phosphotransferase domain containing 1) gene cluster, which express nicotinic acetylcholine receptor subunits (nAChRs) (7-8). Nicotine and its derivatives as a functional components in the tobacco smoke, can bind to nAChRs and could promote tumor cells proliferation, metastasis and inhibit apoptosis through binding to nicotinic acetylcholine receptors (nAChRs) and strongly induce carcinogenesis (9). Activation of nAChRs facilitates the outgrowth of cells with genetic damage and promotes cell proliferation, migration, invasion, and angiogenesis, which stimulates the development of lung cancer cells and suppresses apoptosis by acting as tumor promoters (10). However, debate remains on whether the association is established through a direct effect on a gene that causes lung cancer or facilitated by means of an indirect effect leading to nicotine addiction. Three Genome-wide association studies (GWAS) were conducted in 2008, revealed that CHRNA5-A3 cluster situated on chromosome 15q24-25.1 might be potential loci relevant to both nicotine dependence and smoking related cancer (11-13). It has been reported that 2008 onwards many research studies have reported that CHRNA3 gene polymorphisms are associated with many types of cancers detection and treatment including lung cancer, gastric cancer, esophageal cancer (14-16) etc. CHRNA3 rs938682 variations were strongly associated with lung cancer risk in Europeans (17). CHRNA SNPs (rs16969968 in CHRNA5 and rs1051730 in CHRNA3) were shown to contribute to lung cancer risk in a smoking-independent manner in the Japanese study, (18). Although there are many sophisticated tools applied to study Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A detection, it is very expensive for undeveloped countries. As for known variations in genes, ARMS-PCR system provides an optional way to solve this problem with its additional characteristics such as time-efficient, unbiased, sensitive, accurate, rapid, and reliable. Although there are many sophisticated tools applied. Therefore the aim of this study was to establish a rapid and sensitive molecular based assay like ARMS-PCR system for the detection of CHRNA3 gene-rs938682 G>A gene variations in smokers and nonsmokers.

MATERIALS AND METHODS:

Study Population: This study was conducted on 100 specimens among which 50 were smokers and 50 were nonsmokers.

Inclusion criteria: The study included

- Confirmed subjects of Smokers.
- Both males and females.
- All participants were of Saudi origin.

Exclusion Criteria: The study included

- Non Saudi Arabia smokers were excluded.

Sample collection

- Blood samples were collected from smokers as well as from nonsmokers in EDTA tubes after obtaining a written informed consent form. After assessing the questioner and findings, 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from each smoker as well as from nonsmokers.

Genomic DNA extraction:

- The DNA extraction was done by using DNeasy Blood Kit cat 69506 from Qiagen as per the manufacture's instructions. The QIAamp DNA Blood Mini Kit was designed for processing up to 200µl fresh or frozen human whole blood. The DNA extracted

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 .

Optimization of ARMS-PCR CHRNA3 rs938682G>A genotyping

- The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base changes or small deletions. ARMS are based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele .It is a simple method for detecting any mutation involving single base change.Genotyping for CHRNA3 rs938682 G/A was performed on the genomic DNA using a tetra-primer ARMS PCR approach. The ARMS primers were designed by using Primer3 software as depicted in Table 1.

Table No 1. Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A

CHRNA3 rs938682 Fo1		5'-TAATTCCCTACGAGTATTCCTGCTCCT-3'	630 bp	
CHRNA3 rs938682 Ro1		5'-CTCTAGTATATGTTCTTTGGGCACATGC-3'		
CHRNA3 rs938682 FI-2	**G**	5'-CCATTATCTATAGCTACTTTTGAGCTCAAG-**3'**	300 bp	
CHRNA3 rs938682 2RI-2	**A**	5'-ACGGTCACAGCTATTCATCTCTGCAC**T--3'**	388 bp	

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

The cocktail was prepared for 5 samples as depicted in table 3 .The AS-PCR was performed in a reaction volume of 25uL containing template DNA (50ng), FO -0.25uL , RO -0.25uL, FI-0.25uL, RI -0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix (cat no M7122) (Promega, USA). The final volume of 25 uL was adjusted by adding nuclease free ddH_2O .Finally 2ul of DNA was added from each smoker and nonsmoker .

Table No 2. Preparation of PCR cocktail

Reagent	1x	5 x
PCR master mix	5ul	25ul
Forward outer	0.25 ul	1.25 ul
Reverse outer	0.25 ul	1.25 ul
Forward wild	0.25 ul	1.25 ul
Reverse mutant	0.25 ul	1.25 ul
Nuclease free water	17.0 ul	85ul
Total volume	23ul	110ul

Thermocycling conditions: The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 35 seconds, 60°C for 40 seconds, and 72°C for 45 seconds followed by one elongation step at 72°C for 10 min.

RESULTS:

Study Population: This population-based smoker – control study was done on 100 subjects among whom 50 were smokers and 50 were nonsmoking healthy controls with no history of any type of cancer.

DNA extraction: The extracted DNA was dissolved in nuclease-free water and stored at 4° C until use. The quality of DNA was checked by running in 1% gel electrophoresis as shown in figure 1b. Quality and integrity of DNA were checked by NanoDrop™ (Thermo Scientific, USA) in figure 1a. For pure DNA, A260/280 ratios reported was 1.97 .

Figure 1. :Genomic DNA preparation from peripheral blood of smokers and Nonsmokers.

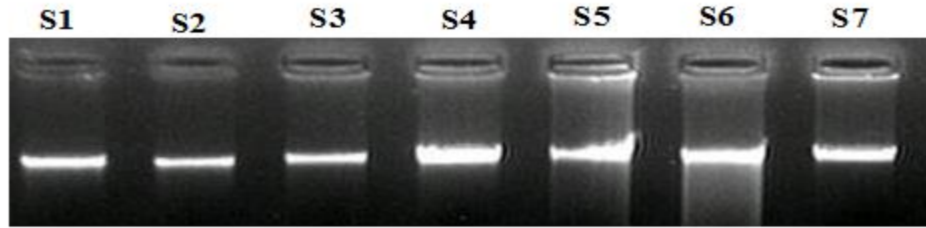
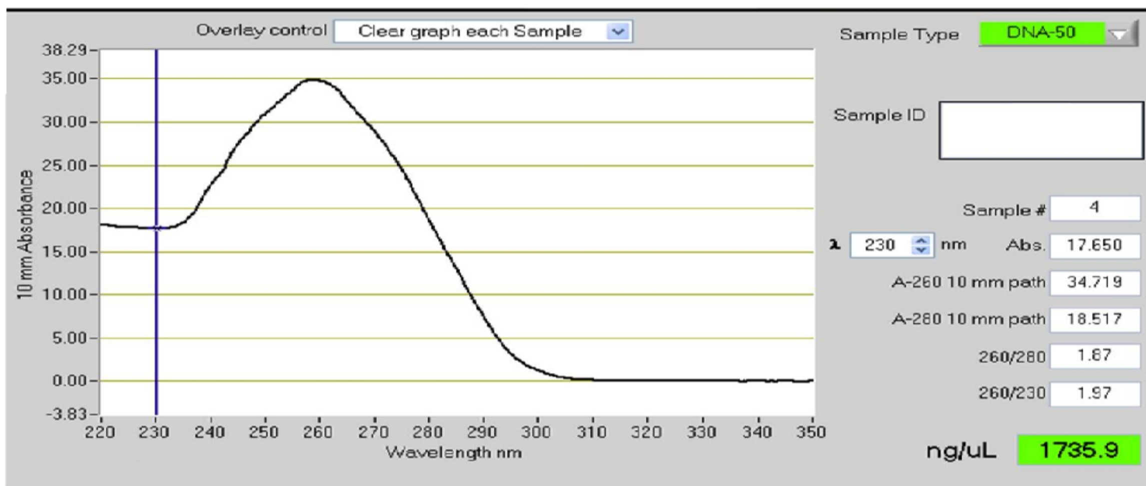
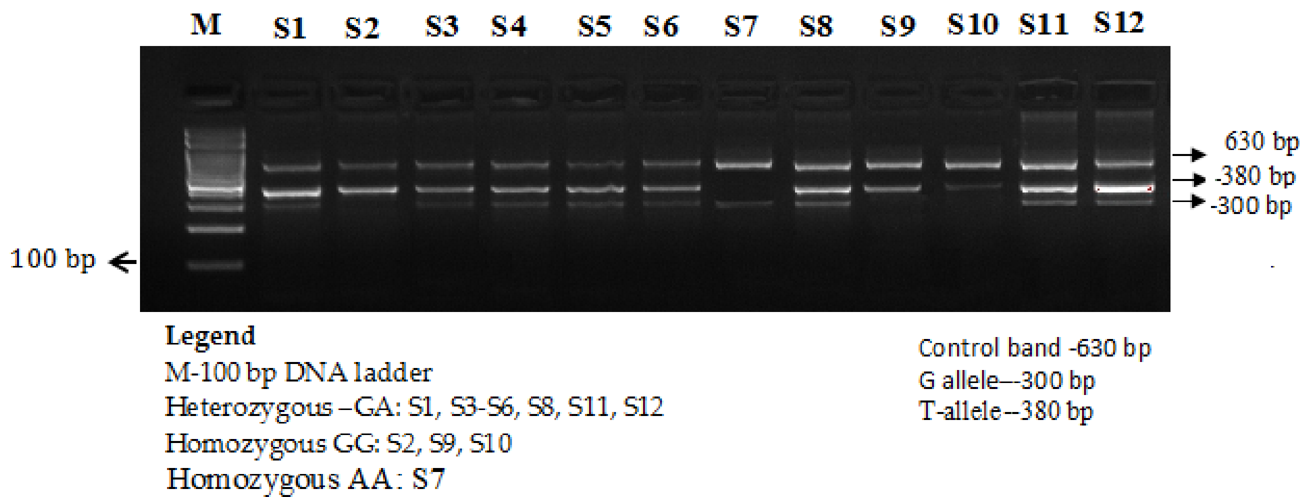


Figure 1b: NanoDrop™ analysis of DNA extracted from peripheral Blood



Optimization of Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A: The primers were designed for tetra-primer amplification was performed on the genomic DNA using a tetra-primer ARMS PCR approach sing primer3 software. The Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A genotyping was detected by using amplification-refractory mutation system PCR .The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. For perfect optimization of CHRNA3 rs938682 G>A a gradient PCR was performed and the best temperature was determined to be at 58°C in the temperature range of 53°C to 61°C tested with a gradient PCR thermocycler. The number of cycles was increased from 30 to 37 cycles, which significantly enhancing the yields of all three PCR products. These changes resulted in a more robust amplification of the mutant allele and a less competing reaction from the control, as shown by the relative intensities of the corresponding bands on agarose gel electrophoresis. The ARMS Primers Fo1 and R2 of CHRNA3 gene amplify a wild-type allele (G allele), generating a band of 300 bp, and primers F2 and R1 generate a band of 388 bp from the mutant allele (A allele) as depicted in figure 2.

Fig 2: Amplification-refractory mutation system –PCR (ARMS-PCR) for Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A gene Polymorphism in smokers and Nonsmokers



It was successfully developed the Allele specific ARMS technique by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known CHRNA3 rs938682 G>A gene variations.

DISCUSSION:

Smoking is a major public health issue and a global health priority. This is believed to be the first study to investigate the sociodemographics and workplace pattern of smoking among a representative sample of male expatriate workers in Saudi Arabia. There is an urgent need to integrate into the Vision 2030 Economic and Development blueprint of Saudi Arabia comprehensive strategies to reduce the prevalence of tobacco smoking. This should include the provision of comprehensive insurance coverage for effective tobacco cessation counselling and therapy with free access and no copayments; enforcement of workplace smoking bans; free individual, group and telephone-based cessation counselling; and targeted anti-smoking initiatives at the identified groups integrated with universally accepted anti-tobacco health messages addressing all segments of the population.

The World Health Organization estimates that if current trends continue, the annual number of deaths from tobacco-related diseases will double from five million in the year 2000 to 10 million in 2020.Nicotine, a naturally occurring alkaloid found in tobacco, mimics acetylcholine, and nicotine's ability to bind to nicotinic cholinergic receptors (nAChRs) underlies the molecular basis of nicotine dependence [susceptibility to tobacco addiction (MIM 188890)]. Chronic nicotine exposure produces long-lasting behavioral and physiological changes that include increased synaptic strength, altered gene expression and nAChR up-regulation (19). Although nAChRs are expressed throughout the central nervous system, the addictive effects of nicotine are thought to be mediated through mesocorticolimbic dopamine (DA) pathways (20). It is believed that the interplay among glutamate, dopamine and gamma-aminobutyric acid (GABA) systems is critical for the reinforcing effects of nicotine (21). Cigarettes are the predominant form of tobacco used worldwide (22), and genetic factors are important to the etiology of nicotine dependence, with estimates of the heritability ranging from 44 to 60% (23).

Cholinergic receptor nicotinic alpha 3 subunit CHRNA3 rs938682 G>A

play a vital role in nervous system and nicotine conduction pathway, recent researches revealed that polymorphisms rs938682 was relevant to neuropsychiatric disease, nicotine addiction and lung cancer.The association of polymorphisms with lung cancer might result from direct genetic susceptibility and indirect smoking carcinogenesis(24-25).Three SNPs (rs8034191, rs1051730 and rs16969968) at 15q25.1 were consistently strongly associated with lung cancer risk in both smokers and nonsmokers in populations of European descent (26). However, the results failed to be replicated in Korean, Japanese and Chinese populations, partly because the minor allelic frequencies (MAF) were extremely low in Asian populations according to the HapMap database (27).

It appears that genetic determinants for the risk of lung cancer are heterogeneous at both the allelic and locus levels among populations of various ethnicities. Genotyping or point mutation assays have been developed as alternatives for genotyping assays. Researchers in the past have used techniques like Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), direct DNA sequencing, Single Strand Conformation Polymorphism (SSCP),and tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) for genotyping of specific polymorphic nucleotide loci. These assays are allele specific and include mutant-allele-specific amplification (MASA), PCR amplification of specific alleles (PASA), and amplification refractory mutation system (ARMS)-PCR (28).These methods utilize the difference in extension efficiency between primers with matched and mismatched 3′ bases. Product detection and identification are the most variable steps that contribute to the throughput capacity of each particular method. The drawback with some of these assays is the detection method, such as fluorescence resonance energy transfer, fluorescence polarization, luminescence, or absorbance (29-30), requiring expensive equipment and reagents which still have limited use in resource-constrained settings. Among these the tetra-primer ARMS-PCR could be a useful tool for genotyping, since SSCP may not be repeatable some times, direct DNA sequencing is a cumbersome, time consuming, technically demanding and costly procedure and the possibility of getting a restriction site for an enzyme could be rare for genotyping by RFLP. For genetic analysis, fast and economical assays that can be performed with standard PCR instruments are highly desirable. Compared tetra-primer ARMS assay with routinely used methods such as typical PCR-RFLP analysis, real time PCR assay and DNA sequencing. No special equipment and only a small amount of standard PCR reagents are needed in tetra-primer ARMS-PCR.ARMS-PCR or T-ARMS methods are rapid, simple, reliable, easy to perform, economical and require minimum level of expertise that can be used for both large- and small-scale genotyping studies. Compared to other genotyping techniques, tetra-primer ARMS-PCR for CHRNA3 rs938682 G>A gene variation reported to be a rapid, reliable, simple and economical assay for SNP genotyping.

CONCLUSION:

This study successfully developed the ARMS-PCR technique for the detection of SNPs in cholinergic receptor nicotinic alpha 3 subunit (CHRNA3 gene-rs938682 G>A) gene variation in smoker and nonsmoker population of Taluk. ARMS method could be useful for time-efficient, unbiased, sensitive, accurate, rapid, and reliable for CHRNA3 gene-rs938682 G>A other gene variations.

Competing interests:-The authors declare that they have no competing interests.

Authors' contributions:-All authors read and approved the final manuscript. Thanks to all authors for their support and help in this study.

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