Ministry of Higher Education and Scientific Research
University of Al-Qadisiyah
College of Biotechnology

Detection of Superoxide Dismutase Gene Variations in Smokers

A Research Project
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By

Aya Ahmed Mortada ,Hawraa Fadhil Asmial and Ramla Jabbar Kadkem

Supervised by
Assist.Prof Dr. Nazar Abdulameer Hamzah

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Summary
Superoxide dismutase (SOD) gene variations were studied in 20 cigarette smokers (different ages and period of smoking) and 10 non-smokers respectively. Sequencing steps were accomplished involved sample collection, DNA extraction, estimation of DNA purity, detection of DNA and RFLP-PCR technique. Results showed presence of wild homozygote genotype only in selected samples.

1. Introduction

Smoke of cigarettes produce reactive oxygen species (ROS) that have ability to destroy of biological molecules such as DNA, proteins and enzymes [Saggu *et al*.,2012]. ROS (O_2^-) can to stimulate DNA damage causes genetic disorder that lead to formation of mutation activity and induce of tumors [Storz, 2005]. Mutation probability and cancer alteration increased by presence of ROS in cells. Lesions of DNA involve bases oxidation, add and delete of bases and nick of its stands result in absent of genome stability [Jackson and Loeb,2001]. Abnormal influences of ROS are avoided by production the antioxidants included of non- enzymatic and enzymatic antioxidant [Janicka *et al*., 2013]. Several enzymatic antioxidants found in human blood such as Lactate Dehydrogenase (LDH), Catalase (CAT), Glutathione peroxidase (GXp), Peroxidase and Superoxide dismutase (SOD) are play important roles as scavenger of ROS [Raddam *et al*., 2017]. In humans, all other mammals, and most chordates Superoxide dismutase are found as three isoforms. According to localization of SOD isoform in the cytoplasm, mitochondria, and extracellular are named into SOD1, SOD2 and SOD3 respectively. SOD1 compose from two polypeptide chains, while SOD2 and SOD3 have four chains of polypeptide. Active site of SOD1 and SOD3 have Cu and Zn metal ions, unlike SOD2 has Mg therefore classified as metalloenzyme. Genes of SOD are present on chromosomes 21, 6, and 4, respectively (21q22.1, 6q25.3 and 4p15.3-p15.1). [Cao *et al*., 2008]. About 1717 SNPs are reported in gene of SOD1 of human by NCBI (latest update 1/11/2017) with or without clinical significances. One of these vartion is A/C +35 exon3/intron3, therefor our work focused to detection of this variants using Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) technique for analysis of digested DNA fragments.

2. Materials and Methods

2.1 Sample collection

Five ml of blood sample were obtained from 20 smoker males, age ranged between19-30 year and 10 of control without history of disease, age ranged between 20-36 years. Sample of blood were collected in EDTA tube and stored under freezing condition (-20◦C) for later DNA extraction.

2.2 DNA extraction

DNA extracted from blood sample according to instructions of geneaid kit as follow briefed procedure:

1. 200µl of blood transferred to 1.5 ml tube.
2. 20µl of proteinase K was added and pipetted 5 times then incubated for 5 mins.
3. 200µl of GSB buffer was added with shaking vigorously then incubated for 5 mins.

4. 200µl of absolute ethanol was added and mixed.

5. GD column was placed in 2 ml collection tube

6. Mixture was transferred into GD column and centrifuged for 1 mins.

7. 400µl of W1 buffer was added and centrifuged for 20 sec.

8. 600µl of wash buffer was added and centrifugated for 30 sec.

9. 100µl of elution buffer was added and let stand for 1 min then centrifugated for 30 secs to elute.

2.3 Estimation of DNA purity

Optical density at 260 and 280 nm using spectrophotometry based to following formula:

DNA purity ratio = OD 260/280

2.3.1 Detection of DNA

2.3.1.1 Solation's preparation

2.3.1.2 Buffer of sample loading

0.25 of blue bromophenol and 40g of sucrose were dissolved in 10 ml of D.W the stored in 4^{o}C.

2.3.1.3 Working solation, TBE 0.5X

500ml of TBE 0.5X was prepared by adding 50ml of TBE (10X) stock solation to final volume of 500ml D.W.

2.3.1.4 Ethidium bromide solation

Previously prepared of 10 µg/ml in water

2.3.1.5 Preparation and running of gel electrophoresis

1. Agarose solation (1%) was prepared by adding 1 g of agarose to 100ml of 0.5 X TBE.

2. Stirred of solation on hot plate until dissolving or (use of microwave for 1min) of agarose and clearing, then cooled to 45^{o}C

3.Ethidium bromide (2.5µl) was added.

4. About 2.5 cm from any end of tray the comb was vertically fixed.

5. the tray was filled (about 6mm depth) by agarose solation.

6. Solidification of gel was allowed at 25^{o}C for 20min.

7. Fixed comb was released.

8. Gel in the tray was placed in chamber of electrophoresis.

9. Chamber of electrophoresis of was submerged by electrophoresis buffer to cover the gel to depth about 1mm.

10. Two µl loading buffer was added to each DNA sample with mixing.

11. Sample (20µl) was loaded carefully by micropipe in agarose wells.

12. leads was attached red to red, black to black in voltage supply unit.

13. Running was performed at 5V/cm until separation of DNA fragments.

2.4 Amplification of SOD1

2.4.1 SOD primer sequencing (macrogen)

Forward-5'CTATCCAGAAAACACGGTGGGCC3'

Reverse-5'TCTATATTCAATCAAATGCTACAAAACC3'

2.4.2 Master Mix ingredients 2X

iNtRON's Maxime PCR PreMix Kit (i-Taq) 2X concentrated solution contain of:

1. *Taq* DNA polymerase (5 unit/µl).

One unit (U) of Taq polymerase is defined as the amount of enzyme required for catalyze the incorporation of 10 nanomoles of deoxyribonucleotides into acid-insoluble material in 30 minutes at 74°C.

2. 2.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP).

3. Reaction buffer (10x).

4. Gel loading buffer (1x).

For each 50 µl reaction of PCR was performed as follow:

1.Thawing of PCR Master Mix (2X) then gently vortex and briefly centrifuge.

2.Above ingredients was placed in thin walled tube of PCR on ice and add the following:

DNA template	4 µl
Forward primer	2.5µl
Reverse primer	2.5µl
Nuclease-free water	To 41µl
Total volume	50µl

3. The samples was spin down and gently vortex.

4. PCR was operated according to basic conditions as follow:

Step	Temp. (°C)	Time	Cycle NO.
Initial denaturation	94	2 min.	1
Denaturation	94	20 sec.	30
Annealing	63	10 sec.	30
Extension	72	30 sec.	30
Final extension	72	5 min	1

2.5 Electrophoresis and visualization

Products of PCR were running on agarose as above then amplicons were detected by visualized in UV transilluminator. The bands were photographed by digital camera.

2.6 Digestion with HhaI restriction enzyme

One unit is defined as the amount of enzyme required to digest 1 µg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 µl1.

Following components were mixed in sterile tube according manufacturer (Thermo Scientific) at 25^{o}C as follow:

Nuclease-free water	17 µl
10X digestion Buffer	2 µl
DNA (PCR product)	10 µl (~0.2 µg)
HhaI enzyme	1 µl
Total volume	30 µl

2. Mix gently and spin down.

3. At 37°C the mixture was heated in a heat block or water thermostat for 5 min.

4. Mixture of reaction loaded in 1% agarose.

5. Fragments of digested DNA stained with ethidium bromide were electrophoresed at 5V/cm and separated bands were observed using gel UV illumination.

3. Results and discussion

Table 1 pointed out to the features of smokers (cigarettes smoking) and control (non-smoking) classified according to the sex, age and period of smoking. ROS and other free radicals related with period of tobacco consumption, thus oxidative stress lesion may be increased with duration of smoke inhalation [Sirisha and Manoha, 2013].

Table 3.1 Features of smokers and non-smokers

feature	**Smoker**	**Non-smokers**
Sex/ male	20	10
Age/ years	19-30	20-36
Duration of smoking/ years	1-7	------

A ratio of purity of DNA was ~1.8-1.9. To determine the purity of nucleic acids (DNA and RNA) the absorbance ratio of 260 nm and 280 nm is used. In general ratio of ~1.8-2 is suitable for DNA purity, if DNA sample contaminated with RNA or proteins the absorbance ratio is more or less compared with optimum ratio [Wu *et al*., 2004]. Smoke of cigarettes consist of different materials can induce formation of superoxide, may be affecting on the expression of gene of the antioxidant enzymes such as Cu-Zn and Mn SOD, Glutathione Peroxidase (GPx), and catalase (CAT) [Russo *et al.*, 2011].

For to the explain possible relationship between association of smoking risk and SOD A/C polymorphism, amplification of the SOD1 by PCR using specific primer. The results of amplification were produced one band may be of 278bp as showed in figure 1.

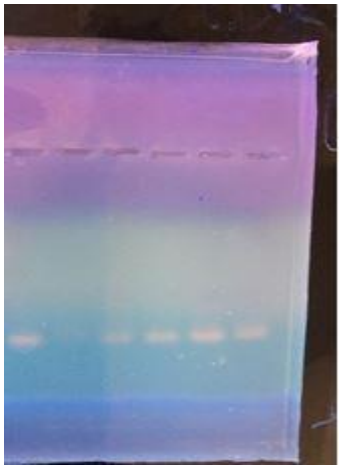

Figure 3.1 The electrophoresis of SOD gene by PCR product, amplification product one band 278bp of smoker and non-smoker

Many SOD genes were detected by PCR technique included Mn-SOD and EC-SOD genes, appeared as 140 and 104 bp bands respectively. In this study, PCR technique used for reflect relationship of SODs genes polymorphism and diabetic neuropathy in type 1 diabetes mellitus [Chistyakov *et al*., 2001]. Iqbal *et al*., 2013 amplify SOD2 gene (434 bp) for analysis of SNP with hearing losing in workers by noise produced in textile factories.

PCR-RFLP was used to detected A→C, +35 exon3/intron3 SOD1 gene polymorphism [Young *et al*., 2005] Specific restriction enzyme Hha I was applied to cut specific sequence 5' CCG$^{\downarrow}$C3' derived from *Haemophilus haemolyticus* [Nair, 2008]. Results of fragmentation of SOD1 gene by Hha I was subjected on agarose to classified into AA (wild homozygote), CC (variant homozygote) and AC (heterozygote). All of studied specimens were appeared as one band of only AA genotype (table 2 and figure 2).

Table 3.2 PCR-RFLP analysis of SOD1 gene polymorphism of smokers and non-smokers

Genotype	**Smokers**	**Non-smokers**
AA	20 (100%)	10 (00%)
CC	0 (0%)	0 (0%)
AC	0 (0%)	0 (0%)
Total	20 (100%)	10 (00%)

Absence of others genotype may be no effect of smoke cigarette on SOD1gene and not alter A to C because slightly short period of smoking or few number of donors. Nithya *et al*., 2016 detect whether the SOD1 +35A/C gene polymorphism is related with diabetes mellitus type 2, about 95%, 3% and 2% were AA wild homozygote, CC variant homozygote and 3% heterozygote type respectively. Small investigated samples also reflect the low frequency of CC and AC in pervious study.

Figure 3.2 The pattern of electrophoresis RFLP-PCR for detection of SOD1 genotype of smoker and non-smoker

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