

The Study of Manganese Superoxide Dismutase Val16Ala Genotypes and its Association with *Helicobacter pylori* in Peptic Ulcer Patients in Kermanshah City, West of Iran

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Abstract

Background: One of the main causes of gastric ulcer and inflammation is *Helicobacter pylori* (*H. pylori*). This bacterium has a global spread. The objective of the present research is to evaluate the genotype relationship between manganese superoxide dismutase (MnSOD) Ala16Val and the risk of the gastric ulcer. **Materials and Methods:** This case-control research was conducted on 75 patients with gastric ulcer and 60 healthy individuals as a control. By using the kit, DNA was extracted from gastric paraffin blocks and control group blood samples. Polymerase chain reaction was implemented to detect glmM and MnSOD genes, and restriction fragment length polymorphism was used to examine MnSOD Ala16Val polymorphism. **Results:** The frequency of glmM gene in patients with gastric ulcer was positive and was 74.66%. Analyzing MnSOD Ala16Val polymorphism indicated that the A/V frequency was higher in both patient and control groups. In addition, the allelic analysis indicated that the allele A was significantly (Sig = 0.000) higher in the patient group compared to that in control group (Sig = 0.000). It also showed that the genotype Ala/Ala can increase the gastric ulcer rate by 32.3 times. **Conclusion:** This research indicated a positive relationship between the MnSOD Ala16Val gene polymorphism and gastric ulcer. Therefore, Ala/Ala genotype could be considered as a risk factor for gastric ulcer disease.

Key words: Gastric ulcer, *Helicobacter pylori*, manganese superoxide dismutase, restriction fragment length polymorphism-polymerase chain reaction

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium which localizes selectively in the epithelium of the stomach and has long-term stability in the acidic environment of the stomach is considered as its characteristics.^[1,2] *H. pylori* is one of the most important causes of chronic inflammation in the gastric mucosa and increases the risk of developing duodenal and gastric ulcer and stomach adenocarcinoma.^[3,4] It is estimated that approximately 0.1% of people infected with *H. pylori* develop gastric mucosa-associated lymphoid tissue lymphoma, 1%–3% develop stomach adenocarcinoma, and approximately 10% develop peptic ulcer disease.^[5]

H. pylori infection has a global spread, so that more than half of the world population is infected with this bacterium. Its frequency of prevalence varies from 50% in developed countries to 80% in developing countries.^[6,7] Approximately 80%–90% of people are infected with this bacterium in Iran.^[8,9] The most people infected with *H. pylori* has no

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Received: 10-05-2018

Revised: 15-06-2018

Accepted: 21-06-2018

clinical symptoms. Numerous studies have indicated that the factors related to host and bacterium play a role in determining the clinical and pathological outcomes developed by bacterium.^[10] However, genetic polymorphism is the most important factor in determining the disease.^[11] Gastric epithelial cells infected with *H. pylori* are able to induce inflammatory factors, followed by the production of great amounts of toxic reactive oxygen species (ROS).^[12,13]

ROS are involved in damage to membranes, mitochondria, and macromolecules such as DNA.^[14] Different antioxidant defense systems protect the cells against ROS.^[15] One of the most important antioxidant agents in the mitochondria is manganese superoxide dismutase (MnSOD) that catalyzes the superoxide radicals in the form of hydrogen peroxide and neutralizes the toxic effects of mitochondrial ROS.^[16] Any defect in cellular antioxidant systems might be involved in the development or increasing the severity of the destructive effects of oxidative stresses.^[17]

Hence, as *H. pylori* infection can induce inflammatory responses and oxidative stresses in its host.^[18] Lack of balance between virulence factors of the bacterium and host defense mechanisms leads to gastric disease in a part of the population.^[19] In other words, weakened antioxidant defense might play a role in the pathogenesis of gastric inflammation, ulcer production or the development of gastric cancer, and other diseases and malignancies.^[20] Genetic polymorphisms change the activity of these vital enzymes and cause imbalance in the cell oxidative load. A wide range of diseases such as various cancers might be developed due to polymorphism in antioxidant agents.^[21] MnSOD in the mitochondria is formed based on different Ala16Val precursors.^[22] The most known polymorphism in these enzymes includes MnSOD Ala16Val in the mitochondrial target sequence.^[21]

It has been shown that single nucleotide changes in the amino acid sequence in the position 16 from valine to alanine might be a source of structural changes in the MnSOD target sequence. It also affects the MnSOD activity in mitochondria.^[23,24]

In research reviewed 79 papers, a potential relationship was reported between the Val16Ala SOD2 genotype and various diseases and disorders such as the stomach, pulmonary, prostate, bladder, and chest cancers, and disorders such as Type 1 diabetes, nephropathy, and chronic kidney disease.^[21] Some research suggests that in some cases of malignancies, the survival rate of the people with Ala/Ala genotype for Ala16Val MnSOD polymorphism is less.^[25]

Regarding the importance of *H. pylori* in the development of peptic ulcer and gastric adenocarcinoma,^[3] the objective of the present research is evaluating the genotypes of MnSOD Ala16Val in the patients with gastric ulcer and in healthy people using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Based on the studies conducted in this regard, PCR-RFLP

has been found as one of the strong methods.^[26] It is the first study on the relation between the role of MnSOD Ala16Val polymorphism and gastric ulcer cases in Iran, and the results of this study could be helpful in determining the prognosis and evaluating the control and therapeutic programs in future.

MATERIALS AND METHODS

Study population

In this observational analytical (control case subgroup) research, 40 men with a mean age of 47 ± 28 and 35 women with a mean age of 47 ± 26 were present in the patient group with gastric ulcer. Paraffin blocks of 75 patients with gastric ulcer were provided from Imam Reza Hospital in Kermanshah, Iran, between 2015 and 2016. The control group consisted of 30 healthy men and 30 healthy women with a mean age of 37 ± 16 and 37 ± 17 , respectively, and their blood samples were collected from the laboratory of Imam Reza Hospital.

The information was obtained from the patient's medical records. The present study was approved by the Ethics Committee of Kermanshah University of Medical Sciences. Written informed consent was obtained from all participants. Written informed consent was obtained from all participants, and the personal information of the patients was kept confidential.

DNA extraction from paraffin tissue and blood samples

About paraffin blocks, deparaffinization was first performed using Xylan,^[27] Genomic DNA was extracted from the paraffin blocks of patients and blood samples of healthy persons using a DNP kit (SINACLON Co.Tehran, Iran), according to the manufacturer's instruction.

Determining the quality and quantity of DNA

The quantity of DNA was measured using the Nanodrop (Thermo) by calculating optical absorption of 260 and 280 nm, and quality was also measured through absorption ratio of 260 to 280 nm. Then, agarose gel was used to analyze the measurement of the band quality regarding the nonexistence of smear.

PCR-RFLP

For molecular (genotype) confirmation of the presence of *H. pylori* in samples related to gastric tissue of people with gastric ulcer, the PCR test was performed based on the glmM gene. This gene plays a role in the synthesis of cell walls of the bacterium.^[28]

The positive samples were examined regarding glmM gene, and the blood samples of control patients were examined to identify and amplify the MnSOD gene. The primer design related to the two considered genes (glmM, MnSOD) was performed using sequences in the gene bank using the Gene Runner Software. The characteristics of primers are presented in Table 1.

Polymerase chain reaction for each gene at the volume of 25 μ l with a target DNA at the amount of 2 μ l (500 ng), 0.2 μ l Taq polymerase (1U), 2.5 μ l buffer (10x), 1.3 μ l MgCl₂ (100 mM), 1.25 μ l forward and reverse primers (10 mM), 16 μ l DDW, and 0.5 μ l dNTP (10 mM) was implemented based on the program, defined in Tables 2 and 3. All the reagents were obtained from SINACLON, Tehran, Iran.

After performing the PCR reaction and ensuring the MnSOD sequence amplification on gel electrophoresis, RFLP technique was used to analyze the MnSOD Ala16Val genotypes. Accordingly, 10 μ l PCR along with 2 μ l 10X buffer and 7.5 μ l DDW underwent enzymatic digestion for 1 day at 60° C under 0.5 μ l BsaWI (Fermentas, St. Leon-Rot, Germany). After enzymatic cutting, to determine the type of polymorphism, the results of reaction on 3% electrophoresis gel were uploaded and observed under ultraviolet light. The pattern of bands obtained from the enzymatic was traced as follows:

Bandwidth for homozygote A/A 196 bp, heterozygote A/V 243 and 196 bp, and homozygote V/V 243 bp. For quality control, 5% of the samples were randomly selected and re-genotyped.

Statistical analysis

Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 18.0, IBM Corporation, Armonk, NY, USA). The level of the correlation between polymorphism and gastric ulcer disease was analyzed using Chi-square (χ^2) at a statistically significant level of $P < 0.05$.

RESULT

Among 77 paraffin blocks in patients with gastric ulcer, 56 (74.66%) were positive regarding the glmM gene [Figure 1], indicating the presence of *H. pylori*. These 56 positive samples have been candidates for determining the

MnSOD Ala16Val genotypes. Figure 2 shows the results of the tracking of MnSOD gene.

PCR-RFLP results and distribution of MnSOD polymorphisms in patients and control groups

The patterns obtained from enzymatic digestion using BsaWI are shown in Figure 3.

The frequency of MnSOD genotypes and alleles in control people and patient people is shown in Table 4. In both of control and patient groups, the frequency of MnSOD genotypes was in Hardy–Weinberg equilibrium.

The results obtained from PCR-RFLP showed that the frequency of A/V genotype was higher in both patient and

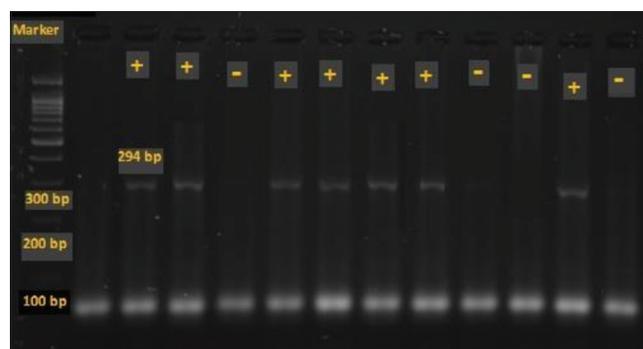


Figure 1: Polymerase chain reaction products of GlmM gene of *Helicobacter pylori*. The positive samples are 294 bp regarding the glmM gene (100 bp marker)

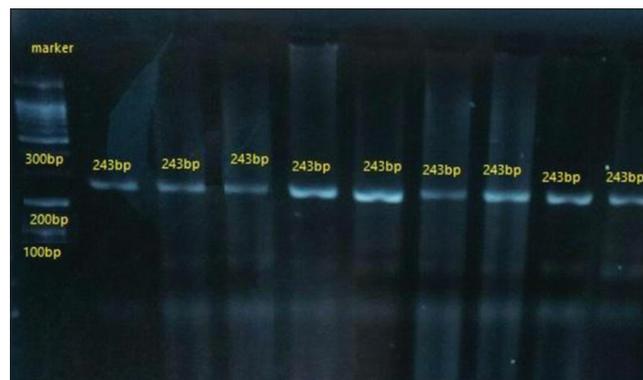


Figure 2: Polymerase chain reaction products of manganese superoxide dismutase gene of *Helicobacter pylori*. Marker with a molecular weight of 100 bp and MnSOD gene with a molecular weight of 243 bp have been shown

Table 1: The oligonucleotide sequence of primers and product sizes

PCR product size (bp)	Sequence (5'–3')		Amplified gene
294 bp	5' - AAGCTTTTAGGGGTGTTAGGGGTTT - 3'	F	glmM
	5' - AAGCTTACTTTCTAACACTAACGC - 3'	R	
234 bp	5' - CGGGCTGTGCTTTCTCGTC - 3'	F	MnSOD
	5' - TCAGCCTGGAACCTACCCTT - 3'	R	

Table 2: PCR program for the *glmM* gene

Duration	Temperature	PCR step
5 min	94°C	Primary denaturation
		35 cycles of:
30 s	94°C	Denaturation
30 s	58°C	Annealing
30 s	72°C	Extention
5 min	72°C	Extention Final

PCR: Polymerase chain reaction

Table 3: PCR program for the MnSOD gene

Duration	Temperature	PCR step
5 min	94°C	Primary denaturation
		35 cycles of:
40 s	94°C	Denaturation
40 s	58°C	Annealing
40 s	72°C	Extention
5 min	72°C	Extention Final

PCR: Polymerase chain reaction, MnSOD: Manganese superoxide dismutase

Table 4: The frequency of MnSOD genotypes

Genotype	Control <i>n</i> =60 (%)	Patient <i>n</i> =56 (%)
A/A	5 (8/37)	13 (23/21)
A/V	38 (63/32)	24 (42/8)
V/V	17 (28/3)	19 (33/9)

MnSOD: Manganese superoxide dismutase

control groups, and the frequency of V allele compared to that of A allele in both groups had the highest frequency. However, the frequency of A allele in patient people (46%) was significantly higher than that in control people (40%) (Sig = 0.000).

The diagram of calculating the odds ratio also showed that the Ala/Ala genotype can increase the rate of disease by 32.3 times, and it can also be considered as a risk factor for gastric ulcer disease.

DISCUSSION

H. pylori is one of the main causes of gastric ulcer and inflammation.^[4] In this study, the *glmM* gene was identified in 56 cases (74.66%) of patients' samples; it indicates a high prevalence of *H. pylori* in patients with gastric ulcer, which is in line with previous studies.^[29] One of the main objectives of this research was determining the polymorphism of MnSOD Ala16Val in patients with gastric ulcer and healthy individuals. In the allelic investigation, it was found that allele V is higher than allele A in both groups, which these results are in line with the results of other researchers.^[18,30]

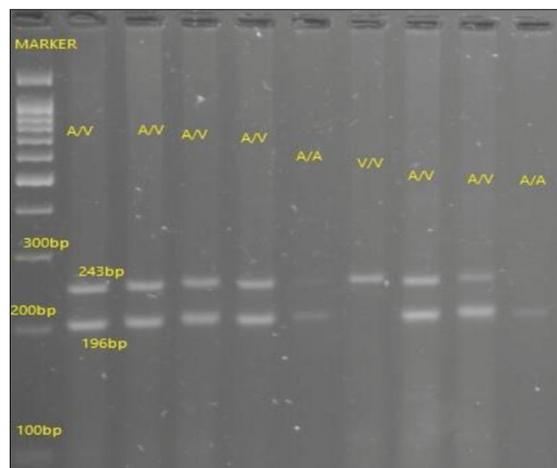


Figure 3: Results of 3% agarose gel electrophoresis of digested polymerase chain reaction products by *Bsa*WI limiting enzyme. Bandwidth A/A 196 bp, A/V 243 and 196 bp, V/V 243 bp, and 100bp DNA marker have been shown

The frequency of allele A in the patients group was significantly higher than that in the control people. We also indicated that the Ala/Ala genotype can increase the rate of gastric ulcer disease by 32.3 times, and it is regarded as a risk factor for gastric ulcer disease. ROS is the agent of damage to membrane, mitochondria, and macromolecules such as DNA.^[14]

MnSOD is the most important antioxidant agent in neutralizing the toxic effects of ROS.^[16] Gastric epithelial cells infected with *H. pylori* are able to induce inflammatory factors, resulting in the production of great amounts of toxic ROS. It also results in inducing tissue necrosis factors and more damage to the mucosa.^[12]

Gastric infected with *H. pylori* compared to healthy tissue contains higher values of ROS values, and there is a direct relationship between bacterium load and ROS values.^[31,32]

Hence, disorder in the oxidant-antioxidant system balance in the stomach might greatly increase the risk of cell death and DNA damage by ROS. In people infected with *H. pylori*, a change in the activity of ROS cleansing enzymes due to the presence of the bacterium might increase the risk of gastric cancer.^[33] Research suggests that AA genotype is associated with increased risk of breast and prostate concussion, immunosenescence profile and damage to DNA in addition, cell surface markers such as apoptosis-related surface receptors in people with genotype VV compared to AA people are less susceptible and appear.^[34] The research conducted by Koistinen *et al.*, on 88 patients with acute myocardial leukemia, showed that the survival rate of the patients with this malignancy with the Ala/Ala genotype for the MnSOD Val16Ala polymorphism is lower than people with Ala/Val genotype.^[25]

Their results suggested that genetic polymorphisms might change the activity of these vital enzymes and cause

imbalance in the cell oxidative load. A wide range of diseases such as various cancers might be developed to polymorphism in antioxidant agents.^[21] Numerous studies have indicated that the factors, related to host and bacterium, are involved in determining the clinical and pathological consequences caused by *H. pylori*.^[10] Individual nature and nutrition affect the antioxidant modulation in aerobics. Diet, alcoholism, and physical activity also affect the enzyme at the molecular level.^[22,35]

In general, the present study revealed that the presence of *H. pylori* is confirmed in the high percentage of the population with gastric ulcer, and Ala/Ala genotype can be considered as a risk factor for gastric ulcer. The results of this study are important for future studies in some aspects, including targeting the factors, which might affect the presence of *H. pylori* and the factors leading to genetic mutations in the host, which cause of these can be traced in the bacterium, host, and lifestyle.

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Source of Support: Nil. **Conflict of Interest:** None declared.