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The repeatability of PCR-RFLP method for study of association between gastric cancer and manganese superoxide dismutase mutant (Val-9Ala)

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Abstract. MnSOD or manganese superoxide dismutase operates as a protective factor. The MnSOD Val-9Ala on mitochondrial target sequence (MTS) has been known as a polymorphism of this gene. The aim of the present expriment was to evaluate the relationship between gastric cancer and MnSOD Val-9Ala gene polymorphism. For this porpuse gastric cancer patients and controls that have been collected from North of Iran, were analyzed. The PCR-RFLP was carried out after DNA extraction. Results showed that the frequencies of MnSOD Ala/Ala, Ala/Val and Val/Val genotypes in healthy individuals were 25.0, 66.0 and 9%, respectively. In patients the frequency of MnSOD Val allele was higher (52 %) compared to that in controls (42 %). The results of this study demonstrated a positive correlation between MnSOD Val-9Ala gene polymorphism with gastric cancer and confirmed the results of Moradi et al. 2015.

Key words: Gastric cancer, MnSOD Val-9Ala, Gene polymorphism.

Introduction

Gastric cancer is the most universal lethal cancer with around 738,000 deaths per year (Jemal et al. 2011). Different frequency of gastric cancer in worldwide can be due to diversity in the genetic conditions, nutritional behaviors and living conditions (Humans 1994).

Gastric cancer is the most numerous disease diagnosed in worldwide and it is the most common lethal cancer in Iran. Northern and northwestern regions in Iran are high risk areas for gastric cancer. Epidemiologic investigations have reported frequent risk factors for gastric cancer, including environmental, genetic factors, adverse living conditions, dietary habits and the prevalence of *Helicobacter pylori* infection (Moges et al. 2006).

If *H. pylori* infect the gastric epithelium cells, the interleukin-8 should be induced and production of too much amounts of toxic reactive oxygen species (ROS) may be occurred. It may induce the interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and some other interleukins (Augusto et al. 2007). Oxidative stress that caused by ROS is involved in human carcinogenesis (Cerutti 1985). ROS generated in normal respiration of cells and during xenobiotics metabolism. It is known as a candidate agents in the growth of cancer and damage to cell membranes, mitochondria and DNA molecule (Egan et al. 2003).

Various of antioxidant systems are concerned in the ROS scavenging, such as the superoxide dismutase or SOD family that catalyzes the dismutation of the superoxide anion to form hydrogen peroxide (H_2O_2) (Ambrosone et al. 2005). The MnSOD is only SOD family and essential for life. It as an antioxidant has a function against ROS in the human mitochondria (Attatippaholkun & Wikainapakul 2013). The MnSOD protein precursor is synthesized in the cell cytoplasm. This protein is associated with a cleavable mitochondrial targeting sequence or MTS at N-terminal (Macmillan-Crow & Cruthirds 2001). A polymorphism has been known in MnSOD (rs4880) gene. This polymorphism is recognized at the codon No. 16 and position - 9 in the complete protein. This leads to the substitution of either alanine (C allele) or valine (T allele) in the mitochondrial targeting sequence. This gene polymorphism of MnSOD is predicted to change the MnSOD secondary structure and may effect on the efficiency of MnSOD mitochondrial transport (Shimoda-Matsubayashi et al. 1996). The new findings revealed that the Ala form of MnSOD is targeted into the mitochondria, whereas the Val-containing protein is partially arrested in the inner-mitochondrial membrane (Sutton et al. 2003).

The relationship between gastric cancer in different ethnicities and polymorphisms of the related candidate genes has been reported. There is one report related to the possible association between the polymorphism of MnSOD and gastric cancer among Iranian populations (Moradi et al. 2015). Thus, the aim of current study was to investigate the MnSOD Val-9Ala genotypes distribution and their association with gastric cancer among a sample of the Iranian population for the second time. This experiment is repeated of the previous experiment to examine the ability and repeatability of the PCR-RFLP technique.

Material and Methods

This experiment is a duplication of the previous experiment (Moradi et al. 2015) to examine the ability and repeatability of the PCR-RFLP technique.

Enzyme and reagents: The GPP DNA isolation kit was supplied from Gen Pajohan, Iran. *BsaW* I as a restriction enzyme, agarose and polymerase chain reaction (PCR) materials were prepared from Fermentas. Specific primers were synthesized by Cinnaclon, Iran.

Participants: Gastric biopsies were taken from 52 gastric cancer patients with the mean age of 67.8± 8.8 years and 100 cancer-free with the mean age of 64.2± 5.1 years as controls. The patients and controls were age and sex matched (Table 1). All gastric cancer patients were from the north and northwest Provinces of Iran who admitted to the medical sciences clinics between May 2011 and June 2012. All sampling, diagnosis and procedures were according to Moradi et al. (2015).

DNA isolation: Genomic DNA was isolated according to Moradi et al. (2015). For this pupose genomic DNA was purified from endoscopic fresh biopsy samples. Tissue specimens were suspended in 550 µl of DNA extraction buffer (2 mM EDTA, 10 mM Tris-HCl (pH 8.0 and 400 mM NaCl). The suspension was incubated at 20-30 ^{o}C for 20 min, then Proteinase K and SDS (400 ng and 0.6%, respectively) were added. The solution was incubated at 55^{o}C for 15 h and DNA

Table 1. Characteristics of gastric cancer patients and cancer-free controls and their distribution of MnSOD Val-9Ala genotypes (χ^2 results).

	Cases		Controls	
Characteristics	n (52)	%	n (100)	%
Age				
Mean age, years [±SD[a]]	67.8 [± 8.84]		64.2 [± 5.14]	
< 50	1 1.93	1.93	16 16	16
50-65	11	21.15	66	66
> 65	40	76.92	18	18
Sex				
Male	30	57.7	62	62
Female	22	42.3	38	38
Smoking statues				
Yes	31	59	55	55
No	21	41	45	45
Oral hygiene				
Good	15	29	72	72
Bad	37	71	28	28
BMI (kg/m^2)	22.2 ± 3.1		22.7 ± 2.9	
H. Pylori				
Positive	39	69		
Negative	16	31		
cagA				
Positive	25	64		
Negative	14	36		
Tumor site				
Cardia cancer	22	42.3		
Non- cardia cancer	25	48		
Others	5	9.7		
Histological type				
Intestinal	36	69.23		
Diffuse	9	17.3		
Others	7	13.4		

was extracted with the GPP isolation kit (Gen Pajoohan, Iran). Isolated DNA was stored at -20 °C until use.

Purified DNA was confirmed by gel electrophoresis on 1% agarose. The concentration and purity of extracted DNA were analyzed acoording to Yari et al. (2010) and Moradi (2014).

Analysis of MnSOD Val-9Ala genotypes: This analysis, primers sequences and DNA amplification was performed according to Moradi et al. (2014). For this puopose the MnSOD Val-9Ala polymorphism was assessed by PCR method and then RFLP analysis.

After PCR-RFLP method, statistical analysis was carried out according to Moradi et al. (2014).

χ^2 analysis: For coparson of our data with Moradi et al. (2014) data, χ^2 analysis was done.

Results

A number of the individuality of the gastric cancer patients are shown in Table 1. The 52 gastric cancer patients and 100 healthy individuals as a control group participated in this study. The control group, 62% men and 38% women with the mean age of 64.2± 5.1 years), were free from cancer signs.

Further, the gastric cancer group consisted of 42.3% women and 57.7% men with the mean age of 67.8± 8.8 years. There was no significant difference between patients and controls in terms of age and sex.

Distribution of MnSOD genotypes and alleles in gastric cancer patients and controls is shown in Table 1. The frequency of MnSOD Ala/Ala, Ala/Val and Val/Val genotypes in healthy individuals were 24.3, 66.7 and 9%, respectively. However, in gastric cancer patients, Ala/Ala, Ala/Val and Val/Val with 24, 48 and 28% were observed ($p>0.01$). In patients the frequency of MnSOD Val allele was higher (52 %) compared to that in controls (42 %). In both of patient and control groups, the frequencies of MnSOD genotypes were in Hardy–Weinberg equilibrium.

χ^2 analysis showed that there was no shgnificant different between our results and Moradi et al. (2014) reports.

Discussion

The present study was carried out for the measurement of PCR-RFLP technique capability and repeatability. For this purpose the methods and materials were according to Mordadi et al. (2014). The expriment in a homogenous population of gastric cancer patients from north and northwest of Iran reports a significantly higher frequency of MnSOD Val allele compared to that in healthy individuals that increased the risk of gastric cancer.

The ROS generated during metabolism of xenobiotics and in normal cellular respiration, leads to membranes damage, mitochondria, and macromolecules including DNA, and thus are candidate agents in the development of human cancer (Margaret et al. 2011). The MnSOD gene as a tumor suppressor gene is localized on chromosome 6. A number of polymorphisms have been reported in the MnSOD gene that some of them are correlated with the increased risk of human cancers. Recent studies confirmed the functional polymorphism of the Val-9Ala MnSOD that Val allele-containing forerunner protein with beta-sheet conformation exhibited impaired transportation, but Ala-containing precursor with alpha helical conformation showed normal transportation. Then, the variant allele (Ala) with higher activity suppresses carcinogenesis. MnSOD polymorphic alleles are widely variable with ethnicity, the frequency of Ala allele is 12% among Japanese and is 14% in Chinese, while it is more general (41-55%) in the population of Caucasian and has been detected in 41% of Jordanian with breast cancer (Shimoda-Matsubayashi et al. 1996, Moradi et al 2014).

There are several available data to investigate the role of Val-9Ala MnSOD variants in susceptibility to gastric cancer. Similar to our study, Abdel-Fattah et. al. (2009) observed that the Val/Val allele of MnSOD Val-9Ala was correlated to a significantly decreased risk of breast cancer disease in Jordanian population (Malak et al. 2009). Also, they indicated that gastritis was characteristic by an oxidative stress with significant absence of MnSOD and GPX expression. In a meta-analysis that reported by Wang it has been suggested that the MnSOD Val-9Ala polymorphism may contribute to cancer development through a disturbed antioxidant balance (Wang et al. 2009).

The current research investigated the relationship between MnSOD Val/Ala polymorphism with the risk of gastric cancer. Results of this research showed that there was a significant positive correlation between the distribution of the MnSOD gene polymorphism and gastric cancer disease. In addition, the results show that the polymorphism of

MnSOD Val-9Ala is an important risk factor that is associated with gastric cancer in a sample of the Iranian population. However, further research with a larger sample size is required to confirm these findings. The present study is the second report on the correlation between the MnSOD genotypes and the susceptibility to gastric cancer disease in the Iranian population. This study can be used as a basis for studying polymorphisms of other important genes in correlation to gastric cancer.

In the current experiment, for all participants, DNA was isolated by the GPP DNA extraction kit and quantity and quality of extracted DNA were evaluated by spectrophotometric assay and gel electrophoresis. To investigate the gene polymorphism, PCR-RFLP was carried out.

χ^2 analysis showed that there was no significant different between our results and Moradi et al. (2015) reports. Then it concluded that the PCR-RFLP technique is powerful, capable and repeatable. It is recomendate to study of association between gene polymorphism and genetics deseases.

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