Serum paraoxonase-1 enzyme activities and oxidative stress levels in patients with esophageal squamous cell carcinoma

Abidin Sehitogulları[1], Mehmet Aslan[2], Fuat Sayır[3], Ali Kahraman[1], Halit Demir[4]

[1]Department of Thoracic Surgery, Van Regional Educational and Research Hospital, Van, Turkey, [2]Department of Internal Medicine, Yuzuncu Yil University, Medical Faculty, Van, Turkey, [3]Medical Faculty, Department of Thoracic Surgery, Yuzuncu Yıl University, Van, Turkey, [4]Faculty of Science, Department of Chemistry, Yuzuncu Yil University, Van, Turkey

Objectives: Oxidative stress is well recognized to play a role in the pathogenesis of many diseases, including cancers. Paraoxonase-1 (PON1) is implicated in the elimination of carcinogenic lipid-soluble radicals produced by lipid peroxidation. Reports on PON1 activities in patients with cancer are conflicting. The aim of this study was to investigate serum antioxidant enzyme activities and oxidative stress levels in patients with esophageal squamous cell carcinoma (ESCC).

Patients and methods: Thirty-two patients with ESCC and 33 healthy controls were enrolled. Serum malondialdehyde (MDA) levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR), paraoxonase, and arylesterase activities were measured spectrophotometrically.

Results: Serum paraoxonase, arylesterase, SOD, activities, GSH-Px, and GR activities were significantly lower in patients with ESCC than in controls (all, $P < 0.05$), whereas serum MDA levels were significantly higher ($P < 0.05$). Serum MDA levels were significantly correlated with paraoxonase ($r = -0.572$, $P < 0.001$) and arylesterase activities ($r = -0.597$, $P < 0.001$) in patients with ESCC.

Conclusions: This study indicated that ESCC is associated with increased oxidative stress and decreased antioxidant enzyme activities. Decreased serum PON1 enzyme activities may play a role in the progression and/or development of ESCC. Further studies are required to clarify these results.

Keywords: Esophageal squamous cell carcinoma, PON1 activity, Glutathione peroxidase, Superoxide dismutase, Malondialdehyde

Introduction

Esophageal cancer is a relatively rare, lethal disease and is the seventh leading cause of cancer death worldwide. Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignant tumors of the esophagus and is associated with a poor prognosis, rapid clinical progression, and a high rate of metastasis. ESCC is also known to be a highly angiogenic tumor.[1]

Reactive oxygen species (ROS), lipid hydroperoxides, and malondialdehyde (MDA) have been found to be involved in the initiation and promotion of multi-stage carcinogenesis.[2] Many studies show that oxidative stress is promutagenic and potentially carcinogenic.[3,4] ROS can directly cause oxidative injury to cells by damaging nucleic acids, proteins, lipids, and cell membranes in tissues.[5] ROS have also been reported to augment tumor cell migration, thereby increasing the risk of invasion and metastasis.[6] MDA is a potential biomarker for oxidative stres.[7] MDA levels have been investigated to examine oxidative stress and have been considered an indicator of oxidative imbalance during the onset of many diseases.[8]

The antioxidant enzyme superoxide dismutase (SOD) catalyzes the dismutation of O_2^- to H_2O_2, thereby preventing the accumulation of this free radical. H_2O_2 can be converted into H_2O and O_2 by catalase and/or glutathione peroxidase (GSH-Px).[9] GSH-Px is one of the body's most potent antioxidant defenses. Glutathione (GSH) and GSH-Px salvage the cell from lipid peroxidation damage, and particularly from damage to the membranes.[10] GSH and GSH-Px also protect cells from damage by free radicals, and specifically lipid peroxides and hydrogen peroxide. These enzymes act as members of the antioxidant system of the cell by degrading hydrogen

Correspondence to: Mehmet Aslan, Medical Faculty, Department of Internal Medicine, Yuzuncu Yil University, 65000 Van, Turkey.
Email: m.aslan301@mynet.com

DOI 10.1179/1351000214Y.0000000091

peroxide before it is converted into a hydroxyl radical.[11]

The human body has a number of endogenous free radical-scavenging systems, including paraoxonase. Paraoxonase-1 (PON1) is a Ca^{2+}-dependent serum esterase that is physically associated with high-density lipoprotein (HDL). PON1, which has antioxidant activity, participates in the metabolism of oxidized, biologically active lipids[12] and is responsible for the HDL-particle-mediated decrease in low-density lipoprotein lipid oxidation.[12] Reduced PON1 activity has been reported in diabetes mellitus, hypercholesterolemia, and cardiovascular disease, which involve increased oxidative stress.[13] Numerous reports have described that there is an association between PON1 enzyme activities and several cancers, including bladder cancer, prostate cancer, oral squamous cell carcinoma, and lung cancer.[14–17] Moreover, several investigators have examined the role of PON1 activity in patients with gastrointestinal cancer, including gastric, colorectal, and pancreatic cancer.[18–21] To the best of our knowledge, limited studies are available regarding the PON1 activity in patients with esophageal cancer,[22] and these studies are conflicting.

Therefore, the aim of this study was to investigate serum MDA levels and SOD, GSH-Px, glutathione reductase (GR), paraoxonase, and arylesterase activities in patients with ESCC.

Methods

Subjects

This prospective case–control study was conducted in the Departments of Thoracic Surgery at Van Regional Educational and Research Hospital and Yuzuncu Yil University. The study included 32 patients with ESCC (20 females and 12 males) and 33 healthy subjects (22 females and 11 males). All patients underwent resection of the primary carcinoma. Sixteen of the patients were receiving chemotherapy or radiotherapy.

A clinical diagnosis of ESCC was confirmed by microscopic examination of the material obtained during biopsy and/or surgery. Eight of the tumors were well-differentiated ESCC, 13 were moderately differentiated, and 11 were poorly differentiated. The depth of tumor invasion was as follows: 8 involved the submucosa, 8 involved the muscularis propria and 16 involved the adventitia or more. The cases with lymph node metastases were classified into two groups: a non-metastatic group ($n = 18$) and a metastatic group ($n = 14$). The tumor location was identified as the upper esophagus ($n = 4$), the middle esophagus ($n = 16$), or the lower esophagus ($n = 12$). Tumors were classified according to the TNM (primary tumor, regional lymph nodes and distant metastasis) classification.[23] The patients were divided into three groups: 15 patients had stage I or IIA disease, 7 patients had stage IIB disease, and 7 had stage III disease. Three patients were deemed inoperable during surgery due to aortic invasion (stage IV).

None of the enrolled patients was receiving regular antioxidant vitamin supplements, such as vitamins E and C. Patients who had an acute infection or an underlying systemic disease were excluded. In addition, the patients were not receiving any drugs and were not smoking or consuming alcohol.

The control subjects were asymptomatic with an unremarkable medical history and with a normal physical examination. None of the control subjects was receiving antioxidant vitamin supplementation, such as vitamins E and C. In addition, the control subjects were not receiving any drugs and were not smoking or consuming alcohol. Furthermore, the control subjects had no known acute or chronic diseases.

Blood collection

Following a 12-hour fasting period, blood samples were obtained in the morning before chemotherapy or radiotherapy. The blood samples were collected into empty tubes and immediately stored on ice at 4°C. The serum was then separated from the cells by centrifugation at 3000 rpm for 10 minutes. The serum samples were stored at −20°C until they were used for the measurement of antioxidant enzyme activities and MDA levels.

Measurement of paraoxonase and arylesterase activities

Paraoxonase and arylesterase activities were measured using commercially available kits (Rel Assay, Gaziantep, Turkey). PON1 activity was assayed using two different substrates.[24] First, the rate of hydrolysis of paraoxon was measured by monitoring the increase in absorbance at 412 nm and 25°C due to the formation of p-nitrophenol. PON1 activity assays were performed in the absence of basal activity and in the presence of NaCl (salt-stimulated activity). The serum sample to be tested was added to a cuvette containing 1.0 mmol paraoxon, 1.0 mmol $CaCl_2$, and 50 mmol glycine/NaOH buffer at pH 10.5. The amount of p-nitrophenol generated was calculated from the molar extinction coefficient at pH 10.5, which was 18 290/mol/cm. PON1 activity was expressed as the U/l required to generate 1 mmol p-nitrophenol in 1 minute under well-established conditions. The second PON1 activity assay was performed using an arylesterase assay with phenyl acetate as the substrate. Aliquots (10 ml) of prediluted (1:3) serum samples were added to a 1.5-ml reaction mixture containing 1.0 mmol of phenylacetate,

9.0 mmol of Tris-HCl buffer, and 0.9 mmol of $CaCl_2$ at pH 8.0 and 25°C. Enzymatic activity was calculated from the molar extinction coefficient, which was 1310/mol/cm. Arylesterase activity was expressed as kU/l and defined as 1 mmol phenol generated per minute under the reaction conditions.[25] Blanks without enzyme were used to correct for the spontaneous hydrolysis of both substrates.

Measurement of serum lipid peroxidation

To determine the amount of lipid peroxidation in the serum, the levels of MDA were analyzed spectrophotometrically using the modified thiobarbituric acid-reactive substance method reported by Yoshioka *et al.*[26] The results were expressed as nmol/ml.

Measurement of serum superoxide dismutase activity

Serum SOD was measured spectrophotometrically according to the GSH-400 and SOD-525 methods and assay conditions developed by the company Bioxytech S.A. (Cedex, France). SOD activation was measured using a Randox RANSOD enzyme kit and an autoanalyzer at 505 nm and 37°C. The rate of inhibition of the superoxide reaction by SOD was calculated according to the definition of McCord and Fridovich.[27]

Measurement of serum glutathione reductase activity

GR catalyzes the reduction of oxidized glutathione by nicotinamide adenine dinucleotide phosphate (NADPH). The spectrophotometric assay for GR is based on observing the decrease in absorbance at 340 nm caused by the conversion of NADPH to $NADP^+$.[28] The enzyme activity was expressed in U/ml.

Measurement of serum glutathione peroxidase activity

The serum GSH-Px activity level was determined spectrophotometrically by using a test reagent kit (Calbiochem-Novabiochem Corp., San Diego, CA, USA). Absorbance was measured at 340 nm. The results were expressed as milliunits of GSH-Px activity per milligram of Hb.

Statistical analysis

The results were expressed as mean and their standard deviations. Nonparametric continuous variables were compared by the Mann–Whitney *U*-test. Parametric variables were compared using Student's *t*-test. Qualitative variables were assessed by the χ^2 test. Correlations were calculated using Pearson's correlation. The results were considered statistically significant when the *P* value was less than 0.05. The data were analyzed using the computing program SPSS® for Windows (version 11.0).

Results

The demographic and clinical data of the ESCC and control groups are shown in Table 1. There were no statistically significant differences between the ESCC patients and controls with respect to age, gender, or body mass index ($P > 0.05$) (Table 1).

Of the 32 ESCC patients, 20 (62.5%) were female and 12 (32.5%) were male. Of the 33 control subjects, 22 (66.5%) were female and 11 (33.5%) were male. The mean age of the ESCC patients was 59 ± 2 years, and the mean age of the control subjects was 60 ± 4 years (Table 1).

Serum paraoxonase, arylesterase, SOD, GSH-Px, and GR activities were significantly lower in patients with ESCC than in controls (all $P < 0.05$), whereas serum MDA levels were significantly higher ($P < 0.05$) (Table 2).

Of the 32 ESCC patients, 4 were identified as having carcinoma in the upper esophagus; 16, in the middle esophagus; and 12, in the lower esophagus. There were no statistically significant differences among tumor localizations, such as upper, middle, and lower esophagus, with respect to serum antioxidant enzyme activities or MDA levels (all $P > 0.05$).

Sixteen of the ESCC patients were receiving chemotherapy or radiotherapy. Both before and after chemotherapy or radiotherapy, there were no statistically significant differences with respect to serum antioxidant enzyme activities or MDA levels (all $P > 0.05$).

Serum MDA levels were significantly correlated with paraoxonase ($r = -0.572$, $P < 0.001$) and arylesterase ($r = -0.597$, $P < 0.001$) activities in patients with ESCC. However, serum MDA levels were not correlated with serum SOD, GSH-Px, or GR activities in ESCC patients (all $P > 0.05$).

Discussion

In this study, we measured the activities of paraoxonase and arylesterase, two enzymes that are particularly important in antioxidative defense in patients with ESCC. In addition, we investigated serum SOD, GSH-Px, and GR activities as an index of antioxidant status in patients with ESCC. Moreover, in this study,

Table 1 Demographic characteristics of study groups

Parameters	Controls ($n = 33$)	Patients ($n = 32$)	*P*
Age (years)	60 ± 4	59 ± 2	NS
Sex (female/male)	22/11	20/12	NS
Body mass index (kg/m^2)	22.01 ± 1.2	22.10 ± 1.2	NS

Values are mean ± SD.
NS, non significant.

Table 2 Antioxidant enzyme activities and oxidative and antioxidant levels in esophageal cancer and controls

Parameters	Controls ($n = 33$)	Patients ($n = 32$)	*P*
Paraoxonase (U/l)	101.27 ± 18.07	61.39 ± 26.46	0.05
Arylesterase (kU/l)	38.32 ± 12.10	19.03 ± 3.14	0.05
Malondialdehyde (nmol/ml)	8.63 ± 2.46	20.17 ± 4.42	0.05
SOD (U/ml)	25.01 ± 2.83	7.39 ± 2.62	0.05
GSHPx (U/ml)	47.32 ± 3.75	22.05 ± 3.98	0.05
GR (U/ml)	13.51 ± 1.08	6.92 ± 1.38	0.05

Values are mean ± SD.
SOD, superoxide dismutase; GSHPx, glutathione peroxidase; GR, glutathione reductase.

we measured the levels of MDA as one of the end-products of lipid peroxidation.

ROS are postulated to be involved in the initiation, promotion. and progression of carcinogenesis, especially in the stages of initiation and promotion.[30] There are certain defense mechanisms in the body that prevent the development of free radicals and the damage that they cause. Antioxidants are substances that prevent, delay, or repair the damage caused by the free oxygen radicals in target tissues and are divided into two groups: enzymatic and non-enzymatic. Antioxidant enzymes include SOD, catalase, GSH, and GSH-Px, and non-enzymatic antioxidants include vitamin E, vitamin C, vitamin A (a-carotene), and selenium.[30,31]

The GSH redox system and SOD are each considered to play a major role in the defense mechanism against oxidative stress.[32,33] SOD catalyzes the transformation of superoxide into less toxic compounds, and GSH serves as a substrate in reactions in which electrophilic compounds, oxidants, and xenobiotics are detoxified.[32,33] GSH-Px, one of the GSH-depleting enzymes, is a more efficient metabolizer of hydrogen peroxide than catalase is.[10] GSH-Px is more important than either SOD or catalase in preventing peroxidation.

The mechanisms underlying the development of esophageal carcinoma are poorly understood. It is well known that oxidative stress induced by environmental carcinogen exposure may affect cellular functions in various pathological conditions, including cancer.[6,7] Oxidative stress may affect several functions in cancer cells and tumor tissues, such as cell proliferation, gene mutation and instability, anticancer agent sensitivity, tumor invasion and metastatic spread.[22] Oxidative stress can also lead to tumor angiogenesis and augment tumor cell migration, increasing the risk of invasion and metastasis.[6] It has been demonstrated that ROS are directly involved in the oxidative damage of cellular macromolecules, such as lipids, proteins, and nucleic acids, in tissues.[5]

MDA is thought to act as a tumor promoter and as a co-carcinogenic agent.[34] The reports in the literature on MDA levels in cancer patients are controversial. Dursun *et al.*[35] investigated MDA levels in esophageal cancer patients. They showed that MDA levels were significantly higher in patients with esophageal cancer than in controls. In addition, Arivazhagan *et al.*[36] reported increased erythrocyte MDA levels in patients with gastric cancer. Bakan *et al.*[37] reported increased plasma MDA levels in patients with gastric cancer. Conversely, several authors reported decreased plasma MDA levels in patients with breast cancer.[38,39] Similarly, Gerber *et al.*[40] reported decreased plasma MDA levels with tumor size and breast cancer progression. In this study, we measured MDA levels as a marker of oxidative stress. We observed that serum MDA levels were significantly increased in ESCC patients compared with levels in healthy subjects.

Reports about the effects of antioxidant enzymes on cancer types are controversial. Dursun *et al.*[35] investigated SOD, GSH-Px, and catalase activities in esophageal cancer patients. They showed that SOD activity was significantly higher and GSH-Px and catalase activities were significantly lower in patients with esophageal cancer than in controls. Conversely, Guven *et al.*[41] reported lower plasma GSH-Px activity in cancer patients with metastasis than in controls and higher plasma SOD activity in cancer patients with metastasis. Similarly, Arivazhagan *et al.*,[36] observed a significant increase in SOD activity and a decrease in GSH-Px activity in patients with gastric cancer compared with control subjects. Skrzydlewska *et al.*[42] reported significantly decreased catalase activity in colorectal cancer. In this study, we observed that serum SOD, GSH-Px, and GR enzyme activities were significantly lower in ESCC patients than in controls.

Antioxidants not only prevent lipid peroxidation but also protect protein, nucleic acids, lipids, and cell membranes.[43] Impaired antioxidant enzyme activity has been associated with the development of cancer. Low antioxidants levels may be due to the scavenging of lipid peroxides and to sequestration by tumor cells.[44] Oberley and Oberley[45] suggested that there is an imbalance in antioxidant enzymes in most cancer cells. Previous studies have reported the beneficial effects of antioxidant supplementation in controlling cancer progression.[46,47] It has been observed that diets rich in fruits and vegetables can decrease both oxidative DNA damage and cancer risk.[48]

Carcinogenic lipid soluble radicals are formed due to lipid peroxidation, and PON1 binds to these radicals. PON1 has been shown to metabolize lipid-soluble radicals[49] and to have both paraoxonase and arylesterase activities. PON1 is an antioxidant enzyme associated with HDL that hydrolyzes lipid peroxides, cell membranes and lipoproteins.[50] Lower PON1 activity may reflect increased oxidative stress in serum and macrophages.[51] PON1 enzyme activities are under genetic

and environmental regulation and appear to vary widely between individuals and populations.[52]

Previous studies have indicated that there is an association between PON1 enzyme activities and several cancers, including bladder cancer, prostate cancer, oral squamous cell carcinoma, and lung cancer.[14–17] Moreover, several investigators have investigated the role of PON1 activity in patients with gastrointestinal cancer, including gastric, colorectal, and pancreatic cancers.[18–21] Limited studies are available regarding PON1 activity in patients with esophageal cancer,[22] and these studies are conflicting. Akcay *et al.*[18] investigated PON1 activity in gastric cancer patients. They showed that PON1 levels were significantly lower in patients with gastric cancer than in the control group. In another study, Akcay *et al.*[19] obtained similar results when comparing pancreatic cancer patients with healthy subjects. Recently, Kodydkova *et al.*[20] showed that PON1 enzyme activity levels were significantly lower in patients with pancreatic cancer. Bulbuller *et al.*[21] reported lower PON1 enzyme activities in patients with colorectal cancer. Similarly, Elkiran *et al.*[14] described lower PON1 activity in patients with lung cancer. More recently, Malik *et al.*[15] reported lower PON1 activity in patients with oral squamous cell carcinoma. Conversely, Cayır *et al.*[22] investigated the possible relationship between the serum activities of PON1 and arylesterase and the clinicopathological characteristics of esophageal cancer and reported increased serum PON1 and arylesterase activities in patients with esophageal cancer compared with healthy controls. Moreover, they observed decreased serum PON1 and arylesterase activities in stage 3 and stage 4 esophageal cancer patients compared with stage 2 esophageal cancer patients. However, Cayır *et al.*[22] did not evaluate MDA levels as a marker of oxidative stress. Similarly, in a recent study, Eroglu *et al.*[16] showed that PON1 enzyme activity was significantly lower in prostate cancer patients than in controls. In contrast, recently, Aydin *et al.*[17] evaluated serum PON1 activity in patients with bladder cancer and stated that there were no significant differences in serum PON1 activity in patients with bladder cancer.

In this study, we found that serum PON1 enzyme activities were significantly decreased in patients with ESCC compared with healthy individuals. Our findings are in agreement with the results of Cayır *et al.*[22] According to published studies, our report is the first to indicate that serum PON1 activity is lower in patients with ESCC. Our findings support prior investigations that suggest that the enzyme PON1 may protect against cancer through its antioxidant properties.[53]

The pathogenetic mechanism underlying the contradictory results obtained in cancer patients is unclear. One of the possible reasons for this discrepancy is the specific tumor biology of esophageal cancer. Genetic differences are also one of the probable explanations for this discrepancy.

Polymorphisms of the PON1 gene have been studied in patients with a large number of different malignancies. It has been emphasized that PON1 polymorphisms might contribute to the increased risk of cancer associated with pollutants and other environmental chemicals.[38] Reports on PON1 polymorphism in patients with cancer are conflicting. Kerridge *et al.*[39] were the first to demonstrate an association between PON1 polymorphisms and cancer in humans. Antognelli *et al.*[54] reported an increased risk of prostatic cancer in patients with the PON192/QQ genotype compared with those with the PON192/RR genotype. Saadat[55] showed that the PON1 M and Q alleles are associated with a higher risk of breast cancer. Conversely, Marchesani *et al.* did not find an association between PON1 polymorphism and prostatic cancer.[56] Van Der Logt *et al.*[57] could not detect a significant difference in the PON1 genotype between colorectal cancer patients and healthy subjects. Similarly, Vecka *et al.*[58] did not find any significant association between PON1 polymorphism and pancreatic cancer. To the best of our knowledge, PON1 polymorphism in patients with esophageal cancer has not yet been investigated. However, we were unable to perform PON1 genotyping analyses due to certain problems.

Our study has several limitations. First, the investigation used a cross-sectional design. Second, the study sample was small, so these observations must be confirmed in a larger patient sample. Third, the activity of PON1 is under genetic and environmental regulation. We were unable to perform PON1 genotyping analyses due to technical challenges.

We conclude that serum antioxidant enzyme activities were significantly lower in ESCC patients compared with healthy subjects. Decreased PON1 activity and increased MDA levels may play a role in the progression and/or development of esophageal cancers. In future studies, it may be important to determine whether PON1 activity plays a causal role in cancer. Further studies are required to clarify the results.

Acknowledgements

The authors thank the staffs of Harran University Clinical Biochemistry for their generous and friendly assistance in every step of this study.

Disclaimer statements

Funding

None.

Conflicts of interest

None.

Authors' contributions

AS and MA: Conception and design;
MA and HD: Analysis and interpretation of the data;
AS, MA and HD: Critical revision of the article for important intellectual content;
AS, MA and HD: Final approval of the article;
AS, FS and AK: Collection and assembly of data.

Ethics approval

The study protocol was performed in accordance with the Helsinki Declaration as revised in 2000. The protocol was approved by the local ethics committee, and informed consent was obtained from each subject.

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