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Determination of glutathione peroxidase activity and oxidized/reduced glutathione ratio in patients with colon cancer

GÖKCE Kemal and DAG Seker *

Department of Biology, Faculty of Science, Sivas Cumhuriyet University, Sivas, Turkey.

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Abstract

Colon cancer (CC) is accepted as the third type of cancer that causes death in the world. Oxidative stress and low glutathione peroxidase (GPx) activity can cause CC. This study aims to show whether GPx and Oxidized (GSSG)/ reduced glutathione (GSH) levels, which are considered oxidative stress markers, are effective in the etiopathogenesis of CC.

Erythrocyte isolation was performed in 3 ml blood sample taken from volunteers aged 18-75 years. Hemoglobin amounts were determined from the standard graph drawn by monitoring the conversion of methemoglobin to cyanmethemoglobin in the presence of cyanide at 540 nm. Glutathione peroxidase activity was determined by spectrophotometric monitoring of NADPH+H+ (reduced nicotinamide adenine dinucleotide phosphate) oxidation at a wavelength of 340 nm. The amounts of oxidized and reduced glutathione were determined by using the standard graph drawn by following the 412 nm wavelength of the formation of 2-nitro-5-thiobenzoic acid, which has a yellow color.

GPx activity of individuals with CC is 5.64 ± 1.49 U/gHb, GSH concentration is 6.96 ± 1.45 nmol/gHb, and GSH/GSSG ratio is 1.04 ± 0.49, GPx activity of healthy individuals is 10.52 ± 2.22 U/gHb, GSH concentration 11.43 ± 1.90 nmol/gHb, and GSH/GSSG: 3.86 ± 1.30, that is, the values of the patient group were significantly lower than the control group.

Current results suggest that GPx activity, GSH concentration and GSH/GSSG ratio can be used as CC markers in the diagnosis and monitoring of disease course, and that the decrease in these parameters may be associated with an increased risk of CC.

Keywords: Colon Cancer; Glutathione; Glutathione Peroxidase; Oxidative Stress

1. Introduction

Reactive oxygen species (ROS) are highly reactive atoms or molecules due to their unshared electrons. Reactive oxygen species produced in many physiological conditions are neutralized by antioxidant defense mechanisms [1]. The shift of the delicate balance between ROS and the antioxidant defense system in favor of pro-oxidant and oxidant substances leads to the development of oxidative stress. The deficiency in the amount of GSH and GPx activity, which have an important role in antioxidant defense, will shift the antioxidant/oxidant balance in the organism to the oxidant side. Therefore, the free radicals formed may cause various diseases by damaging the organism. Cancer is one of these diseases [2]. Substances that can damage DNA and cause cancer are known as free radical (FR) processes. Free radicals induce mutations such as deletion, gene amplification, a rearrangement that cause activation/inactivation of various protooncogenes and/or tumor suppressor genes [3]. DNA damage caused by the effect of FRs can also change the expression of radical scavenger enzymes. Activity changes in enzymes affect antioxidant defense [4]. The radical scavenging activity of GSH facilitates the restoration of detoxication of damage caused by ROS. GSH, GPx, and Glutathione S Transferase (GST) are common substrates for enzymes involved in detoxification reactions of other ROS. In

* Corresponding author: DAG Seker E-mail: sdag@cumhuriyet.edu.tr

detoxification reactions, oxidized GSH must be reduced again to become a co-substrate [5]. This reaction is catalyzed by glutathione reductase (GR) [6,7].

Compared to normal cells, two important features of tumor cells are increased ROS production and decreased antioxidant capacity to scavenge ROS [8,9]. ROS formation above the cell's antioxidant capacity causes a decrease in GSH and an increase in GSSG. The reciprocal variation in GSH and GSSG concentrations is expressed as the GSH/GSSG ratio, which is considered one of the indicators of oxidative stress [10]. It is known that oxidative stress has important effects on cancer development. For example, it is involved in the pathogenesis of many malignant diseases such as colon cancer. It is thought that the superoxide radical and hydrogen peroxide, especially the hydroxyl radical, have an important role in oxidative damage on biomolecules [11]. The incidence of colon cancer is higher than in other parts of the gastrointestinal tract, as dietary red meat's fecal iron and excess dietary fat increase procarcinogens and bile pigments in the stool. The relatively high iron concentrations in feces, combined with the ability of bile pigments to act as iron chelators, which underpin Fenton chemistry, can highly allow efficient hydroxyl radical formation from superoxide and hydrogen peroxide produced by bacterial metabolism. Free radical formation in feces may provide us with an undetermined factor in the etiology of colon cancer [12]. An imbalance occurs in peroxide capacity due to increased production or lack of scavenging, leading to the initiation of carcinogenesis by mutagenesis. The procarcinogenic effect of H_2O_2 may also arise from inflammatory cells associated with cell damage. In this process, GPxs have anticarcinogenic effects [7].

In this study, it was aimed to determine the status of oxidative stress and antioxidant capacity in colon cancer by detecting the changes in the GSH/GSSG ratio, which is thought to be an indicator of oxidative stress, the concentration of GSH, which is an important part of the antioxidant capacity, and the changes in the activity of GPx.

2. Material and methods

2.1. Patient and control groups

This study included 30 healthy subjects and 30 patients diagnosed with colon cancer [Table 1].

2.2. Blood samples

3 ml of venous blood samples from the individuals in the patient and control groups were taken into tubes containing K_3-EDTA, and the tubes were transferred to the laboratory at +4˚C. The erythrocyte isolation process was started immediately in the samples that reached the laboratory. Blood samples were centrifuged at 2500 xg for 10 minutes at +4˚C. After the supernatant was taken, isotonic NaCl solution 3 times its volume was added to the formed elements remaining in the tube. The erythrocytes were washed by gently inverting and centrifuged again at 2500 xg at +4˚C for 10 minutes. This process was repeated 3 times. Then, the erythrocytes were hemolyzed by the freeze-thaw method by adding distilled water at a ratio of 1:5 v/v. After this process, the hemolysate was centrifuged at 22,000 xg at +4˚C for 60 minutes to separate the cell membranes. The formed erythrocyte hemolysates were divided into 1.5 ml Eppendorf tubes to be used in all analyzes and stored at -20˚C [13].

2.3. Determination of glutathione peroxidase activity

GPx activity in erythrocytes was measured by the method of Paglia and Valentine [14]. Hydrogen peroxide was used as a substrate to measure enzyme activity, and the oxidation of NADPH was determined spectrophotometrically at a wavelength of 340 nm. Results are given as U/gHb. Total hemoglobin amount was determined in g/dl by cyanmethemoglobin determination [15].

2.4. Measurement of oxidized and reduced glutathione

Glutathione measurement in erythrocytes was performed according to the principle defined by Beutler, Duron, and Kelly [16]. In the method, sulfhydryl groups form a chromogen compound with the DTNB reagent, and the yellow color formed is read against the reagent blank at 412 nm.

The calibration curve was plotted with 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 nmol/ml GSH solutions prepared from stock GSH solution (10 nmol/ml). The absorbance of the samples was converted to the concentration with the help of this curve, and quantification was made. Erythrocyte GSH values were expressed as nmol GSH/Hb by proportioning to hemoglobin values.

The amount of oxidized glutathione was determined by the method used to measure the amount of total GSH. Differently, only the amount of GSSG was measured by derivatizing the homogenate with 2-Vinylpyridine before use. Obtained absorbance values were converted into concentration values using the standard graph prepared for total GSH.

2.5. Statistical analysis

Results were analyzed using analysis of variance, Tukey test, Kruskal-Wallis test, Mann Whitney U test, and correlation analysis. P<0.05 was considered as the significance level.

3. Results

Thirty patients with colon cancer and 30 healthy control subjects were included in the study. The characteristic features of the patient and control groups are shown in Table 1. The means GPx activity and GSH, GSSG, and GSH/GSSG values of the patient and control groups are shown in Table 2.

Table 1 Distribution of control and patient by mean age of groups, sex and smoking

	Control Group (n=30)	**Colon Cancer (n=30)**
Age	41.46 ± 2.60	56.06 ± 1.75
Sex		
Male	12	16
Female	18	14
Smoking		
Yes	13	11
No	17	19

Table 2 GPx activity, GSH, GSSG concentration and GSH / GSSG ratio of control and patient groups

Parameters ($\bar{X}$ ±SD)	**GPx (U/gHb)**	**GSH(nmol/gHb)**	**GSSG (nmol/gHb)**	**GSH/GSSG**
Control group	10.52 ± 2.22	11.43 ± 1.90	3.09 ± 0.48	3.86 ± 1.30
Colon cancer	5.64 ± 1.49	6.96 ± 1.45	7.20 ± 1.17	1.04 ± 0.49
	*P=0.001	*P=0.001	*P=0.001	*P=0.001

* Statistically significant (p <0.05).

When the GPx activities of the groups were compared, the GPx activities of individuals with colon cancer were statistically significantly lower than those in the control group (p<0.05) [Table 2]. When the GSH values of the groups were compared, it was found that the GSH levels of the patients with colon cancer were lower than the control group, and this was statistically significant (p<0.05) [Table 2].

When the GSSG values of the groups were compared, the GSSG level of individuals with colon cancer was significantly higher than the control group (p<0.05) [Table 2].

When the GSH/GSSG ratios of the groups were compared, as shown in Table 2, the GSH/GSSG ratios of individuals with colon cancer were statistically significantly lower than the control group (p<0.05).

It was determined that the differences between all parameters according to gender in both patient and control groups were statistically insignificant (p>0.05) [Table 3].

Table 3 Comparison of intra-group parameters according to smoking status

Parameters ($\bar{X}$ ± SD)		GPx (U/gHb)	GSH (nmol/gHb)	GSSG (nmol/gHb)	GSH/GSSG
Control group	Smoking	9.27 ± 1.53	11.61 ± 2.06	3.08 ± 0.52	3.98 ± 1.49
	No Smoking	11.47 ±2.22	11.30 ± 1.82	3.10 ± 0.46	3.77 ± 1.17
		*P=0.005	P=0.851	P=0.950	P=0.818
Cancer group	Smoking	5.98 ± 1.24	6.88 ± 1.55	7.22 ± 1.53	1.08 ± 0.73
	No Smoking	5.44 ± 1.62	7.00 ± 1.43	7.18 ± 0.95	1.01 ± 0.31
		P=0.335	P=0.189	P=0.426	P=0.378

* Statistically significant (p <0.05) .

When the GPx activities of the individuals in the control group were compared according to their smoking status, the decrease in the GPx activity of the smokers was statistically significant. At the same time, the differences between the other parameters were statistically insignificant (p<0.05) [Table 3].

When all data of individuals with colon cancer were compared statistically according to smoking status, no significant difference was found (p>0.05) [Table 3].

In individuals with colon cancer, all parameters according to the stage of the disease were statistically insignificant, as shown in Table 3 (p>0.05).

4. Discussion

Under physiological conditions, free oxygen radicals are present in aerobic organisms and are tightly controlled by cellular redox systems and antioxidants. However, due to the increase of oxygen radicals or the insufficiency of the antioxidant system, the cellular redox balance shifts to the oxidation direction, resulting in oxidative stress. The decrease in the amount of GSH and GPx activity, which play an important role in antioxidant defense, may increase due to insufficient scavenging of oxidant substances in the organism and cause many diseases, especially cancer. One of the types of cancer in which FRs may play a role in its etiopathogenesis is CC and RC, which are the third leading cause of death in the world [17,18,19, 20].

In studies on the antioxidant system in patients with CC, erythrocyte GSH levels were decreased in sick individuals compared to healthy individuals [21, 22]. In the study of Navarro *et al*.,[23] by creating artificial tumors in mice, it was observed that there was a significant decrease in the amount of blood GSH and a significant increase in the amount of blood GSSG in mice with tumors when compared to the values before tumor formation.

As mentioned above, many studies with cancer patients show that patients have a significant reduction in the amount of erythrocyte GSH. Depending on this decrease in the amount of GSH, it is expected that there will be an increase in the amount of GSSG. In this study we conducted with patients with colon cancer, we found that the erythrocyte GSH levels of cancer patients decreased significantly compared to healthy individuals. As expected, the erythrocyte GSSG values of cancer patients increased significantly compared to healthy individuals [Table 2].

During scavenging of FRs under oxidative stress, the amount of GSH decreases while the amount of GSSG increases. The reciprocal variation in GSH and GSSG concentrations is expressed by the redox ratio, [GSH]/[GSSG], which is considered one of the hallmarks of oxidative stress. Whenever cells are exposed to high levels of oxidative stress, GSSG accumulates, and the ratio of GSH to GSSG decreases. Therefore, the redox ratio, [GSH]/[GSSG], is also used as a measure of the extent of oxidative stress [24, 25]. In our study, the GSH/GSSG ratio was significantly decreased in individuals with colon cancer compared to healthy individuals. This decrease in the GSH/GSSG ratio indicates that individuals with colon cancer are exposed to significant oxidative stress [Table 2].

The GSH/GPX enzyme system removes reactive oxygen species and reactive nitrogen species (RNS). Glutathione peroxidase, a glutathione-dependent enzyme, conjugates with genotoxic and mutagen electrophilic substances and plays a role in their detoxification [26, 27]. Studies show that the increase of oxidant and genotoxic substances is significantly involved in all stages of chemical carcinogenesis [28]. These studies reported that the amount of glutathione peroxidase decreased significantly in cancerous tissue and blood compared to healthy individuals in

general. Canbay *et al.,*[13] found that the erythrocyte GPx values of patients with laryngeal cancer were significantly lower than those of healthy individuals in their study. Likewise, Canbay *et al.,* [29] reported a significant decrease in the GPx activity of patients with thyroid cancer compared to control group individuals in their study. Hopkins *et al.,* [30] suggested that in a cancer study including colon cancer, erythrocyte GPx activity showed wide variations but was generally lower than normal levels. Saygılı *et al.,* [22] found a highly significant decrease in the patient group when they compared the amount of erythrocyte GPx in the control group and the patient group in the study they conducted in patients with colorectal cancer. In another study conducted in this direction, Scibior *et al.,*[11] reported that the GPx activity in the blood serum of individuals with stomach and colon cancer was significantly lower than in healthy individuals. This study found that the amount of GSH and GPx activity, which are important components of the antioxidant defense system, were lower in the CC group than in the control group [Table 2], and this means that patients with CC have lower antioxidant capacity and higher oxidative stress. Decreased GPx activity in erythrocytes of individuals with CC increases oxidative stress by accumulating H_2O_2 and other oxygen radicals and causes an increase in the effect of oxidizing agents on erythrocytes [31].

Determination of GSH level is a good indicator of antioxidant capacity in various diseases, including cancer and in healthy individuals because GSH plays an important role as a co-substrate for GPx, an enzyme responsible for the removal of H_2O_2 and lipid hydroperoxides, as well as the direct removal of free radicals from the environment [32, 33]. This decrease in erythrocyte GSH levels in patients with colon cancer is also responsible for the decrease in GPx activity [34].

Studies show that smoking, dietary habits, lifestyle, and decrease in selenium amount cause a decrease in GPx enzyme activity [35]. This study showed that GPx activity in smokers in the control group decreased significantly compared to non-smokers, indicating that smoking impairs antioxidant defense. However, there was no significant difference in GPx activity between smokers and nonsmokers in cancer patients [Table 3]. The results we obtained in this study are compatible with the study of [36].

In each group, all parameters according to gender were statistically insignificant. Despite this, as seen in Table 4, GSH and GPx values in women in both control and cancer groups were higher than men, and GSSG values of men were higher than women [Table 4]. These results suggest that antioxidant defense is better in women than in men.

Table 4 Comparison of parameters according to sex

Parameters ($\bar{X}$ ± SD)		**GPx (U/gHb)**	**GSH (nmol/gHb)**	**GSSG (nmol/gHb)**	**GSH/GSSG**
Control group	Female	10.89±2.58	11.87 ±2.30	3.00 ± 0.57	4.19 ±1.58
	Male	9.97 ±1.47	10.77 ± 0.75	3.22 ± 0.26	3.36 ± 0.38
		P=0.553	P=0.472	P=0.099	P=0.138
Cancer group	Female	5.51 ±1.50	7.23 ± 1.67	6.74 ± 1.48	1.20 ± 0.67
	Male	5.75 ±1.52	6.71 ± 1.23	7.59 ± 0.63	0.89 ± 0.21
		P=0.647	P=0.257	P=0.085	P=0.110

* Statistically significant ($p < 0.05$) .

5. Conclusion

Our findings show that the GSH/GPx system, an important part of antioxidant defense, is insufficient in individuals with CC. This insufficiency may be due to the high oxidative stress in CC, which is at a level that forces the system, or because the GSH/GPx system is defective for any reason.

Monitoring the GSH/GPX system and GSH/GSSG levels in cancer patients will provide information about the course of the disease. We think these parameters can be monitored as markers in the follow-up of the path to cancerization processes in healthy individuals.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Statement of ethical approval

Cumhuriyet University, Faculty of Medicine, Scientific Research Assessment Board has been working with the permission of decision 20120-04 / 10.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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