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Increased Glutathione Reductase Expression and Activity in Colorectal Cancer Tissue Samples: An Investigational Study in Mashhad, Iran

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Abstract

Background: Glutathione reductase is an important enzyme in oxidative metabolism that provides reduced glutathione from its oxidized form in the cells. The role of oxidative stress in tumor tissues has led us to investigate the gene expression and activity of this enzyme in tumor and adjacent resected margins of colorectal cancer tissues, one of the most common malignancies in humans.

Methods: We conducted this study on 15 Iranian colorectal cancer patients. RNA was extracted from fresh colon tissues that included tumor and anatomically normal margin tissue. Expression of the glutathione reductase gene was determined using real-time PCR by the ΔΔCt relative quantification method. The gene expression results were standardized with glyceraldehyde 3-phosphate dehydrogenase as the endogenous reference gene. In addition, we measured enzyme activity of glutathione reductase with a commercial kit based on a colorimetric assay.

Results: The tumor tissue had higher expression of glutathione reductase compared to the margin tissue (P=0.005). There was significantly greater glutathione reductase enzyme activity in the tumor tissue (116.9±34.31 nmol/min/ml) compared to the non-cancerous adjacent tissues (76.7±36.85 nmol/min/ml; P=0.003).

Conclusion: These data showed increased glutathione reductase expression and enzyme activity in colorectal tumor tissue. Given the key role of glutathione in synthesis of dNTPs for DNA repair with the glutaredoxin system, the increased glutathione reductase expression and activity might be a reflection of hyperactivity of this enzyme in DNA synthesis and the repair process in colorectal cancer cells.

Keywords: Colorectal cancer, Glutathione reductase, Oxidative stress, Real-time PCR

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in men and second most in women. It is the fourth most common cause of cancer deaths worldwide.[1] The 1.23 million cases, which include 9.7% of overall cancers, indicates the importance of CRC in health care systems.[2] Unfortunately, death occurs in about half of all CRC cases due to its late diagnosis which leads to advanced stages of this malignancy at the time of diagnosis.[3] It has been reported that patients with advanced tumor staging, lymph node invasion, and venous invasion have evidence of elevated oxidative stress factors. Therefore it seems reasonable that higher levels of oxidative stress markers are accompanied by advanced cancer stage.

One of the known factors, glutathione reductase (GR, EC 1.8.1.7) has a critical role in oxidative metabolism through its role in the glutaredoxin system.[4,5] This enzyme produces reduced glutathione (GSH) from its oxidized form (GSSG) in the living cells, thereby GSH provides a reducing intracellular environment.[6] The amount of GSH and its ratio to its oxidized form has an important role in controlling the activity of other redox-sensitive proteins, which suggests a fundamental role for control of cellular function.[7] Considering the role of oxidative stress in malignancy development, one could speculate that changes in expression and activity of the GR enzyme could play a critical role in advancing cancer. The redox poise of the mitochondrial GSH pool is the main factor in mitochondrial response to oxidative damage and redox signaling, however the mechanisms remain uncertain. One possibility is that oxidation of GSH to glutathione disulfide (GSSG) and relevant modifications in the GSH/GSSG ratio induce protein thiols to alter their redox state. This enables the protein function for a reversible response to redox signals and oxidative injury. However, our knowledge about the interaction between the mitochondrial GSH pool and protein thiols function is insufficient.[7,8]

It has been shown that the serum level of oxidative stress factors increased in patients with gastrointestinal tract malignancies, which was probably due to the high level production of reactive oxygen species (ROS). Elevation of lipid peroxidation and significant differences in levels of GSH and other relevant enzyme activities such as glutathione peroxidase (GSHPx), glutathione-S-transferase (GST), and GR were reported in the serum samples of patients with gastrointestinal tract tumors compared to healthy blood donor controls. These observations indicated the occurrence of effective defense mechanisms along with oxidative stress which, in turn, have been linked to GSH metabolism.[8]

In addition, a lower antioxidant activity, higher ROS, and increased lipid peroxidation have been shown in oxidative stress studies in gastrointestinal tract malignancies. There are conflicting results from reports on GSH levels in colorectal carcinoma. Increased GSH and tumor malondialdehyde levels[5] and adversely declined nonenzymatic antioxidant levels that included reduced GSH, vitamin C, and vitamin E have been reported.[9] Similarly, contradictory reports exist on GR activity in CRC. Although studies report increased GR activity,[8-10] others have reported lower GR activities compared to the controls in serum of these patients.[5,11]

Therefore the GR activity and expression in colorectal carcinoma is controversial. Here, we investigate enzyme activity in addition to GR expression levels using different techniques.

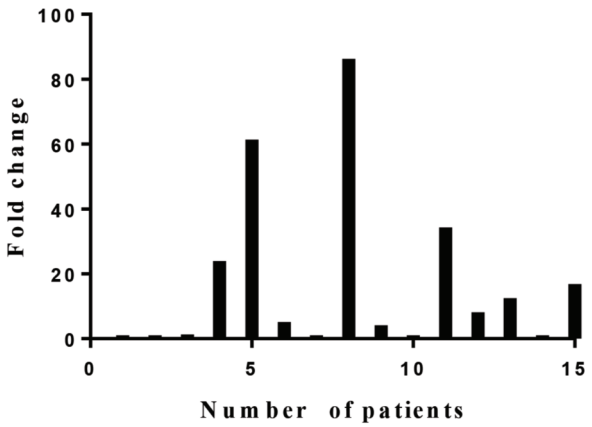

Figure 1. Variation of glutathione reductase (GR) expression in the studied patients using the ΔΔCT method. The relative fold changes in gene expression were higher than one for 10 cases. Numbers in the x-axis indicate each case of colorectal cancer (CRC) evaluated in the study.

Materials and Methods

Patients and samples

This cross-sectional study enrolled 15 patients with CRC who were admitted to the Department of Surgery at Emam Reza university Hospital, Mashhad, Iran. Table 1 lists the participants' demographic and staging characteristics.

All patients received the diagnosis of colorectal carcinoma according to pathology experts' opinions; none had received multivitamins or antioxidant supplements, any chemotherapy or radiotherapy regimen, and no history of other chronic conditions such as diabetes, kidney or cardiovascular diseases were found in patients' records. Tissue samples were provided after surgical resection. The samples were then washed in potassium phosphate buffer (PBS; pH=7.4) in order to remove RBCs and cell debris. Marginal tissue samples were diagnosed as anatomically normal colorectal tissue based on pathology reports.

Table 1. Patients' general characteristics.

Total number of subjects	15
Gender (male/female)	13/2
Mean age (years)	60.8±19.1
Clinical stage (n)	I: 4 II: 7 III: 3 IV: 1

Evaluation of glutathione reductase (GR) gene expression

Total RNA was extracted from 1 g of the CRC samples using an RNeasy plus Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA integrity was evaluated by agarose gel electrophoresis and spectrophotometric analysis of the concentration of extracted RNA by a NanoDrop 2000. A defined amount of RNA (0.3 µg) was applied for cDNA synthesis with the Easy™ cDNA reverse transcription kit (Parstous Biotech cat. no. A101162, Iran). Next, oligonucleotide primers were designed based on sequences obtained from the NCBI Genbank database (Table 2). Then, cDNA was diluted 1:30 with nuclease-free water and used for quantitative real-time PCR.

A total of 2 µl of cDNA was added to 20 µl of a real time-qPCR reaction mixture that consisted of 1x Hotstart Sybr Green PCR Premix (Parstous Biotech cat. no. C101021) and 0.5 pM of each primer. Thermal cyclic reactions were performed in a Rotor Gene 6000 Real-Time PCR Detection System (Corbett Research, Australia). The amplification protocol was as follows: 15 min at 95°C for initial denaturation followed by 40 cycles of 95°C for 15 s, 50°C for 1 min, and 72°C for 30 s.

All reactions were run in triplicate, including the negative controls. No reverse transcriptase (NRT) control was used to confirm that the amplification signal resulted from RNA templates rather than possible genomic DNA contamination. The relative expression of mRNAs was calculated using the ΔΔCt relative quantification method. Gene expression results were standardized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous reference gene.

Measurement of glutathione reductase (GR) enzymatic activity

We measured GR activity in the samples using a glutathione reductase activity assay kit (Cayman, cat. no. 703202). Assessment of GR activity was performed on 10% (w/v) homogenized tissue, which was prepared in 50 mM PBS (pH=7.5) that contained 1 mM EDTA. After 15 min centrifugation at 10000g, the supernatant was used for assessment of GR activity according to the manufacturer's protocol. Based on the kit's instructions and protocol, we measured the enzyme activity in the reaction solution and expressed it as nmol/min/ml; each ml contained 0.1 g of tissue.

Statistical analysis

The statistical evaluations were done with SPSS version 11. The Kolmogorov-Smirnov (KS) test was used to analyze normal data distribution. Paired-sample and independent-sample t-tests were used for data comparison. $P<0.05$ was considered statistically significant.

Table 2. Specific primers used for real-time quantitative PCR.

Primer sequences	Target gene
Forward: GCTTAGGAACCAGCGAT	GR (NM_001195103)
Reverse: AACTTCTAAGTACCTTCATGC	
Forward: GGTTTCTATAAATTGAGCCCG	GAPDH (NM_002046)
Reverse: CCAATACGACCAAATCCGTT	

All primers are from 5´ to 3´;GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GR: Glutathione reductase

Ethical considerations

The Ethical Committee of Mashhad University of Medical Sciences approved this study which was performed based on ethical codes of the Declaration of Helsinki (ethical code: MUMS-900791). Written informed consent was obtained from each patient before enrollment. No additional procedure was performed for sampling.

Results

The data were normally distributed (KS test, $P>0.05$). This study enrolled 15 patients with a mean age of 60.8±19.1 years; 86.6% of the patients were male.

Determination of gene expression in tumor samples and resection margins of colorectal cancer (CRC)

After obtaining the Ct values for the GR and GAPDH genes in the tumor tissue and resection margin samples, we calculated and analyzed ΔCt values for each sample using SPSS software. Tumor tissue samples showed higher expression levels compared to resection margins ($P= 0.005$).

Comparison of gene expression changes in tumor and normal marginal tissues

We calculated ΔΔCt to evaluate changes in gene expression. Evaluation of gene expression based on ΔΔCT showed elevated levels of GR gene expression in all tumor tissues compared to resection margins (Figure 1).

Measurement of glutathione reductase (GR) enzyme activity

Assessment of GR enzyme activity in samples showed significantly higher mean activity in the tumor tissues compared to the enzyme activity in the resection margins (Table 3; $P=0.005$).

Discussion

We found a higher GR gene expression in CRC samples compared to surrounding tissue. In addition, there was significantly greater enzyme activity in tumor tissue compared to the margin controls. The data supported some reports that highlighted the role of glutaredoxin system in colorectal carcinoma patients.[8]

Despite controversial reports of the activity or expression of antioxidant enzymes and the levels of antioxidant molecules in CRC, there is a consensus on a multistep process controlled by oxygen radicals in these cancers, from initiation to the final stages. In addition to tumor cells, inflammatory immune cells like neutrophils and macrophages have been considered as a main source of ROS in the cancer cell microenvironment. Elevated GR activity might be a response of cancer cells to increased ROS.[12]

Increased GR activity in tumor cells may provide special properties that favor tumors. GR is involved in cellular defense against ROS by producing reduced GSH.[13] The GSH content of cancer cells controls possible growth-associated changes such as mutagenic mechanisms, invasive cancer cell, survival and death, and sensitivity to therapy.[14,15] Mechanisms that underlie elevated GR activity might include increased expression of this enzyme by activation of numerous transcription factors. One of the most recognized

Table 3. Enzyme activity of glutathione reductase (GR) in tissue samples of colorectal cancer (CRC).

	Enzyme activity (nmol/min/ml)*		
	Minimum	Maximum	Mean±SD
Tumor tissue	35.92	193.06	116.9±34.31
Normal margin	25.01	124.80	76.7±36.85

$P=0.003$

*Each sample was homogenized in 10 ml of cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA) per gram tissue according to the kit protocol for enzyme assay.

Supplementary Table 1. Detailed characteristics of the colorectal cancer (CRC) patients.

Tumor location	Grade	Gender	Age (years)	Patient number
Rectum	II	Male	70	1
Colon	III	Male	18	2
Rectum	II	Male	65	3
Colon	I	Male	50	4
Colon	II	Male	52	5
Rectum	I	Male	49	6
Colon	IV	Female	90	7
Colon	I	Female	39	8
Colon	III	Male	57	9
Colon	II	Male	86	10
Colon	II	Male	74	11
Rectum	III	Male	45	12
Colon	II	Male	71	13
Colon	II	Male	79	14
Rectum	I	Male	68	15

transcription factors is NF-E2-related factor 2 (Nrf2). This factor has been considered as the first line of defense against oxidative stress. Under oxidative stress conditions this nuclear factor binds to antioxidant response element (ARE), then, an increase in GR expression occurs, and thereby ARE is located in the promoters of genes that encode antioxidant enzymes.[16,17]

Cancer cells seem to have higher levels of GSH than normal cells.[18] Clinically, it has been noted that GSH depletion increases the sensitivity of cancer cells to radiotherapy.[19] Because of high level GR activity and gene expression, CRC sensitivity to chemo- and radiotherapy has been suggested to be increased by GR inhibition.

Significant depletion of GR activity in plasma in CRC patients along with the cancer stage has been mentioned.[11] We could not evaluate this in our study because of the limited number of patients in each group. It has been suggested that cancer cells may overexpress GR to produce more thiols that overcome free radicals and repair oxidative damage of proteins and DNA. In this reduced environment cancer cells can escape from oxidative stress. Through radiotherapy, oxidative stress is generated to help elimination of cancer cells, therefore inhibition of GR is considered a novel approach to enhance cancer sensitivity to irradiation.[15] Various studies that report lower GSH content in tumor tissues compared to the normal tissues. This might reveal that the increased expression and activity of the GR enzyme might not necessarily be adequate to compensate the oxidative stress.[8]

In conclusion, this study showed significantly higher tissue expression and GR activity in colorectal tissues compared to the non-cancerous adjacent tissues. These findings supported a possible role of GR through changes in the level of reduced GSH in CRC development. Technically, we were unable to include more patients into the study due to the limitation of time between tissue preparation and enzyme activity measurment. Therefore, we have suggested additional upcoming investigations to be performed with expanded samples to clarify the correlation of the enzyme particularly with stage and response to therapy.

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Conflict of interests

No conflict of interest is declared.

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