

Involvement of glutathione and glutathione metabolizing enzymes in human colorectal cancer cell lines and tissues

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Received February 21, 2014; Accepted April 30, 2015

DOI: 10.3892/mmr.2015.3902

Abstract. Reduced glutathione (GSH) is an abundant tripeptide present in the majority of cell types. GSH is highly reactive and is often conjugated to other molecules, via its sulfhydryl moiety. GSH is synthesized from glutamic acid, cysteine, and glycine via two sequential ATP-consuming steps, which are catalyzed by glutamate cysteine ligase (GCL) and GSH synthetase (GSS). However, the role of GSH in cancer remains to be elucidated. The present study aimed to determine the levels of GSH and GSH synthetic enzymes in human colorectal cancer. The mRNA and protein expression levels of GSH, the catalytic subunit of GCL (GCLC) and GSS were significantly higher in the following five colon cancer cell lines: Caco-2, SNU-407, SNU-1033, HCT-116, and HT-29, as compared with the normal colon cell line, FHC. Similarly, in 9 out of 15 patients with colon cancer, GSH expression levels were higher in tumor tissue, as compared with adjacent normal tissue. In addition, the protein expression levels of GCLC and GSS were higher in the tumor tissue of 8 out of 15, and 10 out of 15 patients with colon cancer respectively, as compared with adjacent normal tissue. Immunohistochemical analyses confirmed that GCLC and GSS were expressed at higher levels in colon cancer tissue, as compared with normal mucosa. Since GSH and GSH metabolizing enzymes are present at elevated levels in colonic tumors, they may serve as clinically useful biomarkers of colon cancer, and/or targets for anti-colon cancer drugs.

Introduction

Reduced glutathione (GSH) is an abundantly expressed intracellular thiol peptide in aerobic cells, which is known to be involved in cellular detoxification, antioxidant defense, maintenance of thiol status, and modulation of cellular proliferation (1,2). The concentration of intracellular GSH is influenced by numerous factors, and reflects the balance between rates of consumption and *de novo* synthesis. The synthesis of GSH proceeds via two ATP-dependent enzymatic steps: Formation of γ -glutamylcysteine from glutamate and cysteine, which is followed by formation of GSH from γ -glutamylcysteine and glycine. The initial step of GSH biosynthesis is rate-limiting and is catalyzed by glutamate cysteine ligase (GCL), a heterodimer that consists of a 73 kDa catalytic subunit (GCLC) and a 29 kDa modulatory subunit (3). The second step of GSH synthesis is catalyzed by glutathione synthetase (GSS), which is a 52 kDa homodimer. GSH metabolism has a complex role in both cancer and anticancer therapy (4). Furthermore, GSH is important in the detoxification of carcinogens, and elevated levels of GSH may increase the resistance of numerous types of tumor to chemotherapy and radiotherapy (5-7).

Various factors are associated with the initiation and development of cancer, including genetic, environmental and dietary influences (8). Previous studies investigating cancer development have examined various genes, including oncogenes, tumor suppressor genes, DNA repair genes, and genes encoding phase I and II enzymes (9-12). GSH levels have been shown to be elevated in numerous types of human cancer, including bone marrow (13), breast (14,15) and lung (16,17). Therefore, understanding the role of GSH in the development of cancer will be a significant step towards cancer prevention and/or attenuation. The present study examined the expression levels of GSH and the enzymes involved in its biosynthesis in human colon cancer tissues and cell lines.

Materials and methods

Reagents. Anti-GCLC antibody was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-GSS antibody was purchased from Abcam (Cambridge, MA,

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Key words: reduced glutathione, human colorectal cancers, glutamate cysteine ligase, glutathione synthetase

USA). Anti- β -actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals and reagents used in the present study were of analytical grade.

Cell culture. The Caco-2, SNU-407, SNU-1033, HCT-116, and HT-29 human colon cancer cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). The FHC normal human colon cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). All cells were maintained at 37°C in a humidified incubator containing 5% CO₂. The SNU-407, SNU-1033, HCT-116, and HT-29 cells were cultured in RPMI 1640 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Life Technologies), 100 μ g/ml streptomycin (Gibco Life Technologies) and 100 U/ml penicillin (Gibco Life Technologies). The Caco-2 cells were cultured in Minimal Essential medium (Gibco Life Technologies) supplemented with 10% heat-inactivated FBS, 100 μ g/ml streptomycin, and 100 U/ml penicillin. The FHC cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (Gibco Life Technologies) supplemented with 25 nM HEPES (Gibco Life Technologies), 10 ng/ml cholera toxin (EMD Millipore, Billerica, MA, USA), 5 μ g/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 5 μ g/ml transferrin (Sigma-Aldrich), 100 ng/ml hydrocortisone (Sigma-Aldrich) and 10% FBS.

Patient tissue. The colon tissue samples were obtained from 15 human patients (3 female, 12 male; median age 66, age range, 43-86; 1 stage I, 3 stage II and 11 stage III) with colon cancer who were treated at the Jeju National University Hospital (Jeju, Korea). Normal and cancerous sections of tissue (~0.5-1 cm) were collected from each patient during the operation. The present study was approved by the institutional review board for ethics of the Jeju National University Hospital (IRB:2011-38), and written informed consent was obtained from the patients.

Intracellular GSH measurement. The intracellular GSH content was measured using the GSH-400 Colorimetric Assay kit (Oxis Biotech Inc., Portland, OR, USA). The cells and tissues were lysed with lysis buffer and were harvested, homogenized in a metaphosphoric working solution, and subsequently centrifuged at 15,000 x g for 10 min. The obtained supernatant was mixed with an equal amount of trichloroacetic acid (a precipitation reagent provided by the GSH-400 assay kit) and further centrifuged at 15,000 x g for 5 min. A total of 50 μ l R1 solution (from the GSH-400 kit), supplemented with chromogenic reagent in HCl was added to 900 μ l supernatant, prior to being gently centrifuged at 15,000 x g for 5 min. A total of 50 μ l R2 solution (30% NaOH; from the GSH-400 kit) was then added to the solution, and the mixtures were incubated at 25 \pm 3°C for 10 min. Following centrifugation at 15,000 x g for 5 min, the absorbance of the clear supernatant was measured at 400 nm using a Scanning Multi-Well Spectrophotometer (Sunrise; Tecan Group, Ltd., Salzburg, Austria).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was

isolated from the cells using the easy-BLUETM Total RNA Extraction kit (Intron Biotechnology Inc., Seongnam, Korea) and template RNA was amplified using 1 μ l RT reaction buffer, along with primers (Bioneer Corporation, Daejeon, Korea), dNTPs, and 0.5 U Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), in a final volume of 25 μ l. The PCR conditions were as follows: Initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final elongation step at 72°C for 7 min. The following primers were used to amplify the GCLC and GSS cDNA: GCLC forward, 5'-AGTTCAATACAGTTGAGG-3', reverse, 5'-TACTGATCCTATAGTTAT-3' (350 bp); GSS forward, 5'-CTGGAGCGGCTGAAGGACA-3', reverse, 5'-AGCTCTGAGATGCACTGGACA-3' (806 bp); and GAPDH forward, 5'-GTGGGCCCGCCCTAGGCACCAGG-3' and reverse, 5'-GGAGGAAGAGGATGCGGCAGTG-3' (868 bp). Amplifications were performed on a MyCycler Thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified products were separated by electrophoresis on a 1% agarose gel, and stained with RedSafeTM nucleic acid staining solution (Intron Biotechnology Inc.), prior to gel image capture under UV light using Image QuantTM TL analysis software (GE Healthcare Life Sciences, Chalfont, UK).

Immunoblot analysis. The cells were lysed on ice for 30 min in 100 μ l lysis buffer, (120 mM NaCl, 40 mM Tris pH 8.0, and 0.1% NP 40) prior to being centrifuged at 13,000 x g for 15 min. The supernatants were collected and the protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Inc.). Aliquots containing 40 μ g protein were boiled for 5 min prior to being separated by 10% SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes (EMD Millipore) and the blots were blocked with 1% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline with 1% Tween-20 for 1 h. The blots were then incubated with GCLC (PA5-19702; 1:1,000), GSS (ab91591; 1:1,000), or β -actin (sc-47778; 1:2,000) antibodies at 4°C overnight. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary immunoglobulin G antibodies (Pierce Biotechnology Inc., Rockford, IL, USA). The protein bands were detected using an Enhanced Chemiluminescence Western Blotting Detection kit (GE Healthcare Life Sciences). Blots were quantified using ImageJ software, version 1.47 (National Institutes of Health, Bethesda, MD, USA)

Immunohistochemistry. The colon tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. The tissue blocks were cut into 3 μ m sections and mounted onto Superfrost Plus-coated slides (Thermo Fisher Scientific, Inc.). The sections were then deparaffinized in xylene and rehydrated through a series of graded ethanol: 2 min in 100% twice, 2 min in 95%, 2 min in 70% and 2 min in 50%. The staining was performed using a BenchMark XT Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA), according to the manufacturer's instructions. Antigen retrieval was carried out on the immunostainer at 100°C in EDTA buffer for 30 min. The slides were subsequently incubated with anti-GCLC

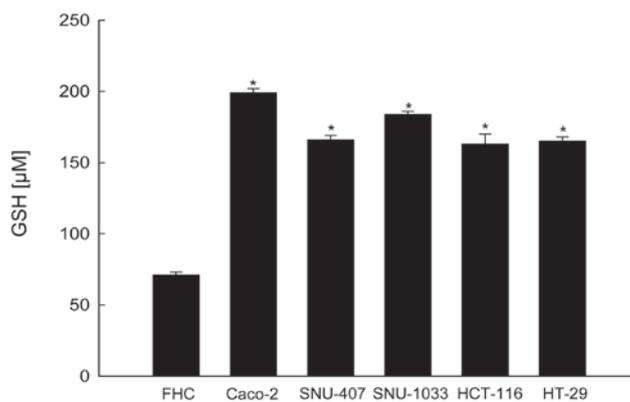


Figure 1. GSH expression levels in FHC normal colon, and Caco-2, SNU-407, SNU-1033, HCT-116 and HT-29 colon cancer cell lines. The GSH levels were measured using a colorimetric assay kit. * $P < 0.05$, vs. FHC cells as determined by analysis of variance and Tukey's tests. GSH, glutathione.

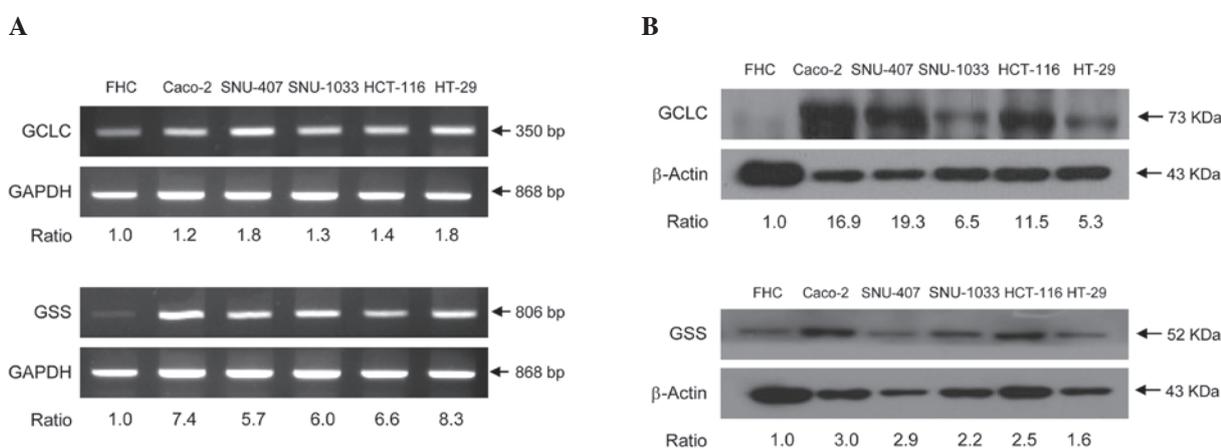


Figure 2. Expression levels of GSH metabolizing enzymes in FHC normal colon, and Caco-2, SNU-407, SNU-1033, HCT-116 and HT-29 cancer cell lines. (A) RT-qPCR analyses of the mRNAs encoding GCLC and GSS. The mRNA expression levels of GAPDH were measured as a loading control. The ratios indicate the expression levels in each cancer cell line relative to those in the normal FHC cell line. (B) Immunoblot analyses of GCLC and GSS proteins. The expression levels of β -actin were measured as a loading control. The ratios indicate the expression levels in each cancer cell line relative to those in the normal FHC cell line. GSH, glutathione; GCLC, glutamate cysteine ligase catalytic subunit; GSS, GSH synthetase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

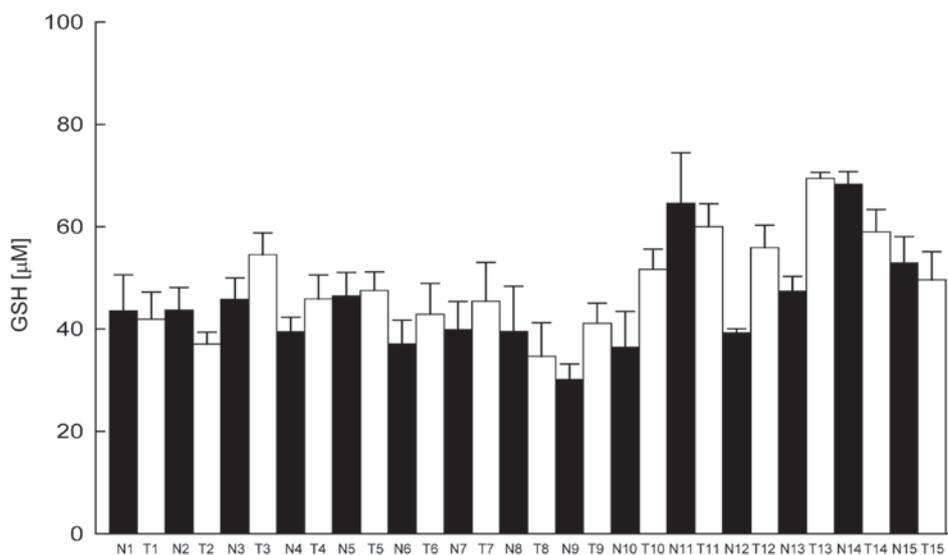


Figure 3. GSH expression levels tissue from patients with colon cancer. The GSH expression levels were measured using a colorimetric assay kit. GSH, glutathione; N, normal; T, tumor.

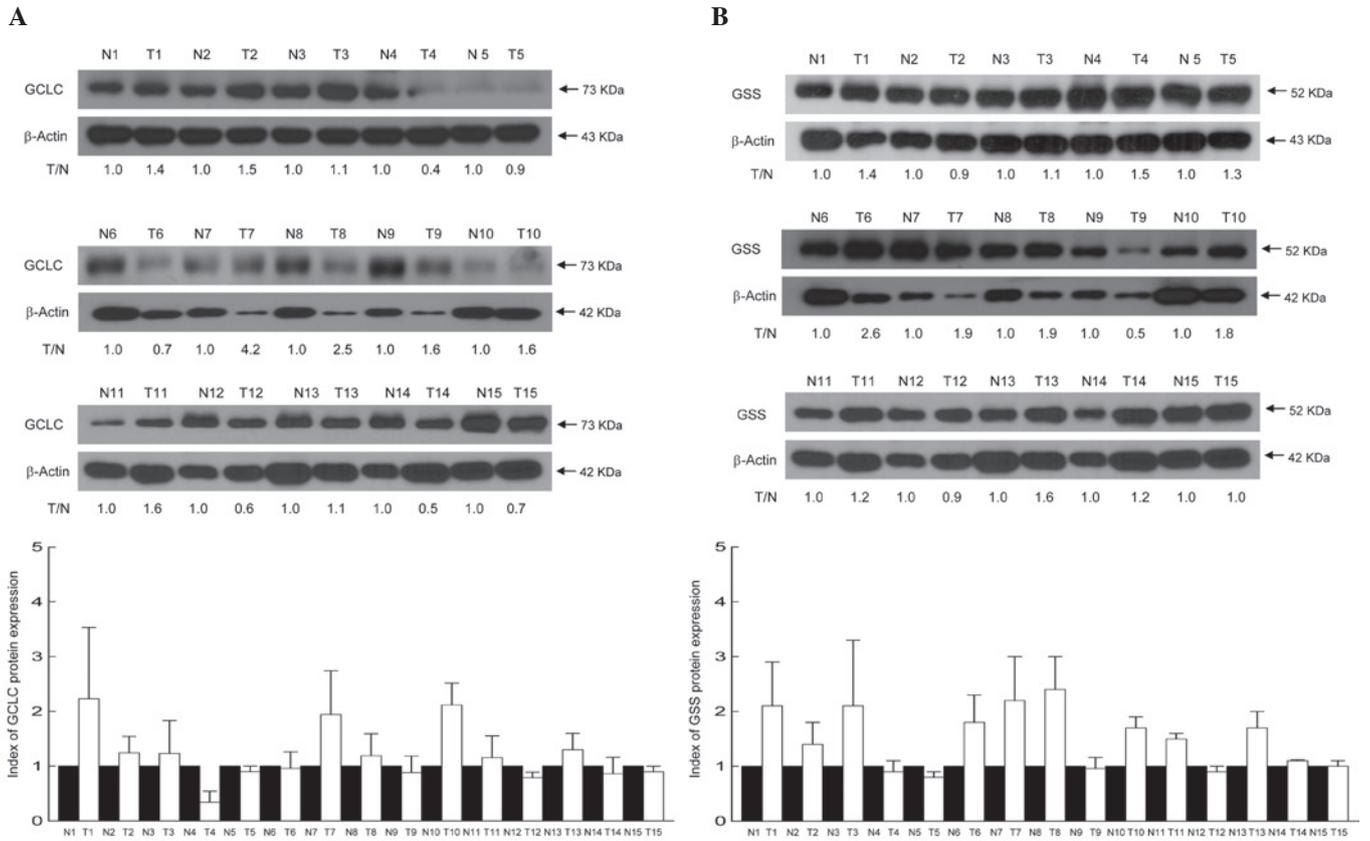


Figure 4. Expression levels of GSH synthetic enzymes in tissue from patients with colon cancer. Immunoblot analyses of (A) GCLC and (B) GSS protein expression. The expression levels of β-actin were measured as a loading control. The numbers below the blots indicate the ratio of expression in the tumor sample relative to the corresponding normal sample N, normal; T, tumor; GCLC, glutamate cysteine ligase catalytic subunit; GSS, GSH synthetase.

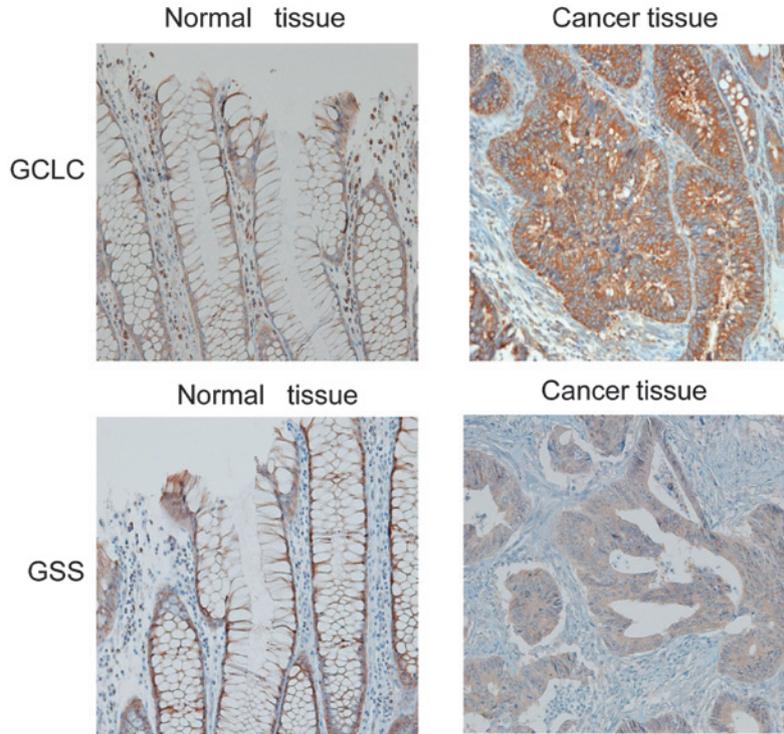


Figure 5. Immunohistochemical analyses of GCLC and GSS in colon carcinoma tissue, and in corresponding normal tissue. Magnification, x100. Low protein expression levels of GCLC and GSS are present in normal mucosa, whereas diffuse and high protein expression levels of GCLC and GSS are present in cancerous tissue. GCLC, glutamate cysteine ligase catalytic subunit; GSS, GSH synthetase.

and anti-GSS antibodies (1:1,000) at 37°C for 32 min, and 3,3'-diaminobenzidine was used as a chromogen. The slides were counterstained with hematoxylin (Sigma-Aldrich) prior to mounting, and were evaluated using a light microscope (Olympus BX51; Olympus, Center Valley, PA, USA) and interpreted by a pathologist.

Statistical analysis. Statistical significance was determined using analysis of variance and Tukey's tests with SigmaStat software, version 3.0 (Systat Software, Inc., San Jose, CA, USA). All values are presented as the mean \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of GSH and GSH metabolizing enzymes in human colon cancer cell lines. The intracellular GSH levels were assessed in a normal human colon cell line (FHC) and five human colon cancer cell lines (Caco-2, SNU-407, SNU-1033, HCT-116, and HT-29) using a commercially available colorimetric assay kit. The intracellular GSH levels were significantly higher in all five of the colon cancer cell lines, as compared with the normal colon cell line (Fig. 1). Concordant with these results, RT-qPCR and immunoblot analyses revealed that the mRNA and protein expression levels of the GSH synthetic enzymes: GCLC and GSS, were also higher in the cancer cell lines, as compared with the normal colon cell line (Fig. 2A and 2B). Although the expression levels of GSH and GSH synthetic enzymes differed among the colon cancer cell lines, the results of the present study suggest that the expression levels of GSH and GSH metabolizing enzymes may serve as useful biomarkers for the detection of colon cancer.

Expression levels of GSH and GSH metabolizing enzymes in normal and colon carcinoma tissue samples. The expression levels of GSH were then examined in the normal and cancerous colon tissue samples of 15 patients undergoing treatment at the Jeju National University Hospital. In nine out of 15 patients, GSH expression levels were higher in the tumor tissue samples, as compared with the corresponding normal tissue samples (Fig. 3). Immunoblot analyses showed that the protein expression levels of GCLC were higher in tumor tissue samples, as compared with the corresponding normal tissue samples in 53% (8/15) of the patients with colon cancer (Fig. 4A). Similarly, the protein expression levels of GSS were higher in tumor tissue samples, as compared with the corresponding normal tissue samples in 67% (10/15) of the patients (Fig. 4B). Furthermore, immunohistochemical analyses of the colon tissue sections revealed low protein expression levels of GCLC and GSS in normal mucosa, whereas diffuse and high expression levels of these proteins were detected in the cancerous tissue (Fig. 5).

Discussion

GSH is a major cellular antioxidant and is crucial for maintaining the balance between oxidation and antioxidant (2,18). GSH is also important in cellular detoxification and is associated with numerous aspects of the immune

response (2). Enhanced resistance to chemotherapeutic drugs and radiotherapy is associated with GSH-conjugation and detoxification (5-7). Previous studies have reported that the expression levels of GSH are elevated in numerous types of human cancer tissue, and increased expression levels of GCLC have also been identified in lung, breast, liver, and other types of cancer (19,20). In addition, GCLC and GSS were highly expressed in the mammary tumors and can be used as prognostic markers for mammary neoplasm (21). Furthermore, a previous study demonstrated that drug-resistance is associated not only with elevated GSH levels, but also with increased activity of GCLC (22).

The results of the present study demonstrated that the GSH levels, and the mRNA and protein expression levels of GCLC and GSS were higher in five human colorectal cancer cell lines, as compared with normal human colon cell lines. Similar results were also observed in cancerous and normal tissue samples, which were collected from 15 patients with colon cancer. The relatively high expression levels of GCLC and GSS in colon cancer tissue, as compared with normal tissue, were confirmed by an immunohistochemical analysis.

In conclusion, the results of the present study suggest that the expression levels of GSH and GSH metabolizing enzymes may serve as clinically useful biomarkers of colon cancer, as well as potential targets for anticancer drugs. Further study is required to elucidate the mechanisms of the GSH metabolizing enzyme upregulation in human colorectal cancer cell lines and tissues.

Acknowledgements

The biospecimens and data used in the present study were provided by the Biobank of Jeju National University Hospital. The present study was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Korea (grant no. 1120340).

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