Expression of Selenium-containing Proteins in Human Colon Carcinoma Tissue

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Abstract. *Selenium may be beneficial in reducing the risk of cancer incidence and mortality in many cancer types such as liver, prostate, colorectal and lung. However, despite the extensive recent research on selenium and selenium-containing proteins, there are still open questions concerning their expression in certain human cancer types, including colorectal carcinoma. Therefore, the expression level of the selenoproteins thioredoxin reductases 1 and 2 (TRXR-1 and TRXR-2) and glutathione peroxidases 1 and 4 (GPX1 and GPX4) in human colon carcinoma tissues was investigated. Up-regulation of TRXR-1 in the colon carcinoma specimens was found both in disease stage-dependent and independent analyses. No differences were found for TRXR-2 expression levels. GPX1 was up-regulated in carcinoma tissues at both the protein and mRNA levels. GPX4 was also up-regulated at the protein level, except for the samples derived from stage III patients. The expression of TRXR-1, GPX1 and GPX4, but not TRXR-2 is differently regulated in cancer as compared to healthy colonic tissue.*

Recent studies have demonstrated that selenium can prevent cancer (1-4). Nutritional supplementation with selenium may be beneficial in reducing the risk of incidence and mortality in many cancer types, especially those of the liver, prostate, colorectum and lung (2, 3). For colorectal cancer, randomized controlled trials have confirmed selenium as one of the four main colon cancer chemopreventive compounds along with calcium carbonate, hormone replacement therapy and nonsterioidal anti-inflammatory drugs (5). However, selenium-containing redox proteins such as thioredoxin reductases (TRXR) and glutathione peroxidases (GPX) are also essential for the carcinogenic process (1, 6-12). In spite of the extensive research on selenium and selenium-containing proteins, there are many open questions concerning their role and expression in different human cancer types, including in colon cancer (3, 7). The selenoproteins TRXR and GPX play important roles both in glutathione and thioredoxin metabolism by maintaining an antioxidative environment within the cell.

TRXR is a part of the thioredoxin system which consists of thioredoxin (TRX) and NADPH (11, 13). Currently three forms of mammalian TRXR, TRXR1, TRXR2 and TRXR3 have been confirmed (9, 10, 14). The TRX system can be found in nearly all living cells and directly influences the reduced intracellular redox environment (15), thereby controlling cellular growth (16) and defense against apoptosis (17). However, the relationship between TRXR activities and tumor growth is less clear and further studies relating enzyme expression with cancer stage and outcome are needed (18, 19). Furthermore, recent investigations have demonstrated TRXR to be a potential novel molecular target for anticancer therapy (9, 20). Especially TRXR-1 is currently under investigation as potential targets for anticancer drug development (10, 14). TRXR-1 reduces oxidized protein substrates in an NADPH-dependent process (20). Furthermore, the TRXR-1/TRX coupled system plays a critical role in the generation of deoxyribonucleotides, which are needed in DNA synthesis and essential for cell proliferation and protects cells against apoptosis (18). The chemical or genetic inhibition of TRX results in a cell cycle arrest (27).

GPXs are believed to have a pivotal role in detoxifying mammalian cells from various damaging peroxides (6, 21). Specifically GPXs catalyze the reduction of hydrogen peroxide, organic hydroperoxides and lipid peroxides by glutathione (22). There are five mammalian GPXs (GPX1,

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GPX2, GPX3, GPX4 and GPX6) (23), which contain selenocysteine at the active site (6). GPX1 and GPX4 are important moderators of oxidative stress, which has been implicated in the pathogenesis of various cancer types (3, 14).

Bringing more insight into the expression of selenium-containing enzymes in carcinogenesis may be valuable for a better understanding of preventive measures. So far, only controversial outcomes have been reported on the expression of different selenoproteins in colon cancer tissue (24, 25). Therefore the expression of the selenoproteins – GPX 1 and 4 and TRXR-1 and 2 was systematically investigated in human colon cancer tissues at different disease stages in comparison with nonmalignant tissue.

Patients and Methods

Patient characteristics. Tumor and normal tissue was collected with informed patient consent from freshly resected, left hemicolectomy specimens of 32 colon cancer patients (8 patients for each tumor stage). For comparison normal tissue was collected from the resection margins. The study was approved by the Local Ethics Committee of University Hospital Mannheim. For the T3 and T4 tumors, the same samples were used for all three different types of expression study, ELISA, real-time PCR and immunohistochemistry. Because of the limited size of the collected samples from the T1 and T2 tumors and the quantity of material required for RNA analysis, real-time PCR and immunochemistry were performed only for the T3 and T4 tumors.

Immunohistochemistry. The following polyclonal antibodies were purchased for immunohistochemistry: a-GPX1, a-GPX4, a-TRXR-1, a-TRXR-2 (LabFrontier, Seoul, South Korea). Paraffin-embedded and frozen tumor and normal colorectal tissue samples were used to obtain 10-μm-thick sections on a Cryostat (Leica, Wetzlar, Germany). The sections were deparaffinized in xylol (3 times for 5 min each) and 100-70% ethanol (3 min) and blocked for endogenous peroxidases using 30% H_2O_2 and methanol for 5 min. Non-specific IgG binding to glass and tissue was prevented by incubation in normal goat serum for 30 min. Afterwards, the sections were overlain with the primary antibody overnight at 4˚C. The next day, the sections were incubated for 30 min with secondary antibodies. The sections were then developed in Nova Red peroxidase substrate (Vector Laboratories, Burlingame, USA) (GPX1, TRXR-2) or bezidine (TRXR-1, GPX4) and counterstained with hematoxylin. The images were analyzed with a Zeiss microscope connected to a charge-coupled device camera (DAGE-MTI, Michigan, MI, USA).

Protein extraction and ELISA. Recombinant human GPX1, GPX4, TRXR-1 and TRXR-2 and their respective anti-human antibodies were purchased from LabFrontier (Seoul, South Korea). The GPX1, GPX4 and TRXR-1 and 2 protein expressions were determined using ELISA assays which have already been fully described and verified (23). The tissues were homogenised in 9 volumes of phosphate-buffered saline (PBS; pH 7.4; 4˚C) containing 0.1% Triton X-100. After centrifugation at 10,000 × *g* for 10 min the resultant supernatants were used for the ELISAs as described previously (23).

RNA purification and real-time PCR. The mRNA expression studies were performed using tumor and normal tissue samples from the stage III and IV cancer patients. The total RNA was extracted from frozen human colon cancer and normal tissues at 37˚C using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) and purified over a cesium chloride column by ultra-centrifugation. The RNA pellet was dissolved in diethyl pyrocarbonate-treated water and quantified spectrophotometrically at A_{260}.

Semi-quantitative real-time PCR was performed on an ABI Prism 7700 Sequence detection System (PE Applied Biosystems, Foster City, CA, USA). The total RNA samples (1 μg) were reverse-transcribed using the QuantiTect Reverse Transcription kit with integrated genomic gDNA-wipeout buffer (Qiagen, Crawley, Surrey, UK) according to the manufacturer's instructions.

The primers for quantitative real-time PCR were purchased from Sigma/Genosys (Steinheim, Germany) the sequences of which were offered by Professor J.R. Arthur. The primer sequences were: *GPX1* forward and reverse primer 5'-TTCCCGTGCAACCAGTTTG-3' and 5'-GGACGTACTTGAGGGAATTCAGA-3'; *GPX4* forward and reverse primer 5'-CTGGCCTTCCCGTGTAACC-3' and 5'-CGGCG AACTCTTTGATCTCTTC-3'; *TRXR-1* forward and reverse primer 5'-GGTGATGGTCCTGGACTTTGTC-3' and 5'-CATTCACACA TGTTCCTCCAAGA-3'; *TRXR-2* forward and reverse primer 5'-CGACGATGGCGGCAAT-3' and 5'-CGTCCGCCACCGGAA-3'.

Statistics. The outcomes of the protein expression studies are illustrated as box-plots showing means and standard deviation. SPSS 10.0.7 software (SPSS, IBM, Ehningen, Germany) was used for the statistical analysis. Differences between groups representing normal and tumor tissues were considered significant if $p<0.05$ using two-sided ANOVA. The relative expression levels of the respective mRNA and the statistical significance were calculated using REST software (26).

Results

In the colon tumor tissues analyzed by ELISA, TRXR-1 was elevated in a stage-dependent manner. Statistically significant up-regulation of TRXR-1 protein was demonstrated in the tumor tissues derived from T1 and T2 colon cancer patients as compared with the corresponding normal tissues (Figure 1A left panel). In the tumor tissue samples of the more locally advanced tumor stages (T3 and T4), an increase in TRXR-1 expression was also observed. However, this increase was not statistically significant. When TRXR-1 protein expression was analyzed independently of the tumor stage by including all tumor samples, the TRXR-1 expression was significantly up-regulated ($p<0.001$) in the tumor tissues (Figure 1A right panel).

Semiquantitive real-time PCR demonstrated an up-regulation of *TRXR-1* mRNA expression by a factor of 2.88 ($p=0.0057$) in the tumor tissue samples compared with normal tissue samples (Table I).

The differences in TRXR-2 protein levels between the normal tissue and tumor samples were not statistically significant at any stage (Figure 1B). Correspondingly, the mRNA expression analysis showed no significant difference

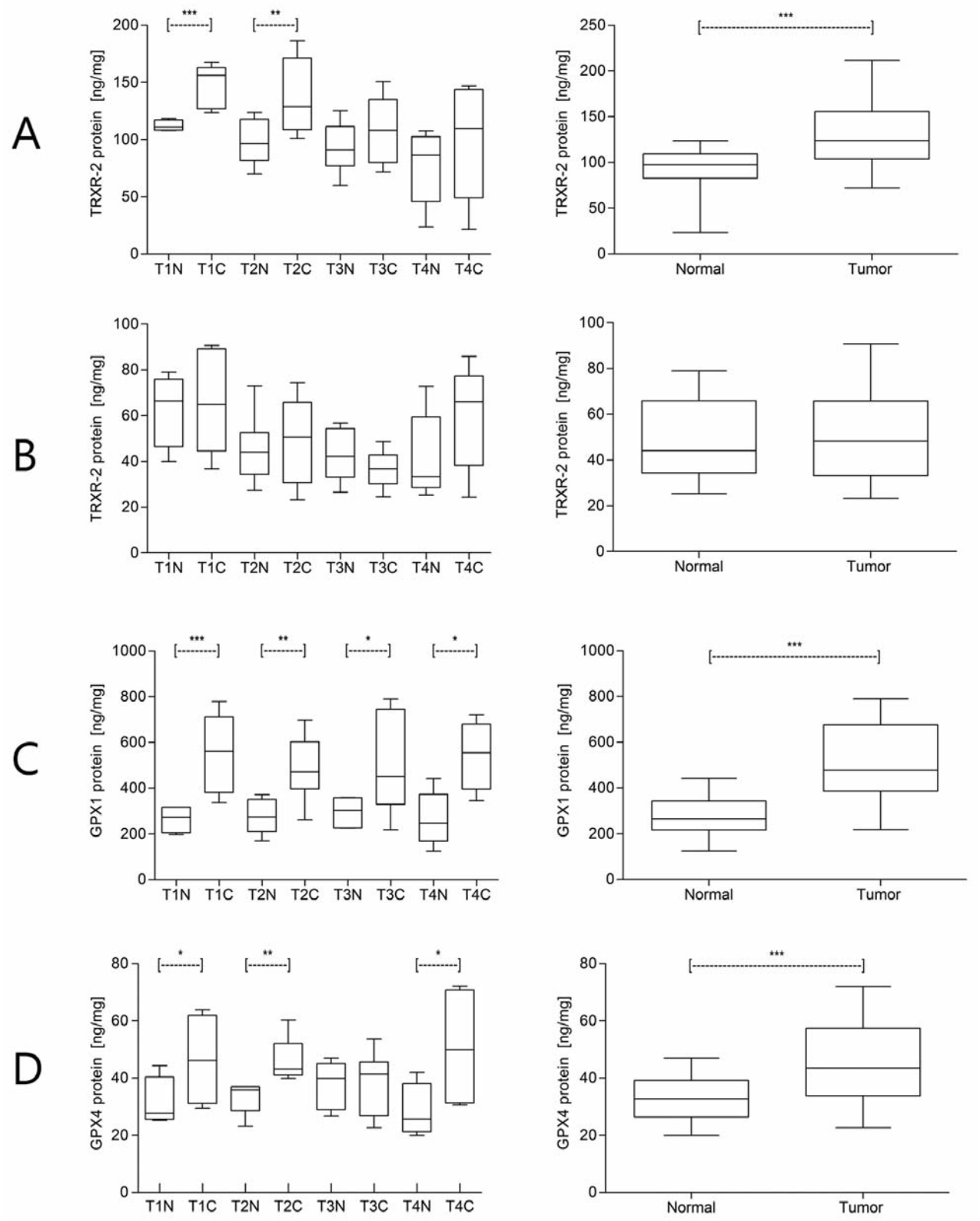

Figure 1. *Protein expression determined by ELISA in colon cancer and matched normal tissue samples. A: TRXR-1, B: TRXR-2, C: GPX1. D: GPX4. Left panels: Comparison of different cancer stages. N: Normal tissue; C: colon cancer tissue. Right panels: Comparison of combined normal and combined colon cancer (T1-T4) samples. *p<0.05, **p<0.01, ***p<0.001.*

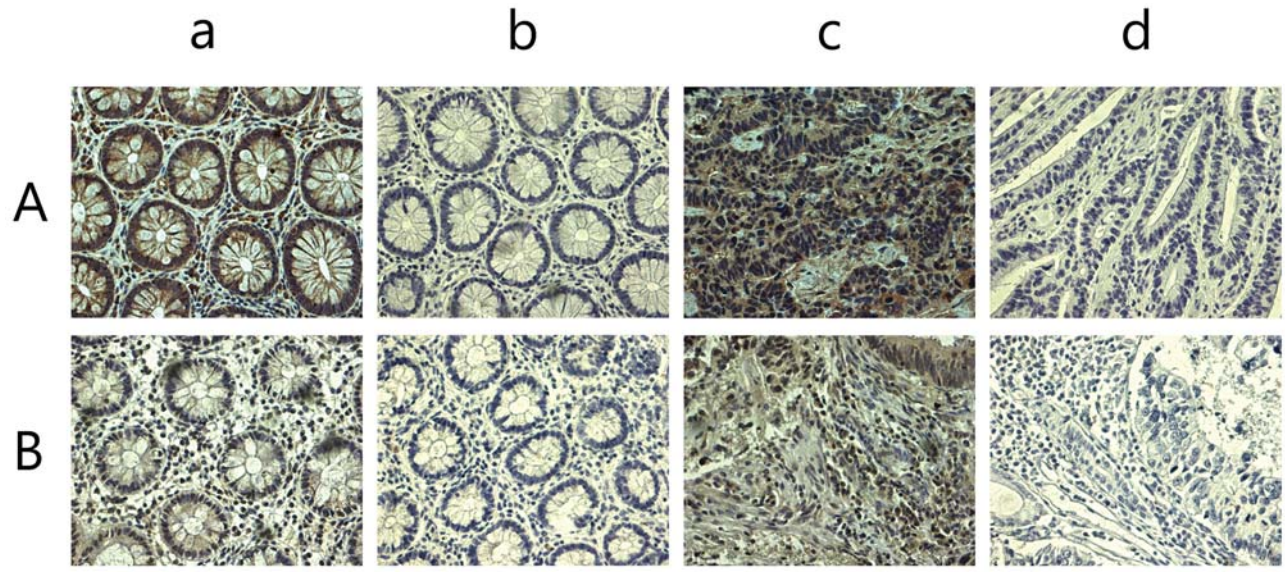

Figure 2. *Immunostaining of normal colon tissue and colon adenocarcinoma from a T4 tumor patient. A: GPX1, B: GPX4; (a) normal colon tissue, (b) negative control of normal tissue, (c) colon cancer tissue, (d) negative control of cancer tissue. (Original magnification, ×40.)*

in the level of *TRXR-2* mRNA in the tumor tissue samples of the T3 and T4 tumors (fold difference 1.24, p=0.528, Table I).

An increase in GPX1 occurred in all the tumor tissue samples compared with the normal controls (Figure 1C left panel). While comparing GPX1 expression in a stage-dependent manner, there was a significant up-regulation of GPX1 over all disease stages. GPX1 was also found to be overexpressed in the tumor specimens when compared to the normal tissue independently of tumor stage (p<0.001, Figure 1C right panel). In parallel, for both the T3 and T4 carcinoma samples the *GPX1* mRNA level was significantly up-regulated by a factor of 3.01 (p=0.0033) compared to normal tissue (Table I).

Within the tumor samples, a higher expression of GPX4 was also found. GPX4 was significantly up-regulated in the tumor samples compared to the normal tissue from each stage except for the T3 tumors (Figure 1D). Higher mRNA expression levels (p=0.0033) were also found in the tumor samples of T3 and T4 carcinoma patients (Table I).

Immunohistochemistry confirmed the stronger GPX1 and GPX4 nuclear and cytoplasmic staining in the colorectal cancer tissues as compared to normal colorectal tissues (Figure 2). TRXR-1 and TRXR-2 immunostaining for the T3 and T4 tumors showed no apparent difference between the colon cancer and normal tissues (data not shown).

Table I. *mRNA expression using semi-quantitative real-time PCR in tumor compared to normal tissue samples of stage III and IV cancer patients. Fold change + indicates up-regulated expression in the tumor samples.*

Symbol	Name	Fold change	*P*-value
TRXR-1	Thioredoxin reductase-1	+2.876	0.0053
TRXR-2	Thioredoxin reductase-2	+1.243	0.528
GPX1	Glutathione peroxidase-1	+3.014	0.0033
GPX4	Glutathione peroxidase-4	+2.304	0.0033

Discussion

We here systematically analyzed the expression of the selenoproteins TRXR-1, TRXR-2, GPX1 and GPX4 in a tumor stage-specific manner since the relationship between the expression of these selenoproteins and tumor progression is still unclear (20). The role of TRXR-1, TRXR-2, GPX1 and GPX4 in tumor and health is further confounded since tumor cells may not necessarily increase expression of the TRXR enzymes, although their catalytic activity may be increased functionally.

TRXR-1 was overexpressed in the early stages of colon cancer. Although the TRXR-1 protein was not overexpressed in the more advanced colon tumors, the stage-independent analysis showed its overall up-regulation. No differences were shown in the expression of TRXR-2 between the tumor tissues and normal tissues. Since TRXR-2 activity in particular can be accurately regulated *in vitro* and *in vivo* (18), this may explain the failure to demonstrate differences in enzyme expression in the more advanced tumors. Thus, to maintain a potent antioxidant environment within cells, the activity although not necessarily the expression, may be regulated.

However, higher levels of TRXR-1 and TRXR-2 were found in the normal tissues derived from T1 carcinoma specimens as compared with the normal tissues derived from the T4 specimens. This may point to a general deficiency of these enzymes in the tissues of cancer patients. Further studies are needed for validation.

GPX1 expression has been found to be decreased or repressed in various *in vitro* and *in vivo* models of cancer and hypermethylation of the *GPX1* promoter in gastric cancer cell lines has recently been demonstrated (14). A recent study showed increased GPX1 levels in malignant human lung tissue in comparison to nonmalignant lung tissue (28). Murawaki *et al*. reported that the level of GPX1 in colon cancer tissue was lower than in its normal counterparts (24) using immunohistochemistry.

In the present study, GPX1 and GPX4 were up-regulated in the colon cancer tissues in comparison with normal tissues of the same patients. In contrast to the previous studies, ELISA and semi-quantitative real-time PCR were carried out rather than immunohistochemical studies alone. Moreover, the present investigations were carried out focusing also on tumor stage differences. Both *GPX1* and *GPX4* mRNA expression reflected the protein changes observed in the stage III and stage IV patients.

Biologically, the up-regulation of *GPX1* and *GPX4* in colon cancer tissues in comparison with healthy tissues could ensure defense against oxidative stress and may support survival mechanisms suppressing apoptosis in the tumor cells, especially within more advanced tumors. It has been postulated that selenoproteins may limit damage in DNA, which has been directly linked to apoptosis resistance (3).

In summary, tumor tissues show a higher expression of TRXR-1, GPX1 and GPX4 than corresponding normal tissues. These selenproteins are key antioxidant players in cancer cell survival and may be potential candidates for anticancer drug development. Studies are currently underway to further clarify the relationship between the selenium status of patients, enzyme activities and levels of expression.

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References

1 Ganther HE: Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. Carcinogenesis *20*: 1657-1666, 1999.

2 Hawk ET and Levin B: Colorectal cancer prevention. J Clin Oncol *23*: 378-391, 2005.

3 Rayman MP: Selenium in cancer prevention: a review of the evidence and mechanism of action. Proc Nutr Soc *64*: 527-542, 2005.

4 Irons R, Tsuji PA, Carlson BA, Ouyang P, Yoo MH, Xu XM, Hatfield DL, Gladyshev VN and Davis CD: Deficiency in the 15-kDa selenoprotein inhibits tumorigenicity and metastasis of colon cancer cells. Cancer Prev Res (Phila) *3*: 630-639, 2010.

5 Lewin MH, Hume R, Howie AF, Richard K, Arthur JR, Nicol F, Walker SW and Beckett GJ: Thioredoxin reductase and cytoplasmic glutathione peroxidase activity in human foetal and neonatal liver. Biochim Biophys Acta *1526*: 237-241, 2001.

6 Arthur JR: The glutathione peroxidases. Cell Mol Life Sci *57*: 1825-1835, 2000.

7 Brown KM and Arthur JR: Selenium, selenoproteins and human health: a review. Public Health Nutr *4*: 593-599, 2001.

8 Mustacich D and Powis G: Thioredoxin reductase. Biochem J *346 Pt 1*: 1-8, 2000.

9 Nguyen P, Awwad RT, Smart DD, Spitz DR and Gius D: Thioredoxin reductase as a novel molecular target for cancer therapy. Cancer Lett *236*: 164-174, 2006.

10 Smart DK, Ortiz KL, Mattson D, Bradbury CM, Bisht KS, Sieck LK, Brechbiel MW and Gius D: Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress. Cancer Res *64*: 6716-6724, 2004.

11 Arner ES and Holmgren A: The thioredoxin system in cancer. Semin Cancer Biol *16*: 420-626, 2006.

12 Ramos A, Lane AN, Hollingworth D and Fan TW: Secondary structure and stability of the selenocysteine insertion sequences (SECIS) for human thioredoxin reductase and glutathione peroxidase. Nucleic Acids Res *32*: 1746-1755, 2004.

13 Crosley LK, Meplan C, Nicol F, Rundlof AK, Arner ES, Hesketh JE and Arthur JR: Differential regulation of expression of cytosolic and mitochondrial thioredoxin reductase in rat liver and kidney. Arch Biochem Biophys *459*: 178-188, 2007.

14 Reeves MA and Hoffmann PR: The human selenoproteome: recent insights into functions and regulation. Cell Mol Life Sci *66*: 2457-2478, 2009.

15 Arner ES and Holmgren A: Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem *267*: 6102-6109, 2000.

16 Powis G, Oblong JE, Gasdaska PY, Berggren M, Hill SR and Kirkpatrick DL: The thioredoxin/thioredoxin reductase redox system and control of cell growth. Oncol Res *6*: 539-544, 1994.

17 Baker A, Santos BD and Powis G: Redox control of caspase-3 activity by thioredoxin and other reduced proteins. Biochem Biophys Res Commun *268*: 78-81, 2000.

18 Powis G, Mustacich D and Coon A: The role of the redox protein thioredoxin in cell growth and cancer. Free Radic Biol Med *29*: 312-322, 2000.

19 Lu J and Holmgren A: Selenoproteins. J Biol Chem *284*: 723-727, 2009.

20 Biaglow JE and Miller RA: The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. Cancer Biol Ther *4*: 6-13, 2005.

21 Chu FF, Esworthy RS and Doroshow JH: Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. Free Radic Biol Med *36*: 1481-1495, 2004.

22 Yamasaki T, Tahara K, Takano S, Inoue-Murayama M, Rose MT, Minashima T, Aso H and Ito S: Mechanism of plasma glutathione peroxidase production in bovine adipocytes. Cell Tissue Res *326*: 139-147, 2006.

23 Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R and Gladyshev VN: Characterization of mammalian selenoproteomes. Science *300*: 1439-1443, 2003.

24 Murawaki Y, Tsuchiya H, Kanbe T, Harada K, Yashima K, Nozaka K, Tanida O, Kohno M, Mukoyama T, Nishimuki E, Kojo H, Matsura T, Takahashi K, Osaki M, Ito H, Yodoi J and Shiota G: Aberrant expression of selenoproteins in the progression of colorectal cancer. Cancer Lett *259*: 218-230, 2008.

25 Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga-Koda L and Sulkowska M: Lipid peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol *11*: 403-406, 2005.

26 Pfaffl MW, Horgan GW and Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res *30*: e36, 2002.

27 Berggren M, Gallegos A, Gasdaska JR, Gasdaska PY, Warneke J and Powis G: Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. Anticancer Res *16*: 3459-3466, 1996.

28 Gresner P, Gromadzinska J, Jablonska E, Kaczmarski J and Wasowicz W: Expression of selenoprotein-coding genes *SEPP1*, *SEP15* and *hGPX1* in non-small cell lung cancer. Lung Cancer *65*: 34-40, 2009.

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