Original Article

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Influence of glutathione levels and activity of glutathione-related enzymes in the brains of tumor-bearing mice

Kaikai Shen[1], Lili Ji[1,2,*], Ying Chen[1], Qianming Yu[1], Zhengtao Wang[1,2,*]

[1] *The MOE Key Laboratory for Standardization of Chinese Medicines and the SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China;*

[2] *Shanghai R&D Centre for Standardization of Chinese Medicines, Shanghai, China.*

Summary **Oxidative stress takes place due to an imbalance between the production of reactive oxygen species (ROS) and the protection provided by cellular antioxidants. High levels of ROS are caused by tumor cells during tumor progression and may affect the functions of other important organs. The present study sought to investigate whether non-primary brain tumors affect reduced glutathione (GSH) levels and the activity of related enzymes in the brain. GSH contents, the activity of glutathione peroxidase (GPx), glutathione-*s*-transferase (GST), glutathione reductase (GR) as well as glutamate cysteine ligase (GCL) were determined in the brains of normal and tumor-bearing mice treated with the chemotherapy drug 5-Fluorouracil (5-Fu) or not. The results in S180 and H22 tumor-bearing mice showed that GSH levels and the activity of GPx, GST, and GCL all decreased while GR activity markedly increased in the brains of tumor-bearing mice compared to those of normal mice. Further investigation found that 5-Fu, a typical chemotherapy drug, significantly inhibited tumor growth but did not improve the loss of redox homeostasis in the brain caused by non-primary brain tumors. Overall, these results suggest that non-primary brain tumors can induce an ROS burden in the brain that cannot be reversed by the chemotherapy drug 5-Fu.**

Keywords: Tumor, brain, reduced glutathione, glutathione peroxidase (GPx), glutathione-*s*-transferase (GST), glutathione reductase (GR), glutamate cysteine ligase (GCL)

1. Introduction

Oxidative stress takes place due to the disturbance of the balance between the formation of reactive oxygen species (ROS) and the defense provided by cellular antioxidants (*1*). Reduced glutathione (GSH), ubiquitously distributed in all mammalian cells, is a reducing sulfhydryl (-SH) tripeptide and plays important roles in the endogenous antioxidant system because it conjugates toxic substances (*2*). Intracellular GSH and its related enzymes, such as glutathione peroxides (GPx), glutathione-*s*-transferase (GST), glutathione reductase (GR), and glutamate cysteine ligase (GCL), constitute the cellular glutathione antioxidant system and represent a crucial defensive system to protect cells against ROS.

The presence of tumors in the human body or in experimental animals is known to affect various functions of vital organs even when the site of the tumor is not close to the organ (*3*). There are two characteristics of tumor cells compared to normal cells: one is the increased generation of ROS and the other is the decreased capacity to eliminate ROS, leading to damage to other normal tissues (*4*). The GSH concentration in the brain is lower than that in the liver, kidney, spleen, and small intestine. Furthermore, there is a large concentration of unsaturated lipids and a high rate of oxidative metabolism in the brain (*5*,*6*), so the brain is especially vulnerable to oxidative stress injury compared to other organs (*7*). However, whether or not a high level of ROS caused by the progression of a non-

**Address correspondence to:*
Dr. Lili Ji and Zhengtao Wang, The MOE Key Laboratory for Standardization of Chinese Medicines and the SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201210, China
e-mail: lily0913@yahoo.cn; wangzht@hotmail.com

primary brain tumor affects the glutathione antioxidant system in the brain remains unclear.

Since brain tissue cannot be obtained from cancer patients with non-primary brain tumors, a mice model with clinical features was established to investigate whether non-primary brain tumors affect GSH levels and the activity of GSH-related enzymes in the brain. The present study sought to compare GSH levels and the activity of GSH-related enzymes in the brains of a control group and model and 5-Fluorouracil (5-Fu) groups using mouse models of transplanted S180 and H22 tumors.

2. Materials and Methods

2.1. *Experimental animals*

Specific pathogen-free male ICR mice (6-8 weeks age) from the Chinese Academy of Science were housed in plastic cages, kept on a light-dark rhythm of 12 h-12 h at a constant temperature (22 ± 1°C) and humidity (65 ± 5%), and allowed free access to water and a standard diet during the experiment. Animal handling procedures were carried out in accordance with Chinese state legislation on the use and care of laboratory animals and this study was approved by the Experimental Animal Ethical Committee of the Shanghai University of Traditional Chinese Medicine. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. *Experimental mice protocol*

Thirty ICR male mice were randomly divided into three groups, with each group consisting of 10 mice. One group was the control group, one was a model group (untreated), and one was a 5-Fu group (treated). Each mouse was subcutaneously injected in the right axilla with S180 or H22 ascites tumor cells (approximately 2×10^6 cells/mouse) except the control group. Twenty-four hours after implantation, the 5-Fu group served as the treatment group and was intraperitoneally administered a 5-Fu dose of 25 mg/kg/2d a total of five times. The control and model groups were treated with the same volume of normal saline. Fourteen days after implantation, the animals were sacrificed. Tumor tissue was totally excised from the animal and accurately weighted. Meanwhile, the brain was excised from the animal and stored at −80°C. The tumor inhibition ratio (%) = [(A-B)/A] × 100, where A is the average tumor weight for the model group and B is that for the 5-Fu group.

2.3. *Measurement of GSH content*

Brain homogenates were prepared in a cold phosphate buffer, pH 7.0, containing 1mM EDTA and 5% metaphosphoric acid, sonicated twice at 4°C, and then centrifuged at 10,000 g at 4°C for 10min. The GSH contents in the brain homogenate were determined in accordance with a reported method (*8*) with minor modifications. The reaction mixture contained 1mM EDTA, NADPH (0.24 mM), glutathione reductase (0.06 Units), DTNB (86 μM), and the sample. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored at 412 nm. GSSG was determined after elimination of GSH with 2-vinylpyridine. The levels of GSH were calculated from the difference between concentrations of total glutathione (GSH + GSSG) and GSSG. Values are expressed as mM/g protein of brain tissue homogenates used.

2.4. *Measurement of GPx enzymatic activity*

Brain homogenates were prepared in ice-cold PBS (pH 7.0), sonicated twice at 4°C, and then centrifuged at 4°C, 3,000 g for 5 min. The supernatant was transferred to new tubes for GPx enzymatic activity assay. GPx activity was determined from brain tissue homogenate in accordance with a previously reported method (*9*) with minor modifications. The enzymatic activity of GPx was expressed as Unit/mg protein, where 1 Unit of GPx activity was defined as 1 μM GSH depleted per minute.

2.5. *Measurement of GST enzymatic activity*

Brain homogenates were prepared in ice-cold PBS (pH 7.0), sonicated twice at 4°C, and then centrifuged at 4°C, 3,000 g for 5 min. The supernatant was transferred to new tubes for GST enzymatic activity assay. GST activity was measured from brain tissue homogenate in accordance with a previously reported method (*10*) with minor modifications. The enzymatic activity of GST was expressed as Unit/mg protein, where 1 Unit of GST activity was defined as 1 μM GSH depleted per minute.

2.6. *Measurement of GR enzymatic activity*

Brain homogenates were prepared in ice-cold PBS (pH 7.0), sonicated twice at 4°C, and then centrifuged at 4°C, 3,000 g for 5 min. The supernatant was transferred to new tubes for GR enzymatic activity assay. GR activity from brain tissue homogenate was assayed in accordance with a reported method (*11*) with minor modifications. The enzymatic activity of GR was expressed as mUnit/mg protein, where 1 Unit of GR activity was defined as 1 mM GSSG catalyzed per minute.

2.7. *Measurement of GCL enzymatic activity*

Brain homogenates were prepared in ice-cold PBS (pH 7.0), sonicated twice at 4°C, and then centrifuged

at 4°C, 10,000 g for 5 min. The supernatant was transferred to new tubes for GCL enzymatic activity assay. GCL activity was assayed in accordance with a previously reported method (*12*). The enzymatic activity of GCL was expressed as Unit/min/mg protein, where 1 Unit of GCL activity was equal to the amount of enzyme that oxidized the reduction of 1 mM NADPH per minute.

2.8. Statistical analysis

Data from all experiments are expressed as means ± S.E.M. Statistical comparisons were subjected to an analysis of variance (ANOVA) and LSD-test using SPSS version 11.5, and $p < 0.05$ was considered a statistically significant difference. All statistical analyses were performed using SigmaPlot version 10.0 software.

3. Results

3.1. *Comparison of GSH levels in the brains of non-tumor-bearing mice and tumor-bearing mice treated with 5-Fu and not treated with 5-Fu*

The results in Figure 1A show that administration of 5-Fu significantly inhibited tumor growth in S180 tumor-bearing mice; the ratio of tumor growth inhibition was 81.9%. Similar results were observed in H22 tumor-bearing mice as shown in Figure 1B. The current results are consistent with those of previous studies indicating that 5-Fu markedly inhibited tumor growth (*13,14*).

GSH is a ubiquitously distributed antioxidant that can exacerbate exogenous toxic injury, enhance defense against oxygen free radicals, and regulate immune function (*15*). GSH levels in the brains of S180 tumor-bearing mice treated with 5-Fu or not and normal mice were investigated. As shown in Figure 2A, the GSH levels in the brains of the model group declined significantly compared to levels in the control group ($p < 0.01$). Meanwhile, the average brain GSH levels of the 5-Fu-treated group increased slightly but not significantly so compared to levels in the model group. Similar results with regard to the changes in GSH levels in the brains of different groups were also observed in H22 tumor-bearing mice, as shown in Figure 2B. The results of Figures 1 and 2 indicate that GSH levels decreased in the brains of mice bearing non-primary brain tumors. The chemotherapy drug 5-Fu significantly inhibited tumor growth but it did not improve GSH levels in the brain.

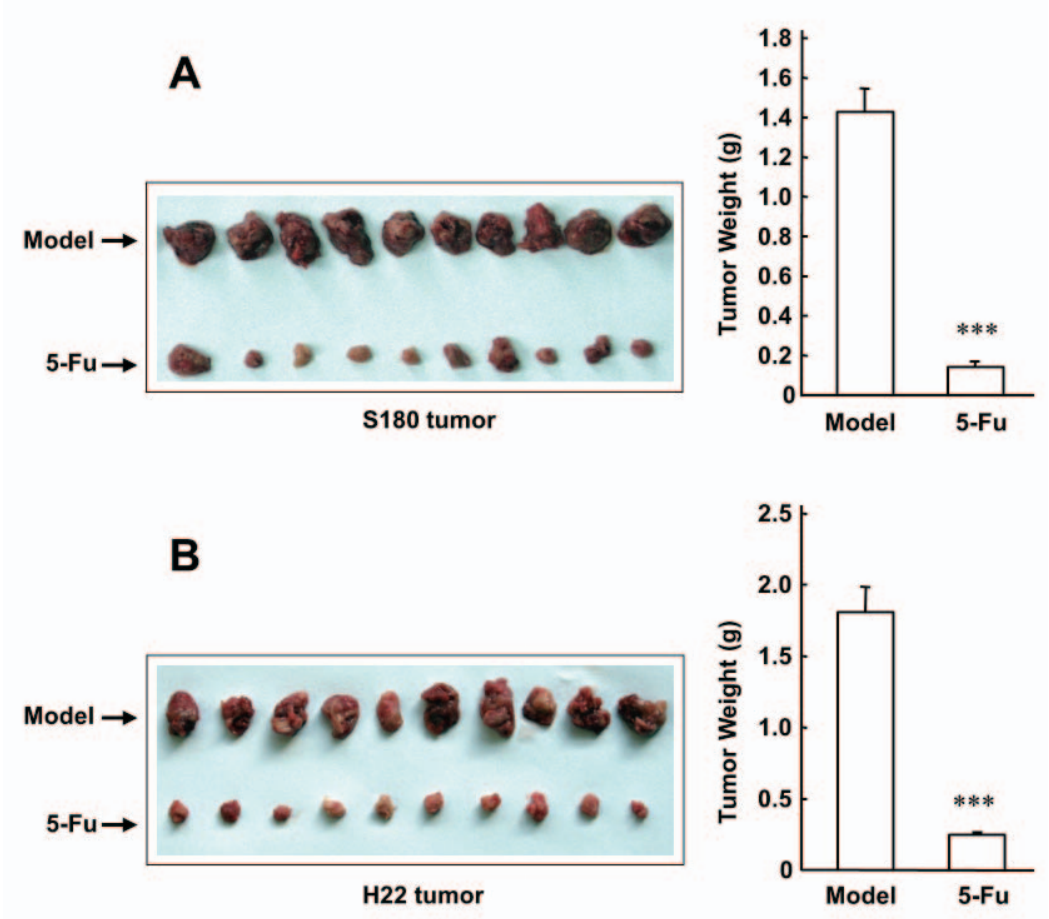

Figure 1. Decreased tumor area in S180 and H22 tumor-bearing mice treated with 5-Fu. (A) S180 tumor-bearing ICR mice; **(B)** H22 tumor-bearing ICR mice. ***, $p < 0.001$ *vs.* the model group.

3.2. *Changes in GPx and GST activity in the brains of non-tumor-bearing mice and treated and untreated tumor-bearing mice*

Previous studies have reported that GSH plays important roles in two forms of chemical detoxification and anti-oxidative defense: one is the inactivation of ROS either *via* direct GSH-ROS interaction or *via* catalysis through GPx, and the other is the accelerated excretion of less toxic GSH-xenobiotic conjugates *via* GST (*16,17*). GPx and GST activity in the brains of S180 tumor-bearing mice treated with 5-Fu or not and normal mice was further investigated. As shown in Figure 3A, in S180 tumor-bearing mice GPx activity

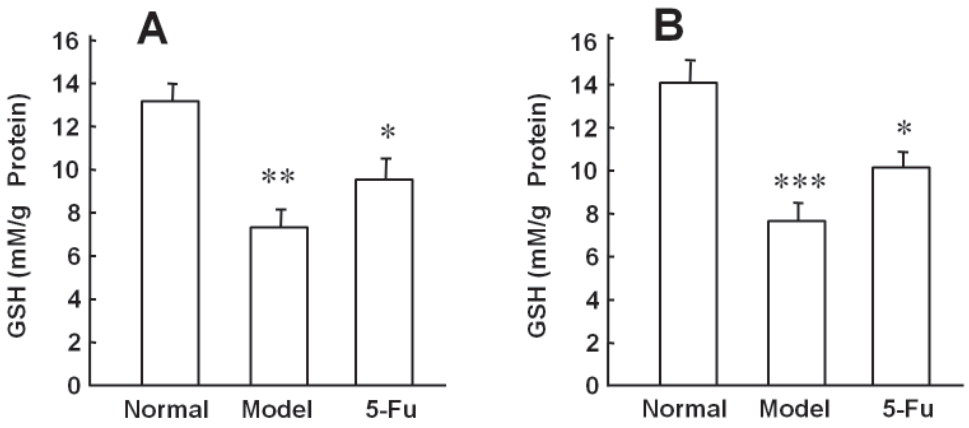

Figure 2. GSH levels in the brain tissues of each group. (A) GSH levels in brains of S180 tumor-bearing mice; **(B)** GSH levels in brains of H22 tumor-bearing mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs.* the control group.

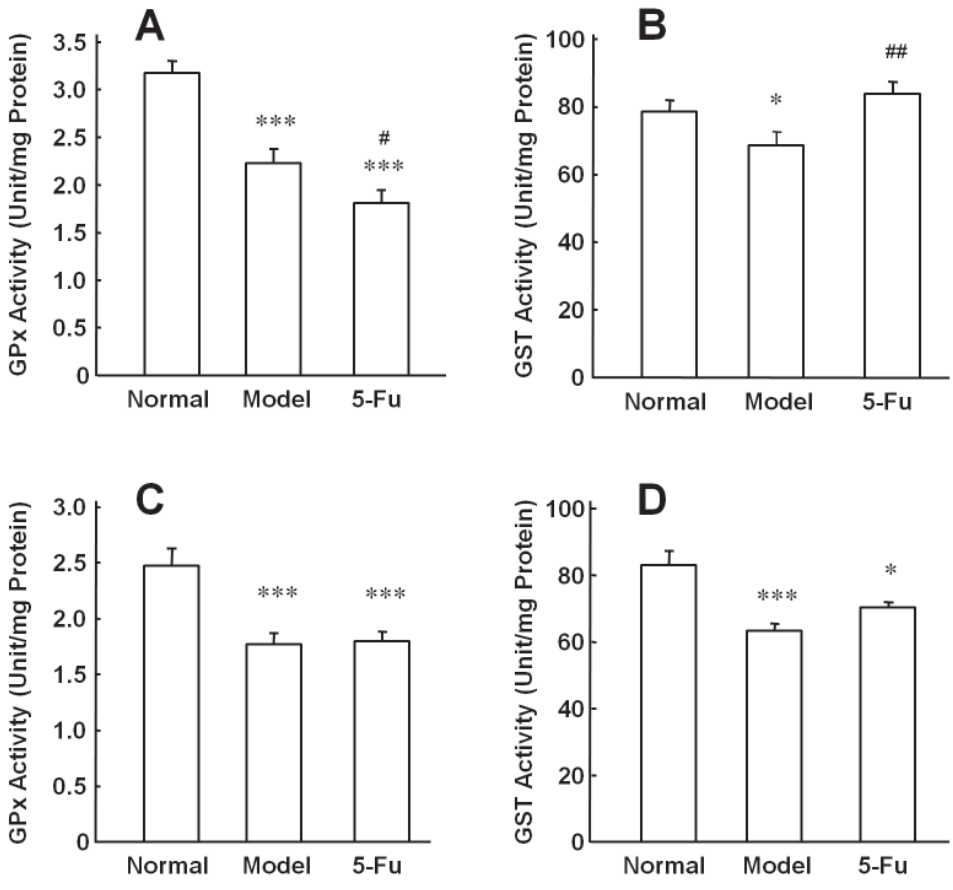

Figure 3. GPx and GST activity in the brain tissues of each group. (A) GPx activity in brains of S180 tumor-bearing mice; (**B)** GST activity in brains of S180 tumor-bearing mice; **(C)** GPx activity in brains of H22 tumor-bearing mice; **(D)** GST activity in brains of H22 tumor-bearing mice. *, $p < 0.05$; ***, $p < 0.001$ *vs.* the control group. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$ *vs.* the model group.

in the brain decreased significantly in the model group compared to the control group (2.23 Unit/mg protein *vs.* 3.18 Unit/mg protein, $p < 0.001$). Moreover, the GPx activity in the 5-Fu group was even lower than that in the model group (1.81 Unit/mg protein *vs.* 2.23 Unit/mg protein, $p < 0.05$). Further, results for H22 tumor-bearing mice (Figure 3C) showed that the model group had GPx activity of 1.77 Unit/mg protein while the 5-Fu-treated group had activity of 1.80 Unit/mg protein; there were no significant differences between the two. GPx activity in the brains of the model group and 5-Fu-treated group was lower than that in the control group, which had activity of 2.47 Unit/mg protein ($p < 0.001$). As shown in Figure 3B, in S180 tumor-bearing mice the GST activity in the brains of the model group declined significantly compared to activity in the control group ($p < 0.05$). Further, the decreased GST activity in tumor-bearing mice was reversed by 5-Fu to a level higher than that in the model group ($p < 0.01$). Similar data were obtained from H22 tumor-bearing mice except for GST activity, which increased little in the 5-Fu-treated group compared to the model group ($p \geq 0.05$) (Figure 3D).

3.3. *Changes in GCL and GR activity in the brains of non-tumor-bearing mice and treated and untreated tumor-bearing mice*

In order to identify the possible reasons for the change in GSH levels, the activity of GR and GCL, which are both main enzymes involved in the synthesis and regeneration of GSH, was measured. GR enzymatic activity was investigated in three groups of mice, *i.e.*, those bearing S180 tumors and administered 5-Fu, those bearing S180 tumors and not administered 5-Fu, and normal mice. The results shown in Figure 4A indicate that GR activity increased slightly in the brains of the model group in comparison to the control group ($p \geq 0.05$). Moreover, GR activity in the brains of the 5-Fu-treated group was much greater than that in brains of the control group ($p < 0.01$). Similarly, results for H22 tumor-bearing mice as shown in Figure 4C indicate that the activity of GR in the brains of mice treated with 5-Fu and mice not treated with 5-Fu was significantly greater than that in the control group, which had activity of 8.87 mUnit/mg protein ($p < 0.01$). Levels of GCL activity in the three groups were also investigated. As shown in

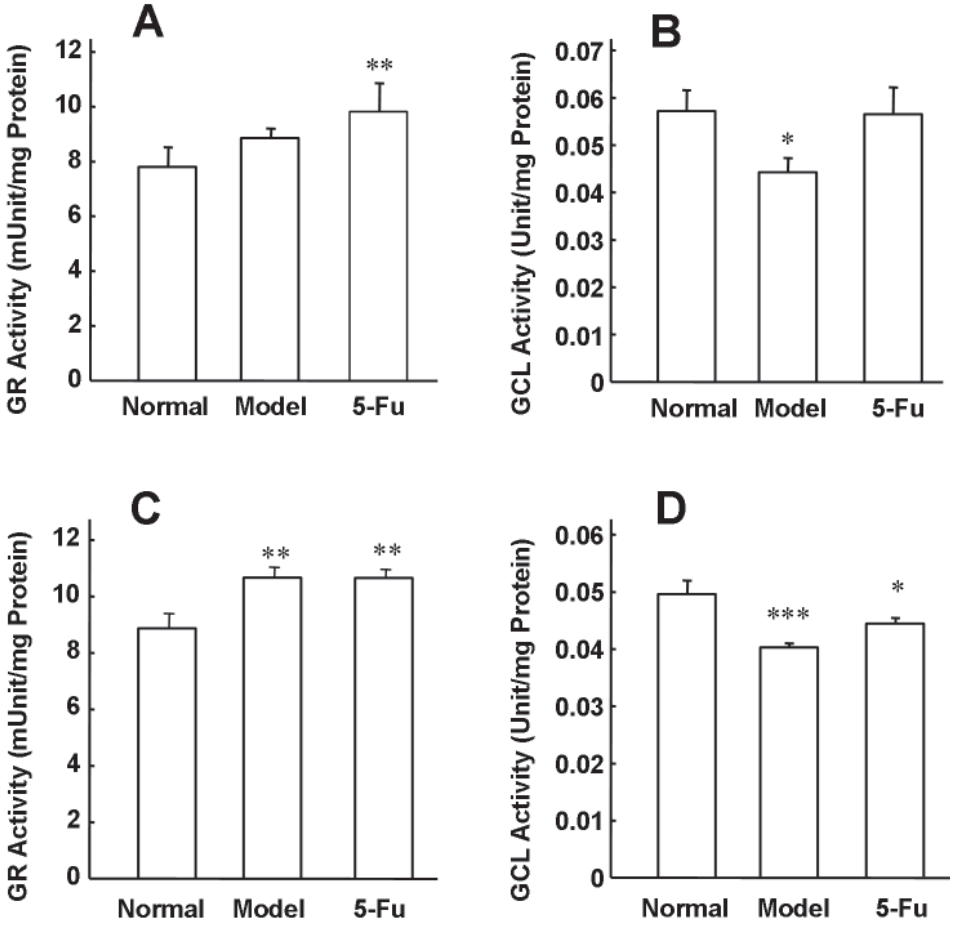

Figure 4. GR and GCL activity in the brain tissues of each group. (A) GR activity in brains of S180 tumor-bearing mice; (**B)** GCL activity in brains of S180 tumor-bearing mice; (**C)** GR activity in brains of H22 tumor-bearing mice; (**D)** GCL activity in brains of H22 tumor-bearing mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs.* the control group.

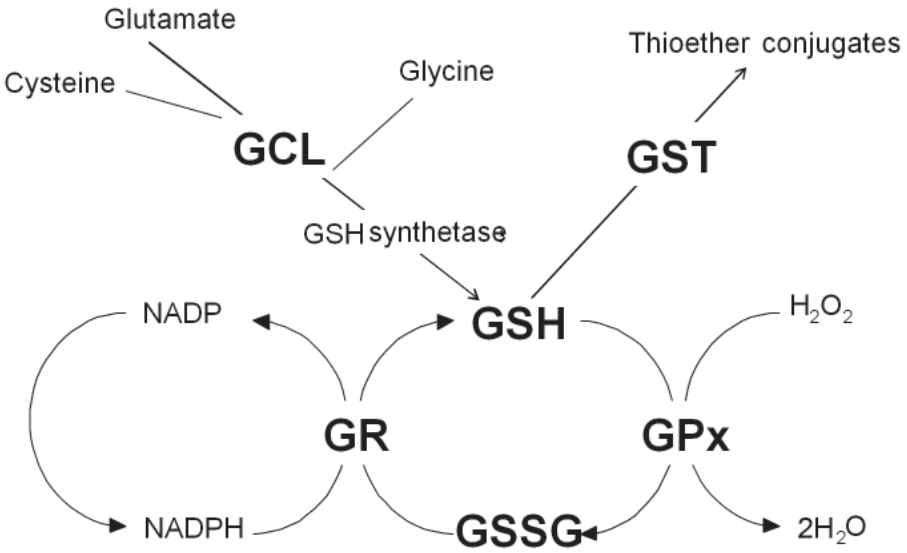

Figure 5. The whole GSH antioxidant system, including GSH, GPx, GST, GR, and GCL, is shown here.

Figure 4B, GCL activity in the brains of S180 tumor-bearing mice decreased slightly compared to that in the model group ($p < 0.05$). In addition, decreased GCL activity was slightly reversed by administration of 5-Fu ($p \geq 0.05$) and almost rose to the level in the control group. Similar results as shown in Figure 4D were also obtained for H22 tumor-bearing mice. Although GCL activity in the brains of the 5-Fu-treated group increased slightly in comparison to the model group, it was still lower than the level in the control group ($p < 0.05$).

4. Discussion

Tumors severely affect human health, and their incidence and mortality rates are increasing worldwide. 5-Fu is a typical chemotherapy drug for nonhematologic malignancies that is widely used in the treatment of a range of cancers (*18,19*). The present study selected 5-Fu since previous studies have used it to treat cancer (*13,14*). Xenografted S180 and H22 cells are typically both chosen when creating *in vivo* tumor models (*20,21*). In this regard, the present study used S180 and H22 ascites tumor cells subcutaneously injected into the right axilla of ICR mice to create a tumor model *in vivo*. The current results showed that 5-FU clearly inhibited S180 and H22 tumor growth.

Cancer cells can produce large amounts of reactive oxygen intermediates such as hydrogen peroxide that may injure surrounding healthy tissue and therefore promote tumor growth and invasion (*4*). Of the main organs in mammals, the brain is particularly susceptive to ROS damage due to its richness in polyunsaturated fatty acids (*22-24*). The whole GSH antioxidant system is shown in Figure 5, and GSH and its related enzymes such as GPx, GST, GCL, and GR are the major components involved in this system. GSH, as a major antioxidant with a high concentration of approximately 2-3 mM, plays a crucial protective role in protecting the brain against ROS (*6*). The present study found that GSH levels in the brain clearly decreased in tumor-bearing mice but slightly increased in the brain with 5-Fu treatment, although the levels were still lower than those in the control group. These results demonstrate that non-primary brain tumors significantly decreased GSH levels in the brain and these decreased levels were not reversed by 5-Fu.

Cytosolic GPx is an enzyme containing four selenium-cofactors that protects tissues from damage by catalyzing the breakdown of hydrogen peroxide and organic hydroperoxides (*25*). The current results showed that brain GPx activity largely decreased in tumor-bearing mice compared to non-tumor-bearing mice, and this decrease was not reversed by administration of 5-Fu. In combination with the results of GSH analysis, results suggest that 5-Fu inhibited tumor growth but did not improve redox homeostasis. GST is a member of a family of detoxification enzymes that metabolizes a variety of carcinogens by conjugating GSH to a wide variety of xenobiotics, which can then be excreted out through thioether formation (*26*). In the present study, brain GST activity declined markedly in tumor-bearing mice compared to non-tumor-bearing mice. As mentioned above, these results suggest that non-primary brain tumors decrease the detoxification activity of xenobiotic and endobiotic compounds in the brains of tumor-bearing mice. Furthermore, decreased GST activity in tumor-bearing mice was reversed by administering 5-Fu, which indicates that 5-Fu may

increase the detoxification activity of GST in the brains of tumor-bearing mice. Previous studies have reported that increased GST activity is closely related to the resistance of tumor cells to cancer drugs used in cancer studies over the past three decades (*27*,*28*). In this regard, the current results indicate that GST activity increased by 5-Fu may enhance the resistance of tumor cells to chemotherapy drugs.

There are two major pathways involved in regulating cellular GSH contents: one is GR, which catalyzes the reduction of GSSG to GSH *via* consumption of NADPH (*11*); the other is GCL, which is the rate-limiting enzyme in GSH synthesis (*29*). The present study found that GR activity was highly elevated in treated and untreated tumor-bearing mice compared to the control group. Increased GR activity may conceivably be due to the body's self-feedback regulation. Results also indicated a marked decrease in GCL activity, and this may augment the depletion of GSH in the brains of tumor-bearing mice. Results also showed that 5-Fu slightly reversed this decline, indicating that 5-Fu may slightly increase GCL activity.

There are reports that oxidative stress is implicated in the pathogenesis of a broad range of neuropathological conditions, including ischemia, Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, stroke, and seizure disorders (*30*-*34*). Additionally, oxidative stress injury caused by ROS seems to be closely related to the loss of neurons during stroke and neurodegenerative diseases, thereby increasing the ROS burden and consequently reducing antioxidant capacity (*35*). The present study found that non-primary brain tumors reduced antioxidant capacity and led to the loss of redox homeostasis in the brain. Excessive free radicals are capable of precipitating brain dysfunction. Although 5-Fu is, as a typical chemotherapy drug, able to inhibit tumor growth, it cannot, however, improve redox homeostasis and even increases the ROS burden in the brain.

Taken together, the current results are the first to show that non-brain primary tumors affect GSH levels and the activity of glutathione-related enzymes in the brain. This study also emphasizes the need for caution when choosing clinical agents for cancer treatment particularly for patients with a brain dysfunction. Further research on the exact mechanism through which how non-primary brain tumors increase the ROS burden in the brain is needed.

Acknowledgements

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