Amino Acids (2011) 41:151–158
DOI 10.1007/s00726-010-0606-3

ORIGINAL ARTICLE

The expression and activity of cystathionine-γ-lyase and 3-mercaptopyruvate sulfurtransferase in human neoplastic cell lines

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Received: 2 January 2010 / Accepted: 17 April 2010 / Published online: 6 May 2010

Abstract The expression and activity of cystathionine γ-lyase (CST) and 3-mercaptopyruvate sulfurtransferase (MPST) were investigated in the human neoplastic cells lines: astrocytoma U373, neuroblastoma SH-SY5Y, melanoma A375, and melanoma WM35. Gene expression analysis demonstrated that the investigated neoplastic cells showed the expression of MPST and what is particularly interesting, the expression of CST. The presence of CST in these cells was confirmed using RT-PCR and western blot analysis. However, in U373 cells, a very low activity of CST was detected. In all the investigated cell lines, the activity of MPST was higher than that of CST, which suggests that in these cells, the main pathway of sulfane sulfur formation is the MPST-catalyzed reaction. RP-HPLC analysis showed a large disparity between the level of cystathionine and GSH in the investigated neoplastic cells. In SH-SY5Y cells, the low level of GSH and low GSH/GSSG ratio corresponded with the highest CST activity. Further investigations could aim at verifying whether the stimulation of CST, at the level of protein or gene expression, could change the proliferation of neoplastic cells.

Keywords Cystathionine-γ-lyase · 3-Mercaptopyruvate sulfurtransferase · Sulfane sulfur · Glutathione · Astrocytoma U373 · Neuroblastoma SH-SY5Y · Melanoma A375 · Melanoma WM35

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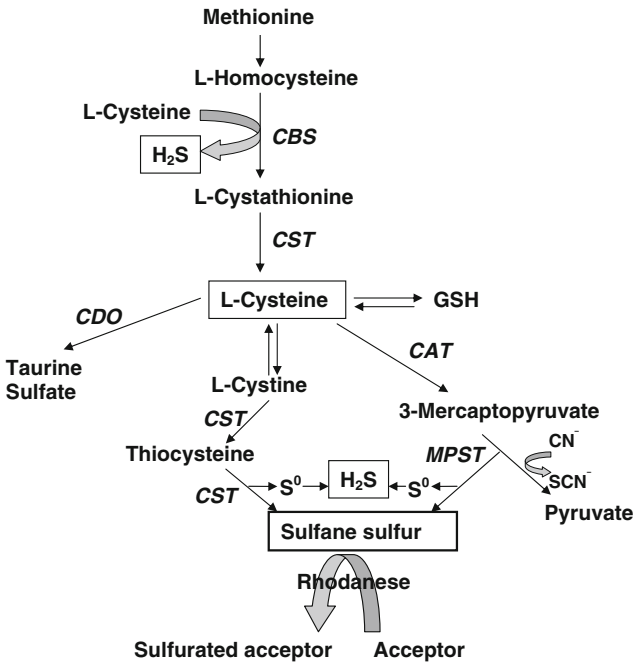
Introduction

Cysteine is metabolized through various pathways leading to its oxidation to the final metabolites, such as sulfate and taurine. Cysteine metabolites containing sulfur at oxidation state ranging from -2 to $+6$, such as hydrogen sulfide (S^{2-}), sulfite (S^{4+}), thiosulfate (S^{2-}, S^{6+}), and hypotaurine (S^{4+}) might be candidates for chemical antioxidants in mitochondria (Ubuka et al. 2008). Sulfane sulfur, a highly reactive sulfur atom covalently bound to another sulfur atom, at oxidation state 0 or -1, (Westley 1980; Ogasawara et al. 1993, 1994) is produced in non-oxidative L-cysteine metabolism, L-cysteine desulfuration (Scheme 1), and plays important roles in biological systems: synthesis of iron sulfur proteins (Bonomi et al. 1985; Tse Sum Bui et al. 2000), detoxification of cyanide (Porter et al. 1996; Baskin et al. 1999; Wróbel et al. 2004), formation of thionucleosides in tRNA (Toohey 1989; Mueller et al. 2001). It exerts anti-cancer (Toohey 1989; Pinto et al. 2006) and antioxidant effects (Ogasawara et al. 1999).

3-Mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) and γ-cystathionase (cystathionine γ-lyase, CST, EC 4.4.1.1) are well-known enzymes participating in the formation of sulfane sulfur containing compounds, while rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) is involved in transferring labile sulfane sulfur atoms from various donors to various acceptors (Westley et al. 1983) (Scheme 1). MPST and CST are also enzymes which participate in a multi-enzyme pathway that can generate H_2S from cysteine, homocysteine and their disulfides (Toohey 2001). CST catalyzes L-cystine (cysteine disulfide) conversion to thiocysteine, pyruvate, and ammonia. Then, thiocysteine, in the presence of thiols, forms H_2S and cystine or the corresponding disulfide or decomposes non-enzymatically to cysteine and inorganic sulfur (Stipanuk



Scheme 1 L-cysteine involving metabolic pathways. *CST* γ-cystathionase, *MPST* 3-mercaptopyruvate sulfurtransferase, *rhodanese* thiosulfate sulfurtransferase, *CBS* cystathionine β–synthase, *CDO* cysteine dioxygenase, *CAT* cysteine aminotransferase. Methionine is converted through *S*-adenosylmethionine (*SAM*) to homocysteine. Transsulfuration of homocysteine to cysteine is catalyzed by *CBS* and *CST*. Cysteine undergoes desulfuration to H_2S or sulfane sulfur by *CST* and by *CAT* in conjunction with *MPST*

and Beck 1982). Cystathionine β-synthase (CBS), a pyridoxal-5′-phosphate-dependent enzyme, also produces H_2S, catalyzing the condensation reaction of homocysteine with cysteine (Chen et al. 2004). MPST converts 3-mercaptopyruvate to pyruvate, ammonia, and elemental sulfur (Shibuya et al. 2009) (Scheme 1). 3-Mercaptopyruvate, a substrate for MPST, is provided through L-cysteine transamination by cysteine aminotransferase (CAT) which is identical with mitochondrial aspartate aminotransferase (Ubuka et al. 1978; Shibuya et al. 2009). Free H_2S may be formed by reduction of the atomic sulfur in the presence of physiologic concentrations of endogenous reducing substances glutathione and cysteine (Ishigami et al. 2009) or released from sulfane sulfur-contaning products such as thiosulfate and thiocysteine (Stipanuk and Beck 1982). Kimura et al. (2010) showed that H_2S protects cells from oxidative stress.

The results of our previous studies (Jurkowska and Wróbel 2008) indicated that the level of sulfane sulfur is lower in astrocytoma U373 cells, as compared with mouse astrocytes. N-Acetylcysteine, an L-cysteine precursor inhibited U373 cells proliferation, a finding that was associated with stimulation of MPST activity and elevation of the level of sulfane sulfur. The observation of defective sulfur metabolism in cancer cells and the anti-cancer effects in vivo of sulfane sulfur precursors suggest that proliferation of malignant cells may be related to a deficiency of sulfane sulfur and the uncontrolled operation of a set of enzymes normally inactivated by sulfane sulfur (Toohey 1989). Few studies have reported a decreased activity of enzymes participating in the non-oxidative metabolism of cysteine in neoplastic cells. It was found that as compared to normal cells the activities of MPST and rhodanese are significantly diminished in the astrocytoma U373 line (Jurkowska and Wróbel 2008), Morris hepatoma 51230 and 7777 (Koj et al. 1977), and Ehrlich ascites tumor cells (EATC) (Włodek et al. 1993; Wróbel and Włodek 1997). A reduced activity of rhodanese was also observed in cancerous tissues of the stomach and the lung (Jamshidzadeh et al. 2001) and in malignant hepatomas (Rosenthal 1955). The activity of CST was not detected in EATC (Włodek et al. 1993). A very low activity was found in astrocytoma U373 cells (Jurkowska and Wróbel 2008) and a decreased activity (Glode et al. 1981a) and expression in leukemic cell lines (Glode et al. 1981b; Krigler et al. 1981).

Redox changes may serve a regulatory role under physiological conditions whereby oxidative stress would increase the flux of homocysteine through the transsulfuration pathway (Mosharov et al. 2000; Persa et al. 2004). The immediate consequence of this would be an increase in cystathionine levels, a direct substrate of CST that could lead to an increase in the concentrations of the downstream metabolites, cysteine, and glutathione. Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) plays an important role in the maintenance of the cellular redox status (Lu 2009). Under metastatic conditions, high levels of GSH can support a rapid cell cycle, an elevated rate of DNA synthesis, and a block in apoptosis (Estrela et al. 2006; Pallardó et al. 2009). Hirono (1961) was the first to show that EATC have higher GSH levels than non-tumor cells. Different types of tumors, including melanoma, bladder carcinoma, lung cancer, colon cancer, and breast tumors that are multidrug- and radiation-resistant, are also found to have a high GSH content (Perry et al. 1993; Berger et al. 1994; Pendyala et al. 1997; Carretero et al. 1999; Honda et al. 2004). Considering the homeostatic redox buffer function of GSH and its role in inactivating some carcinogens (Jakobisiak et al. 2003) and protecting cells against DNA-damaging free radicals and lipid peroxidation (Sadani and Nadkarni 1996), it is plausible that tumor cells may need more GSH for their survival than other cell types (Rosado et al. 2007).

The expression of CST and MPST genes in human neoplastic brain and neural cells or melanoma cells was not investigated earlier. The enzyme CST seems to be important either because of the generation of sulfane sulfur/H_2S or cysteine for glutathione biosynthesis, while MPST can deliver only sulfane sulfur and/or H_2S. The activity and

expression of both enzymes were investigated in the human neoplastic cell lines: astrocytoma U373-derived from a malignant tumor, neuroblastoma SH-SY5Y—a neuroblast-like subclone of SK-N-SH established from a human metastatic neuroblastoma tissue, melanoma A375—a highly invasive cell line from a solid metastatic tumor and melanoma WM35 established from a primary tumor site. Melanomas, gliomas, and neuroblastomas derive embryologically from the neural crest (Carrel et al. 1982). The close relationship between glial cells and melanocytes was demonstrated by Gaynor et al. (1980); Pfreundschuh et al. (1978); Coakham et al. (1980) and Chi et al. (1997). The levels of low-molecular-weight sulfur compounds—GSH, glutathione disulfide (GSSG), cysteine, cystine, and cystathionine—were also determined in these cells. Our study confirmed for the first time the expression of the CST gene in human neoplastic cells.

Materials and methods

Sources of chemicals

Folin–Ciocalteau reagent, NADH, lactate dehydrogenase (LDH), pyridoxal phosphate (PLP), D,L-dithiothreitol (DTT), *N*-ethylmaleimide (NEM), GSH, GSSG, L-cysteine, L-cystine, D,L-cystathionine, bathophenanthrolinedisulfonic acid (BPDS), 2,4-dinitrofluorobenzene, DMEM (Dulbecco's modified Eagle medium), bovine serum albumin (BSA), and Tween 20 were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum was obtained from Gibco Laboratories (Grand Island, NY, USA), sodium 3-mercaptopyruvate and trifluoroacetic acid (TFA) from Flucka Chemie GmbH (Buchs, Switzerland), *N*-methyl-L-lysine from Bachem (Bubendorf, Switzerland). Polyvinylidene difluoride (PVDF) membrane, Blot Marker: Precision Plus Protein™ Standards Dual Color were obtained from Bio-Rad (USA). Anti-human CST mouse monoclonal antibodies were obtained from Abnova (Taiwan). Anti-mouse goat antibodies conjugated with alkaline phosphatase were obtained from Chemicon International (USA). 4-Nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; X-Phos), dNTPs were obtained from Roche (Germany). TRIzol was obtained from Invitrogen (USA). Revert Aid™ H Minus First Strand cDNA Synthesis Kit, marker 100 bp DNA Ladder were obtained from Fermentas (Canada). Polymerase DyNAzyme was obtained from Finnzymes (Finland).

Cell culture

All experiments were performed using the human cell lines: astrocytoma U373, neuroblastoma SH-SY5Y, melanoma A375, and melanoma WM35. U373 cells and SH-SY5Y cells were obtained from the Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University. Melanoma cell lines were obtained from the Department of Cancer Immunology, University School of Medical Sciences at the Great Poland Cancer Center (Poznań, Poland). The U373, SH-SY5Y, A375 and WM35 cells were grown in monolayer in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), in plastic culture dishes (100 mm in diameter), at 37°C, in a humidified atmosphere containing 5% CO_2. After trypsinization (0.25% trypsin/EDTA), the cells were diluted with complete medium (DMEM with 10% FBS) (Ponten and Macintyre 1968).

Cell homogenization

U373, SH-SY5Y, A375, and WM35 cells (2–6 × 10^6) were suspended in 0.1 M phosphate buffer pH 7.5, in the proportion 1 × 10^6 cells/0.04 ml of the buffer, sonicated 3 × 5 s at 4°C (Bandelin Sonoplus GM 70). After centrifugation at 1,600*g* for 10 min, the supernatant was used for the determination of protein concentration and the activity of MPST and CST. For HPLC analyses, the cells were suspended in 10% w/v PCA/1 mM BPDS, sonicated 3 × 5 s at 4°C (Bandelin Sonoplus GM 70). After centrifugation at 4°C at 1,400*g* the supernatant was stored at −70°C.

Assay

MPST activity was assayed according to the method of Valentine and Frankenfeld (1974) with some modifications as described by Wróbel et al. (2004). The enzyme activity was expressed as nmoles of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein. CST activity was determined by Matsuo and Greenberg's method (1958), modified as described in Czubak et al. (2002). The enzyme activity was expressed as nmoles of α-ketobutyrate formed during 1 min incubation at 37°C per 1 mg of protein.

GSH, GSSG, cysteine, cystine, and cystathionine were determined by a reversed-phase HPLC method of Dominick et al. (2001) with some modifications as described by Wróbel et al. (2009). Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Western blot analysis

The cells were suspended in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and proteinase inhibitor cocktail and sonicated 3 × 5 s at 4°C (Bandelin Sonoplus GM 70).

The homogenate was left on ice for 1 h with 0.1% Triton X-100 and 0.03% protamine sulfate and centrifuged at 18,000*g* for 1 h at 4°C. Aliquots of 100 µg of protein extracts were separated in a 6–12% gradient SDS-PAGE (Laemmli 1970) and transferred onto polyvinylidene difluoride (PVDF) membrane. The blots were incubated overnight at 4°C in 0.025 M Tris/HCl, pH 8.4, containing 0.192 M glycine and 20% methanol. The resulting blots were blocked in 2% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 for 2 h at 4°C. After incubation, the membranes were incubated with anti-human CST mouse monoclonal antibodies (1:500) for 12 h at 4°C. The membranes were then briefly washed with PBS and incubated with anti-mouse goat antibodies conjugated with alkaline phosphatase (1:10,000) for 2 h at 4°C. The immune complexes were visualized using NBT and BCIP.

Isolation of total RNA

Total RNA was extracted from the cells using TRIzol, according to the protocol provided by the manufacturer. The extracted RNA was suspended in ribonuclease-free water and was quantified by measuring the absorbance at 260 nm. The integrity of the purified RNA collected by this method was confirmed by observation of the 28S and 18S rRNA bands after agarose gel electrophoresis.

Reverse transcription of RNA

Total RNA from the cell samples was reverse-transcribed using the Revert Aid^{TM} H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions. For reverse transcription (RT), 1 µg of total RNA was mixed with 1 µl Oligo d(T) primer (0.5 µg/µl) and water pretreated with diethylpyrocarbonate (H_2O-DEPC) and incubated for 5 min at 70°C. After preincubation, other components were added to this mixture: 4 µl 5× concentrated RT buffer (250 mM Tris–HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT, pH 8.3 at 25°C), 1 µl RNase inhibitor (20 U/µl), and 2 µl deoxyribonucleotide triphosphates (dNTPs, 10 mM). After incubation at 37°C for 5 min, 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U/µl) in a total volume of 20 µl was added. The mixture was first incubated for 60 min at 42°C, then for the final 10 min at 70°C, and stored at 20°C.

Polymerase chain reaction

Expressions of MPST, CST, and β-actin were analyzed by PCR. Amplification of cDNA samples was run in a 25 µl reaction volume that contained the following: 1 µl of synthesized cDNA, 0.2 µM of each of gene-specific primer pair, 0.04 U/µl DNA polymerase in 10 mM buffer Tris–HCl pH 8.8 (supplemented with 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 0.2 mM of each dNTPs and H_2O-DEPC. For the MPST and β-Actin gene, after an initial 5 min at 94°C denaturation, amplification was performed under the following conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min for 30 cycles, with a final incubation at 72°C for 8 min (Kato et al. 2001). For the CST gene, after an initial 5 min at 94°C denaturation, amplification was performed under the following conditions: 94°C for 30 s, 51°C for 60 s, and 72°C for 8 min for 28 cycles, with a final incubation at 72°C for 10 min. β-actin was used as an internal standard to normalize all samples for potential variations in mRNA content. All PCR products were analyzed by electrophoresis on a 2.5% agarose gel stained with ethidium bromide and directly visualized under UV light and photographed. For MPST, CST, and β-actin cDNA amplification, the previously described primer sequences were used (Kusukawa et al. 2000; Levonen et al. 2000; Kato et al. 2001) (Table 1).

Statistical analysis

Statistical significance of the differences between cells was tested using the Student's *t* test or nonparametric Mann–Whitney's test ($P < 0.05$ was considered significant). The presented results are the means from 3–4 independent experiments ±standard deviation (SD).

Results and discussion

The activity of MPST in cancer cells has been previously repeatedly demonstrated (Koj et al. 1977; Włodek et al.

Table 1 Sequences of oligonucleotide primers used for detection of target genes by RT-PCR

Target gene	Primer	Sequence	Product size (bp)
MPST	MPST 1 (sense)	5′-GACCCCGCCTTCATCAAG-3′	367 bp
	MPST 2 (antisense)	5′-CATGTACCACTCCACCCA-3′	
CST	CST 1 (sense)	5′-GCAAGTGGCATCTGAATTTG-3′	301 bp
	CST 2 (antisense)	5′-CCCATTACAACATCACTGTGG-3′	
β-Actin	β-Actin 1 (sense)	5′-CTGTCTGGCGGCACCACCAT-3′	~300 bp
	β-Actin 2 (antisense)	5′-GCAACTAAGTCATAGTCCGC-3′	

RT–PCR reverse transcriptase-polymerase chain reaction

1993; Wróbel and Włodek 1997; Jurkowska and Wróbel 2008). Gene expression analysis confirmed the expression of MPST in human neoplastic cells: astrocytoma U373, neuroblastoma SH-SY5Y, melanoma A375, and melanoma WM35. Among the investigated cells, the highest MPST expression in SH-SY5Y cells (Fig. 1) corresponded with the highest value of the activity (Table 2) —it is significantly ($P < 0.05$) higher in comparison to the value detected in U373 cells. The expression (Fig. 1) and activity (Table 2) of MPST in both melanoma cell lines (A375 and WM35) are similar.

Most interestingly, in view of the commonly recognized deficiency of CST activity in neoplastic cells (Glode et al. 1981b; Jurkowska and Wróbel 2008; Krigler et al. 1981; Włodek et al. 1993), its expression was also confirmed in all of the investigated neoplastic cells (Fig. 2). The presence of CST in these cells was confirmed using RT-PCR (Fig. 2) and western blot analysis (Fig. 3). The results of the present investigations not only confirmed the expression of CST in all the investigated neoplastic cells but also showed significant differences in the level of CST mRNA depending on the origin of cells.

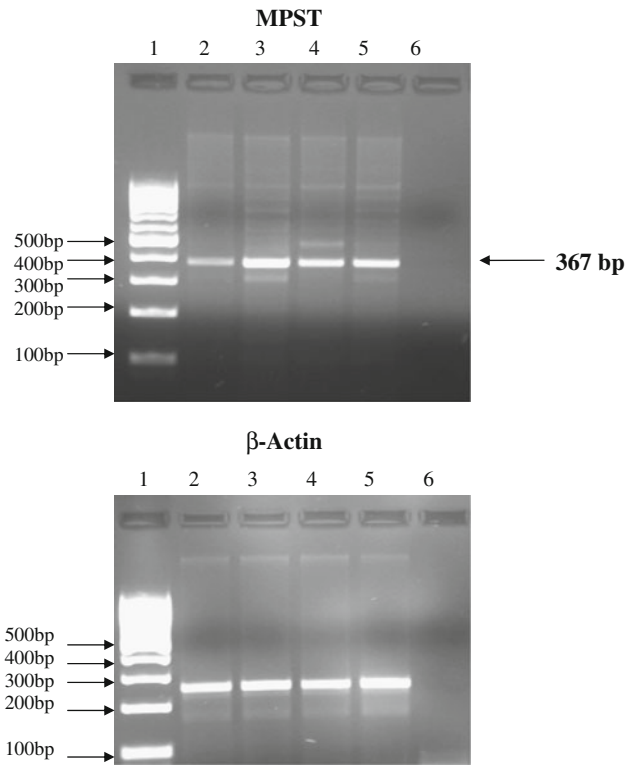


Fig. 1 RT-PCR analysis of the 3-mercaptopyruvate sulfurtransferase (MPST) gene in human neoplastic cells. Three independent experiments were performed with similar results. One set of representative results is shown. *β*-Actin was used as an internal control. *1* molecular mass standard; Cell lines: *2* U373, *3* SH-SY5Y, *4* A373, *5* WM35, *6* empty lane

The results presented in Table 2 show that in all the investigated cell lines, the activity of MPST is higher than CST under optimal assay conditions. Therefore, it is possible to assume that in these cells MPST can be regarded as the principal enzyme involved in sulfane sulfur production.

RP-HPLC analysis showed a large disparity among the levels of GSH in the investigated cells (Table 3). In SH-SY5Y cells, the low level of GSH and low GSH/GSSG ratio corresponded with the highest CST activity (Table 2), which is in accord with the upregulation of the flux of homocysteine through the transsulfuration pathway in a decreased cellular redox status (Mosharov et al. 2000;

Table 2 Activities of 3-mercaptopyruvate sulfurtransferase (MPST) and *γ*-cystathionase (CST) in human neoplastic cells

Human cancer cells	MPST nmole product x mg protein^{-1} x min^{-1}	CST
Astrocytoma U373	145 ± 8	0.50 ± 0.14
Neuroblastoma SH-SY5Y	695 ± 66	1.86 ± 0.55
Melanoma A375	263 ± 30	0.94 ± 0.21
Melanoma WM35	268 ± 29	1.12 ± 0.31

Each value is the mean of 15–112 repeats. Differences between values of the MPST activity were confirmed with Mann–Whitney's test: $P < 0.05$ for SH-SY5Y versus U373, A375, WM35 cell lines

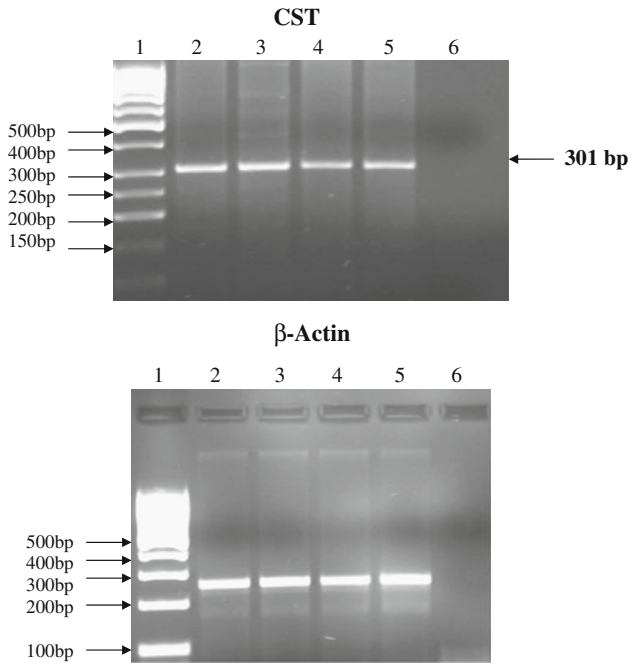


Fig. 2 RT-PCR analysis of the *γ*-cystathionase (CST) gene in human neoplastic cells. Three independent experiments were performed with similar results. One set of representative results is shown. *β*-Actin was used as an internal control. *1* molecular mass standard; Cell lines: *2* U373, *3* SH-SY5Y, *4* A373, *5* WM35, *6* empty lane

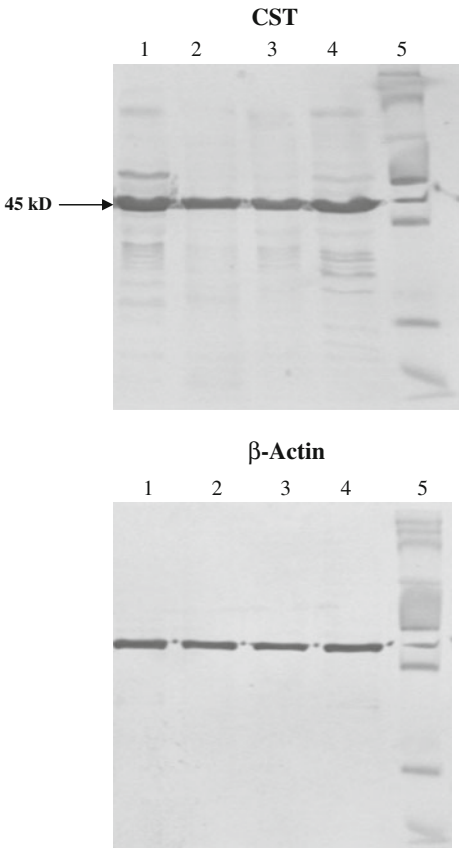


Fig. 3 Western blot analysis of γ-cystathionase (CST) protein in human neoplastic cells. Four independent experiments were performed with similar results. One set of representative results is shown. β-Actin was used as an internal control of protein loading. Cell lines: *1* U373, *2* SH-SY5Y, *3* A373, *4* WM35, *5* molecular mass standard. 100 μg of protein was added to each lane

Persa et al. 2004). The immediate consequence would be an increase in cystathionine levels, a direct substrate of CST. The activity of CST is dependent on cystathionine as a substrate and pyridoxal phosphate (PLP) as a coenzyme. High cystathionine levels in SH-SY5Y and WM35 cells corresponded to a high CST activity (Tables 2, 3). A pyridoxine-induced anti-proliferative effect on cancer cells has been reported (Moussa et al. 1982; DiSorbo et al. 1985; Shultz et al. 1988; Gridley et al. 1989; Molina et al. 1997; Kamat and Lamm 2002; Bidoli et al. 2003; Selhub et al. 2003; Wei et al. 2005; Ren and Melmed 2006), and it could be associated with an increased CST activity and increased production of sulfane sulfur.

The CST/H_2S system has been shown to play an important role in regulating cellular functions in different systems. Yang et al. (2004) used CST stably overexpressed HEK-293 endothelial kidney cells to explore the effect of the CST/H_2S system on cell growth and proliferation. Overexpression of CST resulted in increases in CST mRNA levels, CST proteins, and intracellular H_2S production rates, as well as in inhibition of cell proliferation and DNA synthesis.

Table 3 RP-HPLC analysis of the level of GSH, GSSG, cysteine, cystine, and cystathionine in human neoplastic cells

Human cancer cells	GSH	GSSG	Cysteine	Cystine	Cystathionine
	nmole/10^6 cells				
Astrocytoma U373	14.9	1.7	0.3	2.5	0.4
Neuroblastoma SH-SY5Y	2.37	0.53	0.01	0.19	0.63
Melanoma A375	27.7	2.84	0.31	0.63	0.02
Melanoma WM35	111	8.84	3.03	ND	1.04

Results are the mean from three independent experiments

ND not detectable

Our previous studies (Jurkowska and Wróbel 2008) carried out on U373 cells showed that *N*-acetyl-L-cysteine-dependent inhibition of the proliferation of U373 cells was accompanied by an increase in the level of sulfane sulfur. On the other hand, a dose-dependent increase of GSH level stimulated cells proliferation, while GSH depletion caused cell growth inhibition and enhanced apoptosis in pancreatic cancer cells (Schnelldorfer et al. 2000). CST and MPST participate in both sulfane sulfur generation and in H_2S production (Chen et al. 2004; Kimura et al. 2010; Toohey 2001). The present results indicating the presence of the CST mRNA and its expression, as well as the presence of cystathionine in the neoplastic cells seem to be promising and suggest that stimulation of the expression and/or activity of CST might be possible, which—through an increase in sulfane sulfur levels—might lead to a decrease in cancer cell proliferation, as it was observed in the case of U373 cells (Jurkowska and Wróbel 2008).

Acknowledgments This work was supported by a grant from the National Committee for Scientific Research (KBN) No K/ZBW/000147 and K/ZDS/000450.

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