RESEARCH ARTICLE

Expression and Function of GSTA1 in Lung Cancer Cells

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Abstract

Glutathione S-transferase A1 (GSTA1) appears to be primarily involved in detoxification processes, but possible roles in lung cancer remain unclear. The objective of this study was to investigate the expression and function of GSTA1 in lung cancer cells. Real-time PCR and Western blotting were performed to assess expression in cancer cell lines and the normal lung cells, then verify the A549 cells line with stable overexpression. Localization of GSTA1 proteins was assessed by cytoimmunofluorescence. Three double-strand DNA oligoRNAs (*SiRNAs*) were synthesized prior to being transfected into A549 cells with Lipofectamine 2000, and then the most efficient *SiRNA* was selected. Expression of the GSTA1 gene in the transfected cells was determined by real-time PCR and Western blotting. The viability of the transfected cells were assessed by MTT. Results showed that the mRNA and protein expression of A549 cancer cells was higher than in MRC-5 normal cells. Cytoimmunofluorescence demonstrated GSTA1 localization in the cell cytoplasm and/or membranes. Transfection into A549 cells demonstrated that down-regulated expression could inhibit cell viability. Our data indicated that GSTA1 expression may be a target molecule in early diagnosis and treatment of lung cancer.

Keywords: GSTA1 - A549 - lung cancer - marker - target molecule

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Introduction

Lung cancer is one of the most common and the most deadly types of malignant cancer, especially in the later stages. In 2012, lung cancer is expected to account for 29% of all male cancer deaths and 26% of all female cancer deaths. Because of lung cancer insidious onset, its diagnosis and treatment is far from satisfactory (Siegel et al., 2012). But for early diagnosis of lung cancer patients, the prognosis of surgical treatment obviously improved for the middle-late lung cancer patients. Therefore, the study of lung cancer early diagnosis and treatment, screening and identification of high specificity of lung cancer tumor markers has been the research focus.

Tumor markers, refers to something released by tumor cell or the interaction with the host and tumor in the process of proliferation and tumorigenesis, biosynthesis, reflects the tumor's existence and growth. Some representative significance of lung cancer tumor markers mainly has the following categories (Buccheri et al., 2003; Pollan et al., 2003; Molina et al., 2004; Cho, 2007; Oremek et al., 2007; Xie and Wang, 2013). Embryo antigen (represented by CEA); Glycoprotein class antigen (represented by CA19-9 and IL33); Keratin antigen (represented by CYFRA21-1); Enzymes antigen (represented by NSE); Protein products and antibodies of tumor gene and tumor suppression and so on. However, most of these indicators are no organ specificity, they are not lung cancer specific antigens, but cancer related substances, which not only exist in malignant tumors, but also exist in benign tumors, embryonic organization, even in the normal tissue, lacking of specificity to the diagnosis of lung cancer. Therefore, looking for a kind of high sensitivity, high specificity, can be practical application in clinical diagnosis and treatment of lung cancer biomarkers has become one of the urgent problems in the research of lung cancer.

In the early stage of the study, we selected GSTA1 tumor antigens of lung cancer cells with several lung cancer-specific binding polypeptides by Phage display technology *in vivo* and *in vitro* experiments (Zang et al., 2009). Glutathione S-transferaseA1 (GSTA1) play an important role in the biotransformation of xenobiotics as well as in the detoxification of genotoxic substances that arise from normal constituents of living organisms (Mannervik et al., 2005). In the present study, we identified GSTA1 expressed in cancer cells, especially lung adenocarcinoma cells A549, is much higher than normal cells MRC-5. The same result demonstrated in the tissue. If interference GSTA1 expression, A549 cell proliferation would be inhibited, indicating its potential for a use in early diagnosis or for the approahces to therapy of lung cancer.

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Materials and Methods

Reagents

Lipofectamine 2000 (Invitrogen, USA), RNAiso Plus, PrimeScript™ II 1st Strand cDNA Synthesis Kit and SYBR® *Premix Ex Taq*™ II were purchased from Takara Dalian, JPN. GSTA1 and GAPDH primers (invitrogen, USA.), polyvinylidene difluoride membranes (Millipore, USA), primary antibody anti GSTA1 (Goat anti human IgG), anti β-actin (Mouse anti human IgG), Secondary antibodies, Dnk PAb to Goat IgG HRP), Goat Anti-Mouse IgG H&L (HRP) , Rabbit Anti-Goat IgG H&L (Alexa Fluor® 488) were from abcam (Cambridge, UK), normal donkey serum, Hoechst 33258, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, DiI, Methylthiazolyldiphenyl-tetrazolium br- omide, MTT (Sigma, USA)were from Sigma, USA, Small interfering RNA (*SiRNA*) knockdown of GSTA1 (*SiRNA*-1*SiRNA*-2, *SiRNA*-3), negative control *SiRNA* (*SiRNA*-NC) obtained from Shanghai Integrated Biotech Solutions Co., Ltd (Shanghai, China).

Cell lines

All the cell lines (human lung cancer cell lines LTEP-A2, NCI-H460 (H460), SPCA1 and A549, human hepatocellular carcinoma cell line HepG2, human lung normal cell line MRC-5)used in the study Shanghai Institute of Cellular Biology of Chinese Academy of Sciences (Shanghai, China). For each experiment, cells were maintained in RPMI 1640 medium supplemented with 10% FBS.

RT-PCR analysis of GSTA1 mRNA levels

Total RNA of the cells was isolated with RNAiso Plus following the manufacture's protocol. First-strand cDNA was synthesized with PrimeScript™ II 1st Strand cDNA Synthesis Kit according to the manufacture's instruction, using 3μg of total RNA. Primers for GSTA1 were designed as follows: forward primer, 5'-GCCTCCATGACTGCGTTATT-3'; reverse primer, 5'-CCTGCCCACAGTGAAGA-AGT-3'. Primers for GAPDH were as follows: forward primer, 5'-AACGGATTTGGTCGTATTGGG-3'; reverse primer, 5'-CCTGGAAGATGGTGATGGGAT-3'.Real-time PCR was performed following the manufacture's protocol of SYBR® *Premix Ex Taq*™ II. Gene expression levels were normalized to those of GAPDH.

Protein extraction and Western blot

45μg of cell lysates were separated by 12 % SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by incubating in TBS Tween-20 buffer containing 5% milk for 2h at room tempreture, and then incubated with primary antibodies overnight at 4°C. After three washes in TBST, the membranes were incubated with secondary antibody for 1 h at 37°C.The intensity of the pooled samples bands were determind by densitometric analysis using Image J software.

Cytoimmunofluorescence

Confocal microscopy staining was used to examine GSTA1 subcellular localization in the lung cancer cell A549 .The supernatant of cells grown on confocal special dish was discarded , and then fixed with 4% paraformaldehyde in PBS for 15 mins at room temperature, 0.5% Tween 20 and 0.4% normal donkey serum to block nonspecific binding. Cells were incubated with the primary antibodies (Goat Pab to Glutathione S Transferase alpha) for overnight at 4°C, and then washed in PBS (4×6 min) at room temperature, exposed to Rabbit Anti-Goat IgG H&L for 1 h at 37°C in the dark. After washing again in PBS (4×6 min), Confocal special dish was exposed to Hoechst 33258 for 15mins at room temperature , and then washed in PBS (4×6 min) at room temperature, exposed to DiI for 15 mins, and visualized using a confocal microscopy (Leica, Germany).

Small interfering RNA (SiRNA) knockdown of GSTA1

A549 cells were transiently transfected with *SiRNA* obtained from Shanghai Integrated Biotech Solutions Co., Ltd. *SiRNA*-1 against A549: sense 5'-GGAGCUUGACUCCAGUCUUTT -3'and anti-sense 5'-AAGACUGGAGUCAAGCUCCTT -3'; *SiRNA*-2 against A549: sense 5'-GCCCACAGU-GAAGAAGUUUTT-3' and antisense5'-AAACUUCUUCACUGUGGGCTT -3'; *SiRNA*-3 against A549: sense 5'-GGAGAAGAGUUACCAUCCUTT-3'and

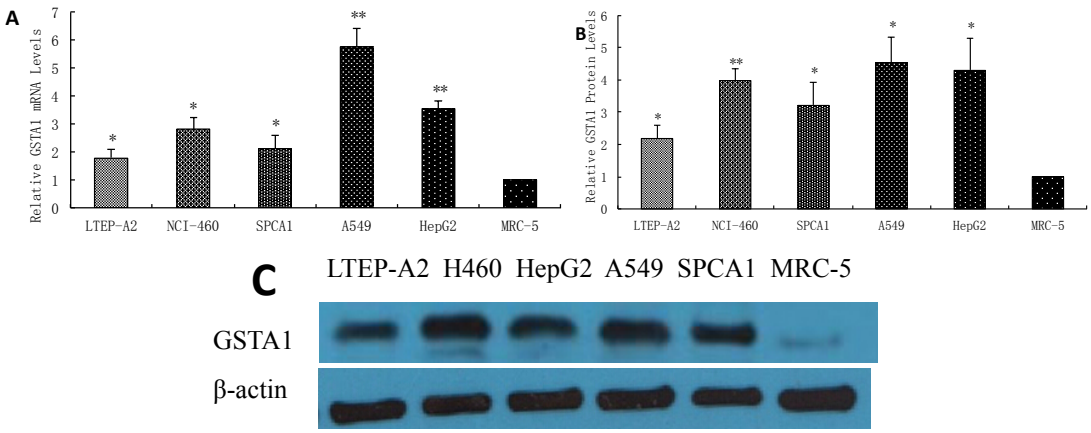

Figure 1. Expression of GSTA1 in different cells. A) Real-time RT-PCR analysis of mRNA levels of GSTA1.*p<0.05, **p<0.01 vs. the MRC-5 group; B) Western blot analysis of GSTA1 protein levels.*p<0.05, **p<0.01 vs. the MRC-5 group;C) Representative images of Western blots performed in B

antisense5'-AGGAUGGUAACUCUUCUCCTT-3'; and *SiRNA*-NC: sense 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense5'-ACGUGACACGU-UCGGAGAATT-3' using Lipofectamine 2000 (lipo) according to the manufacturer's protocol. Following transfection, A549 cells were incubated at 37°C in a CO_2 for 24 and 48 hours before being harvested for quantitative Real time PCR and Western blot analyses in order to select the best sequence of interference efficiency.

Cell viability analysis

MTT is the routine checks of the cell growth was performed to assess the proliferation of the infected A549 cells by the best sequence of interference efficiency. A549 cells were seeded at an initial density of 5×10^3cells/well in 96-well plates for day 1, day 2, day 3, day 4, day 5 post-transfection respectively. Subsequently, 20 μl of the MTT solution in PBS (5mg/ml) was added to the wells. After 4 hours of incubation at 37°C in 5% CO_2, adding 200μl DMSO after abandoning the supernatant. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, USA) for cell viability rate.

Statistical analyses

SPSS 19.0 software was used for all statistical analysis. All data are expressed as the mean±standard deviation (SD). Data from two groups were analyzed by the Student's t-test. $p<0.05$ was considered to indicate a statistically significant difference.

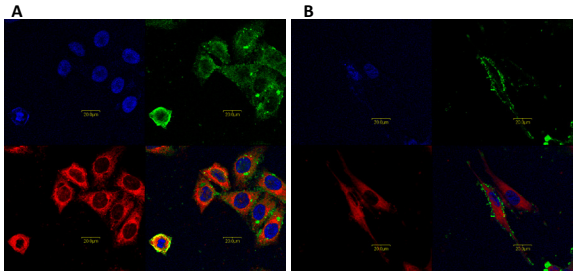

Figure 2. Cellular localization of GSTA1 by confocal microscopy staining. A)Lung cancer A549 cells line; B) Lung normal MRC-5 cells line

Results

Quantitative evaluation of GSTA1 mRNA by Real-time PCR

The levels of GSTA1 in cancer cell lines were high, especially the highly invasive lung adenocarcinoma cell lines A549, whereas the level of GSTA1 in normal lung cell line MRC-5 was low. According to real-time A549 cell lines demonstrated 5.76-fold overexpression of GSTA1 mRNA relative to MRC-5 cell line (Figure 1A).

Western blotting of GSTA1 protein expression

Western blotting was used to evaluate GSTA1 protein expression in eight cancer cell lines. GSTA1 expression was significantly increased in the cancer cell lines, particularly the highly invasive lung adenocarcinoma cell lines A549 (Figure 1B, C). On the basis of the result of Real-time PCR and Western blotting, the GSTA1 mRNA and protein expression in A549 cell lines was the highest among the nine cell lines. Therefore, A549 cell lines were chosen as experimental objects in the next experiment.

Cellular localization of GSTA1 in A549 cells line and MRC-5 cells line

The positive expression of GSTA1 protein showed as green stain in the cytoplasm and/or cell membrane. Confocal microscopy revealed an expression in the cytoplasm and/ or cell membrane of A549 (Figure 2A) and MRC-5 (Figure 2B) that colocalized well with the cell membrane marker DiI .

Small interfering RNA (SiRNA) knockdown of GSTA

The GSTA1 mRNA expression in A549 cells infected with *SiRNA*-1, *SiRNA*-2 and *SiRNA*-3 were all markedly decreased as compared to the *SiRNA*-NC group and lipo group (Figure 3A). The amount of GSTA1 protein was also obviously decreased (Figure 3B). On the basis of the result, among the three *SiRNA*s , *SiRNA*-1 exhibited more intense suppressive effects on GSTA1 mRNA than the other two *SiRNA*s. Therefore, *SiRNA*-1 was chosen for the subsequent experiments.

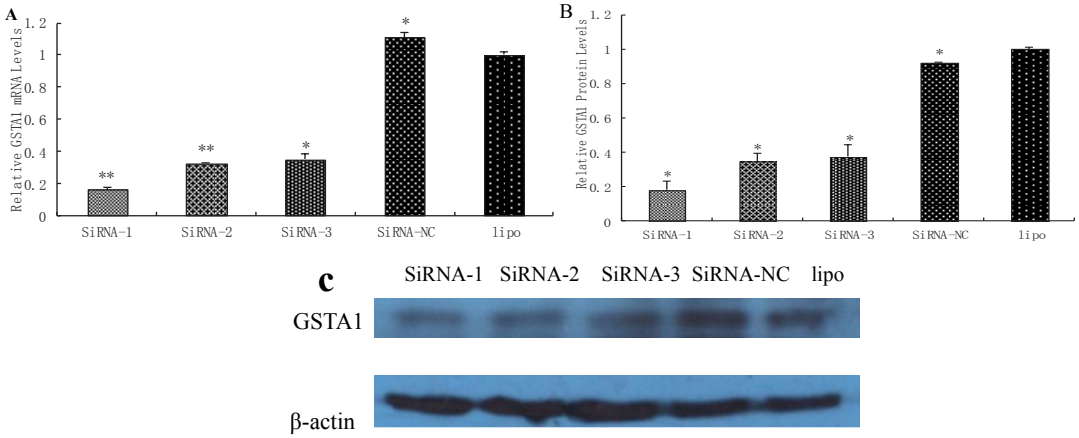

Figure 3. Screening of SiRNAs targeting GSTA1 mRNA. A) Real-time RT-PCR analysis of mRNA levels of GSTA1. *$p<0.05$, **$p<0.01$ vs. the lipo group; B) Western blot analysis of GSTA1 protein levels. *$p<0.05$, **$p<0.01$ vs. the lipo group; C) Representative images of Western blots performed in B. Among the three SiRNA, SiRNA-1 showed more intense effect on suppressing GSTA1 protein and mRNA. The "lipo" in the graph represented A549 cells treated only with liposome, and the expression levels of this group were taken as 1

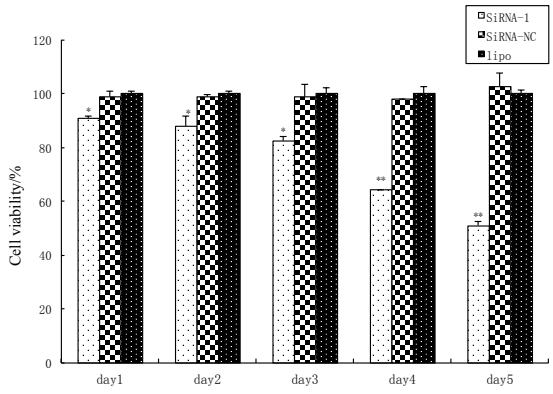

Figure 4. The Effects of siRNA-1 on A549 Cell Viability. **p*<0.05, ***p*<0.01 vs. the lipo group

Cell viability analysis

To examine whether GSTA1 affect the proliferation of A549 cells, the cell viability of the infected A549 cells by the best sequence of interference efficiency *SiRNA*-1 grown in RPMI 1640 medium were analyzed using the experimental method of MTT (day1, day2, day3, day4, day5) (Figure4). The interference of GSTA1 expression, A549 cell proliferation would be inhibited in a time-dependent way.

Discussion

One of the most killer among all cancers in the world is lung cancer (Ferlay et al., 2010). The established etiological factors such as environmental air pollution, usage of tobacco and chronic exposure to chlorophenol related compounds are the major cause for the development of lung cancer (Hecht, 2006; Sturgis and Cinciripini, 2007). GST isoenzymes have become potential therapeutic targets for the treatment of cancer (Laborde, 2010).

The glutathione S-transferases (GSTs) are a multigene family of drug detoxification enzymes that are important in phase II metabolism by catalyzing the conjugation of glutathione to a variety of electrophilic substances (Hayes et al., 2005; Khan et al., 2011). GSTs are also involved in the detoxification of products of lipid peroxidation (Balogh et al., 2008). GST isoenzymes are also known to modulate cell signaling pathways controlling cell proliferation and apoptotic cell death (Holley et al., 2007). Daorueang et al. reported that the mechanism for GST-promoted cell proliferation that accelerates the formation of cholangiocarcinoma (Daorueang et al., 2012). In the present study, at the cellular level, expression of GSTA1 in the eight different cancer cells and the normal lung cells MRC-5 were compared by real-time PCR and western blotting. The results indicated that the GSTA1 level was lower in MRC-5 cells, while A549 cells expressing the highest amount, which were chosen for the next experiment.

Previous studies have shown that GSTA1 over-expression in cell lines with no detectable GSTA1 levels such as the human retinal pigment epithelial (RPE) cells and human lung cancer (H69) cells does not affect growth rate (Liang et al., 2005). In the present study, GSTA1 protein mainly distributed in the cytoplasm and/or cell membrane playing an important role in cell signal transduction and cell structure integrity. While the influence of GSTA1 on A549 cell proliferation was not directly examined in the current study.

RNA interference (RNAi) refers to an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) resulting in the posttranscriptional suppression of gene expression mediated by short double stranded RNA (dsRNA), also called small interfering RNA (*SiRNA*). RNAi provides a potential new way for modulation of oncogenic gene function in cancer cells although the precise mechanism is still unclear (Elbashir, Harborth et al., 2001). In this study, we investigated the possibility if RNAi could silence GSTA1 gene in A549 by real-time PCR and western blotting to select the sequence *SiRNA*-1 of the best interference effect. We also assessed the functional outcome of the GSTA1 silence in A549 in terms of effects on cell proliferation *in vitro*. Our result suggest a role of silencing GSTA1 in inhibiting tumor cell growth.

In conclusion, we proved that GSTA1 was markedly upregulated in human cancer cells and tissues. Decreasing the expression of GSTA1 may lead to a decrease in lung cancer cell invasion ability. The findings suggest that GSTA1 may become the early diagnosis and treatment target of lung cancer.

Acknowledgements

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