Expression of glutathione S-transferase M2 in stage I/II non-small cell lung cancer and alleviation of DNA damage exposure to benzo[a]pyrene

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A B S T R A C T

Glutathione S-transferases (GSTs) are a family of inducible enzymes that are important in carcinogen detoxification. GST-Mu class is showing the high activity towards most polycyclic aromatic hydrocarbon (PAH) epoxide. Our objective is to clarify the expression of GST-M2 in non-small-cell lung carcinoma (NSCLC) patients and to determine the role of GST-M2 in protecting against DNA damage. We detected changes in GST-M2 expression at mRNA levels with a panel of lung cell lines and clinical samples of malignant and paired adjacent non-malignant tissues from 50 patients with stage I or II non-small-cell lung carcinoma using real-time RT-PCR. Comet assay and γ-H2AX were used to clarify whether DNA damage was protected by GST-M2. Our data demonstrate that the expression of GST-M2 in tumor tissues is significantly lower than in paired adjacent non-malignant tissues ($p = 0.016$). Loss of GST-M2 is closely associated with age, gender, T value, N value and cell differentiation ($p < 0.05$) in early stage I/II patients. Downregulation of GST-M2 is mediated through aberrant hypermethylation in lung cancer cell lines. Protection against B[a]P-induced DNA damage by GST-M2 in lung cancer cells was detected by Comet assay and γ-H2AX. In conclusion, DNA hypermethylation altered and reduced GST-M2 expression that resulted in susceptible to benzo[a]pyrene (B[a]P) induced DNA damage. It implies that GST-M2 reduction occurs prior to tumorigenesis.

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1. Introduction

Susceptibility to lung cancer is associated with several environmental factors and xenobiotics, which may be metabolized and detoxified by phase II enzymes, glutathione S-transferases (GSTs). Through exposure to tobacco carcinogenic metabolites, including polycyclic aromatic hydrocarbons (PAHs), the lung is constantly challenged by latent harmful compounds (Kelley et al., 2005). Benzo[a]pyrene (B[a]P), the most extensively studied of the PAHs, is a carcinogen found in cigarette smoke and is considered a cause of lung cancer (Alexandrov et al., 2002), specifically inducing DNA damage (Orlow et al., 2008). B[a]P can be activated to a highly reactive species, benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), by cytochrome P450 enzyme (phase I enzyme). Through highly mutagenic attack with the N2 position of guanine residues in DNA-BPDE can form a stable BPDE-N2-deoxyguanosine (BPDE-dG) adduct, which plays a critical role in tumorigenesis (Alexandrov et al., 2006). The presence of BPDE-DNA adducts in human bronchial cells points to their involvement in induction of mutation in p53, and initiation of human lung cancer (Rojas et al., 2004).

The expression levels of the different GSTs are tissue specific (Konig-Greger et al., 2004). Within each of the GST isoforms there are significant differences in substrate specificity (Kearns et al., 2003). GST isoforms have also been found to possess different activities in tissues such as muscle, testis, brain, heart, blood, liver and upper aerodigestive mucosa (Hayes and Pulford, 1995; Matthias et al., 1999; Tsuchida et al., 1990). In adult human liver, 80% of the total GST proteins are GST-Alpha members, while the Pi-class isozyme, occurs only minimally in GST-M2, a muscle-specific isozyme, occurs only minimally in the epithelium of the terminal airway of malignant tumorous lung lesions (Anttila et al., 1993). Furthermore, a recent cohort study 0378-4274/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
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indicated that the children of mothers who smoked during pregnancy had worse lung function than the children of mothers who did not smoke during pregnancy, and these children showed two alleles at multiple loci transmitted together on the same chromosome (haplotypes) of GST-M2 (Breton et al., 2009). Some reports have indicated that BPDE is a poor substrate for GST-Alph1/Alph2, but a relatively good substrate for GST-M2 (Robertson et al., 1986). GST-M2 expression may associate with lung cancer and exposure to smoke. In addition, our recent study demonstrated that the overexpression of GST-M2 transfectant cells results in reduced BPDE-induced DNA adducts by competitive ELISA and nested-PCR (Weng et al., 2005). The results indicated a role for GST-M2 activity in the prevention of DNA damage. However, the expression of GST-M2 remains undefined in non-small-cell lung carcinomas (NSCLCs).

A aberrant DNA hypermethylation of gene promoter DNA is an important epigenetic mechanism leading to downregulation and silencing of several tumor suppressor genes such as p16, APC and MGM (Peng et al., 2009; Schumann et al., 2005). It can trigger the carcinogenic cascade and lead to cancer progression. In this study, our objective is to clarify the expression of GST-M2 in NSCLC patients and to determine the role of GST-M2 in protecting against DNA damage. Specifically, the aims of this study are (I) to understand the mRNA expression of GST-M2 in lung normal cell and lung cancer cell lines; (II) to analyze the mRNA expression of GST-M2 in association with clinical outcomes of early stage I and II NSCLC patients; (III) to determine the cause of the association of gene expression regulation of GST-M2 with hypermethylation of other processes and (IV) to understand the alleviation of B[a]P-induced DNA damage in NSCLCs.

2. Materials and methods

2.1. Study subjects and follow-up

Between January 2003 and August 2004, we collected lung cancer tissue specimens including cancers tumor and adjacent normal tissues from 50 stage I and stage II NSCLC cases that underwent surgical resection in this prospective study. None of the patients received pre-operative chemotherapy or radiotherapy. Tumor staging was performed according to AJCC (6th edition) criteria (Greene, 2002). None of the patients received pre-operative chemotherapy or radiotherapy. Tumor samples were acquired from the solid part of the mass, avoiding grossly necrotic areas. The non-tumor bearing paired tissues were acquired from the lobar edge of resected lung with a distance of at least 5 cm from the gross tumor margin. The samples were acquired from the Taichung Veterans General Hospital, and informed consent was obtained for every patient.

2.2. Cell lines and culture

Human lung cancer cells and lung cells from the American Type Culture Collection were studied. Lung cancer cell lines (adenocarcinoma cell lines A549, H1299, H1355, H23 and H460; squamous cell carcinoma cell lines CH27 and Calu-1) were cultured on Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Rockville, MD). C1L-0 and its sublines C1L-5 (human lung carcinoma cell lines with different invasive and metastatic capabilities) were obtained from Dr. Pan-Chyr Yang (Chu et al., 1997). The lung normal cell lines (Broblast cell lines MRC-5 and WI-38) were cultured on Basal medium Eagle (BME) (Sigma, St. Louis, MI). The human bronchial epithelial cell line BEAS-2B was cultured on LHC-9 medium (GIBCO, Rockville, MD). All lung cancer cell lines were maintained at 37° C in a 5% CO2-humidified atmosphere on medium containing 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD) and 100 ng/ml each of penicillin and streptomycin (Life Technologies, Inc.).

2.3. Isolation of RNA, RT-PCR and real-time RT-PCR analysis

DNA methylation has a long-standing relationship with gene inactivity and has been implicated in enhanced extent of transcriptional silencing; consistent epigenetic changes in human cancers (Jones and Baylin, 2002). H1355 cells were seeded in low density (1 × 10^4) 16 h before treatment. Cells were treated with beta-propiolactone (BPDE) at a final concentration of 1 and 10 μM for 96 h (Niu et al., 2009). To test the histone acetylation affect specific effect to lung cancer cell lines, we expanded the investigation to assess the effect of TSA. Cells were treated with TSA (1 μM) and harvested 24 h after treatment. DNA damage was assessed by the comet assay.
point. For chromatin isolation, H1355, pcDNA/H1355 and GST-M2/H1355 transfect-
tant cells were grown to 80% prior to exposure to B[a]P 0.2 μM for 48 h. Cells were
harvested in IPB-7 buffer (triethanolamine–HCl, 20 mM; pH 7.8; NaCl, 0.7 M; NP-40,
0.5%; sodium deoxycholate, 0.2%; phenylmethylsulfonyl fluoride, 1 mM; leupeptin,
1 mg/ml; pepstatin, 1 mg/ml; NaF, 1 mM; NaVO₃, 1 mM; trypsin inhibitor 0.1 mg/ml;
ariperon, 1 mg/ml) extraction chromatin protein. The pellet was washed three times
with IPB-7 buffer, homogenized in IPB-7 by sonication and thereafter subjected to
SDS-PAGE and Western blots [Al Rashid et al., 2005; Malmid et al., 2008]. For West-
ern blot assay, equal amounts of chromatin protein from each sample were loaded
onto a 15% SDS-PAGE gel and electrophoresis. Blots were probed with phospho-
histone H2AX (Ser-139) antibody (Upstate Cell Signaling 05636) or total histone 3
(Abcam; ab1791). Equal loading of proteins was confirmed by staining of the blots
with ECL.

2.5. Plasmids, transient transfection and reporter gene assay

The GST-M2 promoter was amplified by PCR with Taq polymerase (Ex taq,
Takara) using genomic DNA from MRC-5, H1355, A549 and H1299 cell
lines (50 ng) as a template, with GST-M2 promoter Kpn I primer 5′-CCTGACCTGGTTAGACGCCTTCTC-3′
and GST-M2 promoter Hind III primer 5′-TTAAGCTTACCCCGCACGCAGCACACG-3′. The PCR reaction included 5 min
denaturation (95°C) followed by 35 cycles, each consisting of denaturation (95°C,
1 min), annealing (55°C, 1 min) and extension (72°C, 2 min) with a final exten-
sion step (10 min). Then, digestion was carried out with Kpnl and Hind III with
cloning upstream of the firefly luciferase reporter in the pGL3-Basic vector (Promega
Corp.). For luciferase assay, H1355 cells (2 × 10⁵) were seeded onto 35-mm wells.
After 18 h, they were transfected with the plasmids described above (0.25 μg/μl)
and β-gal (0.25 μg/μl) with Opti-MEM reduced serum medium (Invitrogen, Cat.
No. 31985). After 20 min incubation, combined diluted DNA and diluted lipofec-
tamine reagent were mixed gently and incubated at room temperature for 30 min.
Then, for each transfection, the diluted complex solution was gently overlaid onto
the rinsed cells. The cells were incubated with the complexes for 5 h at 37°C. The
medium was replaced with fresh, complete medium at 18 h. After carefully removing
from the medium, cells were collected and transcriptional activity was assayed with
luciferase assay system according to the described protocol (Promega). A plasmid
expressing the bacterial β-galactosidase gene was co-transfected in each experi-
ment to serve as internal control of transfection efficiency.

2.6. Statistical analysis

Statistical analysis was performed using the SPSS statistical software program
(version 15.0, SPSS, Inc.). The relative express level of target gene in our interested
variables was also classified as high or low expression groups by their median level
which was calculated by 2^-ΔΔCt. The x² test (two tailed), Mann–Whitney test,
and Wilcoxon signed-rank test were used for nonparametric pair analysis. The x² test
was used to determine the relationships between GST-M2 expression and various
clinical pathologic characteristics including adenoscarcinoma (AD) or squamous cell
carcinoma (SCC), grade of cell differentiation (well, moderate or poor differentia-
tion).

3. Results

3.1. Downregulation of GST-M2 mRNA in lung cancer cells

To identify the mRNA expression of GSTs, we analyzed the expression of six GST molecules including GST-M1, GST-M2, GST-
M4, GST-M5, GST-Alpha and GST-Pi. The mRNA expression profile
analysis was carried out for nine lung cancer cell lines and three normal
lung cell lines using RT-PCR. In Fig. 1A, among the 12 screened cell lines, GST-Alpha and GST-M5 were not detectable in
any lung cells. GST-Pi was abundantly expressed in all cell lines,
except in H1355 cells. GST-M1 was only expressed in some of the
lung cell lines (Fig. 1A). Interestingly, we find that GST-M2 mRNA
was higher expression in normal lung cell lines (MRC-5, WI-38 and
BEAS-2B) than in lung cancer cell lines. GST-M2 mRNA expression
was confirmed by means of real-time RT-PCR experiments (Fig. 1B).
There was a more than seven-fold difference in the expression of
GST-M2 transcript in normal cells and lung cancer cells. There is
GST-M2 genomic expression in DNA of the lung cell lines. GST-M2
DNA was highly expressed in lung cancer and normal lung cell lines
(data not shown), but its transcript was silenced in lung cancer cell
lines (Fig. 1).

3.2. Association of GST-M2 expression with clinical pathology

We investigated the biological and clinic pathologic significance
of GST-M2 in NSCLCs. We carried out real-time RT-PCR on lung
issue specimens of 50 NSCLC patients that underwent curative
surgical resection. On the basis of the American Society of Anes-
thesiologists Physical Status Classification System, case patients
were found to be equally fit for surgery. GST-M2 was detected
with ABI 7000 specific to TagMan probe. Among the 50 NSCLC
patients examined, the median of GST-M2 mRNA expression was
strongly significant high in adjacent non-malignant lung tissues
(2^-ΔCt expression median levels = 3.73) and weakly significant low
in tumor tissues (2^-ΔCt expression median levels = 1.33); (p = 0.016,
Wilcoxon signed-rank test) (Fig. 2). Our data show that loss of
mRNA expression of GST-M2 in lung tumor tissues was in agree-
ment with the pattern in lung tumor cell lines (Fig. 1A). There were
significant associations between clinical pathological factors and the
median of mRNA expression in GST-M2 (p < 0.05, Wilcoxon
signed-rank test) such as patients age (≥67 years), male gender,
T value 1.2, N₀, poor cell differentiation (Table 2). These results
indicate that the mRNA expression of GST-M2 signature is depen-
dent on these clinical parameters in lung cancer. Then, we classified
cases into GST-M2 high and low expression groups. The cutoff value
for patient stratification was determined by the median of total
mRNA expression of all tumor tissues. For example, a case was clas-
sified as high if the mRNA expression of GST-M2 coefficient was
≥3.73 (n = 25); otherwise, it was classified as low (mRNA expres-
sion levels < 3.73; n = 25). We applied the correlation of the high and
low expressions of GST-M2 to the overall survival times of NSCLC
patients. Most of the variables were not significantly associated
with high expression of GST-M2, except for gender (male), cell dif-

Fig. 1. GST-M2 mRNA expression in lung normal and lung tumor cell lines. (A) Rep-
resentative GST-M1, GST-M2, GST-M4, GST-M5, GST-Alpha and GST-Pi expressions
in lung normal and lung tumor cell lines. Expression was analyzed by RT-PCR of total
RNA (2 μg) amplified by RT-PCR. Equal amounts of DNA were loaded, as confirmed
by the intensity of β-actin after ethidium bromide staining. (B) Real-time PCR quan-
tification of GST-M2 by TaqMan analysis. All values have been normalized to the
level of GAPDH and are the averages of three independent readings.

(A) Lung cell Lung cancer cell

(GST-M2) (GST-M1)

(GST-M4) (GST-M5)

(GST-Alpha) (GST-Pi)

(β-actin)
Table 2
Characteristics of patients with NSCLC in relation to expression of the GST M2 gene.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>GST-M2 median (min–max)</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Adjacent non-malignant lung tissues</td>
<td>Tumor tissues</td>
<td></td>
</tr>
<tr>
<td>Age (year) ≤67</td>
<td>24 (48)</td>
<td>4.81 (0.04–49.77)</td>
<td>4.64 (0.04–79.55)</td>
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<tr>
<td></td>
<td>26 (52)</td>
<td>2.61 (0.30–95.61)</td>
<td>0.95 (0.06–73.48)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37 (74)</td>
<td>2.63 (0.04–49.51)</td>
<td>1.55 (0.04–79.99)</td>
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<tr>
<td>Female</td>
<td>13 (26)</td>
<td>6.52 (0.30–49.77)</td>
<td>0.97 (0.12–71.77)</td>
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<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AD</td>
<td>31 (62)</td>
<td>4.02 (0.04–49.51)</td>
<td>1.11 (0.04–79.55)</td>
</tr>
<tr>
<td>SQ</td>
<td>19 (38)</td>
<td>2.63 (0.55–95.61)</td>
<td>2.36 (0.04–38.97)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>33 (66)</td>
<td>4.02 (0.04–49.61)</td>
<td>1.11 (0.04–79.55)</td>
</tr>
<tr>
<td>II</td>
<td>17 (34)</td>
<td>2.90 (0.42–49.77)</td>
<td>1.55 (0.12–38.97)</td>
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<td>T value</td>
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<tr>
<td>1, 2</td>
<td>40 (80)</td>
<td>3.98 (0.04–49.61)</td>
<td>1.74 (0.04–79.55)</td>
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<td>3</td>
<td>10 (20)</td>
<td>2.59 (0.42–49.77)</td>
<td>1.32 (0.12–38.97)</td>
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<tr>
<td>N value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43 (86)</td>
<td>3.95 (0.04–49.61)</td>
<td>1.11 (0.04–79.55)</td>
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<tr>
<td>1</td>
<td>7 (14)</td>
<td>3.50 (1.39–33.14)</td>
<td>2.36 (0.27–9.95)</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well and moderate</td>
<td>26 (52)</td>
<td>3.49 (0.30–95.61)</td>
<td>2.58 (0.06–79.55)</td>
</tr>
<tr>
<td>Poor</td>
<td>24 (48)</td>
<td>4.13 (0.04–49.77)</td>
<td>0.95 (0.04–38.97)</td>
</tr>
</tbody>
</table>

AD, adenocarcinoma; SCC, squamous cell carcinoma. Wilcoxon signed-rank test.

* p < 0.05.

** p < 0.01.

3.3. DNA hypermethylation of GST-M2 promoter correlated with reduced mRNA expression

As shown in Fig. 1A, there is GST-M2 mRNA expression loss in lung cancer cell line. We thought that if different base in promoter region of GST-M2 could affect the expression between high and low GST-M2 expression cells. In order to determine the cause of this GST-M2 mRNA expression loss, we first clarified the occurrence of genomic DNA sequence changes in the promoter region of GST-M2. Analysis of the promoter regions of GST-M2 gene indicated −387 to +244 bp relative to the transcription start site. We identified one base mutation at +128, downstream of the GST-M2 gene transcription start code (Fig. 3A). Lung cancer cell lines (H1355, H1299 and A549) have point mutation T base substitution at +128 (mutant type) in contrast to lung normal cell line that has C base (wild type). To identify the core promoter at different base at +128 bp essential for transcriptional activation, 5′ mutation type of the fragments was prepared and tested by luciferase assay. Transcriptional activity in H1355 cell line with wild type or mutant type exhibited the same activity (Fig. 3B). Moreover, the transcriptional activity increased with more DNA transfection (0.25 and 0.5 μg). Our findings suggest that these sequences change at +128 bp do not affect the activity of the promoter of GST-M2. The point mutation at +128 GCC (Gly) to GGT (Gly) was silent mutation. Therefore, downregulated GST-M2 expression was not due to the mutation in promoter region.

Next, we examined the expression of GST-M2 after treatment with de-methylation reagent 5-Aza in NSCLC cell line. Our data show that GST-M2 mRNA expression is dependent on 5-Aza (10 μM) treatment and is restored in the H1355 cells (Fig. 4A). We confirmed the mean of difference analysis by real-time RT-PCR (**p < 0.01) (Fig. 4B). These data suggest that silence of GST-M2 mRNA expression is associated with promoter DNA hypermethylation. Our data also showed that HDAC inhibitor-TSA at 0.5 μM is not effective in restore the mRNA expression of GST-M2 in lung cancer cell lines. E-cadherin expression was not detected due to DNA hypermethylation and acetylation. Therefore, E-cadherin was shown as positive control for treatment with TSA (Fig. 4A; lane 5). This suggests that histone modifications are not involved in regulating the mRNA expression of GST-M2. As for the model, we also confirmed 5-Aza and 5-Aza (10 μM)/TSA (0.1 or 0.5 μM) treatments in lung cancer cell line analysis by Western blot. There
Fig. 3. Promoter sequences of GST-M2 in MRC-5, H1355, A549 and H1299 cells. (A) The point mutation C to T of GST-M2 promoter located at +128 bp upstream of the GST-M2 transcription start site were identified in H1355, A549 and H1299 lung tumor cell lines. (B) On luciferase activity assay in H1355 cells, the relative measured luciferase levels are depicted on a bar graph, whereby pGL-3 luciferase reporter plasmids containing either C 128 T sequence or wild-type sequence are co-transfected with pGL-3 basic. Each value is the average of at least three independent experiments and the error bars represent the standard deviation.

was no effect on translation of GST-M2 proteins (data not shown). Together, these data suggest that loss mRNA expression of GST-M2 in lung cancer cell lines was through hypermethylation of GST-M2 promoter, but not acetylation of histone, or promoter mutation.

3.4. Alleviation of benzo[a]pyrene-induced DNA damage by GST-M2 in lung cancer cells

To evaluate the protective effect of GST-M2 against B[a]P-induced DNA damage in lung cancer cell line, comet assay and γ-H2AX were carried out. The extent of DNA strands break (measured as tail length, the percentage of migrated DNA and olive tail moment) was determined by scoring cells. In our previously study, we construct the GST-M2 mRNA overexpression in H1355 cell lines (Weng et al., 2005). H1355 cells treated with 0.2 μM B[a]P showed significant increases in DNA strand breaks. When damaged initially, DNA fragments became free to migrate in the electric field toward the anode and the tail length increased (Fig. 5A). There were significant related-time increases in DNA damage at 12, 24 and 48 h. We analyzed the DNA tail moment with 0.2 μM B[a]P at 0, 12, 24 and 48 h in H1355 cells (Fig. 5B black columns), and significant increases were found time-dependent (from 0.6 ± 0.45 μm to 12.1 ± 2.7 μm, 28.3 ± 5.9 μm and 35.4 ± 3.6 μm). Subsequently, in H1355 cells with GST-M2 mRNA overexpression treated with B[a]P there were statistically significant decreases in DNA damage (Fig. 5B hatched columns) at 0, 12, 24 and 48 h, most notably alleviation of DNA strand break (from 1.53 ± 0.21 μm to 4.0 ± 0.5 μm, 5.6 ± 0.6 μm and 14.2 ± 1.39 μm) (a, compare with pcDNA/H1355, p < 0.01, Student’s t-test). We therefore examined DNA fragmentation after pre-treatment with ANS at 200 μM, a high binding capacity for non-substrate compound. The GST-M2 activity could be inhibited by ANS in vitro and in vivo (Mosebi et al., 2003). This caused a significant increase in DNA damage at 12, 24 and 48 h. (Fig. 5B rhombus columns) (from 12.1 ± 0.21 μm to 18.8 ± 1.8 μm and 25.6 ± 1.0 μm) (b, compare with GST-M2 transfectants/H1355, p < 0.05, Student’s t-test). These results indicated that B[a]P-induced DNA strand breakage is time-dependent in lung cancer cells.

Upon DNA double-strand break (DSB) induction in mammals, H2AX become rapidly phosphorylated at serine 139 and termed to form γ-H2AX that is easily identified and serves as a sensitive indicator of DNA DSB formation (Dickey et al., 2009). Therefore, induction of γ-H2AX is found following exposure of cells to suspected DNA-damaging compounds such as cigarette smoke (Albino et al., 2004), polycyclic aromatic compounds, dinitrobenzo[e]pyrene (Mattsson et al., 2009). We detected γ-H2AX chromatin protein on Western blotting at sites flanking DSBs. GST-M2 overexpression in H1355 cells significantly alleviates DNA double-strand breaks (Fig. 6). These results indicate that GST-M2 overexpression can be significantly effective in protecting against B[a]P-induced DNA damage in lung cancer cells.
4. Discussion

Our findings suggest that the significant loss GST-M2 mRNA expression was in lung cancer cell lines, not in lung normal cell lines (Fig. 1A). GST-M2 mRNA expression undergoes de-methylation and restore after exposure to de-methylation agent 5-Aza-2'-deoxycytidine in lung cancer cells (Fig. 4A). Loss of GST-M2 in lung cancer cells causes DNA damage upon exposure to B[a]P (Figs. 5 and 6). Therefore, the data support our hypothesis that the hypermethylation leading to silencing GST-M2 expression in lung cancer cells and become more sensitive to long-term exposure to carcinogenic agents, such as B[a]P and PAH.

In general, GST-M2 mRNA expression was higher in lung normal cell lines (MRC-5, WI-38 and BEAS-2B) than lung cancer cell lines using RT-PCR. It looks like that GST-M2 expression was higher in lung fibroblasts than in epithelial cells (Fig. 1). The data suggested that GST-M2 expression might be higher in stroma or normal
parts than in epithelial cells or tumor parts. Using real-time RT-PCR, we found a significant association for GST-M2 expression between the tumors and adjacent non-malignant lung tissues (p = 0.016) in patients with NSCLCs. There are high levels of GST-M2 in adjacent non-malignant lung tissues but low or absent levels of GST-M2 in tumor tissues (Fig. 2). Aberrant DNA hypermethylation of gene promoter regions is an epigenetic mechanism leading to silencing of tumor suppressor genes in many human cancers (Peng et al., 2009). Downregulation of GST-M2 is mediated through aberrant hypermethylation in lung cancer cell lines (Fig. 4). We consider that there have a higher proportion of GST-M2 hypermethylation in NSCLC patients. We observed that B[a]P-induced DNA strand breakage is time-dependent in H1355 cells and the effect is most severe at 24 h (Fig. 5). These findings are in agreement with previous reports which measured DNA breaks in alveolar macrophages, lung cells, peripheral lymphocytes of Sprague-Dawley rats, exposed to a single dose of B[a]P by endotracheal administration (Garry et al., 2003). H2AX is a key regulator of the DNA damage response, because H2AX plays an essential role in the recruitment and accumulation of DNA repair proteins to sites of DS damage (like replication fork collapse) (Furuta et al., 2003), and leads to the DNA repair response start. Those proteins include 53BP1, MDC1, RAD51, BRCA1, and the MRE11/RAD50/NBS1 (Dickey et al., 2009). The chromatin remodeling complex TOP3α-UBC13, which also participates in DNA repair, is recruited to the DS site by γ-H2AX (Ikura et al., 2007). In our hypothesis that the hypermethylation leading to silencing GST-M2 expression in lung cancer cells became more sensitive to long-term exposure to carcinogenic agents, such as B[a]P and PAH. Thus, GST-M2 prevented BPDE-DNA adducts and γ-H2AX formation did not enhance DNA repair capability for BPDE-DNA adducts. Thus, hypermethylation of tumor suppressor genes forms the basis for development of biomarkers for early detection of lung cancer (Sidransky, 2002). One might conceptualize that biomarkers show high levels of methylation in tumor but very low methylation in adjacent non-malignant lung tissues (Shivapurkar et al., 2007). We are confident that the hypothesis of GST-M2 hypermethylation in NSCLC tissues is supported.

Most studied of the GSTs is GST-M1, which is only expressed in 50% of the population (Seidegard et al., 1988). Similarly, the expression of GST-M1 was inconsistent in our study (Fig. 1A). A study indicated that the proportion of the GST-M1 gene deletion genotype significantly increased in lung cancer patients in Russia (81%). Next, we assessed expression by quantitative real-time PCR in 50 patients. GST-M1 was the least expressed within adjacent non-malignant parts or lung tumor tissues (data not shown). Another study indicated that on immunostaining GST-M1 expression went from being absent to being weak in the bronchial cells of human lung tissues (Anttila et al., 1993). Although the GST-M1 and GST-M2 genes are closely related, sharing 97% of their nucleotides (Konig-Gregor et al., 2004), GST-Mu class is highly diverse.

In conclusion, our data demonstrated a loss of expression of GST-M2 in tumor part of NSCLCs and hypermethylation mediated GST-M2 gene silence. This study provides new insight into the molecular mechanism of GST-M2 expression. Moreover, delineating the alleviation of DNA damage induced by B[a]P manifests a major detoxification role for GST-M2. Thus, quantitative assessment of GST-M2 for levels of methylation within its promoter sequences in NSCLCs appears to be a promising marker in our future study.

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Robertson, I.G., Guttenberg, C., Mannervik, B., Jernstrom, B., 1986. Differences in stereoselectivity and catalytic efficiency of three human glutathione transferases in the conjugation of glutathione with 7 beta,8 alpha-dihydroxy-9 alpha,10 alpha-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. Cancer Res. 46, 2220–2224.


