Oncogenes and Tumor Suppressor

Glutathione S-Transferase Mu2 Suppresses Cancer Cell Metastasis in Non–Small Cell Lung Cancer

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Abstract

Glutathione S-transferase mu2 (GST-M2) is a phase II detoxification enzyme. Low expression of GST-M2 in lung cancers is due to hypermethylation of its promoter. Lung cancer with the GST mu-null genotype is associated with shorter survival. However, a correlation between GST-M2 and important clinical parameters, as well as the migration of GST-M2–defective cells in lung cancer, has not been established. In the present study, we investigate the role of GST-M2 in cell migration and actin disassembly in lung cancer cells. GST-M2 and CCN2 mRNA levels were significantly reduced in non–small cell lung cancer (NSCLC) tumors when compared with matched normal lung tissues in 82 patients with NSCLC. We found that high expressions of both GST-M2 and CCN2 are correlated with favorable survival of patients with lung cancer when compared with similar patients without GST-M2 or CCN2 expression. GST-M2 can induce CCN2 expression by driving the CCN2 proximal promoter. Overexpression of GST-M2 decreases the formation of filopodia, resulting in remodeling of the reorganized cytoskeletons. Overexpression of GST-M2 significantly suppressed cancer cell migration on wound-healing assay. In addition, overexpression of GST-M2 dramatically reduced tumor growth and metastasis in a xenograft mouse model. These data highlight the potential of GST-M2 as a novel tumor suppressor. GST-M2 increases the expression of CCN2 in lung cancer cells, which inhibits cancer cell migration in lung cancer and animal models. *Mol Cancer Res; 11(5); 518–29. ©2013 AACR.*

Introduction

Glutathione S-transferases (GST), a multifunctional family of phase II detoxification enzymes with expression and catalytic activity, have been used as effect modifiers in relationship to environmental exposures (1, 2). GSTs can be divided into 4 human families (isoenzymes), with different but sometimes overlapping substrate specificities. They are termed A (alpha), M (mu), P (pi), and T (theta; ref. 3). Within GST-M, 5 isoforms (denominated: M1–M5) have been identified (4, 5). GST-M has been found to be the most effective in the detoxification following exposure to epoxides such as benzo(a)pyrene and DNA hydroperoxides (6).

GSTs are involved in many important cellular processes such as stress responses, proliferation, apoptosis, oncogenesis, tumor progression, and drug resistance (7–9). Furthermore, an association with the expression of GST-M2 has been identified in the relationship between lung cancer and tobacco exposure (10). These results indicate an important role for GST-M2 activity in the prevention of DNA damage.

In our previous studies, we showed that the expression of GST-M2 in the tumor tissues of patients with early-stage surgical NSCLCs is significantly lower than in paired adjacent nonmalignant tissues (10, 11). The lack of GST-M expression may be linked to susceptibility to cancer. In a recent study of lung cancer, the GST-M1 genotype was found to be associated with lower rates of patient survival (12). The 5-year overall survival rate for patients with NSCLC is a dismal 15% (13) because almost 70% of patients suffer metastasis (14). The correlations among GST-M2, survival rate, and migration in patients with NSCLCs remain unclear.

CCN2 [connective tissue growth factor (CTGF)] is an extracellular matrix (ECM)-associated molecule and a member of the CCN family, which also includes CCN1 [cysteine-rich 61(Cyr61)], CCN3 [nephroblastoma overexpressed (Nov)], and CCN4 [Wisp-1/elm1 and Wisp-2/rCop1]. CCN2 is implicated in many cellular events including...
angiogenesis, skeletogenesis, and wound healing (15–18). CCN2 expression is associated with tumor development and the progression or poor prognosis of esophageal cancer (19). However, some studies have shown that the overexpression of CCN2 suppresses the ability of lung adenocarcinoma cells to carry out in vitro Matrigel invasion, as well as inhibits lung cancer cell proliferation, leading to tumor suppression in NSCLCs (20, 21). Associations have been reported between an increase in CCN2 and better survival rates in patients with esophageal squamous cell carcinoma and chondrosarcoma (22) as well as with decreased metastasis in a colon cancer mouse model (23). In contrast, CCN2 mediates functions such as proliferation, migration, differentiation, and survival, leading to enhanced angiogenesis in endothelial cells (24). The role of CCN2 in different types of cancer is believed to vary considerably, depending on the type of tissue involved.

Previous studies of fibroblasts have shown that CCN2 is induced by TGF-β (25). Regulators such as VEGF, S1P, and hypoxia-inducible factor (HIF)-1α have been reported to induce CCN2 production in different cells (26–28). CCN2 is also upregulated by other fibrogenic cytokines including angiotension II, endothelin 1, and thrombin (29–31). On the other hand, epigenetic silencing by hypermethylation of the CCN2 promoter leads to a loss of CCN2 function, which may be a causative factor in the carcinogenesis of ovarian cancer (32).

Cumulative evidence has established the importance of GSTs as determinants of therapeutic response and survival in patients with cancer (33–35). The aim of the present study is to investigate whether GST-M2 participates in the cell migration process in NSCLCs, by evaluating the CCN2 pathway, a key signaling system that controls migratory responses in lung cancer.

Materials and Methods

Study subjects and tissue collection

The protocol of this study was approved by the Institutional Review Board committee of Taichung Veterans General Hospital (Taichung, Taiwan). Confidentiality issues were explained to the participants and informed consent was obtained. From January 2003 to August 2006, samples were collected, including lung tumor and adjacent normal tissues from 82 stage I/II NSCLC cases undergoing surgical resection. Tumor size, lymph node metastasis, and tumor staging were determined according to the American Joint Committee on Cancer (AJCC; 7th edition) criteria (36). Tumor samples were acquired from the solid part of the tumor. The tissues were stored at −70°C. None of the patients received preoperative chemotherapy or radiotherapy. The overall survival time was calculated from the date of surgery to the time of the last visit or the time of death.

Cell culture and drug treatments

The cell lines used in this study were obtained from the American Type Culture Collection and grown as previously described. Lung cancer cell lines A549 and H1355 were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) and RPMI, respectively. CL1-0 and its sublines and CL1-5 (human lung carcinoma cell lines with either low or high invasive and metastatic capabilities) were obtained from Dr. Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan; ref. 21). Cells were either left untreated or were treated with 8-anilino-1-naphthalene sulfonate (ANS, Sigma Chemical Co.), a nonsubstrate compound with a high GST-binding capacity, at a final concentration of 200 μmol/L, for 0, 6, and 24 hours. Cell lines were maintained at 37°C in a 5% CO2 humidified atmosphere in medium containing 10% FBS (Life Technologies, Inc.) and 100 ng/mL each of penicillin and streptomycin (Life Technologies, Inc.).

RNA interference transfection expression

Lentiviral infection of cell lines was used to stably integrate and express short hairpin RNA (shRNA), which targeted the GST-M2 and CCN2 messenger RNA (mRNA) sequences. Individual clones were identified by the unique number assigned by the RNAi Consortium (TRCN), as follows: TRCN00000072246 was targeted to luciferase (responding sequence: AAAATCAGAATCGGTGATAT) and TRCN0000154400 (responding sequence: GCCAATTCGGAAGACACATTT) and TRCN00000154399 (responding sequence: CCTCGTTCCTTCTCCTGTGTT) were for shGST-M2#48 and shGST-M2#399, respectively, and targeted to GST-M2. TRCN0000061948 (responding sequence: GAGGAATACACAGAAGGGC; responding sequence: CCAATTTGATCGGTGACTA) were for shCCN2#48 and shCCN2#50, respectively, and targeted to CCN2. Cells that stably expressed control or target shRNAs were generated as described previously, using a lentiviral system (10). To double silence the expressions of GST-M2 and CCN2 genes in cells, subcultures of cells (on a 6-well plate) were infected with lentivirus [packaged from equal amounts of plasmids (2.5 μg) in 2 shRNAs against GST-M2#400 and CCN2#48] encoding shRNAs to the GST-M2 and CCN2.

Determination of GST activity

GST activity was evaluated with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, following the formation of the conjugate with GST at 340 nm (ε = 9.6/mMcm), according to the methods used by Weng and colleagues (6). GSH content was determined on fluorometric assay according to the methods of Hissin and Hilf (37). Cells were plated onto 6 cm tissue culture dishes at a density of 1 x 10^5 cells per dish and treated with or without ANS. Following harvesting and washing in PBS, cells were pelleted, resuspended in 100 mmol/L potassium phosphate (pH 6.5), and sonicated. The samples were then centrifuged at 12,000 x g for 10 minutes. The supernatants were collected and used to determine GST activity. Enzyme assays were conducted in a final volume of 0.1 mL phosphate buffer (0.1 mol/L, pH 6.5), 1 mmol/L CDNB, 1 mmol/L GSH, and 30 μg supernatant protein. The reaction was initiated by addition of the aromatic substrate. The change in absorbance, caused by the formation of a glutathione conjugate of CDNB, was
recorded at 340 nm. Enzyme activity was expressed as μmol/min·mg protein.

Confocal microscopy
Following seeding onto a coverslide coated with poly-L-lysine and incubation for 24 hours, cells were washed twice with PBS and fixed in 3.7% paraformaldehyde at room temperature for 15 minutes. After washing an additional 3 times with PBS, cells were permeabilized in 0.05% Triton X-100, pH 7.4, containing 0.05% saponin (Sigma) on ice for 10 minutes. They were then stained with anti-F-actin–conjugated Texas Red phalloidin 1:70 dilution (Invitrogen, T7471), followed by the secondary antibody, and incubated with rhodamine-conjugated phalloidin at room temperature for 30 minutes. Following another wash with PBS, the cells were examined under a confocal 410 Invert Laser Scan Microscope (Carl Zeiss).

Xenograft tumor model
Five-week-old female BALB/AnN.Cg-Foxn1nu/GtNarl nude mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan, R.O.C.). For in vivo tumor growth assays, 6-week-old male immunodeficient nude mice (BALB/c nu/nu mice) weighing 18 to 22 g were used. Animal use was reviewed and approved by the Chung Shan Medical University Experimental Animal Center and Use Committee. Cells were transfected with plasmids (pcDNA) or GST-M2 was injected into the tail vein of the mice (n = 5/group). The mice were housed under pathogen-free conditions, with a 12-hour light/dark cycle, and fed an autoclaved diet with rodent chow.

The mice were inoculated with 1 × 106 viable cells in 100 μL of PBS, via tail vein injection. After 6 weeks, autopsies were conducted. Organs were fixed with 10% formalin in 0.01 mol/L phosphate buffer (pH 7.2) and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) stain for histopathologic examination by light microscopy and metastatic nodules were counted. The body weights of the groups were compared daily following injection of transfected cells into the mice.

Cell-cycle analysis
The cell-cycle distribution was analyzed by flow cytometry. The complete protocol has been described in a previous study (38).

Statistical analysis
All analyses were conducted using SPSS version 18.0 software (SPSS Inc.). The differences in the characteristics of the 2 groups were analyzed using the Student t test for continuous variables or Fisher exact test for categorical variables. This was done for scale variables (expressed as median ± SD) using the Mann–Whitney test and for nominal variables using Fisher exact test. Analysis of the survival data was conducted using the Kaplan–Meier method. P values were 2-sided, and the significance level was 0.05.

Additional methods are detailed in the Supplementary Information online.

Results
Relationships among GST-M2 and CCN2 mRNA expressions and clinical characteristics in NSCLC patients
The expression patterns of GST-M2 and CCN2 genes in NSCLCs were studied by quantifying the levels of GST-M2 and CCN2 mRNA in 82 tumors and their matched normal lung tissues using real-time PCR. The median GST-M2 mRNA expression was significantly higher in adjacent nonmalignant lung tissue than in matched NSCLC tumor tissue (2−ΔCt expression median levels = 23.68 and 14.08, respectively; P = 0.02). CCN2 mRNA was also higher in adjacent nonmalignant lung tissue than in tumor tissue (2−ΔCt expression median levels = 36.1 and 9.87, respectively; P < 0.01; Fig. 1). Correlation analysis showed that expression of GST-M2 and expression of CCN2 are positively correlated (P = 0.047). These results suggested that GST-M2 expression in adjacent normal lung tissues is positively associated with CCN2 expression.

The expressions of both GST-M2 and CCN2 and their influence on the clinical characteristics of patients with NSCLCs were examined using univariate analysis. Patients were divided into 4 groups: high GST-M2 expression with high CCN2 expression (HGST-M2 and HCCN2); high GST-M2 expression with low CCN2 expression (HGST-M2 and LCCN2); low GST-M2 expression with high CCN2 expression (LGST-M2 and HCCN2); and low GST-M2 expression with low CCN2 expression (LGST-M2 and LCCN2). After a 6-year follow-up, Kaplan–Meier analysis showed that the HGST-M2 and HGCCN2 group had a better median survival rate than the HGST-M2 and LCCN2 group (1,060 vs. 707.5 days; 84% vs. 60.0%; P = 0.03; Table 1). In addition, each
Multivariate analysis was conducted after adjusting for GST-
M2 and CCN2 mRNA expressions, gender, tumor stage, and cell differentiation. GST-M2 and CCN2 mRNA expressions, gender, tumor stage, and cell differentiation were significant independent prognostic factors (Table 2; HR = 0.246; P = 0.02, 95% confidence interval (CI), 0.07–0.83 for the H<sub>GST-M2</sub> and H<sub>CCN2</sub> group; HR = 3.667; P = 0.02, 95% CI, 1.20–11.23 for male; HR = 3.148; P = 0.01, 95% CI, 1.31–7.56 for stage II, III; HR = 2.415; P = 0.05, 95% CI, 1.02–5.72 for poor cell differentiation). In particular, patients with high GST-M2 and CCN2 expressions had significantly lower HRs than patients with low GST-M2 and CCN2 expressions. Taken together, these results suggested that high expressions of GST-M2 and CCN2 lead to a better overall survival rate. In addition, high expressions of GST-M2 and CCN2, early stage, female gender, and good cell differentiation are significant favorable prognostic factors in NSCLCs.

**Double silencing of GST-M2 and CCN2 expressions promotes lung cancer cell migration**

We evaluated several shRNAs spanning the DNA sequences of GST-M2 and CCN2 to investigate the migration capacity of these genes in lung cancer cells. The mRNA expressions in shGST-M2#400/CL1-0 (left), shCCN2#48/CL1-0 (middle), and double silenced shGST-M2#400 with shCCN2#48/CL1-0 cells (right) were remarkably low. Gene expression was confirmed by real-time PCR (Fig. 2A).

The shGST-M2#400/CL1-0 and shCCN2#48/CL1-0 cells exhibited 20- (Fig. 2A, black column) and 17-fold (white column) increases in migration capacity, respectively, when compared with shluc/CL1-0 cells, on 18-hour Boyden chamber assay (P < 0.05). Interestingly, the migration capacity following silencing of both GST-M2 and CCN2 decreased significantly.

### Table 1. Univariate analysis of influences of clinical characteristics on overall survival duration of patients with NSCLCs

<table>
<thead>
<tr>
<th>Prognostic Variable</th>
<th>Number</th>
<th>Median survival, d&lt;sup&gt;a&lt;/sup&gt;</th>
<th>All survival, %</th>
<th>Log-rank (P)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-M2 and CCN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L&amp;L</td>
<td>25</td>
<td>707.5</td>
<td>60.0</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>H&amp;L</td>
<td>16</td>
<td>730</td>
<td>51.3</td>
<td>0.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>L&amp;H</td>
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<td>656</td>
<td>81.3</td>
<td>0.03&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
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<td>1060</td>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>29</td>
<td>1411</td>
<td>86.2</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>53</td>
<td>669</td>
<td>62.3</td>
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<td>Age&lt;sup&gt;c&lt;/sup&gt;, y</td>
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<td>≥66.5</td>
<td>41</td>
<td>707.5</td>
<td>73.2</td>
<td>0.63</td>
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<tr>
<td>&lt;66.5</td>
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<td>700</td>
<td>68.3</td>
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<td>730</td>
<td>75.9</td>
<td>0.24</td>
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<td>SCC</td>
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<td>696</td>
<td>60.7</td>
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<td>Stage</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>51</td>
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<td>80.4</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>II, III</td>
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<td>T1</td>
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<td>&gt;1179.5</td>
<td>100</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>T2</td>
<td>59</td>
<td>799.5</td>
<td>72.9</td>
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<td>T3</td>
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<td>50.0</td>
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<td>0.13</td>
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<tr>
<td>N2</td>
<td>6</td>
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<td>33.3</td>
<td></td>
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<tr>
<td>Cell differentiation&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Poor</td>
<td>34</td>
<td>698</td>
<td>58.8</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>No smoking</td>
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<td>730</td>
<td>78.9</td>
<td>0.14</td>
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<tr>
<td>Smoking</td>
<td>44</td>
<td>696</td>
<td>63.6</td>
<td></td>
</tr>
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</table>

Abbreviations: AD, adenocarcinoma; SCC, squamous-cell carcinoma.

<sup>a</sup>Analysis of the survival data was by the Kaplan–Meier method.

<sup>b</sup>The median age of 82 patients is 66.5 years.

<sup>c</sup>The log-rank (P) is compared with low GST-M2 and low CCN2. P values were 2-sided, and the significant level was .05. The all survival time was calculated from the date of surgery to the time of the last visit or death.

<sup>d</sup>Cell differentiation was determined at pathologic examination.

Clinical characteristic, such as female gender, tumor stage I, and good and moderate cell differentiation, significantly influenced the prognosis (Table 1; P = 0.02 for female/male; P = 0.01 for tumor stage I/stage II and III; P = 0.04 for good and moderate cell differentiation/poor differentiation).

### Table 2. Cox regression analysis of various potential prognostic factors in patients with NSCLCs

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR</th>
<th>Unfavorable/favorable</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-M2</td>
<td>0.246</td>
<td>H &amp; H/L &amp; L</td>
<td>0.02</td>
<td>0.07–0.83</td>
</tr>
<tr>
<td>and CCN2</td>
<td>1.158</td>
<td>H &amp; L/L &amp; L</td>
<td>0.77</td>
<td>0.44–3.08</td>
</tr>
<tr>
<td></td>
<td>0.746</td>
<td>L &amp; H/L &amp; L</td>
<td>0.66</td>
<td>0.20–2.76</td>
</tr>
<tr>
<td>Gender</td>
<td>3.667</td>
<td>Male/female</td>
<td>0.02</td>
<td>1.20–11.23</td>
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<tr>
<td>Stage</td>
<td>3.148</td>
<td>II, III/I</td>
<td>0.01</td>
<td>1.31–7.56</td>
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<tr>
<td>Cell</td>
<td>2.415</td>
<td>Poor/well and moderate</td>
<td>0.05</td>
<td>1.02–5.72</td>
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</table>

<sup>a</sup>Statistical significance (P < 0.05). Protective genes were defined as those associated with an HR for death of less than 1.
Figure 2. Double silencing of GST-M2 and CCN2 expressions promotes lung cancer cell migration capability. A, total mRNA levels of GST-M2 and CCN2 were detected by real-time PCR (RT-PCR). Using a lentiviral delivery system, shGST-M2 (RNAi Consortium numbers: 400 and 399, left) and shCCN2 (RNAi Consortium numbers: 48 and 50, middle) were stably expressed in CL1-0 cells. shLuc indicates negative control in luciferase. CL1-0 was cotransfected with 2 stable clone lentivirus plasmids (shGST-M2#400 and shCCN2#48) and further established by selection with puromycin (right). Relative levels of GST-M2 and CCN2 mRNA were quantitated by RT-PCR (bottom). B, the migration capacities of these transfectants were investigated by scraping to generate wounds. Cells that migrated toward the wounded area were photographed every hour (right). C, effects of exogenously added recombinant human connective tissue growth factor (r-hCCN2) on silenced GST-M2 of CL1-0 cells. Cells were cultured in serum-free DMEM in the presence of r-hCCN2 (50, 100 ng). Untreated cells were used as controls. Each column indicates the number of migrating cells after 18, 24, and 38 hours of culture. *, P < 0.05; **, P < 0.01. The results were compared using the Student t-test.
Figure 3. Overexpression of GST-M2 increases the expression of CCN2 in lung cancer cells. A, total mRNA levels of GST-M1 and GST-M2 detected by RT-PCR. Relative levels of GST-M1 (black column) and GST-M2 (white column) mRNA were quantitated by RT-PCR (right). B, GST-M2–transfected lung cancer cells (A549 and H1355) were analyzed on Western blotting. C, effect of GST-M2 on cell migration by wound-healing assay in A549 cells (left) and H1355 cells (right). The migration capabilities of these transfectants were studied by scraping of a culture insert to generate wounds. Photographs were taken at the wound sites. Results were obtained from 3 separate experiments. *, P < 0.05; **, P < 0.01.
showed a dramatic increase to 22-fold that of shluc/CL1-0 cells (Fig. 2B, gray column; \( P < 0.01 \)).

The role of CCN2 without GST-M2 was investigated by adding r-hCCN2 protein (50 and 100 ng) to silence GST-M2/A549/C6 cells. Treatment with the r-hCCN2 protein (50 ng) significantly reduced cell migration at 18 hours (Fig. 2C, black column, migrating cell number decreased from 276 ± 6 to 138 ± 7) but had no effect on cell proliferation. To ensure that these results were not due to alterations in cell growth rate, cell proliferation was determined on Trypan blue exclusion assay. The growth rates of these cells were the same, which suggests that the altered migration capacity of the silenced cells cannot be attributed to changes in their capacity for proliferation (data not shown). Collectively, these results suggested that the loss of GST-M2 and CCN2 promotes the migration of lung cancer cell lines. In addition, the silencing of both GST-M2 and CCN2 significantly increases the migration capacity of lung cancer cells.

**GST-M2 overexpression decreases in vitro lung cancer cell migration**

To confirm whether ectopic GST-M1 or GST-M2 expression reduces cancer cell migration, a lung cancer cell line was created by transfection with or without GST-M1 or GST-M2. GST-M1 and M2 mRNA expressions were confirmed by PCR experiments (Fig. 3A, left) and real-time PCR (Fig. 3A, right). Western blot analysis showed that GST-M2/A549 and GST-M2/H1355 cells express notably higher protein levels of GST-M2 (Fig. 3B). The effects of overexpression of GST-M2 transfectants and pcDNA controls in lung cancer cells were analyzed with respect to migration using a wound-healing assay. Interestingly, migration was greatly reduced in A549 and H1355 cells that overexpress GST-M2 when compared with cells that overexpress pcDNA and GST-M1. However, GST-M1 overexpression did not inhibit cell migration, as shown in Fig. 3C. To rule out the effect of different proliferation rates due to GST-M2 in the migration of lung cancer cells, the growth rates of GST-M2/A549 cells were compared with those of a pcDNA control using a Trypan blue exclusion assay and were found to be the same (data not shown). The cell-cycle distributions were also the same for GST-M2/A549 cells and the pcDNA control. Furthermore, no significant differences in cell-cycle status were found among the lung cancer cells. These data strongly suggested that the inhibition of migration of GST-M2 transfectants is not due to different growth rates but due to the effects of GST-M2 overexpression.

**GST-M2 activity is necessary for the reduction of lung cancer cell migration**

The potential involvement of GST-M2 in the reduction of migration of lung cancer cells was determined by treating cells with or without ANS (200 μmol/L), a GST-M2 inhibitor, followed by Boyden chamber assay. Among GST-M2/A549 cells, there was an 11-fold decrease in the number of migrating cells when compared with the pcDNA/A549 cells (Fig. 4A, control, black columns). Interestingly, after ANS treatment, significant time-related increases were noted in migration capacity of the GST-M2/A549 cells, when compared with the control cells (6 hours; \( P < 0.05 \); migrating cell number increased from 49 ± 4.2 to 178 ± 7.1, white column; 24 hours; \( P < 0.01 \), migrating cell number increased from 49 ± 4.2 to 420 ± 32.5, gray column; Fig. 4A, left). GST activity in GST-M2/A549 cells decreased continuously over time, in response to ANS treatment, with maximal inhibition of 31.8% at 24 hours (from 154.4 to 105.7 μmol/min-mg; Fig. 4B). Taken together, these results suggested that GST-M2 regulates cell migration via a mechanism that is dependent on GST-M2 and its intrinsic glutathione transferase m2 activity.

**Overexpression of GST-M2 increases the transcription of CCN2 in lung cancer cells**

We previously designed a series of primers for lung cancer according to metastasis pathway in the GeneChip (39, 40). CCN2, one of the genes from the list, was considered for the potential relationship of its function to lung cancer metastasis. To determine whether GST-M2 expression is associated with CCN2 expression in lung cancer cells, the GST-M2 and CCN2 mRNA levels in A549 and H1355 cells were examined. Overexpression of GST-M2 significantly increased CCN2 gene expression in lung cancer cells (6.23- ± 1.72-fold compared with pcDNA/A549; 4.62- ± 0.68-fold compared with pcDNA/H1355; Fig. 4C). CCN2 was detected in GST-M2–overexpressing cells but was barely detectable in pcDNA control cells. CCN1 and CCN5 mRNA expressions in GST-overexpressing lung cancer cells were also analyzed. However, neither CCN1 nor CCN5 expressions seemed to be associated with GST-M2 in lung cancer cells (data not shown). Only expression of CCN2 was significantly higher in GST-M2–overexpressing lung cancer cells when compared with other members of the CCN family. Furthermore, the decrease in GST-M2 activity after ANS treatment did not affect the GST-M2 gene expression (Fig. 4D, left) but caused a significant decrease in the CCN2 gene expression (Fig. 4D, right). Taken together, GST-M2 most likely stimulates the production of CCN2.

**GST-M2 reduces actin cytoskeleton rearrangement and cell elongation in lung cancer cells**

The effect of GST-M2 on cancer cell migration was investigated by staining F-actin in transfected cells to determine the association with actin filament arrangement (F-actin, G-actin). Morphologically, pcDNA/A549 cells showed a marked increase in the number of filopodia bundle structures (Fig. 5A, enlargement, arrow). These extensions each had a thick root and were branched at their distal parts. Unlike pcDNA/A549, the GST-M2–overexpressing cells had a rounded morphology and few filopodia (Fig. 5A, bottom). These data suggested that GST-M2 reduces migration capacity by a reduction in the formation of filopodia. GST-M2 transfected into cells spread in the cytosol and showed nuclear localization (Fig. 5B).
CCN2 promoter luciferase activities of GST-M2 expression were compared in stably and transiently transfected cells. The overexpression of GST-M2 on both stable (Fig. 5C, top) and transient (Fig. 5C, bottom) transfection assays resulted in a substantial induction of activity of the CCN2 proximal promoter luciferase in A549 and H1355 cells. CCN2 promoter activity was significantly higher in GST-M2–overexpressing cells than in pcDNA control cells. Overexpression of GST-M2 seemed to lead to overspreading of the cells and excessive focal complexes. GST-M2 may be a regulator of CCN2 and a significant driver of the CCN2 proximal promoter in several lung cancer cell types.

**GST-M2 suppresses in vivo tumor growth and metastasis**

The in vivo effects of GST-M2 expression on tumor growth and metastasis were examined by intravenous injection of GST-M2–overexpressing or pcDNA/A549 cells into BALB/c mice via the tail vein. Large tumors formed and tumor cells significantly invaded pulmonary tissues in the pcDNA groups (Fig. 5D, left). In contrast, mice injected with GST-M2/A549 cells had relatively fewer human lung tumors (Fig. 5D, right).
In the pcDNA/A549 group, 122 ± 16 tumor colonies formed per lung, compared with 3 ± 0.2 in the GST-M2/A549 group (Fig. 5D, bottom). Moreover, no distal metastasis was macroscopically visible in these mice. There were no appreciable differences in body weight between the 2 groups. These data indicated that GST-M2 overexpression significantly suppresses tumor growth in vivo.

Discussion
Tumor metastasis is the main cause of cancer-related death. Identification of the genes that regulate cell migration and metastasis is vital to the understanding of this process. This study showed that GST-M2 has great potential as a tumor suppressor. This finding is supported by the following evidence: (i) strong expressions of GST-M2 and CCN2 are significantly associated with a favorable prognosis in NSCLCs; (ii) GST-M2 overexpression in lung cancer cells is associated with the induced expression of migration-inhibiting gene such as CCN2 in lung tumor; (iii) GST-M2–mediated decreases in the expression of F-actin are accompanied by the development of motile structures such as filopodia and stress fibers; (iv) GST-M2 may be a regulator of the CCN2 promoter and is a...
suppressor of tumor cell migration; and (v) GST-M2 not only suppresses the in vitro migration of lung cancer cells but also strongly inhibits tumor metastasis in an animal model. The evidence suggests that GST-M2 reduces the formation of motile actin cytoskeleton structures and that it is a very potent tumor suppressor in lung cancer.

GST-M has several variants and homologues. This study identified an antibody against GST-M1 that also recognizes GST-M2, due to the highly conserved amino acid motif in these 2 isoforms (Fig. 3B). However, these 2 isoforms of GST inhibited the migration of lung cancer cells to differing degrees. Moreover, variations in the levels of different GSTs may be associated with resistance or susceptibility to chemotherapeutic agents in different cancers (41). Our previous study showed that the mRNA expression of GST-M2 is higher in nonmalignant lung tissues than in tumor tissues and that higher expression of GST-M2 with CCN2 leads to an increase in overall survival (Table 1). Thus, GST-M2 and CCN2 may be potential biomarkers for the outcome of lung cancer.

An established panel of human lung adenocarcinoma cell lines with different invasive activities was used to show that GST-M2 suppresses migration (Fig. 3). A similar recent study indicated that a detoxification phase II acetyltransferase enzyme, N-acetyltransferase 10 (Naa10, also known as ARD1), is also indispensable for the suppression of migration, tumor growth, and metastasis in lung cancer cells (42). This highlights the roles of GST-M2 and ARD1 as conserved and essential functions for these enzymes and as repressors or regulators of tumor cell growth and metastasis.

A reduction in the expression of CCN2 in lung cancer is significantly associated with more advanced tumor stage, lymph node metastasis, and shorter survival (20). This study found that the HR for the HGST-M2 and LC3N2 group (HR, 1.158; 95% CI, 0.435–3.083) was high, when compared with the LGST-M2 and LC3N2 group, but the difference was not statistically significant ($P = 0.769$). The HGST-M2 and LC3N2 group showed no significantly favorable prognostic factors for NSCLC, as shown in Table 2. These data imply that only a high expression of GST-M2 with CCN2 significantly reduces the HR of patients with NSCLC. The result was the same as that shown in Fig. 2. Cells that lost GST-M2 and CCN2 showed significantly increased migration capacity. Furthermore, the migration capacity increased in the absence of GST-M2 but in the presence of CCN2 (shGST-M2/CL-1-0 cells). The addition of the r-hCCN2 protein eliminated the increase in migration capacity (Fig. 2C). These results indicated that excessive amounts of CCN2 inhibit migration capacity. Defects in CCN2 or GST-M2 cells result in increased migration capacity.

The promoter region of CCN2 contains many transcription factor–binding sites, including the specificity protein 1 (Sp–1) and AP-1 response element, as well as STAT, SMAD, and NF-κB recognition elements (43). In our previous report, we showed that Sp–1 induces GST-M2 expression in lung cells but is destroyed by a DNA-hypermethylated epigenetic mechanism (10). Recent studies have proposed that histone deacetylase inhibitors, trichostatin (TSA), and mediated CCN2 regulation are conferred by a short proximal promoter construct that contains 2 Sp–1 sites (44–46). Here, we showed that low expression of GST-M2 with CCN2 leads to poor overall survival (Table 1). However, the relationship between loss of expressions of GST-M2 and CCN2 by an epigenetic mechanism in lung tumor tissues and regulation of Sp–1 binding to GST-M2 and CCN2 in lung cancer cells is still unclear.

CCN2 inhibits metastasis and the invasion of human lung adenocarcinoma via CRMP-1–dependent mechanisms (20), as well as inhibits colorectal cancer through inhibition of the β-catenin/Tcf4/MMP-7 pathway (23). Recent studies have shown that factors such as Ets-1 and Flt1 occupy the CCN2 promoter in fibroblasts (47). β-Catenin and Sox9 have also been shown to be regulated by CCN2 in chondrocytes (48). The present study showed that GST-M2 is a significant driver of the proximal promoter of CCN2 in several lung cancer cell lines, regardless of whether their status is transient or stable. This implies that GST-M2 induces CCN2 or unknown factor(s), which interact with convergent or critical molecules of the signaling pathway.

The current study showed that the loss of GST-M2 and CCN2 expressions in early-stage NSCLCs is associated with a shorter progression-free interval and poorer survival. Neither of these possibilities can be ignored with respect to CCN2 expression in a GST-M2–independent pathway. On the basis of these findings, continuous monitoring of GST-M2 and CCN2 gene expressions will benefit the development of new therapeutic targets for the treatment of NSCLCs. GST-M2 has great potential in cases of lung cancer for the prevention of distant metastases.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.-W. Sung, W.-J. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.-C. Tang, S.-C. Tang
Writing, review, and/or revision of the manuscript: L.-C. Tang, S.-C. Tang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.-L. Ko
Study supervision: C.-P. Hsu, J.-L. Ko

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