Expression of 15-PGDH is downregulated by COX-2 in gastric cancer

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Abstract

Overexpression of cyclooxygenase-2 (COX-2) was frequently detected in gastric cancer and was believed to play a crucial role in gastric carcinogenesis (1–10). COX-2 could inhibit apoptosis of tumor cells and promote proliferation, migration of gastric cancer cells and angiogenesis of gastric cancer. Upregulation of COX-2 might facilitate invasion of gastric cancer and was significantly related to the low survival rate of gastric cancer patients (11–14). COX-2, which can be induced by lipopolysaccharide, inflammatory cytokines, growth factors and some tumor promoters, is the key enzyme to catalyze the synthesis of prostaglandins as the natural tumor therapy to rapidly degrade COX-2-derived prostaglandins, as they are accumulated in gastric cancer with the COX-2 upregulation. 15-Hydroxyprostaglandin dehydrogenase [NAD⁺] (15-PGDH) is the key enzyme in prostaglandin degradation, which mediates the inactivation of prostaglandins by converting the oxidation of 15(S)-hydroxyl group of prostaglandins to the formation of 15-keto metabolites (22). Previous studies on 15-PGDH were mostly in the field of reproductive physiology and inflammation, though 15-PGDH has been studied for some time. What interested us were the studies concerning the relationship between 15-PGDH and tumors in recent years. Recent studies showed that 15-PGDH was often decreased or even absent in some cancers and played a key role as a potential tumor suppressor in the development and progression of some malignant tumors (20,23–30). However, few investigations have been done on the role of 15-PGDH in gastric cancer. In the only two published papers mentioning 15-PGDH expression in gastric cancer, the descriptions were contradictory. By immunohistochemical assay, one of the two studies showed that the 15-PGDH protein expression was absent in 10 of 13 gastric cancers, although robust 15-PGDH immunostaining was present in each patient’s normal gastric mucosa (30). The other study showed that expression of 15-PGDH was not altered in gastric carcinomas (31). Consequently, the ambiguous reports on the 15-PGDH expression in gastric cancer need to be clarified. It is known that COX-2-derived prostaglandins, particularly prostaglandin E2, play an important role in gastric cancer and its level is controlled not only by synthesis but also by degradation (28). Thus, it is also necessary and crucial to determine the relationship between 15-PGDH, the key enzyme in prostaglandin degradation as the natural COX-2 antagonist, and COX-2, the key enzyme to catalyze the synthesis of prostaglandins, in human gastric cancer.

In the present study, using two-dimensional electrophoresis (2-DE), we found that 15-PGDH was one of the significantly upregulated proteins in gastric cancer SGC7901 cells in which the expression of COX-2 was knocked down by RNA interference. Furthermore, the expression of 15-PGDH was decreased when the expression of COX-2 was upregulated in gastric cancer SGC7901 cells. We demonstrated that 15-PGDH is lowly expressed in human gastric cancer and is significantly negatively correlated with COX-2 by immunohistochemical assay. The same results were also found in tissue samples, using western blotting. The low expression of 15-PGDH was found to be also related to the differentiation, tumor, lymph node metastasis (TNM) staging and lymph node metastasis of gastric cancer, using statistical analysis on the results of immunohistochemical studies. All these results of the study indicate that 15-PGDH may be downregulated by COX-2 and act as a tumor suppressor in human gastric cancer. The results presented here will provide clues to the further study of the mechanisms in gastric cancer carcinogenesis and provide a candidate gene for gastric cancer therapy.

Materials and methods

Cell culture

Human gastric adenocarcinoma cell line SGC7901 was obtained from Academy of Military Medical Science (Beijing, China) and preserved in our institute (32). Both cell lines were maintained in RPMI-1640 medium (Gibco Carlsbad, California) and supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and antibiotics in a humidified incubator with a mixture of 5% CO2 and 95% air at 37°C.

Tissue collection

For immunostaining of 15-PGDH and COX-2, paraffin blocks of gastric cancer tissues and adjacent non-tumor tissue specimens from 55 primary gastric cancer patients who received surgical resection in Xijing Hospital were obtained from the Department of Pathology in Xijing Hospital of the Fourth Military Medical University. Paraffin blocks of gastric inflammatory tissues (10 gastritis tissues and 10 gastric ulcer tissues) were also obtained from the Department of...
Fig. 1. Two-dimensional gel analyses and MALDI-TOF MS identification of differential expression proteins from SGC7901 cells transfected with COX-2-siRNA or empty pSilencer vector. (A) Analysis of protein expression level for COX-2 in SGC7901-COX-2/siRNA and SGC7901-pSilencer cells by western blotting.
Pathology in Xijing Hospital. For western blotting, fresh surgical gastric cancer tissues and adjacent non-tumor tissues were obtained from eight patients who underwent surgery in Xijing Hospital. All gastric cancer cases were clinically and pathologically proved. The samples were snap frozen in liquid nitrogen and stored at –80°C until analysis. The study was approved by The Human Research Committee of University and performed with the consent of the patients. Each patient’s age, sex, tumor size, histological type of the neoplasm and TNM staging were obtained from surgical and pathological records.

Small interfering RNA plasmid construction and transfection

Three pairs of hairpin small interfering RNA (siRNA) oligos for COX-2 were designed according to the siRNA design Web site (http://www.ambion.com/techlib/misc/siRNA_finder.html). Target sequences were compared with the human genome database in a BLAST search web to ensure that the chosen sequences were not highly homologous to other coding sequences. For oligo-1: sense, 5'-gacgcagcaagggagatgtaatggtagcttcgaacaatacatactactcttggttggaaa-3' and anti-sense, 5'-agctttcctaaacgcagaccgttgagttcgaagaacctgcagatggactgctac-3'; for oligo-2: sense, 5'-gacgcagcaagggagatgtaatggtagcttcgaacaatacatactactcttggttggaaa-3' and anti-sense, 5'-agctttcctaaacgcagaccgttgagttcgaagaacctgcagatggactgctac-3'; and for oligo-3: sense, 5'-gacgcagcaagggagatgtaatggtagcttcgaacaatacatactactcttggttggaaa-3' and anti-sense, 5'-agctttcctaaacgcagaccgttgagttcgaagaacctgcagatggactgctac-3'. For annealing to form DNA duplexes, 0.01 M each of sense and antisense oligos was used. The duplexes were diluted and the ligated with pSilencer3.1 vector (Ambion, Foster City, California) that was previously digested by the BamHI/HindIII restriction enzyme and gel purified at room temperature for 30 min. The products were transformed into DH5α competent cells. Ampicillin-resistant colonies were chosen, identified by restriction digestion and further confirmed by DNA sequencing. SGC7901 cells were planted in six-well plates and cultured in drug-free RPMI-1640 medium for transfection. The expression plasmids of COX-2-siRNA were transfected into SGC7901 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California), following the manufacturer’s instructions. The cells transfected with pSilencer3.1 vector alone served as negative control. Forty-eight hours later, cells were selected in a growth medium containing G418 (Gibco, 300 μg/ml). The stable cell lines were named SGC7901-COX-2-siRNA and SGC7901-pSilencer, respectively. The expression levels of COX-2 in G418-resistant clones were evaluated by western blotting analysis.

2-DE and protein identification by matrix-assisted laser desorption/ionization time of flight mass spectrometry

SGC7901-COX-2-siRNA and SGC7901-pSilencer cells in log-growth phase were harvested and lysed in lysis buffer (9 M urea, 4% wt/vol 3-[3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate, 1% wt/vol dithiothreitol (DTT), 1% am-photolyte, 40 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml apratrin, 0.5 μg/ml leupeptin and 1 mM ethylenediaminetetraacetic acid) added into each sample, along with DNase and RNase (Sigma–Aldrich, St. Louis, Missouri, both 0.4 mg/ml). The sample was mixed and incubated at 4°C for 30 min, and then centrifuged at 12,000 × g for 10 min. The supernatant was then transferred to a fresh tube and stored –80°C until use. The total protein concentration was measured through Bradford protein assay method with bovine serum albumin as standard.

Isoelectric focusing (IEF) was performed using PROTEAN IEF Cell (Bio-Rad Laboratories, Hercules, California) with 17 cm pH 4–7 immobilized pH gradient (IPG) strips (Bio-Rad Laboratories). Four hundred micrograms of protein taken from each sample was dissolved in 400 μl rehydration solution [8 M urea, 4% wt/vol CHAPS, 65 mM DTT, 0.5% vol/vol pH 4–7 IPG buffer (Amersham Biosciences, Uppsala, Sweden) and trace bromophenol blue] and placed at room temperature for 40 min; they were then applied to IPG strips by 8 M urea, 4% wt/vol CHAPS, 65 mM DTT, 0.5% vol/vol pH 4–7 IPG buffer (Amersham Biosciences, Uppsala, Sweden) and trace bromophenol blue] and placed at room temperature for 40 min; they were then applied to IPG strips by electro-focusing (IEF) was started at 250 V for 30 min (linear ramping mode), 1000 V for 1 h (ramp iding mode) and followed by a ligated with pSilencer3.1 vector (Ambion, Foster City, California) that was previously digested by the BamHI/HindIII restriction enzyme and gel purified at room temperature for 30 min. The products were transformed into DH5α competent cells. Ampicillin-resistant colonies were chosen, identified by restriction digestion and further confirmed by DNA sequencing. SGC7901 cells were selected in a growth medium containing G418 (Gibco, 300 μg/ml) and then put in the same solution, with 20% glycerol and 2% DTT] for 15 min, and then put in the same solution, with 2.5% iodoacetamide replacing DTT, for an additional 15 min with constant shaking. After equilibration, the equilibrated strip was transferred to the top of 10% acrylamide gel and fixed with 0.5% low-melting agarose. Electrophoresis was carried out using PROTEAN II Cell (Bio-Rad) with constant current of 25 mA/gel until the bromophenol blue dye reached the bottom of the gel. Then, the gel was stained with silver to visualize the protein spots in the 2-DE gels. Gels were scanned using the Molecular Imager Gel Doc XR System (Bio-Rad) and analyzed using PDQuest system (Bio-Rad), according to the protocols provided by the manufacturer. The following criteria for differential protein expression were used: spot intensity ≥2-fold increased or decreased in SGC7901-COX-2-siRNA comparison with SGC7901-pSilencer.

Spots of interest were excised and destained thrice with 15 mM K3[Fe(CN)6] and 50 mM Na2S2O3. The gel spots were incubated in digestion solution consisted of 12.5 μg/ml proteases grade trypsin, 10% acetonitrile and 25 mM ammonium bicarbonate for 12–14 h at 37°C. Peptides were extracted from gel spots twice with 50% acetonitrile and 0.2% trifluoroacetic acid and dried in centrifugal evaporator for further matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis. Mass spectrometric analysis was performed using the MALDI-TOF Voyager-DE (Applied Biosystems, Foster City, California). Matrix solution was prepared by saturating 2-cyano-4-hydroxycinnamic acid in acetonitrile/water/trifluoroacetic acid (50:50:0.1) and mixing with peptide solution, at a volume ratio of 3 to 1. The peptide mass profiles produced by MALDI-TOF MS were submitted to the database of ProteinPilot (Applied Biosystems, Foster City, California) (http://www.proteome.com/tools/aldente/). UniProtKB/Swiss-PROT or NCBI protein database were used to match the peptide mass fingerprints. All searches were performed using a mass window between 14 000 and 100 000 Da and Homo sapiens taxon. Modifications were allowed for carboxymethylidomethyl (CAML), phosphorylation (P(ST)), phosphorylation (P(Y)) and oxidation (M(OS)). The thresholds for positive identification of proteins were set as follows: shift max 0.2, slope max 200, internal error max 50, minimum number of hits 4 and P value max 1e−5. Other parameters were unchanged and left at the default.

Sense plasmid construction and transfection

Oligonucleotide primers containing EcoR I or Xba I were synthesized, respectively, for amplification of COX-2 coding sequence. The two primers were as follows: 5'-cgagattctctccctccagccgagac-3' (sense) and 5'-gctcatactagccgagagagaagtattcatccctgatc-3' (antisense). The polymerase chain reaction conditions were as follows: 5 min at 95°C for hot start, followed by 33 cycles of 1 min at 94°C, 3 min at 65°C with a final extension of 10 min at 72°C. The polymerase chain reaction product was excised with Xba I and EcoRI and cloned into pcDNA3.1 vector (Invitrogen). The new vector was named pcDNA3.1-COX-2. The insert sequences were confirmed by DNA sequencing. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Briefly, SGC7901 cells were plated and grown to 70–90% confluence without antibiotics. They were then transfected with pcDNA3.1-COX-2. After 48 h of transfection, G418 (Gibco, 300 μg/ml) was added into medium for stable screening. Cells transfected with empty pcDNA3.1 vector were set as negative control. The two stable cell lines were named SGC7901-COX-2 and SGC7901-pcDNA, respectively.

Immunohistochemical and immunocytochemical staining

The avidin–biotin complex immunoperoxidase method was used to examine COX-2 and 15-PGDH expression by immunostaining. Paraffin-embedded 5 μm thick tissue successive sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min and rinsed in PBS. After blocked with 10% normal animal serum for 30 min, the sections were incubated with anti-15-PGDH primary goat polyclonal antibody (Cayman, Ann Arbor, Michigan; 1:100 dilution) or anti-COX-2 primary monoclonal antibody (Cayman; 1:50 dilution) overnight at 4°C, respectively. Sections were washed with PBS, incubated biotin-labeled goat anti-rabbit or anti-mouse IgG (1:2000 dilution) as the secondary antibody for 30 min and incubated with avidin–biotin–peroxidase complex for 1 h. Primary antibodies were detected with Histostain-SP (Zymed Laboratories, San Francisco, California) incubated with avidin–biotin–peroxidase complex for 1 h. Primary antibodies were detected with Histostain-SP (Zymed Laboratories, San Francisco, California) incubated with avidin–biotin–peroxidase complex for 1 h. Primary antibodies were detected with Histostain-SP (Zymed Laboratories, San Francisco, California) incubated with avidin–biotin–peroxidase complex for 1 h. Primary antibodies were detected with Histostain-SP (Zymed Laboratories, San Francisco, California) incubated with avidin–biotin–peroxidase complex for 1 h. Primary antibodies were detected with Histostain-SP (Zymed Laboratories, San Francisco, California).
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California) according to the manufacturer’s instructions. 3’-3’-Diaminobenzenedia (Sigma–Aldrich) was used as the chromogen for the immunoperoxidase reaction. In control sections, non-immune goat IgG or mouse IgG was used to substitute for the primary antibodies.

For cell staining, SGC7901-COX-2/siRNA and SGC7901-pSilencer cells and SGC7901-COX-2 and SGC9101-pDNA3.1 cells were cultured on glass coverslips for 24 h and fixed with 4% paraformaldehyde. The fixed cells were rinsed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The fixed cells were then stained and examined as tissue sections.

All stained sections and cells were evaluated by two independent investigators in a blind manner. The scoring was based on intensity and extensity. The percentage of positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was scored on a scale of 0–4, in which 0 was used for positive staining in ≤1% of the cells, 1 in 1–25%, 2 in 26–50%, 3 in 51–75% and 4 in 76–100%. The intensity of immunostaining was determined as 0 (negative staining), 1 (weakly staining), 2 (moderately staining) and 3 (strongly staining). The immunoreactive score was calculated by sum of these two parameters and the final score of ≥3 was considered to be positive.

Western blotting analysis

To determine the expression of various proteins in various cells and tissues, western blotting analysis was performed. The collected cells from plates were to extract the proteins in lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.8), 0.1% sodium dodecyl sulfate, 2 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, 5 μg/ml aprotinin and 1 μg/ml leupeptin) at 4°C on ice. Tissues were homogenated in the same lysis buffer to extract the proteins at 4°C on ice. The protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes. The membranes were blocked with 10% fat-free milk at room temperature for 2 h and incubated with anti-COX-2 antibody (1:500 dilution), anti-15-PGDH antibody (1:200 dilution) and anti-β-actin antibody (Sigma–Aldrich; 1:2000 dilution) at 4°C overnight. After three washes for 15 min in tris-buffered saline supplemented with 0.1% Tween-20, the membrane was incubated with the horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG as a secondary antibody (Santa Cruz Biotechnology, California; 1:2000 dilution) for 2 h at room temperature, respectively. Enhanced chemiluminescence (ECL kit, Santa Cruz) was used for reaction. In control sections, non-immune goat IgG or mouse IgG was used to substitute for the primary antibodies.

Table I. MALDI-TOF MS identified proteins in SGC7901-COX-2/siRNA and SGC7901-pSilencer

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession numbera</th>
<th>Protein name</th>
<th>Molecular weight (Da)</th>
<th>pl</th>
<th>Expression in SGC7901-COX-2/siRNA</th>
<th>Biological function</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Q13042</td>
<td>Cell division cycle protein 16 homolog</td>
<td>71 655</td>
<td>5.55</td>
<td>Downregulatedb</td>
<td>Regulation of cell cycle</td>
</tr>
<tr>
<td>2</td>
<td>P63261</td>
<td>Actin, cytoplasmic 2</td>
<td>41 661</td>
<td>5.31</td>
<td>Downregulated</td>
<td>Regulation of cell motility</td>
</tr>
<tr>
<td>3</td>
<td>P31947</td>
<td>14-3-3 protein sigma</td>
<td>27 774</td>
<td>4.68</td>
<td>Downregulated</td>
<td>Adaptor protein; p53-regulated inhibitor</td>
</tr>
<tr>
<td>4</td>
<td>P08670</td>
<td>Vimentin</td>
<td>53 520</td>
<td>5.06</td>
<td>Downregulated</td>
<td>Structure protein</td>
</tr>
<tr>
<td>5</td>
<td>P11142</td>
<td>Heat shock</td>
<td>70 898</td>
<td>5.37</td>
<td>Downregulated</td>
<td>Chaperone</td>
</tr>
<tr>
<td>6</td>
<td>P50135</td>
<td>Histamine N-methyltransferase</td>
<td>33 295</td>
<td>5.18</td>
<td>Downregulated</td>
<td>Inactivation of histamine</td>
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<tr>
<td>7</td>
<td>P20337</td>
<td>Ras-related protein Rab-3B</td>
<td>24 757</td>
<td>4.85</td>
<td>Downregulated</td>
<td>Protein transport</td>
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<tr>
<td>8</td>
<td>Q04760</td>
<td>Lactoylglutathione lyase</td>
<td>20 588</td>
<td>5.25</td>
<td>Upregulatedb</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>9</td>
<td>P15428</td>
<td>15-Hydroxyprostaglandin dehydrogenase [NAD+]*</td>
<td>28 977</td>
<td>5.56</td>
<td>Upregulated</td>
<td>Inactivation of prostaglandins</td>
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<tr>
<td>10</td>
<td>Q07021</td>
<td>Complement component 1 Q subcomponent-binding protein precursor</td>
<td>31 362</td>
<td>4.74</td>
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<tr>
<td>11</td>
<td>Q9UK05</td>
<td>Growth differentiation factor 2 precursor</td>
<td>47 320</td>
<td>6.03</td>
<td>Upregulated</td>
<td>Formation of bone</td>
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<tr>
<td>12</td>
<td>P11021</td>
<td>Heat shock 70 kDa protein 5</td>
<td>72 333</td>
<td>5.01</td>
<td>Upregulated</td>
<td>Chaperone</td>
</tr>
<tr>
<td>13</td>
<td>P48739</td>
<td>Phosphatidylserine transfer protein beta isoform</td>
<td>31 408</td>
<td>6.44</td>
<td>Upregulated</td>
<td>Protein transport</td>
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<tr>
<td>14</td>
<td>P38646</td>
<td>Stress-70 protein</td>
<td>68 759</td>
<td>5.44</td>
<td>Upregulated</td>
<td>Regulation of cell proliferation</td>
</tr>
</tbody>
</table>

Statistical analysis

For statistical analysis, the statistical tests were performed using SPSS version 11.0 software package (SPSS, Chicago, Illinois). The chi-square test of significance and Fisher’s exact probability calculation were used to compare the differences of groups for immunohistochemical scores of 15-PGDH and COX-2 accumulation with clinical pathological parameters. Correlation between 15-PGDH and COX-2 was analyzed using Spearman rank correlation. Student’s t-test and one-way analysis of variance analysis were adopted for other data. P < 0.05 was considered statistically significant.

Results

Differential expression proteins between SGC7901-COX-2/siRNA and SGC7901-pSilencer

To analyze differential expression proteins, we transfected SGC7901 cells with COX-2-siRNA plasmid. After G418 selection, western blotting analysis confirmed that the stable clones transfected with COX-2-siRNA showed lower COX-2 expression compared with empty vector-transfected SGC7901-pSilencer cells. Among them, the COX-2 expression of the first stable clone (siRNA1) was the lowest in all clones, so the first stable clone was used to do the further study (Figure 1A). 2-DE was used to identify the differentially expressed proteins in the two cell lines. In the pH range 4–7, 2-DE maps of SGC7901-COX-2/siRNA and SGC7901-pSilencer displayed ~1000 spots each. Most of 2-DE-separated spots shared identical location, shape and density. Two representative 2-DE maps are shown in Figure 1B. Fourteen differentially expressed protein spots that have been identified between the two cell lines are marked with arrows in Figure 1B. The region of the gels showing differentially expressed proteins between SGC7901-COX-2/siRNA and SGC7901-pSilencer is shown in Figure 1C. The relative expression level of differential expression proteins downregulated or upregulated in SGC7901-COX-2/siRNA cells compared with SGC7901-pSilencer are shown in Figure 1D and E. The MALDI-TOF MS map and database query result of spot 9 are shown in Figure 1F. The information of peptide mass fingerprints was searched in the Swiss-Prot database, and the query result showed that protein spot 9 was NADPH-linked-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The annotation of 14 differential

*aSwiss-Prot accession number.

*bDownregulated showed that spot intensity ≥2-fold decreased in SGC7901-COX-2/siRNA in comparison with SGC7901-pSilencer; upregulated showed that spot intensity ≥2-fold increased in SGC7901-COX-2/siRNA in comparison with SGC7901-pSilencer.
expression proteins is summarized in Table I. Compared with SGC7901-pSilencer, the expression levels of seven proteins, namely cell division cycle protein 16 homolog (CDC16), actin, cytoplasmic 2 (ACTG), 14-3-3 protein sigma (1433S), vimentin (VIME), heat shock cognate 71 kDa protein (HSP7C), histamine N-methyltransferase (HNMT) and Ras-related protein Rab-3B (RAB3B) decreased in SGC7901-COX-2/siRNA, whereas the expression levels of the other seven proteins, namely lactoylglutathione lyase (LGUL), 15-hydroxyprostaglandin dehydrogenase [NAD+] (15-PGDH), complement component 1 Q subcomponent-binding protein precursor (C1QBP), growth differentiation factor 2 precursor (GDF2), heat shock 70 kDa protein 5 (GRP78), phosphatidylinositol transfer protein beta isoform (PIPNB) and stress-70 protein (GRP75) increased in SGC7901-COX-2/siRNA.

Regulation of 15-PGDH by COX-2 in gastric cancer cell lines in vitro

Since the expression of 15-PGDH was low in SGC7901-COX-2/siRNA, we wonder whether COX-2 could regulate the expression of 15-PGDH in gastric cancer cell lines in vitro. To verify this hypothesis, the expression of 15-PGDH was first compared between the SGC7901-COX-2/siRNA and SGC7901-pSilencer. Western blotting and immunocytochemical assay showed that the protein level of 15-PGDH was much higher (128.57%) in SGC7901-COX-2/siRNA with the lower expression of COX-2 than in SGC7901-pSilencer (Figure 2A and B). We then transfected SGC7901 cells with the sense expression plasmid for COX-2. After G418 selection, western blotting analysis confirmed that the stable cell clones transfected with pcDNA3.1-COX-2 showed high COX-2 expression compared with empty vector-transfected SGC7901 cells (Figure 2C). Western blotting and immunocytochemical assay showed that the 15-PGDH expression was much lower (51.72%) in SGC7901-COX-2 cells than in SGC7901-pcDNA3.1 cells (Figure 2C and D). These results demonstrated that alteration of COX-2 could lead to an inverse change of 15-PGDH in gastric cancer cell lines and confirmed that COX-2 could regulate the expression of 15-PGDH in vitro, which was consistent with above findings by 2-DE.

Immunohistochemical expression of 15-PGDH and COX-2 in gastric cancer tissues and their relationship with clinicopathological parameters

The 15-PGDH protein staining exhibited weak or even absent expression in gastric cancer tissues with a total immunoreactive negative rate 80.0% (44/55) in gastric cancer tissues whereas the expression of it was significantly higher in adjacent non-tumor tissues than in gastric cancer tissues ($P < 0.05$) (Figure 3A). Positive immunoreactivity of COX-2 was observed in 76.4% (42/55) of gastric cancer tissues.

Fig. 2. Protein expression level of 15-PGDH and COX-2 in SGC7901 cells transfected with COX-2siRNA, empty pSilencer vector or COX-2-pcDNA3.1, empty pcDNA3.1 vector. (A) Western blotting analysis of protein expression level for 15-PGDH and COX-2. β-Actin was used as loading control. siRNA represents the COX-2siRNA transfectant and vector represents the empty pSilencer vector transfectant. (B) Immunocytochemical assay of COX-2 and 15-PGDH: (a and b) Staining of COX-2 in SGC7901-COX-2/siRNA or SGC7901-pSilencer cells. (c and d) Staining of 15-PGDH in SGC7901-COX-2/siRNA or SGC7901-pSilencer cells. (C) Western blotting analysis of protein expression level for COX-2 and 15-PGDH. β-Actin was used as loading control. SGC7901pc represents the COX-2 sense expression transfectant and vector represents the empty pcDNA3.1 vector transfectant. (D) Immunocytochemical assay of COX-2 and 15-PGDH: (a and b) Staining of COX-2 in SGC7901-COX-2 or SGC7901-pcDNA cells. (c and d) Staining of 15-PGDH in SGC7901-COX-2 or SGC7901-pcDNA cells (original magnification, ×200).
Gastric inflammatory tissues were also involved for 15-PGDH expression and we found descending tendency from normal mucosa, inflammatory tissues, to gastric cancer tissues whereas in case of COX-2, we found ascending tendency (Figure 3A). Since the expression of COX-2 and 15-PGDH was contradictory, we analyzed their correlation in gastric cancer. Spearman analysis showed that there was a significant negative correlation between COX-2 and 15-PGDH immunoreactivity with \( r_s = -0.564, P < 0.01 \) (Table II).

Table II. Relationship between COX-2 and 15-PGDH expression status in gastric cancer

<table>
<thead>
<tr>
<th>15-PGDH expression</th>
<th>COX-2 expression</th>
<th>Total cases (n)</th>
</tr>
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<tbody>
<tr>
<td>Negative</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Total cases (n)</td>
<td>13</td>
<td>42</td>
</tr>
</tbody>
</table>

\( r_s = -0.564, P < 0.01 \).

About their relationship with clinicopathological parameters, statistical analysis showed that neither gender nor age was correlated to the expression of 15-PGDH, whereas the level of 15-PGDH was significantly lower in patients of III–IV stage than in I–II stage \( (P < 0.05) \), its expression had significant difference among differentiation grade \( (P < 0.05) \) and was related with lymph node metastasis \( (P < 0.01) \). For COX-2, although none of gender, age and differentiation grade was related to its expression, the level of COX-2 showed significant difference among different TNM stage \( (P < 0.01, \) well versus moderately; \( P < 0.05, \) well versus poorly) and lymph node metastasis grade \( (P < 0.05) \) (Table III). The results from immunohistochemistry analysis suggested that 15-PGDH may not only be regulated by COX-2 in gastric cancer but also play a suppressive role independently in tumorigenesis of gastric cancer.

Expression levels of COX-2 and 15-PGDH were also examined by western blotting in gastric cancer tissues and adjacent non-tumor tissues taken from eight patients. Each pair of sample was obtained from the same patient. As shown in Figure 3B, 15-PGDH expression was found decreased in gastric cancer tissues whereas COX-2 was overexpressed. Expression of 15-PGDH in 87.5% tumor tissues showed at least a 2-fold decrease whereas COX-2 expression showed...
Lymph node metastasis

TNM stage

Prostaglandins played an important role in carcinogenesis and degradation of cancer. It can stimulate tumor progression by upregulating prostaglandins. However, in fact, 15-PGDH was found decreased or even absent in various kinds of cancers (20,25,27,29,30). Ding et al. (28) detected 15-PGDH expression in 19 pairs of non-small cell lung cancer tissues and adjacent non-tumor tissues. Two-fold decrease was found in 100% of the cancer tissues whereas 10-fold decrease was found in 61% of the cancer tissues compared with the corresponding non-tumor tissues. After being injected with non-small cell lung cancer A549 cells expressing wild-type 15-PGDH, mice displayed a significant decrease in tumor growth compared with the control groups. Yan et al. (30) detected 15-PGDH expression in 21 cases of normal tissues from healthy volunteers and 38 cases of colon cancer tissues. The results showed that, when compared with the normal tissues, 15-PGDH was barely expressed in colon cancer tissues, with at least 17-fold decrease. When 15-PGDH expression was reversed, tumor proliferation significantly slowed down in nude mice. Furthermore, 15-PGDH was found significantly decreased in several colon cancer cell lines. The same results were also found in breast cancer, medullary thyroid cancer and prostate cancer (20,25,29). Moreover, in lung cancer, Tong et al. (47) found that after COX-2 was increased by inducing agents, such as interleukin 1β, tumor necrosis factor-α or phorbol ester, 15-PGDH was significantly downregulated. Meanwhile, adenovirus-mediated restoration of COX-2 rather than COX-1 could inhibit 15-PGDH expression. Additionally, 15-PGDH was significantly increased after inhibition of interleukin 1β-induced COX-2 expression by COX-2siRNA whereas no detectable change of 15-PGDH expression presented after knock down of COX-1 expression by COX-1siRNA. Tong et al. concluded that overexpression of COX-2 but not COX-1 led to the decreased expression of 15-PGDH in lung cancer. However, in the researches on gastric cancer, no coincident conclusion was obtained from the extant studies (30,31). We examined paired expression of COX-2 and 15-PGDH in 55 pairs of gastric cancer tissues and corresponding adjacent non-tumor tissues. 15-PGDH expression was found significantly reduced or even absent in gastric cancer tissues compared with adjacent non-tumor tissues. 15-PGDH decrease was found in 75% tumor tissues (Figure 3C and D). These results were consistent with the results from immunohistochemistry analysis.

Discussion

Though the incidence of gastric cancer has recently decreased in the USA and Western European countries, it is still a major cause of cancerous death in many countries, especially in Eastern Asia, Eastern Europe and Latin America (33). Many studies showed that COX-2 played a pivotal role in gastric cancer (1,11–14). Although some progress has been made, the molecular mechanisms involved in the carcinogenesis and development of COX-2 in gastric cancer are still not fully elucidated. It is a new approach to use the comparative proteomics technique to find the molecules regulated by COX-2, though the mechanisms of COX-2 in gastric cancer have been studied by a variety of ways. In the present study, we successfully constructed the models of gastric cancer cells transfected with COX-2 siRNA and firstly investigate the COX-2-regulated proteins in gastric cancer by using the comparative proteomics approach. Identification of differentially expressed proteins was achieved by 2-DE and MALDI-TOF MS. The functions of some proteins are involved in the metabolism of enzymes. Among them, 15-PGDH, the key enzyme in prostaglandins degradation, was identified as a significantly upregulated protein in SGC7901-COX-2/siRNA. 15-PGDH, as the catabolic enzyme for prostaglandins, acts as a physiological antagonist against the function of COX-2, thus keeping the amount of prostaglandins in balance. In abstracto, we logically presume the expression of 15-PGDH should be upregulated to degrade the redundant prostaglandins if the amount of prostaglandins were abnormally elevated. However, in fact, 15-PGDH was found decreased or even absent in various kinds of cancers (20,25,27,29,30).

Table III. Relationship of immunohistochemical expression of COX-2 and 15-PGDH with clinicopathological parameters in gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Cases (n)</th>
<th>COX-2 (positive)</th>
<th>P value</th>
<th>PGDH (negative)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cases (n)</td>
<td>55</td>
<td>42</td>
<td>NS</td>
<td>44</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
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<td>30</td>
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<td>16</td>
<td>13</td>
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<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>18</td>
<td>15</td>
<td>NS</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>≥50</td>
<td>37</td>
<td>27</td>
<td></td>
<td>31</td>
<td></td>
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<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>21</td>
<td>14</td>
<td>NS</td>
<td>12</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Moderately</td>
<td>21</td>
<td>17</td>
<td>P &lt; 0.05</td>
<td>20</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Poorly</td>
<td>13</td>
<td>11</td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
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<td></td>
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<tr>
<td>I–II</td>
<td>35</td>
<td>23</td>
<td>P &lt; 0.05</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>III–IV</td>
<td>20</td>
<td>19</td>
<td></td>
<td>19</td>
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<td>Lymph node metastasis</td>
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<td></td>
</tr>
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<td>22</td>
<td>14</td>
<td></td>
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<tr>
<td>Yes</td>
<td>33</td>
<td>28</td>
<td></td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

*a* Well versus moderately.  
*b* Well versus poorly.

at least a 2-fold increase in 75% tumor tissues (Figure 3C and D).
an important role as a tumor suppressor considering its more decreased expression in gastric cancer. Simultaneous 15-PGDH and COX-2 staining in successive sections indicated that 15-PGDH expression was negatively correlated to COX-2 expression (P < 0.01). Immunocytochemical and western blotting assay also revealed that 15-PGDH was upregulated in SGC7901-COX-2/siRNA cells whereas it was downregulated in SGC7901-COX-2 cells, further confirming that modulating COX-2 expression could significantly influence 15-PGDH expression and that the decrease of 15-PGDH expression in gastric cancer was regulated by COX-2. The results were in accordance with the finding in lung cancer that 15-PGDH was regulated by COX-2 but not COX-1 (47). Thus, we delineate that COX-2, highly expressed in gastric cancer, not only stimulates the synthesis of prostaglandins but also suppresses the degradation of prostaglandins by down-regulating the expression of 15-PGDH. Due to both disorders of synthesis and degradation, abundant magnified amount of prostaglandins accumulates in vivo, further leading to carcinogenesis and progression of gastric cancer.

In conclusion, 15-PGDH was significantly downregulated in gastric cancer, with its expression negatively related to the differentiation, TNM staging and lymph node metastasis of gastric cancer, which suggested that it might act as a tumor suppressor in gastric cancer. Besides, its expression was also negatively correlated to COX-2 expression. Furthermore, we confirmed that 15-PGDH was regulated by COX-2 by immunochemistry and western blotting. However, the underlying mechanisms are still unknown and we are therefore currently studying them. These findings may have important clinical implications and are valuable for further study of the mechanisms of COX-2-related gastric cancer. Besides, proteomics provides a novel method for detection of COX-2-regulating proteins. Future studies on the other 13 identified proteins may yield equally important information regarding their role relevant to COX-2. Further studies of all these proteins, in addition to 15-PGDH, may yield novel clues for elucidating the mechanisms of carcinogenesis and progression of gastric cancer.

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Expression of 15-PGDH in gastric cancer


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