Galectin-1 Promotes Lung Cancer Progression and Chemoresistance by Upregulating p38 MAPK, ERK, and Cyclooxygenase-2


Abstract

Purpose: This study is aimed at investigating the role and novel molecular mechanisms of galectin-1 in lung cancer progression.

Experimental Design: The role of galectin-1 in lung cancer progression was evaluated both in vitro and in vivo by short hairpin RNA (shRNA)-mediated knockdown of galectin-1 in lung adenocarcinoma cell lines. To explore novel molecular mechanisms underlying galectin-1–mediated tumor progression, we analyzed gene expression profiles and signaling pathways using reverse transcription PCR and Western blotting. A tissue microarray containing samples from patients with lung cancer was used to examine the expression of galectin-1 in lung cancer.

Results: We found overexpression of galectin-1 in non–small cell lung cancer (NSCLC) cell lines. Suppression of endogenous galectin-1 in lung adenocarcinoma resulted in reduction of the cell migration, invasion, and anchorage-independent growth in vitro and tumor growth in mice. In particular, COX-2 was downregulated in galectin-1–knockdown cells. The decreased tumor invasion and anchorage-independent growth abilities were rescued after reexpression of COX-2 in galectin-1–knockdown cells. Furthermore, we found that TGF-β1 promoted COX-2 expression through galectin-1 interaction with Ras and subsequent activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal–regulated kinase (ERK), and NF-κB pathway. Galectin-1 knockdown sensitized lung cancer cells to platinum-based chemotherapy (cisplatin). In addition, galectin-1 and COX-2 expression was correlated with the progression of lung adenocarcinoma, and high clinical relevance of both proteins was evidenced (n = 47).

Conclusions: p38 MAPK, ERK, and COX-2 activation are novel mediators for the galectin-1–promoted tumor progression and chemoresistance in lung cancer. Galectin-1 may be an innovative target for combined modality therapy for lung cancer.

Introduction

Lung cancer is the leading cause of cancer-associated death worldwide. Approximately, 85% of lung cancer is histologically classified as non–small cell lung cancer (NSCLC; ref. 1). The 5-year survival rate of most patients with advanced NSCLCs is only 9% to 15% (2). Combination cytotoxic chemotherapy results in a modest increase in survival at the cost of high toxicity (3). EGFR receptor-tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib and erlotinib, are commonly used for patients with advanced adenocarcinoma. However, clinical reports show that only 10% of patients with NSCLCs respond to gefitinib or erlotinib (4, 5). Therefore, the development of novel molecular approaches is of particular importance for combined modality treatments of lung cancer.

Galectins have high affinity to β-galactoside and share a conserved carbohydrate recognition domain (6, 7). Fifteen mammalian galectins have been identified and divided into 3 subtypes: proto-type, chimera, and tandem-repeat type (6). Galectins are involved in a wide range of physiologic process such as cell adhesion, cell-cycle progression, and apoptosis. Galectin-1, classified as a proto-type galectin, is well known to be involved in the initiation, amplification, and resolution of inflammatory responses (8). In addition, galectin-1 is secreted in large amounts from tumor cells and...
promotes immunosuppression by inducing apoptosis of activated T cells (9). It also contributes to various steps in tumor progression such as transformation, angiogenesis, and metastasis (10). It has been reported that galectin-1 exhibits opposing biologic effects, depending on the physicochemical properties of the protein and the target cell type and status (11). Extracellular galectin-1–induced antiproliferative effects result from the inhibition of the Ras/mitogen-activated protein (MAP)-ERK (MEK)/extracellular signaling–regulated kinase (ERK) pathway and transcriptional induction of p27 (12), whereas intracellular galectin-1 exhibits specificity toward H-Ras and triggers the ERK pathway for tumor transformation (13, 14). Moreover, galectin-1 accumulation in the peritumoral stroma of ovary and breast carcinomas regulates both cancer cell proliferation and invasiveness (15–17). Recent studies report that galectin-1 activates carcinoma-associated fibroblasts and increases MCP-1 secretion of oral squamous cell carcinoma, which promotes tumor progression and metastasis (15). The contradictory effects of galectin-1 on regulating cancer progression still remain to be elucidated.

Galectin-1–expressing lung tumors have been shown to have poor prognosis (18). Invasiveness of tumor cells has shown to correlate positively with the expression level of galectin-1 (19). However, the molecular mechanisms of galectin-1 in progression of lung cancer remain unclear. In this study, we investigated the roles of galectin-1 in lung cancer and further identified which molecular pathway participated in lung tumor progression. We found that knockdown of galectin-1 in lung adenocarcinoma reduced tumor growth in vivo and inhibited cancer migration, invasion, and colony formation in vitro. Furthermore, we surveyed tumorigenic-associated gene profiles regulated by galectin-1 in lung cancer. Galectin-1 could enhance expression of COX-2, and its metabolite, prostaglandin E2 (PGE2), to promote tumor progression in lung cancer. We suggest that TGF-β1 may promote COX-2 expression via Ras/galectin-1 to activate mitogen-activated protein kinase (MAPK)/NF-kB pathway. Our results indicate that MAPK/COX-2 activation is a novel mechanism that contributes to galectin-1–promoted cancer progression and chemoresistance in lung adenocarcinoma and that galectin-1 may be a potential target for drug design in lung cancer therapy.

Materials and Methods

Cell lines

Mouse Lewis lung carcinoma-1 [LLC-1; H-2b; American Type Cell Collection (ATCC no. CRL-1642)] and human lung adenocarcinoma A549 (ATCC no. CCL-185) were obtained from BioResources Collection and Research Center. Human NSCLC cell lines including adenocarcinoma (EKVX, HOP62, NCI-H23, and NCI-H522), large cell carcinoma (HOP92 and NCI-H460), squamous cell carcinoma (NCI-H226), and bronchioloalveolar cell carcinoma (NCI-H322M) cell lines were purchased from the National Cancer Institute (Bethesda, MD). PC-9 lung adenocarcinoma cell line was a kind gift from Dr. A. Maan-Yuh Lin (National Yang-Ming University, Taipei, Taiwan). HEK293T cell line was a kind gift from Dr. Jason C. Huang (National Yang-Ming University).

Mouse model

Male C57BL/6 (H-2b); 6–8 weeks of age) mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). These animals were raised under specific pathogen-free conditions in the Animal Center of National Yang-Ming University in accordance with the regulations of the Animal Care Committee of National Yang-Ming University. To determine the tumor growth in vivo, C57BL/6 mice were subcutaneously inoculated with tumor cells (2 × 10⁵/100 μL cells). Tumor volume was measured with a caliper and calculated as length × width × height (in cm³) at intervals of 3 days.

Tissue microarrays and immunohistochemistry

Tissue array slides were purchased from Biomax (US Biomax, Inc.). The company provided certified documents that all human tissue samples were collected with informed consent from the donors and their relatives. Our research was reviewed for exempt status by the Institutional Review Board. The detailed clinicopathologic characteristics of 82 patients with lung cancer are listed in Supplementary Table S1. The tissue sections were deparaffinized, and then the slides were heated in 10 mmol/L citrate buffer (pH6.0) at 120°C for 20 minutes for antigen retrieval. Nonspecific binding was blocked with 3% H₂O₂ for 5 minutes. Then, they were reacted with galectin-1 or COX-2 primary antibodies at 4°C overnight. The subsequent steps were carried out using LSAB+ System-HRP kit (DAKO). In addition, the
sections were counterstained with hematoxylin. A digital pathology system for stained cells scoring was conducted by Aperio ImageScope (Aperio Technologies, Inc.). The score of tumor cells staining was determined by the sum of percentage and intensity of stained cells to evaluate the protein expression level (20).

Galectin-1 knockdown with short hairpin RNA

HEK293T cells (2.4 × 10⁵/mL) were used to produce lentiviral-expressing short hairpin RNA (shRNA) viruses against galectin-1 (shGa-1) or luciferase as a negative control (shLuc) by cotransfection of pLKO.1-shGal-1, pCMV-ΔR8.91, and pMD.G vectors (National RNAi core Facility, Academia Sinica, Taipei, Taiwan). LLC-1 and A549 cells (10⁵ cells/mL) were infected with shGal-1 or shLuc in the presence of 8 μg/mL protamine sulfate according to the procedures provided from National RNAi core Facility, Academia Sinica. The specific target sequences by shGal-1 are shown in Supplementary Table S2.

Reverse transcription PCR and quantitative reverse transcription PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen), and 5 μg of extracted RNA samples was reverse transcribed into cDNA according to the manufacturer’s protocol for reverse transcription PCR (RT-PCR; Promega). RT-quantitative PCR (RT-qPCR) was carried out by the SYBR Green Mix containing Thermo-Start DNA polymerase (Bio-Rad), according to the manufacturer’s instructions with regard to an ABI7700 System (Applied Biosystems). The specific primers of RT-qPCR are shown in Supplementary Tables S3 and S4.

Immunoprecipitation and Western blotting

The supernatant or whole-cell lysates of A549 cells were harvested and immunoprecipitated by indicated primary antibodies (1 μg) or nonspecific IgG as a negative control which was conjugated to protein G magnetic beads (20 μL; Millipore) at 4°C overnight. The complexes were resolved by 13% SDS-PAGE and then transferred to nitrocellulose. The membrane was blotted with indicated primary antibodies and then with horseradish peroxidase (HRP)-conjugated secondary antibodies. The antibodies used in this study are shown in Supplementary Table S5.

Cytoplasm and nucleus of cells were separated by using the CNMCS Compartamental Protein Extraction Kit (Bio-Chain). Whole-cell lysates (60 μg) or nuclear or cytosolic fractionation of cell lysates (20 μg) were separated by 13% SDS-PAGE and then blotted with indicated antibodies.

Cell proliferation in vitro

shLuc- or shGal-1–infected LLC-1 or A549 cells (2.5 × 10⁴ cells/mL) were seeded onto 96-well plates and cultured in complete medium up to day 5. The cell proliferation was measured at indicated time by CellTiter 96 aqueous nonradioactive cell proliferation assay (MTS assay) according to the manufacturer’s instructions (Promega).

Invasion and migration assay

The invasive and migratory abilities of tumor cells were evaluated by Transwell assay (Costar, 8-μm pore; Corning) as previously described (21). In invasion assay, Transwell inserts were additionally coated with Matrigel (BD Biosciences).

Anchorage-independent growth assay

LLC-1 (1,000 cells per 6-well) or A549 (2,000 cells per 6-well) cells suspended in 0.33% Bacto agar (Sigma-Aldrich) were layered over 0.5% Bacto agar. After incubation for 20 days, these cells were fixed and stained with Giemsa stain for calculating the number of colonies.

Reporter assay

A549 cells were cotransfected with pGL2-COX-2-luc or pGL3-NFκB-luc and pRL-SV40. After 24-hour transfection, the medium was replaced with complete medium containing 5 ng/mL of TGF-β1. Forty-eight hours later, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System Kit according to the manufacturer’s instructions (Promega).

Statistics

Data were expressed as mean ± SD, and statistical significance was assessed by the ANOVA test. For human tissue microarray studies, a nonparametric Mann–Whitney U test was used to test gene expression relevance between different stages. For dual staining of human tissue microarrays, the Spearman correlation method was used to evaluate the association of scores. A significant difference was declared if the P value was <0.05.

Results

Galectin-1 was overexpressed in NSCLC cell lines

Initially, to examine the expression of different types of galectin in lung cancer cells, mRNA levels of galectins in LLC-1 cells were detected and normalized against the expression of those in normal lung isolated from mice. Elevated mRNA levels of most of galectin members were found in LLC-1 cells and the highest expression of galectin-1 among them was noted (Fig. 1A). Furthermore, we confirmed expression of galectin-1 in 9 human lung cancer cell lines, including adenocarcinoma, large cell carcinoma, squamous cell carcinoma, and bronchioloalveolar cell carcinoma. Most of the human lung cancer cell lines highly expressed galectin-1 (Fig. 1B). To investigate whether galectin-1 expression was correlated with the progression of lung adenocarcinoma, immunohistochemical staining was conducted on a tissue microarray containing samples from 47 patients with different stages of lung adenocarcinomas (Fig. 1C; Supplementary Fig. S1A). Higher levels of galectin-1 in stage III lung adenocarcinoma were evidenced compared with stage I or II. In contrast, there was no significant correlation between galectin-1 expressions in different stages of squamous cell carcinomas (Supplementary Fig. S1B).
Next, to identify the sublocalization of galectin-1 protein in A549 lung cancer, cells were fractionated into cytoplasm and nucleus (Fig. 1D, top). In addition, it is well known that galectin-1 protein can be secreted through nonclassical secretory pathway (22). The supernatant of cell culture was immunoprecipitated and Western blotted using anti-Gal-1 antibody to detect levels of secreted galectin-1 (Fig. 1D, bottom). Galectin-1 protein was expressed in the nucleus and cytoplasm of cells. Nevertheless, it was revealed that quite low level of galectin-1 protein was secreted from A549 cells. The secretion level of galectin-1 from A549 cells was only 1.2 pg per 10^6 cells (data not shown).

Suppression of galectin-1 in lung adenocarcinoma cells resulted in reduction of the cell migration, invasion, and anchorage-independent growth in vitro and tumor growth in vivo

To examine the role of galectin-1 in lung cancer progression, lentivirus-mediated delivery of galectin-1 shRNA (shGal-1) was used to reduce galectin-1 expression in mouse LLC-1 and human A549 cell lines. Compared with cells infected with control virus–expressing luciferase shRNA (shLuc), cells infected with shGal-1 virus expressed low levels of galectin-1 (Fig. 2A; Supplementary Fig. S1C). Galectin-1 knockdown significantly inhibited both migratory and invasive abilities of infected cells (Fig. 2B). Furthermore, galectin-1 silencing significantly restrained the anchorage-independent growth of lung cancer cells (Fig. 2C) whereas no effect on the monolayer growth in vitro was observed (Supplementary Fig. S1D). To further evaluate whether galectin-1 could promote tumor growth in vivo, shGal-1- or shLuc-infected LLC-1 cells were injected subcutaneously into mice. As shown in Fig. 2D, tumor growth was reduced in mice injected with shGal-1–infected cells. Therefore, galectin-1 might promote migration, invasion, and anchorage-independent growth of lung cancer cells and tumor growth in mice.

Intracellular galectin-1 promoted expression of COX-2 and PGE2

We further explored which mechanisms contributing to tumor progression were regulated by galectin-1. We analyzed tumorigenic-associated gene profiles by RT-PCR and RT-qPCR in parental, shLuc-, and shGal-1–infected cells. The mRNA levels of COX-2 were significantly
downregulated in both galectin-1 silencing of A549 and LLC-1 cells (Fig. 3A; Supplementary Fig. S2A). VEGF was repressed in LLC-1/shGal-1 but not significantly affected in A549/shGal-1 cells (Fig. 3A; Supplementary Fig. S2A). Furthermore, the COX-2 protein expression and PGE2 release were examined in A549/shGal-1 and LLC-1/shGal-1 cells (Fig. 3B; Supplementary Fig. S2B). We confirmed the reduction of COX-2 protein and PGE2 secretion as a result of galectin-1 knockdown in lung adenocarcinoma cells. In addition, to explore whether exogenous galectin-1 contributed to induction of COX-2 expression, A549 cells were treated with human recombinant galectin-1 (100 ng/mL) and RT-PCR and Western blotting were carried out (Fig. 3C). There was no significant increase in COX-2 expression upon treatment with exogenous galectin-1. Therefore, we suggest that endogenous but not exogenous galectin-1 regulates expression of COX-2.

**COX-2 activation contributed to galectin-1–promoted cancer cell migration, invasion, and anchorage-independent growth**

To confirm the effects of galectin-1 on COX-2 activation, shGal-1–infected A549 cells were transiently transfected with galectin-1 cDNA or pcDNA3 empty vector (shGal-1/Gal-1 or shGal-1/pc). As expected, COX-2 expression (Supplementary Fig. S2C) and PGE2 production (Fig. 4B) were recovered in shGal-1/Gal-1 cells. Moreover, reexpression of galectin-1 led to restore the migratory (Fig. 4C, left) and invasive abilities (Fig. 4C, right) as well as anchorage-independent growth (Fig. 4D) of A549/shGal-1 cells. We also used H522 cells that expressed lower levels of galectin-1 (Fig. 1B) to confirm the effects of galectin-1/COX-2 axis on tumor progression. H522 cells were transfected with galectin-1 cDNA or pcDNA3 empty vector (Supplementary Fig. S3A). We found that mRNA and protein levels of COX-2 were concordantly increased after overexpression of galectin-1 in H522 cells (Supplementary Fig. S3A). In addition, tumor migration, invasion, and anchorage-independent cell growth were significantly upregulated in galectin-1–overexpressed H522 cells (Supplementary Fig. S3B and S3C).

Next, to prove whether COX-2 activation contributed to galectin-1–mediated cancer cell migration, invasion, and in vivo tumor growth, shGal-1–infected A549 cells transiently expressed COX-2 cDNA or empty vector (shGal-1/COX-2 or shGal-1/pc). After overexpression of COX-2, expression of COX-2 (Fig. 4A) and PGE2 (Fig. 4B) was both recovered in shGal-1/COX-2 cells, whereas mRNA and protein levels of galectin-1 did not change, suggesting that galectin-1 was upstream of COX-2. Percentage of migratory and invasive cells was rescued in COX-2–overexpressing A549/shGal-1 cells (Fig. 4C).
anchorage-independent growth was also restored in shGal-1/COX-2 cells as shown in Fig. 4D. These results suggest that COX-2 activation contributes to the galectin-1–mediated tumor progression.

TGF-β1 induced COX-2 transcription via galectin-1 and MAPK/NF-κB activation

The expression of COX-2 is induced by several extracellular stimuli, one of which is growth factors, including TNF, TGF-β1, and HIF-1α. COX-2 transcription is further enhanced by galectin-1, which activates MAPK/NF-κB signaling. Galectin-1 can translocate into the nucleus through the Nuclear Factor Kappa B (NF-κB) pathway, thereby activating COX-2 expression.

Figure 3. Intracellular galectin-1 knockdown repressed COX-2 expression. A, mRNA levels of tumorigenic-associated genes in shLuc- or shGal-1–infected A549 were detected using RT-PCR (top). The expression levels of galectin-1, COX-2, and VEGF were confirmed by RT-qPCR (bottom). B, the protein levels of COX-2 and PGE2 in shLuc– or shGal-1–infected A549 cells were determined by Western blotting and PGE2 Enzyme Immunoassay (EIA), respectively. C, shLuc– or shGal-1–infected A549 cells treated with or without recombinant galectin-1 (rGal-1; 100 ng/mL) for 48 hours. The COX-2 induction in cells was evaluated by RT-PCR (top) and Western blotting (bottom). Results are representative of 3 independent experiments. (*, P < 0.05; **, P < 0.01).

Figure 4. Galectin-1 mediated cancer cell migration, invasion, and anchorage-independent growth in a COX-2–dependent manner. A, A549/shGal-1 cells were transfected with pcDNA3 empty vector (pc) or COX-2 plasmids. The RNA and protein levels of transfectants were analyzed by RT-PCR and Western blotting. B, PGE2 release from transfectants was examined by EIA assay. C, A549/shGal-1 cells reexpressing galectin-1 or COX-2 were subjected to migration and invasion assay. D, A549/shGal-1 cells reexpressing galectin-1 or COX-2 were seeded in soft agar for evaluating the 3-dimensional cell growth. Results are representative of 3 independent experiments. (*, P < 0.05; **, P < 0.01).
TGF, and EGF (23). Recent reports indicate that TGF-$\beta_1$ regulates COX-2 promoter activity through several signaling routes including activation of p38 MAPK, ERK1/2, or Akt pathways (24, 25). NF-$\kappa$B is involved in p38 MAPK- and ERK1/2-induced COX-2 transcription by directly binding to COX-2 promoter. Furthermore, it has been indicated that TGF-$\beta_1$ may trigger a Smad-dependent pathway to control galectin-1 expression in metastatic mammary adenocarcinoma (26). Therefore, we investigated whether galectin-1 knockdown affected the COX-2 expression induced by TGF-$\beta_1$ in lung cancer cells. COX-2 promoter activity was analyzed using luciferase reporter assay upon treatment of TGF-$\beta_1$ for 48 hours. Luciferase reporter assay was conducted to examine COX-2 promoter activity regulated by NF-$\kappa$B.

We next defined the TGF-$\beta_1$ signaling pathways in galectin-1–induced COX-2 expression. We prepared shGal-1–knockdown A549 cells followed by treatment with or without recombinant TGF-$\beta_1$ for 60 minutes. The downstream mediators involved in TGF-$\beta_1$ signaling, including p38 MAPK, NF-$\kappa$B, ERK1/2, c-jun-NH$_2$-kinase (JNK), and Akt, were analyzed by Western blotting. As shown in Fig. 5A, p38 MAPK and ERK1/2 phosphorylation was induced by TGF-$\beta_1$, but the increased levels induced by TGF-$\beta_1$ were much lower in shGal-1–infected cells than those in shLuc-infected cells. The common downstream gene of p38 MAPK and ERK signaling pathways, NF-$\kappa$B/p65, was also reduced upon suppression of galectin-1 expression. Nevertheless, Akt and JNK phosphorylation was not affected by TGF-$\beta_1$ (Fig. 5A).

To confirm the role of NF-$\kappa$B in galectin-1–mediated expression of COX-2, shLuc- or shGal-1–infected cells were transfected with pGL3/NF-$\kappa$B-luc reporter plasmid and treated with TGF-$\beta_1$. The luciferase activity of NF-$\kappa$B reporter was markedly reduced by suppression of galectin-1 while rescued by reexpression of galectin-1 (Fig. 5B). Moreover, the promoter activity of COX-2 suppressed by galectin-1
Galectin-1 knockdown was significantly increased when NF-κB/p65 was overexpressed in shGal-1–infected A549 cells (Fig. 5C). These results suggest that TGF-β1 induces COX-2 expression through galectin-1 and MAPK/NF-κB activation.

**Galectin-1 interacted with H-Ras in lung cancer cells**

It has been reported that intracellular galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation (13). TGF-β1 promotes H-Ras–activated tumor migration and invasion in breast cancer cells (27). Therefore, we investigated whether galectin-1 interacted with Ras in lung cancer cells. The association of galectin-1 and Ras was conducted by co-immunoprecipitation followed by Western blotting. As shown in Fig. 5D, galectin-1 directly interacted with Ras and galectin-1 knockdown reduced the association of galectin-1 and Ras in lung cancer cells.

**Galectin-1 knockdown sensitized lung cancer cells to cisplatin treatment**

COX-2 induction may reduce the response of malignant cells to cytotoxic therapy (28). To investigate the effects of galectin-1 knockdown on chemosensitivity of lung cancer cells, we treated A549/shLuc and A549/shGal-1 cells with cisplatin (IC_{50} = 20 μmol/L) for up to 48 hours (Fig. 6A and B). A549/shLuc cells increased expression of COX-2 protein time dependently after cisplatin treatment. Galectin-1 knockdown in A549 (A549/shGal-1) attenuated cisplatin-induced COX-2 expression (Fig. 6A, top). In addition, galectin-1 knockdown blocked the cisplatin-induced PGE2 production (Fig. 6A, bottom). Furthermore, shGal-1–infected A549 cells were more sensitive to cisplatin treatment than shLuc-infected cells (Fig. 6B). After cisplatin treatment for 72 hours, the percentage inhibition of cell growth was 92% in shGal-1–infected cells whereas only 55% inhibition in shLuc-infected cells (Fig. 6B). These suggest that COX-2 inhibition via galectin-1 knockdown sensitizes lung cancer cells to cisplatin treatment.

**Galectin-1 promoted lung cancer progression and chemoresistance by upregulating p38, ERK, and COX-2 in EGFR-mutated adenocarcinoma cells**

Because A549 cells harbor KRAS mutation, to explore whether galectin-1–regulated tumor progression is KRAS-specific or universal, the PC-9 cell line, which is known to carry an EGFR exon 19 deletion and KRAS wild-type, was used. Upon knockdown of galectin-1, mRNA and protein levels of both galectin-1 and COX-2 were reduced in PC-9 cells (Supplementary Fig. S4A). Galectin-1 knockdown in PC-9 cells resulted in suppression of tumor migration, invasion, and anchorage-independent cell growth (Supplementary Fig. S4B). The expression of p-p38 MAPK, p-ERK, and NF-κB/p65 was downregulated in shGal-1–infected PC-9 cells (Supplementary Fig. S4C). Furthermore, shGal-1–infected lung cancer cells were more sensitive to cisplatin treatment than shLuc-infected cells (Supplementary Fig. S4D). We suggest that either in KRAS-mutated A549 cells or in EGFR-mutated PC-9 cells, galectin-1 appears to...
regulate p38 MAPK, ERK, and COX-2 expression to promote tumor progression.

**Clinical relevance of galectin-1 and COX-2 expression in tissues of lung cancer**

COX-2 is highly expressed in 70% of lung adenocarcinomas and can be detected throughout the progression of a premalignant lesion to the metastatic phenotype (29). To examine the clinical relevance of galectin-1 and COX-2 expression, immunohistochemical staining was conducted on tissue microarrays containing samples from 82 patients with lung cancer, including small cell lung cancer and NSCLC. A positive correlation between galectin-1 and COX-2 expression in lung cancer was revealed (Supplementary Fig. S5A). In addition, galectin-1 expression was correlated with COX-2 expression (Fig. 6C) in 47 patients with lung adenocarcinomas. Higher levels of COX-2 in stage III lung adenocarcinoma were addressed than in stage I (Fig. 6D; Supplementary Fig. S5B). However, in squamous cell carcinomas (n = 17), although clinical relevance between galectin-1 and COX-2 expression was revealed (Supplementary Fig. S5C), neither galectin-1 nor COX-2 expression was correlated with the progression of squamous cell carcinoma (Supplementary Figs. S1A and S5D). Taken together, expression levels of both galectin-1 and COX-2 were correlated with the progression of lung adenocarcinoma, and furthermore, a significant positive correlation between galectin-1 and COX-2 expression in lung cancer was revealed.

**Discussion**

In this study, we showed that endogenous galectin-1 may promote lung cancer progression and chemoresistance by increasing p38 MAPK, ERK, and COX-2 expression. Several reports indicate that galectin-1 exhibits opposing effects in that endogenous galectin-1 has growth-promoting whereas exogenous galectin-1 has growth-inhibiting effects (30, 31). Galectin-1 has been found to induce activated T-cell apoptosis (9, 32–34). However, it remains highly controversial as these cells treated with high concentration of recombinant galectin-1 (35, 36) or concentrated supernatant of tumor cell culture (37) in previous studies. The conditioned supernatant obtained from tumor cell culture contains detectable but quite low level of galectin-1 that is not sufficient for induction of apoptosis. More importantly, induction of T-cell apoptosis requires a cell–physical contact between T cells and tumor cells (9). In this study, we found that lung cancer cells secreted quite low amount of galectin-1 protein and intracellular galectin-1 promoted COX-2 expression. Intriguingly, A549 cells could express α-2,3-sialyltransferase 1 (data not shown) that blocks O-linked glycan elongation to render resistance of exogenous galectin-1 (38). Treatment of recombinant galectin-1 did not induce cell death of A549 cells. We hypothesize that tumor cells may disrupt galectin-1–induced itself cell death and promote tumor progression resulting from loss of susceptibility to exogenous galectin-1 via altering cell surface glycosylation and overexpression of endogenous galectin-1. Intracellular galectin-1 might play much more important roles to regulate lung tumor progression.

In this article, we showed that COX-2 activation contributed to galectin-1–promoted lung cancer progression. COX-2 augments tumor angiogenesis, invasion, and resistance to apoptosis (39). The expression of COX-2 is induced by several extracellular signals including proinflammatory and growth-promoting stimuli. All signals converge to the activation of MAPK and PI3K pathways that regulate COX-2 mRNA expression (23, 24). After treatment of TGF-β1, p38 MAPK and ERK1/2 was induced but abrogated by suppression of galectin-1, indicating that galectin-1 may be an upstream mediator of p38 MAPK and ERK signaling. In addition, the transcriptional activity of COX-2 was induced under TGF-β1 treatment and inhibited by galectin-1 knockdown (Fig. 5A). Therefore, TGF-β1–induced COX-2 mRNA expression was galectin-1–dependent.

Galectin-1 is a critical scaffolding protein and a major regulator of H-Ras nanoclusters that contributes to Ras membrane anchorage and tumor transformation (13). It accord with our finding that galectin-1 and H-Ras are directly associated in A549 cells (Fig. 5D). Furthermore, TGF-β1 activates H-Ras to promote malignant progression of cancer (27). Therefore, we suggest that galectin-1–activated p38 MAPK and ERK pathways, which were induced by TGF-β1, may result from interaction with H-Ras and subsequently upregulated COX-2 expression (Supplementary Fig. S5E). It has been investigated that COX-2 promoter activity is positively regulated by NF-κB translocation. COX-2 expression is induced by TNF-α depending heavily on activation of NF-κB (23, 40). We also found that under the treatment of TGF-β1, galectin-1 would promote NF-κB expression; NF-κB was bound to the response element of COX-2 promoter region to activate COX-2 mRNA transcription (Fig. 5A–C). Besides from promoter deletion analysis, we noted that galectin-1 may induce promoter activity within −330 to −1 proximal region of COX-2 (data not shown). Of note, this promoter region of COX-2 contains NF-κB–binding element. According to these results, we suggest that COX-2 transcription was regulated by TGF-β1/Ras/galectin-1/NF-κB axis (Supplementary Fig. S5E). Ineffective response and high resistance of lung cancer cells to chemotherapy result from induction of COX-2 and PGE2 (28, 41). We found that galectin-1 knockdown may block the cisplatin-induced COX-2 and PGE2 expression and increase the response of cancer cells to cisplatin treatment (Fig. 6A and B). These results suggest that suppression of galectin-1 may reduce the effects of COX-2–induced drug resistance. Moreover, galectin-1 knockdown in lung adenocarcinoma cells reduced p38 MAPK and ERK1/2 phosphorylation induced by TGF-β1 (Fig. 5A). It has been reported that p38 MAPK could induce tumor dormancy that maintains cells in a quiescent state related to drug resistance (42, 43). p38 MAPK activation induces phosphorylation of Hsp27 leading to resistance to chemotherapeutic agents in myeloma (44). STAT1, a downstream mediator of p38 MAPK, has been indicated in the regulation of platinum resistance in ovarian cancer (45). In addition,
ERK signaling also governs drug resistance in human cancer (46). Therefore, inhibition of the common upstream regulator of p38 MAPK, ERK, and COX-2 signaling, galectin-1, will be more effective for cancer therapy. In conclusion, galectin-1 not only is a critical scaffolding protein and a major regulator of H-Ras nanoclusters for tumor transformation (13) but also may contribute to tumor progression and drug resistance through p38, ERK, and COX-2 pathways. We suggest that a nontoxic anti-galectin-1 drug (galecin-1 knockdown) in combination with a toxic chemotherapeutic agent (cisplatin) may serve as a novel therapeutic modality for lung cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were declared.

Authors’ Contributions
Conception and design: L.-Y. Chung, S.-J. Tang, G.-H. Sun, T.-S. Yeh, K.-H. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.-Y. Chou, S.-L. Yu, K.-H. Sun

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