Galectin-1 promotes lung cancer progression and chemoresistance by upregulating p38 MAPK, ERK and cyclooxygenase-2

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Translational relevance

Targeted therapeutic approaches have been indicated to improve standard chemotherapy. EGFR inhibitors have been commonly used in lung cancer treatment. Unfortunately, clinical data show that only 10% of patients with NSCLC respond to EGFR inhibitors. Our studies provide insight into the influence of galectin-1, which is increasingly recognized as an important factor of regulatory proteins, in lung cancer development. Suppression of galectin-1 elicits anti-tumor activity both in vivo and in vitro and inhibits cancer-relevant signaling pathways in NSCLC. Furthermore, galectin-1 is highly expressed in lung cancer tissues from patients with stage III NSCLC, indicating that galectin-1 may be a predictor for outcome of NSCLC patients. Additionally, galectin-1 knockdown sensitized lung cancer cells to platinum-based chemotherapy (cisplatin). These results suggest that galectin-1 may be a biomarker for prognosis and an innovative target for combined modality therapy of lung cancer.
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 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 RT-PCR and Western blot. A tissue microarray containing samples from lung cancer patients was employed to examine expression of galectin-1 in lung cancer.

Results: We found overexpression of galectin-1 in 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 MAPK, ERK and NF-κB pathway. Galectin-1
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knockdown sensitized lung cancer cells to platinum-based chemotherapy (cisplatin).

In addition, galectin-1 and COX-2 expression were correlated with the progression of lung adenocarcinoma, and high clinical relevance of both proteins was evidenced (n = 47).

Conclusions: These findings suggest that 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 of 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) (1). The 5-year-survival rate of most patients with advanced NSCLC is only 9 to 15% (2). Combination cytotoxic chemotherapy results in a modest increase in survival at the cost of high toxicity (3). Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefinib and erlotinib, are commonly used for patients with advanced adenocarcinoma. However, clinical reports show that only 10% of patients with NSCLC respond to gefinib 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 \( \beta \)-galactoside and share a conserved carbohydrate recognition domain (CRD) (6, 7). Fifteen mammalian galectins have been identified and divided into three subtypes: proto-type, chimera, and tandem-repeat type (6). Galectins are involved in a wide range of physiological 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
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tumor cells and promotes immune suppression 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 biological effects depending on the physicochemical properties of
the protein and the target cell type and status (11). Extracellular galectin-1 induced
anti-proliferative effects results from the inhibition of the Ras-MEK-ERK pathway
and transcriptional induction of p27 (12), whereas intracellular galectin-1 exhibits
specificity towards 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 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 \textit{in vivo} and inhibited cancer migration, invasion and colony formation \textit{in vitro}. Furthermore, we surveyed tumorigenic-associated gene profiles regulated by galectin-1 in lung cancer. Galectin-1 could enhance expression of cyclooxygenase-2 (COX-2), and its metabolite, prostaglandin E2 (PGE2) to promote tumor progression in lung cancer. We suggest that transforming growth factor-β1 (TGF-β1) may promote COX-2 expression via Ras/galectin-1 to activate MAPK-NF-κB pathway. Our results indicate that MAPK-COX-2 activation is a novel mechanism that contributes to galectin-1-promoted cancer progression and chemo-resistance in lung adenocarcinoma and 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 Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Human NSCLC cell lines including adenocarcinoma (EKVX, HOP62, NCI-H23, and NCI-H522), large cell carcinoma (HOP-92 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, Taiwan). HEK293T cell line was a kind gift from Dr. Jason C. Huang (National Yang-Ming University, Taiwan).

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 determinate the tumor growth in vivo, C57BL/6 mice were subcutaneously inoculated with tumor cells (2 × 10^5 / 100 µl cells). Tumor volume was measured with a caliper and calculated as length × width × height (in mm^3) at
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intervals of 3 days.

Tissue microarrays and immunohistochemistry

Tissue array slides were purchased from Biomax (US Biomax, Inc., Rockville, MD). 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 IRB. The detailed clinical-pathologic characteristics of 82 patients with lung cancer are listed in Supplementary Table S1. The tissue sections were deparafinized, and then the slides were heated in 10 mM citrate buffer (pH6.0) at 120°C for 20 minutes for antigen retrieval. Non-specific 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, Glostrup, Denmark). In addition, the sections were counterstained with hematoxylin. A digital pathology system for stained cells scoring was performed by Aperio ImageScope (Aperio Technologies, Inc., Vista, CA). 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 shRNA
HEK293T cells (2.4 × 10^5/ml) were used to produce lentiviral expressing 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 (10^5 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 (Taipei, Taiwan). The specific target sequences by shGal-1 are shown in Supplementary Table S2.

**Reverse transcription-PCR and quantitative reverse transcription-PCR (RT-qPCR)**

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) and 5 μg of extracted RNA samples reversely transcribed into cDNA according to the manufacturer’s protocol for RT-PCR (Promega). RT-qPCR was performed by the SYBR Green Mix containing Thermo-Start DNA polymerase (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions with regards to an ABI7700 System (Applied Biosystems, Foster City, CA). The specific primers of RT-qPCR are shown in Supplementary Table S3, S4.

**Immunoprecipitation and Western blot**
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The supernatant or whole cell lysates of A549 cells were harvested and immunoprecipitated by indicated primary antibodies (1 μg) or non-specific IgG as a negative control which conjugated to protein G magnetic beads (20 μl) (Millipore, New Bedford, MA) 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 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 compartmental protein extraction kit (BioChain; Hayward, CA). 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 × 10^4 cells/ml) were seeded onto 96 wells and cultured in complete medium up to day 5. The cell proliferation was measured at indicated time by CellTiter 96 aqueous non-radioactive cell proliferation assay (MTS assay) according to the manufacturer’s instructions (Promega).

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

**Anchorage-independent growth assay**

LLC-1 (1000 cell/6-well) or A549 (2000 cells/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 hours transfection, the medium was replaced with complete medium containing 5 ng/ml of TGF-β1. 48 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 ± S.D. 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 less than 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 nine 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 performed 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 stage 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
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were fractionated into cytoplasm and nucleus (Fig. 1D; top panel). In addition, it is well known that galectin-1 protein can be secreted through non-classical secretary pathway (22). The supernatant of cell culture was immunoprecipitated and Western blotted using anti-galectin-1 antibody to detect levels of secreted galectin-1 (Fig. 1D; bottom panel). 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/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
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anchorage-independent growth of lung cancer cells (Fig. 2C) while no effect on the monolayer growth in vitro (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). Vascular endothelial growth factor (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
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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 performed RT-PCR and Western blot (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.**

In order 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 panel) and invasive abilities (Fig. 4C; right panel) 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, C).

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) were both recovered in shGal-1/COX-2 cells, while 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). The 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 tumor necrosis factor (TNF), transforming growth factor...
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(TGF) and epithelial growth factor (EGF) (23). Recent reports indicate that TGF-β1 regulates COX-2 promoter activity through several signaling routes including activation of p38 mitogen-activated protein kinases (p38 MAPK), extracellular signal-regulated kinase (ERK) 1/2, or Akt pathways (24, 25). NF-κB involves in p38 MAPK- and ERK1/2-induced COX-2 transcription by directly binding to COX-2 promoter. Furthermore, it has been indicated that TGF-β1 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-β1 in lung cancer cells. COX-2 promoter activity was analyzed using luciferase reporter assay upon treatment of TGF-β1 in shLacZ- or shGal-1-infected A549 cells. TGF-β1 increased the COX-2 promoter activity (Supplementary Fig. S2D) and protein levels of galectin-1 and COX-2 (Fig. 5A). However, suppression of galectin-1 in A549 cells significantly blocked TGF-β1-induced COX-2 promoter activity (Supplementary Fig. S2D) and protein levels of galectin-1 and COX-2 (Fig. 5A).

We next defined the TGF-β1 signaling pathways in galectin-1-induced COX-2 expression. We prepared shGal-1-knockdown A549 cells followed by treatment with or without recombinant TGF-β1 protein for 60 minutes. The downstream mediators involved in TGF-β1 signaling, including p38 MAPK, NF-κB, ERK1/2, JNK and Akt,
were analyzed by Western blot. As shown in Fig. 5A, p38 MAPK and ERK1/2 phosphorylation was induced by TGF-β1, but the increased levels induced by TGF-β1 were much lower in shGal-1-infected cells compared with those in shLuc-infected cells. The common downstream gene of p38 MAPK and ERK signaling pathways, NF-κB-p65, was also reduced upon suppression of galectin-1 expression. Nevertheless, Akt and JNK phosphorylation were not affected by TGF-β1 (Fig. 5A).

To confirm the role of NF-κB in galectin-1-mediated expression of COX-2, shLacZ- or shGal-1-infected cells were transfected with pGL3-NF-κB-luc reporter plasmid and treated with TGF-β1. The luciferase activity of NF-κB 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 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 performed by co-immunoprecipitation followed by Western blot. 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 μM) 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 panel). In addition, galectin-1 knockdown blocked the cisplatin-induced PGE2 production (Fig. 6A; bottom panel). Furthermore, shGal-1-infected A549 cells were more sensitive to cisplatin treatment compared with shLuc-infected cells (Fig. 6B). After cisplatin treatment for 72 hours, the percentage inhibition of cell growth was 92% in shGal-1-infected cells while only 55% inhibition in shLuc-infected cells (Fig. 6B). These suggest that COX-2 inhibition via galectin-1 knockdown sensitizes lung

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

Since 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 a 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 were downregulated in shGal-1-infected PC-9 cells (Supplementary Fig. S4C). Furthermore, shGal-1-infected lung cancer cells were more sensitive to cisplatin treatment compared with 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 pre-malignant lesion to the metastatic phenotype (29). To examine the clinical relevance of galectin-1 and COX-2 expression, immunohistochemical staining was performed on tissue microarrays containing samples from 82 patients with lung cancer, including small cell and non-small cell lung cancer. A positive correlation between galectin-1 and COX-2 expression in lung cancer was demonstrated (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 compared with stage I (Fig. 6D; Supplementary Fig. S5B). However, in squamous cell carcinomas (n = 17), although clinical relevance between galectin-1 and COX-2 expression were revealed (Supplementary Fig. S5C), neither galectin-1 nor COX-2 expression was correlated with the progression of squamous cell carcinoma (Supplementary Fig. 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 demonstrated that endogenous galectin-1 may promote lung cancer progression and chemoresistance by increasing p38MAPK, ERK and COX-2 expression. Several reports indicate that galectin-1 exhibits opposing effects that endogenous galectin-1 has growth-promoting while exogenous galectin-1 has growth-inhibition (30, 31). Galectin-1 has been found to induce activated T cell apoptosis (9, 32-34). However, it remains highly controversial since 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 cell-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) which 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 resulted from loss of susceptibility to exogenous galectin-1 via altering
Galectin-1 activates COX-2 in lung cancer 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 paper, we demonstrated 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 pro-inflammatory 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 Ras membrane anchorage and tumor transformation (13). It accords with our finding that galectin-1 and H-Ras 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
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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 NFκB 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 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).
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reported that p38 MAPK could induce tumor dormancy which 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 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 may contribute to tumor progression and drug resistance through p38, ERK and COX-2 pathways. We suggest that a non-toxic anti-galectin-1 drug (galectin-1 knockdown) in combination with a toxic chemotherapeutic agent (cisplatin) may serve as a novel therapeutic modality for lung cancer.
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Galectin-1 activates COX-2 in lung cancer

...metastatic mammary adenocarcinoma cells: implications for tumor-immune escape.


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Figure Legends

**Figure 1. Galectin-1 was overexpressed in NSCLC cell lines.** A, The mRNA levels of galectins (Gal) in mouse LLC-1 normalized against mRNA levels of lung tissue of C57BL/6 mice were detected by RT-qPCR. B, Total RNA of human NSCLC cells was extracted and galectin-1 mRNA levels were detected using RT-PCR. Whole cell lysates of human NSCLC cells were prepared for Western blot (WB) using anti-Gal-1 antibody. AC: adenocarcinoma; LC: large cell carcinoma; SC: squamous cell carcinoma; BAC: bronchioloalveolar cell carcinoma. C, Immunohistochemical (IHC) staining was performed using an antibody to galectin-1 on a tissue microarray containing samples from 47 patients with stage I (n = 15), stage II (n = 19), and stage III (n = 13) lung adenocarcinomas. Galectin-1 expression levels were verified according to the score of tumor staining in different stages of lung adenocarcinomas. D, Whole cell lysates of A549 cells were fractionated. Nuclear (N) and cytosolic (C) galectin-1 expression were detected by Western blot. USF-2 and GAPDH were the nuclear and cytosolic internal control respectively. In the right panel, A549 cells (8 × 10^4 cells/ml) were cultured for 2 days, and then the cultured medium was replaced with serum free medium. After 24 hours, supernatants (Sup) of cells (5 × 10^6) were subjected to immunoprecipitate (IP) and Western blot (WB) using anti-Gal-1 antibody. WCL, whole cell lysate control. Results are representative of three independent
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Figure 2. Galectin-1 knockdown in lung adenocarcinoma resulted in reduction of migration, invasion, anchorage-independent growth and *in vivo* tumor growth. A, Galectin-1 was knockdown in LLC-1 and A549 cell lines by lentiviral shGal-1 infection. Luciferase shRNA lentivirus (shLuc) was used as a control. The expression levels of galectin-1 were detected using RT-qPCR and Western blot. B, shLuc- or shGal-1-infected cells (2 × 10^4) were seeded onto upper side of transwell inserts and incubated for 24 hours at 37°C to determine the migratory and invasive abilities. C, shLuc- or shGal-1-infected cells were seeded in soft agar for evaluating anchorage-independent tumor growth. D, shLuc- or shGal-1-infected LLC-1 cells were inoculated subcutaneously into C57BL/6 mice (n = 6). Tumor volume was measured every 3 days. Results are representative of three independent experiments. (*, *P* < 0.05; **, *P* < 0.01)

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 panel). The expression levels of galectin-1, COX-2 and VEGF were confirmed by RT-qPCR (bottom panel). B, The protein levels of COX-2 and PGE2 in shLuc- or shGal-1-infected A549 cells were determined by
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Western blot and PGE2 EIA assay, respectively. C, shLuc- or shGal-1-infected A549 cells treated with or without recombinant galectin-1 (100 ng/ml) for 48 hours. The COX-2 induction in cells was evaluated by RT-PCR (top panel) and Western blot (bottom panel). Results are representative of three 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 blot. B, PGE2 release from transfectants was examined by EIA assay. C, A549/shGal-1 cells reexpressed galectin-1 or COX-2 were subjected to migration and invasion assay. D, A549/shGal-1 cells reexpressed galectin-1 or COX-2 were seeded in soft agar for evaluating the three-dimensional cell growth. Results are representative of three independent experiments. (*, \( P < 0.05; **, P < 0.01 \))

**Figure 5.** TGF-β1 drove galectin-1-regulated COX-2 transcription though p38 MAPK and ERK pathway in lung adenocarcinoma. A, shLuc- or shGal-1-infected cells were treated with or without TGF-β1. One hour later, Western blot was performed. B, shLacZ- or shGal-1-infected A549 cells were cotransfected with
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pcDNA3 (pc) or galectin-1 (Gal-1), pNF-κB-luc and pRL-SV40. 24 hours later, cells were treated with TGF-β1 for 48 hours. Luciferase reporter assay was performed to investigate NF-κB promoter activity regulated by galectin-1. C, shLacZ- or shGal-1-infected A549 cells were cotransfected with pcDNA3 (pc) or NF-κB-p65 (p65), pXC918-COX-2-luc and pRL-SV40. 24 hours later, cells were treated with or without TGF-β1 for 48 hours. Luciferase reporter assay was performed to examine COX-2 promoter activity regulated by NF-κB. D, shLuc- or shGal-1-infected A549 cells were subjected to immunoprecipitation (IP) using anti-Ras antibody (right panel) or anti-Gal-1 antibody (left panel), and then immunoblotted using anti-Gal-1 antibody or anti-Ras antibody. IgG, negative control. WCL, whole cell lysate control. Results are representative of three independent experiments. (*, \( P < 0.05 \); **, \( P < 0.01 \))

**Figure 6. Effects of galectin-1 knockdown on drug sensitivity and clinical relevance of galectin-1 and COX-2 expression in lung adenocarcinoma.** A, COX-2 expression and PGE2 production were examined by Western blot and EIA assay upon treatment of 20 μM (IC50) of cisplatin for up to 48 hours. CDDP, cisplatin. B, shLuc- or shGal-1-infected A549 cells were cultured in 96-wells and treated with 20 μM of cisplatin for up to 72 hours. Cell growth rate was examined by MTS assay. C, Statistic analysis of galectin-1 expression was compared with COX-2 in lung adenocarcinoma tissues (n = 47). D, COX-2 expression levels were verified in
different stages of lung adenocarcinomas (n = 47).
Figure 1

A

RT-qPCR

**

Normal lung

LLC-1

B

Gal-1

GAPDH

Gal-1

GAPDH

C

Gal-1 expression (score)

n = 16

n = 19

D

Gal-1

GAPDH

USF-2

anti-Gal-1

WCL

light chain

Gal-1

WB:
Figure 2

A  RT-qPCR

B  % of migrated cells

C  % of invaded cells

D  Colony formation (%)

**shLuc**  **shGal-1**

**GAPDH**

**LLC-1**  **A549**

**shRNA:**

- Luc  Gal-1

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A

Gal-1
COX-2
TGF-β1
VEGF
MMP-2
MMP-9
HIF-1α
GAPDH

RT-qPCR

B

A549

shRNA:

Luc
Gal-1

COX-2
GAPDH

C

rGal-1:

Gal-1

COX-2
GAPDH

rGal-1:

shLuc
shGal-1

Gal-1

COX-2
GAPDH
Figure 4
Figure 5

A

TGF-β1
Gal-1
COX-2
p-p38
NF-κB p65
p-IL-β-α
p-ERK1/2
p-Akt
p-JNK
GAPDH

B

NFκB reporter
RLU (Fold change)

shLuc shGal-1

C

COX-2 reporter
RLU (Fold change)

shLuc(shGal-1)

D

IP: anti-Gal-1

WB: Ras

WB: Gal-1

IP: anti-Ras

WB: shLuc

WB: shGal-1

WB: WCL

Light chain

Light chain

Light chain

Ras

Ras

Ras
Figure 6

A

B

C

D

Gal-1 expression (score)

COX-2 expression (score)

stages of lung adenocarcinoma

n = 15

n = 19

n = 13

r = 0.758

P < 0.001

n = 47

P = 0.023
Galectin-1 promotes lung cancer progression and chemoresistance by upregulating p38 MAPK, ERK and cyclooxygenase-2


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