Galectin-1 promotes lung cancer tumor metastasis by potentiating integrin α6β4 and Notch1/Jagged2 signaling pathway

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Lung cancer is a major cancer, leading in both incidence and mortality in the world, and metastasis underlies the majority of lung cancer–related deaths. Galectin-1, a glycans-binding protein, has been shown to be overexpressed in lung cancer and involved in tumor-mediated immune suppression. However, the functional role of galectin-1 in lung cancer remains unknown. We demonstrate that ectopic expression of galectin-1 in a low-metastatic CL1-0 lung cancer cell line promotes its migration, invasion and epithelial-mesenchymal transition. Conversely, we also show that suppression of galectin-1 expression in highly invasive CL1-5 and A549 cells inhibits migration and invasion of lung cancer cell and causes a mesenchymal-epithelial transition. These effects may be transduced by increasing the expression of integrin α6β4 and Notch1/Jagged2, which in turn co-operates in the phosphorylation of AKT. The effects of galectin-1 on cancer progression are reduced when integrin β4 and Notch1 are absent. Further study has indicated that galectin-1 knockdown prevents the spread of highly metastatic Lewis lung carcinoma in vivo. Our study suggests that galectin-1 represents a crucial regulator of lung cancer metastasis. Thus, the detection and targeted treatment of galectin-1-expressing cancer serves as a new therapeutic target for lung cancer.

Introduction

Lung cancer is the leading cause of cancer mortality, and tumor metastasis is the most life-threatening event that lung cancer patients must face (1,2). Approximately 40–50% of patients with advanced lung cancer will develop metastasis, dramatically increasing mortality rates (3), and clinical treatment of lung cancer remains challenging due to this high incidence of tumor metastasis. Therefore, understanding the process contributing to cancer metastasis is beneficial for the development of effective therapy strategies.

Integrins, large, complex transmembrane glycoproteins belonging to adhesion receptors, have been associated with receptor tyrosine kinases in regulating cell survival, mitogenesis and cell migration (4,5). Integrins provide anchorage for cell motility and invasion by directly binding to components of the extracellular matrix (6). Ligation of integrins with extracellular matrix components activates their downstream signaling molecules, focal adhesion kinase (FAK) or Src family kinases, which in turn affects the shape and migratory properties of cells (7,8). Cancer cell upregulation of α6β1, α6β4, αvβ3, αvβ5 and αvβ5 is correlated with metastatic features in melanoma, breast carcinoma, prostate and lung cancers (9,10). FAK, a non-receptor protein kinase, transduces signals from integrins and is hyperactivated in various cancers. The kinase activity of FAK is modulated by autophosphorylation at Y397 on activation of β integrins. For full kinase activation, additional sites, such as γ576 and γ577 in the activation loop, are phosphorylated by Src family kinases (7,11). Subsequently, activated Src family kinase phosphorylates the Crk/p130Cas complex and paxillin at Y118 and Y31, leading to cancer migration (11).

Notch signaling pathway involves communication among adjacent cells, one expressing a ligand (either Delta or Jagged) and the other expressing Notch receptor (12,13). Ligation of Delta or Jagged protein to Notch leads to a proteolytic cleavage cascade releasing the notch intracellular domain (NICD), which translocates to the nucleus from the membrane and activates transcription of target genes such as hairy and enhancer-of-split 1 (Hes1) (13,14). Notch signaling is an evolutionarily conserved pathway that plays an important role in some features of stem cells, and abnormal expression of this pathway is often found in various types of cancers (15,16). In addition, cancers expressing elevated levels of both Notch1 and its ligand Jagged2 are associated with patients having the lowest overall survival rates, suggesting a synergistic effect of these expression changes on tumor progression (17). Notch antagonists, such as gamma-secretase inhibitors, are being developed as potential anticancer agents.

Protein–glycan interactions play important functions in several aspects of cancer biology, including cancer transformation, growth, metastasis, angiogenesis and immune response (18,19). Galectins are glycan-binding proteins characterized by their high affinity for N-acetyllactosamine sequences via conserved carbohydrate recognition domains. Galectin-1, the first protein discovered in the family, has been shown to be overexpressed in many malignancies, including lymphoma, oral, colon, bladder, ovarian, astrocytoma, liver, pancreatic and melanoma carcinomas (20–23). Dysregulation of galectin-1 in cancer has also been correlated with the aggressiveness of these tumors (22,23). Our previous studies not only demonstrated that lung cancer cells secrete high amounts of galectin-1 to mediate dendritic cell anergy (24) but also demonstrated that lung cancer–derived galectin-1 is responsible for stimulating tumor-associated dendritic cells’ migration and upregulating the expression of maturation markers (25). Galectin-1 has also been implicated in regulating immune responses by controlling T-cell survival, cytokine secretion and transendothelial migration (26). Inhibition of galectin-1 reduces lung metastasis by increasing number of T cells in a mouse model (26,27). Intracellular galectin-1 has been reported to regulate cancer development by protein–protein interactions with oncopgenic Ras (28,29). Recent studies have also reported that galectin-1 contributes to cancer growth and chemoresistance (30). However, whether galectin-1 is involved in lung cancer progression and metastatic potential per se has not yet been determined. Consequently, the role of galectin-1 in mediating lung cancer tumor development has been investigated in the current study.

Materials and methods

Cell cultures

Human lung adenocarcinoma cell lines, the less invasive (CL1-0) and the highly invasive (CL1-5), were generously provided by Dr Pan-Chyr Yang of the Department of Internal Medicine, National Taiwan University Hospital, and cultured in RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco BRL, Life Technologies) (31,32). Mouse...
Fig. 1. Galectin-1 is overexpressed in highly invasive lung cancer cells. The amount of galectin-1 in low-invasive isogenic CL1-0 and CL1-5 cells at mRNA (A) and protein (B) levels. (C) The expression of galectin-1 increases after repeated transplantation in vivo. (D) The migratory ability of LLC increases after repeated transplantation. (E) The efficacy of galectin-1 siRNA. (F) Knockdown of galectin-1 decreases cell migration in LLC-4. Cells were collected and the
Lewis lung carcinoma (LLC, CL1-1642) was obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 1% antibiotics.

Gene knockdown and overexpression

Knockdown of galectin-1, integrin β4 and Notch1 in CL1-0 and/or A549 cells was performed using a lentiviral expression system provided by the National RNAi Core Facility (Taipei, Taiwan). The lentiviruses were produced by cotransfecting HEK293T with pLKO-AS2, pLKO-AS2-galectin-1, pLKO-AS2-integrin β4 or pLKO-AS2-Notch1 shRNA, and two packaging plasmids (pCMV-DR8.91 and pMD.G). Stable clones were established via treatment with puromycin. The efficacy of galectin-1, integrin β4 and Notch1 plasmid was assessed by real-time PCR. The sequences of the shRNA for galectin-1, integrin β4 and Notch are as follows: galectin-1, 5′-AATTCTGGATCCACCTGCGACG-3′; integrin β4, 5′-TTCAATTTGATGACACCCTC-3′; Notch1, 5′-ATTGTCTTGTCCAGCGACG-3′. Cells were transfected with pCMV, pCMV-galectin-1 plasmid or pCMV-integrin β4 plasmid (Origene, Rockville, MD) and stable clones were established by treatment with G418.

For transient mouse galectin-1 inhibition, LLC-4 cells were transfected either with MISSION® siRNA Universal Negative Controls or galectin-1 siRNA (Sigma-Aldrich). The efficacy of small interfering RNA (siRNA) was assessed by quantitative reverse transcription (qRT)-PCR.

Cell migration and invasion

Cell migration was assessed by scratch wound-healing assay. Cells were allowed to grow into full confluence in 24-well plates. The following day, a uniform scratch was made down the center of the well using a micropipette tip, followed by washing once with phosphate-buffered saline. Photographic imaging was performed using the Olympus 1×50 inverted microscopes. Cell migration and invasion were also assessed by QCM™ 24-well Cell Migration Assay and Invasion System (Millipore), according to the manufacturer’s instructions.

Immunoblot/immunoprecipitation

Cells were lysed on ice for 15 min in a M-per solution. Cell lysis was centrifuged at 14 000g for 15 min, and the supernatant fraction collected for immunoblot analysis. Equivalent amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10–12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated with the desired primary antibody for 1–16 h. The membranes were then treated with appropriate peroxidase-conjugated secondary antibody and the immunoreactive proteins were visualized via enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions. The primary antibodies used in this study targeted N-cadherin and E-cadherin, Notch1, Jagged2, AKT, FAK, p-FAK and NICO, which were obtained from Cell Signaling Technology; Lyn, p-Lyn, integrin β4 and integrin α6 antibody were obtained from Abcam, Ltd. (Cambridge, UK). Glyceraldehyde-3-phosphate dehydrogenase was obtained from Upstate Biotechnology (Lake Placid, NY). For IP, cell lysates (200 μg of total protein) were incubated with 2 μg of anti-FAK or anti-integrin β4 antibody, and then with 20 μl of protein A-agarose beads (Millipore, Bedford, MA) for 2 h at 4°C. Phosphorylated FAK,integrin β4 and integrin α6 was detected by incubating the blots with specific antibodies.

Real-time RT–PCR

RNA isolation was performed using the TRIZol reagent (Invitrogen). Complementary DNA (cDNA) was prepared using an oligo (dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. qPCR was performed using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR mixture contained 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 μl cDNA and RNase-free water with a total volume of 20 μl. The PCR was carried out with a denaturation step at 95°C for 10 min, for 40 cycles at 95°C for 15 s and then at 60°C for 1 min. The following primers were used for target genes: galectin-1, 5′-ctctcgggtgcggcttctg-3′ and 5′-aggaaccttgctgctgaa-3′; integrin β4, 5′-ccctcttcctctcaacagcaac3′ and 5′-actctcgagccgagaaag-3′; Notch1, 5′-ctctgagccgagaaag-3′ and 5′-tggaggtgcctcag-3′; Jagged2, 5′-aggaaccttgctgctgaa-3′ and 5′-tggaggtgcctcag-3′; Hey-1, 5′-gtcagctgctgctgctgaa-3′ and 5′-ttggtgctgctgctgctgaa-3′; GAPDH, 5′-gtaggcaacagttgctgctg-3′ and 5′-ttggtgctgctgctgctgaa-3′. All PCRs were performed in triplicate and normalized to internally control glyceraldehyde-3-phosphate dehydrogenase mRNA. Relative expression was presented using the 2−ΔΔCT method.

Microarray

Microarray experimental procedures were carried out following the manufacturer’s protocols. Total RNA (1 μg) was amplified by an Agilent Quick Amp Labeling Kit (Agilent Technologies, CA). RNA was labeled by either Cy5 or Cy3 in an in vitro transcription process. About 0.825 μg of Cy-labeled cRNA was cleaved to an average size of about 50–100 nucleotides by incubation with fragmentation buffer (Agilent Technologies) at 60°C for 30 min. Equal Cy-labeled cRNA was pooled and hybridized to Agilent Whole Human Genome 4x44k oligo microarray (Agilent Technologies, CA) at 65°C for 17 h. After washing and drying by nitrogen gun blowing, microarrays were scanned by an Agilent microarray scanner (Agilent Technologies, CA) at 535 nm for Cy3 and 625 nm for Cy5. Scanned images were analyzed by Feature Extraction Software 10.5 (Agilent Technologies, CA), an image analysis and normalization software used to quantify signals and background intensity for each feature. The data were then substantially normalized by the rank-consistency-filtering LOWESS method.

Animal model

LLC cells were transplanted into C57BL/6 mice by tail vein injection. Animals were killed on day 20 after LLC transplantation, and LLC cells in the lungs were isolated by mincing, collagenase type I digestion and filtration. The LLC cells were cultured in a growth medium containing 10% fetal bovine serum and 1% penicillin–streptomycin and expanded for second-round transplantation. The subline of the first-round transplantation was designated as LLC-1, and sublines from rounds 2, 3 and 4 of transplantation were designated as LLC-2, -3 and -4, respectively.

LLC and galectin-1-knockdown LLC (clone 1 and 2) were transplanted into C57BL/6 mice by tail vein injection. Animals were killed on day 24 and the number of tumor nodules was recorded for analysis of lung cancer incidence.

Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons of the results were made using analysis of variance. Significant differences (P < 0.05) between the means of the two test groups were analyzed by Student’s t-test.

Results

Galectin-1 is overexpressed in highly invasive lung cancer cell lines, both in vitro and in vivo

To assess the role of galectin-1 in lung cancer, we investigated galec- tin-1 expression in a pair of isogenic lung cancer cell lines, CL1-0 (low invasiveness) and CL1-5 (high invasiveness). As shown in Figure 1A, the level of galectin-1 mRNA in CL1-5 cells is higher than that in CL1-0. Immunoblot analysis also reveals that high levels of galectin-1 presented in CL1-5, consistent with the results of qRT–PCR (Figure 1B).

To evaluate the requirements for galectin-1 in vivo cancer metastasis, we repeatedly transplanted LLC cells into mice by tail vein injection, thereby creating the LLC-1, LLC-2, LLC-3 and LLC-4 cell-line series. Galectin-1 expression was assessed by qRT–PCR. As shown in Figure 1C, the expression of increased galectin-1 varies with the number of transplantations (LLC-4 > LLC-3 > LLC-2 > LLC-1). The migratory ability of LLC cell lines is also enhanced by varying the number of transplantations (LLC-4 > LLC-3 > LLC-2 > LLC-1; Figure 1D). These data are consistent with the expression of galec- tin-1. In addition, knockdown of galectin-1 in LLC-4 cells by siRNA transfection decreases the migration of LLC-4, suggesting that galec- tin-1 is involved in the enhancement of migratory ability in mouse lung cancer cells (Figure 1E and F).

Overexpression of galectin-1 promotes epithelial-mesenchymal transition

We first studied whether endogenous galectin-1 expression was required for lung adenocarcinoma cell progression. We decreased amount of mRNA and protein was assessed by qRT–PCR and immunoblot analysis, respectively. LLC-4 cells were transfected with either control siRNA or mouse galectin-1 siRNA (10 nM). The invasiveness and migration ability of LLC and the cell sublines were quantified by QCM™ 24-well Cell Migration and Invasion assay. About 10% of fetal bovine serum acts as a chemotraactant for cancer migration and invasion. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the LLC-1 and LLC sublines, as analyzed by Student’s t-test (∗P < 0.05).
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The expression of galectin-1 by shRNA transfection in CL1-5 and A549 cells. Knockdown of galectin-1 in CL1-5 and A549 cells was determined by qRT–PCR and immunoblot analysis (Supplementary Figure 1A and B, available at Carcinogenesis Online). As shown in Figure 2A, knockdown of galectin-1 switched CL1-5 and A549 cells from a spindle-like fibroblastic morphology to cobblestone-like cells.

Overexpression of galectin-1 cDNA changes cell shape (C) and causes EMT (D) in CL1-0 cells. EMT-related protein levels were determined by immunoblot analysis. Results are representative of at least three independent experiments.

Fig. 2. Galectin-1 promotes EMT in lung cancer cells. Knockdown galectin-1 changes cell morphology (A) and causes MET (B) in both CL1-5 and A549 cells. Overexpression of galectin-1 cDNA changes cell shape (C) and causes EMT (D) in CL1-0 cells. EMT-related protein levels were determined by immunoblot analysis. Results are representative of at least three independent experiments.
Fig. 3. Galectin-1 is involved in the enhancement of cell migration and invasion. The migratory capacity of lung cancer cells is inhibited by galectin-1 shRNA transfection, as determined by wound-healing assay (A) and transwell assay (B). Inhibition of galectin-1 expression by shRNA decreases invasiveness (C). The migratory capacity of lung cancer cells is increased by galectin-1 cDNA transfection, as determined by wound-healing assay (D) and transwell assay (E). Overexpression of galectin-1 cDNA increases invasiveness (F). Cell migration was assessed by wound-healing assay. The invasiveness and migration ability of galectin-1 overexpressing and knockdown lung cancer cells were quantified as described above. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference among cells transected with control or expression plasmid, as analyzed by Student’s t-test (*P < 0.05).
Fig. 4. Galectin-1 regulates the integrin α6β4/FAK/Lyn/AKT pathway. (A) Microarray-based gene expression and Venn diagram. The levels of integrin α6 (B) and β4 (C) mRNA in galectin-1 knockdown and galectin-1 overexpressing lung cancer cells. The expression of integrin α6, integrin β4, FAK, Lyn and AKT in galectin-1-knockdown and galectin-1 overexpressing lung cancer cells, as determined by immunoblot analysis (D). The interaction of integrin α6 and
with well-organized cell contacts and polarity. Change in epithelial-mesenchymal transition (EMT) is an important process for cancer cells to develop the ability to metastasize (9,10). We, therefore, assessed the effect of galectin-1 on EMT. Inhibition of galectin-1 expression increased epithelial markers, including E-cadherin, claudin-3 and ZO-1, and reduced mesenchymal markers such as α-smooth muscle actin (α-SMA) in both CL-1-5 and A549 cells (Figure 2B), suggesting that galectin-1 is involved in the regulation of EMT.

To further validate the metastasis promoting activity of galectin-1, we generated overexpressing galectin-1 human lung cancer CL-1-0 cells stably expressing galectin-1 cDNA and confirmed exogenous protein expression by immunoblot analysis (Supplementary Figure 2, available at Carcinogenesis Online). Galectin-1 overexpressing CL-1-0 cells displayed a spindle-like fibroblastic morphology, one of the major characteristics of EMT (Figure 2C). The expression of epithelial markers, including E-cadherin, claudin-3 and ZO-1, is reduced in galectin-1 expressing cells (Figure 2D). In contrast, α-SMA, which is positively correlated with EMT, showed enhanced expression in galectin-1 overexpressing cells (Figure 2D), suggesting that galectin-1 is involved in the regulation of EMT.

**Galectin-1 was involved in the enhancement of cell migration and invasion consistent with EMT in lung cancer cells**

Because cell motility is an important factor regulating cancer invasiveness and metastasis, the effect of galectin-1 on cell motility was characterized by wound healing, transwell migration and Matrigel invasion assays. Scratch wound-healing assay showed that knockdown of galectin-1 remarkably decreases the migratory ability of both CL-1-5 and A549 cells (Figure 3A). The transwell migration assay indicated that inhibition of galectin-1 led to a marked decreased in cell motility compared with control shRNA plasmid–transfected CL-1-5 and A549 cells (Figure 3B). Similarly, the invasion assay showed that galectin-1-knockdown cells exhibited a significantly lower invasiveness than control cells (Figure 3C).

Scratch wound-healing assay indicated that overexpression of galectin-1 increases cell migration of CL-1-0 cells (Figure 3D). The transwell migration assay indicated that upregulation of galectin-1 leads to increased cell motility compared with pCMV-transfected CL-1-0 cells (Figure 3E). Similarly, the invasion assay showed that galectin-1 overexpressing cells exhibit significantly enhanced invasiveness than do pCMV vector-transfected cells (Figure 3F).

**The regulation of galectin-1 on expression of integrin α6β4 and the activation of FAK, PI3K/AKT signaling pathway**

To identify the downstream molecules regulated by galectin-1, we used microarray to determine the genes in which mRNA levels change due to galectin-1 overexpression or knockdown. We were specifically interested in those genes in which expression was reduced in galectin-1-knockdown cells but enhanced in galectin-1 overexpressing cells (Supplementary Table 1, available at Carcinogenesis Online, Figure 4A). We identified integrin α6 and β4 as candidate targets of galectin-1. qRT–PCR and immunoblot analysis also further confirmed that the expression of both integrin α6 and β4 at mRNA and protein levels was reduced in galectin-1-knockdown A549 and CL-1-5 cells, whereas overexpression of galectin-1 increased the amount of integrin α6 and β4 in CL-1-0 cells (Figure 4B–D). Immunoprecipitation data observed for the association of integrin α6 and β4 were enhanced in galectin-1 overexpression CL-1-0 cells, whereas the interaction was reduced in galectin-1-knockdown lung cancer cells (Figure 4E).

**Integrin β4 knockdown reverses galectin-1-mediated cell migration, invasion and EMT**

Galectin-1 increases integrin β4 expression and activates FAK/AKT signaling. We, therefore, examined whether the oncogenic effects of galectin-1 could be reversed by integrin β4 silencing. We transfected galectin-1 overexpressing CL-1-0 cells with integrin β4 shRNA plasmid, and the efficacy of shRNA was validated by immunoblot analysis (Figure 5A). Knockdown of integrin β4 decreases galectin-1 overexpression-mediated FAK and Lyn phosphorylation, cell migration, invasion and EMT (E-cadherin downregulation and α-SMA upregulation; Figure 5A and B). However, inhibition of integrin β4 partially decreases the activation of AKT (Figure 5A).

**The involvement of Notch1/Jagged2 in galectin-1-induced cancer progression**

Other factors that increase in galectin-1 overexpressing cells, but decrease in galectin-1-knockdown cells, are Notch1 and its ligand Jagged2, as determined by microarray and qRT–PCR (Supplementary Table 1, Figures 4A and 6A). The results also revealed that Notch transactivator NICD in the nuclear fraction and levels of Notch target Hey-1 mRNA were elevated in CL-1-0 cells expressing galectin-1, whereas NICD and Hey-1 were decreased in galectin-1-knockdown cells (Figure 6A and B). To determine whether Notch activation contributes to galectin-1-mediated cancer progression, we eliminated Notch1 by shRNA transfection in galectin-1 overexpressing CL-1-0 cells (Figure 6D). The data show that eliminating Notch1 decreases cell migration and invasion (Figure 6C), as well as EMT (Figure 6D) in galectin-1 overexpressing CL-1-0 cells. Moreover, inhibition of Notch1 also partially decreases the phosphorylation of AKT. These data suggest that Notch1 plays a critical role in galectin-1-induced lung cancer progression via AKT signaling.

**Inhibition of galectin-1 expression decreases lung metastasis of mice transplanted with LLC**

To test whether galectin-1 is responsible for lung cancer metastasis in vivo, we injected LLC and galectin-1-knockdown LLC cells into the tail veins of mice. Mice were killed 24 days after cell injection and the metastatic tumor nodules, which had formed in the lungs, were examined. As shown in Figure 7A, knockdown of galectin-1 decreased lung metastasis by 2-fold. Interestingly, vector plasmid–transfected LLC was observed in cancer cells invading the surrounding tissue, whereas the invasiveness of LLC was decreased by inhibiting galectin-1 (Figure 7B).

β4 (E). Cells were collected and the mRNA and the protein assessed by qRT–PCR and immunoblot analysis. For (E), whole-cell lysates were prepared and immunoprecipitation was performed with anti-integrin β4 antibody overnight at 4°C. The immunoprecipitates were subjected to immunoblot analysis with integrin α6 antibody. The immunoblot analysis of integrin β4 antibody was used for each immunoprecipitation to confirm whether equal immunoprecipitates were assessed. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference among the indicated comparison groups, as analyzed by Student’s t-test (*P < 0.05).
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Fig. 5. Integrin β4 knockdown decreases galectin-1-mediated cell migration, invasion and EMT. Knockdown of integrin β4 decreases FAK/AKT phosphorylation, E-cadherin downregulation, α-SMA upregulation (A), cell migration and invasion (B) in galectin-1 overexpressing CL1-0 cells. Ectopic expression of integrin β4 reverses the phosphorylation of FAK and AKT (C), cell migration and invasion (D) in galectin-1-knockdown CL1-5 cells. Galectin-1 overexpressing CL1-0 cells were transfected with control shRNA or integrin β4 shRNA, and stable colonies were created by G418 and puromycin selection. Galectin-knockdown CL1-5 cells were transfected with pCMV or pCMV-integrin β4 overexpressing plasmid, and stable colonies were created by G418 and puromycin selection. The invasiveness and migration ability of lung cancer cells were quantified as described above. Cells were collected and the protein assessed by immunoblot analysis. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference among the indicated comparison groups, as analyzed by Student’s t-test (*P < 0.05).
Fig. 6. The involvement of Notch1/Jagged2 in galectin-1-mediated cancer progression. (A) The mRNA levels of Notch1, Jagged2 and Hey-1 in galectin-1-knockdown CL1-5 and galectin-1 overexpressing CL1-0 cells. (B) The levels of Notch1, Jagged2 and NICD. (C) Knockdown of Notch1 reduced galectin-1-mediated cell migration and invasion. Knockdown of Notch1 decreases AKT phosphorylation, as well as EMT mediating by galectin-1 overexpression (D).
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Discussion

The vast majority of cancer deaths are due to metastasis, rather than the influence of the primary cancer (3). A series of in vitro and in vivo investigations were performed to assess the role of galectin-1 in the regulation of lung cancer cell migration and invasion, and the results showed that galectin-1 overexpression significantly enhances and contributes to cell motility, invasiveness and EMT in lung cancer.

The processes of cancer metastasis are complex. In the first step of metastasis, cancer cells dissociate from a primary tumor by losing cell–cell contact and transforming into a mesenchymal phenotype, resulting in increased motility and invasive abilities (33,34). The conversion of an epithelial phenotype to a mesenchymal phenotype is achieved by the downregulation of proteins related to tight junctions and the upregulation of focal adherin factors (35). It has been demonstrated that the switch from an epithelial phenotype to a mesenchymal phenotype is associated with metastasis and a poor prognosis for lung cancer patients (36,37). Our results demonstrate that ectopic expression of galectin-1 in CL1-0 cells increases the capacity for migration, invasion and EMT (E-cadherin, claudin-3 and ZO-1 downregulation, and α-SMA upregulation). In contrast, in metastatic CL1-5 or A549 cells that express high levels of endogenous galectin-1, transfection of specific shRNA against galectin-1 decreases migration and invasion abilities and causes MET (E-cadherin, claudin-3 and ZO-1 downregulation).

Fig. 7. Knockdown of galectin-1 decreases lung metastasis in vivo. (A) Inhibition of galectin-1 reduces the number of tumor nodules in the lungs of the mice. (B) Inhibition of galectin-1 decreases the invasiveness of LLC in mice. The knockdown of galectin-1 in LLC was carried out using galectin-1 shRNA lentiviral particles. Galectin-1 mRNA was assessed by qRT–PCR. Scramble shRNA and galectin-1 shRNA-transfected LLC were injected into mice via the tail vein. After 24 days, non-tumorous and tumorous regions of the lungs were harvested. The asterisk indicates a significant difference in LLC cells transfected with control shRNA plasmid versus galectin-1 shRNA plasmid (*P < 0.05) as analyzed by Student’s t-test.

Galectin-1 overexpressing CL1-0 cells were transfected with control shRNA or Notch1 shRNA, and stable colonies were created by G418 and puromycin selection. The invasiveness and migration ability of lung cancer cells were quantified as described above. Cells were collected and the protein was assessed by immunoblot analysis. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference among the indicated comparison groups, as analyzed by Student’s t-test (*P < 0.05).
upregulation, and α-SMA downregulation). Furthermore, knockdown of galectin-1 in LLC decreases the metastatic recurrence of LLC. Our previous study also reported that lung cancer patients express high amounts of galectin-1 compared with healthy donors (24,25). These results from experimental cell studies, mouse models and clinical patient sections strongly suggest that galectin-1 is an important mediator responsible for enhancing the development of lung cancer. 

Integrins, the family of heterodimeric transmembrane adhesion receptors, have been reported to be upregulated in various tumors, including lung cancer (9,38). Alteration of the expression or activation of individual integrins increases transduction of progrowth and promigratory cell signaling in tumors. Integrins are predominantly expressed in epithelial cells, where they serve as laminin receptors, and the α6 and β4 subunits are both upregulated during cancer progression (39,40). Interaction of integrin α6β4 with several tyrosine kinase receptors, such as IGFR, EGF and Met, triggers oncogenic signaling, leading to increased cancer growth, migration and invasion (41,42). After ligation, integrins have several adhesion complex--associated signaling molecules, such as FAK, that mediate their biological functions and also lead to cancer proliferation, migration and invasion (43,44). In addition, AKT, an important regulator of cancer metastasis, is activated following the tyrosine phosphorylation of FAK (45). In this study, we have shown that galectin-1 increases the expression and association of integrin β4 with α6 subunits, which in turn increases phosphorylation of FAK and AKT. Ectopic expression of integrin β4 restores cell migration, invasion and FAK activation in galectin-1-knockdown cancer cells, whereas elimination of integrin β4 prevents galectin-1-mediated cancer progression, suggesting that galectin-1 promotes cancer progression through integrin α6β4 and the FAK/AKT signaling pathway.

The Notch signaling pathway has been implicated in the pathogenesis of various cancers, including lung cancer (46). Elevated expression of Notch1 and its ligand in lung cancer supports the hypothesis that this pathway plays a crucial role in this type of cancer development. Notch signaling is known to be an activator of AKT signaling in various types of cells (47,48). The trigger of Notch pathway regulates anoikis and EMT in human papilloma virus–derived cancers by activating AKT signaling (49). Our study shows that ectopic expression of galectin-1 not only increases the levels of Notch and its ligand Jagged2 but also enhances AKT activation. In contrast, eliminating Notch by shRNA transfection partially decreases AKT activation induced by galectin-1 overexpression. The functional role of Notch1 on galectin-1-mediated cancer progression is also revealed by the inhibition of Notch1 decreasing the oncogenic potential of galectin-1-mediated cell migration, invasion and EMT.

Many studies have indicated that the oncogenic role of Notch may be related to a complex cross talk between Notch and other developmental or metastasis-related signaling pathways, such as sonic hedgehog, TGF-β, Wnt and integrin (50,51). Integrin regulates Notch expression and activation by GSK phosphorylation and cavelin1-lipid raft pathway (52,53). Conversely, Notch pathway enhances integrin–extracellular matrix affinity via R-Ras activation (53,54). Our findings demonstrate that both integrin α6β4 and Notch modulate AKT phosphorylation, which partially decrease during integrin β4 or Notch1 knockdown, supporting the hypothesis of co-operation between the two pathways. However, the mechanisms for this cross talk require further study and elucidation.

Taken together, our findings provide strong evidence that galectin-1 promotes lung cancer metastasis by using a loss-of-function mechanism. The role of galectin-1 in promoting activation of AKT after triggering integrin α6β4 and Notch1/Jagged2 is correlated with these two membrane receptors in metastatic phenotypes of tumor cells. Therefore, inhibition of galectin-1 may serve as a potential target for the development of therapies against early metastatic events.

Supplementary material
Supplementary Table 1 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/.

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