Am J Transl Res 2019;11(6):3862-3878
www.ajtr.org /ISSN:1943-8141/AJTR0095768

Original Article

Galectin-1 induces metastasis and epithelial-mesenchymal transition (EMT) in human ovarian cancer cells via activation of the MAPK JNK/p38 signalling pathway

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Received April 21, 2019; Accepted May 19, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: Background: It has been reported that Galectin-1 (Gal-1) indicates bad prognosis of patients with ovarian cancer, and Gal-1 overexpression promotes metastasis of ovarian cancer cells. Nevertheless, the underlying mechanisms of the Gal-1-mediated enhancement of metastasis are still unclear. Furthermore, little is known about whether Gal-1 affects epithelial-mesenchymal transition (EMT) in ovarian cancer. Methods: The human SKOV3-ip and SKOV3 cell lines were transfected with Gal-1 siRNAs and LV-Gal-1 lentivirus, respectively. Cell migration and cell invasion abilities were examined by transwell assays. Protein or mRNA levels of Gal-1, p-JNK1/2, t-JNK1/2, p-p38, t-p38 and EMT markers were detected via immunohistochemistry, qRT-PCR and western blot in SKOV3-ip as well as SKOV3 cells. A xenograft tumour model was used in vivo to ascertain whether upregulation of Gal-1 in ovarian cancer cells can enhance metastasis in vivo. Results: In a total of 107 human ovarian cancer tissues, higher Gal-1 expression strongly associated with higher histological grade, more lymph node metastases and more advanced FIGO stage, while lower E-cadherin expression strongly associated with higher histological grade, more lymph node metastases and more advanced FIGO stage. In vitro assays revealed that Gal-1 promoted migration and invasion of ovarian cancer cells, as well as EMT. Additionally, the results showed that Gal-1 enhanced EMT, migration and invasion by activating the MAPK JNK/p38 signalling pathway. Moreover, in vivo bioluminescence imaging revealed that Gal-1 modulated ovarian cancer metastasis in nude mice. Immunochemistry of xenograft tumour tissues confirmed that Gal-1 may modulate metastasis and EMT via the MAPK JNK/p38 signalling pathway. Additionally, treatment of Gal-1 mice with the MAPK JNK/p38 signalling pathway antagonists SB203580 or SP600125 reduced cancer metastasis. Conclusion: Gal-1 enhances metastasis and EMT of ovarian cancer cells via promoting the activation of the MAPK JNK/p38 signalling pathway, suggesting the possibility that Gal-1 is a molecular target to prevent and cure ovarian cancer metastasis.

Keywords: Galectin-1 (Gal-1), ovarian cancer, epithelial-mesenchymal transition (EMT), MAPK JNK/p38 signalling pathway

Introduction

Ovarian cancer is a serious threat to the physical and mental health of women [1]. Mostly, terminal ovarian cancer was diagnosed in patients, so their prognosis remains poor [2, 3]. Although increasing numbers of genes and signalling pathways, some of which are potential therapeutic targets, have been shown to be strongly correlated with the pathogenesis of ovarian cancer, practical and effective therapeutic targets have not been discovered, and the patient prognosis of ovarian cancer is still poor. For this reason, it is of vital importance to search for biomarkers related to early screening, diagnosis and prognosis.

Most tumour-related deaths in patients suffering from solid tumours are not due to the primary tumour but rather to metastasis or invasion. Most patients with ovarian cancer are diagnosed at terminal stages, and this cancer

type features high invasiveness [2]. The process of metastasis involves the dissemination of primary tumour cells to other places via complicated, multi-stage biological activities. Various reports have discovered that epithelial-mesenchymal transition (EMT) has a vital effect on the metastasis and invasion of tumour cells, manifested as the upregulation of mesenchymal markers, like vimentin, as well as the downregulation of epithelial-related genes, like E-cadherin [4]. EMT takes place in carcinoma progression, and the tumour cells become more aggressive [5]. Meanwhile, after EMT, tumour cells gain more metastatic and invasion potential, similar to embryonic mesenchymal cells, with an increased ability to invade adjacent stroma to form new tumour foci [5, 6].

Galectin-1 (Gal-1), which is encoded by the human LGALS1 gene, is part of the family of carbohydrate-binding proteins [7]. In the cell, Gal-1 takes part in sugar-dependent interactions with other proteins [8]. Out of the cell, autocrine sugar-dependent and paracrine interactions with β-galactoside-containing glycoconjugates can activate Gal-1 [9, 10]. Studies have shown that overexpression of Gal-1 correlates with cancer malignancy in various human malignant tumours [11-13], like ovarian cancer [14] and gastric cancer [15]. Moreover, our previous studies demonstrated that overexpression of Gal-1 associated with bad prognosis of epithelial ovarian cancer patients, and Gal-1 could enhance tumour progression as well as chemoresistance of ovarian cancer cells [16].

Previous researches have demonstrated that Gal-1 enhances carcinoma invasion by triggering the activation of the MAPK JNK/p38 signalling pathway [17, 18]. MAPKs include a class of serine/threonine kinases, which can be triggered by growth or stress factors, thus playing a vital part in signal transduction inside the cell. Upon stimulation, ERK, SAPK/JNK, or p38, which belong to MAPK protein subgroups, can be activated. It has been previously reported that MAPKs play vital roles in various biological activities related to malignant tumours, such as ovarian cancer [3, 19, 20]. At present, convincing evidence shows that the MAPK JNK/p38 signalling pathway closely correlates with EMT in several cancers, such as ovarian cancer [21, 22].

In the current research, we explored whether Gal-1 promotes EMT in ovarian cancer via triggering activation of the MAPK JNK/p38 signalling pathway. In our previous research, Gal-1 expression levels were higher in human ovarian cancer tissues than in normal ovarian tissues, and higher expression of Gal-1 was correlated with bad prognosis [16]. In the current research, we detected the relationship of Gal-1 as well as E-cadherin expression in human EOC tissues with clinicopathological characteristics of EOC patients. Based on the clinical results, in vitro as well as in vivo experiments were performed to detect whether the overexpression or silencing of Gal-1 in ovarian cancer cells affects cell migration, invasion and EMT and to analyse the underlying mechanisms. Our results reveal that Gal-1 promotes metastasis and enhances EMT in ovarian cancer by triggering activation of the MAPK JNK/p38 signalling pathway.

Materials and methods

Clinical samples and cell lines

A total of 107 human ovarian cancer tissues were obtained from patients who had surgery in the Obstetrics and Gynecology Hospital of Fudan University from 2016 to 2017, and patients' clinical data were obtained afterwards. The classification of clinical staging and histological grading of ovarian cancer were determined according to the FIGO 2014 system. Approval from the research ethics committee was obtained prior to the study. In addition, written informed consent from the patients were obtained before experiment for the use of their samples.

Human ovarian cancer cell lines (A2780/cp, A2780, SKOV3, SKOV3-ip and Hey cells) were obtained from ATCC. Cells were cultured based on the instructions provided. The above-listed cells were cultured in a humidified incubator with 5% CO_2.

Reagents and antibodies

The reagents SB203580 and SP600125 were obtained from Beyotime Biotechnology (Jiangsu, China). Anisomycin was obtained from MCE MedChemExpress (NJ, USA). Anti-Galectin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-t-p38, anti-p-p38, anti-t-

JNK1/2, and anti-p-JNK1/2 antibodies (Cell Signalling Technology, Danvers, MA, USA); and anti-GAPDH antibody (Cell Signalling Technology, Danvers, MA, USA) were used in this study.

Immunohistochemistry

Immunohistochemical (IHC) assay of ovarian cancer tissues was done according to the published protocol [1]. Five-micrometre sections of paraffin-embedded human ovarian cancer tissues were prepared for staining. After dewaxing, the sections were rehydrated, and antigen retrieval and endogenous peroxidase blocking were performed. The slides were incubated with primary antibodies overnight at 4°C. Afterwards, the slides were incubated with HRP-conjugated secondary antibodies for one hour at 37°C. Then, the slides were stained with a DAB staining kit (Guge Biotech, Wuhan, China) and haematoxylin (Guge Biotech, Wuhan, China). The staining density of the slides was judged as shown below: negative staining means negative or weak staining (less than 20% of cells showed light-brown staining); positive staining means moderate or strong staining (more than 20% cells showed brown or dark-brown staining).

Lentiviral production and transduction

The lentiviral vector carrying the Gal-1 gene (LV-Gal-1) and the negative control lentiviral vector were obtained from Genepharma (Shanghai, China). The lentiviral vectors were transfected into SKOV3 cells with 5 μg/ml polybrene (Genepharma, Shanghai, China). To obtain stably transfected cells, puromycin (Sigma-Aldrich) was added into the culture medium.

Transfection of siRNA

Gal-1 siRNAs (Genechem, Shanghai, China) were used to downregulate Gal-1 expression. The two siRNA sequences are shown below: Gal-1 siRNA-1 (5'-UUGCUGUUGCACACGAUGGUGUUGG-3'); Gal-1 siRNA-2 (5'-GCUGCCAGAUGGAUACGAAUUTGGA-3'). SKOV3-ip cells were transfected with Gal-1 siRNAs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Four to six hours post transfection, the cell culture medium was changed. After 48 hours of transfection, SKOV3-ip cells were used for further examination.

qRT-PCR

Total RNA was extracted by RNA Lysis Buffer (TaKaRa, Dalian, China). cDNA was obtained by reverse transcription using the RT Reagent Kit (TaKaRa, Dalian, China). Real-time PCR was carried out with the SYBR Green Real-Time PCR Master Mix (TaKaRa, Dalian, China). GAPDH was used as an internal reference. Primers used in this study are as follows: Gal-1 (forward) CTCCTGACGCTAAGAGCTTCG and (reverse) CCAGGCTGGAAGGGAAAGAC; MMP7 (forward) TTCCTCCACTCCATTTAGCA and (reverse) ACATTTATTGACATCTACCC); uPA (forward) TGATTACCCAAAGAAGGAGG and (reverse) GCAAGGCAATGTCGTTGT); Snail (forward) TCGGAAGCCTAACTACAGCGA and (reverse) AGATGAGCATTGGCAGCGAG; Slug (forward) AAGCATTTCAACGCCTCCAAA and (reverse) GGATCTCTGGTTGTGGTATGACA; FN (Fibronectin) (forward) CCATCGCAAACCGCTGCCAT and (reverse) CCATCGCAAACCGCTGCCAT; E-cadherin (forward) GCGTCCTGGCAGAGTGAATTTT and (reverse) GGCCTTTTGACTGTAATCACAAA); N-cadherin (forward) ATCCTACTGGACGGTTCG and (reverse) TTGGCTAATGGCACTTGA); and GAPDH (forward) TGACTTCAACAGCGACACCCA) and (reverse) CACCCTGTTGCTGTAGCCAAA.

Immunofluorescence assay

Cultured SKOV3-ip and SKOV3 cells were fixed with 4% polymerized formaldehyde for 15 min. 5% BSA was used for blocking for 1 hour, then cells were incubated with primary antibody diluted at 1:50 in 5% BSA overnight at 4°C. After washing with PBS, cells were incubated with secondary antibody diluted at 1:500 in 5% BSA for 2 hour at room temperature. The cells were washed with PBS and incubated with DAPI for 10 min at room temperature. Images were obtained using a confocal microscope.

Migration and invasion assays

For invasion assays, before seeding cells, 60 μl of Matrigel (BD Biosciences, San Diego, CA, USA) was placed on the upper surface of the 24-well transwell chamber (Corning, New York, USA). Cells (10^4) in 100 μl of RPMI 1640 medium were seeded in the upper chamber, and the lower chamber was filled with 600 μl of medium with 20% FBS. Twenty-four hours after incubation, cells remaining on the upper surface were removed using a cotton swab, while the invad-

ed cells were fixed, stained and photographed. Five random fields of cells were selected and counted for further calculation. For tumour cell migration assays, the transwell chamber was not pretreated with Matrigel, while the other procedures were the same as in the tumour cell invasion assays.

Western blot

Cells were lysed with RIPA buffer (Beyotime, Shanghai, China) to obtain total protein. Then, 30-50 μg of protein was separated in 10% SDS/PAGE gels and transferred to PVDF membranes, which were blocked with 5% fat-free milk. The membranes were then incubated overnight at 4°C with a primary antibody and incubated at room temperature for one hour with a secondary antibody conjugated with horseradish peroxidase. In the end, the protein bands were examined with chemiluminescence assay.

Tumour xenograft and bioluminescence imaging

A total of 16 female nude mice (6 weeks old) were fed in a standard environment. SKOV3 cells stably transfected with firefly luciferase and LV-Gal-1/LV-GFP were generated, and 5 × 10^6 cells were injected intraperitoneally into nude mice. For all 16 nude mice, 4 were injected with SKOV3-LUC-GFP cells, and the other 12 were injected with SKOV3-LUC-Gal-1 cells. Mice injected with SKOV3-LUC-Gal-1 cells were divided into three groups at random (4 mice per group): (a) vehicle control, (b) intraperitoneal (i.p.) treatment with p38 inhibitor (SB203580), and (c) i.p. treatment with JNK inhibitor (SP-600125). Three weeks later, an i.p. injection of luciferin (Promega) was given to the mice, and luciferase activity detected in an In-Vivo Xtreme II imaging system (Bruker, Germany). Afterwards, the nude mice were decapitated and the whole xenografted tumours were surgically removed and fixed in formalin for immunohistochemistry staining (IHC).

Statistical analyses

SPSS 16.0 (IBM, USA) was used for the statistical analyses. Continuous data was expressed as the mean ± SD, and analysed by independent t-test between two groups. Among multiple groups, one-way ANOVA was applied, and Turkey test was applied as a post hoc test. The categorical data were compared via the Chi-squared or Fisher's exact tests as appropriate. A p value < 0.05 was regarded as statistically significant.

Results

High expression of Gal-1 is closely correlated with EMT and metastasis in human ovarian cancer tissues

To explore the relationship between Gal-1 expression and EMT in ovarian cancer, immunohistochemistry assays were carried out to detect the expression levels of Gal-1 and E-cadherin in 107 cases of epithelial ovarian cancer tissues (**Figure 1**). **Table 1** demonstrates the clinicopathological characteristics of these patients and the relationship between these features and Gal-1 as well as E-cadherin expression. Higher Gal-1 expression was closely associated with higher histological grade, more lymph node metastases and more advanced FIGO stage, while lower E-cadherin expression was closely associated with higher histological grade, more lymph node metastases and more advanced FIGO stage. Moreover, the Spearman rank correlation analysis demonstrated a negative correlation between the expression of Gal-1 and E-cadherin in ovarian cancer (**Table 2**). In conclusion, these clinical data suggest that high expression of Gal-1 closely correlated with EMT and metastasis in human ovarian cancer tissues.

Gal-1 enhances the migration as well as invasion of ovarian cancer cells

To explore whether Gal-1 can promote the metastasis of ovarian cancer, qRT-PCR was used to examine Gal-1 expression in five ovarian cancer cell lines: A2780/cp, A2780, SKOV3, SKOV3-ip and Hey cells (**Figure 2A**). Among these cells, SKOV3-ip cells had the highest expression of Gal-1, while SKOV3 cells showed the lowest level of Gal-1 expression (**Figure 2A**). As Galectins can exert different, often contradictory functions in cancer depending of their intracellular/extracellular localization, immunofluorescence assay was performed to determine whether Gal-1 was expressed in cytosolic and/or nuclear compartments in SKOV3-ip and SKOV3 cells. Results showed that Gal-1 was located in cytosolic compartments of both cells (**Figure 2B**).

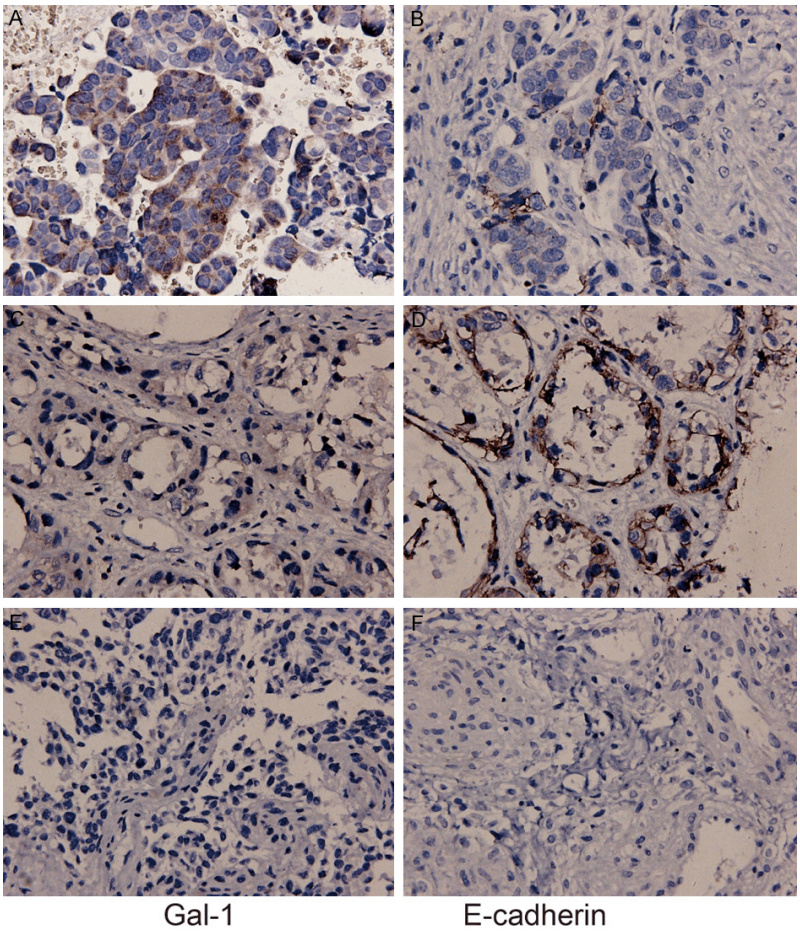


Figure 1. Representative images of immunohistochemically Gal-1 and E-cadherin staining in human ovarian cancer tissues. Typical image of positive cytosolic Gal-1 staining (A) and typical image of negative E-cadherin staining (B) of a same sample. Typical image of negative Gal-1 staining (C) and typical image of positive E-cadherin staining (D) of a same sample. Negative control of Gal-1 (E) and E-cadherin (F) staining.

Then, we detected the effect of Gal-1 on cell motility and transmigration of SKOV3-ip and SKOV3 cells via transwell migration as well as invasion assays. Because SKOV3-ip cells had the highest expression of Gal-1, siRNAs were applied to silence Gal-1 expression in SKOV3-ip cells. Gal-1 siRNAs significantly reduced the mRNA and protein expression of Gal-1 (> 70%; **Figure 2C**), and cell migration as well as invasion abilities were dramatically decreased in Gal-1 siRNA-transfected SKOV3-ip cells after comparing to negative control cells (**Figure 3A**). Additionally, a lentiviral vector (LV-Gal-1) was used to upregulate Gal-1 expression in SKOV3 cells (**Figure 2D**). Upregulation of Gal-1 notably enhanced cell migration as well as invasion ability after comparing to control (GFP) in SKOV3 cells (**Figure 3C**). Moreover, in order to determine whether the observed phenotype in ovarian cancer cells was mediated by intracellular or secreted gal-1, a blocking experiment was performed. As showed in **Figure 3B**, Gal-1 antibody did not affect cell migration and invasion, which suggested that intracellular Gal-1 mediated the observed phenotype. These above data indicate that intracellular Gal-1 may play a vital part in increasing the migration as well as invasion abilities of ovarian cancer cells.

Gal-1 promotes EMT in ovarian cancer cells

Accumulating evidence has shown that EMT of primary carcinomas can initiate metastasis [23], and EMT plays a key part in cancer invasion as well as metastasis [24]. To explore the influence of Gal-1 on EMT in human ovarian cancer cells, we detected EMT-related mRNA and protein levels via qRT-PCR and western blot in Gal-1 siRNA-transfected SKOV3-ip cells and SKOV3-Gal-1 cells. In Gal-1 siRNA-transfected SKOV3-ip cells, we observed notably increased mRNA levels of E-cadherin but significantly decreased levels of MMP7, uPA, fibronectin (FN), N-cadherin, Snail and Slug (**Figure 4A**). At the protein level, E-cadherin was increased, while N-cadherin and vimentin were significantly decreased (**Figure 4C**). In contrast, SKOV3-Gal-1 cells showed decreased mRNA levels of E-cadherin but increased mRNA levels of MMP7, uPA, fibronectin (FN), N-cadherin, Snail and Slug (**Figure 4B**). Simultaneously, the E-cadherin protein level decreased in SKOV3-Gal-1 cells, while N-cadherin and vimentin increased in SKOV3-Gal-1 cells (**Figure 4D**). These data strongly indicate that Gal-1 initiates EMT in

Table 1. Relationship between Gal-1 and E-cadherin immunostaining and the clinicopathological features of 107 patients with ovarian cancer cases assessed using the chi-square test

Parameters	n	Gal-1			E-cadherin		
		+	-	*p* value	+	-	*p* value
Age (years)							
< 60		34	26	0.936	30	30	0.647
≥ 60		27	20		24	23	
Tumor Size							
< 5 cm		25	18	0.847	26	17	0.09
≥ 5 cm		36	28		28	36	
Histological subtype							
Serous		38	27	0.903	36	29	0.426
HGSC		33	24		32	25	
LGSC		5	3		4	4	
Other		23	19		18	24	
Histological grade							
Low		19	28	0.002	38	9	< 0.001
High		42	18		16	44	
FIGO stage							
I+II		23	30	0.005	34	19	0.005
III+IV		38	16		20	34	
Lymph Nodes Metastasis							
NO		23	29	0.009	35	17	< 0.001
YES		38	17		19	36	

HGSC: High-grade serous carcinoma; LGSC: Low-grade serous carcinoma.

Table 2. Relationship between Gal-1 and E-cadherin expressions in 107 human primary ovarian cancer tissues

	Gal-1		R	*p* value
	+	-		
E-cadherin				
+	19	35	-0.441	< 0.001
-	42	11		

ovarian cancer cells. In summary, the above-mentioned results demonstrate that Gal-1 plays a vital part in the EMT-MET plasticity of ovarian cancer cells.

Gal-1 promotes EMT via the MAPK JNK/p38 signalling pathway in ovarian cancer cells

Previous studies suggested that the activated MAPK JNK/p38 pathway could induce EMT in malignant tumours [25]. Consequently, we explored whether the MAPK JNK/p38 pathway participated in the regulation of EMT by Gal-1 in Gal-1 siRNA-transfected SKOV3-ip cells and SKOV3-Gal-1 cells. In Gal-1 siRNA-transfected SKOV3-ip cells, the levels of p-JNK/t-JNK and p-p38/t-p38 were dramatically reduced (**Figure 5A**), but these levels were significantly promoted in SKOV3-Gal-1 cells (**Figure 5B**). The above results suggest that Gal-1 can trigger the activation of the MAPK JNK/p38 signalling pathway. To test whether the MAPK JNK/p38 pathway correlates with the regulatory effect of Gal-1 on EMT in ovarian cancer cells, we tested the relationship between Gal-1-induced EMT and the MAPK JNK/p38 signalling pathway with the MAPK p38 antagonist SB203580, MAPK JNK antagonist SP600125 and MAPK JNK/p38 pathway agonist anisomycin. In Gal-1 siRNA-transfected SKOV3-ip cells, anisomycin significantly decreased E-cadherin expression and upregulated N-cadherin and vimentin expression (**Figure 5C**). Moreover, in SKOV3-Gal-1 cells, both SB-203580 and SP600125 significantly reduced N-cadherin and vimentin expression and upregulated E-cadherin expression (**Figure 5D**). Additionally, in Gal-1 siRNA-transfected SKOV3-ip cells, anisomycin significantly enhanced cell migration and invasion abilities (**Figure 6A** and **6B**). In control siRNA-transfected SKOV3-ip cells, anisomycin did not affect cell migration or invasion abilities, possibly because the basal phosphorylation levels of MAPK JNK/p38 were already relatively high. At the same time, in SKOV3-Gal-1 cells, both SB203580 and SP-600125 significantly decreased migration and invasion abilities (**Figure 6C** and **6D**). In SKOV3-GFP cells, SB203580 or SP600125 did not have the same effects, possibly because the basal phosphorylation levels of MAPK JNK/p38 were relatively low. In summary, the above data demonstrate that Gal-1 may promote the metastasis of ovarian cancer cells and regulates EMT via the MAPK JNK/p38 pathway.

Upregulation of Gal-1 promotes the metastasis of EOC in a nude mouse model

To verify the relationship between Gal-1 expression and tumour cell metastasis, a xenograft

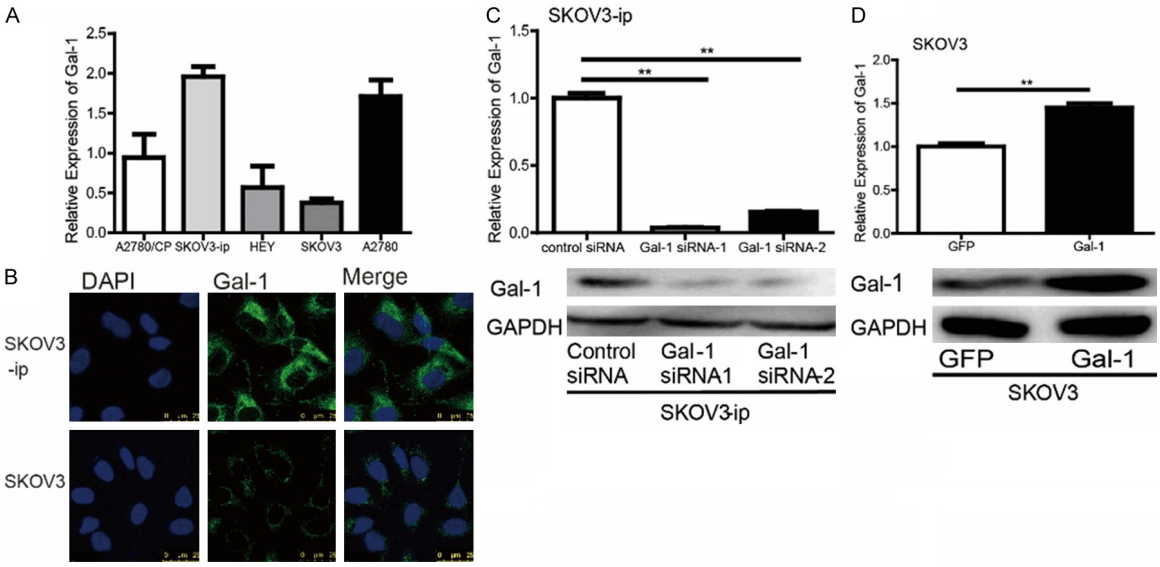


Figure 2. Expression and location of Gal-1 in different ovarian cancer cells. A. Gal-1 expression in the A2780/cp, A2780, SKOV3, SKOV3-ip and HEY cell lines was detected by qRT-PCR. B. Cytosolic expression of Gal-1 via immunofluorescence assay in SKOV3-ip and SKOV3 cells. C. Silencing of Gal-1 in SKOV3-ip cells decreased Gal-1 expression, which was detected by qRT-PCR and western blot. D. Overexpression of Gal-1 in SKOV3 cells increased Gal-1 expression, which was detected by qRT-PCR and western blot. **, $P < 0.01$.

tumour model was tested. Before cell injection, we found no significant difference in the luminescence intensity of SKOV3-LUC-GFP and SKOV3-LUC-Gal-1 cells (**Figure 7A**). Twenty-one days after injection, primary and metastatic tumours were examined on the basis of the luminescence of luciferase. Photon counts increased in the primary and metastatic sites in the SKOV3 Gal-1 group (**Figure 7B**). Three mice injected with SKOV3-Gal-1 cells developed metastases (multiple small metastatic nodules) under the diaphragm and on the surface of the liver, but no mice injected with SKOV3-GFP cells had metastatic tumours (**Figure 7C**). Moreover, we measured numbers of tumor implants and tumor weights. As showed in **Figure 7E**, there was significant difference between GFP group and Gal-1 group. These data suggest that the upregulation of Gal-1 can promote the metastasis of SKOV3 cells in vivo.

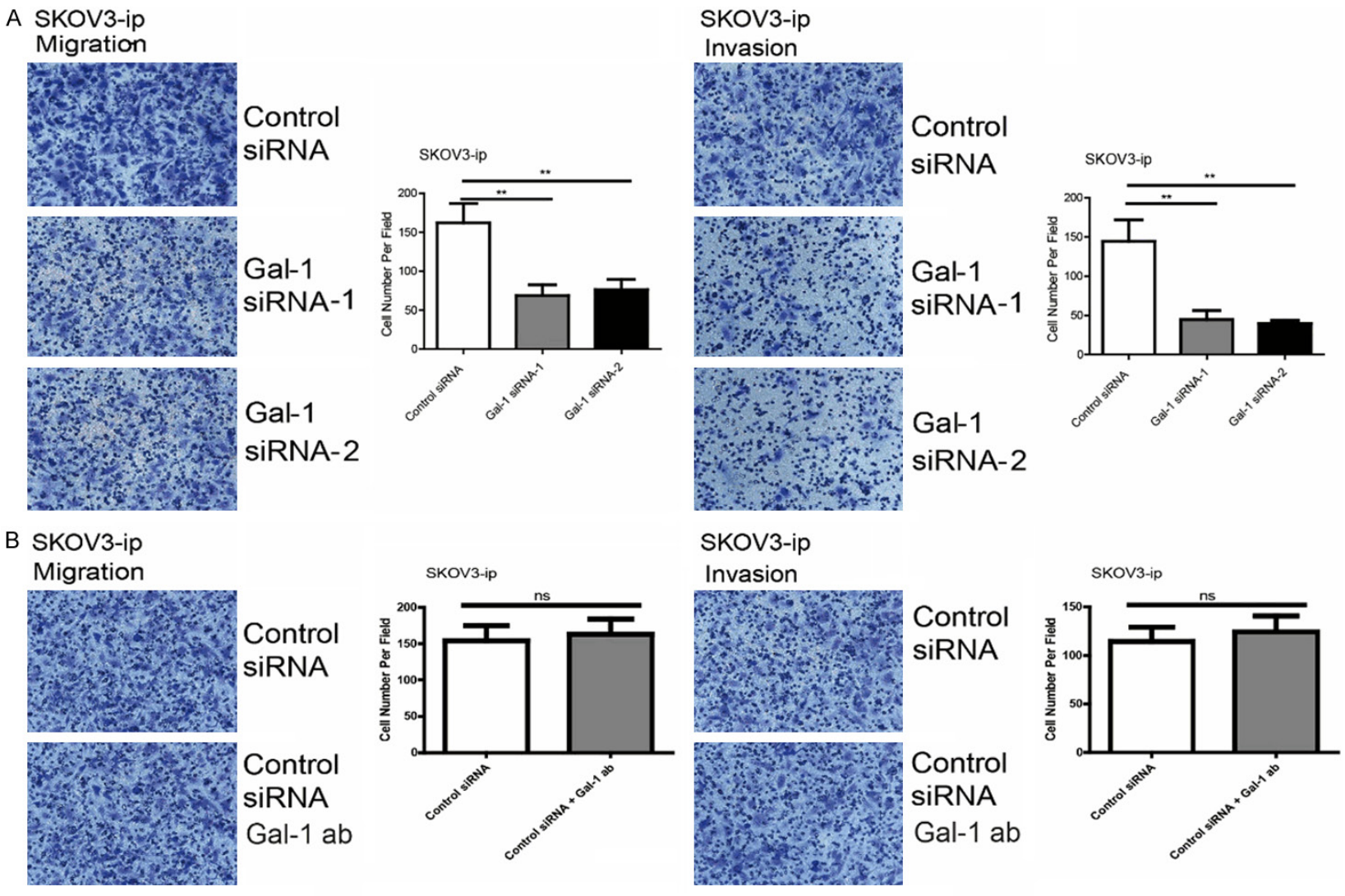
Next, we investigated the protein levels of Gal-1, E-cadherin, N-cadherin, vimentin, p-p38 and p-JNK in xenografted tumour slices. We found that the SKOV3-Gal-1 group with high Gal-1 expression had high levels of vimentin, N-cadherin, p-p38, and p-JNK and low levels of E-cadherin. On the contrast, xenografted tumour tissues from the SKOV3-GFP group with low Gal-1 expression exhibited strong E-cadherin expression and weak vimentin, N-cadherin, p-p38, and p-JNK expression (**Figure 7D**).

Additionally, we found that treatment of Gal-1 mice with SB203580 or SP600125 reduced cancer metastasis (**Figure 7E**), further suggesting that Gal-1 promoted the metastasis of ovarian cancer cells via the MAPK JNK/p38 pathway.

Discussion

The development and progression of ovarian cancer is a complex, multi-stage process accompanied by various genetic changes, the overexpression of oncogenes, the downregulation of tumour suppressor genes, and the acquisition of metastatic capability [26]. A majority of deaths caused by ovarian cancer are due to tumour metastasis, tumour recurrence, and delayed diagnosis of advanced stage disease [27]. Nevertheless, sensitive diagnostic markers, effective drug targets and potent treatment strategies are still lacking, which results in the high mortality of ovarian cancer patients.

Galectins are soluble proteins that are widely expressed in a lot of cell types and mediate their functions both inside and outside the cells. Up to now, a total of 11 galectins have


A
SKOV3-ip
Migration
Control siRNA
Gal-1 siRNA-1
Gal-1 siRNA-2
SKOV3-ip
Cell Number Per Field
Control siRNA
Gal-1 siRNA-1
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SKOV3-ip
Invasion
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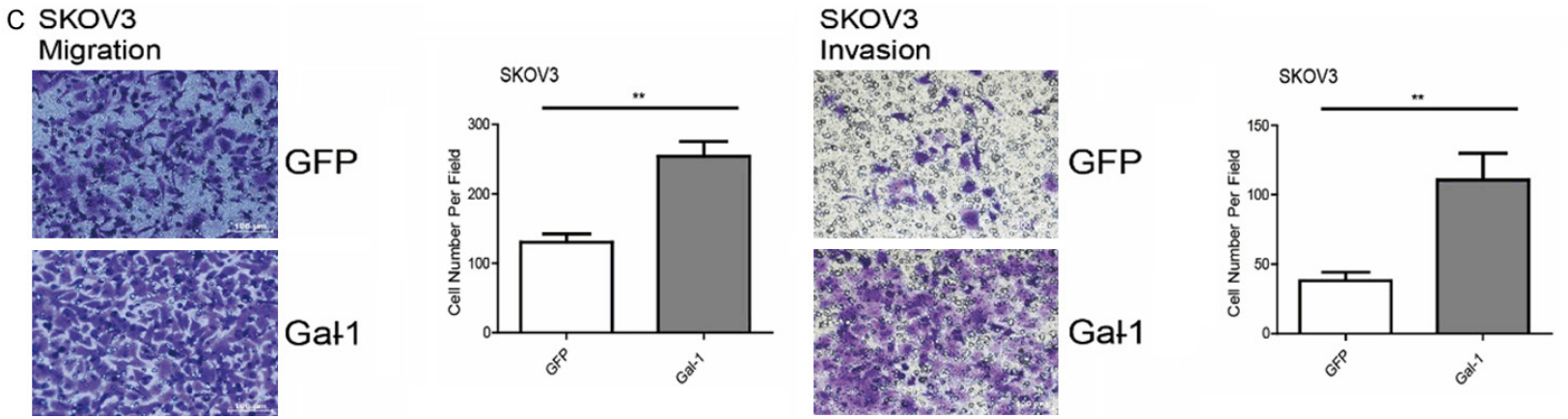
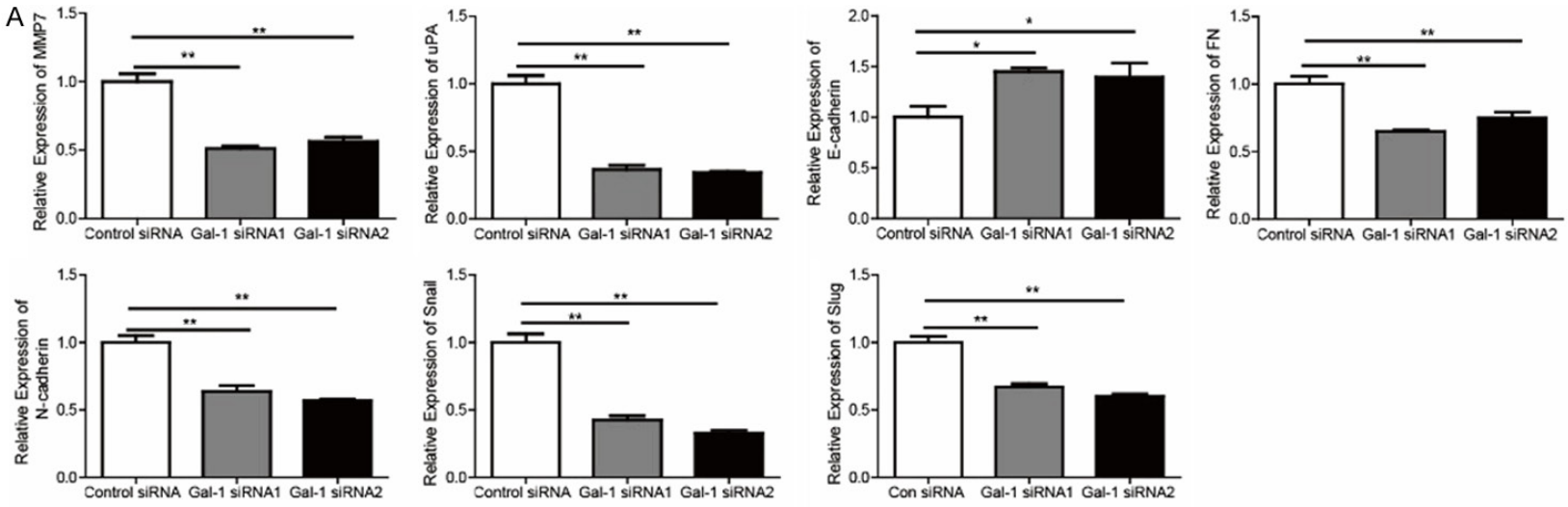


Figure 3. Gal-1 promotes cell migration and invasion in different ovarian cancer cells in vitro. A. Silencing of Gal-1 via siRNAs in SKOV3-ip cells decreased migration to and invasion of the bottom of transwell filters. B. SKOV3-ip cells transfected with control siRNA were treated with or without anti-galectin-1 antibodies (Gal-1 ab; 2 μg/ml). After 48 h, migration and invasion assay were performed. There was no significant difference between two groups. C. Overexpression of Gal-1 via lentivirus in SKOV3 cells increased migration to and invasion of the bottom of transwell filters. Ns, none significant; **, $P < 0.01$.



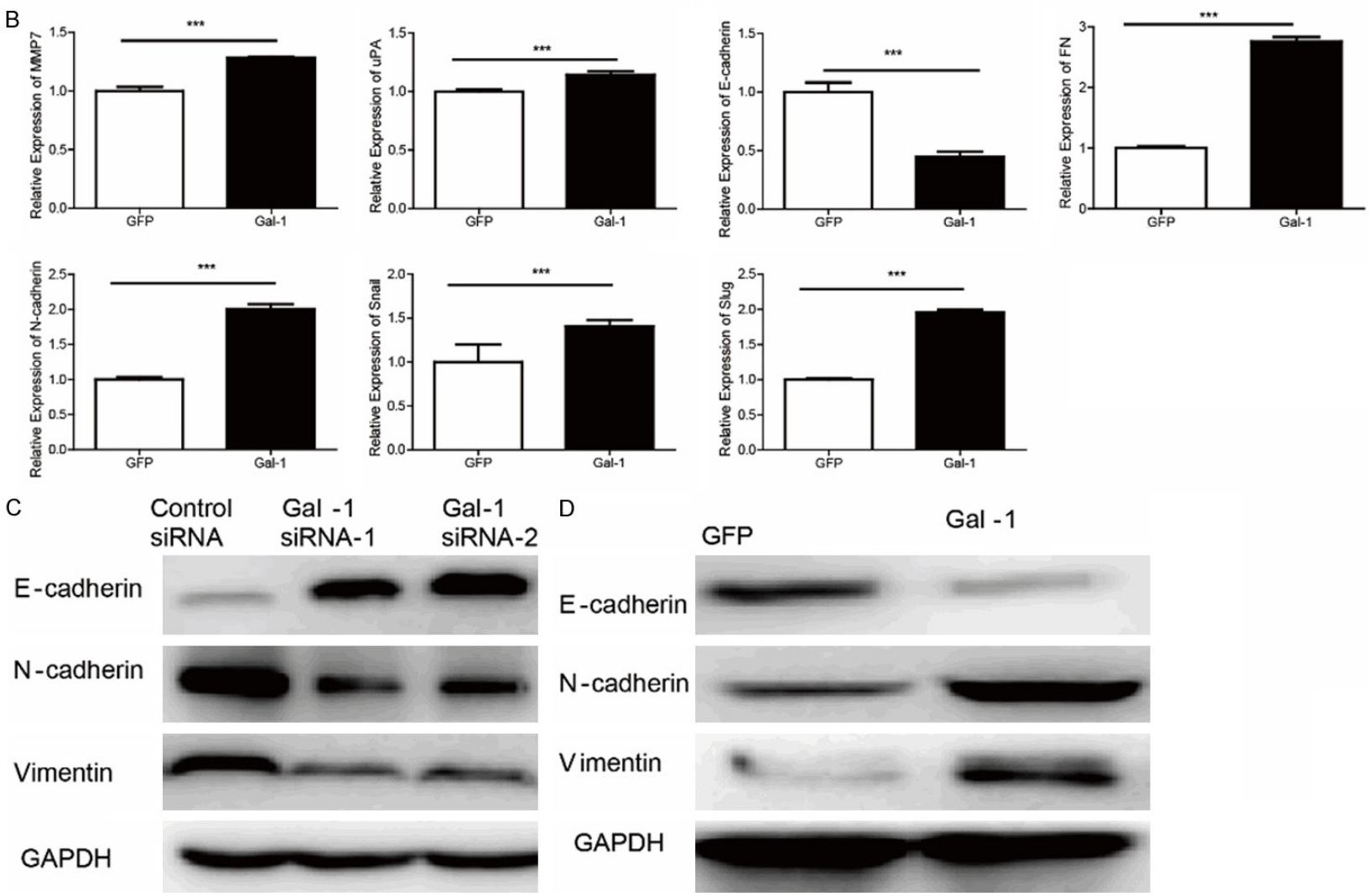


Figure 4. Gal-1 regulates the transition between epithelial and mesenchymal phenotypes in human ovarian cancer cells. A. The mRNA expression levels of epithelial and mesenchymal markers were assessed in Gal-1-silenced SKOV3-ip cells by qRT-PCR. B. The mRNA expression levels of epithelial and mesenchymal markers were assessed in Gal-1-overexpressing SKOV3 cells by qRT-PCR. C. Silencing of Gal-1 in SKOV3-ip cells markedly increased E-cadherin expression and decreased vimentin and N-cadherin expression as detected by western blot. D. Gal-1 overexpression in SKOV3 cells markedly decreased E-cadherin expression and increased vimentin and N-cadherin expression as detected by western blot. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

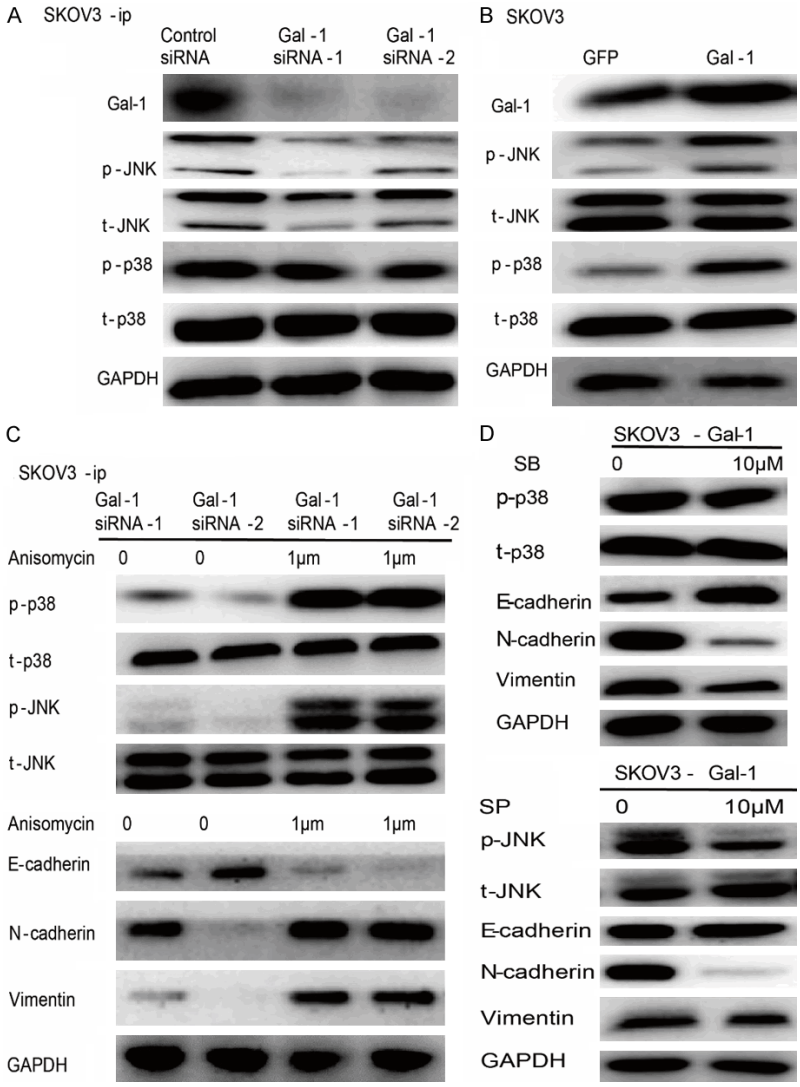


Figure 5. Gal-1 regulates EMT via activation of the MAPK JNK/p38 signalling pathway in ovarian cancer cells. A. Effects of silencing Gal-1 expression on MAPK JNK and p-38 phosphorylation in SKOV3-ip cells as detected by western blot. B. Effects of Gal-1 overexpression on MAPK JNK and p38 phosphorylation in SKOV3 cells as detected by western blot. C. Effects of anisomycin on the MAPK JNK/p38 signalling pathway and EMT markers in Gal-1-silenced SKOV3-ip cells as detected by western blot. D. Effects of the MAPK p38 antagonist SB203580 and the MAPK JNK antagonist SP600125 on the MAPK JNK/p38 signalling pathway and EMT markers in Gal-1-overexpressing SKOV3 cells as detected by western blot.

been found in humans, acting both intracellularly and extracellularly. Among them, Galectin-1, Galectin-3 and Galectin-9 have drawn the most attention of investigators, focusing on cell biology and immunology [28]. These galectins have diverse effects on tumours, including activating oncogenic signal pathways [29-31], regulating tumour cell growth or apoptosis [32], modulating cell migration and suppressing immune responses [33].

We previously reported that Gal-1 increases the progression as well as drug-resistance of human ovarian cancer [16]. The purpose of this study was to further explore how Gal-1 regulates ovarian cancer. This study demonstrated that higher Gal-1 expression closely associated with higher histological grade, more lymph node metastases and more advanced FIGO stage. Moreover, results showed a negative correlation between the expression of Gal-1 and E-cadherin in ovarian cancer. In vitro, we first detected the expression of Gal-1 in several epithelial ovarian cancer cells. Gal-1 expression was the lowest in SKOV3 cells and the highest in SKOV3-ip cells. SKOV3 and SKOV3-ip cells are a pair of epithelial ovarian cancer cells. SKOV3 is a low metastasis cell line, while SKOV3-ip is highly metastatic. In epithelial ovarian tissues, results indicated that higher Gal-1 expression closely associated with higher histological grade, more lymph node metastases and advanced FIGO stage. The results of the in vitro experiments coincide with the results for cancer tissue, as the lower metastatic SKOV3 cells had lower expression of Gal-1, while the more highly metastatic SKOV3-ip cells had higher expression of Gal-1. Next, we changed Gal-1 expression in SKOV3 and SKOV3-ip cells. Upregulation of Gal-1 in ovarian cancer cells led to EMT and enhanced migration and invasion, while downregulation of Gal-1 had the opposite effects. Furthermore, we revealed crosstalk between Gal-1 and the MAPK JNK/p38 signalling pathway. In addition, upregulation of Gal-1 enhanced migration and invasion of human ovarian cancer cells in the nude

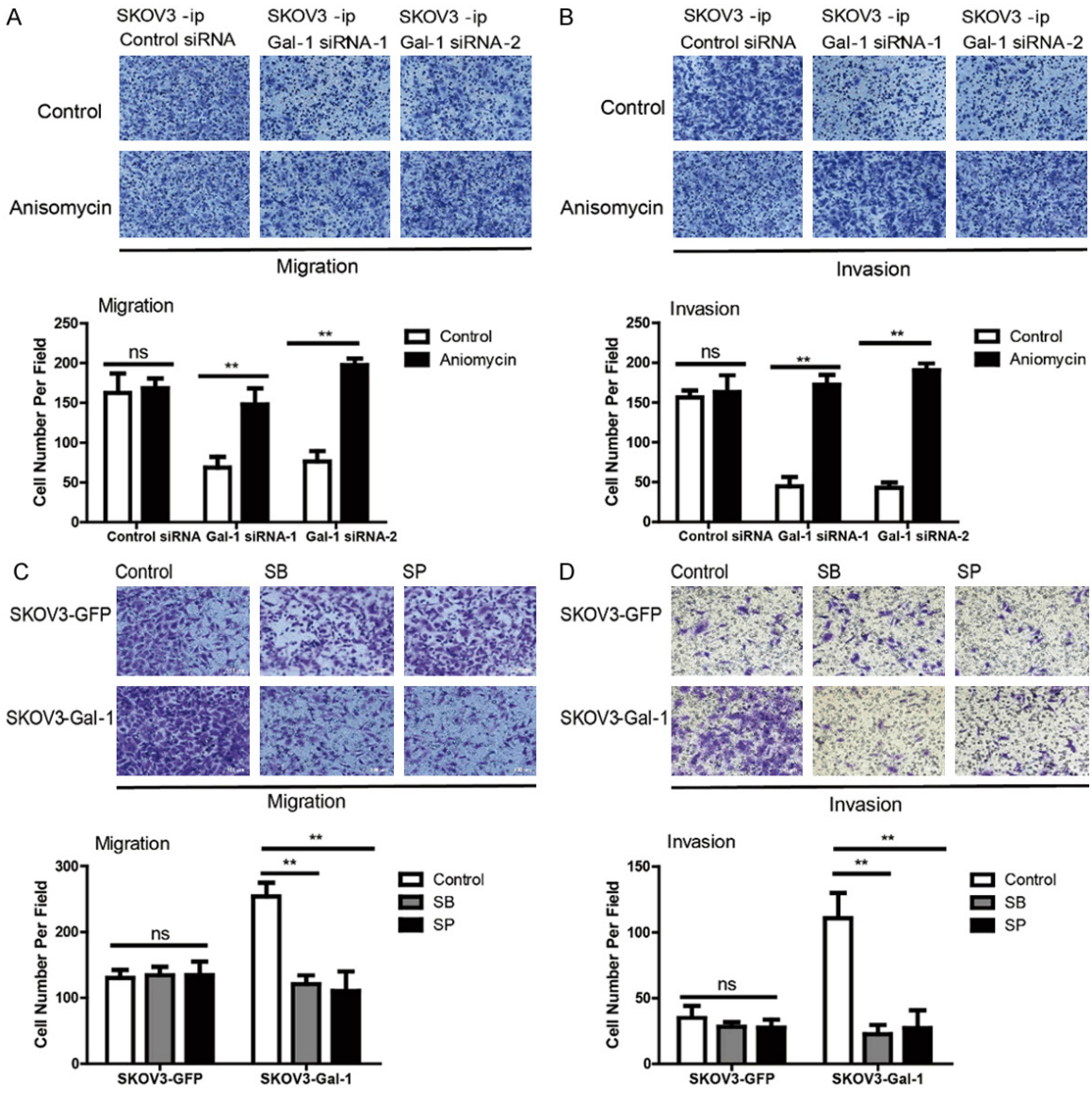


Figure 6. Effects of the MAPK JNK/p38 signalling pathway on cell migration and invasion regulated by Gal-1. A and B. Representative crystal violet staining of SKOV3-ip cells that migrated to or invaded the bottom of transwell filters with or without anisomycin. Anisomycin increased migration to and invasion of the bottom of transwell filters of Gal-1-silenced SKOV3-ip cells. C and D. Representative crystal violet staining of SKOV3 cells that migrated to or invaded the bottom of transwell filters with or without SB203580 and SP600125. SB203580 and SP600125 decreased migration to and invasion of the bottom of transwell filters of Gal-1-overexpressing SKOV3 cells. **, $P < 0.01$, ***, $P < 0.001$.

mouse model. Based on these data, we conclude that Gal-1 enhances metastasis and EMT in ovarian cancer by regulating the MAPK JNK/p38 signalling pathway (**Figure 8**).

Gal-1 is a part of the galectin family of proteins with conserved carbohydrate-recognition domains [34]. Galectin-1 is upregulated in many cancers like colon [35], breast [36], lung, ovarian [37] and prostate cancer [38]. In most instances, high expression of Gal-1 expression is related to tumour metastasis. Gal-1 takes part in many cancer-causing processes like transformation [39], metastasis [39, 40], cell proliferation [41] and cell migration [35]. It has also been reported that Gal-1 participates in tumour angiogenesis [42]. The current research reveals a new role of Gal-1 in ovarian cancer metastasis via EMT induction by activating the MAPK JNK/p38 signalling pathway. Ectopic

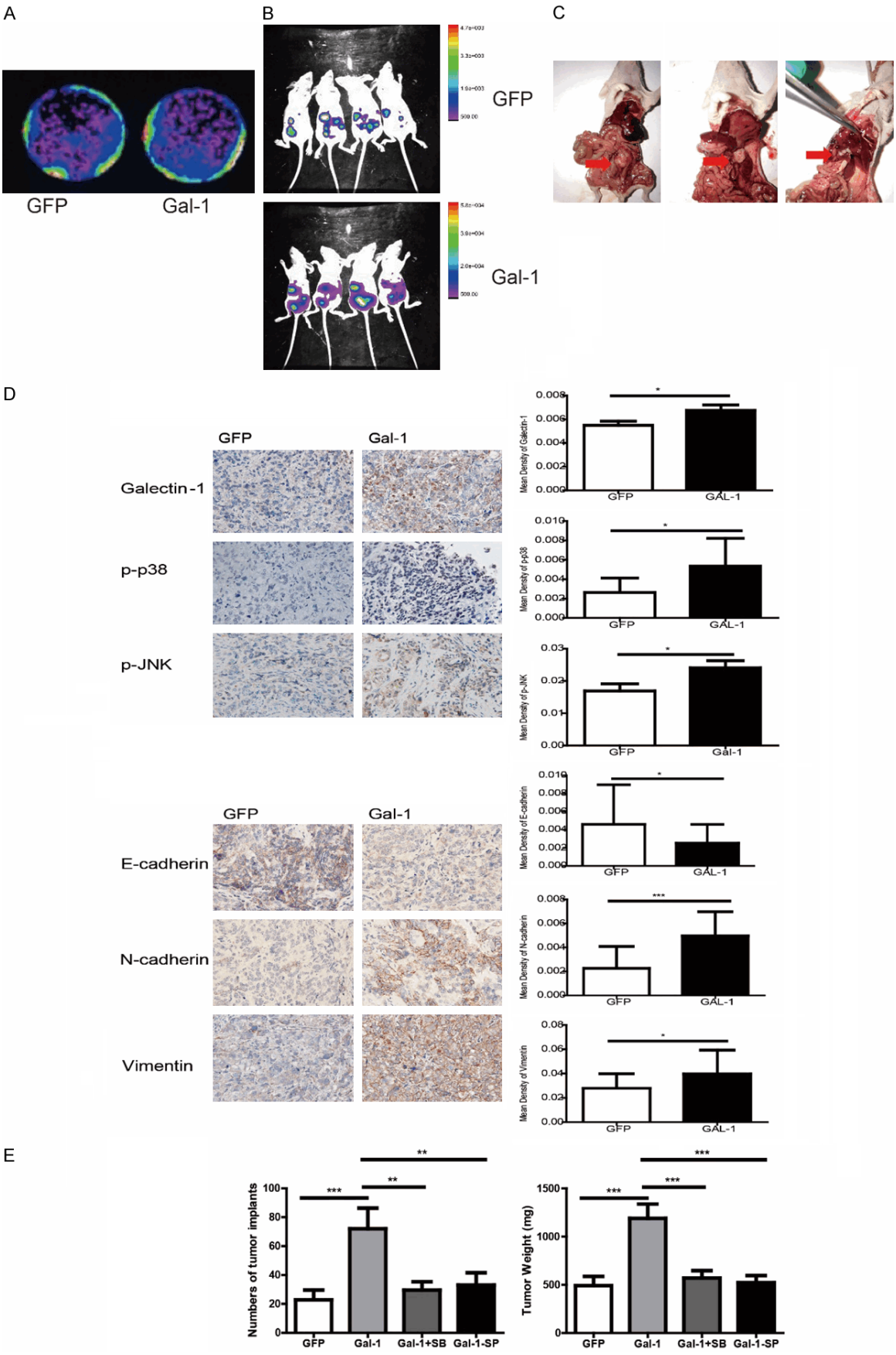

A
GFP
Gal-1
B
GFP
Gal-1
C
D
GFP
Gal-1
Galectin-1
p-p38
p-JNK
E-cadherin
N-cadherin
Vimentin
Mean Density of Galectin-1
Mean Density of p-p38
Mean Density of p-JNK
Mean Density of E-cadherin
Mean Density of N-cadherin
Mean Density of Vimentin
GFP
GAL-1
E
Numbers of tumor implants
Tumor Weight (mg)
GFP
Gal-1
Gal-1+SB
Gal-1-SP

Figure 7. Gal-1 in SKOV3 cells accelerates tumour cell metastasis in mouse tumour xenografts in vivo. A. The initial bioluminescence images of SKOV3-LUC-GFP and SKOV3-LUC-Gal-1 cell lines. B. The bioluminescence images of xenografted tumours were taken 21 days after injection. C. Anatomic images showing metastases on the liver surface and sub-mesentery in mice with SKOV3-LUC-Gal-1 cell xenograft tumours. D. Representative IHC staining of Gal-1, p-JNK, p-p38, E-cadherin, N-cadherin and vimentin in the xenografted tumour tissues of nude mice. E. Number of tumour implants and tumour weight in the four groups. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

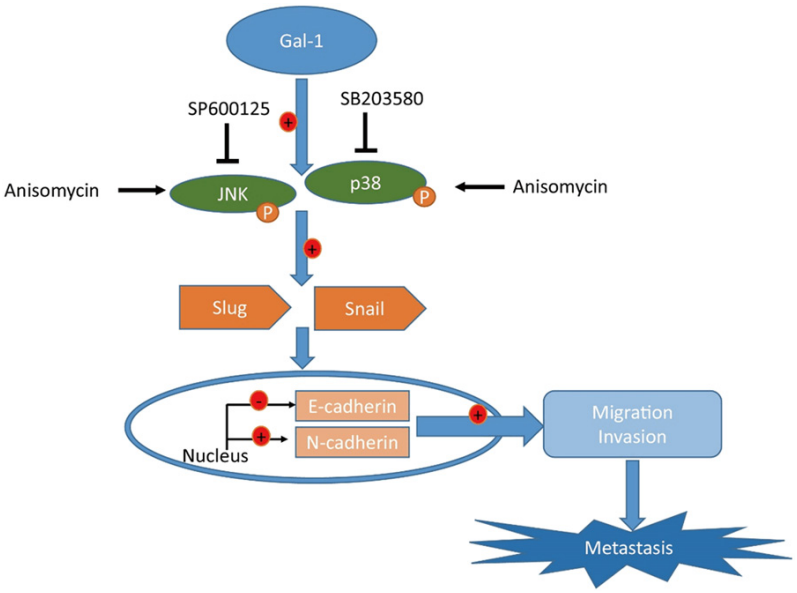


Figure 8. Schematic diagram of the relationships among Gal-1, MAPK JNK/p38 signalling pathway and EMT. Gal-1 can activate the MAPK JNK/p38 signalling pathway, which increases the expression of Slug and Snail, thus promoting cell migration and invasion by increasing the expression of N-cadherin and decreasing the expression of E-cadherin.

upregulation of Gal-1 in ovarian cancer cells promotes EMT and simultaneously enhances migration as well as invasion in vitro and in vivo.

EMT has been reported to have an important influence on the dissemination of malignant cells during ovarian cancer progression [43]. Therefore, exploring the underlying mechanisms of EMT may contribution considerably to the discovery of new treatments for ovarian cancer. Previous paper demonstrated that the overexpression of Gal-1 leads to greater H-Ras-GTP membrane association, more Raf-1 recruitment sites, MEK-ERK pathway activation and increased cell transformation [9]. The Ras-ERK signalling pathway leads to EMT and is necessary for the maintenance of the mesenchymal state of carcinoma cells. This signalling pathway works together with other signalling pathways to increase the expression of EMT-related genes, such as mesenchymal genes and transcriptional repressors of epithelial genes. In addition, Gal-1 triggers NF-κB activation in kidney cancer, leading to CXCR4 expression [44]. Moreover, the SDF-1/CXCR4 axis induces EMT in glioblastoma [45]. Our study suggested that the upregulation of Gal-1 in ovarian cancer cells led to the upregulation of EMT-related mRNAs and proteins. Moreover, deactivation of MAPK JNK and MAPK p38 reversed the EMT induced by Gal-1 overexpression, indicating that Gal-1 led to EMT via activating the MAPK JNK/p38 signalling pathway. Therefore, we conclude that the MAPK JNK/p38 signalling pathway is very important in the progression of ovarian cancer mediated by Gal-1.

Several upstream signalling pathways like the MAPK, PI3K/AKT and TGF-β pathways, can regulate EMT [46-48]. Accumulating evidence has indicated that EMT is correlated to chemoresistance and that suppressing EMT reverses chemoresistance [49, 50]. The MAPK JNK/p38 signalling pathway plays vital roles in tumour invasion and metastasis [51]. Moreover, many studies have indicated that the MAPK JNK/p38 signalling pathway induces EMT [52], which has an influence on tumour metastasis. Also, reports demonstrate that the MAPK JNK/p38 pathway affects the expression of EMT-related proteins, including E-cadherin as well as vimentin [21]. These previous reports suggest that activation of the MAPK JNK/p38 signalling pathway may lead to Gal-1-induced EMT in ovarian cancer. Currently, results indicate that the upregulation of Gal-1 leads to EMT activation of the MAPK JNK/p38 signalling pathway. Furthermore, despite a small cohort, clinical results of human ovarian cancer patients also indicate that overexpression of Gal-1 is strongly

associated with metastasis and EMT. In addition, Hsu et al. [53] describes that Gal-1 promotes lung cancer tumour metastasis through Notch1/Jagged signalling pathway. In the future, we can also explore whether Gal-1 can activate the Notch/Jagged pathway, and whether an inhibitor of Notch/Jagged pathway can reverse the Gal-1 induced ovarian cancer metastasis.

In brief, we have identified a novel biological effect of Gal-1 in ovarian cancer and revealed that the upregulation of Gal-1 triggers activation of the MAPK p38/JNK signalling pathway, leading to enhanced ovarian cancer migration and invasion via EMT. Consequently, our research indicates that targeting Gal-1 in ovarian cancer may serve as a promising treatment. Moreover, Gal-1 may function as a molecular biomarker in predicting metastasis and prognosis in ovarian cancer.

Acknowledgements

This work was supported in part by the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality to Dr. Pengnan Zhang (15ZR1404700) and Dr. Hong Sun (No. 16411963600).

Disclosure of conflict of interest

None.

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