

Original Article

MiR-548c inhibits lung cancer cell proliferation through suppression of Galectin-3-mediated TLR4 signaling pathway

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Abstract: Lung cancer is one of the leading causes of cancer-related mortality worldwide. MicroRNAs (miRNAs) are endogenous non-coding small RNAs that repress the expression of a broad array of target genes. Many efforts have been made to therapeutically target miRNAs in lung cancer treatments. In the present study, we revealed that low miR-548c expression in lung cancer was correlated with poorer prognosis in patients with lung cancer; miR-548c could inhibit lung cancer cell viability and proliferation. Moreover, miR-548c inhibited LGALS3 and TLR-4 expression through direct targeting; LGALS3 acted as a ceRNA to modulate TLR-4 expression by sponging miR-548c expression. Through miR-548c suppression of Galectin-3 and TLR-4, miR-548c exerted its inhibitory effect on lung cancer cells. What's more, Galectin-3-mediated TLR-4 signaling activation was involved in miR-548c regulation of lung cancer cell proliferation. Taken together, this miR-548c/Galectin-3/TLR-4 axis might represent an attractive target for the treatment of lung cancer.

Keywords: Lung cancer, proliferation, miR-548c, TLR-4, Galectin-3

Introduction

Lung cancer is one of the leading causes of cancer incidence and cancer-related mortality worldwide, with a 5-year survival rate <20% [1, 2]. Histologically, lung cancer can be classified into nonsmall cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The more aggressive SCLC accounts for 10-15% and NSCLC accounts for about 85% of lung cancer patients [3, 4]. Unfortunately, most lung cancer patients treated with standard cytotoxic chemotherapy ultimately develop drug-resistance. Therefore, there is an urgent need to develop more effective strategies or targeted agents for lung cancer treatment.

MicroRNAs (miRNAs) are a class of small, single-stranded, evolutionarily conserved nonprotein coding RNAs (19-22 nucleotide-long), they regulate gene expression at the posttranscriptional and translational levels by targeting the 3'UTR of messenger RNAs (mRNAs) [5]. miRNAs play an important role in the regulation of many biological processes, including cellular devel-

opment, growth, proliferation, differentiation, apoptosis and stem cell self-renewal. A dysregulation of miRNA profiling often correlates with the onset and progression of diseases, such as immune disorders and cancers [3]. With respect to carcinogenesis, miRNAs can exert either an oncogenic or tumor suppressor role in lung cancer [6-8]. Among these miRNAs, miR-21, miR-155 and miR-17-92 are well-studied miRNAs with oncogenic activity (oncomiRs), and the let-7 family, miR-15a, miR-16, miR-29b and miR-34a are miRNAs with tumor suppressor roles [7, 9]. Of interest, several universe miRNAs (including miR-548a/b/c, etc.) belong to the same miR-548 family [10] and have been implicated in tumorigenesis [11, 12], however their regulatory functions in lung cancer remain largely unknown.

In addition to miRNAs, Studies have demonstrated several mechanisms, such as drug inactivation, cell-associated drug reduction, deregulation of anti-apoptotic regulator, DNA damage tolerance/repair, and deregulation of growth factor receptors, which result in hyperprolifera-

tion in lung cancer [13, 14]. Recently, activation of TLR4 has been implicated in cancer cell proliferation. In lung cancer cells, TLR4 could exert an essential function in cancer cell proliferation and confer tumor growth and migration [15, 16].

Galectin-3, a member of the Galectin family, is a multifunctional protein [17]. Recently, compelling evidence has pointed out that Galectin-3 is involved in many physical and pathologic processes, and is closely correlated with tumorigenesis, development and metastasis, including lung cancer [18, 19]. Notably, a previous study demonstrated that galectin-3 could act as an endogenous paracrine TLR4 ligand and galectin-3 inhibition led to neuroprotective and anti-inflammatory effects [20]. In human synovial fibroblasts, galectin-3 was a positive sensor-regulator of TLR4 signaling and modulated TLR4-induced IL-6 secretion [21]. However, whether galectin-3 regulates TLR-4 in lung cancer to affect the progression of lung cancer, could galectin-3 and TLR-4 be regulated by miRNA, these all remain unclear.

In the present study, we investigated the biological functions and underlying mechanism of miR-548c in lung cancer, and provide *in vitro* evidence that miR-548c inhibits cell viability and proliferation of lung cancer cells. We further demonstrate that miR-548c inhibits lung cancer cell proliferation through direct targeting Galectin-3 and TLR-4, and suppressing galectin-3-induced TLR-4 signaling activation. Taken together, miR-548c/galectin-3/TLR-4 might present a novel target in lung cancer treatment.

Materials and methods

Tissue samples, cell lines and cell transfection

We collected 83 paired primary lung cancer tissues and the matched adjacent normal tissues. We obtained all samples from patients who underwent surgical resection at Xiangya Hospital, Central South University (Changsha, China). The tissues were snap-frozen in liquid nitrogen, and then stored at -80°C. This project was approved by the Ethic Committee of Xiangya Hospital, Central South University.

The immortalized human bronchial epithelial cell, BEAS-2B, and the human lung cancer cell

lines, A549, H1299, H23, H460, H1976, and H1755 were purchased from American Tissue Culture Collection and cultured in RPMI 1640 (Gibco, Life Technologies) containing 10% calf serum (Biofluids).

The expression of miR-548c was achieved by transfection of miR-548c mimics or miR-548c inhibitor (Genepharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen). A pcDNA3.1/Galectin-3 or pcDNA3.1/TLR-4 was used to achieve the overexpression of Galectin-3 or TLR-4 (GeneCopoeia, Guangzhou, China). Cells were plated in 6-well plates or 96-well plates, transfected, incubated for 24 h or 48 h and used for further assays or RNA/protein extraction.

RNA extraction and SYBR green quantitative PCR analysis

We extracted total RNA from cells using Trizol reagent (Invitrogen, CA, USA) and detected mature miR-548c expressions in cells using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China). We used expression of RNU6B as an endogenous control. The data were processed using a $2^{-\Delta\Delta CT}$ method.

MTT assay

A modified MTT assay was used to evaluate cell viability. 24 h after seeded into 96-well plates (5000 cells per well), cells were transfected with miR-548c mimics/miR-548c inhibitor, or co-transfected with pcDNA3.1/Galectin-3 or pcDNA3.1/TLR-4 and miR-548c mimics. 48 h after transfection, 20 μ l MTT (at a concentration of 5 mg/ml; Sigma-Aldrich) was added, and the cells were incubated for an additional 4 h in a humidified incubator. 200 μ l DMSO was added after the supernatant discarded to dissolve the formazan. OD_{490 nm} value was measured. The viability of the non-treated cells (control) was defined as 100%, and the viability of cells from all other groups was calculated separately from that of the control group.

BrdU incorporation assay

DNA synthesis in proliferating cells was determined by measuring 5-Bromo-2-deoxyUridine (BrdU) incorporation. BrdU assays were performed at 24 h and 48 h after transfecting A549 and H1299 cells with miR-548c mimics/

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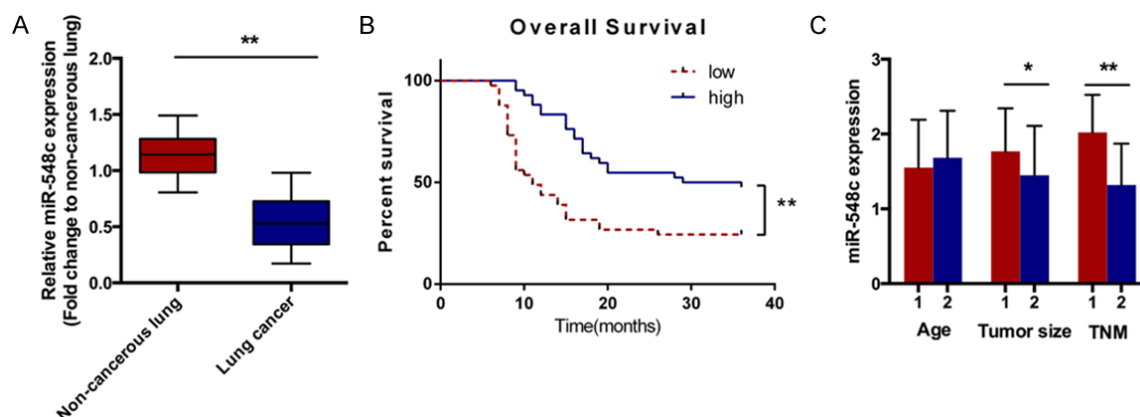


Figure 1. MiR-548c expression in lung cancer tissues and decreased miR-548c levels associates with worse prognosis in patients with lung cancer. A. Expression levels of miR-548c in 83 paired lung cancer tissues and non-cancerous lung tissues were determined using real-time PCR assays. B. Kaplan-Meier overall survival curves for 83 patients with lung cancer classified according to relative miR-548c expression level. C. Expression levels of miR-548c in patients of different age, tumor size and TNM stages with lung cancer. Age: 1: age <50, 2: age ≥50; Tumor size: 1: size <3, 2: size ≥3; TNM: 1: TNM = I, 2: TNM = II, III, IV. The data are presented as mean ± SD of three independent experiments. The data are presented as mean ± SD of three independent experiments. *P<0.05, **P<0.01.

miR-548c inhibitor, or co-transfected with pcDNA3.1/Galectin-3 or pcDNA3.1/TLR-4 and miR-548c mimics. After seeding the infected cells in 96-well culture plates at a density of 2×10^3 cells/well, they were cultured for 24 h or 48 h, and incubated with a final concentration of 10 μ M BrdU (BD Pharmingen, San Diego, CA, USA) for 2 h to 24 h. When the incubation period ended, we removed the medium, fixed the cells for 30 min at RT, incubated them with peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) for 60 min at RT, washed them three times with PBS, incubated the cells with peroxidase substrate (tetramethylbenzidine) for 30 min, and measured the absorbance values at 450 nm. Background BrdU immunofluorescence was determined in cells not exposed to BrdU but stained with the BrdU antibody.

ELISA

After 48 h transfection of miR-548c mimics or inhibitor, the medium of transfected cells was harvested. The Galectin-3 levels was determined using Human Galectin-3 Quantikine ELISA Kit (R&D system, USA) as described by the manufacturer.

Western blot analysis

The expression of Galectin-3, TLR-4, MyD88 and p-p65 in lung cancer cells was detected by performing immunoblotting. We lysed cultured or transfected cells in RIPA buffer with 1%

PMSF, and loaded protein onto an SDS-PAGE minigel and transferred them onto PVDF membrane. After probed with the following antibodies: Galectin-3 (mouse monoclonal, Cat# A3A12, Abcam, MA, USA), TLR-4 (rabbit polyclonal, ab47093, Abcam), MyD88 (rabbit polyclonal, ab2064, Abcam), p-p65 (rabbit polyclonal, ab86299, Abcam) and GAPDH (mouse monoclonal, Cat# 6C5, Abcam) at 4°C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody (1:5000). ECL Substrates were used to visualize signals (Millipore, MA, USA). GAPDH was used as an endogenous protein for normalization.

Luciferase reporter assay

Human embryonic kidney 293T cells (China Center for Type Culture Collection, Wuhan, China) were seeded into a 24-well plate. A wild-type and mutated LGALS3 (gene name of Galectin-3) 3'UTR (wt-LGALS3 and mut-LGALS3 containing a 4 bp mutation in the predicted binding sites of miR-548c) or TLR-4 3'UTR (wt-TLR-4 3'UTR and mut-TLR-4 3'UTR containing a 5 bp mutation in the predicted binding sites of miR-548c) luciferase reporter gene vector was constructed. After cultured overnight, cells were co-transfected with the indicated vector and miR-548c mimics and miR-548c inhibitor, respectively. Luciferase assays were performed 48 h after transfection using the Dual

Table 1. Correlation of the expression of miR-548c with clinicopathologic features

Characteristics		miR-548c expression		P-value
		High expression	Low expression	
Age	<50	20	23	0.440
	≥50	22	18	
Gender	Female	17	15	0.716
	Male	25	26	
Differentiation	Poor	24	18	0.228
	Well, moderate	18	23	
Tumor size (maximum diameter cm)	≥3	15	25	0.021
	<3	27	16	
Lymph node metastasis	Negative	26	18	0.100
	Positive	16	23	
TMN stage	I	23	12	0.019
	II/III/IV	19	29	

Luciferase Reporter Assay System (Promega, WI, USA).

Statistical analysis

Data were exhibited as mean ± SD of three independent experiments and processed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). By using the Student's paired test we compared the expression of miR-548c in lung cancer tissues and the paired adjacent normal colonic tissues. The differences between groups were evaluated using the one-way ANOVA. *P* values of <0.05 were considered statistically significant.

Results

MiR-548c expression in lung cancer tissues and decreased miR-548c level associates with worse prognosis in patients with lung cancer

MiR-548c has been reported to be down-regulated in cancer tissues [22]. In the present study, we firstly evaluated the expression levels of miR-548c in 83 paired lung cancer tissues and the adjacent normal tissues using real-time PCR assays. Results showed that miR-548c expression was significantly down-regulated in lung cancer tissues, compared to the matched adjacent normal tissues (**Figure 1A**). To investigate the functional role of miR-548c in the lung cancer pathological process, we evaluated the correlation of miR-548c expression and clinical parameters in lung cancer. 83

paired of cases of lung cancer tissues were divided into two groups: a high miR-548c expression group (above the median miR-548c expression, *n* = 42) and a low miR-548c expression group (below the median miR-548c expression, *n* = 41). To determine the potential relationship between miR-548c expression and the patients' prognosis, Kaplan-Meier analysis and log-rank test was used

to evaluate the effects of miR-548c expression on overall survival (OS). The results indicated that patients with lower miR-548c expression had a significantly poorer OS compared to patients with higher miR-548c expression (*P* < 0.01) (**Figure 1B**). Low expression of miR-548c in lung cancer showed to be related to larger tumor size (*P* = 0.021) and advanced TNM stage (*P* = 0.019) as exhibited in **Figure 1C** (age: 1: age <50, 2: age ≥50; Tumor size: 1: size <3, 2: size ≥3; TNM: 1: TNM = I, 2: TNM = II, III, IV) and **Table 1**. Moreover, A COX risk proportional regression model was further used to analyze the survival and pathological characteristics of 83 paired of cases. The results of univariate analysis showed that tumor size, TNM stage and miR-548c expression caused significant differences in over survival time; the results of multivariate analysis showed that miR-548c expression caused differences in survival time were statistically significant (*P* = 0.000, HR = 0.140; 95% CI: 0.057-0.346) (**Table 2**). These data indicated that miR-548c expression was significantly down-regulated in lung cancer tissues; low miR-548c expression was associated with poorer prognosis of patients with lung cancer.

MiR-548c expression in lung cancer cell lines and the functional role of miR-548c in proliferation of lung cancer cell

Since we demonstrated the lower expression of miR-548c in lung cancer tissues, and the cor-

MiR-548c inhibits lung cancer cell proliferation

Table 2. Univariate and multivariate analysis for factors related to overall survival using the COX proportional hazard model

Characteristics		Univariate analysis			Multivariate analysis		
		p-value	HR	95% CI	p-value	HR	95% CI
Age	45.783±15.375	0.253	0.990	0.972-1.007	N.A		
Gender	Female vs male	0.725	0.904	0.515-1.586	N.A		
Differentiation	Poor vs Well, moderate	0.100	0.633	0.368-1.091	N.A		
Tumor size (maximum diametercm)	<3 vs ≥3	0.000	0.309	0.176-0.543	0.000	0.251	0.138-0.457
Lymph node metastasis	Negative vs Positive	0.089	0.624	0.362-1.074	0.954	0.984	0.568-1.705
TMN stage	I vs II/III/IV	0.000	0.160	0.081-0.316	0.000	0.229	0.109-0.479
MIR-548c	1.080±0.418	0.000	0.073	0.031-0.174	0.000	0.140	0.057-0.346

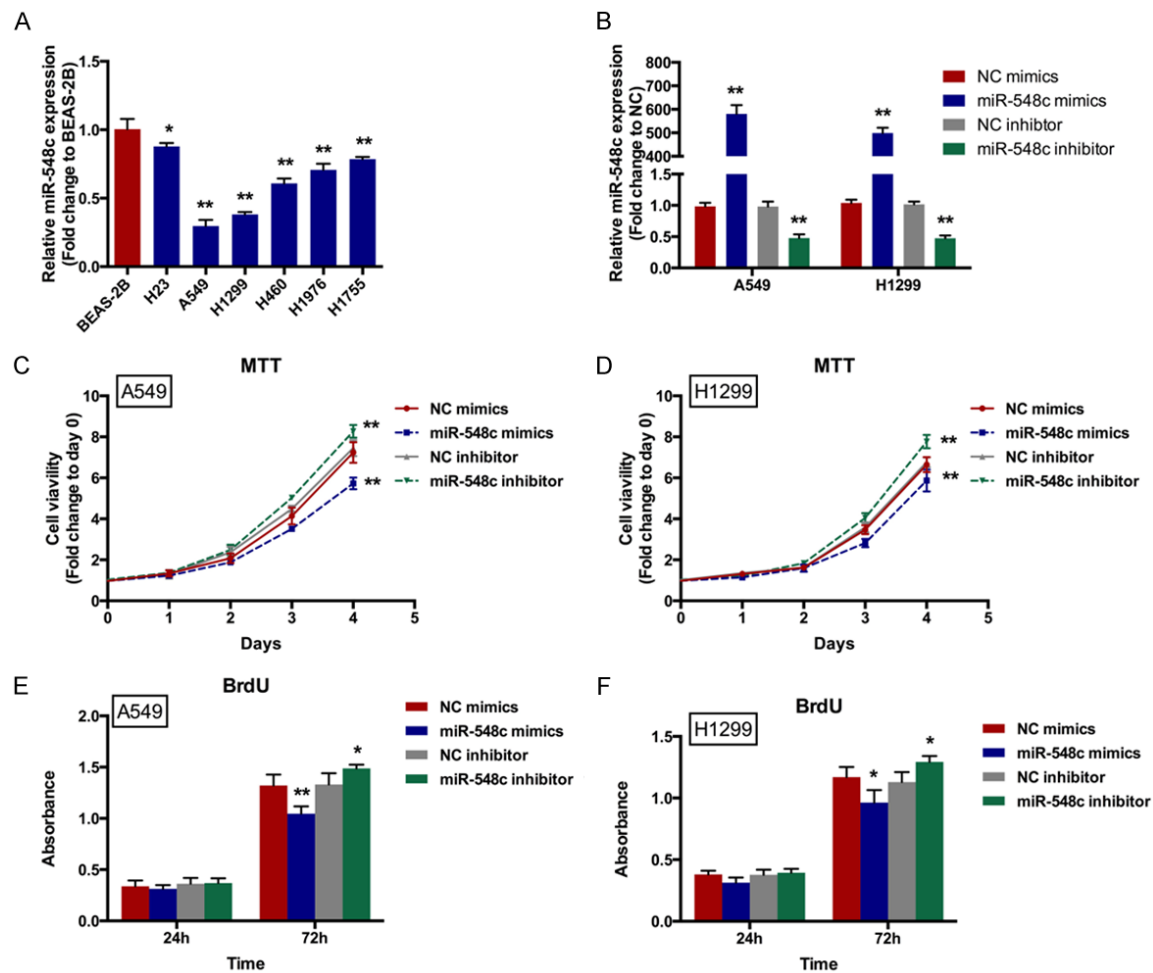


Figure 2. MiR-548c expression in lung cancer cell lines and the functional role of miR-548c in proliferation of lung cancer cell. A. Expression levels of miR-548c in six lung cancer cell lines, A549, H1299, H23, H460, H1976, and H1755 and a normal cell line BEAS-2B using real-time PCR assays. B. miR-548c mimics or miR-548c inhibitor was transfected into A549 and H1299 cells to achieve ectopic miR-548c expression or miR-548c inhibition, as verified using real-time PCR assays. C and D. A549 and H1299 cells were transfected with miR-548c mimics or miR-548c inhibitor; the cell viability was determined using MTT assays. E and F. A549 and H1299 cells were transfected with miR-548c mimics or miR-548c inhibitor; the cell proliferation was determined using BrdU assays. The data are presented as mean $\pm$ SD of three independent experiments. * P <0.05, ** P <0.01.

relation of low miR-548c expression with poor prognosis of patients with lung cancer; we

further investigated the expression of miR-548c in lung cancer cells and its functions in

MiR-548c inhibits lung cancer cell proliferation

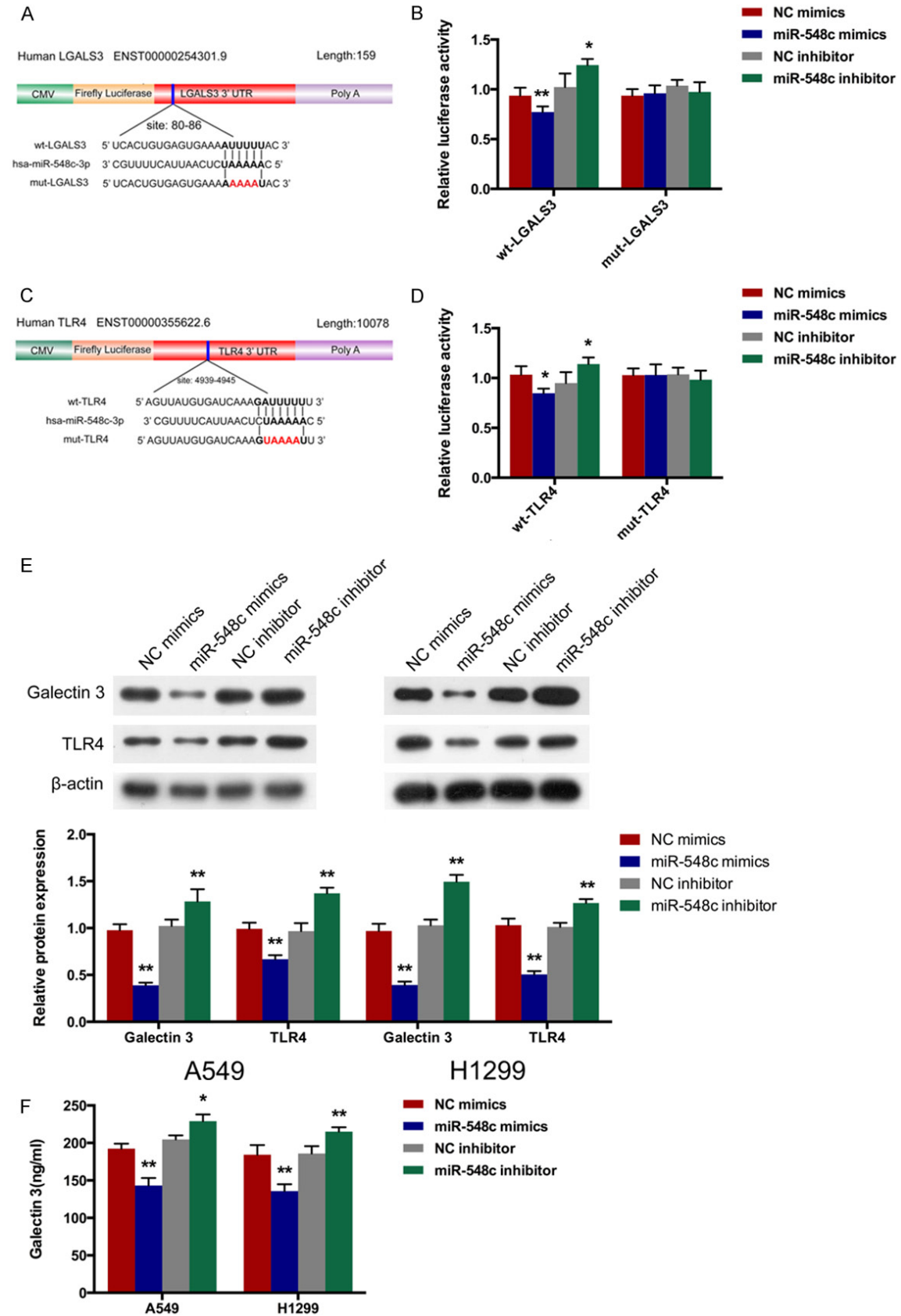


Figure 3. A549 and H1299 cells were transfected with miR-548c mimics or miR-548c inhibitor; the cell viability was determined using MTT assays. A and C. A wild-type and mutated LGALS3 3'UTR (wt-LGALS3 and mut-LGALS3 containing a 4 bp mutation in the predicted binding sites of miR-548c) or TLR-4 3'UTR (wt-TLR-4 3'UTR and mut-TLR-4 3'UTR containing a 5 bp mutation in the predicted binding sites of miR-548c) luciferase reporter gene vector was constructed. B and D. 293T cells were co-transfected with the indicated vectors and miR-548c mimics or miR-548c inhibitor, respectively. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay to determine the luciferase activity. E. miR-548c mimics or miR-548c inhibitor was transfected into A549 and H1299 cells; the protein levels of Galectin-3 and TLR-4 in A549 and H1299 cells were determined using Western blot assays. F. miR-548c mimics or miR-548c inhibitor was transfected into A549 and H1299 cells; Galectin-3 secretion was determined using ELISA assays. The data are presented as mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

lung cancer cell proliferation. Results showed that miR-548c expression was significantly down-regulated in all the six lung cancer cell lines, more strongly down-regulated in A549 and H1299 cells (**Figure 2A**). Due to the low miR-548c expression in A549 and H1299 cell lines, we selected the two cell lines as further cell model. MiR-548c mimics or miR-548c inhibitor was transfected into A549 and H1299 cells to achieve ectopic miR-548c expression or miR-548c inhibition, as verified using real-time PCR assays (**Figure 2B**). Further, the cell viability and proliferation of the two cell lines were determined using MTT and BrdU assays. Results showed that ectopic miR-548c expression significantly suppressed the cell viability of A549 and H1299 cell, whereas miR-548c inhibition promoted the cell viability of A549 and H1299 cell (**Figure 2C** and **2D**). Consistent results were observed in BrdU assays: miR-548c overexpression suppressed the cell proliferation, whereas miR-548c inhibition promoted the cell proliferation (**Figure 2E** and **2F**). These data indicated that miR-548c negatively regulates lung cancer cell proliferation.

MiR-548c regulated Galectin-3 and TLR-4 expression by directly targeting the 3'UTR of LGALS3 and TLR-4

MiRNAs regulate gene expression at the post-transcriptional and translational levels by targeting the 3'UTR of messenger RNAs (mRNAs) to participate in the progression of cancers [5]. Here, we used online tools to predict the candidate downstream target genes of miR-548c. As exhibited in **Figure 3A** and **3C**, a possible binding site of miR-548c existed in the 3'UTR of LGALS3 (gene name of Galectin-3) and TLR-4, respectively (**Figure 3A** and **3C**). To validate the binding, we constructed a wild-type and mutated LGALS3 3'UTR (wt-LGALS3 and mut-LGALS3 containing a 4 bp mutation in the predicted binding sites of miR-548c) or a wild-type and mutated TLR-4 3'UTR (wt-TLR-4 and mut-TLR-4

containing a 5 bp mutation in the predicted binding sites of miR-548c) luciferase reporter gene vector. The indicated vectors were co-transfected into 293T cells with miR-548c mimics or miR-548c inhibitor, respectively; the luciferase activity was determined using the Dual Luciferase assays. Results showed that the luciferase activity of the wt-LGALS3 or wt-TLR-4 vectors were significantly suppressed by miR-548c mimics, amplified by miR-548c inhibitor; after mutations at the predicted miR-548c binding sites in the 3'UTR of LGALS3 or TLR-4, the changes of the luciferase activity were abolished (**Figure 3B** and **3D**). These data indicated that miR-548c might inhibit LGALS3 and TLR-4 expression through direct targeting. Interestingly, we found that LGALS3 and TLR-4 3'UTR shared an almost identical binding site in miR-548c, suggesting that LGALS3 might compete with TLR-4 for miR-548c binding.

We further transfected miR-548c mimics or miR-548c inhibitor into A549 and H1299 cells; then the protein levels of Galectin-3 and TLR-4 were determined using the Western blot assay. Results showed that Galectin-3 and TLR-4 proteins were reduced by miR-548c mimics, whereas increased by miR-548c inhibitor (**Figure 3E**), indicating the negative effect of miR-548c on Galectin-3 and TLR-4 proteins. Further, Galectin-3 secretion was determined using ELISA assays. Consistent with the results from Western blot assays, Galectin-3 secretion in both A549 and H1299 cells was suppressed by miR-548c mimics, whereas promoted by miR-548c inhibitor (**Figure 3F**). These data indicated that miR-548c negatively regulates Galectin-3 and TLR-4 through direct targeting LGALS3 and TLR-4.

MiR-548c acted on lung cancer cell proliferation through Galectin-3 and TLR-4, respectively

We demonstrated that miR-548c negatively regulates lung cancer cell proliferation; further,

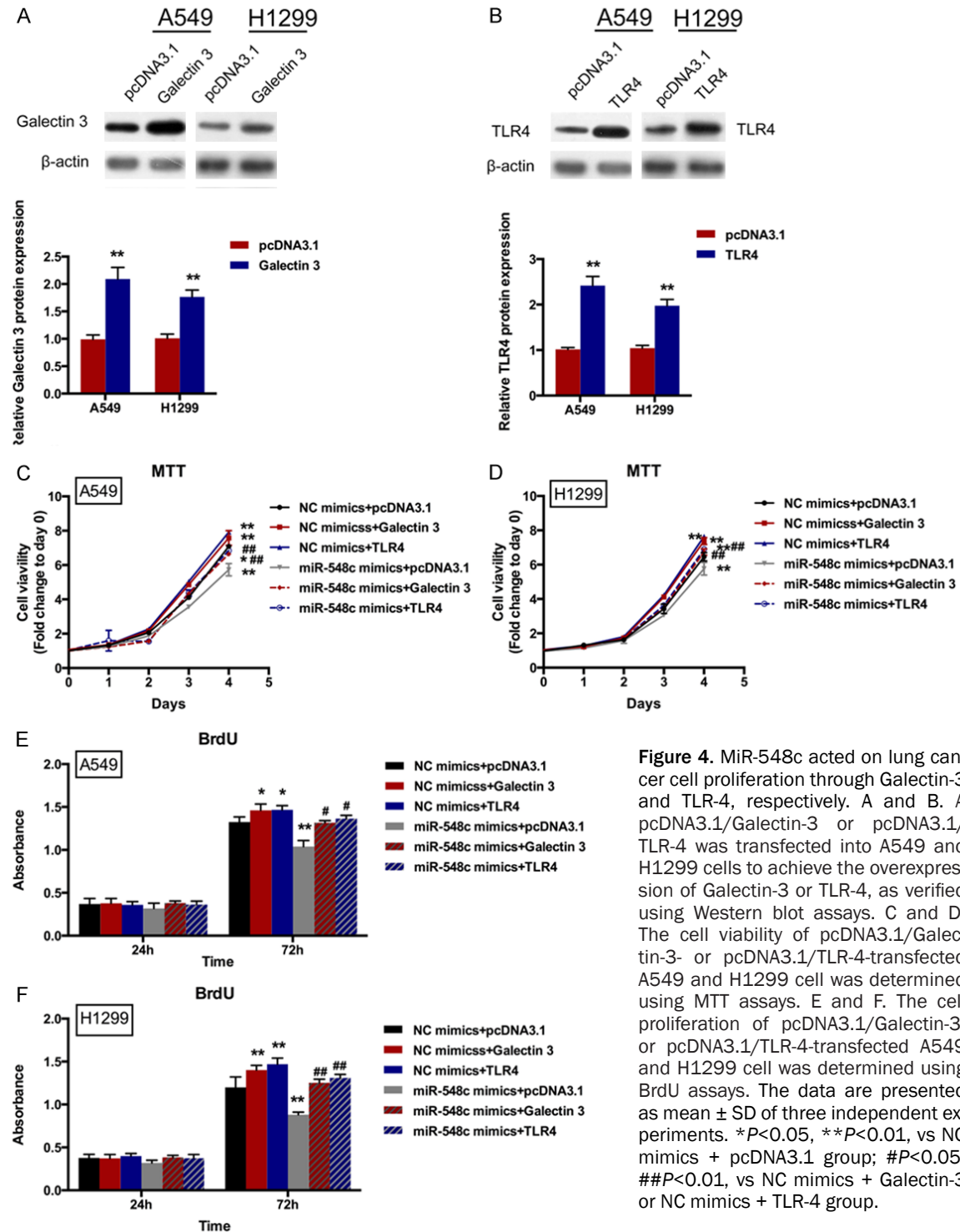


Figure 4. MiR-548c acted on lung cancer cell proliferation through Galectin-3 and TLR-4, respectively. A and B. A pcDNA3.1/Galectin-3 or pcDNA3.1/TLR-4 was transfected into A549 and H1299 cells to achieve the overexpression of Galectin-3 or TLR-4, as verified using Western blot assays. C and D. The cell viability of pcDNA3.1/Galectin-3- or pcDNA3.1/TLR-4-transfected A549 and H1299 cell was determined using MTT assays. E and F. The cell proliferation of pcDNA3.1/Galectin-3- or pcDNA3.1/TLR-4-transfected A549 and H1299 cell was determined using BrdU assays. The data are presented as mean $\pm$ SD of three independent experiments. * P <0.05, ** P <0.01, vs NC mimics + pcDNA3.1 group; # P <0.05, ## P <0.01, vs NC mimics + Galectin-3 or NC mimics + TLR-4 group.

we revealed that miR-548c negatively regulates Galectin-3 and TLR-4 proteins through direct targeting. Here, we investigated whether miR-548c acts on lung cancer cell proliferation through regulation of Galectin-3 and TLR-4. A549 and H1299 cells were transfected with

pcDNA3.1/Galectin-3 or pcDNA3.1/TLR-4 to achieve forced Galectin-3 and TLR-4 expression, as verified using Western blot assays (Figure 4A and 4B). Further, A549 and H1299 cells were co-transfected with miR-548c mimics and pcDNA3.1/Galectin-3 or pcDNA3.1/

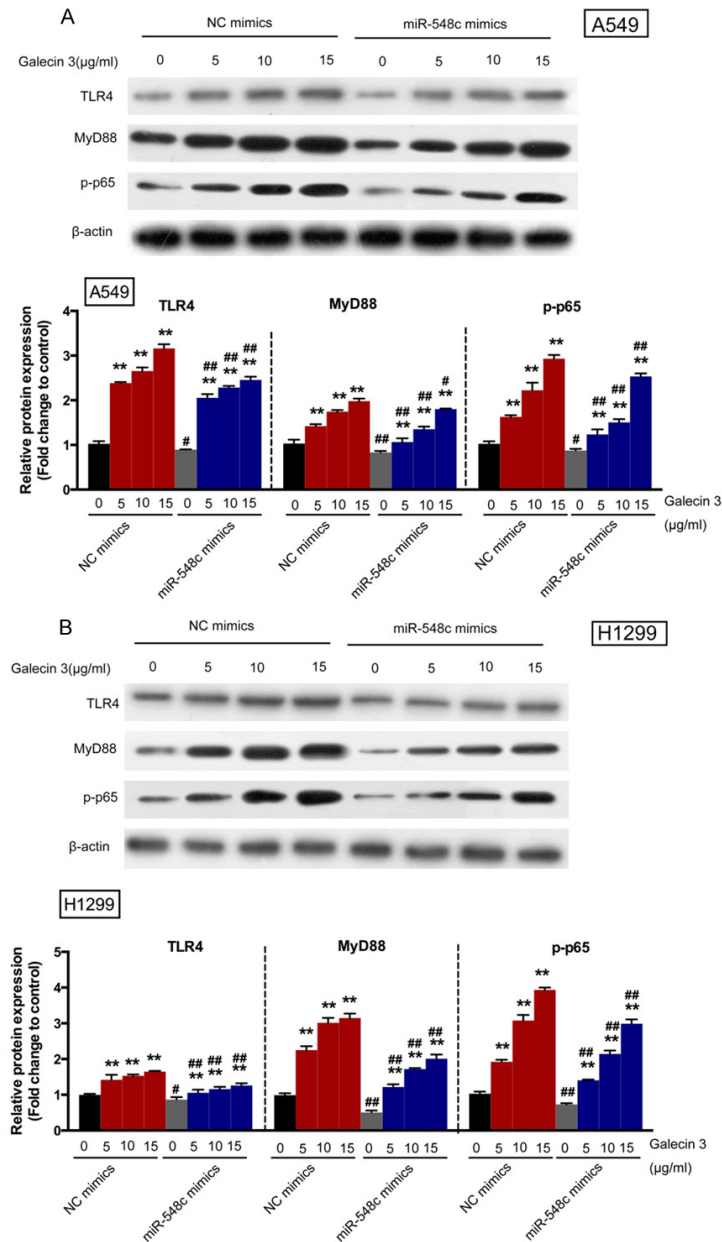


Figure 5. MiR-548c suppressed ectopic Galectin-3-mediated TLR-4 signaling activation. A and B. MiR-548c mimics- or NC mimics-transfected A549 and H1299 cells were exposed to a series of doses of Galectin-3 (0, 5, 10, 15 μ g/ml); then the protein levels of TLR-4, MyD88 and p-p65 were determined using Western blot assays. ** $P < 0.01$, vs NC mimics + 0 μ g/ml Galectin-3 group or miR-548c mimics + 0 μ g/ml Galectin-3 group; # $P < 0.05$, ## $P < 0.01$, vs NC mimics + 0-15 μ g/ml Galectin-3 group.

TLR-4, respectively; the cell viability and proliferation was then determined using MTT and BrdU assays. Results showed that the cell viability and proliferation of A549 and H1299 cells were promoted by either forced Galectin-3 or TLR-4 expression, whereas suppressed by ectopic miR-548c expression; the promotive

effects of Galectin-3 or TLR-4 on lung cancer cell proliferation could be partially reversed by ectopic miR-548c expression (Figure 4C-F). These data suggested that miR-548c inhibits lung cancer cell proliferation through Galectin-3 or TLR-4.

MiR-548c suppressed ectopic Galectin-3-mediated TLR-4 signaling activation

We demonstrated that 3'UTR of LGALS3 might compete with 3'UTR TLR-4 for miR-548c binding. Further, we validated the interaction between Galectin-3 and TLR-4 in lung cancer cells. MiR-548c mimics- or NC mimics-transfected A549 and H1299 cells were exposed to a series of doses of Galectin-3 (0, 5, 10, 15 μ g/ml); then the protein levels of TLR-4, MyD88 and p-p65, important factors in the TLR-4 signaling, were determined using Western blot assays. Results showed that the protein levels of TLR-4, MyD88 and p-p65 were down-regulated by Galectin-3 treatment in a dose-dependent manner, either in miR-548c mimics- or NC mimics-transfected A549 and H1299 cells (Figure 5A and 5B); however, in miR-548c mimics-transfected cells, although the indicated proteins were increased, the amplitudes of the increase were significantly slighter compared with NC mimics-transfected lung cancer cells (Figure 5A and 5B). Since the elevated protein content indicates TLR-4 pathway activation, these data suggested that miR-548c suppresses ecto-

pic Galectin-3-mediated TLR-4 signaling activation.

Discussion

MiRNAs are promising predictive/prognostic biomarkers or therapeutic targets in human

tumors [23, 24]. Recent findings have identified miR-548c family members as universe miRNA, and illustrated the possibility that these miRNAs exert broad effects on many features of tumor cells through a broad range of targets and pathways [25]. Although one such miRNA, miR-548c, has been shown to inhibit cancer cell growth [12, 22], its cellular functions, the underlying mechanisms and clinical significance have not been comprehensively studied in lung cancer.

In the present study, we firstly found that miR-548c was under-expressed in lung cancer tissues and cell lines compared with the adjacent normal tissues and normal cell line. Low miR-548c expression was correlated with poorer prognosis of patients with lung cancer. Moreover, by conducting the ectopic miR-548c expression or miR-548c inhibition, we revealed that miR-548c inhibited the cell viability and proliferation of lung cancer cell line. These findings reveal for the first time that in lung cancer cells, miR-548c is a tumor-suppressive miRNA that inhibits cancer cell viability and proliferation. However, the mechanism(s) still remains unclear.

Galectins are a family of animal lectins with diverse biological activities. They function both extracellularly, by interacting with cell-surface and extracellular matrix glycoproteins and glycolipids, and intracellularly, by interacting with cytoplasmic and nuclear proteins to modulate signalling pathways. There is direct evidence that Galectin-3 expression is necessary for the initiation of the transformed phenotype of tumors. Following the inhibition of Galectin-3 expression, breast carcinoma cells and thyroid papillary carcinoma cells lose their characteristic transformed phenotypes in cell culture [26, 27]. Conversely, the introduction of Galectin-3 cDNA into a normal thyroid follicular cell line induces a transformed phenotype [28]. Recently, Galectin-3 has been reported to induce ovarian cancer cell survival and chemoresistance via TLR4 signaling activation [29]. In addition to Galectin-3, TLR-4 signaling itself has been also regarded as an essential pathway in the pathopoiesis of tumors [30]. Since we revealed that miR-548c plays an inhibitory role in lung cancer cell proliferation; here we further investigated whether Galectin-3 and TLR-4 are involved in miR-548c regulation of lung cancer cell proliferation.

As predicted by online tools, the 3'UTR of LGALS3 (encoding Galectin-3 protein) and TLR-4 possessed potential miR-548c binding site; these direct bindings were further confirmed using luciferase assays. Further, we found that miR-548c reduces Galectin-3 and TLR-4 proteins through direct binding to the 3'UTR of LGALS3 and TLR-4, respectively. Interestingly, we observed that LGALS3 and TLR-4 shared an almost identical binding site in miR-548c, which suggested that LGALS3 might compete with TLR-4 for miR-548c binding, and further affect TLR-4 expression and lung cancer cell proliferation.

To further investigate the functional roles of Galectin-3 and TLR-4 in lung cancer pathology, a series of functional assays were employed. The results of these assays were consistent with previous studies, forced Galectin-3 or TLR-4 expression significantly promoted cancer cell proliferation [30-33]. Importantly, ectopic miR-548c expression could partially reverse the promotive effects of Galectin-3 and TLR-4 on cancer cell proliferation, indicating that miR-548c regulates lung cancer cell proliferation through Galectin-3 and TLR-4. Since we hypothesized that LGALS3 might compete with TLR-4 for miR-548c binding to regulate TLR-4 expression by sponging miR-548c expression, here we further investigated whether Galectin-3 regulates TLR-4 and TLR-4 signaling-related factors. After conducting a series of doses of Galectin-3 treatment on NC mimics- or miR-548c mimics-transfected lung cancer cell lines, we monitored the protein levels of TLR-4, MyD88 and p-p65 to evaluate the functions of Galectin-3 in activation of TLR-4 signaling. As exhibited by Western blot assays, TLR-4, MyD88 and p-p65 proteins were increased by Galectin-3 treatment in a dose-dependent manner; however, in miR-548c mimics-transfected lung cancer cells, the amplitudes of proteins increases were slighter than those of the NC mimics-transfected lung cancer cells. These all confirmed our earlier hypothesis that LGALS3 compete with TLR-4 for miR-548c binding to promote TLR-4 expression by sponging miR-548c expression; moreover, miR-548c inhibits lung cancer cell proliferation through suppressing Galectin-3-induced TLR-4 signaling activation.

Taken together, our study suggested that high expression of miR-548c in lung cancer appears

to correlate with better prognosis in patients with lung cancer. LGALS3 acts as a ceRNA to promote TLR-4 expression by sponging miR-548c expression in lung cancer cells. Through suppressing Galectin-3-induced TLR-4 signaling activation, miR-548c inhibits lung cancer cell proliferation and might further affect lung cancer progression. Since miR-548c directly regulates Galectin-3 and TLR-4 in lung cancer cells, this miR-548c/Galectin-3/TLR-4 axis might also represent an attractive target for the treatment of lung cancer.

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Disclosure of conflict of interest

None.

Authors' contribution

Xing Chen, Yingji Chen, Qinghua Hu, Xuliang Chen, Lingjin Huang. Xing Chen: writing the article, operating the experiments. Yingji Chen: operating the experiments. Qinghua Hu and Xuliang Chen: Collecting and analyzing clinical samples. Lingjin Huang: guiding the experiments and editing the manuscript.

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