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LncRNA HAGLROS accelerates the progression of lung carcinoma *via* sponging microRNA-152

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Abstract. – OBJECTIVE: The aim of this study was to analyze the expression profiling of long non-coding RNA (lncRNA) HAGLROS and microRNA-152 in lung carcinoma (LCa), and to explore their regulatory effects on the malignant progression of LCa.

PATIENTS AND METHODS: The expression of HAGLROS in 44 paired LCa tissues and matched adjacent tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between HAGLROS expression and clinical indexes of LCa patients was analyzed. Furthermore, HAGLROS expression in LCa cell lines was detected as well. The HAGLROS over-expression and knockdown models were established in A549 and SPC-A1 cells by transfection of pcDNA-HAGLROS and anti-HAGLROS, respectively. The biological influences of HAGLROS on LCa cells were evaluated through a series of functional experiments. Furthermore, the potential relationship between HAGLROS and microRNA-152 was analyzed.

RESULTS: HAGLROS was highly expressed in LCa tissues compared with adjacent normal tissues. LCa patients with a higher expression of HAGLROS presented significantly worse tumor stage, a higher rate of lymphatic metastasis, and a lower survival. The knockdown of HAGLROS significantly attenuated the proliferative and migratory abilities of LCa cells. Meanwhile, HAGLROS over-expression obtained the opposite results. MicroRNA-152 was negatively correlated with HAGLROS in LCa. Rescue experiments showed that the knockdown of microRNA-152 reversed the regulatory effects of HAGLROS on proliferative and migratory abilities of LCa cells.

CONCLUSIONS: HAGLROS expression is correlated with tumor stage and lymphatic metastasis of LCa patients. Furthermore, HAGLROS accelerates proliferation and migration of LCa cells by regulating microRNA-152.

Key Words:
HAGLROS, MicroRNA-152, Lung carcinoma (LCa).

Introduction

Lung carcinoma (LCa) is a global health problem that seriously threatens human lives[1-3]. In 2018, the number of newly diagnosed LCa patients ranked second among all types of malignancies in the United States, involving 117,920 male and 106,470 females. Currently, LCa is a leading cause of tumor death, with a mortality of 27% (158,080 cases)[4]. In China, the morbidity and mortality of LCa have greatly increased in recent years, severely affecting families and society[5]. It is known that the risk factors for LCa are diverse, and the most significant include smoking, air pollution, and long-term exposure to carcinogens[6,7]. The pathogenesis of LCa has not been fully elucidated[8,9]. Therefore, it is necessary to search for novel diagnostic and prognostic markers of LCa[9].

A large number of non-coding RNA (ncRNA) transcripts have been found in eukaryotes. Based on the length, they are divided into long non-coding RNAs (lncRNAs) and short ncRNAs (siRNAs, miRNAs, piRNAs)[10,11]. LncRNAs are more than 200 nt long, with no protein-encoding function. They are capable of regulating gene expressions at multiple levels and serving as scaffolds for modifying chromatin complexes[12,13]. For example, lncRNA HAGLROS is abnormally expressed in many types of malignancies, presenting a carcinogenic role[14,15]. However, the exact role of HAGLROS in the malignant progression of LCa remains unclear.

LncRNAs can act as miRNA sponges to further mediate miRNA-targeted miRNAs[16,17]. Meanwhile, lncRNAs serve as co-activators by interacting with RNA binding proteins or altering localization and activities of proteins[18]. Therefore, the explorations on lncRNA-miRNA-mRNA regulatory axis in tumors are of great significance[15-18]. Bioinformatics prediction has proposed microRNA-152 as the target of HAGLROS involving in the progression of LCa. Previous studies[19,20] have reported that microRNA-152 is involved in many types of tumors. In this paper, the expression patterns of HAGLROS and microRNA-152 in LCa tissues and cell lines were determined. Furthermore, we explored the biological functions of HAGLROS/microRNA-152 axis in the progression of LCa.

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Patients and Methods

Subjects and LCa Samples

44 paired LCa tissues, and adjacent normal tissues were surgically resected from LCa patients. No patient received preoperative anti-tumor therapies. The clinical indexes of patients were collected for further analyses. Informed consent was obtained from patients and their families before the study. This study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University.

Cell Culture

Pulmonary epithelial cell line (BEAS-2B) and LCa cell lines (A549, H1299, PC-9, H358, and SPC-A1) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Life Technologies, Thermo Scientific, Waltham, MA, USA) and maintained in a 37°C, 5% CO_2 incubator. Culture medium was replaced every 2-3 days. The cell passage was conducted after reached the 90% of confluence.

Cell Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). The cells were preseeded into 6-well plates. The cell transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at 40% of confluence. At 48 h, the cells were harvested for subsequent experiments.

Cell Proliferation Assay

Cells were first seeded into 96-well plates at a density of 2×10^3 cells per well. At appointed time points, the absorbance (A) at 450 nm of each sample was detected according to the instructions of cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Finally, cell viability curve was plotted.

Transwell Migration Assay

Transfected cells for 48 h were first adjusted to a dose of 5.0×10^5/mL. 200 μL/well suspension was added in the upper side of the transwell chamber (Millipore, Billerica, MA, USA). Meanwhile, 700 μL of medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, the migratory cells of the lower chamber were fixed with methanol for 15 min and stained with crystal violet staining for 20 min. The number of penetrating cells was counted under a microscope. 5 fields were randomly selected for each sample.

Colony Formation Assay

Cells were first seeded into 6-well plates at a density of 2.5×10^3 cells per well, followed by culture for 2 weeks. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min and stained with Giemsa solution for 10 min. After removing the staining solution, formed colonies were washed, air dried and observed under a microscope. Finally, the number of formed colonies was counted.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was purified by DNase I treatment and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaKa, Otsu, Shiga, Japan). Subsequently, the obtained cDNA was subject to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaKa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. The qRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Three replicates were set for each sample, and the relative level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. iQ5 2.0 (Bio-Rad, Hercules, CA, USA) was applied for the data analysis. The primer sequences used in this study were as follows: HAGLROS, F: 5'-GGAACGGCTCCTTACTTTC-3', R: 5'-CGTAGGGTGTCCGCCGTAGA-3'; microRNA-152, F: 5'-GCCTATAAACATCCGACTG-3', R: 5'-GATCGcTGTCGTGGAAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

The cells were co-transfected with NC/microRNA-152 mimics and pmirGLO-HAGLROS-WT/pmirGLO-HAGLROS-MUT/pmirGLO using Lipofectamine 2000. 24 h later, the co-transfected cells were harvested. The luciferase activity was detected using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for the data analysis. The data were expressed as mean ± standard deviation. A *t*-test was used to compare the difference between the two groups. The one-way ANOVA was performed to compare the differences among different groups, followed by a post-hoc test (Least Significant Difference). The receiver operating characteristic (ROC) curve was used to assess the diagnostic value of HAGLROS on LCa patients by calculating AUC. $p<0.05$ was considered statistically significant.

Results

High Expression of HAGLROS in LCa

HAGLROS was significantly up-regulated in LCa tissues when compared with adjacent normal tissues, suggesting its potential carcinogenic role in LCa (Figure 1A, 1B). Identically, HAGLROS was highly expressed in LCa cell lines relative to pulmonary epithelial cell line (Figure 1C). The ROC curve was used to assess the prognostic sensitivity of HAGLROS in LCa. The calculated AUC indicated the prognostic value of HAGLROS on LCa (AUC=0.898, 95%CI=0.800-0.997, Figure 1D).

HAGLROS Expression Was Correlated With Pathological Staging and Lymphatic Metastasis of LCa Patients

Based on the expression of HAGLROS, LCa patients enrolled in the study were divided into high expression group and low expression group. The results showed that high expression of HAGLROS was positively correlated with tumor stage and lymphatic metastasis; by contrast, it was not associated with age, gender, and distant metastasis of LCa patients (Table I).

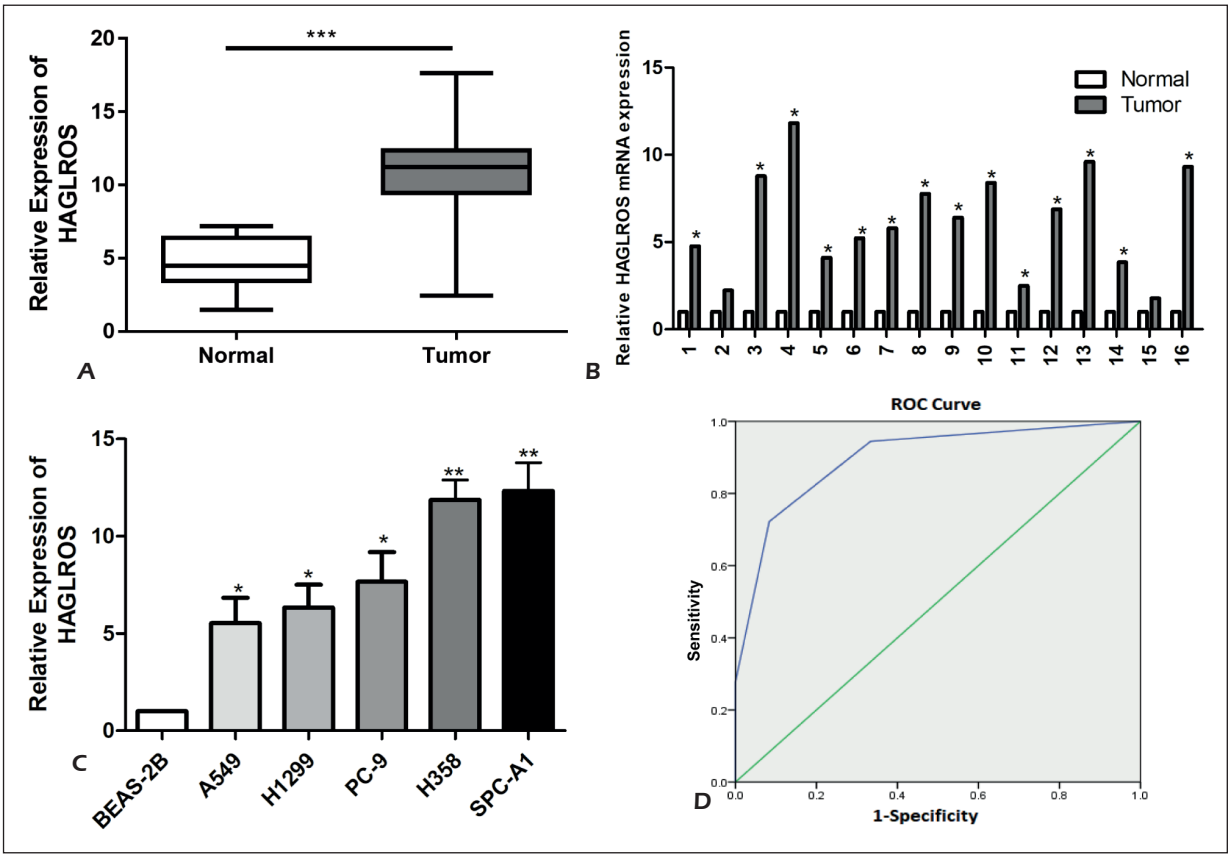

Figure 1. High expression of HAGLROS in LCa. ***A***, Relative level of HAGLROS in LCa tissues and adjacent normal tissues (n=44). ***B***, Relative level of HAGLROS in 16 paired LCa tissues and adjacent normal tissues. ***C***, Relative level of HAGLROS in pulmonary epithelial cell line (BEAS-2B) and LCa cell lines (A549, H1299, PC-9, H358 and SPC-A1). ***D***, ROC curve was introduced for sensitivity and specificity of HAGLROS in LCa patients.

Table I. Association of HAGLROS expression with clinicopathologic characteristics of lung cancer.

Parameters	Number of Cases	HAGLROS expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.507
<60	20	12	8	
≥60	24	12	12	
Gender				0.263
Male	26	16	10	
Female	18	8	10	
T stage				0.042
T1-T2	27	18	9	
T3-T4	17	6	11	
Lymph node metastasis				0.019
No	28	19	9	
Yes	16	5	11	
Distance metastasis				0.084
No	32	20	12	
Yes	12	4	8	

Knockdown of HAGLROS Attenuated Proliferative and Migratory Abilities of LCa Cells

For *in vitro* assays, we first constructed pcDNA-HAGLROS and anti-HAGLROS and transfected them into A549 and SPC-A1 cells, respectively (Figure 2A). The transfection efficacy was verified by qRT-PCR. The results indicated that the transfection of pcDNA-HAGLROS in A549 cells significantly elevated cell viability, colony formation, and migration abilities (Figure 2B-2D, left). Conversely, the transfection of anti-HAGLROS in SPC-A1 cells obtained the opposite trends (Figure 2B-2D, right).

HAGLROS Bound to MicroRNA-152 in LCa

Based on the predicted binding sequences between HAGLROS and microRNA-152 (data not shown), the Dual-Luciferase reporter gene assay was conducted to verify their relationship *in vitro*. A decreased luciferase activity was observed in LCa cells co-transfected with microRNA-152 mimics and pmirGLO-HAGLROS-WT. These results demonstrated the direct binding condition between microRNA-152 and HAGLROS (Figure 3A). QRT-PCR indicated that microRNA-152 was lowly expressed in both LCa tissues and cell lines (Figure 3B, 3C). Moreover, microRNA-152 expression was negatively correlated with HAGLROS expression in LCa patients (Figure 3D). The transfection of pcDNA-HAGLROS significantly down-regulated microRNA-152 level in A549 cells. However, the microRNA-152 expression was significantly up-regulated after the transfection of anti-HAGLROS in SPC-A1 cells (Figure 3E).

HAGLROS/MicroRNA-152 Axis in LCa Cells

Since the relationship between HAGLROS and microRNA-152 was proved, we then speculated whether microRNA-152 was involved in HAGLROS-mediated LCa progression. Subsequent results indicated that, after the co-transfection of microRNA-152 inhibitor, the down-regulated HAGLROS in LCa cells transfected with anti-HAGLROS was up-regulated (Figure 4A). The knockdown of HAGLROS attenuated the viability of LCa cells, which could be reversed after microRNA-152 knockdown (Figure 4B). Similarly, the reduced migration and colony formation abilities of LCa cells with HAGLROS knockdown were reversed after co-transfection of microRNA-152 inhibitor (Figure 4C-4D).

Discussion

LncRNAs were initially considered as non-functional transcription noises. With advanced technologies, especially in the application of sequencing, lncRNAs have been well concerned in the regulation of biological processes[10-12]. Currently, multiple lncRNAs have been identified to be crucial in tumor biology. These differentially expressed lncRNAs in tumors may serve as prognostic hallmarks[12-15]. Meanwhile, the researches on abnormally expressed lncRNAs in LCa contribute to enhance the diagnostic efficacy and prognosis of LCa patients.

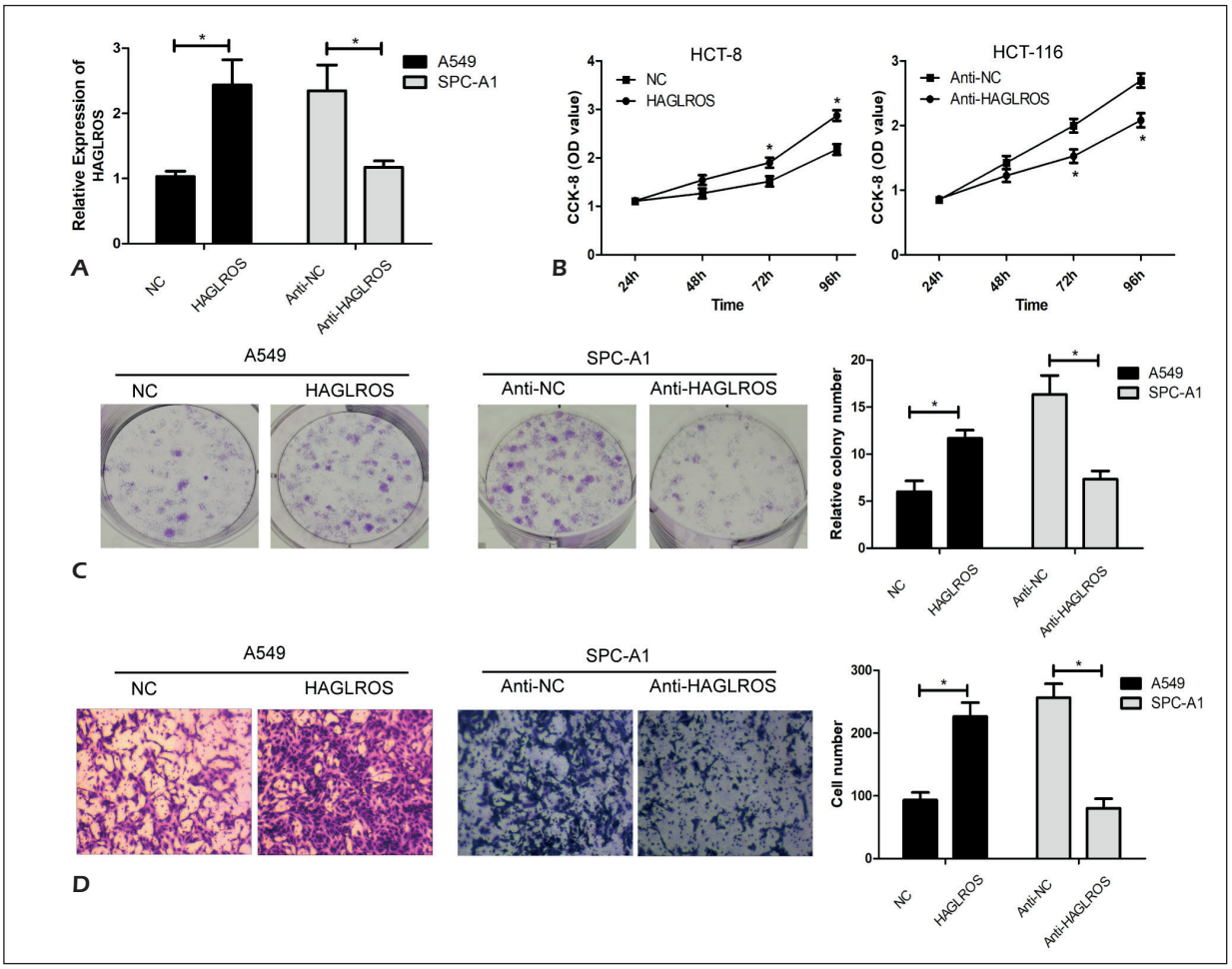

Figure 2. Knockdown of HAGLROS attenuated proliferative and migratory abilities of LCa cells. ***A***, Transfection efficacy of pcDNA-HAGLROS and anti-HAGLROS in A549 and SPC-A1 cells, respectively. ***B***, CCK-8 assay showed the viability of A549 cells transfected with NC or pcDNA-HAGLROS *(left)* and that of SPC-A1 cells transfected with anti-NC or anti-HAGLROS *(right)*. ***C***, Colony formation assay showed the number of formed colonies in A549 cells transfected with NC or pcDNA-HAGLROS *(left)* and that in SPC-A1 cells transfected with anti-NC or anti-HAGLROS *(right)*. ***D***, Transwell assay showed the migration ability of A549 cells transfected with NC or pcDNA-HAGLROS (left) and that of SPC-A1 cells transfected with anti-NC or anti-HAGLROS (right) (Magnification × 40).

In this study, we first collected paired LCa tissues and adjacent normal tissues from patients. Forward and reverse primers of HAGLROS were synthesized based on the sequences of HAGLROS searched from the database. The HAGLROS level in both LCa tissues and cell lines was determined by qRT-PCR. As the data revealed, the HAGLROS expression was significantly higher in LCa tissues than in adjacent normal tissues. Moreover, the HAGLROS expression was correlated with tumor stage and lymphatic metastasis, rather than age, gender, and distant metastasis of LCa patients. Subsequently, *in vitro* experiments demonstrated that the knockdown of HAGLROS significantly attenuated the proliferative and migratory abilities of LCa cells. The above results suggested that HAGLROS served as an oncogene in LCa.

Current studies[16-18] have indicated that lncRNAs can sponge microRNAs to downregulate their levels in tumors. For example, lncRNA HULC sponges miR-372 and abolishes its inhibitory effect on the downstream gene PRKACB. This may eventually attenuate the progression of liver cancer through PKA pathway[21]. Here, HAGLROS was negatively correlated with microRNA-152 in LCa. Meanwhile, their binding relationship was verified through the Dual-Luciferase reporter gene assay. Importantly, HAGLROS regulated the malignant progression of LCa by interacting with microRNA-152. Our findings confirmed that HAGLROS endogenously competed with microRNA-152 to stimulate the progression of LCa.

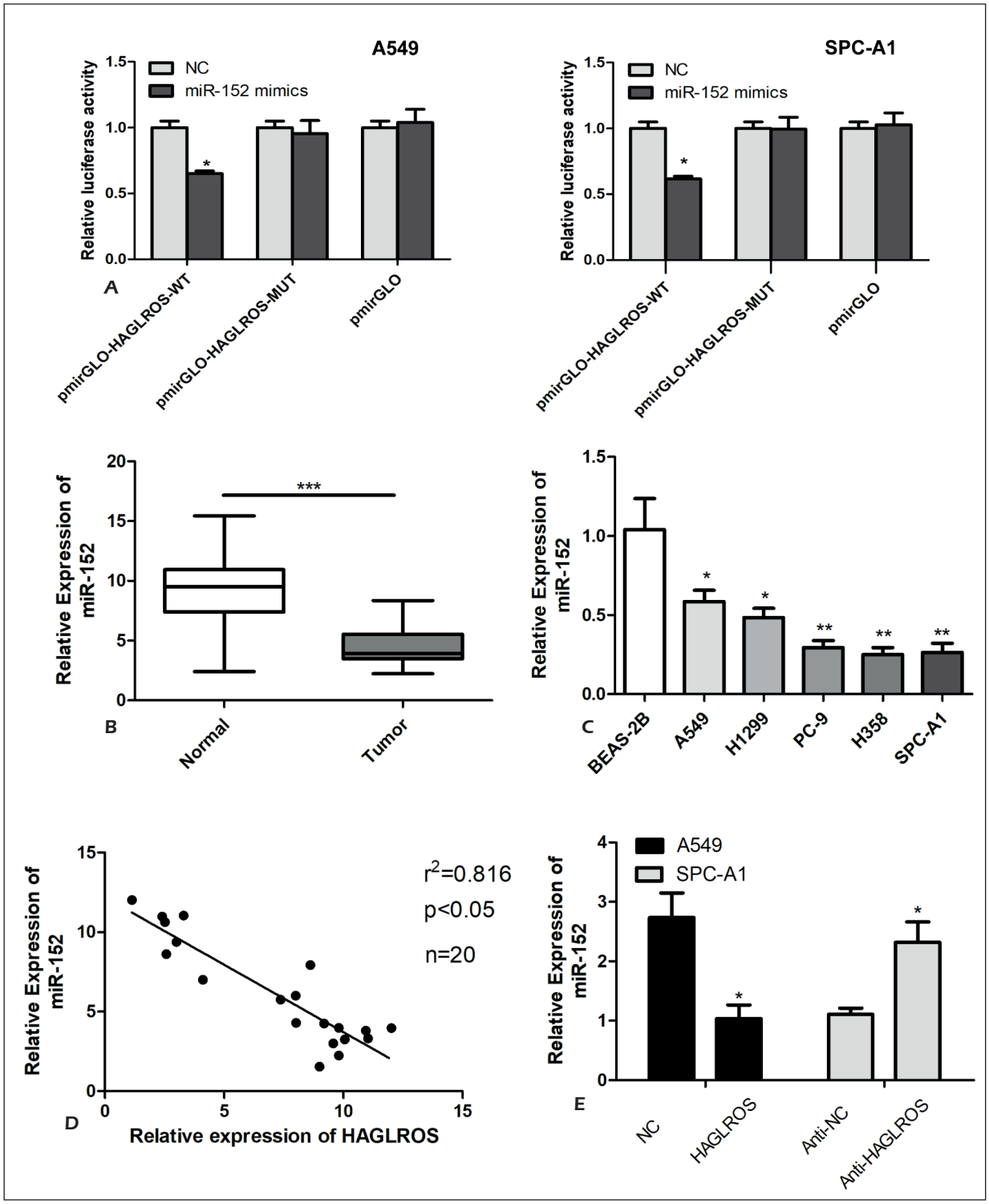

Figure 3. HAGLROS bound to miR-152 in LCa. ***A***, Relative luciferase activity in A549 and SPC-A1 cells co-transfected with NC/miR-152 mimics and pmirGLO-HAGLROS-WT/pmirGLO-HAGLROS-MUT/pmirGLO. ***B***, Relative level of miR-152 in LCa tissues and adjacent normal tissues (n=44). ***C***, Relative level of miR-152 in pulmonary epithelial cell line (BEAS-2B) and LCa cell lines (A549, H1299, PC-9, H358 and SPC-A1). ***D***, A negative correlation was observed between HAGLROS expression and miR-152 expression in 20 LCa patients. ***E***, Relative level of miR-152 in A549 cells transfected with NC or pcDNA-HAGLROS *(left)* and that in SPC-A1 cells transfected with anti-NC or anti-HAGLROS *(right)*.

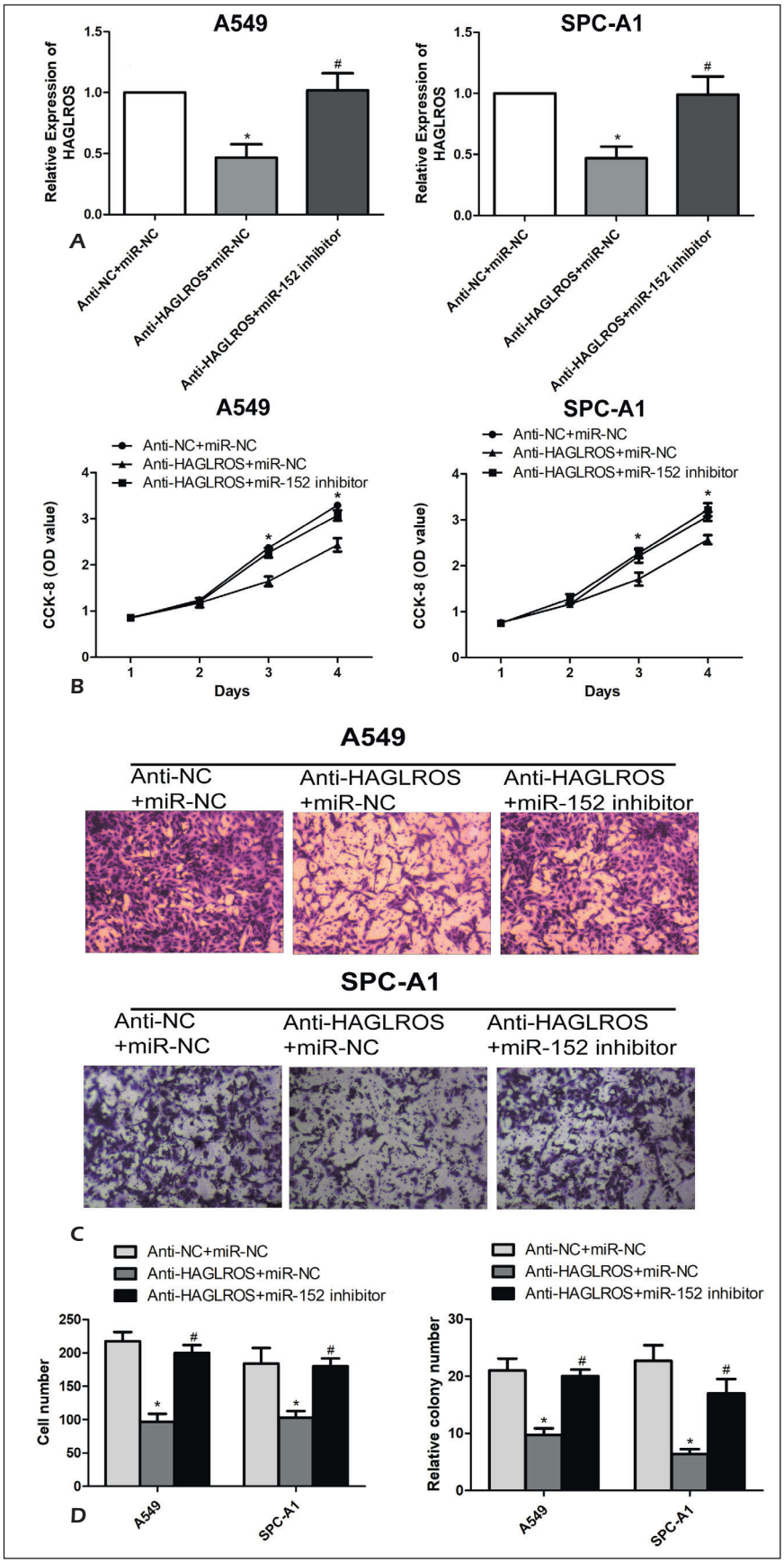

Figure 4. HAGLROS/miR-152 axis in LCa cells. A, 549 and SPC-A1 cells were transfected with anti-NC+miR-NC, anti-HAGLROS+miR-NC or anti-HAGLROS+miR-152 inhibitor. ***A***, Relative level of HAGLROS in each group. ***B***, CCK-8 assay showed cell viability in each group. ***C***, Transwell assay showed cell migration in each group (Magnification × 40). ***D***, The number of formed colonies in each group.

Conclusions

We revealed that the HAGLROS level is remarkably correlated with tumor stage and lymphatic metastasis of LCa. Furthermore, it accelerates the proliferation and migration of LCa cells by regulating microRNA-152.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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