



The biological functions of LGR5 in promoting non-small cell lung cancer progression

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Background: The Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), which is used as a marker of adult stem cells and colorectal cancer stem cells (CSCs), is closely associated with the progression of non-small cell lung cancer (NSCLC). This study aimed to identify the clinical significance and biological function of LGR5 in NSCLC.

Methods: Quantitative reverse transcription polymerase chain reaction (RT-PCR) was applied to detect the expression of LGR5 and stemness-related genes in 22 NSCLC patients, and the clinical significance of LGR5 in NSCLC progression was estimated by statistical analysis. LGR5 overexpressing A549- and H1299-transfected cells were established, and CCK-8 and clone formation assays were used to test the proliferation ability. A wound-healing assay was utilized to clarify the migration ability. The invasion ability was confirmed via the Transwell assay kit.

Results: LGR5 expression was markedly higher in NSCLC tissues than in the matched adjacent normal tissues, and had a trend to associate with tumor size, lymph node metastasis, and TNM stage. The proliferation rates, clone formation rates, wound healing rates, number of invasive cells, and the NOTCH1 expression of the LGR5 overexpressing groups, were significantly higher than those of the control groups.

Conclusions: LGR5 plays an essential role in NSCLC tumorigenesis and is closely associated with the proliferation, metastasis, and invasion of NSCLC cells. LGR5 may promote NSCLC progression via NOTCH1 and could be a new target for gene-targeted therapies for NSCLC.

Keywords: Proliferation; metastasis; invasion; leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5); non-small cell lung cancer (NSCLC)

Submitted Jul 27, 2018. Accepted for publication Jan 17, 2019.

doi: 10.21037/tcr.2019.01.24

View this article at: <http://dx.doi.org/10.21037/tcr.2019.01.24>

Introduction

Lung cancer is the leading cause of cancer mortality in men and the second leading cause of cancer mortality in women, and accounted for 18.4% of total global cancer mortalities in 2018 (1). With increasing pollution (2) and

overabundance of fine ambient particles (PM_{2.5}) (3), lung cancer poses significant public health problems in China, especially non-small cell lung cancer (NSCLC), which accounts for more than 80% of all lung cancers (4). Due to the poor prognosis and lack of an effective early diagnostic

method for NSCLC, new targets for diagnosis and treatment are urgently needed (5).

Evidence has shown that the presence of cancer stem cells (CSCs) is one of the reasons for the poor therapeutic effect (6,7). A recent study indicated that NSCLC also harbored CSC populations, which can lead to tumor recurrence, therapy resistance, and distant metastasis (8-11).

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a member of the G-protein-coupled receptor superfamily, which has been proven to be a stem cell marker in colorectal cancers (CRC) (12) and a potentiator of the Wnt signaling pathway (13,14). It was reported that LGR5 had a strong correlation with tumor growth, invasion, and poor prognosis in CRC. Also, liver metastases derived from the primary CRCs were dependent on Lgr5⁺ CSCs (15-17). LGR5⁺ cells in intestinal adenomas were also found to be highly proliferative and correlated with tumor expansion and invasion (18). A previous study found that LGR5 was also expressed in NSCLC and was related to cancer progression (19). However, the mechanism for LGR5 in the modulation of the biological function of NSCLC cells remains unknown. Therefore, the goal of this study was to analyze LGR5 expression in human NSCLC tissues and evaluate the relationship between LGR5 expression and clinical parameters. We also investigated the role of LGR5 in the proliferation, migration, and invasion of NSCLC *in vitro*.

Methods

Clinical samples

A total of 22 NSCLC tissues and matched adjacent normal tissues were collected from January 2015 to March 2017 at the Fifth People's Hospital of Suzhou (Suzhou, China). None of the patients had received preoperative chemotherapy or radiotherapy. The median age was 64 years (range, 42-77 years), and the sample included 17 males and 5 females. Disease stage was classified according to the criteria recommended by the International Association for the Study of Lung Cancer (IASLC). Permission for this study was granted by the Ethics Committee of The Fifth People's Hospital of Suzhou (No. 2016010), and the study was conducted according to the 2013 revision of the Declaration of Helsinki guidelines. Informed consent was obtained from all patients involved in this study.

Cell lines and culture

Human NSCLC cell lines A549 and H1299 were obtained from the American Type Culture Collection and cultured in RPMI-1640 (GE Healthcare HyClone, USA) supplemented with 10% FBS (GE Healthcare HyClone, USA).

LGR5 transfection in A549 and H1299 cells

The full-length LGR5 cDNA was amplified by PCR, and the primer sequences were as follows: 5'-CCAACTTGTGCCAACCGGTCGCCACCATGGACACCTCCCGGC TCGG TGTGCTC-3' and 5'-AATGCCAACTCTGAGC TTGAGACATGGGACAAATGCCACAGAGG-3'.

The LGR5 DNA fragment was cloned into the AgeI and NheI sites of the lentivirus vector GV341 (Gene Chem, China), which was then transfected into A549 and H1299 cells. Cells transfected with the empty vector were used as controls. Stable LGR5-expressing clones were selected by puromycin (Gene Chem, China).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells and tissues by the TRIzol method. 1µg of total RNA was used for cDNA synthesis, and cDNA was prepared by using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Quantitative RT-PCR analysis was performed on StepOne Plus (ABI) using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA). The primer sequences for GAPDH were 5'-ATCATCCCTGCCTCTACTGG-3' and 5'-TTTCTAGACGGCAGGTCAGGT-3', and those for LGR5 were 5'-GCAAACCTACGTCTGGACAA-3' and 5'-TGATGCTGGAGCTGGTAAAG-3'. The primer sequences for NOTCH1, SMAD2, NANOG, and OCT4 were 5'-GAGGCGTGGCAGACTATGC-3' and 5'-CTTGTA CTCCGT CAGCGTGA-3'; 5'-CCGACACACCGAGATCCTAAC-3' and 5'-GAGGTGGCGTTTCTGGAATATAA-3'; 5'-TTTGTGGGCCTGAAGAAACT-3' and 5'-AGGGCTGTCCTGAATAAGCAG-3'; and 5'-CTTGAATCCC GAATG GAAAGGG-3' and 5'-GTGTATATCCCAGGGTGATCCTC-3', respectively. The reaction system was as follows: 10 µL of Power SYBR Green PCR Master Mix, 1 µL of primer, 8 µL of H₂O,

and 1 μL of template cDNA. The mRNA expression was calculated using the $2^{-\Delta\text{Ct}}$ method.

Flow cytometry analysis

The expression of LGR5 in transfected A549/H1299 cells was measured using the rat anti-human LGR5 antibody (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The cells were suspended at a density of 1×10^6 cells/mL in Hank's balanced salt solution (HBSS) with 1% FCS, incubated with the antibody (10 μL) for 30 min, and then washed once by HBSS with 1% FCS. Flow cytometry was performed on an FC 500 analyzer (Beckman Coulter). Data were analyzed by FlowJo (San Carlos, CA, USA).

Cell proliferation assay

Transfected cells were seeded in 96-well plates at a density of 3×10^3 cells per well, and cell proliferation was examined after 0, 24, 48, 72, and 96 h. At each time point, 10 μL of CCK8 (Vazyme, China) solution was added to each well and incubated for 2 h at 37 °C. A microplate reader (Thermo Fisher Scientific, USA) was used to detect the optical density at a wavelength of 450 nm.

Clone formation assay

Transfected cells were seeded in 6-well plates at a density of 2×10^2 cells per well. The medium was replaced every 3 days, and the cells were cultured for 12 days. The clones were fixed with a 4% paraformaldehyde solution for 15 min and stained with 0.5% crystal violet for 5 min (Sigma). A clone was defined as >50 cells. Clone formation rate as a percentage was determined according to the following formula: clone formation = (the number of clones/the number of seeded cells) $\times 100\%$. The detection was performed 3 times.

Wound-healing assay

Transfected cells were seeded at a density of 2×10^4 cells per well in 24-well plates and grown to full confluence overnight. The cells were scratched by a plastic tip, washed with PBS 3 times to remove the detached cells, and maintained in a 0.5% FBS-containing RPMI-1640. The cells were incubated at 37 °C in a 5% CO₂ incubator for 24 h. Images of migrating cells were taken at 0, 12, and 24 h after

scratching. The widths of the scratches were measured by Image-Pro Plus. The wound-healing assay was performed at least 3 times.

Invasion assays

The upper portion of the Transwell inserts with an 8 μm pore size was coated with 30 μL of Matrigel diluted 1:4 in serum-free RPMI-1640 and incubated at 37 °C for 4 h. A total of 2×10^4 cells were plated on coated inserts in serum-free medium, and the coated inserts were placed in a 24-well plate with 500 μL RPMI-1640 containing 20% FBS in the bottom chamber. Noninvaded cells and gel were removed from the upper chamber after 24 h incubation at 37 °C in a 5% CO₂ incubator. The cells on the bottom of the membrane were fixed with anhydrous ethanol and then stained with crystal violet (Sigma). The number of invasive cells was counted in 5 random fields per filter at 100-fold magnification. This experiment was performed in triplicate.

Western blot

Cells were washed 3 times in cold PBS and lysed in RIPA buffer. The proteins were separated on 10% SDS-PAGE gels and then blotted onto PVDF membranes. The membrane was blocked with 5% milk and incubated with anti-NOTCH1 antibody (Abcam, UK) overnight and then incubated with HRP-conjugated secondary antibody (Abcam, UK) for 2 h. The proteins were detected using an ECL reagent kit (Thermo Fisher Scientific, USA).

Statistical analysis

Statistical analysis was performed using the SPSS 22.0 software (SPSS Inc., Chicago, USA). *T*-test, one-way ANOVA, and variance analyses were used as the statistical methods for the analysis of significant differences, and a *P* value of <0.05 was considered statistically significant.

Results

LGR5 expression was markedly higher in NSCLC tissues than in matched nontumor lung tissues

To explore the expression pattern of LGR5 and its clinical significance in NSCLC, we detected its expression by quantitative RT-PCR in NSCLC patients. LGR5

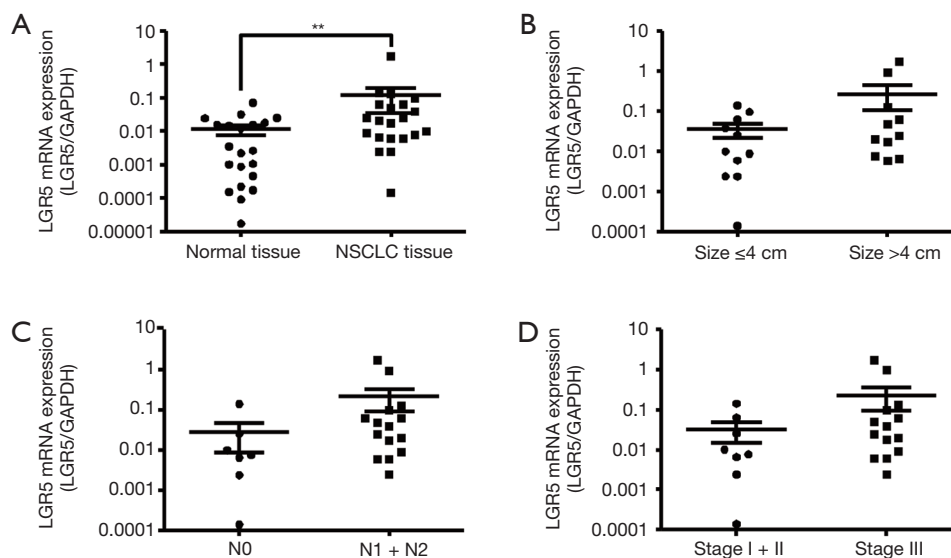


Figure 1 LGR5 expression in NSCLC tissues. (A) LGR5 expression in NSCLC tissues and matched nontumor lung tissues ($P=0.0071$); (B) LGR5 expression in NSCLC tumors of different sizes ($P=0.2934$); (C) LGR5 expression in different lymph node statuses of NSCLC ($P=0.1210$); (D) LGR5 expression in different TNM stages of NSCLC ($P=0.2601$). Statistical analysis was performed by a nonparametric t -test. **, $P<0.01$. LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; NSCLC, non-small cell lung cancer.

expression was significantly higher in NSCLC tissues than in adjacent nontumor lung tissues ($P=0.0071$) (Figure 1A). LGR5 expression showed no significant differences based on tumor size, lymph node status or TNM stage ($P=0.2934$, 0.1210 and 0.2601 , respectively) (Figure 1B,C,D).

The expression of LGR5 was detected in transfected cells

Stable LGR5-overexpressing cells (A549/LGR5 and H1299/LGR5) were established to investigate the role of LGR5 in tumor promotion in NSCLC. Cells transfected with an empty vector were used as controls (A549/Mock and H1299/Mock). Quantitative RT-PCR and flow cytometry were used to examine the expression of LGR5 in the transfected cells. A higher expression of LGR5 mRNA was detected in A549/LGR5 and H1299/LGR5 cells compared with that in the controls ($P<0.0001$, $P=0.0006$, respectively) (Figure 2A). The expression of LGR5 in these cell lines was then assessed by flow cytometry, and the frequency of positive cells reached 97.2% and 97.7% in A549/LGR5 and H1299/LGR5 cells, respectively (Figure 2B). The results showed that we established high-LGR5-expression cell lines. When detecting the stemness-related gene expression in the transfected cells, we only found that the expression of NOTCH1 increased in both

A549/LGR5 and H1299/LGR5 cells compared to that in A549/Mock and H1299/Mock cells ($P=0.0036$, $P=0.0192$, respectively) (Figure 2C,D).

LGR5 promoted the proliferation and clone formation ability of NSCLC cells

To identify the biological function of LGR5 in NSCLC cell growth, we evaluated the proliferation and clone formation abilities of A549/Mock, A549/LGR5, H1299/Mock, and H1299/LGR5 cells. The proliferation of these cells was detected at 0, 24, 48, 72, and 96 h by CCK-8 assay. The results showed that compared with the mock cells, LGR5-overexpressing cells had a higher rate of proliferation ($P=0.0002$, $P=0.004$) (Figure 3A,B). Next, we used the clone formation assay to test the cell proliferation ability further. The investigation showed that overexpression of LGR5 promoted clone formation ($P=0.0059$, $P=0.0018$) (Figure 3C).

LGR5 enhanced the migration of NSCLC cells

As previously reported, LGR5 can promote the invasion and migration of colorectal cancer (16), esophageal squamous cell carcinoma (20) and gastric cancer (21). To further

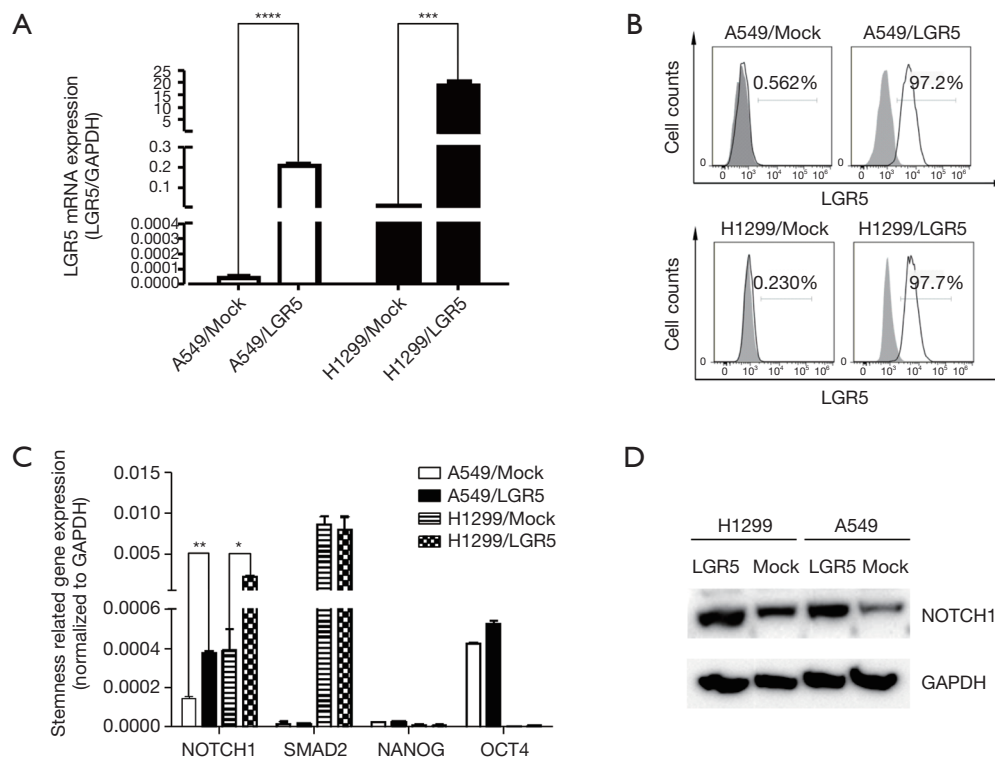


Figure 2 Overexpression of LGR5 in human NSCLC cell lines. (A) LGR5 mRNA expression was estimated by qRT-PCR. The expression of LGR5 in A549/LGR5 and H1299/LGR5 cells was higher than that in matched Mock cells ($P < 0.0001$, $P = 0.0006$, respectively); (B) the expression of LGR5 was detected by flow cytometry. Gray = isotype control, black line = expression of LGR5; (C) the stemness-related gene expression was detected in the transfected cells. The expression of NOTCH1 was higher in both A549/LGR5 and H1299/LGR5 cells compared to that in A549/Mock and H1299/Mock cells ($P = 0.0036$, $P = 0.0192$); (D) the expression of NOTCH1 was detected by Western blot in the transfected cells. NOTCH1 expression was increased in LGR5-overexpressing NSCLC cell lines. Statistical analysis by unpaired *t*-test with Welch correction. ***, $P < 0.001$, ****, $P < 0.0001$. LGR5, Leucine-rich repeat-containing G-protein-coupled receptor 5; NSCLC, non-small cell lung cancer.

confirm the role of LGR5 in NSCLC cell migration, we performed a wound-healing assay with LGR5-transfected and Mock-transfected NSCLC cells. LGR5 enhanced the migration of A549 cells after 12 h ($P = 0.0029$) (Figure 4A) and H1299 cells after 12 and 24 h ($P = 0.0387$, $P = 0.0414$, respectively) (Figure 4B).

LGR5 increased the invasion ability of NSCLC cells

A Transwell invasion assay was used to determine the number of invasive cells to compare the invasion ability of LGR5-transfected NSCLC cells with that of the Mock-transfected control cells. The results showed that both the LGR5-transfected A549 cells (Figure 5A) and LGR5-

transfected H1299 cells (Figure 5B) had increased invasion ability compared to the control cells.

Discussion

Lung cancer, which is the leading cause of cancer-related death, is a hazard to human health worldwide. The current 5-year relative survival rate of lung cancer is 18% (22). Little improvement has been made in the survival of lung cancer compared with that of most other cancers. Finding useful therapeutic targets for NSCLC has significant clinical value. Previous studies have shown that LGR5 is a stem cell marker in CRC and can promote tumor progression in various cancers, including CRC (17), ovarian

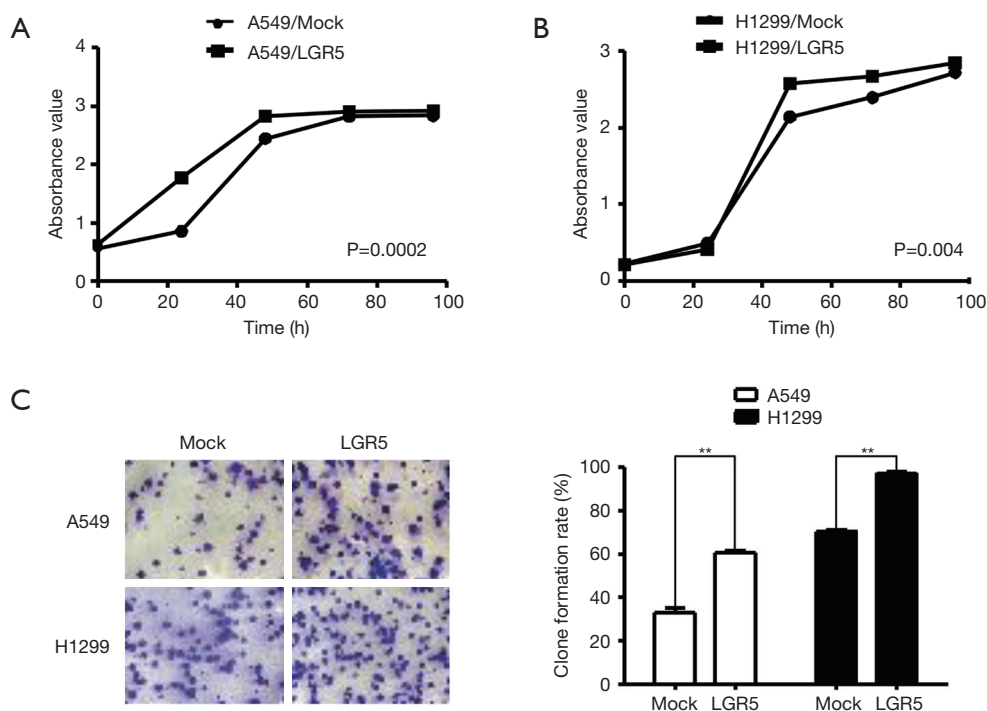


Figure 3 Overexpression of LGR5 promoted the proliferation of NSCLC cells. (A) Overexpression of LGR5 promoted the proliferation of A549 cells ($P=0.0002$); (B) overexpression of LGR5 promoted the proliferation of H1299 cells ($P=0.004$); (C) comparison of the clone-forming ability of each group. LGR5 promoted the clone formation of A549 and H1299 cells ($P=0.0059$ and $P=0.0018$, respectively). Statistical analysis by one-way ANOVA with replicate measures or unpaired t -test. **, $P<0.01$. LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; NSCLC, non-small cell lung cancer.

cancer (23), and cervical cancer (24). Although many studies have found that LGR5 was significantly increased in NSCLC tissues and was associated with the poor prognosis of NSCLC (19,25), the mechanism of LGR5 in modulating the biological functions of NSCLC cells remains unknown.

In the present study, we confirmed that LGR5 expression was markedly higher in NSCLC tissues than in matched nontumor lung tissues. However, the LGR5 expression only showed a trend associated with larger tumor size, lymph node metastasis, and higher TNM stage because of the small sample size. These results suggest that LGR5 plays an essential role in NSCLC development.

Using LGR5-overexpressing NSCLC cells, this study further investigated the molecular function of LGR5 in NSCLC. The results revealed that the proliferation ability of LGR5-overexpressing cells was significantly higher than that of the control groups. To further explore the role of LGR5 in cell proliferation and population

dependence, we applied a clone formation assay to test the clone formation rates. The clone formation rates of LGR5-overexpressing cells were significantly higher than those of the control groups. These results demonstrated that the upregulated expression of LGR5 in NSCLC could promote proliferation and clone formation, which may lead to stronger tumorigenicity of tumor cells. Using a wound-healing assay and a Transwell assay, we found that the migration and invasion abilities of LGR5-overexpressing cells were higher than those in the control groups. These results suggest that an upregulated LGR5 in NSCLC may promote metastasis and invasion. To demonstrate whether LGR5 expression is associated with stemness in NSCLC, we detected the expression of the stemness-related gene in LGR5-overexpressing cells. Only the expression of NOTCH1 was increased in both A549/LGR5 and H1299/LGR5 cells compared to that in A549/Mock and H1299/Mock cells. The NOTCH signaling pathway participates

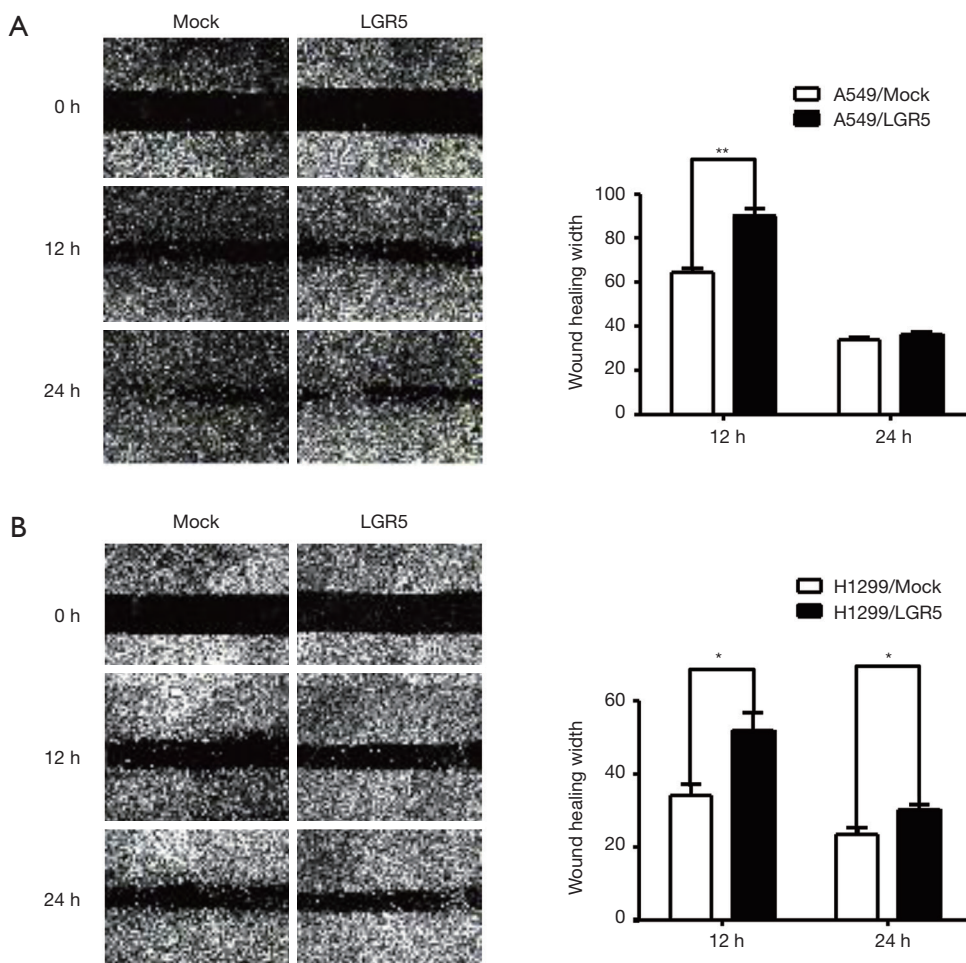


Figure 4 LGR5 enhanced the migration of NSCLC cells. A wound-healing assay was used to examine the effect of LGR5 on the migration of A549 (A) and H1299 cells (B). Statistical analysis by unpaired *t*-test. *, $P < 0.05$; **, $P < 0.01$. LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; NSCLC, non-small cell lung cancer.

in regulating cell proliferation and differentiation (26,27). A previous study showed that LGR5 could promote ovarian cancer progression via NOTCH1 signaling (23) and that the NOTCH signaling pathway could control the proliferation and differentiation of *Lgr5*⁺ cells (28). These results suggest that LGR5 could also promote NSCLC progression via the NOTCH signaling pathway.

Conclusions

In summary, LGR5 expression was higher in NSCLC

patients, was closely associated with tumorigenicity, metastasis, and invasion in NSCLC cells and could increase NOTCH1 expression. The results suggested that the function of LGR5 in promoting NSCLC progression may be correlated with NOTCH1. Our study explored the function of LGR5 in the biological behavior of NSCLC for the first time. These results imply that LGR5 might be a new target for gene-targeted therapies for NSCLC. Further studies are needed to explore the molecular mechanisms and signaling pathways involved in regulating LGR5 dysregulation in NSCLC.

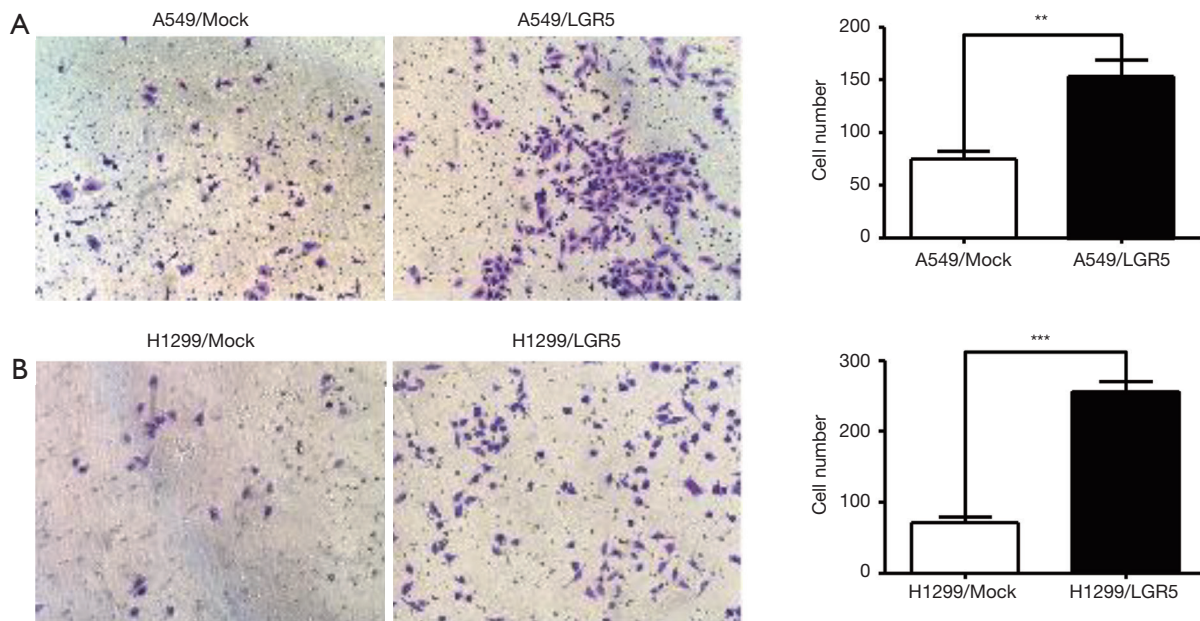


Figure 5 LGR5 increased the invasion ability of NSCLC cells. A Transwell invasion assay was used to examine the effect of LGR5 on the invasion of A549 (A) and H1299 cells (B). Statistical analysis by unpaired *t*-test. **, $P < 0.01$; ***, $P < 0.001$. LGR5, Leucine-rich repeat-containing G-protein-coupled receptor 5; NSCLC, non-small cell lung cancer.

Acknowledgments

Funding: This work was supported by the Science and Technology Development Foundation of Nanjing Medical University (grant number 2016NJMU145), the Science and Technology Plan of Suzhou, China (grant number SYS201369, SS201657), and the Health Plan of Jiangsu, China (grant number H201652, H2017068).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.01.24>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all patients. The study was approved by the Ethics Committee of The Fifth People's Hospital of Suzhou (No. 2016010).

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Cite this article as: Gao F, Xu JC, You XR, Gao X, Wei JL, Li SX, Zhu CL, Yang C. The biological functions of LGR5 in promoting non-small cell lung cancer progression. *Transl Cancer Res* 2019;8(1):203-211. doi: 10.21037/tcr.2019.01.24