

Leucine-rich repeat-containing G protein-coupled receptor 4 promotes proliferation, invasion and migration, and inhibits apoptosis in non-small cell lung cancer cells

XIAOYU DUAN^{1,2}, XIANGSEN LIU², YUCHEN SHAN², SONGFU ZHOU²,
MING LOU², ZHAOJIA GAO² and KAI YUAN^{2,3}

¹School of Medicine, Dalian Medical University, Dalian, Liaoning 116044, P.R. China; ²Department of Cardiothoracic Surgery, The Third Affiliated Hospital of Nanjing Medical University, Changzhou Second People's Hospital, Changzhou, Jiangsu 213161, P.R. China; ³Heart and Lung Disease Laboratory, The Third Affiliated Hospital of Nanjing Medical University, Changzhou Second People's Hospital, Changzhou, Jiangsu 213161, P.R. China

Received April 10, 2025; Accepted August 21, 2025

DOI: 10.3892/ol.2025.15304

Abstract. Leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4/GPCR48) belongs to subfamily B of the GPCR superfamily. LGR4 is a transmembrane signaling molecule involved in organogenesis across multiple tissue systems and contributes to cancer progression. To the best of our knowledge, however, the specific involvement of LGR4/GPCR48 in non-small cell lung cancer (NSCLC) has not been comprehensively studied. The present study aimed to elucidate the mechanisms by which LGR4 promotes the progression of NSCLC. First, the expression and survival of LGR4 were analyzed by downloading relevant data from The Cancer Genome Atlas database. Subsequently, immunohistochemistry was conducted to evaluate the association between LGR4 expression levels and prognostic outcomes in tissue samples from NSCLC. LGR4 levels were further determined across NSCLC cell line subtypes via western blotting and reverse transcription-quantitative PCR. LGR4 expression was silenced in the A549 and H226 cell lines using small interfering RNA. Flow cytometry, Cell Counting Kit-8 assays and Transwell assays were then used to study the effects on tumor cells after LGR4 knockdown. Finally, to elucidate the oncogenic mechanisms of LGR4, Gene Set Enrichment Analysis (GSEA) 4.3.3 software was utilized to identify signaling pathways potentially

activated by the elevated expression of LGR4 in NSCLC tumor samples. The present findings indicated that LGR4 was significantly overexpressed in NSCLC and associated with worse clinical outcomes. Additionally, silencing LGR4 promoted apoptosis in A549 and H226 cells, while also suppressing cell proliferation, invasion and metastasis in NSCLC. Moreover, GSEA indicated that elevated expression of LGR4 in NSCLC may trigger activation of critical signaling pathways, such as the Wnt/ β -catenin, TGF- β and PI3K/AKT/mTOR pathways. Therefore, LGR4 could represent a promising biomarker and a potential target for therapy in the management of NSCLC.

Introduction

According to GLOBOCAN statistics, lung cancer is the most frequently diagnosed cancer globally and a major contributor to cancer-related deaths worldwide, accounting for ~12.4% of all new cancer cases and 18.7% of all cancer deaths in 2022 (1). Non-small cell lung cancer (NSCLC), primarily consisting of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), is the predominant form of lung cancer (2). In previous years, substantial strides have been made in the treatment of lung cancer, including advancements in screening, diagnosis and minimally invasive treatments. Additionally, progress in radiotherapy, including stereotactic ablative radiotherapy, has become an effective approach for treating lung cancer (3-5). Meanwhile, the emergence of new targeted therapies and immunotherapies has markedly improved the survival rate of patients with NSCLC (6,7). Patients with NSCLC who are eligible for targeted therapy and immunotherapy now have a longer survival time, with a 5-year survival rate ranging from 15.0 to 62.5%, depending on the biomarkers (8). This underscores the importance of identifying new molecular markers and therapeutic targets for NSCLC.

Leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4/GPCR48) belongs to the GPCR family and can regulate developmental pathways through typical G protein signaling (9). LGR4 is also involved in cell

Correspondence to: Dr Kai Yuan, Heart and Lung Disease Laboratory, The Third Affiliated Hospital of Nanjing Medical University, Changzhou Second People's Hospital, 68 Gehu Middle Road, Wujin, Changzhou, Jiangsu 213161, P.R. China
E-mail: yuankai1978@163.com

Key words: leucine-rich repeat-containing G protein-coupled receptor 4, non-small cell lung cancer, immunohistochemistry, Gene Set Enrichment Analysis, overall survival, proliferation, invasion, migration, apoptosis

proliferation and organ development. For example, LGR4 deficiency decreases the migration and proliferation of eyelid epidermal keratinocytes, and it also disrupts postnatal intestinal crypt development, leading to defective epithelial proliferation and abnormal Paneth cell differentiation (10). Moreover, numerous studies have reported that LGR4 promotes tumor progression, such as in colorectal (11), breast (12) and prostate (13) cancer. A recent study indicates that the R-spondin (RSPO)-LGR4/5-E3 ubiquitin-protein ligase ZNRF3 (ZNRF3)/E3 ubiquitin-protein ligase RNF43 (RNF43) signaling complex critically regulates Wnt/ β -catenin signaling in hepatic biology (14). Dysregulation of the RSPO-LGR4/5-ZNRF3/RNF43 complex, commonly caused by RSPO overexpression or loss-of-function mutations in ZNRF3/RNF43, constitutes a major oncogenic driver in hepatocellular carcinoma that induces constitutive Wnt/ β -catenin activation (15). However, another study indicates that LGR4 potentiates breast cancer metastasis via a Wnt-independent mechanism, wherein it directly interacts with epidermal growth factor receptor (EGFR) and suppresses E3 ubiquitin-protein ligase CBL-mediated ubiquitination, thereby impeding EGFR degradation and augmenting EGFR signaling activation (16). Nevertheless, the function of LGR4 in NSCLC requires further investigation.

The present study aimed to investigate the role of LGR4 in NSCLC and potential mechanisms underlying its involvement in tumor progression. Understanding the contribution of LGR4 may provide insights into its significance in NSCLC pathophysiology and its potential as a therapeutic target.

Materials and methods

Analysis of The Cancer Genome Atlas (TCGA) data. NSCLC mRNA sequencing data were retrieved from TCGA, comprising 1,043 tumor samples and 110 normal samples from TCGA-LUAD and TCGA-LUSC (cancer.gov/tcga). The data were analyzed using R software (version 4.3.3; R Foundation) to determine LGR4 gene expression levels in NSCLC. Patients were divided into high and low LGR4 expression groups based on the median expression. Kaplan-Meier survival analysis was performed using the log-rank test.

Tissue samples from patients. To evaluate the prognostic relevance of LGR4 expression in NSCLC, two independent tissue microarrays (TMA1 and TMA2) were utilized to validate its association with clinical outcomes. TMA1 (cat. no. ZL-lug1201) included 60 pairs of NSCLC tumors and paracancerous tissue, sourced from Superbiotek, for comparing LGR4 expression. TMA2 comprised samples from 140 patients with NSCLC and 10 normal controls (28 female, 112 males) who underwent surgery at the Department of Thoracic Surgery, Zhongshan Hospital, Fudan University (Shanghai, China) between January and December 2005. The normal control lung tissue samples were resected from patients undergoing surgery for benign pulmonary diseases (such as pulmonary bulla) and confirmed tumor-free by histopathological examination. Clinical follow-ups were conducted until July 2013 and stages Ia-IIIa, according to the American Joint Committee on Cancer and Union for International Cancer Control criteria (17).

Immunohistochemical (IHC) staining. The TMA of both groups was stained immunohistochemically with rabbit anti-LGR4 (Proteintech Group, Inc.; cat. no. 20150-1-AP; 1:400). Tissue sections were baked at 59°C for 60 min and then immersed sequentially in xylene and an ethanol series for 10 min each. After hydration and washing in distilled water for 10 min, the sections were exposed to 3% H₂O₂ for 10 min. Antigen retrieval was performed by diluting the Antigen Retrieval Solution (Weiao; cat. no. WH1034; 1:1 and heating it in a pressure cooker to boiling (~100°C). Tissue sections were steamed for 2 min 30 sec, then cooled to room temperature and washed twice with 1X PBS (Weiao, China; cat. no. WB6020) for 15 min each. The sections were blocked with 5% BSA (Weiao, China; cat. no. WH2051) at room temperature for 35 min, then incubated overnight with the primary antibody (rabbit anti-LGR4; Proteintech, Wuhan, China; cat. no. 20150-1-AP; 1:400) at 4°C. After four washes with PBS (15 min each), the sections were incubated with the secondary antibody (HRP-conjugated goat anti-rabbit IgG; ImmunoWay, China; cat. no. RS0002; 1:100) at 37°C for 35 min, followed by further washing. The color reaction was developed according to the manufacturer's instructions, followed by hematoxylin counterstaining at room temperature for 1 min and a 10 min wash. Finally, dehydration of the sections was carried out through an ethanol gradient and xylene, followed by mounting with a sealing agent.

The average optical density (AOD) values from TMA1 were analyzed using a paired Student's t-test with GraphPad Prism software (Dotmatics; version 10.1.2). For TMA2, AOD values were divided into high- and low-expression groups based on the median cutoff. Clinicopathological characteristics are summarized in Table I, and univariate and multivariate Cox regression analyses were performed to evaluate whether LGR4 expression serves as an independent prognostic factor in NSCLC. Kaplan-Meier survival analysis was then conducted to assess the prognostic significance of LGR4 expression during the clinical follow-up period.

Cell and cell line culture. Human healthy lung epithelial cells (BEAS-2B; cat. no. TCH-C132), and NSCLC cell lines A549 (cat. no. TCH-C116) and H226 (cat. no. TCH-C279) were obtained from Haixing Biosciences and cultured in DMEM (cat. no. BL305A, Biosharp) supplemented with 10% fetal bovine serum (FBS; cat. no. SLB-13011-8611; Zhejiang Tianhang Biotechnology Co., Ltd.) and 1% penicillin-streptomycin at 37°C.

Small interfering RNA (siRNA) transfection. Cells were transfected with one of three siRNAs targeting LGR4 from the 'siRNA 3-in-1 package' (Ruibo Bio, Shanghai, China), and with a non-targeting scrambled control si-NC. The sequences (5'-3') were as follows: si-LGR4 #1: forward GAAAGACUCAA AGUUCUAAAC, reverse UAGAACUUUGAGUUCUUUCAA. si-LGR4 #2: forward GGUAGUUCUGCAUCUUCUAA, reverse AUGAAGAUGCAGACUACCAAG. si-LGR4 #3: forward GCUGCGCGGACUGCUGAAGG, reverse UUC AGCAGUCGCGCGCAGCGG. si-NC: forward UUCUUC GAAGGUGUCACGUTT, reverse ACGUGACACCUUCGA AGAATT. A549 and H226 cells were seeded 3x10⁵ into 6-well plates, each with 2 ml of complete medium as aforementioned.

Table I. Clinicopathological characteristics of 126 patients with non-small cell lung cancer.

Characteristic	Total (n=126)	High LGR4 expression (n=63)	Low LGR4 expression (n=63)	P-value
Mean age, years	59.8±10.2	59.1±10.6	60.5±9.8	0.455
Sex, n (%)				0.826
Male	100 (79.4)	51 (81.0)	49 (77.8)	
Female	26 (20.6)	12 (19.0)	14 (22.2)	
Histological type, n (%)				0.017
Adenocarcinoma	48 (38.1)	17 (27.0)	31 (49.2)	
Squamous cell carcinoma	78 (61.9)	46 (73.0)	32 (50.8)	
Tumor stage, n (%)				0.304
I	48 (38.1)	20 (31.7)	28 (44.4)	
II	40 (31.7)	21 (33.3)	19 (30.2)	
III	38 (30.2)	22 (34.9)	16 (25.4)	
Ki-67 (%)				0.434
Negative	89 (70.6)	42 (66.7)	47 (74.6)	
Positive	37 (29.4)	21 (33.3)	16 (25.4)	
Tumor location, n (%)				0.284
Left	59 (46.8)	26 (41.3)	33 (52.4)	
Right	67 (53.2)	37 (58.7)	30 (47.6)	

LGR4, leucine-rich repeat-containing G-protein coupled receptor 4.

This allowed the cells to reach ~50% confluence by the time of transfection. Cells were transfected using Lipo 8000™ Transfection Reagent (cat. no. C0533 Beyotime Institute of Biotechnology, China). Cells were transfected with siRNAs at a final concentration of 50 nM at 37°C (CLM-170B-8-CN, ESCO, Singapore). Transfection efficiency was evaluated by reverse transcription-quantitative PCR (RT-qPCR) at 48 h post-transfection.

RT-qPCR. Total RNA was isolated from the NSCLC cell lines and BEAS-2B cells using the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech Co., Ltd.), following the protocol provided by the manufacturer. cDNA synthesis was performed using the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions. Any genomic DNA present was removed and then the extracted RNA was reverse-transcribed into cDNA. Next, gene-specific primers were designed for the target genes using SnapGene software (version 7.2.1, GSL Biotech LLC), ensuring that the primers only amplified the desired gene fragment and avoided spanning splice junctions. Primer specificity was validated using the NCBI Primer-BLAST tool (version 2.53.0, ncbi.nlm.nih.gov/tools/primer-blast/) to ensure primers would not amplify non-target sequences. Finally, the primers were synthesized through Agena Bioscience, Inc., and the sequences of the primers are provided in Table II.

RT-qPCR was conducted with the Taq Pro Universal SYBR quantitative PCR (qPCR) Master Mix (Vazyme Biotech Co., Ltd.) under the following thermal cycling conditions: An initial denaturation at 95°C for 30 sec followed by 40 cycles

consisting of denaturation at 95°C for 5 sec and extension at 60°C for 60 sec. Gene expression levels were determined using the 2^{-ΔΔCq} method (18), with GAPDH serving as the endogenous control.

Western blot assay. Western blotting was used to evaluate the protein expression of LGR4. Proteins were extracted from A549 and H226 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) with protease and phosphatase inhibitors, quantified by BCA assay. 10% Cell-Free Acrylamide System (CFAS) separation gels were prepared by combining CFAS PAGE Separation Gel A (cat. no. PE004-A, Zhonghui Hecai, China) and Separation Gel B (cat. no. PE004-B, Zhonghui Hecai, China) at a 1:1 ratio, following the manufacturer's instructions (10% CFAS PAGE Rapid Gel Preparation Kit, PE004, Zhonghui Hecai, China), and thoroughly homogenized before use. Proteins were transferred onto PVDF membranes using 1x rapid transfer buffer and blocked with 5% non-fat dried milk in 1x TBST (0.1% Tween-20, Biosharp, China) at room temperature for 2 h. Membranes were incubated with rabbit polyclonal primary antibodies against LGR4 (cat. no. ER63609; HuaAn Biotech, China; 1:400) and GAPDH (cat. no. R1210-1; HuaAn Biotech, China; 1:400) overnight at 4°C, washed with 1x TBST (0.1% Tween-20) 5x7 min, then incubated with HRP-linked goat anti-rabbit IgG (cat. no. A0208; Beyotime Biotechnology, China; 1:10,000) at room temperature for 80 min, followed by the same TBST washing cycles 5x7 min. Protein signals were detected using Super ECL Chemiluminescent Substrate (cat. no. BL520A, Biosharp, China). Images were analyzed using Tanon Image software (<https://tanon.cnreagent.com/>).

Cell Counting Kit-8 (CCK-8) assay. To examine the effect of LGR4 on the proliferative capacity of NSCLC cells, cell proliferation in A549 and H226 cells was measured at 0, 24, 48 and 72 h after transfection with si-LGR4 using the CCK-8 assay. A total of 2,500 cells (A549 and H226) were seeded in 100 μ l of complete medium into each well of a 96-well plate. Then 10 μ l enhanced CCK-8 reagent (cat. no. C0041; Beyotime Institute of Biotechnology) was dispensed into each well. Cells without the culture medium and CCK-8 solution served as the blank control. After incubating both the experimental and control groups in a cell incubator for 2 h, absorbance was measured at 450 nm.

Flow cytometry apoptosis assay. Following transfection, A549 and H226 cells were digested with 0.25% trypsin (without EDTA and phenol red; cat. no. T1350; Beijing Solarbio, China) for 3 min at room temperature until the cells detached. The detached cells were centrifuged at 1,000 $\times$ g for 5 min at room temperature. Thereafter, the supernatant was discarded and the cells were collected. After washing twice with PBS, the cells were stained with Annexin V-FITC and propidium iodide using the Cell Apoptosis Detection Kit (cat. no. C1062M, Beyotime Biotechnology, China), and incubated on ice in the dark for 20 min. Finally, apoptosis was then analyzed using BD Accuri™ C6 software (version 1.0.264.21, BD Biosciences) on the FACS Accuri C6 flow cytometer (BD Biosciences, USA).

Transwell assay. A549 and H226 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS (cat. no. SLB-13011-8611, Sijiqing, China) and 1% penicillin-streptomycin (cat. no. BL505A, Biosharp, China). Transwell inserts with an 8- μ m pore size, 24-well format (cat. no. 3422, Corning, USA) were used for both migration and invasion assays. For invasion assays, the upper surface of the inserts was precoated with Matrigel (cat. no. 082704; Mogengel) at 37°C for 30 min; uncoated inserts were used for migration assays. A total of $\sim 5 \times 10^4$ cells were seeded into the upper chambers containing serum-free DMEM/F12, while 500 μ l of medium supplemented with 10% FBS was added to the lower chambers as a chemoattractant. After 48 h of incubation at 37°C with 5% CO₂, non-migrated or non-invaded cells remaining on the upper membrane surfaces were removed with a cotton swab. Cells on the lower membrane surfaces were fixed with methanol for 30 min and stained with 0.1% crystal violet (cat. no. BL802A, Biosharp, China) for 20 min at room temperature. Migrated or invaded cells were quantified in five random fields under an inverted light microscope (x400; Model CKx53, Olympus, Japan).

Gene Set Enrichment Analysis (GSEA). To explore the possible oncogenic mechanisms of LGR4 in NSCLC, The LGR4 expression matrix was extracted from TCGA NSCLC dataset (<https://www.cancer.gov/tcga>), comprising 1,043 tumor samples, using R software (version 4.3.3; R Foundation), and subsequently converted into gct and cls files for GSEA. Thereafter, GSEA 4.3.3 software (Broad Institute) was employed to analyze the signaling pathways activated in tumor samples exhibiting high LGR4 expression. High and low expression groups were defined using the median LGR4 expression as the cut-off (4.7589). Specifically, the KEGG canonical

pathway gene set (c2.cp.kegg.v2023.1.Hs.symbols.gmt, ftp://ftp.broadinstitute.org/pub/gsea/msigdb/human/gene_sets/c2.cp.kegg.v2023.1.Hs.symbols.gmt) and the HALLMARK gene set (h.all.v2025.1.Hs.symbols.gmt, ftp://ftp.broadinstitute.org/pub/gsea/msigdb/human/gene_sets/h.all.v2025.1.Hs.symbols.gmt) were used. Pathways with normalized enrichment score >1, $P < 0.05$ and FDR <0.05 were regarded as significantly enriched.

Statistical analysis. Intergroup All quantitative data are presented as the mean $\pm$ SD from at least three independent experiments. Two-group comparisons were performed using paired or unpaired Student's t-tests. Multi-group comparisons were analyzed by one-way ANOVA with Dunnett's post hoc test. Time-course experiments, such as CCK8 assays, were evaluated by two-way ANOVA with Šídák's multiple comparisons test. Survival probability estimations were performed with Kaplan-Meier methodology and log-rank testing. Prognostic factor screening used uni- and multivariate Cox proportional hazards models. Statistical analyses were performed with GraphPad Prism (version 10.1.2; Dotmatics) and R software (version 4.3.3; R Foundation). $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were repeated at least three times.

Results

LGR4 expression is upregulated in NSCLC. The present investigation into the role of LGR4 in NSCLC began with an evaluation of its expression using data obtained from the TCGA database. The analysis revealed that LGR4 expression was significantly higher in NSCLC samples than in healthy lung tissue ($P < 0.001$; Fig. 1A). Kaplan-Meier survival curves were plotted to determine the prognostic impact of LGR4. The findings indicated that patients with elevated LGR4 expression had shorter overall survival times compared with those with low expression ($P = 0.013$; Fig. 1B). To further confirm these results, the expression of LGR4 in NSCLC cell lines was assessed by western blotting and RT-qPCR. Both the protein and mRNA protein levels of LGR4 were significantly elevated in NSCLC compared with those in healthy tissues (Fig. 1C and D), with differences considered statistically significant (both $P < 0.05$). Significant downregulation of LGR4 was also consistently observed across all three siRNA knockdown groups via qPCR analysis (all $P < 0.05$; Fig. 1E). Among the three siRNAs targeting LGR4, si-LGR4 #1 and #2 showed comparable knockdown efficiency in preliminary experiments. To maintain consistency in subsequent functional assays, si-LGR4 #2 was used for all following experiments.

Expression of LGR4 in NSCLC tissues. IHC staining of two independent TMAs revealed predominant cytoplasmic localization of LGR4 within tumor cells. Analysis of TMA1 demonstrated elevated LGR4 expression in both LUAD and LUSC specimens compared with matched adjacent paracancerous tissues (Fig. 2A and B). Consistent with this finding, IHC evaluation of TMA2 displayed increased LGR4 levels in malignant tissues compared with those in healthy lung tissues (Fig. 2C). Matched-pair analysis of 60 tumor/paracancerous samples from TMA1 confirmed significantly higher LGR4

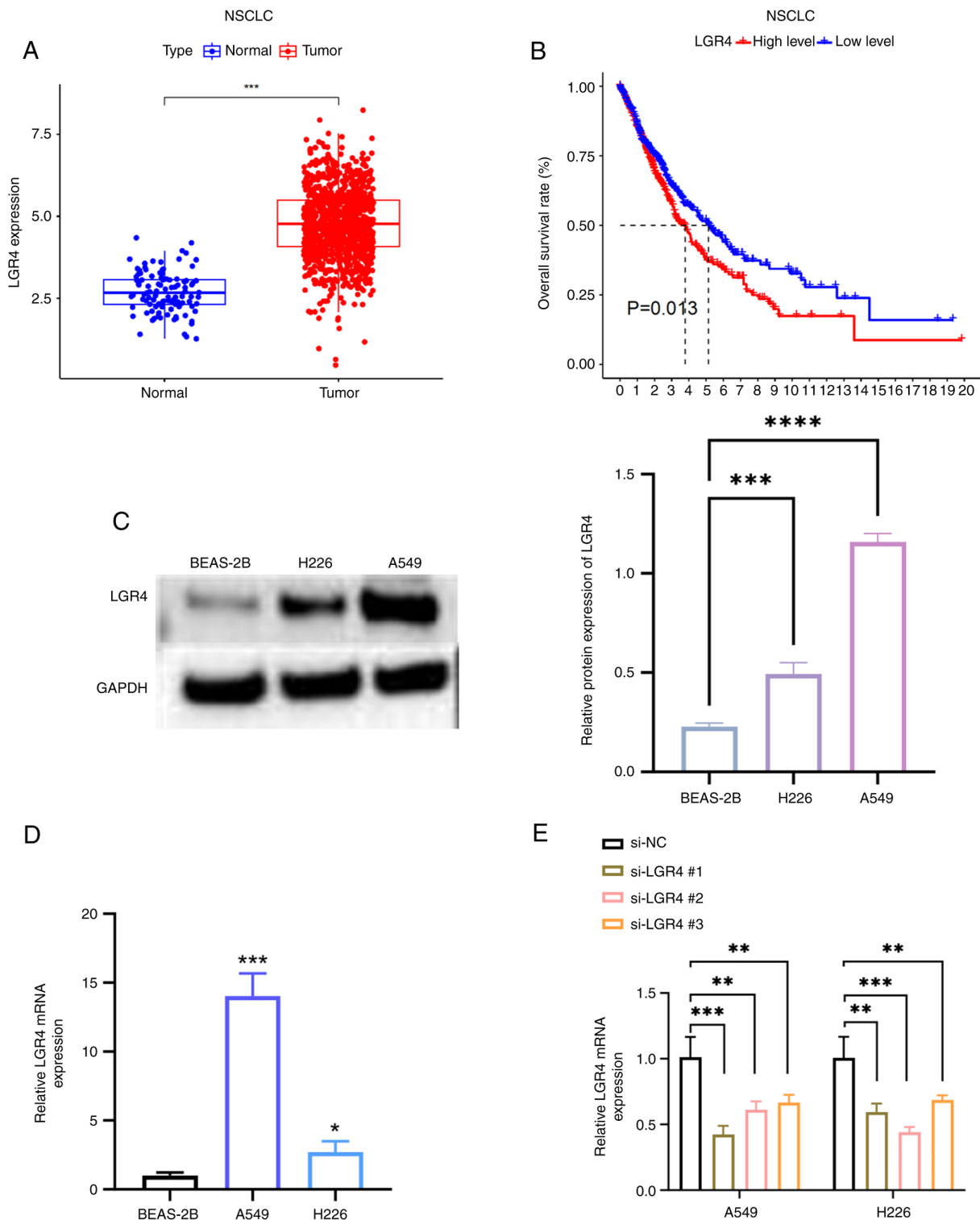


Figure 1. LGR4 is highly expressed in NSCLC. (A) Unpaired analysis. TCGA [NSCLC (n=1,043) vs. healthy tissues (n=110); unpaired Student's t-test]. (B) High LGR4 expression is associated with a poor prognosis in patients with NSCLC (data fromTCGA). (C) Western blot analysis showed the expression level of LGR4 protein in NSCLC cell lines. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. (D) RT-qPCR analysis showed the expression level of LGR4 mRNA in NSCLC cell lines (one-way ANOVA followed by Dunnett's multiple comparison test). (E) RT-qPCR analysis showed that si-LGR4 transfection significantly reduced LGR4 expression in A549 and H226 cells compared with the si-NC group (one-way ANOVA followed by Dunnett's multiple comparison test). LGR4, leucine-rich repeat-containing G-protein coupled receptor 4; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; si-NC, small-interfering RNA negative control.

expression in the cancerous component vs. the relevant para-cancerous counterpart (P<0.0001; Fig. 2D). Moreover, LGR4 expression was significantly increased in tumor specimens compared with healthy lung tissue (n=10) (P<0.0001; Fig. 2E).

Association of LGR4 expression with clinicopathological characteristics. IHC staining of TMA2 resulted in tissue core detachment in some cases. Therefore, patients lacking evaluable staining due to detachment were excluded, leaving 126 patients

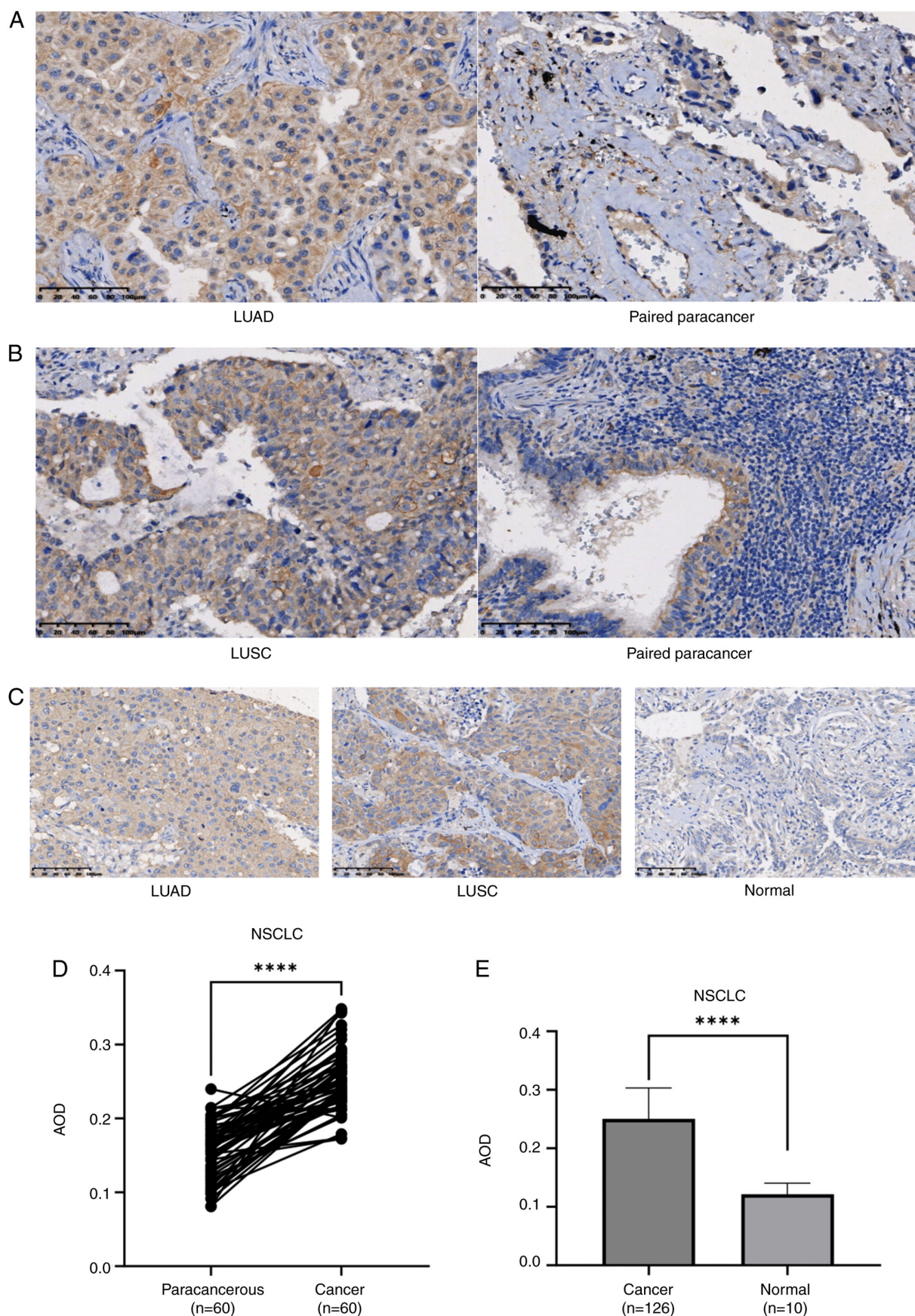


Figure 2. LGR4 is overexpressed in tumor tissues. (A) Lung adenocarcinoma tissue (x200 magnification) and adjacent non-tumor tissue (x200 magnification). (B) LUSC tissue (x200 magnification) and adjacent non-tumor tissue (x200 magnification). (C) LUAD tissue (x200 magnification), LUSC tissue (x200 magnification) and healthy tissue (x200 magnification). Scale bar, 100 μ m. (D) Comparison of LGR4 expression in TMA1 (paired Student's t-test). (E) LGR4 expression in TMA2 (unpaired Student's t-test). **** $P < 0.0001$. LGR4, leucine-rich repeat-containing G-protein coupled receptor 4; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; TMA1, tissue microarray 1; TMA2, tissue microarray 2; NSCLC, non-small cell lung cancer; HR, hazard ratio; AOD, average optical density.

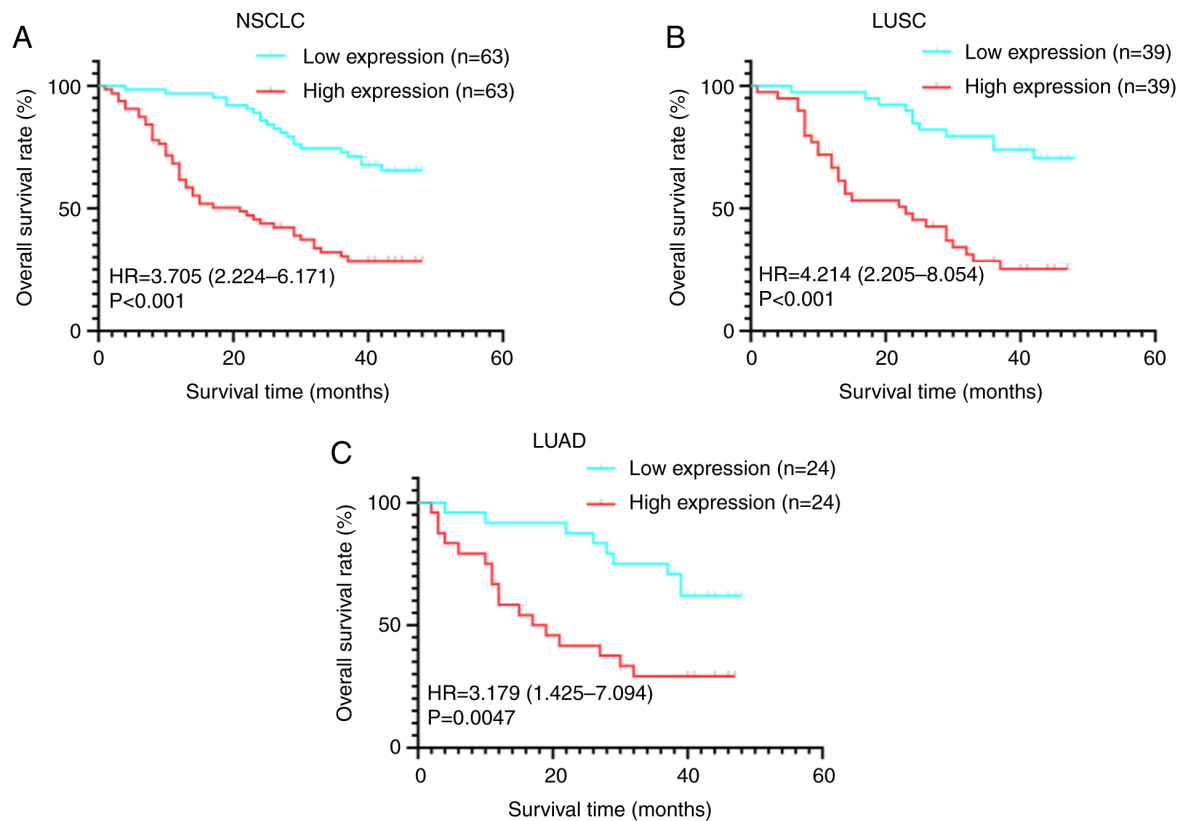


Figure 3. Kaplan-Meier survival curves. High LGR4 expression is associated with poor prognosis in (A) NSCLC ($P<0.001$), (B) LUAD ($P<0.001$) and (C) LUSC ($P=0.0047$). LGR4, leucine-rich repeat-containing G-protein coupled receptor 4; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

for analysis. The baseline clinicopathological characteristics are detailed in Table I. Patients were stratified into high- and low-expression groups based on the median AOD value of LGR4 (0.2512). As presented in Table I, LGR4 expression demonstrated a significant association with histological tumor type ($P=0.017$; Table I). However, no significant associations were observed between LGR4 expression levels and patient age ($P=0.455$), sex ($P=0.826$), TNM tumor stage ($P=0.304$), Ki-67 expression status ($P=0.434$) or tumor location ($P=0.284$).

Prognostic value of LGR4 in NSCLC. Analysis of long-term follow-up data from TMA2 demonstrated that patients with high LGR4 expression exhibited significantly shorter overall survival times compared with those with low LGR4 expression (Fig. 3A, $P<0.001$). Furthermore, this adverse association was consistently observed in both LUAD and LUSC subtypes, where high LGR4 expression was associated with poorer survival outcomes relative to low expression groups (both $P<0.05$; Fig. 3B and C). Subsequent univariate and multivariate Cox proportional hazards regression analyses confirmed elevated LGR4 expression as an independent predictor of poor prognosis in patients with NSCLC ($P<0.001$; Table III). Ultimately, both high LGR4 levels and advanced tumor stage (stage II and III; both $P<0.05$) were identified as independent adverse prognostic factors.

Knockdown of LGR4 inhibits the proliferation of A549 and H226 cells. To investigate whether LGR4 knockdown could inhibit the proliferation of NSCLC cells, a CCK-8 assay

Table II. Primer sequence for reverse transcription-quantitative PCR.

Gene name and primer direction	Primer sequence (5'-3')
LGR4 forward	ACTCAAAGTTCTAACGCTCCAG
LGR4 reverse	AAAGCACTCAGCCCTCGAATG
GAPDH forward	GGAGCGAGATCCCTCCAAAT
GAPDH reverse	GGCTGTTGTCATACTTCTCATGG

LGR4, leucine-rich repeat-containing G-protein coupled receptor 4.

was conducted. The results revealed a significant reduction in the proliferation of both A549 and H226 cells at 24, 48 and 72 h following si-LGR4 transfection, ($P<0.001$; Fig. 4A and B).

Knockdown of LGR4 promotes apoptosis in A549 and H226 cells. Flow cytometry analysis indicated that silencing LGR4 considerably increased the apoptotic rate in both A549 and H226 cells relative to the si-NC control group, suggesting that LGR4 depletion promotes apoptosis in these NSCLC cell lines (both $P<0.001$; Fig. 4C and D).

Knockdown of LGR4 inhibits invasion and migration of A549 and H226 cells. To determine if LGR4 contributes to the

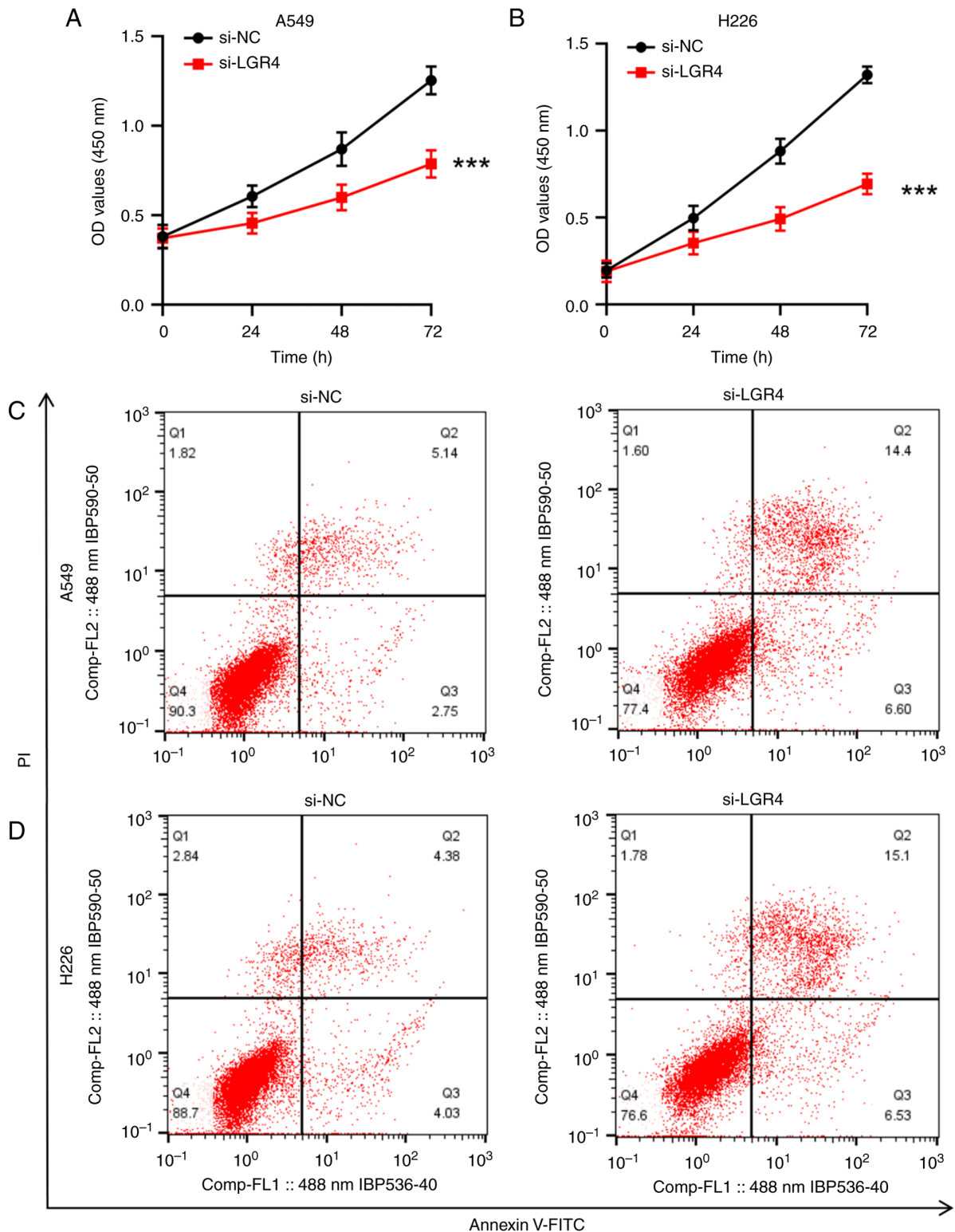


Figure 4. LGR4 promotes the proliferation and inhibits the apoptosis of non-small cell lung cancer cell lines *in vitro*. CCK-8 assay showed that LGR4 knock-down inhibited the proliferation of (A) A549 cells and (B) H226 cells (two-way ANOVA with Šídák's multiple comparisons test). *** $P < 0.001$ vs. si-NC. Flow cytometry showed that LGR4 knockdown promoted apoptosis in (C) A549 cells and (D) H226 cells. LGR4, leucine-rich repeat-containing G-protein coupled receptor 4; CCK-8, Cell Counting Kit-8; OD, optical density; si-NC, small interfering RNA negative control; PI, propidium iodide.

invasive and migratory behavior of NSCLC cells, the effect of LGR4 silencing on the invasion and migration abilities of A549 and H226 cells was evaluated using a Transwell assay. The results demonstrated that transfection with si-LGR4 significantly decreased both the invasion and migration abilities of

A549 and H226 cells, indicating that LGR4 depletion inhibits these key cellular processes (all $P < 0.05$; Fig. 5A and B).

Enrichment analysis of LGR4. GSEA demonstrated that elevated LGR4 expression in tumor samples was significantly enriched

Table III. Univariate and multivariate analysis.

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	0.98 (0.96-1.01)	0.321	0.99 (0.97-1.03)	0.833
Sex (Male)	1.14 (0.62-2.09)	0.681	1.16 (0.62-2.15)	0.648
Ki-67 (Positive)	1.41 (0.84-2.35)	0.193	1.75 (0.98-3.14)	0.061
LGR4	3.55 (2.10-5.99)	<0.001	4.31 (2.43-7.63)	<0.001
Histological type				
Squamous cell carcinoma	1.00 (Reference)	NA	1.00 (Reference)	NA
Adenocarcinoma	1.10 (0.67-1.81)	0.702	2.22 (1.21-4.09)	0.011
Tumor stage				
I	1.00 (Reference)	NA	1.00 (Reference)	NA
II	2.23 (1.20-4.16)	0.012	2.30 (1.20-4.40)	0.012
III	2.08 (1.10-3.95)	0.024	1.96 (1.01-3.83)	0.049
Tumor location				
Left	1.00 (Reference)	NA	1.00 (Reference)	NA
Right	1.37 (0.84-2.26)	0.209	1.01 (0.58-1.74)	0.988

HR, hazard ratio; CI, confidence interval; LGR4, leucine-rich repeat-containing G-protein coupled receptor 4.

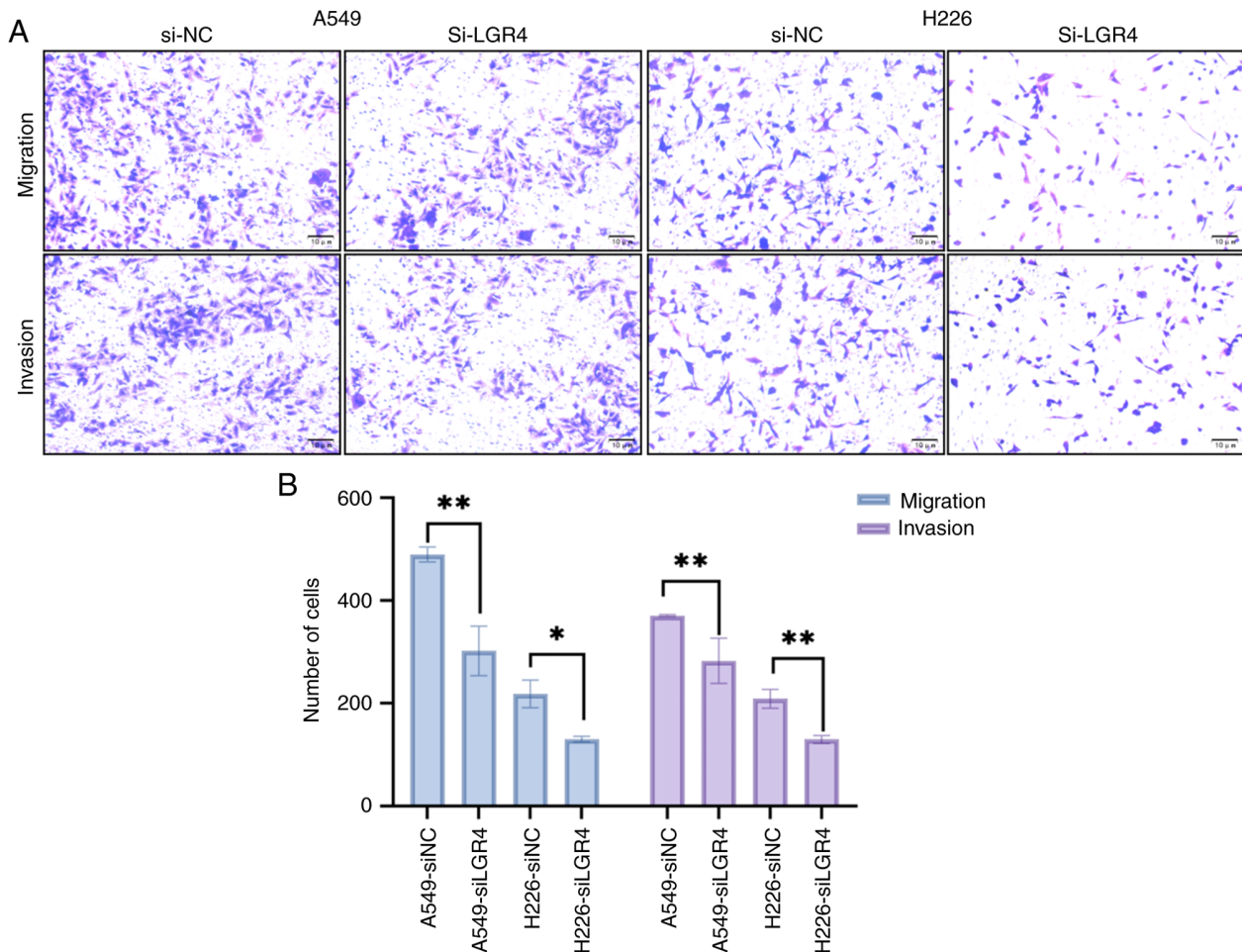


Figure 5. Transwell assay results. (A) Images of Transwell assays performed in A549 and H226 cells transfected with si-NC or si-LGR4. (B) LGR4 knockdown inhibited the invasion and migration of A549 and H226 cells compared with the si-NC group (scale bar, 10 μm) (unpaired Student's t-test; *P<0.05 and **P<0.01). LGR4 promotes the invasion and migration of non-small cell lung cancer cell lines *in vitro*. LGR4, leucine-rich repeat-containing G-protein coupled receptor 4; si-NC, small interfering RNA negative control.

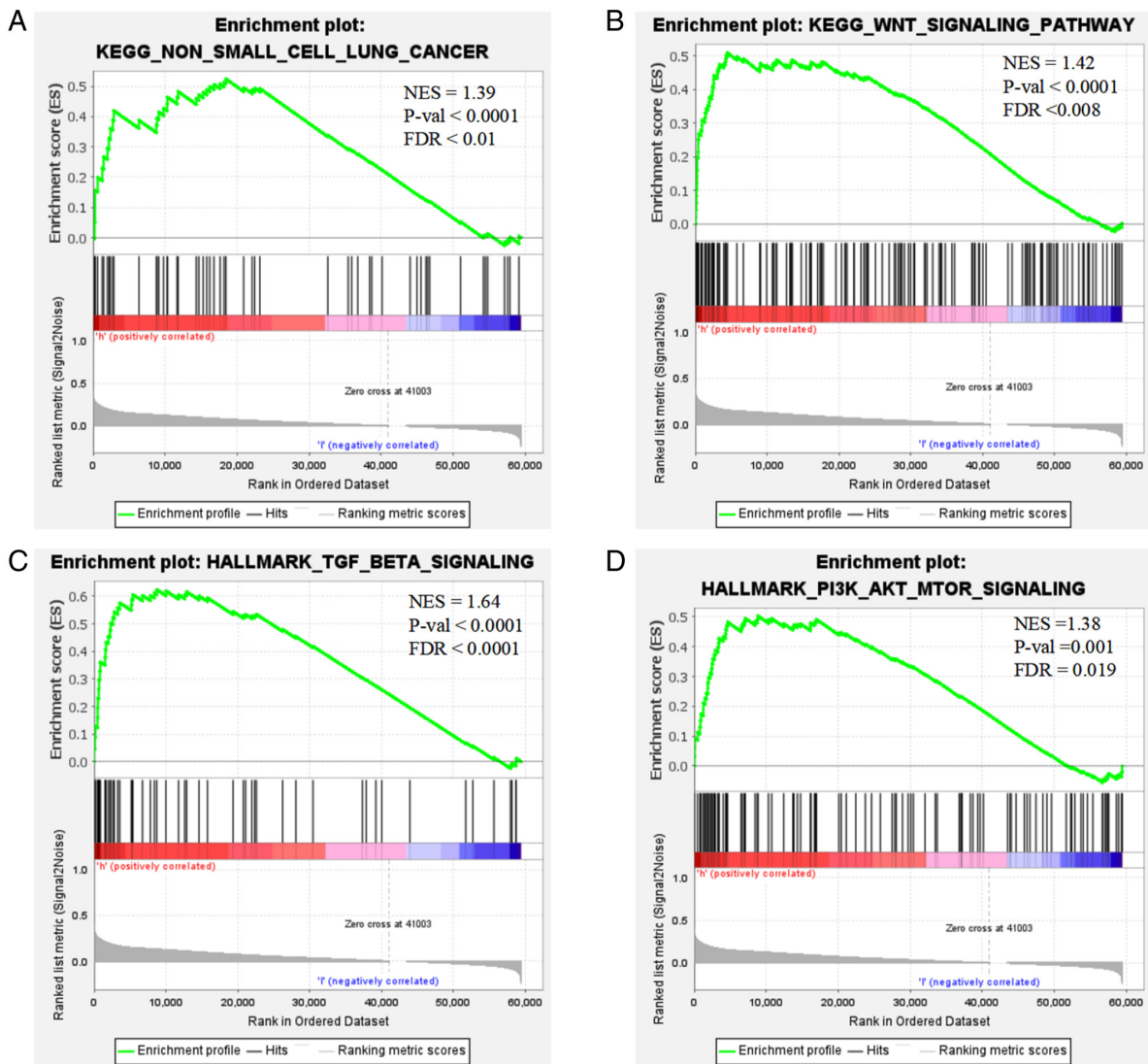


Figure 6. GSEA of high LGR4 expression in non-small cell lung cancer datasets. (A) KEGG non-small cell lung cancer pathway was significantly enriched in the high LGR4 expression group, indicating high LGR4 expression was associated with overall upregulation of genes in the KEGG non-small cell lung cancer pathway compared with low LGR4 expression. (B) The Wnt signaling pathway was significantly enriched in the LGR4 high-expression group. (C) The PI3K/AKT/mTOR signaling pathway showed significant positive enrichment in the LGR4 high-expression group. (D) The TGF- β signaling pathway was enriched in high LGR4-expressing NSCLC samples. LGR4, Leucine-rich repeat-containing G-protein coupled receptor 4; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, Normalized Enrichment Score; FDR, False Discovery Rate.

in the KEGG 'non-small cell lung cancer' pathway (Fig. 6A) and was associated with activation of 'Wnt signaling pathway', 'PI3K/AKT/mTOR signaling' and 'TGF- β signaling' (Fig. 6B-D).

Discussion

GPCRs constitute the most extensive class of cell surface receptors that mediate signal transduction, and are notable drivers of tumor progression and metastasis (19). As a member of the GPCR family, LGR4 exerts various regulatory effects in multiple cell types and acts on numerous targets. In the liver, it controls key gluconeogenic enzymes (such as phosphoenolpyruvate carboxykinase) via Wnt/ β -catenin, affecting glucose output (20). In osteoblasts, LGR4 regulates glycolysis, and its loss reduces bone formation and strength (21). Abnormal LGR4 signaling is highly influential in cancer and other disease, with loss-of-function mutations associated with osteoporosis, electrolyte imbalance, and reduced body weight, and gain-of-function mutations linked

to increased bone density, insulin resistance, and obesity (22). A growing body of evidence indicates that LGR4 is integral to both the development and progression of various cancer types. For instance, Steffen *et al* (23) observed that the transcriptional and translational levels of LGR4 and LGR6 are upregulated in gastric cancer, with LGR4 expression significantly associated with lymph node metastasis. Additionally, Cui *et al* (24) found that the expression of LGR4, LGR5 and LGR6 is elevated in gastrointestinal cancer, with LGR5 being enriched in cancer stem cells. Research has demonstrated that RSPO2 and receptor activator of nuclear factor κ -B ligand co-opt LGR4 as a shared receptor to synergistically over-activate osteoclastogenesis, thereby disrupting bone homeostasis and establishing a premetastatic niche conducive to tumor colonization, which ultimately drives breast cancer bone metastasis. Targeting LGR4 may represent a novel therapeutic strategy to intercept this process (25). Upregulation of microRNA (miR)-449b significantly suppresses the growth and invasive capacity of NSCLC by modulating

LGR4 (26). The association between LGR4 expression and the development of NSCLC remains to be elucidated.

In the present study, LGR4 expression was initially confirmed in NSCLC tissues using immunohistochemistry. A marked elevation of LGR4 was detected in NSCLC tissues when benchmarked against normal controls and histologically healthy adjacent areas. Notably, relatively high LGR4 expression was observed in some non-cancerous tissues. This may be attributed to the physiological roles of LGR4 in healthy tissue homeostasis and regeneration. In addition, the adjacent non-tumor tissues may be influenced by tumor-associated factors such as inflammation or hypoxia, which could upregulate LGR4 expression. Alternatively, individual variation and tissue heterogeneity might also contribute to this observation. Furthermore, higher LGR4 expression was linked to a poor prognosis in patients with NSCLC, consistent with the bioinformatics analysis in the present study. Univariate and multivariate analyses established high LGR4 expression as an independent predictor of poor prognosis in NSCLC. The functional role of LGR4 was then investigated in two NSCLC cell lines. Relative to healthy lung epithelial cells, LGR4 expression was significantly elevated in the A549 lung adenocarcinoma cell line and the H226 squamous cell carcinoma cell line. In addition, LGR4 knockdown increased apoptosis and decreased proliferation, invasion and migration abilities in both A549 and H226 cells.

The present enrichment analysis revealed that high LGR4 expression levels may be associated with the activation of major signaling pathways, including the Wnt, PI3K/AKT/mTOR and TGF- β signaling pathways. Moreover, the significant enrichment of LGR4 within the KEGG 'non-small cell lung cancer' pathway indicates that LGR4 may be a potential key driver in NSCLC progression. Numerous studies have reported that LGR4 and its homologs LGR5 and LGR6 influence cellular signaling primarily through the Wnt pathway via their interaction with the ligand R-spondin (27-30). Furthermore, research has shown that LGR4 can regulate the expression of TGF- β 1, thereby triggering the TGF- β 1/Smad signaling pathway and driving multiple myeloma progression (31). Additionally, according to a study by Liang *et al* (32), overexpression of LGR4 upregulates mTOR and the key effector AKT in the PI3K/AKT pathway. Building on these findings, it is proposed that LGR4 may promote NSCLC progression through the activation of these pathways. These results from previous studies imply that LGR4 could be strongly associated with the initiation and progression of NSCLC.

In conclusion, LGR4 may represent a promising therapeutic approach for patients with NSCLC. However, the present study is confined to *in vitro* experiments, and animal studies have yet to be conducted. In the present research, bioinformatics analysis was utilized to predict the potential signaling pathways that LGR4 may activate in NSCLC. To further validate the reliability of LGR4 as a diagnostic biomarker, our future studies will focus on exploring the upstream regulatory molecules of LGR4 and conducting additional experiments for further confirmation.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Changzhou High-Level Medical Talents Training Project (grant no. 2022CZBJ069), the Changzhou Sci&Tech Program (grant no. CZ20220025), the '333 Project' of Jiangsu Province (grant no. BRA2020157) and the 333 High-Level Talent Training Project (grant no. 2022-2).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XD was responsible for the conceptualization, methodology, investigation, formal analysis, writing the original draft, and reviewing and editing the manuscript. XL provided the software, analyzed and curated the data (which involved collection, organization, cleaning, and preservation of research data, including the archiving of experimental results, preparation of data tables, and processing and management of images/raw data), and provided experimental supervision. YS and SZ interpreted data. ML and ZG performed the experiments. KY conceived and designed the study, supervised the project, and revised the manuscript for important scientific content. All authors have read and approved the final manuscript. XD and KY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The Research Ethics Committee of The Third Affiliated Hospital of Nanjing Medical University (Changzhou, China) reviewed and approved the study [approval no. (2023) KY422-01]. All participating patients and their families voluntarily provided written informed consent. All tissue samples were anonymized following ethical and legal guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I and Jemal A: Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 74: 229-263, 2024.
2. Siegel RL, Miller KD, Wagle NS and Jemal A: Cancer statistics, 2023. *CA Cancer J Clin* 73: 17-48, 2023.
3. National Lung Screening Trial Research Team; Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, Fagerstrom RM, Gareen IF, Gatsonis C, Marcus PM and Sicks JD: Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med* 365: 395-409, 2011.
4. Timmerman R, Paulus R, Galvin J, Michalski J, Straube W, Bradley J, Fakiris A, Bezjak A, Videtic G, Johnstone D, *et al*: Stereotactic body radiation therapy for inoperable early stage lung cancer. *JAMA* 303: 1070-1076, 2010.

5. Sun B, Brooks ED, Komaki R, Liao Z, Jeter M, McAleer M, Balter PA, Welsh JD, O'Reilly M, Gomez D, *et al*: Long-term outcomes of salvage stereotactic ablative radiotherapy for isolated lung recurrence of non-small cell lung cancer: A phase II clinical trial. *J Thorac Oncol* 12: 983-992, 2017.
6. Reck GJ, Rodríguez-Abreu D, Robinson AG, Hui R, Csősz T, Fülöp A, Gottfried M, Peled N, Tafreshi A, Cuffe S, *et al*: Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* 375: 1823-1833, 2016.
7. Shaw AT, Kim DW, Nakagawa K, Seto T, Crinó L, Ahn MJ, De Pas T, Besse B, Solomon BJ, Blackhall F, *et al*: Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med* 368: 2385-2394, 2013.
8. Riely GJ, Wood DE, Etttinger DS, Aisner DL, Akerley W, Bauman JR, Bharat A, Bruno DS, Chang JY, Chirieac LR, *et al*: Non-Small cell lung cancer, version 4.2024, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw* 22: 249-274, 2024.
9. Filipowska J, Kondegowda NG, Leon-Rivera N, Dhawan S and Vasavada RC: LGR4, a G protein-coupled receptor with a systemic role: From development to metabolic regulation. *Front Endocrinol (Lausanne)* 13: 867001, 2022.
10. Ordaz-Ramos A, Rosales-Gallegos VH, Melendez-Zajgla J, Maldonado V and Vazquez-Santillan K: The role of LGR4 (GPR48) in normal and cancer processes. *Int J Mol Sci* 22: 4690, 2021.
11. Zheng H, Liu J, Cheng Q, Zhang Q, Zhang Y, Jiang L, Huang Y, Li W, Zhao Y, Chen G, *et al*: Targeted activation of ferroptosis in colorectal cancer via LGR4 targeting overcomes acquired drug resistance. *Nat Cancer* 5: 572-589, 2024.
12. Yue Z, Yuan Z, Zeng L, Wang Y, Lai L, Li J, Sun P, Xue X, Qi J, Yang Z, *et al*: LGR4 modulates breast cancer initiation, metastasis, and cancer stem cells. *FASEB J* 32: 2422-2437, 2018.
13. Liang F, Zhang H, Cheng D, Gao H, Wang J, Yue J, Zhang N, Wang J, Wang Z and Zhao B: Ablation of LGR4 signaling enhances radiation sensitivity of prostate cancer cells. *Life Sci* 265: 118737, 2021.
14. Annunziato S, Sun T and Tchorz JS: The RSPO-LGR4/5-ZNRF3/RNF43 module in liver homeostasis, regeneration, and disease. *Hepatology* 76: 888-899, 2022.
15. Planas-Paz L, Orsini V, Boulter L, Calabrese D, Pikiolek M, Nigsch F, Xie Y, Roma G, Donovan A, Marti P, *et al*: The RSPO-LGR4/5-ZNRF3/RNF43 module controls liver zonation and size. *Nat Cell Biol* 18: 467-479, 2016.
16. Yue F, Jiang W, Ku AT, Young AIJ, Zhang W, Souto EP, Gao Y, Yu Z, Wang Y, Creighton CJ, *et al*: A Wnt-independent LGR4-egfr signaling axis in cancer metastasis. *Cancer Res* 81: 4441-4454, 2021.
17. Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR and Winchester DP: The eighth edition AJCC cancer staging manual: Continuing to build a bridge from a population-based to a more 'personalized' approach to cancer staging. *CA Cancer J Clin* 67: 93-99, 2017.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
19. Dorsam RT and Gutkind JS: G-protein-coupled receptors and cancer. *Nat Rev Cancer* 7: 79-94, 2007.
20. Fang Q, Ye L, Han L, Yao S, Cheng Q, Wei X, Zhang Y, Huang J, Ning G, Wang J, *et al*: LGR4 is a key regulator of hepatic gluconeogenesis. *Free Radic Biol Med* 229: 183-194, 2025.
21. Yang YY, Zhou YM, Xu JZ, Sun LH, Tao B, Wang WQ, Wang JQ, Zhao HY and Liu JM: Lgr4 promotes aerobic glycolysis and differentiation in osteoblasts via the canonical Wnt/ β -catenin pathway. *J Bone Miner Res* 36: 1605-1620, 2021.
22. Yang L, Wang J, Gong X, Fan Q, Yang X, Cui Y, Gao X, Li L, Sun X, Li Y and Wang Y: Emerging roles for LGR4 in organ development, energy metabolism and carcinogenesis. *Front Genet* 12: 728827, 2022.
23. Steffen JS, Simon E, Warneke V, Balschun K, Ebert M and Röcken C: LGR4 and LGR6 are differentially expressed and of putative tumor biological significance in gastric carcinoma. *Virchows Arch* 461: 355-365, 2012.
24. Cui J, Toh Y, Park S, Yu W, Tu J, Wu L, Li L, Jacob J, Pan S, Carmon KS and Liu QJ: Drug conjugates of antagonistic R-spondin 4 mutant for simultaneous targeting of leucine-rich repeat-containing g protein-coupled receptors 4/5/6 for cancer treatment. *J Med Chem* 64: 12572-12581, 2021.
25. Yue Z, Niu X, Yuan Z, Qin Q, Jiang W, He L, Gao J, Ding Y, Liu Y, Xu Z, *et al*: RSPO2 and RANKL signal through LGR4 to regulate osteoclastic premetastatic niche formation and bone metastasis. *J Clin Invest* 132: e144579, 2022.
26. Yang D, Li JS, Xu QY, Xia T and Xia JH: Inhibitory effect of MiR-449b on cancer cell growth and invasion through LGR4 in non-small-cell lung carcinoma. *Curr Med Sci* 38: 582-589, 2018.
27. de Lau W, Barker N, Low TY, Koo BK, Li VS, Teunissen H, Kujala P, Haegbarth A, Peters PJ, van de Wetering M, *et al*: Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* 476: 293-297, 2011.
28. Luo J, Yang Z, Ma Y, Yue Z, Lin H, Qu G, Huang J, Dai W, Li C, Zheng C, *et al*: LGR4 is a receptor for RANKL and negatively regulates osteoclast differentiation and bone resorption. *Nat Med* 22: 539-546, 2016.
29. de Lau W, Peng WC, Gros P and Clevers H: The R-spondin/Lgr5/Rnf43 module: Regulator of Wnt signal strength. *Genes Dev* 28: 305-316, 2014.
30. Han XH, Jin YR, Tan L, Kosciuk T, Lee JS and Yoon JK: Regulation of the follistatin gene by RSPO-LGR4 signaling via activation of the WNT/ β -catenin pathway in skeletal myogenesis. *Mol Cell Biol* 34: 752-764, 2014.
31. Yi Z, Ma T, Liu J, Tie W, Li Y, Bai J, Li L and Zhang L: LGR4 promotes tumorigenesis by activating TGF- β 1/Smad signaling pathway in multiple myeloma. *Cell Signal* 110: 110814, 2023.
32. Liang F, Yue J, Wang J, Zhang L, Fan R, Zhang H and Zhang Q: GPCR48/LGR4 promotes tumorigenesis of prostate cancer via PI3K/Akt signaling pathway. *Med Oncol* 32: 49, 2015.



Copyright © 2025 Duan et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.