

Expression of G protein-coupled receptor 56 is associated with tumor progression in non-small-cell lung carcinoma patients

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Background: G protein-coupled receptor 56 (GPR56) is an adhesion G protein-coupled receptor with essential functions for cell physiology and survival, and its expression correlates with prognosis in a number of malignancies. The aim of this study is to determine the relationship of GPR56 expression with clinicopathological parameters and prognosis in non-small-cell lung carcinoma (NSCLC).

Methods: The levels of GPR56 were evaluated by immunohistochemistry in 157 NSCLC tissue samples. The association between GPR56 and clinicopathological parameters was evaluated by χ^2 test. Univariate and multivariate analyses were performed to demonstrate the prognosis role of GPR56. The function of GPR56 in NSCLC cell lines was also explored through overexpression and knockdown studies.

Results: The expression level of GPR56 in tumor tissues was significantly correlated with the TNM stage of NSCLC ($P=0.005$). Univariate and multivariate analyses revealed that GPR56 can act as an independent prognostic factor for overall survival. Furthermore, through overexpression and knockdown experiments, we confirmed that GPR56 can promote the proliferation and invasion of NSCLC cells.

Conclusion: GPR56 plays an important role in tumor development and may serve as a promising target for prognostic prediction in NSCLC.

Keywords: G protein-coupled receptor 56, non-small-cell lung carcinoma, prognosis, proliferation, invasion

Introduction

Lung cancer is the most leading cause of cancer-associated death in the world.^{1,2} The main types of lung cancer are small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC); ~85% of lung cancers are classified as NSCLC.^{3,4} NSCLC can be further categorized into squamous cell carcinoma (SCC) and nonsquamous carcinoma (such as adenocarcinoma [AC] and large-cell carcinoma). NSCLC is hard to diagnose at early stage, and the 5-year overall survival (OS) is <15% for the patients with advanced TNM stage.⁵⁻⁷ One of the dominant reasons for the treatment failure is the frequently adjacent invasion and lymph node metastasis. Therefore, exploring the pathogenesis of NSCLC and elucidating potential biological markers will be very important for predicting the treatment response as well as prognosis of NSCLC patients.

G protein-coupled receptor is one of the largest membrane protein families, and G protein-coupled receptor 56 (GPR56) belongs to the adhesion subfamily of G protein-coupled receptors. GPR56 can interact with collagen III,⁸ transglutaminase 2,⁹ and

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CD81,¹⁰ and thus participate in the cell survival, proliferation, adhesion, and migration. Moreover, reverse transcription polymerase chain reaction, Northern blot analyses, and functional results revealed that GPR56 expression was inversely correlated with metastatic potential in melanoma cell lines, and the expression level of GPR56 was downregulated in the high metastatic cell lines compared with that in the low metastatic cell lines.^{11,12} However, later, there were several reports indicating that GPR56 high expression was positively involved in promoting the tumorigenesis of glioma and digestive cancers.^{13,14}

Therefore, GPR56 may play different roles in distinct cancers and the function of GPR56 in lung cancer has not been reported. Here, we explored the expression pattern of GPR56 in NSCLC and demonstrated its correlation with clinicopathological characteristics as well as its effect in cell proliferation and invasion.

Patients and methods

Patients

NSCLC specimens were obtained from 157 patients who underwent the curative surgical resection at Yidu Central Hospital of Weifang (Weifang, People's Republic of China) and Jinan Central Hospital (Jinan, People's Republic of China) from 2006 to 2010. All patients were diagnosed with primary NSCLC (AC or SCC) based on pathological examinations. This study enrolled 53 women and 104 men (median age: 64 years, range: 36–77 years). None of the patients received any preoperative chemotherapy or radiation therapy. The histological grade and clinical stage of the tumors were identified according to the 7th edition of the TNM classification of the International Union Against Cancer. All specimens were fixed in 10% formalin and embedded in paraffin. The study was approved by the ethical committee of Yidu Central Hospital of Weifang and Jinan Central Hospital. Written informed consent was obtained from all patients.

Immunohistochemical staining

Paraffin sections from NSCLC tissues and adjacent nontumor tissues were deparaffinized in xylene and rehydrated in a descending ethanol series (100%, 95%, 90%, 80%, 70%) and double-distilled water. Antigen retrieval was performed in citrate buffer and boiled for 10 minutes. Sections were then treated with 3% hydrogen peroxide and 1% bovine serum albumin to block the nonspecific binding and endogenous peroxidase activity. After blocking, the sections were incubated with GPR56 antibody (Sigma-Aldrich Co., St Louis, MO, USA,

HPA046065, dilution 1:100) overnight at 4°C. After phosphate-buffered saline washing, the sections were incubated with the secondary antibody and streptavidin–horseradish peroxidase complex at room temperature. DAB staining kit (Tiangen, Beijing, People's Republic of China) and hematoxylin counterstaining was used for detecting the immunoreactivity.

Immunohistochemical evaluation

The criteria used for quantitating immunohistochemical staining results included the staining intensity and the percentage of positive cells. The intensities of positive staining were scored by 0–3: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. As for the percentage of positive cells, staining was scored as 0 (<10%), 1 (11%–25%), 2 (26%–50%), and 3 (51%–100%) for evaluation. In this study, the expression pattern of GPR56 was divided into negative expression (both the staining and percentage scores were <2) and positive expression (either the staining or percentage score was ≥2). The results were assessed by two pathologists independently following the double-blinded principles.

Cell culture

The human lung carcinoma cell lines A549, H520, and normal bronchial epithelial cell line human bronchial epithelial (HBE) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin sodium, and 100 µg/mL streptomycin sulfate at 37°C with 5% CO₂.

Western blot

Cells were lysed with the NP40 lysis buffer (1 M Tris-HCl pH 7.5, 1% Triton X-100, 10% sodium dodecyl sulfate, 1% NP-40, 0.5 M EDTA, 0.5% sodium deoxycholate, 10 µg/mL aprotinin, 1 mM PMSF, 10 µg/mL leupeptin) on ice for 30 minutes, and then centrifuged at 13,000× *g* for 30 minutes. Protein concentration of the supernatant was then determined using the bicinchoninic acid protein assay. And equal amounts of proteins (50 µg) with 2× sodium dodecyl sulfate-loading buffer were electrophoresed and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Immobilon; EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin and then incubated overnight at 4°C with the primary antibodies (anti-GPR56 rabbit polyclonal antibody, ab172361, Abcam,

Cambridge, UK; anti-glyceraldehyde 3-phosphate dehydrogenase mouse monoclonal antibody, sc-365062, Santa Cruz Biotechnology, Dallas, TX, USA). After washing with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) for three times, the membranes were incubated with secondary antimouse or antirabbit antibodies (1:5,000; CW Bio, Beijing, People's Republic of China) for 1 hour at room temperature. The results were visualized by chemiluminescence detection system (NEN Life Science Products, Boston, MA, USA).

Enzyme-linked immunosorbent assay

The quantification of GPR56 expression level in the cell lysate was measured with the sandwich enzyme-linked immunosorbent assay (ELISA) kit for human GPR56 (ab172361, Abcam, Cambridge, UK) according to the manufacturer's instructions.

Overexpression and siRNA transfection

For overexpression, the cDNA of GPR56 was cloned into the pcDNA3.1 vector (Thermo Fisher Scientific, Waltham, MA, USA) to generate the GPR56 expression plasmid. The expression plasmid was verified by sequencing and transfected into the A549 and H520 cells using Lipofectamine 2000 (Invitrogen) at ~70% cell confluence. The pcDNA3.1 vector was used as a negative control.

The GPR56 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology. In brief, Opti-MEM diluted siRNAs were mixed with Lipofectamine 2000 and incubated for 20 minutes in room temperature, and then transfected into cells with 30%–50% confluence using Opti-MEM for 4–6 hours. The target sequences were as follows: siGPR56: 5'-AGAUUACAUCUUCUCUAUGGCAAGC-3'; Scrambled siRNA: 5'-UUCUCCGAACGUGUCACGU-3'. The transfection efficiency of GPR56 was confirmed by Western blotting and ELISA.

Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was used to assess cell proliferation. Briefly, cell clones (200/well) were seeded and cultured in 96-well plates for 1, 2, 3, and 4 days at the indicated time points, and 20 μ L of 0.5 mg/mL MTT (Sigma-Aldrich Co.) was added into the medium and incubated for 4 hours, followed by adding 150 μ L of dimethyl sulfoxide for an additional 15 minutes. The absorbance at 490 nm was measured on a microplate

reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each experiment was repeated for at least three times.

Cell invasion assay

Transwell assays were performed to analyze the cell invasion capacity. Briefly, transwell membranes were coated with Matrigel™ Basement Membrane (BD Biosciences, San Jose, CA, USA). Cells were reseeded onto the upper precoated chamber (3×10^4 cells per well) in 100 μ L of serum-free medium. Lower wells of the chamber contained 10% fetal bovine serum-containing medium. Cells were incubated for another 16 hours at 37°C in 5% CO₂. The cells in the lower chambers were then fixed, stained, and counted. Each experiment was repeated for at least three times.

Statistical analysis

The relationships between GPR56 expression and clinicopathological features were analyzed using the Person Chi-square test and OS curves were performed using the Kaplan–Meier method, and the differences were examined using log-rank tests. Cox proportional-hazards regression analysis was applied in order to estimate univariate and multivariate hazard ratios for OS. All *P*-values were two-sided, and *P* < 0.05 was considered as statistically significant. All statistical analyses were carried out by SPSS 20.0 software (IBM Incorporation, Armonk, NY, USA).

Results

Patients' characteristics

The clinical characteristics of the patients are presented in Table 1. The median patient age was 64 years (range: 36–77 years) and the majority were male (66.2%). More than half of the patients (55.4%) were present or previous smokers. The median follow-up was 78 months (range 53–104 months). There were 88 ACs and 69 SCCs. Survival information of the patients was obtained through letters and phone calls. The 5-year OS for all the NSCLC patients was 64.4%, and the median survival time was 67 months.

Expression of GPR56 in NSCLC tissues and its correlations with clinical parameters

Immunohistochemical staining indicated that GPR56 protein was mainly located in the cytoplasm and membrane of NSCLC tissues as well as in the adjacent nontumor tissues (Figure 1). The positive rate of GPR56 expression was 72.7%

Table 1 Correlations between GPR56 expression and clinicopathological features in NSCLC patients

Variables	Cases (n=157)	GPR56 expression		P-value
		Negative (n=46) (%)	Positive (n=111) (%)	
Sex				0.588
Female	53	17 (32.1)	36 (67.9)	
Male	104	29 (27.9)	75 (72.1)	
Age (years)				0.858
≤60	87	26 (29.9)	61 (70.1)	
>60	70	20 (28.6)	50 (71.4)	
Smoking				0.897
Yes	90	26 (28.9)	64 (71.1)	
No	67	20 (29.9)	47 (70.1)	
Tumor size (cm)				0.930
≤3	64	19 (29.7)	45 (70.3)	
>3	93	27 (29.0)	66 (71.0)	
Histological type				0.532
Adenocarcinoma	88	24 (27.3)	64 (72.7)	
Squamous cell carcinoma	69	22 (31.9)	47 (68.1)	
Differentiation				0.411
Well/moderate	98	31 (31.6)	67 (68.4)	
Poor	59	15 (25.4)	44 (74.6)	
TNM stage				0.005*
I/II	72	29 (40.3)	43 (59.7)	
III/IV	85	17 (20.0)	68 (80.0)	
LN metastasis				0.499
Negative	89	28 (31.5)	61 (68.5)	
Positive	68	18 (26.5)	50 (73.5)	

Note: *Statistically significant.

Abbreviations: GPR56, G protein-coupled receptor 56; LN, lymph node; NSCLC, non-small-cell lung carcinoma.

(64/88) and 68.1% (47/69) in AC and SCC, respectively. Both are significantly higher than that in adjacent nontumor tissues (21.0%, 17/81; and 35.5%, 22/62 – data not shown).

Moreover, GPR56 protein expression level was found to be correlated with the TNM stage of NSCLC ($r=0.222$, $P=0.005$). However, no significant relationship was detected between GPR56 expression level and other clinicopathological characteristics, such as age, tumor size, histological type, and differentiation (all $P>0.05$, Table 1).

Univariate and multivariate analyses

The Kaplan–Meier univariate survival analysis revealed that tumor differentiation, lymph node metastasis, TNM stage, and GPR56 expression were significantly associated with OS of NSCLC. Patients with negative GPR56 expression demonstrated longer OS compared with those with positive GPR56 expression (mean, 73.9 vs 49.1 months; $P<0.001$; Figure 2, Table 2). The 5-year OS rate of the GPR56-negative group was 82.7% while that for the GPR56-positive group was only 52.7%.

In addition, the Cox regression multivariate analysis results indicated GPR56 can act as an independent prognostic factor (hazard ratio = 2.489, 95% confidence interval = 1.219–5.086, $P=0.012$). Other independent prognostic factors include TNM stage and lymph node metastasis (Table 3).

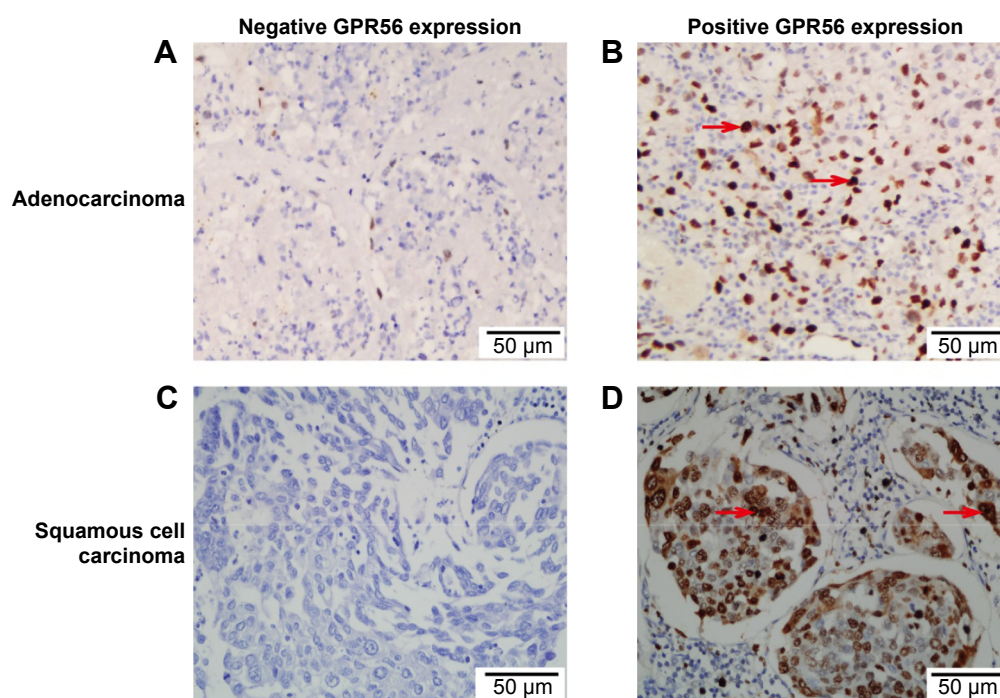


Figure 1 (Continued)

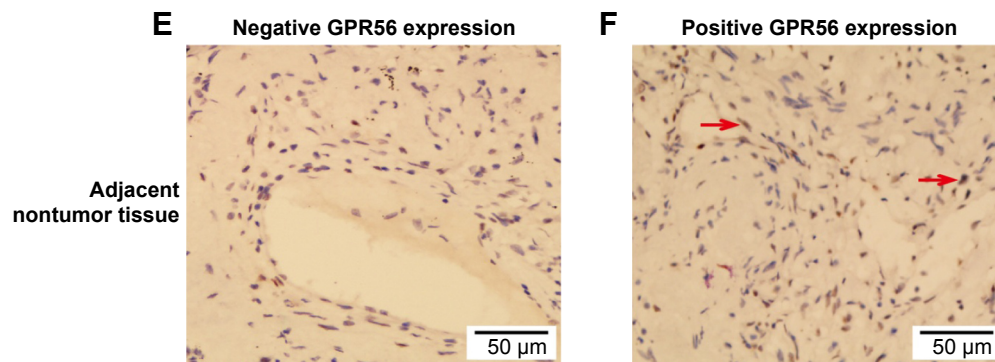


Figure 1 Representative immunohistochemistry results of GPR56 expression in NSCLC and adjacent nontumor tissues.

Notes: Negative (A) and positive (B) GPR56 expression in adenocarcinoma. Negative (C) and positive (D) GPR56 expression in squamous cell carcinoma. Negative (E) and positive (F) GPR56 expression in adjacent nontumor tissues. Arrows point to the strong IHC staining.

Abbreviations: GPR56, G protein-coupled receptor 56; IHC, immunohistochemical; NSCLC, non-small-cell lung carcinoma.

Expression of GPR56 in NSCLC cell lines and its effect on cell proliferation and invasion

Western blot analysis showed detectable GPR56 protein levels in AC cell line A549 and SCC cell line H520. Both are significantly higher than the expression level in HBE

cells (normal bronchial epithelial cell line) (Figure 3A, left). ELISA experiment revealed similar results (Figure 3A, right). Thus, we performed the overexpression and knockdown of GPR56 in both A549 and H520 cell lines, and the transfection efficiency of overexpression and siRNA was confirmed by Western blot (Figure 3B) and ELISA (Figure 3C).

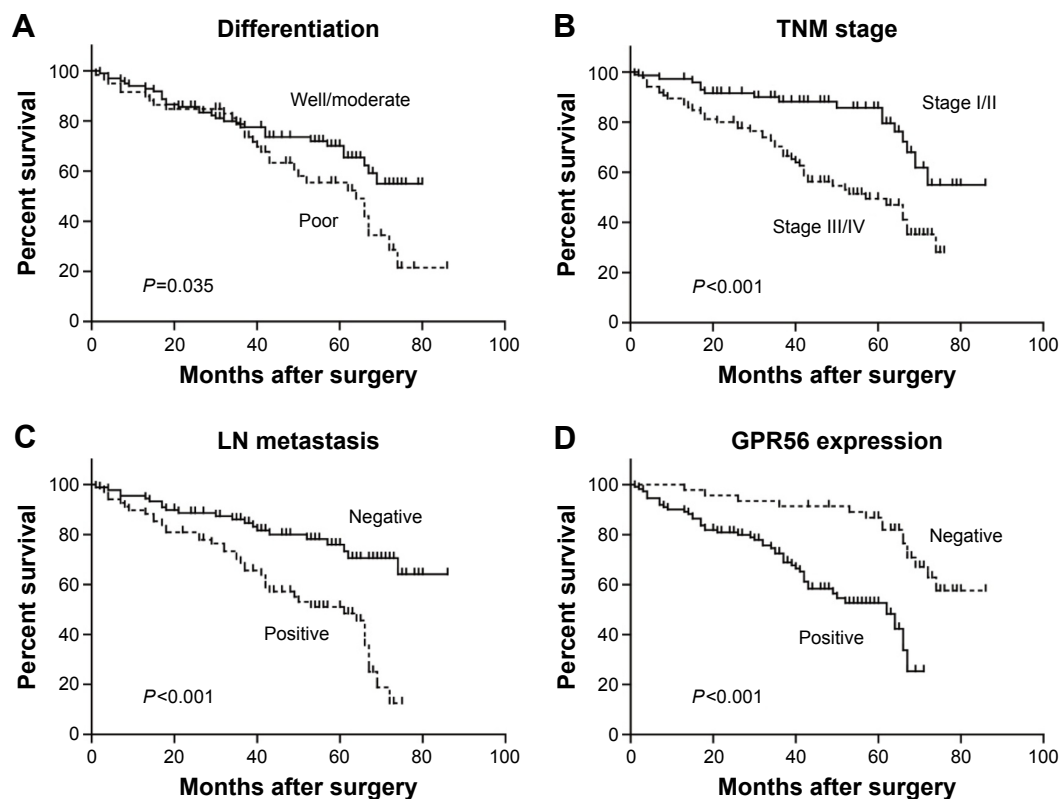


Figure 2 Survival analysis of NSCLC patients.

Notes: Kaplan–Meier curves showing overall survival (OS) for all patients according to differentiation (A), TNM stage (B), lymph node metastasis (C), and GPR56 expression (D). OS was worse in tumors with poor differentiation, advanced TNM stage, positive lymph node metastasis, and positive GPR56 expression (all $P<0.05$).

Abbreviations: GPR56, G protein-coupled receptor 56; LN, lymph node; NSCLC, non-small-cell lung carcinoma; OS, overall survival.

Table 2 Univariate analysis of clinicopathological factors for the overall survival in 157 NSCLC patients

Variables	Overall survival time (months)		P-value
	Mean \pm SD	Median	
Sex			0.171
Female	60.8 \pm 3.7	NR	
Male	58.5 \pm 2.9	66.0	
Age (years)			0.209
≤ 60	58.0 \pm 3.1	66.0	
> 60	60.2 \pm 3.2	NR	
Smoking			0.370
Yes	56.7 \pm 2.8	67.0	
No	62.6 \pm 3.7	72.0	
Tumor size (cm)			0.547
≤ 3	57.4 \pm 3.3	67.0	
> 3	61.8 \pm 3.2	74.0	
Histological type			0.264
Adenocarcinoma	58.5 \pm 3.3	64.0	
Squamous cell carcinoma	60.7 \pm 3.0	69.0	
Differentiation			0.035*
Well/moderate	61.7 \pm 2.7	NR	
Poor	55.2 \pm 3.7	64.0	
TNM stage			$<0.001^*$
I/II	71.2 \pm 3.1	NR	
III/IV	50.3 \pm 2.8	57.0	
LN metastasis			$<0.001^*$
Negative	70.0 \pm 3.0	NR	
Positive	48.9 \pm 2.9	61.0	
GPR56 expression			$<0.001^*$
Negative	73.9 \pm 2.9	NR	
Positive	49.1 \pm 2.4	62.0	

Note: *Statistically significant.

Abbreviations: GPR56, G protein-coupled receptor 56; LN, lymph node; NR, not reached; NSCLC, non-small-cell lung carcinoma; SD, standard deviation.

As shown by the MTT assay, GPR56 siRNA-transfected A549 and H520 cells showed significantly less cell growth than negative control siRNA-transfected cells, while the GPR56 overexpression can increase the cell proliferation ability (Figure 3D). Besides, the invasion capacity of GPR56 siRNA-transfected cells was decreased compared with that of control cells while the GPR56 overexpression can enhance

Table 3 Cox multivariate analysis of variables affecting overall survival in NSCLC patients

Variables	Hazard ratio	95% confidence interval	P-value
Differentiation	1.351	0.810–2.253	0.249
TNM stage	2.686	1.461–4.938	0.001*
LN metastasis	3.173	1.822–5.525	$<0.001^*$
GPR56 expression	2.489	1.219–5.086	0.012*

Note: *Statistically significant.

Abbreviations: GPR56, G protein-coupled receptor 56; LN, lymph node; NSCLC, non-small-cell lung carcinoma.

the invasion process, as shown by the Transwell assays (Figure 3E). Apparently, the effect of GPR56 on A549 cells was much more obvious than that in H520 cells (Figure 3D and E); this may be caused by the genotype differences between the two cell lines.

Discussion

Despite advances in the diagnosis and treatment in the past decades, lung cancer remains the leading cause of death from cancer worldwide and one of the leading causes of death from all causes.¹⁵ Usually, patients with NSCLC have a poor prognosis because most of them present with advanced or metastatic disease at the time of diagnosis.^{16,17} Further understanding of the molecular basis of NSCLC, including the discovery of disease-specific biomarkers, would greatly improve our ability to diagnose, provide prognostic information, and potentially guide treatment of patients. In this study, we explored the prognostic significance of age, sex, smoking, tumor size, histological type, tumor differentiation, lymph node metastasis, and TNM stage in NSCLC patients. Poorer differentiation, lymph node metastasis, and advanced TNM stage were identified as unfavorable prognostic factors, consistent with other reports.^{18–21}

GPR56 was preferentially expressed in neuronal progenitor cells of the cerebral cortical ventricular and subventricular zones during periods of neurogenesis.²² Later, it was indicated that GPR56 can activate the RhoA pathway and functions in the neural progenitor cell migration.²³ Moreover, Xu et al¹¹ reported that GPR56 was downregulated in tumors derived from highly metastatic human melanoma cell lines; overexpression of GPR56 suppressed tumor growth and metastasis. They also figured out that GPR56 can bind specifically to tissue transglutaminase (TGM2), which was implicated as an inhibitor of tumor progression, and thus suppress tumor growth and metastasis. However, the function of GPR56 in glioma seems to be different in that it can promote tumor growth and metastasis.¹⁴ Therefore, the expression and function of GPR56 may differ in distinct tissues or tumors. Here, we analyzed the expression of GPR56 in human NSCLC as well as its role in regulating the NSCLC tumor progression.

We found that GPR56 showed comparable expression level in lung AC and SCC tissues, both higher than adjacent nontumor tissues. Furthermore, it is interesting to note that positive GPR56 expression was closely associated with advanced TNM stage, indicating the oncogenetic role of GPR56 in NSCLC. Correspondingly, the presence of GPR56 showed a remarkable prognostic impact by univariate and

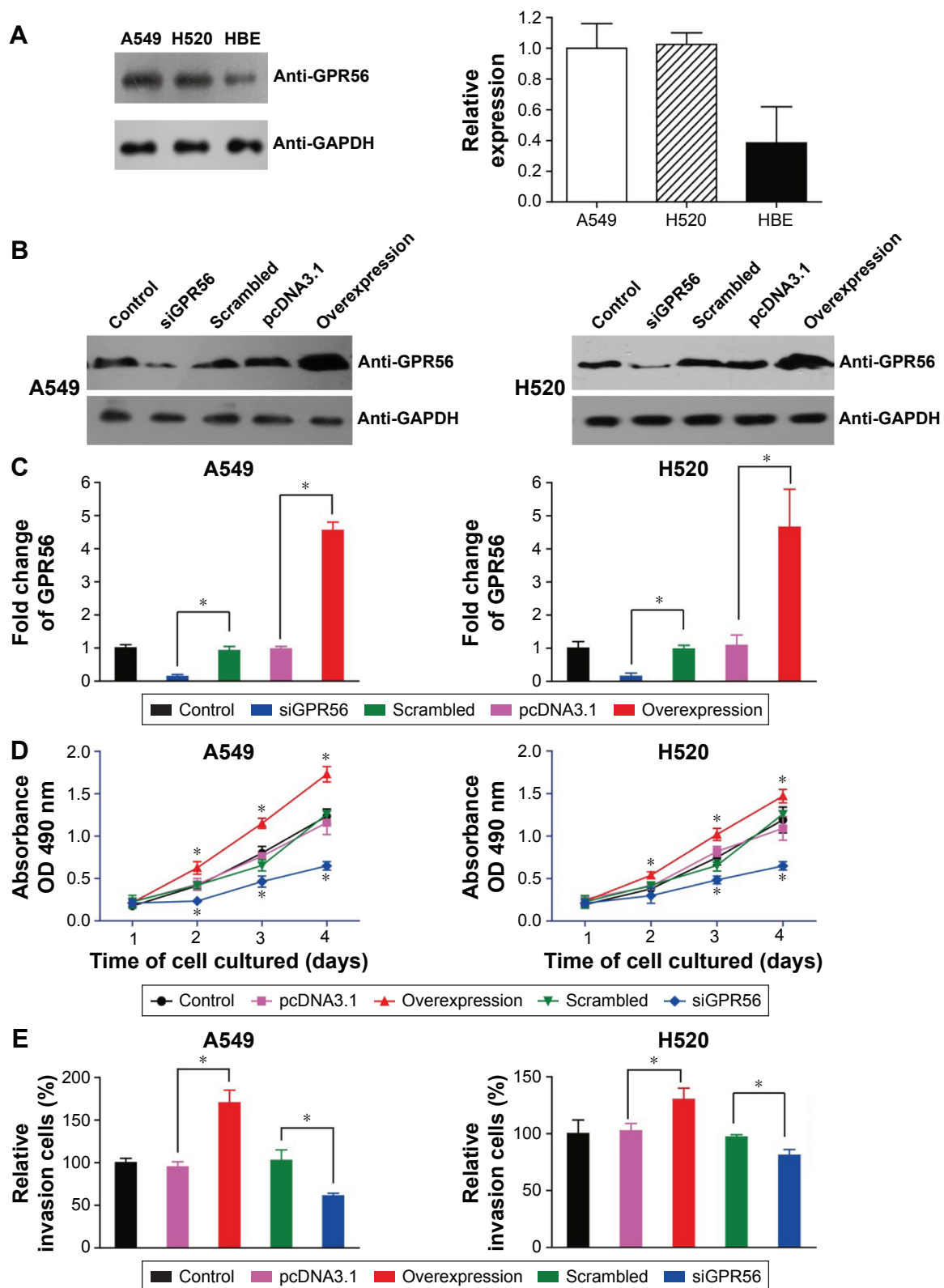


Figure 3 Expression and functions of GPR56 in NSCLC cell lines.

Notes: (A) GPR56 expression in adenocarcinoma cell line A549, squamous carcinoma cell line H520, and normal bronchial epithelial cell line HBE was demonstrated by Western blot (left) and ELISA (right). Western blot (B) and ELISA (C) results showed the efficiency of GPR56 overexpression and knockdown in A549 and H520 cell lines, respectively. With overexpression and knockdown, GPR56 was proven to be able to positively regulate cell proliferation (D, MTT assay) and invasion (E, Transwell assay) in cell lines A549 and H520. * $P < 0.05$. The data represent mean \pm standard deviation of three independent experiments.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPR56, G protein-coupled receptor 56; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; NSCLC, non-small-cell lung carcinoma; SD, standard deviation.

multivariate analyses. Positive GPR56 expression exhibited a poorer outcome compared to those with negative GPR56 expression. In addition, the in vitro experiment revealed that higher expression of GPR56 can significantly upregulate the capacity of cell proliferation and invasion, further demonstrating its unfavorable role in the outcomes of NSCLC patients.

Conclusion

The positive expression of GPR56 was associated with advanced TNM stage of NSCLC. Univariate and multivariate analyses showed that GPR56 can act as an independent biomarker of prognosis. In vitro studies also verified the role of GPR56 in regulating NSCLC cell proliferation and invasion.

Disclosure

The authors report no conflicts of interest in this work.

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