学位論文

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香川大学大学院医学系研究科

機能構築医学専攻

喜田裕介

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Inhibition of Cell-surface Molecular GPR87 With GPR87-suppressing Adenoviral Vector Disturb Tumor Proliferation in Lung Cancer Cells

YUSUKE KITA[1], TETSUHIKO GO[1], NARIYASU NAKASHIMA[1], DAGE LIU[1], YOSHIMASA TOKUNAGA[1], XIA ZHANG[2], TAKAYUKI NAKANO[1], KAZUHITO NII[1], SUNG SOO CHANG[1] and HIROYASU YOKOMISE[1]

Departments of [1]General Thoracic Surgery and [2]Urology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Abstract. *Background/Aim: GPR87 is a member of the cell surface molecular G protein-coupled receptors (GPCR) family and suggested to contribute to the viability of human tumor cells. Its tumor-specific expression and cell surface location make it a potential molecule for targeted therapy. In the present study, we aimed to examine the effect of silencing GPR87 expression and explore the possibility of establishing gene therapy against GPR87-overexpressing lung cancer. Materials and Methods: Twenty malignant cell lines were investigated and GPR87-overexpressing H358 and PC9 lung cancer cells were subjected to inhibiting experiments. A short hairpin siRNA targeting the GPR87 gene was transformed into an adenoviral vector (Ad-shGPR87). Real-time RT-PCR and western blot analyses were performed to evaluate gene and protein expression. Tumors derived from human H358 cells were subcutaneously implanted in nude mice for in vivo experiments. Results and Conclusion: About 50% (10/20) malignant cells showed GPR87-overexpression, especially for lung cancer cells (70%, 7/10). Ad-shGPR87 effectively down-regulated the GPR87 expression, and significantly inhibited the cell proliferation in GPR87-overexpressing H358 and PC9 cells. Treatment with Ad-shGPR87 exerted a significant antitumor effect against the GPR87-expressing H358 xenografts. In addition, the gene expression of H3.3, a recently proved activator for GPR87 transcription, was positively correlated with GPR87 gene expression. Furthermore, a significant decrease of KRAS and c-Myc expression was observed in both cell lines after Ad-shGPR87 infection. In conclusion, GPR87 may play a critical role in cancer cell proliferation, and indicate its potential as a novel target for lung cancer treatment.*

Correspondence to: Dr. Dage Liu, Department of General Thoracic Surgery, Faculty of Medicine, Kagawa University, 1750-1, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Tel: +81 87891219, Fax: +81 878912192, e-mail: dgliu@med.kagawa-u.ac.jp

Key Words: GPR87, shRNA, gene therapy, adenoviral vector, cell surface marker, NSCLC.

Lung cancer has generally a poor prognosis and remains the leading cause of cancer-related death worldwide (1). In recent years, some advances have been achieved in non-small cell lung cancer (NSCLC) treatment with molecular-targeted therapies such as the small-molecules gefitinib (2), erlotinib (3), and afatinib (4). On the other hand, immune therapy with checkpoint inhibitors, such as anti-PDL-1 and anti-CTLA-4, are other sorts of targeted therapies that have shown efficacy in lung cancer treatment (5). However, these targeted treatments are only applicable to a fraction of patients, a relevant increase of costs (6), development of resistance and recurrence are still a considerable problem in patients who even responded (7-9). Hence, it is necessary for us to identify novel targets for NSCLC treatment.

Ideal targets for cancer therapy should have characteristics such as, i) play a key role in cancer cell growth and survival, ii) be more abundant in cancer cells than normal cells, iii) be easier to approach from drugs, for example, be located on the surface of the cell. Recently, by analyzing microarray gene expression data, several high expression cell-surface markers (CA9, CA12, CXorf61, GPR87, LYPD3, and SLC7A11) were selected based on differential mRNA expression in lung tumors *vs.* normal lung and other normal tissues (10). Among these potential targets, G protein-coupled receptor 87 (GPR87) is a newly deorphanized member of the G protein-coupled receptor family and has been suggested to contribute to human tumor cells viability (11). In our previous clinical study, we found that 51.2% (63/123) of NSCLC tumors were GPR87-positive and the overexpression of GPR87 correlates with the worse tumor differentiation and high tumor proliferation in NSCLC (12).

In the present study, GPR87 gene expression was further investigated in 20 malignant cell lines including lung cancer cell lines. The GPR87-overexpressing cells were more

Table I. *GP87 and H3F3A gene expression in 20 malignant cell lines.*

Cell line	General character	GPR87		H3F3A	
		Gene expression (%)	Group	Gene expression (%)	Group
A549	Lung, adenocarcinoma	0.91±0.043	–	64.34±5.12	–
COLO668	Lung, small cell	3.78±0.07	–	98.13±6.88	–
EBC-1	Lung, squamous	99.38±7.96	+	100.00±0.00	–
H358	Lung, adenocarcinoma	363.39±32.06	+	412.59±7.22	+
LK-2	Lung, squamous	6.13±0.90	–	380.46±14.89	+
LUDLU	Lung, squamous	65.79±6.20	+	132.10±15.30	+
MAC10	Lung, adenocarcinoma	50.00±7.07	+	116.71±11.61	+
PC-9	Lung, adenocarcinoma	390.00±11.55	+	515.90±52.24	+
RERF-LC-AI	Lung, squamous	127.39±0.18	+	441±31.11	+
RERF-LC-KJ	Lung, adenocarcinoma	19.06±2.16	+	51.23±8.59	–
H2052	Malignant mesothelioma	18.74±0.41	+	103.67±0.22	–
H28	Malignant mesothelioma	0.03±0.02	–	11.68±1.50	–
Meso 1	Malignant mesothelioma	17.67±0.10	–	60.73±8.16	–
Meso 4	Malignant mesothelioma	109.71±12.18	+	572.66±86.34	+
MSTO	Malignant mesothelioma	32.98±2.03	+	146.18±1.01	+
DLD-1	Colon, adenocarcinoma	0.03±0.01	–	74.64±2.71	–
Hela	Cervix, adenocarcinoma	0.33±0.001	–	85.89±4.16	–
HT1080	Fibrosarcoma	2.84±0.06	–	90.90±38.82	–
NUGC3	Gastric cancer	0.25±0.01	–	38.07±13.43	–
ZR75-30	Brest, ductal carcinoma	2.48±0.17	–	212.09±17.01	+
Total		93.83±140.38		210.79±184.67	

Data were expressed as mean±SD. +: high gene expression group, –: low gene expression group.

frequently observed in lung cancer cells (70%, 7/10). With an adenoviral vector expressing short hairpin RNA (shRNA) targeting GPR87 (Ad-shGPR87), inhibition of GPR87 gene expression effectively inhibited the cell proliferation in GPR87-expressing lung cancer cell lines both *in vitro* and *in vivo*. Regarding the mechanism of GPR87, we found that the gene expression of a recently reported activator for GPR87 transcription, H3.3, was positively correlated with GPR87 in the malignant cells. Moreover, the K-Ras signal pathway seems to be responsible for the cell proliferation role of GPR87. The present study indicated that GPR87 may become a very good candidate of targeted therapy when developing new treatment for patients with GPR87-overexpressing lung cancer.

Materials and Methods

Cell lines. Twenty malignant cell lines, including 10 lung cancer cell lines, 5 malignant mesotheliomas and 5 other cell lines, were investigated for GPR87 gene expression (Table I). Two GPR87-overexpressing cell lines, H358 cell and PC9 cell were selected for silencing experiments. Cell lines were cultured in RPMI 1640 or DMEM medium (SIGMA-ALDRICH, St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

Construction of adenoviral vectors. A replication-deficient recombinant adenoviral vector expressing shRNA targeting GPR87 [forward strand: 5'-GPR87-siRNA sense strand (GUAAGGGAGAUACCUACCUTT) + loop (TAGTGCTCCTGGTTG) + GPR87-siRNA antisense strand + polymerase III terminator (TTTTTT)] under the control of the human U6 promoter (Ad-shGPR87) was constructed using the COS-TPC method as described previously (13, 14). An adenoviral vector expressing shRNA against the scrambled sequence (5'-UCUUA AUCGCGUAUAAGGCTT-3') was also constructed (Ad-shScramble) as a negative control. Constructed adenoviral vectors were amplified in 293 HEK cell line and purified by CsCl ultracentrifugation method.

Quantitative RT-PCR. Total cellular RNA was extracted using the acid guanidinium thiocyanate procedure with TRizol RNA isolation reagents (Life Technologies, Carisbad, CA, USA). First-strand cDNA was synthesized with TaqMan reverse transcriptase kit (Applied Biosystems, Branchburg, NJ, USA). TaqMan real-time quantitative PCR was performed with the ABI Step One Plus (Applied Biosystems, Foster City, CA, USA). The primers and probes were purchased from Applied Biosystems with the Assays-on-Demand gene expression products (GPR87: Hs00225057_m1; KRAS: Hs00364282_m1; MYC: Hs00154308_m1; AKT1: Hs00178289_m1, Applied Biosystems). The comparative threshold cycle method was used to calculate the gene expression in the sample relative to the value in the cells using GAPDH as internal control (ID 4326317E, Applied Biosystems) for normalization gene expression among samples. Each assay was performed in triplicate and repeated three times with consistent results.

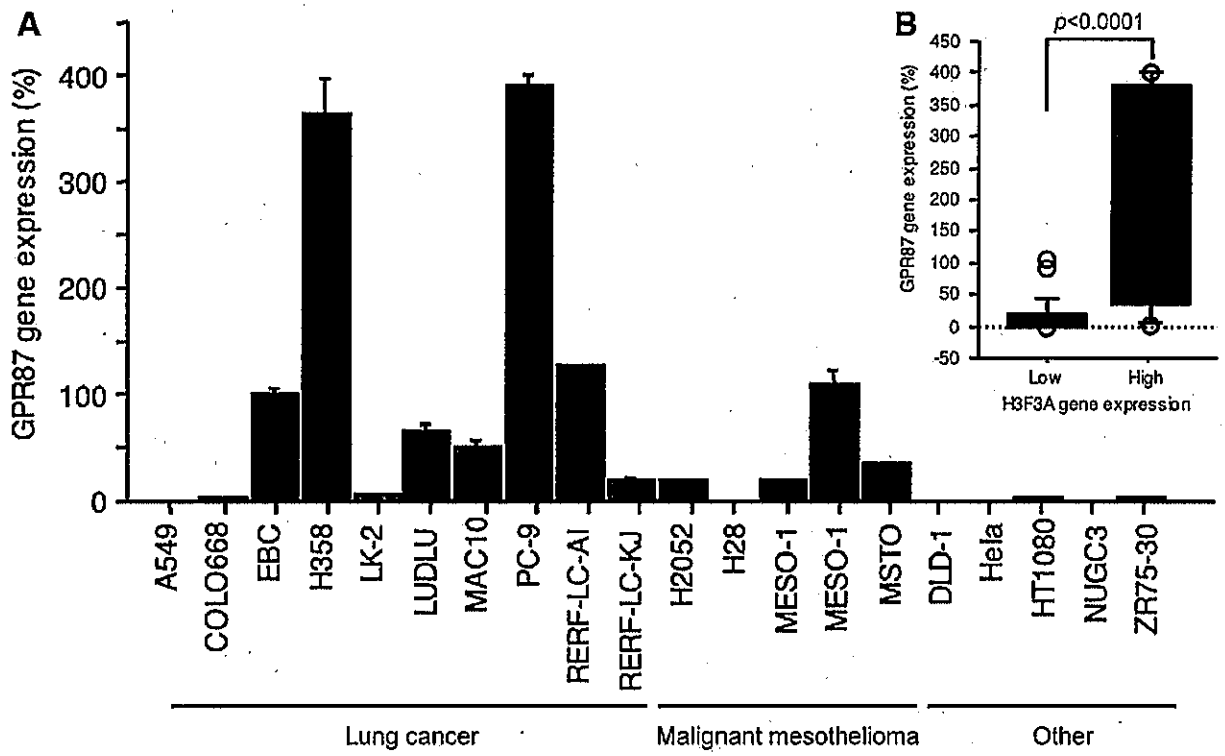

Figure 1. *GPR87 gene expression in human malignant cell lines (A). GPR87 gene expression in malignant cell lines according to the H3F3A gene status (B). Comparison was carried out with the independent Student's t-test.*

Western blotting. Western blotting was performed using an antibody against GPR87 (ab77517, Abcam, UK) at the recommend concentration. A lysis buffer specially for extracting the cellular membrane incorporated proteins (Cell-LyEX MP, Toyo Ink Group, Tokyo, Japan) was used for detecting GPR87 during sample collection. Protein samples (40 μg) were diluted into a 30 μl solution of lysis buffer containing 2-mercaptoethanol and heated at 95°C for 5 min. The protein sample was then separated by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes. Membranes were set in an iBind Flex Western Systems (SLF2000, Thermo Fisher Scientific, Waltham, MA, USA) allow perform hands-free blocking, antibody binding, and washes for western detection workflows according to the manufacturers description. The membranes were stripped and re-probed with an rabbit anti-human GAPDH antibody (sc25778, Santa Cruz Biotechnology, Dallas, TX, USA, 1:10,000) as a loading control. Each experiment was repeated three times with consistent results.

Cell viability assay. Tumor cells were seeded into 96-well plates at a concentration of 4,000 cells/well and subjected for experiment after 24 h. The cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method using a Cell Proliferation Kit I (Roche, Mannheim, Germany) at different time points. For the MTT assay, tumor cells were incubated with 10 μl of MTT labeling reagent for 4 h, and then further incubated with 100 μl of solubilization solution overnight. Finally, the cell viability in each well was measured in terms of optical density at a wavelength of 570 nm, with 750 nm for reference wavelength. Each cell viability assay was performed in triplicate and repeated three times.

Tumor xenograft model in nude mice. The *in vivo* experiment was performed as described previously (14, 15). First, H358 cells were subcutaneously implanted into the back of nude mice for tumor formation. After tumor xenografts formation, approximately 8 mm^3 fragments of tumors were prepared and implanting subcutaneously into the back of 6-week-old male nude mice (BALB/cA Jcl-nu, Yokohama, Japan). When the tumor volume reached approximately 200 mm^3, 12 mice were numbered and randomly divided into 2 groups of 6 each, with a method of random digits table, including Ad-shGPR87 treatment group and control group treated with Ad-shScramble. Intratumoral injection with adenoviral vectors (at 2×10^9 PFU, respectively) was performed every four days for 28 days. The tumor volume was calculated by the following formula: tumor volume=(length)×(width)^2×0.5. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals from Kagawa University.

Statistical analysis. Data are expressed as the mean±SD. Statistical significance regarding values was assessed by the *t*-test or Pearson's correlation coefficient accordingly. A p-value<0.05 was considered to indicate statistical significance.

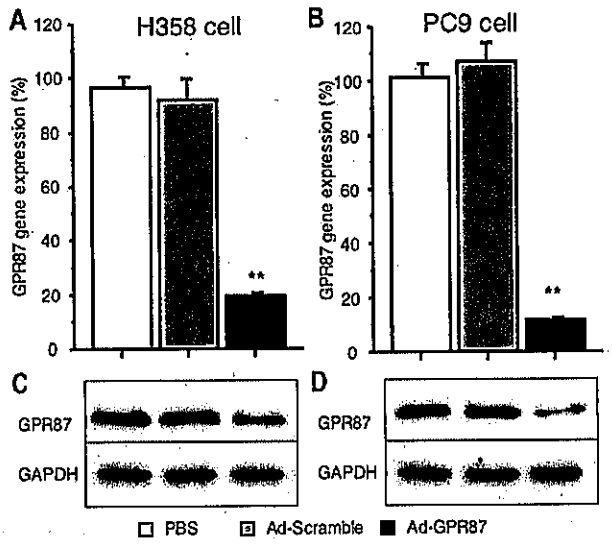

Figure 2. *GPR87 gene and protein expression in GPR87-overexpressing human lung cancer cells after adenoviral vector infection. GPR87 gene expressions in lung cancer H358 (A) cells and PC9 (B) cells. GPR87 protein expression in H358 (C), and PC9 (D) cells. Sample collection for RNA and protein analysis was performed at 5 days after transduction with adenoviral vectors at a multiplicity of infection MOI of 20 and one of three experiments with similar results is shown. MOI, multiplicity of infection; **p<0.005 versus Ad-shScramble treatment.*

Results

GPR87 is overexpressed in lung cancer cell lines. The normalized GPR87 gene expression ratio was evaluated in 10 human NSCLC cells and 10 malignant cell lines by real-time PCR. The GPR87 gene expression varied seriously (from 0.18% to 400.0%, mean±SD: 93.8±140.4%; median: 18.7%) in these malignant cells. With the median value as the cut-off value, 50% (10/20) of malignant cells appeared as GPR87-overexpression cells. The GPR87-overexpression cells were more frequently observed in the lung cancer cells (70%, 7/10) when comparing with the other malignant cells (3/10, 30%) (Figure 1A and Table I).

Significant correlation between GPR87 and H3F3A gene expression in malignant cells. As H3.3 was recently reported to be deposited at a specific intronic region of GPR87, where it modifies the chromatin status and directly activates GPR87 transcription, the relation between H3.3 and GPR87 gene expression was also explored. The gene expression of H3F3A, encoding H3.3, was evaluated in the same cell lines as listed above. The H3F3A gene expression varied seriously (from 10.6% to 633.7%, mean±SD: 210.8±184.7%; median: 113.4%) in these malignant cells also. With the median as cut-off value, 45% (9/20) of malignant cells showed high H3F3A gene expression. The high H3F3A gene expression cells were more frequently observed in the lung cancer cells (60%, 6/10) in comparing with the other malignant cells (30%, 3/10) (Table I). A significantly positive correlation was fund between H3F3A and GPR87 gene expression (R^2=0.575, p=0.005), the GPR87 gene expression was significantly higher in the H3F3A-high expression cells than that in H3F3A-low expression cells (172.5±162.7% *vs.* 14.8±28.5%, p<0.0001) (Figure 1B).

Ad-shGPR87 efficiently down-regulated the GPR87 expression. Two GPR87-overexpressing tumor cells, H358 cells and PC9 cells, were transfected with Ad-shGPR87 at a multiplicity of infection (MOI, PFU/cell) of 10. The Ad-shGPR87 effectively knocked-down the GPR87 gene expression in both of these GPR87-overexpressing tumor cells (p<0.005 *versus* Ad-shScramble) (Figure 2A and B). The down-regulation of GPR87 protein expression was also detected after transfection with Ad-shGPR87 in both these two GPR87-overexpressing tumor cells (Figure 2C and D).

Ad-shGPR87 inhibits the growth of GPR87-overexpressing tumor cells. The inhibitory effect of Ad-shGPR87 was investigated in GPR87-overexpressing H358 and PC9 cells. The percentages of viable cells significantly decreased in both GPR87-overexpressing tumor cells from day 5 after Ad-shGPR87 transfection (p<0.005 *versus* Ad-shScramble) (Figure 3A and B). Ad-shGPR87 strongly reduced the percentage of viable cells in these two GPR87-overexpressing tumor cell lines in a time-dependent manner. These results indicate the essential role of GPR87 in regulating cell proliferation of NSCLC cells.

Ad-shGPR87 inhibits the growth of tumor xenograft delivered from GPR87-overexpressing H358 cell. The GPR87-overexpressing H358 tumor xenograft was prepared in nude mice. Tumors in the Ad-shGPR87-treated group were significantly smaller than that in Ad-shScramble-treated group (Figure 4A). Tumor volumes at day 28 were 452.6±60.9 mm^3 in the Ad-shScramble-treated group and 194.8±41.5 mm^3 in the Ad-shGPR87-treated group (Figure 4B). The Ad-shGPR87 treatment significantly inhibited the growth of H358 xenografts in comparison to that of Ad-shScramble treatment from 18 days after treatment (p<0.05). Based upon these results, Ad-shGPR87 was proven to have anti-tumor effects *in vivo*.

Down-stream gene changes cells after GPR87 inhibition in lung cancer cell. For investigating the mechanism of GPR87 in regulating cell proliferation, Ras and Akt signaling were analyzed. Both cells showed a significant decrease in KRAS and c-Myc gene expression 5 days after Ad-shGPR87 transfection (Figure 5A and B). On the other hand, after Ad-

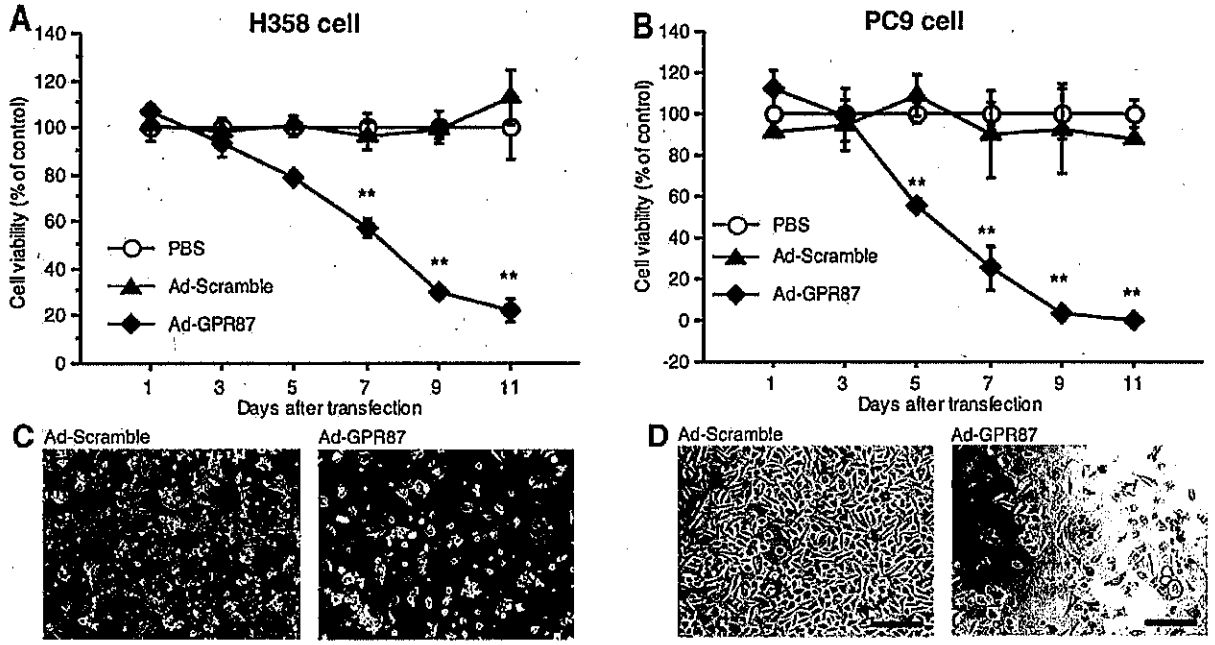

Figure 3. *Ad-shGPR87 in vitro inhibits the proliferation of GPR87-overexpressing human lung cancer cells. Cell viability evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after transduction with adenoviral vectors in H358 (A) cells and PC9 cells (B). A significant reduction of viable cells can be also directly observed in H358 (C) and PC9 (D) cells 5 days after Ad-shGPR87 infection (20 MOI). MOI: Multiplicity of infection; **p<0.005 versus Ad-shScramble treatment.*

shGPR87 transfection, Akt and cyclin-D1gene expression decreased slightly in H358, but not in the PC9 cell (Figure 5C and D).

Discussion

G protein-coupled receptors (GPCRs) are a large protein family of receptors containing a seven-membrane-spanning helix connected by three intracellular loops with an extracellular NH2 terminus and an intracellular COOH terminus (16). GPCRs have been the prime targets for cancer therapy accounting for 50% of all drug targets (16, 17). Among these GPCRs, GPR87 (also known as GPR95) is a novel orphan receptor originally reported by Nonaka *et al.* (18) and recently deorphanized as a lysophosphatidic acid (LPA) receptor (19, 20).

The cell surface location, cancer-specific expression and role in facilitating cell viability of GPR87, which indicate a suitable candidate molecule for diagnosis and treatment, recently attracted more attention. By using Affimetrix Chip technology, Glatt *et al.* reported the cancer-specific distribution of GPR87 in human for the first time (21). They found that GPR87 is highly expressed in squamous cell carcinomas (SCC) of the lung, cervix, skin, urinary bladder, testis, and head and neck, and in large-cell carcinomas and adenocarcinomas of the lung and transitional cell carcinomas of the urinary bladder, but expressed at low levels in most tissues with the exception of placenta, and head and neck tissues. They also found that loss-of-function studies using siRNA in human cancer cell lines lead to antiproliferative effect and induction of apoptosis. Subsequent clinical studies further confirmed these finding in cancers of the bladder (13), lung (12, 21) and pancreas (22). The role of GPR87 in promoting cell growth was further well proved in normal cells (23, 24) and tumor cells including hepatocellular carcinoma (25), bladder cells (15), pancreatic cancer (22) and human squamous carcinoma (20). Overexpression of GPR87 was frequently found in these cancers and patients with GPR87-overexpression tumor had a significant worse overall survival compared to patients with lower GPR87 expression.

Inconsistent to these studies, in the present study, high GPR87 expression was found in 70% of lung cancer cells (7/10), and 50% (10/20) of malignant cell lines (Table I). Knockdown of GPR87 gene with Ad-shGPR87 effectively down-regulated the GPR87 gene expression and inhibited the cell proliferation in lung cancer cells (Figures 2 and 3). The role of GPR87 in keeping the viability of human cancer was

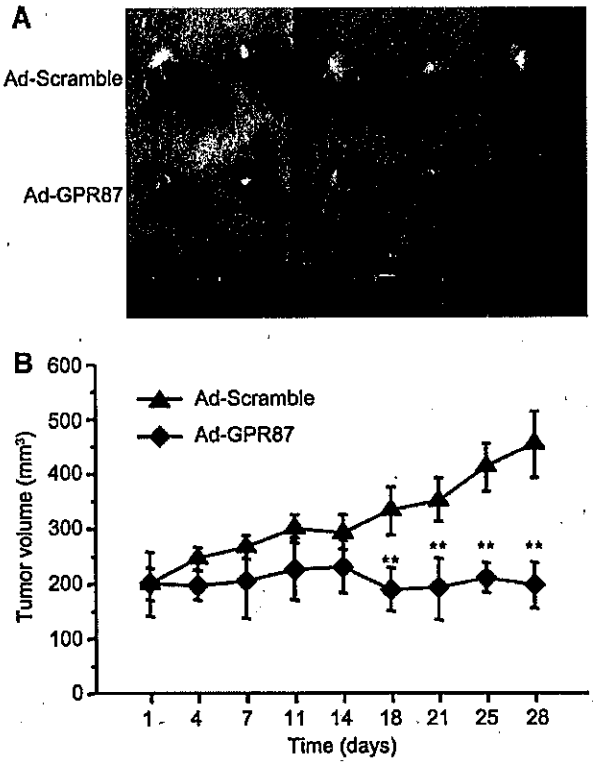

Figure 4. *Ad-shGPR87 in vivo inhibits tumor growth of xenografs. Tumor appearances (A) and volumes (B) of GPR87-expressing H358 xenografts in nude mice after adenoviral vector treatment. The volumes of the treatment group were shown as mean values±SD and comparison between the Ad-shGPR87 and Ad-shScramble group was carried out with the independent Student's t-test on each individual day. *p<0.05; **p<0.005, vs. Ad-shScramble treatment.*

confirmed in lung cancer cell lines. Our present results and those of other studies provided strong support on that GPR87 plays an important role in cancer cell survival and is essential for the development and maintenance of lung cancer tumor.

Though increasing studies on GPR87 have been recently reported, the mechanism for GPR87 in gene regulation and enhancing cell proliferation is still not well understood. With regards to the regulation of gene expression, GPR87 has been reported to be up-regulated by the tumor suppressor p53 in the cancer cell lines MCF7 and ROC (23, 26). In another study, p53 was suggested as a downstream effector of GPR87 in wild-type p53 bladder cells (15). In a most recent study, Park *et al.* provided new evidence for GPR87 gene regulation by the histone variant H3.3 with ChIP-PCR assay. They confirmed a specific H3.3 binding position to the GPR87 intron and proved a direct activation of GPR87 transcription *via* its deposition at the specific intronic region (24). In the present study, by mRNA expression analysis, we confirmed the positive correlation between GPR87 and H3F3A gene in malignant cell lines. The GPR87 gene expression was significantly high in H3F3A-overexpressing cells than in low H3F3A-expressing cells (Figure 1B).

Regarding the downstream signal pathway of GPR87, it is still not clearly understood. Several signaling pathways including p53 (23, 26), KRAS, IP3-Akt (15) and NF-κB (22, 27) pathways were suggested to be concerned to the GPR87 signal transduction in different cell lines. In bladder cells, inhibition of cell proliferation by GPR87 knockdown was only observed in wild-type p53 cell lines but not in p53-mutant cells (15). However, in the present study, inhibition of cell proliferation was observed in both H358 and PC9 lung cancer cell which have been used as p53-mutent cells (28). With PCR analysis, the KRAS and c-Myc gene expression significantly decreased correspondingly in both H358 and PC9 cell after GPR87 inhibition (Figure 5A and B). Ras pathway seems to be responsible for the GPR87-mediated cell proliferation in lung cancer cells. Akt was also investigated, but inconsistent gene expression ware observed in these two lung cancer cell lines. Although there was a significant decrease in Akt and cyclin-D1 gene expression in H358 cell, but not in PC9 cells (Figure 5C and D), further studies are necessary to clarify the pathways involved in GPR87-mediated survival.

Recently targeted therapy is gaining importance due to its specificity towards cancer cells while sparing toxicity to off-target cells (29). Most targeted therapies include either small molecules or monoclonal antibodies (30). Small-molecule compounds are typically developed for targets that are located inside the cell because such agents are able to enter cells relatively easily. Monoclonal antibodies are relatively large and are generally used only for targets that are outside cells or on the cell surface. On the other hand, gene therapy is another important approach for targeted therapy (31). RNA interference (RNAi) is a useful tool for cancer gene therapy, however successful application of RNAi to cancer gene therapy depends on the efficient delivery of siRNA into the cells (32-34). Stable siRNA molecules can be produced *via* shRNA expressed under the control of the RNA polymerase III-dependent promoter (35, 36). Furthermore, adenoviral vectors have been widely used for the expression of transgenes, not only under experimental conditions (37, 38) but also in a clinical setting (39, 40).

There are still several disadvantages in the present study. Though a close positive correlation between GPR87 and H3F3A gene expression was confirmed in the malignant cell lines, direct evidence such as inhibiting of H3F3A gene is necessary to clarify its role in GPR87 gene regulation. On the other hand, regarding the mechanism under GPR87-mediated cell proliferation, the signal findings of downstream genes were all based on the inhibiting experiment with p53-mutant cells, thus further studies with

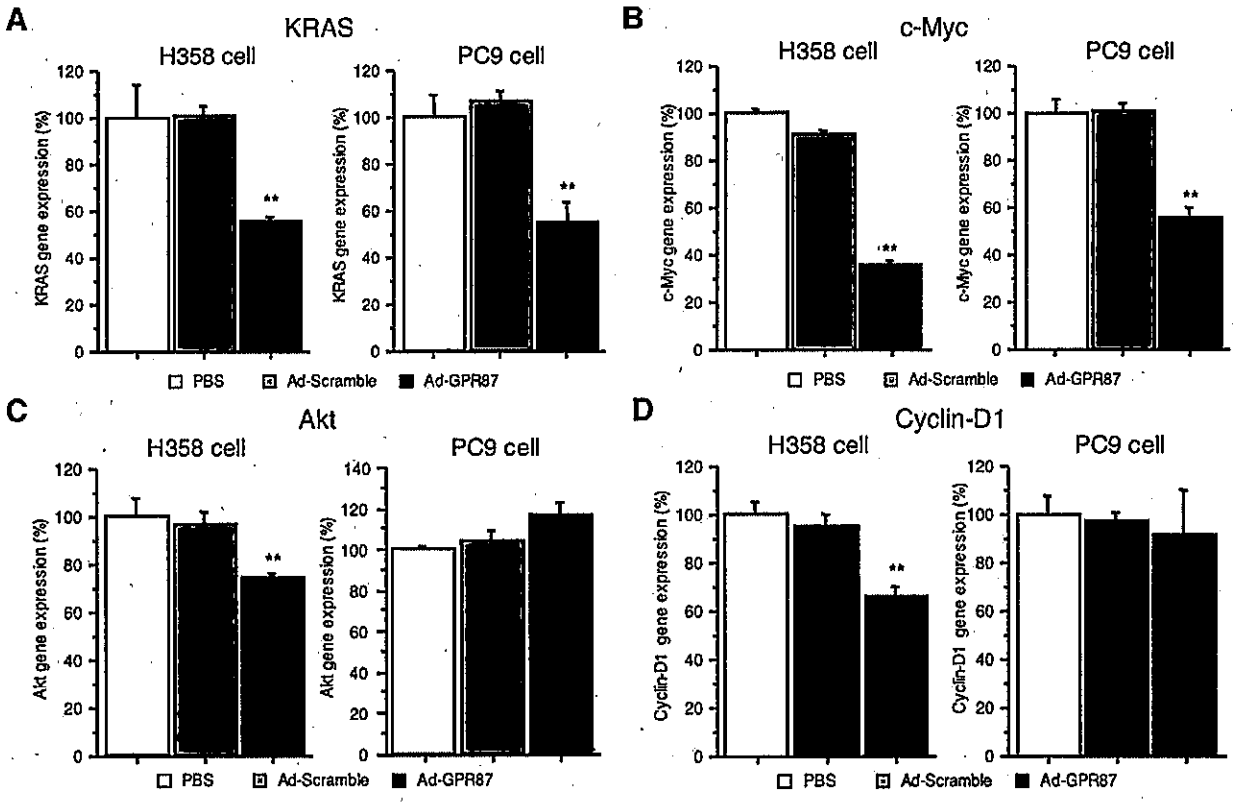

Figure 5. *Changes in downstream genes after GPR87 inhibition in lung cancer cells. A significant decrease in KRAS and c-Myc gene expression was observed after Ad-shGPR87 transfection (A and B). On the other hand, Akt and cyclin-D1gene expression decreased slightly in H358, but not in the PC9 cell (C and D). Samples for RNA analysis was collected at 5 days after transduction with adenoviral vectors at a multiplicity of infection MOI of 20 and one of three experiments with similar results is shown. MOI, Multiplicity of infection; **p<0.005 versus Ad-shScramble treatment.*

p53-wild cell lines should be designed to investigate these changes for complete understanding of this mechanism in lung cancer cells. Moreover, when thinking about the therapeutic application of GPR87 in cancer therapy, the construction of monoclonal antibodies should be taken into consideration in future studies.

In summary, we report that GPR87 expression was significantly high in most malignant cell lines, and positively correlated with H3F3A gene expression. In the present study, we constructed an adenoviral vector expressing shRNA targeting GPR87 and demonstrated that this vector exerted effective activity against GPR87-expressing cells both *in vitro* and *in vivo*. Our findings suggest that GPR87 plays a vital oncogenic role in cancer progression and highlight its potential as a novel target for lung cancer therapy.

Availability of Data

The datasets used/or analyzed in this study are available by the corresponding author on reasonable request.

Conflicts of Interest

None declared.

Authors' Contributions

YK and DL designed the study; YK and NN performed research; YT, XZ and TN provided sample collection and contributed to data interpretation; YK and DL wrote the manuscript, and KN, SSC, TG and HY revised the manuscript and participated in interpreting the data.

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