ANTICANCER RESEARCH *40*: 6835-6844 (2020)
doi:10.21873/anticanres.14705

ADGRF4 Regulates Non-small Cell Lung Cancer Cell Invasiveness

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Abstract. *Background/Aim: Adhesion G protein-coupled receptors (aGPCRs) have a crucial role in cancer. However, the role of ADGRF4, one of aGPCRs, in cancer has yet to be revealed. Therefore, we investigated its role in lung cancer, a leading cause of cancer-related deaths worldwide. Materials and Methods: ADGRF4 gene expression pattern in lung cancer were analyzed by in silico analyses. RNA sequencing was conducted to investigate gene expression pattern altered by ADGRF4 knockdown. Lung cancer cell lines were subjected to cell migration and invasion assays. Results: In silico analysis data indicated a major role of ADGRF4 in lung cancer. RNA sequencing data showed that ADGRF4 gene silencing in lung cancer cells altered global expression pattern. ADGRF4 gene silencing reduced lung cancer cell invasiveness. Furthermore, PPP2C gene expression was most significantly down-regulated by ADGRF4 gene silencing. PPP2C overexpression rescued cell invasiveness inhibited by ADGRF4 gene silencing, and PPP2C gene silencing blocked lung cancer cell invasiveness. Conclusion: ADGRF4 regulates lung cancer cell invasiveness via PPP2C.*

Lung cancer is the leading cause of cancer death in the world and histopathologically divided by non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (1, 2). Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers (1). Although knowledge on gene mutations in NSCLC lead to development of therapeutics targeting proteins often mutated, one of hurdles to therapeutic success is lack of knowledge for molecular markers of NSCLC (3-7).

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Key Words: ADGRF4, GPR115, PPP4C, lung cancer, cell invasiveness.

Many newly developed drugs target G protein-coupled receptors (GPCRs) (8-10). In cancer, many GPCR-targeting drugs including cabergoline, degarelix, lanreotide, mogamulizumab and sonidegib were approved by the United States Food & Drug Administration (11). Recent GPCR research in cancer suggests some GPCRs as potential targets for cancer treatment (11-13). However, drugs targeting GPCRs for lung cancer treatment are yet to be developed, which is likely due to absence of knowledge for GPCRs specifically expressed in lung cancer. More recently, large-scale analyses revealed expression levels of GPCRs in lung cancer (11, 14, 15). Thus, further studies for GPCRs in lung cancer will help develop drugs for lung cancer treatment.

Adhesion G protein-coupled receptors (aGPCRs) have long N-terminal regions containing GPCR-autoproteolysis including (GAIN) domain (16-18). This domain mediates autocatalytic cleavage at GPCR proteolytic site (GPS) (16, 17). Exceptionally, long N-terminal extracellular region is thought to promote cell adhesion and migration (16, 17, 19). Because most aGPCRs are orphan receptors, searching ligands is difficult for aGPCRs researches (16, 17). Adhesion G protein-coupled receptor F4 (ADGRF4, also named GPR115) is a member of aGPCRs (16, 17, 20, 21). However, it is recently revealed that ADGRF4 does not undergo autoproteolysis (16, 17, 21). ADGRF4 is highly expressed in normal skin tissue (20, 22). However, there is no research on ADGRF4 in cancer (18).

In this study, we first demonstrated a role of ADGRF4 in lung cancer. ADGRF4 regulates lung cancer cell invasiveness by altering global gene expression pattern, especially *PPP4C*.

Materials and Methods

Cell culture, cloning and transfection. Lung cancer cell lines H460, A549 and H1299 were purchased from the National Cell Bank at Seoul National University Hospital (Seoul, Republic of Korea). Cells were cultured in DMEM with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotics and antimycoplasma (Sigma-Aldrich, Merck, Kenilworth, NJ, USA). *PPP4C* (CCDS10669.1) information for cloning work was obtained

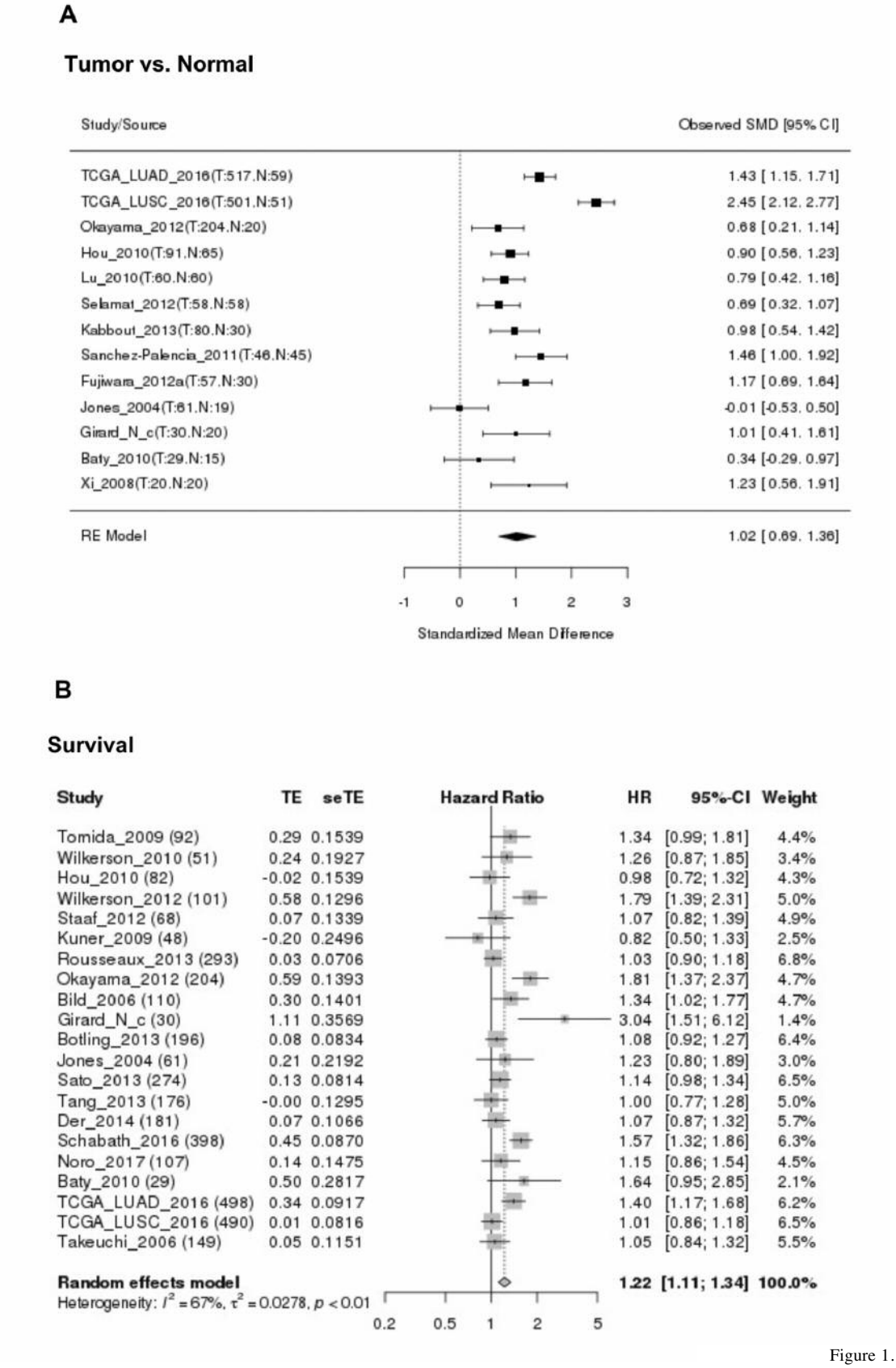


Figure 1. *Continued*

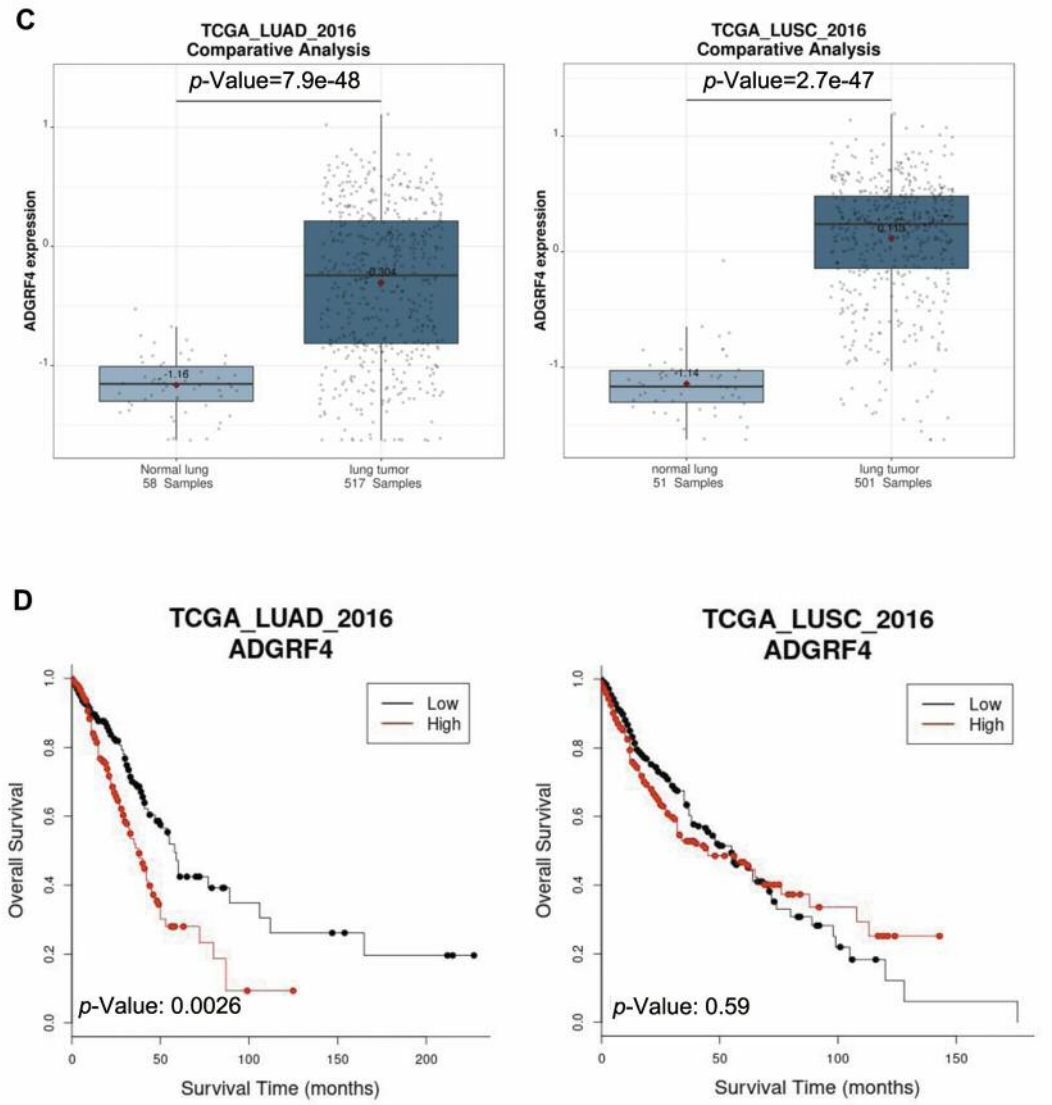


Figure 1. *ADGRF4 expression in lung cancer. (A) ADGRF4 expression level in tumor vs. normal tissues in meta-analysis. (B) Lung cancer patient survival with ADGRF4 expression level in meta-analysis. (C) ADGRF4 expression levels in TCGA_LUAD_2016 and TCGA_LUSC_2016. (D) Overall survival of lung cancer patients with ADGRF4 expression level.*

from Consensus CDS protein set (CCDS) database (https://www.ncbi.nlm.nih.gov/CCDS/). *PPP4C* cDNA was amplified by PCR using primers (forward 5'-AAGCTTATGGCGGAGAT-3', reverse 5'-TCACAGGAAGTCTTAAG-3') and then inserted into HindIII/EcoRI site in pcDNA3.1(+) plasmid.

Gene analyses. The Lung Cancer Explorer (LCE) web application (http://lce.biohpc.swmed.edu/) was used for *in silico* meta-analyses. Meta-analysis results, expression levels and survival plots for *ADGRF4* gene were gained in LCE website. QuantSeq 3'-mRNA-Seq was performed to analyze an effect of *ADGRF4* in gene expression pattern. Total RNA was isolated using Trizol reagent (Invitrogen). RNA was qualified by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, the Netherlands), and RNA quantification was conducted using ND-2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Library construction was done by QuantSeq 3'-mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturer's instruction. High-throughput sequencing was conducted as single-end 75 sequencing using NextSeq 500 (Illumina, San Diego, CA, USA). Bowtie 2 was used for the alignment of QuantSeq 3'-mRNA-Seq reads. Differential gene expression was determined using coverage in Bedtools. The read count data were done by quantile normalization method using EdgeR within R with Bioconductor. Data results and images were produced in the excel-based differentially expressed gene analysis (ExDEGA) tool (ebiogen, Seoul, Republic of Korea). The RNA sequencing data in this study have been deposited in NCBI's Gene Expression Omnibus and are

accessible through GEO Series accession number GSE153554 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153554).

Knockdown and real-time PCR. *ADGRF4* gene silencing was conducted by transfecting *ADGRF4* siRNAs (GPR115 predesign Chimera RNAi, Abnova, Taoyuan, Taiwan, ROC). *PPP4C* siRNAs (PPX siRNA) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). For either *PPP4C* overexpression or knockdown, 3 x 10^6 cells were transfected with Lipofectamine 2000 for 2 days. For real-time PCR, primers were used as follows: *LOC10929631* (F: 5'-GAAATTGTGCCTCCTTCGCC-3', R: 5'-TTGAATCTAGGCAGC TCCGC-3'), *LINC00410* (F: 5'-CATGGTTTCCAGAGGCGTCA-3', R: 5'-CAGCCTCCATCAATGCCGTA-3'), *ITGB3* (F: 5'-GAGGAGTCAGGGAGAGCTGA-3', R: 5'-CCCAGCCAACTCA TGGGAAT-3'), *PLA2G10* (F: 5'-GTGCAAGTGTGACCAGGA GA-3', R: 5'-CGGCTCACATAGGAACTGGG-3'), *KIF1A* (F: 5'-CACTGACACCAACACTGTGC-3', R: 5'-TCGTTCAGGTTG ACGAGGTG-3'), *CD101* (F: 5'-AGAGAGGCTCCAGTCCTCAG-3', R: 5'-CTTCCCACACAACTTGCTGC-3'), *ARVCF* (F: 5'-TGAGAACGAGGGTGTCAAGC-3', R: 5'-CCTTGGTGGAACC ACTCCTC-3'), *HSPB8* (F: 5'-TTCCCAGACGACTTGACAGC-3', R: 5'-GCCAATTGCGCTATCCTGTG-3'), *MRPL39* (F: 5'-GTGGCGGGATCAAATGGAGA-3', R: 5'-AGCCAGAATGGA CTTCCTGC-3'), *PPP4C* (F: 5'-AAGGAGAGCGAAGTCAAGGC-3', R: 5'-CCTGTGGTGTCTTCTGGGTC-3').

Cell viability, migration and invasion assays. A total of 110^3 cells were cultured in 96 well plates and then subjected to WST-1 assays (Daeil lab, Seoul, Republic of Korea). Cells were cultured in triplicate, and the assays were performed in triplicate. For cell migration, cells cultured at about 90% confluency on 6-well plates were scratched and took a picture after incubating for 12 h. The assays were performed in triplicate. For cell invasion, 110^5 cells were seeded on the chamber (Corning, Corning, NY, USA), and the chamber was put in 24-well dishes. The chamber and wells were filled with 1% FBS and 10% FBS, respectively. After incubating for 16 hours, the chambers were stained with 0.5% crystal violet for 5 min and then washed twice with water. The assays were done in triplicate and independently repeated in triplicate. Data were analyzed by two-tailed student t-test.

Western blots. Protein was extracted using RIPA buffer (150 mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA). 30 A total of 30 μg of protein were loaded on 8~12% SDS-PAGE and transferred to nylon membrane. Anti-ADGRF4 antibody was purchased from LSBio (Seattle, WA, USA). Anti-PPP4C antibody was obtained from Bethyl (Montgomery, TX, USA). Anti-alpha tubulin antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Membranes were incubated for 1 hour with appropriate primary antibodies at 1:1,000 dilution. Appropriate secondary antibodies were incubated for another 1 hour at 1:10,000 dilution.

Statistical analysis. All experiments were performed in triplicate. Values are expressed as the mean±standard deviation. Differences between groups were analyzed using one-way analysis of variance or an unpaired Student's *t*-test. Tukey's HSD was conducted as a post-hoc test. $p<0.05$ was considered to indicate a statistically significant difference. Graphs were made in Excel (Microsoft, Redmond, WA, USA). SPSS v21.0 (IBM, Armonk, NY, USA) was used for statistical analysis.

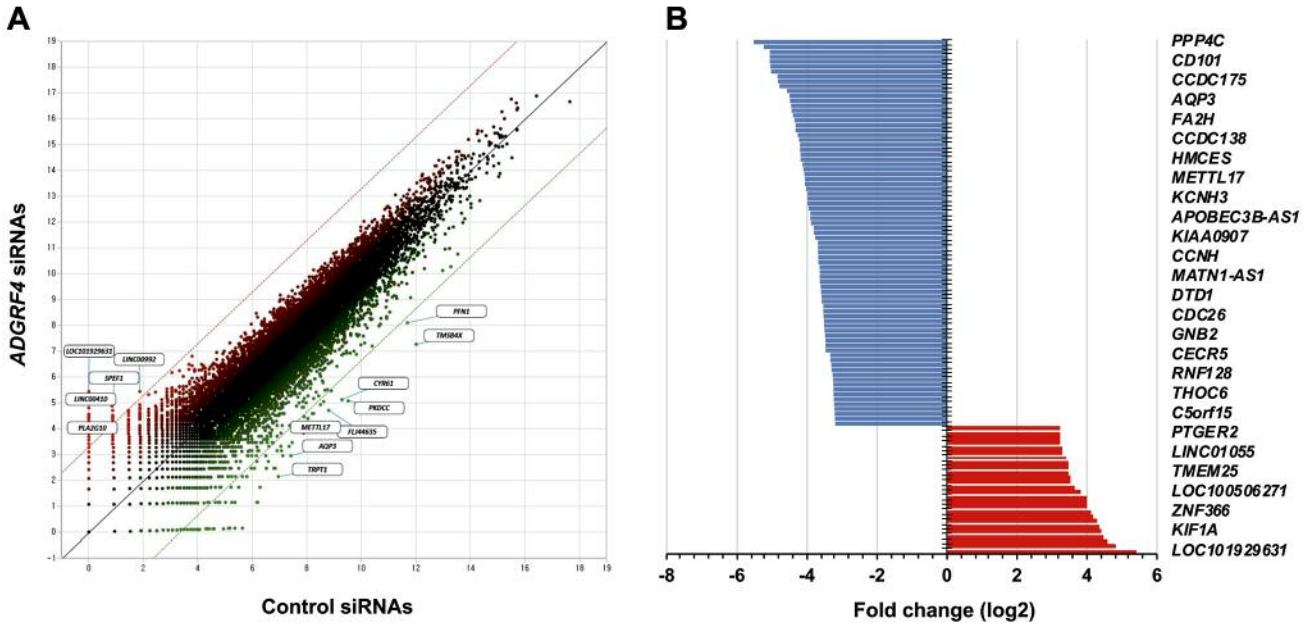
Results

ADGRF4 is highly expressed in lung cancer. In LCE (http://lce.biohpc.swmed.edu/lungcancer/), meta-analysis by centralizing data collection with *ADGRF4* gene showed high expression of the *ADGRF4* gene in tumor samples (Figure 1A) and *ADGRF4* gene association with survival of lung cancer patients (Figure 1B). In TCGA_LUAD_2016 and TCGA_LUSC_2016 datasets, *ADGRF4* gene was highly expressed in tumor samples (Figure 1C). However, survival association of *ADGRF4* gene was only found in TCGA_LUAD_2016 dataset (Figure 1D), suggesting a certain role of *ADGRF4* gene in lung adenocarcinoma. However, a certain role of *ADGRF4* gene in squamous cell carcinoma cannot be ignored because of its high expression level.

ADGRF4 regulates global gene expression pattern in lung cancer cells. We next sequenced mRNAs extracted from A549 cells knocked-down with either control siRNAs or *ADGRF4* siRNAs (GSE153554). *ADGRF4* gene silencing altered the expression of genes involved in various cellular events. Filtering by ten-fold threshold selected 105 genes from total 20,739 genes (Figure 2A). *ADGRF4* gene silencing up-regulated 26 genes and down-regulated 79 genes (Figure 2B). Top 5 up-regulated and down-regulated genes were chosen (Figure 2C), and their expression levels were confirmed by real-time PCRs (Figure 2D).

ADGRF4 regulates lung cancer cell invasiveness. To examine the role of ADGRF4 in NSCLC cells, ADGRF4 expression was knocked-down by transfecting A549, H460 and H1299 NSCLC cells with either control siRNAs or *ADGRF4* siRNAs (Figure 3A). When cell migration was examined in scratching assays, *ADGRF4* silencing altered cell migration rate by approximately 25-35% (Figure 3B). In the invasion assays, *ADGRF4* silencing also reduced invaded cell numbers by approximately 35% to 65% (Figure 3C). However, *ADGRF4* silencing did not alter cell proliferation when we examined its effect for 72 h (data not shown). Therefore, we conclude that ADGRF4 is required for lung cancer cell invasiveness.

ADGRF4 requires PPP4C for lung cancer cell invasiveness. *PPP4C* gene encodes PPP4C, serine/threonine protein phosphatase 4 catalytic subunit. As our gene expression data showed that *ADGRF4* gene silencing altered *PPP4C* expression level most significantly (Figure 2C), we tested PPP4C involvement for ADGRF4 role in lung cancer cell invasiveness. In A549 cells where *ADGRF4* gene was silenced, *PPP4C* overexpression rescued the invasiveness inhibited by *ADGRF4* knockdown (Figure 4A). Next, the cells were knocked-down with control or *PPP4C* siRNAs and examined the invasiveness. Expectedly, *PPP4C* gene silencing reduced the invasiveness



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Gene	Fold change (log2)
LOC101929631	5.43
LINC00410	4.80
ITGB3	4.56
PLA2G10	4.49
KIF1A	4.42
CD101	-5.06
ARVCF	-5.06
HSPB8	-5.06
MRPL39	-5.22
PPP4C	-5.51

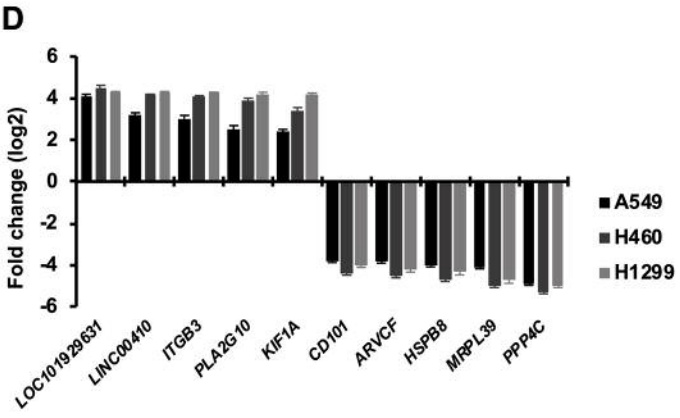

Figure 2. *ADGRF4 knockdown in A549 cells alters global gene expression pattern. (A) Scatter plot to show gene expression pattern in cells transfected with control siRNAs or ADGRF4 siRNAs. Red and green dots indicate genes up- and down-regulated by ADGRF4 knockdown. Dash lines indicate a range for ten-fold change of expression levels. (B) Genes of which expression level over ten-fold ($p<0.05$) were selected. (C) Top 10 genes altered by ADGRF4 knockdown. (D) Real-time PCR analyses to confirm RNA sequencing results.*

(Figure 4B). Thus, our data indicate that ADGRF4 involves PPP4C in regulation of lung cancer cell invasiveness.

Discussion

aGPCRs have been recently highlighted because of their numerous roles in cancer (13, 16, 19). Furthermore, it has been reported that members of group VI of aGPCR family have multiple roles of in cancer (13, 14, 16, 19). However, a role of ADGRF4, one of the members of group VI of aGPCRs, has not been reported in cancer. Our *in silico* study let us start defining a role of ADGRF4 in lung cancer and consolidated our simple hypothesis: ADGRF4 plays a role for lung cancer. Therefore, in this study, we report that ADGRF4 is required for lung cancer cell invasiveness.

Genomic data collections for lung cancer focus on genes such as *TP53*, *KRAS* and *EGFR* (23-28). Those superstar genes veil significance of other genes which are not too much altered in either expression level or in mutation status. In GPCR screening in cancers including lung cancer, ADGRF4 is veiled by other GPCRs that appeared much more critical to drive cancer biological events (12-14, 29, 30). When we investigated its gene expression levels in whole-gene expression panels from the TCGA website (https://www.cancer.gov/tcga), ADGRF4 looked to be easily ignored because of its relatively low differential expression level. However, LCE, the web-based analysis tool

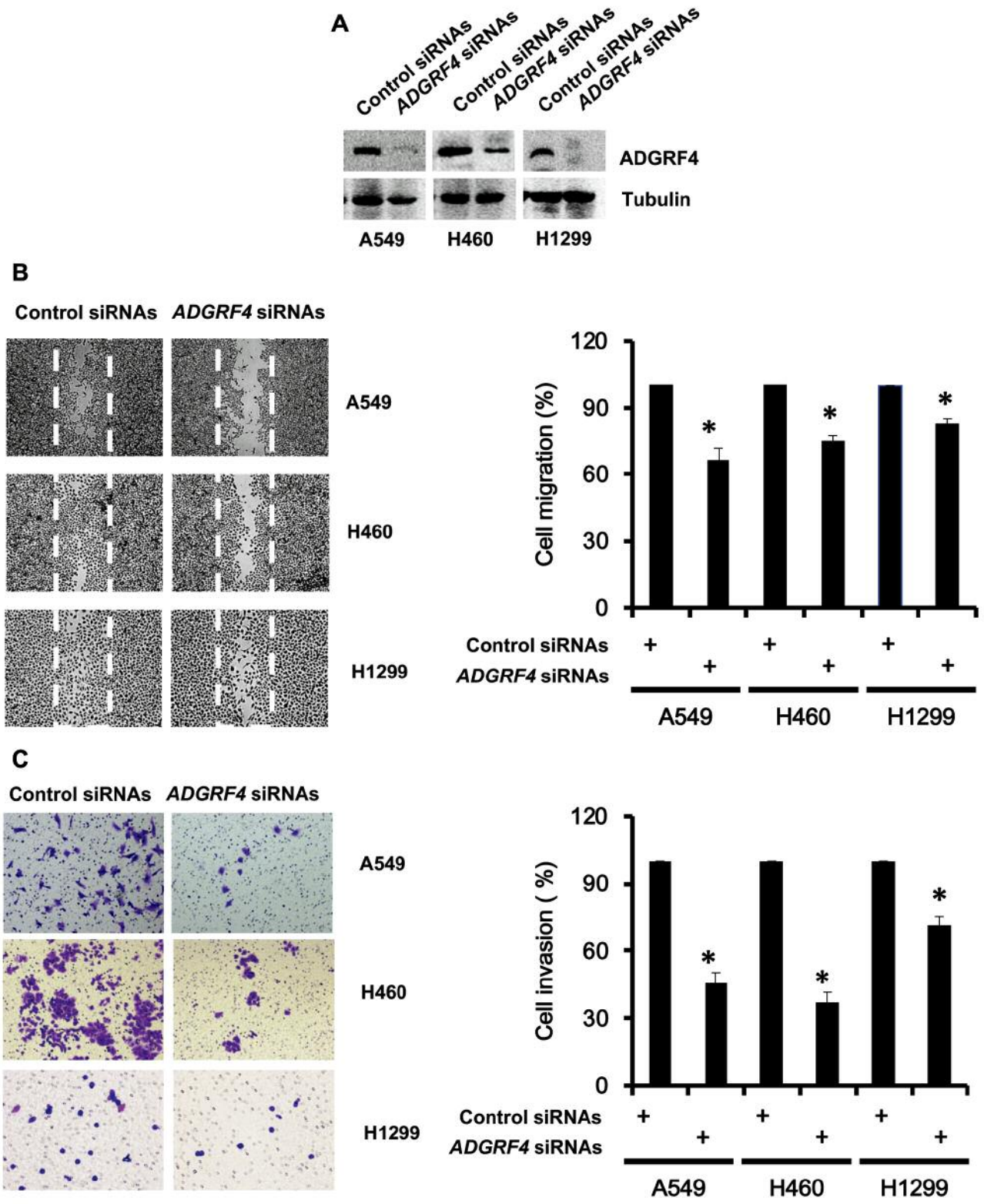

Figure 3. *ADGRF4 regulation of lung cancer cell invasiveness. (A) Western blots to confirm ADGRF4 knockdown in lung cancer cell lines. (B) An effect of ADGFR4 knockdown on lung cancer cell migration. Scratching assays were conducted for 24 h (left), and the migrated cells were counted to measure the effect of ADGFR4 knockdown on cell migration (right), *p<0.05. (C) An effect of ADGRF4 knockdown on lung cancer cell invasion. Invasion assays were performed for 24 h (left) and then the invaded cells were counted to measure the effect of ADGRF4 knockdown on cell invasiveness (right), *p<0.05.*

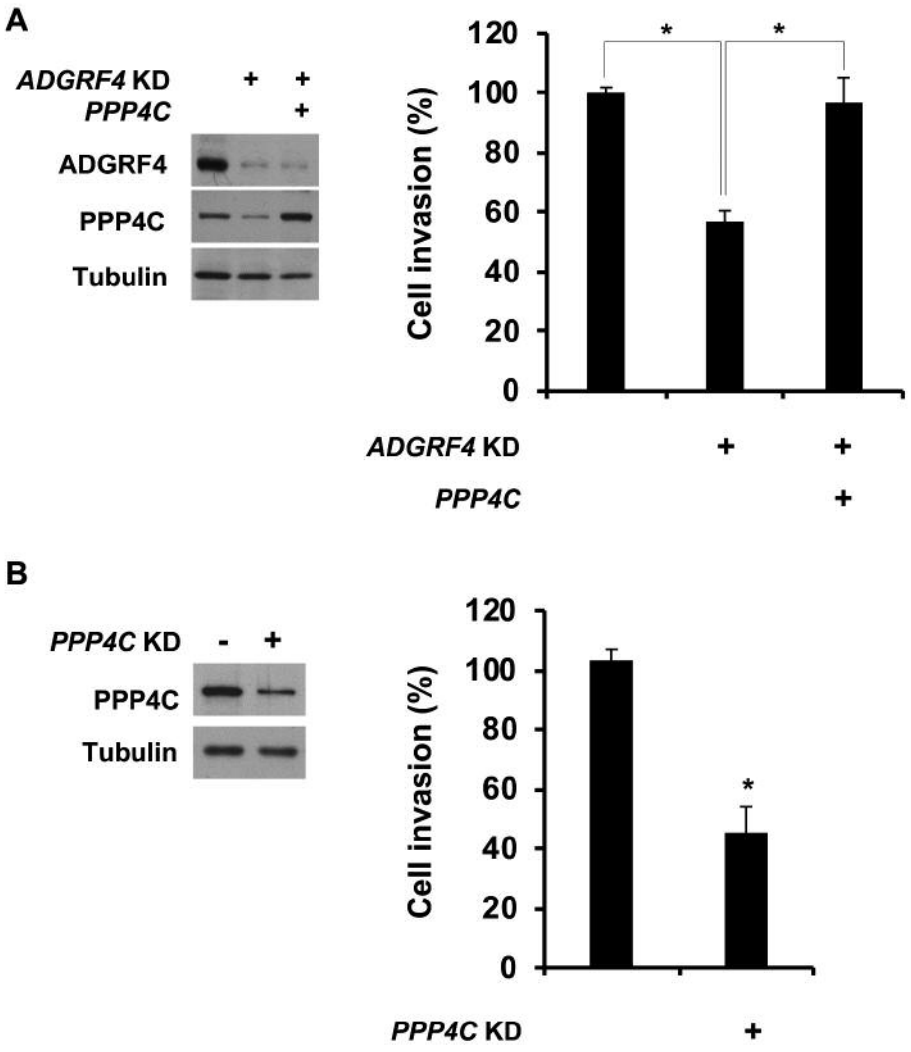

Figure 4. *ADGRF4 requires PPP4C for lung cancer cell invasiveness. (A) A549 cells were transfected with control siRNAs or ADGRF4 siRNAs together with pcDNA3.1(+) empty vector or PPP4C plasmid for 48 h. Western blot confirmed gene silencing or overexpression (left). The invasion assays were conducted for another 24 h (right), *p<0.05. (B) A549 cells were knocked-down with control siRNAs or PPP4C siRNAs. PPP4C silencing was confirmed by western blot (left). The invasiveness was conducted for 24 hours post transfection (right), *p<0.05.*

with lung cancer genomic data collection (http://lce.biohpc.swmed.edu/lungcancer/), provided meaningful results for ADGRF4 from multiple analyses including *ADGRF4* gene expression, survival associated with *ADGRF4*, correlation with other remarkable genes. Importantly, those analyzed results derived from samples of patients.

NSCLC exhibits high level of *ADGRF4* gene expression, which may be, in part, due to 6p12 amplification, because *ADGRF4* and syntenic genes including *ADGRF1* (*GPR110*), *ADGRF2* (*GPR111*), *ADGRF5* (*GPR116*) are located on human chromosome 6p12.3 region (16, 31-34). *ADGRF4*/*CALR* by t(6;19)(p12;p13) by translocation between *ADGRF4* at 6p12.3 and *CALR* at 19p13.13 is frequently found in leukemia and lymphoma (35). However, it is rare in lung cancer. One of *ADGRF4* paralogs, *ADGRF3* (*GPR113*) is located on chromosome 2p23.3 region, which is frequently deleted in lung cancer (36, 37). Therefore, if *ADGRF4* and its syntenic genes compensate *ADGRF3* role, we can imagine that *ADGRF3* and its one of paralogs, *GPR128*, may work for metastasis (18, 19, 30). In this study, we did not focus on underlying mechanisms affecting *ADGRF4* expression such as genome status, transcription factors and epigenetic states. Interestingly, DNA methylation seems to affect both *ADGRF4* and *ErbB* genes in all cancer types (38). In addition, DNA methylation at CpG island around exon 1 of TRIM58 is linked to *ADGRF4* gene expression in lung squamous cell carcinoma (39). Thus, clear

mechanisms regulating *ADGRF4* gene expression in lung cancer remains to be answered.

In this study, *ADGRF4* gene silencing in A549 lung cancer cells dramatically altered genes. Among top ten highly altered genes, we focused on a dramatic reduction of *PPP4C* mRNA because of the correlation between high *PPP4C* gene expression level and cancer malignancy (40, 41). Our study found that *PPP4C* overexpression rescued *ADGRF4* gene silencing-mediated inhibition of the invasiveness and *PPP4C* gene silencing reduced the invasiveness. Thus, it is plausible that ADGRF4 requires PPP4C for lung cancer malignancy.

Conclusion

ADGRF4 expression is higher in lung tumor samples than adjacent normal tissue samples. Moreover, it is associated with lung cancer patient survival. However, its role in lung cancer has not been examined. In this study, we first found that ADGRF4 regulates lung cancer cell invasiveness by modulating gene expression pattern. Therefore, we conclude that ADGRF4 has a critical role in lung cancer. This study will provide its utility as diagnostic marker and therapeutic target.

Conflicts of Interest

The Authors declare there are no conflicts of interest.

Authors' Contributions

Yoon JH conducted experimental work, analyzed data and wrote the manuscript. Cho SG designed experiments, analyzed data and wrote the manuscript. All Authors read and approved the final manuscript.

Acknowledgements

This work was supported by the Korea National University of Transportation in 2019 and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03034996). This study is part of the thesis of master's degree for Yoon JH.

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Received September 1, 2020
Revised October 22, 2020
Accepted October 27, 2020