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Targeting diacylglycerol kinase α impairs lung tumorigenesis by inhibiting cyclin D3

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Abstract

Background: Diacylglycerol kinase α (DGKA) is the first member discovered from the diacylglycerol kinase family, and it has been linked to the progression of various types of tumors. However, it is unclear whether DGKA is linked to the development of lung cancer.

Methods: We investigated the levels of DGKA in the lung cancer tissues. Cell growth assay, colony formation assay and EdU assay were used to examine the effects of DGKA-targeted siRNAs/shRNAs/drugs on the proliferation of lung cancer cells in vitro. Xenograft mouse model was used to investigate the role of DGKA inhibitor ritanserin on the proliferation of lung cancer cells in vivo. The downstream target of DGKA in lung tumorigenesis was identified by RNA sequencing.

Results: DGKA is upregulated in the lung cancer cells. Functional assays and xenograft mouse model indicated that the proliferation ability of lung cancer cells was impaired after inhibiting DGKA. And cyclin D3(CCND3) is the downstream target of DGKA promoting lung cancer.

Conclusions: Our study demonstrated that DGKA promotes lung tumorigenesis by regulating the CCND3 expression and hence it can be considered as a potential molecular biomarker to evaluate the prognosis of lung cancer patients. What's more, we also demonstrated the efficacy of ritanserin as a promising new medication for treating lung cancer.

KEYWORDS
DGKA, lung cancer, CCND3, tumorigenesis

INTRODUCTION

Recent research has shown that lung cancer is the leading cause of mortality from cancer in men and women in the United States, although fewer cases have been reported relative to those of prostatic cancer in men and mammary cancer in women for the first time.[1] About 85% of patients with primary lung cancer have non–small cell lung cancer (NSCLC).[2] Despite significant advancements in the lung cancer prevention and treatments, numerous challenges remain in improving patient outcomes.[3] Hence, it is important to search for new and unique pathogenesis of lung cancer and develop novel treatment methods.

The diacylglycerol kinase family consists of ten mammalian diacylglycerol kinase isozymes that were later classified into 5 subtypes. DGKA is the first member of this family to be purified and cloned.[4] In the cytosol, DGKA transfers the γ-phosphate of adenosine 5′-triphosphate (ATP) to the second messenger, diacylglycerol, converting it into another pivotal second messenger, the phosphatidic acid.[5] Hepatocellular carcinoma, colon carcinoma and melanoma cell lines have been demonstrated express high levels of DGKA. In particular, cancer cells express it strongly, while normal cells express it barely or not at all.[6–8] It has been reported DGKA promotes the

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migration of endometrial cancer cells which was evaluated by cell migration assay in vitro.[9] Invasion assay in vitro confirmed that the inhibition of DGKA reduces the number of invasive cells in breast cancer.[10] Tunel assay in vitro demonstrated that DGKA inhibits TNA-α-induced apoptosis in melanoma.[7] Li et al. revealed that *DGKA* is involved in the resistance of ovarian cancer cells to cisplatin through in vivo experiments (in vivo xenografts mouse model) and in vitro experiments (such as apoptosis assay, cell viability assay and colony farmation assay).[11] By stimulating the RAS-Raf-MEK or atypical PKC/β1 integrin signaling pathways, DGKA was found to be related to the invasion of hepatocellular carcinoma and breast cancer cells, respectively.[6,12] By activating the transcription factor c-JUN, DGKA confers cisplatin resistance in ovarian cancer cells through the induction of WEE1 expression.[11] In a recent study, the human erythroid leukemia cell line K562 showed an increase in the DGKA expression, which drives K562 cells over the G1/S checkpoint.[13] Flores et al.[14] reported that DGKA accelerates the G1 to S transition induced by interleukin-2, which in turn promotes the proliferation ability of T cells. According to another study, tumor angiogenesis is aided by DGKA, which stimulates the proliferation and migration of endothelial cells.[15] This mechanism was further illustrated by Cutrupi et al.[16] who determined that hepatocyte growth factor promotes DGKA binding to Src. Since the DGKA expression levels differ greatly between multiple tumor cells and normal cells and they can accelerate the proliferation of T lymphocytes and endothelial cells, we wondered if there is a similar difference in the DGKA expression levels between lung cancer tissues and normal lung tissues and whether such a difference would speed up the proliferation of lung cancer cells.

Janssen introduced ritanserin, an inverse agonist for the 5-HT_2 receptor, in 1987.[17] Ritanserin is used mostly for treating schizophrenia and depression, and improving sleep quality and alcohol addiction. It is one of the first medicines to have been clinically approved for the treatment of schizophrenia as well as used in several clinical trials toward reducing depression and promoting positive emotions in patients with depressive schizophrenia.[17,18] Studies have shown that ritanserin can reduce alcohol intake or the desire to drink of volunteers.[19,20] In addition, for those who have insomnia or mental health problems, ritanserin can improve their slow-wave sleep.[21,22] However, ritanserin has recently emerged as a novel DGKA antagonist. It has been reported that ritanserin can decrease DGKA kinase activity while simultaneously increasing the kinase's affinity for ATP.[23] The fact that ritanserin inhibits the DGKA activity suggests that it may have a wide variety of purposes not yet discovered.

In our research, we found that DGKA is aberrantly elevated in patients with lung cancer and correlates with poor prognosis. The results of this study indicated that small interfering RNAs (siRNAs) targeting the DGKA expression could prevent lung cancer by impairing the production of CCND3. The DGKA inhibitor ritanserin was found to have a significant anti-lung cancer effect. In conclusion, DGKA may be an attractive therapeutic target for lung cancer, and ritanserin is a promising antitumor medicine.

MATERIALS AND METHODS

Cell culture

The two lung cancer cell lines used in this research (i.e., A549 and H1299) were obtained from the American Type Culture Collection and cultivated in the Dulbecco's Modified Eagle Medium/F12 medium or PRIM-1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL penicillin, respectively. A549 and H1299 cells were cultured in a humid incubator of 5% carbon dioxide at 37°C.

Plasmid transfection

The Flag-CCND3 plasmid was purchased from Wzbio. Lipofectamine 2000 reagent (Invitrogen) was used to transfect plasmid into cells. The cells were then transfected with plasmids for 24 h for the subsequent functional experiments.

RNA interference

Lipofectamine RNAiMAX reagent (Invitrogen) was used to transfect the indicated siRNAs (50 nM) to A549 and H1299 cells for 48 to 72 h. The following scramble and targeting DGKA siRNAs sequences were used in the present research:

Scramble, 5′-ACGUGACACGUUCGGAGAATT−3′;
SiDGKA#1, 5′-AUAUGUACCACUUUACUCAUC (dT) (dT)-3′;
SiDGKA#2, 5′-UUGAGCUUGGUAUAGAUUUGG (dT) (dT)-3′.

Establishment of stable DGKA-knockdown cell lines for lung cancer

The shDGKA-plvx-puro was purchased from OLIGOBIO. We used the virus particles to infect A549 and H1299 cells with 10 μg/mL polybrene. Next, we used a puromycin-containing medium to select stably silenced cells, which were confirmed by immunoblotting with an anti-DGKA antibody. The sequences of short hairpin RNAs (shRNAs) against DGKA was as follows:

Scramble: 5-TTCTCCGAACGTGTCACGT-3;
shDGKA#1: 5-CGGCCAGAAGACAAGTTAGAA-3;
shDGKA#2: 5-GCTCTGGAAGTTCCAGTATAT-3.

Western blotting

After digestion and centrifugation, we used the cell lysis buffer (Beyotime, P0013) to lyse A549 and H1299 cell precipitates. Subsequently, the cell lysate was centrifuged in a cryogenic

centrifuge at 14 000 *g* for 25 min. Next, 5× sodium dodecyl sulfate (SDS) buffer was added to the supernatant obtained after centrifugation and the solution was heated in a metal water bath at 100°C for 10 min. Total cell proteins from the previous step were isolated in SDS-polyacrylamide gel electrophoresis gels through an electrophoretic reaction and then transferred to the polyvinylidene fluoride (PVDF) membranes via the transmembrane process. Next, the applicable primary antibodies against DGKA (Proteintech, 11 547-1-AP, 1:6000), CCND3 (Proteintech, 26 755-1-AP, 1:1000), MAD2L1 (Proteintech, 10 337-1-AP, 1:1000), PKMYT1 (Proteintech, 67 806-1-Ig, 1:5000), CCND1 (Proteintech, 60 186-1-Ig, 1:10000), ESPL1 (ABclonal, A15366, 1:1000), E2F2 (Affinity, AF4100, 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Servicebio, GB11002, 1:1000) were cultured overnight with PVDF membranes at 4°C. Finally, the PVDF membranes were cultured with secondary antibodies for 1 h and 30 min under room temperature and then tested through enhanced chemiluminescence.

Real-time quantitative polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Invitrogen). Reverse transcription was performed with the ABScript III RT Master Mix for quantitative polymerase chain reaction (qPCR) (ABclonal, RK20428). Real-time qPCR was performed with the Genious 2X SYBR Green Fast qPCR Mix (ABclonal, RK21, 204). The relative target gene expression quantity was estimated by using the ΔCt method. The primers used in this research were listed in Table 1.

TABLE 1 Primers used in this study.

Name of primer	Sequence (5′ to 3′)
GAPDH -F	AGAAGGCTGGGGCTCATTTG
GAPDH -R	AGGGGCCATCCACAGTCTTC
DGKA -F	CTTGGCAAACAGGGACTGAG
DGKA -R	CTTCCGAGACTTGGCATAGG
CCND3 -F	CCTGGATCGCTACCTGTCTT
CCND3 -R	GTCGGTGTAGATGCACAGTTTT
CCND1 -F	GCGAGGAACAGAAGTGCG
CCND1 -R	TGGAGTTGTCGGTGTAGATGC
E2F2 -F	GGCAACTTTAAGGAGCAGACA
E2F2 -R	CAGAGGGTGGAGGTAGAGGG
PKMYT1 -F	GCTGGGTGACTTCGGACTGC
PKMYT1 -R	CACTTCCAGGATGGTGAGGC
ESPL1 -F	TCTGCTACCACGACTTTACG
ESPL1 -R	GATCCCGCTCGATACCTT
MAD2L1 -F	ATACGGACTCACCTTGCT
MAD2L1 -R	CAGGACCTCACCACTTTC
MCM4 -F	GCTATCCCTCTTGACTTTG
MCM4 -R	GACTGCTCACTTGCCACTA
PLK1 -F	TGCTCAAGCCGCACCAGA
PLK1 -R	CCAACACCACGAACACGA

RNA sequencing

Lipofectamine RNAiMAX (Invitrogen) was used to transfect A549 cells with scramble or DGKA-targeting siRNAs for 48 h. TRIzol Reagent (Invitrogen) was used to extract total RNA from the cell lysates and then sequenced by Shenzhen BGI Gene. The genes that met the conditions of fold change >2 and Q value <0.01 were considered to have been remarkably differentially expressed.

Cell growth and colony formation assays

For the cell growth assay, the cells treated with ritanserin for 48 h or transfected with scramble or DGKA-targeting siRNAs were spread into 6-well plates at the density of 10 000 cells/well. Next, the cells in each well were collected and counted every 2 days. For the colony formation assay, we seeded cells into 6-well plates (density: 1000 cells/well) and followed the same pretreatment as that for the cell growth assay and cultured the cells for 7 to 14 days. Finally, we used a methanol and crystal violet solution to fix and stain the colonies in the 6-well plates, and the colonies with >50 cells were counted under a microscope.

5-Ethynyl-2′-deoxyuridine experiment

The 5-ethynyl-2′-deoxyuridine (EdU) Assay Kit (Beyotime, C0078S) was used to conduct the EdU assay in accordance with the specifications of the kit manufacturer. Briefly, the cells disposed of by ritanserin for 48 h or transfected with scramble and targeting siRNAs were cultured in a 96-well plate and collected the next day. Next, the cells were incubated in a 37°C incubator for ∼1 h and 30 min after the addition of 1000× dilution of the EdU working solution. Next, we fixed the cells with 4% paraformaldehyde for 15 min. Subsequently, the cells were incubated in the permeable solution containing 0.3% Triton X-100 in PBS. The cells were incubated with the Click Additive Solution for 30 min and with the Hoechst 3342 reagent for 10 min, respectively. Finally, the relevant pictures were captured at 20× magnification under a fluorescence microscope.

Cell Counting Kit 8 assay

We used the cell count kit-8 (Beyotime, C0037) to detect the half-maximal inhibitory concentration (IC_{50}) of ritanserin (MedChemExpress, 87 051–43-2). Briefly, the cell suspension was added to a 96-well plate at a density of 4000 cells/well. On the second day, a different concentration gradients

of ritanserin were added to the adherent cells for 48 h. Then, the treated cells were cultured with 80-times diluted cell counting kit 8 reagent in a thermostatic incubator. The absorbance at 450 nm was measured with an enzyme-labeling apparatus every 30 min until the maximal absorbance reached ~1.

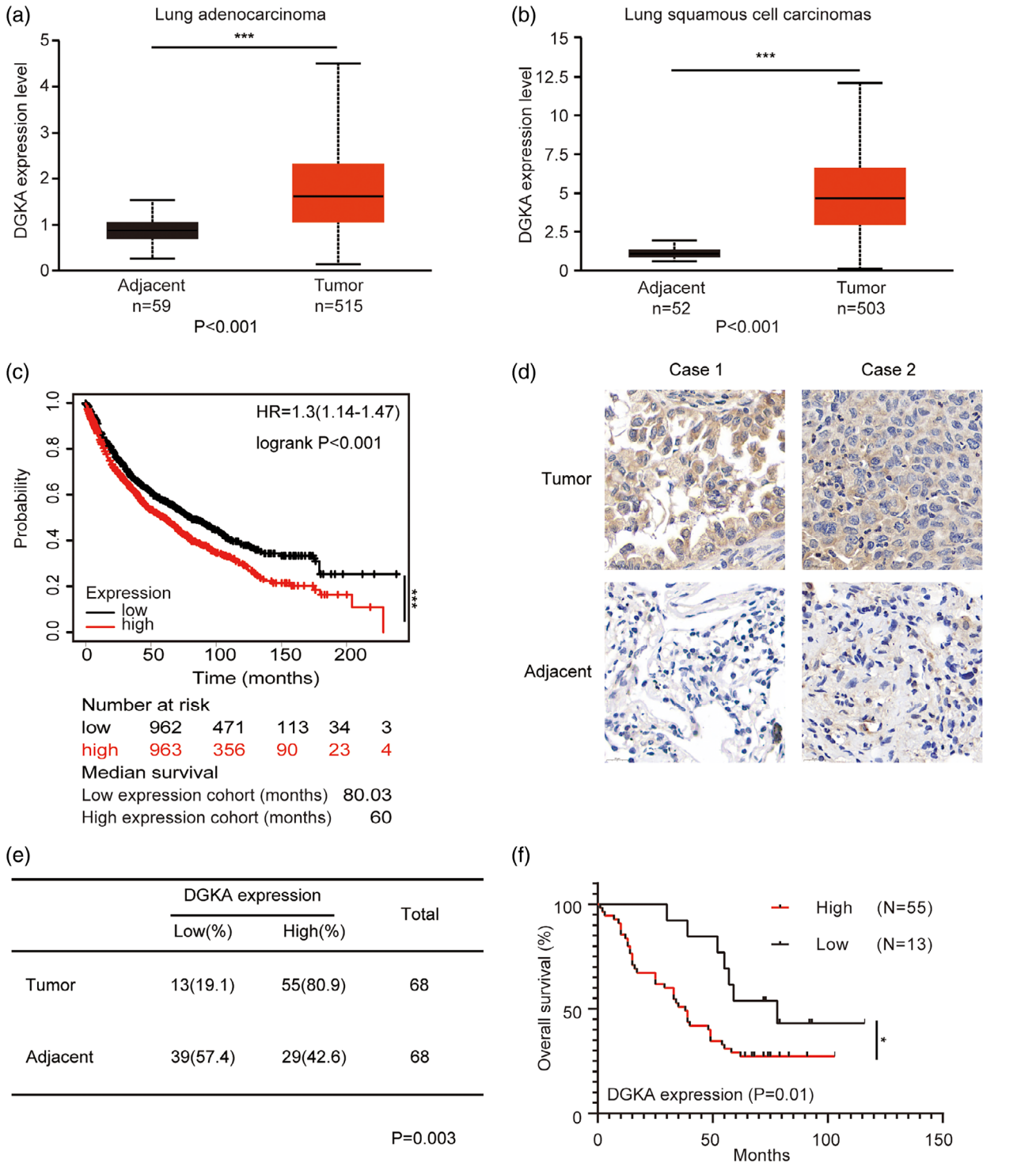

FIGURE 1 DGKA is overexpressed in the lung cancer and predicts a poor prognosis for the patients of lung cancer. (a)–(b) Comparison of the *DGKA* mRNA levels between NSCLC (lung adenocarcinoma and lung squamous cell carcinoma) and adjacent normal tissues acquired from UALCAN database. ***$p < 0.001$. (c) Kaplan–Meier Plotter database shows that high *DGKA* expression is associated with a poor prognosis in NSCLC. (d) Representative IHC staining images for DGKA in lung adenocarcinoma tissues and adjacent normal lung tissues. Scale bar, 20 μm. (e) Statistical analysis of IHC staining to evaluate DGKA expression in a lung adenocarcinoma tissue microarray. (f) High levels of DGKA are associated with unfavorable overall survival in lung cancer patients.

Immunohistochemical staining

We purchased a lung adenocarcinoma tissues microarray composed of lung adenocarcinoma tissues and the paired adjacent normal tissues from Outdo Biotech. Immunohistochemical (IHC) staining was performed as described previously.[24] All -IHC images were scored one-four and zero-three based on the proportion of the area of positive staining cells in the total area of each field and the intensity of positive staining, respectively. The staining score of every IHC image was calculated by multiplying these 2 values.

Animal experiment

A total of 10 5-week-old BALB/c nude mice acquired from the Wuhan Mouse Bailey Biotechnology were randomly allocated to 2 groups and injected with 5×10^6 A549 cells into the right axilla. When the tumor volumes reached approximately 150 mm^3 in nude mice, each nude mouse in the ritanserin-administered and control groups was injected with 500 μL of ritanserin (10 mg ritanserin was dissolved in 2.5 mL of 0.5% sodium carboxymethyl cellulose saline solution) and 500 μL vehicle via gavage once a day, respectively. From the first day of drug administration, the tumor volumes and body weights of each nude mouse were measured blindly every 3 days. The formula for calculating the tumor volumes was as follows: $V = (\text{Length} \times \text{Width}^2/2)$. The excised tumor tissue was stained by IHC staining.

Statistics analysis

The experiments performed in this study were independently repeated three times. Unless otherwise specified, mean ± standard deviation was used in the charts to express the quantitative data in this study. Student's *t*-test was applied to assess whether the discrepancy between the groups was statistically significant. The overall survival time was assessed by using the Kaplan–Meier (K-M) method. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

DGKA is upregulated in NSCLC and associated with adverse outcomes

Using the UALCAN database, we searched for the DGKA expression in lung cancer to learn more about the protein's function in NSCLC. According to the UALCAN data, the RNA levels of *DGKA* are higher in the two most frequent forms of NSCLC tissues when compared to that in the adjacent normal tissues (Figures 1(a),(b)). Furthermore, the Kaplan-Meier Plotter database shows that the median overall survival time for the *DGKA* low expression group is 80.03 months, whereas for the *DGKA* high expression cohort, it is only 60 months (Figure 1(c)). This suggests that high *DGKA* expression correlates with poor prognosis in NSCLC patients. Based on the above database, *DGKA* seems to be closely related to the occurrence of lung cancer. This hypothesis was tested by staining immunohistochemically 68 pairs of carcinoma and adjacent normal tissues in a lung adenocarcinoma tissue microarray. The clinical features of 68 adenocarcinoma patients and the levels of DGKA expression were shown in Table 2. The results of the IHC staining demonstrated a substantial increase in the DGKA content in lung adenocarcinoma when compared to that in paired normal tissues (Figures 1(d),(e)). Additionally, we evaluated how the DGKA protein affects the prognosis of patients with lung adenocarcinoma. Kaplan-Meier survival curve suggested that patients with higher DGKA protein levels may have shorter overall survival time (Figure 1(f)). In summary, it was concluded that patients with NSCLC with a high level of DGKA protein have a poor prognosis.

DGKA silencing reduces the proliferation ability of NSCLC cells

We proposed that increased expression of DGKA in NSCLC is related to the initiation and development of lung cancer. Therefore, using two distinct siRNAs targeting DGKA, we significantly decreased the DGKA protein levels in two NSCLC cell lines (Figure 2(a)). The proliferation ability of lung cancer cells was substantially impaired when endogenous DGKA was silenced (Figures 2(b),(c)). Owing to its similarity to thymidine, EdU can be used to monitor DNA

TABLE 2 The relationship between DGKA expression and clinical parameters of lung cancer patients.

	DGKA immunostaining		
Characteristics	**Low**	**High**	***p* value**
Gender			
Male	9	30	0.3718
Female	4	25	
Age, years			
≤60	9	28	0.3543
>60	4	27	
AJCC stage			
I	8	13	0.0727
II	3	21	
III	2	19	
IV	0	1	
Tumor size			
T1 (≤3 cm)	7	16	0.1407
T2 (3 ∼ 5 cm)	6	24	
T3 (5 ∼ 7 cm)	0	7	
T4 (>7 cm)	0	8	
Lymph node-positive rates			
≤50%	10	44	0.7057
>50%	3	10	

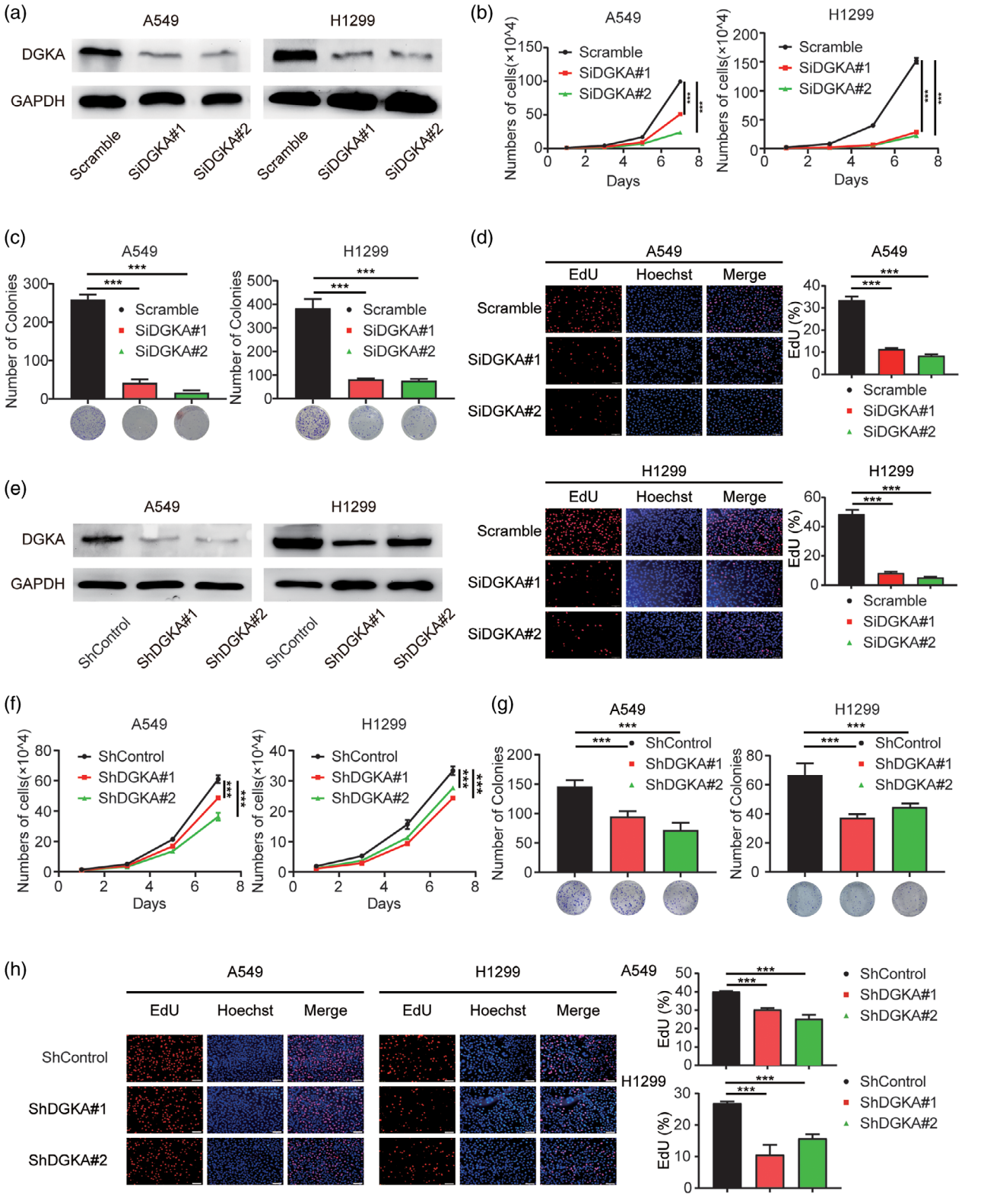

FIGURE 2 Loss of DGKA suppresses the proliferation ability of NSCLC. (a) A549 and H1299 cells were collected and analyzed by western blotting after transfected with the targeting siRNAs for 48 to 72 h. (b) The growth of A549 and H1299 cells were inhibited after DGKA was silenced ($n = 3$). (c) Colony formation ability was prominently decreased in DGKA-depleted cells. ***$p < 0.001$ ($n = 3$). (d) DGKA silencing reduced the proliferation of A549 and H1299 cells according to the results of EdU assay. ***$p < 0.001$ ($n = 3$). Scale bar: 50 μm. (e) Western blotting showed that DGKA was efficiently knocked down in A549 and H1299 cells by shRNAs transfection. (f) The growth of stable DGKA-deplete A549 and H1299 cells was inhibited. ***$p < 0.001$ ($n = 3$). (g) Colony formation ability was reduced in the shDGKA groups compared with the shControl group. ***$p < 0.001$ ($n = 3$). (h) EdU assay showed that stable knockdown of DGKA could inhibit the proliferation of A549 and H1299 cells. ***$p < 0.001$ ($n = 3$). Scale bar: 50 μm.

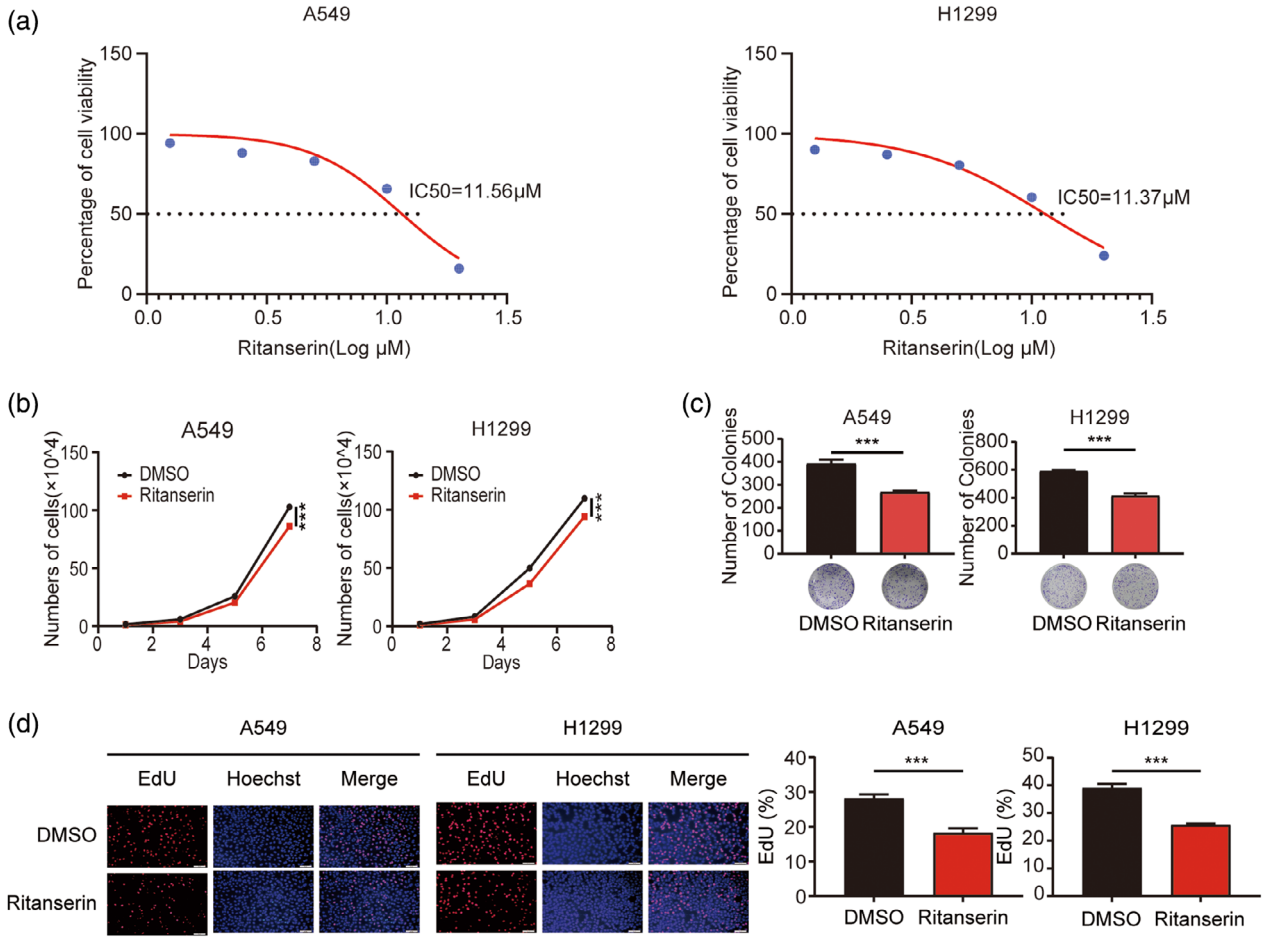

FIGURE 3 The proliferation ability of A549 and H1299 cells could be inhibited by ritanserin. (a) Half-maximum inhibitory concentration of ritanserin in A549 and H1299 cells. ($n = 3$). (b) Ritanserin could inhibit the growth of NSCLC cells. ($n = 3$). (c) The colony formation ability of NSCLC cells was impaired after 48 h of treatment with ritanserin (the concentration of ritanserin was the IC50 of A549 and H1299 cells). ($n = 3$). (d) EdU assay showed that the proliferation ability of A549 and H1299 cells was significantly decreased after treated with ritanserin for 48 h. ***$p < 0.001$ ($n = 3$). Scale bar: 50 μm.

replication by injecting it into the newly synthesized DNA instead of thymidine. The significant reduction in the ratio of EdU-positive cells after DGKA depletion indicated that the proliferation ability of NSCLC cells was severely damaged (Figure 2(d)). To complement these in vitro findings, we established two stable DGKA-knockdown NSCLC cell lines using two different shRNA sequences (Figure 2(e)), on which we performed functional experiments in vitro. The results of functional experiments confirmed that stable DGKA knockdown decreased the proliferation ability of NSCLC cells (Figures 2(f)–(h)).

Ritanserin could dampen the proliferation ability of NSCLC cells in vitro and in vivo

To bolster the clinical translational value of this study, we noticed that ritanserin is a small molecule inhibitor that competitively inhibits the affinity of DGKA for ATP. Our subsequent functional experiments made use of half-maximum inhibitory concentration (IC_{50}) of ritanserin (Figure 3(a)). Our experiments showed that ritanserin inhibited the growth and proliferation ability of NSCLC cell lines (Figures 3(b)–(d)). In addition, ritanserin significantly slowed the growth of xenograft tumors in the ritanserin-administered group when compared with that in the control group (Figures 4(a),(b)). The tumor weights were reduced in the ritanserin-administered group when compared to that in the control group (Figure 4(c)). The expression of Ki-67 was downregulated by ritanserin as revealed by IHC staining (Figure 4(d)). There was no evident difference in the hematoxylin and eosin staining outcomes of the several organs that are essential for life between the ritanserin-administered group and the control group (Figure 4(e)). Notably, ritanserin did not affect the weights of nude mice of the ritanserin-administered group (Figure 4(f)). These results suggested that ritanserin is an effective and safe drug for lung cancer.

DGKA silencing reduces cyclin D3 protein levels in NSCLC cells

Two distinct DGKA-targeting siRNAs were used to transfect A549 cells for RNA sequencing to demonstrate the molecular mechanism of DGKA in lung tumorigenesis. When

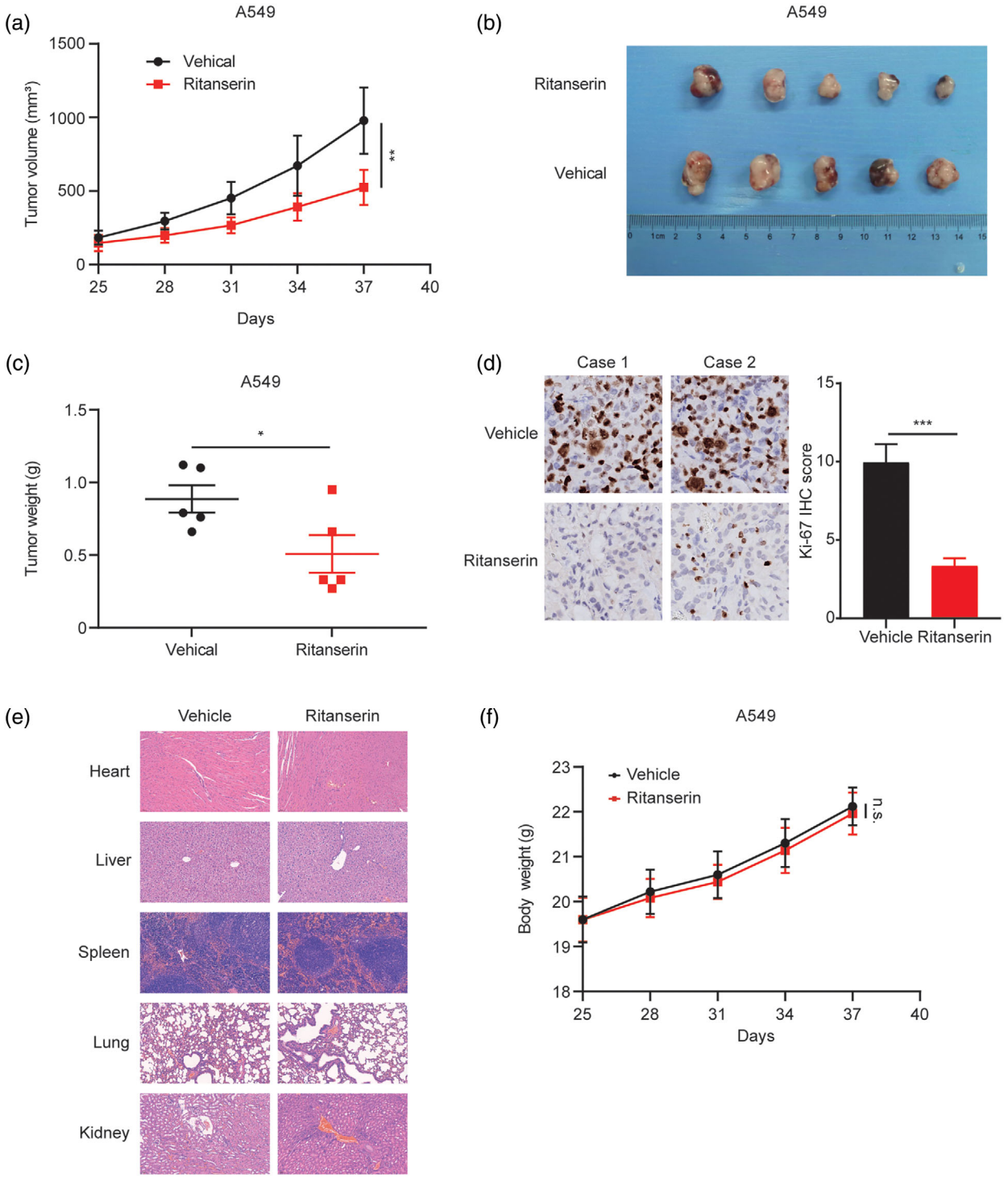

FIGURE 4 The DGKA inhibitor ritanserin inhibits lung tumorigenesis in vivo. (a) Growth curves of xenograft tumors in the ritanserin-administered group and vehicle group. Tumor volumes were measured every 3 days. Data was shown as the mean tumor volume ± standard error of the mean ($n = 5$ mice/group). $^{**}p < 0.01$. (b) Images of xenografts formed from A549 cells with the indicated treatment. (c) Weights of xenograft tumors when the animal experiment was over. $^{*}p < 0.05$ ($n = 5$ mice/group). (d) Representative images of IHC staining for Ki-67 in xenograft tumors in the ritanserin-administered group and vehicle group and statistical histograms of the IHC score. $^{**}p < 0.01$($n = 3$). Scale bar: 10 μm. (e) The hematoxylin and eosin staining images of heart, liver, spleen, lung, and kidney of nude mice in the ritanserin-administered group and vehicle group. Scale bar 50 μm. (f) Body weights of mice in the ritanserin-administered group and vehicle group. n.s. = not significant. ($n = 5$ mice/group).

compared to the scramble group, one siRNA dramatically changed the levels of 2133 genes, and the other siRNA changed the expression of 1140 genes (Figure 5(a)). A total of 739 genes were shared by the two siRNAs, whose expression was significantly altered (Figure 5(b)). We finally analyzed 739 genes through Kyoto Encyclopedia of Genes and

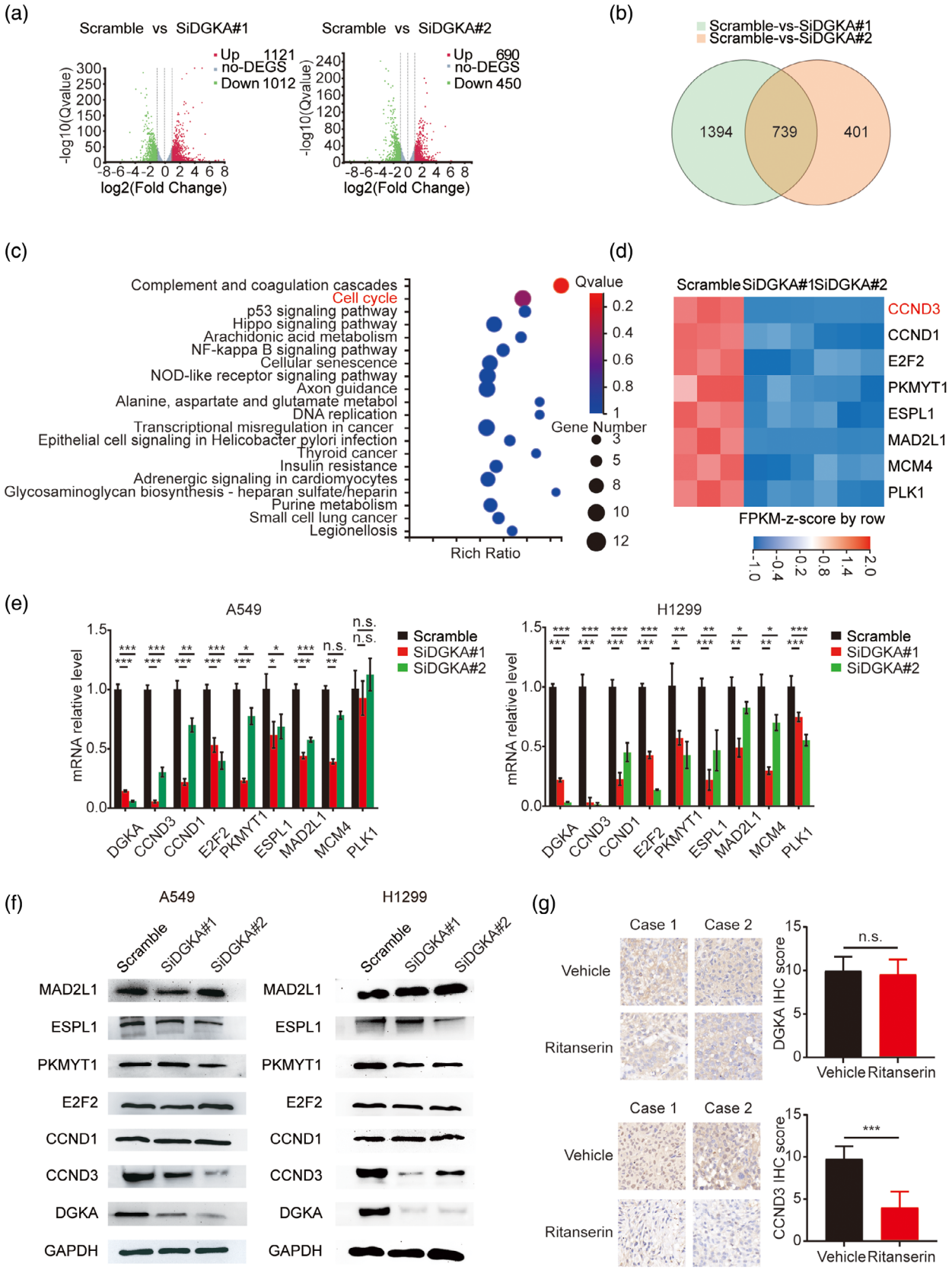

FIGURE 5 Legend on next page.

Genomes (KEGG) pathway enrichment analysis and found that 8 genes associated with the cell cycle pathway were significantly enriched (Figure 5(c)), including the genes *CCND3*, *CCND1*, *E2F2*, *PKMYT1*, *ESPL1*, *MAD2L1*, *MCM4*, and *PLK1* (Figure 5(d)). We used real-time qPCR to detect the mRNA levels of these 8 genes in NSCLC cells transfected with scramble siRNAs and targeted *DGKA* siRNAs to confirm whether this 8 genes were changed. Except for *MCM4* and *PLK1*, the changes in the remaining genes were consistent with the results of RNA sequencing (Figure 5(e)). Next, we performed western blotting to confirm whether the protein level changes in the 6 genes in DGKA knockdown lung carcinoma cells matched the alterations detected by RNA sequencing. We found that RNA sequencing and the protein expression variations of CCND3 were consistent between DGKA-silenced A549 and H1299 cells (Figure 5(f)). Except for CCND3, the results of RNA sequencing were not validated by western blotting for most of the identified genes. The reason may be that there is sometimes a difference between the RNA level and the protein level. On the one hand, the transition from gene to protein involves two stages of transcription and translation, both of which occur at different times and places. On the other hand, the newly synthesized mRNA needs to go through a complex process of post-transcriptional processing and modification, translation, post-translational processing and modification, etc., during which even a little change may have an influence on the levels of proteins. In addition, the results of IHC staining of xenograft tumors showed that the staining intensity of CCND3 in the ritanserin-administered group was observably reduced by ritanserin when compared with that in the control group (Figure 5(g)). According to the aforementioned validation studies, CCND3 may be the downstream target of DGKA in promoting lung cancer.

Overexpression of CCND3 could partly rescue the proliferation ability of NSCLC cells diminished by the loss of DGKA

We conducted rescue studies, such as plate colony formation and EdU assays, to establish whether CCND3 plays a role in DGKA-mediated lung cancer promotion. Within DGKA-depleted A549 and H1299 cells, we overexpressed CCND3 exogenously (Figure 6(a)). Then, we performed plate colony formation and EdU assays and found that the CCND3 protein overexpression could partially restore the proliferation ability of A549 and H1299 cells impaired by the loss of DGKA (Figures 6(b),(c)). These foregoing findings demonstrated that DGKA reduced the proliferation ability of NSCLC cells by regulating CCND3.

DISCUSSION

For the first time, this research identified DGKA as the target for the accelerated risk of lung cancer. We found that DGKA is significantly upregulated in the lung cancer tissues when compared to that in paired adjacent normal lung tissues and that such elevated levels of this protein are associated with a poor survival prognosis of patients. We also determined that DGKA promotes lung cancer in part by promoting the CCND3 expression. Additionally, ritanserin, a small molecule inhibitor of DGKA, has the effect of attenuating the proliferation ability of NSCLC cells.

The diacylglycerol kinases (DGKs), are a family of membrane lipid kinases in which DGKA plays a pivotal role.[25] Although high levels of DGKA expression in T cells were initially reported, subsequent research revealed that a considerable fraction of cancer cells also exhibited this anomalous pattern.[26,27] We were interested in the expression levels of DGKA in lung cancer and whether it stimulated the proliferation of lung cancer cells because of its abnormal expression in tumors and its potential to expedite the proliferation of T cells and endothelial cells. The IHC staining results of lung cancer tissues in this research indicated that the DGKA protein levels in the normal lung tissues were markedly lower than that in the lung cancer tissues. Furthermore, there was a negative correlation between the DGKA expression and patient survival. We also validated that DGKA silencing and the medication targeting DGKA could both reduce the growth and proliferation of NSCLC cells. Recently, Fu et al.[28] demonstrated that increased DGKA expression in the metastatic lesions of patients with non-small cell lung cancer. And DGKA enhances the metastatic ability of H460 and H1975 cells without affecting their proliferation ability. This seems to be contrary to our research results. Maybe it is because that both the cell lines and the sequences of shDGKAs we used were different. In addition, Fu et al. also found that shDGKAs slightly increased the apoptosis of H460 and H1975 cells. This suggests that inhibiting DGKA has an antitumor effect. Thus, from the findings of this research, DGKA's oncoprotein status suggested that it may serve as a latent therapeutic target and prognostic biomarker for NSCLC.

FIGURE 5 DGKA silencing downregulates the expression of CCND3. (a) Volcano plot showing differentially expressed genes in DGKA-depleted vs. scramble siRNA-transfected A549 cells. (b) Venn diagram showing the number of genes in each indicated set. (c) Enrichment of differentially expressed genes in the pathways is illustrated in a Kyoto Encyclopedia of Genes and Genomes enrichment bubble chart. Y-axis represents the pathways, and the X-axis represents rich ratio. (d) Clustering heatmap of eight deregulated genes affected by loss of DGKA. (e) A549 and H1299 cells were transfected with the indicated siRNAs. After 48 h, the cells were harvested, and the levels of indicated mRNAs were measured by real-time qPCR. $^{***}p < 0.001$ ($n = 3$). (f) Knockdown of DGKA led to decreased protein levels of CCND3 in A549 and AH1299 cells. (g) Representative images of IHC staining for DGKA and CCND3 in xenograft tumors in the ritanserin-administered group and vehicle group and statistical histograms of the IHC score. $^{*}p < 0.05$ ($n = 5$). Scale bar: 10 μm.

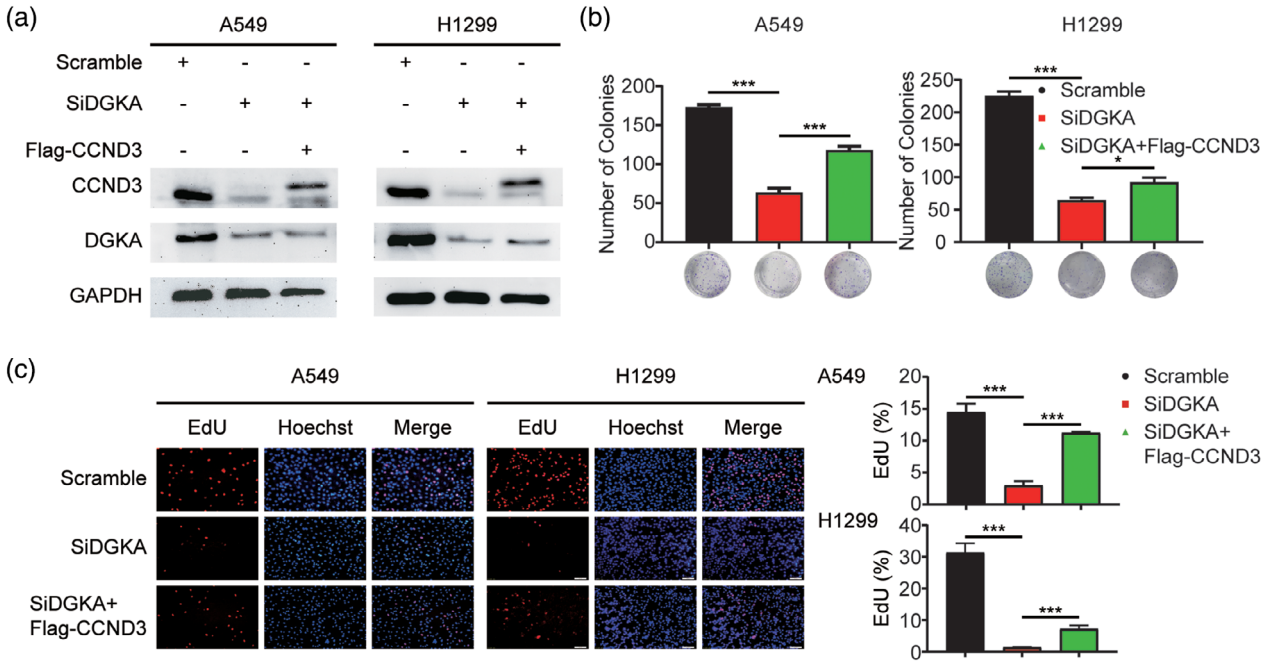

FIGURE 6 The inhibitory effect of DGKA silencing on the proliferation of lung cancer cells is partly dependent on CCND3. (a) A549 and H1299 cells were harvested and analyzed by western blotting after transfected with the siRNAs targeted DGKA and Flag-CCND3. (b) A549 and H1299 cells transfected with the targeting siRNAs and Flag-CCND3 were cultured in a 6-well plate in triplicate and grown for 7 to 14 days. ***$p < 0.001$ ($n = 3$). (c) EdU assay was used to detect the proliferation of A549 and H1299 cells transfected with the indicated siRNAs and Flag-CCND3. ***$p < 0.001$ ($n = 3$). Scale bar: 50 μm.

Further, we conducted RNA sequencing and validation experiments to search for the downstream target of DGKA in accelerating lung cancer. Reducing the CCND3 mRNA and protein levels were another consequence of DGKA silencing in both A549 and H1299 cells. As a result, CCND3 may play a crucial role as a DGKA effector molecule. Rescue assays validated our hypothesis that DGKA needs CCND3 to some extent to regulate the proliferation of lung cancer cells. Since DGKA increases the expression of CCND3, it may contribute to the acceleration of lung cancer development. CCND3, a D-type cyclin, activates CDK4/CDK6 in mammalian cells, facilitating the smooth transition from the G1 to the S phase of the cell cycle.[29,30] Breast carcinoma,[31] bladder carcinoma,[32] leukemia,[33] and melanoma[34] are only some of the tumor types where CCND3 is abnormally expressed in numerous investigations. In breast cancer, lymphoma, and superficial melanoma, high levels of CCND3 were found to be associated with reduced overall survival or progression-free survival and other prognostic parameters.[31,34,35] Multiple microRNAs (miRNAs) (miR-34a,[36] miR-592,[37] and miR-195[38]) have been shown to regulate CCND3 in ways that either promote or inhibit the growth and proliferation of bladder cancer, colorectal carcinoma, and NSCLC cells. These studies provide substantial evidence that CCND3 is critically important for the proliferation of tumor cells.

As an inverse agonist for the 5-HT_2 receptor, ritanserin has been used clinically to treat psychological illness and mental disorders.[17] Ritanserin, however, is also an inhibitor of DGKA because of its structural similarity to lipid kinase inhibitors.[23] Furthermore, ritanserin is promising as a novel small-molecule medication for treating cancer.[39] Ritanserin increases the radiosensitivity of gliomas by blocking nuclear factor-κβ-mediated DNA damage responses, as reported by Inan Olmez et al.[40] Moreover, intracranial xenografts in mice created from a mesenchymal glioma stem cell line showed dramatically increased survival after treatment with ritanserin.[41] After determining the IC_{50} value in A549 and H1299 cell lines, we conducted a series of functional experiments in vitro, which demonstrated that ritanserin inhibits the proliferation ability of NSCLC cells. Analogously, this efficacy of ritanserin was also demonstrated in vivo as well. Importantly, ritanserin did not cause evident toxic effects on the vital organs such as the heart, liver, spleen, lung, and kidney in nude mice. These findings suggested that ritanserin could be an effective drug for patients with lung cancer.

CONCLUSIONS

In conclusion, we demonstrated that increased DGKA in lung cancer tissues aided in tumor growth by inducing the CCND3 expression, which drives cell division. Therefore, patients with lung cancer may consider DGKA as a therapeutic and prognostic target. Excitingly, ritanserin, a DGKA inhibitor, has been exhibited to have an inhibitory effect on the proliferation of lung cancer cells, suggesting that it may be a wonderful latent anticancer drug.

AUTHOR CONTRIBUTIONS

Conception and design: Jian Wang and Gang Wu. *Collection and assembly of data*: Dong Zhou, Tao Liu, and Xinrui Rao. *Data analysis and interpretation*: Dong Zhou, Xiaohua Jie,

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CONFLICT OF INTEREST STATEMENT

There are no potential competing interests to disclose.

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