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Potassium voltage-gated channel subfamily D member 2 induces an aggressive phenotype in lung adenocarcinoma

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The study aimed to investigate the roles of potassium voltage-gated channel subfamily D member 2 (KCND2) in lung adenocarcinoma (AD). RNA sequencing data from The Cancer Genome Atlas (TCGA) database showed that the expression of KCND2 was elevated in lung AD samples compared to the normal samples, and its upregulation was significantly associated with the unfavorable clinic outcome of lung AD patients. Cell proliferation and transwell assays revealed that the growth, migration, and invasion of lung AD cells, which was crucial to cancer aggressiveness, were markedly inhibited after the depletion of KCND2. Importantly, we demonstrated that the depletion of KCND2 suppressed the biological behaviors of lung AD cells via restraining the expression of four tumor-related genes including PCNA, CDH2, SNAI1, and MMP2. Overall, KCND2 promotes the aggressiveness of lung AD and can be considered as a potential predictor of the prognosis of lung AD patients. Downregulation of KCND2 may contribute to the therapy of lung AD.

Key words: lung adenocarcinoma, KCND2, proliferation, migration and invasion

As the leading cause of cancer-related death, lung cancer has led to 26.4% of the total cancer deaths in 2018 in China [1]. Lung cancer is histologically classified into two categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. Lung adenocarcinoma (AD) is the most common type of NSCLCs, which accounts for 40% of lung cancers [3]. Because of easy metastasis at early stage and lack of en early diagnosis, lung adenocarcinoma has an extremely poor prognosis. The 5-year survival rate of lung adenocarcinoma patients is less than 15% [4]. Therefore, it is imperative to discover more novel predictive and diagnostic molecules.

Voltage-gated potassium (Kv) channels modulate the cell membrane potential in excitable cells, particularly in neurons and myocytes [5]. Some evidence implicated that Kv channels regulate cell volume, the cellular processes of proliferation, cell division, and apoptosis, and its expression could be activated in non-excited cells in some specific circumstances like hyperpolarization [6–9]. Recently, lines of evidence have manifested that Kv channels are remodeled in many types of cancers, supporting a potential application of targeting Kv channels in cancer therapies [10]. Kv1.3 [11], Kv9.3 [12], Kv3.1, and Kv3.4 [13] in lung AD cells were involved in cell proliferation. Among Kv channels, Kv channel subfamily D (KCND, Kv4) consisting of Kv4.1, Kv4.2, and Kv4.3, are A-type Kv channels, which features transient inactivation [10]. It is reported that the Kv4 subfamily plays a critical role in tumorigenesis. For example, Kv4.1 siRNA inhibited cell proliferation in gastric and breast cancers [14]. Kv4.2, the most studied member of the Kv4 subfamily encoded by the KCND2 gene, is dysregulated in several tumors, such as gastric cancer [15], neuroblastoma cells [16], and bladder cancer [17]. Upregulation of Kv4.3 mediated by large T antigen-induced cell apoptosis and necrosis [18]. In addition, our present findings detected that KCND2 was dysregulated in lung AD. Based on these investigations, we hypothesize that KCND2 plays important roles in lung AD.

In our study, KCND2 was investigated as a promoter of lung AD malignancy. By bioinformatics analysis, we detected an upregulation of KCND2 in lung AD tissues, which was associated with a dismal prognosis of lung AD patients. By cell proliferation and transwell assays in KCND2-defected lung AD cells, we demonstrated that knockdown of KCND2 inhibited cell growth and mobility. In addition, we observed that KCND2 contributed to cellular behaviors via activating a panel of proliferative and mesenchymal markers.

Materials and methods

Bioinformatics analysis. RNA sequencing (RNA-seq) data of 535 lung AD samples and 59 normal samples were downloaded from The Cancer Genome Atlas (TCGA) database. The correlation of KCND2 and the proliferation-related protein (PCNA) and mesenchymal markers (SNAI1, CDH2, and MMP2) was analyzed by the Gene expression Profiling Interactive Analysis (GEPIA) database.

Cell culture and transfection. The lung AD cell lines Calu-3 and SPC-A-1 were bought from the Chinese Academy of Medical Sciences Cell Bank (Shanghai, China). Normal lung cell line BEAS2B and human bronchial epithelial (HBE) cell line were from the American Type Culture Collection (ATCC). The four cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The lung AD cell lines Calu-3 and SPC-A-1 were transfected with one of the three siRNAs (si-NC: 5'-AATTCTCCGAACGTGTCACGT-3'; si-KCND2 #1: 5'-CGATGAAGAACTGGCCTTCTTCT-3'; si-KCND2#2: 5'-ACCTGTTATACAGAGTAATATCT-3'), among which, si-KCND2 #1 and si-KCND2#2 were KCND2 siRNAs, and si-NC was used as a control. The transfection was mediated by Lipofectamine 2000 (Thermo Scientific, USA) according to the manufacturer's protocol. After 24 h of transfection, the RNA expression level was determined.

Real-time quantitative PCR (RT-qPCR). TRIzol reagent (Invitrogen, Thermo Scientific, USA) was used to extract total RNA, which was subsequently reversely transcribed into cDNAs using a PrimeScript reagent Kit with gDNA Eraser (Takara). StepOnePlus Real-Time PCR System (Life Technologies, USA) with SYBR Green kit (Thermo Fisher, MA, USA) was used to determine the levels of cDNAs. Gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were repeated for each sample.

Cell proliferation determination and colony formation assays. Cells (1000 cell/well) were cultured in 96-well plates for 0, 24, 48, and 72 h. Cell proliferation was determined using 10 µl Cell CCK-8 reagent (Beyotime Biotechnology, Shanghai, China), and the absorbance at 450 nm was measured with a microplate reader. For colony formation assay, the cells were implanted in a 60 mm dish (400 cells/dish) and cultured for 1–2 weeks at 37 °C. The colonies were fixed with 4% paraformaldehyde for 30 min and stained by 0.1% crystal violet for 20 min. The numbers of the colonies were counted under a microscope.

Cell migration and invasion assays. The 24-transwell chambers were used to determine cell mobility. For migration assay, about 100 µl cell suspension (1.0×10^5 cells) were seeded into the transwell insert and 500 µl complete medium were added to the lower chamber. After incubation overnight at 37 °C, the migrated cells were fixed with 4% paraformaldehyde for 30 min and stained by 0.1% crystal violet for 20 min. The migrated cells were counted using a microscope. For invasion assays, the upper layer of the chamber was pre-coated with Matrigel.

Western blots. RIPA lysis buffer was used to extract total proteins from lung AD cell lines. The 20 µg proteins were loaded onto 10% SDS-PAGE for separation and transferred onto a 0.45 µm polyvinylidene fluoride (PVDF) membrane. The 5% skim milk in TBST was used to block the membrane. Then the membrane was incubated with appropriate primary antibodies overnight at 4 °C. Primary antibodies against Kv4.2 (1:1000, #74748), GAPDH (1:1000, #5174), PCNA (1:1000, #13110), N-cadherin (1:1000, #13116), Snail (1:1000, #3895), and MMP2 (1:1000, #40994) were obtained from Cell Signaling Technology (MA, USA). Then the membrane was washed with TBST three times and incubated with secondary antibodies. Finally, the membrane was exposed to an ImageQuant LAS 4000 system (GE Healthcare) with ECL (Pierce, Thermo Scientific, USA) as a chemiluminescent reagent.

Statistical analysis. SPSS 22.0 (IBM, USA) was utilized for statistical analysis. The correlation between KCND2 and overall survival of lung AD patients was evaluated by the survival curves plotted by the Kaplan-Meier method. The log-rank test was used for comparison of the difference of the two survival curves. For comparison of two groups, we utilized a Student's t-test, whereas one-way analysis of variance (ANOVA) was for three or more groups followed by Bonferroni's post hoc test. The difference between two or more groups was significant when the p-value was <0.05.

Results

KCND2 is upregulated in lung adenocarcinoma and is associated with poor outcome of lung AD patients. Using the lung AD RNA-seq data from TCGA, we examined the KCND2 expression profile between lung AD (n=535) and normal lung tissues (n=59). Figure 1A showed that KCND2 was significantly upregulated in lung AD samples ($p<0.0001$). Similarly, the upregulation of KCND2 was identified in lung AD cell lines (Calu-3 and SPC-A-1) compared to normal lung cell lines BEAS2B and HBE (Figure 1B, $p<0.01$). To uncover the roles of KCND2 in lung AD clinical features, 472 lung AD samples with complete clinical information were selected and divided into two groups according to the median of KCND2 expression. By the chi-square test, we detected that the high expression of KCND2 was closely associated with gender ($p=0.034$) and node metastasis stage (Table 1, $p=0.001$). Subsequently, the effects of KCND2 expression on the outcome of 472 lung AD patients were assessed via the survival curves (Figure 1C). The results revealed that the overall survival of the KCND2 high expression group was shorter than that of the KCND2 low expression group, suggesting a negative effect of KCND2 expression on the prognosis of lung AD.

The knockdown of KCND2 inhibited the proliferation of lung AD cells. To investigate the function of KCND2 in

Table 1. 472 lung AD samples divided into two groups according to the median of KCND2 expression.

Characteristics	Expression of KCND2		p-value
	Low	High	
Age			0.353
<60	60	69	
≥60	176	167	
Gender			0.034*
female	139	116	
male	97	120	
Pathologic-Stage			0.069
I+II	195	179	
III+IV	41	57	
Pathologic-T			0.337
T1+T2	202	209	
T3+T4	34	27	
Pathologic-N			0.001*
N0	174	140	
N1+N2	62	96	
Pathologic-M			0.118
M0	222	229	
M1	14	7	

*p<0.05; Abbreviations: Lung AD: lung adenocarcinoma; Pathologic-T: Pathologic tumor status; Pathologic-N: Pathologic node metastasis; Pathologic-M: Pathologic distant metastasis

cellular biology behaviors, KCND2 siRNA (si-KCND2-1 or si-KCND2-2) or the control siRNA was transfected into the lung AD cell lines (Calu-3 and SPC-A-1). As shown in Figures 2A–2D, si-KCND2-1 and-2 significantly decreased the mRNA and protein expression levels of KCND2 in Calu-3 and SPC-A-1 cells, and the si-KCND-2 stood out as the siRNA with better knockdown efficiency ($p<0.01$). Thus, the si-KCND2-2 transfected cells were used for the following experiments. CCK-8 and colony formation assays were conducted to investigate the roles of KCND2 in lung AD cell growth. After transfection for 48 h and 72 h, the OD values were significantly lower in the KCND2 siRNA transfected cells compared to the si-NC group, and their gap became wider in a time-dependent way (Figures 3A, 3B, $p<0.01$). Consistently, the colony numbers were less in the si-KCND2 group than that in the si-NC group (Figures 3C, 3D). The results indicated that silencing KCND2 could suppress the proliferation of lung AD cells.

The knockdown of KCND2 restrained the migration and invasion of lung AD cells. According to the inhibitory effect of KCND2 on lung AD cell growth, the function of KCND2 in cell mobility was also measured by the Transwell assays. The migratory and invasive numbers of the Calu-3 cells transfected with KCND2 siRNA were notably decreased compared to the si-NC group (Figures 4A, 4B, $p<0.01$). Similarly, the reduction of the migratory and invasive numbers was also detected in the SPC-A-1 cells after the KCND2 knockdown (Figures 4C, 4D, $p<0.01$). The results suggested that KCND2 impaired the mobility of lung AD cells.

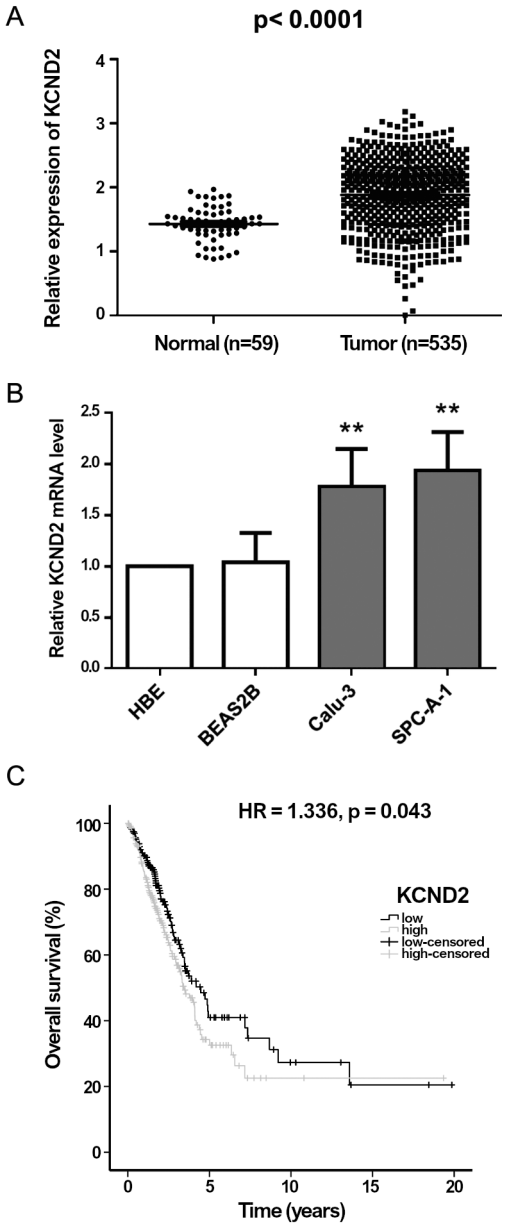

Figure 1. KCND2 is upregulated in lung AD and is associated with poor outcomes of lung AD patients. A) The mRNA expression data of KCND2 obtained from TCGA were much higher in lung AD tissues than in normal tissues, p<0.0001. B) The mRNA expression levels of KCND2 in Calu-3 and SPC-A-1 cells were significantly higher than in normal cell lines (n=3). Data were presented as mean ± SD, **p<0.01 vs. control groups. C) High expression of KCND2 is associated with unfavorable survival outcomes of lung AD patients, hazard ratio (HR)=1.336, p=0.043.

The knockdown of KCND2 inhibited the expression of several tumor-associated genes. It is known that proliferation-related protein (PCNA) is a crucial regulator of cell growth and SNAI1, CDH2, and MMP2 play critical roles in the process of epithelial to mesenchymal transition (EMT) and metastasis [19, 20]. To uncover the mechanisms of how

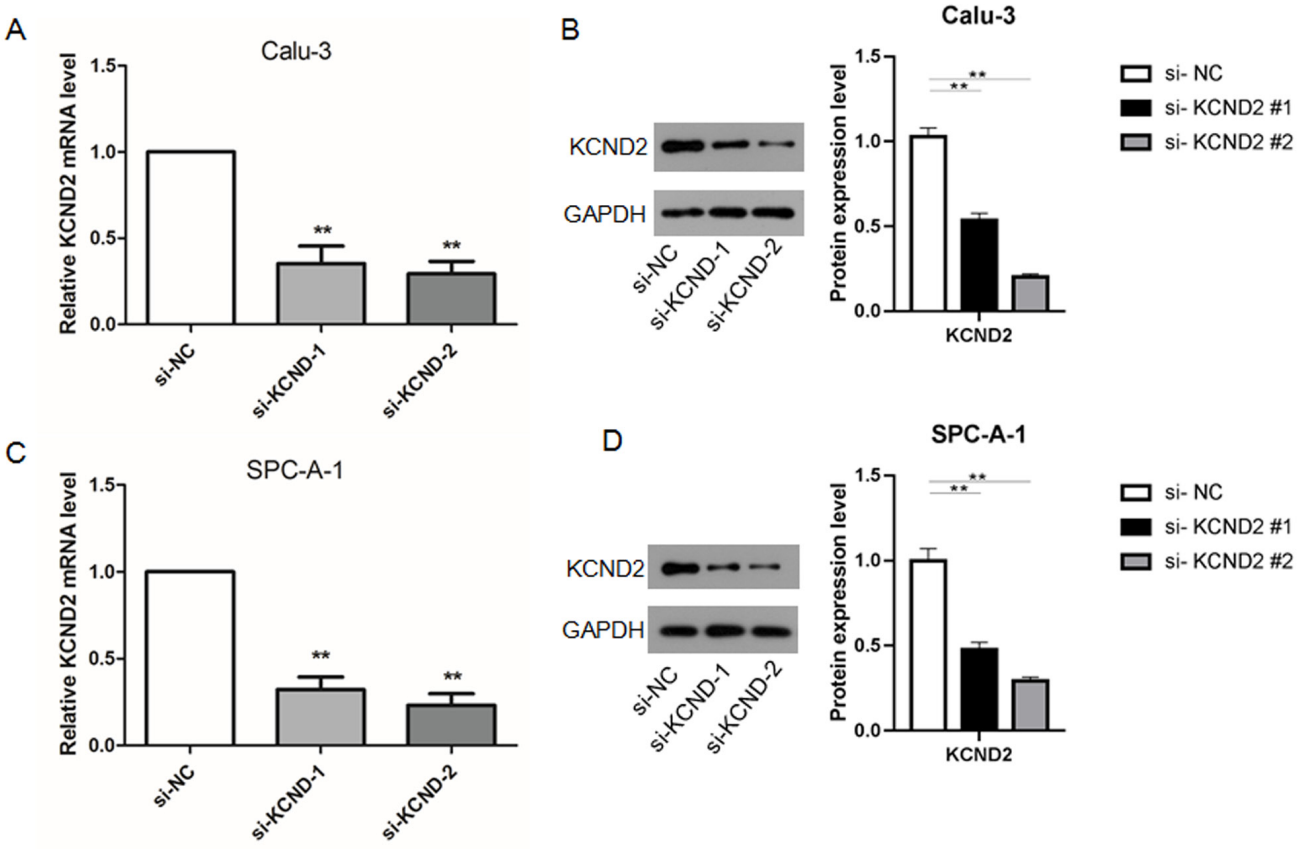

Figure 2. Lung AD cells (Calu-3 and SPC-A-1) were transfected with si-KCND2-1, si-KCND2-2, or si-NC. A, C) KCND2 mRNA expression was determined by RT-qPCR in Calu-3 (A) and SPC-A-1 (C) transfected with si-KCND2-1, si-KCND2-2, or si-NC. Protein levels of KCND2 were determined by western blot in Calu-3 (B) and SPC-A-1 (D) transfected with si-KCND2-1, si-KCND2-2, or si-NC. The number of independent experiments = 3, data are presented as mean ± SD. **p<0.01 vs. control groups

KCND2 regulated the biological behaviors of lung AD cells, we analyzed the correlation between KCND2 expression and the expression of the four tumor-associated genes in Calu-3 and SPC-A-1 cells transfected with KCND2 siRNA. The scatter plots showed that KCND2 was positively correlated with the PCNA (R=0.29, p=7.2e–11), CDH2 (R=0.25, p=2e–08), SNAI1 (R=0.14, p=0.0018), and MMP2 (R=0.32, p=3.6e–13) (Figure 5A). Subsequently, the correlations were confirmed by the western blotting results. The protein expression levels of PCNA, CDH2, SNAI1, and MMP2 in Calu-3 and SPC-A-1 cells were significantly declined with the depletion of KCND compared to the si-NC groups (Figures 5B, 5C, p<0.01). Therefore, KCND may contribute to the biological behaviors of lung AD cells through targeting PCNA, SNAI1, CDH2, or MMP2.

Discussion

In this study, we illustrated that KCND2 was significantly upregulated in lung AD samples and closely related to a dismal prognosis of lung AD patients. In line with our results, several Kv channels were reported to be highly expressed in many cancers. For example, the Kv10.1 expression level was increased in brain metastases and correlated with a poor prognosis of patients [21]. Importantly, high KCND2 expression was reported to be associated with decreased overall survival of gastric cancer [15]. In addition, we also detected that there was a significant correlation between KCND2 high expression and gender and node metastasis. Moreover, the presence of metastasis is closely related to the therapy and prognosis of patients. Consequently, the KCND2 expression may be useful in the treatment and prediction of stages and survival outcomes of lung AD patients.

Further, the increased KCND2 expression suggested that KCND2 might have a role as a promoter in lung AD. The notion was confirmed with the subsequent loss of function of KCND2. Cell growth and mobility were significantly inhibited in the KCND2-defected cells. To date, no study has reported the function of KCND2 in lung AD, whereas one study reported its role in gastric cancer [15]. However, there are multiple studies about the roles of its family members in several cancers [10, 15, 22]. A recent review summarized

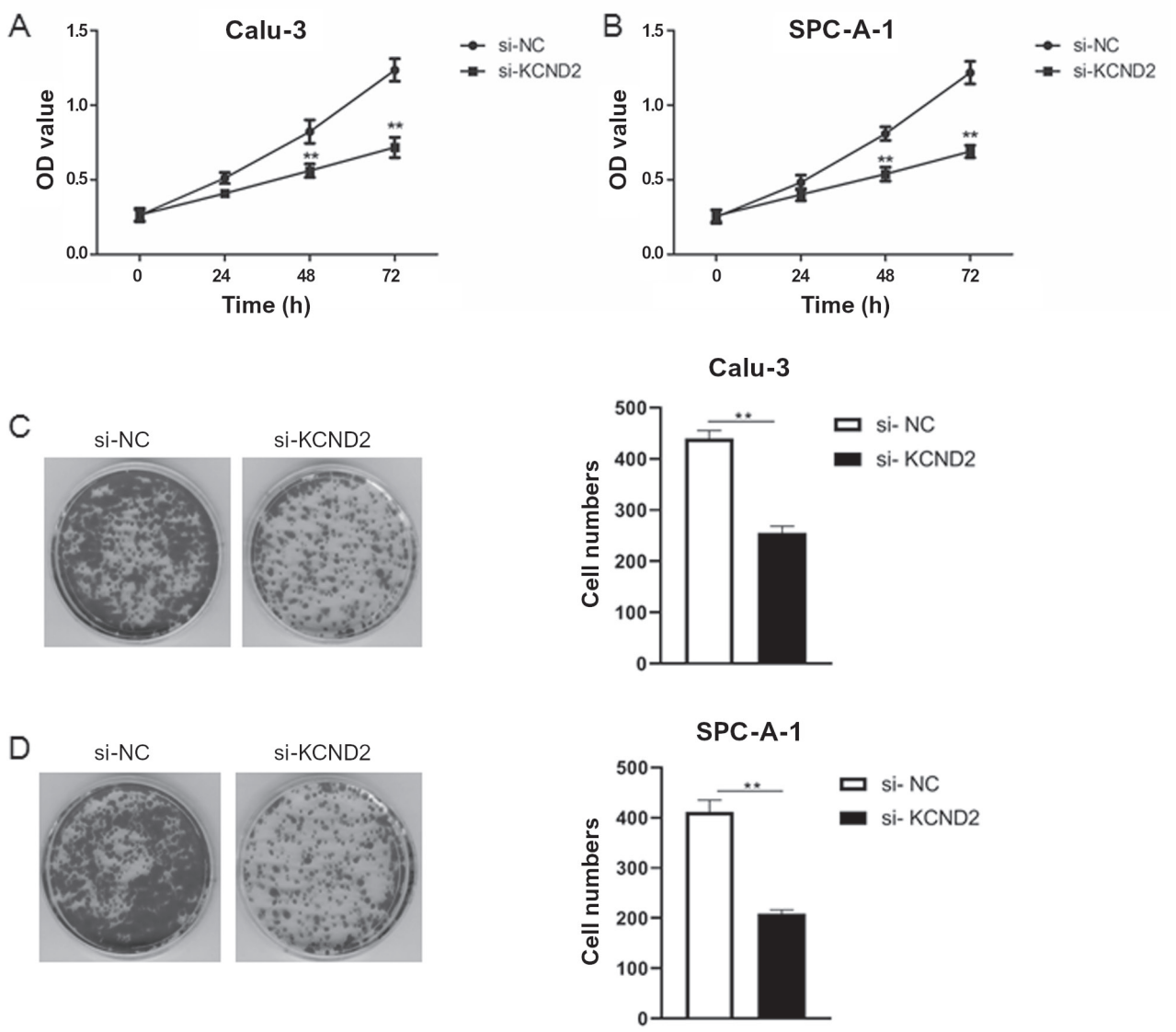

Figure 3. The knockdown of KCND2 inhibited the proliferation of lung AD cells. A, B) The proliferation of Calu-3 (A) and SPC-A-1 (B) transfected with si-KCND2 was measured by CCK-8 assay. C, D) the colony formation of Calu-3 (C) and SPC-A-1 (D) transfected with si-KCND2 was markedly decreased. The number of independent experiments = 3, data are presented as mean ± SD. **p<0.01 vs. control groups

that Kv channels including Kv1.3 [23], Kv1.5 [24, 25], Kv1.1 [22, 26], Kv2.1 [27], Kv7.1 [28], Kv10.1 [21, 29], Kv3.4 [30], Kv4.1 [14, 31], Kv9.3 [12], and Kv3.1 [13] were reported to be aberrantly expressed and regulated cancer cell proliferation. Among them, Kv 3.4 and Kv9.3 promote migration and invasion of lung AD cells [12, 13]. All the existing reports laid a foundation for our results that KCND2 contributed to lung AD cell viability and mobility, which is essential to the cancer metastasis process.

To investigate the molecular mechanisms of how KCND2 regulates biological behaviors of lung AD cells, the protein expression of four tumor-related genes including PCNA, SNAI1, CDH2, and MMP2 were determined. The results revealed that the expressions of these four molecules were positively correlated with KCND2, and they were decreased after the depletion of KCND2 in lung AD cells. Proliferating cell nuclear antigen (PCNA) is critical to cell growth. Wang et al. found that PCNA promoted cell proliferation and hindered apoptosis of NSCLC cells [19]. Besides, PCNA is highly expressed and considered as a cell proliferation-related marker in many cancers [32–35]. Therefore, KCND2 facilitated the lung AD cell viability possibly via targeting PCNA. Besides, the most important function of PCNA is regulating DNA replication and cell cycle, and there are

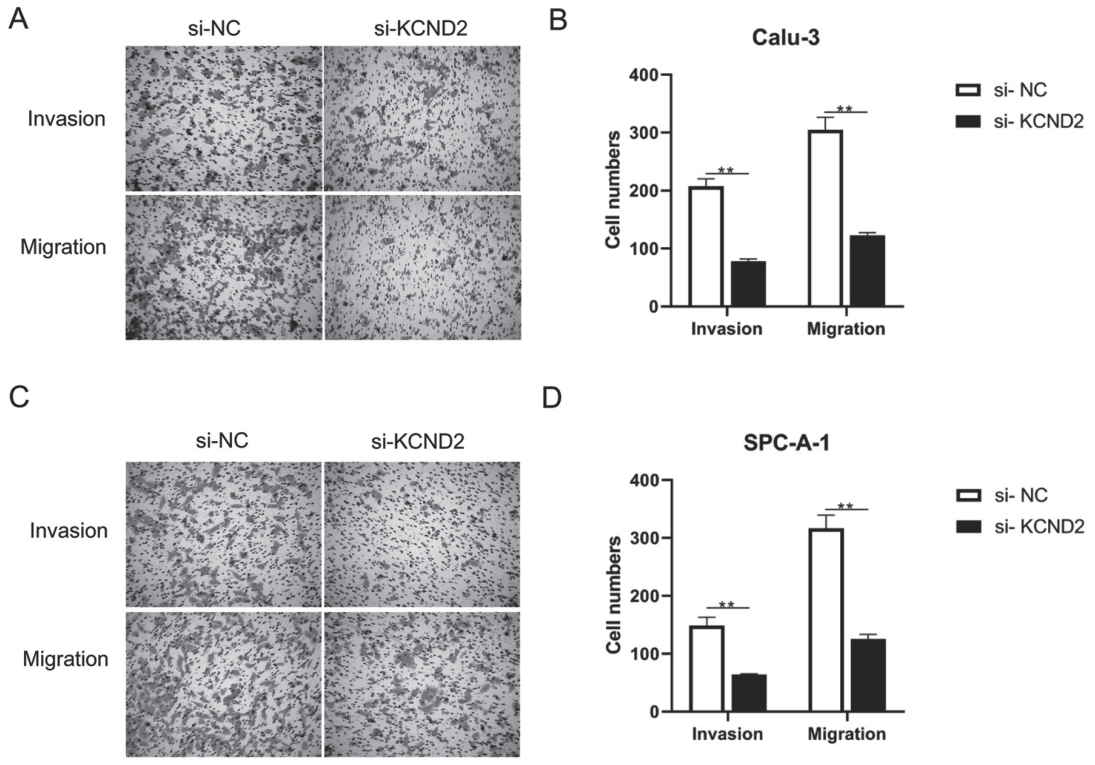

Figure 4. The knockdown of KCND2 restrained the migration and invasion of lung AD cells. A, C) Migration and invasion ability of Calu-3 (A) and SPC-A-1 (C) cells transfected with si-KCND2 were identified by the Transwell assay, magnification ×200. B, D) The number of migrating and invading Calu-3 (B) and SPC-A-1 (D) cells transfected with si-KCND2 was markedly declined. The number of independent experiments = 3, data are presented as mean ± SD. **p<0.01 vs. control groups

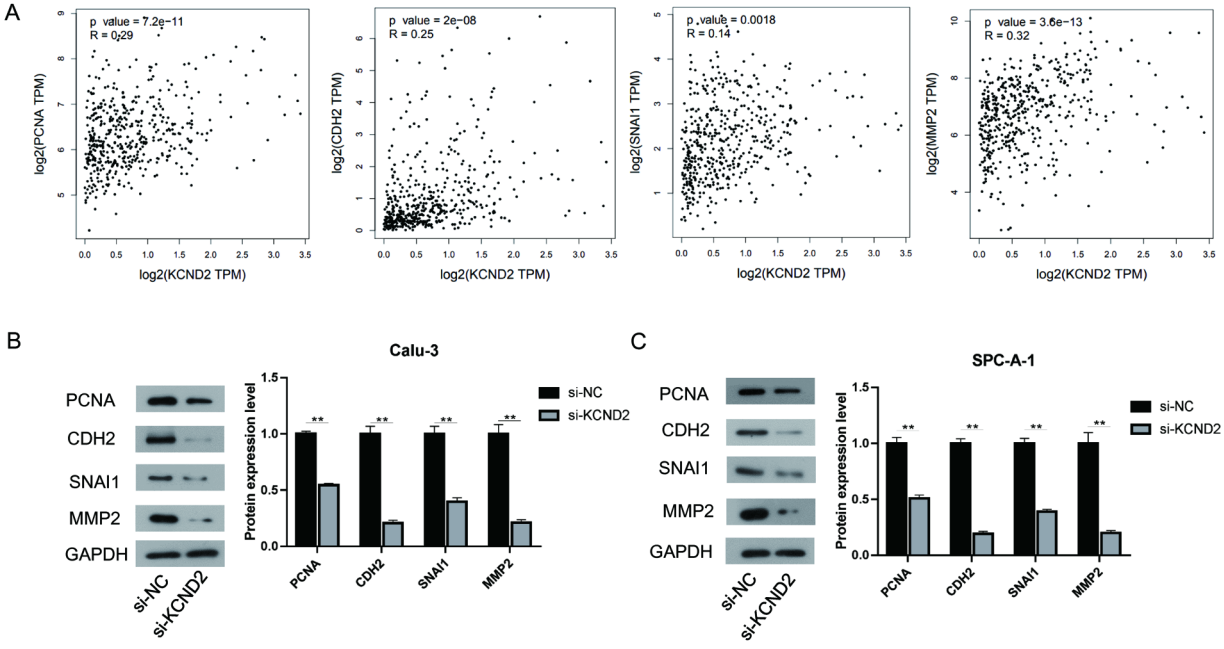

Figure 5. The knockdown of KCND2 inhibited the expression of four tumor-associated genes. A) The correlations between KCND2 and four tumor-associated genes including PCNA, CDH2, SNAI1, and MMP2 were assessed by the Gene expression Profiling Interactive Analysis (GEPIA) database. B) The protein levels of the four proteins were declined in Calu-3 cells transfected with si-KCND2 compared to the si-NC group. C) The protein levels of the four proteins were declined in SPC-A-1 cells transfected with si-KCND2 compared to the si-NC group. The number of independent experiments = 3, data were presented as mean ± SD. **p<0.01 vs. control groups

many reports that Kv channels suppressed cell proliferation probably through impairing the cell cycle process [36–38]. Reduced expression of different Kv channels including Kv1.3, Kv9.3, Kv10.1, and Kv4.1 arrest cancer cells at different transitions [12, 14, 31, 39]. Therefore, KCND2 probably interrupts certain phases of cell cycle progression via PCNA, which awaits to be investigated.

Additionally, SNAI1, a member of the zinc-finger transcription factor family, and matrix metalloproteases (MMPs) are critical regulators of EMT, which is involved in the invasion and metastasis of cancers [40]. A well-known function of SNAI1 is inducing the EMT process, in which tight junction proteins including occludin and claudins are decreased and thus epithelial cells are converted into moveable mesenchymal cells [41]. In this way, SNAI1 plays an important role in metastasis and spread of cancers [41]. For example, SNAI1 was reported to support the invasion of lung carcinoma by directly targeting claudin and inducing EMT [40]. Matrix metalloprotease-2 gene (MMP2), a member of MMPs is able to hydrolyze proteins of extracellular matrix (ECM), which entitles cells to migration and invasion [42]. Its expression and activity are regulated in a complex manner. As a novel target of nuclear EGFR-mediated transcriptional activation, MMP2 expression is decreased by the recruitment of another nuclear EGFR target – PML (promyelocytic leukemia protein), leading to decreased cell invasion and lung cancer metastasis [43]. MMP2/MMP9 signaling was diminished by NMI (N-myc and STAT interactor), a potential tumor suppressor in lung cancer [44]. On the other side, Minn et al. demonstrated that MMP2 was involved in lung metastasis of breast cancer, specifically enriched at the metastatic site [45]. CDH2, a type of calcium-dependent cell adhesion protein, plays important roles in mediating cell-cell adhesion, which is crucial in EMT [46]. The upregulation of CDH2 empowers the mobility of tumor cells, which is closely related to the aggressiveness of the tumor [47]. CDH2 was proved to significantly promote cell mobility by inducing EMT in lung cancer [48]. Overall, the reduction of SNAI1, MMP2, and CDH2 levels were associated with reduced cell mobility, suggesting that KCND2 impaired lung AD cell mobility via targeting the three EMT-related genes.

In conclusion, KCND2 was highly expressed in lung AD tissues and cancer cells and was associated with a dismal prognosis of lung AD patients. The downregulation of KCND2 markedly repressed the growth and mobility of lung cancer cells, suggesting that KCND2 could be considered as a tumor promoter for lung AD. Furthermore, four tumor-related genes including PCNA, SNAI1, MMP2, and CDH2 were downregulated in KCND2-defect lung AD cells, which implicated that KCND2 supported the aggressiveness of lung AD probably via regulating the four proteins. Overall, KCND2 played a favorable role in tumorigenesis of lung AD and could be considered as a therapeutic target and predictor of clinical outcome of lung AD patients.

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