


# KRT6A Promotes EMT and Cancer Stem Cell Transformation in Lung Adenocarcinoma

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## Abstract

**Aim:** Keratin 6A is a type II cytokeratin which is important in forming nail bed, filiform papillae, the epithelial lining of oral mucosa, and esophagus; recently, keratin 6A was found hyperexpressed in different types of cancer. But, the biological function of keratin 6A in lung adenocarcinoma still remains unclear. Therefore, in current study, we investigated the biological role of keratin 6A in lung adenocarcinoma. **Methods:** By utilizing The Cancer Genome Atlas database, we investigated the expression profile of keratin 6A and its relationship with other clinical parameters in lung adenocarcinoma. The biological function of keratin 6A in lung adenocarcinoma was also investigated by using A549 and PC-9 lung cancer cell lines *in vitro*. **Results:** Our data indicate that, compared with normal lung tissue samples, keratin 6A was hyperexpressed in lung adenocarcinoma. Moreover, keratin 6A hyperexpression was positively correlated with lymph node positive and aggressive tumor T stage. Keratin 6A knockdown inhibited the cell proliferation, migration, and colony formation ability but not cell death in lung adenocarcinoma cells. In addition, we found keratin 6A exerted its phenotype via promoting cancer stem cells (CXCR4<sup>high</sup>/CD133<sup>high</sup>) transformation and epithelial–mesenchymal transition. **Conclusion:** In conclusion, current study suggests that hyperexpressed keratin 6A in lung adenocarcinoma promotes lung cancer proliferation and metastasis via epithelial–mesenchymal transition and cancer stem cells transformation.

## Keywords

cancer stem cells, EMT, KRT6A, lung adenocarcinoma, metastasis

## Abbreviations

CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; FACS, flow cytometry; FBS, fetal bovine serum; GSEA, gene set enrichment analysis; KRT6A, keratin 6A; LUAD, lung adenocarcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; TBST, Tris-buffered saline and Tween 20; TCGA, The Cancer Genome Atlas.

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## Introduction

Lung cancer is the leading cause of cancer-related deaths in men and one of the most common forms of cancer in women, based on data from World Health Organization lung cancer killed more than 1 million people annually worldwide (<https://www.who.int/news-room/fact-sheets/detail/cancer>). Among the various histological subtypes, lung adenocarcinoma (LUAD) is the most prevalent subtype, which accounts the most in all cases diagnosed with lung cancer.<sup>1</sup> The 5-year survival rate of lung cancer is still less than 15% in most of the countries,<sup>2</sup> compared to the effect of the primary tumor itself, the devastating spread of cancer cells in metastases contributes the most to the lung cancer–related death,<sup>3</sup> and the process of epithelial–mesenchymal transition (EMT) is highly related to tumor metastasis. Cancer stem cells (CSCs) are a

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group of cells that are capable of self-renewal, initiate tumor generation, and undergo differentiation heterogeneously.<sup>4,5</sup> Epithelial–mesenchymal transition could also induce tumor cells to develop stem cell characteristics; during EMT these CSCs could invade surrounding tissues and even forming metastasis in distant tissues.

The keratin 6A (KRT6A) gene, located at chromosome 12q13.13, which encodes the cytokeratin 6A protein. Keratin 6A is a family member of type II keratin proteins, biologically KRT6A could lead to epidermalization of squamous epithelium,<sup>6,7</sup> and KRT6A plays an important role in EMT in nasopharyngeal carcinoma,<sup>6</sup> KRT6A mutation plays an important role in pachyonychia congenita and could serve as a diagnosis marker.<sup>7-9</sup>

In present study, the *in vitro* KRT6A silencing model was constructed to investigate the biological role of KRT6A. Our data indicate that KRT6A knockdown could significantly undermine the cancer cells proliferation and migration; moreover, KRT6A was involved with CSC transformation and EMT in LUAD. Taken from the perspective of tumor metastasis, our research helps further to understand the formation of LUAD, aiming to improve the survival for LUAD patients.

## Methods

### Data Sources

Briefly, the level 3 data from The Cancer Genome Atlas (TCGA) LUAD were downloaded.<sup>10</sup> This data set (TCGA\_LUAD) contains the clinical parameters including, tumor-node-metastasis stage, age, grading, pathology type, and so on, from 515 LUAD patients, consisted of 59 adjacent normal lung tissues and 515 cancer tissues. For gene set enrichment analysis (GSEA), the gene expression profiles from the top 10% samples (25 tumor samples) exhibiting the highest levels of KRT6A expression, together with the bottom 10% samples (25 tumor samples), with the lowest levels of KRT6A expression were submitted for GSEA following the official instructions.<sup>11,12</sup>

### Cell Lines, Cell Culture, si-RNA, Plasmids, and Transfection

The lung cancer cell line, A549 was obtained from the ATCC (American Type Culture Collection) and PC-9 was obtained from NanJing KeyGen Biotech Co, Ltd. A549 and PC-9 cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Inc, Waltham, MA; cat. no. 12491-015) supplemented with 10% fetal bovine serum (FBS; cat. no. 10500064; Gibco; Thermo Fisher Scientific, Inc) and penicillin/streptomycin cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Transfection was performed using Lipofectamine 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc) following the manufacturer's protocol. A nontargeting small interfering RNA (siRNA) 5'-CCTAAGGTTAAGTCGCC CTCGCTC-3' was used as a negative control, for KRT6A siRNA knockdown experiments the si-RNA targeting sequence, 5'-CCAGCAGGAAGAGCUAUA-3' was

introduced.<sup>6</sup> Transfection efficiency was evaluated by using quantitative reverse transcription PCR (qRT-PCR) and Western blot 48 hours after transfection.

### Flow Cytometry Analysis

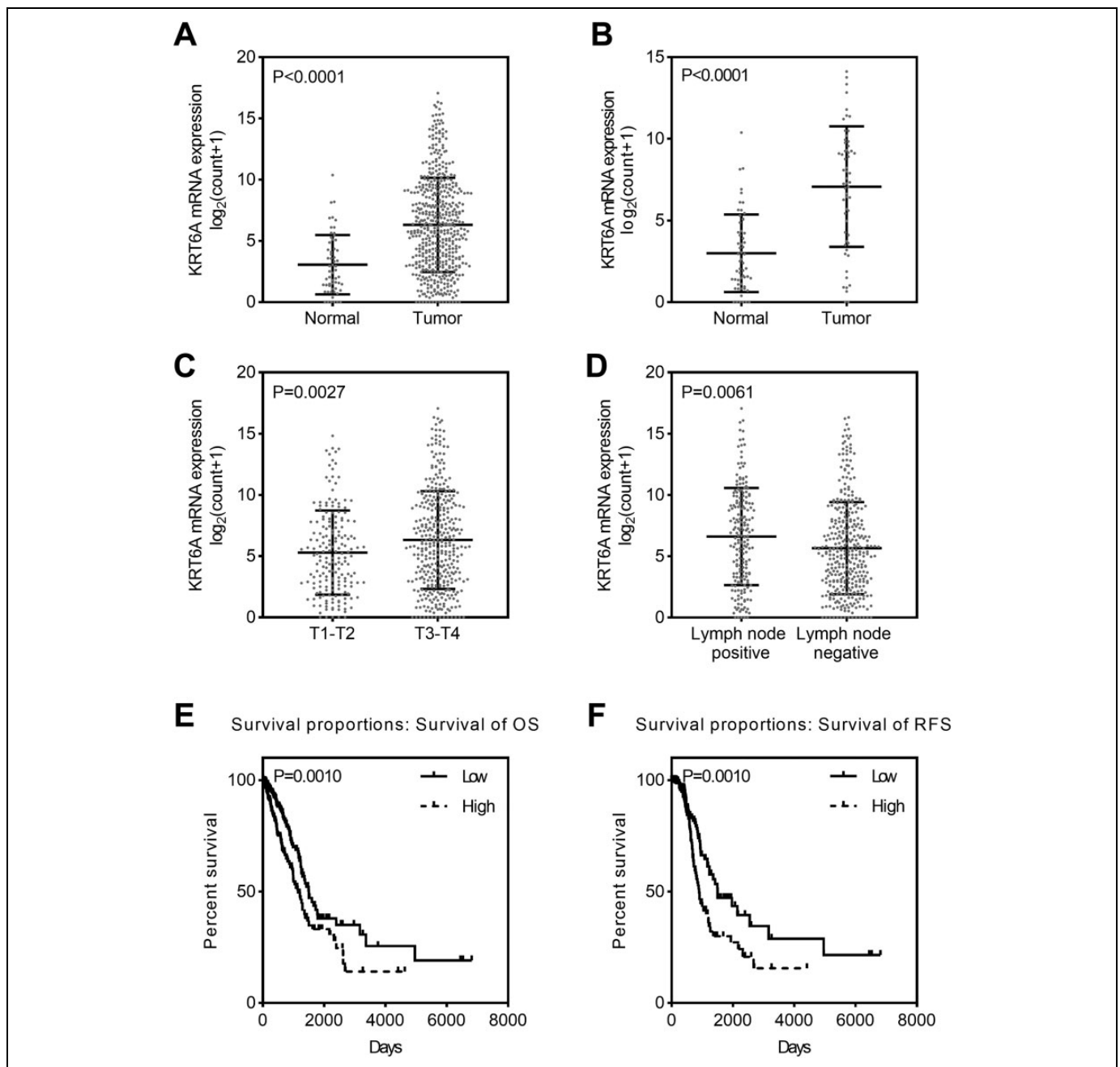
For flow cytometry (FACS) staining, cells were washed and resuspended at a concentration of  $1 \times 10^6$  cells/mL in FACS buffer (3% FBS in PBS with 0.5 mM EDTA). Then, cells were preincubated with Fc-block and subsequently stained with antibodies (CXCR4, Abcam, ab124824; CD133, Abcam, ab252553) for 2 hours and fluorescently labeled second antibodies for 1 hour. Propidium iodide (PI) staining was used for viability gating, and PI was added before loading the samples to flow cytometer. Flow analysis was performed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo (version 10; FlowJo LLC), and CXCR4<sup>high</sup>/CD133<sup>high</sup> subpopulations were identified as CSCs (Q2 gate). Each assay was performed in triplicate.

### RNA Preparation and qRT-PCR

TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc) was used for total RNA extraction. After that the RNA were reverse transcript to complementary DNA, using a Reverse Transcription kit (cat. no. RR036A; Takara Biotechnology, Co, Ltd, Nojihigashi, Japan), following the official instructions. Quantitative reverse transcription PCR analyses were performed with SYBR Select Master Mix (cat. no. 4472908; Applied Biosystems; Thermo Fisher Scientific, Inc) on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc). And qRT-PCR thermos-cycling conditions consisted of 95°C for 10 minutes, followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Each sample was run in triplicate and the relative expression of the target gene was normalized based on the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level using the  $2^{-\Delta\Delta C_q}$  method.<sup>10</sup> The primers in the study were as follows: GAPDH, Forward 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse 5'-GGCTGTTGTCATACTTCTCATGG-3', KRT6A Forward 5'-TCACCGTCAACCAGAGTCTC-3' Reverse 5'-GAACCTTGTTCTGCTGCTCC-3'.

### Protein Preparation and Western Blot Analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Nanjing, China) and quantified with a BCA kit. Equal amount (10 µg) of protein was loaded onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. After electrophoresis, protein were electrotransferred to negative control (NC) membranes. After that the membranes were blocked in 2% bovine serum albumin in Tris-buffered saline and Tween 20 (TBST) for 1 hour at room temperature and incubated with first antibodies (KRT6A, Abcam ab18586, E-Cad, Abcam, ab40772; N-Cad, Abcam, ab18203; Vimentin, MAB2105, R & D; Catenin,

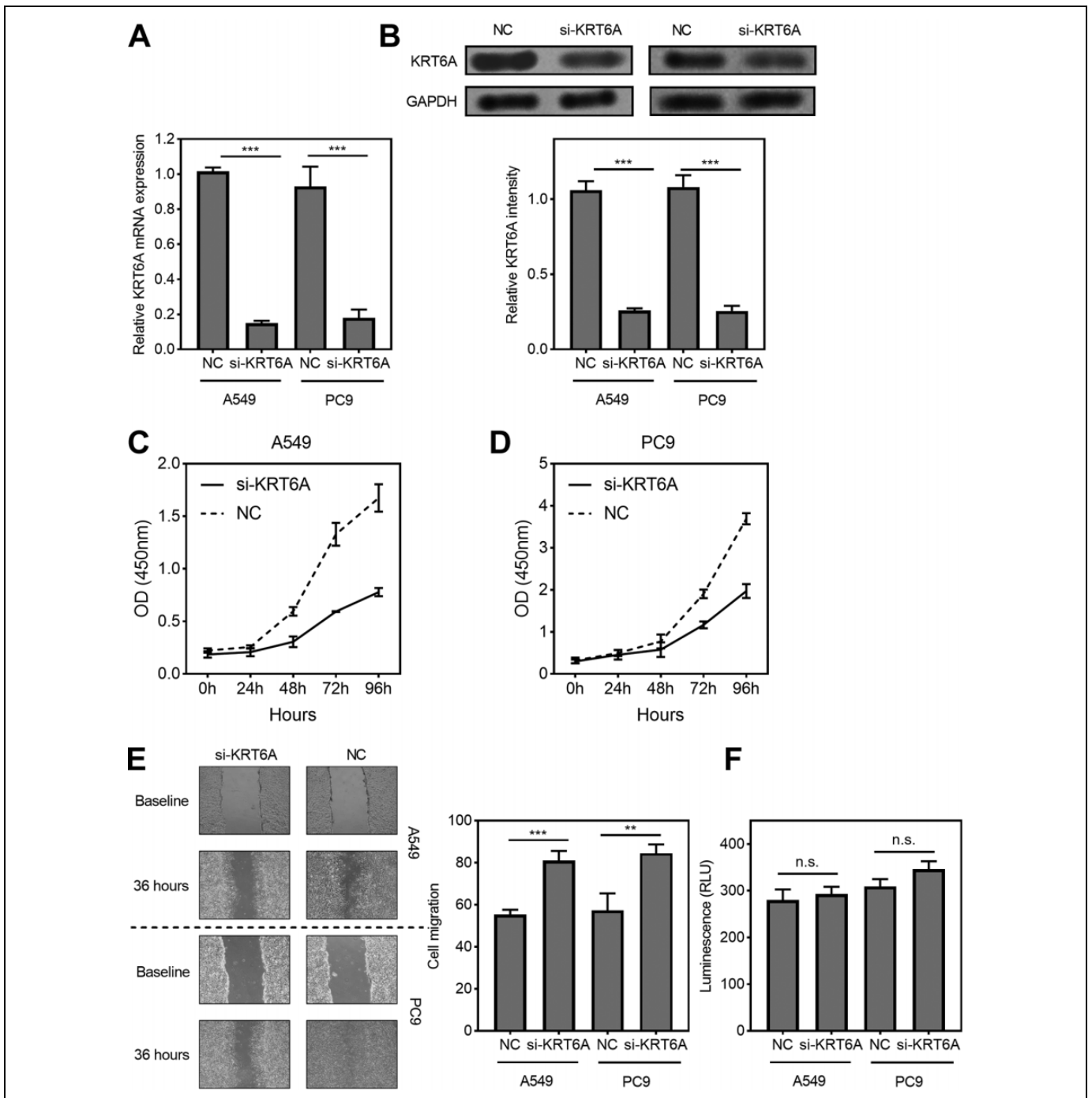


**Figure 1.** Keratin 6A is hyperexpressed in LUAD. A, KRT6A was hyperexpressed in LUAD compared with normal adjacent tissues,  $P < .0001$ . B, From paired samples, the difference between cancer and normal tissues was more significant, the KRT6A level was higher in tumor samples compared with adjacent normal samples in paired samples. C, Patients with more aggressive T stage have significantly higher KRT6A expression. D, Patients with lymph node metastasis have higher KRT6A expression. E and F, KRT6A expression could predict patients survival, patients with higher KRT6A expression have poor prognosis in both OS and RFS. KRT6A indicates keratin 6A; LUAD, lung adenocarcinoma; OS, overall survival; RFS, recurrence-free survival.

#2309, CST) at  $4^{\circ}\text{C}$  overnight. After that wash membranes in TBST and incubated with horseradish peroxidase-coupled second antibodies for 2 hours in room temperature. At last, membranes were treated with enhanced chemiluminescence Western blotting substrate (Pierce; cat. no., 32106) at room temperature for 1 minute, then scanned with ChemiDoc blot scanner (BioRad, Hercules, California). All experiments were repeated in triplicate.

### Cell Proliferation, Death, and Migration Assays

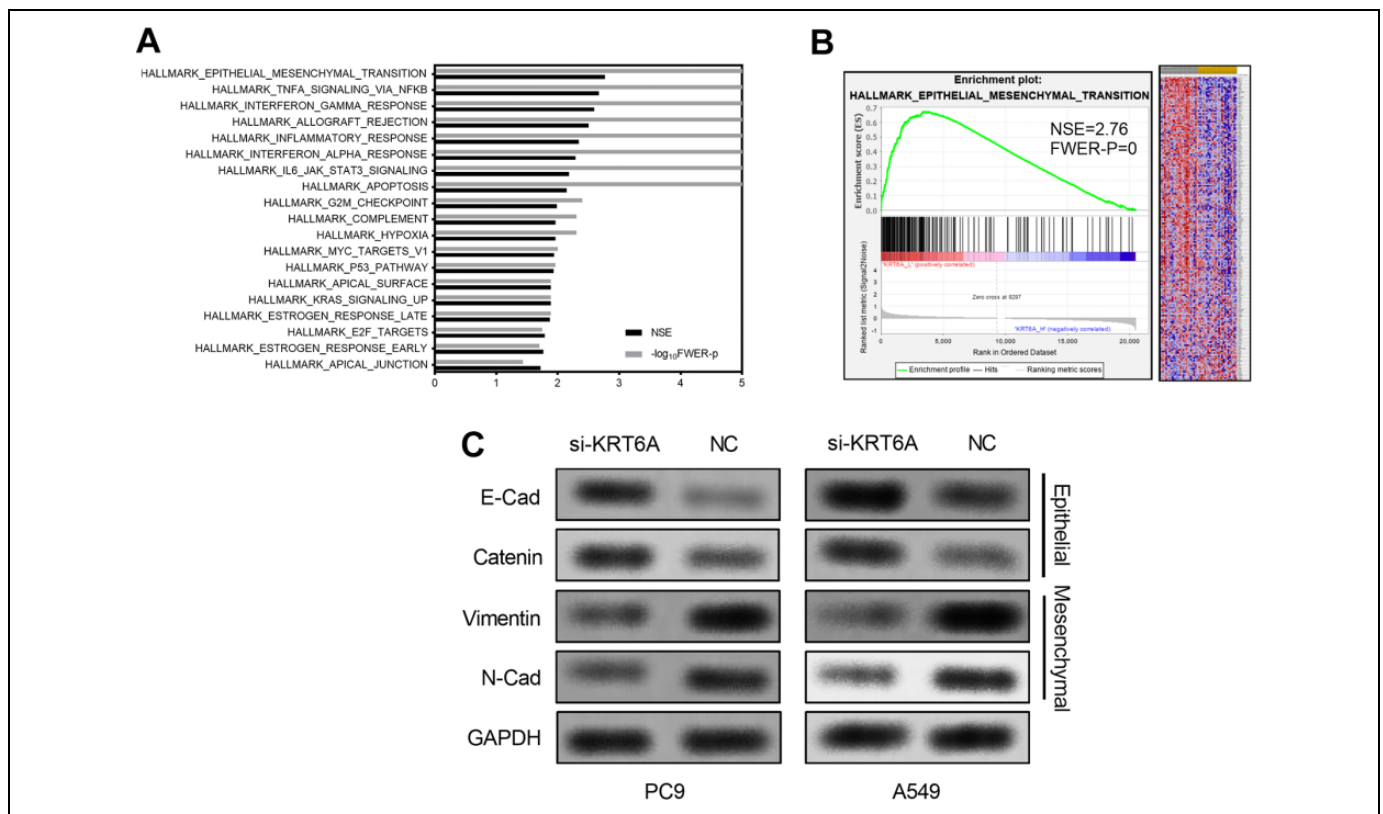
For measuring the cell proliferation rate, the Cell Counting Kit-8 (cat. no. CK04; Dojindo Molecular Technologies, Inc, Rockville, MD) was introduced. Two thousand cells were seeded into wells of 96-well plates with  $100\ \mu\text{L}$  of medium; after several days, the reagent was added and the absorbance rate was measured at a wavelength of  $450\ \text{nm}$  using an ELx800



**Figure 2.** Keratin 6A knockdown undermines the proliferation and migration ability of lung cancer cells. A and B, KRT6A was knocked down by siRNA, siRNA significantly knocked down KRT6A mRNA expression and protein level in A549 and PC-9 cell lines (the Western blots were quantified by Image J). C and D, CCK8 assay shown si-KRT6A could significantly undermine the proliferation of A549 and PC-9 cells. E, Wound healing assay shown that KRT6A knockdown significantly undermined the migration ability in lung cancer cells. F, By measuring the supernatant LDH level, the cell death rate was determined, no significant difference was found between si-KRT6A and NC groups, which indicate KRT6A knockdown did not influence the cell death rate between NC and KRT6A knockdown group. CCK8 indicates Cell Counting Kit-8; KRT6A, keratin 6A; mRNA, messenger RNA; NC, negative control; siRNA, small interfering RNA.

Universal Microplate Reader (Norgen Biotek Corp). For cell death measurement, the LDH-Glo Cytotoxicity Assay (Promega) was introduced based on the official instructions, 48 hours after transfection, the cell culture supernatant was

collected and the relative supernatant lactate dehydrogenase (LDH) concentration was measured with LDH-Glo using Universal Microplate Reader. Each experiment was repeated in triplicate in different time points. For evaluation of the



**Figure 3.** Keratin 6A is involved in EMT. A and B, For GSEA, the transcriptome data of top 10% KRT6A expression and bottom 10% KRT6A expression were submitted for the GSEA from TCGA\_LUAD data set, our data indicate that KRT6A was correlated with EMT pathway, normalized enrichment score (NES) = 2.76, FDR = 0. C, By Western blot, several EMT markers were measured, KRT6A knockdown could significantly decrease N-Cad and Vimentin level but increase E-Cad and Catenin level when compared with negative control groups. Our data indicate KRT6A knockdown could influence the EMT in lung cancer cells. EMT indicates epithelial–mesenchymal transition; GSEA, gene set enrichment analysis; KRT6A, keratin 6A.

migration ability, the wound healing assay was introduced and transfected cells were seeded in 6-well plates with si-KRT16 or si-NC; after 24 hours, an artificial scratch wound on a confluent monolayer of cells was created with a 200- $\mu$ L pipette tip, then serum-free medium was added, and cells were imaged at baseline and 24 hours. The migration ability was measured by cell moving percentage. Experiments were repeated in triplicate.

### Statistical Analysis

GraphPad Prism 8.01 (GraphPad Software, Inc, San Diego, CA) was used for statistical analyses and the production of graphs. Data are presented as the mean  $\pm$  standard deviation. Data analyzed using Student *t* test, or 1-way analysis of variance with post hoc test.  $P < .05$  was considered as statistical significant.

## Results

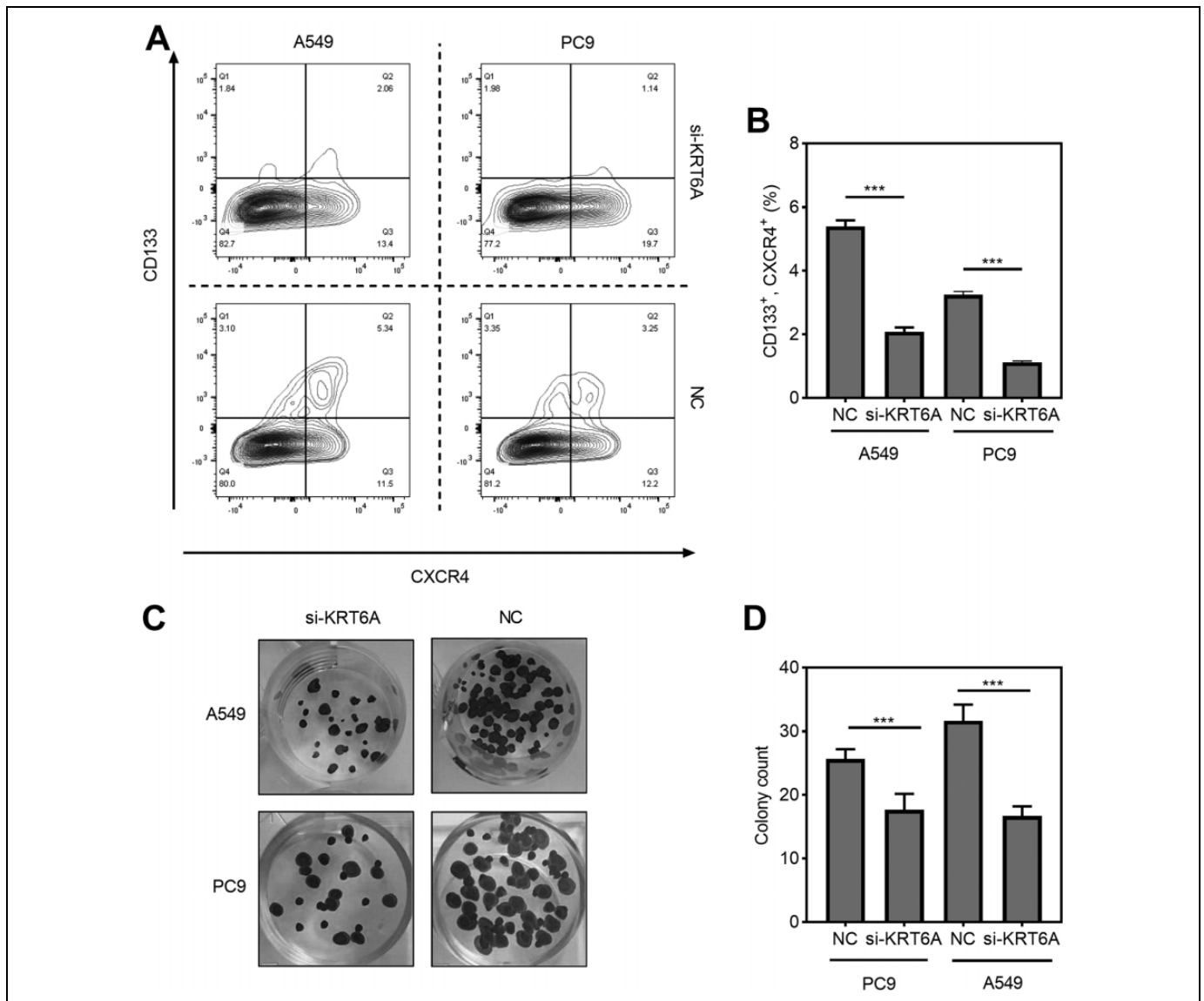
### Keratin 6A Is Hyperexpressed in LUAD and Associated With Poor Prognosis

Using TCGA LUAD data set, the expression of KRT6A in LUAD was determined, as shown in Figure 1A and B, LUAD

tumors express significantly higher KRT6A levels compared with adjacent normal samples ( $P < .0001$ ). And patients with aggressive T stage (III-IV) exhibited significantly higher KRT6A expression when compared with I to II T stage patients ( $P < .001$ ; Figure 1C). In addition, KRT6A expression was associated with lymph node metastasis, lymph node positive patients express higher levels of KRT6A ( $P < .001$ ; Figure 1D). For survival analysis, as shown in Figure 1E, the KRT6A higher expression was positively associated with poor overall survival in LUAD patients ( $P = .001$ ), and similar result was also observed in survival analysis with recurrence-free survival (Figure 1F,  $P = .001$ ). In summary, our data indicate KRT6A was hyperexpressed in LUAD, and KRT6A high was associated with poor prognosis in LUAD patients.

### Keratin 6A–Knockdown Inhibits Lung Cancer Cell Proliferation and Migration Ability

To investigate the biological role of KRT6A in LUAD, we knockdown the KRT6A expression in LUAD cell lines (A549 and PC9), the efficiency of siRNA-knockdown was determined by qRT-PCR and Western blot (Figure 2A and B), the si-RNA could significantly knockdown the KRT6A



**Figure 4.** Keratin 6A is involved in CSCs transformation. A and B, CXCR4<sup>high</sup>/CD133<sup>high</sup> cells were identified as CSCs, compared with si-KRT6A groups, NC groups have significantly expanded CSCs population (Q2). C and D, The colony formation assay shown that compared with si-KRT6A A549 and PC-9 cells the normal control cancer cells (NC groups) have greater biological advantage in forming colonies. CSC indicates cancer stem cell; KRT6A, keratin 6A; NC, negative control.

expression and level in both cell lines. After that we evaluated the impact of KRT6A on cancer cell proliferation and cell death, as presented in Figure 2C and D, the NC groups shown higher cell counts, and on the other hand, the results of Luminescent Cell Viability Assay (which measured the LDH level in the supernatant) indicated no significant difference in the cell death rate between the NC and the si-KRT6A group (Figure 2F). These results suggest that knockdown of KRT6A decreased the proliferation ability of lung cancer cells but not cell death rate. Last, the migration ability of A549 and PC9 cells was determined using wound healing assays. As demonstrated in Figure 2E, the migration ability of both A549 and PC9 cells was significantly undermined by knocking down of KRT6A ( $P < .001$ ). In summary, the knockdown of KRT6A

could significantly undermine the proliferation, migration ability of the lung cancer cells, but not their cell death rate.

#### *Keratin 6A Exerts Its Oncogenic Activity Via EMT and CSCs Transformation*

For further investigation, GSEA was introduced. Patients from the TCGA LUAD data set were sorted according to the KRT6A expression, the top 10% and bottom 10% KRT6A samples were enrolled for GSEA. GSEA results suggest that KRT6A could significantly influence EMT pathway (Figure 3A and B). Therefore, the level of EMT markers (E-Cad, N-Cad, catenin, and vimentin) was analyzed by Western blot in lung cancer cells; our results demonstrated that in si-KRT6A group, the

level of epithelial markers (E-Cad and catenin) was significantly higher, whereas the mesenchymal markers (N-cad and vimentin) expression levels were significantly lower when compared with the negative control group (Figure 3C).

The EMT program is also as a critical regulator of the CSCs transformation phenotype; thus, we detected the surface markers for CSCs in A549 and PC-9 cells. The CSCs are minor subpopulations in tumor tissues, but CSCs are uniquely capable of seeding new tumors, which could cause distant metastasis and cancer progression lead to severe cancer-related complication. The CXCR4<sup>high</sup>/CD133<sup>high</sup> subpopulations were identified as CSCs subgroup. As shown in Figure 4A, the CXCR4<sup>high</sup>/CD133<sup>high</sup> CSCs were around 3% to 5% in viable/singlet cells. After KRT-6A knockdown, the CD133 and CXCR4 were costained for flow cytometry, as shown in Figure 4A and B, in both cell lines NC group have significantly expanded Q2 population (CSC, CXCR4<sup>high</sup>/CD133<sup>high</sup>), when compared with si-KRT6A groups. The phenotype for CSCs transformation could be translated to the colony formation ability of CSCs. Thus, we measured the colony formation ability between si-KRT6A cells and normal wild type A549 and PC-9 cells, as shown in Figure 4C and D, the NC groups have significantly higher colony numbers ( $P < .001$ ). Our data indicate the KRT6A serves as an oncogene by promoting EMT and CSCs transformation.

## Discussion

The EMT is a transdifferentiation process that epithelial cells lose their cell–cell adhesion and cell polarity, and gain migratory and invasive properties to become mesenchymal-like stem cells, EMT occurs in the initiation of metastasis in cancer progression.<sup>13,14</sup> Cancer stem cells express specific cell-surface markers including CD24, CD44, CD133, CD200, EpCAM, ABCB5, and THY1.<sup>15,16</sup> But the CSC markers are diverse between different cancer types, for example, CD133<sup>high</sup> is a good CSC marker for lung and colon carcinoma but not for breast and prostate carcinoma.<sup>4</sup> And for CXCR4, it has been reported that CXCR4<sup>high</sup> could serve as a marker for CSCs in lung cancer<sup>17</sup> and associated with radioresistant.<sup>18</sup> Tu Z reported that CD133/CXCR4 coexpression was found to be an independent prognostic factor and positively associated with EMT.<sup>19</sup> Thus, in current study, CD133 and CXCR4 were selected as CSCs markers.

In current study, we demonstrated that KRT6A is hyperexpressed in LUAD and especially hyperexpressed in lymph node positive patients' samples. Patients with higher KRT6A expression shown poorer prognosis compared with low KRT6A expression patients in LUAD. For exploring the biology impact of KRT6A in LUAD, the siRNA KRT6A knockdown *in-vitro* model was introduced, we found that KRT6A knockdown significantly undermined the LUAD cells' proliferation, migration, and colony formation ability but not the cell death rate. For further mechanism exploring, GSEA was utilized, we identified KRT6A knockdown might influence EMT as downstream mechanism in LUAD

(NES = 2.76, family-wise error rate (FWER)-P = 0). Thus, several EMT markers were detected, we found that KRT6A knockdown significantly increased the E-Cad expression level but decreased N-Cad level; moreover, the KRT6A knockdown groups have significantly smaller CSCs population which indicate KRT6A knockdown significantly undermine the CSC transformation ability.

Our data indicate KRT6A overexpression in LUAD promotes lung cancer cells proliferation, migration, and colony formation ability via EMT and CSC transformation, and KRT6A could serve as a prognosis marker in LUAD.

## Authors' Note

B.Y., W.Z., and M.Z. contributed equally to the manuscript. All data and materials are available.

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B.Y. and R.Z. designed the study. B.Y., W.Z., and X.W. accomplished the whole experiment. M.Z. performed the Bioinformatic analysis. S.P. and B.Y. performed the statistical analysis and B.Y., X.W., and R.Z. wrote the manuscript. All authors read and approved the final manuscript.


## Declaration of Conflicting Interests

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