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RESEARCH PAPER

p27 inhibits CDK6/CCND1 complex formation resulting in cell cycle arrest and inhibition of cell proliferation

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ABSTRACT

p27 plays critical roles in cell proliferation, differentiation, and apoptosis, which have been well studied in mammals and *Drosophila*. However, the mechanisms underlying *p27* regulation of the cell cycle have not been thoroughly researched. In this study, Genevestigator, Kaplan–Meier Plotter, and the Human Protein Atlas databases were used to analyze the expression of *p27*, cell division protein kinase 6 *(CDK6)*, and cyclin D1 (*CCND1)*, as well as its prognostic value in different tumor tissues and corresponding normal tissues. Quantitative PCR and immunohistochemistry were used to detect the expression of *p27, CDK6*, and *CCND1* in the tissues of cancer patients. The effects of *p27, CDK6*, and *CCND1* on the proliferation of lung cancer cells were examined by the MTT assay, and flow cytometry was used to investigate the mechanism by which *p27* affected cell proliferation. Immunofluorescence, co-immunoprecipitation, and Western blotting were used to determine if *p27* interacted with *CDK* and *CCND1* to regulate the cell cycle. The results showed that *p27, CDK6*, and *CCND1* played different roles in tumorigenesis and development, which are in accordance with *CDK6* and *CCND1* in affecting the cell cycle and cell proliferation. *p27* regulated the cell cycle and inhibited cell proliferation by affecting formation of the cell cycle-dependent complex CDK6/CCND1, but did not directly affect the expression of *CDK6* and *CCND1*. Moreover, *CCND1* did not regulate the cell cycle alone, but rather, functioned together with *CDK6*. This study provides insights into the effects of *p27* on tumor formation and development, and the underlying regulatory mechanisms.

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Cancer; cell proliferation; cell cycle; *p27*; *CDK6*; *CCND1*

Introduction

If the cell cycle is not constrained by homeostatic mechanisms, abnormalities in tissues and organs, and even cancer lesions can occur [1]. Cell cycle regulation is very complex and involves a variety of mechanisms which keep the organism in a steady state [2]. Cyclin-dependent kinase inhibitors (CKIs) play important roles in regulating the cell cycle, and genes that encode CKIs are highly conserved among all eukaryotes. Progression through the cell cycle is regulated by the coordinated activities of cyclin/cyclin-dependent kinase (CDK) complexes [3]. One level of regulation of these complexes is provided by their binding to CKIs [4].

p27 is a CKI [5] and cell cycle regulator which is involved in both cell fate determination and tissue growth [6]. Because CKIs can inhibit cell proliferation, they play essential roles as tumor suppressor genes [7]. The expression of *p27* is associated with the occurrence and development of most tumors. *p27* encodes an inhibitor of CCNE/CDK2 complexes in *Drosophila* similar to vertebrate Cip/Kip inhibitors [8], which accumulate in the G1 phase and are gradually degraded in the S and G2 phases of the cell cycle (Figure 9(a)) [9,10]. In the nucleus, *p27* acts as an inhibitor of cyclin/CDK2 complexes in the G0 and early G1 phases, and CCNE/CDK2 phosphorylates and binds to *p27* before the S phase. Subsequently *p27* is ubiquitylated by SCF and degraded in the cell [9] or translocated to the cytoplasm, and then phosphorylated at S10 by the KPC complex. Finally, it is degraded by the ubiquitin pathway [10].

p27 affects formation of the cell cycle checkpoint complex (CCNE/CDK2); however, there has been less research on its effects on the CCND1/CDK6 cell cycle checkpoint complex. This study provides

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Supplemental data can be accessed here

insights into the effects of *p27* on the CCND1/CDK6 complex, cell proliferation, and tumor formation.

Results

Expression of p27, CDK6, and CCND1 in drosophila, mice, and humans

We extracted data on the transcript expression of *p27, CDK6*, and *CCND1* from the Genevestigator database (https://genevestigator.com/gv/doc/tools.jsp) for *Drosophila*, mice, and humans (Figure 1). During the growth process of mice and *Drosophila*, the expression of *p27, CDK6*, and *CCND1* generally remained the same, which proved that these three genes are closely associated with the growth and development of mice and *Drosophila* (Figure 1(a,b)). With analyzing expression in human tissue, high levels of p27, CDK6, and CCND1 were found in the lung, stomach, heart, and other tissues, indicating that they play more important roles in humans than mice and *Drosophila* (Figure 1(c)). Functional clustering analysis of these three genes showed that their main functions were regulation of the cell cycle (Figure 1(d)). Cell cycle regulation involves cyclin-dependent protein serine/threonine activity, CDK activity, and G/S transition of the mitotic cell cycle (Figure 1(d)). Based on the results mentioned above, we presumed that the close interaction among p27, CDK6, and CCND1 affect the growth and development of mice, *Drosophila*, and humans by regulating the cell cycle. Cancer is a disorder caused by dysfunction of the cell cycle, so our subsequent experiments focused on the impact of these three genes in cancer development.

Functions of p27, CDK6, and CCND1 in tumors

In our mutational gene analysis of tumors (www.cbioportal.org/), we found that mutations in *p27* were associated with changes in *CDK6* and *CCND1* in gastric, lung, and breast cancers (Figure 2(a)). The results were in accordance with those shown in Figure 1. P27, CDK6, and CCND1 were closely associated with regulation of the growth and development of mice, *Drosophila*, and humans as well as cancer development. The expression of these three genes in lung, gastric, and breast cancers was analyzed using data from Proteinatas (www.proteinatlas.org). The results showed that p27 was highly expressed in normal tissues and that CDK6 was more expressed in tumor tissues; the difference in CCND1 expression between normal and cancer tissues was not clear (Figure 2(b)). Next, we focused our analyzes on the effects of *p27, CDK6*, and *CCND1* expression on the survival of cancer patients (lung, gastric, and breast cancers) (http://www.kmplot.com/). The results showed a correlation between *p27* expression and overall survival (OS) (Figure 2(c)). When we restricted our analysis to tumor type, a positive influence on OS was observed with the expression of *p27*. At the same time, analyzes of *CDK6* and *CCND1* showed a correlation between their gene expression and OS rates (Figure 2(d,e)). Specifically, high *CDK6* expression was correlated with decreased OS and a poor prognosis (Figure 2(d)). However, high *CCND1* expression was correlated with increased OS and a favorable prognosis (Figure 2(e)). These results were in accordance with the difference in gene expression observed between cancer patients and healthy controls (Figure 2(b)).

Expression of p27, CDK6, and CCND1 in lung cancer patients

In the data analysis mentioned above, *p27* had reduced expression in cancer tissues. To verify these results, we detected changes in *p27* mRNA expression in cancer tissues and corresponding paracancerous tissues from five patients with lung squamous cell carcinoma (Figure 3(a)). There was significant downregulation of *p27* in four patients compared with the corresponding adjacent tissues, with accompanying changes in *CDK6* and *CCND1* expression (Figure 2(a)). The expression of these two genes influenced the survival of cancer patients (Figure 2(d,e)). Therefore, we also detected changes in the expression of CDK6 and CCND1 in the tissues from these five patients (Figure 3(b,c)). CDK6 was significantly upregulated in the tumor tissues of four cancer patients, but the expression of CCND1 did not show a stable trend. At the same time, the expression of p27 was detected in the cancer and paracancerous tissues of four lung squamous cell

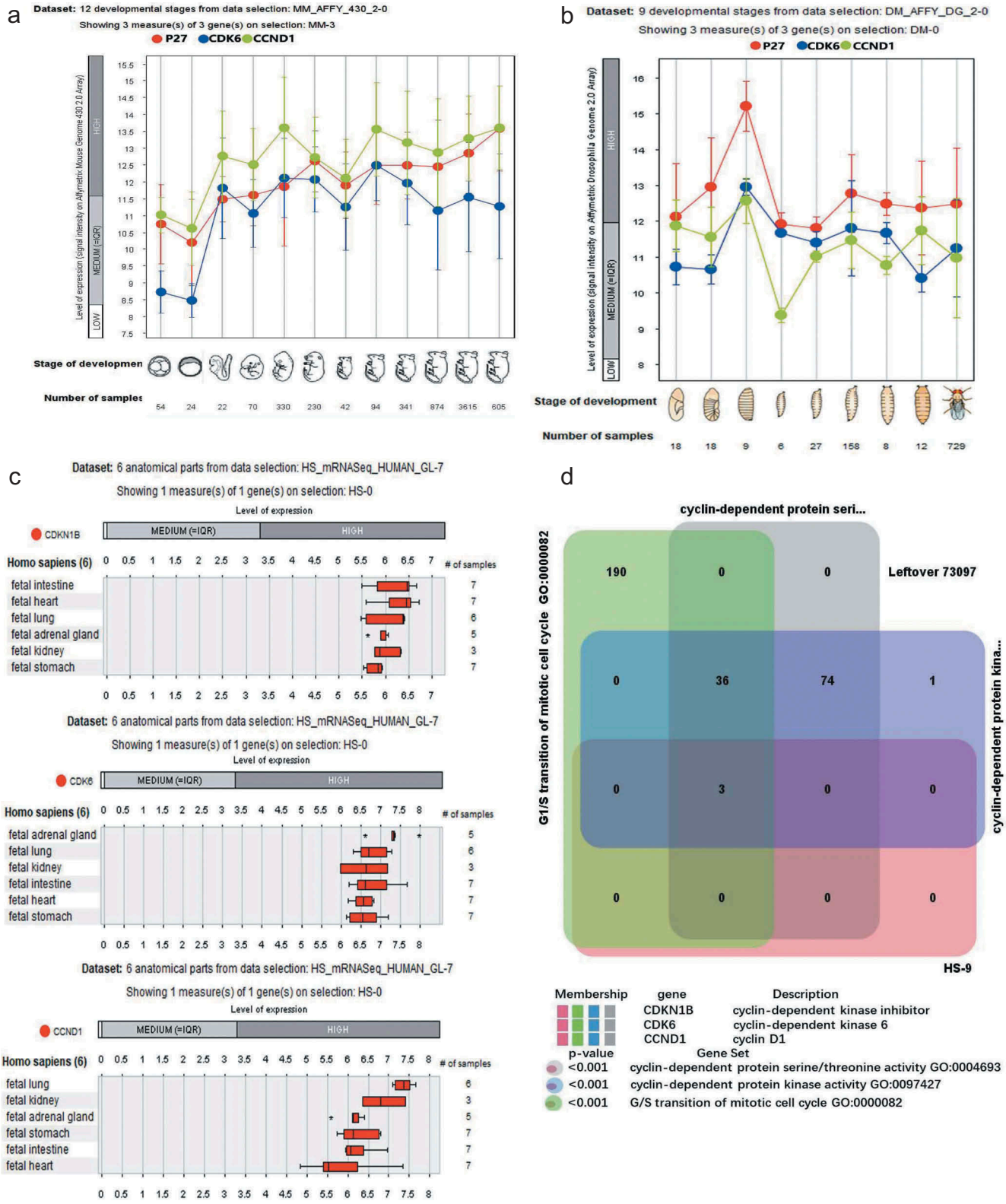

Figure 1. Expression of *p27, CDK6*, and *CCND1* in *Drosophila*, mice, and humans. (a) Twelve developmental stages from data selections: MM-AFFY-430 −2–0 Showing three measures of *P27, CDK6*, and *CCND1* in mice. The 12 stages were: prenatal_0–1, prenatal_2–4, prenatal_7–8.5, prenatal_9–11, prenatal_11.5–15, prenatal_16-18, postnatal_0, postnatal_1–3, postnatal_4–15, postnatal_16–63, adult_64-255, adult_256-9999. (b) Nine developmental stages from data selections: DM-AFFY-DG −2–0 Showing three measures of *P27, CDK6* and *CCND1* in *Drosophila*. The nine stages are: germ band elongation stage embryo, germ band retraction stage embryo, late stage embryo, first instar larval stage, second instar larval stage, third instar larval stage, prepupal development, pupal development, and adult development. (c) Detection of mRNA expression of p27, CDK6, CCND1 in six human organs. The organs included the stomach, lung, heart, kidney, adrenal gland, and intestine. (d) Analysis of p27, CDK6, CCND1 functions in humans. The functions of three genes involved in the regulation including cyclin-dependent protein serine/threonine activity, cyclin-dependent protein kinase activity, and G/S transition of mitotic cell cycle.

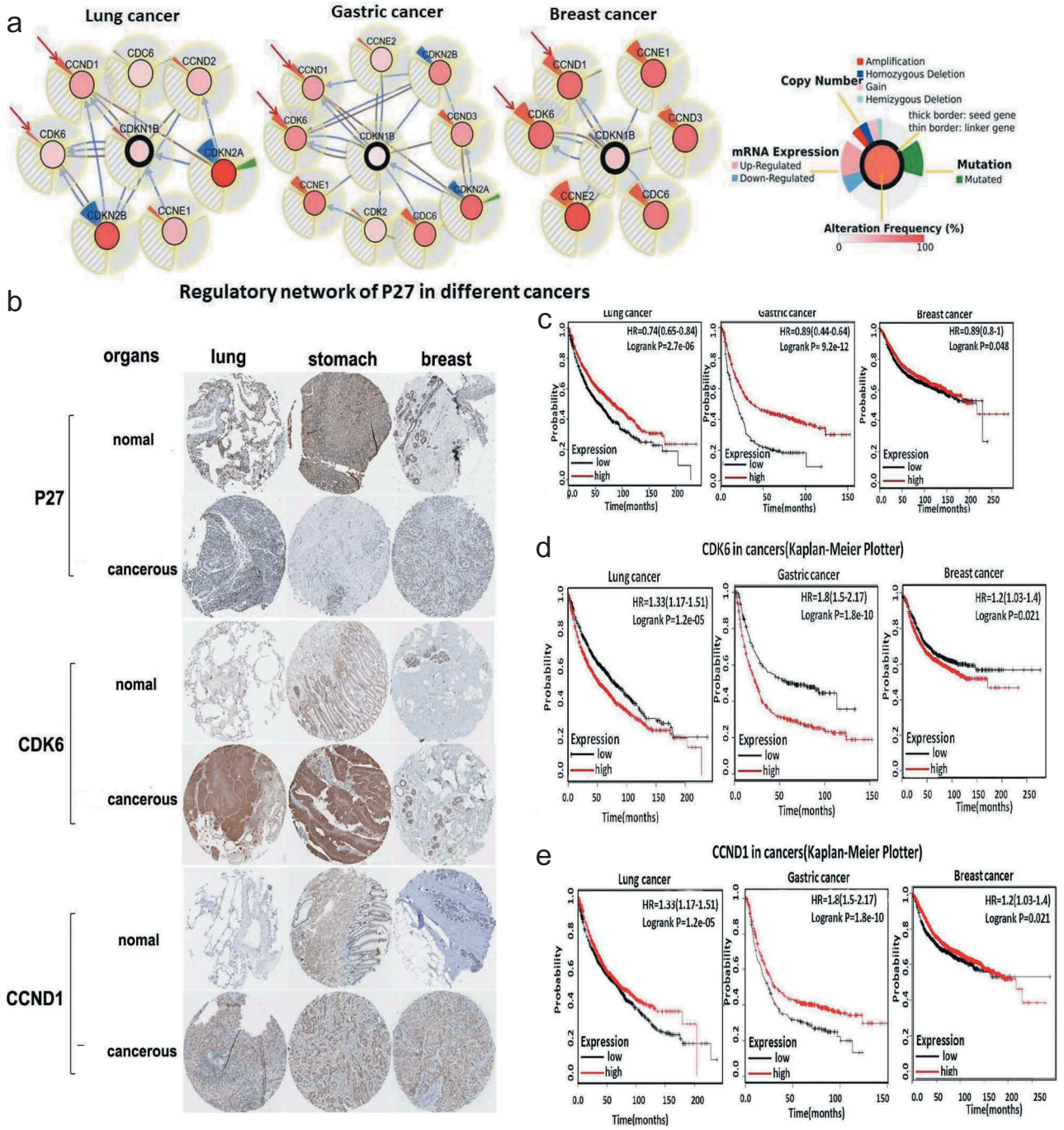

Figure 2. Functions of p27, CDK6, and CCND1 in tumors. (a) Analysis of Mutations in Other Genes in p27-mutated tumors (www.cbioportal.org/), the marked genes were significant changes in the range, the outer circle showed the result of genes copy number analysis, expression change analysis, mutation analysis. The inner ring belongs to P27 mutation frequency analysis. (b) Analysis of the expression of p27, CDK6, and CCND1 in tumors and corresponding normal tissues. Tumors including lung cancer, gastric cancer and breast cancer. The analysis data were derived from the immunohistochemical results of the database of Proteinatas (www.proteinatlas.org). (c) P27 in gastric, lung, and breast cancer (Kaplan–Meier Plotter). Kaplan–Meier plots showing OS in adenocarcinomas. In red: patients with expression above the median and in black, patients with expressions below the median. Kaplan–Meier plots revealed the correlation between gene expression and OS rate. (d) *CDK6* in gastric, lung, and breast cancers (Kaplan–Meier Plotter). Kaplan–Meier plots showing OS in adenocarcinomas. In red: patients with expression above the median and in black, patients with expressions below the median. Kaplan–Meier plots revealed the correlation between gene expression and OS rate. (e) *CCND1* in gastric, lung, and breast cancers (Kaplan–Meier Plotter). Kaplan–Meier plots showing OS in adenocarcinomas. In red: patients with expression above the median and in black, patients with expressions below the median. Kaplan–Meier plots revealed the correlation between gene expression and OS rate.

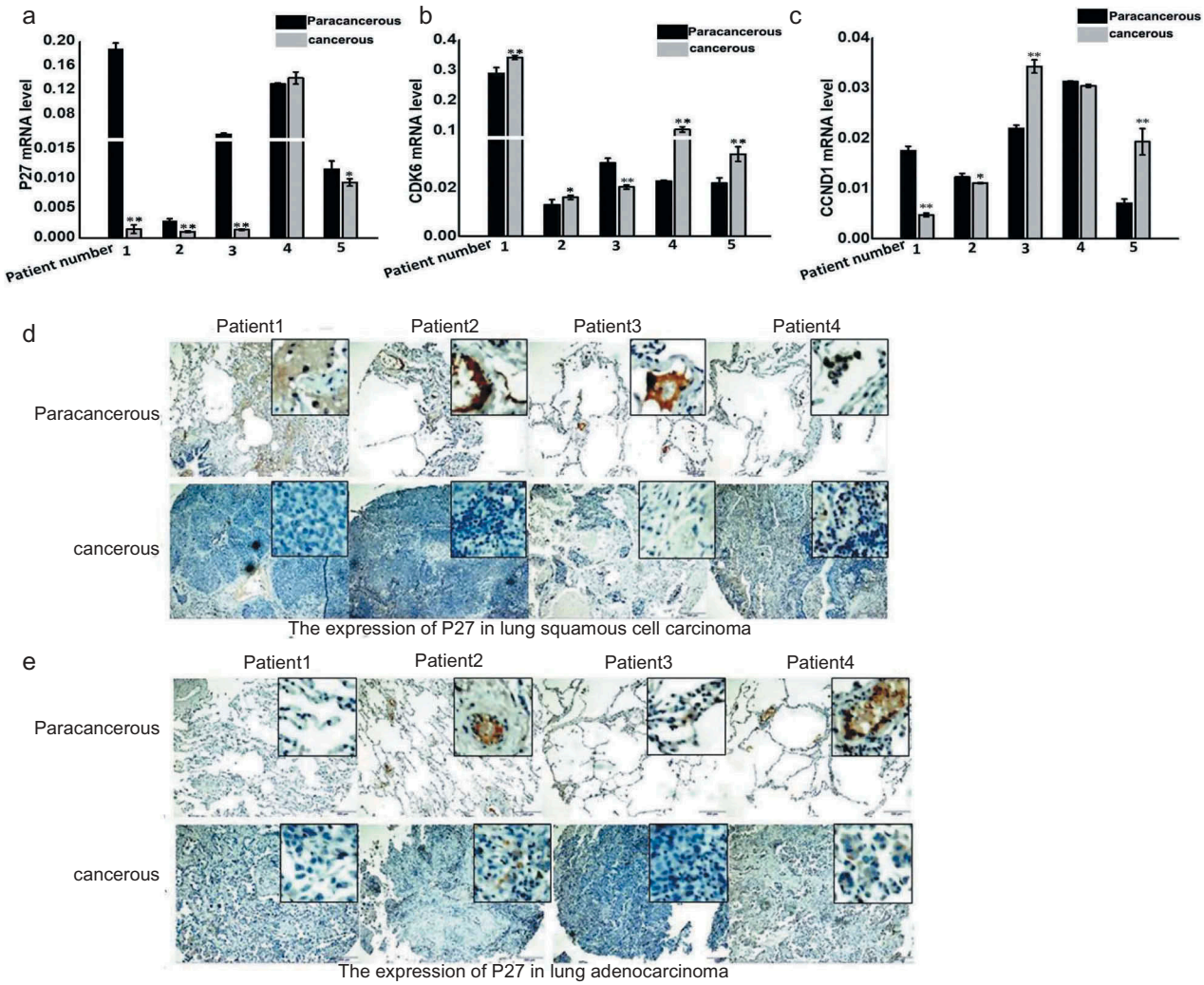

Figure 3. Expression of p27, CDK6, and CCND1 in lung cancer patients. (a) Expression of p27 in cancer tissues and the corresponding adjacent tissues of five lung cancer patients. (b) Expression of CDK6 in cancer tissues and the corresponding adjacent tissues of five lung cancer patients. (c) Expression of CCND1 in cancer tissues and the corresponding adjacent tissues of five lung cancer patients. (d) Expression of p27 was detected in cancer tissues and paracancerous tissues of four lung squamous cell carcinoma patients by IHC. (e) Expression of p27 was detected in cancer tissues and paracancerous tissues of four lung adenocarcinoma patients by IHC.

carcinoma patients and lung adenocarcinoma patients, as determined by immunohistochemistry (IHC) (Figure 3(d,e)). The downregulated expression of p27 was found in the cancer tissues of most patients with lung cancer.

p27, CDK6, *and* CCND1 *affected the proliferation of A549 lung cancer cells and HTB182 lung cancer cells*

Quantitative PCR (qPCR) was used to detect the expression of *p27*, *CDK6*, and *CCND1* after changing the expression levels of related genes in A549 cells (Figures 4(a) and 5(a)). Meanwhile, we detected cell proliferation at four time points (0, 24, 36, and 48 h) with flow cytometry and the MTT assay [11]. At 24, 36, and 48 h, there were many more cells with high *p27* expression than low expression (Figures 4(b) and 5(b)). The same result was obtained for the optical density (OD) value of cells. *P27* significantly decreased the OD value of cells in the same period (Figures 4(c) and 5(c)). *P27* inhibited cell proliferation while *CDK6* effectively increased the number of cells and the OD value of cells compared with cells during the same period. However, *CCND1* had no obvious effects on cell proliferation (Figures 4(b,c) and 5(b,c)). When *CDK6* and *CCND1* were simultaneously upregulated, the rate of cell proliferation increased (Figures 4(d) and 5(d)). However, when

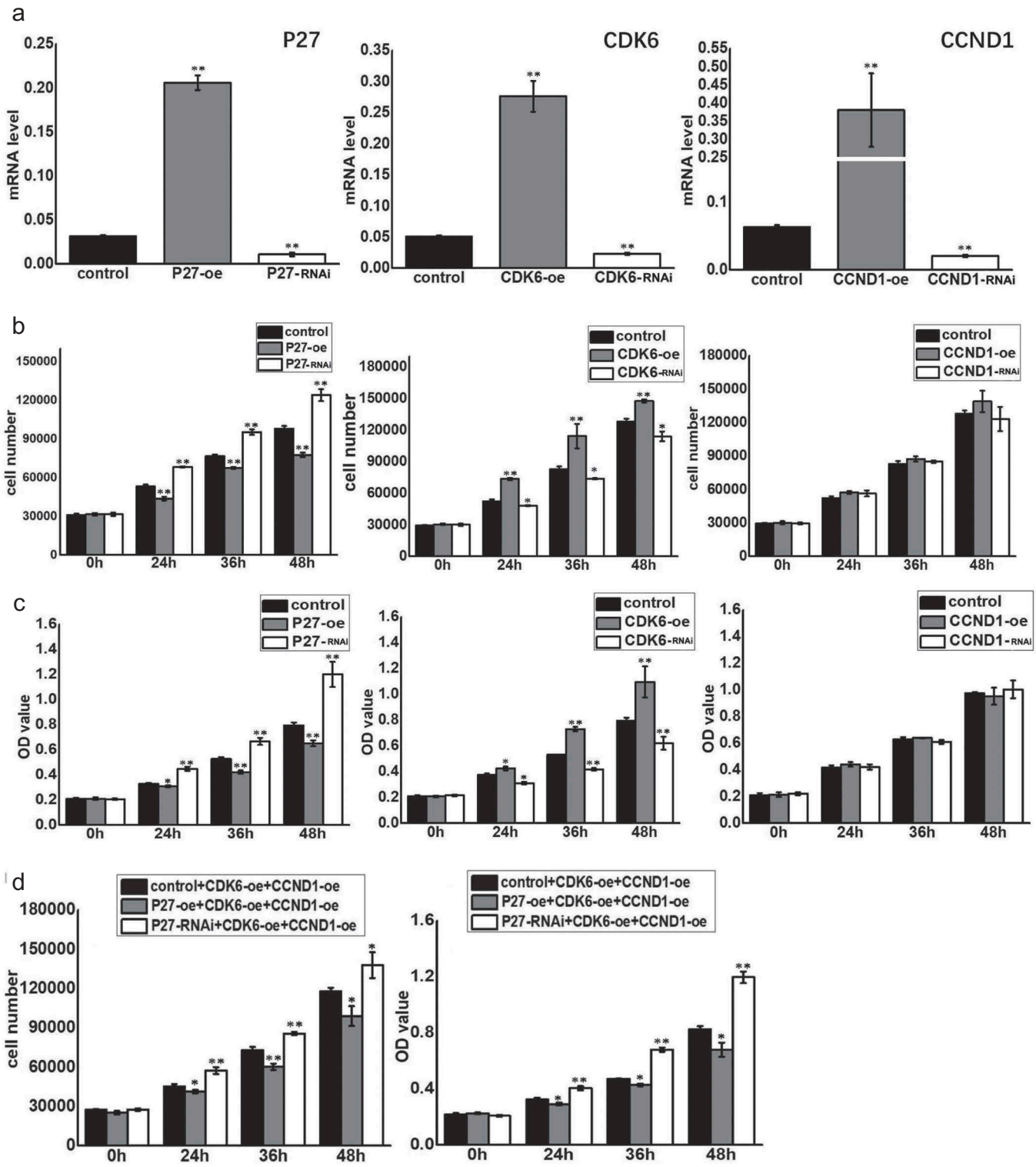

Figure 4. P27, CDK6 and CCND1 affected cell proliferation in A549 cells. (a) qPCR was used to detect the expression of p27, CDK6 and CCND1 after changing the expression levels of these genes. (b) The number of cells was detected by flow cytometry at 0, 24, 36, and 48 h after changing the expression of the related genes. Control: blank load transfection group as control group; P27-oe: over-expressed P27 in A549; P27-RNAi: Short Hairpin RNA on Expression of P27 in A549; CDK6-oe: over-expressed CDK6 in A549; CDK6-RNAi: Short Hairpin RNA on Expression of CDK6 in A549; CCND1-oe: over-expressed CCND1 in A549; CCND1-RNAi: Short Hairpin RNA on Expression of CCND1 in A549. Horizontal axis represents the hours after cell transfection. The vertical axis represents the cell number. (c) MTT reduction assay was used to reflect the proliferation at 0, 24, 36, and 48 h after changing the expression of related genes. Horizontal axis represents the hours after cell transfection. The vertical axis represents the OD value of cell proliferation. (d) Flow cytometry and MTT methods were used to detect cell proliferation at 0, 24, 36, and 48 h after changing the expression of related genes.

p27 was upregulated, the positive effects of their simultaneous upregulation on cell proliferation were reduced (Figure 4(d)). The inhibitory effects on cell proliferation were relieved while *p27* expression was downregulated (Figures 4(d) and 5(d)).

P27, CDK6, *and* CCND1 *affected the cell cycle in A549 cells and HTB182 cells*

To determine the mechanism by which *p27, CDK6*, and *CCND1* regulated cell proliferation, we monitored changes in the cell cycle by flow cytometry when the expression levels of these genes were changed. When *p27* was overexpressed, cell proliferation was negatively regulated, the number of cells in the G1 phase increased, and the number of cells in the S and G2 phases decreased. When *p27* was knocked down, the opposite results were observed (Figures 6(a) and 7(a)). The high expression of *p27* increased the proportion of cells in the G1 phase and blocked cells from entering the S and G2 phases, thereby inhibiting cell proliferation. We also examined the effects of *CDK6* and *CCND1*, which form the cell cycle-dependent complex, on cell cycle changes. High *CDK6* expression promoted the cell transition from the G1 phase to the S phase (Figures 6(b) and 7(b)). However, *CCND1* had no apparent effects on cell cycle switching (Figures 6(c) and 7(c)). When both *CDK6* and *CCND1* were overexpressed at the same time, cells entered the S phase more rapidly from the G1 phase (Figures 6(d) and 7(d)). However, when *p27* was upregulated in addition to *CDK6* and *CCND1* upregulation, cell cycle progression from the G1 phase to S phase slowed down and underwent cell cycle arrest in the G1 phase. When the expression of *p27* was downregulated, cell cycle arrest was significantly relieved (Figures 6(d) and 7(d)). These data confirmed that *p27* affects the cell cycle transition via *CDK6* and *CCND1*.

P27 *affected formation of the CDK6/CCND1 complex*

The expression of *CDK6* and *CCND1* was detected after *p27* was upregulated or downregulated, respectively (Figure 8(a)). The results showed that *p27* did not inhibit the expression of *CDK6* and *CCND1*. According to the experimental results mentioned above (Figures 4(b,c), 5(b,c), 6(c) and 7(c)), upregulation of *CCND1* did not have a significant effect on cell cycle progression and cell proliferation. However, the upregulation of *CDK6* and *CCND1* accelerated cell cycle progression from the G1 phase to S phase, and cell proliferation was also faster than that of *CDK6* alone (Figures 4(d), 5(d), 6(d) and 7(d)). The effects of p27 on the protein expression of CDK and CCND1 was detected by co-immunoprecipitation and Western blotting experiments, which were also used to detect the role of *p27* on formation of the CDK6/CCND1 cell cycle complex (Figure 8(b)). Changes in *p27* expression did not cause changes in CDK or CCND1 protein expression; however, the binding efficiency between CDK and CCND1 changed, indicating the varies in formation of the cell cycle complex. Furthermore, *p27* impacted formation of the CDK6/CCND1 cell cycle-dependent complex, as determined by immunofluorescence (Figure 8(c)). Specifically, *p27* inhibited the formation of cell cycle-dependent complexes in the nucleus. Retinoblastoma protein (Rb) is a downstream target gene of the CDK6/CCND1 complex [12]. Formation of the CDK6/CCND1 complex can induce Rb phosphorylation and promote the transition from the G1 phase to S phase [13], thereby promoting cell proliferation. P27 inhibited formation of the CDK6/CCND1 cell cycle-dependent complex. Phosphorylation of Rb was detected when p27 expression changed (Figure 8(d)). Together, the experimental results showed that upregulation of P27 can suppress the phosphorylation of Rb by blocking formation of the CDK6/CCND1 cell cycle-dependent complex in A549 and HTB182 cells.

Discussion

The results of this study clearly showed that *p27* has conservative functions in regulating the cell cycle (Figure 9); we detail how this occurs (Figure 9(b)). *P27* did not affect the expression of genes associated with cell cycle-dependent complexes, but the binding efficiency. *P27* has been identified as an important regulator of cell cycle [14]. Mutations in *p27* result in changes in cell cycle regulation [15], leading to a defective cell cycle and uncontrolled cell proliferation which can induce tumors [16,17]. Here, we showed that *p27* is also involved in regulation

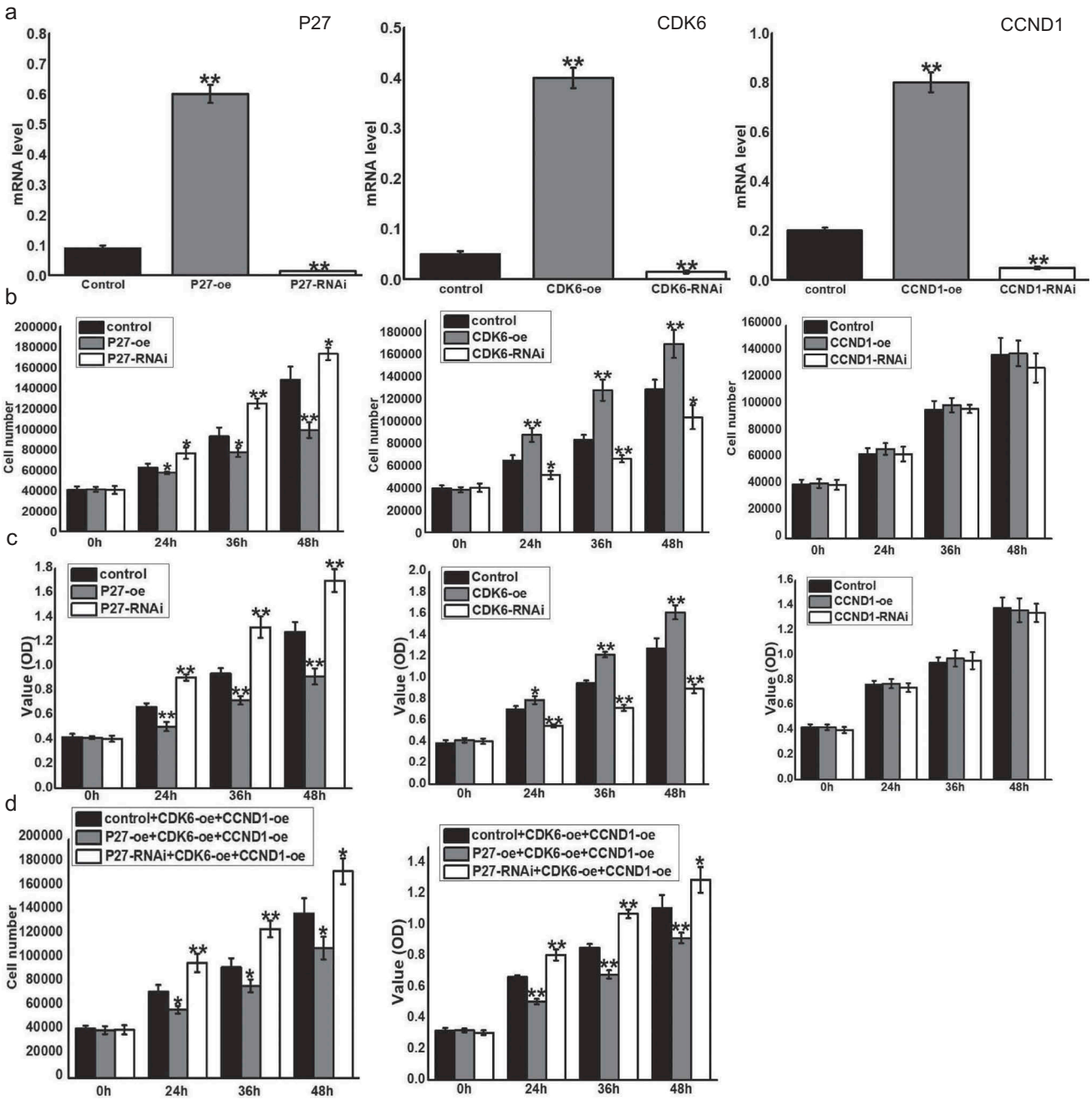

Figure 5. P27, CDK6 and CCND1 affected the proliferation of HTB182 cells. (a) qPCR was used to detect the expression of *p27, CDK6* and *CCND1* after changing the expression levels of these genes. (b) The number of cells was detected by flow cytometry at 0, 24, 36, and 48 h after changing the expression of related genes. Control: blank load transfection group as control group; P27-oe: over-expressed P27 in HTB182; P27-RNAi: Short Hairpin RNA on Expression of P27 in HTB182; CDK6-oe: over-expressed CDK6 in HTB182; CDK6-RNAi: Short Hairpin RNA on Expression of CDK6 in HTB182; CCND1-oe: over-expressed CCND1 in HTB182; CCND1-RNAi: Short Hairpin RNA on Expression of CCND1 in HTB182. Horizontal axis represents the hours after cell transfection. The vertical axis represents the cell number. (c) MTT reduction assay was used to reflect cell proliferation at 0, 24, 36, and 48 h after changing the expression of related genes. Horizontal axis represents the hours after cell transfection. The vertical axis represents the OD value of cell proliferation. (d) Flow cytometry and MTT methods were used to detect cell proliferation at 0, 24, 36, and 48 h after changing the expression of related genes.

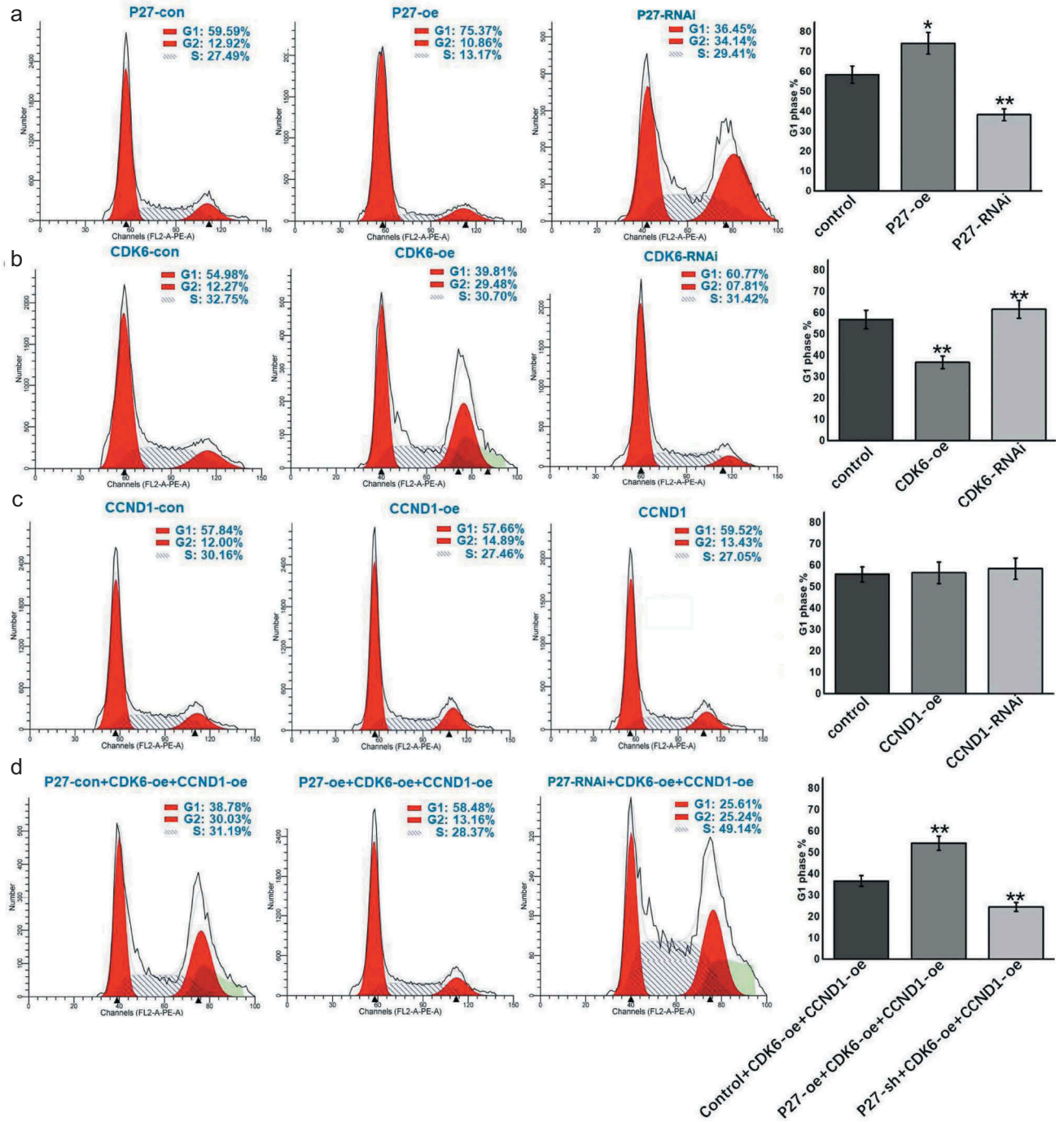

Figure 6. P27, CDK6 and CCND1 regulate the cell cycle in A549 cells. (a) Flow detection of cell cycle after changes to p27 expression. (b) Flow detection of cell cycle after changes to CDK6 expression. (c) Flow detection of cell cycle after changes to CCND1 expression. (d) Flow detection of cell cycle after changes to p27, CDK6, CCND1 expression

of CDK6/CCND1 complex formation. However, *p27* did not change the expression levels of *CDK6* or *CCND1*. The goal of our future studies may be to determine the mechanisms that affect complex formation.

P*27* is regarded as an important target for anticancer therapy [18]. In this study, positive effects on OS were observed with upregulation of *p27*. In addition, the cycle-dependent complex-associated gene *CDK6* promoted cell proliferation and negatively

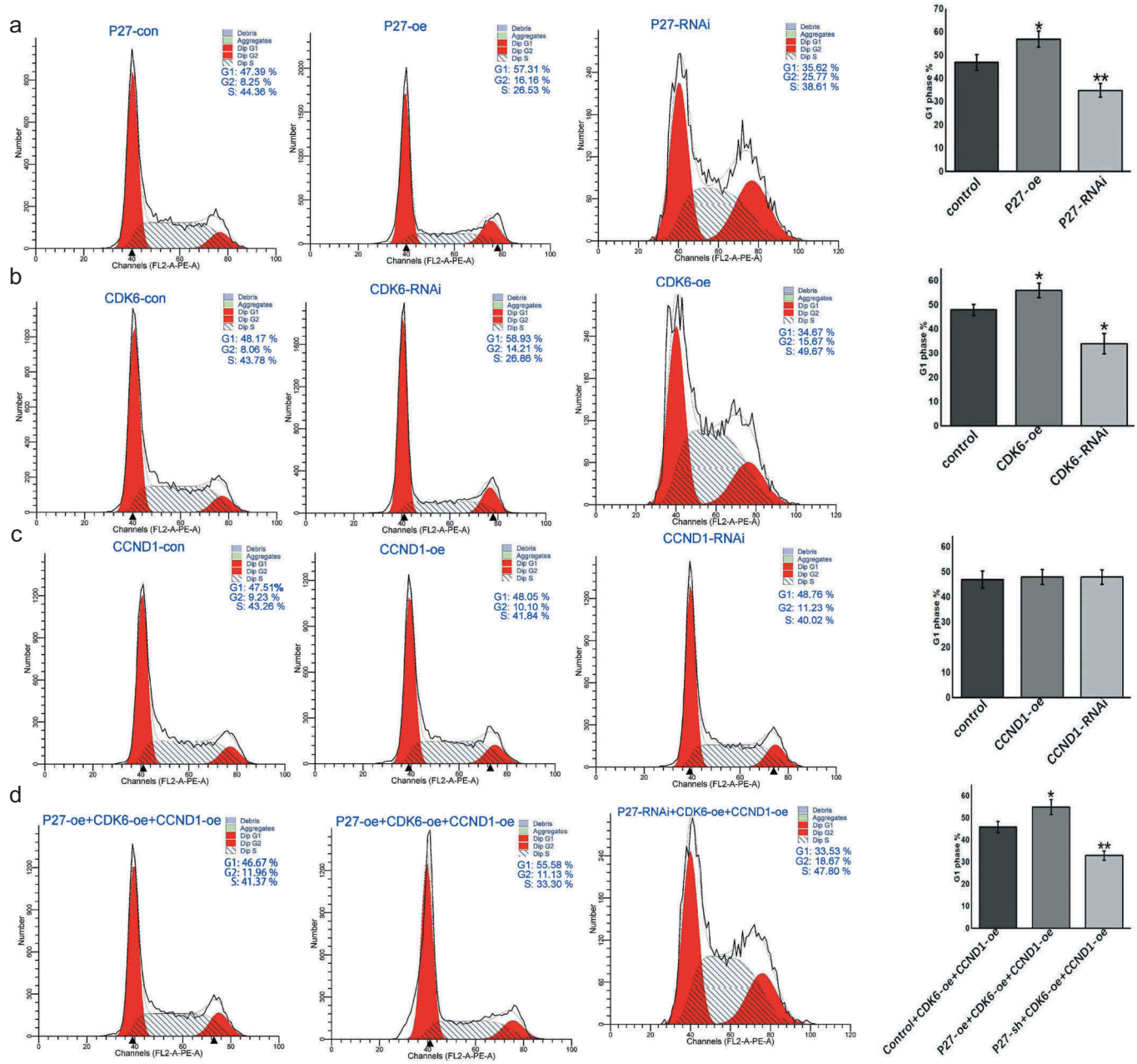

Figure 7. P27, CDK6 and CCND1 regulate the cell cycle in HTB182 cells. (a) Flow detection of cell cycle after changes to P27 expression. (b) Flow detection of cell cycle after changes to CDK6 expression. (c) Flow detection of cell cycle after changes to CCND1 expression. (d) Flow detection of cell cycle after changes to P27, CDK6, CCND1 expression.

affected the survival rate of cancer patients. However, another gene, *CCND1*, showed the opposite effect.

In summary, the results of this study demonstrated the effects of *p27* on the regulation of the cell cycle and cell proliferation. In addition, the mechanisms underlying regulation of the cell cycle by *p27* were discussed in depth, and the regulatory relationship between *p27* and the cell cycle-dependent complex genes *CDK6* and *CCND1* was explored. This study provides fundamental knowledge and information which can be used as a basis to further explore the cell cycle.

Material and methods

Cells and treatment

The A549 human lung cancer cells were supplied by the Cell Bank of Chinese Academy of Sciences

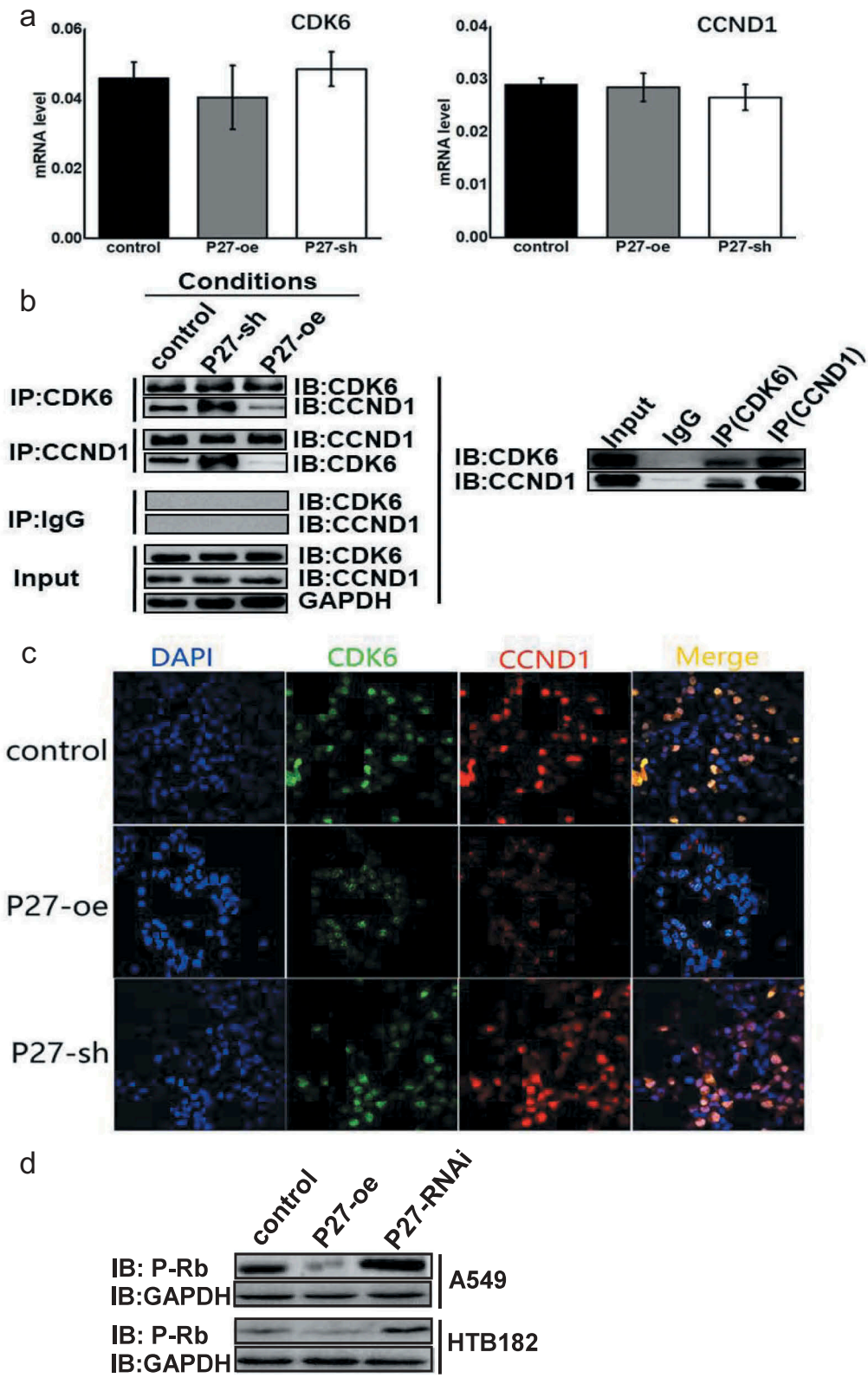

Figure 8. P27 affects the formation of CDK6/CCND1 complex. (a) qPCR was used to detect the expression of *CDK6* and *CCND1* after regulating the expression of P27 in A549 cells. (b) Co-immunoprecipitation and Western blotting were combined to examine the effects of p27 on formation of the CDK6/CCND1 complex after regulating the expression of P27 in A549 cells. (c) The distribution of CDK6 and CCND1 was detected by immunofluorescence after regulating the expression of p27 in A549 cells. (d) Phosphorylation of Rb was detected when the expression of p27 was changed in A549 and HTB182 cells.

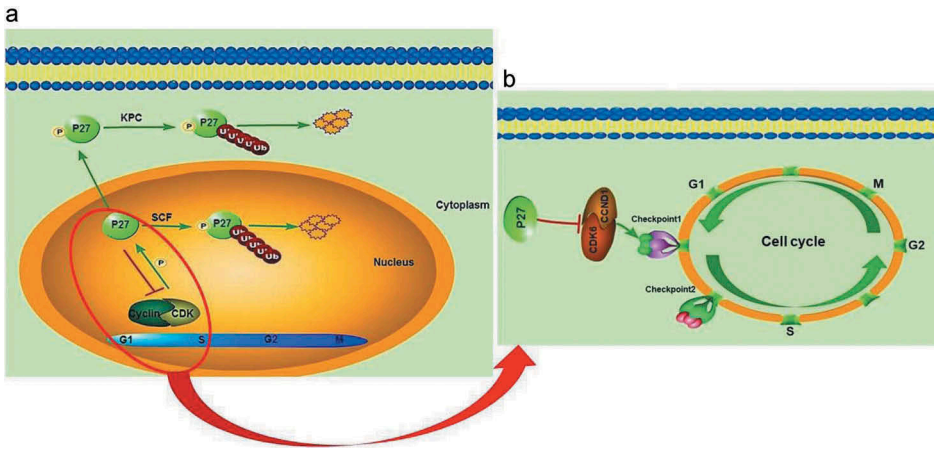

Figure 9. The roles of p27 in cell cycle regulation and tumor development. (a) The mechanism of p27 in cell cycle. P27 is ubiquitylated and degraded in late G1, S and G2 phases by SCFSkp2 in the nucleus. P27 phosphorylated at S10 is ubiquitylated by the KPC complex when exported to the cytoplasm. (b) Contributions of the study of cell cycle regulation by P27. The mechanism of p27 in cell cycle. P27 induces cell cycle arrest in the G1 phase by inhibiting the formation of cell cycle-dependent complexes CDK6/CCND1.

(Shanghai, China). For qPCR, fresh cells were frozen by immersion in liquid nitrogen and stored at −80 °C. Each experiment was performed in triplicate.

RNA extraction and cDNA synthesis

Total RNA was extracted from eggs using the MicroElute Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). Total RNA from A549 cells was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was verified spectrophotometrically with an A260/A280 ratio between 1.8 and 2.0. cDNA was synthesized from 2 μg DNase-treated total RNA samples with oligo (dT) primers and PrimeScript™ Reverse Transcriptase (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. cDNA was diluted 10-fold and used in the qPCR experiments.

Fluorescence qPCR

Fluorescence qPCR experiments were performed using a qPCR system (CFX96; Bio-Rad, Hercules, CA, USA) with the SYBR Premix EX Taq Kit (Takara, Dalian, China), according to the manufacturer's recommendations. β-actin was used as an internal control to eliminate variation in cDNA quality. Transcript abundance was calculated based on the mean of three replicates.

Cell transfection

When the cells were more than 70% confluent, they were transfected using PolyJet TM DNA Transfection Reagent (SignaGen Laboratories, Rockville, MD, USA) with the following plasmids: empty vector, *p27* overexpression vector (*p27*-oe), *p27* interference vector (*p27*-RNAi), *CDK6* overexpression vector (*CDK6*-oe), *CDK6* interference vector (*CDK6*-RNAi), *CCND1* overexpression vector (*CCND1*-oe), and *CCND1* interference vector (CCND1-RNAi). Experiments were conducted 48 h post-transfection. Related gene sequences are listed in Supplement 1.

Analysis of cell proliferation and the cell cycle

After transfection for 0, 24, 48, and 72 h, the changes in cell number and the cell cycle were detected by flow cytometry. The MTT cell viability assay was used to evaluate cell proliferation at 48 h post-transfection [19]. Briefly, the cells were placed into 96-well plates and then 10 μL MTT Reagent (Beyotime Biotechnology, Beijing, China) was added to each well, followed by a 4 h incubation in the cell culture incubator. Finally the absorbance

in each well was measured at 570 nm using a microtiter plate reader. In the cell proliferation and cell cycle assays, three replicates were used for each treatment group.

Immunoprecipitation

After cells were harvested using cell lysis buffer (containing protease inhibitors), they were incubated for 30 min at 4 °C, and centrifuged at 12,000 xg for 30 min. A small amount of lysate was used for Western blot analysis, and the remaining lysate was used for immunoprecipitation with 1 mg antibody and 10–50 uL protein A/G beads. After overnight incubation at 4 °C with shaking, the lysate was centrifuged at 3,000 xg for 5 min, and the protein A/G beads were washed three times with 1 mL lysis buffer. Finally 2x sodium dodecyl sulfate (SDS) sample buffer was added to the beads followed by SDS-polyacrylamide gel electrophoresis. The proteins were analyzed by either Western blotting or mass spectrometry.

Immunofluorescence

Changes in the distribution of CDK6 and CCND1 were detected by the immunofluorescence assay. The following primary antibodies were used: rabbit anti-CDK6 and rat anti-CCND1 (Cell Signaling Technology [CST], Danvers, MA, USA). The nucleus was stained with DAPI (Beyotime Biotechnology). Fluorescent-conjugated secondary antibodies were also obtained from Beyotime Biotechnology. Confocal microscopy was performed using the Olympus TCS SP5 confocal microscope (Olympus, Tokyo, Japan) with a 40x/1.25 oil objective.

Gene expression analysis

We used Firebrowse (http://www.firebrowse.org/) to detect *p27* expression in different tumor tissues and their corresponding normal tissues, and to compare changes in expression levels of *p27* [20]. Firebrowse allows scientists to gain access to terabytes of data and close to 1,000 reports. To systematize analyzes from The Cancer Genome Atlas (TCGA) pilot and to scale their execution to the dozens of remaining diseases to be studied, Firebrowse now sits atop ~ 40 terabytes of TCGA data and reliably executes more than 6,000 pipelines per month.

Kaplan–Meier plotter analysis

The prognostic value of *p27* in lung, gastric, and breast cancers have been analyzed using the Kaplan–Meier Plotter (http://kmplot.com/analysis/) [21], a database that integrates gene expression data and clinical data [22]. To date, the Kaplan–Meier Plotter contains information on 22,277 genes and their effects on the survival of 2,977 breast, 1,065 gastric, and 1,715 lung cancer patients. We focused our analysis on OS. To this end, the patient samples were divided into two groups: high and low *p27* expression, and compared using the Kaplan–Meier survival plot. The hazard ratio with 95% confidence intervals and log rank p value were calculated. We analyzed the best specific probes (JetSet probes) that recognized *p27* to reduce our false discovery rate, and selected $p < 0.05$ as the threshold.

Immunohistochemistry

All specimens were fixed in 4% formalin and embedded in paraffin, and cut into 4 μm thick sections, followed by immunohistochemistry using antibodies against p27 (CST).

Highlights

(1) *p27, CDK6*, and *CCND1* affect growth and development.
(2) *p27* regulates the cell cycle by affecting CDK6/CCND1 complex formation.
(3) Upregulation of *CCND1* alone is unable to effectively promote cell proliferation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

Experiments were conceived and designed by Fangyin Dai, Niannian Li and Jie Zeng. Experiments were performed by Niannian Li, Jie Zeng, Fuze Sun, Xiaoling Tong, and Gang Meng. Data were analyzed by Jie Zeng, Niannian Li, Chunman Wu, Xin Ding, and Lanlan Liu. Reagents, materials, and tools were prepared by Jie Zeng. The manuscript was prepared by Niannian Li. Xiaoling Tong, Minjin Han, Cheng Lu, and FangyinDai revised the manuscript.

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