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Systematic cancer-testis gene expression analysis identified CDCA5 as a potential therapeutic target in esophageal squamous cell carcinoma

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ABSTRACT

Background: Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies with poor prognosis. Cancer-testis genes (CTGs) have been vigorously pursued as targets for cancer immunotherapy, but the expressive patterns and functional roles of CTGs remain unclear in ESCC.

Methods: A systematic screening strategy was adopted to screen CTGs in ESCC by integrating multiple public databases and RNA expression microarray data from 119 ESCC subjects. For the newly identified ESCC prognosis-associated CTGs, an independent cohort of 118 patients with ESCC was recruited to validate the relationship via immunohistochemistry. Furthermore, functional assays were performed to determine the underlying mechanisms.

Findings: 21 genes were recognized as CTGs, in particular, *CDCA5* was aberrantly upregulated in ESCC tissues and significantly associated with poor prognosis (HR = 1.85, 95%CI: 1.14–3.01, $P = .013$). Immunohistochemical staining confirmed that positive CDCA5 expression was associated with advanced TNM staging and a shorter overall survival rate (45.59% vs 28.00% for CDCA5−/+ subjects, $P = 1.86 \times 10^{-3}$). H3K27 acetylation in *CDCA5* promoter might lead to the activation of *CDCA5* during ESCC tumorigenesis. Functionally, in vitro assay of gain- and loss-of-function of CDCA5 suggested that CDCA5 could promote ESCC cells proliferation, invasion, migration, apoptosis resistance and reduce chemosensitivity to cisplatin. Moreover, in vivo assay showed that silenced *CDCA5* could inhibit tumor growth. Mechanistically, *CDCA5* knockdown led to an arrest in G2/M phase and changes in the expression of factors that played fundamental roles in the cell cycle pathway.

Interpretation: CDCA5 contributed to ESCC progression and might serve as an attractive target for ESCC immunotherapy.

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1. Introduction

Esophageal cancer is the sixth leading cause of cancer-related death and the ninth most frequently diagnosed cancer worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is the main histology subtype and accounts for >95% of all esophageal cancer cases in China [2]. Although the prognosis of ESCC has profited from the development of diagnostic techniques and therapeutic modalities over the past decades, it remains poor with a 5-year overall survival (OS) rate ranging from 10% to 30% [3]. Therefore, it is extremely important to identify effective novel therapeutic strategies to improve the survival rate of patients with ESCC, particularly when current therapies are exhausted.

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Research in context

Evidence before this study

Cancer-testis antigens (CTAs) are promising immunotherapeutic targets of malignancies given their resilient immunogenicity and tumor-restricted expression pattern. However, systematic analysis of the specific CTAs involvement in ESCC has not been conducted.

Added value of this study

In this study, we performed a systematic screening for cancer-testis genes (CTGs) in ESCC by integrating multiple public databases with our own data. As a result, 21 CTGs were identified, 13 of which were novel. *CDCA5* was aberrantly expressed in ESCC tumor tissues and showed significant association with poor ESCC prognosis. Mechanistically, we found that *CDCA5* might be activated by the gain of H3K27ac. Furthermore, knockdown of *CDCA5* inhibited tumor growth both in vitro and in vivo through the cell cycle pathway.

Implications of all the available evidence

These findings expanded our understanding of the systematic expression of CTGs in ESCC and how CTGs drove ESCC progression. Moreover, this study proposed novel CTGs as potential targets for ESCC immunotherapy for use in the clinics.

In recent years, novel therapies for the treatment of malignant tumors have been proposed and developed due to an improved understanding of the fundamental mechanisms underlying tumor genomics and biology [4,5]. Immunotherapy is a novel treatment strategy that has emerged as an effective and promising option for various types of cancers [6]. The targeting of immune checkpoints and agonists of T-cell activation in melanoma and lung cancer have made their way into clinical practice; however, data regarding ESCC remain immature, and immunotherapy should be used within the framework of the clinical trial [7]. Nevertheless, ESCC might be excellent candidate disease for immunotherapy, in light of the abundant somatic mutations found in tumors, which might make the cancer cells more susceptible to recognition by the immune system due to neoepitope presentation on their surfaces that enhances tumor immunogenicity [7,8].

Cancer testis antigens (CTAs) are a large family of tumor-associated and immunogenic antigens that are highly expressed in cancer cells but limited in normal cells, except for cells in reproductive tissues, such as testis, ovary, and placenta [9,10]. The specific expression patterns and immunogenicity of CTAs make them perfect molecular target candidates for cancer immunotherapy [11–13]. Over the past decades, clinical trials using CTA-targeted therapeutic vaccines (such as MAGE-A and NY-ESO-1 antigens) have shown positive clinical efficacy, well-established safety and tolerability in various cancers [13–15]. However, the immunogenicity of different CTAs and their distribution in heterogeneous tumors vary significantly [13,16]. Previous studies have identified several CTAs that not only participate in the development of ESCC but also exhibit potential as therapeutic targets for ESCC based on a candidate gene strategy [17–21]. For example, the induction of NY-ESO-1 immunity and preferable outcomes were observed in a clinical trial of patients with ESCC vaccinated with NY-ESO-1 [22]. Similarly, a cancer vaccine therapy using three HLA-A24-restricted epitope peptides derived from three CTAs (TTK, LY6K and IMP-3) demonstrated satisfactory safety, strong immunogenicity and a high rate of disease control for patients with advanced ESCC [23]. These promising findings motivated us to explore the specific CTAs in ESCC and to provide effective immunotherapies for ESCC.

In our previous study, we established a maneuver to systematically explore the molecular landscape of cancer testis genes (CTGs) and successfully identified 876 novel CTGs across 19 cancer types using transcriptomics data from multiple independent public-available databases; unfortunately, the ESCC was not included [24]. In the present study, we performed a systematic analysis based on public-available databases and our published RNA microarray data from 119 paired ESCC samples to identify specific CTAs in ESCC. Associations between our identified CTGs and ESCC prognosis were evaluated, followed by validation in an independent ESCC cohort with 118 specimens. Furthermore, functional assays were conducted to evaluate the biological functions and potential therapeutic value of novel CTGs in ESCC. Overall, this study would provide us a deeper insight into the roles of CTGs in ESCC and prospective immunotherapeutic targets for ESCC treatment.

2. Materials and methods

2.1. Databases used in this study

Several public-available databases were used in this study, including GTEx, HBM and HPM. Transcriptomics data from GTEx, HBM and our NJMU-seq were analyzed to screen the testis-specific genes (TSGs). We used the proteomic data from the HPM database to define the testis-specific proteins (TSPs). RNA expression microarray data from 119 paired ESCC samples were further adopted to determinate candidate CTGs in ESCC. Detailed information about these databases and samples have been described in our previous papers [24,25].

2.2. Study subjects

A total of 118 ESCC patients were recruited from the First Affiliated Hospital of Nanjing Medical University. All patients received esophagectomy between January 2002 and December 2003. Patients were diagnosed as having primary ESCC by a pathological examination and received no chemo-or radio-therapy prior to surgery. This study was approved by the medical ethics committees of the First Affiliated Hospital of Nanjing Medical University.

2.3. Immunohistochemistry

Tissue microarrays (TMAs) of the 118 subjects were constructed from paraffin-embedded tissue blocks. For each tumor, a representative area was carefully selected from a hematoxylin and eosin-stained section. For each case, normal tissue and cancer tests were repeated twice. We used the avidin-biotin complex method to perform the immunohistochemistry (IHC) analysis. In brief, xylene was used for deparaffinization followed by rehydrating (ethanol) and rinsing (0.1 M PBS, pH 7.4). After incubating at 95 °C for 20 min in a citrate buffer (10 mM, pH 6.0), the sections were cooled to 30 °C and rinsed using 0.1 M PBS. Then, 3% hydrogen peroxide was used to inactivate the endogenous peroxidase and binding sites were blocked by incubating in 10% normal animal serum for 30 min. Sections were then incubated at 4 °C for 24 h with a primary antibody for CDCA5 (Abcam, ab210610; 1/200) and followed by incubation with the 2-step Polymer Detection System (Polink-2 Plus, GBI, USA) at room temperature. Dako Envision system was used for the detection with diaminobenzidine (DAB) as the chromogen. Finally, the specimens were lightly counterstained using Mayer's hematoxylin and then dehydrated and mounted. We established the negative controls by replacing the specific primary antibody with animal serum.

Two experienced pathologists who were blind to these subjects were responsible for the final IHC scores, which were calculated by combining both staining intensity and extent. Staining intensity was

divided into four grades: 0 (no), 1 (weak), 2 (moderate), and 3 (strong), and staining extent was graded as 0 (≤10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). Samples with an IHC score ≥ 3 were considered as positive; otherwise, they were designated as negative.

2.4. Cell culture and transfection

The human normal esophageal epithelial cell line HET-1a and the ESCC cell lines KYSE30, ECA109, KYSE70, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510 and TE-1 were purchased from Genechem Co. Ltd. (Shanghai, China). HET-1a cells were cultured in DMEM (Gibco, USA), and other cells were cultured in RPMI-1640 medium (Gibco, USA); 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin G sodium/streptomycin sulphate were also used with a humidified atmosphere consisting of 95% air and 5% CO_2 at 37 °C.

Short hairpin (shRNA) RNAs for human CDCA5 were cloned into a phU6-MCS-Ubiquitin-EGFP-IRES-puromycin plasmid (GV280, Genechem, China). To knock down endogenous CDCA5, we used two lentivirus vector sequences 5′-GCAGTTTGATCTCCTGGTT-3′ (named KD1), and 5′-AGAAACAGAAACGTAAGAA-3′ (named KD2) and one scrambled control sequence 5′-TTCTCCGAACGTGTCACGT-3′ (named NC). The transfection of the plasmids was performed using Lipofectamine 3000 reagent (Invitrogen, USA). Lentiviral infection was used to obtain stable cell lines with a low expression of sh-CDCA5 in KYSE30 and Eca109 cells. For the chemosensitivity array to cisplatin and mouse xenograft assay, only ESCC cells of KD2 group were used because of its optimum knockdown efficiency.

KYSE150 cells, which have a low CDCA5 expression, were transfected with pcDNA-CDCA5 (seq: 5′-GAGGATCCCCGGGTACCGGTC GCCACCATGTCTGGGAGGCGAACGCG-3′, named OE) or a negative control (named NC) sequence as before. The qualified ones were selected with 0.5 mg/l puromycin for 10 days.

2.5. RNA extraction, reverse transcription, and real-time PCR

The Total RNA Isolation Reagent - SuperfecTRI (Pufei Biotech, China) was used to extract the total RNA. The concentration and quality of RNA were evaluated using Nano Drop 2000C Spectrophotometer (Thermo, USA) and agarose gel electrophoresis. Complementary DNA (cDNA) was generated by using the M-MLV Reverse Transcriptase Kit (Promega, China) according to the manufacturer's protocol. The primers (RiboBio, Guangzhou, China) for human CDCA5 were as follows: F: 5′-AGAAAG TCAGGCGTTCCTACAG-3′ and R: 5′-GGGAGATTCCAGGGAGAGTCAT-3′. Real-time PCR was performed on the LightCycler480 System (Roche, USA) using SYBR Prime Script RT-PCR Kits (Takara, Japan). The expression of *CDCA5* was calculated using the $2^{-\Delta\Delta}$Ct method with GAPDH as the reference gene.

2.6. Cell function tests

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Genview, USA) and colony formation assay. Flow cytometry analysis (Guava easyCyte HT, Millipore, USA) was used to detect the cell cycle distribution. Cell apoptosis was assessed by an Annexin V-FITC early apoptosis detection kit (eBioscience, USA) with Annexin V and PI double staining according to the manufacturer's instructions. Tumor cell invasion and migration activities were performed using Transwell plates (8 μm; Corning, USA). Exactly 40 μL ECM gel (Sigma, USA) was added to each Transwell for cell invasion assay. The effect of CDCA5 on the chemosensitivity of the ESCC cells to cisplatin (DDP) was assessed by MTT assay. Cells were treated with 10 μM DDP for 48 h. All assays were performed in triplicate.

2.7. Chromatin immunoprecipitation

Formaldehyde treatment crosslinks proteins to DNA to ensure co-precipitation. Cells were lysed and sonication was performed to shear the chromatin to manageable size. The average fragment size of DNA was confirmed by gel electrophoresis to ensure fragment sizes of 200–1000 bp. We used One-Day Chromatin Immunoprecipitation Kits (Cat#17-408, EZ-Magna ChIP, Millipore) for the Chromatin immunoprecipitation (ChIP) assay according to the manufacturer's protocol. Anti-Histone H3 (acetyl K27) antibody-ChIP Grade was obtained from Abcam (ab177178). Protein-DNA crosslinks were reversed and DNA was purified to remove the chromatin proteins and prepare the DNA for real-time PCR analysis. We designed 2 pairs of primers and the sequences were: Primer 1: F: 5′-AGGAAGCCAATACCGCCTTG —3′ and R: 5′-ACTGCCTGGTAGCCAATCAC-3′; Primer 2: F: 5′-TATCACCCCAAGGT CCGACT-3′ and R: 5′-ACTGCCTGGTAGCCAATCAC-3′.

2.8. Mouse xenograft assay

A total of 4×10^6 KYSE30 or Eca109 cells (in which *CDCA5* was stably expressed or knocked down, respectively) in 0.1 ml phosphate-buffered saline were subcutaneously injected into the right or left oxter of female BALB/c nude mice at 4 weeks of age (SLAC Laboratory Animal Co., Ltd., Shanghai, China). Tumor size was measured with calipers every three days. Mice were euthanized by anesthesia 21 days after injection. The tumors were removed from the mice and then measured. All animal studies were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Nanjing Medical University.

2.9. Western blot

Proteins from the KYSE30 and Eca109 cells were extracted using a lysis buffer (P0028, Beyotime) after being lysed in a RIPA lysis buffer (P0013, Beyotime). The cell lysates were boiled in 5× SDS-PAGE loading buffer for 10 min and then resolved by 8% SDS-PAGE. These lysates were transferred to a nitrocellulose membrane. Antibodies for CCNA2, CCNB1, CDC25A, PCNA and CDCA5 were purchased from Abcam. Tubulin (Maixin, China) was used as the internal reference and bound antibodies were visualized using an ECL kit (P0018, Beyotime).

2.10. Statistics

χ^2 test was used to compare the difference distributions of categorical variables between subgroups. Associations between clinicopathological factors and the prognosis of ESCC patients were evaluated based on Cox proportional hazards regression analysis. The OS or disease-free survival was defined as from the time of operation to the date of death or disease relapse. Age, sex, TNM staging, tumor differentiation grade and tumor location were adjusted when appropriate. Differential gene expression analysis was performed using paired Student's *t*-test. Pearson correlation was adopted to conduct the gene co-expression analysis using the RNA expression microarray data of 119 ESCC samples. A correlation coefficient > 0.5 and a P value < .05 were defined as significantly correlated. Gene Ontology (GO) analysis was performed based on the DAVID Bioinformatics Resources 6.8 database (https://david.ncifcrf.gov/home.jsp). All analyses were performed using R 3.5.1 software.

3. Results

3.1. Characteristics of study subjects

In this study, multiple public databases were combined with the RNA expression microarray data from 119 ESCC samples to screen for CTGs in ESCC, followed by IHC validation in 118 recruited ESCC cases. The characteristics of study subjects are shown in Table 1. The OS rate

Table 1
Characteristics of subjects in screening and validation stages.

Characteristics	RNA expression array samples: 119					IHC Validation: 118				
	Dead (N = 73)	Alive (N = 46)	OS (%)	HR (95%CI)[a]	P[a]	Dead (N = 73)	Alive (N = 45)	OS (%)	HR (95%CI)[a]	P[a]
Gender					0.505					0.652
Male	58 (79.45%)	40 (86.96%)	40.82%	1.00		46 (63.01%)	30 (66.67%)	39.47%	1.00	
Female	15 (20.55%)	6 (13.04%)	28.57%	1.22 (0.69–2.15)		27 (36.99%)	15 (33.33%)	35.71%	1.12 (0.69–1.80)	
Age					0.059					0.886
≤60 years	38 (52.05%)	31 (67.39%)	44.93%	1.00		34 (46.58%)	22 (48.89%)	39.29%	1.00	
>60 years	35 (47.95%)	15 (32.61%)	30.00%	1.56 (0.99–2.48)		39 (53.42%)	23 (51.11%)	37.10%	1.03 (0.65–1.64)	
T stage					0.898					0.035
T1-2	18 (24.66%)	10 (21.74%)	35.71%	1.00		10 (13.70%)	13 (28.89%)	56.52%	1.00	
T3-4	55 (75.34%)	36 (78.26%)	39.56%	0.97 (0.57–1.64)		63 (86.30%)	32 (71.11%)	33.68%	1.95 (1.00–3.79)	
N stage					0.002					1.85×10^{-5}
N_0	24 (32.88%)	30 (65.22%)	55.56%	1.00		33 (45.21%)	36 (80.00%)	52.17%	1.00	
N_+	49 (67.12%)	16 (34.78%)	24.62%	2.16 (1.32–3.53)		40 (54.79%)	9 (20.00%)	18.37%	2.76 (1.74–4.40)	
Differentiation										
G1	14 (19.18%)	9 (19.57%)	39.13%	1.00		27 (36.99%)	19 (42.22%)	41.30%	1.00	
G2	36 (49.32%)	28 (60.86%)	43.75%	0.87 (0.47–1.61)	0.655	37 (50.68%)	23 (51.11%)	38.33%	1.05 (0.64–1.72)	0.849
G3	23 (31.50%)	9 (19.57%)	28.13%	1.36 (0.70–2.66)	0.360	9 (12.33%)	3 (6.67%)	25.00%	1.98 (0.93–4.21)	0.078
Location										
Upper	11 (15.07%)	3 (6.52%)	21.43%	1.00		4 (5.48%)	1 (2.22%)	20.00%	1.00	
Middle	40 (54.79%)	29 (63.04%)	42.03%	0.64 (0.33–1.25)	0.193	18 (24.66%)	12 (26.67%)	40.00%	0.47 (0.16–1.41)	0.179
Lower	22 (30.14%)	14 (30.43%)	38.89%	0.71 (0.35–1.48)	0.365	51 (69.86%)	32 (71.11%)	38.50%	0.48 (0.17–1.34)	0.161
CDCA5					0.013					1.86×10^{-3}
Negative (Low expression)	30 (41.10%)	30 (65.22%)	50.00%	1.00		37 (50.68%)	31 (68.89%)	45.59%	1.00	
Positive (High expression)	43 (58.90%)	16 (34.78%)	27.12%	1.85 (1.14–3.01)		36 (49.32%)	14 (31.11%)	28.00%	2.27 (1.36–3.82)	

[a] Based on cox proportional hazards regression analysis, age, gender, TNM stage, tumor differentiation grades and tumor location were adjusted when appropriate.

of the patients in the screening dataset was 38.66% (46/119), which was similar to that in the IHC validation dataset (38.14%, 45/118). Age, sex, tumor differentiation grade and tumor location showed no significant association with the prognosis of ESCC patients, whereas lymph node stage was significantly associated with ESCC prognosis. Samples with positive lymph node metastasis had lower OS rate (55.56% vs 24.62% for N−/N+ subjects, respectively, Cox proportional hazards regression analysis, $P = .002$) in the screening dataset. A consistent result was also observed in the validation dataset (52.17% vs 18.37% for N−/N+ patients, respectively, Cox proportional hazards regression analysis, $P = 1.85 \times 10^{-5}$).

3.2. Systematic screening for CTGs in ESCC

A flow chart of this study is shown in Fig. 1a. Briefly, 32,080 probes were included in the RNA expression microarray. Probes without gene symbols or transcript IDs, or probes with a detection rate ≤ 80% in all samples were excluded. As a result, 25,890 probes were initially retained. Of these probes, genes that were not contained in the Gencode v19 database were further removed. Then, the genes that belonged to the 1336 testis-specific genes (C1 class) identified by our previous study were selected [24]. Consequently, 764 probes (corresponding to 690 TSGs) were conserved as candidate CTGs in ESCC (Table S1). Of these genes, 21 genes showed aberrant expression (Paired Student's *t*-test, fold change ≥ 2 & $P < .05$) in ESCC tumor tissues and were therefore considered as CTGs in ESCC (Table S2 and Fig. S1a). Among them, 8 genes (*MAGEA4*, *KIF2C*, *CABYR*, *CAGE1*, *MAGEA2B*, *MAGEA11*, *PRAME* and *MAGEA12*) were recognized as CTGs in previous studies [11,26–29], however, 13 genes were newly identified as ESCC specific CTGs in this study.

3.3. Higher CDCA5 mRNA expression was associated with poorer ESCC prognosis

To gain insight into the roles of the identified 21 CTGs in the prognosis of ESCC, survival analysis was performed. Notably, *CDCA5* was the unique one that was overexpressed in ESCC tumor tissues and associated with the poor ESCC prognosis (Fig. S1b). As shown in Fig. 1b & c, *CDCA5* was upregulated in 99.16% (118/119) of all ESCC tumor tissues compared with adjacent normal tissues (Paired Student's *t*-test, $P = 3.79 \times 10^{-52}$). Furthermore, we identified the restricted pattern of CDCA5 over-expression in testis and tumor tissues by analyzing public-available databases, and the expression of *CDCA5* in various normal tissues and tumor tissues was shown in Fig. S2. Patients with higher *CDCA5* expression (the median expression value was set as the cut-off) showed a significantly increased mortality risk (Cox proportional hazards regression analysis, HR = 1.85, 95%CI: 1.14–3.01, $P = .013$) and reduced OS rate (27.12% vs 50.00%, Table 1, Fig. 1d) compared with those with lower *CDCA5* expression.

3.4. CDCA5 expression was validated in an independent cohort of ESCC patients

For the validation of CDCA5 at the protein level, 118 ESCC patients were recruited from our hospital. As shown in Table 1, the medium survival time was 23 months with 73 deaths in the validation dataset. IHC detection showed that CDCA5 was aberrantly elevated in ESCC tumor tissues (Fig. 1e). As a result, 50 of the 118 ESCC samples showed positive expression of CDCA5 and were correlated with poorer prognosis than the negative cases (Cox proportional hazards regression analysis, HR = 2.27, 95%CI: 1.36–3.82, $P = 1.86 \times 10^{-3}$, Table 1 and Fig. 1f). The OS rate was 45.59% for CDCA5- subjects, while 28.00% for the CDCA5+ patients. In addition, CDCA5 expression was significantly associated with the disease-free survival and the cumulative relapse rate of ESCC (Cox proportional hazards regression analysis, HR = 1.85, 95%CI: 1.17–2.94, $P = .007$, Fig. S3). The disease-free survival rate was 44% for the CDCA5- subjects, while 23.2% for the CDCA5+ patients.

3.5. Positive expression of CDCA5 was associated with advanced TNM stages

To uncover the possible mechanism by which *CDCA5* modulated ESCC prognosis, we analyzed the associations between CDCA5 protein expression and clinicopathological characteristics. Positive expression

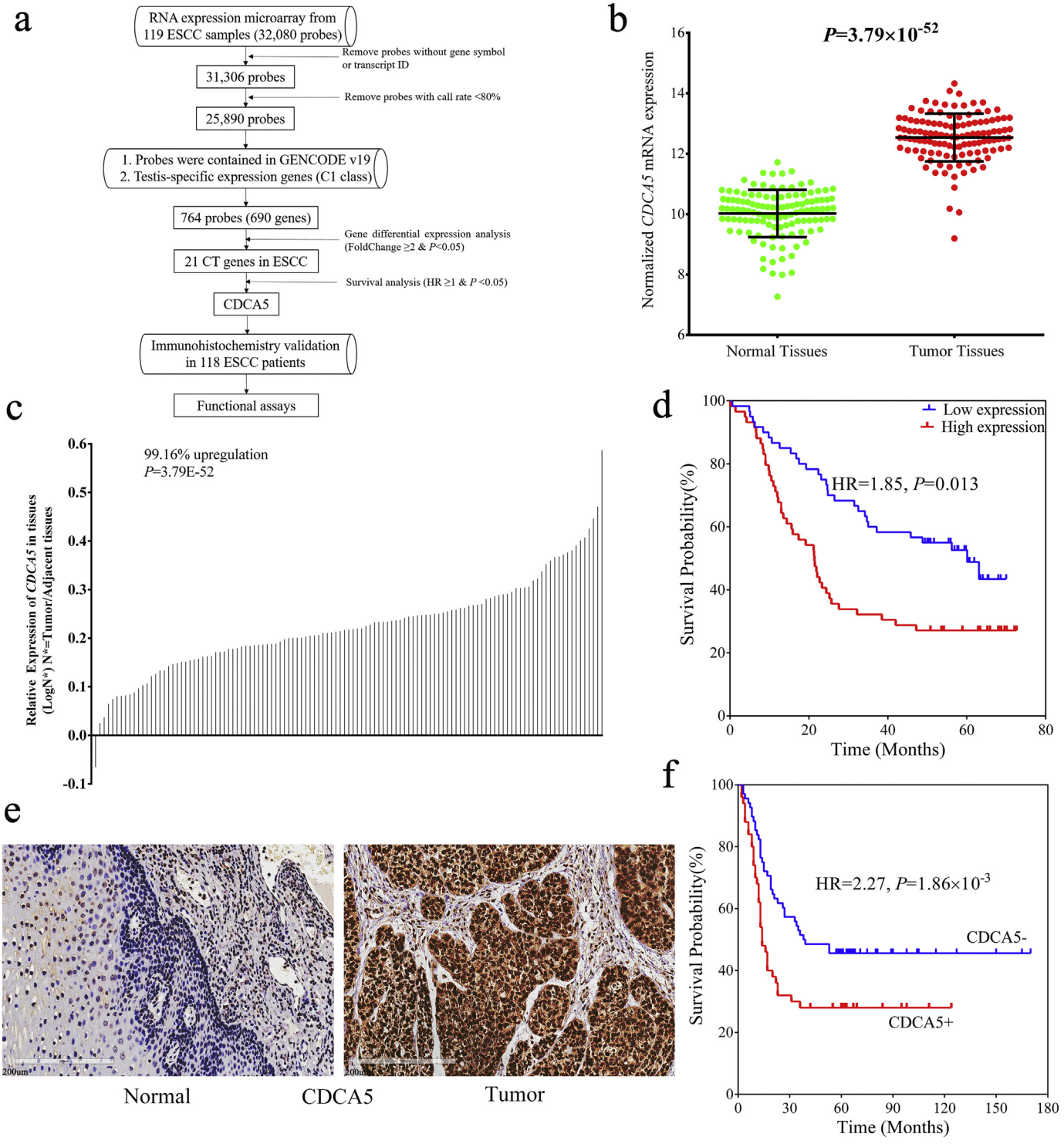


Fig. 1. *CDCA5* was aberrantly upregulated in ESCC tumor tissues and significantly associated with poor ESCC prognosis. (a) Flowchart of this study; (b–c) *CDCA5* was significantly elevated in ESCC tumor tissues based on the RNA expression microarray data from 119 paired ESCC samples (Mean ± SD, Paired Student's *t*-test, $P < .001$); (d) ESCC patients with higher *CDCA5* mRNA expression showed poorer prognosis (Log-rank test, $P = .013$); (e) Expression of CDCA5 protein was increased in ESCC tumor tissues compared with adjacent normal tissues (Bar represents 200 μm); (f) Positive CDCA5 expression was significantly associated with poor prognosis of ESCC in the IHC validation dataset (Log-rank test, $P = 1.86 \times 10^{-3}$).

of CDCA5 was significantly associated with T stage (χ^2 test, $P = .007$), N stage (χ^2 test, $P = .018$) and TNM stage (χ^2 test, $P = .008$, Table 2). Patients with positive CDCA5 expression had a higher percentage of advanced T stage (92.0% vs 72.1%), N stage (54.0% vs 32.4%) and TNM stage (52.0% vs 27.9%). These findings suggested that *CDCA5* might modulate the prognosis of ESCC patients by promoting tumor cells proliferation and migration.

3.6. *CDCA5* was epigenetically activated in ESCC cells

Previous studies have revealed the roles of *CDCA5* in other cancer types [30–32]. However, the role of *CDCA5* in ESCC is not well understood. *CDCA5* expression was aberrantly elevated in ESCC cells (Fig. 2a). Of these cells, KYSE30 and ECA109 cells showed the most abundant expression of *CDCA5* and were therefore selected as the

Table 2
Associations between CDCA5 protein expression and clinicopathological factors.

Factors	N = 118	Negative (n = 68)	Positive (n = 50)	P[a]
Gender				0.937
Male	76	44 (64.7%)	32 (64.0%)	
Female	42	24 (35.3%)	18 (36.0%)	
Age				0.397
≤60 years	56	30 (44.1%)	26 (52.0%)	
>60 years	62	38 (55.9%)	24 (48.0%)	
T stage				0.007
T_{1-2}	23	19 (27.9%)	4 (8.0%)	
T_{3-4}	95	49 (72.1%)	46 (92.0%)	
N stage				0.018
N_0	69	46 (67.6%)	23 (46.0%)	
N_+	49	22 (32.4%)	27 (54.0%)	
Differentiation				0.604
G1	46	29 (42.6%)	17 (34.0%)	
G2	60	33 (48.5%)	27 (54.0%)	
G3	12	6 (8.9%)	6 (12.0%)	
Location				0.397
Upper	5	2 (2.9%)	3 (6.0%)	
Middle	30	15 (22.1%)	15 (30.0%)	
Lower	83	51 (75.0%)	32 (64.0%)	
TNM stage				0.008
I–II	73	49 (72.1%)	24 (48.0%)	
III–IV	45	19 (27.9%)	26 (52.0%)	

[a] Based on χ^2 test.

model cells for the following functional assays. Epigenetic modifications such as methylation, acetylation and phosphorylation were one of the most common mechanisms of regulation of the CTGs expression [13,33,34]. To suggest the possible activation mechanism of *CDCA5* in ESCC, we performed functional annotations based on the ENCODE database. As shown in Fig. 2b, the high enrichment of H3K27Ac (acetylation of histone H3 at lysine 27), which represents an active enhancer marker, was observed at the promoter of *CDCA5*, which suggested that the acetylation of the *CDCA5* promoter might enhance the promoter activity of *CDCA5*. As we expected, the anti-H3K27Ac levels in both KYSE30 (Student's *t*-test, $P < .01$) and Eca109 (Student's *t*-test, $P < .05$) cells were significantly higher than that in Het-1A cells. These findings suggested that *CDCA5* promoter acetylation might at least partially account for the activation and upregulation of *CDCA5* during ESCC tumorigenesis.

3.7. Knockdown of CDCA5 inhibits ESCC cells proliferation, migration and invasion, cell cycle arrest and promotes cell apoptosis

ESCC cells with suppressed *CDCA5* expression were obtained by using two lentivirus vectors (Fig. S4). As shown in Fig. 2c–d, the knockdown of CDCA5 significantly reduced cell viability and proliferation in both KYSE30 and ECA109 cells (Student's *t*-test, $P < .001$). In addition, results from the flow-cytometry analysis indicated that cells (both KYSE30 and ECA109) with *CDCA5* knockdown had higher percentage in G2/M phase (Student's *t*-test, $P < .01$, Fig. 2e), suggesting that the knockdown of *CDCA5* could induce cell cycle arrest in ESCC. Furthermore, inhibition of *CDCA5* in KYSE30 and ECA109 cells significantly promoted the cell apoptosis (Student's *t*-test, $P < .001$, Fig. 3a). These findings provided biological evidence that *CDCA5* was associated with tumor proliferation. Moreover, consistent with results shown in Table 2, knockdown of *CDCA5* significantly repressed the invasion (Student's *t*-test, $P < .001$, Fig. 3b) and migration (Student's *t*-test, $P < .001$, Fig. 3c) of ESCC cells.

3.8. Overexpression of CDCA5 promotes ESCC cells proliferation, invasion and migration

CDCA5 overexpression assays were performed to further confirm the effect of *CDCA5* on the proliferation, invasion and migration of ESCC cells. As shown in Fig. 3d, *CDCA5* was successfully overexpressed in KYSE150 cells (Student's *t*-test, $P < .001$). The MTT assay showed that the overexpression of *CDCA5* promoted ESCC cells proliferation (Fig. 3e, Student's *t*-test, $P < .001$). In addition, the invasion and migration of ESCC cells with CDCA5 overexpression were significantly enhanced compared with the NC group (Fig. 3f, Student's *t*-test, $P < .001$). Both the knockdown and overexpression of CDCA5 assays proved that CDCA5 functioned as an oncogene and could promote the progression of ESCC.

3.9. CDCA5 knockdown enhances the chemosensitivity of ESCC cells to cisplatin

Furthermore, we explored the effect of *CDCA5* on the sensitivity of ESCC cells to cisplatin. As shown in Fig. 4a, KYSE30 and Eca109 cells were cultured in 10 μM, 20 μM, 50 μM and 100 μM DDP for 48 h. The concentration of 10 μM DDP was chosen to perform the following assays. The cell viability of ESCC cells with *CDCA5* knockdown by using lentivirus was significantly decreased compared with the viability of the NC cells (Fig. 4b–c, Student's *t*-test, $P < .001$). Furthermore, *CDCA5* knockdown cells treated with DDP showed a significantly reduced cell viability than *CDCA5* knockdown cells or DDP culture cells alone (both KYSE30 and Eca109 cells, Student's *t*-test, $P < .001$). This finding suggested that *CDCA5* could induce cisplatin resistance and that the inhibition of *CDCA5* could enhance the sensitivity of ESCC cells to cisplatin.

3.10. Inhibition of CDCA5 suppresses tumor growth in vivo

As described above, *CDCA5* has been proven to promote the progression of ESCC in vitro. Furthermore, we evaluated the effects of *CDCA5* on tumor in vivo by a mouse xenograft assay. Three models were constructed: (1) NC: without transfection; (2) Vector: transfected with an empty vector; (3) CDCA5-KD: transfected with cells that *CDCA5* was stable knockdown (KYSE30 or ECA109). As shown in Fig. 4d–e, tumor size was measured every three days and the CDCA5-KD group (both KYSE30 and ECA109 cells) had smaller tumor size than the NC or Vector group (Student's *t*-test, $P < .01$). Similarly, tumor weight in CDCA5-KD group was significantly lower compared with that in NC or Vector group (Student's *t*-test, $P < .01$). The knockdown efficiency of *CDCA5* in established xenograft tumors is shown in Fig. 4f. All these findings suggested that knockdown of *CDCA5* could inhibit tumor growth in vivo and *CDCA5* might be a potential therapeutic target for the treatment of ESCC.

3.11. CDCA5 exerted its activity via influencing the cell cycle pathway

To explore the underlying mechanisms and the potential pathway through which *CDCA5* promoted ESCC progression. As shown in Fig. 5a, 30 genes were aberrantly expressed (Fold change ≥ 2 & $P < .05$) and showed significant correlation with the expression of *CDCA5* in ESCC tumor tissues. GO analysis suggested that *CDCA5* might mainly participate in cell cycle related biological processes according to the KEGG database (Fig. 5b). Specifically, *CCNA2*, *CCNB1*, *CDC25A* and *PCNA* were considered as the key members in the cell cycle pathway and showed closely co-expression with *CDCA5* (Fig. 5c and e). Western blot assay confirmed that the expression of CCNA2, CCNB1, CDC25A and PCNA was greatly decreased in CDCA5-knockdown cells (both KYSE30 and ECA109, Fig. 5e). These findings suggested that *CDCA5* could promote the progression of ESCC by activating the cell cycle pathway.

4. Discussion

Recent prominent advances in the identification and characterization of novel specific molecular targeting has enhanced the development of innovative cancer treatment strategies, including new types of therapeutic agents or antibodies and cancer vaccines [35,36].

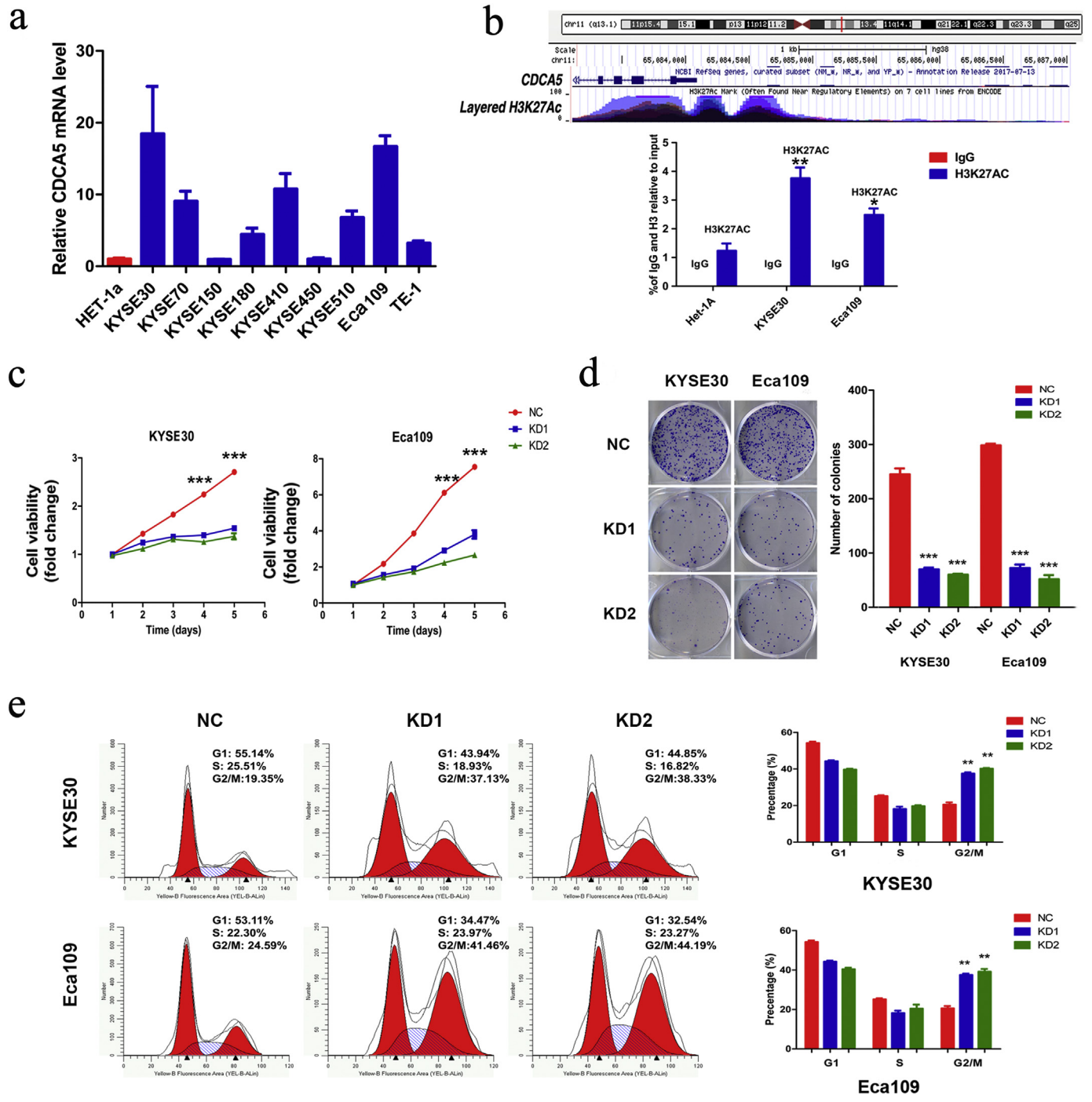
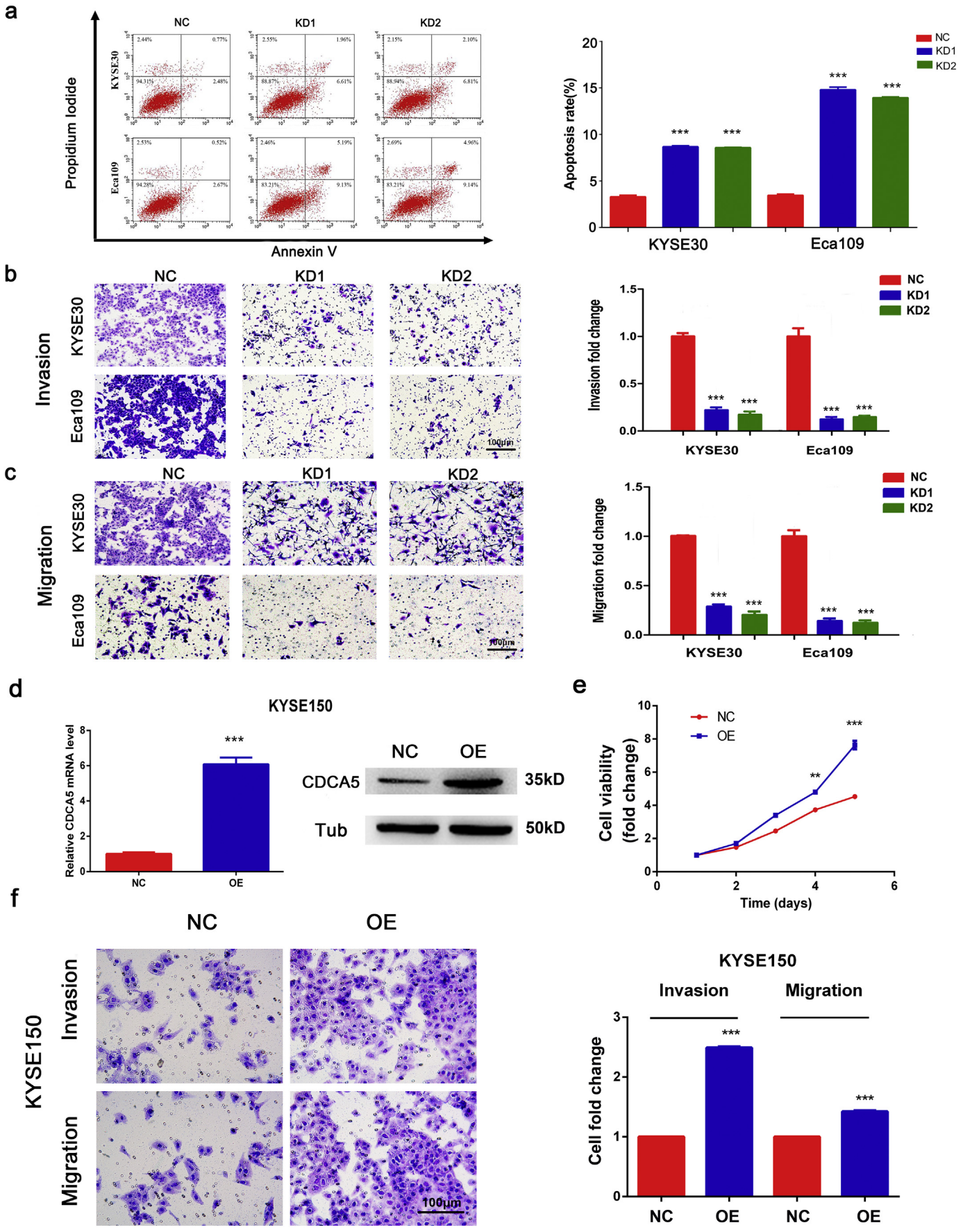


Fig. 2. *CDCA5* promotes tumor proliferation, cell cycle arrest and its potential regulation. (a) Expression of *CDCA5* mRNA in HET-1a and ESCC cell lines; (b) H3K27Ac (acetylation of histone H3 at lysine 27) was enriched at the promoter of *CDCA5* gene (Mean ± SD, Student's *t*-test, $^{*}P < .05$, $^{**}P < .01$); (c) *CDCA5* knockdown inhibited proliferation in both KYSE30 and Eca109 cells (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (d) The amount of colony formation of ESCC cells was decreased in the *CDCA5* knockdown group compared with the control (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (e) KYSE30 and Eca109 cells transfected with shRNAs-CDCA5 exhibited a G2/M phase arrest (Mean ± SD, Student's *t*-test, $^{**}P < .01$).

Molecular targeted drugs are expected to be highly specific to malignant cells, with minimal adverse effects due to their well-defined mechanisms. One attractive strategy to achieve this goal is to combine the power of a comprehensive analysis to effectively screen for genes that are overexpressed in cancer cells but minimally expressed in normal organs. CTAs are multifunctional protein group with specific expression patterns in various types of cancer cells; they are considered as unique and promising cancer biomarkers and targets for cancer therapy [13]. Using our systematic approach, 21 CTGs were identified. Of these CTGs, 8 have been recognized, while the other 13 CTGs were considered as novel in ESCC.

Of the 8 reported CTGs, *MAGEA4*, *MAGEA2B*, *MAGEA11* and *MAGEA12* belong to the *MAGEA* family, which is a well-known highly conserved family of CTGs located on the human X-chromosome q28 [13]. MAGEA proteins are processed by the intracellular proteasome and their peptides are presented by the MHC class I molecules on the surface of cancer cells thereby making them ideal cancer specific antigens. Growing evidence supports MAGEA protein involvement in the regulation of the processes that underlie cancer cell survival, tumor formation, and metastasis [37,38]. For instance, MAGEA4 inhibits the apoptosis of cancer cells by suppressing endogenousp53, or by enhancing malignant progression via p53-independent pathways [39]. Simultaneously, the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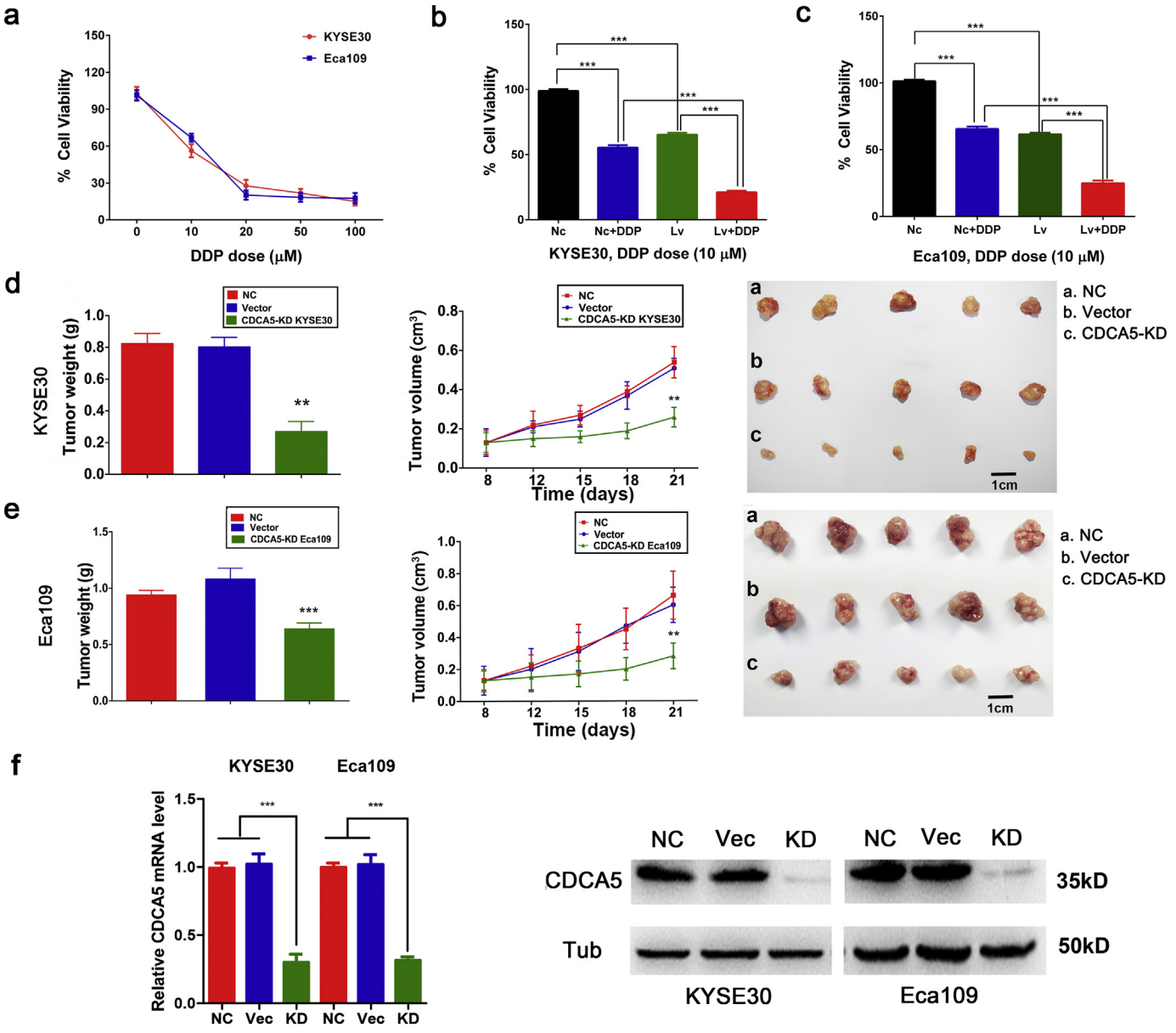

Fig. 4. Knockdown of *CDCA5* enhances the chemosensitivity of ESCC cells to cisplatin and inhibits the tumor growth in vivo. (a) Dose-response curves showing the effect of DDP at different concentrations (0, 10 μM, 20 μM, 50 μM and 100 μM) on the cells viability of KYSE30 and Eca109; (b–c) Inhibition of *CDCA5* enhanced the chemosensitivity of KYSE30 (b) and Eca109 (c) cells to cisplatin (Mean ± SD, Student's *t*-test, $^{***}P < .001$); Lv: lentivirus transfection; (d–e) *CDCA5* knockdown significantly inhibited tumor growth in vivo (both Eca109 and KYSE30 cells, (Mean ± SD, Student's *t*-test, $^{**}P < .01$, $^{***}P < .001$)); (f) The *CDCA5* knockdown efficiency in established xenograft tumors.

overexpression of TWIST1, a bHLH transcription factor, may transcriptionally up-regulate the expression of MAGEA4 to activate cancer cell migration-invasion program [40]. In addition, MAGEA11 can activate androgen receptor signaling by forming a molecular bridge between transcriptionally active androgen receptor dimers to contribute to cancer cell growth [41]. Most recently, MAGEA11 was found to promote oncogenesis through interactions with retinoblastoma-related protein p107 and the E2F1 transcription factor, which is important for cell cycle progression and apoptosis [42]. Regarding the other 4 reported CTGs (*KIF2C*, *CABYR*, *CAGE1* and *PRAME*), although previous studies have discovered that they may play a critical role in tumorigenesis [43–46], the potential molecular mechanisms, especially those in ESCC, are not yet well understood. More studies are warranted to further explore their functions in ESCC.

Of the thirteen identified novel CTGs, several CTGs have been reported as having essential roles in cancers. For example, *AURKA* encodes a cell cycle-regulated kinase and participates in the regulation of the cell cycles in multiple cancers, including esophageal cancer [47–49]. Similarly, *TPX2* encodes microtubule-associated protein and takes part in the normal assembly of the microtubules involved in the tumorigenesis of colorectal cancer, bladder cancer and hepatocellular carcinoma during apoptosis [50–52]. Importantly, *CDCA5* expression was significantly

Fig. 3. *CDCA5* promotes ESCC cells proliferation, migration and invasion. (a) *CDCA5* knockdown induced apoptosis in both KYSE30 and Eca109 cells according to the flow cytometric analysis (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (b) *CDCA5* knockdown inhibited ESCC cells invasion in KYSE30 and Eca109 cells, (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (c) Knockdown of *CDCA5* inhibited the migration of tumor cells in KYSE30 and Eca109 cells, (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (d) The efficiency of *CDCA5* overexpression in KYSE150 (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (e) Overexpression of *CDCA5* promoted the proliferation of KYSE150 cells (Mean ± SD, Student's *t*-test, $^{***}P < .001$); (f) *CDCA5* overexpression promoted KYSE150 cells invasion and migration (Mean ± SD, Student's *t*-test, $^{***}P < .001$).

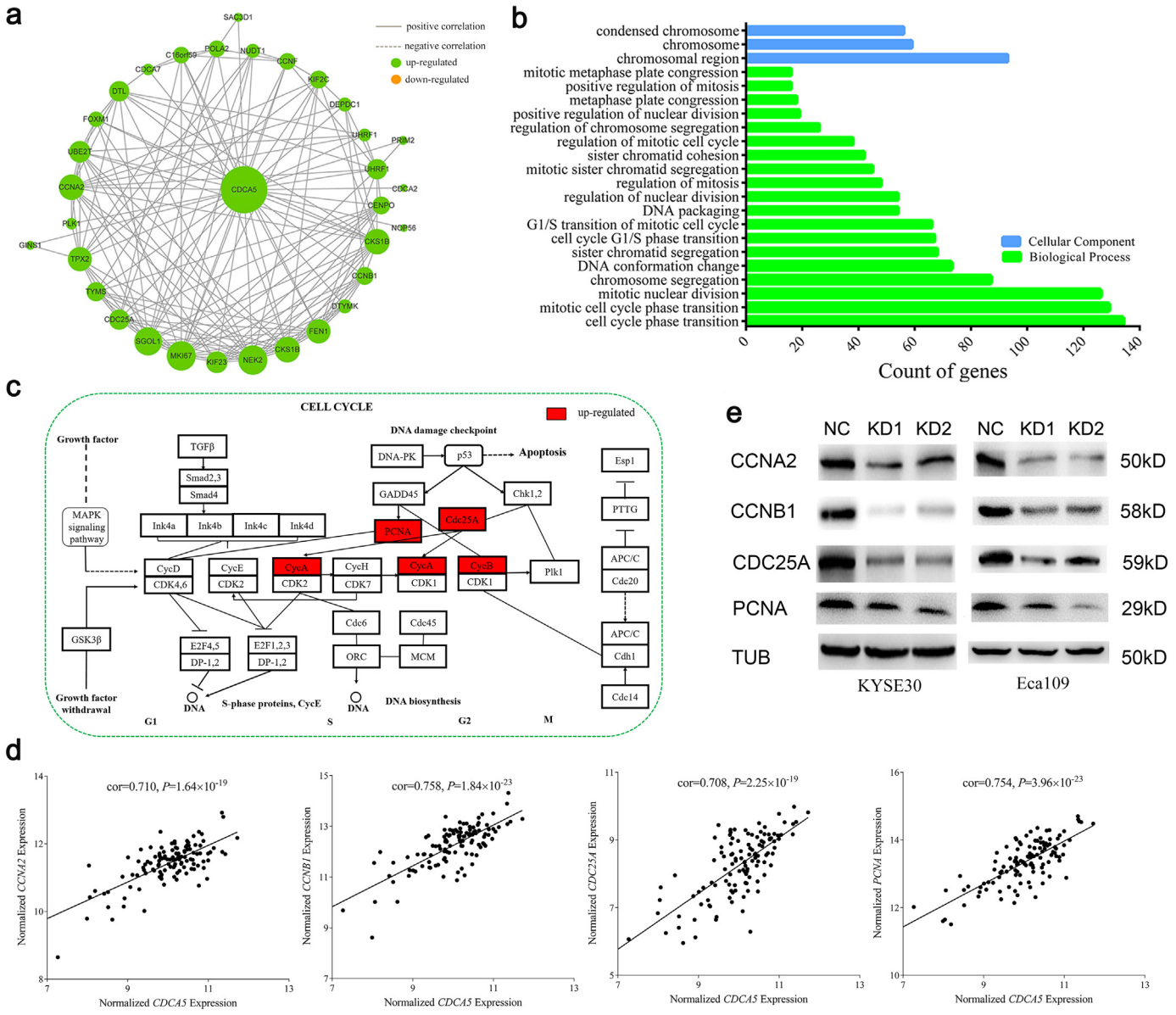

Fig. 5. *CDCA5* promotes ESCC progression through the cell cycle pathway. (a) Genes co-expressed with *CDCA5* in the RNA microarray data; (b) Gene Ontology analysis (GO) of differentially expressed genes in the RNA microarray based on DAVID Bioinformatics Resources 6.8; (c) CycA (*CCNA2*), CycB (*CCNB1*), *CDC25A* and *PCNA* participates in the cell cycle pathway according to the KEGG database; (d) Expression of *CCNA2*, *CCNB1*, *CDC25A* and *PCNA* was positively correlated with *CDCA5* mRNA expression in the RNA microarray data (Pearson correlation analysis, $P < .001$); (e) Western blot assay showed that knockdown of CDCA5 inhibited the expression of CCNA2, CCNB1, CDC25A and PCNA in both KYSE30 and Eca109 cells.

increased in ESCC tumor tissues and was correlated with poor clinical outcomes. The *CDCA5* gene, located on chromosome11q, is a critical regulator of sister-chromatid cohesion and separation during cell division [53,54]. The aberrant upregulation of *CDCA5* has been observed in human cancers during tumor progression, and indicated poor prognosis such as lung cancer, hepatocellular carcinoma, and breast cancer [31,55,56]. For the first time, we found that the expression of *CDCA5* was aberrantly elevated in ESCC tumor tissues and higher CDCA5 expression was significantly associated with the poorer prognosis of ESCC patients.

Regarding the underlying mechanisms, CDCA5 was identified as an evolutionarily conserved small molecular protein that contributes to maintenance of sister chromatid cohesion from the S phase to the onset of anaphase [57]. It has been reported that high levels of *CDCA5* expression promoted lung cancer cells proliferation interaction with ERK kinase through the activation of the MAPK pathway [31]. In another study, the upregulation of *CDCA5* was confirmed to increase cell viability and proliferation in gastric cancer malignant progression via influencing cyclin E1 [58]. However, the tumorigenic functions of *CDCA5* in ESCC have remained largely unclear thus far. The present study found that the knockdown of *CDCA5* strongly inhibited the proliferation, migration and invasion of ESCC cells. Consistently, the overexpression of *CDCA5* promoted ESCC cells proliferation, invasion and migration. What's more, *CDCA5* knockdown enhanced the chemosensitivity of ESCC cells to cisplatin and led to a significant reduction in the tumor volumes and weights of mice tumors. All of these findings suggested that *CDCA5* might serve as a potential immunotherapeutic target for ESCC intervention. Recent evidence indicates that the regulation of the cell cycle is extremely important to the cell because a dysregulated cell cycle leads the cell to grow autonomously, which is thought to be a fundamental hallmark of cancer, especially the checkpoint pathways [30,59]. In this study, we present the first evidence that *CDCA5* knockdown induced G2/M phases arrest and apoptosis in ESCC. The results support the putative role of CDCA5 in chromatid separation during the G2/M transition [60]. Notably, the GO enrichment analysis demonstrated that the term “cell cycle” ranks first among CDCA5-related

potential pathways. Moreover, the key members of cell cycle pathway (*CCNA2*, *CCNB1*, *CDC25A* and *PCNA*) showed significant co-expression with *CDCA5*. Consistent with this finding, in vitro experiments revealed that inhibition of CDCA5 significantly suppressed the expression of CCNA2, CCNB1, CDC25A and PCNA, and these genes appear to function with cell cycle pathway to govern ESCC development. Regarding the regulation of *CDCA5*, we found that the *CDCA5* promoter was highly acetylated in ESCC cells. Highlighting the finding, we speculated that the acetylation modification of the *CDCA5* promoter might be the potential activation mechanism of *CDCA5* in ESCC. Further functional assays are warranted to support our speculation.

In conclusion, we outline a schematic of CTGs in ESCC by integrating public databases and our data. A total of 13 novel CTGs were successfully identified in ESCC. Moreover, *CDCA5* was characterized as a novel tumor-promoting gene and was significantly associated with unfavorable ESCC prognosis, suggesting that it might be a favorite prognostic biomarker in the clinic and a prospective immunotherapeutic target for ESCC vaccines.

Author contributions

J. Xu, C. Zhu, Y. Yu and W. Wu participated in data analysis and drafting of the manuscript; J. Cao, Z. Li, J. Dai and C. Wang contributed to the analysis and the making of tables and figures; Y. Tang, Q. Zhu, J. Wang, W. Wen and L. Xue performed the functional assays; F. Zhen, J. Liu, C. Huang, F. Zhao, Y. Zhou and Z. He were involved in material and technical support; X. Pan, H. Wei, Y. Zhu, Y. He and J. Que took part in the collection of samples; J. Luo, L. Chen and W. Wang designed this study and revised the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

None.

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