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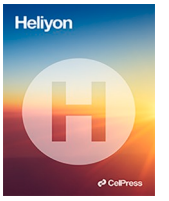

Research article

NEK2 promotes the migration, invasion, proliferation of ESCC and mediates ESCC immunotherapy

Shaorui Gu [a,1], YakuFujiang Yasen [a,1], Mengying Wang [b,1], Baiqing Huang [a], Yongxin Zhou [a,**], Wenli Wang [a,*]

[a] Department of Cardiothoracic Surgery, Shanghai Tongji Hospital Affiliated With Tongji University, Shanghai, 200065, PR China
[b] Department of Anesthesiology, Shuguang Hospital Affiliated With Shanghai University of Traditional Chinese Medicine, Shanghai, PR China

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ABSTRACT

Purpose: Esophageal squamous cell carcinoma (ESCC) is a disease with a high incidence rate and high mortality worldwide. The Never in Mitosis A (NIMA) family member NIMA-related kinase 2 (NEK2) plays an important role in mitosis. However, the role of NEK2 in the pathogenesis of ESCC remains unclear.

Patients and methods: The expression and function of NEK2 in TCGA and GEO data sets were analyzed by bioinformatics. We verified the expression of NEK2 in ESCC tissues and cell lines by Western blotting and immunohistochemical methods and further explored the relationship between tumor stage and NEK2 expression. The differences in NEK2 expression and survival in patients with EC were verified by bioinformatics analysis. ESCC cell lines with stable knockdown of NEK2 were established by lentivirus-mediated shRNA delivery. The effects of NEK2 on ESCC cells were analyzed on the cytological level with assays including CCK-8, EdU, cell scratch, Transwell migration and invasion, colony formation, flow cytometry and apoptosis assays. Tumor growth was measured in a mouse xenograft model.

Results: We found that NEK2 is highly expressed in ESCC tissues and ESCC cells and that the high expression of NEK2 is associated with poor tumor healing. Knockdown of the NEK2 gene inhibits the migration, proliferation, invasion and cell cycle of ESCC cells. Biologic analysis shows that NEK2 is involved in biological processes such as progression and apoptosis of esophageal cancer, and is related to E2F.Mechanistically, NEK2 knockdown decreases the expression levels of E2F1 and IGF2. NEK2 competes with the transcription factor E2F1 to bind CDC20, resulting in decreased degradation and increased expression of E2F1. IGF2 expression is also increased, which promotes the expression of thymidylate synthase, further promoting the drug resistance of ESCC cells. NEK2 is associated with immune infiltration in esophageal cancer.

Conclusion: NEK2 is highly expressed in ESCC and can promote the migration, proliferation and invasion of ESCC cells. NEK2 mediates ESCC immunotherapy.

* Corresponding author. Tongji Hospital Affiliated to Tongji University, 389 Xincun Road, Putuo District, Shanghai, PR China.
** Corresponding author. Tongji Hospital Affiliated to Tongji University, 389 Xincun Road, Putuo District, Shanghai, PR China.
E-mail addresses: zhou6302@tongji.edu.cn (Y. Zhou), anderson840913@163.com (W. Wang).
[1] Contributed equally.

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1. Introduction

Esophageal carcinomas (ESCA) is one of the most common malignancies in the world, with the sixth-highest mortality rate worldwide [1,2]. ESCA is divided into two major categories: squamous carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) is most commonly seen in southern Africa and eastern Asia [3,4]. At present, the main treatments for ESCA are surgery and postoperative radiotherapy and chemotherapy [5]. The incidence of postoperative complications such as anastomotic leakage and pulmonary infection is high, and the 5-year survival rate in China and the United States is approximately 15–20 % [6,7]. In view of the early metastasis, high postoperative recurrence rate, poor prognosis and other characteristics of ESCC, research on the molecular mechanisms of ESCC is very important for disease treatment [8,9].

Never in Mitosis A (NIMA)-related kinase 2 (NEK2) is a serine/threonine kinase that plays an important role in cell cycle regulation [10]. The expression of NEK2 is periodic; it is low in G1 phase and peaks in G2 phase and S phase [11]. NEK2 is located in the centrosome and has been found to have three splice isomers: NEK2A, NEK2B and NEK2C [12]. NEK2B can regulate centrosomal separation via phosphorylation of centrosomal-binding proteins such as C-Nap1. In addition, NEK2 regulates microtubule stability, participates in chromosome aggregation during meiosis, and maintains chromosome coherence [13]. Related studies have shown that NEK2 is highly expressed in a variety of cancers, such as prostate cancer, breast cancer, liver cancer, and diffuse large B-cell lymphoma [14]. NEK2 plays important roles in the genesis, development and drug resistance of tumors. In breast cancer, high expression of NEK2 leads to premature centrosomal segregation, promoting diffusion of centrosomal substances, and aneuploidy formation after chromatin destabilization [15]. However, the molecular mechanism by which NEK2 promotes the occurrence and development of esophageal cancer has not been fully clarified [16].

As a member of the E2F transcription factor family, E2F1 (E2F transcription factor 1) is involved in the cell cycle, DNA damage, cell proliferation, apoptosis and other activities and is regulated by the protein retinoblastoma (Rb). E2FL aggregates in the G1/S phase of the cell cycle, and increases in its expression promote cell cycle progression [17,18]. Insulin-like growth factor 2 (IGF2), a mitotic peptide hormone, is highly expressed in a variety of cancers and is associated with poor prognosis [19]. IGF2-mediated activation of IGF1R or receptor can promote tumorigenesis. NEK2 physically binds MAD2 and cell division cyclin 20 (CDC20) and phosphorylates CDC20 to regulate the SAC, thereby regulating chromosome segregation. CDC20 activates APC to form an E3 ubiquitin ligase complex, APC-CDC20, which is involved in E2F1 degradation [20,21]. The relationship among NEK2, E2F1 and CDC20 is not clear at present. We hypothesized that NEK2 can compete with E2F1 to bind to CDC20 such that E2F1 is not consumed but rather preserved and that E2F1 can bind to the IGF2 promoter to promote an increase in IGF2 expression.

Previous studies have shown that, NEK2 phosphorylates PD-L1 to maintain its stability, resulting in poor efficacy of PD-L1 targeted immunotherapy for pancreatic cancer [22]. The high expression of NEK2 in bone marrow progenitor cells suppresses T-cell immunity in multiple myeloma [23]. Currently, the role of NEK2 in immunotherapy for ESCC has not been confirmed.

2. Material and methods

2.1. Patients and tissue specimens

Sixty-two patients and surgical tissue samples were selected from among patients with ESCC who underwent radical operation in the thoracic surgery department of Tongji Hospital of Shanghai From January 1, 2010, to December 31, 2015. All patients were followed up during the study period. This study was approved by the Ethics Committee of Tongji Hospital Affiliated to Tongji University (2019-LCYJ-006). Before participating in this study, each patient signed an informed consent form.

The criteria for patient selection are as follows: (1) Patients who have been definitively diagnosed with esophageal squamous cell carcinoma by two pathologists; (2) Each patient has undergone radical resection of esophageal cancer with no residual cancer cells at the upper and lower resection margins; (3) No preoperative radiotherapy or chemotherapy has been received; (4) No synchronous or metachronous cancers other than esophageal squamous cell carcinoma; (5) Regular postoperative follow-up for 6 months.

2.2. Bioinformatics analysis

The pan cancer analysis used RNAseq data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases. Data in TPM (scripts per million reads) format is obtained from UCSC XENA database (https://xenabrowser.net/datapages/) and be handled through the Toil [24] process. The subgroup analysis of esophageal cancer was completed by using the online tool of ualcan (http://ualcan.path.uab.edu/index.html). TCGA dataset manipulation, Gene Set Enrichment Analysis (GSEA)and Gene Ontology (GO) function annotation were performed using R (4.2.0 version) package “clusterProfiler”. The Hallmarks gene set in the gene set database MSigDB Collections was used for Gene Set Enrichment Analysis (GSEA) analysis.

For data analysis, normalized data in GEO datasets (GEO accession numbers GSE23400, GSE38129, GSE44021) and the corresponding probe annotation data were downloaded from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/gds). Microarray data manipulation and analysis were performed in R 4.2.0. According to the list of given genes based on a large number of genomics and proteomics data, we used the GeneMANIA database (http://www.genemania.org) to predict the 50 most relevant genes of NEK2-E2F1-IGF2 regulatory axis.

2.3. Cell lines and cell culture

In this study, human esophageal cancer cell lines (TE1, TE5, KYSE-410, KYSE-150, Eca-109) and esophageal epithelial cells (Het-1A) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in medium containing 10 % serum and penicillin/streptomycin liquid (penicillin 100 U/ml, streptomycin 100 μg/ml). The culture medium consisted of 90 % RPMI 1640 and 10 % FBS (Gibco, USA). All cells were cultured in the incubator with 95 % O2 and 5 % CO2 at 37 °C. A lentiviral NEK2 short hairpin RNA (shRNA) was purchased from Genechem (GIEL0236793, China). The shRNA sequence targeting human NEK2 complementary DNA was 5'-GCAGACGAGCAAAGAAGAAAT-3'.

2.4. Antibodies

An anti-NEK2 antibody (ab227958, Abcam, USA), an anti-GAPDH antibody (ab181602, Abcam, USA), a recombinant anti-E2F1 antibody (ab179445, Abcam, USA), and an anti-IGF2 antibody (ab177467, Abcam, USA) were used in the current study. The related reagents included the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-al) (MedChemExpress, USA) and the protein synthesis inhibitor cycloheximide (CHX) (Abcam, USA).

2.5. Immunohistochemistry

Paraffin sections were immersed in xylene for dewaxing, hydrated through a graded ethanol series, and then placed into ethylenediaminetetraacetic acid (EDTA) repair solution (pH = 9.0). The antigens were repaired via heating in a water bath at 100 °C for 15 min. Hydrogen peroxide (3 %) was added to the sections to block endogenous peroxidase. The sections were blocked in serum and incubated overnight with primary antibodies at 4 °C. The sections were incubated with horseradish peroxidase-labeled second antibodies the next day. The staining was developed with diaminobenzidine (DAB). After restaining with hematoxylin, the slices were dehydrated with graded ethanol and fixed with neutral gum. Immunohistochemical staining intensity was scored according to the following scale: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The positive cell frequency was scored as follows: 0, <5 %; 1, 5–25 %; 2, 26–50 %; 3, 51–75 %; and 4, >75 %. In data analysis, a total score of 0–7 was considered low expression, and a score of 8–12 was considered high expression.

2.6. Quantitative real time-PCR

Knockdown efficiency in tumor cells was tested by quantitative real-time PCR. First, RNA was extracted from ESCC cells by the TRIzol method (Ambion). The optical density (OD) value was determined with a micro spectrophotometer, and the RNA concentration was calculated. The RNA was stored in a freezer at −80 °C until use. The extracted RNA was reverse-transcribed into cDNA with a PrimeScript™ RT Reagent Kit with gDNA Eraser, and the cDNA was amplified by quantitative real-time PCR using TB Green Premix Ex Taq™ II (Takara). GAPDH was used as an internal reference to reduce the experimental error. The primers for qRT-PCR are described in Annex 1.

2.7. Western blotting

Cells were placed in a mixture of RIPA Lysis Buffer (Epizyme, China) and Phenylmethanesulfonyl fluoride (PMSF). After centrifugation, the supernatant was obtained, and the protein concentration was determined by Bicinchoninic Acid (BCA) method. Protein samples were separated via 10 % SDS-PAGE. After electrophoresis, the proteins in the gel were transferred onto a PVDF membrane. The PVDF membrane was incubated with a mixture of primary antibodies and primary antibody buffer at 4 °C overnight. On the second day, it was incubated with secondary antibodies for 1 h to develop. All Western blot reagents were purchased from Epizyme.

2.8. CCK-8 cell proliferation assay

Cell proliferation was detected by using a CCK-8 (Dojindo Laboratories, Japan). First, cells were cultured in 96-well plates at a density of 5000 cells per well in a mixture of 10 % serum and 90 % RPMI-1640 medium. After the indicated culture duration, 10 μl of CCK-8 solution was added to each well, and the plates were incubated in an incubator for 2 h. The absorbance at 450 nm was measured with an ELISA reader.

2.9. EdU cell proliferation assay

Cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 4 × 103 cells per well and cultured to the normal growth stage. The cells were incubated in 50 μM EdU (5-ethynyl-2′-deoxyuridine) medium (RiboBio, Guangzhou, China) for 6 h. Then, the cells were fixed and stained with EdU. The cells were restained with Hoechst 33342 reaction solution and observed under a fluorescence microscope.

2.10. Colony formation assay

Cells were seeded on a 6-well plate at a density of 400 cells per well. After 15 days of culture in a 5 % CO2 incubator at 37 °C, the cells were washed with PBS and stained with 0.1 % crystal violet. The colony formation was observed under a microscope.

2.11. Wound healing assay

Cells were seeded on a 6-well plate at a density of 5×10^5 cells per well and cultured in a 5 % CO_2 incubator at 37 °C. After they reached confluence, the cells were scratched with a pipette, then washed with PBS three times, and cultured in serum-free medium for 24 h. Finally, the cells were observed under a microscope, sampled and photographed.

2.12. Migration and invasion assays

Transwell chambers (Corning, USA) were used for migration and invasion experiments. In the migration experiment, 5×10^4 cells were placed in the upper compartment with 1 % FBS and RPMI-1640 medium. In the invasion experiment, 5×10^4 cells suspended in 1 % FBS and RPMI 1640 were inoculated into the upper compartment, which was coated with 300 μg/ml Matrigel (BD Biosciences, USA). RPMI-1640 medium containing 10 % FBS was placed in the lower compartment. The cells in the upper chamber were wiped off. The cells in the lower compartment were fixed with methanol for 10 min and then stained with crystal violet. The cells were counted and imaged under a microscope.

2.13. Flow cytometry

The flow cytometry experiments included a cell cycle test and an apoptosis test. A FITC/PI Kit (Kaiji, Nanjing) was used to detect apoptosis. After cell culture, the supernatant was removed, and the cells were resuspended with PBS. Binding buffer (500 μl), annexin

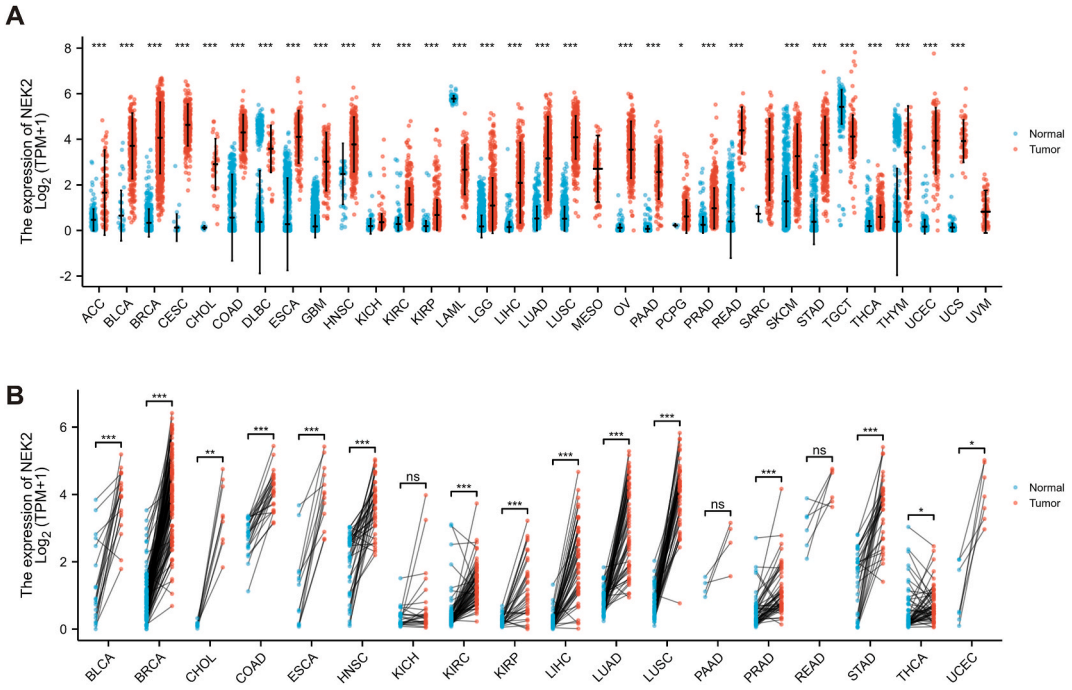


Fig. 1. The expression of NEK2 in pan carcinoma. (A) The expression of NEK2 in pan carcinoma and corresponding normal tissues from TCGA database and normal tissues from GETx database. (B) The expression of NEK2 in pan carcinoma and corresponding normal tissues extracted from TCGA database. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$). ACC: Adrenal cortical carcinoma, BLCA: Bladder Urothelial Carcinoma, BRCA: Breast invasive carcinoma, CESC: Cervical squamous cell carcinoma, CHOL: Cholangio carcinoma, COAD: Colon adenocarcinoma, DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, ESCA: Esophageal carcinoma,GBM: Glioblastoma multiforme, HNSC: Head and Neck squamous cell carcinoma, KICH: Kidney Chromophobe, KIRC: Kidney renal clear cell carcinoma, KIRP: Kidney renal papillary cell carcinoma, LAML: Acute Myeloid Leukemia,LGG: Brain Lower Grade Glioma, LIHC: Liver hepatocellular carcinoma, LUAG: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma, MESO: Mesothelioma,OV: Ovarian serous cystadenocarcinoma,PAAD: Pancreatic adenocarcinoma, PCPG: Pheochromocytoma and Paraganglioma, PRAD: Prostate adenocarcinoma, READ: Rectum adenocarcinoma,SARC: Sarcoma, SKCM: Skin Cutaneous Melanoma,STAD: Stomach adenocarcinoma, TGCT: Testicular Germ Cell Tumors, THCA: Thyroid carcinoma,THYM: Thymoma, UCEC: Uterine Corpus Endometrial Carcinoma,UCS: Uterine Carcinosarcoma, UVM: Uveal Melanoma.

V-FITC (5 μl) and PI(Propidium Iodide) (5 μl) were added in turn. In the cell cycle experiment, the digested and centrifuged cells were fixed with 80 % ethanol, incubated at 37 °C for 30 min(s) after addition of 100 μl of RNase(ribonuclease) (50 μg/ml). PI (400 μg, 50 μg/ml) was then added, after which the cells were stained at 4 °C for 30 min and detected by flow cytometry (Beckman).

2.14. Xenograft animal models

Twelve female Balb/c nude mice aged 3–4 weeks were purchased from Shanghai Jie-si-jie Experimental Animal Company (Shanghai, China). The nude mice were randomly divided into two groups (n = 6): the TE1-negative control (NC) group and the TE1-NEK2-knockdown (KD) group. ESCC cells (5×10^6) were subcutaneously injected into the right armpits of nude mice under lidocaine local anesthesia. The longest diameter of each tumor was measured every week. At the fourth week, the nude mice were killed, and the tumor tissue was taken out for measurement. The animal experiments were approved by the Ethics Committee of Tongji Hospital Affiliated to Tongji University (2019-LCYJ-006).

2.15. Statistical analysis

All the statistics were analyzed with GraphPad Prism and SPSS 22. The paired samples were compared by Student's T-test, the measurement data of unknown distributions were compared by rank sum test, and the count data were compared between groups by

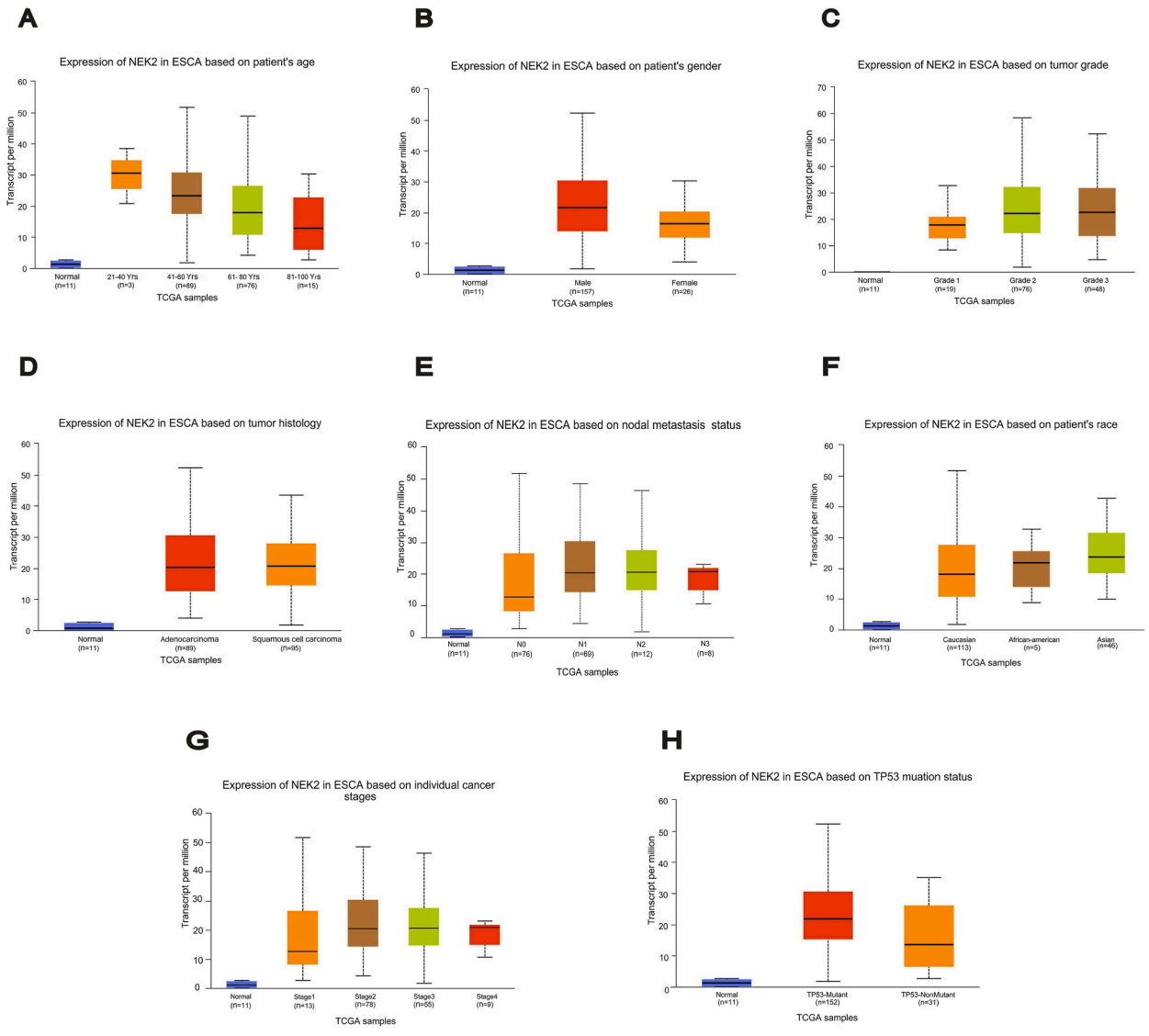


Fig. 2. The relationship between NEK2 expression and clinical subtypes of esophageal adenocarcinoma. (A) Age, (B) Gender, (C) Tumor grade, (D) Tumor histology, (E) Nodal metastasis status, (F) Race, (G) Tumor stage, (H) TP53 mutation status.

the exact probability method. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$). All the experiments were repeated three times.

3. Results

3.1. NEK2 level is elevated in esophageal carcinoma

We analyzed the expression of NEK2 in pan carcinoma based on the Cancer Genome Atlas (TCGA) database at first. Compared with GTEx and TCGA normal tissues, NEK2 also has significant expression in TCGA cancer samples, including ESCA (Fig. 1A). Compared with matched normal tissues, highly expressed NEK2 was detected in different types of tumors (TCGA tumors vs TCGA normal), including ESCA (Fig. 1B). We analyzed the relationship between NEK2 expression and clinical subtypes of ESCA using the online tool of ualcan. The results showed that NEK2 expression in ESCA was significantly different from that in normal tissues in Age (Fig. 2A), Gender (Fig. 2B), Tumor grade (Fig. 2C), Tumor histology (Fig. 2D), Nodal metastasis status (Fig. 2E), Race(Fig. 2F), Tumor stage (Fig. 2G), TP53 mutation status (Fig. 2H) and other subgroups. The expression of NEK2 was significantly higher in male, Asian, younger, higher-grade and TP53-mutant esophageal tumors.

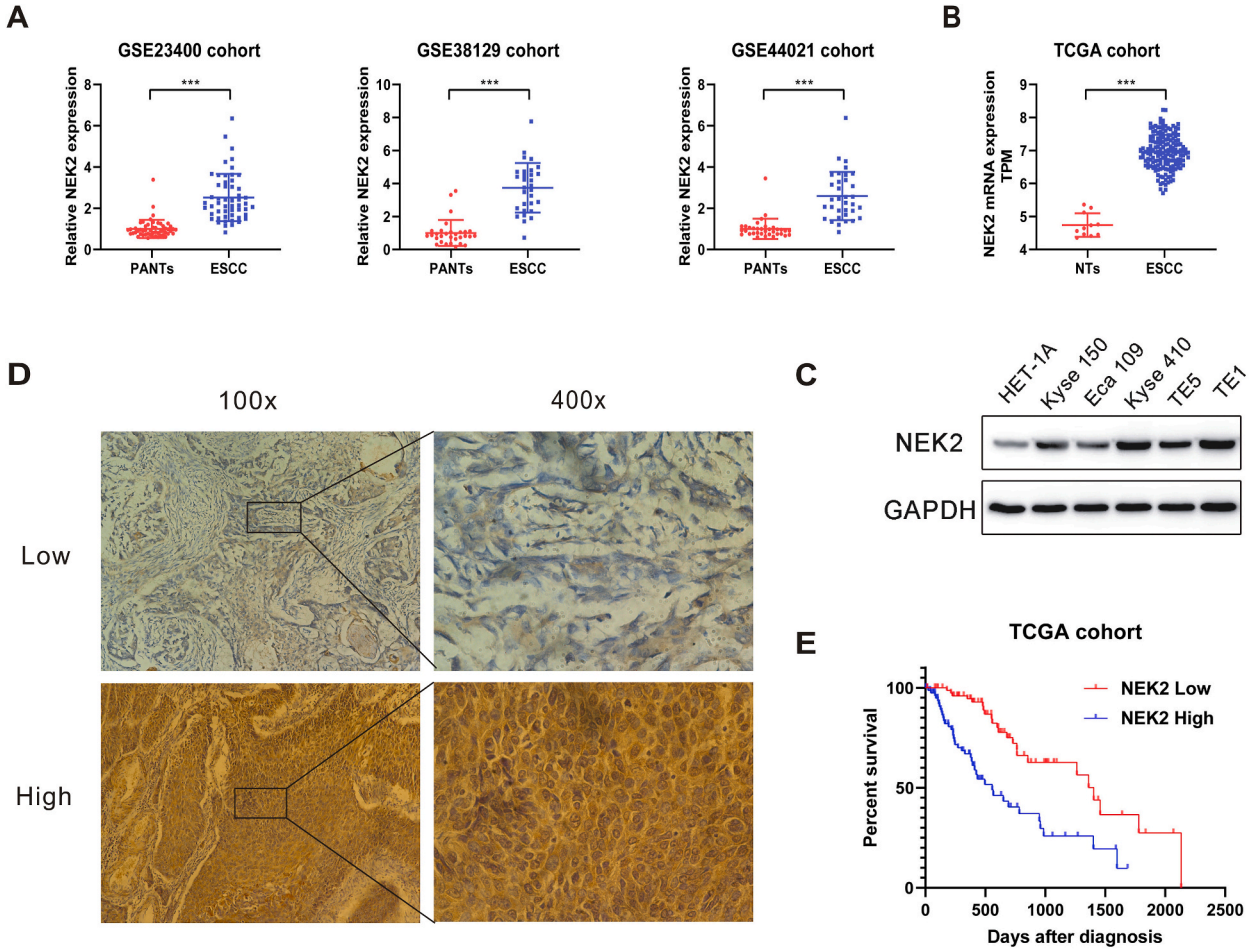


Fig. 3. NEK2 is highly expressed in ESCC cell lines and tissues of patients with ESCC and is related to the prognosis of patients. (A) The differences in NEK2 expression between normal esophageal tissue (NT) and ESCC tissue in GEO datasets (GEO accession numbers GSE23400, GSE38129, GSE44021) were analyzed. The data were normalized to NEK2 expression in NTs and are presented as the means and SDs. Statistical significance was determined by a modified *t*-test (implemented via the limma package in R). (B) The difference in NEK2 mRNA expression between NT and ESCC tissue in TCGA was analyzed by RNA sequencing. (C) The expression of NEK2 in the esophageal cell line Het-1A and a group of ESCC cell lines was detected by Western blotting, with GAPDH as an internal reference. The data were normalized to NEK2 expression in Het-1a cells and are presented as the means and SDs ($n = 3$). (D) The expression of NEK2 in ESCC tissue was higher than that in normal tissue, as indicated by immunohistochemistry. Magnification: 100 × , 400 × . (E) Survival analysis using TCGA data showed that high expression of NEK2 was associated with poor prognosis. Kaplan-Meier survival analysis showed that high expression correlated with short survival. Data are expressed as means ± SEM of three independent experiments. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$).

3.2. NEK2 is highly expressed in ESCC and is associated with prognosis

We analyzed the gene expression data of ESCC patients selected from the Gene Expression Omnibus (GEO) database firstly. We found that the expression of NEK2 in ESCC tissues was significantly different from that in normal esophageal tissues and that NEK2 was significantly overexpressed in ESCC tissues (Fig. 3A). We selected 11 normal human esophageal tissues and 161 esophageal cancer tissues from the TCGA database and found that the expression of IFI6 was significantly upregulated in esophageal squamous cell carcinoma tissues (Fig. 3B). Subsequently, we verified the protein expression of NEK2 in five ESCC cell lines and one esophageal squamous epithelial cell line. Western blot analysis showed that the expression levels of NEK2 in the five ESCC cell lines were higher than that in the esophageal squamous epithelial cell line (Fig. 3C). Postoperative pathological sections from 62 patients with esophageal cancer (EC) were analyzed for the expression of NEK2 protein. A positive result was indicating by brown or yellow staining of tumor cells, while a negative result was indicated by blue staining (Fig. 3D). Immunohistochemical staining showed that NEK2 was highly expressed (staining score $\geq$8) in 62 cases of esophageal cancer, accounting for 56.4 % of cases (35 cases), Statistical analysis showed that the expression level of NEK2 was correlated with the invasion depth, lymph node metastasis, tumor differentiation and TNM stage of ESCC (Table 1). Finally, survival analysis showed that the survival rate of patients with high expression of NEK2 was significantly lower than that of patients with low expression of NEK2 (Fig. 3E).

3.3. Gene set enrichment analysis (GSEA) analysis enriched the functions of NEK2

We used R package "DESeq2" to analyze the RNAseq data of TCGA esophageal cancer. Using $p < 0.05$ and $|\log FC| \geq 1$ as cut-off criteria, differential genes were analyzed by dividing them into high and low groups according to NEK2 expression (Fig. 4A). GSEA analysis mainly enriched the functions of NEK2: E2F target, proliferation and differentiation of esophageal epidermal cells, immune response, hypoxia, apoptosis, etc. (Fig. 4B). In GO analysis, the up-regulated genes mainly enrich esophageal epithelial proliferation, differentiation, granulocyte chemotaxis, humoral immunity, etc. (Fig. 4C and D). The above enrichment analysis can help us further study the role of NEK2 in ESCA, suggesting that E2F may be the downstream target of NEK2.

3.4. NEK2 knockdown inhibits the migration, invasion and cell cycle progression of ESCC cells and promotes apoptosis

To further verify the effect of NEK2 on ESCC at the cytological level, we constructed stable TE1-NEK2-KD and KYSE-410-NEK2-KD cell lines via lentivirus-mediated shRNA transfection. The knockdown efficiency of NEK2 was verified by Western blotting (Fig. 5A) and qRT-PCR (Annex 2). Firstly, we carried out CCK-8 and EdU proliferation experiments. The results showed that proliferation ability was significantly decreased in TE1 and KYSE-410 cells transfected with shRNA via lentivirus (Fig. 5B and E). To verify the effect of NEK2 on the long-term proliferation of ESCC cells, we carried out a colony formation experiment. The results showed that TE1-NEK2-KD and KYSE-410-NEK2-KD cells were less able to form colonies than normal TE1 and KYSE-410 cells (Fig. 5C and D).

Subsequently, in order to verify the effects of NEK2 on the migration and invasion of ESCC cells, we carried out Transwell migration and invasion experiments. As expected, the migration and invasion ability of normal ESCC cells was significantly stronger than that of ESCC cells subjected to lentivirus-mediated shRNA transfection. The experimental data thus showed that NEK2 promoted the migration and invasion ability of ESCC cells (Fig. 6A,B&C). In addition, a cell scratch experiment was carried out, and similar results

Table 1
Expression of NEK2 in 62 patients with ESCC.

Feature	NEK2 Expression		*P*-value
	Low	High	
Gender			
Male	11	9	0.283
Female	17	25	
Age (years)			
<60	11	13	0.773
≥60	16	22	
Differentiation			
G1	12	4	0.002**
G2	13	21	
G3	2	10	
Invasive depth			
T1/2	15	6	0.002**
T3/4	12	29	
Lymph node metastasis			
Negative	17	18	0.033*
Positive	6	21	
TNM stage			
I	10	4	0.006**
II	11	13	
III/IV	6	18	

Notes: $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$).

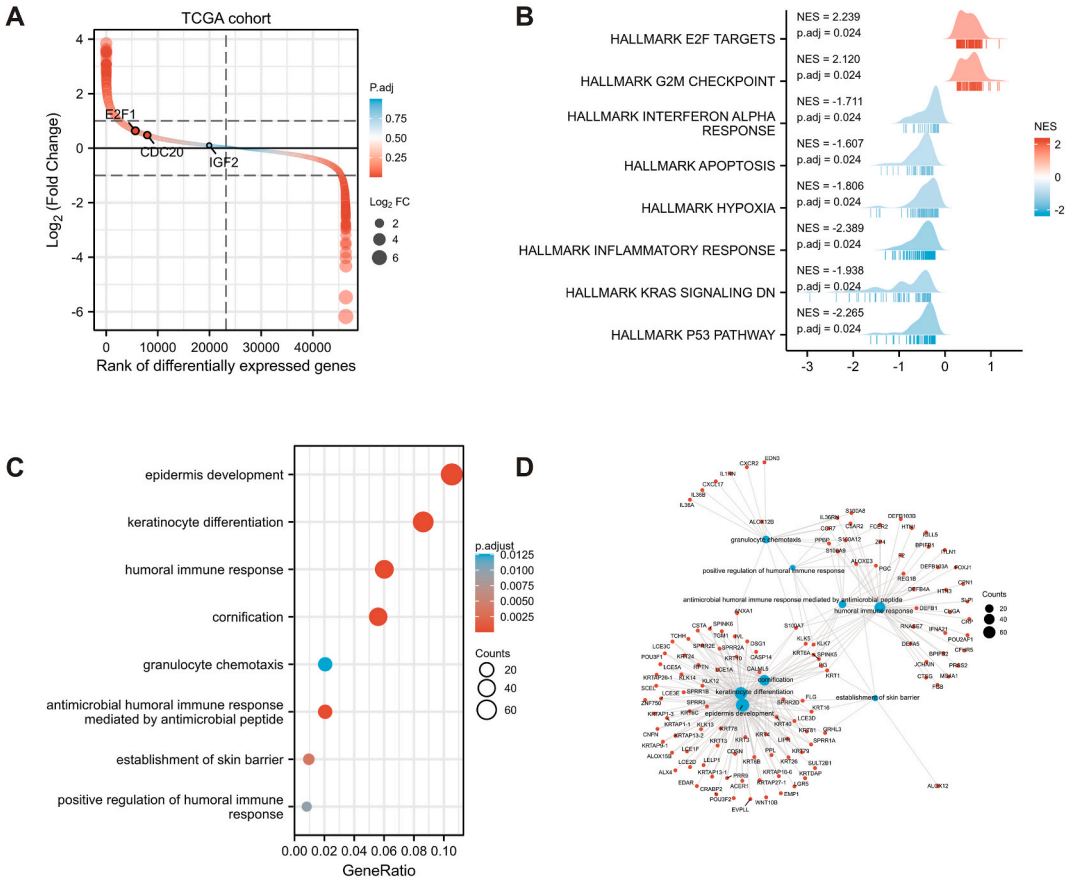


Fig. 4. Gene set enrichment analysis (GSEA) analysis enriched the functions of NEK2. (A) Rank of differentially expressed genes in TCGA esophageal cohort was shown by high NEK2 expressed group versus low groups. (B) GSEA analysis mainly enriched the main functions of NEK2. (C&D) GO functional annotation suggested that NEK2 plays a role in the physiological processes of esophageal epithelial proliferation, differentiation, granulocyte chemotaxis, and humoral immunity. (Blue dots denote GO items, red dots denote genes, and line segments denote inclusion connections.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were obtained (Fig. 5F).

Finally, we verified by flow cytometry that the apoptosis levels of EC cells with NEK2 knockdown were higher than those of normal tumor cells (Fig. 6D and F). In the cell cycle progression experiment, G2-/M-phase arrest was observed in ESCC cells after NEK2 knockdown (Fig. 6E and G).

3.5. *NEK2 promotes drug resistance of ESCC cells through the E2F1-IGF2 pathway*

Previous studies have shown that NEK2 can physically bind to CDC20 and Mad2. In addition, NEK2 can regulate the spindle assembly checkpoint (SAC) by phosphorylating CDC20 and Mad2, thus regulating chromosome segregation. CDC20 can specifically bind to the transcription factor E2F1 to degrade protein. In addition, the transcription factor E2F1 can directly bind to the IGF2 promoter to activate IGF2 transcription. IGF2 can promote the expression of thymidylate synthase through the PI3K/Akt pathway and further enhance the chemoresistance of ESCC cells to 5-fluorouracil (5-FU). The relationship among NEK2, E2F1 and IGF2 is not clear. We hypothesized that NEK2 can compete with E2F1 to bind CDC20 such that the transcription factor E2F1 can be preserved and that E2F1 can activate IGF2 transcription, promote the production of thymidylate synthase, and promote the drug resistance of ESCC cells.

To verify the relationship among NEK2, E2F1 and IGF2, we selected samples from TCGA to analyze the relationship between NEK2 and IGF2 RNA expression. The results showed that there was a positive correlation between NEK2 and IGF2 RNA expression (Fig. 7A). Western blotting assay showed that NEK2, E2F1 and IGF2 were highly expressed in EC cells. After NEK2 knockdown, the expression levels of E2F1 and IGF2 were also decreased. Compared with TE1-NEK2-KD cells, MG132-treated TE1-NEK2-KD cells exhibited higher expression levels of E2F1 and IGF2. While MG132 + CHX-treated TE1-NEK2-KD cells exhibited significantly lower expression of E2F1 and IGF2 (Fig. 7B and D). TE1-NC and TE1-NEK2-KD cells were cultured with the protein synthesis inhibitor CHX (10 μg/ml) for 2, 4 or 8 h. Proteins were extracted for Western blotting. With extension of culture time, the protein expression of E2F1 and IGF2 decreased, and E2F1 protein expression and IGF2 protein expression were positively correlated (Fig. 7C and E). The gene gene interaction network

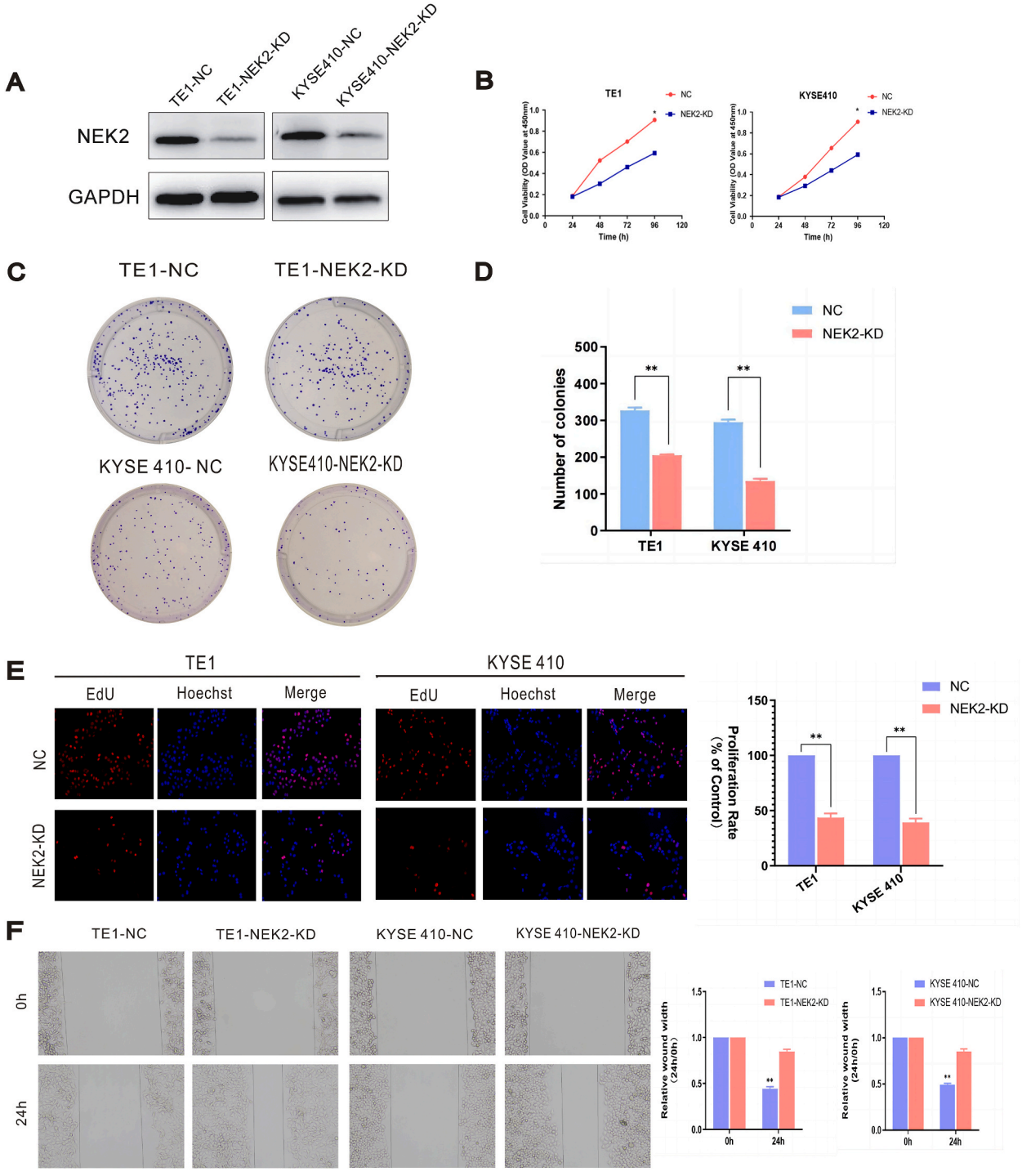
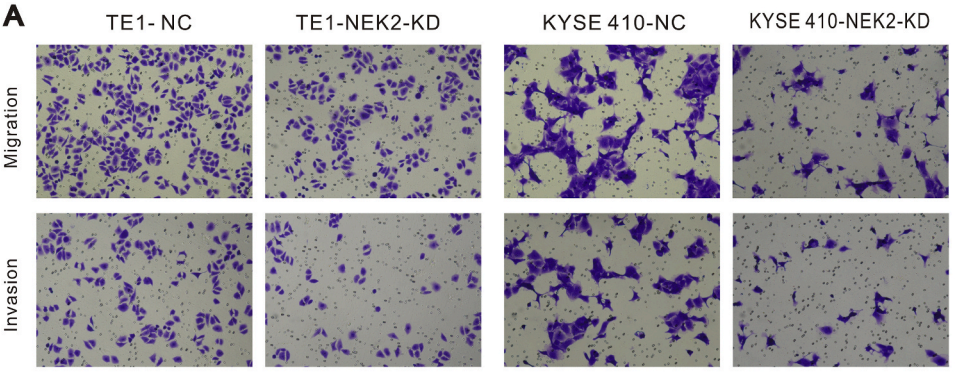
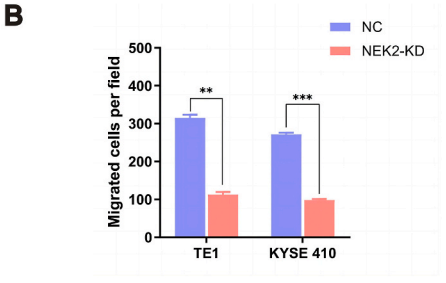
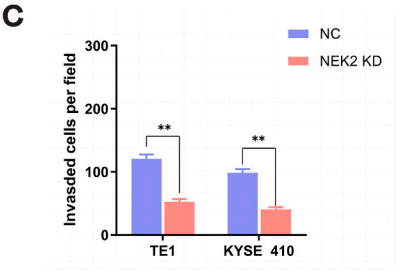
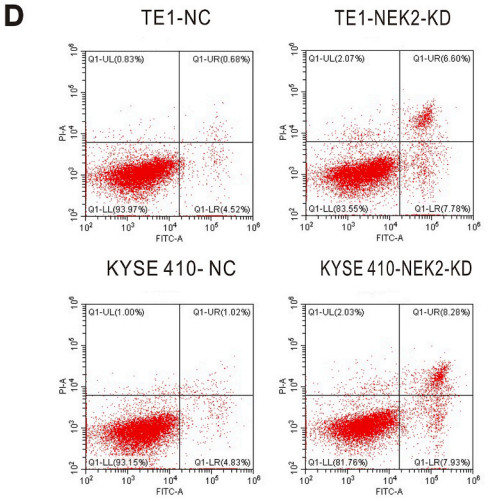


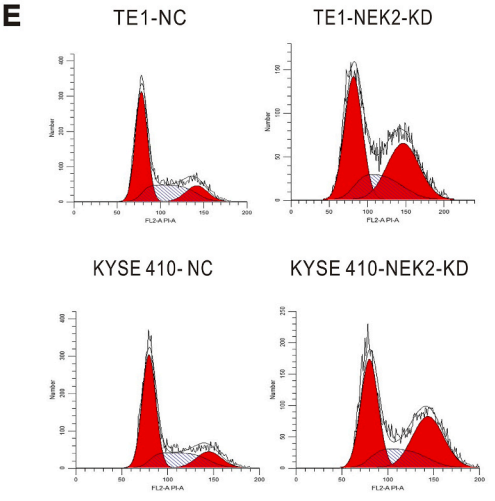
Fig. 5. NEK2 knockdown inhibits the migration, invasion and proliferation of ESCC cells. (A) Western blot showing NEK2 expression in TE1 and KYSE-410 cells transfected with NEK2-shRNA lentivirus. GAPDH served as an internal control. (B) CCK-8 assay results showing that NEK2 knockdown inhibited proliferation of TE1 and KYSE-410 cells. (C&D) Colony formation assay results showing that NEK2 knockdown inhibited TE1 and KYSE-410 cell colony formation. (E) EdU cell proliferation assay results showing that the proliferation ability of TE1 and KYSE-410 cells decreased after NEK2 knockdown. EdU: red, Hoechst 33342: blue. Statistical significance was determined by two-tailed Student's t-test. (F) Cell scratch assay results showing that NEK2 knockdown inhibited migration of TE1 and KYSE-410 cells. Data are expressed as means ± SEM of three independent experiments. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

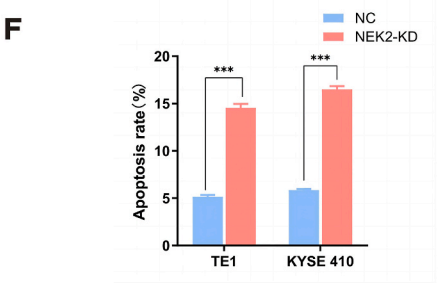












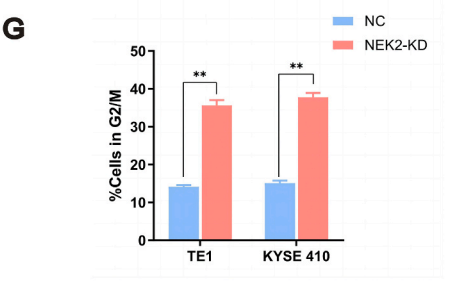


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Fig. 6. NEK2 knockdown inhibits the migration, invasion and cell cycle progression of ESCC cells and promotes apoptosis (A, B & C) Transwell migration and invasion assay results showing that NEK2 knockdown inhibited proliferation and of TE1 and KYSE-410 cells. (D&F) Flow cytometry results showing that NEK2 knockdown promoted apoptosis of TE1 and KYSE-410 cells. (E&G) Flow cytometry results showing that TE1 and KYSE-410 cells accumulated in G2/M phase of the cell cycle after NEK2 knockdown. Data are expressed as means ± SEM of three independent experiments. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$).

of NEK2-E2F1-IGF2 regulatory axis was constructed by GeneMANIA. The results showed that the 50 most frequently changed genes were closely related to the NEK2-E2F1-IGF2 regulatory axis (Fig. 7G). In addition, functional analysis showed that NEK2-E2F1-IGF2 regulatory axis and its similar genes were significantly related to regulation of translation involved in G1/S transition of mitotic cell cycle, platinum drag resistance, regulation of apoptotic signaling pathway, DNA binding translation activator activity, RNA polymerase II specific and other processes (Fig. 7H).

These results showed that NEK2 was highly expressed in ESCC cells, that E2F1 activated IGF2 transcription, and that E2F1 and IGF2 were highly expressed. After NEK2 knockdown, CDC20 combined with E2F1 to promote E2F1 degradation and thus prevent IGF2 transcription. E2F1 protein expression and IGF2 protein expression decreased and were proportional. Culture with MG132 inhibited the degradation of E2F1 and IGF2 proteins and increased the protein expression levels in TE1-NEK2-KD cells. It can be inferred that NEK2 can compete with E2F1 to bind CDC20 and that E2F1 can be retained to activate IGF2 transcription and promote the production of thymidylate synthase, thus promoting the drug resistance of ESCC cells.

3.6. NEK2 knockdown inhibits tumor growth in nude mice

To verify the effect of NEK2 on tumor growth in nude mice, we injected TE1 cells and TE1-NEK2-KD cells into the armpits of separate groups of nude mice. Tumor volume was measured weekly. The results showed that tumor growth was inhibited after NEK2 knockdown (Fig. 8B). After 4 weeks of tumor growth, the tumors were removed from the nude mice and photographed (Fig. 8A). The tumor mass in the TE1-NC group was significantly higher than that in the TE1-NEK2-KD group (Fig. 8C).

3.7. NEK2 is associated with immune infiltration in esophageal cancer

We analyzed TCGA esophageal cancer data through the TIMER tumor immune infiltration analysis database (https://cistrome.shinyapps.io/timer/). We found a negative correlation between the expression of NEK2 gene and the degree of dendritic cell infiltration, and there was no significant difference compared to other immune cell infiltration (Fig. 9A). This result suggests that high expression of NEK2 in esophageal tumors may inhibit the antigen presentation of dendritic cells to tumor cells. We divided esophageal cancer samples into high and low groups based on 50 % NEK2 expression levels, and then conducted differential analysis. Using $p < 0.001$ and $|\log FC| \geq 2$ as cut-off criteria, a total of 95 genes were selected (Fig. 9B). The differentially expressed genes were subjected to GDSC drug sensitivity analysis and found to be associated with Talazoparib, Olaparib, and AICAR. The differentially expressed genes were subjected to GDSC drug sensitivity analysis and found to be associated with multiple drugs (Fig. 9C).

4. Discussion

NEK2 was first known as a multifunctional protein regulating the cell cycle [25]. In recent years, it has gradually been found that NEK2 is overexpressed in a variety of cancers, including breast cancer, cervical cancer, ovarian cancer, prostate cancer and other cancers, and that it participates in the occurrence, development and drug resistance of tumors [26]. However, the specific mechanism has remained unclear. To further explore the role of NEK2 in tumor development, we conducted relevant experiments. Compared with normal esophageal tissues, ESCC tissues highly expressed NEK2, and the degree of NEK2 expression was correlated with the T, N and M stages of ESCC. We revealed that the high expression of NEK2 was associated with poor prognosis of ESCC by bioinformatics analysis. Knockdown of NEK2 decreased the migration, invasion and proliferation of ESCC cells; increased apoptosis; and arrested the cell cycle in G2/M phase. In vitro, the proliferation ability of the NEK2-knockdown cell line was weaker than that of the normal ESCC cell line.

E2F1, a transcription factor, is frequently amplified in ESCC. The protein RB can inactivate E2F1, but dissociation of the Rb protein from E2F1 can restore the transcriptional activity of E2F1, thus promoting cell cycle progression from G1 phase to S phase [27]. CDC20 can specifically bind to E2F1 and promote the degradation of E2F1 [28]. Our experimental study showed that E2F1 expression was lower in NEK2-knockdown ESCC cells than in normal ESCC cells, which indicated that NEK2 could regulate the expression of E2F1. Because NEK2 can physically bind to CDC20 and Mad2 or phosphorylate CDC20, we conclude that NEK2 can compete with E2F1 to bind CDC20 and thus to protect E2F1 from degradation.

Thus far, many studies have shown that elevated serum IGF2 levels are associated with increased risk of a variety of cancers, such as lung cancer, breast cancer, and colorectal cancer [29]. Previous studies have confirmed that overexpression of IGF2 can significantly increase the expression of thymidylate synthase and phosphorylated Akt [30]. Overexpression of thymidylate synthase is closely related to the drug resistance of tumor cells to chemotherapy. In previous experiments, knockdown of IGF2 has been found to reduce thymidylate synthase synthesis and thus to enable construction of a stable 5-FU-resistant ESCC cell line [21]. Therefore, it can be concluded that IGF2 plays an important role in 5-FU resistance of ESCC cells. The Western blotting results showed that the expression of E2F1 and IGF2 in ESCC cells was proportional, which confirms that E2F1 can directly bind to the promoter of IGF2 and promote the transcription of IGF2.

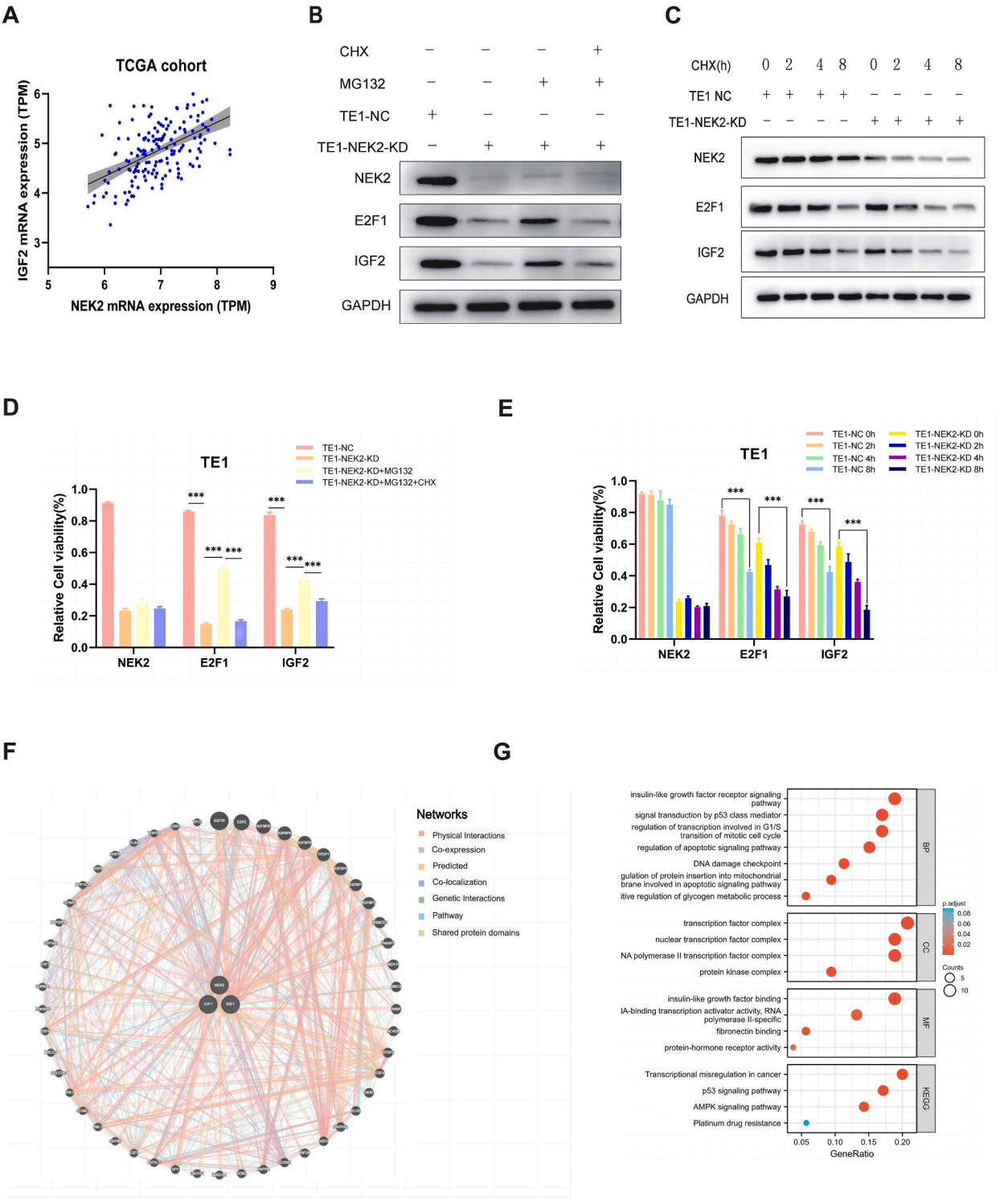

Fig. 7. NEK2 promotes drug resistance of ESCC cells through the E2F1-IGF2 pathway. (A) The correlation between NEK2 mRNA expression and IGF2 mRNA expression in EC tissues was analyzed with TCGA data. (B&D) Western blot analysis was used to detect the protein expression of E2F1 and IGF2 in TE1-NC and TE1-NEK2-KD cell lines cultured with or without MG132 or MG132 + CHX. (C&E) The protein expression of E2F1 and IGF2 in TE1-NC and TE1-NEK2-KD cell lines cultured with or without CHX for 0, 2, 4 or 8 h was detected by Western blot analysis. (F) 50 most relevant genes of NEK2-E2F1-IGF2 regulatory axis were predicted by the GeneMINIA website. (G) GO and KEGG analysis showed that NEK2-E2F1-IGF2 regulatory axis was mainly related to some tumor progression processes. Data are expressed as means ± SEM of three independent experiments. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$,***$P < 0.001$).

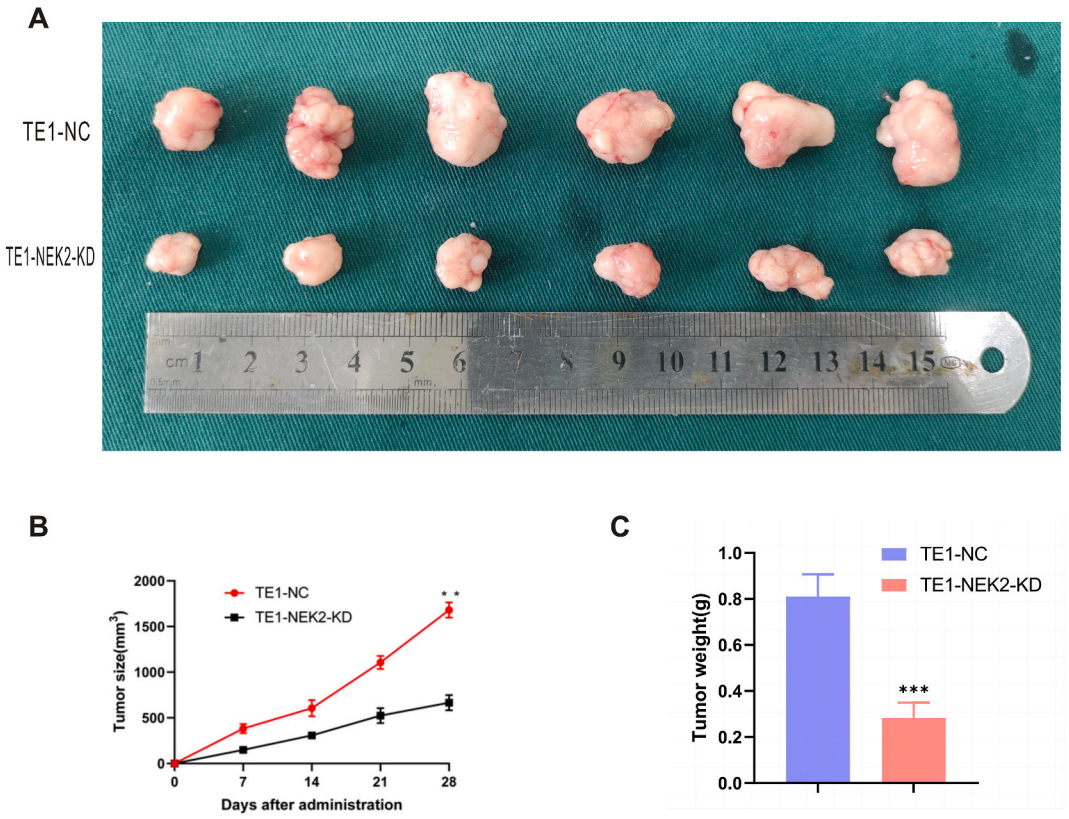

Fig. 8. NEK2 knockdown inhibits tumor growth in nude mice. (A) Photos of tumor growth in nude mice after subcutaneous injection of TE1-NC and TE1-NEK2-KD cells for 4 weeks. (B) Changes in tumor volume after subcutaneous injection of TE1-NC and TE1-NEK2-KD cells. (C) Comparison of tumor masses in nude mice after subcutaneous injection of TE1-NC and TE1-NEK2-KD cells for 4 weeks. Data are expressed as means ± SEM of three independent experiments. $P < 0.05$ was considered to indicate statistical significance (*$P < 0.05$, * *$P < 0.01$, ***$P < 0.001$).

The expression of NEK2 gene is negatively correlated with the degree of dendritic cell infiltration, and there is no significant difference compared to other immune cell infiltration. This indicates that high expression of NEK2 in tumors may inhibit the antigen presentation of dendritic cells to tumor cells. In esophageal cancer samples, the difference in expression with NEK2 is associated with drug sensitivity. In addition to esophageal tumor cells, the expression of NEK2 and PD-L1 has also been observed in macrophages and dendritic cells. Defects in NEK2 lead to the suppression of PD-L1 expression and an enhancement of lymphocyte infiltration. Based on this, we speculate that NEK2 may also regulate the expression of PD-L1 in these suppressive cell populations. Previous experiments have demonstrated that the inhibition of NEK2 triggers anti-pancreatic cancer immunity by targeting PD-L1. However, the specific mechanism of how NEK2 regulates immunotherapy in ESCC remains to be further studied.

5. Conclusion

In conclusion, our experimental results show that NEK2 is highly expressed in ESCC and that it can promote the proliferation, migration and invasion of ESCC cells, thus playing an important role in regulating cell cycle progression. High expression of NEK2 is associated with poor prognosis in patients with ESCC. High expression of NEK2 in esophageal tumors may inhibit the antigen presentation of dendritic cells to tumor cells.

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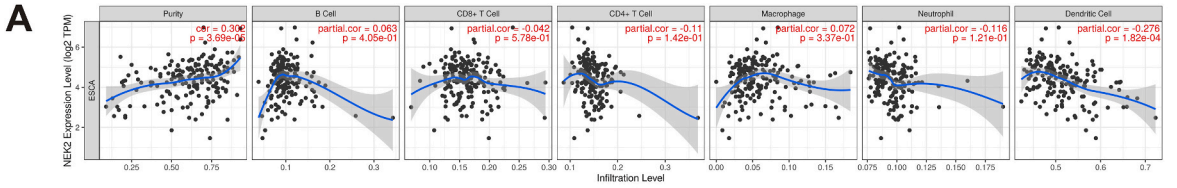
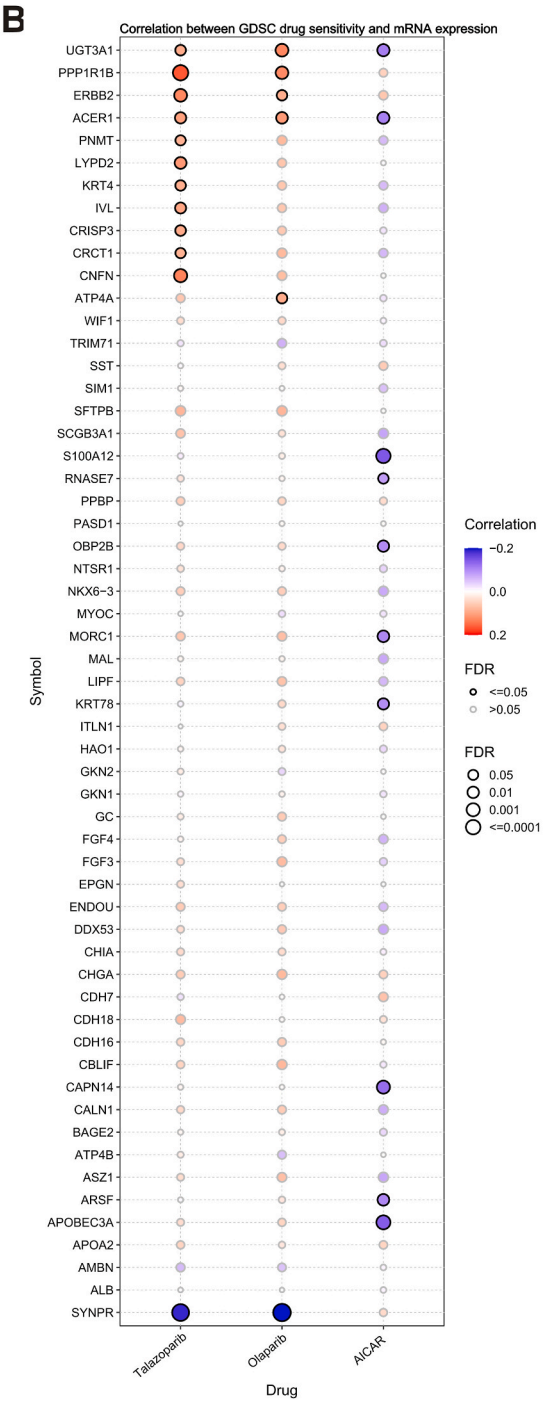
Data available statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.





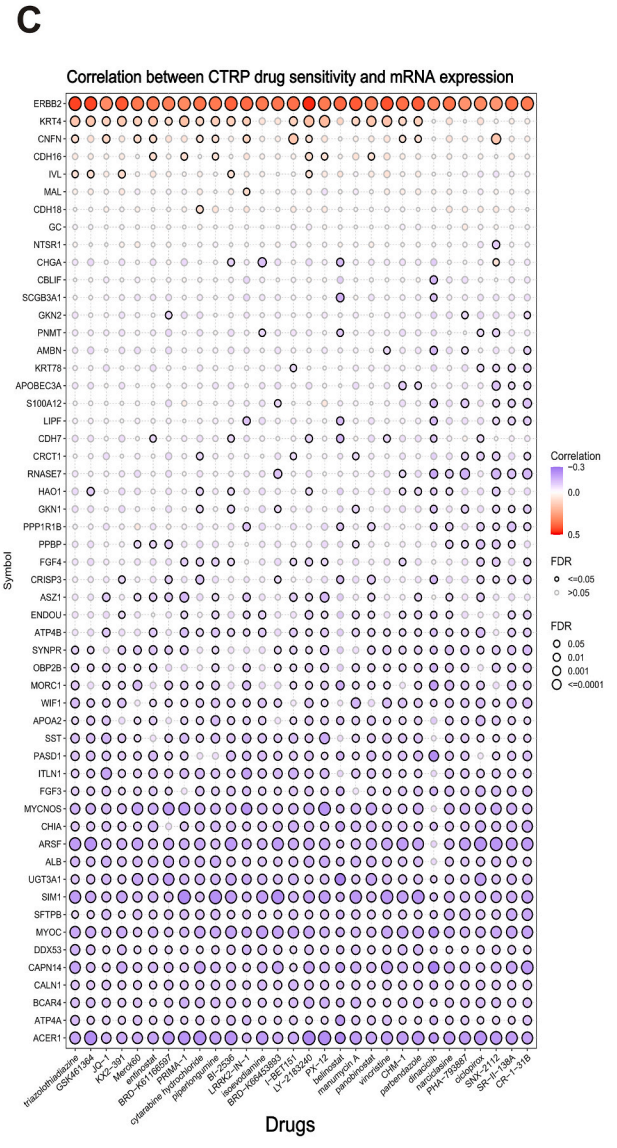


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Fig. 9. NEK2 is associated with immune infiltration in esophageal cancer. (A) Negative correlation between NEK2 gene expression and dendritic cell infiltration in esophageal cancer patient data through TIMER tumor immune infiltration analysis database (https://cistrome.shinyapps.io/timer/). (B&C) Divided esophageal cancer samples into high and low groups based on 50 % expression of NEK2, screened 95 differential genes, and conducted GDSC drug sensitivity analysis.

CRediT authorship contribution statement

Shaorui Gu: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **YakuFujiang Yasen:** Validation, Resources, Data curation. **Mengying Wang:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Baiqing Huang:** Visualization, Methodology, Data curation. **Yongxin Zhou:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation. **Wenli Wang:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

ESCC： Esophageal squamous cell carcinoma
ESCA： Esophageal carcinomas
NIMA： Never in Mitosis A
NEK2： NIMA-related kinase 2
IGF2： Insulin-like growth factor 2
CDC20： Cell division cyclin 20
E2F1： E2F transcription factor 1
PI3K： phosphatidylinositol 3-kinase
PKB/AKT： protein kinase B
TCGA： the Cancer Genome Atlas
GTEx： Genotype-Tissue Expression
GSEA： Gene Set Enrichment Analysis)
GO： Gene Ontology
TCGA： The Cancer Genome Atlas
GTEx： Genotype-Tissue Expression
GO： Gene Ontology
CHX： cycloheximide
EDTA： ethylenediaminetetraacetic acid
DAB： Diaminobenzidine
PMSF： Phenylmethanesulfonyl fluoride
BCA： Bicinchoninic Acid
EdU： 5-ethynyl-2′-deoxyuridine
PI: Propidium Iodide
ACC: Adrenal cortical carcinoma
BLCA: Bladder Urothelial Carcinoma
BRCA: Breast invasive carcinoma
CESC: Cervical squamous cell carcinoma
CHOL: Cholangio carcinoma
COAD: Colon adenocarcinoma
DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
ESCA: Esophageal carcinoma
GBM: Glioblastoma multiforme
HNSC: Head and Neck squamous cell carcinoma
KICH: Kidney Chromophobe
KIRC: Kidney renal clear cell carcinoma
KIRP: Kidney renal papillary cell carcinoma
LAML: Acute Myeloid Leukemia
LGG: Brain Lower Grade Glioma
LIHC: Liver hepatocellular carcinoma
LUAG: Lung adenocarcinoma

LUSC: Lung squamous cell carcinoma
MESO: Mesothelioma
OV: Ovarian serous cystadenocarcinoma
PAAD: Pancreatic adenocarcinoma
PCPG: Pheochromocytoma and Paraganglioma
PRAD: Prostate adenocarcinoma
READ: Rectum adenocarcinoma
SARC: Sarcoma
SKCM: Skin Cutaneous Melanoma
STAD: Stomach adenocarcinoma
TGCT: Testicular Germ Cell Tumors
THCA: Thyroid carcinoma
THYM: Thymoma
UCEC: Uterine Corpus Endometrial Carcinoma
UCS: Uterine Carcinosarcoma
UVM: Uveal Melanoma

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29682.

References

[1] F.L. Huang, S.J. Yu, Esophageal cancer: risk factors, genetic association, and treatment, Asian J. Surg. 41 (2018) 210–215.
[2] H. Tang, L. Jiang, C. Zhu, R. Liu, Y. Wu, Q. Yan, M. Liu, Y. Jia, J. Chen, Y. Qin, V.H. Lee, S. Luo, Q. Wang, X.Y. Guan, Loss of cell adhesion molecule L1 like promotes tumor growth and metastasis in esophageal squamous cell carcinoma, Oncogene 38 (2019) 3119–3133.
[3] J. Lagergren, E. Smyth, D. Cunningham, P. Lagergren, Oesophageal cancer, Lancet. 390 (2017) 2383–2396.
[4] Z. Liu, S. Gu, T. Lu, K. Wu, L. Li, C. Dong, Y. Zhou, IFI6 depletion inhibits esophageal squamous cell carcinoma progression through reactive oxygen species accumulation via mitochondrial dysfunction and endoplasmic reticulum stress, J. Exp. Clin. Cancer Res. 39 (2020) 144.
[5] W. Cao, H. Lee, W. Wu, A. Zaman, S. McCorkle, M. Yan, J. Chen, Q. Xing, N. Sinnott-Armstrong, H. Xu, M.R. Sailani, W. Tang, Y. Cui, J. Liu, H. Guan, P. Lv, X. Sun, L. Sun, P. Han, Y. Lou, J. Chang, J. Wang, Y. Gao, J. Guo, G. Schenk, A.H. Shain, F.G. Biddle, E. Collisson, M. Snyder, T.G. Bivona, Multi-faceted epigenetic dysregulation of gene expression promotes esophageal squamous cell carcinoma, Nat. Commun. 11 (2020) 3675.
[6] Y. Zheng, Z. Chen, Y. Han, L. Han, X. Zou, B. Zhou, R. Hu, J. Hao, S. Bai, H. Xiao, W.V. Li, A. Bueker, Y. Ma, G. Xie, J. Yang, S. Chen, H. Li, J. Cao, L. Shen, Immune suppressive landscape in the human esophageal squamous cell carcinoma microenvironment, Nat. Commun. 11 (2020) 6268.
[7] M.L. Qiu, J.B. Lin, X. Li, R.G. Luo, B. Liu, J.W. Lin, Current state of esophageal cancer surgery in China: a national database analysis, BMC Cancer 19 (2019) 1064.
[8] Y. Song, H. Liu, C. Cui, X. Peng, C. Wang, X. Tian, W. Li, Silencing of peroxiredoxin 1 inhibits the proliferation of esophageal cancer cells and promotes apoptosis by inhibiting the activity of the PI3K/AKT pathway, Cancer Manag. Res. 11 (2019) 10883–10890.
[9] D.C. Codipilly, Y. Qin, S.M. Dawsey, J. Kisiel, M. Topazian, D. Ahlquist, P.G. Iyer, Screening for esophageal squamous cell carcinoma: recent advances, Gastrointest. Endosc. 88 (2018) 413–426.
[10] Y. Fang, X. Zhang, Targeting NEK2 as a promising therapeutic approach for cancer treatment, Cell Cycle 15 (2016) 895–907.
[11] Y. Li, L. Chen, L. Feng, M. Zhu, Q. Shen, Y. Fang, X. Liu, X. Zhang, NEK2 promotes proliferation, migration and tumor growth of gastric cancer cells via regulating KDM5B/H3K4me3, Am. J. Cancer Res. 9 (2019) 2364–2378.
[12] T.D. Melo-Hanchuk, M.B. Martins, L.L. Cunha, F.A. Soares, L.S. Ward, J. Vassallo, J. Kobarg, Expression of the NEK family in normal and cancer tissue: an immunohistochemical study, BMC Cancer 20 (2020) 23.
[13] X.L. Liu, H.M. Liu, N. Han, F.H. Li, F. Sun, D.M. Fan, Q. Xu, PCAT1 promotes the proliferative and migratory potentials of ovarian cancer via targeting NEK2, Eur. Rev. Med. Pharmacol. Sci. 23 (2019) 8239–8248.
[14] H. Shen, W. Yan, J. Yuan, Z. Wang, C. Wang, NEK2B activates the wnt pathway and promotes triple-negative breast cancer chemothezrapy-resistance by stabilizing β-catenin, J. Exp. Clin. Cancer Res. 38 (2019) 243.
[15] Y. Zheng, Y. Li, J. Lian, H. Yang, F. Li, S. Zhao, Y. Qi, Y. Zhang, L. Huang, TNF-α-induced Tim-3 expression marks the dysfunction of infiltrating natural killer cells in human esophageal cancer, J. Transl. Med. 17 (2019) 165.
[16] J. Ferlay, M. Colombet, I. Soerjomataram, C. Mathers, D.M. Parkin, M. Piñeros, A. Znaor, F. Bray, Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods, Int. J. Cancer 144 (2019) 1941–1953.
[17] Y. Wang, G. Wang, Y. Ma, J. Teng, Y. Wang, Y. Cui, Y. Dong, S. Shao, Q. Zhan, X. Liu, FAT1, a direct transcriptional target of E2F1, suppresses cell proliferation, migration and invasion in esophageal squamous cell carcinoma, Chin. J. Cancer Res. 31 (2019) 609–619.
[18] K. Zhang, B. Zhang, Y. Bai, L. Dai, E2F1 promotes cancer cell sensitivity to cisplatin by regulating the cellular DNA damage response through miR-26b in esophageal squamous cell carcinoma, J. Cancer 11 (2020) 301–310.
[19] J.N. Chun, M. Cho, S. Park, I. So, J.H. Jeon, The conflicting role of E2F1 in prostate cancer: a matter of cell context or interpretational flexibility? Biochim. Biophys. Acta Rev. Canc 1873 (2020) 188336.
[20] B. Li, W.W. Xu, X.Y. Guan, Y.R. Qin, S. Law, N.P. Lee, K.T. Chan, P.Y. Tam, Y.Y. Li, K.W. Chan, H.F. Yuen, S.W. Tsao, Q.Y. He, A.L. Cheung, Competitive binding between Id1 and E2F1 to CDC20 regulates E2F1 degradation and thymidylate synthase expression to promote esophageal cancer chemoresistance, Clin. Cancer Res. 22 (2016) 1243–1255.
[21] S. Müller, N. Bley, B. Busch, M. Glaß, M. Lederer, C. Misiak, T. Fuchs, A. Wedler, J. Haase, J.B. Bertoldo, P. Michl, S. Hüttelmaier, The oncofetal RNA-binding protein IGF2BP1 is a druggable, post-transcriptional super-enhancer of E2F-driven gene expression in cancer, Nucleic Acids Res. 48 (2020) 8576–8590.
[22] X. Zhang, X. Huang, J. Xu, E. Li, M. Lao, T. Tang, G. Zhang, C. Guo, X. Zhang, W. Chen, D.K. Yadav, X. Bai, T. Liang, NEK2 inhibition triggers anti-pancreatic cancer immunity by targeting PD-L1, Nat. Commun. 12 (1) (2021) 4536.
[23] Y. Cheng, F. Sun, D.V. Alapat, V. Wanchai, D. Mery, W. Guo, H. Cao, Y. Zhu, C. Ashby, M.A. Bauer, I. Nookaew, E.R. Siegel, J. Ying, J.R. Chen, D. Gai, B. Peng, H. Xu, C. Bailey, Hadidi S. Al, C. Schinke, S. Thanendrarajan, M. Zangari, M. Chesi, P.L. Bergsagel, F. van Rhee, S. Janz, G. Tricot, J.D. Shaughnessy Jr., F. Zhan, High NEK2 expression in myeloid progenitors suppresses T cell immunity in multiple myeloma, Cell Rep Med 4 (10) (2023 Oct 17) 101214.

[24] J. Vivian, A.A. Rao, F.A. Nothaft, C. Ketchum, J. Armstrong, A. Novak, B. Paten, Toil enables reproducible, open source, big biomedical data analyses, Nat. Biotechnol. 35 (4) (2017) 314–316.
[25] A.M. Fry, L. O'Regan, S.R. Sabir, R. Bayliss, Cell cycle regulation by the NEK family of protein kinases, J. Cell Sci. 125 (Pt 19) (2012) 4423–4433.
[26] B. Frett, R.V. Brown, M. Ma, W. Hu, H. Han, H.Y. Li, Therapeutic melting pot of never in mitosis gene a related kinase 2 (Nek2): a perspective on Nek2 as an oncology target and recent advancements in Nek2 small molecule inhibition, J. Med. Chem. 57 (14) (2014) 5835–5844.
[27] M.T. Pickering, B.M. Stadler, T.F. Kowalik, miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression, Oncogene 28 (1) (2009) 140–145.
[28] S. Nath, A. Chowdhury, S. Dey, A. Roychoudhury, A. Ganguly, D. Bhattacharyya, S. Roychoudhury, Deregulation of Rb-E2F1 axis causes chromosomal instability by engaging the transactivation function of Cdc20-anaphase-promoting complex/cyclosome, Mol. Cell Biol. 35 (2) (2015) 356–369.
[29] J. Brouwer-Visser, G.S. Huang, IGF2 signaling and regulation in cancer, Cytokine Growth Factor Rev. 26 (3) (2015) 371–377.
[30] G. Liu, W. Guo, G. Chen, W. Li, Y. Cui, J. Qin, J. Peng, Lnc-MCEI mediated the chemosensitivity of esophageal squamous cell carcinoma via miR-6759-5p to competitively regulate IGF2, Int. J. Biol. Sci. 16 (2020) 2938–2950.