Bioinformatics and experimental validation of an AURKA/TPX2 axis as a potential target in esophageal squamous cell carcinoma

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Received January 13, 2023; Accepted April 3, 2023

DOI: 10.3892/or.2023.8553

Abstract. Aurora kinase A (AURKA), a serine/threonine kinase that regulates mitotic processes, has garnered significant interest given its association with the development of several types of cancer. In the present study, it was shown that AURKA expression was significantly upregulated in esophageal squamous cell carcinoma (ESCC) and could serve as a diagnostic and prognostic indicator based on data obtained from The Cancer Genome Atlas (TCGA) and immunohistochemical analysis. In addition, AURKA was functionally associated with ESCC cell proliferation and colony formation *in vitro* and knockdown of AURKA inhibited ESCC tumor growth *in vivo*. Both bioinformatics analysis and pull-down assays demonstrated that TPX2 interacted with AURKA, and their expression was correlated. AURKA cooperated with TPX2 to regulate ESCC progression via the PI3K/Akt pathway. Furthermore, AURKA or TPX2 expression levels were negatively associated with the infiltration of cytotoxic cells, $CD8^+$ T cells and mast cells, but positively associated with Th2 cells. The present study provided a relatively comprehensive understanding of the oncogenic roles of AURKA in ESCC based on data obtained from TCGA combined with experimental analysis.

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Abbreviations: AURKA, Aurora kinase A; ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; IP, immunoprecipitation; GEO, Gene Expression Omnibus

Key words: ESCC, AURKA, TPX2, therapeutic target, bioinformatics

Introduction

Esophageal carcinoma is one of the leading causes of cancer-related death worldwide. According to the Global Cancer Statistics 2020, esophageal carcinoma is the seventh most common and sixth most lethal type of cancer (1). Two most common subtypes of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, and the prevalence varies by country (2). ~90% of esophageal cancer cases are ESCC, which has a poor prognosis and a high mortality rate (3). It is worth noting that China accounts for ~50% of all esophageal cancer cases worldwide, and in certain areas, ESCC is one of the leading causes of cancer-related death (4,5). The prognosis of patients with ESCC is poor, with <20% of patients surviving >5 years after diagnosis (6). Therefore, there is a need to identify novel therapeutic targets in ESCC.

Aurora kinase A (AURKA) is a serine/threonine kinase that is involved in mitosis progression, centrosome maturation/separation, and mitotic spindle function. In addition to its important role in the cell cycle, an increasing number of studies have shown that AURKA participates in cancer development and progression in several types of cancer and upregulated AURKA expression has consistently been shown to be associated with a poor prognosis, including in stomach (7), bladder (8), breast (9), colorectal (10) and ovarian cancer (11). AURKA can promote cancer cell proliferation and migration, inhibit apoptosis, and is associated with resistance to radiotherapy and chemotherapy. Given its significance in cancer development, AURKA may serve as a candidate for kinase inhibition in the management of cancer. Over the past decades, a range of AURKA inhibitors have been developed and several of these have been assessed in clinical studies (12).

Although several studies have indicated that AURKA can act as a valuable biomarker and therapeutic target, there is a lack of comprehensive research on AURKA expression, function, and the molecular mechanism involved in ESCC. In the present study, it was revealed that AURKA expression was upregulated in ESCC, and its clinical value in cancer diagnosis was determined based on data obtained from The Cancer Genome Atlas (TCGA) and tissue array analysis. In addition, AURKA was identified to enhance ESCC cell proliferation and

colony formation *in vitro* and promoted ESCC tumor growth *in vivo*. Both bioinformatics analysis and pull-down assays identified TPX2 as an interacting and correlated protein of AURKA. AURKA cooperated with TPX2 to promote ESCC proliferation through the PI3K/Akt pathway. These findings highlighted the AURKA/TPX2 axis as a useful candidate for ESCC diagnosis and therapy.

Materials and methods

Cell culture and reagents. All ESCC cell lines (KYSE30, KYSE70, KYSE140, KYSE150, KYSE410, KYSE450, KYSE510 and EC109) and 293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured at 37˚C in a 5% CO_2 humidified incubator and were not maintained for >2 months. All ESCC cells were maintained in RPMI-1640, while 293T cells were cultured in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% FBS (Biological Industries). All cell lines were mycoplasma-free and were authenticated using short tandem repeat DNA typing. The human immortalized normal esophageal epithelial cell line SHEE was kindly gifted by Dr Enmin Li from the Laboratory of Tumor Pathology (Shantou University Medical College, Shantou, Guangdong, China). Transfection was performed using Lipofectamine® 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) for ESCC cell lines and Simple-Fect Reagent (Signaling Dawn Biotech) for 293T cells according to the manufacturer's protocol. AURKA inhibitor alisertib was purchased from Selleck Chemicals (cat. no. S1133).

The antibodies used in the present study were as follows: Anti-AURKA (cat. no. ab13824; Abcam), anti-Flag (cat. no. F1804; MilliporeSigma), anti-HA (cat. no. 3724; Cell Signaling Technology, Inc.), anti-His (cat. no. ab137839; Abcam), anti-phosphorylated (p)-PI3K (Tyr467) (cat. no. sc-293115; Santa Cruz Biotechnology, Inc.), anti-p-AKT (Ser473) (cat. no. 4060; Cell Signaling Technology, Inc.), anti-p-Akt (Thr308) (cat. no. 13038; Cell Signaling Technology, Inc.), anti-PI3Kp85 (cat. no. 4257; Cell Signaling Technology, Inc.), anti-Akt (pan) (cat. no. 4691; Cell Signaling Technology, Inc.), anti-p21 (cat. no. sc-397; Santa Cruz Biotechnology, Inc.), anti-p27 (cat. no. 3686; Cell Signaling Technology, Inc.), anti-p53 (cat. no. sc-126; Santa Cruz Biotechnology, Inc.), anti-TPX2 (cat. no.122455; Cell Signaling Technology, Inc.), anti-RPS6 (cat. no. 2217; Cell Signaling Technology, Inc.), anti-HSP27 (cat. no. sc-13132; Santa Cruz Biotechnology, Inc.), anti-VDAC (cat. no. 4866; Cell Signaling Technology, Inc.), anti-RPL7 (cat. no. 2415; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. HRP-60004; ProteinTech Group, Inc.).

AURKA knockdown using short hairpin (sh)RNA. Gene knockdown was performed using specific shRNAs kindly provided by Professor Xiang Li from Zhengzhou University. The target sequence for shAURKA-1 is 'TGGCTCTTAAAG TGTTATTTA' and for shAURKA-3 is 'GCAGAGAACTGC TACTTATAT'. Briefly, 4 μg pMD2.G, 4 μg psPAX2 packaging plasmids, and 4 μg pLKO.1 plasmid containing the specific shRNAs were transfected into 293T cells. A total of 2 days after transfection, the viral particles were collected and filtered using a 0.45-mm filter. To obtain stable AURKA knockdown ESCC cell lines, the lentivirus was transfected with a MOI of 50 using 8 mg/ml polybrene (cat. no. 107689; MilliporeSigma) and selected using 2 μg/ml puromycin.

Western blotting. Cells were lysed using RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.25% deoxycholic acid disodium salt, 1% NP40, 150 mM NaCl and 0.1% SDS). After sonication, cells were centrifuged at 12,000 x g for 15 min at 4˚C. Protein concentration was determined using a BCA assay. Then, 30 μg of cell extracts were subjected to 10-12% SDS-PAGE electrophoresis and transferred to PVDF membranes. Following protein transfer, the membranes were blocked with 5% milk and then incubated with specific primary antibodies (1:1,000) at 4˚C overnight followed by a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. nos. ab6721 and ab6728; Abcam) at room temperature for 2 h. Protein bands were visualized using an enhanced chemiluminescence reagent (cat. no. 32106; Thermo Fisher Scientific, Inc.).

Immunoprecipitation (IP). Cell lysates were prepared using lysis buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, protease inhibitors and PMSF for 30 min at 4˚C and centrifuged at 12,000 g for 15 min at 4˚C. Co-IP was performed using 2 μg antibodies and 500 mg lysate. Cell lysates were incubated with specific antibodies at 4˚C overnight and then combined with 20 μl protein A/G agarose beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) for 3 h at 4˚C. The beads were washed twice with lysis buffer and twice with PBS, and the immune complexes were eluted with sample loading buffer for 5 min at 95˚C. The immunoprecipitated proteins were assessed using western blotting.

MTT and colony formation assays. Viable cells were seeded into 96-well transparent walled plates at a density of 1-2x10^3 cells per well, depending on the growth kinetics of the specific cell line. Cell proliferation was determined using MTT assays according to the manufacturer's protocol (cat. no. M1020; Beijing Solarbio Science & Technology Co., Ltd.). Namely, 10% of 5 mg/ml MTT solution was added to cells for a duration of 4 h at the time point of 0, 24, 48, 72, or 96 h. The medium was then discarded, and 110-μl formazan solution was added to dissolve the MTT crystals. Cell proliferation was next measured at an absorbance of 490 nm. For the colony formation assay, 200 viable cancer cells were seeded into six-well plates in 2-ml cell culture medium in triplicate. After 1-2 weeks depending on the growth kinetics size of the cell line, colonies were stained with 0.2% crystal violet at room temperature for 15 min and images were captured under the light microscope. The number of colonies which contained at least 20 cells was counted manually and analyzed.

Soft agar colony formation assay. A total of 8x10^3 viable cancer cells were seeded in triplicate in 1-ml cell culture medium with 2 mM glutamine, 5 μg/ml gentamycin and 0.3% soft agar. The mixture was layered onto 0.5% solidified agar in cell culture medium in six-well plates. After 1-3 weeks depending on the growth kinetics of the cells, images of the colonies were captured under a light microscope with x100

magnification and counted using the Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.).

Immunohistochemical (IHC) analysis. A human ESCC tissue array was purchased from Shanghai Outdo Biotech Company. The slides were deparaffinized in xylene, rehydrated using a series of decreasing alcohol solutions, immersed in 3% hydrogen peroxide (cat. no. PV-6002 Kit; OriGene Technologies, Inc.) for 10 min to block endogenous peroxidase activity, and antigen-retrieval was performed using a pressure cooker for 3 min in citrate buffer (pH=6). Subsequently, non-specific binding was blocked using 10% normal goat serum (cat. no. ZLI-9022; OriGene Technologies, Inc.) at room temperature for 30 min. Then, the slides were incubated overnight at 4˚C in a humidified chamber with an anti-AURKA antibody (1:200). After washing, the slides were incubated with the horseradish peroxidase-labeled secondary antibody (cat. no. PV-6002 Kit; OriGene Technologies, Inc.) for 30 min at room temperature. Slides were stained using DAB Kit (cat. no. ZLI-9017; OriGene Technologies, Inc.) and finally counterstained with hematoxylin. After staining, images of the sections were captured under a light microscope and analyzed using Image-Pro Plus.

Purification of recombinant AURKA protein. AURKA was subcloned into the pet28a vector and transformed into *E. coli* strain BL-21. Then, protein expression in BL21 was induced at 16˚C overnight using 0.5 mM isopropyl-D-thiogalactopyranoside. Cells were collected and resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonylfluoride, 1% Triton X-100). After sonication, the supernatants of the cell lysates were incubated with NI-NTA agarose (Qiagen GmbH) at 4˚C for 4 h. The beads with bound AURKA were washed four times with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 60 mM imidazole). Protein purity was determined using SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.

Pull down assay and mass spectrometry. The purified AURKA-NI-NTA agarose complex was incubated with KYSE410 cell lysates for 4 h at 4˚C. The complex was subsequently washed four times in washing buffer (50 mM NaH2PO4, 300 mM NaCl, 60 mM imidazole) and subjected to SDS/PAGE. After CBB staining, discrepant gel lanes were cut down and prepared for mass spectrometry analysis.

Xenograft mouse model. The present study was approved (approval no. NYISTIRB-2021-005) by the Ethics Committee of Nanyang Institute of Technology (Nanyang, China). A total of 12 6-8-week-old NU/NU female mice (weight, 22±2 g; Beijing Vital River Laboratory Animal Technology Co., Ltd.) were used for animal experiments. All mice were housed in a specific pathogen-free sterile environment with a constant temperature of 25˚C, a relative humidity of 50-70%, with free access to sterilized food and autoclaved water supply and mice health and behaviour were monitored daily. Mice were randomly divided into three groups according to body weight (scramble group: n=4; shAURKA-1 group: n=4; shAURKA-3 group: n=4). KYSE450 cells infected with the indicated lentivirus ($1x10^7$ cells) were injected subcutaneously into the right flank of each mouse. The tumor volumes were determined using calipers to measure the longest (length) and shortest (diameter) every 4 days and were calculated as follows: length x $diameter^2$ x0.5. The humane endpoint was determined as a tumor volume of 1 cm^3 and all the mice were alive until this time point. A total of 4 weeks after tumor injection, all mice were humanely sacrificed by intraperitoneal injection of 2% sodium pentobarbital (100 mg/kg), and the death was confirmed by the cessation of breathing and heartbeat. Next, the tumor samples were harvested from sacrificed mice, and tumor weight was measured.

Data acquisition. All bioinformatics data on esophageal carcinoma were obtained from TCGA and data analysis was performed using R version 3.6.3. AURKA and TPX2 expression in paired tissues were visualized using the ggplot2 package and ROC curves for AURKA and TPX2 were generated using the pROC package. For the correlation heat map, data was obtained from TIMER2.0 (timer.cistrome.org/) and visualized using the ggplot2 and ggdendro packages. The expression of AURKA and TPX2 and its clinic correlation was from UALCAN (http://ualcan.path.uab.edu). AURKA-binding proteins were derived from STRING (https://string-db.org/) and GEPIA2.0 (http://gepia2.cancer-pku.cn/#index) was used to get AURKA-correlated genes. The GSE161533 dataset, downloaded from Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/), contained the expression profiles from paired normal tissues, para-tumor tissues, and tumor tissues of 28 ESCC patients. For Gene Ontology (GO) (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) functional enrichment, the clusterProfiler package was used for analysis, and the org.Hs.eg.db package for ID transition. For GSEA, data was obtained from MSigDB Collections (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) and the clusterProfiler package was used for analysis. The ggplot2 and ggdendro packages were used for the visualization of the enriched data. Immune cell infiltration analysis was performed using the R package GSVA and ggplot2.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6 (Dotmatics). All statistical tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference. All data are presented as the mean ± SD. Gene expression in paired tissues was analyzed using a Student's paired t-test. Gene expression in unpaired tissues was analyzed using an unpaired Student's t-test. Kaplan-Meier analysis was used for the overall survival analysis followed by the log-rank test. An unpaired Student's t-test was also used to analyze the data of the functional assays between control and AURKA knockdown or overexpression cells.

Results

AURKA expression is increased in esophageal carcinoma. First, AURKA expression was explored in cancer based on data obtained from TCGA. Upregulation of AURKA was observed in several cancer types and AURKA exhibited different mutation rates in the different types of various cancer

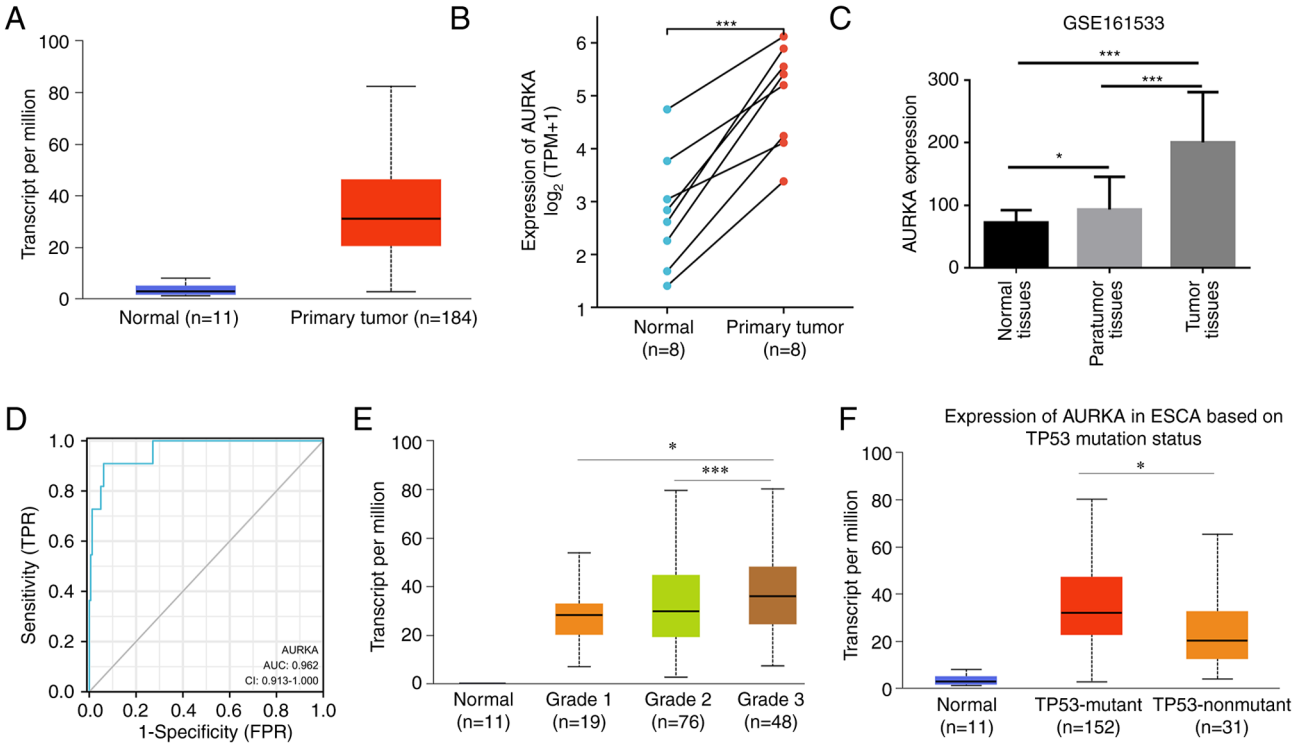

Figure 1. Expression of AURKA in esophageal carcinoma and its relationship with pathological status. (A) Expression of AURKA in unpaired normal esophageal tissues and esophageal carcinoma tumor tissues derived from the UALCAN website. (B) Expression of AURKA in paired normal esophageal tissues and esophageal carcinoma tumor tissues based on data obtained from TCGA. (C) Expression of AURKA in paired normal tissues, para-tumor tissues and ESCC tumor tissues (n=28) from GSE161533. (D) AURKA levels in normal and cancerous tissues were used for ROC analysis based on TCGA data. (E) Expression of AURKA in esophageal carcinoma based on tumor grade according to the UALCAN website. (F) Expression of AURKA in esophageal carcinoma based on TP53 mutation status according to the UALCAN website. *P<0.05 and ***P<0.001. AURKA, Aurora kinase A; TCGA, The Cancer Genome Atlas; ESCC, esophageal squamous cell carcinoma.

(Fig. S1A and B). As shown in Fig. 1A, AURKA mRNA expression was significantly higher in primary esophageal carcinoma tumor tissues than in the respective normal tissues. In addition, the expression levels of AURKA were upregulated in esophageal carcinoma tumor tissues compared with the paired normal tissues based on the data obtained from TCGA (Fig. 1B). Based on the data obtained from GEO, AURKA expression was highest in ESCC tumor tissues compared with the paired para-tumor tissues and normal tissues, and the para-tumor tissues exhibited higher AURKA expression levels than the normal tissues (Fig. 1C). Furthermore, ROC diagrams for AURKA demonstrated that AURKA expression levels served as an excellent biomarker for stratifying esophageal carcinoma patients from healthy individuals (AUC=0.962, Fig. 1D). Next, the relationship between AURKA expression and certain clinicopathological characteristics in TCGA was assessed. It was revealed that patients with tumor grade 3, which is poorly differentiated, exhibited higher AURKA expression than grade 1 and grade 2 tumors (Fig. 1E). Notably, it was found that AURKA expression was associated with p53 mutation status. In p53 mutant esophageal carcinoma tissues, AURKA revealed significantly higher expression than p53 non-mutant tissues (Fig. 1F).

AURKA expression is correlated with a poorer prognosis. To further explore AURKA expression and its significance in ESCC, AURKA expression was measured in a human ESCC tissue array using IHC staining. The results demonstrated that the expression levels of AURKA were significantly upregulated in ESCC tissues compared with the paired (Fig. 2A and B) and unpaired (Fig. 2C) cancer adjacent tissues. Next, the association between AURKA expression and the survival of patients with ESCC was examined. ESCC patients with upregulated AURKA expression levels had a significantly shorter overall survival than those with lower AURKA expression levels based on the Kaplan-Meier analysis (Fig. 2D). Furthermore, the clinicopathologic correlation analysis revealed that AURKA expression was significantly higher in patients with a larger tumor size, lymph node metastasis, or more advanced clinical stage cancer (Fig. 2E; Table I). These data showed a critical role for AURKA upregulation in the progression of ESCC. To further validate the expression of AURKA in ESCC, western blotting was used to measure AURKA protein levels. It was identified that in 80% of ESCC tumor tissues, AURKA expression was significantly higher than that in the paired adjacent normal tissues (Fig. 2F). Finally, AURKA expression in ESCC cell lines and in the SHEE normal esophageal epithelial cells was also determined. A general trend of increased AURKA expression in ESCC cell lines compared with the control SHEE cells was observed (Fig. 2G). Collectively, these results suggested that AURKA expression in ESCC was upregulated and was correlated with patient survival and clinical stage, serving as an informative prognostic factor in ESCC.

AURKA promotes ESCC proliferation via the PI3K/Akt pathway. To investigate the contribution of AURKA in ESCC progression, the cell proliferation and colony formation

Table I. Correlation between AURKA expression and clinicopathologic characteristics of esophageal squamous cell carcinoma.

Characteristics	Expression levels of AURKA: Low (n=47) (%)	Expression levels of AURKA: High (n=47) (%)	P-value
Sex			0.194
Male	33 (70.21)	37 (78.72)	
Female	14 (29.79)	10 (21.28)	
Age, years			0.169
≤60	12 (25.53)	17 (36.17)	
>60	35 (74.47)	30 (63.83)	
Histological grade			0.876
Well/moderately	34 (72.34)	33 (70.21)	
Poorly	13 (27.66)	14 (29.79)	
T classification			0.02
T1	4 (8.51)	1 (2.13)	
T2	6 (12.77)	5 (10.64)	
T3	37 (78.72)	38 (80.85)	
T4	0 (0.00)	3 (6.38)	
N classification			<0.0001
N0	27 (57.45)	16 (34.04)	
N1	15 (31.91)	13 (27.66)	
N2	5 (10.64)	13 (27.66)	
N3	0 (0.00)	5 (10.64)	
Clinical stage			<0.0001
I	7 (14.90)	2 (4.26)	
II	20 (42.55)	13 (27.66)	
III	20 (42.55)	26 (55.32)	
IV	0 (0.00)	6 (12.76)	

T classification means tumor burden discrepancy and N classification means lymph nodes metastasis status. P-value was evaluated by Chi square analysis. AURKA, Aurora kinase A.

potential of ESCC cells after AURKA overexpression was first assessed. The results indicated that AURKA was effectively transfected into KYSE450, KYSE30, and KYSE150 cells based on the western blotting (Fig. S2A). Additionally, ectopic overexpression of AURKA significantly increased cell growth based on the results of the MTT assay (Fig. S2B). Moreover, an increased number and larger colonies were formed in ESCC cells overexpressing AURKA (Fig. S2C).

To further confirm the oncogenic role of AURKA in ESCC, specific shRNAs targeting AURKA were used to generate stable AURKA-knockdown cells. Both shAURKA-1 and shAURKA-3 effectively reduced AURKA expression in ESCC cells (Fig. 3A). Next, MTT and soft agar colony formation assays were used to assess whether AURKA affected cell proliferation and colony formation of the ESCC cells. The cell growth and colony formation capacity were significantly reduced following AURKA knockdown (Fig. 3B-D).

Next, the AURKA downstream signaling pathway in ESCC was assessed based on EGF stimulation. Western blotting revealed that AURKA knockdown reduced phosphorylation of Akt and PI3K (Fig. 3E). Furthermore, increased expression of p21, p53, and p27 was also observed after knockdown of AURKA expression (Fig. 3E). Finally, to determine whether AURKA could contribute to ESCC tumor growth *in vivo*, control cells or AURKA knockdown cells were subcutaneously injected into nude mice. The results showed that AURKA knockdown significantly inhibited tumor growth based on the reduced tumor volume and tumor weight (Fig. 3F-H). Taken together, AURKA was found to be associated with the acquisition of an oncogenic phenotype in ESCC.

Targeting AURKA with alisertib inhibits ESCC cell growth. To further verify the significance of targeting AURKA in ESCC, the antitumor effects of alisertib, an AURKA inhibitor, were determined. The MTT assay demonstrated that alisertib treatment significantly inhibited cell proliferation in a concentration-dependent manner and the inhibitory effects at 100 nM alisertib were ~85% in KYSE30 cells and 70% in KYSE450 cells (Fig. 4A). Furthermore, alisertib significantly inhibited KYSE30 and KYSE450 cell colony formation (Fig. 4B). In the anchorage-independent cell growth assay, alisertib exhibited inhibitory effects consistent with its suppressive effects on cell proliferation in KYSE30 and KYSE450 cells (Fig. 4C).

AURKA is involved in multiple biological processes and pathways in ESCC. To study the molecular mechanism of AURKA in ESCC tumorigenesis, the AURKA-binding proteins and the genes correlated with AURKA expression were screened. First, a total of 50 AURKA-binding proteins were obtained, which were supported by experimental evidence using STRING. The interaction network of these proteins is shown in Fig. 5A. Next, to identify the AURKA-binding proteins, a His-pull down assay was used. AURKA was purified using a prokaryotic expression system as demonstrated in Fig. S3A, and the purified AURKA protein was verified by western blotting (Fig. S3B). A total of 239 proteins were identified via mass spectrometry following the His pull-down assay (Fig. S3C and Table SI). Immunoprecipitation assays were performed to verify several AURKA-interacting proteins based on the mass spectrometry analysis. The results revealed that AURKA could bind with RPS6, HSP27, VDAC and RPL7 (Fig. S3D).

Next, the GEPIA2.0 tool was used to obtain the top 100 genes that were correlated with AURKA expression in esophageal carcinoma. The five most positively related genes were CSTF1 (R=0.71, P<0.001), VAPB (R=0.7, P<0.001), TAF4 (R=0.69, P<0.001), RAE1 (R=0.66, P<0.001) and TPX2 (R=0.66, P<0.001) (Fig. 5B). Furthermore, the heat map of the correlation of these genes in various types of cancer showed that TPX2 was correlated with AURKA in different types of cancer (Fig. 5C). Data obtained from GEPIA2 and STRING was combined with the results of the His-pull-down assay to perform KEGG and GO enrichment analyses. As demonstrated in Fig. 5D, the GO enrichment analysis data indicated that the majority of these genes were associated with the biological process or pathways of cell-substrate junction (including cell adhesion molecule binding, cadherin binding, focal adhesion) and cell cycle regulation (such as cell cycle

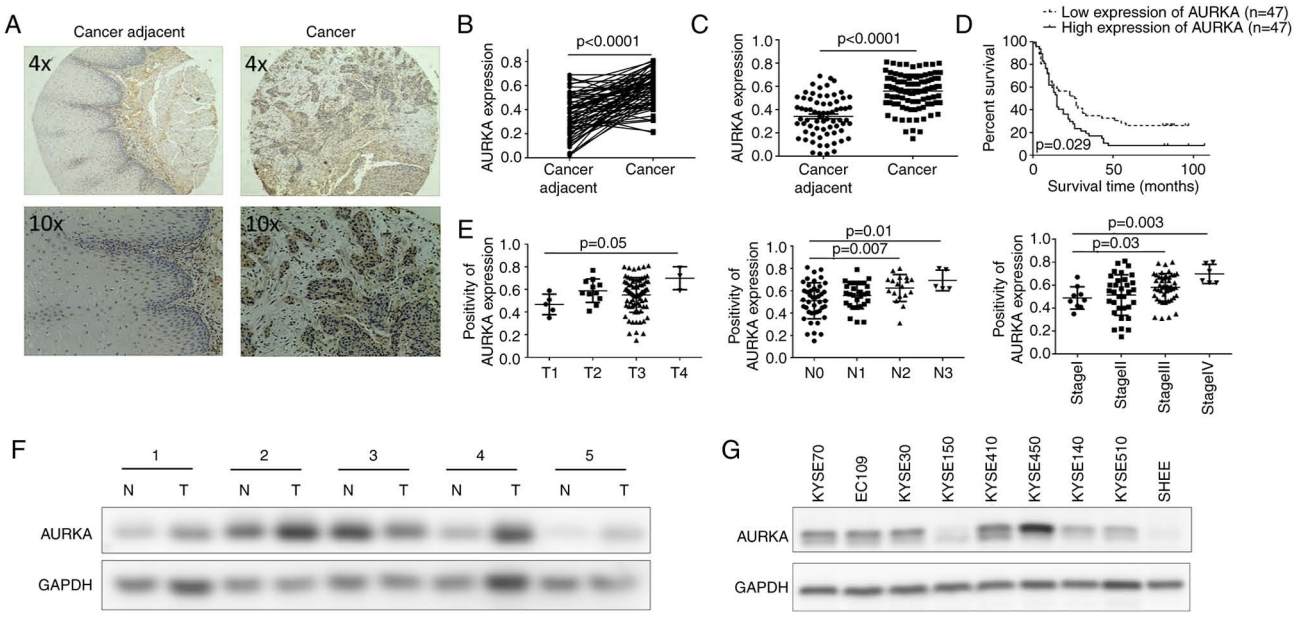

Figure 2. Upregulated expression of AURKA in ESCC is correlated with a poorer prognosis. (A) AURKA expression in ESCC cancer tissues and the adjacent tissues was verified by IHC, and the representative images are shown. (B and C) Expression of AURKA in (B) paired and (C) unpaired ESCC tissues was statistically analyzed based on the IHC staining. (D) Relationship between AURKA expression levels and the overall survival in the patients with ESCC. (E) Relationship between AURKA expression levels and the clinal stage of patients with ESCC. (F) Expression of AURKA in 5 paired human clinical samples with ESCC in the adjacent normal tissues and tumor tissues. (G) Expression of AURKA in normal esophageal epithelial cell SHEE and ESCC cell lines. AURKA, Aurora kinase A; ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry.

checkpoint, mitotic nuclear division and spindle organization). The KEGG data suggested that 'cellular senescence', 'ubiquitin mediated proteolysis', 'cell cycle', 'hippo signaling pathway' and 'platinum drug resistance' may be involved in the effect of AURKA on tumor pathogenesis (Fig. 5E).

AURKA-mediated effects on ESCC progression involve TPX2. Among AURKA-binding and AURKA expression-correlated proteins, an intersection analysis of STRING, GEPIA, and His-pull-down proteins showed one common member, namely, TPX2 (Fig. 6A). Additionally, aberrantly high TPX2 expression was observed in several different types of cancer (Fig. S4), and TPX2 expression was higher in esophageal carcinoma compared with the normal controls (Fig. 6B and C). According to a GEO dataset, TPX2 expression was upregulated in ESCC tumor tissues compared with the para-tumor tissues and normal tissues (Fig. 6D). Additionally, western blotting revealed increased TPX2 expression in ESCC cell lines compared with the normal esophageal epithelium SHEE cells (Fig. 6E). The association between TPX2 expression and clinicopathological features was further evaluated based on the UALCAN database. Similar to AURKA, TPX2 demonstrated significantly higher expression in p53 mutant tissues than in the p53 wild-type tissues (Fig. 6F). Furthermore, TPX2 expression was correlated with lymph node metastasis and advanced-clinical stage cancer (Fig. 6G and H). Next, TPX2 promoter methylation status was examined in esophageal carcinoma based on data obtained from TCGA, and the results showed that TPX2 expression was negatively associated with promoter methylation at the -513 region (Fig. 6I). More importantly, TPX2 was found to serve as an excellent diagnostic indicator for esophageal carcinoma (AUC=0.969, Fig. 6J). Finally, the correlation between AURKA and TPX2 expression was assessed. Based on GEPIA, AURKA expression was positively associated with TPX2 in esophageal carcinoma (R=0.66, P<0.001, Fig. 6K). Based on the GSE161533 dataset, TPX2 expression was associated with AURKA expression in ESCC tumor tissues (R=0.81, P<0.001, Fig. 6L).

Next, GSEA enrichment was performed to analyze the potential function of TPX2 in ESCC. The results showed that TPX2 was involved in cell cycle checkpoints, in mitotic G1 phase and in G1 to S transition (Fig. 6M). Additionally, TPX2 was related to AURKA activation in ESCC (Fig. 6M). To further confirm whether TPX2 was both an AURKA-interacting and AURKA-correlated protein, an immunoprecipitation assay was performed, and the results revealed that AURKA could bind with TPX2 in KYSE410 and KYSE450 cells (Fig. 7A). Furthermore, decreased expression of TPX2 was observed following AURKA knockdown (Fig. 7B). To further confirm TPX2 was involved in the oncogenic role of AURKA, the function of TPX2 in ESCC was also measured. It was shown that TPX2 knockdown significantly reduced ESCC cell proliferation and colony formation (Fig. 7C-F). Additionally, the expression of PI3K/Akt pathway-related proteins were evaluated after TPX2 knockdown, and the results demonstrated that the activity of the PI3K/Akt pathway was reduced when TPX2 expression was knocked down (Fig. 7G). Accordingly, AURKA may co-operate with TPX2 to regulate ESCC progression via the PI3K/Akt pathway.

AURKA or TPX2 expression levels are associated with tumor immune cell infiltration. Immune cells in the tumor microenvironment affect cancer patient survival. Among these immune cells, T cell-mediated immune responses have been used as therapeutic targets clinically (13,14). Thus, the relationship between AURKA and TPX2 expression levels with the levels of $CD4^+$ T cells was examined. There was a significantly

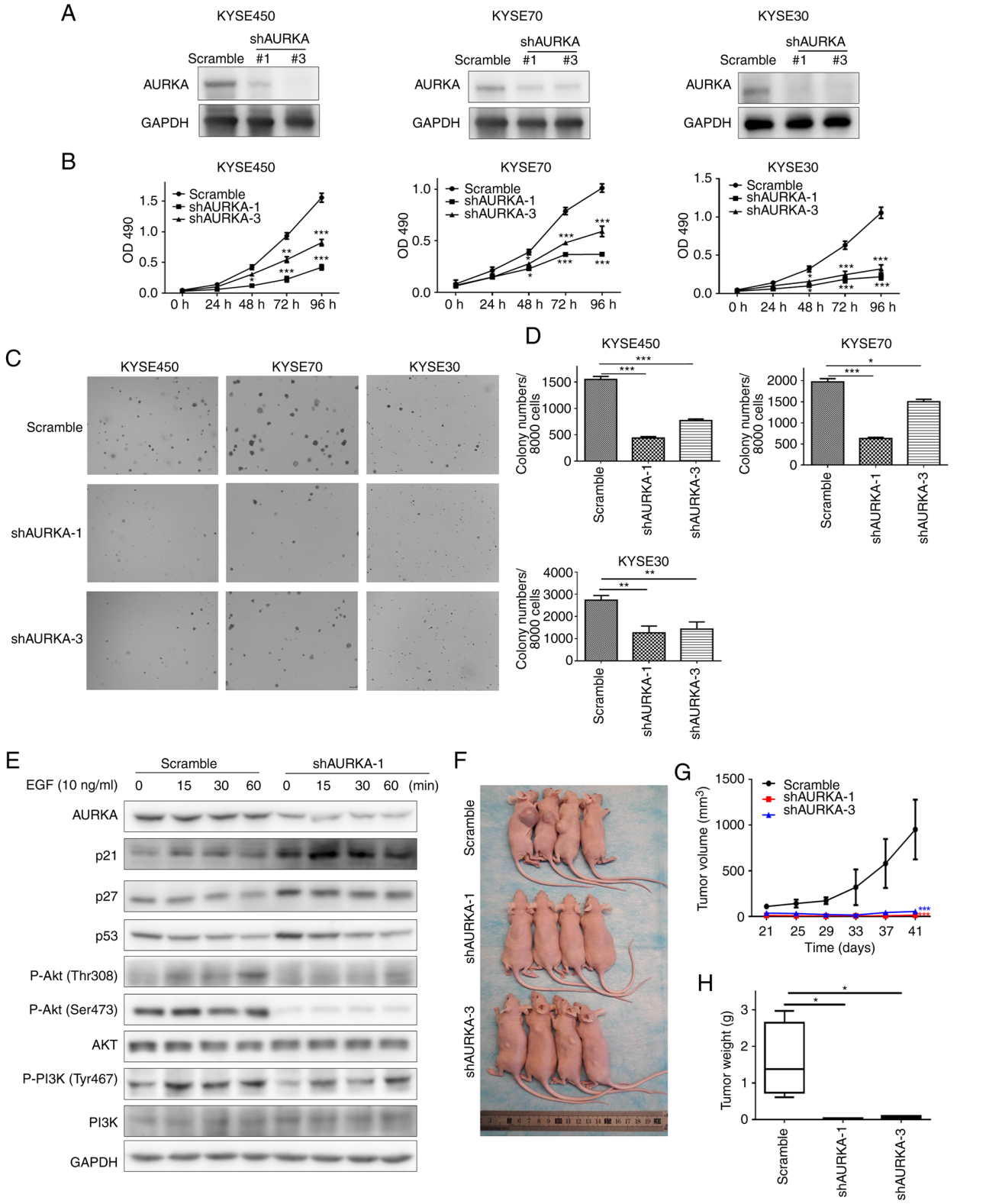

Figure 3. AURKA knockdown suppresses esophageal squamous cell carcinoma oncogenic properties *in vitro* and *in vivo*. (A) AURKA knockdown efficiency was detected through western blotting. (B-D) Cell proliferation was measured by (B) MTT and (C and D) soft agar colony formation assays. (E) The expression of the PI3K/Akt pathway related proteins in scramble and AURKA knockdown cells was detected following EGF stimulation. (F-H) A xenograft mouse model was established in nude mice subcutaneously implanted with scramble or AURKA knockdown KYSE450 cells. (F) Images of the xenografted mice. (G) Tumor volumes and (H) tumor weights were measured following tumor excision. *P<0.05, **P<0.01 and ***P<0.001. Data are presented as the mean ± SD. AURKA, Aurora kinase A; sh-, short hairpin; p-, phosphorylated.

positive relationship between AURKA and TPX2 expression with the infiltration of Th2 cells in humans pan-cancer (Fig. S5). Next, the relationship between AURKA and TPX2 expression levels with the different subsets of immune cells was examined. AURKA and TPX2 expression levels were positively associated with Th2 cells, T helper cells, and Tgd

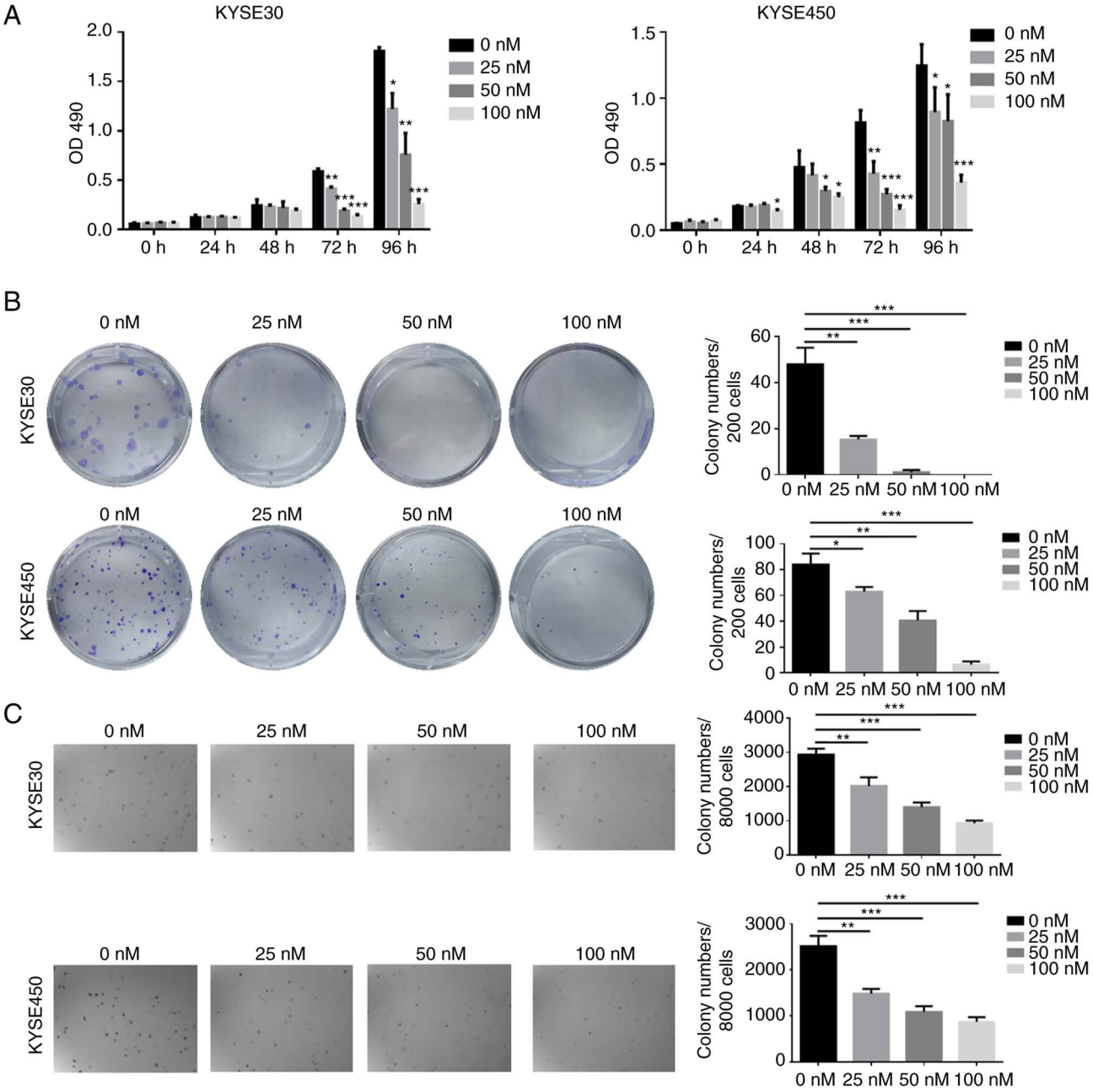

Figure 4. An inhibitor of Aurora kinase A, alisertib, inhibits esophageal squamous cell carcinoma cell growth. (A and B) Effect of alisertib on the proliferation of KYSE30 and KYSE450 cells was assessed after 24, 48, 72, and 96 h using an (A) MTT assay and (B) plate colony formation assay. (C) Effect of alisertib on the anchorage-independent colony growth of cells was evaluated. *P<0.05, **P<0.01 and ***P<0.001. Data are presented as the mean ± SD.

(Fig. 8A and B). In addition, AURKA was positively associated with Th17 cells (Fig. 8A). AURKA and TPX2 expression were negatively associated with the majority of the remaining types of immune cells including mast cells, cytotoxic cells, and $CD8^+$ T cells, amongst others (Fig. 8A and B). The correlation analysis between AURKA and TPX2 expression with the Th2 cells, cytotoxic cells, $CD8^+$ T cells, and mast cells is shown in Fig. 8C and D. Collectively, these results strongly suggested that AURKA and TPX2 exerted vital roles in tumor immunity.

Discussion

ESCC is an aggressive upper gastrointestinal tumor whose 5-year survival is <20%, given that several cases are diagnosed in the first instance at an advanced stage at the time of diagnosis and the lack of specific targeted drugs. Although the molecular signatures of ESCC have been well defined including the most frequently mutated and amplified genes, there are still no effective therapeutic targets (5,15,16). Therefore, studies elucidating the actionable targets in ESCC are urgently required. AURKA upregulation has been indicated in several types of cancer and was shown to be inversely related to disease prognosis. It has been reported that upregulation of AURKA in ESCC was related to distant lymph node metastasis and a poorer prognosis (4). The results of the present study highlighted the oncogenic role of AURKA in ESCC both through bioinformatics analysis and experimental verification. Upregulated AURKA expression

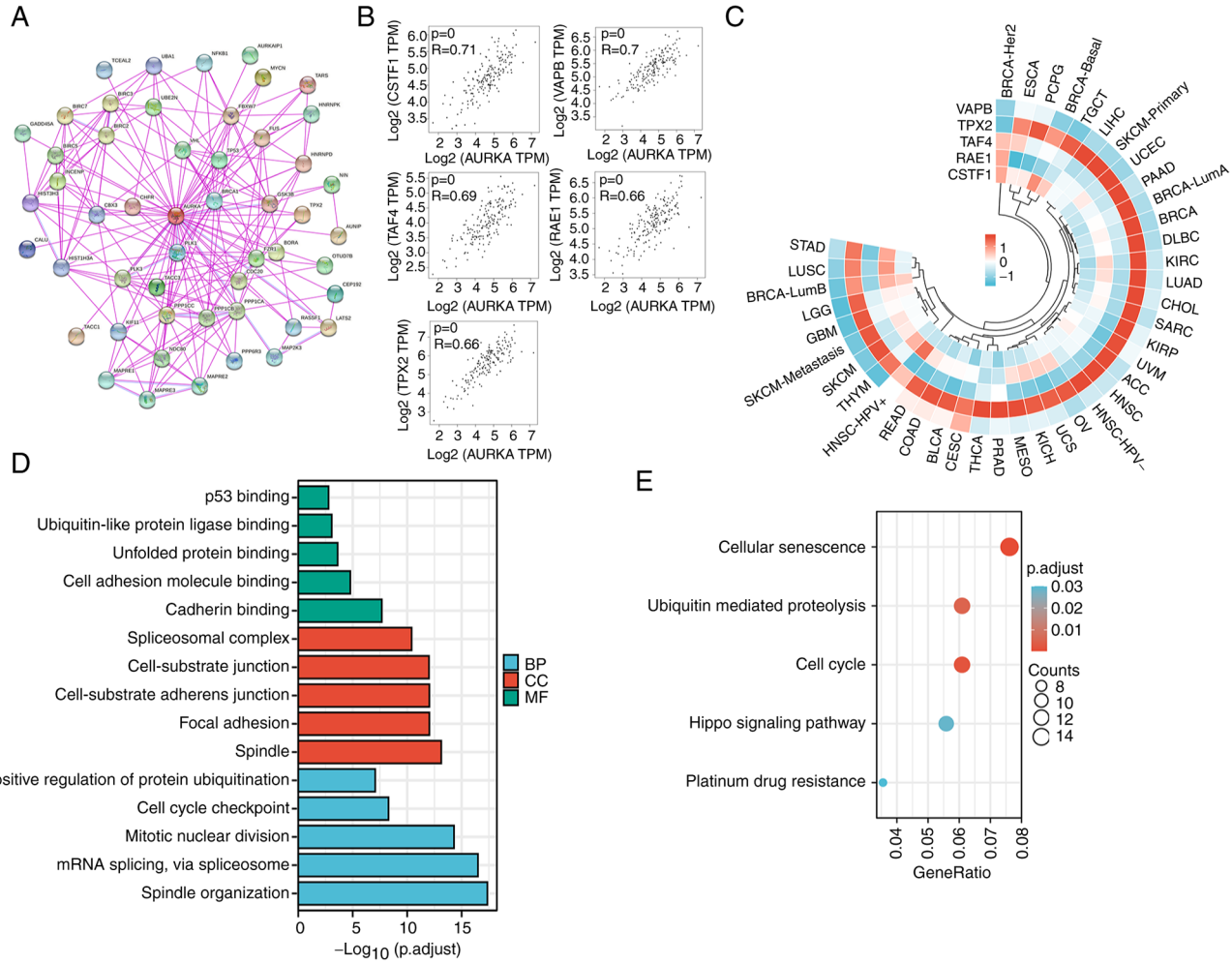

Figure 5. AURKA-related gene enrichment analysis. (A) Network of experimentally determined AURKA-interacting proteins based on STRING. (B) Correlation analysis of AURKA with CSTF1, VAPB, TAF4, RAE1 and TPX2 in esophageal squamous cell carcinoma using GEPIA2. (C) Correlation heat map of AURKA with CSTF1, VAPB, TAF4, RAE1 and TPX2 in various types of cancer. (D and E) The possible AURKA-interacting and AURKA-correlated proteins were subjected to (D) Gene Ontology and (E) Kyoto Encyclopedia of Genes and Genomes enrichment analysis. AURKA, Aurora kinase A.

in ESCC was associated with advanced-stage disease and a poorer prognosis, indicating that AURKA may serve as an excellent therapeutic target. AURKA was initially shown to serve as a regulator of mitosis and was subsequently considered to regulate a malignant phenotype of cancer cells. The results also showed that AURKA promoted ESCC cell proliferation, and AURKA knockdown significantly suppressed ESCC tumor growth. These results strongly indicated that AURKA can be used as a prognostic and diagnostic biomarker for ESCC.

TPX2 has been studied as a factor critical for mitosis and spindle assembly. Additionally, TPX2 was verified as a potential therapeutic target in pancreatic (17), breast (18), and cervical cancer (19), amongst others. Based on the data obtained from TCGA, TPX2 was identified to be significantly upregulated in esophageal cancer and can serve as a diagnostic marker. Previous studies indicated that TPX2 can stimulate autophosphorylation and autoactivation of AURKA, resulting in AURKA activation (20,21). Bioinformatics analysis from TCGA data and pull-down assays suggested that TPX2 may both interact with AURKA and was correlated with AURKA in esophageal cancer. It was further verified that AURKA interacted with TPX2 through immunoprecipitation assays. In addition, AURKA expression was positively correlated with TPX2 based on data obtained from TCGA, and AURKA knockdown decreased TPX2 protein expression. AURKA and TPX2 may form a positive feedback loop to regulate ESCC progression, although this requires further confirmation. In colorectal cancer, AURKA was found to coordinate with TPX2 to drive tumor progression (22,23). Additionally, TPX2 was reported to regulate the PI3K/Akt pathway to promote hepatocellular carcinoma progression and act as a STAT3 regulator (24,25). Based on the results of the present study, AURKA knockdown inhibited the EGF-stimulated PI3K/Akt pathway activation, and TPX2 knockdown also impeded PI3K/Akt signaling activation. According to the AURKA interactome from previous studies, AURKA-interacting proteins were enriched in the PI3K/Akt pathway (12). Thus, AURKA may cooperate with TPX2 to promote ESCC development through the PI3K/Akt pathway.

AURKA is a serine/threonine kinase that is crucial in regulating the cell cycle. p53, a primary tumor suppressor, induces cell cycle arrest through transcriptional downregulation of

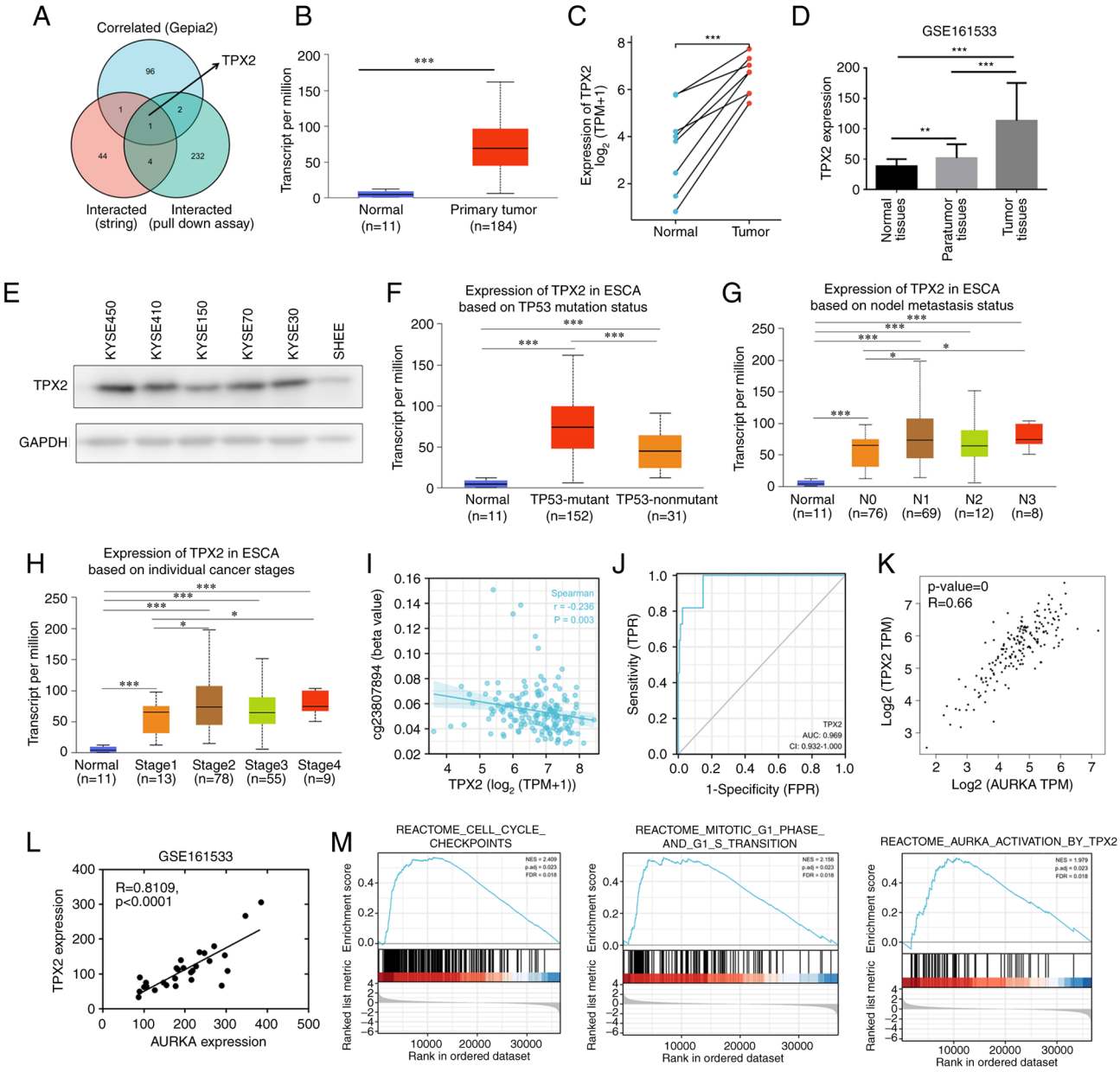

Figure 6. AURKA expression is associated with TPX2 which is upregulated in ESCA. (A) An intersection analysis of the AURKA-binding and correlated genes was performed. (B) TPX2 expression in normal and esophageal carcinoma tissues was obtained from the UALCAN database. (C) Expression of TPX2 in paired normal esophageal tissues and esophageal carcinoma tissues based on TCGA data. (D) Expression of TPX2 in the paired normal tissues, para-tumor tissues and ESCC tumor tissues (n=28) from the GSE161533 dataset. (E) TPX2 expression in ESCC cells and SHEE normal esophageal epithelial cells was measured by western blotting. (F-H) The relationship between TPX2 expression and (F) TP53 mutation status, (G) nodal metastasis and (H) cancer stage is shown. (I) TPX2 expression and promoter methylation correlation was analyzed. (J) TPX2 levels in normal and cancerous tissues was used for ROC analysis based on TCGA data. (K) Relationship between TPX2 and AURKA expression based on GEPIA2.0. (L) The relationship between TPX2 and AURKA expression from GSE161533 sequencing data was analyzed. (M) GSEA enrichment analysis of TPX2 in esophageal carcinoma was performed using the data obtained from TCGA. *P<0.05, **P<0.01 and ***P<0.001. AURKA, Aurora kinase A; ESCA, esophageal carcinoma; TCGA, The Cancer Genome Atlas.

several cell cycle-associated genes and therefore impedes tumor cell cycle progression (26-28). In the present study, it was revealed that AURKA knockdown increased p53 expression, suggesting that AURKA may regulate the cell cycle through p53 in ESCC. In several types of cancer, TP53 is the most commonly mutated gene, and the majority of tumors have lost p53 function due to mutations (29). In ESCC, p53 mutations have been observed in as high as 93% of patients (5). According to the UALCAN database, AURKA expression was significantly higher in p53 mutant tissues compared with p53 non-mutant tissues. Thus, AURKA may result in the loss of the tumor-inhibiting role of p53. Furthermore, it was demonstrated that AURKA suppression also induced p21 and p27 expression, which are both tumor suppressors and CDK inhibitors. Taken together, it was shown that AURKA regulated cell cycle related genes and thus promoted ESCC proliferation.

A series of inhibitors targeting AURKA have been explored as previously described in a review by the authors, amongst which alisertib is the most popular (12). Alisertib has finished phase III clinical assessment in patients with relapsed/refractory peripheral T-cell lymphoma and showed

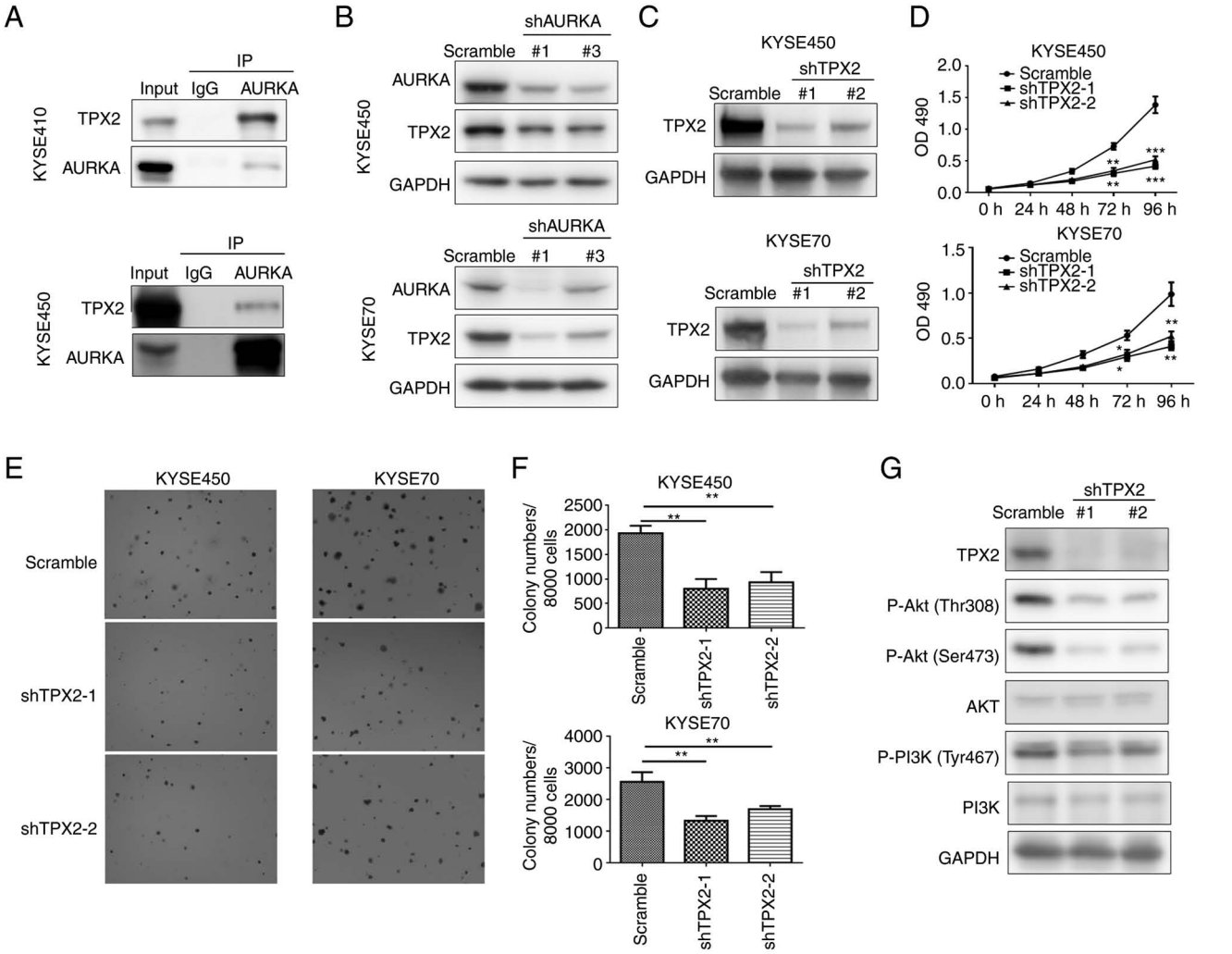

Figure 7. AURKA interacts with TPX2 to promote esophageal squamous cell carcinoma progression. (A) Lysates from KYSE410 and KYSE450 cells were immunoprecipitated with an AURKA antibody and then immunoblotted with the AURKA and TPX2 antibodies. (B) TPX2 expression was measured by western blotting following AURKA knockdown in KYSE450 and KYSE70 cells. (C) TPX2 knockdown efficiency was detected through western blotting. (D-F) Cell proliferation was measured by (D) MTT and (E and F) soft agar colony formation assays. (G) The expression of PI3K/Akt pathway-related proteins was detected following TPX2 knockdown. *P<0.05, **P<0.01 and ***P<0.001. Data are presented as the mean ± SD. AURKA, Aurora kinase A; sh-, short hairpin; p-, phosphorylated; IP, immunoprecipitation.

no significant improvement over chemotherapy (30). However, alisertib showed relatively good effects in solid tumors, including breast and small-cell lung cancer in a multicenter phase II study (31). In the present study, it was demonstrated that 25 nM alisertib significantly inhibited ESCC cell proliferation and colony formation. These data support the necessity of further investigation of alisertib in ESCC therapy. Another strategy targeting AURKA is to disrupt the interaction between AURKA and its activators including TPX2. Withanone, a herbal ligand isolated from ashwagandha, exerted an anticancer effect by binding to the TPX2/AURKA complex and dissociate TPX2 from AURKA (32). Therefore, targeting the AURKA/TPX2 axis in ESCC is a promising strategy for ESCC therapy.

The tumor microenvironment has been gaining increasing interest in research circles in recent years. Tumor-infiltrating lymphocytes have been suggested to be an independent predictor of a patient's prognosis in several types of cancer (33-35). AURKA was reported to participate in TCR activation (36) and determine CD8^{+} T cell cytotoxic activity and antiviral responses (37). Furthermore, AURKA inhibition promoted CD8^{+} T-cell infiltration and activation by inducing IL-10 production (38). In breast cancer, AURKA inhibitor alisertib eliminated myeloid cell-mediated immunosuppression and enhanced anti-PD-L1 therapy (39). It was also found that AURKA inhibitors enhanced T-cell cytotoxicity *in vitro* and could potentiate antitumor immunity *in vivo* (40). CD4^{+} T cells and CD8^{+} T cells are crucial members of the tumor microenvironment that participate in specific antitumor immune responses (41). Among the different subsets of CD4^{+} T cells, Th1 and Th2 cells are the most important classes of CD4^{+} T cells. Based on the results of the present study, both expression levels of AURKA and TPX2 were positively associated with Th2 cell infiltration and negatively associated with cytotoxic cells and CD8^{+} T cell infiltration. Th2 cell aggregation resulted in the dysfunction of CD8^{+} T cells and cytotoxic T cells and ultimately contributed to immune escape. Therefore, the role of AURKA or TPX2 in tumorigenesis may be associated with increased Th2 cell and decreased CD8^{+} T and cytotoxic T cell infiltration, which require further

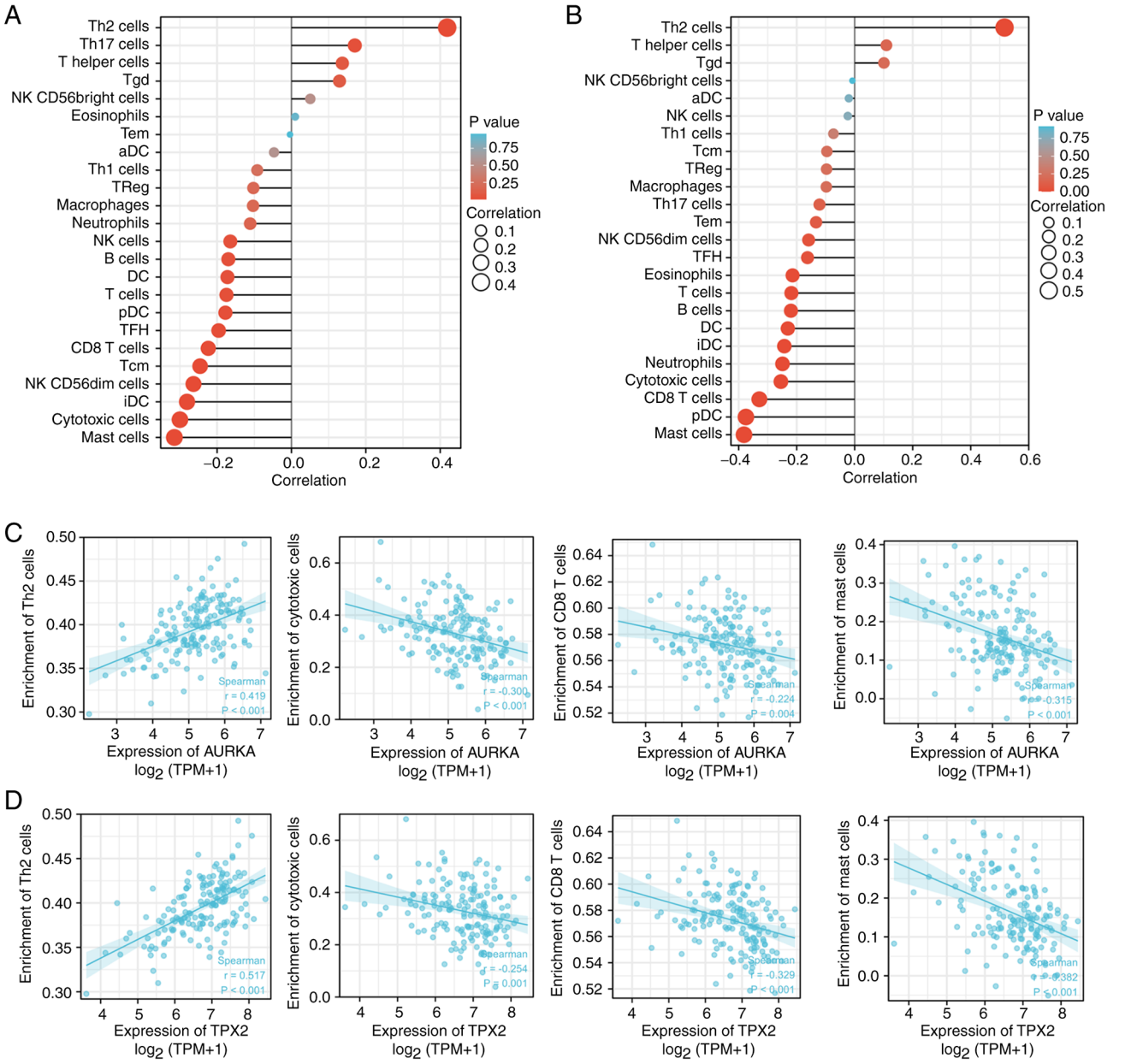

Figure 8. Correlation analysis between AURKA and TPX2 expression with immune cell infiltration. (A and B) The correlation between (A) AURKA or (B) TPX2 expression and immune cells was analyzed based on data obtained from TCGA. (C and D) The correlation between (C) AURKA and (D) TPX2 expression and Th2 cells, cytotoxic cells, $CD8^+$ T cells and mast cells was analyzed based on data obtained from TCGA. AURKA, Aurora kinase A; TCGA, The Cancer Genome Atlas.

study. Mast cells are immune cells present in all classes of vertebrates that have also been identified in tumor tissues. The pro- or antitumorigenic roles of mast cells in different tumors are cancer type-specific (42,43). It was reported that mast cells expressing IL-17 was predictive of a favorable prognosis in ESCC (44). It was observed that there was a negative correlation between AURKA or TPX2 expression and infiltration of mast cells, which may indicate the possible pro-tumorigenic role of mast cells in ESCC. Collectively, these observations may help to reveal the role of the AURKA/TPX2 axis in the tumor microenvironment and provides a reference for a deeper study of AURKA or TPX2 in tumor immunity.

In conclusion, an AURKA/TPX2 axis was elucidated that may be used as a potential target in ESCC using both bioinformatics analysis and experimental validation. AURKA expression was upregulated in ESCC and was predictive of poorer patient survival. Upregulated AURKA expression promoted ESCC cell proliferation and tumor growth via the PI3K/Akt pathway. Moreover, targeting AURKA with alisertib inhibited ESCC cell proliferation and colony formation. Mechanistic analysis suggested AURKA interacted and correlated with TPX2 which was upregulated in ESCC. Furthermore, both TPX2 and AURKA were positively associated with Th2 cell infiltration and negatively associated with cytotoxic, $CD8^+$ T and mast cell infiltration. These findings may assist in elucidating the role of the AURKA/TPX2 axis in tumorigenesis and development, whilst providing a reference for the realization of more precise and personalized therapy in the future.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 82273058 and 82002592), the Science and Technology Project of Henan Province (grant no. 222102310156), the Doctoral Research Start-up Fund Project of Nanyang Institute of Technology (grant no. NGBJ-2022-05) and the Talent Program of Central China: Science and Technology Innovation Leading Talent (grant no. 234200510006).

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

HB and LH designed the project. RJD performed the cell functional experiments and data analysis and wrote the article. KL, ZJZ and YLH performed the molecular biology experiment. KLG and HZ focused on the animal experiment. ZGC and XLZ finished the bioinformatic analysis and revised the article. All authors read and edited the manuscript. All authors read and approved the final manuscript. RJD and LH confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. NYISTIRB-2021-005) by the Ethics Committee of Nanyang Institute of Technology (Nanyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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