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To verify the biological characteristics of disulfidptosis associated gene ADORA2B in esophageal cancer

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Abstract

Esophageal cancer is an aggressive malignant tumor. Statistics show that esophageal cancer has claimed the lives of approximately 300,000 people worldwide. Many patients are diagnosed as stage 3 or 4 when they visit a doctor; however, the prognosis may not be accurate because the disease's early signs are not always evident. The gene ADORA2B may be important in the diagnosis and prognosis of esophageal cancer, as adenosine (ADO) is implicated in the proliferation and spread of many malignancies. Through the use of bioinformatics analysis, this study sought to discover and validate particular genes and putative pathways linked to the course and prognosis of esophageal cancer. Utilizing integrated transcriptomics and single-cell proteomics, the involvement of immune cells in the tumor microenvironment was examined, while bioinformatics was used to investigate the expression, function, and survival data of ADORA2B. Western blot (WB) and qRT-PCR were then used to determine the expression level of ADORA2B in the postoperative tissues of patients with esophageal cancer. Tests using Transwell, Edu, and CCK8 were performed to ascertain its capacity for erosion, migration, invasion, and proliferation. Flow cytometry was used to quantify apoptosis. The results of this investigation validate ADORA2B as a potential therapeutic target and diagnostic biomarker.

Keywords ADORA2B, Esophageal cancer, Prognosis

Introduction

The processes of esophageal cancer, which is the sixth most common cause of cancer-related deaths worldwide, have not received much attention. Esophageal squamous cell carcinomas (ESCC) account for about 90% of esophageal cancer cases. The resulting death rate is significant due to poor prognosis, which is partly because ESCC is frequently discovered when the disease has progressed, necessitating a gastroscopy and chest computed tomography (CT). Despite significant improvements in prognosis brought about by surgery and all-encompassing treatment, the five-year survival rate for ESCC remains around 20% [1–3]. Consequently, early screening is especially crucial, and the discovery of novel molecular biomarkers may shed light on additional molecular mechanisms influencing the onset and progression of esophageal cancer. These findings may also have implications for the identification of biomarkers and the development of more sophisticated treatments.

A vital part of a cell's cycle is cell death, which differs between healthy cells and tumor cells. Researchers have discovered a distinct type of ferroptosis or cell apoptosis in recent years, which we refer to as disulfidptosis. This type of cell death is characterized by excessive disulfide accumulation in solute carrier family 7 member 11 (SLC7A11) cells. Disulfidptosis denotes the programmed cell death mechanism

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initiated by the excessive buildup of disulfide bonds in cellular proteins, leading to protein misfolding, endoplasmic reticulum stress, and cellular dysfunction [4, 5]. This study clarifies the connection between cell destiny and metabolism and could aid in the creation of fresh anti-cancer treatment approaches.

Adenosine A2A (ADORA2A) and A2B (ADORA2B) receptors, both G-coupled GPCRS, make up the ADORA2 adenosine receptor, known as ADORA2B. Research has demonstrated that ADORA2B is present in cardiomyocytes, fibroblasts, epithelial cells, and many immune cell types. It also functions as a potent anti-inflammatory modulator in a variety of illness models [6, 7]. Recent research has demonstrated that the ADO receptor (ADOR) is also essential for the spread of tumors. ADORA2B is one such receptor that is crucial to signal transduction [8]. Research on lung cancer reveals that ADORA2B is highly expressed and overexpressed in lung adenocarcinomas, which suggests that individuals with this type of cancer received poor prognoses [9]. In one investigation on tumor immunity, blocking ADO-producing enzymes and ADOA2A or A2B receptors boosted anti-tumor immunity and stopped tumor growth, whereas blocking ADORA2B dramatically increased anti-tumor immunity [10]. ADORA2B stimulates cell proliferation, migration, and angiogenesis, according to research on head and neck squamous cell carcinoma and breast cancer [11]. Nevertheless, the role of ADORA2B in cancer, particularly esophageal cancer, remains underexplored. This study aims to investigate the specific function of ADORA2B in esophageal cancer pathogenesis. By investigating these mechanisms, our goal is to provide a comprehensive understanding of how ADORA2B contributes to esophageal cancer, potentially offering new therapeutic targets.

Based on RNA-seq and scRNA-seq in the TCGA and GEO databases, this study examined ADORA2B expression, prognostic significance, immune infiltration, and co-expression gene network in ESCA patients using an online database and the R software program. In order to eliminate batch effects, we combined the GSE196756 data sets into a single metadata set. We then performed analysis on this larger sample size dataset. Specifically, we used Western Blot and qPCR to confirm the findings in our ESCA tissues. Additionally, we knocked down ADORA2B in ESCA cell lines for the first time, confirming its role as an ESCA tumor promoter in vitro. Furthermore, we discovered that ADORA2B is essential to ESCA. According to the findings, ADORA2B may be a predictive biomarker and a target for ESCA treatment.

Materials and Methods

Esophagus Cancer Samples from Datasets

Numerous forms of esophageal cancer gene expression analysis are available through the Cancer Genome Atlas (TCGA) online database (https://portal.gdc.cancer.gov). The ESCA dataset was obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov), and the RStudio DESeq2 package was utilized to assess the differences in expression between tumor and paracancerous tissues. The cut-off points for screening the differential expression of mRNA related to esophageal cancer were set at $|\log2FC|>1$ and $P<0.05$. The genes were found with differential expression (Deseq).

The GEO database included single cell sequencing data for six samples from three patients who had esophageal cancer (GSE196756). The raw data for GSE196756 was processed in R using the Seurat package. The following standards were applied while excluding cells: (1) greater than 20% of genes connected to mitochondria, and (2) gene expression ranges from fewer than 300 to more than 7,000. Initially, we standardized the data via the NormalizeData function within the Seurat package, accounting for discrepancies in sequencing depth across each sample. The Harmony method was employed for batch effect correction.The execution of these procedures significantly mitigated the possible bias in single-cell RNA-seq analysis results, hence improving the study's reliability and accuracy [12]. The cells were subsequently clustered into nine distinct cell populations utilizing the FindClusters function. Revivification and visualization of gene expression were performed using tSNE and UMAP analyses.

Clinical Specimens

The study included 30 patients with esophageal squamous cell carcinoma who underwent radical surgery at Xinjiang Cancer Hospital between January 2017 and January 2020. All patient tissue samples were promptly frozen in liquid nitrogen or stored in a freezer set at −80 °C. Through pathological investigation, cancer and paracancer tissues were chosen from patients with comprehensive follow-up data. The Affiliated Cancer Hospital of Xinjiang Medical University's Ethics Committee granted approval and received signed informed permission from all participants (Ethics number: K-2024092).

Functional Enrichment Analysis and Construction of the Hub Gene-Pathway Network

The relevance of these genes in biological processes and the cell pathways was better understood due to enrichment analysis using the Kyoto Encyclopedia of Gens and Genomes (KEGG) and GeneOntology (GO). The GEIPA

database's top 100 genes linked to ADORA2B were chosen for correlation enrichment analysis.

TIMER Database Analysis

TIMER2.0 is available at http://timer.cistrome.org. We investigated the relationship between ADORA2B and ESCA immune cell infiltration using a "gene module" in our study. It was deemed statistically significant ($P<0.05$).

GSCA Database Analysis

Genome with the correlation analysis between drug is GSCA through an online website (http://bioinfo.life.hust.edu.cn/GSCA) analysis, is a user-friendly web server and a useful hypothesis test of the resources of the GSA method to be used in the genome, Pharmacogenomic and immunogenomic levels.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent was used to extract total RNA from ESCC tissues, and the quality of the extracted RNA was assessed. Using whole RNA samples, the first strand cDNA was created. SYBR premix was used for the RT-PCR experiment. The above comprehensive operating processes are completed in compliance within guidelines. The internal reference is GAPDH, and the $2^{-\Delta\Delta Ct}$ technique was applied. By comparing each sample's CT value with three replicates, the data was computed. The SPSS26.0 software was used to examine the experimental data. It was deemed statistically significant ($P<0.05$). Table 1 lists certain primer sequences.

Western Blot

After transfection, the 48-h ESCA cells were cultured for protein expression. Subsequently, total protein was extracted and analyzed by Western blot.After measuring the protein content with BCA and obtaining a protein sample (60 μg/well), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used. Using the wet transfer approach, the protein was moved to the NC membrane and sealed with 5% skim milk powder. Wash the membrane with TBST three times and the secondary antibody, IgG labeled with horseradish peroxidase (HRP), was added after the protein was incubated for an additional night at 4 °C with ADORA2B(1:300) and GAPDH(1:1000) (both from Proteintech). 1:5000 was allowed to incubate for one hour at room temperature, after which the secondary antibodies were removed, the chemiluminescent reagent (ECL) acquired its color, and the relative expression level of the target protein was determined by utilizing GAPDH as the internal reference.

Table 1 Primer sequence

Gene	Primer sequences
ADORA2B	F1:ACTTCTACGGCTGCCTCTTCCTC R1:TATACCTGAGCGGGACACAGATGG
GADPH	F:TGACTTCAACAGCGACACCCA R:CACCCTGTTGCTGTAGCCAAA

Cell Culture

The Xinjiang Medical University Cancer Research Institute's cell bank provided the human esophageal cancer cell line (KYSE150, ECA109). Si-ADORA2B, a small interfering RNA, was acquired from Shanghai Jima Co., LTD. RPMI-1640 (Gibco) culture medium with 10% fetal bovine serum (FBS), 37 °C, and 5% CO2 was used. Trypsin was used to break down the cells for passage culture once the cell density reached 80%. After the treatment time of 48 h, cells were extracted for analysis. Table 2 lists the siRNA sequence for Si-ADORA2B.

CCK8

Using a CCK8 test kit, cell proliferation was identified. The previous night, 1×103 cells/well of transfected ECA109 or KYSE150 cells with si-ADORA2B or negative control NC were added to 96-well plates. Every day after that, 100 μ culture media with 10 μ CCK8 reagent was applied to every well. After transfection, the proliferation rate was assessed at 0, 12, 24, and 48 h, and the absorbance at 450 nm was calculated.

Edu

The DNA synthesis rate was evaluated using the EDU imaging kit (Beyotime, Beijing, China) according to the manufacturer's protocols. Take logarithmic phase cells and cultivate them in 6-well plates at a density of 2×10^5 cells per well, then prepare an EdU working solution by diluting the EdU stock solution in a cell culture medium at a ratio of 1:1000. Use the prepared EdU medium for cell fixation and staining. Finally, acquire and analyze

Table 2 Sequence of ADORA2B plasmid

	Sense(5′–3′)	Antisense(5′–3′)
NC	UUCUCCGAACGUGUCACG UTT	ACGUGACACGUUCGG AGAATT
si-ADORA2B[1]	GGCCAUUCUUCUGUCACA UTT	AUGUGACAGAAGAAU GGCCTT
si-ADORA2B[2]	GAUGGAACCACGAAUGAA ATT	UUUCAUUCGUGGUUC CAUCTT
si-ADORA2B[3]	CCAAGCAGAUGUCAAGAG UTT	ACUCUUGACAUCUGC UUGGTT
si-ADORA2B[4]	UGGUGAUCUACAUUAAGA UTT	AUCUUAAUGUAGAUC ACCATT

images under a fluorescence microscope (ECLIPSE Ji, Nikon) with a magnification of 200 μm.

Transwell

In Transwell cells (Corning, NY, USA), 2×10^5 oesophageal cancer cells transfected with NC or si-ADORA2B were injected. The upper layer was supplemented with serum-free media, while the lower layer received 20% fetal bovine serum. Following a 48-h incubation period, the cells were stained with crystal violet solution, fixed with 4% paraformaldehyde, and photographed using an optical microscope. The Transwell membrane is precoated with Matrigel in the cell invasion test, which is comparable to the cell migration test (Corning, Inc.).

Flow experiment After being collected into a centrifuge tube, the medium from the 6-well plate was sorted into NC and Si-ADORA2B. After stopping the medium's digestion and centrifuging, pancreatic enzymes without EDTA were introduced. 400 ul of Annexin V resuspension cells were introduced. After adding 1 × Annexin V and 5ul of Annexin FITC dye to the suspension, it was carefully mixed and left to incubate for 15 min at 2–8 °C in a dark environment. After adding the PI dye solution, it was thoroughly stirred, left to sit at 2 to 8 °C and shielded from light for 2 to 5 min, and then the machine test was performed.

Statistical Analysis The experimental data were examined using the SPSS 26.0 software. The mean ± standard deviation (x's) represents continuous variables that follow a normal distribution, while the median (interquartile distance (IQR)) represents non-normal variables. The T-test, one-way ANOVA, Mann–Whitney U test, and Wilcoxon rank sum test were used to compare the data. For categorical variables, Fisher exact tests or chi-square tests were used. For the survival analysis, the Kaplan–Meier method and the log-rank test were employed. Cox regression analysis, both univariate and multivariate, was used to examine the prognostic factors. Statistics were deemed significant if $P<0.05$.

Results

Within the TCGA-ESCA team, our research team first performed R analysis on 15 identified disulfidptosis genes, including FLNA, FLNB, MYH9, TLN1, ACTB, MYL6, MYH10, CAPZB, DSTN, IQGAP1, ACTN4, PDLIM1, CD2AP, INF2, and SLC7A11. Through the use of Pearson correlation analysis, the genes linked to disulfidptosis were found. With $|r|>0.4$ as the cutoff, 4044 disulfidptosis-related mRNA were identified. In parallel, ESCA and normal tissues underwent differential expression analysis, yielding 2420 differential genes (DEseq) specific to esophageal cancer (Fig. 1A). 1101 differentially expressed mRNAs linked to disulfide death were found by intersecting DEseq and disulfidptosis-related gene (Fig. 1B). Using univariate Cox regression analysis, the top 10 prognostic disulfidptosis-related mRNA were determined (Fig. 1C), from which ADORA2B was ultimately chosen and discussed in conjunction with existing research. Analysis of the TCGA database revealed differential expression of ADORA2B across a majority of malignant tumors(Fig. 1E) and considerably overexpressed in ESCA ($P<0.05$) (Fig. 1D). These findings imply that the ADORA2B gene's elevated expression may have a significant regulatory role in the onset and progression of esophageal cancer.

Clinical Stage and Survival Probability of High ADORA2B Expression

To explore the clinical stage and survival rate of ADORA2B high expression, we downloaded and organized the RNAseq data of the STAR process of the TCGA-ESCA project, extracted the data in TPM format, and visualized it using the ggplot2 R package. We found that expression was correlated with Barretts ADORA2B esophagus, pathological stage I—IV, Residual tumor, Reflux, histotype, age, weight, and tumor central location (F ig. 2A-I). Columnar metaplasia and Histilogy grade (Fig. 3A, B) showed a significant difference compared with normal healthy controls, suggesting that ADORA2B plays an important role in the clinical progression of patients with esophageal cancer.

Disease-Specific Survival (DSS) and Overall Survival (OS) were used to build the survival curve (Fig. 3C, D) and prognosis list. The findings demonstrated that the survival rate of patients with esophageal cancer was significantly impacted by the upregulation of ADORA2B expression. A total survival analysis of ADORA2B in esophageal cancer was carried out to have a better understanding of the prognostic value and probable mechanisms of ADORA2B expression in ESCA. In ESCA, positive overall survival (OS:HR = 0.57,95%CI from 0.35 to 0.94, P = 0.026) and disease-specific survival (DSS) (HR = 0.53,95%CI from 0.30 to 0.96, P = 0.037) were strongly correlated with increased ADORA2B expression. It was established that a positive prognosis for ESCA was closely linked to higher expression of ADORA2B (Fig. 3E, F). ROC curves were utilized to assess ADORA2B's diagnostic value in ESCA. Figure 3G's ROC curve analysis demonstrated ADORA2B's good predictive accuracy for ESCA (AUC = 0.863, CI = 0.728 – 0.997). Based on ADORA2B and other independent clinical risk variables, we developed a nomographic prediction to offer a quantitative way for predicting outcomes in patients with ESCA. As an illustration, Fig. 3H displays a total score of 100 for ESCA patients with high ADORA2B expression (0

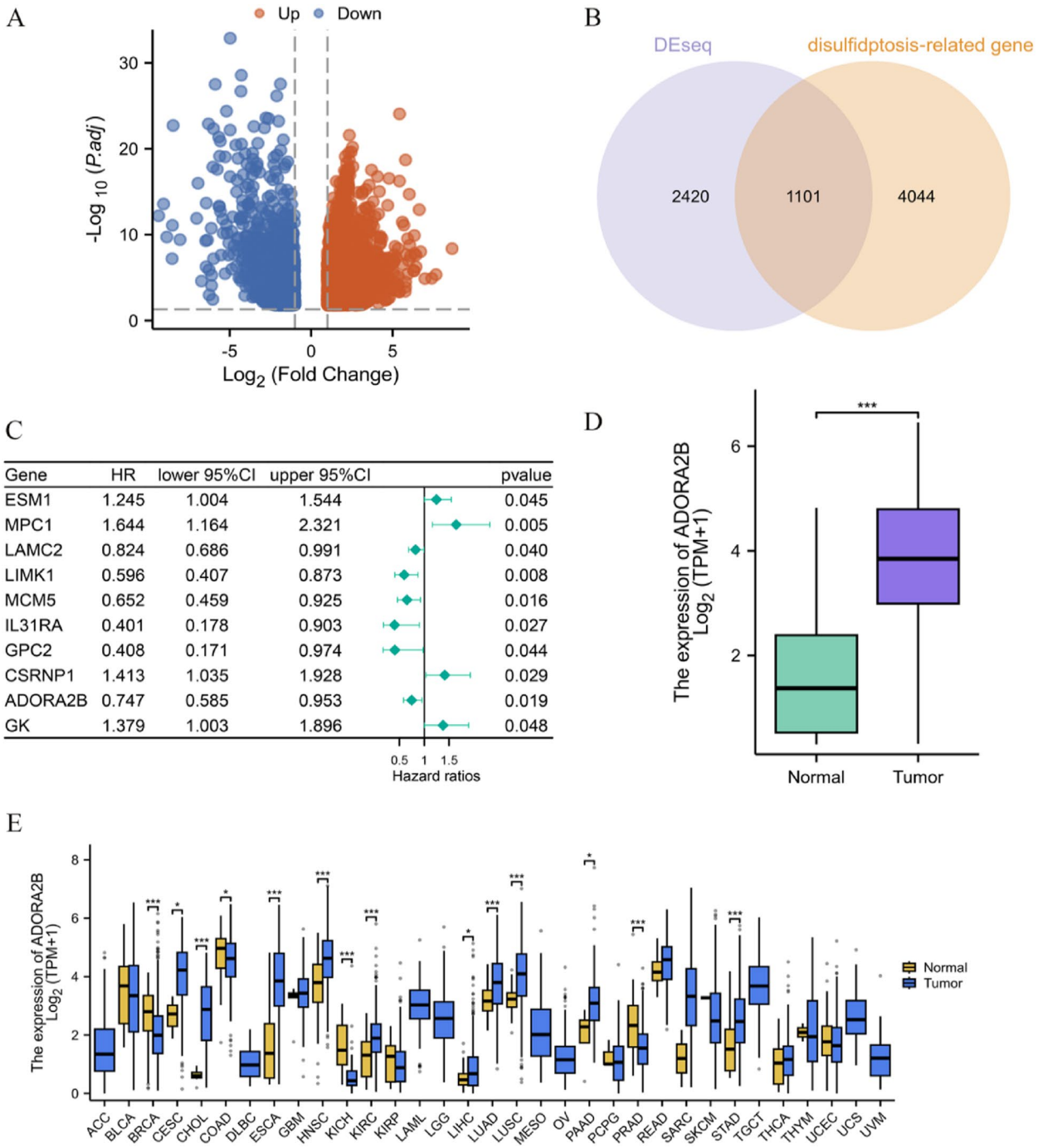

Fig. 1 **A** Differential genes of disulfide death genes associated with esophageal cancer expressed in volcanic maps by Pearson correlation analysis; **B** Venn Diagram expressed 1101 genes at the intersection of esophageal cancer differential gene and disulfide death gene; **C** The top 10 genes were screened by COX regression analysis ($P < 0.05$); (D, E) Shengsheng verified the difference of ADORA2B expression in esophageal carcinoma tissues and pancarcinoma($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

points), a female (0 points), pathological stage T3 (70 points), esophageal adenocarcinoma (0 points), and Barrett's esophagus (30 points). The survival rates after one year, three years, and five years were 80.0%, 40%, and fewer than 20%, respectively. According to the findings, ADORA2B shows promise as a biomarker for differentiating esophageal cancer from nearby controls.

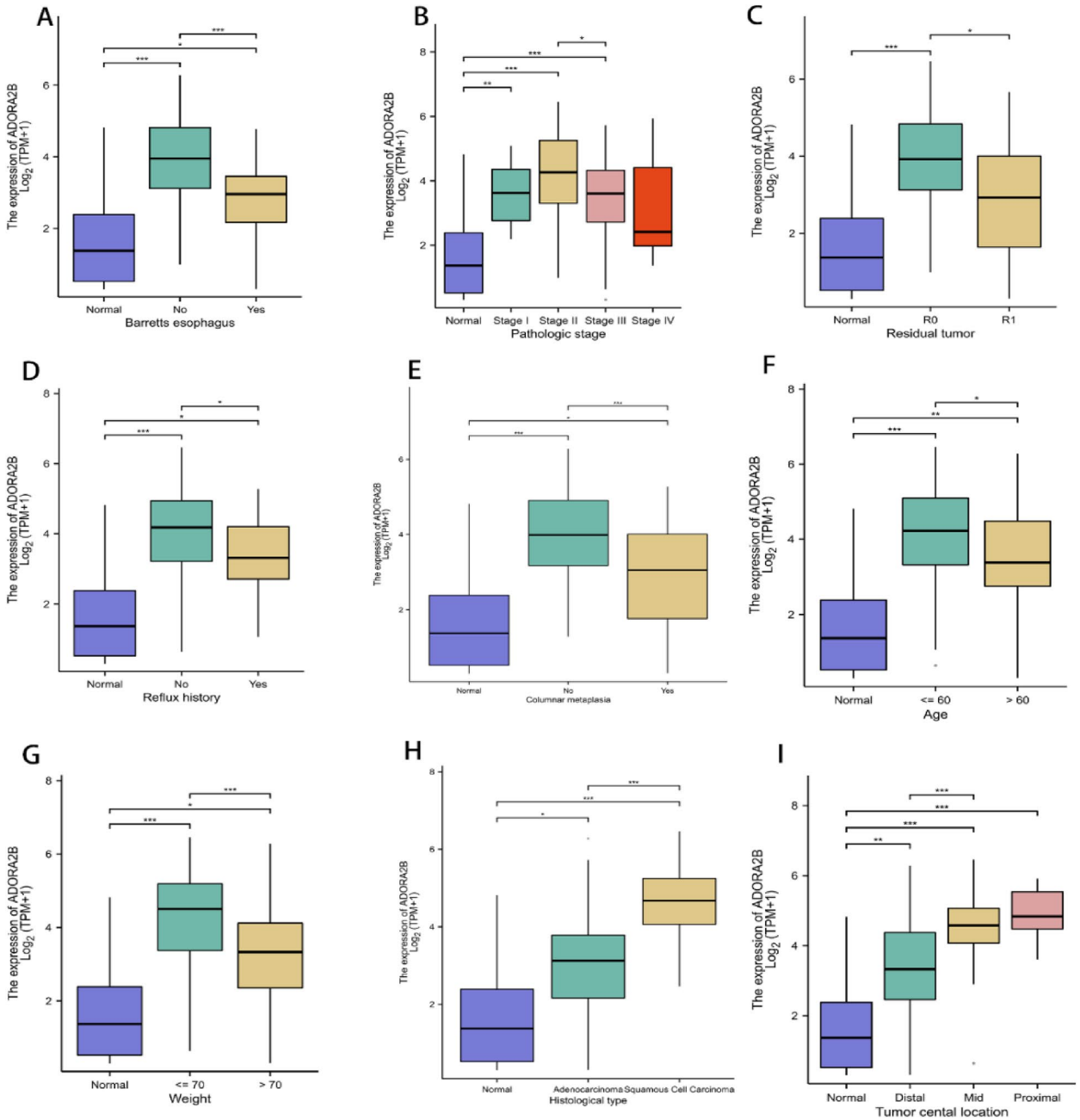

Fig. 2 **A-I** The expression of ADORA2B is correlated with Barretts esophagus, pathological stage, Residual tumor, Reflux, history type, age, weight, and tumor central location($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

We built the ADORA2B's protein–protein interaction (PPI) network by utilizing the STRING database to learn more about the protein's function in cancer. These include ENTPD1, ADCY5, GNAS, GNB3, etc. (Fig. 4A). The GENEMANIA online database was used to build a gene interaction network. The findings demonstrated a robust association between 20 genes and ADORA2B (Fig. 4B). The top 100 genes linked to ADORA2B were found in the GEPIA2 dataset. Three datasets underwent KEGG and GO enrichment analysis. ADORA2B was found to be strongly correlated with regulation of body fluid levels, skin development, keratinocyte proliferation, skin morphogenesis, adenylate cyclase activation of dopamine receptor signaling pathways, intercellular connections, basement membrane, dopaminergic synapses, etc. ($P < 0.05$). The KEGG analysis

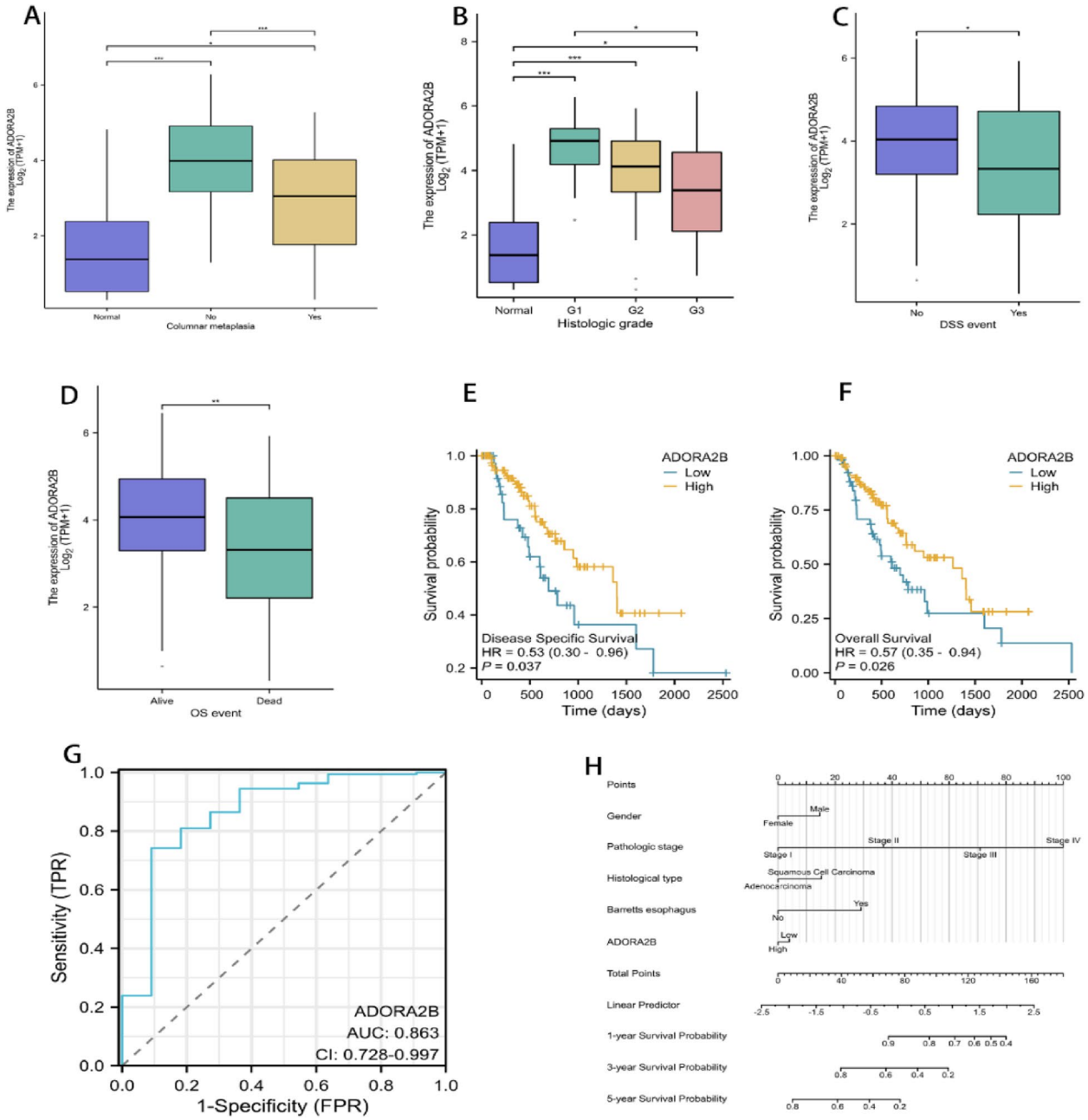

Fig. 3 **A**,**B** ADORA2B has correlation difference with column metaplasia and Histological grade; **C**,**D** DSS, OS-related survival analysis was performed for ADORA2B in esophageal cancer; (Figure **E**,**F**) Poor prognosis in ESCA is strongly associated with high expression of ADORA2B; **G** ROC curve was used to verify the diagnostic value of ADORA2B in ESCA; **H** A nomogram forecast was made for ADORA2B($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

revealed that ADORA2B is implicated in the etiology of tumors through the following mechanisms: "cadherin binding," "GTpase activity," "G-protein-coupled receptor binding," "G-protein beta/gamma subunit complex binding," and "cadherin binding is involved in cell–cell adhesion" (Fig. 4C-F). The correlation analysis between ADORA2B expression and co-expressed genes in esophageal carcinoma (ESCA) from TCGA was displayed (Fig. 4G, H).

To thoroughly investigate any possible connection between immune infiltration and ADORA2B expression in ESCA, the association between ADORA2B and immunological infiltration of associated immune cells was investigated using TIMER2.0 analysis. The

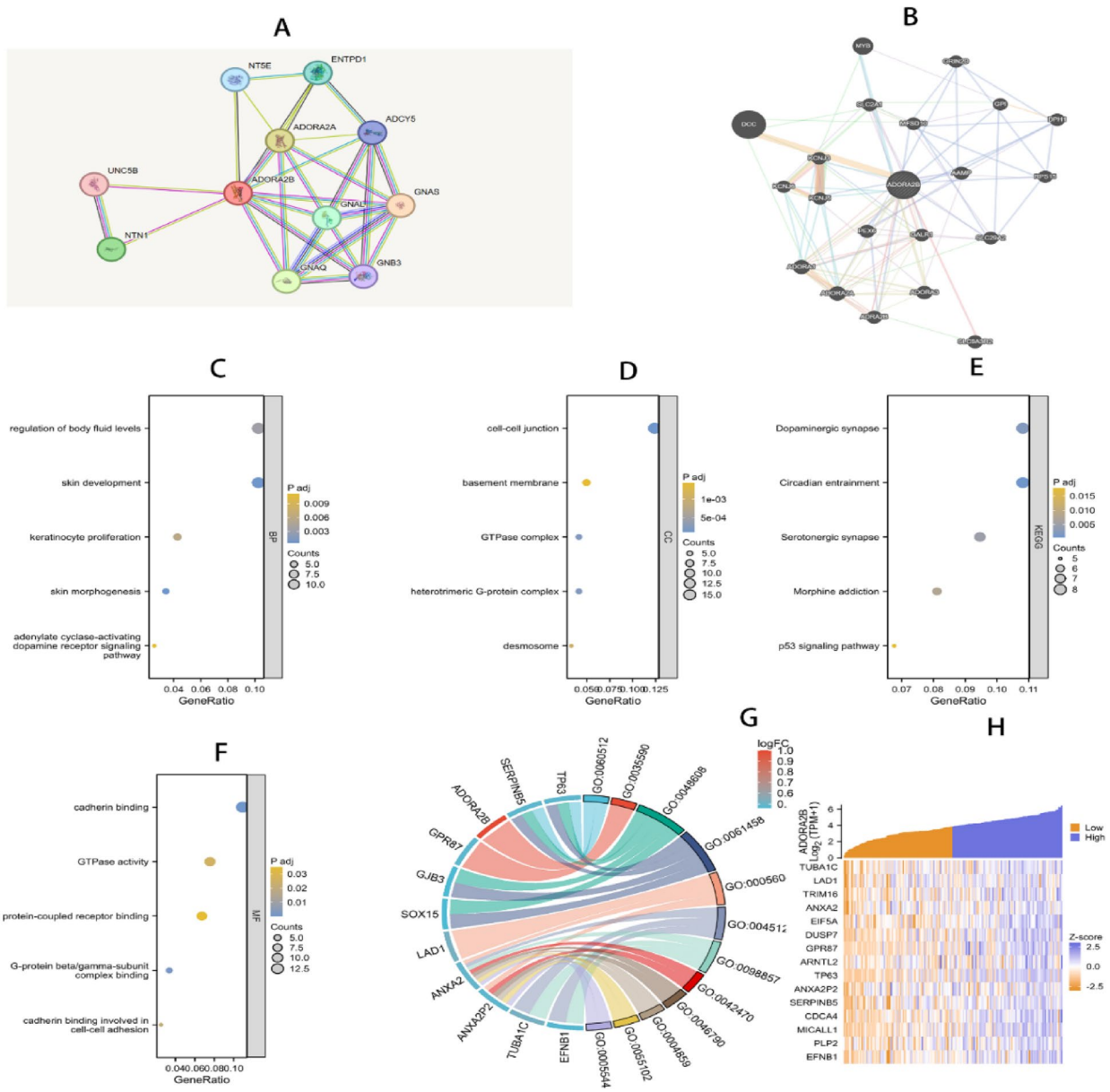

Fig. 4 **A**, **B** ADORA2B related genes; **C**-**F** GO and KEGG enrichment analysis; **G**, **H** ADORA2B related gene string map and heat map

immunological response to ESCA is significantly regulated by increased expression of ADORA2B (Fig. 5A). To evaluate the impact of ADORA2B on the tumor microenvironment (TME), a correlation between immune cell infiltration level and expression level (TPM) of ADORA2B has been demonstrated by Spearman correlation analysis quantified by ssGSEA, as illustrated in Fig. 5B-H. ADRA2B was positively correlated with Tcm ($R=0.333$, $P<0.001$), CD8 T cells ($R=-0.322$, $P<0.001$), Eosinophils ($R=-0.548$, $P<0.001$), mast cells ($R=-0.328$, $P<0.001$), pDC cells ($R=-0.424$, $P<0.001$), Tem cells ($R=-0.362$, $P<0.001$), Th17 cells ($R=-0.401$, $P<0.001$) showed a significant negative correlation.

In order to investigate the connection between ADORA2B expression and cells, we examined the GSE196756. In this study, we employed a systematic approach to identify and classify cell populations based on scRNA-seq data from the GSE196756 dataset. Specifically, cells were clustered using Seurat's FindClusters function, with the clustering granularity adjusted by setting the resolution parameter to 0.5 and Selecting the top ten genes. To enhance the accuracy of our annotations,

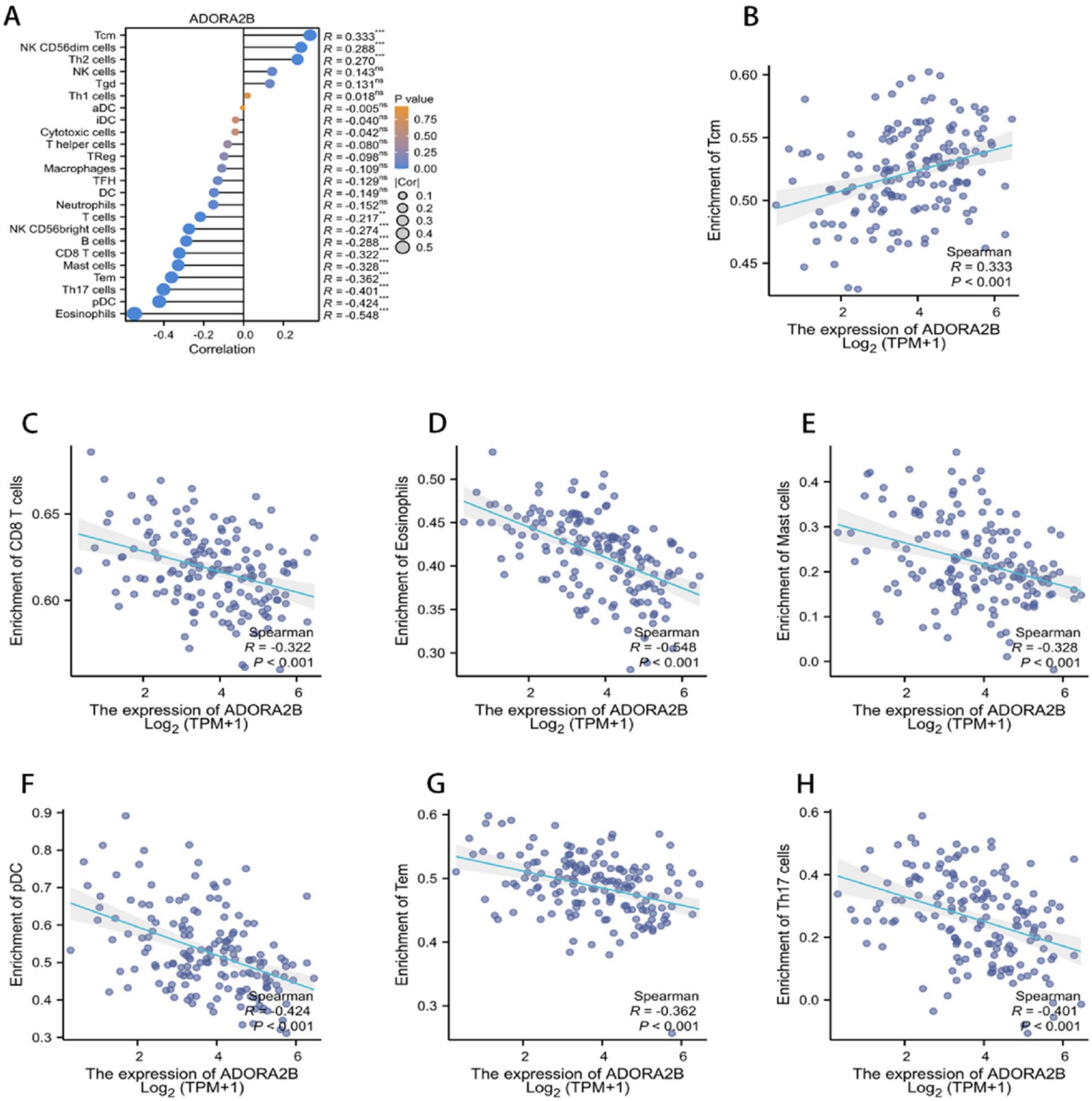

Fig. 5 **A** Immunoinfiltration of ADORA2B associated with 24 types of immune cells; **B**-**H** Immune correlation of ADORA2B in esophageal cancer in TIMER2.0 database

we relied on marker genes identified through CellMarker2.0 (http://bio-bigdata.hrbmu.edu.cn/CellMarker/index.html) and related references, achieving high precision in cell population classification. Based on the expression of marker genes, the cell population was split into 9 groups, which were then further separated into 6 cell populations: based on the expression of cell markers, Cluster 0 is defined as T cell, cluster I, VIII is defined as B cell, cluster II is defined as Monocyte cell, cluster III is defined as macrophages, cluster IV is defined as FGFR1 High NME5-epithelial cell. Clusters V,VI,VII are defined as Tumor cells. According to the CellMarker website, we investigated the subsequent characteristic cell markers in ESCA. macrophages (CD163), B cells (CD19), (CD79A), malignant tumor cells (CD68), (AREG), (VCAN), T cells (CD4), and (CD8) (FIG 6A, B). The findings demonstrated that ADORA2B was expressed in all four types of cells, with malignant tumor cells expressing it the most.

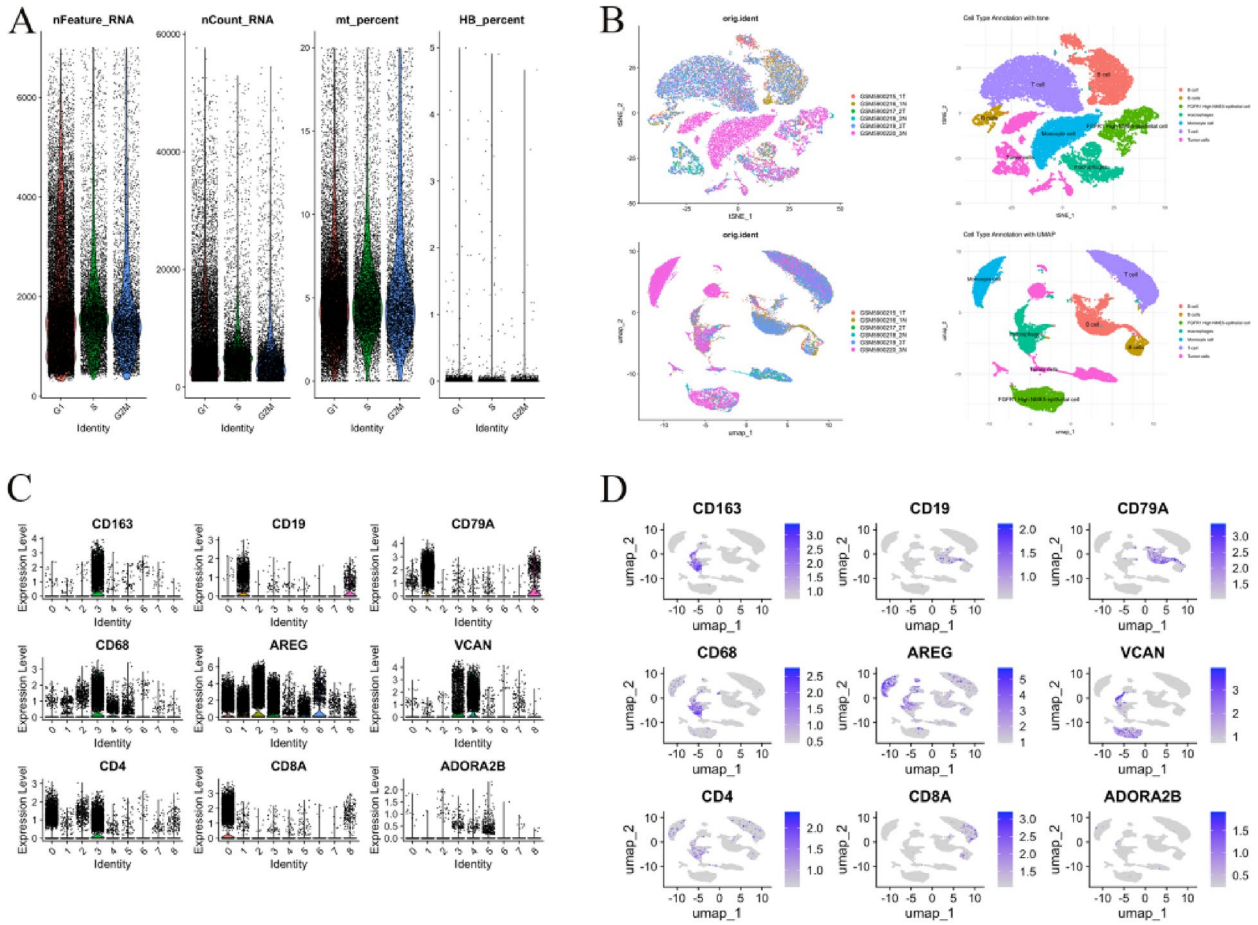

Fig. 6 ADORA2B expression was analyzed in single cells. **A** The GSE196756 of ESCA patients was screened. **B** Cell markers for cluster annotation. **C**-**D** Expression of ADORA2B in tissues of 3 ESCA patients

When ESCA samples were compared to normal samples, ADORA2B expression was primarily up-regulated in Monocyte cell, FGFR1 High NME5-epithelial cell, macrophages, and malignant tumors (Fig. 6C, D).

We obtained expression data from cancer drug sensitivity Genomics (GDSC) on 860 cell lines for 265 small compounds of IC50, correlating ADORA2B, and its top 20 genes. Data on their drug sensitivity and expression were pooled. To gather information on the relationship between gene expression and drug IC50, a Pearson correlation analysis was performed on 481 small molecules, their matching ADORA2B, and the top 20 gene expressions associated with these compounds in 1001 cell lines from Therapeutic Response Portal Genomics (CTRP). When it comes to the relationship between drug sensitivity and drug IC50, the following figures all show that ADORA2B exhibits a positive correlation and significance (Fig. 7A, B).

This study used 30 cases of esophageal cancer tissues and compared RT-qPCR with normal tissues to investigate the precise mechanism of ADORA2B in esophageal cancer. ESCA tumors were found to have high expression of ADORA2B (Fig. 7C). WB experiments conducted on 12 pairs of cancer and paraneoplastic samples from patients suffering from esophageal cancer demonstrated that ADORA2B expression was significantly higher at the translation level in the tumor group compared to the paraneoplastic group (Fig. 7D). The oncogenic involvement of ADORA2B in esophageal cancer has been tentatively confirmed.

According to our findings, The TCGA database analysis shows that esophageal cancer has a considerably high expression of ADORA2B. The ADORA2B gene and protein expression in ESCC was also shown to be higher than in nearby normal tissues, according to our qPCR and WB results. The effect of ADORA2B knockdown on the proliferation, migration, invasion, and death of esophageal cancer cells was subsequently demonstrated by cell tests including CCK8 cell proliferation, Transwell, EDU cell proliferation, and flow

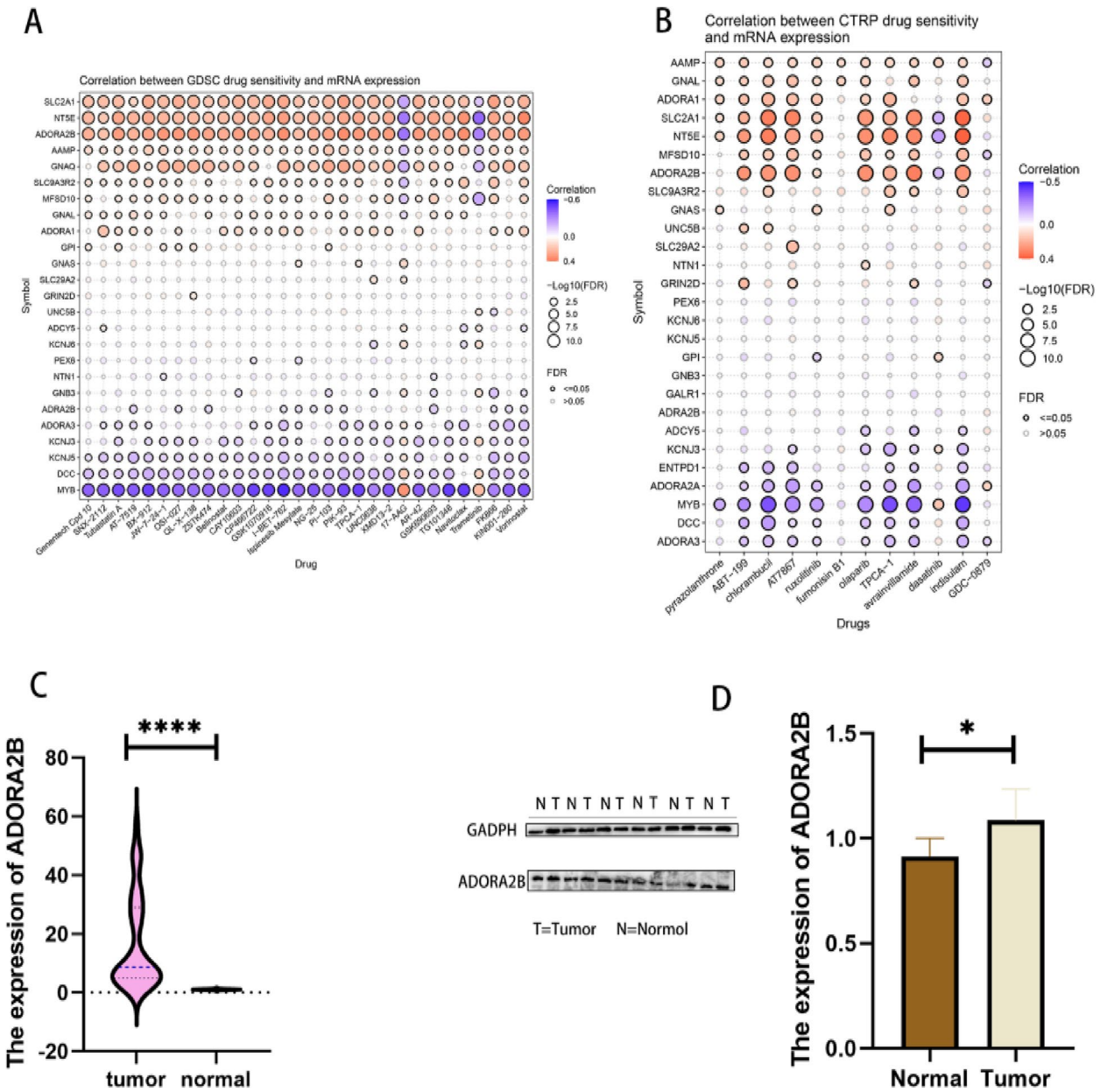

Fig. 7 **A**-**B** The ADORA2B drug sensitivity was positively correlated with the semi-inhibitory concentration of the drug, which was significant. **C**-**D** The expression of ADORA2B in esophageal cancer was verified by qRT-PCR and WB experiments($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$)

cytometry, which demonstrated the carcinogenic effect of this gene.

In this study, 30 patients with esophageal cancer had their ADORA2B expression correlated with their age, smoking status, tumor size, clinical stage, and differentiation type. ADORA2B expression was found to be linked with T division, N classification, clinical stage, and pathological stage, according to a single factor Chi-square analysis (Fisher exact test). In this investigation, 30 ESCC patients were categorized based on the degree of gene expression, as indicated in Table 3, using a retrospective univariate analysis.

We carried out knockdown experiments targeting ADORA2B expression in KYSE150 and ECA109 cell lines to confirm the function of ADORA2B expression in ESCC. In esophageal cancer cells, we knocked down ADORA2B using four different forms of Si-RNA. Two Si-ADORA2B1 and Si-ADORA2B2 successfully silenced ADORA2B at the protein level (Fig. 8A). Figure 8B, C of the Edu findings illustrate the reduction in cell viability

Table 3 Relationship between ADORA2B expression and clinical factors

Character	n	ADORA2B expression		X^2	P
		Low (n)	High (n)		
ALL	30	15	15		
Age				0.536	0.714
< 60	14	6	8		
≥ 60	16	9	7		
Gender				0.682	0.409
Male	22	12	10		
Female	8	3	5		
Smoking				0.556	0.456
Negative	18	8	10		
Positive	12	7	5		
Tumor size (cm)				2.400	0.245
< 5	20	12	8		
≥ 5	10	3	7		
T classification				6.879	0.021
T1 + T2	5	5	0		
T3	23	10	13		
T4	2	0	2		
N classification				14.691	0.001
N0	12	11	1		
N1	11	2	9		
N2	6	2	4		
N3	1	0	1		
M classification				3.333	0.169
M0	24	14	10		
M1	6	1	5		
pTNM stage				11.174	0.002
I + II	10	9	1		
III	16	6	10		
IV	4	0	4		
Pathological differentiation				10.694	0.003
High	5	0	5		
Middle	16	7	9		
Low	9	8	1		
Tumor embolus				1.677	0.390
Negative	23	13	10		
Positive	7	4	3		

seen in ECA109 and KYSE150 cells with ADORA2B deletion.

We then investigated the impact of ADORA2B knockdown on the capacity of ESCA cells to proliferate, invade, and migrate. To this end, we transfected 1000 cells into each hole of a 96-well plate, resulting in esophageal cancer cells KYSE150 and ECA-109. After culture, the absorbance at 450 nm was measured 0, 12, 24, and 48 h later. As demonstrated in Fig. 9A, we were able to confirm through the CCK8 experiment that ADORA2B knockdown could statistically significantly decrease the ability of esophageal cancer cells to proliferate. Regarding the Transwell experiment, it was confirmed that following transfection with ECA109 and KYSE150 cell lines, there were significantly less migrating and invading cells in the ADORA2B knockdown group than in the NC group (both $P < 0.05$) (Fig. 9B,C).

In our experiments, we employed the conventional Annexin V/PI double staining method to differentiate between early and late apoptotic cells. The extent of apoptosis was quantified with precision using a gating strategy. Flow cytometry software was utilized to determine the proportions of early and late apoptotic cells. Using flow cytometry, it was demonstrated that the ADORA2B knockdown group experienced more cell death following transfection with esophageal cancer cells than the NC group ($P < 0.05$). This suggests that ADORA2B prevents esophageal cancer cells from undergoing apoptosis (Fig. 10A-D).

Discussion

Patients with esophageal cancer frequently receive late-stage treatment and poor prognoses due to the lack of distinct early symptoms, evident indicators, and early diagnostic markers. To increase the survival duration of patients with esophageal cancer, it is crucial to identify additional molecular markers for prognostic assessment [13, 14]. Recently, in research on lung adenocarcinoma, PDAC, hepatocellular carcinoma, and breast cancer, ADORA2B proved detrimental. Through ADORA2B, adenosine signaling affects the tumor microenvironment in breast cancer cells, amplifying the tumor-causing function of cancer-associated fibroblasts. This is linked to a higher risk of metastasis and a worse prognosis [15, 16]. Also, our findings indicate that ADORA2B expression exhibits a negative correlation with CD8 + T cells and eosinophils in esophageal cancer (ESCA), suggesting that ADORA2B may play a crucial role in immunosuppressive processes. Previous studies have demonstrated that ADORA2B can directly inhibit the immune system in other tumor microenvironments (TME) through several mechanisms: upregulation of ADORA2B may enhance the secretion of immunosuppressive factors such as IL-10 and TGF-β via the adenosine signaling pathway, thereby suppressing the cytotoxic activity of CD8 + T cells [17]. Additionally, ADORA2B can promote macrophage polarization towards the M2 phenotype and regulate the proliferation of regulatory T cells (Tregs), further contributing to the formation of immunosuppressive microenvironments [7].

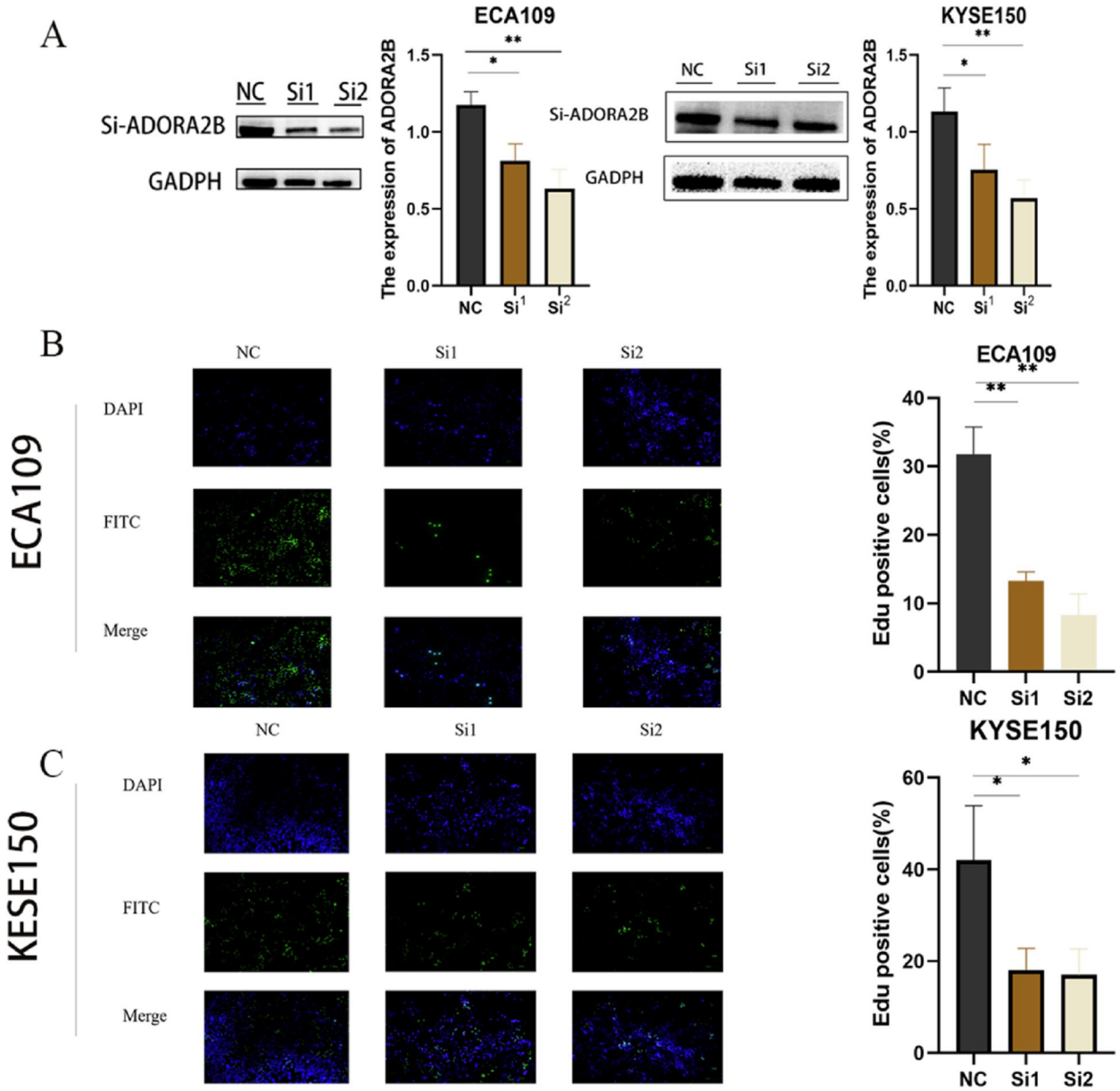

Fig. 8 **A** knockdown experiment targeting ADORA2B expression, and (**B**, **C**) EDU cell experiment verified the proliferative activity of esophageal cancer cells transfected with ADORA2B($*p<0.05$, $**p<0.01$, $***p<0.001$)

Moreover, ADORA2B may also participate in immune regulation indirectly: hypoxia within the tumor microenvironment induces ADORA2B expression, leading to widespread suppression of immune cell function, not limited to CD8 + T cells and eosinophils [18]. As for the mechanism of ADORA2B's influence on cell behavior, we have previously discussed the role of ADORA2B in "cadherin binding," "GTpase activity," "Cadherin Binding," "Gtpase activity," "Cadherin Binding. "G-protein-coupled receptor binding "and other pathways, recent literature confirms that ADORA2B activates the G protein-coupled receptor (GPCR) signaling pathway, thereby promoting cell migration and invasion. ADORA2B also indirectly influences cell adhesion to the ECM by regulating the expression and function of adhesion molecules such as integrins and cadherins on the cell membrane, enabling cells to bind more effectively to the ECM and thus affecting their migratory capacity [19, 20]. Furthermore, PI3K/Akt pathway modulation by ADORA2B in

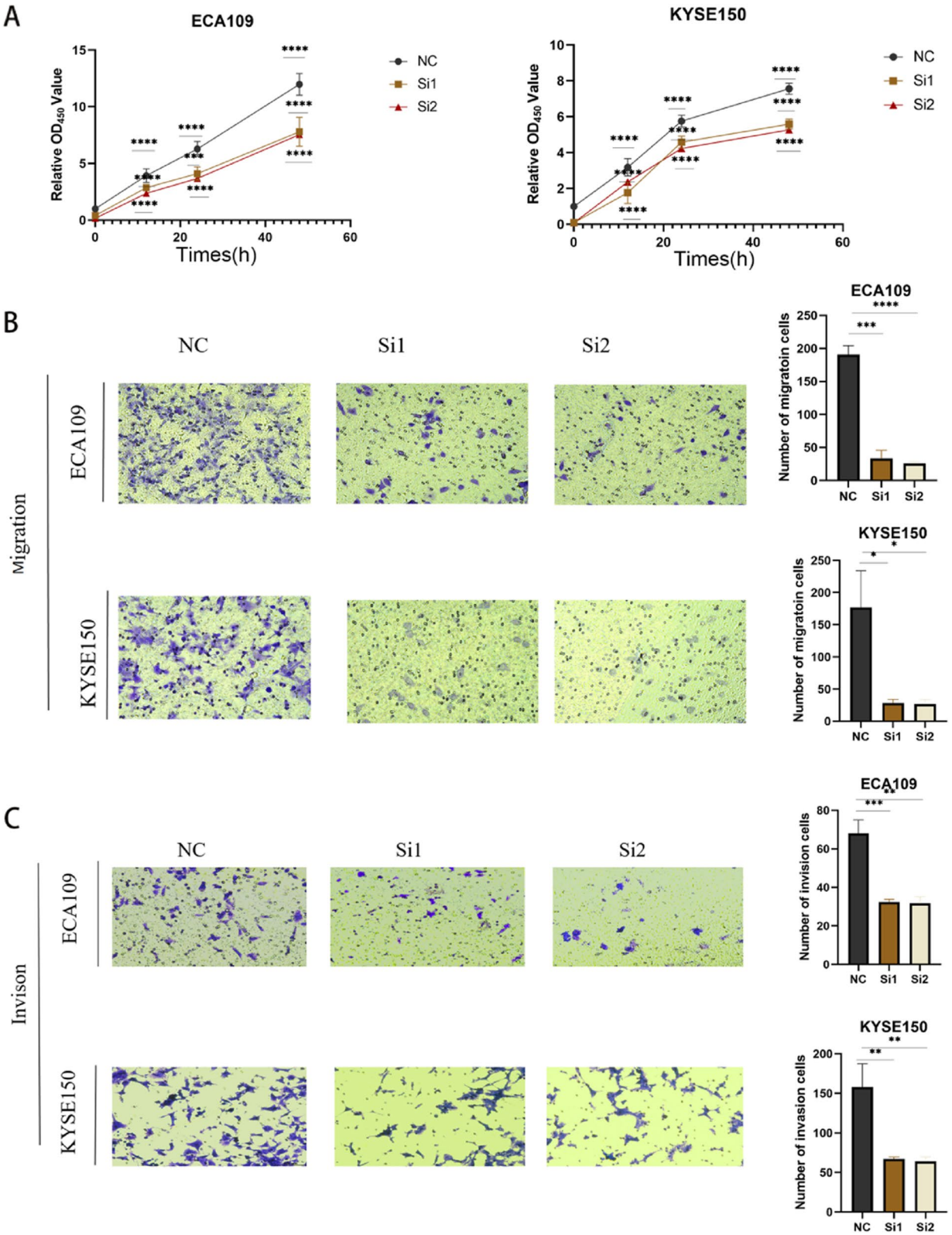

Fig. 9 **A**-**C** After ADORA2B was knocked down, the Transwell function test in CCK8 verified the effect on the proliferation, migration and invasion of esophageal cancer cells($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

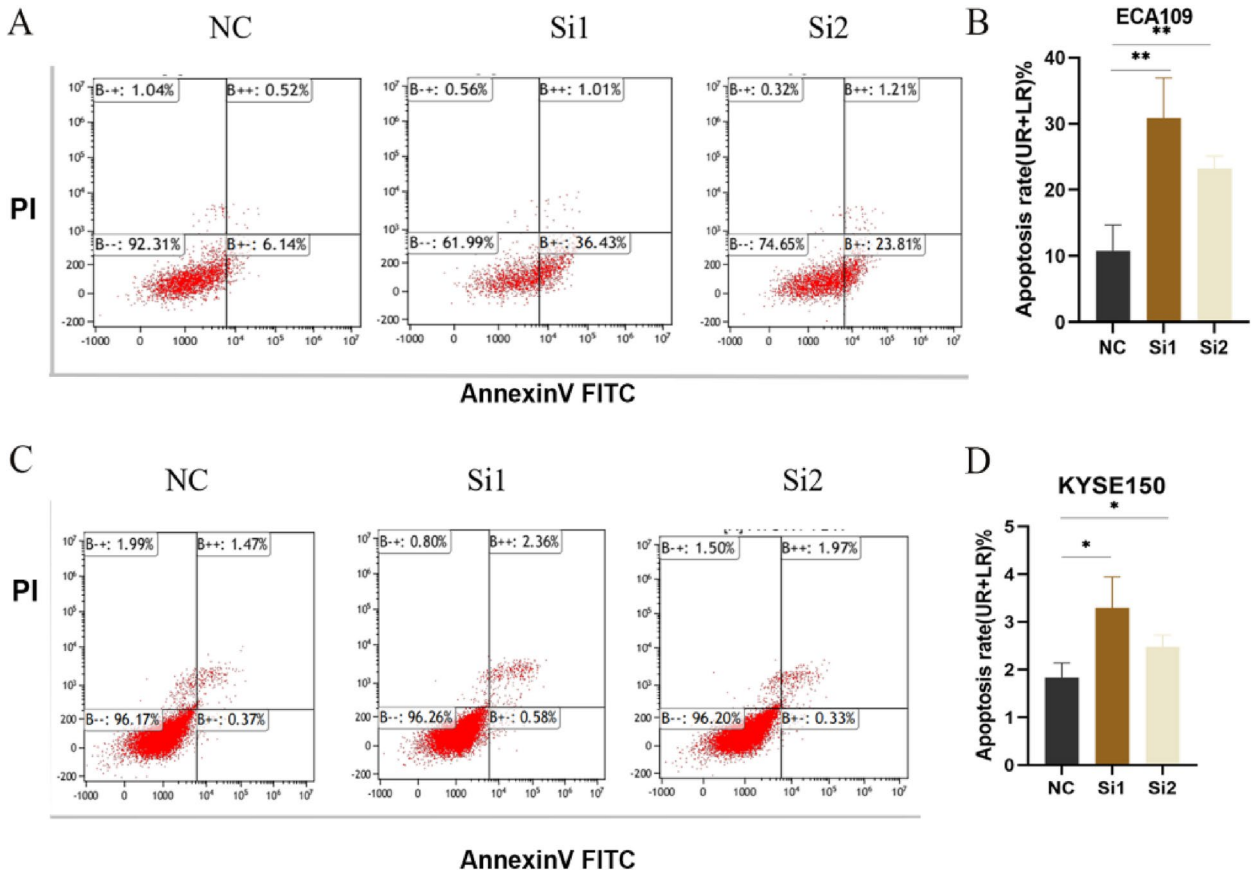

Fig. 10 **A**-**D** After knockdown of ADORA2B, the effect on apoptosis of esophageal cancer cells was verified by flow cytometry($*p<0.05$, $**p<0.01$, $***p<0.001$)

tumor cell migration [21].These mechanisms indicate that ADORA2B's role extends beyond simple signal transduction; it regulates cell migration and invasion through multi-level interactions between cells and the ECM, as well as between cells themselves.

This work has some limitations even though it methodically showed that the expression level of ADORA2B in ESCA is higher than that in nearby tissues. First off, there were no animal function assays in this study; it solely relied on the findings of PCR, WB, cell, and bioinformatics analyses. The findings of the data analysis require additional confirmation. Additionally, the molecular mechanism underlying the elevated expression of ADORA2B in ESCA and the associated downstream targeting pathway mechanism remain unverified. ADORA2B may function by directly suppressing effector immune cells and indirectly altering the immunological microenvironment; however, the precise mechanism requires additional investigation. This research group will assess its functional behavior in the subsequent study to elucidate its significance in ESCA immunosuppression.

Conclusion

To summarize, this study used an online database to thoroughly examine ADORA2B's expression and predictive importance in esophageal cancer. It also included experimental verification of the protein's high expression and clinical significance in esophageal cancer. To determine the theoretical underpinnings of ADORA2B's crucial biological role in esophageal cancer, ADORA2B was shown to be a pivotal gene based on bioinformatics analysis and experimental results. This study used the transfection of ECA109 and KYSE150 to examine the tumor promoting activity of ADORA2B in two ESCC cell lines. Both in vitro and in vivo studies were conducted to examine ADORA2B's potential in malignancies. Additionally, Recent studies have elucidated that ADORA2B can augment anti-tumor immunity by mitigating adenosine-mediated immunosuppression, thereby enhancing the functionality of effector T cells and natural killer cells, which is advantageous for cancer therapy [17]. Furthermore, research has demonstrated that combining ADORA2B inhibitors with immune checkpoint inhibitors (e.g., anti-PD-1) can synergistically enhance

anti-tumor immunity [22]. In summary, ADORA2B holds considerable promise in tumor immunotherapy but also poses risks related to immune-related adverse events and diminished protective effects in hypoxic settings. By employing strategies such as patient stratification, precise targeting, combination therapies, and dynamic monitoring, ADORA2B can be safely and effectively integrated into clinical practice.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12876-025-03768-4.

Supplementary Material 1.

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Not applicable.

Authors' contributions
The author Yixiao Cui wrote the manuscript and made revisions, in addition to participating in the study's design and data collecting, performing in vitro experiments, and conducting bioinformatics analysis. Yuhan Deng gathered data and helped to write the manuscript. Zhenhua Wu carried out data gathering. Xiaohong Sun oversaw the study's design, data interpretation, paper preparation, and final manuscript approval for publication. The final manuscript was read and approved by all writers.

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Data availability
Data Availability Statement The data sets utilized in this analysis are available online.

Declarations

Ethics approval and consent to participate
The Affiliated Cancer Hospital of Xinjiang Medical University's Ethics Committee granted approval and received signed informed permission from all participants (Ethics number: K-2024092). This research adheres to the Helsinki Declaration's tenets. Written informed consent has been signed by each patient.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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