ENT Updates
https://ojs.ukscip.com/index.php/ENTU

Article

Cuproptosis-related Gene CDKN2D is Linked to Prognosis in Esophageal Cancer

Xingnong Xu [1,2], Lei Ma [1] and Shichao Liu [3,*]

[1] School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China
[2] Department of Pharmacy, Yancheng Third People's Hospital, Yancheng 224000, China
[3] College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China
[*] Correspondence: LSC10251025@163.com; Tel.: +86 18545078137

Received: 30 June 2025; **Revised:** 17 July 2025; **Accepted:** 21 July 2025; **Published:** 8 August 2025

Abstract: Cuproptosis, a novel type of regulated cell death, is controlled through protein fatty acylation and has been linked to mitochondrial metabolic processes. Nonetheless, continued research efforts are essential to determine how CDKN2D (Cyclin-dependent kinase inhibitor 2D), a gene associated with cuproptosis, influences the tumor immune microenvironment and progression of esophageal cancer (EC). Gene expression levels of CDKN2D in EC and matched normal tissues were assessed using data from The Cancer Genome Atlas (TCGA). To confirm these findings, we conducted validation analyses utilizing datasets from the Gene Expression Omnibus (GEO) and the Human Protein Atlas (HPA). To ascertain if CDKN2D expression levels were associated with clinical outcomes, we conducted a multivariable regression analysis supplemented by Kaplan-Meier survival estimates. The protein-protein interaction network related to CDKN2D was created using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins). To characterize the immunological relevance of CDKN2D in EC, we performed comprehensive bioinformatic analyses to assess its correlation with tumor-infiltrating immune cells. Three complementary computational approaches-Gene Ontology (GO) functional annotation, conventional GSEA, and ssGSEA (single-sample GSEA) were integrated to evaluate the immunomodulatory potential of CDKN2D in EC. Transcript abundance level of the CDKN2D in EC samples is considerably higher than in normal tissue samples. Analyses involving both single variables and multiple variables indicate that there is no significant statistical difference in overall survival (OS) between EC patients with high CDKN2D expression and those with low CDKN2D expression ($p > 0.05$). Cyclin-dependent kinase 4/6 (CDK4/6) is a critical protein that interacts with the CDKN2D gene, and ECs with high CDKN2D expression are bound to a considerable volume of infiltrating immunocytes. Elevated CDKN2D expression in EC correlated with disease progression and modified immune infiltration patterns.

Keywords: CDKN2D; Esophageal Cancer; Immune Cells; CDK4/6

1. Introduction

As a malignant tumor impacting the gastrointestinal tract, esophageal cancer is the seventh most widespread cancer globally and the sixth most common cause of cancer related mortality [1,2]. Esophageal carcinoma, among the most lethal malignancies, primarily comprises two key subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), each manifesting unique epidemiological behaviors, pathogenic mechanisms, and molecular characteristics [3,4]. Chemotherapy combined with immunotherapy has become the recommended first-line treatment for esophageal cancer [5]. Early stage EC usually has a positive prognosis, with a sur-

vival rate over five years above 90%, while patient survival after five years for advanced stage patients is relatively low [6]. Drug resistance is one of the primary causes of failure in EC treatment and poor prognosis in patients. It is closely related to multiple gene mutations and epigenetic changes. Individualized treatment based on molecular typing is a key strategy to overcome drug resistance [7–9]. In recent decades, there have been a range of developments in the therapeutic techniques for this disease. Currently, treatment options for EC are limited. New therapies targeting the onset of esophageal cancer need to be developed [4,10].

A recently discovered cell death mechanism, cuproptosis occurs when copper ions (Cu^{2+}) accumulate to toxic levels within cells. Chelation of copper ions to lipid-modified TCA (Tricarboxylic Acid Cycle) proteins induces their oligomerization, leading to iron-sulfur cluster protein depletion, subsequent proteotoxic stress, and eventual cellular demise. Its biological mechanism mainly involves abnormal mitochondrial metabolism and protein function [11,12]. The CDKN2D gene is a component of the INK4 family. CDKN2D forms a complex with CDK4/6 to prevent its phosphorylation, thereby inhibiting cells from entering the S phase and exerting the role of cell cycle regulation. The abnormality of its function may be related to the pathogenesis of multiple cancers [13,14]. In pancreatic and cervical tumor, the expression intensity of CDKN2D is tightly linked to tumor progression, and its downregulation may promote the expansion and movement of malignant cells [14,15]. The level of *CDKN2A* expression has been shown to correlate with copper-induced death in a range of cancers, and its high expression is associated with strong tumor invasiveness, proliferation ability and poor patient outcomes [16–18]. The CDKN2D gene is not clear in the copper death mechanism, but as a CDK inhibitor, it may have a certain functional or expression correlation with *CDKN2A* in cell cycle regulation. The biological function of CDKN2D in esophageal carcinoma has not yet been elucidated.

This investigation utilized transcriptomic and clinical datasets from GEO, TCGA, and HPA to examine correlations between CDKN2D expression levels, clinicopathological parameters, and survival outcomes in EC patients. Subsequently, we extracted datasets from TIMER and GEPIA to analyze potential correlations between CDKN2D expression patterns, immune cell infiltration levels, and related immunological gene signatures. Protein-protein interaction networks involving CDKN2D were subsequently constructed using the STRING database. Lower *CDKN2D* gene levels were linked to reduced immune cell invasion in EC tissue, indicating a poor prognosis. Therefore, it is reasonable that *CDKN2D* gene defects might weaken the anti-tumor immunity effect in EC. Targeted combined immunotherapy associated with CDKN2D may be a feasible treatment in EC.

2. Materials and Methods

2.1. Data Source

As a major component of the NCI's cancer genomics program, TCGA systematically catalogues clinical and molecular figures across diverse malignancies. Our investigation specifically retrieved EC-related clinical annotations and transcriptomic data (RNA-seq) from this curated database. The fragments per kilobase per million mapped fragments (FPKM) method, as implemented in HTSeq, was utilized to ascertain transcript expression levels. Additionally, for further analysis, the RNA-Seq gene expression level 3 HTSeqFPKM data from patients with EC and the corresponding clinical data were converted into transcripts per million (TPM) reads. Since the database is publicly accessible, no approval from the local ethics committee was deemed necessary.

2.2. GEO and HPA Datasets

Hosted by the National Center for Biotechnology Information (NCBI), the GEO is one of the most extensive repositories of microarray data worldwide, serving as a premier resource for transcriptomic studies. The HPA compiles comprehensive transcriptomic and proteomic data from a variety of human samples, encompassing tissue, cellular, and pathological atlases. Currently, the platform archives cellular localization data across 44 normal human tissues and 20 high-incidence malignancies. The database further encompasses immunohistochemical protein expression profiles for both malignant and non-malignant human tissue specimens.

2.3. Statistical Evaluation of Clinical Outcomes, Alongside the Creation and Appraisal of Predictive Models

The analysis of prognostic factors, including overall survival (OS), disease-specific survival (DSS), and progression-free survival (PFS), was conducted using clinical data sourced from the TCGA through the module for deriving clinical insights available on the Xiantao platform. The Cox proportional hazards regression model and Kaplan-Meier survival curves were employed in these assessments. The CDKN2D expression groups were dichotomized using median cutoff values. Associations with clinicopathological features were evaluated through Wilcoxon rank-sum tests and logistic regression modeling. The impact of CDKN2D gene expression on survival and other clinical variables was assessed using a multivariate Cox regression analysis. Statistical significance was set at $p < 0.05$. The outputs from the Cox model were integrated with significant multivariate predictors to generate survival estimates for 1, 3, and 5 years. The prediction accuracy was assessed using calibration plots, where perfect concordance aligns with the 45° reference line.

2.4. In-depth Investigation of Protein-Protein Interactions

The STRING platform served as the primary analysis tool, offering extensively integrated and consolidated protein-protein interaction (PPI) data. After uploading the CDKN2D expression data to STRING, we obtained information from the PPI network. The significance threshold was identified by a confidence score above 0.7.

2.5. Enrichment Profiling

Functional annotation of genes associated with CDKN2D was performed through GO enrichment analysis utilizing clusterProfiler (v3.6.3) in R, examining three core ontology domains: cellular components (CC), molecular functions (MF), and biological processes (BP). Significance thresholds were set at enrichment coefficient > 1.5, minimum gene count > 3, and p-value < 0.01. Pathway analysis employed GSEA methodology with 1,000 genome permutations, considering pathways significant at FDR (false discovery rate) < 0.25 and adjusted $p < 0.05$. The findings were interpreted using NES (normalized enrichment scores) and corrected p-values, with visualization conducted through clusterProfiler [19].

2.6. Immunological Infiltration Profiling

In a study by Tao W et al. [19], marker genes for 24 distinct immune cell types were identified. Tumor infiltration by these immune cells was evaluated using the ssGSEA method. The Spearman rank correlation model was employed to juxtapose the levels of immune cell invasion within subgroups characterized by superior versus minimal CDKN2D gene transcription, thereby assessing the relationship among CDKN2D activity and immunocyte invasion. The Xiantao tool was utilized to analyze the connection between CDKN2D expression and immune infiltration, as well as the correlation between immune cell levels and CDKN2D expression subgroups. This included data on immune infiltration, Spearman correlation analysis via the Xiantao tool, and the Wilcoxon signed-rank test. We adopted the conventional 0.05 cutoff for determining significance.

2.7. Intergenic Association Analysis

GEPIA serves as a web-based tool that provides access to data from cancer types and health specimens sourced from TCGA and GTEx. It primarily centers on RNA-seq analysis. The platform includes 60,498 gene types and 198,619 isoform types. An investigation in GEPIA examined the interrelation among CDKN2D gene activity and various immune cell markers. CDKN2D gene expression is plotted on the x-axis, with relevant gene expression profiles plotted on the ordinate. Furthermore, TIMER data supported gene expression patterns strongly linked to CDKN2D as observed in GEPIA. Statistical significance was set at a p-value less than 0.05.

3. Results

3.1. *CDKN2D* Gene Expression Exhibited an Increase in Tumors Relative to Normal Samples

Pan-cancer screening revealed CDKN2D overexpression as a frequent oncogenic event (**Figure 1A**). TCGA analysis of 163 EC/11 normal samples showed significantly elevated CDKN2D transcripts in tumors (**Figure 1B**). This

upregulation was confirmed in tumor-adjacent tissue comparisons (8 pairs, **Figure 1C**). To verify the diagnostic potential of CDKN2D, we generated ROC curves comparing its expression in EC tumors versus (a) GTEx-normalized tissues and (b) adjacent non-cancerous tissues. The resulting AUC of 0.525 (95% CI: 0.471–0.580) suggests limited discriminatory capacity (**Figure 1D**). In EC tissues, the expression of CDKN2D protein was notably increased compared to normal tissue samples (**Figure 1E**). Similarly, HPA data demonstrated that CDKN2D protein expression was enhanced in EC versus normal control (**Figure 2**).

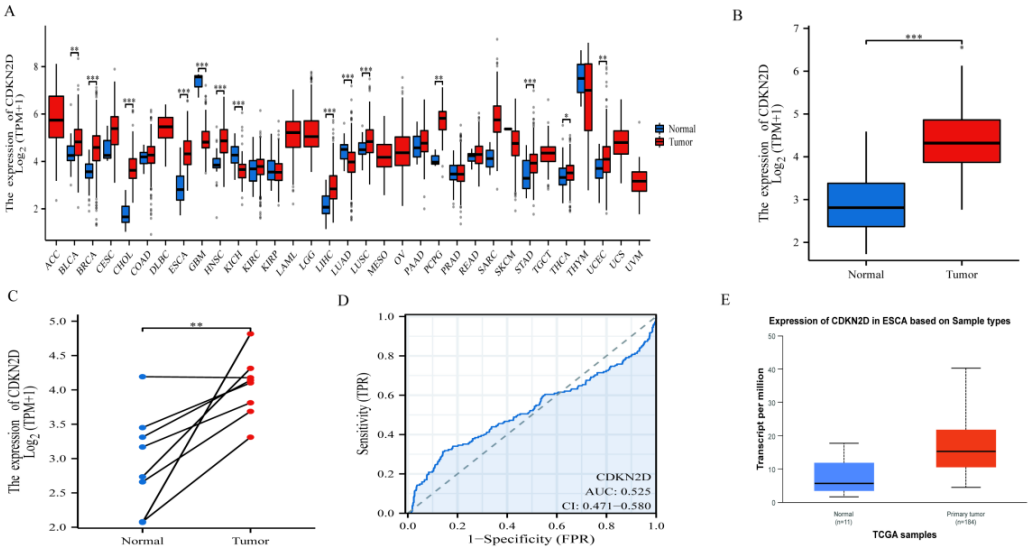


Figure 1. CDKN2D oncogenic expression profile. (**A**) Pan-cancer vs. normal tissue comparison. (**B**) EC tumor-adjacent tissue differential expression. (**C**) Matched tumor-normal paired analysis. (**D**) ROC curve analysis (GTEx controls vs. EC samples). (**E**) Tumor-specific protein upregulation.

Notes: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and ns, no statistical difference.

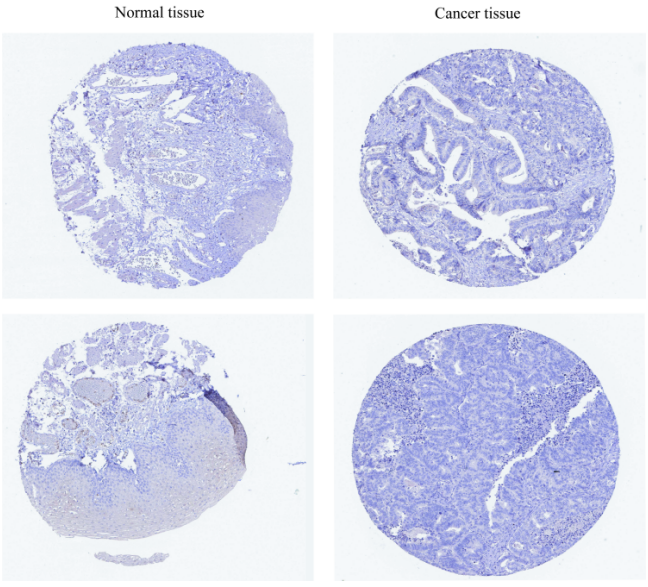


Figure 2. HPA data on CDKN2D expression.

An assessment of CDKN2D gene expression using the HPA showed that CDKN2D protein levels were notably increased in EC compared to normal control.

3.2. CDKN2D Expression in Relation to Clinical Parameters

Using the Z-score criterion, the expression levels of CDKN2D in tumor samples were determined. The EC cohort was subsequently divided into expression-based subgroups (low/high). Kruskal-Wallis and Wilcoxon signed-rank examinations were applied to analyze the correlation between CDKN2D expression and clinical indicators. Compared to the normal group, tumor staging (T/N/M) showed significant positive correlations with elevated CDKN2D levels (**Figures 3A-F**). These associations were further validated through multivariate Cox regression analysis. Higher pathologic T stage, pathologic N stage, clinical N stage, and pathologic stages, as well as gender (male) were associated with higher CDKN2D expression, particularly pathologic stage ($p < 0.001$) (**Table 1**). Interestingly, univariate analysis demonstrated significant relationships between CDKN2D expression and clinical indicators (**Table 2**). Collectively, these results establish CDKN2D as a potential biomarker for the occurrence of EC.

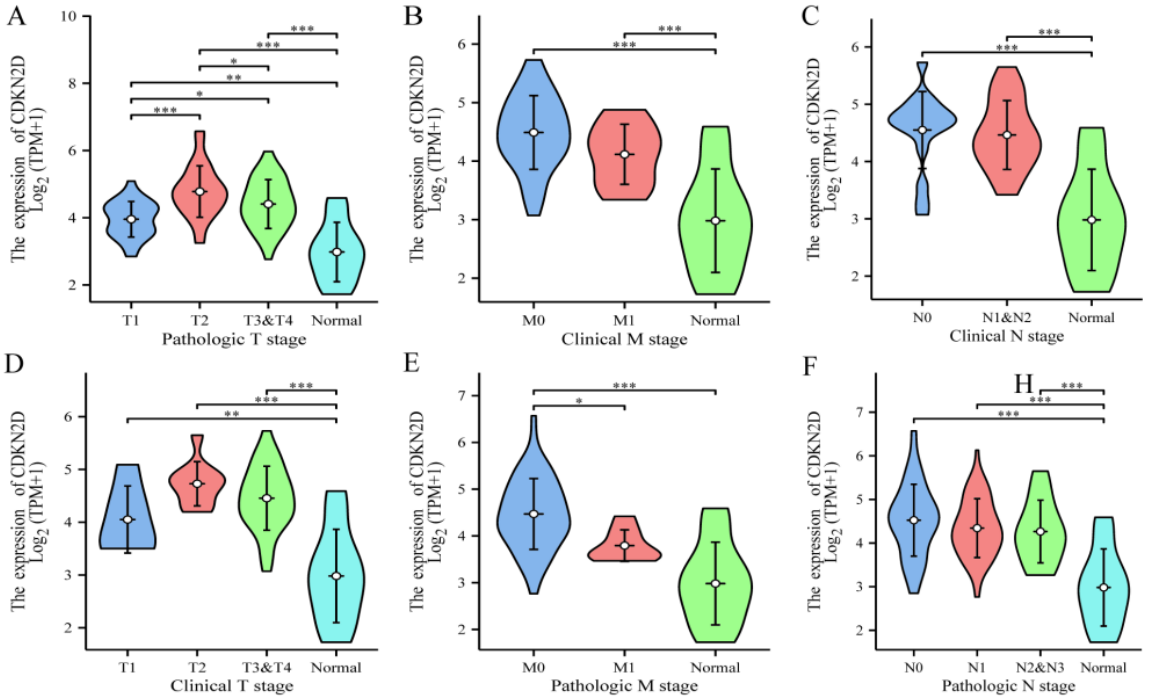


Figure 3. Correlation of CDKN2D expression with clinical-pathological characteristics of EC. The correlation among CDKN2D expression and T stage (**A**, **D**), N stage (**C**, **F**), M stage (**B**, **E**).

Notes: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Table 1. Association of CDKN2D expression with clinicopathological characteristics in patients with EC.

Characteristics	Total(N)	HR(95% CI) Univariate Analysis	*p* value Univariate Analysis	HR(95% CI) Multivariate Analysis
Pathologic T stage	145			
T1&T2	64	Reference		
T3&T4	81	1.312 (0.756–2.277)	0.334	
Pathologic N stage	144			
N0&N1	129	Reference		
N2&N3	15	1.944 (0.904–4.181)	0.089	
Pathologic stage	142			
Stage I&Stage II	85	Reference		Reference
Stage IV&Stage III	57	3.223 (1.807–5.747)	< 0.001	2.936 (1.629–5.293)
Clinical T stage	52			

Table 1. *Cont.*

Characteristics	Total(N)	HR(95% CI) Univariate Analysis	p value Univariate Analysis	HR(95% CI) Multivariate Analysis
T1&T2	16	Reference		
T4&T3	36	0.764 (0.311–1.876)	0.556	
Clinical N stage	43			
N0	17	Reference		
N1&N2	26	1.333 (0.403–4.409)	0.638	
Gender	163			
Female	23	Reference		Reference
Male	140	2.338 (0.935–5.846)	0.069	1.874 (0.655–5.365)
Age	163			
<= 60	83	Reference		
> 60	80	0.858 (0.525–1.402)	0.541	
CDKN2D	163			
Low	81	Reference		
High	82	0.857 (0.521–1.410)	0.544	

Table 2. Logistic regression analysis of CDKN2D expression.

Characteristics	Total (N)	OR (95% CI)	p value
Pathologic T stage (T3&T4 vs. T1&T2)	145	0.892 (0.462–1.722)	0.734
Pathologic N stage (N2&N3 vs. N0&N1)	144	0.408 (0.132–1.262)	0.120
Pathologic M stage (M1 vs. M0)	129	0.119 (0.014–0.997)	0.050
Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II)	142	0.547 (0.277–1.078)	0.081
Clinical T stage (T3&T4 vs. T1&T2)	52	0.750 (0.224–2.507)	0.640
Clinical N stage (N1&N2 vs. N0)	43	0.359 (0.092–1.399)	0.140
Clinical M stage (M1 vs. M0)	52	0.405 (0.102–1.604)	0.198
Clinical stage (Stage III&Stage IV vs. Stage I&Stage II)	54	0.500 (0.165–1.517)	0.221
Primary therapy outcome (PR&CR vs. PD&SD)	94	2.365 (0.811–6.895)	0.115
Gender (Male vs. Female)	163	1.695 (0.689–4.171)	0.251
Age (> 60 vs. <= 60)	163	0.539 (0.289–1.003)	0.051

3.3. CDKN2D Expression as a Prognostic Indicator in EC

Survival analyses using TCGA data revealed no significant prognostic associations for CDKN2D expression levels: OS (HR = 0.86, 95% CI 0.52–1.41, $p = 0.544$), DSS (HR = 0.77, 95% CI 0.43–1.39, $p = 0.389$), or PFS (HR = 0.90, 95% CI 0.58–1.40, $p = 0.636$) (**Figures 4A-C**).

In this study, it was found that patients with EC had increased risk scores and higher levels of CDKN2D expression. Notably, those with higher risk scores also displayed significant CDKN2D expression. The relationship between CDKN2D expression and numerous cohorts was further verified. Specifically, CDKN2D expression was discovered to be elevated in tumors with pathologic stage III–IV [HR = 3.223, 95% CI: 1.807–5.747, $p < 0.001$] (**Figure 4D**). Moreover, a prognostic risk stratification tool for EC was constructed, considering factors such as gender, TNM stages, age, pathologic stage, and CDKN2D expression (**Figure 4E**). We also applied a calibration chart to assess the accuracy of the model's predictions (**Figure 4F**). The expression of CDKN2D does not seem to improve the accuracy of one-year survival predictions.

3.4. Assembling Protein-protein Interaction Networks

Protein network analysis via STRING revealed CDKN2D-interacting partners implicated in EC development, providing insights into its functional role in tumor biology. Analysis of research results shows the topmost ten proteins along with their associated gene names, scores, including *CDKN1A*, *CDKN1B*, *CDKN1C*, *CCNL2*, *CDK2*, *CDK4*, *CDK6*, *CCND1*, *CCND2* and *CCND3* (**Figure 5**).

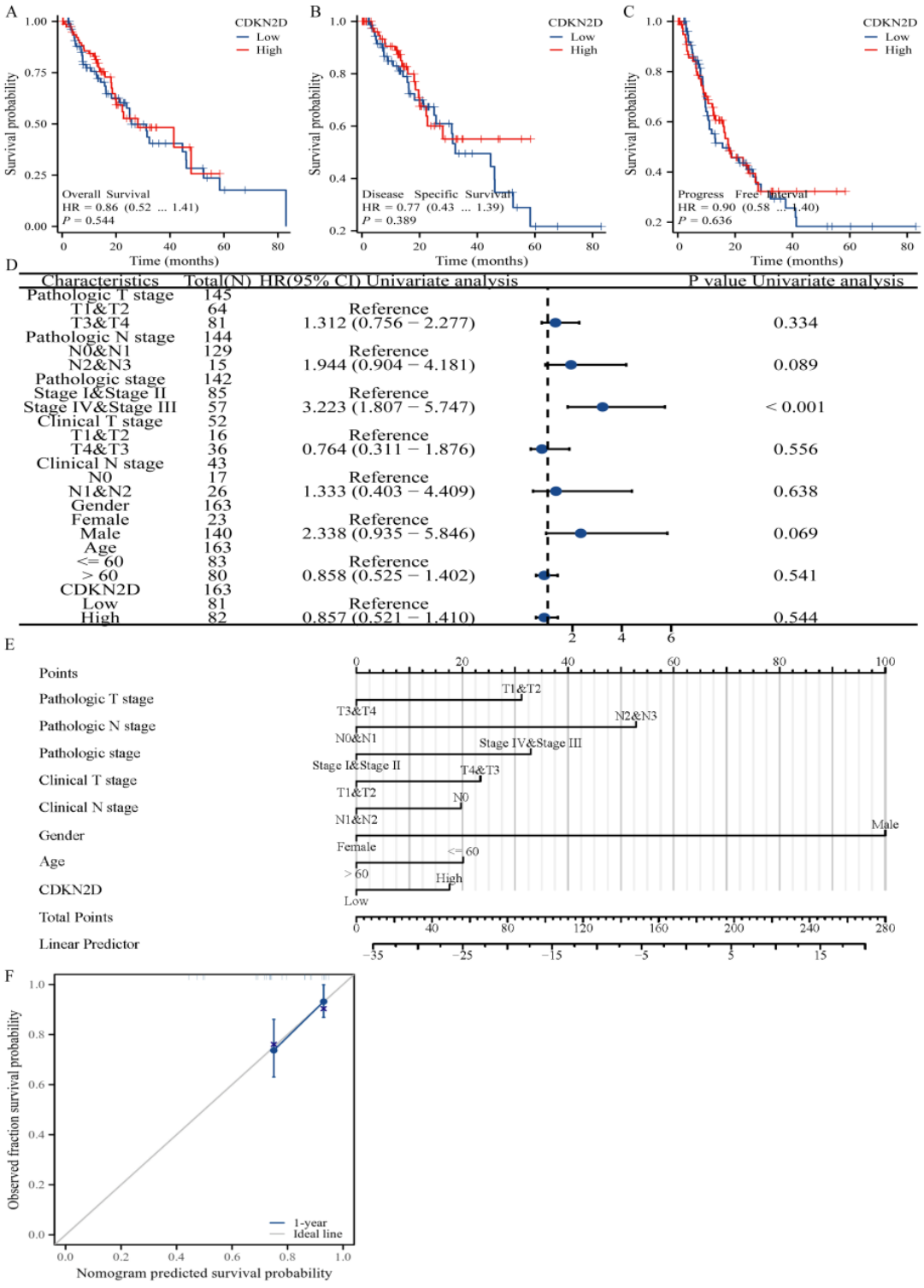
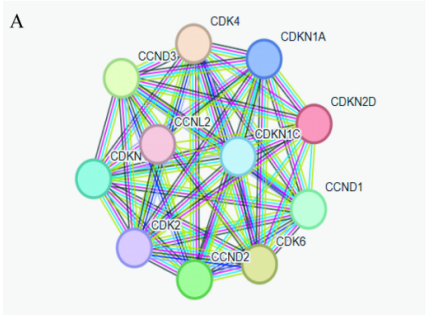


Figure 4. Prognostic analysis of CDKN2D expression. Patients exhibiting lower CDKN2D expression did not demonstrate significantly favorable prognostic outcomes compared to those with higher CDKN2D expression, characterized by OS (**A**), PFS (**B**), and DSS (**C**) (both log-rank $p > 0.05$). (**D**) Prognostic evaluation based on CDKN2D expression across various clinical features. (**E**) A nomogram derived from multivariate analysis incorporating clinical features linked to CDKN2D. (**F**) A calibration graph illustrating the model's predictive accuracy, as assessed through multi-variable Cox regression method.



B

Gene Symbol	Score
CCND1	0.952
CCND2	0.963
CCND3	0.978
CCNL2	0.819
CDK2	0.847
CDK4	0.999
CDK6	0.999
CDKN1A	0.854
CDKN1B	0.925
CDKN1C	0.878

Figure 5. CDKN2D-interacting proteins in EC. (**A**) Proteins that bind to CDKN2D. (**B**) Their co-expression values.

3.5. Whole-transcriptome Association Study with CDKN2D Expression

Transcriptomic profiling of CDKN2D in EC revealed significant associations with 1,437 downregulated and 2,595 upregulated genes (logFC > 1, adjusted $p < 0.05$; **Figure 6A**). A heatmap visualization highlighted the 20 most differentially expressed genes (DEGs, logFC > 2, padj < 0.01; **Figure 6B**). GO analysis identified intermediate filament organization, intermediate filament cytoskeleton organization, and keratinization as the predominant biological processes linked to CDKN2D activity (**Figure 6C**).

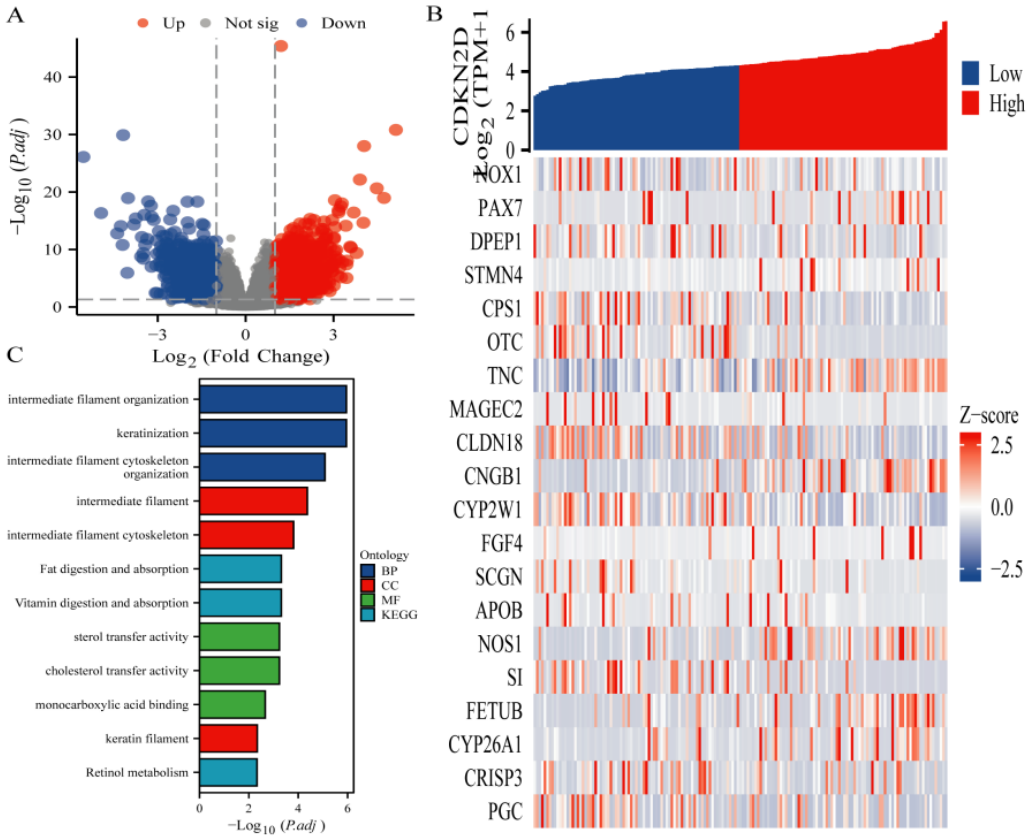


Figure 6. Analysis of CDKN2D gene expression and GO enrichment. (**A**) A volcano plot based on CDKN2D highlights DEGs. (**B**) A heat map generated using CDKN2D expression levels shows genes that were either upregulated or downregulated. (**C**) GO enrichment results of DEGs, filtered based on CDKN2D gene expression, were scrutinized using the metascape data repository.

3.6. Exploring CDKN2D Expression Through GSEA

TCGA-based GSEA revealed significantly enriched pathways (FDR< 0.25) when comparing CDKN2D expression groups, with the top-ranked pathway identified by NES shown in **Figure 7**. GSEA revealed CDKN2D-high phenotypes were significantly associated with [Reactome] Scavenging of Heme From Plasma (**A**), [Reactome] CD22 Mediated BCR Regulation (**B**), [Reactome] Antigen Activates B Cell Receptor BCR Leading To Generation of Second Messengers (**C**), [Reactome] Fceri Mediated Ca^{2+} Mobilization (**D**), [Reactome] Role of LAT2 Ntal Lab On Calcium Mobilization (**E**), [Reactome] Fceri Mediated Mapk Activation (**F**), [Reactome] Fcgr Activation (**G**), [Reactome] Creation of C4 and C2 Activators (**H**), [Reactome] Contribution of phospholipids to phagocytic processes (**I**), [Reactome] Initial Triggering of Complement (**J**), [Reactome] Fceri Mediated Nf Kb Activation (**K**), and [Reactome] Potential Therapeutics For Sars (**L**).

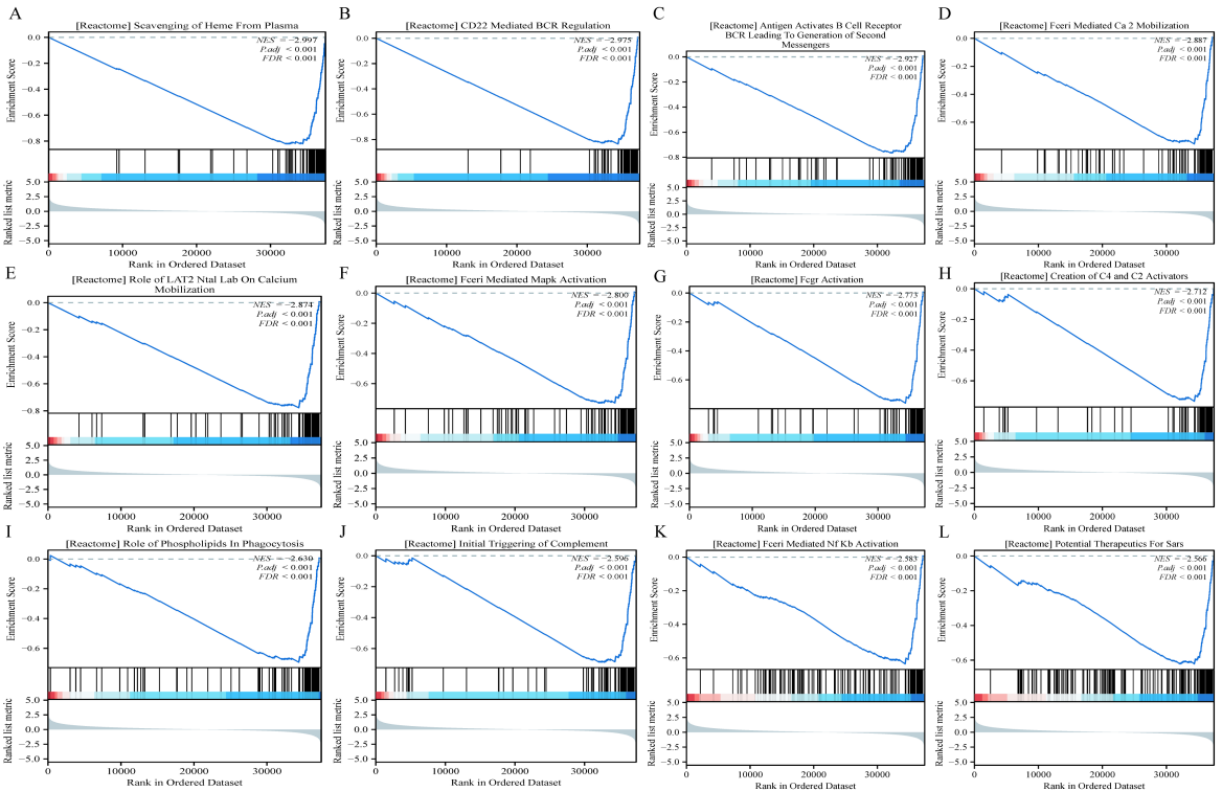


Figure 7. The insights of the GSEA. GSEA results showed that [Reactome] Removal of Heme from Plasma (**A**), [Reactome] CD22-dependent B-cell receptor regulation (**B**), [Reactome] antigen-induced BCR signaling (**C**), [Reactome] Fceri-mediated calcium flux (**D**), [Reactome] LAT2/NTAL calcium regulation (**E**), [Reactome] Fceri-Mapk activation (**F**), [Reactome] Fcgr Activation (**G**), [Reactome] complement C4/C2 activation (**H**), [Reactome] phospholipid-dependent phagocytosis (**I**), [Reactome] Initial Triggering of Complement (**J**), [Reactome] FCERI-NF-κB signaling (**K**), and [Reactome] Potential Therapeutics For Sars (**L**).

3.7. CDKN2D-dependent Modulation of Immune Cell Recruitment in Tumor Tissues

Our investigation revealed significant immunogenomic associations between CDKN2D expression and 24 immune cell populations in EC. CDKN2D demonstrated: (i) positive correlations with Tcm, NK CD56dim cells, NK cells, Th2 cells, and (ii) negative correlations with Th17 cells, eosinophils, B lymphocytes, Tem (**Figures 8A, E-L**). Distinct CDKN2D expression patterns were observed across various immune infiltrates, particularly in B cells, eosinophils, NK cells, Th17 cells, and Tcm (**Figures 8B-D**).

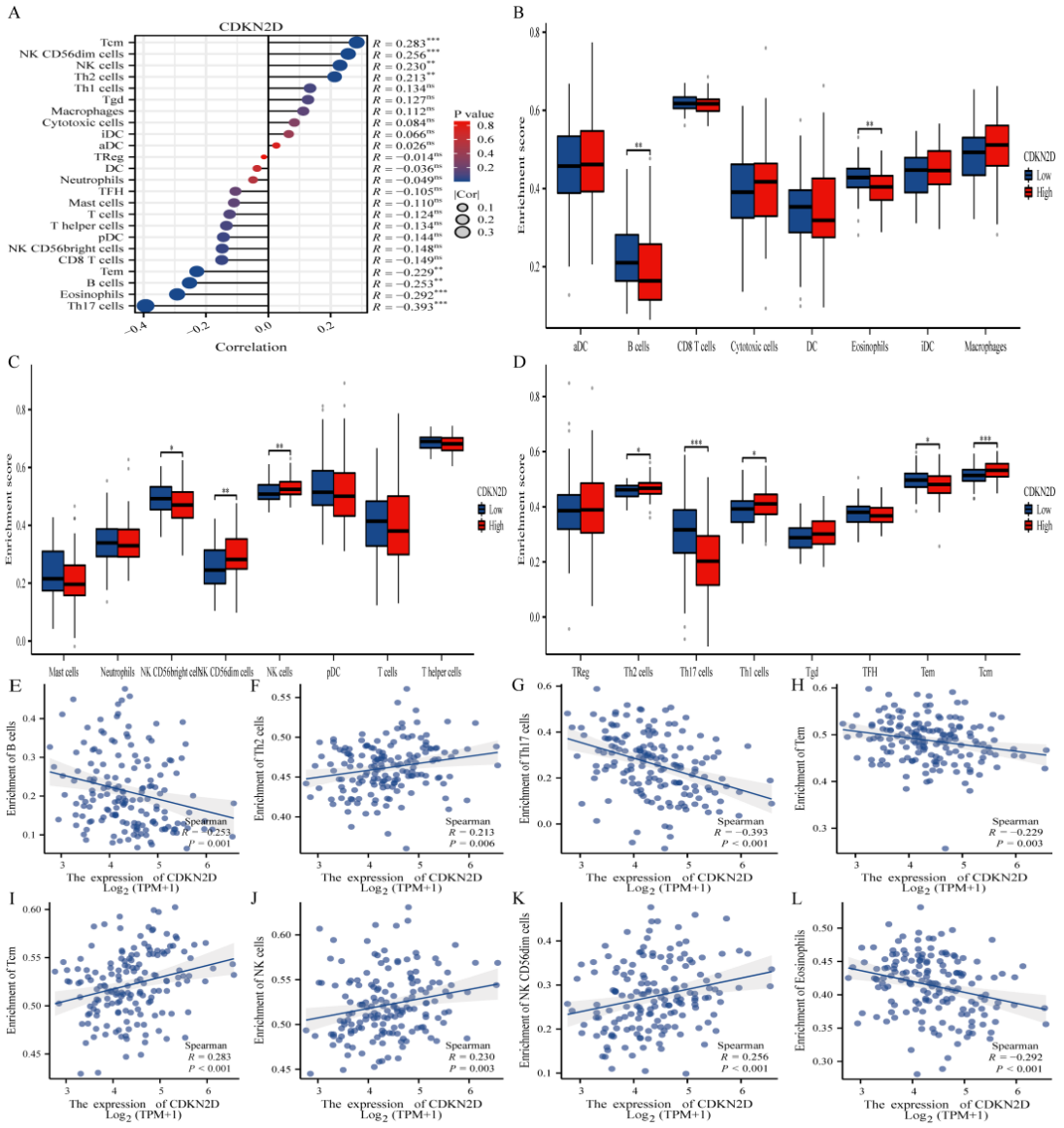


Figure 8. Correlation between CDKN2D and immune cell invasion. (**A**) Analysis of the correlation between CDKN2D expression and immune cell invasion profile. (**B–D**) Variations in the enrichment levels of specific immune cell subsets in groups with high and low CDKN2D gene expression. (**E–L**) Relationships between the CDKN2D gene expression and tumor microenvironment characteristics.

Notes: Ns is the abbreviation of no significance. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

Esophageal carcinoma constitutes a significant global health burden, ranking among the top ten most prevalent malignancies worldwide, with an estimated annual incidence exceeding 470,000 cases. Although recent years have seen notable progress in therapeutic approaches, including the advent of targeted therapies and immunotherapeutic regimens, clinical outcomes remain suboptimal, with five-year survival rates consistently below 25% [20]. This therapeutic inadequacy largely stems from the disease's characteristic late-stage presentation, with approximately 60–70% of cases diagnosed at advanced stages (III or IV), making curative intervention challenging. The insidious onset of symptoms, coupled with the absence of robust early detection biomarkers, significantly contributes to delayed diagnosis. Furthermore, the anatomical complexity of the esophagus and its proximity to critical structures often limit radical treatment options. Emerging evidence suggests that molecular heterogeneity between esophageal squamous cell carcinoma and adenocarcinoma subtypes may underlie differential treatment responses, compound-

ing therapeutic challenges. Current multimodal treatment strategies, while showing incremental improvements in locoregional control, frequently fail to address systemic micrometastases present in advanced disease. These clinical realities underscore the urgent need for improved early detection methods and the development of more effective systemic therapies, customized based on tumor molecular signatures [20,21]. Our investigation revealed significant associations between CDKN2D expression and the progression of EC. Utilizing Cox proportional hazards regression, we developed a prognostic stratification model that effectively categorized EC patients into distinct risk groups. Notably, elevated CDKN2D expression correlated with adverse clinical outcomes, as high-risk patients exhibited reduced survival rates. Both univariate and multivariate analyses confirmed CDKN2D as a promising prognostic biomarker. These findings suggest that CDKN2D may be useful in risk assessment and in developing personalized therapeutic strategies for EC.

Cuproptosis has emerged as a distinct modality of regulated cell death, exhibiting fundamental differences from both apoptotic and ferroptotic pathways. The defining pathophysiological features include: (i) supra-physiological intracellular copper (Cu^{2+}) concentrations that disrupt metal homeostasis, (ii) obligatory copper-dependent cytotoxicity, and (iii) profound impairment of mitochondrial energy metabolism. This cell death mechanism exhibits preferential cytotoxicity against cells with increased metabolic activity, particularly those in the S-phase. Interestingly, CDKN2D-induced cell cycle inhibition during the G1-to-S transition may confer relative resistance by decreasing cellular metabolic demand, thereby indirectly reducing susceptibility to cuproptosis [22]. In hepatocellular carcinoma (HCC), the cuproptosis-related gene *CDKN2A* displays significant clinical relevance. It is positively correlated with Th2 cell infiltration and the presence of cancer-associated fibroblasts (CAFs), whereas it is negatively correlated with the abundance of $CD8^+$ T cells. Experimental research indicates that *CDKN2A* is significantly overexpressed in hepatocellular carcinoma (HCC) cell lines. Functional analysis reveals that knocking down *CDKN2A* not only suppresses HCC cell proliferation but also concurrently restrains migratory and invasive capacities. Furthermore, it triggers G2/M phase cell cycle arrest and enhances apoptotic cell death [23]. Considering the crucial role of the *CDKN2A* gene within the same family in conferring resistance to copper-induced cell death and in promoting disease progression, the knockdown of *CDKN2A* renders cells susceptible to copper-induced cell death [22,24]. Our findings suggest that CDKN2D may confer cytoprotective effects against cuproptosis in EC, analogous to its established anti-apoptotic functions. The significantly elevated expression of CDKN2D in tumor tissues compared to normal tissues (**Figures 1B-C**) suggests a potential role in evading copper-dependent cell death. Clinically, elevated CDKN2D expression was correlated with reduced overall survival (**Figures 4A-C**), potentially indicating a selection advantage for tumor cells that resist copper toxicity. This aligns with the metabolic adaptation hypothesis, where CDKN2D-mediated cell cycle modulation (G1/S arrest) may decrease mitochondrial susceptibility to copper overload. The observed survival disparity may arise from two mechanisms: (1) direct copper detoxification through pathways regulated by CDKN2D, and (2) indirect metabolic reprogramming that promotes tumor persistence. These findings suggest that CDKN2D could serve as both a clinical prognostic marker and a potential target for treatments aimed at modulating cuproptosis sensitivity in EC.

The development and malignant progression of tumors are critically influenced by complex interactions between dysregulated metabolic pathways and immunosuppressive microenvironmental conditions. These interconnected processes cooperatively drive tumor immune evasion and sustained proliferation [23,25]. Cancer cells rewire their metabolic pathways to meet the bioenergetic and biosynthetic demands of rapid proliferation, while simultaneously shaping an immunosuppressive microenvironment that facilitates immune evasion [26]. For example, changes in lipid metabolism—including increased lipogenesis and accumulation of lipid droplets—are characteristic features of HCC, contributing to tumor growth, chemoresistance, and metastatic potential [27]. Similarly, dysregulated fatty acid oxidation (FAO) has been implicated in immune suppression, particularly by promoting the expansion of regulatory T cells (Tregs) and impairing the function of cytotoxic T cells, thereby fostering lymph node metastasis [28]. These metabolic adaptations sustain not only tumor survival but also modulate immune cell infiltration and function. Elevated lipid metabolism in cancer-associated fibroblasts (CAFs) can secrete immunosuppressive cytokines, while fatty acid oxidation (FAO)-driven metabolic crosstalk between tumor cells and myeloid-derived suppressor cells (MDSCs) further dampens antitumor immunity. Metabolic-immune interactions create an ecological niche that facilitates tumor progression and therapeutic resistance. Understanding these mechanisms could reveal new targets for combined metabolic and immunotherapeutic interventions in cancer treatment [23,25–28]. Our integrated multi-database analysis (GEO/TCGA/HPA) revealed markedly elevated CDKN2D tran-

script levels in EC specimens compared to normal controls. Clinically, patients with overexpression of CDKN2D have shown significantly poorer survival outcomes, indicating its potential as both a diagnostic marker and a focus of clinical attention in the progression of EC. This association remained significant even after multivariate adjustment.

Protein-protein interaction network analysis using STRING revealed CDK4, CDK6, and CCND3 as key functional partners of CDKN2D in EC, uncovering critical cell cycle regulatory mechanisms. CDK4 and CDK6, members of the cyclin-dependent kinase family, act as key regulators of the G1/S phase transition by phosphorylating the retinoblastoma protein (Rb), which in turn releases E2F transcription factors to initiate DNA replication [29]. CCND3, a D-type cyclin, functions as an essential regulatory subunit that binds to and activates CDK4/6 kinases during the mid-G1 phase. These CDK4/6-CCND3 complexes then phosphorylate downstream targets, thereby promoting cell cycle progression and proliferation [30]. In contrast, CDKN2D acts as a potent cyclin-dependent kinase inhibitor (CKI), competitively binding to CDK4/6-cyclin complexes and effectively blocking their kinase activity through various mechanisms: (i) direct steric hindrance of the catalytic domain, (ii) prevention of cyclin binding, and (iii) alteration of substrate recognition. This inhibition maintains Rb in its active, hypophosphorylated state, enforcing G1 phase arrest and suppressing uncontrolled proliferation. The opposing functions of these interacting partners—with CDK4/6-CCND3 promoting and CDKN2D restricting cell cycle progression—establish a delicate balance that is crucial for maintaining genomic integrity. Dysregulation of this equilibrium, particularly through CDKN2D downregulation or CDK4/6-CCND3 overexpression, represents a common oncogenic driver in the pathogenesis of EC [31]. These findings emphasize the therapeutic potential of targeting this regulatory axis, as CDK4/6 inhibitors have shown promise in preclinical models of EC. However, their interaction during EC requires additional investigation. ROC curve analysis demonstrated moderate diagnostic potential for CDKN2D in EC (AUC = 0.525, 95% CI 0.471–0.580), supporting its utility as a putative biomarker, elevated CDKN2D expression correlated significantly with advanced tumor stage, nodal metastasis, and reduced overall survival. Gene ontology analysis further implicated CDKN2D in keratinocyte differentiation pathways, particularly in the reorganization of the intermediate filament cytoskeleton (FDR < 0.05), suggesting a role in tumor structural integrity and progression.

The current prognostic evaluation in EC primarily depends on conventional clinicopathological parameters, such as TNM staging, histological classification, and lymph node involvement status. Nevertheless, emerging molecular profiling studies have identified novel biomarkers that could refine risk stratification beyond these traditional metrics [32,33]. Notably, recent investigations have shown that increased cuproptosis-related risk signatures are associated with heightened immune cell infiltration in various types of cancer [34,35]. The findings suggest that (i) copper-dependent cell death pathways might modulate anti-tumor immunity via metabolic-immune crosstalk, and (ii) the integration of molecular signatures with clinical parameters could enhance prognostic accuracy. The observed association between cuproptosis sensitivity and the abundance of tumor-infiltrating lymphocytes suggests potential therapeutic opportunities for copper-modulating agents in immunologically "cold" tumors. Furthermore, machine learning approaches that incorporate both genomic and immunological features hold promise for developing more precise survival prediction models for patients with EC [36].However, the existing body of research on the relationship between the CDKN2D gene and immunocytes in EC is insufficient. In this study, we innovatively explored the correlation between CDKN2D expression in EC and 24 distinct immune cell subtypes. Our results indicate that the expression of the CDKN2D gene is significantly and consistently correlated with the infiltration levels of Tcm, NK CD56dim cells, NK cells, and Th2 cells in EC, underscoring its potential role in immune regulation within the tumor microenvironment. Consistent with previous findings by Sahar et al., demonstrating a positive correlation between CDKN2D and T cell infiltration [37], our comprehensive analysis revealed significant associations between elevated CDKN2D expression and increased abundance of multiple immune cell populations, including B lymphocytes, CD8+ cytotoxic T cells, tumor-associated macrophages, and dendritic cells [37, 38]. Notably, we observed strong correlations with immune checkpoint markers *CTLA-4* and *PD-1*, suggesting CDKN2D may participate in establishing an immunosuppressive niche. These discoveries suggest that CDKN2D could function as: (i) a potential predictor for immunotherapy response, as its expression reflects the immune-active yet exhausted phenotype, and (ii) a regulator of immune cell recruitment via cytokine signaling pathways. The simultaneous upregulation of both effector immune cells and inhibitory checkpoints suggests a complex, dual role in tumor immunology—promoting initial immune recognition while also facilitating immune escape mechanisms. This paradoxical relationship warrants further investigation into CDKN2D's precise mechanisms in shaping the tumor-immune interface [38,39].

While this investigation provides valuable insights into CDKN2D's role in EC, several limitations should be acknowledged. Firstly, our findings were derived primarily from retrospective bioinformatics analyses of public databases, which may introduce selection biases. Prospective validation using well-annotated clinical cohorts is essential to confirm the prognostic and diagnostic significance of CDKN2D. Secondly, the study lacks functional validation—future work should include *in vitro* assays and *in vivo* models to elucidate CDKN2D's mechanistic contributions to tumor progression, immune modulation, and therapeutic resistance. Additionally, while we identified correlations between CDKN2D and immune infiltration, the precise molecular pathways underlying these observations remain unexplored.

Conclusions

In summary, CDKN2D upregulation in EC may impact the progression of the disease via pivotal molecular functions and pathways. Moreover, CDKN2D expression correlates with the infiltration levels of various immune cell types. To bridge the gap between computational insights and clinical relevance, future work should employ laboratory experiments to validate CDKN2D's mechanistic contributions to EC cells and their microenvironment.

Author Contributions

All members of this article participated in the first draft of the article writing, X.X. responsible for most of the writing of the article, and the experimental data visualization, L.M. and S.L. to assist me to write articles, and the experimental data acquisition, S.L. is responsible for the data analysis, S.L. in charge of the experiment, S.L. responsible for the supervision of experiment.

Funding

This work was supported by collaborative innovation research project of jiangsu pharmaceutical and vocational college [grant number: 20239120].

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data utilized in this study are accessible through online repositories. Details regarding the specific repository names and corresponding accession numbers are provided within the article and Supplementary Material.

Conflicts of Interest

The authors attest that the study was conducted without any commercial or financial interests that might be considered a potential conflict of interest.

References

1. Lingling, L.; Dongxian, J.; Qiao, Z.; et al. Integrative proteogenomic characterization of early esophageal cancer. *Nat. Commun.* **2023**, *14*, 1666.
2. Xiaofeng, C.; Xiang, X.; Danping, W.; et al. Neoadjuvant sintilimab and chemotherapy in patients with potentially resectable esophageal squamous cell carcinoma (KEEP-G 03): An open-label, single-arm, phase 2 trial. *J. Immunother. Cancer* **2023**, *11*, e005830.
3. Lei, C.; Wenjing, Z.; Dan, C.; et al. RBM4 dictates ESCC cell fate switch from cellular senescence to glutamine-addiction survival through inhibiting LKB1-AMPK-axis. *Signal Transduct. Target Ther.* **2023**, *8*, 159.
4. Xu, Z.; Yuxiang, W.; Xi, Z.; et al. Intact regulation of G1/S transition renders esophageal squamous cell carcinoma sensitive to PI3Kα inhibitors. *Signal Transduct. Target Ther.* **2023**, *8*, 153.

5. Qing, W.; Wei, Q.; Xiaoli, S. Small-molecule inhibitors, immune checkpoint inhibitors, and more: FDA-approved novel therapeutic drugs for solid tumors from 1991 to 2021. *J. Hematol. Oncol.* **2022**, *15*, 143.
6. Paridisha, D.; Rajkumar, D.; Elisha, T.S.; et al. Esophageal cancer in Northeast India: A comprehensive review of risk factors, diagnosis, and therapeutic approaches. *J. North East Counc.* **2024**, *25*, 38–53.
7. Alei, F.; Ning, Y.; Ruoying, Y.; et al. Prognostic implications of six altered genes in Asian non-surgical esophageal carcinoma patients treated with chemoradiotherapy. *Onco Targets Ther.* **2022**, *15*, 41–51.
8. Hana, Q.S.; Alanoud, A.; Sara, A.M.; et al. Epigenetic modifications: Key players in cancer heterogeneity and drug resistance. *Transl. Oncol.* **2024**, *39*, 101821.
9. Bujiang, W.; Haojun, S.; Haizhong, J.; et al. Early diagnostic potential of APC hypermethylation in esophageal cancer. *Cancer Manag. Res.* **2018**, *10*, 181–198.
10. Yong, X.; Yaxing, S.; Donglei, W.; et al. CircBCAR3 accelerates esophageal cancer tumorigenesis and metastasis via sponging miR-27a-3p. *Mol. Cancer* **2022**, *21*, 145.
11. Liyun, C.; Junxia, M.; Fudi, W. Copper homeostasis and cuproptosis in health and disease. *Signal Transduct. Target Ther.* **2022**, *7*, 378.
12. Paul, A.C.; Donita, C.B. Cuproptosis: Cellular and molecular mechanisms underlying copper-induced cell death. *Mol. Cell* **2022**, *82*, 1786–1787.
13. Hongyan, Z.; Yujie, G.; Yong, W.; et al. LncRNA CRNDE promotes the progression and angiogenesis of pancreatic cancer via miR-451a/CDKN2D axis. *Transl. Oncol.* **2021**, *14*, 101088.
14. Keyi, Z.; Xiao, N.; Xiaoling, M.; et al. LINC01012 upregulation promotes cervical cancer proliferation and migration via downregulation of CDKN2D. *Oncol. Lett.* **2023**, *25*, 124.
15. Wioletta, R.K.; Radosław, W.; Jacek, P.S.; et al. The genetic and endoplasmic reticulum-mediated molecular mechanisms of primary open-angle glaucoma. *Int. J. Mol. Sci.* **2020**, *21*, 4171.
16. Aimin, J.; Peng, L.; Ming, C.; et al. A new thinking: Deciphering the aberrance and clinical implication of copper-death signatures in clear cell renal cell carcinoma. *Cell Biosci.* **2022**, *12*, 209.
17. Shuai, Z.; Shuxian, C.; Wangrui, L.; et al. Integrated machine learning and bioinformatic analyses used to construct a copper-induced cell death-related classifier for prognosis and immunotherapeutic response of hepatocellular carcinoma patients. *Front. Pharmacol.* **2023**, *14*, 1188725.
18. Kuanhao, T.; Juihu, H.; Lite, L.; et al. The cross-communication of cuproptosis and regulated cell death in human pathophysiology. *Int. J. Biol. Sci.* **2024**, *20*, 218–230.
19. Tao, W.; Yufeng, L.; Qing, L.; et al. Cuproptosis-related gene FDX1 expression correlates with the prognosis and tumor immune microenvironment in clear cell renal cell carcinoma. *Front. Immunol.* **2022**, *13*, 999823.
20. Nathaniel, D.; Christopher, M.J.; Kohei, Y.; et al. Advances in diagnosis and management of cancer of the esophagus. *BMJ* **2024**, *385*, e074962.
21. Hong, Y.; Feng, W.; Christopher, L.H.; et al. Oesophageal cancer. *Lancet* **2024**, *404*, 1991–2005.
22. Meilin, L.; Yu, T.; Zhixin, L.; et al. Biological characterization and clinical significance of cuproptosis-related genes in lung adenocarcinoma. *BMC Pulm. Med.* **2025**, *25*, 13.
23. Chenglin, L.; Ying, L.; Nasra, M.A.; et al. The role of innate immune cells in the tumor microenvironment and research progress in anti-tumor therapy. *Front. Immunol.* **2023**, *13*, 1039260.
24. Baowen, X.; Kaiyong, Y.; Xin, H.; et al. Cuproptosis-related gene CDKN2A as a molecular target for IPF diagnosis and therapeutics. *Inflamm. Res.* **2023**, *72*, 1147–1160.
25. Vinicio, M.; Edwin, B.; John, D.M. Towards immunotherapy-induced normalization of the tumor microenvironment. *Front. Cell Dev. Biol.* **2022**, *10*, 908389.
26. Ziheng, Z.; Yajun, H.; Yuefeng, C.; et al. Immunometabolism in the tumor microenvironment and its related research progress. *Front. Oncol.* **2022**, *12*, 1024789.
27. Bo, H.; Xiaobo, Y.; Xinting, S. Construction of a lipid metabolism-related and immune-associated prognostic signature for hepatocellular carcinoma. *Cancer Med.* **2020**, *9*, 7646–7662.
28. Li, M.; Xian, H.C.; Tang, Y.J.; et al. Fatty acid oxidation: Driver of lymph node metastasis. *Cancer Cell Int.* **2021**, *21*, 339.
29. Henggang, W.; Cheng, W.; Yajun, L.; et al. miR-138-5p suppresses glioblastoma cell viability and leads to cell cycle arrest by targeting cyclin D3. *Oncol. Lett.* **2020**, *20*, 264.
30. Dorian, V.Z.; Kanishka, P.; Lluis, F. Beyond cell cycle regulation: The pleiotropic function of CDK4 in cancer. *Semin. Cancer Biol.* **2024**, *98*, 51–63.
31. Sofie, N.; Karoline, K.; Veronika, S. The role of CDK6 in cancer. *Int. J. Cancer* **2020**, *147*, 2988–2995.
32. Jomrich, G.; Paireder, M.; Gleiss, A.; et al. Comparison of inflammation-based prognostic scores in a cohort of patients with resectable esophageal cancer. *Gastroenterol. Res. Pract.* **2017**, *12*, 1678584.

33. Xiaofeng, Y.; Jun, C.; Dingsen, S.; et al. Advanced esophageal cancer with bone metastases: Prognostic biomarkers and palliative treatment. *Heliyon* **2024**, *10*, e23510.
34. Junjie, L.; Shuoyi, M.; Yantao, Z.; et al. Prognostic value and immune infiltration analyses of cuproptosis-related genes in hepatocellular carcinoma. *Acta Mater. Med.* **2023**, *2*, 386–399.
35. Dongmei, L.; Songyang, L.; Jie, L.; et al. Characterization of cuproptosis identified immune microenvironment and prognosis in acute myeloid leukemia. *Clin. Transl. Oncol.* **2023**, *25*, 2393–2407.
36. Xiangxu, W.; Shizhou, D.; Lihong, W.; et al. Cuproptosis-mediated patterns characterized by distinct tumor microenvironment and predicted the immunotherapy response for gastric cancer. *ACS Omega* **2023**, *8*, 10851–10862.
37. Sahar, Y.; Monireh, M.; Somayeh, Y.; et al. The mirror like expression of genes involved in the FOXO signaling pathway could be effective in the pathogenesis of human lymphotropic virus type 1 (HTLV-1) through disruption of the downstream pathways. *BMC Res. Notes* **2023**, *16*, 147.
38. Hui, L.; Songhao, J.; Kun, G.; et al. INK4 cyclin-dependent kinase inhibitors as potential prognostic biomarkers and therapeutic targets in hepatocellular carcinoma. *Biosci. Rep.* **2022**, *42*, 1082.
39. Karam, K.; Lucas, M.; Anamaria, A.R.; et al. Melanoma targeted therapies beyond BRAF-mutant melanoma: Potential druggable mutations and novel treatment approaches. *Cancers* **2021**, *13*, 5847.