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High *WFDC3* gene expression is associated with poor prognosis and reduced immune cells infiltration in pancreatic adenocarcinoma

A study using the TCGA database and bioinformatics analysis

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Abstract

Whey-acidic-protein (WAP) four-disulfide core domain protein 3 (WFDC3) is one of the WAP family proteins. This protein family is associated with the development of solid tumors and affects the tumor immunological microenvironment. However, the prognostic value of WFDC3 in pancreatic adenocarcinoma (PAAD) and its effect on the tumor immune microenvironment is yet to be clarified. The Cancer Genome Atlas database and Genotype-Tissue Expression database were used to analyze the differential expression of WFDC3 between the tumor and adjacent tissues. The clinical significance of WFDC3 was analyzed in The Cancer Genome Atlas and International Cancer Genome Consortium database using WFDC3 transcripts and clinical information. In order to elucidate the underlying mechanisms, gene set enrichment analysis was conducted to determine potential activated pathways. Immune score evaluation and publicly available pharmacogenomics database [the Genomics of Drug Sensitivity in Cancer] were utilized to quantify immune cell infiltration and the effect on chemotherapeutic drug sensitivity. WFDC3 levels were higher in PAAD tissues than in normal pancreatic tissues. High levels of WFDC3 expression progressively increased as PAAD tumor stages progressed. Patients with elevated WFDC3 expression showed a poor prognosis. The gene set enrichment analysis analysis revealed that glutamate, arginine, and proline, and histidine metabolism levels were elevated in patients with a high WFDC3 expression phenotype. B, $CD4^+$ T, and $CD8^+$ T cell infiltration was diminished in PAAD tissues with elevated WFDC3 expression. According to pharmacogenomics, PAAD tissues with high WFDC3 expression are susceptible to gemcitabine. WFDC3 is highly expressed in PAAD, and patients with a high level of WFDC3 expression have a shorter overall survival time, indicating a poorer prognosis. High expression of WFDC3 may lead to the development of PAAD by affecting the amino acid metabolism and the tumor immunological microenvironment. WFDC3 may serve as a potential diagnostic and prognostic biomarker for PAAD patients.

Abbreviations: DEGs = differentially expressed genes, DFS = disease free survival, GDSC = Genomics of Drug Sensitivity in Cancer, GO = Gene Ontology, GSEA = gene set enrichment analysis, GTEx = Genotype-Tissue Expression, IC50 = half-maximal inhibitory concentration, ICGC = International Cancer Genome Consortium, KEGG = Kyoto Encyclopedia of Genes and Genomes, OS = overall survival, PAAD = pancreatic adenocarcinoma, PFS = progression free survival, ROC = receiver operating characteristic curve, SLC = solute carrier family, TCGA = The Cancer Genome Atlas, WAP = Whey-acidic-protein, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

Keywords: amino acid metabolism, pancreatic adenocarcinoma, TCGA, tumor immune microenvironment, WFDC3

1. Introduction

Pancreatic adenocarcinoma (PAAD) is an aggressive digestive tract tumor, difficult to diagnose and treat, with a poor prognosis. The incidence of PAAD is 14th in the world and has become the 7th most common cause of cancer-related deaths worldwide due to its high level of malignancy.[1] Radical surgical resection is an effective treatment method for patients with PAAD for the cure and long-term survival. However, a majority of PAAD patients do not qualify for curative surgery due

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to the advanced stage of the disease at the time of diagnosis.[2] In recent years, with the development of adjuvant, neoadjuvant, and other comprehensive tumor therapy, the prognosis of PAAD patients has improved significantly, and the 5-year survival rate has increased from 5% to 10%.[3,4] Nonetheless, due to individual differences, comprehensive treatment may not benefit all patients. Therefore, exploring individualized treatment models of PAAD and finding novel diagnostic markers and therapeutic targets for PAAD patients is an urgent requisite.

Proteins are composed of amino acids, the basic building blocks, and are essential for the biosynthesis of nucleotides, glutathione, glucosamine, and polyamines. Some amino acid degradation products are involved in energy-generating processes, such as the TCA cycle.[5] Amino acids are also vital nutrients for immune and tumor cells. Moreover, tumor cells use amino acids for proliferation and invasion[5] and for immune escape.[6,7] The metabolites of amino acids and the lack of specific amino acids in the tumor microenvironment inhibit the immune function of immune cells, especially the activation and function of effector T cells.[8] In carcinomas, the balance of amino acid metabolism is disrupted, leading to a decline in amino acid levels, which in turn promotes tumor cell growth.[6] Several molecules encoding amino acid transporter proteins, such as solute carrier family (SLC) 1, member 5 (SLC1A5), SLC6A14, SLC7A1, SLC7A2, and SLC7A3, are involved in amino acid metabolism and also affect tumor cell growth.[9–17] Since amino acid metabolism affects tumor growth, small molecule inhibitors targeting amino acid metabolism have made some progress.[18] However, whether there are other molecules that affect amino acid metabolism in pancreatic tumors is yet unclear.

The gene encoding whey-acidic-protein (WAP) four-disulfide core domain protein 3 (WFDC3), also known as WAP14, is located on human chromosome 20q12-13.1; the protein belongs to the WAP protein family.[19] High expression of WFDC3 is associated with systemic lupus erythematosus (SLE) pathogenesis-like disease.[20] The WAP protein family is expressed in numerous solid cancers and affects patient prognosis. The current studies have shown that WFDC2 is overexpressed in various malignant tumors, including lung, and ovarian cancers.[21,22] High levels of WFDC2 activate nuclear factor kappa B (NF-κB) in the oncogenic and inflammatory pathways that promote tumorigenesis.[23] WFDC2 expression is also associated with poor prognosis in these patients.[23] On the other hand, WFDC1 expression is considerably reduced in lung, liver, and other malignant tumors, while its overexpression can limit the growth of these cancers.[24,25] Regarding the therapeutic significance of WAP proteins, the expression status of WFDC3 in PAAD, its predictive value in PAAD patients, and the underlying molecular mechanism remain unknown.

In this study, we used bioinformatics to analyze the expression of WFDC3 in PAAD, clarify the significance of abnormal expression of WFDC3 in PAAD, and determine its potential value in prognosis and diagnosis. Also, the related functions and pathways were investigated. Our results indicated that WFDC3 is upregulated in PAAD tissues, which might affect the prognosis of the patients by regulating amino acid metabolism and the immune microenvironment of tumor cells.

2. Materials and Methods

2.1. Analysis of gene expression datasets

The mRNA-sequencing data (level 3, Illumina HiSeq 2000 RNASequencing platform) of PAAD and relevant clinical data were obtained from The Cancer Genome Atlas (TCGA) database portal (https://portal.gdc.cancer.gov/) and the current-release (V8) Genotype-Tissue Expression database was obtained from the portal https://www.gtexportal.org/home/datasets, which included 179 PAAD samples and 328 adjacent controls. International Cancer Genome Consortium (ICGC) database (https://dcc.icgc.org/releases/current/Projects) consisting of 262 samples was used as an external verification of the survival analysis of WFDC3 in PAAD. The differences in *WFDC3* mRNA expression between healthy biopsies and malignant tissues were assessed using paired-sample *t*-tests. The differences in WFDC3 expression levels between tumor grades (I–IV) were examined using one-way analysis of variance. Patients from each database were divided into WFDC3 high- and low-expression groups according to the *WFDC3* transcript level. The differences between the groups with high and low WFDC3 expression were examined in terms of overall survival (OS), disease-free survival (DFS), and progression-free survival (PFS) using Kaplan–Meier analysis. The predictors for survival were assessed using univariate and multivariate Cox proportional hazard regression models. The data were examined using functional enrichment to further verify the underlying function of prospective targets.

2.2. Identification of differentially expressed genes (DEGs)

We employed the R program to examine microarray data of TCGA linked to PAAD. Bioconductor was used to construct the volcano plot (https://bioconductor.org/biocLite.R). The top 50 regulated genes in TCGA were used to create heatmaps using the "pheatmap" function of the R statistical program.

2.3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

The ClusterProfiler package (version: 3.18.0) in R was used to examine the GO function of possible targets, including molecular function, biological pathways, and cellular components and enrich the KEGG pathway to better understand the carcinogenesis of mRNA. Heatmaps were created using the R software's pheatmap package, and boxplots were created using the R software's ggplot2 package.

2.4. Gene set enrichment analysis (GSEA)

In this study, GSEA was used to examine the PAAD expression profile that was retrieved from TCGA. GSEA v4.2.3 for Windows was used to conduct GSEA (http://software.broadinstitute.org/gsea/). All gene set data for this study were downloaded from the GSEA website (www.broadinstitute.org/gsea/) to determine the signaling pathways that are active in pancreatic cancer. The GSEA findings were illustrated using an enrichment map. After designing 1000 different combinations of the gene set permutations for the study, the enrichment score (ES) and false discovery rate (FDR) values were used to rank the enriched pathways.

2.5. Immune score evaluation

RNA sequencing expression (level 3) profiles and corresponding clinical information for PAAD were downloaded from TCGA database (https://portal.gdc.com). Immuneeconv, a R software package that includes 6 of the most recent methods, including TIMER, xCell, MCP-counter, CIBERSORT, EPIC, and quanTIseq, was used to evaluate the validity of immune score evaluation findings on various WFDC3 levels. These algorithms had a distinct advantage and have been benchmarked. All the above analysis methods and R package were implemented by R foundation for statistical computing (2020) version 4.0.3 and software packages ggplot2 and pheatmap. Gene Set Cancer Analysis (GSCA, http://bioinfo.life.hust.edu.cn/GSCA/#/) is an online program that integrates >10,000 multidimensional

genomic datasets across 33 cancer types from TCGA and >750 small molecule medicines from Genomics of Drug Sensitivity in Cancer (GDSC) and The Cancer Therapeutics Response Portal. An immunogenomic study was performed using ImmuCellAI algorithm of GSCA with 24 immunes cells to examine the link between WFDC3 expression and immune cell infiltration.

2.6. Correlations analysis

Spearman correlation analysis was used to describe the correlation between WFDC3 expression and amino acid transporter. *P*-values < 0.05 indicated a statistically significant difference (*P < .05). The R software package ggstatsplot was used to visualize the correlation between WFDC3 expression and amino acid transporter.

2.7. Gene expression and drug sensitivity

TCGA dataset (https://portal.gdc.com) was utilized to retrieve the RNA-sequencing expression (level 3) profiles and the corresponding clinical data for WFDC3. Based on the largest publicly available pharmacogenomics database [GDSC, https://www.cancerrxgene.org], we predicted the chemotherapeutic response for each sample. The prediction procedure was implemented using the "pRRophetic" R package. Using ridge regression, the half-maximal inhibitory concentration (IC50) of the samples was calculated. All parameters were configured with their default values. The batch effect of combat, tissue type, and the mean value of duplicate gene expression were calculated. R foundation for statistical computing (2020) version 4.0.3 was used to implement the aforementioned analysis methods and R package.

3. Results

3.1. Expression of WFDC3 in PAAD tissues was significantly higher than that in adjacent normal tissues

According to *WFDC3* transcript levels, patients in the TCGA database were classified into high- and low-expression groups and the correlation between WFDC3 expression and clinicopathological characteristics is shown in Table 1. WFDC3 expression values were compared between tumor and adjacent tissues, and the results showed that the expression of WFDC3 was significantly increased in tumor tissues compared to that in adjacent tissues (P < .01, Fig. 1A). The WFDC3 levels were measured in accordance with the phases in TCGA to analyze the impact of WFDC3 on PAAD development. Pancreatic adenocarcinoma can be divided into 4 grades according to the degree of malignancy of tumor cells (grades I–IV). *WFDC3* mRNA levels increased progressively with tumor grade, and there was a significant difference in WFDC3 levels between tumor grades (Fig. 1B). To evaluate the expression of WFDC3 in pan-cancer, we searched the TCGA database and found that the *WFDC3* gene is highly expressed in various cancer types, including PAAD, colon adenocarcinoma, cholangiocellular carcinoma, and stomach adenocarcinoma (Fig. 1C).

3.2. High WFDC3 expression indicated poor prognosis in patients with PAAD

The predictive significance of WFDC3 expression in PAAD patients was assessed using Kaplan–Meier analysis. According to TCGA, which contained OS, DFS, and PFS information, patients with high levels of WFDC3 expression had significantly lower OS and PFS than those with low levels of WFDC3 expression (P = .002, Fig. 2A; P = .014, Fig. 2D). DFS was marginally better in individuals with low levels of WFDC3 expression compared to those with high levels of WFDC3 expression (P = .082; Fig. 2C). In another PAAD database, ICGC, patients with WFDC3-high tumors had worse OS compared to those with WFDC3-low tumors (P < .001; Fig. 2B). These findings implied that high levels of *WFDC3* mRNA expression might predict a poor outcome for PAAD patients. The receiver operating characteristic curve analysis was conducted to evaluate the diagnostic value of WFDC3 mRNA levels for PAAD. The area under the receiver operating characteristic curve was 0.575 and 0.615 in TCGA and ICGC databases, respectively, indicating a diagnostic value of WFDC3 (Fig. 2E and F).

Moreover, we used univariate and multivariate Cox regression analysis to assess the correlation between high WFDC3 expression and other variables associated with OS. In the univariate Cox analysis, WFDC3 expression, KRAS status, lymph node metastasis, and depth of invasion were identified as factors for the poor outcome of PAAD patients; the hazard ratio (HR) values were 1.939, 2.052, 2.160, and 2.035, respectively (Fig. 3A). For multivariate analysis, all factors that demonstrated prognostic relevance in the univariate study were included. The results indicated that positive lymph node metastasis is an independent predictor of poor OS (HR 1.767; 95% confidence interval [CI]: 1.017–3.069; P = .043, Fig. 3B). Patients with high levels of WFDC3 expression had slightly shorter median survival than patients with low levels of WFDC3 expression (P = .071, HR 1.506; 95% CI: 0.966–2.350, Fig. 3B). However, multivariate analysis found no significant correlation between other clinicopathological characteristics and OS (Fig. 3B).

Table 1

Correlations between WFDC3 expression and clinicopathological features in pancreatic adenocarcinoma patients from TCGA database.

Variables	Cases	WFDC3 expression		*P*
		Low	High	
Age				
<60 yr	55	29	26	.518
≥60 yr	124	58	66	
Gender				
Female	80	40	40	.500
Male	99	49	50	
Smoking				
Smoking	79	35	44	.126
Nonsmoking	66	30	36	
Race				
Asian	11	7	4	.939
Black	6	5	1	
White	158	75	83	
Grade				
G1/G2	127	63	64	.205
G3/G4	50	24	26	
Depth of invasion				
T1/T2	31	20	11	.115
T3/T4	146	67	79	
Lymph node metastasis				
Negative	47	26	21	.781
Positive	124	56	68	
Uncertain	4	3	1	
Distance metastasis				
Negative	80	36	44	.943
Positive	5	3	2	
Uncertain	94	50	44	
Survival				
Alive	86	51	35	**.021**
Dead	93	38	55	

P values in bold was statistically significant.

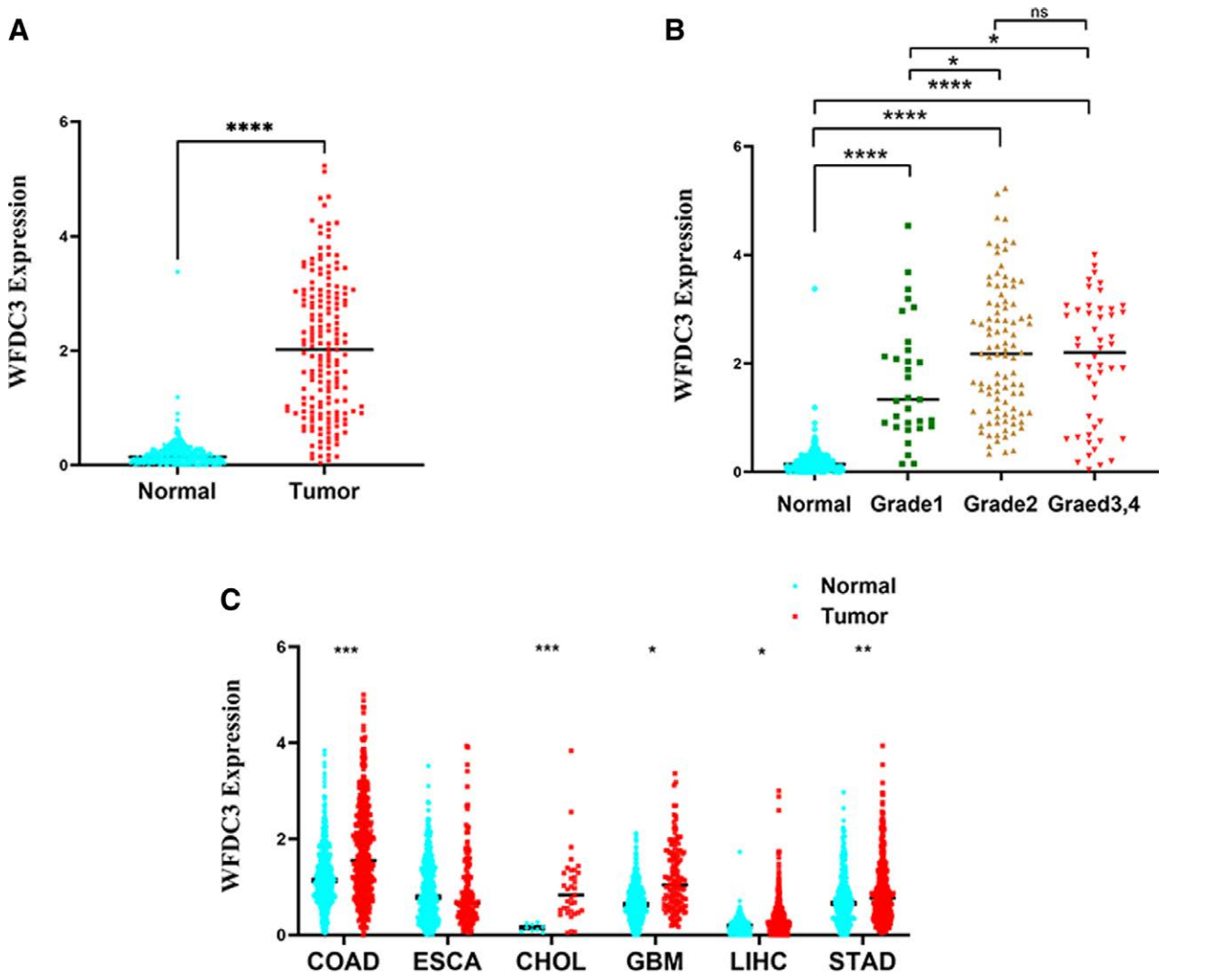

Figure 1. Expression of WFDC3 in human PAAD tissues. (A) *WFDC3* mRNA level was significantly higher in PAAD tissues than in adjacent normal PAAD tissues from TCGA and GTEx databases. (B) *WFDC3* mRNA level was increased gradually with tumor progression. (C) WFDC3 is highly expressed in a variety of malignancies. GTEx = Genotype-Tissue Expression, PAAD = pancreatic adenocarcinoma, TCGA = The Cancer Genome Atlas, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

3.3. Identification and GO annotation analyses of DEGs

In the TCGA database of PAAD, 1060 DEGs (748 upregulated and 312 downregulated) were found in the WFDC3 high- and low-expression groups (Fig. 4A). The heatmaps were for the top 50 top up- and downregulated genes, respectively, were constructed in the TCGA database (Fig. 4B). KEGG analysis indicated that these upregulated DEGs were mainly involved in pathways, such as p53 signaling, small cell lung cancer, transcriptional misregulation in cancer, proteoglycans in cancer, and central carbon metabolism associated with tumorigenesis and the pathways that modulate the microenvironment of the cells, including histidine metabolism, IL-17 signaling pathway, and glycolysis/gluconeogenesis. The GO analysis identified the DEGs related to epidermis development, skin development, epidermal cell differentiation, keratinocyte differentiation, extracellular structure organization, and extracellular matrix organization (Fig. 4C).

3.4. High expression of WFDC3 determined by GSEA may increase amino acid metabolism in tumor cells

As high WFDC3 expression is related to carcinogenesis and promotes the metabolism of the tumor microenvironment, we investigated the molecular pathways that were affected by WFDC3. Four gene sets were utilized in the GSEA analysis: the hallmark gene sets, the oncogenic signatures sets, the GO gene sets, and the KEGG gene sets. Nod-like receptor signaling pathway and KRAS pathway were enriched in the high WFDC3 expression group, along with biological activities, such as glutamate metabolic process, arginine and proline metabolism, histidine metabolism, innate immune response, and interferon-gamma (IFN-γ) response. These results revealed that elevated WFDC3 expression might promote pancreatic carcinogenesis by influencing the amino acid metabolism and immunological microenvironment of tumor cells (Fig. 5).

3.5. Immune landscape of different WFDC3 expressions in PAAD patients

The immune landscape was effectively examined in TCGA, and the range of immune cell infiltration in WFDC3 high- and low-expression groups was compared. We assessed the correlation between the risk score and the TIICs in PAAD generated by the CIBERSORT algorithm. The CIBERSORT-based heat map of immune responses revealed that M0 macrophages, naive B cells, plasma B cells, activated mast cells, regulatory T cell

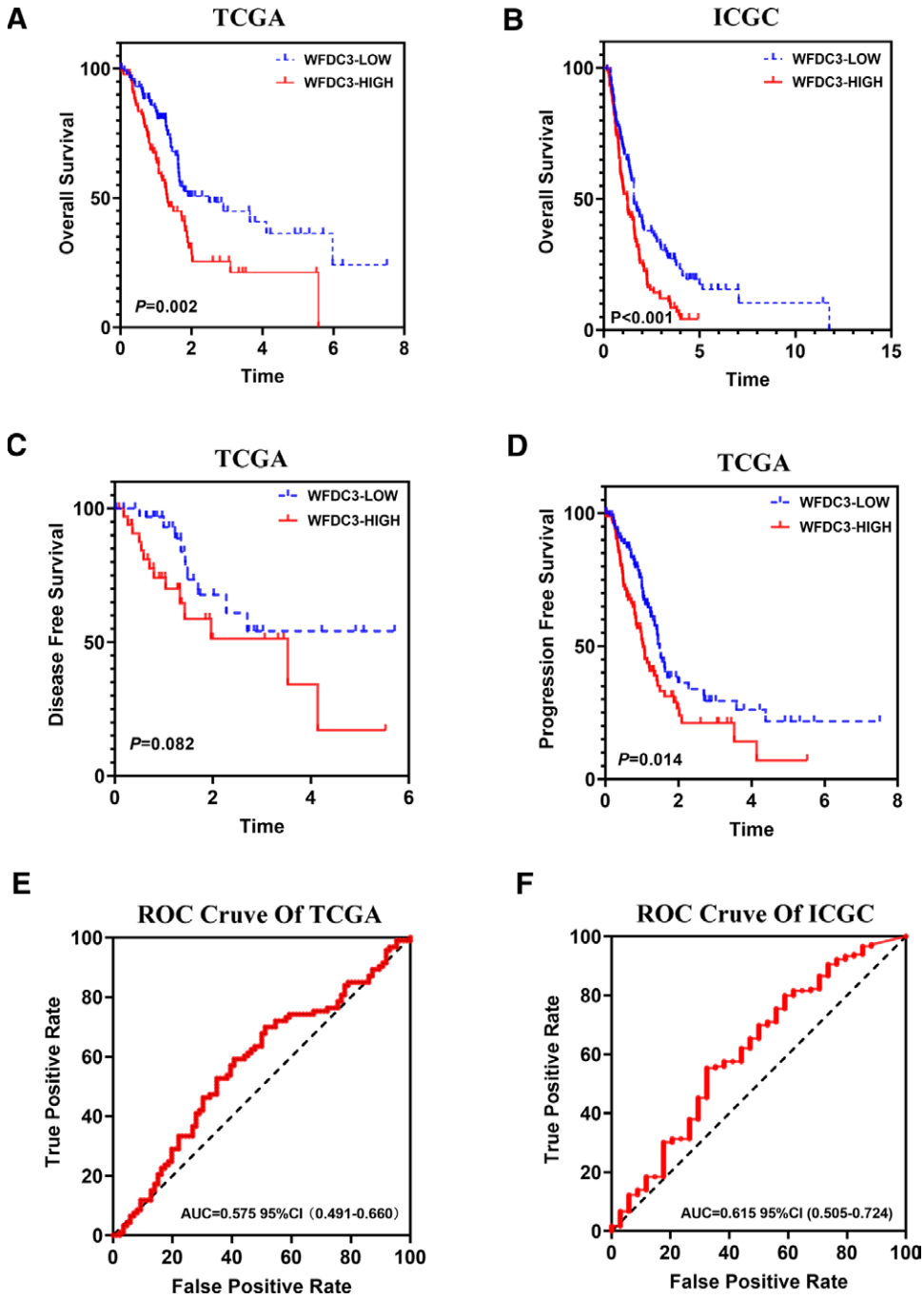

Figure 2. High WFDC3 expression is associated with a significantly poor prognosis. (A) Patients in the high WFDC3 group have a significantly worse OS than those in the low WFDC3 group by analyzing TCGA database. (B) The identical OS results were also found in the ICGC database. (C) Patients in the group with high WFDC3 expression had a significantly lower DFS than those in the group with low WFDC3 expression. (D) Patients in the group with high WFDC3 have a significantly shorter PFS than those in the group with low WFDC3. (E–F) ROC curves of WFDC3 for predicting the OS of PAAD by studying the TCGA and ICGC databases. OS = overall survival, ROC = receiver operating characteristic curve, TCGA = The Cancer Genome Atlas, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

(Tregs), CD8+ T cells, and activated CD4+ memory T cells were significantly different between the WFDC3 high- and low-expression groups. Specifically, the percentage of naive B cells, plasma B cells, activated mast cells, and CD8 + T cells decreased in the WFDC3 high-expression group, while the percentage of M0 macrophages and Tregs increased (Fig. 6A). In each sample, the percentage of immune cells that infiltrated the tumor was also displayed (Fig. 6B). Correlation analysis also indicated that high expression of WFDC3 was negatively connected with CD4+ T cell (Cor = –0.31; $P < .001$; Fig. 6C), CD8 + T cell (Cor = –0.3; $P < .001$; Fig. 6D), and B cell (Cor = –0.24; $P < .001$; Fig. 6E) infiltration, while favorably correlated with nTregs (Cor = 0.3; $P < .001$; Fig. 6F) infiltration. These findings implied that high WFDC3 expression might inhibit the activation of immune cells, resulting in immunosuppression and tumor formation. Since immunotherapy is still in the experimental stage for the treatment of pancreatic cancer,[26] we explored whether the etiology of WFDC3 immunosuppression in pancreatic cancer is related to immunological checkpoints. Next, we investigated the expression of immune checkpoint-related genes *CD274*, *CTLA4*, *HAVCR2*, *LAG3*, *PDCD1*, *PDCD1LG2*, *TIGIT*, and *SIGLEC15* in PAAD tissues with different expression levels of

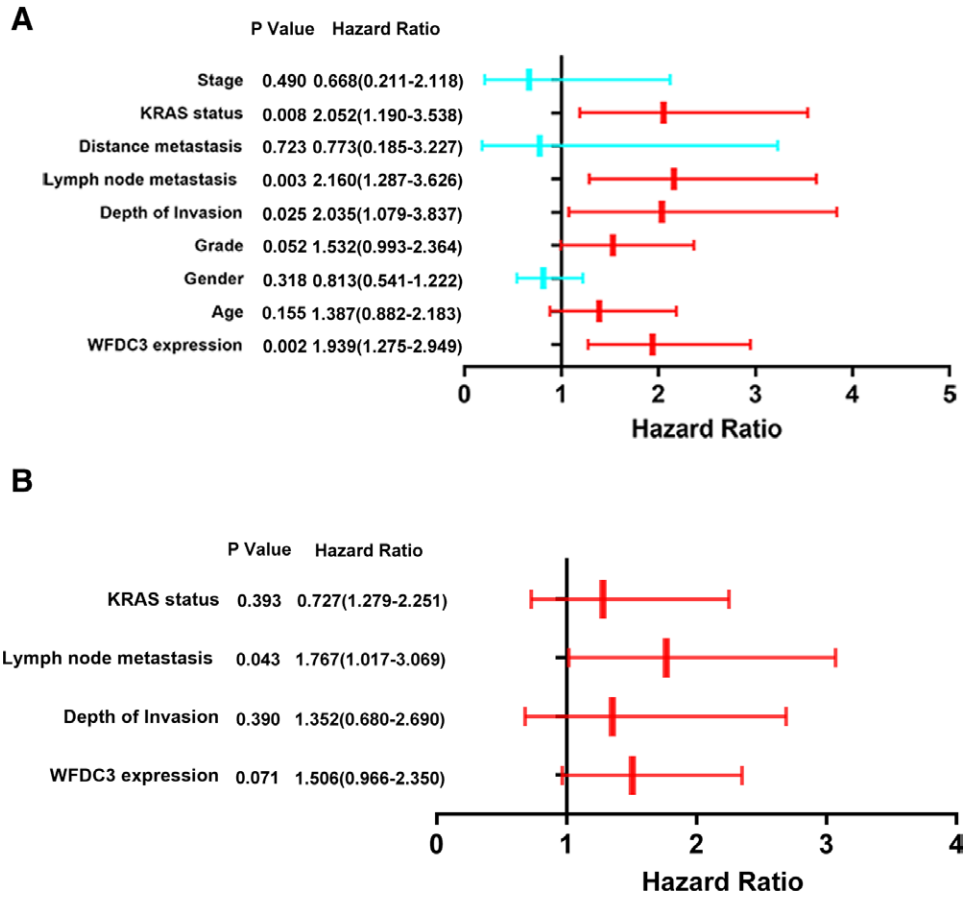

Figure 3. The impact of WFDC3 on overall survival (OS) in TCGA database patients with PAAD, using both univariate and multivariate analysis. (A)WFDC3 expression (P = .002, HR = 1.939), KRAS status (P = .008, HR = 2.052), lymph node metastasis (P = .003, HR = 2.160), and depth of invasion (P = .025, HR = 2.035) were all associated with OS in PAAD in a univariate Cox analysis. (B) Lymph node metastasis is correlated with overall survival (P = .043, HR = 1.767) based on a multivariate Cox analysis. PAAD = pancreatic adenocarcinoma, TCGA = The Cancer Genome Atlas, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

WFDC3. The outcomes differed from what was anticipated; CD274, HAVCR2, LAG3, and SIGLEC15 levels in pancreatic cancer tissues with varied WFDC3 expression were not significantly different. However, CTLA4, PDCD1, PDCD1LG2, and TIGIT expression levels tended to rise in the low WFDC3 expression group (Fig. 6G). This suggested that the immunosuppression of PAAD cells effectuated by the high expression of WFDC3 cannot be achieved by increasing the expression of immunological checkpoints and triggering immune escape in tumor cells.

3.6. WFDC3 is related to amino acid transporter expression, and high WFDC3 expression may be sensitive to gemcitabine treatment

To further elucidate the therapeutic importance of WFDC3 for pancreatic cancer, we investigated the expression correlation between WFDC3 and amino acid transporters and the link between WFDC3 and antitumor medications using the public pharmacogenomics database (GDSC, https://www.cancerrxgene.org/). The results indicated that SLC1A5 (Cor = 0.34, P < .001; Fig. 7A), SLC6A14 (Cor = 0.26, P = .001; Fig. 7B), and SLC7A5 (Cor = 0.21, P = .005; Fig. 7C), which encoded amino acid metabolic transporters, displayed an upward trend with increasing WFDC3 expression, while SLC7A2 (Cor-0.40, P < .001; Fig. 7D) demonstrated a downward trend. This phenomenon showed that WFDC3 and some amino acid transporters may have a synergistic effect, consequently influencing the metabolism of amino acids. Furthermore, we investigated the correlation between WFDC3 expression and the sensitivity of anticancer medicines. Nonetheless, the database lacks information on anticancer medications that target amino acid metabolism. Hence, we determined whether the expression of WFDC3 influences the sensitivity of traditional pancreatic cancer chemotherapy agents, such as gemcitabine, 5-fluorouracil, paclitaxel, and cisplatin. In tissues with high WFDC3 expression, gemcitabine and paclitaxel had lower IC50 (P = .023, Fig. 7E; P < .001, Fig. 7F). 5-fluorouracil did not demonstrate a statistically significant difference, but the IC50 value was lower in the high WFDC3 expression group (P = .074, Fig. 7G). Nonetheless, the IC50 value for cisplatin was higher in the group with increased WFDC3 expression than in the group with low expression (P = .028; Fig. 7H). The current findings suggested that PAAD tissues with high WFDC3 expression may be responsive to treatment based on gemcitabine.

4. Discussion

This study used bioinformatics analysis to confirm that WFDC3 is substantially expressed in PAAD. Additionally, patients with high WFDC3 expression have a poor prognosis. Gene function and pathway analysis indicated that WFDC3 induces immunosuppressive suppression in pancreatic tissue by inhibiting the progression of amino acid metabolism in the tumor microenvironment, hence promoting tumor development. Pharmacological sensitivity analysis revealed that high WFDC3 expression benefits from gemcitabine-based chemotherapeutic treatments. The

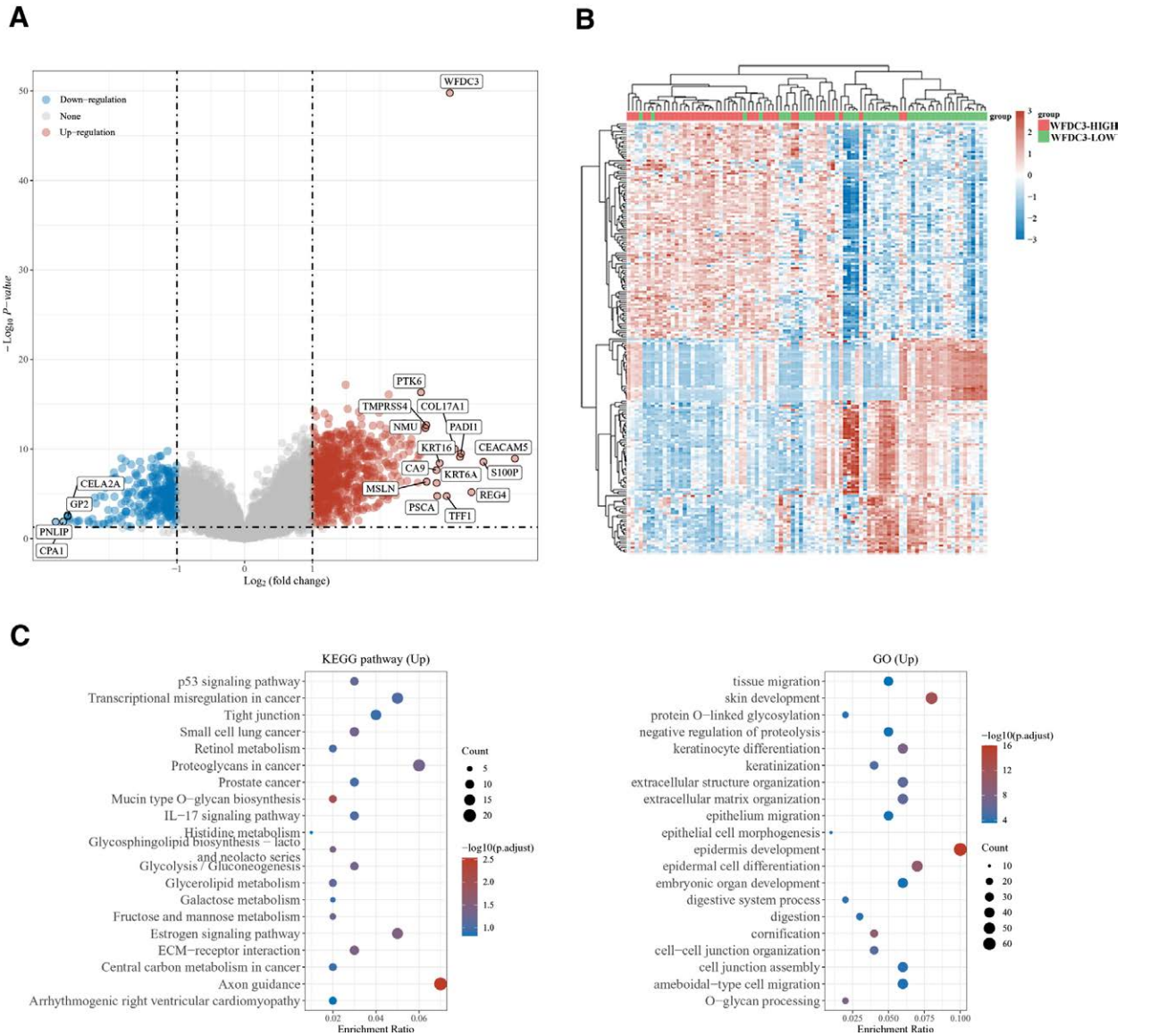

Figure 4. Differential genes and function enrichment analyses in the high WFDC3 expression group. (A) Volcano plot: the volcano plot was constructed using the fold change values and P-adjust. Red dots indicate upregulated genes; blue dots indicate downregulated genes; gray dots indicate not significant. (B) Heatmap: a heatmap displaying the differential gene expression, where the patterns of gene expression in the various tissues are represented by distinct hues. This figure displayed the top 50 genes whose expression levels increased and the top 50 genes whose expression levels decreased. (C) The leading 20 GO terms and KEGG pathways. GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

current findings suggested that WFDC3 may serve as a molecular marker for predicting the prognosis and chemosensitivity of PAAD. WFDC3 has not been investigated in pancreatic cancer. Our research reveals for the first time the expression of WFDC3 in PAAD, as well as its impact on patient prognosis, immune microenvironment, and immune cell infiltration. It established a certain theoretical basis for future research on this gene.

WFDC3 is a member of the WAP four-disulfide core domain protein family. The gene encoding this protein was found for the first time in 2002 by Clauss et al[19] The genes encoding the WAP family of proteins are located on chromosome 20q12-13.1, which was identified in 26 tissues, including the testis, lung, liver, and kidney of humans. It regulates the generation of serine protease inhibitors and natural immune responses,[19,27,28] has been associated with the development of SLE, and may impact the expression of estrogen receptors.[20,29] No studies have yet established a connection between WFDC3 and the development of gastrointestinal cancers. Nonetheless, several members of the WFDC family are directly associated with the development of various solid tumors. WFDC2 is highly expressed in ovarian cancer; it promotes tumor cell proliferation, invasion, and metastasis and inhibits tumor apoptosis by activating the ERK and JAK-STAT pathways.[22,30–34] This protein is also strongly expressed in lung cancer, and patients with high WFDC2 expression have a poor prognosis.[35] In pancreatic and non-small-cell lung cancer, WFDC4 (also known as SLPI) expression is increased substantially.[36,37] In addition, WFDC4 overexpression is frequently linked to aggressive, high-risk, or metastatic cancers originating from diverse organs.[38–42] Elafin, also known as WFDC14, is a well-known member of the WAP family of proteins. It is overexpressed in the cells and tissues of various cancerous tumors, including lung, bladder, and skin cancer.[43–45] In addition, it stimulates the growth of tumors by activating the MEK-ERK pathways.[46] In contrast to other members of the WFDC family, WFDC1 demonstrated a correlation between oncogenesis and genes that prevent tumor growth,[47] especially in the case of liver cancer, wherein the tumor suppressor genes display

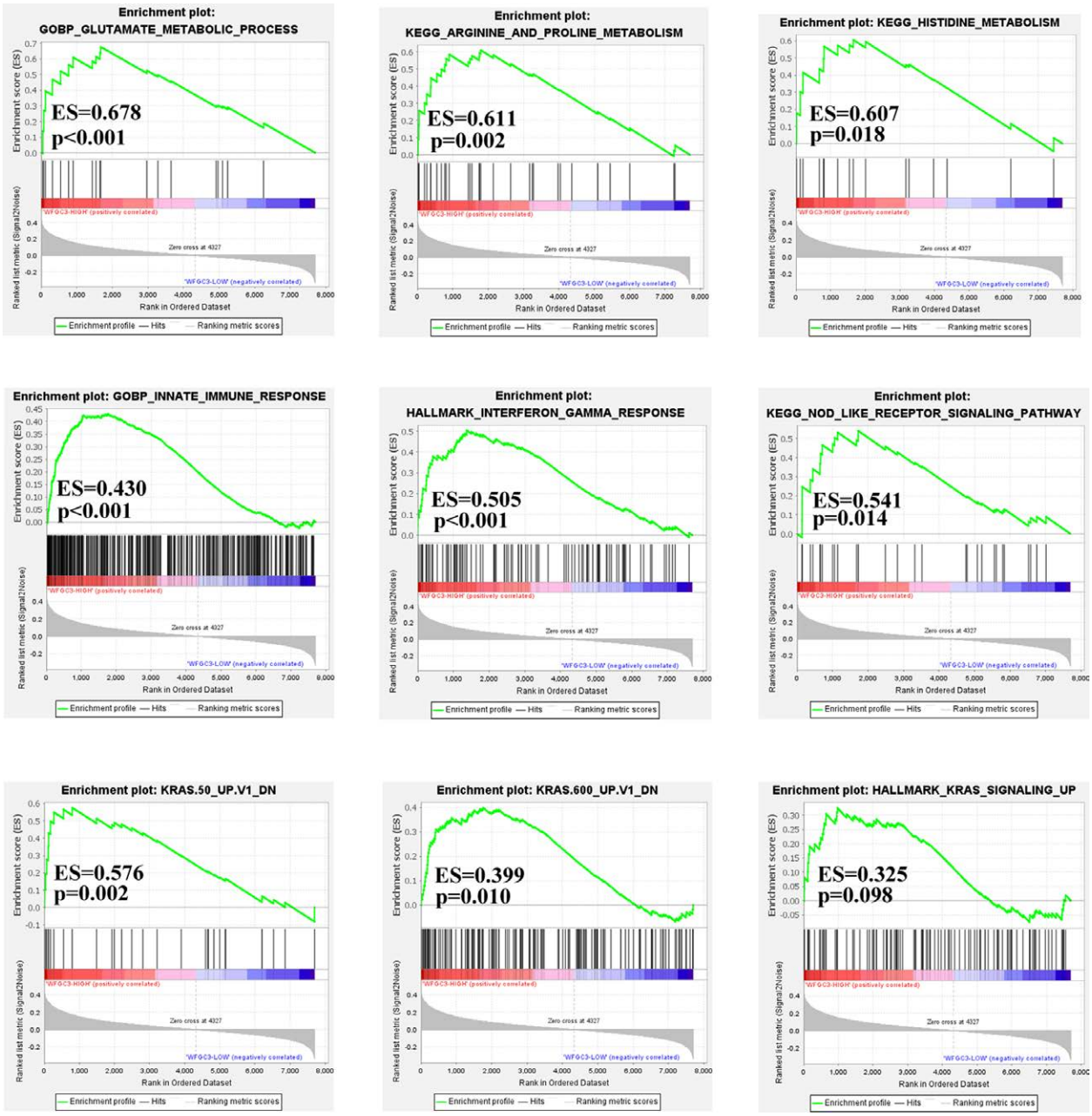

Figure 5. Gene set enrichment analysis (GSEA) in the high WFDC3 expression group. Glutamate-metabolic process, arginine and proline metabolism, histidine metabolism, innate immune response, nod-like signaling pathway, KRAS.50, and KRAS.600 were the significantly enriched signaling pathways. ES = enrichment scores; FDR = false discovery rate, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

their properties.[25] Regarding the importance of the WFDC protein family in carcinogenesis and development, we analyzed the TCGA database to determine the expression of WFDC3 in PAAD and its effect on patient survival. The publicly available database was examined, and the results showed that the levels of WFDC3 transcript in PAAD tissues were considerably greater than those in the corresponding normal tissues. The high levels of WFDC3 transcripts gradually grew in accordance with the phases of the tumor. In addition, we discovered that the expression of WFDC3 is elevated in a range of solid tumors. Patients with PAAD who exhibited a high expression of WFDC3 had a lesser chance of survival, indicating a poor clinical outcome.

The tumor immunological microenvironment refers to the milieu of blood vessels, immune cells, inflammatory cells, signaling chemicals, and extracellular matrix surrounding the tumor.[48] This phenomenon is essential for protecting against pathogen invasion and maintaining tissue homeostasis.[49] Recent studies have shown that WFDC family proteins suppress inflammatory response and regulate the internal environment homeostasis. Specific components of the inflammatory response, such as tumor necrosis factor-alpha (TNF-α) and prostaglandin E2 (PGE2), can boost the expression of SLPI.[50] Some WFDC protein family compounds, such as SLPI, contain NF-κB binding sites, which contribute to inflammatory reactions and promote tumor progression.[51] In this study, the expression of WFDC3 was associated with numerous cell metabolisms and the activation of tumor-related pathways. WFDC3 increases polysaccharide and lipid metabolism in the microenvironment of tumor cells, especially amino acid metabolism. In the WFDC3 high-expression group, glutamine, arginine, histidine, and proline metabolism levels were increased. Additionally, KEGG analysis revealed that WFDC3 was implicated in the formation of a variety of cancers, and enrichment analysis revealed that the molecules associated with KRAS and Nod-like receptor signaling pathways increased

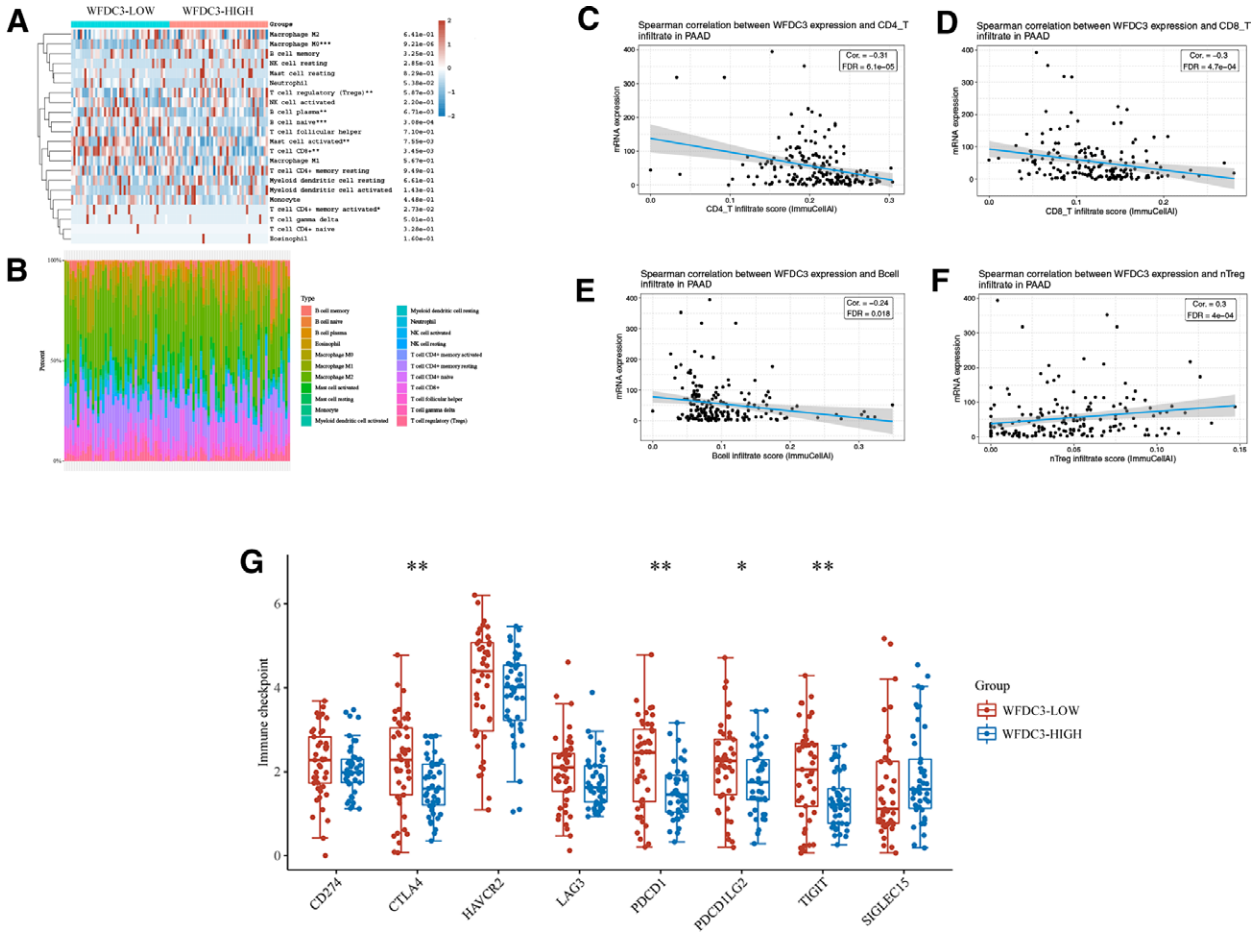

Figure 6. The distribution of immunological score, immune infiltration cells, and immune checkpoint genes in PAAD tissues with high and low WFDC3 expression. (A) Immune cell score heatmap. Red and blue indicate high and low WFDC3 expression, respectively, and immune cells distribution in different samples. (B) The proportion of tumor-infiltrating immune cells present in each sample. (C–F) Spearman was used to examine the relationships between WFDC3 gene expression and immunological score. (G) The expression distribution of immune checkpoint genes in PAAD tissues with high and low WFDC3 expression. (*P < .05, **P < .01; * represents significance levels.) PAAD = pancreatic adenocarcinoma, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

in a WFDC3 expression-dependent manner. The KRAS mutation is associated with the development of PAAD,[52] whereas the Nod-like receptor signaling pathway is associated with carcinogenesis and affects the immunological milieu of tumor cells.[53] Since the Warburg effect was hypothesized,[54] the chemicals that are metabolized in tumor microenvironments are under intensive focus. Among these, the anomalies of amino acid metabolites and critical enzymes are associated with the development of different types of cancers.[55,56] Glutamine is a nonessential amino acid that is abundant in the human body. Previous studies have shown that tumor cells can increase the decomposition of their own glutamine, and its metabolite ammonia can further stimulate the release of glutamine in fibroblasts, which tumor cells can take up to maintain their proliferation.[57] Arginine is essential for the proliferation, activation, and antitumor function of T cells,[58] and the high expression of arginase and SLC7A2 in tumor cells can limit the uptake of arginine by T cells, resulting in arginine deficiency in T cells and impairing their antitumor immune function.[15] Proline plays a crucial role in the metabolism of tumors. The mTOR signaling pathway is overactive in tumor cells that rely on exogenous proline,[59] and blocking proline synthesis can decrease tumor growth.[60] The metabolism of amino acids can affect the function of immune cells. Previous studies have shown that the expression of SLC1A5 and SLC7A5 is greater on activated $CD8^+$ T cells than on nonactivated $CD8^+$ T cells.[61] Asymmetry in amino acid transporter distribution impacts T cell differentiation.[62] In the current study, we found that the high expression of WFDC3 in PAAD tissue increases amino acid metabolism and decreases immune cell infiltration, such as B cells and effector T cells. Similarly, the expression of WFDC3 and the other genes encoding amino acid transporters was upregulated. Consequently, we hypothesized that pancreatic cancer tissues with high WFDC3 expression enhances the uptake of amino acids by raising the expression of the amino acid transporter-related molecules, hence, competitive suppression of immune cell amino acid absorption. As a result, immune cells fail to perform their antitumor functions. However, this phenomenon must be confirmed by other molecular biology experiments.

Immune checkpoint inhibitors have become the focal point of antitumor therapy due to their efficacy against various malignancies.[63] Single-agent immune checkpoint blockade is promising in other cancers, but early trials in PAAD have shown disappointing results.[64,65] Although elevated WFDC3 expression in PAAD tissues is associated with an immunosuppressed state, this was not the result of an increased expression of immunological checkpoints and immune escape. This finding disproves the hypothesis that WFDC3 can predict the success of immunotherapy for PAAD. Another study demonstrated that WFDC14 induces treatment resistance in ovarian cancer by reducing the effect of cisplatin on tumor cell apoptosis,[66] which suggested that the WAP family proteins may affect chemosensitivity. In this study, we employed online tools and deduced that the IC50 value of cisplatin was greater and that of gemcitabine and paclitaxel was lower in the PAAD tissues with high expression of WFDC3. Together, these findings demonstrated that PAAD with high WFDC3 expression was sensitive to gemcitabine.

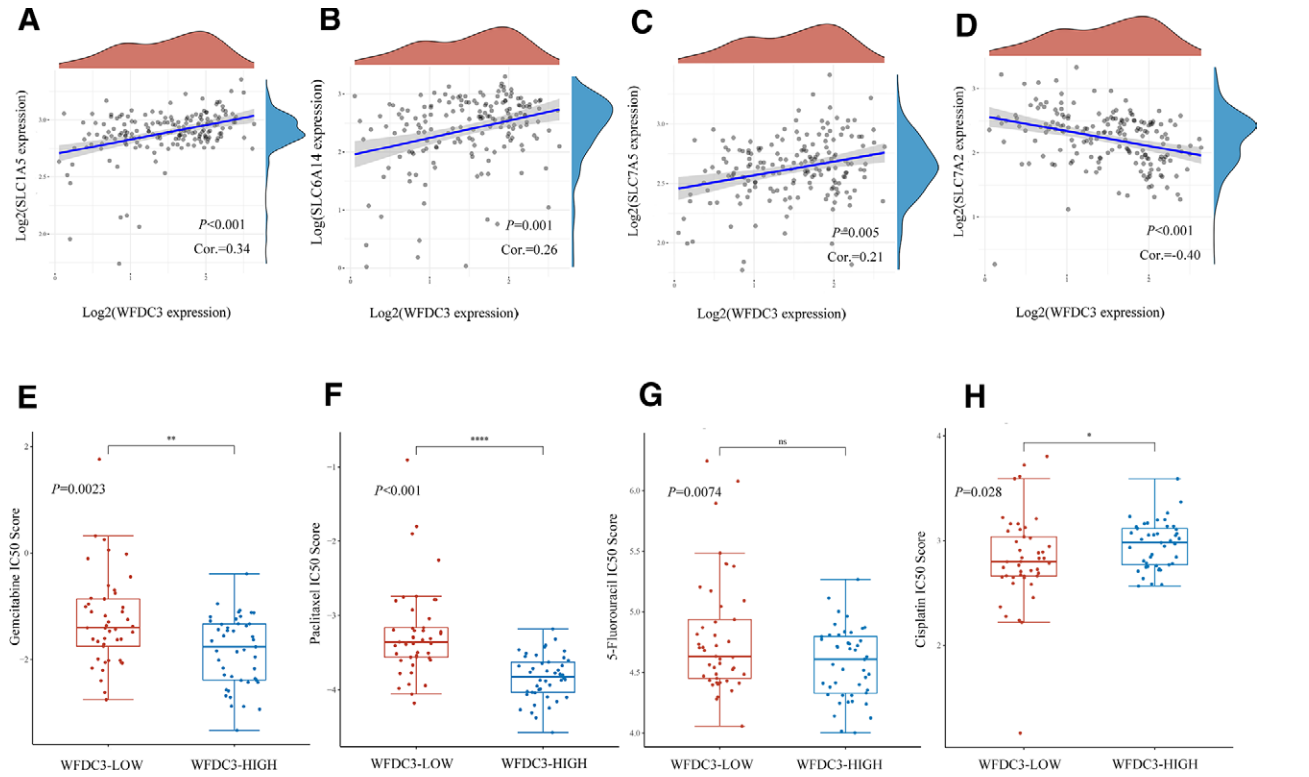

Figure 7. Spearman correlation analysis between WFDC3 and amino acid transporter genes and the effect of WFDC3 on IC50 of chemotherapeutic drugs. (A–D): Correlation between WFDC3 and solute carrier family (SLC) genes. (E–H): Different WFDC3 expression levels influence the IC50 values of gemcitabine, paclitaxel, 5-fluorouracil, and cisplatin in PAAD tissues. ($^{ns}P \geq 0.05$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$; * represents significance levels.) PAAD = pancreatic adenocarcinoma, WFDC3 = Whey-acidic-protein four-disulfide core domain protein 3.

Patients with high WFDC3 expression may benefit from gemcitabine-based treatment but need to be validated by additional drug sensitivity tests and clinical trials.

In conclusion, the transcriptome and genome data evaluation showed that WFDC3 is significantly expressed in PAAD and that individuals with high WFDC3 expression had a poor prognosis. WFDC3 influences the metabolites in the tumor microenvironment, especially amino acid metabolism, and limits immune cell penetration into tumor tissues. Moreover, PAAD tissues with elevated WFDC3 expression are susceptible to gemcitabine treatment. These results indicated that WFDC3 could serve as a diagnostic and prognostic biomarker in PAAD patients.

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