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Research article

Elevated of NDUFA4L2 expression in colon adenocarcinoma is correlated with an unfavorable prognosis and increased immune cell infiltration

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ABSTRACT

Background: Colon adenocarcinoma (COAD) is a prevalent malignancy worldwide, yet, its underlying pathogenesis and genetic characteristics are still unclear. Previous studies have suggested that NADH dehydrogenase 1 alpha subcomplex subunit 4-like 2 (NDUFA4L2) may affect tumor progression across various cancers. However, this effect on COAD has rarely been reported. Thus, this study investigated NDUFA4L2's prognostic and diagnostic relevance and explored its potential connection with immune cell infiltration in COAD.

Methods: To achieve this, RNA sequencing data from Cancer Genome Atlas (TCGA) was analyzed to assess NDUFA4L2's prognostic value in COAD, and factors relevant to the prognosis of COAD, including NDUFA4L2, were scrutinized using Kaplan-Meier analyses as well as univariate and multivariate Cox regression. A nomogram model was created to project prognosis based on the results of multivariate Cox analysis. Furthermore, gene set enrichment analysis (GSEA) was employed to pinpoint key NDUFA4L2-related pathways, and single-sample GSEA (ssGSEA) on TCGA data was employed to investigate the connections of NDUFA4L2 with cancer immune infiltrations.

Results: Our findings revealed significant associations of high NDUFA4L2 expression with poor overall survival, progression-free interval, and disease-specific survival of COAD patients. GSEA indicated close links of NDUFA4L2 with several signaling pathways implicated in tumorigenesis, including extracellular matrix receptor interaction, the intestinal immune network for immunoglobulin A production, natural killer (NK) cell-mediated cytotoxicity, pathways in cancer, cell adhesion molecules, mitogen-activated protein kinase signaling pathway, Hedgehog signaling pathway, transforming growth factor beta signaling pathway, and chemokine signaling pathway.

Abbreviations: COAD, colon adenocarcinoma; NDUFA4L2, NADH dehydrogenase 1 alpha subcomplex subunit 4-like 2; TCGA, The Cancer Genome Atlas; GSEA, gene set enrichment analysis; ssGSEA, single-sample GSEA; NK, natural killer; OS, overall survival; ROS, reactive oxygen species; ccRCC, clear renal cell carcinoma; CRC, colorectal carcinoma; HPA, Human Protein Atlas; GEO, Gene Expression Omnibus; TPM, transcripts per million reads; IHC, immunohistochemical; ROC, receiver operating characteristic; DEG, differentially expressed gene; FDR, false discovery rate; DSS, disease-specific survival; PFI, progression-free interval; AUC, area under the ROC curve; IgA, immunoglobulin A; CAMs, cell adhesion molecules; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor beta; DCs, dendritic cells; iDCs, immature dendritic cells; HCC, hepatocellular carcinoma.

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Additionally, ssGSEA identified a positive link between increased NDUFA4L2 expression and higher infiltration degree of various immune cells, such as immature dendritic cells, macrophages, NK cells and dendritic cells.
Conclusions: Collectively, our findings demonstrate the association of increased NDUFA4L2 expression with adverse prognosis and heightened immune cell infiltration in COAD patients.

1. Introduction

Colon adenocarcinoma (COAD) ranks among the primary causes of cancer-related death [1]. The current standard therapeutic strategies for COAD encompass surgical resection, chemotherapy, and radiotherapy [2]. Despite the effectiveness of targeted drugs and immunotherapies, the 5-year overall survival (OS) for COAD is persistently low, primarily due to late-stage diagnosis, aggressive progression and frequent metastasis [3–5]. Therefore, there is a compelling demand to inspect the molecular mechanisms of COAD and recognize novel biomarkers for survival assessment and targeted therapy.

NDUFA4L2, an increasingly expressed electron transport chain complex I subunit under hypoxic conditions has garnered attention. It can inhibit the activity of complex I, leading to reduced mitochondria oxygen species (ROS) levels under a hypoxic environment [6]. However, the functions and roles of NDUFA4L2 in cancer development information are not fully understood. Studies have shown that NDUFA4L2 knockdown cells can survive under hypoxic conditions while exhibiting increased ROS accumulation in mitochondria, suggesting its ability to suppress ROS production and mitigate, oxidative stress in cancer cells [7]. Additionally, NDUFA4L2 expression has been associated with Warburg-like shift from mitochondrial respiration towards glycolysis and increased lysosomal diameter and decreased lysosomes in RCC4 cells, indicating that NDUFA4L2 regulates mitochondrial and lysosomal activities in clear renal cell carcinoma (ccRCC) cells. Furthermore, elevated NDUFA4L2 transcription and translation have been observed in colorectal carcinoma (CRC) [8]. However, NDUFA4L2 expression and its potential prognostic relevance in COAD have not been extensively elaborated.

Therefore, this study utilized COAD datasets from Human Protein Atlas (HPA), Cancer Genome Atlas (TCGA), and Gene Expression Omnibus (GEO) to explore the correlation between NDUFA4L2 levels and prognosis, as well as clinicopathological features in COAD patients. Additionally, this study also investigated the potential functions of NDUFA4L2, developed a nomogram to predict patients' prognosis, and assessed the impact of NDUFA4L2 on immune infiltration in COAD. Our findings revealed the significant role of NDUFA4L2 in COAD, highlighting the underlying mechanisms behind its increased expression and potential correlation with tumor-immune infiltrations.

2. Methods

2.1. Data sourcing and manipulation

Clinical information and level 3 HTSeq-FPKM data 41 normal and 480 COAD samples were retrieved from TCGA (https://portal.gdc.cancer.gov/). After excluding those without clinical information, FPKM data were transfigured to the transcripts per million reads (TPM) to ensure consistency and subsequently analyze following the TCGA publication guidelines. Additionally, GSE37364 and GSE33133 datasets from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) were utilized to certify NDUFA4L2 expression.

2.2. NDUFA4L2 over-expression in COAD patients

NDUFA4L2 expression in non-paired and paired tissues of COAD patients was compared using immunohistochemical (IHC) staining with HPA078265 antibody based on HPA (https://www.proteinatlas.org) datasets. Additionally, the diagnostic utility of NDUFA4L2 was assessed by employing the R package (1.18.0) through the receiver operating characteristic (ROC) curve analysis.

2.3. Differentially expressed gene (DEG) analysis

HTseq-Counts were categorized into low- and high-NDUFA4L2 groups, with the cutoff being the median NDUFA4L2 level. DEGs in COAD were identified in accordance with the adjusted $P < 0.05$ and $|\log_2\text{-fold change (FC)}| > 1.5$ and analyzed by employing DESeq2 package in R version 1.36.0 [9].

2.4. Gene set enrichment analysis (GSEA)

GSEA was implemented to assess the functional and pathway disparities between two groups exhibiting varying NDUFA4L2 levels using R package clusterProfiler (version 4.4.4) [10,11] by referencing to c5.all.v2022.1.Hs.symbols.gmt (Gene ontology) and c2.cp.kegg.v7.5.1.symbols.gmt (KEGG Pathway Database) from the MSigDB Collections (https://www.gseamsigdb.org/gsea/msigdb/index.jsp), with 1000 permutations set for each analysis. Gene sets were considered substantially enriched if they had a false discovery rate (FDR) below 0.25 and an adjusted P-value below 0.05.

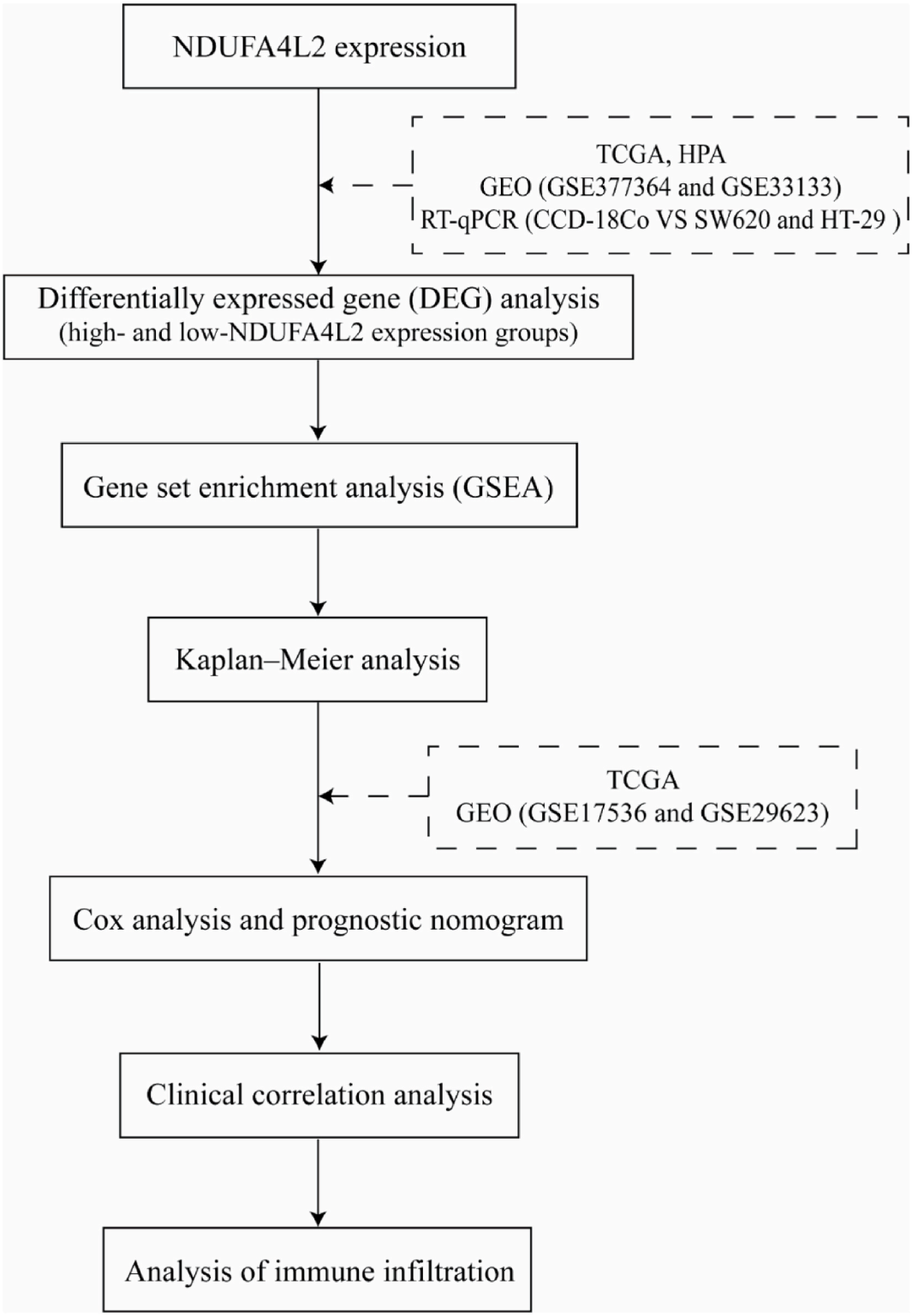

Fig. 1. The flow chart of this study.

2.5. Nomogram construction and evaluation

The rms package in R version 6.3–0 was utilized to construct a survival-predicting nomogram at 1-, 3-, and 5-year intervals, incorporating all independent prognostic factors identified via multivariate analysis. Their performance was ascertained using the calibration curve and consistency index (C-index).

2.6. Immune infiltration analysis

To explore the link between NDUFA4L2 and marker genes of 24 distinct immune cell types, an immune infiltration study was executed using the ssGSEA [12] approach in the GSVA package of R version 1.46.0 [13]. Immune cell infiltration in the low- and high-NDUFA4L2 groups was examined using the Wilcoxon rank sum test, its connection with NDUFA4L2 level was assessed using Spearman correlation.

2.7. Cell culture

CCD-18Co, SW620 and HT-29 cell lines were from Otwo Biotech Inc. (Shenzhen, China) and cultured in McCoy's 5a (Procell) and DMEM (Biosharp) with 10 % fetal bovine serum (Sijiqing) and 1 % penicillin/streptomycin (Sangon Bioteck), respectively.

Table 1
Clinicopathological features of patients with COAD.

Characteristics	Low expression of NDUFA4L2	High expression of NDUFA4L2	P value
n	239	239	
Gender, n (%)			0.067
Female	103 (21.5 %)	123 (25.7 %)	
Male	136 (28.5 %)	116 (24.3 %)	
T stage, n (%)			**0.030**
T1	7 (1.5 %)	4 (0.8 %)	
T2	49 (10.3 %)	34 (7.1 %)	
T3	161 (33.8 %)	162 (34 %)	
T4	21 (4.4 %)	39 (8.2 %)	
N stage, n (%)			**0.008**
N0	151 (31.6 %)	133 (27.8 %)	
N1	58 (12.1 %)	50 (10.5 %)	
N2	30 (6.3 %)	56 (11.7 %)	
M stage, n (%)			0.133
M0	178 (42.9 %)	171 (41.2 %)	
M1	27 (6.5 %)	39 (9.4 %)	
Pathologic stage, n (%)			**0.049**
Stage I	51 (10.9 %)	30 (6.4 %)	
Stage II	92 (19.7 %)	95 (20.3 %)	
Stage III	64 (13.7 %)	69 (14.8 %)	
Stage IV	27 (5.8 %)	39 (8.4 %)	
Residual tumor, n (%)			0.138
R0	178 (47.6 %)	168 (44.9 %)	
R1	1 (0.3 %)	3 (0.8 %)	
R2	8 (2.1 %)	16 (4.3 %)	
CEA level, n (%)			0.882
≤ 5	97 (32 %)	99 (32.7 %)	
>5	52 (17.2 %)	55 (18.2 %)	
History of colon polyps, n (%)			0.918
No	135 (33.1 %)	127 (31.1 %)	
Yes	76 (18.6 %)	70 (17.2 %)	
Colon polyps present, n (%)			0.080
No	78 (31.3 %)	84 (33.7 %)	
Yes	52 (20.9 %)	35 (14.1 %)	
OS event, n (%)			**0.035**
Alive	197 (41.2 %)	178 (37.2 %)	
Dead	42 (8.8 %)	61 (12.8 %)	
DSS event, n (%)			**0.011**
No	205 (44.4 %)	193 (41.8 %)	
Yes	22 (4.8 %)	42 (9.1 %)	
PFI event, n (%)			**0.004**
No	189 (39.5 %)	161 (33.7 %)	
Yes	50 (10.5 %)	78 (16.3 %)	
Age, median (IQR)	69 (60, 75.5)	69 (57, 78)	0.935

2.8. Real-time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated using RNAeasy™ Animal RNA Isolation Kit (Beyotime Biotechnology) and converted to cDNA using BeyoRT™ III First Strand cDNA Synthesis Kit (Beyotime Biotechnology). qPCR was executed using BeyoFast™ SYBR Green qPCR Mix (2X) (Beyotime Biotechnology) on a QuantStudio apparatus (Applied Biosystems) with the following amplification program: an initial denaturation step 95°C for 2 min, followed by 40 cycles of 15s at 95 °C for denature and 30s at 60°C for annealing/extension. Primers 5′-ATGATCGGCTTAATCTGCCTG-3′ and 5′-TCCGGGTTGTTCTTTCTGTCC-3′ were used for NDUFA4L2, and primers 5′-GGAGCGA-GATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCATGG -3′ were for GAPDH.

2.9. Statistical analysis

Data were visualized using the ggplot 2 package in R version 3.3.6. Differences in NDUFA4L2 expression levels between COAD patients and normal controls were compared using the Wilcoxon rank sum test and Wilcoxon signed rank test. The association of clinicopathological characteristics with NDUFA4L2 levels was scrutinized using the Kruskal-Wallis test, followed by Dunn's post hoc test and logistic regression. The impact of NDUFA4L2 expression on COAD prognosis was determined using Kaplan-Meier method and

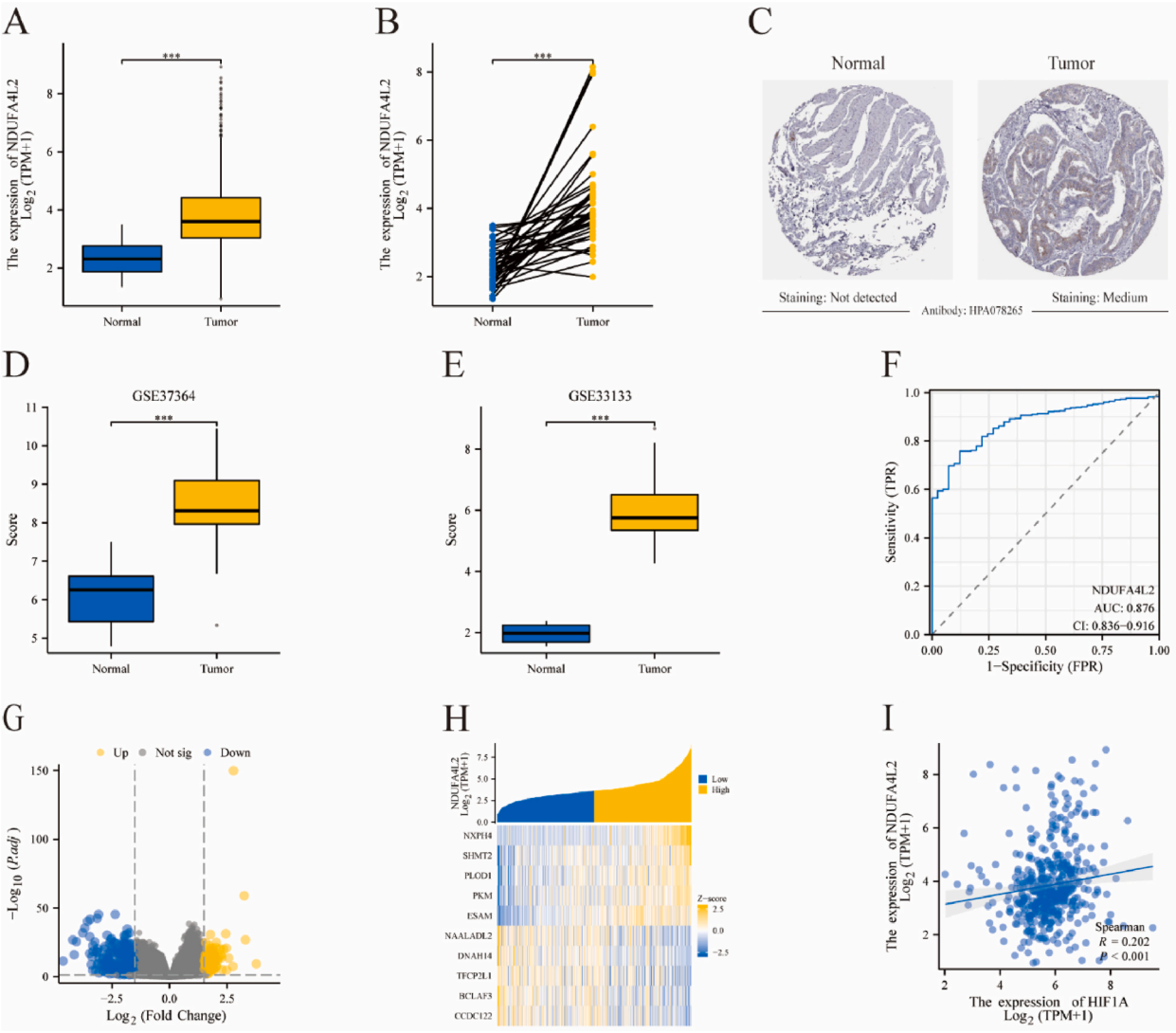

Fig. 2. Differential expression levels of NDUFA4L2 in COAD from the TCGA data. (A) Expression levels of NDUFA4L2 in COAD and normal samples. (B) Expression levels of NDUFA4L2 in paired tumor and normal samples. (C) The protein expression of NDUFA4L2 in COAD and normal colon tissues. (D) Expression levels of NDUFA4L2 in GSE37364 (E) Expression levels of NDUFA4L2 in GSE 33133 (F) Diagnostic ROC curve of NDUFA4L2. (G) The volcano plot of differentially expressed mRNAs. (H) The heat map of the 10 genes correlated to NDUFA4L2. (I) Positive correlation trend between NDUFA4L2 and HIF-1α mRNA expression in the TCGA data. *P < 0.05, **P < 0.01, ***P < 0.001.

validated using the OSppc web server [14]. NDUFA4L2's prediction ability for 5-, 3-, and 1-year OS was predicted using the time-dependent analysis of the ROC curve, and the impacts of NDUFA4L2 level and clinicopathological characteristics on OS were scrutinized using the univariate and multivariate Cox regression analyzes. Statistical significance in RT-qPCR was evaluated using one-way analysis of variance (ANOVA). Statistical analyses were fulfilled using R (4.2.1), and a significance threshold of $P < 0.05$ (two-tailed) was applied to determine significance.

3. Results

3.1. Clinical characteristics of the included COAD patients

Fig. 1 shows the study's flow chart. The clinical information of 478 COAD patients was collected, including residual tumor, gender, N stage, M stage, T stage, carcinoembryonic antigen level, history of colon polyps, pathologic stage, age, disease-specific survival (DSS), OS, colon polyps present, and progression-free interval (PFI) (Table 1). The correlation analysis revealed that NDUFA4L2 exhibited a trending link with OS ($P = 0.035$), DSS ($P = 0.011$), T stage ($P = 0.030$), N stage ($P = 0.008$), pathologic stage ($P = 0.049$), and PFI ($P = 0.004$), as determined by the chi-square test. However, NDUFA4L2 expression was not correlated with other clinicopathologic characteristics.

3.2. NDUFA4L2 is upregulated in COAD

NDUFA4L2 exhibited higher levels in 480 COAD samples relative to 41 normal samples ($P < 0.001$) (Fig. 2A). NDUFA4L2 level was dramatically augmented in tumor tissues among the 41 paired samples ($P < 0.001$) (Fig. 2B). Consistent results were noticed in additional analyses using GSE37364 (Fig. 2D) and GSE33133 datasets (Fig. 1E), supporting the validity of the TCGA data. To assess NDUFA4L2 protein levels, IHC staining results obtained from the HPA were analyzed. These results demonstrated the absence of NDUFA4L2 IHC staining in normal colon tissues but revealed medium intensity in COAD tissues (Fig. 2C). Furthermore, NDUFA4L2's diagnostic potential in COAD was evaluated using ROC curve analysis, indicating a reasonably accurate predictive capability (area under the ROC curve [AUC] = 0.876, CI = 0.836–0.916) (Fig. 2F). Subsequently, 286 DEGs were uncovered in high- and low-NDUFA4L2 groups, based on its median level, using adjusted $P < 0.05$ and $|\log_2\text{-fold change (FC)}| > 1.5$ as the cutoff, including 74 upregulated and 212 downregulated. The distribution of DEGs was visualized in the volcano plot shown in Fig. 2G. The correlation between NDUFA4L2 and the top 10 DEGs was illustrated in a heatmap (Fig. 2H). Pearson correlation analysis demonstrated a positive correlation of NDUFA4L2 level with HIF-1α level (Fig. 2I).

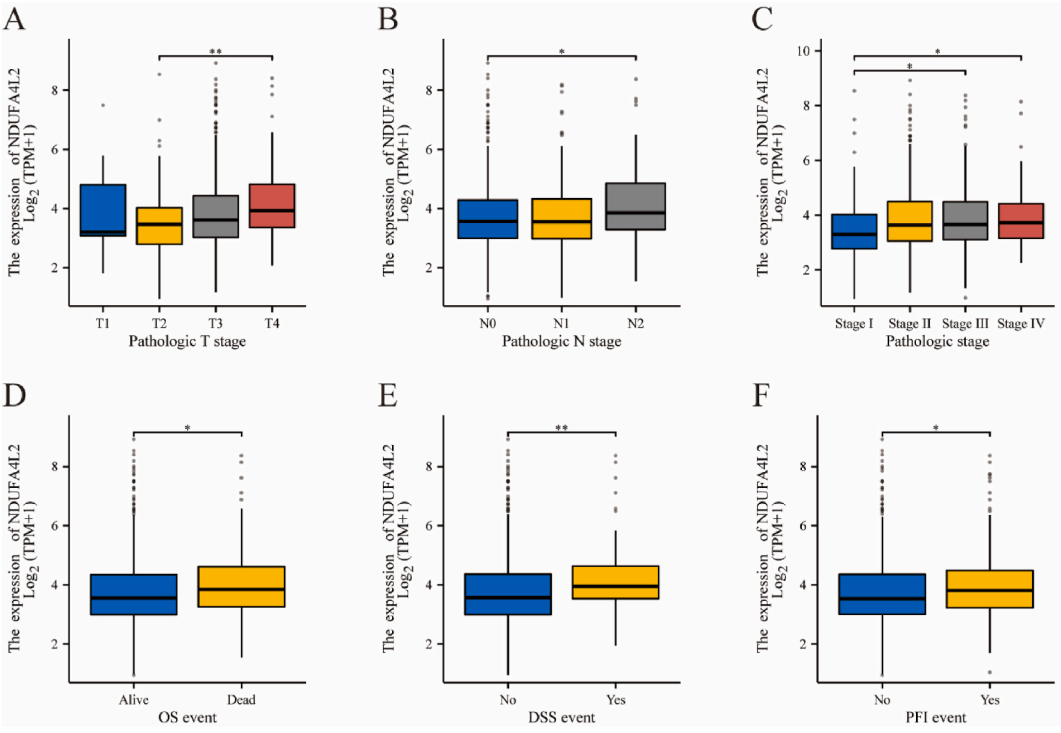

Fig. 3. Association between NDUFA4L2 expression and clinicopathological characteristics, including (A) T stage, (B) N stage, (C) pathologic stage, (D) OS event, (E) DSS event, and (F) PFI event. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.3. Association between NDUFA4L2 expression and clinical features

The connection between clinicopathological characteristics and NDUFA4L2 expression was revealed using the Kruskal-Wallis test, followed by Dunn's t post hoc test and logistic regression, which demonstrated dramatic correlations of NDUFA4L2 expression with T stage, N stage, pathologic stage (Fig. 3A–C), OS, DSS, and PFI events (Fig. 3D–F). Logistic regression analysis found dramatic correlations of NDUFA4L2 expression with the T stage (OR = 1.628, 95 % CI: 1.033–2.588, P = 0.037) and N stage (OR = 2.006, 95 % CI: 1.245–3.280, P = 0.005) (Table 2).

3.4. High NDUFA4L2 expression is connected with adverse outcomes in COAD

Evaluation of NDUFA4L2's prognostic value in COAD patients through Kaplan–Meier analysis signaled that augmented NDUFA4L2 level was linked to unfavorable OS (HR = 1.69, 95 % CI: 1.14–2.51, P = 0.009). Consistent results were discerned for DSS (HR = 2.07, 95 % CI: 1.23–3.46, P = 0.006) and PFI (HR = 1.90, 95 % CI: 1.33–2.91, P < 0.001) (Fig. 4A–C). Additionally, Kaplan–Meier survival plots in GSE175336 and GSE29623 datasets also revealed that high NDUFA4L2 expression group had poorer OS (Fig. 4D and E). Assessment of the accuracy of NDUFA4L2 expression in projecting 5-, 3-, and 1-year OS using time-dependent ROC analysis yielded AUC values of 0.561, 0.612, and 0.659 respectively, illustrating NDUFA4L2 being a reliable prognostic indicator for assessing OS of COAD patients (Fig. 4F).

3.5. Cox regression analyses and prognostic nomogram

Cox regression analysis to assess the independent prognostic factors for OS revealed that age (P = 0.028), pathologic stage (P < 0.001), T stage (P = 0.004), N stage (P < 0.001), M stage (P < 0.001), and NDUFA4L2 expression (P = 0.009) were correlated with OS (Table 3). Among them, age (P = 0.002), T stage (P = 0.047), NDUFA4L2 expression (P = 0.027), and M stage (P = 0.002) were independent prognostic factors for OS (Fig. 5A). Additionally, analysis of NDUFA4L2 levels in relation to the risk score and survival time revealed a clear association of higher risk scores with worse prognosis (Fig. 5B). Furthermore, a predictive nomogram was developed to estimate the 1-, 3-, and 5-year OS according to the multivariate Cox analysis (Fig. 5C). The survival model C-index was 0.745 (0.716–0.774), and the 5-, 3-, and 1-year OS calibration curves signified a satisfactory prediction effect of the nomogram (Fig. 5D).

3.6. Function and pathway enrichment analysis by GSEA

A GSEA was conducted using the MSigDB collection to identify relevant biological functions associated with different NDUFA4L2 expression levels in COAD. The top 20 gene ontology (GO) terms that positively correlated with high NDUFA4L2 levels and the top 20 GO terms that negatively correlated with high levels of NDUFA4L2 based on their normalized enrichment scores are presented in Fig. 6A and B, respectively. GSEA indicated several key signaling pathways related to tumorigenesis were positively connected with high NDUFA4L2 expression phenotype, including antigen processing and presentation, chemokine signaling, the intestinal immune network for immunoglobulin A (IgA) production, NK cell-mediated cytotoxicity, leukocyte transendothelial migration, the Hedgehog

Table 2
Univariate and multivariate Cox regression analyses for OS.

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95 % CI)	P value	Hazard ratio (95 % CI)	P value
Gender	477		0.626		
Female	226	Reference			
Male	251	1.101 (0.746–1.625)	0.627		
Age	477		**0.024**		
≤ 65	194	Reference		Reference	
>65	283	1.610 (1.052–2.463)	**0.028**	2.179 (1.332–3.564)	**0.002**
T stage	476		**< 0.001**		
T1&T2	94	Reference		Reference	
T3&T4	382	3.072 (1.423–6.631)	**0.004**	3.311 (1.013–10.817)	**0.047**
N stage	477		**< 0.001**		
N0&N1	391	Reference		Reference	
N2	86	3.419 (2.286–5.113)	**< 0.001**	1.529 (0.893–2.619)	0.122
M stage	414		**< 0.001**		
M0	348	Reference		Reference	
M1	66	4.193 (2.683–6.554)	**< 0.001**	2.322 (1.363–3.955)	**0.002**
Pathologic stage	466		**< 0.001**		
Stage I&Stage II	267	Reference		Reference	
Stage II &Stage IV	199	2.947 (1.942–4.471)	**< 0.001**	1.685 (0.927–3.064)	0.087
NDUFA4L2	477		**0.008**		
Low	238	Reference		Reference	
High	239	1.691 (1.141–2.507)	**0.009**	1.646 (1.059–2.560)	**0.027**

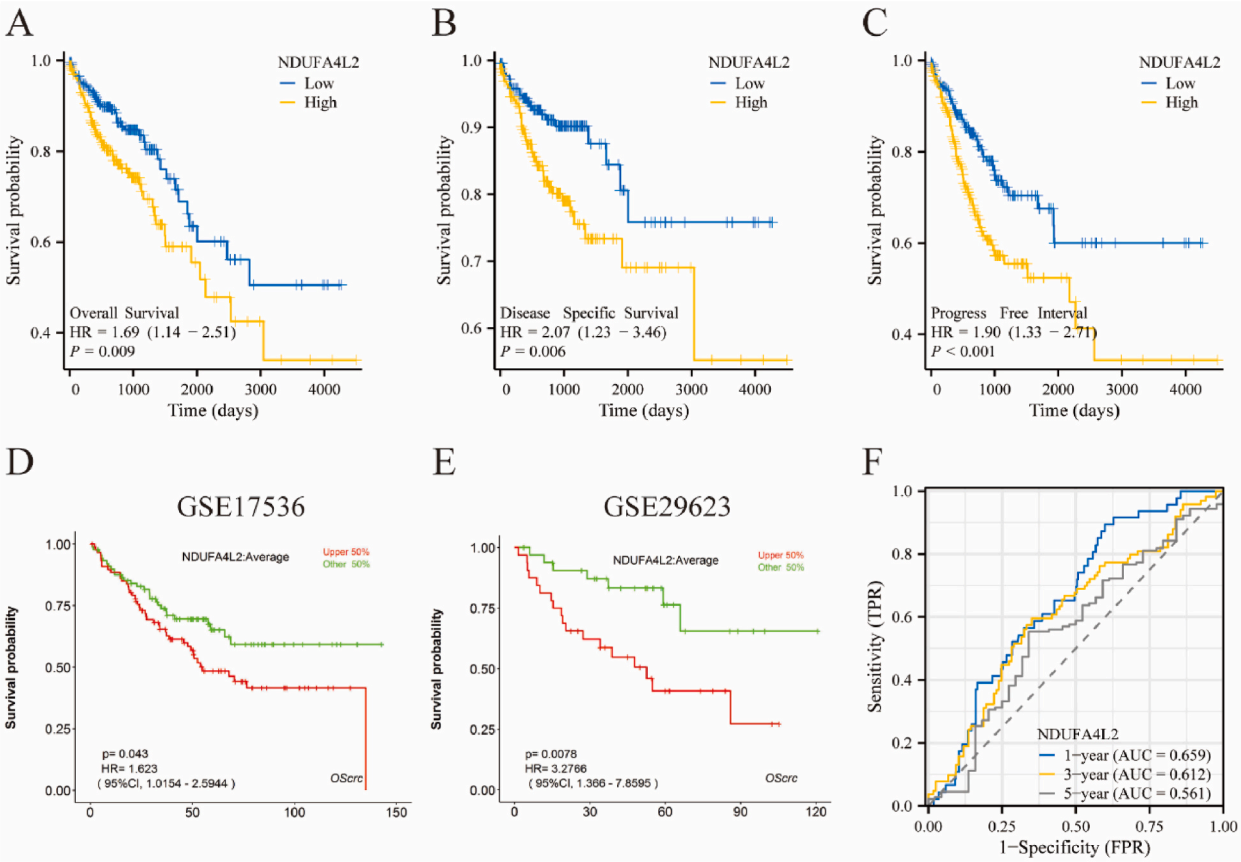

Fig. 4. Prognostic value of NDUFA4L2 for patients with COAD. (A–C) The prognostic value of NDFA4L2 in OS, DSS, and PFI of COAD. (D, E) The prognostic value of NDFA4L2 in OS of COAD in GSE17536 and GSE29623 (F) Time-dependent ROC curves of the predictive value of NDFA4L2 for OS.

Table 3
Association between NDUFA4L2 expression and clinical pathological characteristics (logistic regression).

Characteristics	Total(N)	Odds Ratio (OR)	P value
T stage (T3&T4 vs. T1&T2)	477	1.628 (1.033–2.588)	**0.037**
N stage (N2 vs. N0&N1)	478	2.006 (1.245–3.280)	**0.005**
M stage (M1 vs. M0)	415	1.397 (0.824–2.393)	0.218
Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II)	467	1.311 (0.908–1.895)	0.149
Residual tumor (R1&R2 vs. R0)	374	2.211 (0.998–5.258)	0.058
CEA level (>5 vs.≤5)	303	1.015 (0.634–1.628)	0.949
History of colon polyps (YES vs. NO)	408	0.979 (0.652–1.468)	0.918
Colon polyps present (YES vs. NO)	249	0.657 (0.385–1.110)	0.118

signaling pathway, cytokine–cytokine receptor interaction, cell adhesion molecules (CAMs), extracellular matrix (ECM) receptor interaction, pathways in cancer, the mitogen-activated protein kinase (MAPK) signaling pathway, and the transforming growth factor beta (TGF-β) signaling pathway (Fig. 6C and D). These findings suggest that NDUFA4L2 may contribute to COAD progression via various cancer-related signaling pathways in COAD.

3.7. Connection between NDUFA4L2 level and immune infiltration

ssGSEA showed a positive connection of NDUFA4L2 level with various immune cell populations, including NK cells, dendritic cells (DCs), NK CD56dim cells, immature dendritic cells (iDCs), macrophages, Th1 cells, cytotoxic cells, neutrophils, mast cells, Treg, T follicular helper cells, activated DCs, NK CD56bright cells, eosinophils, T cells, plasmacytoid DCs, CD8 T cells, T gamma delta, B cells, and Th2 cells while negatively correlated with T central memory cells (Fig. 7A). Examination using Spearman correlation showed a weakly ($R < 0.5$) but significantly ($P < 0.05$) linear correlation of NDUFA4L2 level to the infiltration level, which was quantified by the

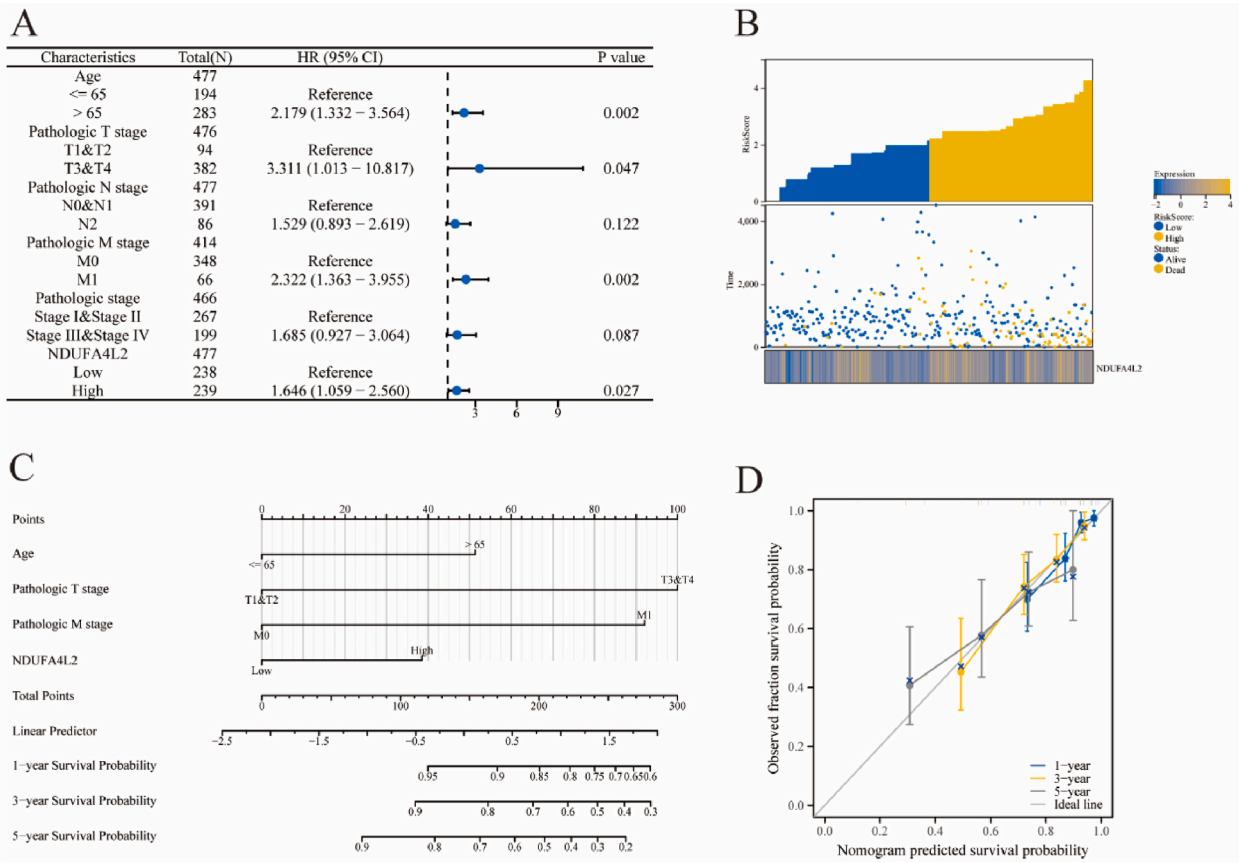

Fig. 5. Prognostic prediction model of NDUFA4L2 in COAD. (A) Multivariate Cox regression visualization by the forest plot. (B) NDUFA4L2 expression distribution and survival status. (C) Nomogram for 1-, 3-, and 5-year OS of patients with COAD. (D) Calibration plots for 1-, 3-, and 5-year OS prediction.

ssGSEA score, of NK cells (R = 0.496), iDCs (R = 0.402), DCs (R = 0.398), and macrophages (R = 0.398), among 24 immune cells (Fig. 7C). Consistently, Wilcoxon rank sum test revealed significantly higher infiltration levels of NK cells, iDCs, DCs, and macrophages in high NDUFA4L2 group ($P < 0.001$) (Fig. 7B), indicating that NDUFA4L2 is essential in the immune infiltration of COAD. Moreover, the correlations among the percentages of 24 tumor-infiltrating immune cell subpopulations were evaluated and illustrated in a heat map (Fig. 7D).

3.8. Validation of the expression of NDUFA4L2 mRNA by RT-qPCR

As depicted in Fig. 8, using RT-qPCR, NDUFA4L2 level was significantly augmented in SW620 and HT-29 cell lines relative to the normal CCD-18o cell line ($P < 0.001$).

4. Discussion

Currently, there is limited available information on NDUFA4L2's role in diseases. However, some studies have shed light on its involvement in certain contexts. For instance, in cardiac stem cell research, it has been demonstrated that hypoxia can enhance their survival in a serum-free medium by activating the HIF-1α/NDUFA4L2 pathway. This activation suppresses ROS production in a mitochondrial function-dependent manner. Additionally, NDUFA4L2 has been shown to accelerate pulmonary artery smooth muscle cell proliferation by modulating HIF-1α and its downstream p38-5-lipoxygenase signaling pathways. This, in turn contributes to pulmonary vascular remodeling and pulmonary arterial hypertension [15,16]. Although there have been reports on NDUFA4L2 expression and function in tumors, the available literature is sparse and lacks comprehensive substantiation. The relationship of NDUFA4L2 expression with its role in COAD stay largely unknown. Therefore, investigated NDUFA4L2's potential role in COAD. Analyzing the TCGA dataset uncovered the overexpression of NDUFA4L2 in COAD tissues, which exhibited a significant correlation with a poorer OS. Consistent with these findings, our analysis of the HPA database revealed higher NDUFA4L2 expression in COAD tissues. Through further examination of the TCGA dataset, we validated that NDUFA4L2 expression acts as an independent prognostic factor for COAD, providing support to recent reports and underscoring the potential significance of NDUFA4L2 expression in the

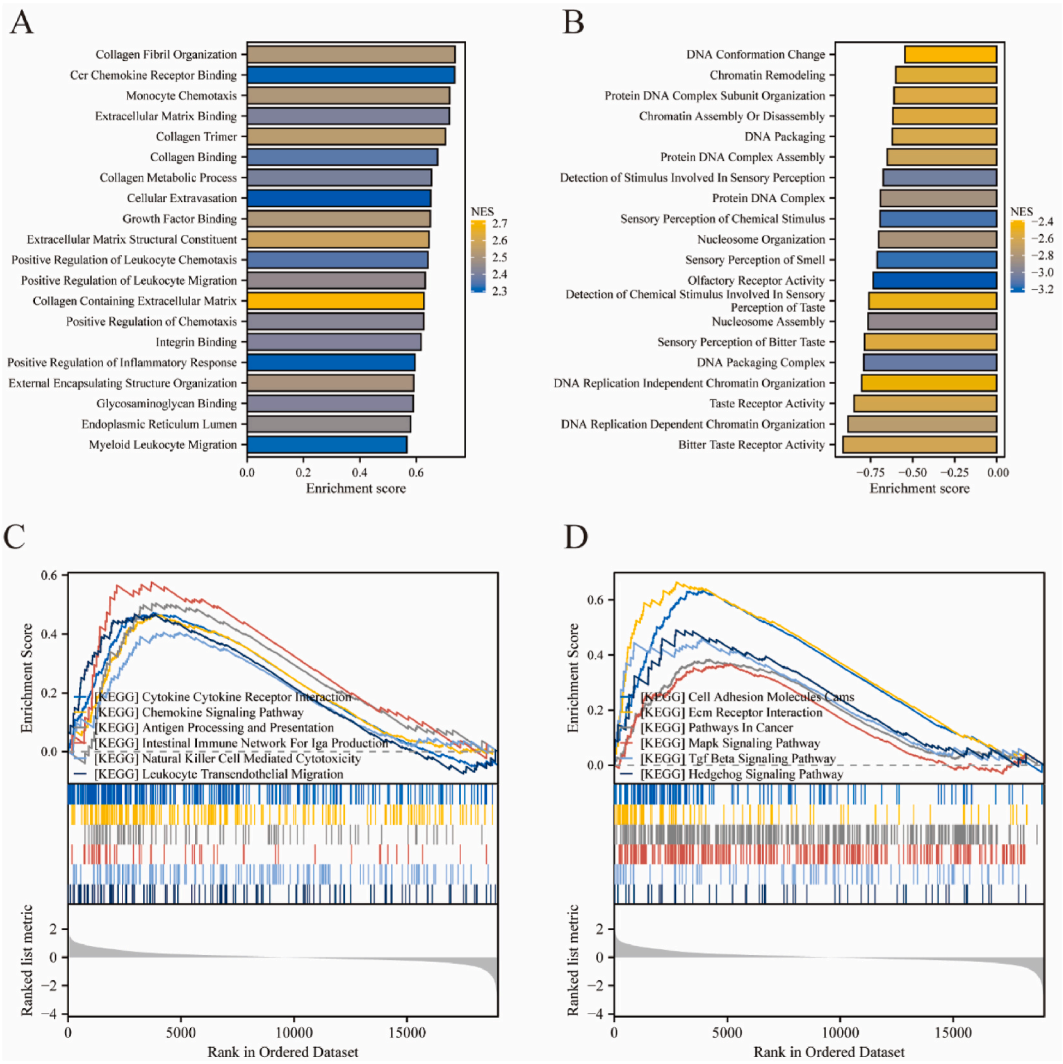

Fig. 6. Enrichment analysis of NDUFA4L2 in COAD. (A, B) GSEA of DEGs in low- and high- NDUFA4L2-expression samples. (C, D) GSEA of DEGs in low- and high- NDUFA4L2-expression samples.

prognosis of COAD [8]. Furthermore, our analyses revealed the association between NDUFA4L2 expression and several critical tumorigenesis-related signaling pathways. Further a positive correlation of NDUFA4L2 to infiltration levels of iDCs and NK cells was observed. These novel insights into the potential involvement of NDUFA4L2 in immune cell infiltration further highlight its promising application as a biomarker for cancer therapy and prognosis.

NDUFA4L2 functions as an inhibitory subunit of the mitochondrial oxidative respiratory chain complex I, exerting essential control over metabolic reprogramming and oxidative stress in malignant tumors [7,17]. Research has shown aberrant NDUFA4L2 expression in various cancer types, including ccRCC, glioblastoma, hepatocellular carcinoma (HCC), and CRC. Additionally, a high NDUFA4L2 expression level has been linked to poor prognosis [8,18–21]. NDUFA4L2 is highly augmented in HER2-positive breast cancer with trastuzumab resistance. Augmentation of NDUFA4L2 level leads to an increase in the Warburg effect and aerobic glycolysis while reducing oxygen consumption and suppressing ROS production [22]. Furthermore, mitochondrial NDUFA4L2 is regulated by HIF-1α, which is involved in the adaption to hypoxia by decreasing ROS production, oxygen consumption, and complex I activity in mitochondria [7]. In HCC cells, NDUFA4L2 is modulated by HIF-1α, and its inactivation results in elevated mitochondrial activity, ROS accumulation, oxygen consumption, and ultimately apoptosis [23]. NDUFA4L2, induced by HIF-1α , improve osteosarcoma cell survival, metastasis, and epithelial-mesenchymal transition by inhibiting ROS generation. Additionally, as a target of HIF-1α in ccRCC, NDUFA4L2 downregulation exhibits significant antiproliferative effects [6,24]. NDUFA4L2 level is enhanced in both human non-small cell lung cancer cell lines and tissues under hypoxic conditions and is modulated by HIF-1α and its silencing significantly increases mitochondrial ROS production and inhibits NSCLC viability [25].

Our study revealed a positive connection between HIF-1α and NDUFA4L2 levels, supporting the notion that HIF-1α induces NDUFA4L2 expression, thereby suggesting the involvement of HIF-1α/NDUFA4L2 pathway in COAD progression. However,

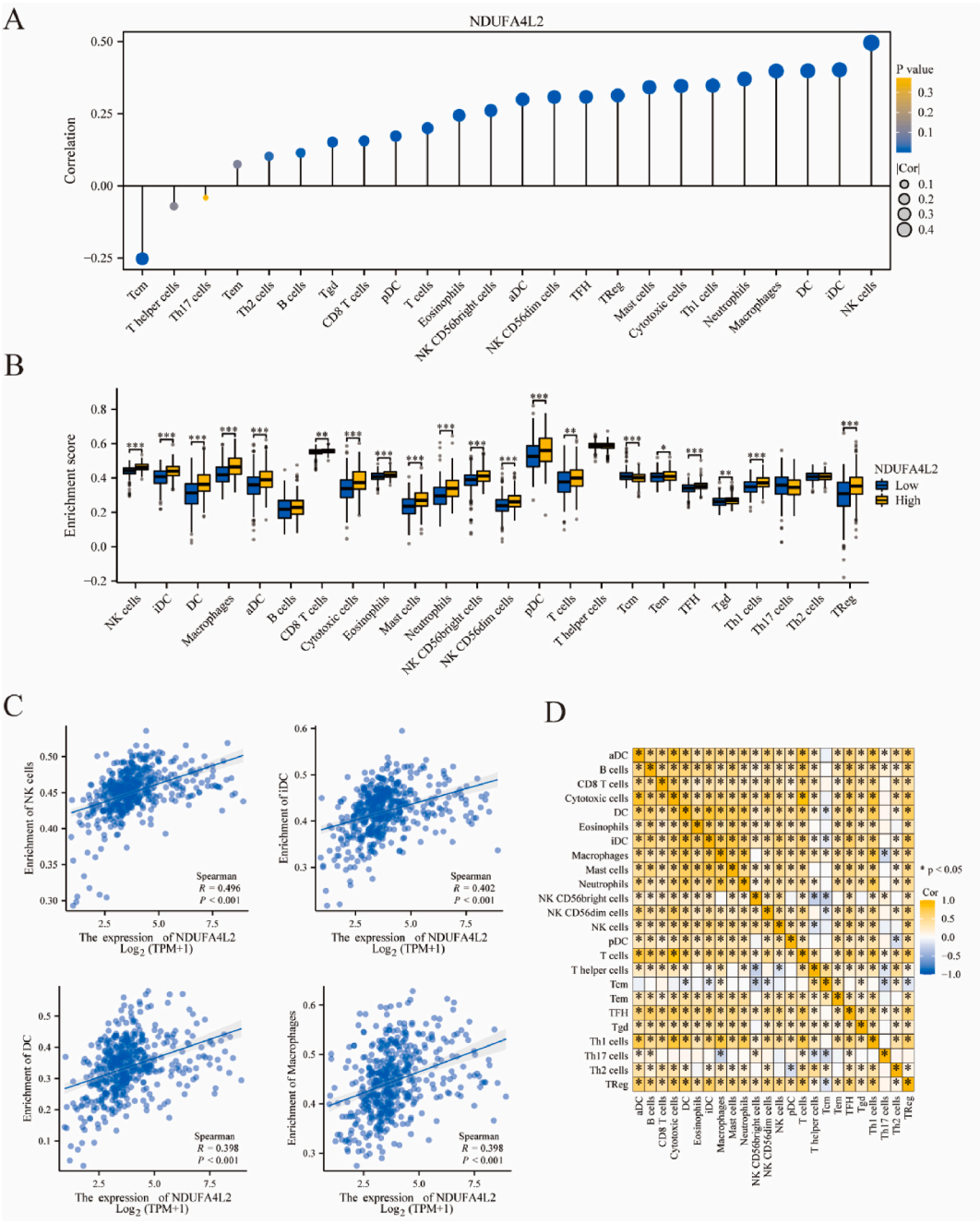

Fig. 7. NDUFA4L2 expression and immune infiltration. (A) Correlation between NDFA4L2 expression and the relative abundances of 24 immune cells. (B) Comparison of the different immune cells infiltration levels under high- and low- NDUFA4L-expression conditions. (C) The positive correlation between NDUFA4L2 expression and NK cells, iDCs, DCs and macrophages. (D) Heatmap of 24 immune infiltration cells in COAD. *P < 0.05, **P < 0.01, ***P < 0.001.

conflicting findings have been reported in other studies. For instance, NFIB has been found to induce sorafenib resistance in HCC cells by promoting NDUFA4L2 transcription, which decreases ROS production. Additionally, enhanced glycolysis promotes bladder cancer progression, metastasis, and gemcitabine resistance by modulating NDUFA4L2 expression through NXPH4 [20,26]. Furthermore, Pearson correlation analysis of clinical ccRCC samples revealed an reverse correlation between NDUFA4L2 level and HIF-1α expression but a positive correlation with ELK1 level, indicating that ELK1 may potentially induce NDUFA4L2 expression [18]. Thus, investigations are required to further evaluate NDUFA4L2's role in COAD progression.

Due to the lack available data on NDUFA4L2 function, its potential involvement in various biological pathways was explored using

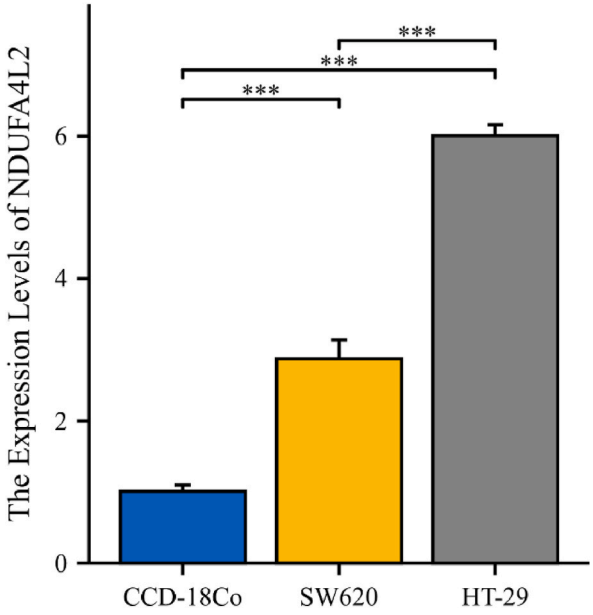

Fig. 8. Validation of the expression of NDUFA4L2 mRNA in CCD-18Co, SW620 and HT-29 cell lines by RT-qPCR analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

functional annotation. The analysis revealed that NDUFA4L2 might be implicated in cytokine-cytokine receptor interaction, antigen processing and presentation, the chemokine signaling pathway, NK cell mediated-cytotoxicity, the intestinal immune network for IgA production, leukocyte transendothelial migration, CAMs, ECM receptor interaction, pathways in cancer, the MAPK signaling pathway, the TGF-β signaling pathway, and the Hedgehog signaling pathway. Several chemokines, including CCL2, CCL3, CCL5, CXCL1, CXCL2, and CXCL8, have been linked to the risk and progression of colon cancer [27]. Inhibiting the intestinal immune network for IgA production signaling pathway suppresses HCC proliferation and migration [28]. The cell surface glycoproteins CAMs are entangled in cell-cell and cell-ECM interactions [29]. In cancer, adhesion to the ECM is essential for cell migration, and various CAMs play significant roles in mediating these interactions. The ECM serves as the natural niche for cells, and CAM-mediated interactions with surface ligands activate key signals involved in cell proliferation, differentiation, and dissemination [30]. Numerous studies have demonstrated the critical involvement of the ECM receptor interaction signaling pathway in CRC development and metastasis [31–33]. TGF-β, a secreted multifunctional cytokine, activates its type I and type II membrane receptors and downstream SMAD [34]. Dysregulated TGF-β signal transduction leads to various tumors, including esophageal cancer, CRC, gastric cancer, HCC, and pancreatic cancer, [35]. Mutations in TGF-β or SMAD can result in an aberrant TGF-β signaling pathway, especially CRC metastasis [36]. MAPK signaling pathway impacts tumor cell proliferation, apoptosis, and metastasis. Activation of ERK2 and downregulation of p38 MAPK have been observed in primary tumors, promoting CRC metastasis to the liver and lung, respectively [37]. However, more investigations are necessary to unravel the potential regulatory mechanisms of NDUFA4L2 in COAD.

Our investigation on the links between NDUFA4L2 and immune infiltration levels in COAD revealed a strong connection between NDUFA4L2 levels and various immune cells, including NK cells, iDCs, DCs, and macrophages. Tumors with high levels of NDUFA4L2 showed a significant infiltration of these immune cells. Immune cells, stromal cells, cancer cells, and ECM within the tumor microenvironment (TME) collectively influence immune evasion, tolerance, and tumor growth [38]. Within the tumor microenvironment (TME), tumors and tumor-associated cells/factors exert control over DC function, enabling tumor cells to evade immune recognition and suppression. Tumor-infiltrated DCs frequently exhibit reduced or deficient function, leading to immunosuppression [39,40]. Infiltrated NK cells in the TME also affect immunotherapy, and targeting NK cells can enhance antitumor immune responses. It has been demonstrated that infiltrated NK cells exhibit impaired function and phenotype leading to their dysfunction or exhaustion in the TME [41–43]. DCs, as highly specialized antigen-presenting cells, is critical in connecting innate and adaptive immune responses [44]. Macrophages, key players within the innate immune system, are crucial in promoting tumor metastasis and growth by accelerating angiogenesis, immunosuppression, and cancer cell proliferation and producing numerous inflammatory cytokines, chemokines, and growth factors [45,46]. In summary, our results suggest that NDUFA4L2 is crucial role in recruiting and regulating immune infiltrating cells in COAD.

While this work enhanced our understanding of NDUFA4L2 in COAD, it is not without limitations. First, additional research with large sample size and prospective design is necessary to mitigate selection and recall biases and generate more robust results. Second, the mRNA and protein expression of NDUFA4L2 should be validated at cellular and clinical levels. Lastly, further *in vitro* and animal tests are required to confirm the underlying mechanism through which NDUFA4L2 contributes to COAD.

5. Conclusions

This study revealed that NDUFA4L2 expression is elevated in COAD tumor tissues, and high NDUFA4L2 expression is connected with unfavorable prognosis and augmented immune cell infiltration in COAD patients suggesting that NDUFA4L2 shows promise as a prognostic biomarker and target for immunotherapy in COAD. However, further experimental validation is needed to elucidate the precise biological effects of NDUFA4L2.

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Data availability statement

Data included in article/supp. material/referenced in article.

Ethics declarations

Not applicable.

CRediT authorship contribution statement

Qingbu Mei: Writing – original draft. **Ping Chen:** Formal analysis. **Ying Lv:** Formal analysis. **Lihong Zheng:** Validation. **Dan Liu:** Validation. **Minglong Zhang:** Resources, Software, Visualization. **Wanquan Liu:** Conceptualization, Supervision, Writing – review & editing. **Penghui Li:** Software, Visualization.

Declaration of competing interest

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

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