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RESEARCH

KLF12 as a potential biomarker for lateral pelvic lymph node metastases in advanced rectal cancer

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Abstract

Rectal cancer accounts for approximately 40% of colorectal cancer cases, and lateral pelvic lymph node (LPLN) metastasis in rectal cancer significantly increases the local recurrence rate. Despite its clinical significance, studies on the molecular biology of LPLN metastasis are relatively scarce. In this study, we aimed to elucidate the underlying mechanisms by identifying hub regulatory genes in LPLN tissues and analyzing differentially expressed genes shared between tumor and pericarcinomatous tissues within our clinical cohort. To investigate the biological functions of these hub regulatory genes, we performed GSEA, GO, and KEGG pathway analyses on mRNA-Seq data. Among the identified hub genes, KLF12 emerged as a pivotal regulatory gene in rectal cancer. We further explored its clinical relevance and biological function. Our findings, validated using public databases, clinical cohort data, and immunohistochemistry (IHC), identified KLF12 as a specific marker for LPLN. Additionally, KLF12 expression exhibited a strong correlation with disease-free survival (DFS). According to clinical data, significant differences in KLF12 expression exist between groups based on factors such as age, gender, tumor location, pathological N stage, and postoperative tumor residue. Both treatment outcomes (DFS) and receiver operating characteristic curves (AUCs) were significantly associated with KLF12 expression. Furthermore, KLF12 demonstrated a strong association with immune cell infiltration, immune checkpoint expression, and immunophenoscore (IPS), indicating its potential regulatory role in immunotherapy. Functional molecular experiments revealed that KLF12 overexpression inhibited the proliferation, migration, and invasion of SW620 cells. In conclusion, leveraging mRNA-Seq data, TCGA database analysis, immune infiltration data, and biological function assessments, we confirmed that KLF12 could serve as an effective predictive marker and potential therapeutic target for LPLN metastasis. These findings suggest that KLF12 may be instrumental in assessing predictive risk and identifying novel therapeutic targets for patients with rectal cancer.

Keywords Rectal cancer · Lateral pelvic lymph node · KLF12 · Marker · Target

Introduction

In 2022, there were 1,926,118 newly diagnosed colorectal cancer cases worldwide, with rectal cancer accounting for nearly 40% of these cases [1]. The lateral pelvic lymph node (LPLN) is a typical site for rectal cancer metastasis, playing a crucial role in local recurrence [2]. However, treatment approaches for lateral pelvic lymph node metastases (LPLN-M) in rectal cancer vary considerably between Eastern and Western nations. Western researchers generally consider LPLN-M a systemic disease and prefer neoadjuvant chemoradiotherapy (nCRT) over lateral pelvic lymph node dissection (LPLND) to manage local recurrence in advanced low rectal cancer. Despite this approach, studies have demonstrated that nCRT alone is insufficient to control local recurrence, with a substantial proportion of patients still presenting with LPLN metastases post-treatment [3]. In contrast, Japanese researchers advocate a proactive strategy, recommending prophylactic LPLND for patients with stage T3/T4 rectal cancer located below the peritoneal reflection. The Japanese Clinical Oncology Group (JCOG) 0212 trial revealed that while prophylactic LPLND

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significantly reduces local recurrence in the lateral pelvic region, the pathological LPLN-M rate is only 7%. This low rate suggests that the strategy could result in overtreatment, leading to unnecessary surgical trauma for patients with pathologically LPLN-negative (LPLN-N) [4]. Consequently, both Eastern and Western treatment paradigms for LPLN-M exhibit notable limitations and shortcomings.

To address these challenges, Chinese researchers have adopted a more selective approach, limiting LPLND to patients with clinically confirmed LPLN-M. However, current diagnostic methods that depend exclusively on imaging characteristics and clinicopathological parameters have demonstrated limited accuracy in identifying LPLN-M [5–7]. This diagnostic uncertainty often results in either missed diagnoses or unnecessary surgical interventions, highlighting a critical need for more precise molecular markers. Despite extensive research on various cancers, the molecular mechanisms underlying LPLN-M remain largely unexplored, highlighting a critical gap in our knowledge of rectal cancer progression.

Utilizing comprehensive transcriptome analysis of our clinical cohort and The Cancer Genome Atlas (TCGA) database, we identified KLF12 as a promising candidate gene based on several key findings. First, KLF12 exhibited a consistently distinct expression profile between LPLN-M and non-LPLN-M tissues. Second, previous studies have implicated KLF12 in cancer metastasis by modulating epithelial-mesenchymal transition (EMT) and cellular invasion, though its role in LPLN-M remains unexamined [8–11]. Utilizing RNA sequencing (RNA-Seq) for comprehensive transcriptome profiling, we analyzed mRNA expression in rectal cancer tissues, pericarcinomatous tissues, and LPLN tissues from our clinical cohort. We integrated these findings with public data from The Cancer Genome Atlas (TCGA) to identify differentially expressed genes (DEGs). Among these DEGs, KLF12 emerged as a hub gene with a potentially significant role in LPLN-M, as supported by clinical feature analysis and immune infiltration studies.

To further investigate the clinical and molecular significance of KLF12, we conducted quantitative polymerase chain reaction (qPCR), immunohistochemistry, colony formation, and transwell assays to assess its expression and biological functions in rectal cancer. Our results demonstrate that KLF12 is a promising biomarker for predicting LPLN-M and plays a regulatory role in rectal cancer progression, suggesting potential applications in both diagnosis and targeted therapy.

Methods

Tissue sample collection

Freshly excised rectal cancer and lateral pelvic lymph node (LPLN) tissues were obtained from the Colorectal Surgery Department at the Cancer Hospital affiliated with the Chinese Academy of Medical Sciences. Prior to sample collection, all patients provided informed consent, as officially recorded by the hospital administration. Notably, no patients received treatment before surgery. The study protocol was approved by the Ethics Committee of the Cancer Hospital affiliated with the Chinese Academy of Medical Sciences (NCC 2017-YZ-026, Oct 17, 2017) and registered at ClinicalTrials.gov (NCT04850027).

RNA sequencing

RNA sequencing was performed by mapping the reads to the GENCODE (human-hg19, mouse-mm10) or Ensembl gene annotations using Bowtie2 version 2.1.0. Gene expression levels were quantified using RSEM v1.2.28 and normalized using the TMM (trimmed mean of M-values) method. Differentially expressed genes (DEGs) were identified using the R package "edgeR," based on the threshold criteria of $P < 0.05$ and $|\log FC| \geq 1$. The significance of canonical pathways was assessed using Fisher's exact test with the false discovery rate (FDR) correction.

Data gathering and preparation

Data from the TCGA-READ adenocarcinoma project were downloaded and processed, including STAR-processed RNA sequencing data and clinical information extracted from the TCGA database (https://portal.gdc.cancer.gov). Data filtering involved the removal of non-essential clinical and redundant metadata, and the processed data were log-transformed using the $\log2(\text{value} + 1)$ method.

Estimation of immune score and immune-infiltrating cell percentage

The expression profile of the TCGA-READ dataset was analyzed using the R packages GSVA, CIBERSORT, and GSEABase to estimate the percentage of immune-infiltrating cells. The CIBERSORT algorithm was applied to determine cell composition in complex tissues based on gene expression profiles.

Drug sensitivity analysis

The R package pRRophetic, along with gene expression data from the READ cohort, was used to predict the IC50 values of 138 drugs from the Genomics of Drug Sensitivity in Cancer (GDSC) database. Statistical tests were performed to assess the significance of IC50 differences among subtypes, and drugs with significant variations were selected for visualization using IC50 heatmaps and distribution box plots.

IHC staining examination

Immunohistochemistry (IHC) was performed using an SP kit (No. SP-9000, ZSGB-BIO, China) following established protocols. The anti-KLF12 antibody (1:200) was purchased from ABclonal (No. A20469, China). IHC images were magnified 20 × for detailed examination.

Cell culture

The human colorectal cancer (CRC) cell line SW620 was obtained from the ATCC Cell Bank in the USA. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 100 µg/ml streptomycin (Thermo Scientific, Waltham, MA, USA). Cultivation was conducted in an incubator maintained at 37 °C under ambient CO_2-free conditions.

Lentiviral transduction and transfection

To overexpress KLF12 in the SW620 cell line, recombinant lentiviral vectors (pRP[Exp]-EGFP-CMV or pRP[Exp]-EGFP-CMV-KLF12) and packaging plasmids (psPAX2 and pMD2.G) were utilized (Supplementary File 1). The transfection of cells with these overexpression plasmids was performed using Lipofectamine 2000 (12,566,014, Invitrogen, Shanghai, China), following the manufacturer's protocol (Supplementary File 2).

Real-time quantitative polymerase chain reaction (qPCR) and colony formation assay

The sequences of the KLF12 primers were as follows: Forward: CCTTTCCATAGCCAGAGCAGTAC, Reverse: CTG GCGTCTTGTGCTCTCAATAC. For tissue samples, 1 ml of RNA extraction buffer (Servicebio, G3013) was added and homogenized using a high-speed microtherm homogenizer (Servicebio, KZ-III-F) until no visible tissue mass remained. For tumor cells, Trizol reagent was used for RNA extraction, followed by chloroform, isopropanol, and 75% ethanol treatment (Chemical Reagent Co. Ltd). Servicebio® RT Enzyme Mix Strand cDNA Synthesis and SYBR Green qPCR Master Mix (Servicebio, G3320) were used for qPCR. For the colony formation assay, cells were seeded at a density of 10^3 cells per well in 6-well plates and cultured for 10 days in DMEM supplemented with 10% FBS. Colonies containing fewer than 30 cells were excluded from analysis.

Transwell assay

The invasive and migratory capabilities of SW620 cells were evaluated using Transwell assays. For the migration assay, 700 µl of DMEM containing 20% serum was added to the lower chamber of a Transwell plate (Corning, NY, USA), while 1.5×10^5 cells suspended in 200 µl of serum-free DMEM were seeded in the upper chamber. After a 24-h incubation at 37 °C, the Transwell chamber was removed, washed with PBS, fixed for 30 min, and stained with 0.5% crystal violet. Cells on the upper side of the filter were gently detached before the chamber was washed with PBS.

Statistical analysis

All statistical analyses were performed using R software (v3.5.3) and GraphPad Prism (v8.01, GraphPad Software, La Jolla, CA, USA). Student's t test was used for comparisons, with statistical significance set at $P < 0.05$.

Results

Tumor microenvironment and immune infiltration in tumor and lymph node tissues between LPLN-N and LPLN-M groups

We investigated the tumor microenvironment by assessing the ESTIMATE score, stromal score, and tumor purity. As depicted in Fig. 1, immune and stromal scores were significantly higher in lymph node tissues from the LPLN-N group compared to the LPLN-M group. Conversely, tumor purity was elevated in LPLN-N tumor tissues compared to LPLN-M tumor tissues.

To further explore immune infiltration, we employed single-sample Gene Set Enrichment Analysis (ssGSEA). In tumor tissues, inflammation-promoting factors, type I interferon (IFN) response, and chemokine receptor (CCR) signaling were significantly enriched in the LPLN-M group (Fig. 1A). In lymph node tissues, CCR signaling, human leukocyte antigen (HLA) expression, type II IFN response, and macrophage-related pathways were significantly enriched in the LPLN-M group (Fig. 1B).

Differential expression genes between LPLN-M and LPLN-N

We analyzed mRNA expression profiles in rectal cancer, adjacent tissues, and LPLN tissues using mRNA sequencing (mRNA-Seq). Initially, 2,633 differentially expressed genes (DEGs) were identified between tumor and adjacent tissues. To investigate gene expression patterns associated with LPLN metastasis status, patients were classified into LPLN-M-positive and LPLN-M-negative groups. A total of 1,951 DEGs were identified between the LPLN-N and LPLN-M groups. Additionally, 1,671 DEGs were detected in tumor tissues between the LPLN-N and LPLN-M groups.

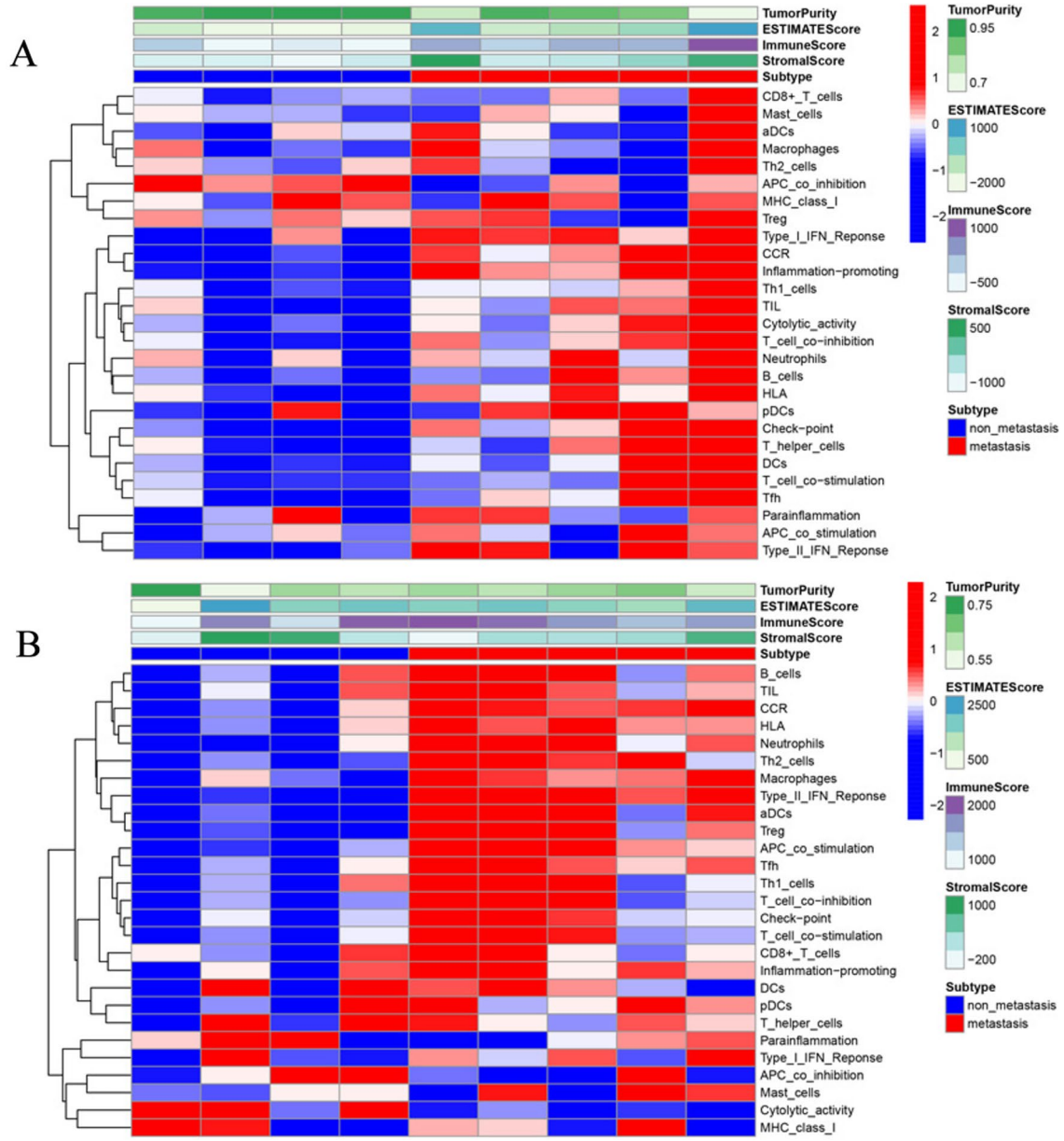

Fig. 1 Single-sample Gene Set Enrichment Analysis (ssGSEA) and Tumor Microenvironment (TME) analysis of rectal cancer and lymph node tissues between the LPLN-N and LPLN-M groups. **A** Heatmap of tumor tissues. **B** Heatmap of lymph node tissues

The functional significance of DEGs was explored using Gene Ontology (GO) enrichment analysis. In DEGs between tumor and adjacent tissues, significant enrichment was observed in biological process (BP) terms related to mitotic cell cycle phase transition, sister chromatid segregation, RNA catabolism, DNA replication, and chromosome segregation (Fig. 2A). Cellular component (CC) terms such as chromosomal region, chromosome centromeric region, spindle, and kinetochore were enriched, while molecular function (MF) terms included ATPase activity, ubiquitin-like protein ligase binding, and DNA-dependent ATPase activity.

For DEGs between the LPLN-N and LPLN-M tumor groups, enrichment was observed in protein targeting and ribosome biogenesis (BP), ribosomal subunit and ribosome-related structures (CC), and structural constituents of ribosomes and cadherin binding (MF) (Fig. 2B). In lymph node tissues between the LPLN-N and LPLN-M groups, DEGs were enriched in DNA-templated transcription initiation and chromosome organization (BP), lipid droplet and lysosomal membrane (CC), and carbohydrate kinase activity and transcription coregulator activity (MF) (Fig. 2C).

Pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database revealed that

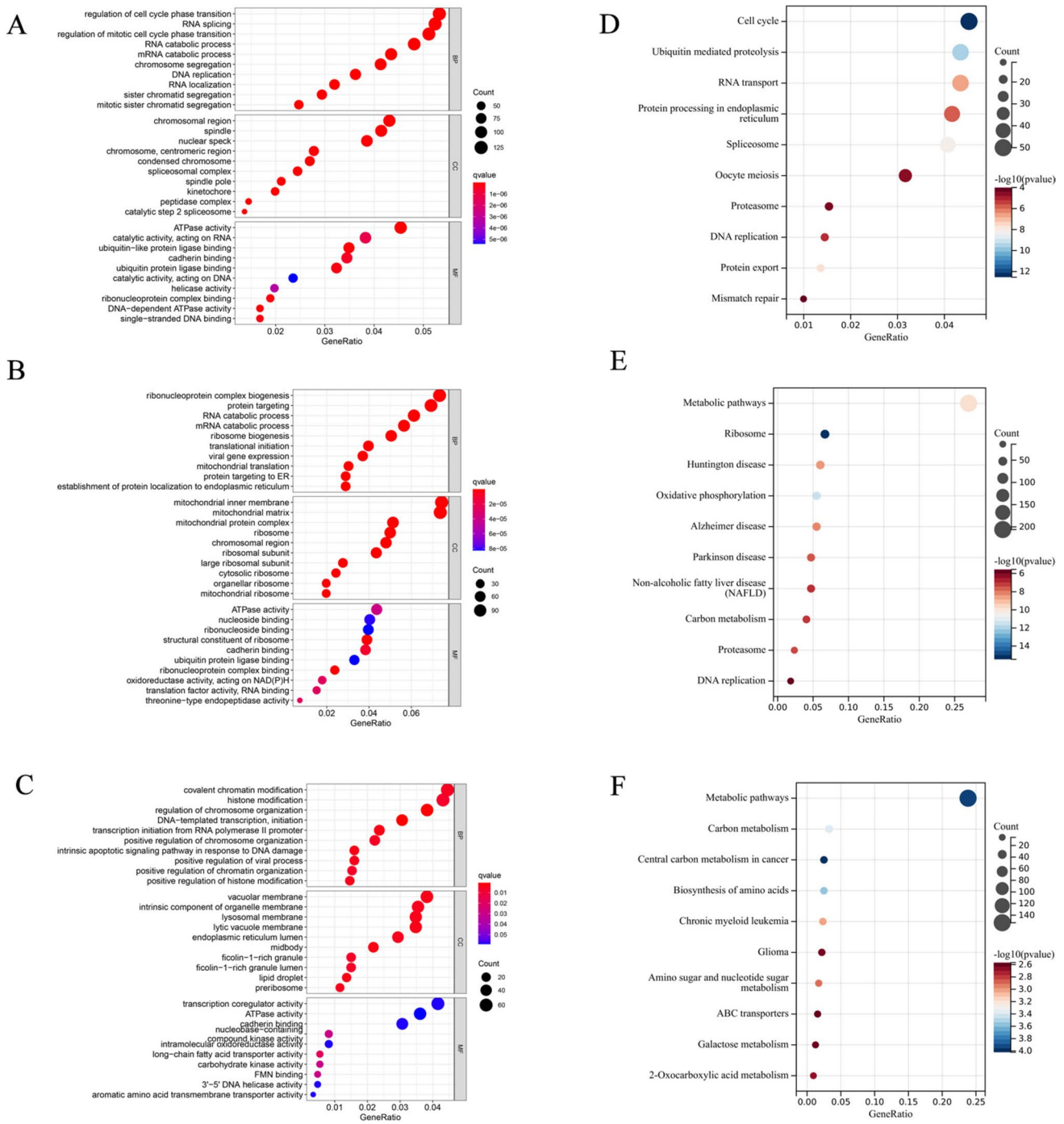

Fig. 2 Biological enrichment analysis of differentially expressed genes (DEGs) across various tissue types. Gene Ontology (GO) enrichment bubble plots of DEGs: **A** GO enrichment of DEGs between tumor and adjacent tissues. **B** GO enrichment of DEGs in tumor tissues between the LPLN-N and LPLN-M groups. **C** GO enrichment of DEGs in lymph node tissues between the LPLN-N and LPLN-M groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment bubble plots of DEGs: **D** KEGG enrichment of DEGs between tumor and adjacent tissues. **E** KEGG enrichment of DEGs in tumor tissues between the LPLN-N and LPLN-M groups. **F** KEGG enrichment of DEGs in lymph node tissues between the LPLN-N and LPLN-M groups

DEGs between tumor and adjacent tissues were significantly enriched in pathways related to cell cycle regulation and RNA transport (Fig. 2D). In tumors between the LPLN-N and LPLN-M groups, DEGs were enriched in metabolic pathways, ribosomal activity, and oxidative phosphorylation (Fig. 2E). In lymph nodes between the LPLN-N and

LPLN-M groups, DEGs were significantly associated with metabolic pathways, carbon metabolism, and 2-oxocarboxylic acid metabolism (Fig. 2F).

Biological function of hub DEGs between metastatic and non-metastatic LPLN

In this study, we analyzed differentially expressed genes (DEGs) between tumor and adjacent tissues, as well as between metastatic and non-metastatic groups, to identify shared DEGs. As illustrated in Fig. 3A, 52 genes were found to be overlapping among all three comparisons. To elucidate the functional characteristics of these genes, we performed Gene Ontology (GO) enrichment analysis. These genes were significantly enriched in biological process (BP) terms related to DNA replication and transcription-coupled nucleotide excision repair. Regarding cellular component (CC) terms, they were associated with the replication fork and nuclear replication machinery. For molecular function (MF) terms, significant enrichment was observed in β-tubulin binding and drug binding (Fig. 3B).

Additionally, KEGG pathway analysis revealed that these genes were involved in cellular senescence, the mTOR signaling pathway, and DNA replication (Fig. 3C), suggesting their potential role in tumor progression and metastasis.

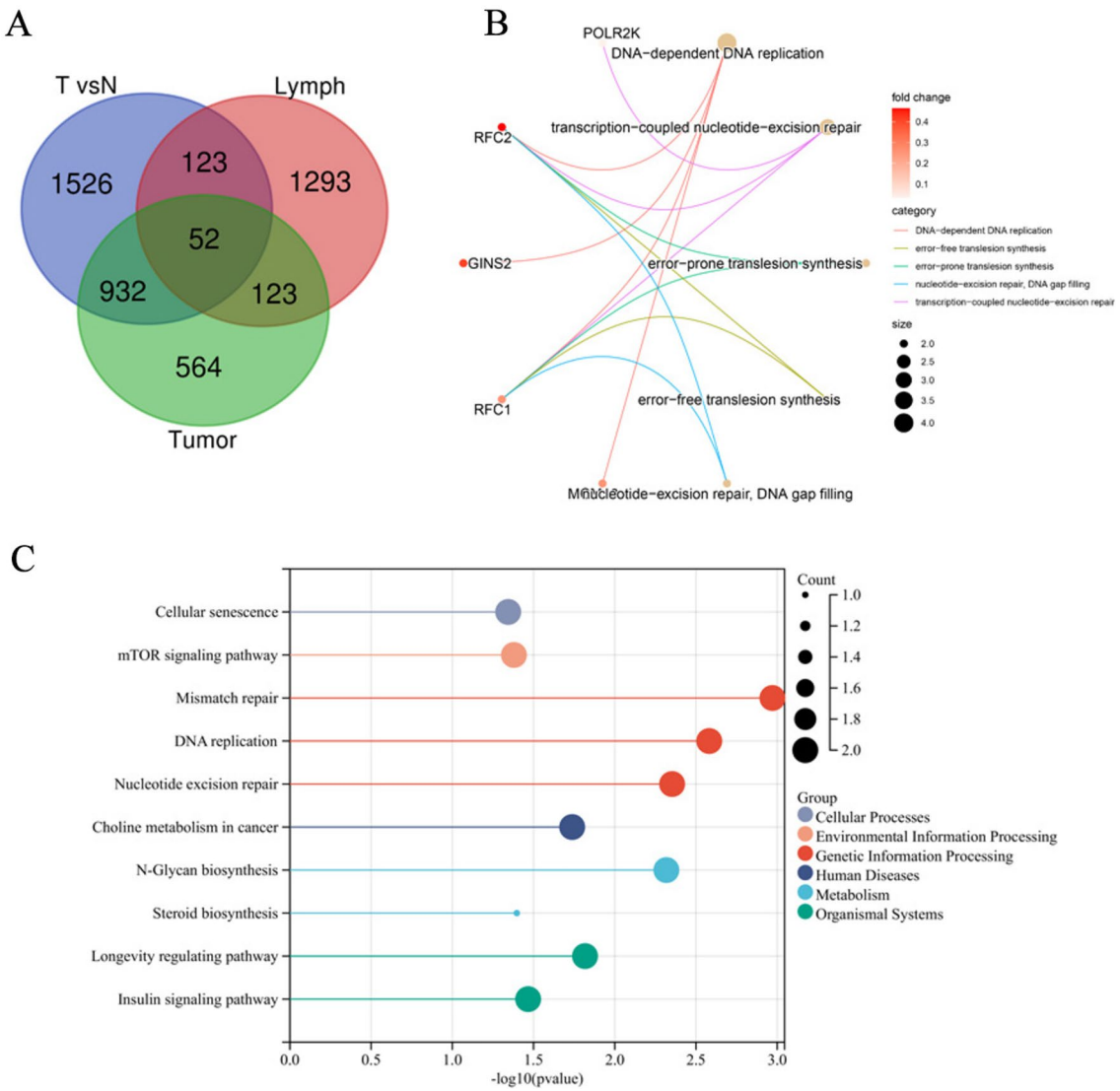

Fig. 3 Identification of hub genes and biological enrichment analysis. **A** Venn diagram displaying commonly expressed genes among rectal cancer tissues, adjacent tissues, and lymph node tissues in the LPLN-N and LPLN-M groups. **B** GO enrichment circular plot of hub genes. **C** KEGG enrichment lollipop plot of hub genes. T: tumor tissues, N: adjacent tissues

KLF12 clinical characteristics in rectal cancer

Among the 52 identified genes, KLF12 emerged as a key hub gene. We assessed its prognostic significance in lymphatic invasion using data from the TCGA database. As depicted in Fig. 4A, KLF12 expression was significantly associated with overall survival (OS), where higher expression correlated with a more favorable prognosis in rectal cancer cases involving lymphatic invasion. However, no significant difference in OS was observed between rectal cancer patients with high and low KLF12 expression (Fig. 4B).

To further investigate KLF12 expression in relation to clinical features, we analyzed TCGA rectal cancer datasets. The area under the time-dependent receiver operating characteristic (ROC) curve (AUC) was calculated to evaluate the diagnostic performance of KLF12, yielding an AUC of 0.726 (Fig. 4C). Our analysis revealed that patients aged > 65 years (Fig. 4D), male patients (Fig. 4E), and those with rectal cancer (Fig. 4F) exhibited significantly lower KLF12 expression.

Furthermore, KLF12 expression was able to distinguish between high- and low-expression groups based on anatomic neoplasm subdivision (Fig. 4G), pathologic N stage (Fig. 4H), and residual tumor status (Fig. 4I). Disease-free survival (DSS) analysis demonstrated that KLF12 expression was significantly associated with treatment outcomes, further supporting its potential clinical relevance (Fig. 4J).

Immune infiltration of KLF12 expression in rectal cancer

To investigate the relationship between KLF12 expression and the tumor immune microenvironment, we used the CIBERSORT method to assess the infiltration patterns of 24 immune cell types in the rectal cancer TCGA dataset. The results revealed notable variations in immune cell infiltration associated with KLF12 expression (Fig. 5A). Specifically, KLF12 expression was significantly correlated with the infiltration levels of T helper cells (Fig. 5B), macrophages (Fig. 5C), TFH cells (Fig. 5D), NK cells (Fig. 5E), Tgd cells (Fig. 5F), Th17 cells (Fig. 5G), Tem cells (Fig. 5H), Tcm cells (Fig. 5I), and CD56 bright cells (Fig. 5J).

Additionally, we used the ESTIMATE program to predict the tumor microenvironment (TME) score. KLF12 expression significantly differentiated stromal score, immune score, and ESTIMATE score (Fig. 5K), suggesting a potential role in shaping the TME.

KLF12's relationship with immunotherapy and chemotherapy

We evaluated the potential of KLF12 as a predictor of immunotherapy response by analyzing immune checkpoint expression and the immunophenoscore (IPS) in rectal cancer samples from the TCGA database, as accessed through the TCIA database. Immune checkpoints regulate immune activation to maintain self-tolerance and prevent autoimmunity. Our findings indicate that KLF12 expression correlates with the expression levels of multiple immune checkpoints, including NRP1, TNFSF4, TNFRSF14, CD28, and TIGIT (Fig. 6A).

The IPS is an indicator of tumor immunogenicity and potential response to immunotherapy. Notably, IPS scores for the categories ips_ctla4_neg_pd1_neg, ips_ctla4_neg_pd1_pos, and ips_ctla4_pos_pd1_neg were significantly lower in the high KLF12 group compared to the low KLF12 group (Fig. 6B–E), indicating that high KLF12 expression may be predictive of enhanced immunotherapy response.

Furthermore, we investigated KLF12-associated chemosensitivity by calculating IC50 values for 138 chemotherapy drugs from the GDSC database, using the rectal cancer TCGA expression profile. Our analysis revealed a notable association between KLF12 risk scores and the IC50 values of 28 drugs. To illustrate this relationship, we selected four representative drugs for scatter plot visualization (Fig. 6F–I).

Biological role of KLF12 in rectal cancer expression

To further investigate the biological role of KLF12 in rectal cancer, we examined fresh cancer tissues and utilized immunohistochemistry (IHC) to compare its expression between the LPLN-M and LPLN-N groups. The results revealed that KLF12 expression was significantly elevated in the LPLN-M positive group relative to the LPLN-M negative group (Fig. 7A, Supplementary Material 3).

Based on these IHC findings, we performed disease-free survival (DFS) and overall survival (OS) analyses for patients in the LPLN-M positive group. The analysis demonstrated a significant association between KLF12 expression and DFS (Fig. 7B), whereas no statistically significant correlation was observed with OS (Fig. 7C) in rectal cancer patients (Table 1).

To elucidate the functional roles of KLF12 in rectal cancer cells, we conducted colony formation assays to examine cell proliferation and transwell assays to assess invasion and migration potential. Our experimental findings demonstrated that KLF12 overexpression inhibited the proliferation, migration, and invasion of SW620 cells (Fig. 7D–F).

Discussion

Lateral pelvic lymph node metastasis (LPLN-M) in rectal cancer is a key determinant in treatment strategies, as its presence significantly increases the risk of local recurrence [12]. Current diagnostic approaches predominantly

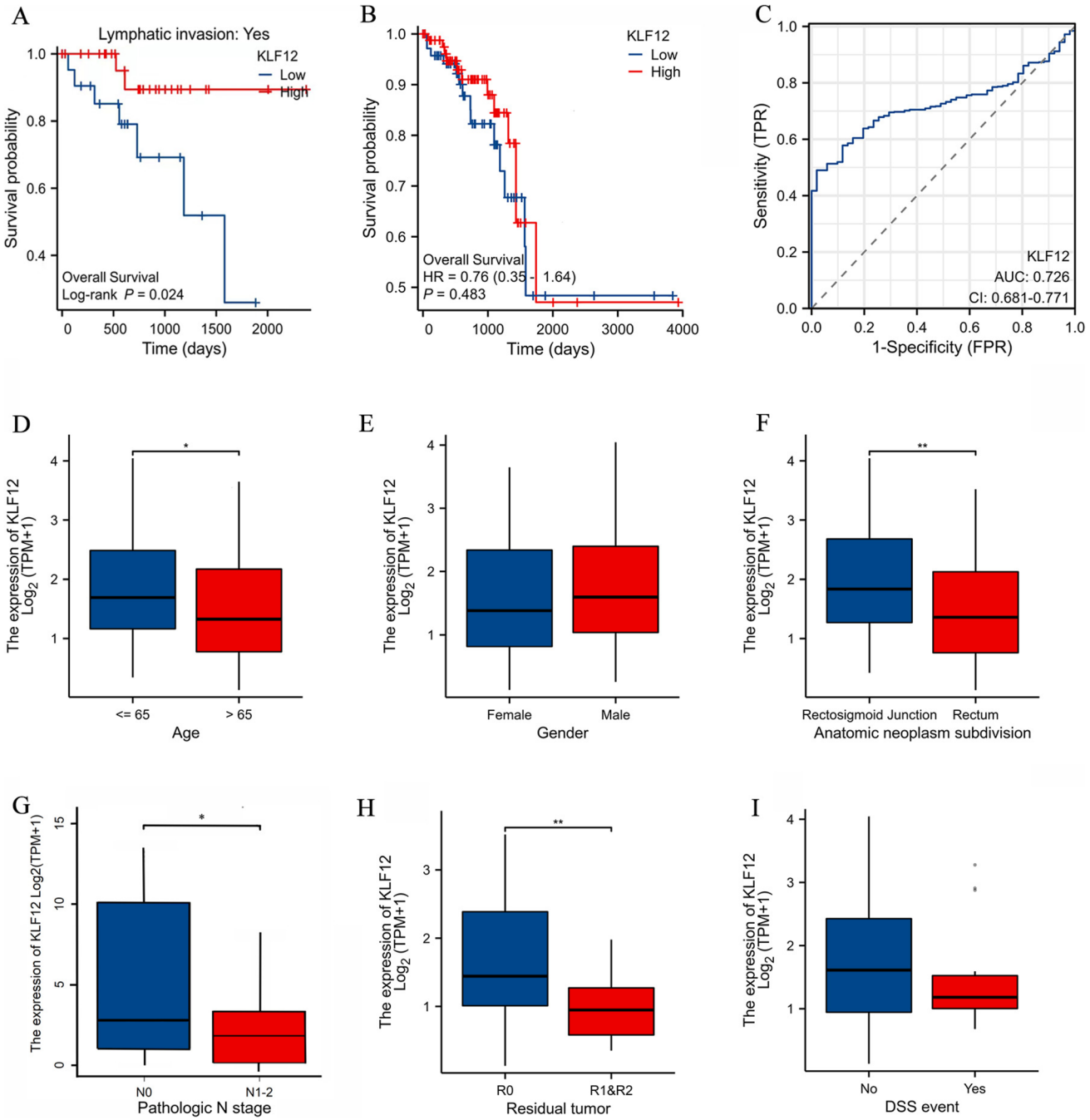

Fig. 4 Clinical significance of KLF12 in rectal cancer. **A** Kaplan–Meier survival curve illustrating the relationship between KLF12 expression and overall survival in rectal cancer with lymphatic invasion. **B** No statistically significant difference in overall survival (OS) was observed between patients with high and low KLF12 expression. **C** Receiver Operating Characteristic (ROC) curve showing the predictive efficacy of KLF12, with an area under the curve (AUC) of 0.726. KLF12 expression was significantly lower in **D** older colorectal cancer (CRC) patients and **E** male patients. KLF12 expression effectively differentiated between high and low groups based on **F** anatomic neoplasm subdivision, **G** pathologic N stage, **H** residual tumor status, and **I** disease-specific survival (DSS)

rely on imaging; however, molecular markers offer a novel avenue for enhancing LPLN-M detection [13]. However, LPLN dissection is associated with potential complications, including nerve injury, bleeding, and lymphorrhea [14, 15]. Neoadjuvant therapy is the standard first-line treatment for patients with lymph node metastasis. Indeed, in rare cases where rectal cancer complications like obstruction or perforation occur, direct surgical intervention becomes necessary, and LPLN dissection can be performed simultaneously. However, accurately locating

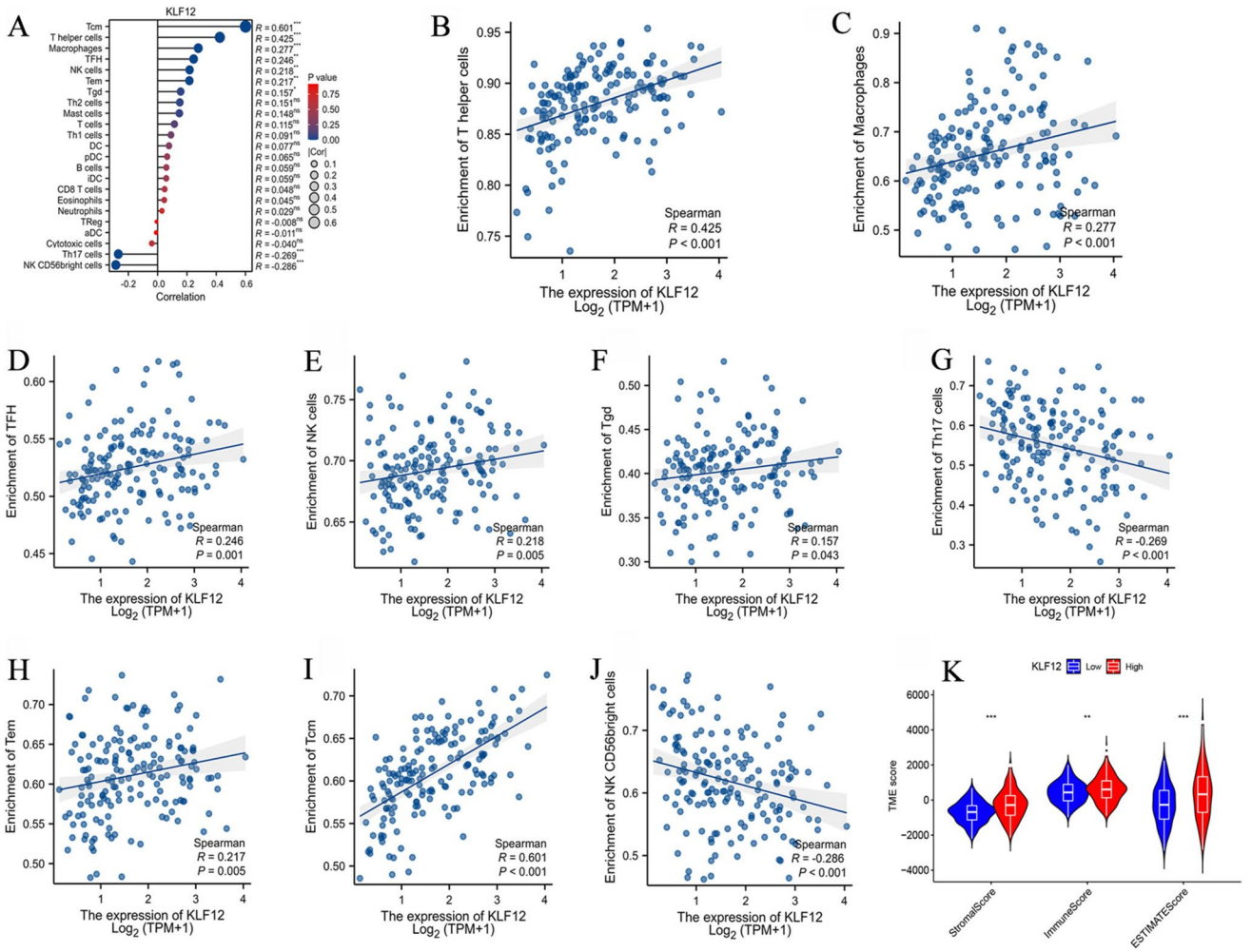

Fig. 5 Correlation between KLF12 expression and immune cell infiltration in rectal cancer. **A** Overview of significant differences in immune cell infiltration associated with KLF12 expression. KLF12 expression was significantly correlated with the infiltration of **B** T helper cells, **C** macrophages, **D** TFH cells, **E** NK cells, **F** Tgd cells, **G** Th17 cells, **H** Tem cells, **I** Tcm cells, and **J** CD56 bright cells. **K** KLF12 expression was able to effectively differentiate stromal score, immune score, and ESTIMATE score

and clearing the metastatic lymph nodes during dissection itself is quite challenging. As critical immune organs responsible for lymphocyte differentiation and maturation [16], metastatic lymph nodes play a key role in guiding surgical decisions, emphasizing the importance of precise LPLN diagnosis [17]. Thus, developing a simple and reliable diagnostic method for lymph node metastasis is of paramount importance.

Our study reveals that lymph node metastasis is marked by increased immune cell infiltration but diminished antigen-presenting capacity, potentially weakening anti-tumor immunity and promoting further metastasis. For the first time, we identify potential biomarkers in primary rectal tumor tissues that are associated with LPLN-M, offering novel biopsy indicators for its diagnosis. Integrating these biomarkers with imaging techniques could significantly improve the diagnostic accuracy of lymph node metastasis.

In this study, we first analyzed differences in immune cell infiltration between metastatic and non-metastatic lymph nodes, as well as between tumor tissues with and without lymph node metastasis. To identify predictive biomarkers for lymph node metastasis, we conducted mRNA sequencing (mRNA-seq) on tumor, adjacent, and lymph node tissues. Initially, we identified differentially expressed genes (DEGs) between tumor and adjacent tissues, followed by an analysis of DEGs in tumor and lymph node tissues between LPLN-N and LPLN-positive (LPLN-M) cases. The biological functions of these DEGs primarily involve DNA transcription, thereby influencing gene expression and protein synthesis. From these DEGs, we identified a shared gene set, termed the hub gene set, and highlighted KLF12 as a potential biomarker for predicting LPLN-M.

The Kruppel-like factor (KLF) family of transcription factors regulates key oncogenic processes, including

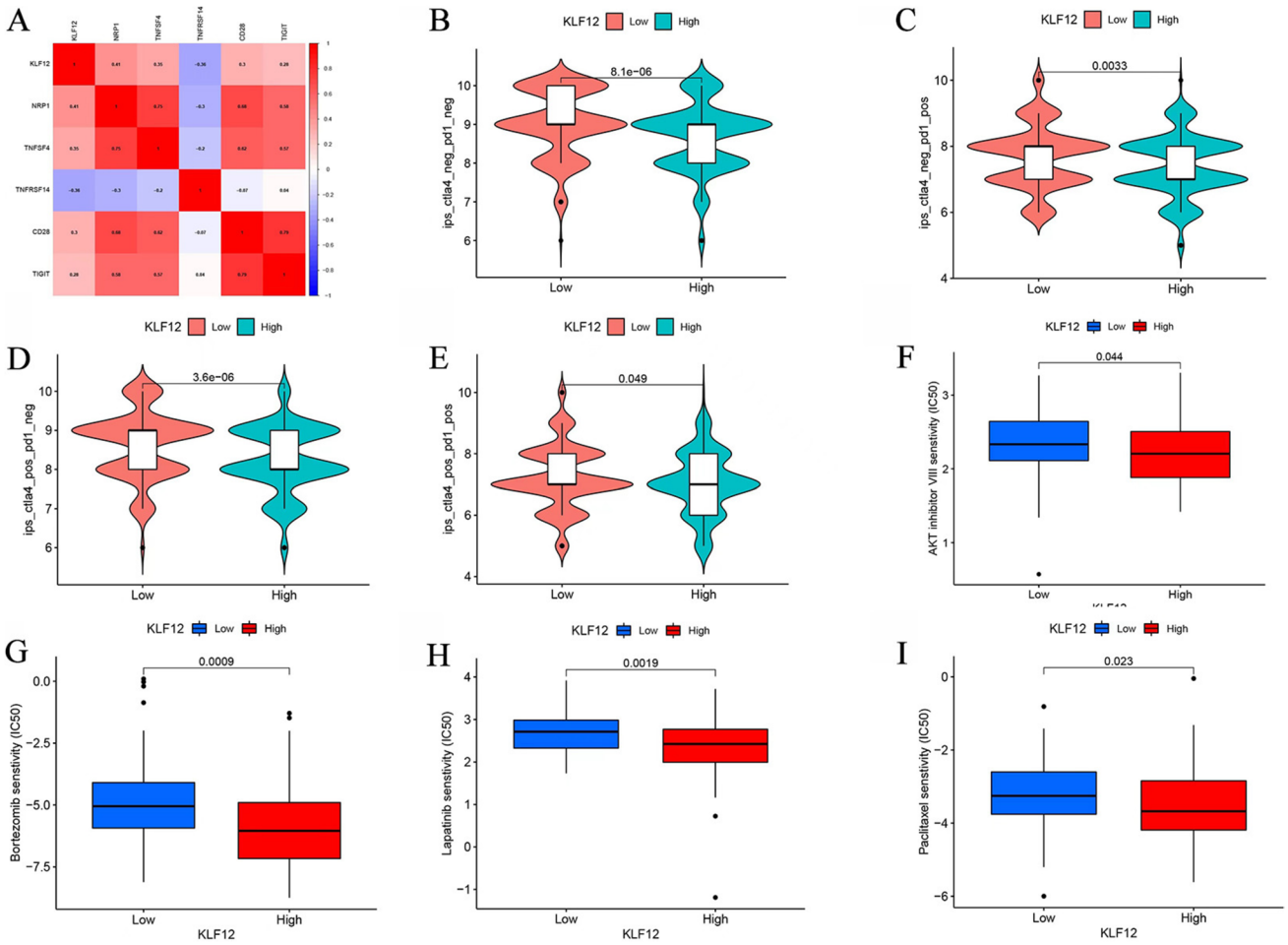

Fig. 6 Association between KLF12 expression and the tumor immune microenvironment. **A** KLF12 expression was correlated with multiple immune checkpoint molecules, including NRP1, TNFSF4, TNFRSF14, CD28, and TIGIT. **B**–**E** Immunophenoscore (IPS) analysis revealed that tumors with low KLF12 expression exhibited significantly lower IPS values across four categories: ips_ctla4_neg_pd1_neg, ips_ctla4_neg_pd1_pos, ips_ctla4_pos_pd1_neg, and ips_ctla4_pos_pd1_pos. **F**–**I** Scatter plots displaying correlations between KLF12 risk scores and IC50 values of chemotherapy drugs, with four drugs randomly selected for visualization

proliferation, survival, therapy resistance, motility, and invasiveness [18–20]. KLF12, originally identified as a transcriptional repressor of AP-2, has been implicated in multiple malignancies. Studies suggest that KLF12 promotes breast cancer cell proliferation by downregulating p21 transcription through both p53-dependent and independent pathways [21]. Conversely, in bladder cancer, KLF12 functions as a tumor suppressor by downregulating ENO2, a potential therapeutic target [22]. KLF12 also plays a critical role in poorly differentiated gastric cancer, highlighting its potential as a therapeutic target [23].

In our study, KLF12 exhibited robust diagnostic performance for LPLN-M, with an area under the curve (AUC) of 0.726. Analysis of disease-specific survival (DSS) events revealed a strong correlation between KLF12 expression and therapeutic response. Moreover, immunohistochemistry (IHC) findings demonstrated a significant association between KLF12 expression and disease-free survival (DFS) in rectal cancer patients. These findings indicate that KLF12 serves as a reliable biomarker for predicting rectal cancer prognosis and DFS in LPLN-M patients. However, KLF12 expression does not show a strong correlation with overall survival (OS), likely due to the multifactorial nature of OS, which is influenced by factors such as distant metastasis, treatment response, and comorbidities [24]. Additionally, the availability of effective salvage therapies for recurrence may reduce the impact of LPLN-M on OS [25].

Subsequent analyses of mRNA sequencing (mRNA-Seq) data, including Gene Set Enrichment Analysis (GSEA), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, revealed a strong association between immune infiltration, immunotherapy, and LPLN-M, particularly regarding PD-L1 expression and the PD-1 checkpoint mechanism in cancer. We further examined

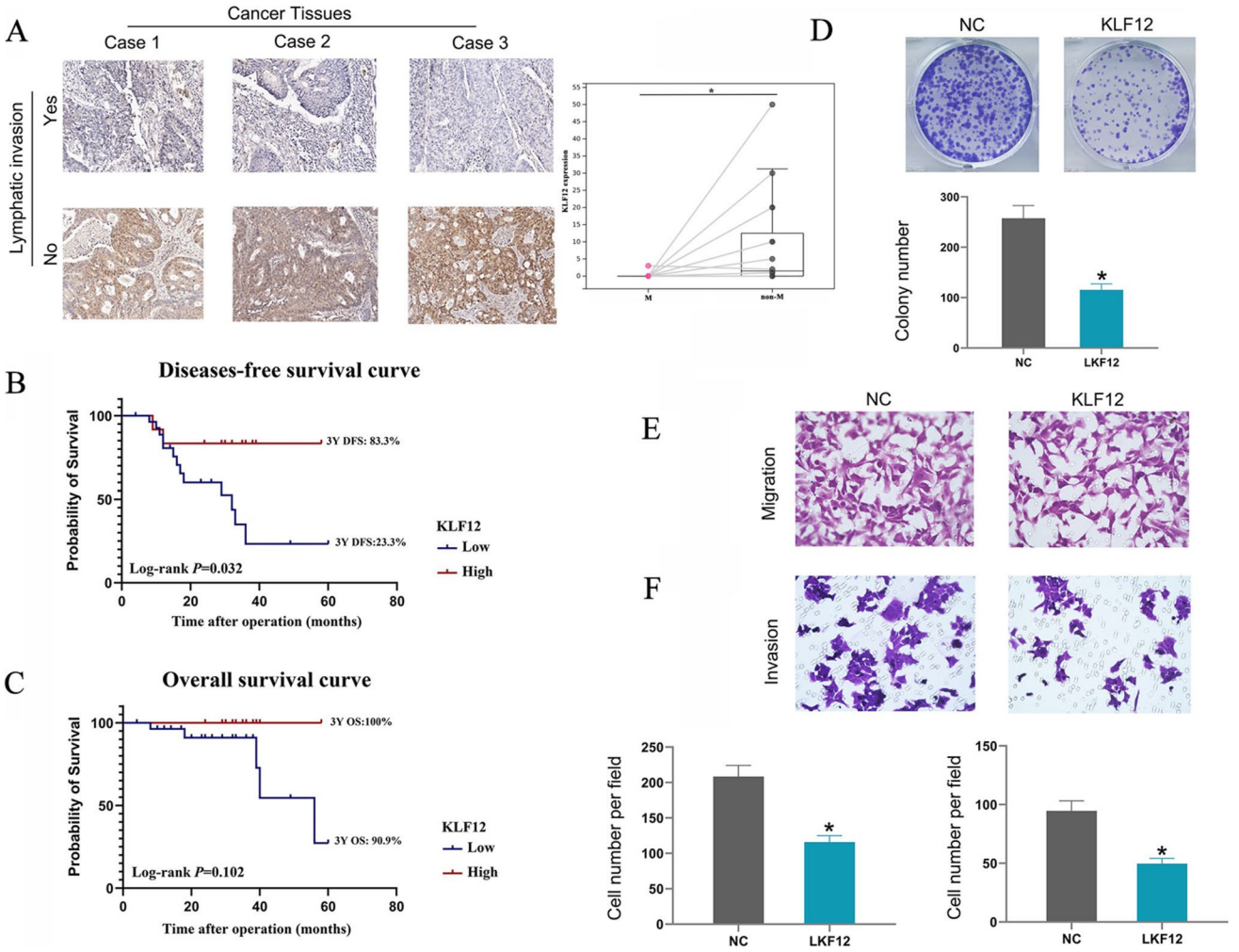

Fig. 7 Expression and biological function of KLF12 in rectal cancer. **A** Immunohistochemistry (IHC) staining and positive percentage analysis demonstrated significantly higher KLF12 expression in the LPLN-M positive group compared to the LPLN-M negative group. **B** Higher KLF12 expression was significantly associated with improved disease-free survival (DFS), **C** but not with overall survival (OS). Functional experiments showed that KLF12 overexpression significantly suppressed **D** cell proliferation, **E** migration, and **F** invasion in SW620 cells

the relationship between KLF12 expression and the tumor immune microenvironment. Our correlation analysis identified statistically significant associations between KLF12 expression and immune cell infiltration ($P < 0.05$). However, many correlation coefficients were below 0.3, suggesting that KLF12 functions within a broader regulatory network. Despite these modest correlations, we observed significant differences in immune cell infiltration, immune checkpoint expression, and immunophenoscore (IPS) between high and low KLF12 expression groups, suggesting that patients with high KLF12 expression may derive greater benefit from immunotherapy. These findings align with previous studies associating KLFs with immune cell regulation in cancer [26–28].

Functional assays demonstrated that KLF12 overexpression suppressed the proliferation, migration, and invasion of SW620 cells, highlighting its previously unrecognized potential as a therapeutic target for rectal cancer. However, our study has certain limitations. Due to the rarity of these specimens, our sample size is limited. In future studies, we aim to expand the sample size and develop a predictive model to validate KLF12's diagnostic performance. Additionally, since our study primarily focuses on immune infiltration and KLF12 function, we plan to refine experimental approaches and further investigate the molecular mechanisms underlying KLF12's role.

In conclusion, our study provides the first comprehensive molecular-level analysis of LPLN-M in rectal cancer, identifying KLF12 as a potential biomarker for predicting LPLN-M.

Table 1 Clinical characteristics of KLF12 in the LPLN-M positive group

Characteristic	*N*	KLF12		*P* value
		Low ($n=28$)	High ($n=12$)	
Sex				0.490
Male	20	13 (46.4)	7 (58.3)	
Female	20	15 (53.6)	5 (41.7)	
Age (years)				0.629
≥60	19	14 (50.0)	5 (41.7)	
<60	21	14 (50.0)	7 (58.3)	
Tumor location				0.418
Low rectum	27	20 (71.4)	7 (58.3)	
Middle rectum	13	8 (28.6)	5 (41.7)	
Tumor differentiation				0.411
Moderate	9	5 (17.9)	4 (33.3)	
Poor	31	23 (82.1)	8 (66.7)	
Tumor stage				0.341
T1–T2	6	3 (10.7)	3 (25.0)	
T3–T4	34	25 (89.3)	9 (75.0)	
Nodal stage				0.124
N0	11	10 (35.7)	1 (8.3)	
N1–N2	29	18 (64.3)	11 (91.7)	
LLN metastasis				0.209
Unilateral	37	27 (96.4)	10 (83.3)	
Bilateral	3	1 (3.6)	2 (16.7)	
LLN locations				1.000
Internal iliac and obturator metastases	35	24 (85.7)	11 (91.7)	
External or common iliac metastases	5	4 (14.3)	1 (8.3)	
Perineural invasion				0.157
No	26	16 (57.1)	10 (83.3)	
Yes	14	12 (42.9)	2 (16.7)	
Lymphatic invasion				0.007
No	28	16 (57.1)	12 (100.0)	
Yes	12	12 (42.9)	0 (0)	

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate None.

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