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Upregulation of CDKN2B in 5-fluorouracil-resistant colorectal cancer cells: a pilot study of its dual role and potential clinical relevance

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Research Article

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Abstract

Background

Resistance to 5-fluorouracil (5-FU) is almost inevitable during chemotherapy for advanced colorectal cancer (CRC). Early identification of resistant patients and overcoming resistance remain major clinical challenges.

Objective

This study aimed to investigate the role of CDKN2B in 5-FU resistance in CRC and to explore its potential as a predictive biomarker.

Methods

The 5-FU-resistant cell line HT-29/5FU-R was established by concentration-gradient induction. Differentially expressed genes were screened by transcriptome sequencing, and CDKN2B expression was verified by qPCR and Western blot. The expression, methylation status, prognostic value, and association with 5-FU response of CDKN2B were analyzed using the TCGA-COAD cohort (286 tumors and 41 normal tissues) and two GEO chemotherapy cohorts (GSE28702, n = 83; GSE72970, n = 124). CD8 + T-cell infiltration was evaluated using TIMER2.0.

Results

A total of 3,293 differentially expressed genes were identified (1,780 upregulated and 1,513 downregulated). CDKN2B was upregulated in resistant cells (fold change 2.31, $p = 0.0006$) and confirmed at the mRNA and protein levels. In TCGA-COAD, CDKN2B was significantly downregulated in tumor tissues compared with normal tissues ($p = 1.3\times10^{-23}$), and its expression was not associated with overall survival ($p = 0.26$). In GSE28702, non-responders showed higher CDKN2B expression, an association that reached significance in primary-tumor biopsies ($p = 0.048$); this association was not replicated in the validation cohort GSE72970. CDKN2B expression was negatively correlated with promoter methylation ($r = -0.245$, $p < 0.001$) and modestly associated with CD8 + T-cell infiltration.

Conclusion

CDKN2B is upregulated in 5-FU-resistant CRC and is epigenetically regulated by promoter methylation. CDKN2B is not a prognostic marker, but it is a candidate predictive biomarker of fluoropyrimidine response that requires prospective validation. CDKN2B may help identify patients who are unlikely to

benefit from 5-FU-based chemotherapy and guide the selection of later-line treatment, and it may also serve as a candidate target for overcoming 5-FU resistance.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide, with high morbidity and mortality [1,2]. For advanced and metastatic CRC, treatment options remain limited, and combination chemotherapy based on 5-fluorouracil (5-FU), together with targeted agents, is still the mainstay of treatment [3]. However, almost all patients eventually develop primary or acquired resistance to 5-FU during treatment, which reduces chemotherapy efficacy, increases the risk of recurrence and metastasis, and leads to treatment failure and poor prognosis. In clinical practice, two questions are therefore of particular concern: how to identify patients who are resistant to 5-FU at an early stage, so that later-line regimens can be selected in a timely manner; and how to overcome 5-FU resistance itself. Although several factors have been reported to modulate 5-FU resistance in CRC, including aberrant expression of microRNAs [4], downregulation of connexin 43 (CX43) [5], and dysbiosis of the gut microbiota [6], the key molecular mechanisms and regulatory targets remain incompletely understood, and sensitive and specific biomarkers for predicting chemotherapy response are still lacking. Identifying reliable biomarkers of 5-FU resistance and clarifying their mechanisms is therefore of direct clinical significance, both for patient stratification and for the development of strategies to reverse resistance.

Cell cycle dysregulation is an important driver of malignant phenotypes and chemotherapy resistance in tumors. In CRC, the link between cell cycle regulation and chemotherapy efficacy has long been recognized: chronomodulated delivery of 5-FU and oxaliplatin according to circadian cell cycle rhythms improved treatment outcomes in randomized clinical trials [7], and inhibition of the cell cycle kinase CDK1 was recently shown to overcome oxaliplatin resistance in preclinical models [8]. However, whether 5-FU resistance in CRC is associated with cell cycle regulators, particularly cyclin-dependent kinase inhibitors (CKIs), remains largely unknown. CDKN2B (also known as p15INK4b) is a member of the cyclin-dependent kinase inhibitor family. It regulates the G1/S transition of the cell cycle and helps maintain the balance between cell proliferation and apoptosis. Genetic and epigenetic alterations of CDKN2B have been reported in various tumors [9,10]. CDKN2B has also been shown to regulate cell proliferation, invasion, and drug sensitivity in several cancer types, including hepatocellular carcinoma and glioma [11, 12]. However, the expression pattern, prognostic value, and association with chemotherapy response of CDKN2B in 5-FU-resistant CRC have not been systematically investigated. To our knowledge, the relationship between CDKN2B and 5-FU resistance in CRC has not been reported so far.

In this study, we established the 5-FU-resistant human CRC cell line HT-29/5FU-R by concentration gradient induction, and screened for differentially expressed genes by transcriptome sequencing. CDKN2B was identified as a candidate molecule, and its upregulation in resistant cells was confirmed by qPCR and Western blot. We then analyzed the expression, promoter methylation, prognostic significance, and association with 5-FU chemotherapy response of CDKN2B using the TCGA-COAD cohort and two

GEO chemotherapy cohorts, and evaluated its relationship with immune cell infiltration. The aim of this study is to evaluate the potential of CDKN2B as a predictive biomarker for 5-FU resistance in CRC, which may help identify resistant patients and guide the selection of later-line treatment, and to provide a candidate molecular target for overcoming 5-FU resistance.

2.Materials and Methods

2.1 Primary Materials

The human colorectal cancer cell line HT-29 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). 5-Fluorouracil (5-FU) was purchased from MedChemExpress (Monmouth Junction, NJ, USA; purity 99.93%). McCoy's 5A medium, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Pricella (Wuhan, China). The CCK-8 reagent was purchased from Dojindo (Kumamoto, Japan). Reverse transcription and qPCR kits were purchased from Accurate Biology (Hunan, China). Primers were synthesized by Tsingke Biotechnology (Beijing, China). The CDKN2B primary antibody (Rabbit, 1:1000) was purchased from Proteintech (Wuhan, China), and the HRP-conjugated secondary antibody (Goat Anti-Rabbit, 1:10000) was from Zhongshan Golden Bridge Biotechnology (Beijing, China).

2.2 Establishment of the 5-FU-Resistant Cell Line HT-29/5FU-R

HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃ in a humidified incubator with 5% CO2.The 5-FU-resistant cell line HT-29/5FU-R was established by stepwise escalation of FU concentration. Briefly, parental HT-29 cells were exposed to 5-FU starting at one-fifth of the parental IC50. The surviving cells were passaged, and the drug concentration was gradually increased. This process was continued until the cells proliferated stably at the parental IC50 concentration; the resulting resistant cells were named HT-29/5FU-R.

2.3 Evaluation of 5-FU Resistance by CCK-8 Assay

Cells were seeded in 96-well plates at 5×10^3 cells per well (100μL medium per well). After 24h of adhesion, the medium was replaced with fresh medium containing 5-FU at serial concentrations (0, 4, 8, 16, 32, 64, 128, and 256μL, followed by incubation for 72h. CCK-8 reagent (10μL) was then added to each well. After incubation at 37°C for 2h, the absorbance at 450 nm was measured with a microplate reader. Cell viability (%) = (A treated − A_blank)/(A_control − A_blank) ×100%. The IC50 values were calculated using GraphPad Prism 9.0. All experiments were performed in triplicate. 5-FU sensitivity was compared between parental and resistant cells, and the resistance index was calculated.

2.4 Transcriptome Analysis

Parental and resistant cells were seeded at 1×10^5 cells per dish and cultured for 48h. Total RNA was extracted, and its purity and integrity were examined. Transcriptome sequencing was performed on an

Illumina platform. After quality control, clean reads were aligned to the human reference genome (GRCh38). Differentially expressed genes (DEGs) between the two groups were screened using DESeq2, with thresholds of |log2 fold change|>1 and adjusted $p < 0.05$. Gene Ontology (GO) enrichment analysis was then performed for the DEGs.

2.5 RNA Extraction and qPCR

Total RNA was extracted with TRIzol reagent and reverse-transcribed into cDNA

according to the manufacturer's instructions. qPCR was performed using the SYBR Green method, with β-actin as the internal reference. Relative expression was calculated by the 2^−ΔΔCt method. The primer sequences were as follows: CDKN2B forward 5'-GCCTAGAGGGAATTCGAACGA-3' and reverse 5'-TGCGTATGCAGCGATACCAC-3'; β-actin forward 5'-CATTGCCGACAGGATGCAG-3' and reverse 5'-AAGCATTTGCGGTGGACGAT-3'.

2.6 Western Blot Analysis

Cells were lysed in RIPA buffer containing protease inhibitors, and protein concentrations were determined by the BCA assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with the CDKN2B primary antibody (1:1000) overnight at 4°C, followed by the HRP-conjugated secondary antibody (1:10000). GAPDH was used as the loading control. Bands were visualized by enhanced chemiluminescence, and band intensities were quantified using ImageJ software.

2.7 Database Analysis

2.7.1 TCGA data analysis

Gene expression data (RNA-seq, HiSeqV2, RSEM normalized, log2(x + 1)) and clinical information of colon adenocarcinoma (COAD) were downloaded from the UCSC Xena platform (https://xenabrowser.net). A total of 286 primary tumor and 41 adjacent normal samples were included. CDKN2B expression was compared between tumor and normal tissues, and 26 paired tumor-normal samples were analyzed separately. Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic value of CDKN2B. The associations of CDKN2B expression with AJCC stage, age, and gender were also examined. For survival analysis, patients were divided into high- and low-expression groups according to the median CDKN2B level. Overall survival (OS) was estimated by the Kaplan-Meier method, and univariate and multivariate Cox proportional hazards regression was performed.

2.7.2 GEO data analysis

Two independent cohorts, GSE28702 and GSE72970, were obtained from the Gene Expression Omnibus(https://www.ncbi.nlm.nih.gov/geo/) (GEO). GSE28702 contains 83 biopsy samples from unresectable colorectal cancer patients before mFOLFOX6 treatment, with response annotated as

responder (n = 42) or non-responder (n = 41). GSE72970 contains 124 metastatic colorectal cancer patients treated with fluoropyrimidine-based regimens, with response evaluated by RECIST (CR, n = 8; PR, n = 55; SD, n = 42; PD, n = 19) and with progression-free survival (PFS) and OS data available. Both datasets were profiled on the Affymetrix HG-U133 Plus 2.0 platform (GPL570), and CDKN2B expression was represented by probe set 207530_s_at. Objective response rate (ORR) and progressive disease (PD) rate were compared between high- and low-expression groups (median split).

2.7.3 DNA Methylation Analysis of CDKN2B

The DNA methylation profile of the CDKN2B locus in TCGA-COAD was analyzed

using MethSurv(https://biit.cs.ut.ee/methsurv/)[13], based on Illumina Human

Methylation450 data with matched gene expression and clinicopathological annotations.

Spearman correlation analysis was used to assess associations between CpG-site

methylation, CDKN2B expression, and clinicopathological variables.

2.7.4 Tumor Immune Infiltration Analysis

The correlation between CDKN2B expression and CD8 + T-cell infiltration in TCGA-COAD (n = 456) was evaluated using TIMER2.0 (http://timer.cistrome.org/) [14] with multiple deconvolution algorithms (TIMER, EPIC, MCP-counter, CIBERSORT, CIBERSORT-ABS, quan TIseq, xCell, ABIS, ConsensusTME, ImmuCellAI, and TIDE). Partial Spearman correlation was computed for each algorithm, and $p \leq 0.05$ was considered significant.

2.8 Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (IBM, USA) and R software (version 4.3.0). Continuous data from in vitro experiments are presented as mean ± SD, and each experiment was independently repeated three times; two-group comparisons were performed using the Student's t-test. For transcriptome analysis, differentially expressed genes were identified using DESeq2, and *p* values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure. The reliability of cluster analysis was evaluated by the bootstrap method with 1000 resamplings. Principal component analysis (PCA) was used to visualize the overall expression differences between groups. For public database analyses, continuous variables were compared between two groups using the Mann-Whitney U test, paired samples using the Wilcoxon signed-rank test, and multiple groups using the Kruskal-Wallis test. Survival curves were generated by the Kaplan-Meier method and compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using the Cox proportional hazards regression model. Categorical variables, including objective response rate and progressive disease rate, were compared using Fisher's exact test, and the ordered trend across RECIST categories was tested after scoring CR < PR < SD < PD. Spearman correlation analysis was applied to assess the

relationships between CDKN2B expression, CpG-site methylation, and immune infiltration. All statistical tests were two-tailed, and a p value < 0.05 was considered statistically significant.

3. Results

3.1 Construction and validation of the HT-29/5FU-R resistant cell line

3.1.1 5-FU sensitivity of colorectal cancer cell lines

To select a suitable parental cell line, the sensitivity of three colorectal cancer cell lines (HT-29, HCA7, and RKO) to 5-FU was measured by CCK-8 assay after 72 h of treatment. The three cell lines differed markedly in 5-FU sensitivity (Fig. 1A-C): HT-29 was the most sensitive (IC50 = 19.49μM), RKO showed intermediate sensitivity (IC50 = 40.22μM), and HCA7 was the most resistant (IC50 = 75.11μM). HT-29 was therefore chosen as the parental line for establishing the resistant model.

3.1.2 Induction and verification of HT-29/5FU-R cells

The HT-29/5FU-R resistant line was established by stepwise escalation of 5-FU concentration, starting from one-tenth of the parental IC50, over 4–5 months. The IC50 of HT-29/5FU-R cells reached 78.6μM, about 4.26-fold higher than that of parental cells (18.47μM), with a clear rightward shift of the dose-response curve (Fig. 1D), indicating a stable resistant phenotype.

3.1.3 Transcriptome sequencing and quality assessment

Parental and HT-29/5FU-R cells in the logarithmic growth phase were subjected to transcriptome sequencing, with three biological replicates per group. Pearson correlation analysis showed tight clustering of replicates within each group and a clear separation between the two groups (Fig. 1E). In PCA, PC1 and PC2 explained 71.05% and 9.12% of the variance, respectively, and the two groups were completely separated along PC1 (Fig. 1F), indicating good reproducibility and substantial transcriptional differences. Among the differentially expressed genes, CDKN2B was significantly upregulated in HT-29/5FU-R cells (fold change = 2.31, p = 0.0006; Fig. 1G).

3.2 Screening of differentially expressed genes and resistance-related pathways

3.2.1 Identification of DEGs and CDKN family expression

Differentially expressed genes (DEGs) between HT-29/5FU-R and parental cells were screened with the criteria of |log2FC| > 1 and adjusted p < 0.05. A total of 3,293 DEGs were identified, including 1,780 upregulated and 1,513 downregulated genes (Fig. 2A). Heatmap analysis confirmed the overexpression

of CDKN2B in resistant cells, and other CDKN family members, such as CDKN1A and CDKN2A, showed a similar upregulation trend (Fig. 2C).

3.2.2 GO and KEGG enrichment analysis

GO enrichment analysis showed that the DEGs were mainly involved in cell cycle regulation, viral transcription, cellular component organization, development, and macromolecule biosynthesis, with cell cycle regulation being the most prominently enriched category (Fig. 2D). KEGG analysis showed enrichment in the cell cycle, p53 signaling, apoptosis, FoxO signaling, fatty acid degradation, MAPK signaling, and cAMP signaling pathways, among which the cell cycle, p53 signaling, apoptosis, and fatty acid degradation pathways were the most significant (Fig. 2B).

3.2.3 Validation of CDKN2B upregulation

The upregulation of CDKN2B was verified at the mRNA and protein levels. Under drug-free conditions, qPCR showed significantly higher CDKN2B mRNA in HT-29/5FU-R cells than in parental cells ($p < 0.01$; Fig. 2E), and Western blot confirmed increased CDKN2B protein expression (Fig. 2F). The results of transcriptome sequencing, qPCR, and Western blot were consistent.

3.3 Clinical relevance of CDKN2B in TCGA and GEO cohorts

3.3.1 CDKN2B is downregulated in colorectal cancer tissues but relates to tumor stage

We next examined CDKN2B expression in clinical samples using the TCGA-COAD cohort. Unexpectedly, CDKN2B expression was significantly lower in tumor tissues than in normal tissues (286 vs 41 samples, $P = 1.3\times10^{-23}$; Fig. 3A), and this was confirmed in 26 paired tumor-normal samples ($P = 6.0\times10^{-8}$; Fig. 3B). CDKN2B expression distinguished tumor from normal tissue with an AUC of 0.983 (Fig. 3C). Among clinicopathological factors, CDKN2B expression differed across AJCC stages ($P = 0.018$; Fig. 3E), but was not related to age ($P = 0.40$; Fig. 3F) or gender ($P = 0.17$; Fig. 3G). These findings, together with the promoter hypermethylation of CDKN2B in tumors (Section 3.3.4), indicate that CDKN2B is largely silenced during colorectal carcinogenesis.

3.3.2 CDKN2B expression is not associated with overall survival

Kaplan-Meier analysis showed no significant difference in overall survival between the high- and low-CDKN2B groups (median 70.1 vs 99.9 months, log-rank $P = 0.26$; Fig. 3D). Cox regression confirmed that CDKN2B was not an independent prognostic factor (univariate HR = 1.31, 95% CI 0.81–2.12, $P = 0.264$; multivariate HR = 1.08, 95% CI 0.66–1.77, $P = 0.770$; Fig. 3H), whereas AJCC stage and age

independently predicted survival. CDKN2B is therefore not a prognostic marker for the natural course of CRC.

3.3.3 High CDKN2B expression shows a consistent trend toward chemoresistance in two GEO cohorts

We further asked whether CDKN2B expression is related to chemotherapy response. In GSE28702 (mFOLFOX6, n = 83), CDKN2B expression was comparable between responders and non-responders in the full cohort (P = 0.53; Fig. 4A); however, in primary-tumor biopsies (n = 56), non-responders showed significantly higher CDKN2B levels (p = 0.048; Fig. 4B). The objective response rate was lower in the CDKN2B-high group (47.6% vs 53.7%, P = 0.66; in the primary subgroup 42.9% vs 67.9%, p = 0.11; Fig. 4C). In the validation cohort GSE72970 (fluoropyrimidine-based regimens, n = 124), expression was again similar between response groups (p = 0.64; Fig. 4E); however, no significant association with treatment response was observed: the objective response rate (54.8% vs 46.8%, p = 0.47) and the progressive disease rate (9.7% in CDKN2B-high vs 21.0% in CDKN2B-low tumors, p = 0.13; Fig. 4F) did not differ significantly between the two groups, and progression-free and overall survival were also comparable (p = 0.50 and 0.82; Fig. 4G, 4H). Thus, the association between high CDKN2B expression and non-response observed in GSE28702, particularly in primary tumors, was not replicated in GSE72970. The predictive value of CDKN2B therefore remains hypothesis-generating and requires validation in prospective cohorts.

3.3.4 Promoter methylation is associated with CDKN2B expression

MethSurv analysis of the CDKN2B locus in TCGA-COAD showed that CDKN2B expression differed significantly across sample types ($p = 2.33\times10^{-59}$) and was associated with gender, histological type, and tumor stage (all $p < 0.05$; Fig. 5A). Notably, methylation at one promoter CpG site was significantly negatively correlated with CDKN2B expression ($r = -0.245$, $p < 0.001$), indicating that promoter methylation participates in the epigenetic regulation of CDKN2B in CRC.

3.3.5 CDKN2B expression correlates with CD8 + T-cell infiltration

TIMER2.0 analysis revealed a modest, algorithm-dependent association between CDKN2B expression and CD8 + T-cell infiltration (Fig. 5B). Positive correlations were observed with TIMER, ConsensusTME, TIDE, and xCell (central memory) estimates, whereas EPIC and naive CD8 + T-cell estimates showed negative correlations, suggesting a potential link between CDKN2B and the tumor immune microenvironment.

4. Discussion

5-Fluorouracil (5-FU) remains the backbone of chemotherapy for colorectal cancer (CRC). FOLFOX and FOLFIRI are still the standard first-line regimens for advanced disease, but nearly all patients will eventually face primary or acquired resistance to 5-FU, which is a major cause of treatment failure [15, 16]. Known mechanisms, such as altered drug metabolism and thymidylate synthase amplification, explain only part of the clinical resistance phenotypes [17]. In daily practice, two questions matter most: how to identify patients who are unlikely to benefit from fluoropyrimidine-based chemotherapy before treatment, and how to overcome resistance once it develops. In this study, we established a 5-FU-resistant cell line (HT-29/5FU-R, 4.26-fold increase in resistance) and identified CDKN2B as a candidate regulator of 5-FU resistance through transcriptome sequencing and public database analysis.

CDKN2B encodes p15INK4b, a member of the INK4 family, which inhibits CDK4/6, prevents Rb phosphorylation, and arrests cells at the G1/S transition [18]. The gene is located in the 9p21 tumor susceptibility locus and is frequently inactivated by methylation or mutation in human tumors [19]. Its role in 5-FU resistance in CRC has not been reported. We found that CDKN2B was significantly upregulated in 5-FU-resistant cells, with consistent results at the mRNA and protein levels. The underlying mechanism is plausible: 5-FU kills mainly proliferating cells in S phase [20]. When CDKN2B is upregulated, it blocks the G1-to-S transition, so fewer cells enter S phase and escape the S-phase-specific cytotoxicity of 5-FU [21, 22]. In addition, prolonged cell cycle arrest gives cells time to activate DNA damage repair, which attenuates 5-FU-induced damage and promotes survival[23]. Similar cell cycle-dependent protection has been described for CDKN1A/p21 [24].

We also observed that CDKN1A and CDKN2A were upregulated together with CDKN2B in the resistant cells. CDKN2A and CDKN2B are clustered at 9p21 and are often co-regulated by epigenetic mechanisms, while CDKN1A inhibits a broader range of CDKs [24, 25]. Coordinated upregulation of these CKIs may form a redundant inhibitory network that stabilizes the resistant phenotype of HT-29/5FU-R cells [26].

An unexpected finding of this study is the dual expression pattern of CDKN2B. In the TCGA-COAD cohort, CDKN2B was significantly downregulated in tumor tissues compared with normal mucosa ($P = 1.3\times10^{-23}$), consistent with its tumor-suppressive role. Mechanistically, promoter hypermethylation is a well-established epigenetic mechanism for CDKN2B silencing [27, 28], which supports our observed negative correlation between CDKN2B methylation and expression($r = -0.245$, $p < 0.001$) in CRC.

In contrast, CDKN2B was upregulated 2.31-fold in the 5-FU-resistant cell line, and in GSE28702, tumors with higher CDKN2B expression tended to respond worse to fluoropyrimidine-based chemotherapy, an association that reached significance in primary-tumor biopsies but was not replicated in the GSE72970 cohort. We therefore propose a context-dependent, dual role of CDKN2B in CRC: it is silenced by methylation during carcinogenesis, but under the selective pressure of chemotherapy, cells that re-express CDKN2B gain a survival advantage because cell cycle arrest protects them from S-phase-specific killing. A similar functional duality has been described for p53 and other cell cycle regulators [29]. To our knowledge, this is the first study to link CDKN2B to 5-FU resistance in CRC.

Equally important, our data indicate what CDKN2B is not associated with overall survival in TCGA-COAD, nor with progression-free or overall survival in GSE72970, so it should not be regarded as a prognostic marker. This does not reduce its clinical value. For patients with advanced CRC, the more urgent question is not how the disease will progress naturally, but whether the tumor will respond to chemotherapy. A biomarker can be predictive of treatment response without being prognostic, as exemplified by KRAS status and anti-EGFR therapy. CDKN2B may belong to this category, although the current evidence is hypothesis-generating: the association between high CDKN2B expression and non-response reached significance only in the primary-tumor subgroup of GSE28702 (P = 0.048) and was not replicated in GSE72970.

These findings have direct clinical implications. CDKN2B expression in biopsy or surgical specimens can be measured by immunohistochemistry or qPCR, both of which are routine techniques in pathology departments. If confirmed in prospective cohorts, CDKN2B could be used before first-line treatment to help stratify patients: those with high CDKN2B expression, who are less likely to respond to fluoropyrimidine-based regimens, might be considered for intensified combination regimens, early enrollment in clinical trials, or earlier use of later-line agents such as regorafenib or TAS-102; patients with low expression could proceed with standard FOLFOX or FOLFIRI with greater confidence. Combining CDKN2B expression with its promoter methylation status may further improve predictive accuracy, because methylation determines whether the gene can be re-expressed under treatment pressure. Our immune infiltration analysis additionally suggested that CDKN2B-high tumors are not necessarily immune-excluded, which means that cell cycle-mediated resistance may coexist with an inflamed microenvironment and does not exclude immunotherapy-based strategies; this observation was algorithm-dependent and needs validation by immunohistochemistry.

Our results also raise a caution for the use of CDK4/6 inhibitors in CRC. Because CDKN2B mediates resistance by inhibiting CDK4/6, pharmacological CDK4/6 inhibition in CDKN2B-high tumors may mimic this effect, strengthen G1 arrest, and theoretically blunt 5-FU efficacy. In contrast, in CDKN2B-low tumors with hyperactive CDK4/6, these inhibitors might have a different impact on chemosensitivity. This hypothesis requires experimental validation but should be considered in the design of future trials combining cell cycle inhibitors with fluoropyrimidines.

This study has several limitations. First, the resistance model was established in a single cell line, and confirmation in additional CRC cell lines and in vivo models such as xenografts and organoids is needed. Second, the TCGA and GEO analyses were retrospective, the cohorts were modest in size, and the chemotherapy regimens included oxaliplatin- or irinotecan-containing combinations rather than 5-FU monotherapy. Third, microarray data from a single probe set may not fully reflect protein levels, and the methylation and immune infiltration analyses show correlations rather than causation. Despite these limitations, the core finding of this study rests on convergent evidence: CDKN2B upregulation was identified by transcriptome sequencing, independently verified at the mRNA and protein levels, and supported by a consistent direction of association in an independent clinical cohort. Prospective validation in larger, well-annotated cohorts, confirmation of protein expression in clinical specimens, and

functional dissection of the downstream pathways (including possible crosstalk with p53 and MAPK signaling) will be important next steps before CDKN2B can inform treatment decisions.

In conclusion, this study reveals a dual role of CDKN2B in colorectal cancer: it is silenced by promoter methylation during carcinogenesis but re-expressed under chemotherapeutic stress, where it contributes to 5-FU resistance, most likely by arresting cells in G1 and protecting them from S-phase-specific killing. CDKN2B is not a prognostic marker, but it is a candidate predictive biomarker of fluoropyrimidine response. If validated prospectively, CDKN2B, alone or combined with its methylation status, may help identify patients unlikely to benefit from standard chemotherapy and guide the selection of alternative strategies, which is the clinical problem that motivated this study.

Abbreviations

5-FU
5-Fluorouracil
HT-29/5FU-R
5-FU-resistant HT-29 cell line
CCK-8
Cell Counting Kit-8
CDKN1A
Cyclin-dependent kinase inhibitor 1A
CDKN2A
Cyclin-dependent kinase inhibitor 2A
CDKN2B
Cyclin-dependent kinase inhibitor 2B
CDK4/6
Cyclin-dependent kinase 4/6
CI
Confidence Interval
cDNA
complementary DNA
CR
Complete Response
CRC
Colorectal Cancer
CX43
Connexin 43
DCR
Disease Control Rate
DEGs
Differentially Expressed Genes

FC
Fold Change
FBS
Fetal Bovine Serum
GEO
Gene Expression Omnibus
GO
Gene Ontology
HR
Hazard Ratio
IC50
Half-maximal Inhibitory Concentration
KEGG
Kyoto Encyclopedia of Genes and Genomes
miRNAs
microRNAs
ORR
Objective Response Rate
OS
Overall Survival
PCA
Principal Component Analysis
PD
Progressive Disease
PR
Partial Response
Rb
Retinoblastoma
RECIST 1.1
Response Evaluation Criteria in Solid Tumors 1.1
SD
Stable Disease
TAS-102
trifluridine/tipiracil
TCGA-COAD
The Cancer Genome Atlas-Colon Adenocarcinoma
qPCR
quantitative real-time PCR

Declarations

Ethical Statement

It includes only cell line experiments and analyses of publicly available, de-identified data, with no direct involvement of human participants.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Yuqin Zhang conducted experiments, established the HT-29/5FU-R cell line, validated findings, and drafted the manuscript. Mengxing Diao proofread the manuscript, offered clinical insights, and revised clinical content.Liping Wang handled TCGA and GEO data analysis, contributed to writing and revisions, and funded the study. Corresponding author.

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The linguistic polishing of this manuscript was assisted by AI tools. All experimental design, data analysis and manuscript writing were independently completed by the authors. We thank the staff of the laboratory for their technical support during the cell experiments.

Data Availability

Raw sequencing datasets analyzed during this study are available in the TCGA database and the GEO repository (accession numbers: GSE28702, GSE72970). The cellular experimental datasets generated in our laboratory are available from the corresponding author upon reasonable request. No custom code was generated in this research.

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Figures

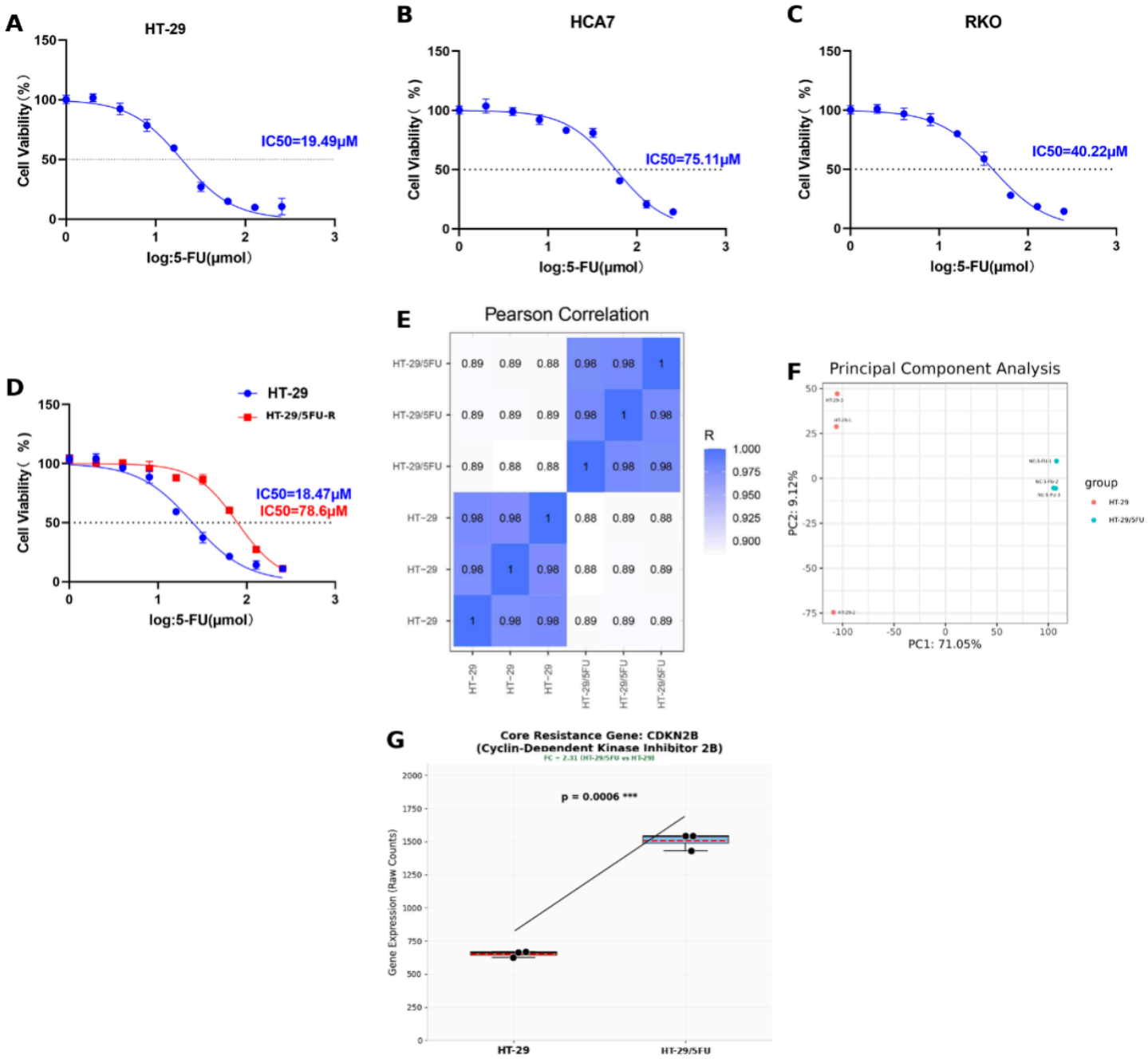

Figure 1

Establishment of 5-FU-resistant colorectal cancer cells and identification of CDKN2B.

(A-C) Dose-response curves of three parental colorectal cancer cell lines treated with 5-FU for 72 h (CCK-8 assay). (D) 5-FU sensitivity of parental HT-29 and resistant HT-29/5FU-R cells (IC_{50} fold change = 4.26). (E) Pearson correlation analysis of biological replicates. (F) Principal component analysis (PCA) of parental and resistant cells. (G) Volcano plot of transcriptome sequencing data; CDKN2B was upregulated in HT-29/5FU-R cells (fold change = 2.31, p = 0.0006). Data are presented as mean ± SD. ***p< 0.001.

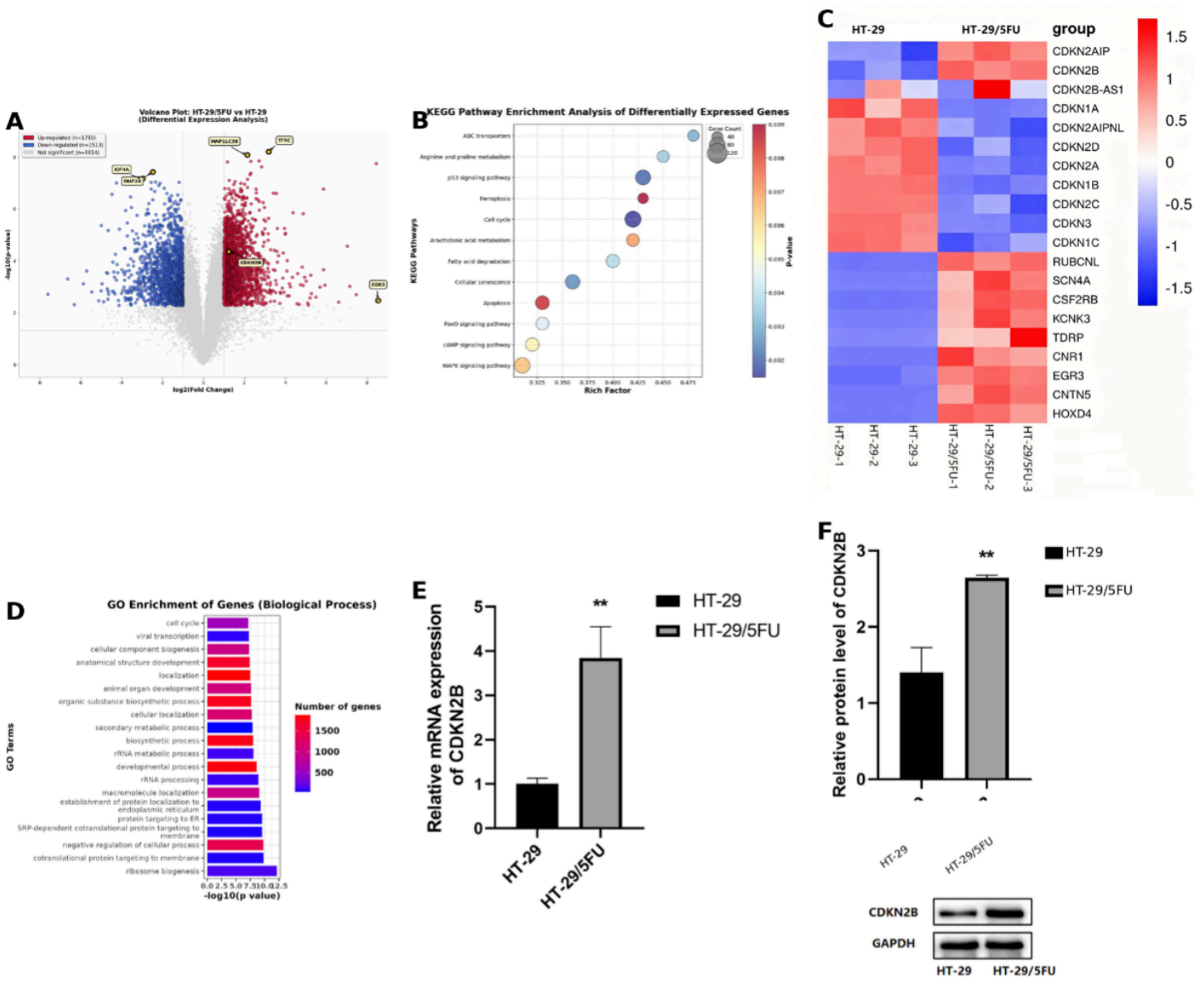

Figure 2

Transcriptomic profiling and validation of elevated CDKN2B in 5-FU-resistant HT-29 cells.

(A) Volcano plot showing differentially expressed genes (DEGs) between HT-29/5FU-R and parental HT-29 cells. X-axis: $\log_2$(fold change); Y-axis: -$\log_{10}$(P-value). Red, significantly upregulated genes; blue, significantly downregulated genes; gray, non-significant genes. CDKN2B and other key genes are marked. (B) KEGG enrichment analysis of DEGs. X-axis: Rich factor; Y-axis: enriched pathways. Circle size represents gene count, and color indicates P-value. (C) Heatmap showing expression of CDKN family genes and related regulators. Red: high expression; blue: low expression. (D) GO biological process enrichment of DEGs. X-axis: -$\log_{10}$(P-value); Y-axis: GO terms. Bar length and color intensity represent gene numbers enriched in each term. (E) qPCR verification of CDKN2B mRNA. CDKN2B mRNA was markedly upregulated in HT-29/5FU-R cells. (F) Western blot detection of CDKN2B protein. CDKN2B

protein expression increased in HT-29/5FU-R cells. GAPDH served as internal reference. Data are shown as mean±SD (n=3). **p < 0.01 vs. HT-29 group.

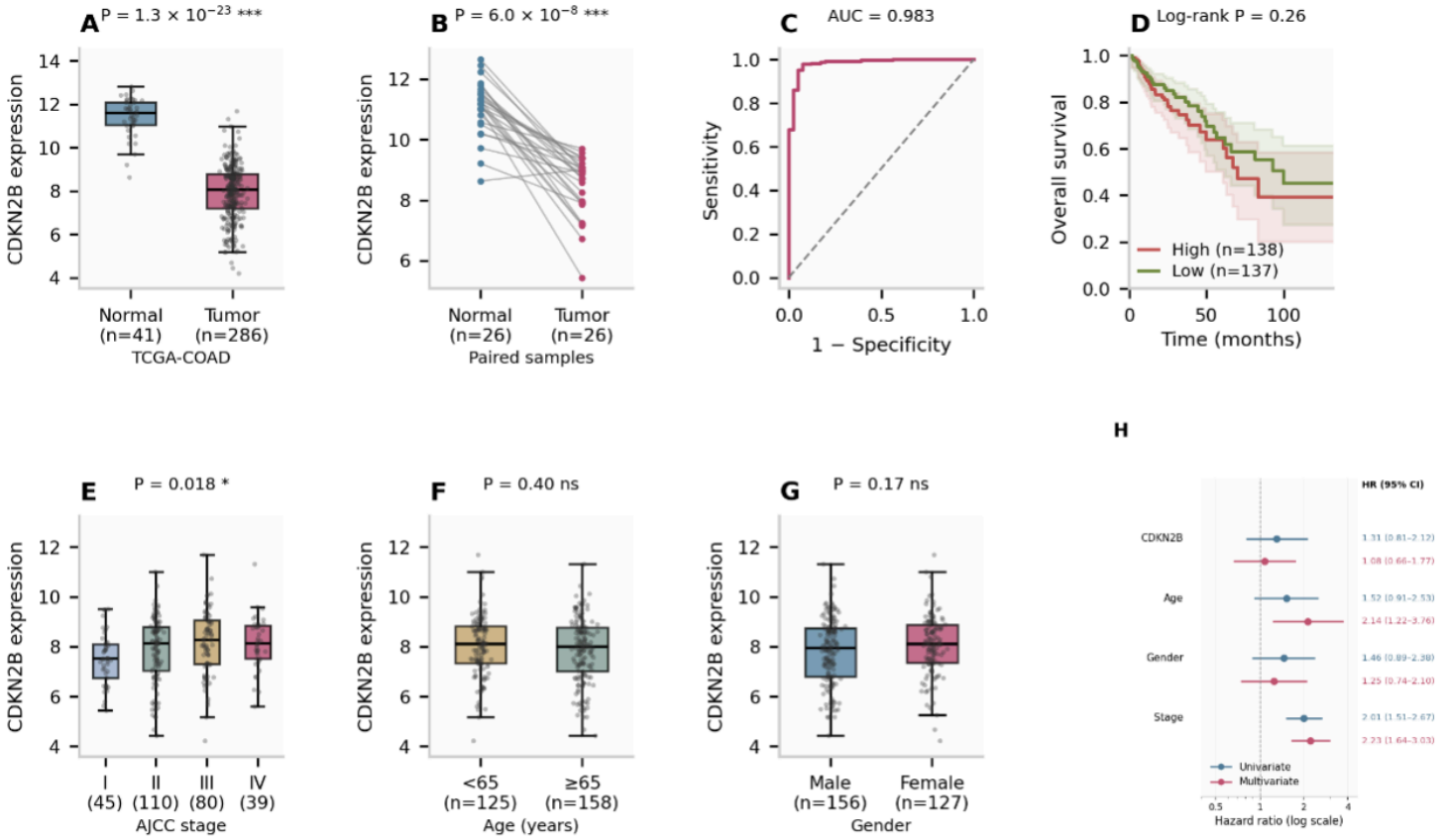

Figure 3

CDKN2B expression and its clinical relevance in the TCGA-COAD cohort.

(A) CDKN2B expression in primary tumors (n = 286) and normal tissues (n = 41); Mann–Whitney U test. (B) CDKN2B expression in 26 paired tumor-normal samples; Wilcoxon signed-rank test. (C) ROC curve of CDKN2B expression for distinguishing tumor from normal tissue. (D) Kaplan-Meier overall survival curves stratified by median CDKN2B expression; log-rank test. (E-G) CDKN2B expression according to AJCC stage (Kruskal-Wallis test), age, and gender (Mann-Whitney U test). (H) Forest plot of univariate and multivariate Cox regression for overall survival. *P < 0.05, **P < 0.001; ns, not significant.

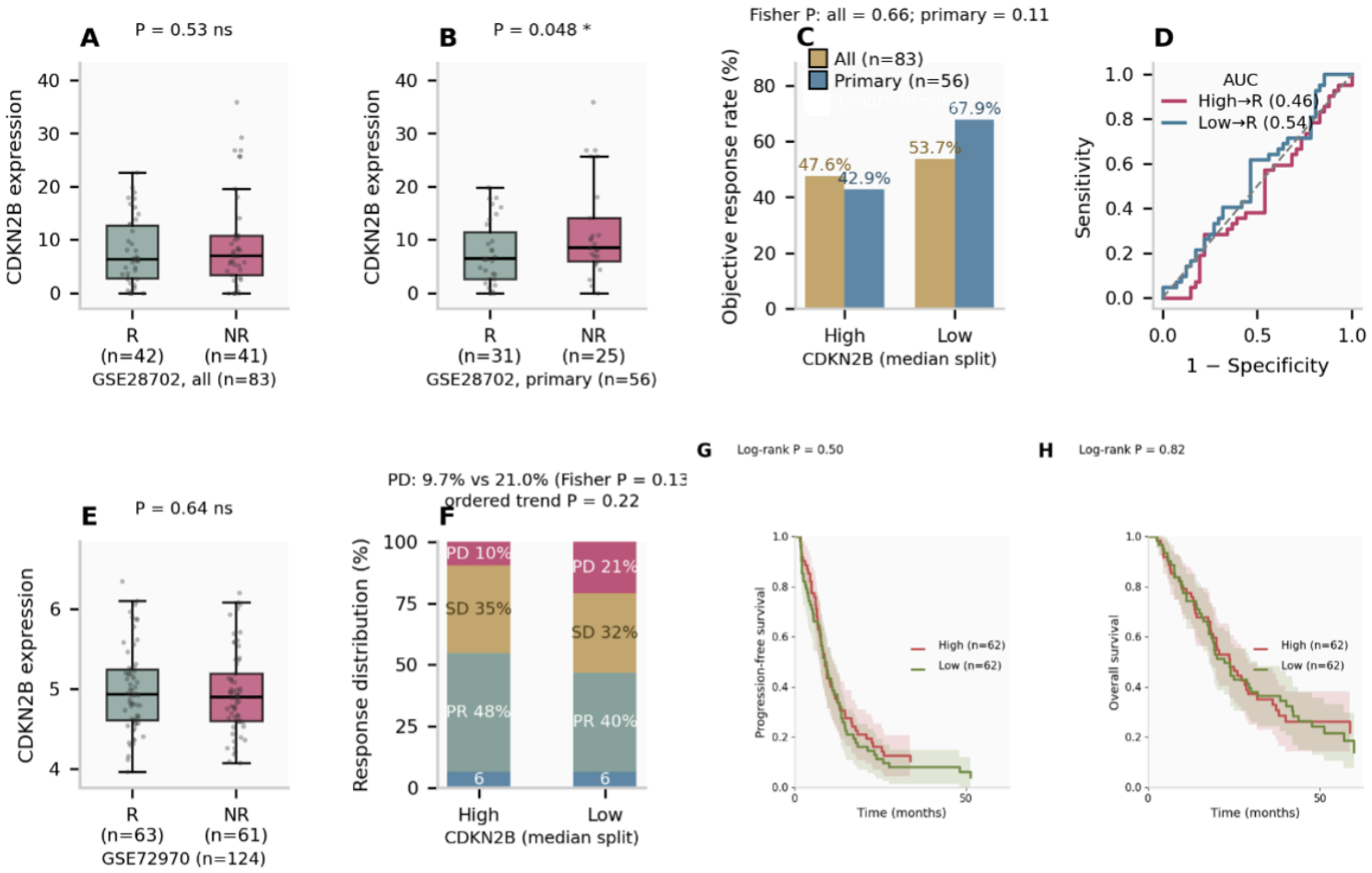

Figure 4

Association of CDKN2B expression with chemotherapy response in two independent GEO cohorts.

(A-D) GSE28702 (mFOLFOX6, n=83). (A) CDKN2B expression in responders (R) and non-responders (NR); (B) analysis restricted to primary-tumor biopsies (n=56); (C) objective response rate in CDKN2B-high and -low groups (median split); Fisher's exact test; (D) ROC curves for response prediction. (E-H) GSE72970 (fluoropyrimidine-based regimens, n=124). (E) CDKN2B expression in R and NR; (F) distribution of RECIST categories (CR/PR/SD/PD) in CDKN2B-high and -low groups; (G, H) Kaplan–Meier curves of progression-free survival and overall survival; log-rank test. CDKN2B expression was represented by probe set 207530_s_at (GPL570). *P < 0.05; ns, not significant.

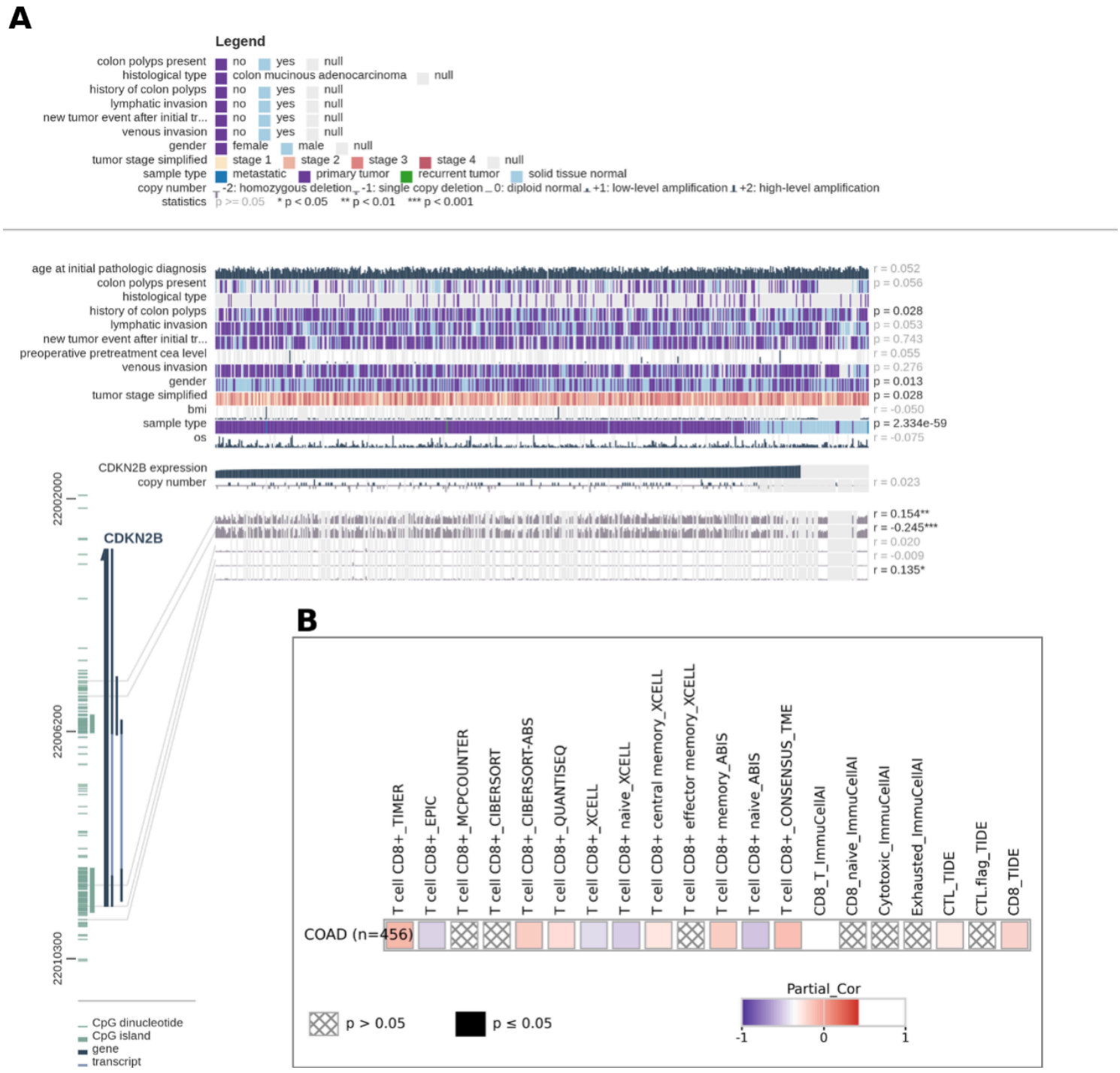

Figure 5

Methylation landscape of the CDKN2B locus and its association with CD8+ T-cell infiltration in TCGA-COAD.

(A) DNA methylation profile of CDKN2B (MethSurv). Upper panel: clinicopathological characteristics; middle panel: CDKN2B expression and copy number status; lower panel: methylation at five promoter CpG sites relative to CpG islands and gene structure. Right margin: associations of clinical variables with CDKN2B expression and Spearman correlations between CpG-site methylation and expression. (B) Partial correlations between CDKN2B expression and CD8+ T-cell infiltration (n=456) by multiple

algorithms (TIMER2.0). Red, positive; blue, negative; filled cells, $p \leq 0.05$; cross-hatched cells, $p > 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.