Role of CDX2 in the prognosis of colorectal cancer across stages 0-IV

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Abstract. Caudal-type homeobox 2 (CDX2) proteins function as tumour suppressors in colorectal cancer (CRC), but their prognostic value remains controversial. In the present study, a total of 453 eligible patients diagnosed with CRC and having undergone surgery between January 2017 and January 2020 were included after applying exclusion criteria. CDX2 expression was classified as either absent/low (negative or +) or high (++ or +++). Clinicopathological characteristics, 5-year overall survival (OS) and disease-free survival (DFS) were compared across groups using Kaplan-Meier analysis with log-rank tests. Associations between categorical variables were assessed via Spearman's correlations, while univariate and multivariate analyses were performed using Cox regression models. CDX2 expression was only correlated with tumour-node-metastasis (TNM) stage (P=0.001). Tumour carcinoembryonic antigen expression was associated with E-cadherin (P<0.01) and microsatellite instability (P<0.01). High CDX2 expression was associated with significantly improved OS [stage II: P=0.013; hazard ratio (HR)=0.472; 95% CI, 0.257-0.867; stage III: P<0.001; HR=0.413; 95% CI, 0.271-0.628] and DFS (stage II: P=0.042; HR=0.606; 95% CI, 0.370-0.994; stage III: P<0.001; HR=0.468; 95% CI, 0.334-0.655). In the pooled-stage analysis, high CDX2 expression was also associated with improved OS (HR=0.511; 95% CI, 0.371-0.703) and DFS (HR=0.581; 95% CI, 0.448-0.753), with all pooled P-values <0.001. Cox regression identified chemotherapy, TNM stage, differentiation status, Ecadherin expression and CDX2 expression as independent prognostic factors. In conclusion, high CDX2 expression is associated with favourable prognosis in stages II and III CRC and represents an independent prognostic marker for both OS and DFS.

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Introduction

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract and a leading cause of cancer-related mortality worldwide (1); for example ~153,020 individuals were diagnosed with CRC and ~52,550 died from the disease in the United States in 2022 (2). Prognosis in patients with CRC is influenced by various clinical factors, including tumour stage, necrosis, vascular invasion, differentiation status, immune markers such as the Ki-67 proliferation index, serum carcinoembryonic antigen (CEA) levels and inflammatory markers (3). However, the tumour-node-metastasis (TNM) staging system, defined by the American Joint Committee on Cancer (AJCC), remains the primary framework for prognostic assessment and treatment planning (4). In previous work, several immunohistochemical markers with potential prognostic relevance in CRC have been evaluated (5-8).

Caudal-type homeobox 2 (CDX2) is an intestinal transcription factor, typically localised to the nuclei of intestinal epithelial cells, which serves a critical role in intestinal development and homeostasis (9,10). As a homeobox gene, CDX2 participates in embryonic development, contributes to the maintenance of dynamic homeostasis within the gut and functions to inhibit proliferation and tumour formation in colon cancer cells (11-13). More recently, CDX2, along with SATB homeobox 2, has been increasingly associated with intestinal inflammation. CDX2 has been shown to modulate inflammasome activity through the expression of tripartite motif containing 31, an inhibitor of NLR family pyrin domain containing 3 (14). In murine colonic epithelium, loss of CDX function results in enhanced macrophage infiltration and elevated expression of proinflammatory cytokines, including TNFα, IL1β and IL6 (14,15). Both CDX1 and CDX2 are essential for maintaining intestinal epithelial homeostasis. In humans, loss of CDX2 is linked to more aggressive CRC

subtypes (16). Correspondingly, in an APCmutant murine model, concurrent loss of CDX1 and CDX2 leads to increased polyp formation throughout the intestinal tract (16). Unlike microsatellite instability (MSI) and Ecadherin, which demonstrate stagerestricted prognostic effects (17,18), CDX2 has been preliminarily reported to show stable prognostic relevance across both early and advanced tumour stages (19). Expression levels of CDX2 are associated with tumour differentiation, invasion depth and lymph node metastasis in a stagedependent manner, an association not fully captured by MSI or Ecadherin alone (20). Based on archived specimens, the nextgeneration multitarget stool DNA assay has shown promising performance characteristics, indicating it has high sensitivity for CRC and advanced precancerous lesions, with excellent specificity for CRC screening and will undergo further evaluation in a prospective clinical validation study (21).

Genes such as solute carrier family 17 member 9 (SLC17A9) function as competitive endogenous RNAs that sequester microRNA (miR)12263p, thereby facilitating CRC progression (22). These findings establish SLC17A9 as a novel prognostic biomarker and a potential therapeutic target for CRC (21). Inhibitors of STAT3, particularly those targeting the DNAbinding domain, have been shown to hold therapeutic potential in CRC (23). To address this, subsequent experiments disrupted the trefoil factor 3-Janus kinase 2/STAT3-CDX2 pathway, revealing notable downstream changes that mechanistically link these molecular interactions to CRC progression (24).

CRCs which lose CDX2 expression are known to display aggressive clinical behaviour yet are frequently accompanied by a high density of tumourinfiltrating lymphocytes (25). CDX2low CRC has been associated with worse survival and differential responses to adjuvant chemotherapy (26). The serrated pathway of colorectal carcinogenesis may be linked to gastrictype metaplasia in the colon and alterations in CDX2 expression (1). CDX2, a transcription factor involved in gastrointestinal differentiation, has been suggested as a biomarker for favourable outcomes (such as high CDX2 expression being associated with well and highly differentiated tumours) and may also help identify subtypes responsive to targeted therapies (27). Reduced CDX2 expression is associated with worse survival and several adverse prognostic factors, including advanced tumour stage, higher tumour grade and BRAF mutation (28). Variations in CDX2 expression may also correspond to differences in treatment outcomes. For instance, loss of CDX2 detected by immunohistochemistry (IHC) has been associated with worse prognosis and could help identify patients who are more likely to benefit from chemotherapy (29). Moreover, CDX2positive cancer of unknown primary constitutes a distinct subgroup that tends to respond favourably fluorouracilbased therapy, supporting the role of CDX2 as a predictive biomarker for treatment selection (30). Although loss of CDX2 expression has been linked to worse clinical outcomes and appears to predict benefit from adjuvant chemotherapy in stage II and III CRC, its prognostic relevance in stage II disease, particularly in relation to established clinical risk factors such as MSI status, BRAF mutation status and tumour budding, remains inadequately validated (31).

Building on the established link between CDX2 and CRC, the present study hypothesises that high CDX2 expression exerts a protective effect in CRC and correlates with a favourable prognosis. To test this hypothesis, a retrospective analysis of patients who underwent CRC cancer surgery was performed, including stratified analyses across different TNM stages. To date, few studies (32,33) have comprehensively evaluated the prognostic role of CDX2 beyond AJCC 8th edition stratification, and investigations examining the association between CDX2 expression and both overall survival (OS) and diseasefree survival (DFS) remain limited. Addressing this gap represents a primary aim of the present study.

It is well established that CDX2 is commonly expressed in normal colorectal epithelium, whereas its loss or reduced expression is frequently observed in colorectal tumour tissue and is associated with unfavourable prognosis (34). To further explore this association, the relationship between differential CDX2 expression and prognosis in patients with stage 0-IV CRC treated within a defined period was examined as the first objective of the present study. Additional objectives were to determine whether CDX2 expression correlates with TNM stage, differentiation status, tumour CEA (TCEA) levels, tumour p53 status and other clinicopathological variables and to evaluate whether CDX2 serves as an independent prognostic factor in CRC.

Materials and methods

Patients. A total of 802 patients with CRC who underwent surgery between January 2017 and January 2020 in the Colorectal Surgery Department of Huzhou Central Hospital (Huzhou, China) were initially identified using the medical records system (version 4.37; Hangzhou Lianzhong Medical Science Co., Ltd.) and Haitai software (version 3.0; Nanjing Haitai Medical). After applying the inclusion and exclusion criteria, 453 cases were included in the final analysis. Tumour staging was performed according to the AJCC 8th edition (4). For analytical purposes, TNM staging is presented in two formats: i) Conventional staging (denoted as 'TNM'), which includes subgroups not used in the final analysis; and ii) a combined grouping that merges overall stages and combines stages 0 and I (denoted as 'TNM1').

The inclusion criteria were: i) A diagnosis of CRC confirmed by colonoscopy, computed tomography or pathological examination, regardless of whether the initial diagnosis was made within or outside the Colorectal Surgery Department of Huzhou Central Hospital; ii) patients who underwent colorectal surgery (radical or nonradical) at Huzhou Central Hospital; iii) a diagnosis of primary tumour recurrence or mortality attributable to the primary tumour during the present study period; iv) availability of complete and detailed clinical and pathological records; and v) complete followup data with accurate documentation. The exclusion criteria were: i) The presence of severe cardiac, cerebral, hepatic or pulmonary disease that rendered the patient unfit for surgery; ii) mortality due to nonCRC causes, or pathological confirmation of nonadenocarcinoma malignancies, such as gastrointestinal stromal tumours, neural tumours, lymphomas or melanomas; and

(iii) incomplete clinicopathological data or insufficient followup information. Based on these criteria, 349 cases were excluded.

Follow-up. Patients were routinely followed up at the outpatient clinic 2 weeks after surgery. Subsequent follow-ups were scheduled every 3 months during the first year, every 6 months during the second year and annually for the following 3 years, for a total follow-up period of 5 years postoperatively. Followup data were obtained through telephone interviews and outpatient medical records. The endoffollowup date was January 2025, with a median followup duration of 58 months.

CDX2 detection by IHC (Elivision method). For CDX2 immunohistochemistry, formalin-fixed paraffin-embedded tissue sections of CRC tissues (4 μm thickness) were used. Tissue fixation was performed using 10% neutral buffered formalin at room temperature for 24-48 h. After deparaffinization and rehydration, antigen retrieval was performed. Pretreatment was conducted with BOND Epitope Retrieval Solution 2 (cat. no. AR9640; Leica Biosystems) at 100˚C for 30 min. The sample was incubated with a blocking agent containing 3% hydrogen peroxide for 5 min at room temperature. Immunohistochemical staining for CDX2 was performed on an automated immunostainer (BONDIII; Leica Biosystems) using the BOND Refine detection kit (cat. no. AR9640; Leica Biosystems). A CDX2 antibody (clone EPR2764Y; Epredia; cat. no. RM2116; dilution 1:500) served as the primary antibody. The primary antibody was incubated at room temperature for 20 min. Subsequently, the BOND Refine detection kit was applied according to the manufacturer's instructions, with the secondary antibody incubated at room temperature for 6 min. Slides were scanned using a NanoZoomer XR scanner (Hamamatsu Photonics K.K) under x200 and x400 objective magnifications. The resulting digital images were analysed with QuPath software (35). Cellular detection was performed using the software's builtin cell detection function. Intensity features were incorporated to calculate Haralick's texture features, and smoothed features (within a 20 μm radius) were generated using the corresponding smoothing function. An object classifier was trained to distinguish tumour cells from stromal cells. The intensity of 3,3'diaminobenzidine (DAB) chromogen staining was quantified for each tumour cell using QuPath, with the DAB threshold set in accordance with the manufacturer's protocol (BONDIII; Leica Biosystems).

CDX2 classification. For each cell, DAB staining intensity was classified into four categories according to predefined thresholds. The CDX2 scoring cutoffs were established by the pathology department in line with the CDX2 kit protocol (cat. no. AR9640; Leica Biosystems) and recent literature (9): A DAB intensity value <0.25 was classified as negative (-); 0.25-0.55 as weakly positive (+); 0.55-0.85 as moderately positive (++); and >0.85 as strongly positive (+++). Considering that cases with complete CDX2 negativity are comparatively uncommon and do not encompass the full spectrum of TNM staging categories (36), CDX2 (-) samples and CDX2 (+) samples were consolidated into a unified group, defined as CDX2 absent/low, which classification approach has been previously utilized by other researchers (36). For binary group comparison and to facilitate subsequent analyses, cases classified as CDX2 (-) and CDX2 (+) were combined into a 'CDX2 absent/low' group (Fig. 1A and B), while cases classified as CDX2 (++) and CDX2 (+++) were combined into a 'CDX2 high' group (Fig. 1C and D). Each case was assessed independently by two experienced pathologists who were blinded to clinical outcomes. In cases of discordance, a third senior pathologist adjudicated to minimise interobserver bias. Receiver operating characteristic curve analysis was performed to evaluate the diagnostic efficacy of this classification. The resulting area under the curve values were 0.75 for OS and 0.72 for DFS (both >0.5), confirming its discriminative power in terms of sensitivity and specificity (Fig. S1).

Statistical analysis. All clinical and followup data were entered into SPSS version 29.0 (IBM Corp.). Missing values were imputed with the series mean prior to analysis. Continuous clinicopathological variables were compared between the CDX2 absent/low and CDX2 high expression groups using independentsamples Student's ttests. Categorical data were analysed with crosstabulation and Pearson's χ^2 test. Ordinal categorical variables were assessed using Spearman's correlation. The primary endpoints were defined as the time from surgery to mortality or until 5-years postoperatively, which served as the study cut-off. Analysis of 5 year OS and DFS was performed using Kaplan-Meier curves with logrank tests; risk tables were generated according to CDX2 expression levels. Correlation analyses and relation maps were performed in SPSS 29, while correlation heatmaps were visualised using R 4.4.1 R (Posit Software, PBC). The R packages 'corrplot' (https://cran.r-project.org/package=corrplot), 'ggplot2' (https://cran.r-project.org/package=ggplot2), 'survival' (https://cran.r-project.org/package=survival) and 'survminer' (https://cran.r-project.org/package=survminer) were employed for visualisation. Univariate and multivariate Cox regression analyses were conducted incorporating clinical, pathological and biochemical variables. Omnibus tests and the enter method were used in the Cox regression analysis. In the multivariate analysis, all variables were initially included using the enter method. To minimize bias, continuous variables were supplemented with the statistically recognized sequence mean imputation for missing values. Additionally, cases with missing values of the categorical variable CDX2 were excluded to reduce bias. Fisher's exact test was used if expected values in cells were <5. P<0.05 was considered to indicate a statistically significant difference.

Results

General data. The present study included 453 patients, of whom 266 were men (58.7%) and 187 women (41.3%). Participant age ranged from 25 to 90 years, with a median of 66 years. The CDX2 absent/low group consisted of 217 cases (47.9%), including 123 men (27.2%) and 94 women (20.8%). The CDX2 high group included 236 cases (52.1%), with 143 men (31.6%) and 93 women (20.5%). No significant difference

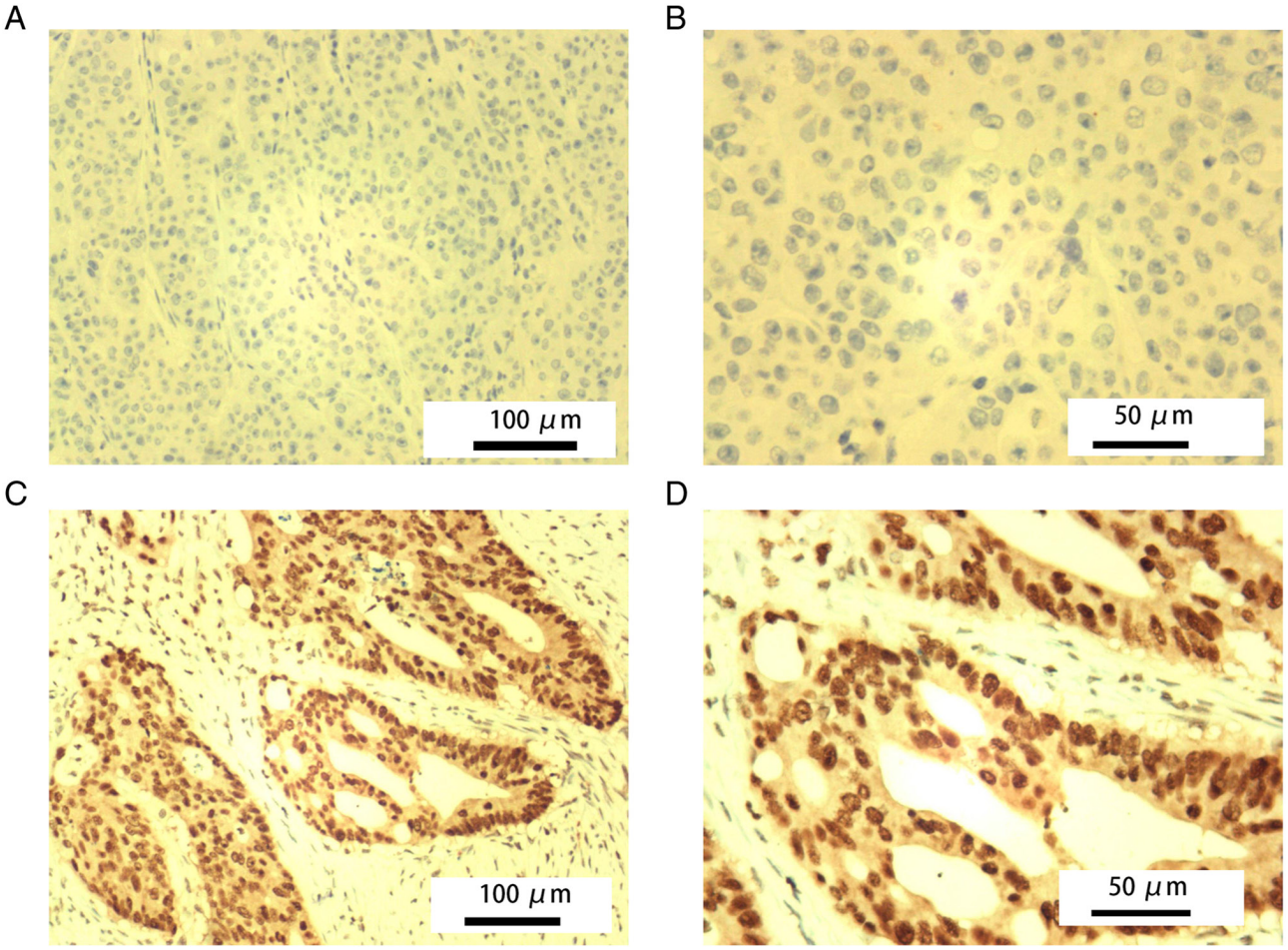


Figure 1. Representative images of CDX2 expression in colorectal cancer. (A) CDX2 absent/low expression at x200 magnification. (B) CDX2 absent/low expression at x400 magnification. (C) CDX2 high expression at x200 magnification. (D) CDX2 high expression at x400 magnification. CDX2, caudal-type homeobox 2.

in sex distribution was observed between the two groups (χ^2=0.713; P=0.398). The mean ages were 64.9±11.2 years in the CDX2 absent/low group and 65.9±10.7 years in the CDX2 high group; this difference was not statistically significant (t=-0.97; P=0.334). Baseline characteristics are summarised in Table I.

Clinicopathological features between CDX2 absent/low and CDX2 high groups. No significant differences were found between the CDX2 absent/low and CDX2 high groups in the following continuous variables: Symptom duration (23.3±11.8 vs. 22.1±8.2 months; P=0.193), number of lymph nodes harvested (15.7±6.56 vs. 15.7±9.69; P=0.999), minimum tumour size (3.3±1.5 vs. 3.2±1.5cm; P=0.415) and maximum tumour size (4.6±1.8 vs. 4.6±1.8 cm; P=0.898). Similarly, no significant differences were found in levels of carbohydrate antigen (CA) 72-4 (CA72-4), CA19-9, CA15-3, CA125, CEA, preoperative C-reactive protein (CRP), preoperative albumin, treatment cost, receipt of chemotherapy, surgical approach (laparoscopy), TCEA, differentiation grade, MSI status, E-cadherin expression or topoisomerase II α (Top2) expression (all P>0.05). However, significant differences between the two groups were identified in TNM1 stage (P=0.012) and tumour p53 (tp53) expression (P=0.017). Detailed results are presented in Table I.

Correlations between CDX2 and other variables. Pairwise associations between all categorical variables listed in Table II were analysed using Spearman's rank correlation. Significant correlations were found between tp53 and differentiation (P=0.016) and between tp53 and TNM1 stage (P=0.024). Significant correlations were also observed between TCEA and E-cadherin (P<0.001), between E-cadherin and MSI (P<0.001), between tumour differentiation and TNM1 stage (P<0.001) and between TNM1 stage and CDX2 expression (P=0.001). Correlation coefficients and P-values are provided in Table II and visualised in a correlation matrix (Fig. 2A). To further clarify these relationships, crosstabulation analyses confirmed strong associations between CDX2 expression (absent/low vs. high) and moderate differentiation, TNM1 stages II and III and poor/undifferentiated differentiation. A strong association was also observed between moderate differentiation and TNM1 stages II and III (Fig. 2B).

Systematic cluster analysis of CDX2, TNM1, TCEA and MSI. A systematic cluster analysis was conducted due to the correlations identified between the four variables analyzed. The results demonstrated that CDX2 clustered most closely with MSI (coefficient=258), followed by MSI with TCEA (coefficient=495), and finally MSI with TNM1 (coefficient=876).

Table I. Clinicopathological features of CDX2 classifications.

Variables	CDX2 absent/low (n=217)	CDX2 high (n=236)	t/χ^2	P-value (two-sided)
Sex			0.713	0.398
Men	123 (27.2)	143 (31.6)		
Women	94 (20.8)	93 (20.5)		
Age, years	64.9±11.2	65.9±10.7	-0.97	0.334
Symptom duration, days	23.3±11.8	22.1±8.2	1.30	0.193
Laparoscopy			3.466	0.063
Yes	125 (27.6)	156 (34.4)		
No	92 (20.3)	80 (17.7)		
Lymph nodes harvested	15.7±6.6	15.7±9.7	0.001	0.999
Tumour size, cm				
Min	3.3±1.4	3.2±1.5	0.816	0.415
Max	4.6±1.8	4.6±1.8	-0.128	0.898
CA72-4, U/ml	5.7±11.4	6.5±24.4	-0.450	0.653
CA19-9, U/ml	96.9±500.3	45.3±143.4	1.466	0.144
CA15-3, U/ml	9.2±4.4	9.5±7.7	-0.636	0.525
CA125, U/ml	17.9±17.8	18.1±4.7	-0.061	0.951
CEA, ng/ml	16.3±63.1	16.3±54.7	0.000	1.000
Preoperative CRP, mg/l	7.8±16.2	7.7±21.3	0.069	0.945
Preoperative album, g/l	37.6±5.3	37.8±4.7	-0.558	0.578
Cost, thousand RMB	43.4±9.8	43.0±10.5	0.435	0.664
Chemotherapy			0.261	0.610
Yes	142 (31.3)	149 (32.9)		
No	75 (16.6)	87 (19.2)		
TNM1			11.011	0.012[a]
0 and I	28 (6.2)	45 (9.9)		
II	71 (15.7)	96 (21.2)		
III	103 (22.7)	88 (19.4)		
IV	15 (3.3)	7 (1.5)		
Differentiation			1.223	0.543
Poor and undifferentiated	92 (20.3)	94 (20.8)		
Moderate	121 (26.7)	134 (29.6)		
High	4 (0.9)	8 (1.8)		
MSI			0.183	0.668
Low	162 (35.8)	172 (38.0)		
High	55 (12.1)	64 (14.1)		
TP53			10.186	0.017[a]
Negative	55 (12.1)	40 (8.8)		
+	31 (6.8)	46 (10.2)		
++	32 (7.1)	54 (11.9)		
+++	99 (21.9)	96 (21.2)		
TCEA			3.107	0.389[b]
Negative	2 (0.4)	1 (0.2)		
+	141 (31.1)	165 (36.4)		
++	11 (2.4)	16 (3.5)		
+++	63 (13.9)	54 (11.9)		
E-cadherin			3.639	0.303[b]
Negative	1 (0.2)	2 (0.4)		
+	79 (17.4)	99 (21.9)		
++	55 (12.1)	65 (14.3)		
+++	82 (18.1)	70 (15.5)		

Table I. Continued.

Variables	CDX2 absent/low (n=217)	CDX2 high (n=236)	t/χ^2	P-value (two-sided)
Top2			0.678	0.712
+	37 (8.2)	34 (7.5)		
++	162 (35.8)	180 (39.7)		
+++	18 (4.0)	22 (4.9)		

Data presented as means ± SD or n (%). [a]P<0.05; [b]Fisher's exact test was used if expected values in cells were <5. TNM1 indicates total stage of American Joint Committee on Cancer-8 after combining substages. CDX2, caudal-type homeobox transcription factor 2; CRP, Preoperative C-reactive protein; TP53, tumour p53; TCEA, tumour carcinoembryonic antigen; MSI, microsatellite instability; TOP2, topoisomerase II α; TNM, tumour-node-metastasis.

Table II. Spearman (two tailed) correlation analysis matrix between CDX2 and other variables.

Variable	Result	TP53	TCEA	MSI	E-cadherin	Top2	Differentiation	TNM1	CDX2
TP53	Coef.	1.000	0.019	-0.043	0.036	0.090	0.113[a]	0.106[a]	0.012
	P-value	/	0.688	0.357	0.444	0.055	0.016[a]	0.024[a]	0.806
TCEA	Coef.	0.019	1.000	0.074	0.298[c]	-0.040	-0.017	0.048	-0.062
	P-value	0.688	/	0.117	<0.001[c]	0.391	0.718	0.308	0.188
MSI	Coef.	-0.043	0.074	1.000	0.191[c]	-0.041	-0.035	0.011	0.020
	P-value	0.357	0.117	/	<0.001[c]	0.384	0.453	0.814	0.669
E-cadherin	Coef.	0.036	0.298[b]	0.191[c]	1.000	0.051	-0.003	-0.015	-0.075
	P-value	0.444	<0.01	<0.001[c]	/	0.277	0.951	0.757	0.110
Top2	Coef.	0.090	-0.040	-0.041	0.051	1.000	-0.003	0.018	0.038
	P-value	0.055	0.391	0.384	0.277	/	0.947	0.696	0.423
Differentiation	Coef.	0.113[a]	-0.017	-0.035	-0.003	-0.003	1.000	-0.212[c]	0.034
	P-value	0.016[a]	0.718	0.453	0.951	0.947	/	<0.001[c]	0.470
TNM1	Coef.	0.106[a]	0.048	0.011	-0.015	0.018	-0.212[c]	1.000	-0.151[b]
	P-value	0.024[a]	0.308	0.814	0.757	0.696	<0.001[c]	/	0.001[b]
CDX2	Coef.	0.012	-0.062	0.020	-0.075	0.038	0.034	-0.151[b]	1.000
	P-value	0.806	0.188	0.669	0.110	0.423	0.470	0.001[b]	/

[a]P<0.05, [b]P<0.01 and [c]P<0.001. TNM1 indicates total stage of American Joint Committee on Cancer-8 after combining substages. CDX2, caudal-type homeobox transcription factor 2; TP53, tumour p53; TCEA, tumour carcinoembryonic antigen; MSI, microsatellite instability; TOP2, topoisomerase II α; TNM, tumour-node-metastasis; coef, coefficient.

As illustrated in Fig. 3, CDX2 exhibited a high degree of similarity with MSI.

OS and DFS analysis by CDX2 expression across CRC stages 0-IV. Kaplan-Meier survival analysis with logrank tests was used to compare 5year OS and DFS between CDX2 expression groups. For OS, high CDX2 expression was associated with significantly improved survival in stage II (P=0.013) and stage III CRC (P<0.001; Fig. 4B and C), but not in stage I (P=0.11) or stage IV (P=0.69; Fig. 4A and D). In the pooled-stage analysis (all stages combined), the CDX2 high group showed significantly improved OS compared with the CDX2 absent/low group (P<0.001; Fig. 4E). Similarly, for DFS, high CDX2 expression was associated with significantly improved outcomes in stage II (P=0.042) and stage III (P<0.001) disease (Fig. 5B and C), but not in stage I (P=0.16) or stage IV (P=0.94) (Fig. 5A and D). In the pooledstage analysis, the CDX2 high group exhibited significantly improved DFS compared with the CDX2 absent/low group (P<0.001; Fig. 5E).

Univariate and multivariate Cox regression analyses of OS and DFS. Cox regression models were used for univariate and multivariate analyses. Univariate analysis identified the following variables as significantly associated with worse OS: Not laparoscopic surgery (vs. laparoscopy), no chemotherapy, higher TNM1 stage, worse differentiation, MSIstable status, negative and positive Ecadherin expression and low CDX2 expression (all P<0.05). Sex, TCEA and Top2 expression were not significant predictors of OS (all P>0.05). For DFS, significant variables included not laparoscopic surgery, no chemotherapy, higher TNM1 stage, worse differentiation, negative and positive Ecadherin expression and low CDX2 expression (all P<0.001). Notably, MSI status was significant for OS (P=0.015) but not for DFS (P>0.05). Detailed results

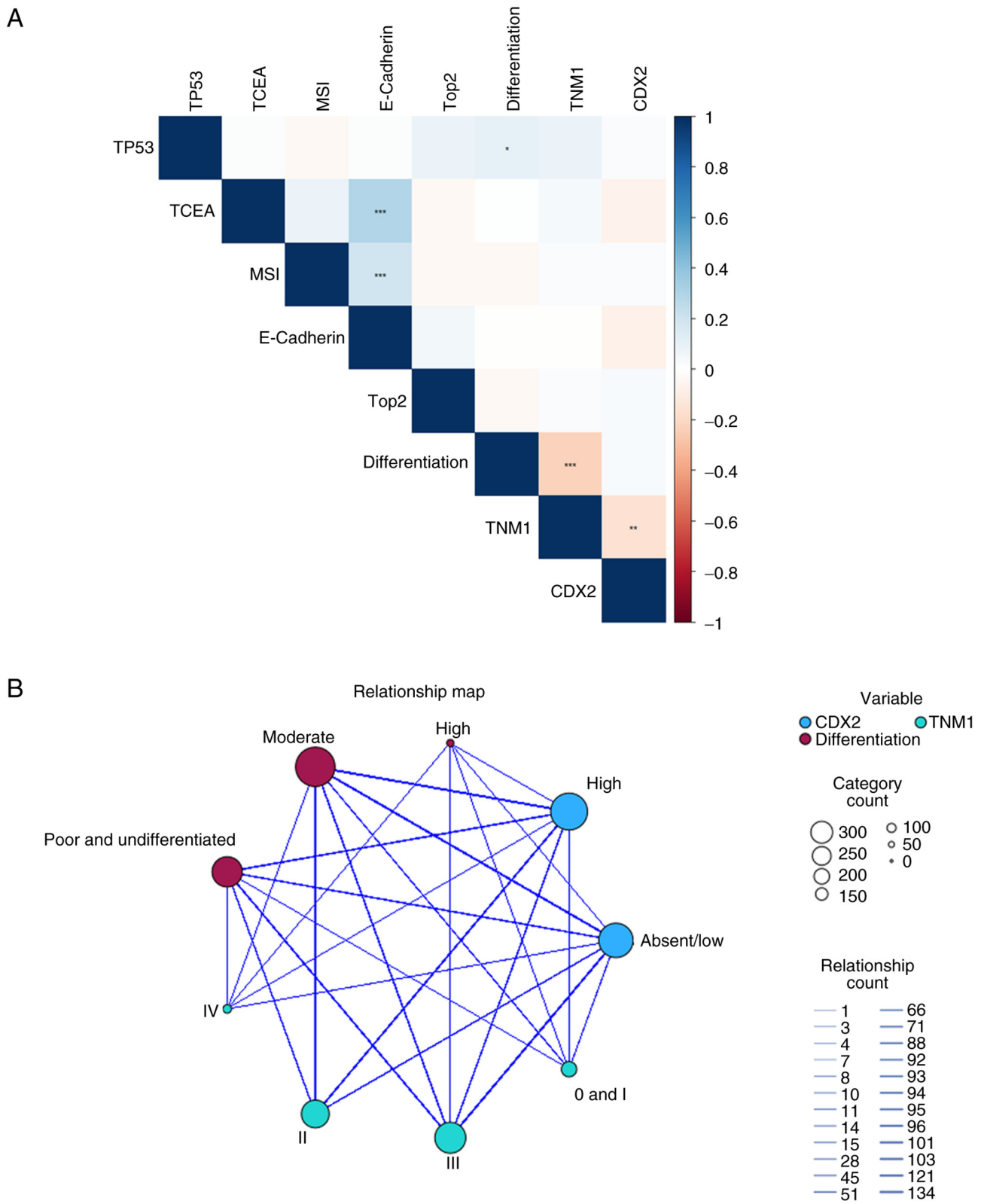
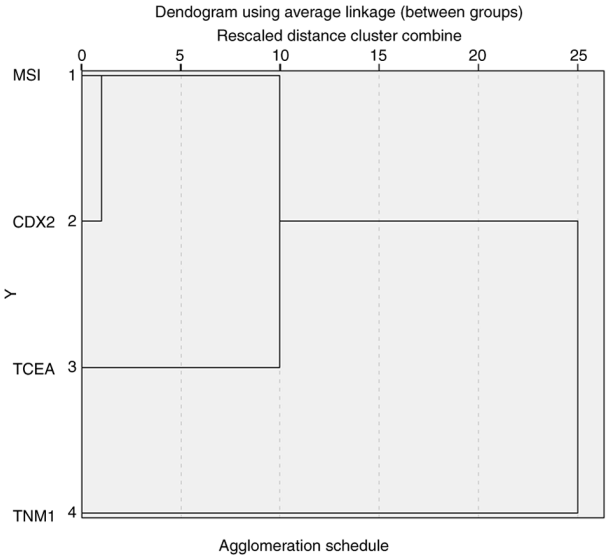


Figure 2. Correlation and relational analysis of CDX2. (A) Spearman correlation heatmap between CDX2 and variables including TP53, TCEA, MSI, Ecadherin, Top2, differentiation and TNM1 stage, generated using R 4.4.1. Blue indicates positive correlations, red indicates negative correlations and white indicates nearzero correlation. Asterisks denote statistical significance: *P<0.05; **P<0.01; ***P<0.001. (B) Relation map between CDX2 expression (absent/low vs. high), TNM1 stage and differentiation, generated using SPSS 29. Circle size corresponds to the number of cases; line thickness indicates the strength of association. CDX2, caudal-type homeobox 2; MSI, microsatellite instability; TCEA, tumour carcinoembryonic antigen; TP53, tumour p53; TNM, tumour-node-metastasis; Top2, topoisomerase II α.

are presented in Table III. Variables that were significant in both univariate OS and DFS analyses were included in the multivariate models. Laparoscopy was not retained as an independent factor in the multivariate models (OS, P=0.393;



Agglomeration schedule

	Cluster combined			Stage cluster first appears		
Stage	Cluster 1	Cluster 2	Coefficients	Cluster 1	Cluster 2	Next stage
1	1	2	227.000	0	0	2
2	1	3	474.500	1	0	3
3	1	4	866.000	2	0	0

Figure 3. Hierarchical cluster analysis with coefficient table. Systematic cluster analysis was performed using SPSS 29. The results indicate that CDX2 clusters most closely with MSI (coefficient=227), followed by the linkage between MSI and TCEA (coefficient=475), and finally between MSI and TNM1 (coefficient=866). CDX2, caudal-type homeobox 2; MSI, microsatellite instability; TCEA, tumour carcinoembryonic antigen; TNM, tumour-node-metastasis.

DFS, P=0.087). The remaining factors, chemotherapy, TNM1 stage, differentiation grade, Ecadherin expression and CDX2 expression, were statistically significant independent prognostic factors for both OS and DFS (all P<0.05), confirming their role in CRC prognosis. Detailed multivariate results are shown in Table IV.

Discussion

CDX2, a nuclear homeobox transcription factor encoded by the CDX homeobox gene (10,37), is established as a specific and sensitive immunohistochemical biomarker in colorectal adenocarcinoma (38). Its loss of expression is associated with highgrade tumours and advanced disease stage in CRC (38-41). Foundational research across the past decades has characterised CDX2 as a tumour suppressor (42-44), while more recent clinical studies link high CDX2 expression to a favourable prognosis in CRC (1,9). However, these prior investigations have predominantly focused on OS, without examining DFS or conducting stagespecific analyses across TNM stages. Moreover, they have not conclusively established CDX2 as an independent prognostic factor in CRC. The relationships between CDX2 expression and other clinical or immunohistochemical variables also remain underexplored.

A previous study has reported notable associations between tumour histological differentiation and various factors, including perineural invasion, vascular invasion, mismatchrepair deficiency, p53 status, CDX2 expression and Ecadherin (41). The influence of CDX2 on prognosis merits further investigation, particularly as biological and molecular heterogeneity may confound the assessment of other prognostic markers (45). CDX2negative phenotypes are associated with poorly differentiated histological subtypes (46). Addressing these gaps was a primary aim of the present study.

As negative CDX2 expression occurs in ~7.8% of cases (47), CDX2 (-) and CDX2 (+) cases were combined into a 'CDX2 absent/low' group, and CDX2 (++) and CDX2 (+++) cases into a 'CDX2 high' group to enable more robust statistical comparisons.

In the present cohort, CDX2 absent/low expression accounted for 47.9% of cases, and CDX2 high expression for 52.1%. This incidence of CDX2 absent/low (47.9%) aligns with the reported rate of <48% for CDX2low expression alone in prior literature (48), a concordance attributable to the inclusive classification combining negative and low cases. No significant differences were observed between the CDX2 expression groups regarding sex, age, tumour size, lymph node yield, differentiation grade or preoperative serum levels of CA72-4, CA19-9, CA15-3, CA125, CEA, CRP and albumin. However, a significant association was identified between CDX2 expression and pooled TNM1 stage, with a higher prevalence of CDX2 absent/low expression in stages III and IV, a relationship not clearly detailed in existing literature (49).

CRC prognostication traditionally relies on the Union for International Cancer Control/AJCC TNM staging system. Nonetheless, stageindependent survival variations observed in clinical practice suggest that prognosis, risk stratification and guidance for neoadjuvant/adjuvant therapy are governed by a complex interplay of stage, pathological features and biomarkers. Histological characteristics, such as tumour budding, perineural invasion and lymph node metrics, alongside molecular markers such as MSI, KRAS, BRAF and CDX2, can enhance prognostic assessment and optimise adjuvant treatment (50).

Although a higher frequency of CDX2 absent/low expression was noted in tissues with elevated p53 expression and an initial analysis suggested significance, subsequent Spearman correlation analysis did not confirm a statistically significant association, a finding consistent with a prior report indicating p53 has no association with CDX2 expression (45). Nevertheless, strong relationships were observed between CDX2 expression (absent/low vs. high) and moderate tumour differentiation; between CDX2 expression and TNM1 stages II-III; and between CDX2 expression and poor differentiation/undifferentiated. A pronounced association was also evident between moderate differentiation and TNM1 stages II-III. These specific interrelationships have not been thoroughly examined in previous studies (45,49-57). Systematic cluster analysis in the present investigation revealed that CDX2 clustered closely with MSI, yet TNM1 stage retained the dominant influence. This finding challenges suggestions that prognostic markers for CRC could supplant TNM staging in therapeutic decision-making (58) and reinforces the role of TNM as the cornerstone for clinical management in CRC (49).

Correlation analysis demonstrated an inverse relationship between CDX2 expression and TNM stage: Elevated CDX2 expression was associated with more favourable outcomes, whereas advanced TNM stage predicted worse prognosis, results that align with the aforementioned survival data. In the

Table III. Univariate analysis for OS and DFS by COX regression.

	OS			DFS		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Sex			0.836			0.352
Men	1			1		
Women	0.968	0.709-1.321		1.128	0.875-1.453	
Laparoscopy			<0.001[c]			<0.001[c]
Yes	1			1		
No	1.683	1.238-2.287		1.690	1.313-2.177	
Chemotherapy			<0.001[c]			<0.001[c]
No	1			1		
Yes	1.846	1.304-2.614		1.897	1.423-2.528	
TNM1			<0.001[c]			<0.001[c]
0 and I	1			1		
II	5.336	1.913-14.882		2.959	1.559-5.615	
III	15.451	5.682-42.035		8.905	4.817-16.461	
IV	54.387	18.452-160.302		25.218	12.1.2-52.551	
Differentiation			<0.001[c]			<0.001[c]
Poor and undifferentiated	1			1		
Moderate	0.400	0.292-0.547		0.466	0.361-0.602	
High	0.221	0.054-0.896		0.154	0.038-0.621	
MSI			0.015[a]			0.165
Low	1			1		
High	0.888	0.625-1.261		0.811	0.604-1.090	
TCEA			0.545			0.498
Negative	1			1		
+	0.568	0.140-2.301		0.838	0.208-3.380	
++	0.769	0.170-3.472		1.202	0.278~5.202	
+++	0.678	0.165-2.795		0.946	0.232-3.863	
E-cadherin[d]			0.030[a]			0.019[a]
Negative and +	1			1		
++	1.447	1.007-2.081		1.337	0.990-1.806	
+++	0.882	0.650-1.285		0.848	0.624-1.154	
Top2			0.201			0.398
+	1			1		
++	0.705	0.481-1.034		0.808	0.582-1.121	
+++	0.777	0.429-1.408		0.759	0.452-1.275	
CDX2			<0.001[c]			<0.001[c]
Absent/low	1			1		
High	0.437	0.319-0.599		0.515	0.399-0.665	

Counting variables in tables were included in univariate analysis by COX regression. [a]P<0.05; [b]P<0.01; [c]P<0.001; [d]n numbers of negative CDX2 expression were too small so they were combined with + to avoid bias. CDX2, caudal-type homeobox transcription factor 2; DFS, disease-free survival; HR, hazard ratio; MSI, microsatellite instability; OS, overall survival; TCEA, tumour carcinoembryonic antigen; TNM1 indicates total stage of American Joint Committee on Cancer-8 after combining substages,TNM, tumour-node-metastasis; Top2, topoisomerase II α.

pooled analysis of TNM1 stages, high CDX2 expression was associated with greater OS and DFS, partially corroborating previous findings indicating that high CDX2 expression may be associated with a good prognosis of CRC (1,9). However, stagespecific stratification revealed that the prognostic benefit of high CDX2 expression for both OS and DFS was confined to stages II and III. This may be explained by the limited discriminative power of prognostic markers in earlystage (I)

Table IV. Multivariate analysis by Cox regression of OS and DFS for colorectal cancer.

	OS			DFS		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Laparoscopy			0.393			0.087
Yes	1			1		
No	1.151	0.833-1.589		1.259	0.967-1.641	
Chemotherapy			0.015[a]			0.018[a]
No	1			1		
Yes	0.616	0.417-0.910		0.672	0.483-0.935	
TNM1			<0.001[b]			<0.001[b]
0 and I	1			1		
II	5.588	1.947-16.037		2.987	1.520-5.868	
III	16.575	5.688-48.30		9.283	4.646-18.549	
IV	53.232	16.747-169.198		24.222	10.746-54.598	
Differentiation			<0.001[b]			<0.001[b]
Poor and undifferentiated	1			1		
Moderate	0.534	0.386-0.740		0.613	0.470-0.799	
High	0.292	0.069-1.228		0.221	0.054-0.909	
E-cadherin[c]			0.043[a]			0.040[a]
Negative and +	1			1		
++	1.476	1.019-2.138		1.348	0.993-1.828	
+++	0.926	0.632-1.357		0.895	0.656-1.222	
CDX2			<0.001[b]			<0.001[b]
Absent/low	1			1		
High	0.482	0.347-0.669		0.557	0.427-0.725	

Variables that have significant difference by univariate analysis were put into multivariate analysis by Cox regression. [a]P<0.05; [b]P<0.001; [c]n numbers of negative CDX2 expression were too small so they were combined with + to avoid bias. CDX2, caudal-type homeobox transcription factor 2; DFS, disease-free survival; HR, hazard ratio; OS, overall survival; TNM, tumour-node-metastasis.

disease and the universally adverse prognosis characteristic of stage IV CRC (59).

The present study demonstrates that established factors such as chemotherapy, TNM1 stage, differentiation grade and immunohistochemical markers including MSI, Ecadherin and CDX2 are notable prognostic determinants in CRC (7,49,60-62). TCEA, by contrast, did not emerge as an independent prognostic factor in the present study cohort, contradicting a finding from an earlier study (6). This discrepancy highlights the limitation of relying on singlemarker assessment and highlights the necessity for a multimodal prognostic approach. In this context, the combined utility of preoperative serum CEA and postoperative TCEA has recently been explored (8). Other serum tumour markers (such as CA724, CA199, CA153 and CA125) showed no significant variation across CDX2 expression groups and were therefore not included in the univariate or multivariate Cox regression models. As the prognostic value of these markers is well-documented, they were not the focus of the present analysis. Nevertheless, the potential synergistic prognostic value of combining these markers with CDX2 and CEA warrants future investigation, as a panel of complementary biomarkers could enhance prognostic precision.

The emphasis on CDX2 expression and its correlations aligns with the increasing focus on molecular subtyping in CRC management. As a key intestinal differentiation marker, CDX2 could serve as an anchor for constructing multimodal prognostic models that integrate clinical, pathological and molecular data. Validation of combined biomarker panels and refinement of riskstratification strategies will require future studies with larger, multicentre cohorts.

Treatment stratification based on CDX2 expression levels represents a promising strategy for personalising therapy in gastrointestinal malignancies. CDX2 exerts contextdependent effects on tumour biology: High expression is often associated with favourable responses to chemotherapy and immunotherapy, whereas low expression is associated with drug resistance and aggressive phenotypes (20,63). Stratifying patients by CDX2 status could therefore guide therapeutic selection, with highCDX2 tumours potentially benefiting from conventional cytotoxic agents and lowCDX2 tumours necessitating combinatorial approaches targeting compensatory pathways such as epithelialmesenchymal transition. This stratified paradigm not only aims to optimise outcomes by minimising ineffective treatments and toxicity but also addresses tumour heterogeneity, thereby advancing precision

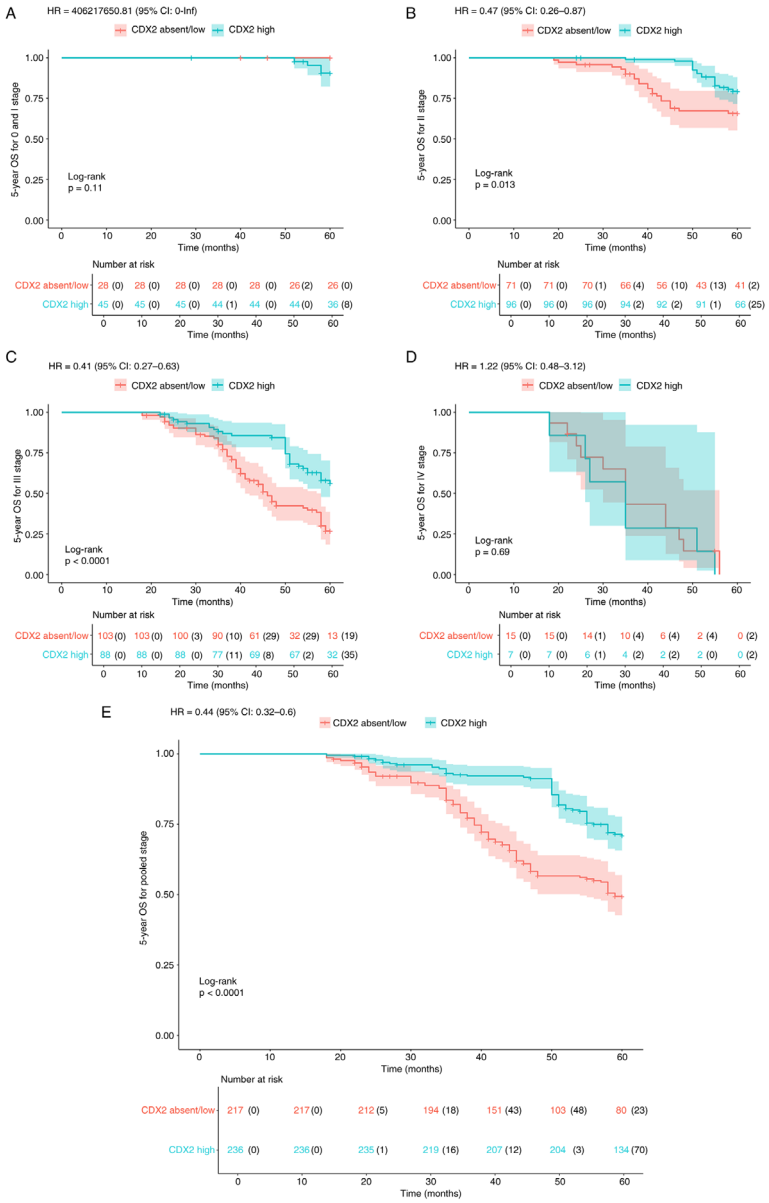


Figure 4. Kaplan-Meier curves for 5-year OS stratified by CDX2 expression across colorectal cancer stages and in the pooled analysis, with numberatrisk tables. (A) Stage 0 and I (P=0.11). (B) Stage II (P=0.01). (C) Stage III (P<0.001). (D) Stage IV (P=0.69). (E) Pooledstage analysis (P<0.001). OS, overall survival; HR, hazard ratio.

oncology approaches grounded in specific molecular profiles rather than empirical algorithms (64-66).

The present study has several limitations. External validation in an independent cohort or publicly available dataset would substantially enhance the robustness and reproducibility of the prognostic role of CDX2 identified in present research. In the present study, the analysis was based on a single-centre cohort with strict inclusion and exclusion criteria to ensure the homogeneity of the study population. Publicly available datasets, including The Cancer Genome Atlas and Gene Expression Omnibus databases (https://www.cancer.gov/ccg/research/genome-sequencing/tcga; https://www.ncbi.nlm.nih.gov/geo/) were used to attempt to validate the present findings (data not shown); however, a majority of these datasets lack standardised CDX2 IHC data (the primary detection method of CDX2 in the present study) or consistent follow-up endpoints, which limits the direct comparability of data. External validation is a key step to confirm the prognostic value of CDX2 and this should be prioritised in future research agendas. To ensure balanced datasets, CDX2 (-) and CDX2 (+) cases were combined into a composite 'CDX2 absent/low' group. Although this approach deviates from the reported prevalence of CDX2low expression (≤48%) in contemporary literature (1), it was methodologically required to avoid excluding key subgroups during TNMstage analyses, especially in a largescale study where such exclusions could affect feasibility. Two specific statistical limitations should be noted: First, no correction for multiple testing was applied across the comparisons performed; and second, formal protocols for handling missing data were not implemented. Further limitations include the relatively modest sample size, the singlecentre design and the classification of CDX2 expression based on IHC rather than genetic testing. These

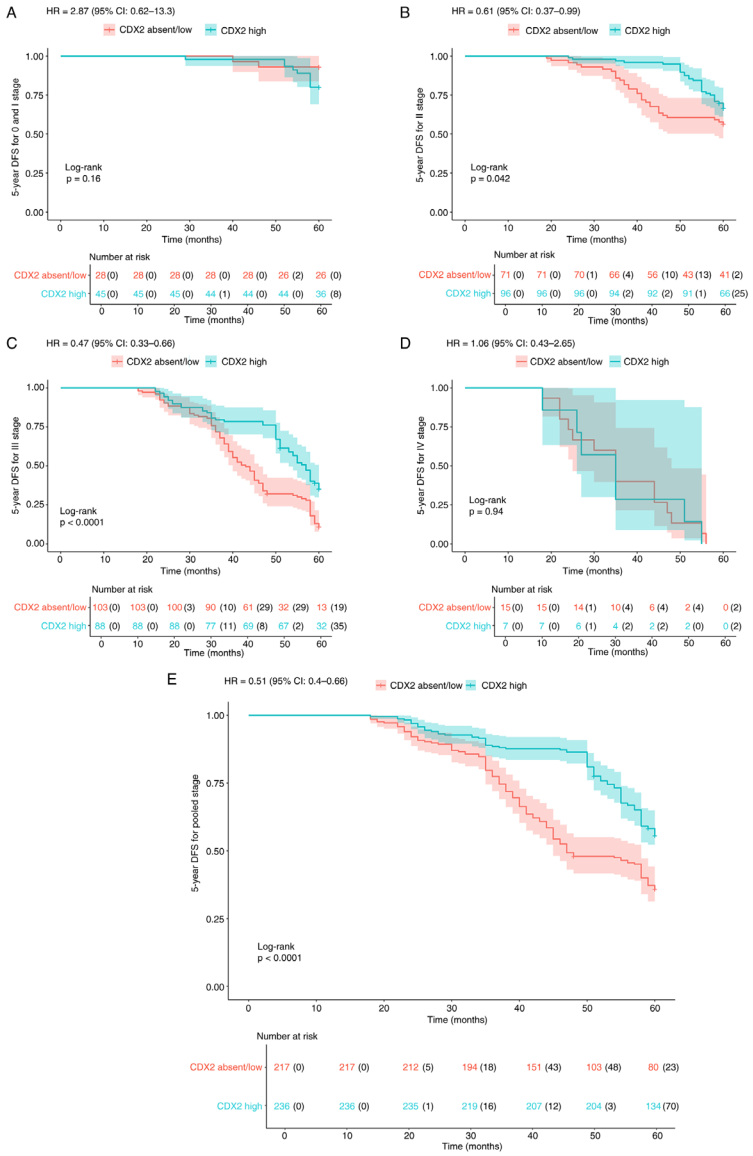


Figure 5. Kaplan-Meier curves for 5 year DFS stratified by CDX2 expression across colorectal cancer stages and in the pooled analysis, with numberatrisk tables. (A) Stage 0 and I (P=0.16). (B) Stage II (P=0.04). (C) Stage III (P<0.001). (D) Stage IV (P=0.94). (E) Pooledstage analysis (P<0.001). CDX2, caudal-type homeobox 2; DFS, disease-free survival; HR, hazard ratio.

factors increase potential type I error, risk bias and limit generalizability. Consequently, findings require validation in larger, multi-centre studies using standardized IHC and prospective data. Serum markers CA724, CA199, CA153, CA125 and CEA were recorded as continuous variables in the present study and showed no significant association with CDX2 expression groups; they were therefore not included in the Cox regression analysis. As a potential direction for future research, these markers could be categorised into normal and elevated groups according to established reference values, creating categorical variables suitable for Cox regression modelling.

In conclusion, elevated CDX2 expression is associated with significantly improved OS and DFS in patients with stage II and III CRC, as well as in pooledstage analyses. CDX2 represents an independent prognostic biomarker in CRC. These findings, however, require validation in largescale, multi-centre prospective studies. Future research should focus on the following: i) Evaluating the combined prognostic utility of CDX2 with serum biomarkers such as CEA, CA199 and CA724; ii) assessing its synergistic effects with immunohistochemical markers including p53 status, MSI and Ecadherin expression; and iii) establishing standardised scoring systems for CDX2 expression.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GT, YF and HQ contributed to conceptualisation, formal analysis, investigation, methodology, supervision and the writing of the original draft, as well as reviewing and editing the manuscript. SYu, KG, JL, WY and SYa were involved in conceptualisation, methodology and supervision. GT, YF and HQ were responsible for data curation and validation. GT and YF confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of Huzhou Central Hospital (approval no. 20250901702). The need for written informed consent from all participants for the use of their tissue samples and medical records for research purposes was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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