Multicenter validation of CEACAM6 and FOXP3 as robust prognostic biomarkers in colon cancer: Combined immunohistochemical and transcriptomic analysis

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Abstract. Colon cancer remains a leading cause of cancer-related mortality worldwide, with metastatic disease exhibiting particularly poor prognosis. To identify robust prognostic biomarkers, the present multicenter study employed immunohistochemistry and reverse transcription-quantitative PCR to evaluate CEACAM6 and FOXP3 expression in 301 colon cancer specimens from three tertiary hospitals in China, analyzing their associations with tumor-infiltrating lymphocytes, clinicopathological features and patient outcomes. Key findings revealed that early-stage (I-II) tumors exhibited significantly higher infiltration of $CD3^+$, $CD8^+$ and $CD45RO^+$ T cells compared with advanced-stage (III-IV) tumors ($P<0.001$), while FOXP3 and CEACAM6 expression were significantly elevated in late-stage and poorly differentiated tumors ($P<0.001$). Notably, CEACAM6 overexpression correlated inversely with $CD3^+$, $CD8^+$ and $CD45RO^+$ T-cell infiltration but positively with $FOXP3^+$ Tregs. Transcriptomic analysis further confirmed upregulation of CEACAM6 and FOXP3 mRNA in advanced-stage tumors ($P<0.001$). Kaplan-Meier survival analysis demonstrated that high CEACAM6 and FOXP3 expression were associated with significantly shorter overall survival ($P<0.001$). Multivariate Cox regression identified TNM stage, tumor differentiation, CEACAM6 and FOXP3 as independent prognostic factors. The present study provides robust multicenter validation of CEACAM6 and FOXP3 as critical biomarkers in colon cancer, highlighting their roles in immune evasion and tumor progression. These findings support their potential integration into clinical risk stratification and the development of targeted immunotherapies. Further mechanistic and prospective studies are warranted to explore their therapeutic applications.

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Key words: CEACAM6, FOXP3, colon cancer, tumor microenvironment, tumor-infiltrating lymphocytes, prognostic biomarkers, multicenter study

Introduction

Colon cancer remains a leading cause of cancer-related mortality worldwide, accounting for nearly 10% of all cancer deaths (1). While localized tumors exhibit favorable outcomes with >90% 5-year survival rates, metastatic disease continues to portend a dismal prognosis, with median survival under 30 months despite advances in systemic therapy (2). This stark contrast underscores the critical need for robust prognostic biomarkers and novel therapeutic strategies to improve patient stratification and treatment outcomes.

Recent breakthroughs in tumor immunology have reshaped our understanding of colon cancer progression. The tumor immune microenvironment, specifically the infiltration density and spatial organization of $CD8^+$ cytotoxic T lymphocytes and $CD45RO^+$ memory T cells, represents an independent prognostic factor beyond conventional TNM staging (3-5). Conversely, $FOXP3^+$ regulatory T cells (Tregs) contribute to immunosuppression and correlate with tumor recurrence (6,7). However, despite these advances, current immune score systems remain incomplete, failing to incorporate emerging players such as CEACAM6, a glycoprotein increasingly implicated in immune evasion and metastasis (8-10).

Simultaneously, CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6) has emerged as a key player in colon cancer progression (11,12). Originally identified as a marker of poor differentiation, CEACAM6 is now recognized to promote metastasis through multiple mechanisms including resistance to anoikis, enhancement of epithelial-mesenchymal transition and modulation of tumor-stroma interactions (13,14). Most recently, CEACAM6 has been implicated in immune evasion by upregulating PD-L1 expression and recruiting myeloid-derived suppressor cells (MDSCs) (15). These findings suggest that CEACAM6 may

serve as both a prognostic marker and a potential therapeutic target in colon cancer.

A multicenter, dual-platform investigation was conducted, across three tertiary hospitals in China, analyzing 301 patients with colon cancer. Notably, the present study represents a significant expansion beyond our previous single-center pilot investigation (16), which analyzed 120 patients from one institution. The current multicenter design, larger sample size (301 vs. 120), incorporation of long-term overall survival (OS) data (follow-up until 2025), and multivariate Cox regression analysis which established CEACAM6 and FOXP3 as independent prognostic factors, collectively provide a higher level of evidence and move beyond initial correlation to establish prognostic value. Furthermore, the samples used herein are entirely new and non-overlapping with the previous publication, having been collected between 2015 and 2020 from three distinct medical centers. The present study investigated the associations of tumor-infiltrating lymphocytes (TILs), $FOXP3^+$ regulatory T cells, and CEACAM6 expression with clinicopathological characteristics and prognosis in colon cancer, while exploring their roles in tumor progression and providing a theoretical foundation for immunotherapy development.

Materials and methods

Patient specimens. A total of 301 paraffin-embedded colon cancer tissue samples were collected from July 2015 to June 2020. The sample inclusion period was limited to June 2020 to ensure a minimum of 5 years of follow-up data for OS analysis, with the final follow-up date being June 30, 2025. This approach guaranteed the availability of complete and robust survival data for meaningful Kaplan-Meier and Cox regression analyses. The specimens for the present study were obtained through a collaborative effort among three independent tertiary hospitals. A total of 161 cases were collected from the Affiliated Hospital of Jiangnan University (Wuxi, China), while Jiangsu Provincial Veterans Hospital (Wuxi, China) and the First Affiliated Hospital of Soochow University (Suzhou, China) contributed 30 and 110 cases, respectively. It should be noted that these institutions share no direct administrative affiliation; their collaboration was solely facilitated by academic connections within our research team. To ensure consistency across all samples, each hospital collected specimens from their own patient archives using identical predefined inclusion and exclusion criteria. Prior to specimen acquisition, written informed consent was obtained from all participants for the use of their tissues in scientific research. Patients with prior exposure to radiotherapy, chemotherapy, or immunotherapeutic interventions were excluded from the study cohort. Histopathological examination confirmed the diagnosis of colon adenocarcinoma in all cases, with tumor staging (TNM classification) and histological grading performed in strict accordance with the Union for International Cancer Control guidelines (8th edition) (17,18). The present retrospective multicenter study was conducted with approval from the research Ethics Committee of all participating hospitals in accordance with the Declaration of Helsinki.

IHC staining for CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3. Sections (4-μM) were prepared from formalin-fixed, paraffin-embedded (FFPE) tissue blocks and subjected to standard deparaffinization through three xylene washes (5 min each) followed by graded ethanol rehydration (100, 95, 75 and 50%; 5 min each). Antigen retrieval was performed by heat-induced epitope retrieval in 10 mmol/l sodium citrate buffer (pH 6.0) at 100˚C for 15 min, after which endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol (10 min, 25˚C). Non-specific binding was minimized by 10 min blocking with 10% normal horse serum (Wuhan Boster Biological Technology) at room temperature, followed by 2 h incubation in a humidified dark chamber with the following antihuman antibodies: Monoclonal mouse IgG against CD3 (cat. no. sc-20047), polyclonal rabbit IgG against CD4 (cat. no. sc-7219) and CD8 (cat. no. sc7188), (all from Santa Cruz Biotechnology, Inc.; 1:100); monoclonal mouse IgG2a against CD45RO (cat. no. ab86080; Abcam; 1:10), monoclonal rabbit IgG against CEACAM6 (cat. no. ab134074; Abcam; 1:400) and monoclonal mouse IgG3 against FOXP3 (cat. no. ab450; Abcam; 1:50). After PBS washing, sections were incubated with biotinylated polyclonal goat antimouse/rabbit IgG secondary antibodies (cat. no. K5007; Dako; Agilent Technologies, Inc.; 1:2,000) for 1 h under identical conditions, then developed with 3,3'-diaminobenzidine tetrahydrochloride hydrate and counterstained with hematoxylin (5 min) at room temperature. Five random high-power fields per section were analyzed by light microscopy (BX53; Olympus Corporation), excluding necrotic areas, with human tonsillar FFPE sections as positive controls (obtained with donor consent) and PBS substitution as negative controls, all procedures being performed in accordance with institutional biosafety protocols and manufacturer specifications.

Scoring system for IHC. IHC expression of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 was evaluated using a validated two-tiered scoring system incorporating both staining intensity and cellular distribution (19). All slides underwent blinded evaluation by two independent pathologists. The staining intensity was scored as 0 (achromatic), 1 (light yellow), 2 (brownish yellow) or 3 (brown). In addition, the percentage of positive cells was scored as 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), or 4 (>75%). The two scores were added together, and the samples were assigned to one of four levels as follows: (-), score 0-1; (+), score 2; (++), score 3-4; or (+++), score ≥5. () and (+) were defined as negative expression, (++) as weak expression and (+++) as strong expression. Discrepant scores between the two pathologists were resolved through joint re-evaluation until a consensus was reached.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from FFPE tissue sections using the RNeasy FFPE Kit (cat. no. 73504; Qiagen, Inc.). Briefly, freshly cut sections were deparaffinized using the manufacturer's proprietary solution, followed by tissue lysis in an optimized buffer to release nucleic acids. To reverse formalin-induced cross-linking, samples were heated at 80˚C, then treated with DNase to eliminate genomic DNA contamination. Lysates were mixed with Buffer RBC, and ethanol was added to facilitate RNA binding to RNeasy MinElute spin columns. Purified

Table I. Primer pairs used for reverse transcription-quantitative PCR.

Gene name (GenBank no.)	Sequence	Product size, bp
CD3 (NM_000732.4)	F: 5'-GGGAGTCTTCTGCTTTGCTG-3'	153
	R: 5'-TTGTTCCGAGCCCAGTTTC-3'	
CD4 (NM_000616.4)	F: 5'-GTGAACCTGGTGGTGATG-3'	122
	R: 5'-GAGACCTTTGCCTCCTTG-3'	
CD8 (NM_001768.7)	F: 5'-ATGGCCTTACCAGTGACCG-3'	104
	R: 5'-AGGTTCCAGGTCCGATCCAG-3'	
CD45RO (NM_002838)	F: 5'-TCTGCTGGAACTGACACG-3'	168
	R: 5'-CTCATTAACATTTAGCTTTG-3'	
CEACAM6 (BC005008.1)	F: 5'-TCCAGCAATCCACACAAGAG-3'	144
	R: 5'-GGACAGGAGCACTTCCAGAG-3'	
FOXP3 (NM_014009.3)	F: 5'-TCCCAGAGTTCCTCCACAAC-3'	122
	R: 5'-ATTGAGTGTCCGCTGCTTCT-3'	
β-actin (NM_001101.3)	F: 5'-CACTGTGCCCATCTACGAGG-3'	154
	R: 5'-AATGTCACGCACGATTTCC-3'	

F, forward; R, reverse.

RNA was eluted in a minimum of 14 μl of RNase-free water. First-strand cDNA was synthesized from total RNA using the RevertAid™ First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. qPCR was performed with FastStart Universal SYBR Green Master (Rox) kit (cat. no. 4913914001; Sigma-Aldrich; Merck KGaA) under the following conditions: Initial denaturation at 95˚C for 1 min; 45 cycles of 95˚C for 20 sec, 58˚C for 30 sec, and 68˚C for 45 sec. β-actin served as the endogenous control. Relative gene expression was calculated via the $2^{-\Delta\Delta Cq}$ method using Cq values normalized to β-actin (20). All primers were synthesized by Sangon Biotech Co., Ltd., as shown in Table I.

Statistical analysis. All statistical analyses were carried out using SPSS (v26; IBM Corporation). Normality of continuous variables was assessed using Shapiro-Wilk tests. Normally distributed continuous variables are presented as the mean ± standard deviation. For comparisons of mRNA expression levels between groups, the independent samples t-test was used after confirming normality (Shapiro-Wilk test, P>0.05) and homogeneity of variances (Levene's test, P>0.05). The χ^2 test was employed to assess the association between expression levels and patient characteristics. Survival was analyzed by Kaplan-Meier curves, and differences were assessed using the logrank test. Variables were selected by univariate and multivariate Cox regression analyses, with forest plots visualized using R (version 4.5.1). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. From July 2015 to June 2020, a total of 301 patients with colon cancer were enrolled. The study population comprised 141 patients (46.8%) with early-stage disease (stage I-II) and 160 patients (53.2%) with advanced-stage disease (stage III-IV). Sex distribution showed a male predominance (n=174, 57.8%) compared with female patients (n=127, 42.2%). Tumor laterality was distributed with 159 cases (52.8%) presenting as left-sided tumors and 142 cases (47.2%) as right-sided tumors. Histopathological examination revealed poorly differentiated tumors in 96 cases (31.9%), while the majority exhibited well-to-moderately differentiated features (n=205, 68.1%). The mean age at the time of surgery was 64.94±10.63 years (range: 32-93 years).

Association between CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 expression and clinicopathological data. IHC analysis of colon cancer specimens revealed distinct spatial distribution patterns of TILs, with infiltration densities significantly associated with tumor stage and differentiation (Table II). Early-stage tumors (I-II) exhibited significantly higher infiltration of $CD3^+$ (P<0.001), $CD8^+$ (P=0.001), and $CD45RO^+$ T-cells (P<0.001) compared with advanced-stage (III-IV) tumors, whereas $CD4^+$ T-cell infiltration showed an inverse correlation with tumor progression (P=0.014). Similarly, well-to-moderately differentiated tumors displayed significantly greater infiltration of $CD3^+$ (P<0.001) and $CD45RO^+$ T-cells (P<0.001) than poorly differentiated tumors (Fig. 1), while $CD4^+$ T-cell density was reduced in higher-grade tumors (P=0.020). By contrast, no significant associations were found between TIL subsets and patient age (all P>0.05) or tumor location (P>0.05). Notably, sex differences were observed only for $CD45RO^+$ T-cells (P=0.017), with female patients exhibiting higher infiltration levels than males.

IHC staining demonstrated strong FOXP3 and CEACAM6 expression in 65.4% (119/182) and 61.8% (115/186) of cases, respectively (Table III). Both FOXP3 and CEACAM6 showed no significant associations with patient age, sex, or tumor location (P>0.05). Notably, elevated expression of both FOXP3 and CEACAM6 was observed in advanced-stage (III-IV) tumors (P<0.001) and poorly differentiated colon cancer (P<0.001)

Table II. Relationship between clinicopathological parameters and $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T-cell infiltrations in patients with colon cancer.

		$CD3^+$ T cell				$CD4^+$ T cell				$CD8^+$ T cell				$CD45RO^+$ T cell			
Characteristic	n	Strong	Negative/ weak	χ^2	P-value	Strong	Negative/ weak	χ^2	P-value	Strong	Negative/ weak	χ^2	P-value	Strong	Negative/ weak	χ^2	P-value
Age, years				0.440	0.507			0.17	0.681			2.025	0.155			0.031	0.860
≤60	93	57	36			29	64			22	71			29	64		
>60	208	119	89			60	148			66	142			67	141		
Sex				2.549	0.110			0.01	0.909			2.269	0.132			5.653	0.017
Male	174	95	79			51	123			45	129			46	128		
Female	127	81	46			38	89			43	84			50	77		
Tumor site				1.957	0.162			1.03	0.310			0.017	0.896			2.427	0.119
Left colon	159	87	72			43	116			47	112			57	102		
Right colon	142	89	53			46	96			41	101			39	103		
Differentiation				14.42	<0.001			5.45	0.020			3.692	0.055			27.099	<0.001
Well/moderately	205	135	70			52	153			67	138			85	120		
Poorly	96	41	55			37	59			21	75			11	85		
Stage				16.93	<0.001			6.02	0.014			10.529	0.001			27.165	<0.001
I and II	141	100	41			32	109			54	87			66	75		
III and IV	160	76	84			57	103			34	126			30	130		

Table III. Relationship between clinicopathological parameters and CEACAM6 and FOXP3 expression levels in patients with colon cancer.

		CEACAM6				FOXP3			
Characteristic	n	Strong	Negative/ weak	χ^2	P-value	Strong	Negative/ weak	χ^2	P-value
Age, years				0.019	0.891			1.479	0.224
≤60	93	35	58			32	61		
>60	208	80	128			87	121		
Sex				0.716	0.398			0.819	0.366
Male	174	70	104			65	109		
Female	127	45	82			54	73		
Tumor site				0.172	0.678			0.831	0.362
Left colon	159	59	100			59	100		
Right colon	142	56	86			60	82		
Differentiation				41.540	<0.001			40.138	<0.001
Well/moderately	205	53	152			56	149		
Poorly	96	62	34			63	33		
Stage				81.049	<0.001			71.310	<0.001
I and II	141	16	125			20	121		
III and IV	160	99	61			99	61		

Table IV. Relationship between CEACAM6 and CD3, CD4, CD8, CD45RO and FOXP3 expression levels in patients with colon cancer.

		CEACAM6			
T cell infiltration	n	Strong	Negative/weak	χ^2	P-value
CD3				28.670	<0.001
Strong	176	45	131		
Negative/weak	125	70	55		
CD4				11.414	0.001
Strong	89	47	42		
Negative/weak	212	68	144		
CD8				16.598	<0.001
Strong	88	18	70		
Negative/weak	213	97	116		
CD45RO				53.279	<0.001
Strong	96	8	88		
Negative/weak	205	107	98		
FOXP3				168.711	<0.001
Strong	119	99	20		
Negative/weak	182	16	166		

(Fig. 1). These results suggested that FOXP3-positive regulatory T cells and CEACAM6 may contribute to tumor progression and aggressive biological behavior in colon cancer.

Relationship between CEACAM6 expression and T cell infiltration markers. Significant associations were observed between CEACAM6 expression and T cell infiltration markers (CD3, CD4, CD8, CD45RO and FOXP3) in patients with colon cancer (P<0.05) (Table IV). These findings suggested that CEACAM6 expression is significantly correlated with altered T cell infiltration patterns in colon cancer, particularly showing a strong inverse association

Table V. Univariate analysis of prognostic factors in patients with colon cancer.

Characteristics	HR (95% CI)	P-value
TNM Staging	15.530 (7.833-30.790)	P<0.001
Differentiation	4.092 (2.800-5.981)	P<0.001
CD3	0.521 (0.356-0.761)	P=0.001
CD4	2.236 (1.529-3.271)	P<0.001
CD8	0.377 (0.225-0.633)	P<0.001
CD45RO	0.143 (0.072-0.282)	P<0.001
CEACAM6	45.560 (22.580-91.930)	P<0.001
FOXP3	30.030 (15.750-57.280)	P<0.001

HR, hazard ratio; CI, confidence interval.

with CD3, CD8 and CD45RO but a positive association with FOXP3.

mRNA expression levels of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 in stage I-II vs. stage III-IV colon cancer. A total of 50 tumor blocks were randomly selected from 301 paraffin-embedded specimens for mRNA extraction, with successful RNA isolation achieved in 35 cases (16 stage I-II and 19 stage III-IV samples). Consistent with IHC findings, transcript levels of CD3, CD8 and CD45RO were significantly downregulated in advanced-stage (III-IV) tumors compared with early-stage (I-II) lesions (P<0.001; Fig. 2). By contrast, CD4 mRNA expression showed no significant intergroup difference (P=0.457; Fig. 3). Both CEACAM6 and FOXP3 mRNA levels were significantly elevated in advanced-stage tumors (III-IV) relative to early-stage disease (P<0.001; Fig. 2).

Association between CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 expression and the prognosis of patients with colon cancer. In the present study, patient follow-up was conducted until June 30, 2025, with OS as the primary endpoint (maximum follow-up duration: 118 months). Kaplan-Meier analysis demonstrated that patients with weakly positive or negative expression of CD3 (median OS: 90 months) and CD45RO (median OS: 85 months) exhibited significantly shorter survival compared with those with strongly positive expression (P=0.001 and P<0.001; Fig. 3). By contrast, strong positivity for CD4 (median OS: 59 months), CEACAM6 (median OS: 35 months), and FOXP3 (median OS: 36.5 months) was associated with poorer outcomes relative to weak/negative expression (P<0.001; Fig. 3).

Association of survival outcomes with tumor histological grade and TNM staging classification. Kaplan-Meier survival analysis revealed significant prognostic differences based on tumor differentiation and TNM staging. Patients with poorly differentiated tumors demonstrated significantly worse outcomes, with a median OS of 40 months compared with those with well-to-moderately differentiated tumors (P<0.001; Fig. 4). Similarly, advanced-stage (III-IV) cases showed significantly reduced survival (median OS: 49 months) relative to early-stage (I-II) patients (P<0.001; Fig. 4). These findings

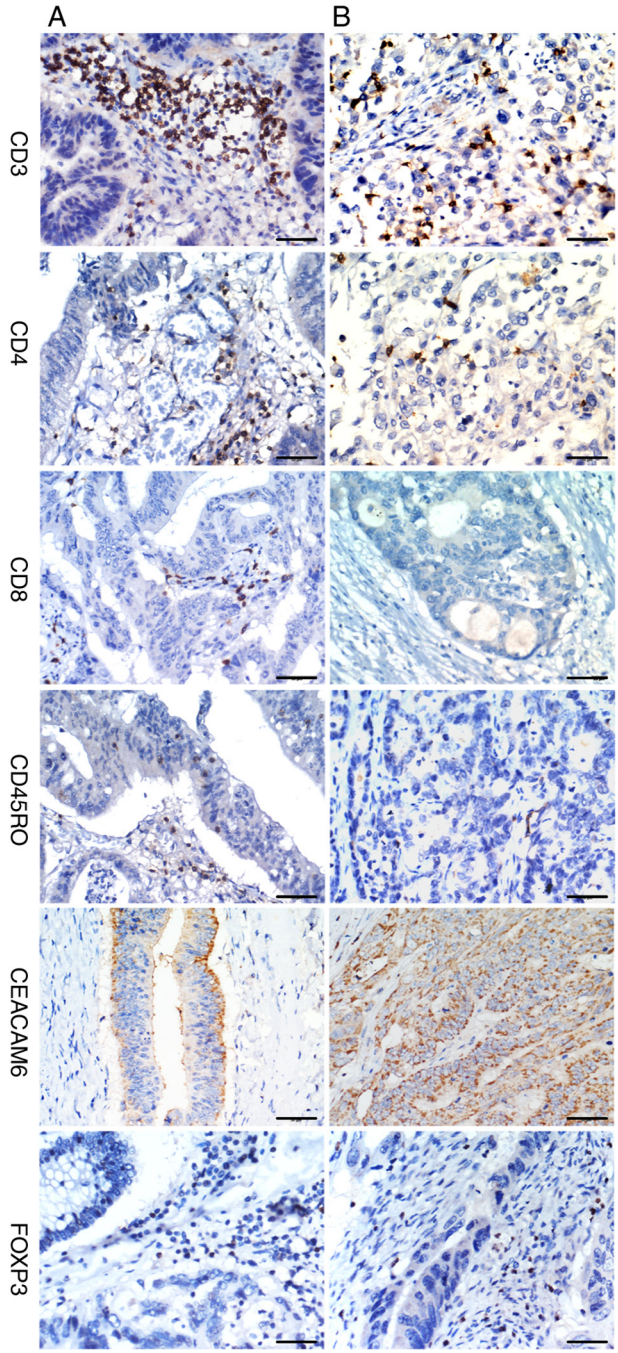

Figure 1. Representative immunohistochemical staining of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 in colon cancer (magnification, x400). Scale bar, 50 μm. (A) Well/moderately differentiated; (B) Poorly differentiated.

underscore the strong association between pathological grade, clinical stage and survival outcomes in this cohort.

Multivariable Cox regression analysis of prognostic factors in patients with colon cancer. Univariate Cox regression analysis identified multiple significant prognostic factors for colon cancer outcomes (Table V). The statistically significant variables from univariate Cox analysis were then included in multivariate Cox regression modeling. This analysis identified four independent prognostic factors (Fig. 5): TNM staging [hazard ratio (HR)=4.437; 95% confidence interval (CI):

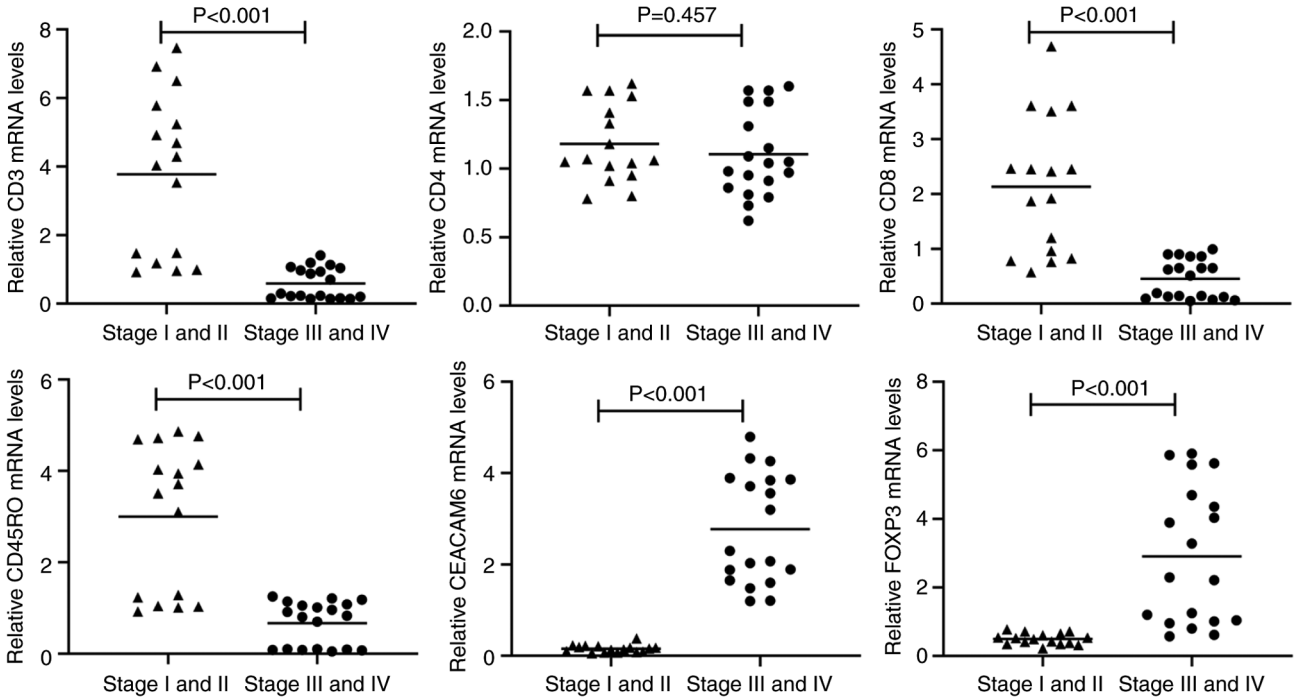

Figure 2. mRNA expression levels of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 in stage I-II vs. stage III-IV colon cancer.

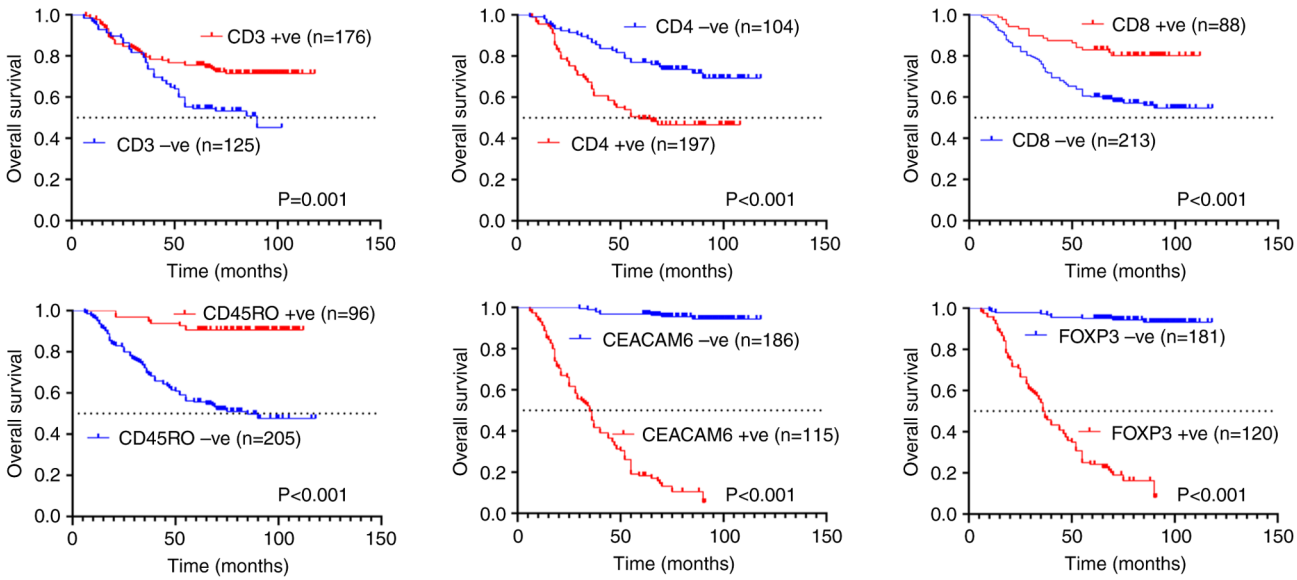

Figure 3. Association between survival time and expression levels of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 in patients with colon cancer. KaplanMeier curves for overall survival in patients with colon cancer. The P-values were determined using logrank test. -ve, negative/weak expression; +ve, strong expression.

2.142-9.189; P<0.001), tumor differentiation (HR=2.425; 95% CI: 1.635-3.600; P<0.001), CEACAM6 expression (HR=9.516; 95% CI: 4.133-21.914; P<0.001) and FOXP3 levels (HR=3.345; 95% CI: 1.572-7.118; P=0.002).

Discussion

The antitumor immune response plays a pivotal role in colon cancer progression, with T cell infiltration serving as a key determinant of clinical outcomes (21). Colon tumors are generally immunogenic and frequently exhibit infiltration by T lymphocytes (22). Multiple studies have shown that reduced T cell infiltration in colon tumors is inversely associated with disease stage and predicts improved OS (23,24). Our findings in poorly differentiated tumors and advanced pathological stages further support this observation. Specifically, it was found that increased infiltration of $CD3^+$, $CD8^+$ and $CD45RO^+$ T cells was associated with improved prognosis, whereas $CD4^+$ T cells showed paradoxical associations. However, the present data highlight limitations of current immune score systems by demonstrating that CEACAM6 and FOXP3 refine prognostic stratification beyond TIL density. For example,

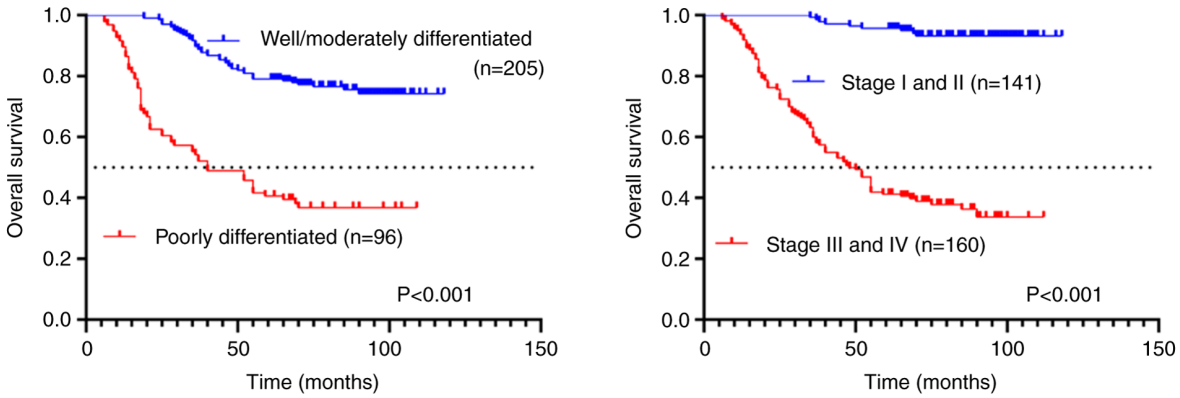

Figure 4. Association of survival outcomes with tumor histological grade and TNM staging classification. Kaplan-Meier curves for overall survival in patients with colon cancer. The Pvalues were determined using logrank test. -ve, negative/weak expression; +ve, strong expression.

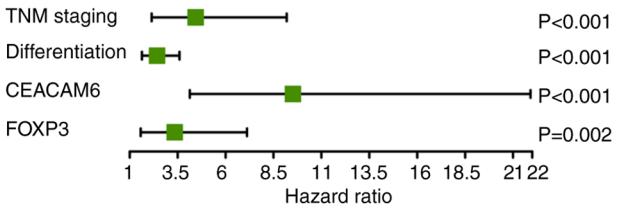

Figure 5. Forest plot displaying hazard ratios with 95% confidence intervals for various prognostic factors in the study. All P-values indicate statistically significant associations with the outcome.

patients with tumors exhibiting high CEACAM6 or FOXP3 expression exhibited worse outcomes regardless of $CD8^+$ T-cell levels, suggesting that biomarker panels integrating immune checkpoints and stromal factors may improve risk prediction (16).

FOXP3 is a highly specific marker for Tregs (25). Tumors frequently exhibit increased Treg infiltration, which contributes to immunosuppression by dampening anti-tumor immune responses (26). Multiple studies have demonstrated that elevated $CD4^+CD25^+FOXP3^+$ Treg infiltration correlates with poor prognosis in various malignancies (27,28). In the present study, transcriptional analysis revealed significant downregulation of CD3, CD8 and CD45RO mRNA levels in advanced-stage tumors, contrasting with the upregulation of CEACAM6 and FOXP3 expression. This differential expression pattern further supports their respective roles in tumor suppression and disease progression. Importantly, multivariate analysis identified CEACAM6 and FOXP3 as independent prognostic factors, underscoring their potential for refining risk stratification in clinical practice. These findings align with recent studies demonstrating that $FOXP3^+$ Treg enrichment in advanced-stage tumors highlights their therapeutic potential as targets for novel immunotherapies designed to disrupt immunosuppressive networks (29,30).

The strong association between CEACAM6 overexpression and advanced tumor stage, poor differentiation, and reduced survival aligns with its documented roles in metastasis and therapy resistance (12). Our findings build upon previous observations by revealing an inverse correlation between CEACAM6 expression and tumor-infiltrating $CD3^+$, $CD8^+$ and $CD45RO^+$ lymphocytes, suggesting its potential role in mediating immune exclusion - a key immunological feature associated with tumor aggressiveness. Mechanistically, CEACAM6 has been shown to contribute to an immunosuppressive microenvironment through multiple pathways. Experimental evidence indicates that CEACAM6 can facilitate the recruitment of MDSCs through interactions with CEACAM1 on immune cells (8). Furthermore, CEACAM6 signaling through Src family kinases and PI3K/AKT pathways can lead to the upregulation of PD-L1 expression on tumor cells (12,15). These mechanisms were demonstrated in murine models where CEACAM6 overexpression resulted in increased PD-L1 levels and MDSC infiltration, while a CEACAM6-targeted vaccine combined with anti-PD-1 antibody synergistically enhanced antitumor immunity (15). These findings position CEACAM6 as a dual biomarker and therapeutic target, particularly for immune checkpoint inhibitor-resistant cases.

The independent prognostic significance of CEACAM6 and FOXP3 supports their integration into clinical decision-making. Patients exhibiting overexpression of either CEACAM6 or FOXP3 may benefit from intensified adjuvant therapy such as anti-CEACAM6 CAR-T cells or antibody-drug conjugates (31,32), or agents modulating Treg function. Targeting CEACAM6 holds promise but requires caution due to its expression on normal granulocytes and epithelial cells, potentially leading to on-target/off-tumor toxicities such as neutropenia or dermatitis (12). Targeting $FOXP3^+$ Tregs systemically carries the risk of inducing autoimmune adverse events. Strategies focusing on specific Treg depletion within the tumor via CTLA-4 inhibition or targeting activation markers might offer improved windows of safety (27,28). Combining CEACAM6 or FOXP3/Treg-targeted strategies with PD-1/PD-L1 blockade represents a rational approach to overcome immune resistance (31). Conversely, tumors lacking both biomarkers but showing high $CD8^+$ T-cell infiltration may be suitable for de-escalation strategies. Future studies should investigate dynamic changes in these biomarkers during treatment to guide adaptive therapeutic approaches.

Our findings on the role of CEACAM6 in promoting immune suppression are mechanistically supported by the study of Pinkert *et al* (8), which showed that inhibiting the CEACAM6-CEACAM1 interaction

potentiates T cell-mediated cytotoxicity. Similarly, Li *et al* (9) identified CEACAM6 as a critical driver of lung cancer metastasis, underscoring its importance in advanced-stage disease across cancer types. Our observations regarding FOXP3$^+$ Tregs align with clinical evidence from Martinez-Rios *et al* (26), who demonstrated Treg-associated immunosuppression in colorectal cancer, further supporting the conserved nature of this mechanism. It should be noted, however, that the exclusive use of a patient cohort from Chinese tertiary hospitals represents a limitation of the present study, potentially restricting the generalizability of our conclusions to other ethnic and geographic populations. Variations in genetic background, environmental exposures and regional clinical practices may influence tumor immune biology and biomarker performance. Therefore, large-scale, prospective multinational studies incorporating diverse ethnicities and geographic regions will be essential to validate the universal prognostic value of CEACAM6 and FOXP3 and to establish globally applicable clinical thresholds.

Our multicenter study establishes CEACAM6 and FOXP3 as robust, independent prognostic biomarkers in colon cancer, validated through integrated IHC and transcriptomic analyses. The findings not only corroborate emerging evidence on their roles in tumor progression and immune evasion but also reveal novel mechanistic insights with direct clinical implications. CEACAM6 potentially contributes to an immunosuppressive microenvironment by recruiting MDSCs and upregulating PD-L1 (15), providing a mechanistic rationale for the observed immune exclusion and suggesting combination immunotherapy strategies targeting these pathways alongside CEACAM6 itself (32).

While our multicenter design enhances generalizability, limitations include the retrospective nature which limited the availability of consistent data on adjuvant therapies, comorbidities and molecular subtypes such as microsatellite instability status, factors that should be incorporated into future prospective analyses and RNA extraction challenges from FFPE samples (35/50 success rate). The mRNA subset was randomly selected, mitigating selection bias, but future studies should utilize more robust platforms like NanoString or digital spatial profiling for transcriptomic analysis, the latter also enabling spatial resolution of interactions within the tumor microenvironment. Prospective validation using liquid biopsies or fresh-frozen specimens is warranted. Additionally, mechanistic studies, potentially utilizing single-cell RNA sequencing or spatial transcriptomics to precisely map cellular interactions, are needed to clarify whether CEACAM6 directly modulates FOXP3$^+$ Treg recruitment or vice versa.

In conclusion, CEACAM6 and FOXP3 were established as critical biomarkers for colon cancer prognostication, with dual roles in immune evasion and tumor progression. Their incorporation into clinical practice could optimize risk stratification and therapeutic targeting, particularly in the era of precision immunotherapy.

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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

YYL and XXP conceived and designed the study, contributed to data collection and analysis, participated in the interpretation of results, and critically revised the manuscript. YYL prepared the initial draft of the manuscript. JXY contributed to the development of the research methodology and provided supervision throughout the study. JZM carried out a part of the data analysis. YYL, JXY, JZM and XXP 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 retrospective study analyzed anonymized data and was approved by the research ethics committees of the Affiliated Hospital of Jiangnan University (approval no. 2025-154; Wuxi, China), the First Affiliated Hospital of Soochow University (approval no. 2025-140; Suzhou, China) and Jiangsu Provincial Veterans Hospital (approval no. 2025-001; Wuxi, China). Written informed consent was obtained from all participants for the use of their tissues in scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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