Journal of Inflammation Research Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

A Longitudinal Dynamic Change in LMR Can Be a Biomarker for Recurrence in Fusobacterium Nucleatum-Positive Colorectal Cancer Patients

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Purpose: This study assessed lymphocyte-to-monocyte ratio (LMR) changes to predict postoperative recurrence in Fusobacterium nucleatum-positive (Fn-positive) CRC patients.

Patients and Methods: Clinical information and paraffin tissue specimens were collected from a retrospective cohort of 332 patients. The abundance of Fn in tumor tissue was measured using a quantitative polymerase chain reaction. We evaluated the prognostic value and diagnostic performance of the dynamic changes of LMR from pre-operative to post-treatment (pr-LMR-po) and the dynamic alterations of LMR from pre-operative to post-treatment to pre-end of follow-up (pr-LMR-f) in predicting recurrence in Fn-positive CRC.

Results: In the total cohort and adjuvant therapy group cohort, pr-LMR-po independently predicted recurrence-free survival in Fn-positive CRC patients. In the adjuvant therapy group, pr-LMR-po (High-High vs Low-Low: HR: 3.896, 95% CI: 1.503–10.095, p=0.005) was particularly significant. Meanwhile, pr-LMR-f can serve as a predictive biomarker for Fn-positive CRC recurrence, especially in the adjuvant therapy group cohort where the c-statistic for pr-LMR-f was 0.825 (95% CI: 0.804–0.8251), with a sensitivity of 83.6% and a specificity of 79.3%. Compared to the overall adjuvant therapy group cohort, the prognostic performance of pr-LMR-f was superior in the Fn-positive CRC adjuvant therapy group cohort (AUC: 0.825 VS 0.711). Finally, we constructed a prediction model combining pr-LMR-f and CEA. After internal validation using the bootstrap resampling, the model had an AUC of 0.9295, a sensitivity of 94%, and a specificity of 72.7% in the Fn-positive CRC adjuvant therapy group cohort.

Conclusion: This study found that pr-LMR-po predicts Fn-positive CRC prognosis, and pr-LMR-f may predict Fn-positive CRC recurrence.

Keywords: longitudinal dynamic changes in LMR, Fusobacterium nucleatum, colorectal cancer, recurrence

Introduction

In terms of cancer prevalence, colorectal cancer (CRC) is among the most frequently occurring malignancies worldwide. Based on global cancer statistics in 2020, CRC was the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths.[1,2]

In China, survival rates of CRC patients have been steadily improving with advancements in diagnostic and therapeutic technologies, including curative surgery, adjuvant chemotherapy, radiation therapy, targeted therapy, and immunotherapy.[3] Despite curative surgery, recurrence occurs in 40% of stage I–III CRC patients, with 80% experiencing recurrence within two years and 95% within five years. Standard monitoring methods like CEA, ctDNA, colonoscopy,

Received: 27 August 2024
Accepted: 29 November 2024
Published: 25 December 2024

and imaging have drawbacks such as invasiveness, cost, and low sensitivity. An urgent need exists for a clinically reproducible, easily detectable, and cost-effective monitoring approach to evaluate disease status and forecast prognosis in post-surgical CRC patients.

A combination of genetic and epigenetic alterations and environmental factors causes CRC.[4] The gut microbiota plays a significant role among these ecological factors.[5] Fusobacterium nucleatum (Fn), an opportunistic pathogen from the oral cavity, can migrate to the gut through blood circulation and the intestinal route.[6,7] Research has indicated that the levels of Fn DNA are higher in CRC tumor tissues than in nearby normal tissues.[8]

Furthermore, research using fecal metagenomic sequencing has demonstrated a consistent increase in the relative abundance of Fn from intramucosal carcinoma to advanced CRC stages, with a statistical significance.[9] It has been observed that patients with higher levels of Fn DNA have a poorer prognosis compared to those who are negative for Fn.[8] Additionally, Fn has been detected in liver and lymph node metastases of CRC, and studies have revealed that the strains present in metastatic tissues are the same as those found in primary tumors.[10] The presence of Fn in CRC can cause genetic and epigenetic modifications, stimulate tumor cell proliferation and metabolism, and alter the tumor microenvironment, thereby promoting the progression of CRC.[7]

The prognosis of solid tumors is heavily influenced by immune cells in the tumor microenvironment (TME).[11–13] Fn can selectively recruit myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs), and tumor-associated macrophages (TAMs) by upregulating pro-inflammatory cytokines and chemokines, ultimately reshaping the immune microenvironment of the tumor.[14] The abundance of Fn is negatively associated with tumor-infiltrating lymphocytes[15] and induces the polarization of TAMs from an M1 to an M2 phenotype.[16,17] Furthermore, there is a correlation between the TME and circulating immune cells. Research has revealed a positive association between $CD3^+$ T cells and $CD8^+$ T cells in tumor tissue and $CD4^+$ T cells and $CD8^+$ T cells in peripheral blood.[18,19] Additionally, research has shown a correlation between TAMs in tumor tissue and monocytes in the bloodstream.[20] Several studies suggest monitoring lymphocyte-to-monocyte ratio (LMR) dynamics in peripheral blood could prognosticate CRC and its recurrence.[21] However, no evidence suggests whether dynamic LMR changes can prognosticate CRC or serve as a recurrence biomarker, especially in Fn-positive CRC patients.

This study aims to evaluate dynamic LMR changes' predictive value for prognosis and recurrence in Fn-positive CRC patients, seeking a non-invasive, cost-effective biomarker for CRC monitoring, aiming for more precise diagnostic and treatment indicators.

Materials and Methods

Study Population

This study includes clinical data from 332 stage I–III CRC patients who underwent surgery at Affiliated Hospital of Jiangnan University between January 2016 and August 2018. Inclusion criteria: CRC pathological diagnosis, confirmed TNM stage I–III (AJCC 8th edition, 2017), available longitudinal complete blood counts, CEA data, and paraffin-embedded tissue samples. Exclusion criteria: CRC related to inflammatory bowel disease, familial adenomatous polyposis, other malignancy history, hematologic, autoimmune disorders, schistosomiasis history, acute infection, or post-neoadjuvant therapy. This study was approved by the Ethics Committee of Jiangnan University Affiliated Hospital and adhered to the tenets of the Helsinki Declaration.

Data Collection

Data on lymphocyte, monocyte counts, and CEA were collected at three points for each patient: pre-surgery (half a month before surgery), post-surgery (two months after surgery without adjuvant therapy), one month after adjuvant therapy (chemotherapy or chemoradiotherapy), and before recurrence/ one year before non-recurrence follow-up end. Patients were followed for ≥5 years or until recurrence, with recurrence confirmed by serologic markers and imaging/biopsy. Recurrence-free survival (RFS) is from surgery to recurrence or follow-up end.

We assessed LMR changes from pre-op to follow-up's end at various times, including pre-op, post-treatment, and pre-follow-up end. Each time, optimal CEA and LMR cutoffs were identified using ROC curves and the Youden index. LMR ≥ cutoff was "High," < cutoff was "Low."

DNA Extraction

Genomic DNA was extracted from whole CRC tissue sections in formalin fixed paraffin-embedded (FFPE) tissues blocks using the TIANquick FFPE DNA Kit (TIANGEN, China) following the manufacturer's protocol. Concentration and purity were assessed with Nanodrop One (Thermo Fisher Scientific, America). Four samples (concentration <10 ng/μL or OD 260/280 ratio out of 1.8–2.0) were discarded. DNA was stored at −20 °C before PCR amplification.

Quantitative Real-Time PCR

qPCR detected relative Fn (NusG gene) and PGT gene abundances in colorectal cancer tissues. Reactions on a 96-well plate included 100 ng DNA, 0.4 μM primers, 0.2 μM probes (Sangon Biotech, China), and 1x ChamQ Geno-SNP Probe Master Mix (Vazyme, China), totaling 20 μL. DNA was amplified on a LightCycler 480 II: 95°C for 5 mins, then 45 cycles at 95°C for 10 secs and 60°C for 1 min. Duplication was averaged for Ct values. Spearman correlations for Ct duplicates were 0.984 (Fn, n=137) and 0.994 (PGT, n=195). Fn levels were calculated relative to PGT using the 2-ΔCt method (ΔCt = Fn Ct - PGT Ct).

The primers and probes for each experiment are as follows:[15,22] Fusobacteria forward primer, 5′-CAACCATT ACTTTAACTCTACCATGTTCA-3′; Fusobacteria reverse primer, 5′-GTTGACTTTACAGAAGGAGATTATGTA AAAATC-3′; Fusobacteria FAM probe, 5′-TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC-3′; PGT forward primer, 5′-ATCCCCAAAGCACCTGGTTT-3′; PGT reverse primer, 5′-AGAGGCCAAGATAGTCCTGGTAA-3′; PGT VIC probe, 5′-CCATCCATGTCCTCATCTC-3′.

Statistical Analysis

ROC curves and the Youden index defined the optimal LMR and CEA cutoffs. Categorical variables were analyzed using Chi-square and Fisher's exact tests. Patients were categorized into four groups based on pre- and post-treatment LMR changes: Low-Low, High-High, Low-High, and High-Low. Additionally, LMR levels at pre-operative, post-treatment, and before follow-up end were classified into four groups: the normal group (all LMR≥ cutoff), the first group (one LMR < cutoff), the second group (two LMRs < cutoff), and the third group (all LMR < cutoff).

Kaplan-Meier curves and Log rank tests were used for survival analysis. Multivariate Cox regression, adjusted for factors significant in univariate analysis, was used to assess RFS. Logistic regression identified independent recurrence risk factors in colorectal cancer. R 4.1.2 was used for model fitting, nomogram display, model validation, and prediction effect evaluation. All statistical tests were two-sided; Analyses were conducted with IBM SPSS 26 and R 4.1.2; $p < 0.05$ was significant.

Results

Baseline Characteristics of CRC Patients

Of the 811 patients who underwent radical surgery, 332 had available clinical data and Fn DNA results (Figure 1). Using qPCR, 41.3% of tissue samples were classified as Fn-positive; 46% experienced recurrence (Table 1). Fn-positivity was higher in right colon cancers, with most cases at the T4 stage and having lymph node metastasis (LNM) (N1: 45.6%, N2: 50%) (Table 1). These traits were consistent in the adjuvant therapy group with Fn-positive CRC (Supplementary Table 1). However, differences in T and N stages were noted in the surgery-only group. This may be related to the predominance of early-stage patients and fewer cases in the surgery-only group (Supplementary Table 2).

Distribution Characteristics of pr-LMR-po Change Group

In Fn-positive CRC patients, the Low-Low group predominates in the pr-LMR-po change group (46.7%, 64/137), with a 65.6% recurrence rate (42/64) in the total cohort. Similarly, in the adjuvant therapy cohort, 42.4% (42/99) are Low-Low group, with a 76.2% recurrence (32/42). In contrast, in the surgery-only cohort, the Low-Low patients have a 50% recurrence rate (5/10). This may be related to the relatively fewer Fn-positive patients (Supplementary Table 3).

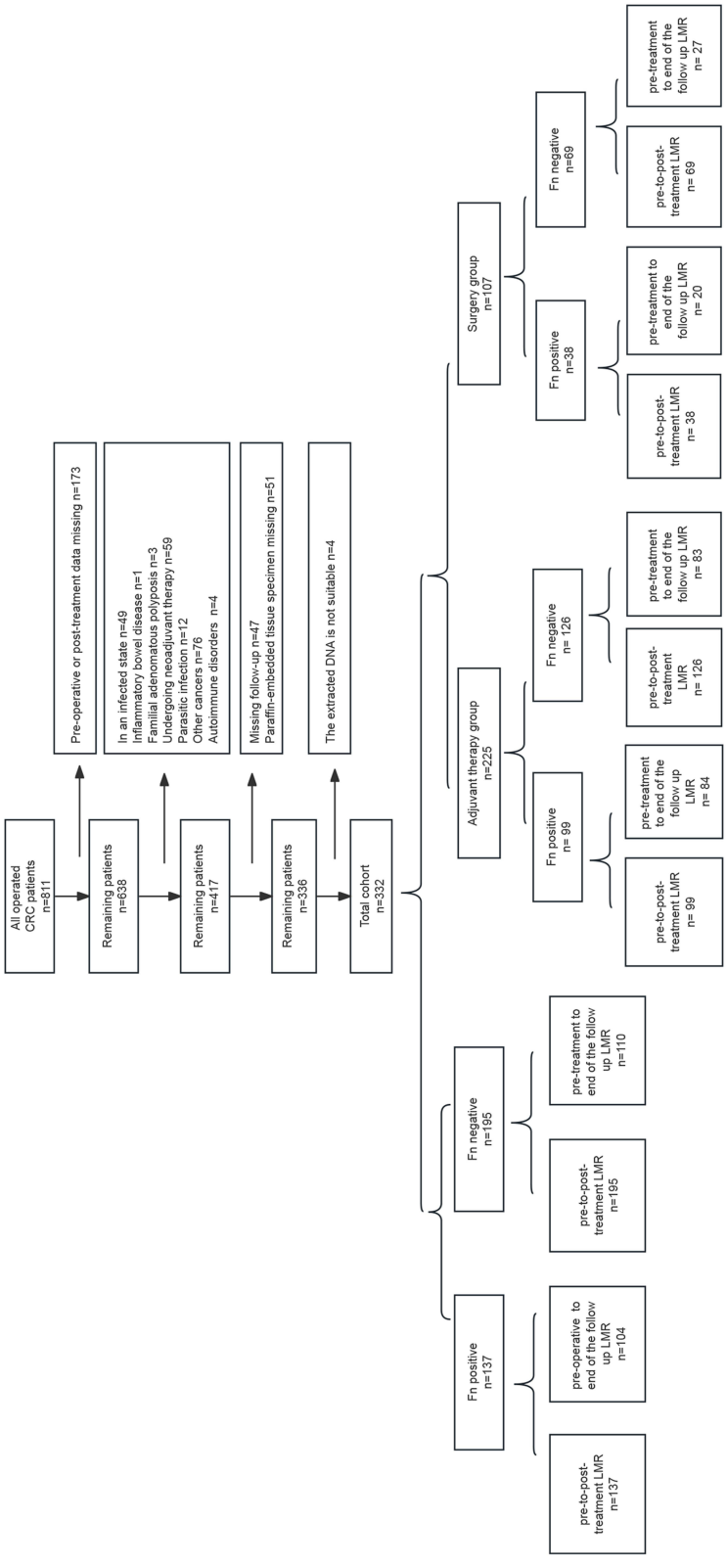

Figure 1 Flowchart of the patient cohort based on inclusion and exclusion criteria.

Table 1 Baseline Clinicopathologic Characteristics of Patients by Fn DNA Test in the Total Cohort

Clinicopathological Characteristics	Fn-Positive n=137 (%)	Fn-Negative n=195 (%)	Fn-Positive Patient (n =137)		Fn-Negative Patient (n=195)	
			Relapse n=63 (%)	No Relapse n=74 (%)	Relapse n=58 (%)	No Relapse n=137 (%)
Age						
<=70	120 (42.4)	163 (57.6)	55 (45.8)	65 (54.2)	50 (30.7)	113 (69.3)
>70	17 (34.7)	32 (65.3)	8 (47.1)	9 (52.9)	8 (25.0)	24 (75.0)
Gender						
Female	51 (37.8)	84 (62.2)	20 (39.2)	31 (60.8)	20 (23.8)	64 (76.2)
Male	86 (43.7)	111 (56.3)	43 (50.0)	43 (50.0)	38 (34.2)	73 (65.8)
T stage						
1	3 (37.5)	5 (62.5)	0 (0.0)	3 (100.0)	1 (20.0)	4 (80.0)
2	23 (35.4)	42 (64.6)	6 (26.1)	17 (73.9)	13 (31.0)	29 (69.0)
3	19 (33.9)	37 (66.1)	12 (63.2)	7 (36.8)	12 (32.4)	25 (67.6)
4	92 (45.3)	111 (54.7)	45 (48.9)	47 (51.1)	32 (28.8)	79 (71.2)
N stage						
0	70 (36.8)	120 (63.2)	15 (21.4)	55 (78.6)	27 (22.5)	93 (77.5)
1	41 (45.6)	49 (54.4)	25 (61.0)	16 (39.0)	19 (38.8)	30 (61.2)
2	26 (50.0)	26 (50.0)	23 (88.5)	3 (11.5)	12 (46.2)	14 (53.8)
Grade						
High	2 (22.2)	7 (77.8)	0 (0.0)	2 (100.0)	0 (0)	7 (100)
Mod	96 (41.2)	137 (58.8)	45 (46.9)	51 (53.1)	39 (28.5)	98 (71.5)
Low	39 (43.3)	51 (56.7)	18 (46.2)	21 (53.8)	19 (37.3)	32 (62.7)
Site						
Right-sided colon	34 (44.2)	43 (55.8)	14 (41.2)	20 (58.8)	13 (30.2)	30 (69.8)
Left-sided colon	35 (40.2)	52 (59.8)	16 (45.7)	19 (54.3)	13 (25.0)	39 (75.0)
Rectum	68 (40.5)	100 (59.5)	33 (48.5)	35 (51.5)	32 (32)	68 (68.0)
Adjuvant therapy						
Yes	99 (44.0)	126 (56.0)	56 (56.6)	43 (43.4)	40 (31.7)	86 (68.3)
Chemotherapy	85 (43.8)	109 (56.2)	48 (56.5)	37 (43.5)	34 (31.5)	74 (68.5)
Chemoradiotherapy	14 (45.2)	17 (54.8)	8 (57.1)	6 (42.9)	5 (29.4)	12 (70.6)
No	38 (35.5)	69 (64.5)	7 (18.4)	31 (81.6)	18 (26.1)	51 (73.9)

Abbreviations: Mod, moderate; n, number; Fn, Fusobacterium nucleatum.

In the total cohort of Fn-negative CRC patients, the pr-LMR-po change group mainly comprises the Low-High group (88.7%, 173/195), but the recurrence rate is only 28.9% (50/173). However, in both the adjuvant therapy group cohort and surgery-only group cohort, the majority of patients belong to the Low-Low group (48.4% vs 53.6%, 61/126 vs 37/69), with a lower recurrence rate (36.1% vs 37.8%) (Supplementary Table 3).

Distribution Characteristics of pr-LMR-f Change Group

In the pr-LMR-f change group of Fn-positive CRC patients, the third group is most common in both total (32.8%, 45/137) and adjuvant therapy cohorts (31.3%, 31/99), with high recurrence rates (84.4% and 90.3%, respectively). Conversely, in the surgery-only cohort, the normal group prevails (18.4%, 7/38) with no recurrences, while the third group sees a 66.7% recurrence rate (4/6) (Supplementary Table 3).

In Fn-negative CRC patients within the pr-LMR-f change group, the first group leads in the total cohort (40%, 78/195), showing a 41% recurrence rate. In the adjuvant therapy cohort, the first and second groups are significant (19.05%). At the same time, the third group has a 57.1% recurrence rate (Supplementary Table 3).

Correlation Between pr-LMR-po Dynamic Changes and Clinical Characteristics

In the total cohort of Fn-positive CRC patients, pr-LMR-po changes were linked only to tumor location (p=0.042), with the Low-Low group found in both the left (54.3%, 19/35) and right colon (47.1%, 16/34) (Supplementary Table 4). However, in the adjuvant therapy cohort of Fn-positive CRC patients, pr-LMR-po changes were associated with age (p=0.013) and adjuvant therapy type (p=0.009), with 44.7% (38/85) in the Low-Low group receiving only chemotherapy (Supplementary Table 5). Analysis on the surgery-only group was not done due to few Fn-positive patients.

The Correlation Between pr-LMR-f Dynamic Changes and Clinical Characteristics

In the total Fn-positive CRC cohort, no significant correlation was found between pr-LMR-f changes and gender (p=0.159) or T stage (p=0.06) (Supplementary Table 6). However, these changes were significant in the adjuvant therapy cohort with T stage (p=0.004) and CEA (p=0.016). The third group was prevalent in the T4 stage (41%) with high CEA (51.4%), while the normal group mainly was T2 (54.5%) (Supplementary Table 7). Limited data in the surgical group prevented further analysis.

The Dynamic Changes of pr-LMR-po are Independent Prognostic Factors for Clinical Recurrence in CRC Patients

Upon performing Cox regression analysis with both univariate and adjusted covariates, we observed that pre-LMR (HR=1.534, 95% CI: 1.054–2.233, p=0.025), pos-LMR (HR=1.938, 95% CI: 1.285–2.924, p=0.002), and pr-LMR-po (Low-Low group: HR=2.543, 95% CI: 1.456–4.442, p=0.001) all emerged as significant adverse prognostic factors for CRC recurrence in the total cohort (Supplementary Tables 8–10). To account for the influence of postoperative adjuvant therapy and different stages on peripheral immune cells, we analyzed the predictive value of dynamic changes in LMR within the adjuvant treatment and the surgery-only groups. Among those receiving adjuvant therapy, only pos-LMR (HR=1.715, 95% CI: 1.114–2.640, p=0.014) and pr-LMR-po (Low-Low group: HR=2.536, 95% CI: 1.310–4.907, p=0.006) demonstrated statistical significance (Supplementary Tables 8–10). In the surgery-only group cohort, only pr-LMR-po (HR=6.406, 95% CI: 1.410–29.098, p=0.016) was identified as a prognostic factor for CRC recurrence, whereas pre-LMR (p=0.075) and pos-LMR (p=0.052) did not exhibit statistical significance (Supplementary Tables 8 and 10).

The Dynamic Changes of pr-LMR-po are Independent Prognostic Factors for Clinical Recurrence in Fn-Positive CRC Patients

Furthermore, our findings revealed that Fn-positive was associated with a significantly increased risk of CRC recurrence, both in the total cohort and the adjuvant therapy group cohort (HR=1.733, p=0.003 vs HR=2.24, p<0.001) (Supplementary Table 9). However, in the surgery-only group cohort, Fn-positive (p=0.365) did not show statistical significance in CRC recurrence (Supplementary Table 8).

Based on the results of the Fn tests, we divided the patients into Fn-positive and Fn-negative subgroups; we conducted further analysis to examine the correlation between dynamic changes in pr-LMR-po and CRC recurrence in different Fn statuses.

In the total cohort of the Fn-positive subgroup, univariate Cox analysis indicated significant relationships between T stage (T4 vs T2: p=0.043), N stage (p<0.001), adjuvant therapy (p<0.001), pre-LMR (p=0.001), pos-LMR (p<0.001),

and pr-LMR-po (p<0.001) with the clinical recurrence of CRC (Supplementary Table 11). After adjusting for confounding factors, pre-LMR (HR=2.484, 95% CI: 1.333–4.63, p=0.004), pos-LMR (HR: 3.171, 95% CI: 1.679–5.99, p<0.001), and pr-LMR-po LMR (Low to Low: HR=4.553, 95% CI: 1.781–11.641, p=0.002) continued to demonstrate potential for predicting CRC recurrence (Tables 2 and 3).

In the adjuvant therapy group cohort, only the N stage (p<0.001), pre-LMR (p=0.024), pos-LMR (p=0.001), and pr-LMR-po LMR (p=0.001) showed statistical significance. However, in the surgery-only group cohort, only the N stage and pos-LMR (p=0.014) were statistically significant (Supplementary Table 11). The Cox multivariate analysis indicated that in the adjuvant therapy group cohort, pos-LMR (HR=3.079, 95% CI: 1.586–5.980, p=0.001) and pr-LMR-po (Low to Low: HR=3.896, 95% CI: 1.503–10.095, p=0.005) could be considered prognostic factors for CRC. In the surgery-only group cohort, pos-LMR (HR=12.539, 95% CI: 1.428–110.084, p=0.023) showed statistical significance, while pr-LMR-po did not show statistical significance, possibly due to the small sample size (Table 3).

Table 2 Multivariate Cox Proportional-Hazard Regression Analysis of Fn[a] Positive Patients' Recurrence-Free Survival

Characteristics	Multivariate Analysis			
	Total Cohort		Adjuvant Therapy Group	
	HR[d] (95% CI[e])	p-value	HR (95% CI)	p-value
pre-LMR[b]				
T stage			–	
4	1 (Referent)			
3	1.924 (0.984–3.762)	0.056		
2	0.954 (0.393–2.319)	0.918		
1	0	0.979		
N stage				
0	1 (Referent)		1 (Referent)	
1	2.897 (1.483–5.657)	<0.001	2.558 (1.263–5.182)	0.009
2	6.24 (3.117–12.494)	<0.001	5.351 (2.607–10.987)	<0.001
Adjuvant therapy			–	
No	1 (Referent)			
Yes	2.127 (0.939–4.819)	0.07		
Pre-LMR	2.484 (1.333–4.63)	0.004	1.621 (0.920–2.855)	0.094
pr-LMR-po change group[c]				
T stage			–	
4	1 (Referent)			
3	1.575 (0.803–3.091)	0.077		
2	0.827 (0.341–2.01)	0.676		
1	0	0.987		

(*Continued*)

Table 2 (Continued).

Characteristics	Multivariate Analysis			
	Total Cohort		Adjuvant Therapy Group	
	HR[d] (95% CI[e])	p-value	HR (95% CI)	p-value
N stage				
0	1 (Referent)		1 (Referent)	
1	2.769 (1.432–5.355)	0.002	2.519 (1.242–5.108)	0.01
2	6.853 (3.383–13.881)	<0.001	5.612 (2.715–11.6)	<0.001
Adjuvant therapy			–	
No	1 (Referent)			
Yes	2.11 (0.922–4.83)	0.077		
pr-LMR-po change group				
High-High	1 (Referent)		1 (Referent)	
Low-High	1.369 (0.42–4.46)	0.602	1.15 (0.345–3.834)	0.820
High-Low	1.952 (0.639–5.96)	0.241	2.441 (0.860–6.928)	0.094
Low-Low	4.553 (1.781–11.641)	0.002	3.896 (1.503–10.095)	0.005

Abbreviations: [a]Fusobacterium nucleatum; [b]preoperative lymphocyte-to-monocytes ratio; [c]the dynamic changes of lymphocyte-to-monocytes ratio from preoperative to post-treatment; [d]Hazard Ratio; [e]Confidence Interval.

Table 3 Multivariate Cox Proportional-Hazard Regression Analysis of Patients' Recurrence-Free Survival by Fn[a]

Characteristics	Multivariate Analysis					
	Total Cohort		Adjuvant Therapy Group		Surgery Group	
	HR[c] (95% CI[d])	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Fn-positive						
pos-LMR[b]						
T stage			–		–	
4	1 (Referent)					
3	1.224 (0.641–2.341)	0.540				
2	0.78 (0.323–1.885)	0.581				
1	0	0.978				
N stage						
0	1 (Referent)		1 (Referent)		1 (Referent)	
1	2.742 (1.401–5.366)	0.003	2.619 (1.293–5.305)	0.007	3.987 (0.781–20.357)	0.096
2	6.364 (3.155–2.838)	<0.001	5.654 (2.757–11.597)	<0.001	43.946 (2.481–778.425)	0.010
Adjuvant therapy			–		–	
No	1 (Referent)					

(*Continued*)

Table 3 (Continued).

Characteristics	Multivariate Analysis					
	Total Cohort		Adjuvant Therapy Group		Surgery Group	
	HR[c] (95% CI[d])	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Yes	2.122 (0.919–4.896)	0.078				
Pos-LMR	3.171 (1.679–5.99)	<0.001	3.079 (1.586–5.980)	0.001	12.539 (1.428–110.084)	0.023
Fn-negative						
Pos-LMR						
N stage			–			
0	1 (Referent)				1 (Referent)	
1	2.212 (1.215–4.026)	0.009			2.326 (0.658–8.224)	0.19
2	2.498 (1.263–4.941)	0.009			0.835 (0.109–6.397)	0.862
Pos-LMR	2.837 (1.266–6.357)	0.011			3.02 (0.979–9.32)	0.055

Abbreviations: [a]Fusobacterium nucleatum; [b]post-treatment lymphocyte-to-monocytes ratio; [c]Hazard Ratio; [d]Confidence Interval.

The Prognostic Potential of Dynamic Changes in pr-LMR-po in the Fn-Negative CRC Group

Regarding Fn-negative CRC patients, single-factor Cox analysis indicated that pos-LMR significantly correlated with outcomes in the total and surgery-only cohorts. Nevertheless, pre-LMR and pr-LMR-po did not show significance in the three cohorts (Supplementary Table 12). Upon adjustment for confounding factors, pos-LMR (HR=2.837, 95% CI: 1.266–6.357, p=0.011) remained a significant prognostic factor for CRC recurrence only in the total cohort (Table 3).

Survival Differences with Different Fn Status and Patients' Subgroups

According to the Kaplan-Meier curves, the results show that in the total cohort and the adjuvant therapy group cohort, Fn-positive patients have a poorer prognosis compared to Fn-negative patients (Figure 2). However, in the surgery-only group cohort, there was no statistically significant difference in prognosis between Fn-positive and Fn-negative patients (p=0.361). In the total cohort, the 75% RFS for Fn-positive patients was 18.2 months, while it was 38.6 months for Fn-negative CRC patients.

We further analyzed the prognosis of Fn-positive CRC patients using the dynamics change of pr-LMR-po. In both the adjuvant therapy group cohort and the total cohort, pr-LMR-po was able to stratify Fn-positive CRC patients further and identify high-risk individuals. Taking the adjuvant therapy group cohort as an example, the 75% RFS for Fn-positive patients was 13.4 months, while it was 26.9 months for Fn-negative CRC patients. Further analysis of the pr-LMR-po for Fn-positive CRC patients revealed that the Low-Low group had a poorer prognosis than the High-High group. The 75% RFS for the Low-Low group was 7.8 months, while it was 48.1 months for the High-High group. The 75% RFS for the Low-High group was 15.2 months, and for the High-Low group, it was 13.8 months (Figure 2).

These findings support the hypothesis that pr-LMR-po can further stratify Fn-positive CRC patients and identify high-risk individuals more prone to recurrence.

pr-LMR-f Change Group is an Independent Risk Factor for CRC Recurrence

We further analyzed whether the dynamic changes in pr-LMR-f can be used as a biomarker for CRC recurrence. Logistic regression univariate analysis showed that N stage, f-LMR, and pr-LMR-f were significantly associated with clinical recurrence in different cohorts (Supplementary Table 13). After adjusting for confounding factors, we found that f-LMR and pr-LMR-f were independent risk factors for CRC recurrence in all cohorts (Supplementary Table 12). In the adjuvant treatment group, f-LMR

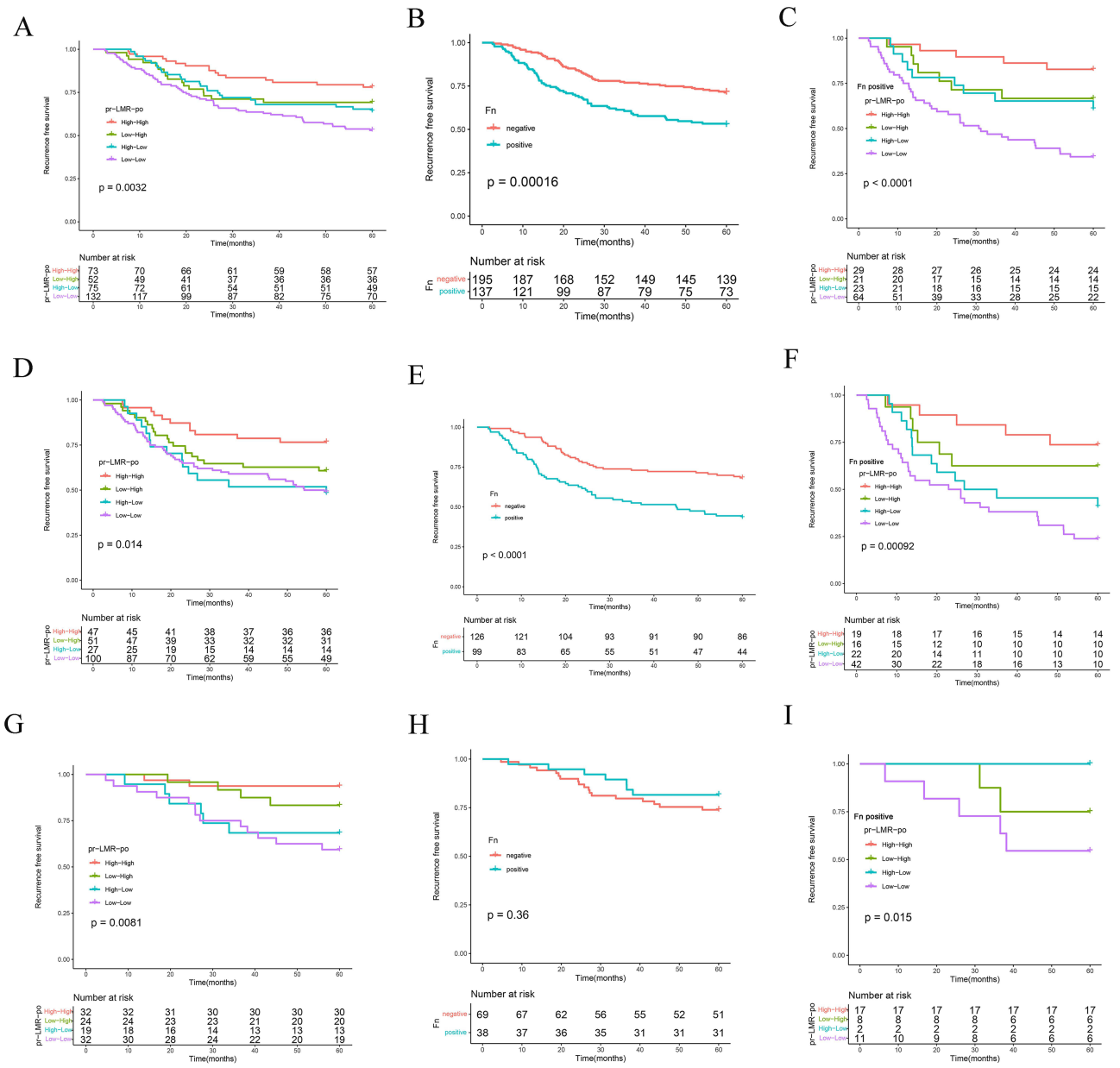

Figure 2 Kaplan-Meier survival curves illustrating the relationship between RFS and pr-LMR-po changes.

Notes: (**A**) pr-LMR-po changes group in the total cohort. (**B**) KM curve of Fn in the total cohort. (**C**) Dynamic changes of pr-LMR-po changes group in the total cohort. (**D**) pr-LMR-po changes group in the adjuvant therapy cohort. (**E**) KM curve of Fn in the adjuvant therapy cohort. (**F**) Dynamic changes of pr-LMR-po changes group in the adjuvant therapy cohort. (**G**) pr-LMR-po changes group in the surgery cohort. (**H**) KM curve of Fn in the surgery cohort. (**I**) Dynamic changes of pr-LMR-po changes group in the surgery cohort.

(OR=4.468, 95% CI: 2.205–9.054, p<0.001) and pr-LMR-f (normal group vs third group: OR=6.132, 95% CI: 2.141–17.562, p=0.001) were significantly associated with CRC recurrence (Supplementary Table 14).

pr-LMR-f are an Independent Risk Factor for CRC Recurrence in Fn-Positive Patients

In Fn-positive CRC, logistic univariate analysis revealed that N stage and f-LMR were statistically significant in all three cohorts (Table 4). However, pr-LMR-f (normal group vs third group: p<0.001) showed significance only in the total cohort and the adjuvant therapy group cohort, as there were not enough patient cases for analysis in the surgery group cohort (Table 4). After adjusting for confounding factors, we found that in the total cohort of Fn-positive CRC, f-LMR (OR=9.225, 95% CI: 3.239–26.272, p<0.001) and pr-LMR-f (normal group vs third group: OR=18.638, 95% CI: 3.039–114.309, p=0.002) were independent risk factors for clinical recurrence. The same characteristics were observed in the adjuvant therapy group cohort.

Table 4 The Result of the Univariate Logistic Regression Analysis with Fn[a] Positive

Variables	Univariate Analysis					
	Total Cohort		Adjuvant Therapy Group		Surgery Group	
	OR[d] (95% CI[e])	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Fn-positive						
Age						
<=70	1 (Referent)		1 (Referent)		1 (Referent)	
>70	1.051 (0.38–2.907)	0.924	1.905 (0.462–7.845)	0.372	0.694 (0.07–6.905)	0.756
Gender						
Female	1 (Referent)		1 (Referent)		1 (Referent)	
Male	1.55 (0.767–3.132)	0.222	1.339 (0.57–3.146)	0.503	0.8 (0.153–4.184)	0.792
N stage						
0	1 (Referent)		1 (Referent)		1 (Referent)	
1	5.729 (2.453–13.382)	<0.001	3.808 (1.45–10.001)	0.007	9.333 (1.27–68.597)	0.028
2	28.111(7.422–106.471)	<0.001	16.5 (4.131–65.897)	<0.001	1.508E+10(0)	1.0
Grade						
High	1 (Referent)		1 (Referent)		0	0.999
Mod	1.56 (0.426–5.715)	0.502	1.08 (0.222–5.251)	0.924	0.714 (0.11–4.653)	0.725
Low	1.5 (0.377–5.965)	0.565	0.8 (0.153–4.184)	0.792	1 (Referent)	
Site						
Right colon	1 (Referent)		1 (Referent)		0	0.999
Left colon	1.203 (0.464–3.121)	0.704	0.796 (0.271–2.342)	0.678	2.7 (0.448–16.255)	0.278
Rectum	1.347 (0.586–3.096)	0.483	1.462 (0.551–3.881)	0.446	1 (Referent)	
f-LMR[b]						
High	1 (Referent)		1 (Referent)		1 (Referent)	
Low	13.333 (5.098–34.871)	<0.001	13.736(4.558–41.392)	<0.001	16.667 (1.361–204.033)	0.028
pr-LMR-f change group[c]						
normal group	1 (Referent)		1 (Referent)		1 (Referent)	
first group	0.952 (0.197–4.611)	0.952	1.067 (0.209–5.444)	0.938	–	0.999
second group	9.524 (2.019–44.914)	0.004	16 (2.634–97.186)	0.003	–	0.999
third group	21.333 (4.317–105.433)	<0.001	24.889(4.186–148)	<0.001	–	0.999

Notes: [a]Fusobacterium nucleatum; [b]lymphocyte-to-monocytes ratio of pre-end of follow-up; [c]the dynamic changes of lymphocyte-to-monocytes ratio from preoperative to post-treatment to pre-end of follow-up; [d]Odds Ratio; [e]Confidence Interval.

However, in the surgery-only group cohort, f-LMR (p=0.172) did not show statistical significance (Table 5). There was no multicollinearity among the variables.

In Fn-negative CRC, f-LMR (p=0.028) and pr-LMR-f (normal group vs second group: p=0.042) were statistically significant only in the total cohort. At the same time, they did not show significance in the adjuvant therapy group cohort and surgery group cohort (Supplementary Tables 15 and 16).

Table 5 The Result of the Multivariate Logistic Regression Analysis in the Fn-Positive[a] CRC Patients

Variables	Multivariate Analysis					
	Total Cohort		Adjuvant Therapy Group		Surgery Group	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
f-LMR[b]						
N stage						
0	1 (Referent)		1 ((Referent)		1 (Referent)	
1	4.308 (1.417–13.097)	0.010	2.603 (0.755–8.977)	0.13	–	0.999
2	14.222 (2.642–76.552)	0.002	10.104 (1.721–59.305)	0.01	–	1
f-LMR						
High	1 (Referent)		1 (Referent)		1 (Referent)	
Low	9.225 (3.239–26.272)	<0.001	10.57 (3.299–33.866)	<0.001	6.667 (0.437–101.732)	0.172
pr-LMR-f change group[c]						
N stage					-	
0	1 (Referent)		1 (Referent)			
1	8.647 (2.325–32.16)	0.001	4.395 (1.102–17.532)	0.036		
2	23.366 (3.752–145.514)	0.001	12.882 (1.916–86.615)	0.009		
pr-LMR-f change group					–	
normal group	1 (Referent)		1 (Referent)			
first group	0.589 (0.087–3.998)	0.588	0.897 (0.151–5.309)	0.904		
second group	7.259 (1.126–46.793)	0.037	15.453 (2.144–111.403)	0.007		
third group	18.638 (3.039–114.309)	0.002	19.503(2.851–133.435)	0.002		

Notes: [a]Fusobacterium nucleatum positive; [b]lymphocyte-to-monocytes ratio of pre-end of follow-up; [c]the dynamic changes of lymphocyte-to-monocytes ratio from preoperative to post-treatment to pre-end of follow-up.

pr-LMR-f Has the Potential to Function as a Biomarker for Monitoring the Recurrence of Fn-Positive CRC

F-LMR and pr-LMR-f are independent risk factors for CRC recurrence. We further evaluated the diagnostic performance of these markers. The results showed that in the total or adjuvant treatment group cohort, the specificity of f-LMR and pr-LMR-f was relatively low, ranging from 64.4% to 74.3%. In comparison, the sensitivity of CEA ranged from 67.2% to 69.2% (Table 6).

However, further stratified analysis revealed that in Fn-positive CRC patients, both f-LMR and pr-LMR-f had significantly increased AUC values compared to the overall CRC cohort, regardless of whether in the total or adjuvant treatment group cohort. Especially in the adjuvant therapy group cohort, we performed internal validation using Bootstrap, and the pr-LMR-f showed better diagnostic performance in Fn-positive CRC patients with a C-statistic of 0.825 (95% CI: 0.804–0.8251), sensitivity of 83.6%, and specificity of 79.3%. This result was superior to the unstratified adjuvant therapy group cohort's pr-LMR-f, which had an AUC of 0.711 (95% CI: 0.653–0.711), sensitivity of 74.2%, and specificity of 62.2% (Figure 3 and Table 5).

Additionally, in the adjuvant therapy group cohort of Fn-positive CRC, pr-LMR-f (AUC: 0.825, sensitivity: 83.6%, specificity: 79.3%) showed superior diagnostic performance compared to f-LMR (AUC: 0.787, sensitivity: 78.2%, specificity: 79.3%) (Figure 3 and Table 6). Furthermore, analysis of the DCA curve revealed that pr-LMR-f had better

Table 6 The Sensitivity, Specificity, and AUC of Markers for CRC Recurrence

	Total Cohort			Adjuvant Therapy Group			Surgery Group		
All patients	**AUC**	**Sen[a] (%)**	**Spe[b] (%)**	**AUC**	**Sen (%)**	**Spe (%)**	**AUC**	**Sen (%)**	**Spe (%)**
f-LMR[c]	0.694	74.3	64.6	0.705	66.7	74.3	0.691	90.0	48.1
pr-LMR-f[d]	0.704	71.7	64.4	0.711	74.2	62.2	0.712	95.0	44.4
CEA	0.819	69.2	94.6	0.813	67.1	95.5	0.855	78.9	92
Model 2	0.898	91.3	70	0.90	90.6	72.9	0.920	78.9	90.5
Model 1	0.895	82.7	78.8	0.894	81.2	79.7	0.941	73.7	100
Fn-positive[e]									
fLMR	0.775	85.2	69.8	0.787	78.2	79.3	0.801	83.3	76.9
pr-LMR-f	0.801	88.5	65.1	0.825	83.6	79.3	0.833	83.3	76.9
CEA	0.818	69.1	94.6	0.819	68	95.8	0.862	80	92.3
Model 2	0.933	100	65.6	0.908	92	72.7	0.940	80	90
Model 1	0.925	98.2	59.4	0.930	94	72.7	–	–	–
Fn-negative[f]									
fLMR	0.598	73.1	46.6	–	–	–	–	–	–
pr-LMR-f	0.642	87.9	36.5	–	–	–	–	–	–

Notes: [a]Sensitivity; [b]Specificity; [c]lymphocyte-to-monocytes ratio of pre-end of follow-up; [d]the dynamic changes of lymphocyte-to-monocytes ratio from preoperative to post-treatment to pre-end of follow-up; [e]Fusobacterium nucleatum positive. [f]Fusobacterium nucleatum positive; Model 1, CEA+ pr-LMR-f; Model 2, CEA+ fLMR.
Abbreviations: AUC, Area Under the Curve; CRC, colorectal cancer.

clinical net benefit than f-LMR and CEA when the threshold probability was between 0.5–0.85 (Figure 4). In summary, pr-LMR-f demonstrated good diagnostic performance and can be a biomarker for Fn-positive CRC patients.

Development, Validation, and Evaluation of the Model

Given the good diagnostic performance of pr-LMR-f and f-LMR in Fn-positive CRC, we further constructed diagnostic models, Model 1 and Model 2, by combining CEA with pr-LMR-f and f-LMR, respectively, and presented the corresponding nomogram (Supplementary Figure 1). In the adjuvant therapy group cohort and total cohort, Model 2 exhibited better calibration performance in the calibration curve analysis compared to Model 1 (Supplementary Figure 2). The models were internally validated using the bootstrap method.

In the total cohort, Model 2 demonstrated a higher internal validation C-statistic of 0.9327 (95% CI: 0.9111–0.9327), while Model 1 had an AUC of 0.9247 (95% CI: 0.8946–0.9253). However, both models showed low specificity (65.6% vs 59.4%) (Supplementary Figure 3 and Table 6).

In the adjuvant therapy group cohort, the C-statistic for Model 1 was 0.9295 (95% CI: 0.8841–0.9295), with a sensitivity of 94% and a specificity of 72.7%, while for Model 2, it was 0.9077 (95% CI: 0.8868–0.9077), with a sensitivity of 92% and a specificity of 72.7% (Supplementary Figure 3 and Table 6).

The decision curve analysis (DCA) (Figure 4) showed that for threshold probabilities between 0 and 1, the use of Model 1 and Model 2 resulted in better clinical net benefit compared to "treat-all", "treat-none", or using CEA or pr-LMR-f alone, both in the total cohort and the adjuvant therapy group cohort. Furthermore, in the total cohort, Model 1 had higher clinical net benefit than Model 2 for threshold probabilities between 0 and 0.65. In contrast, for threshold probabilities between 0.65 and 0.9, Model 1 had a slightly lower clinical net benefit than Model 2. However, in the adjuvant therapy group cohort, only for threshold probabilities between 0.75 and 0.8, Model 1 had a slightly lower

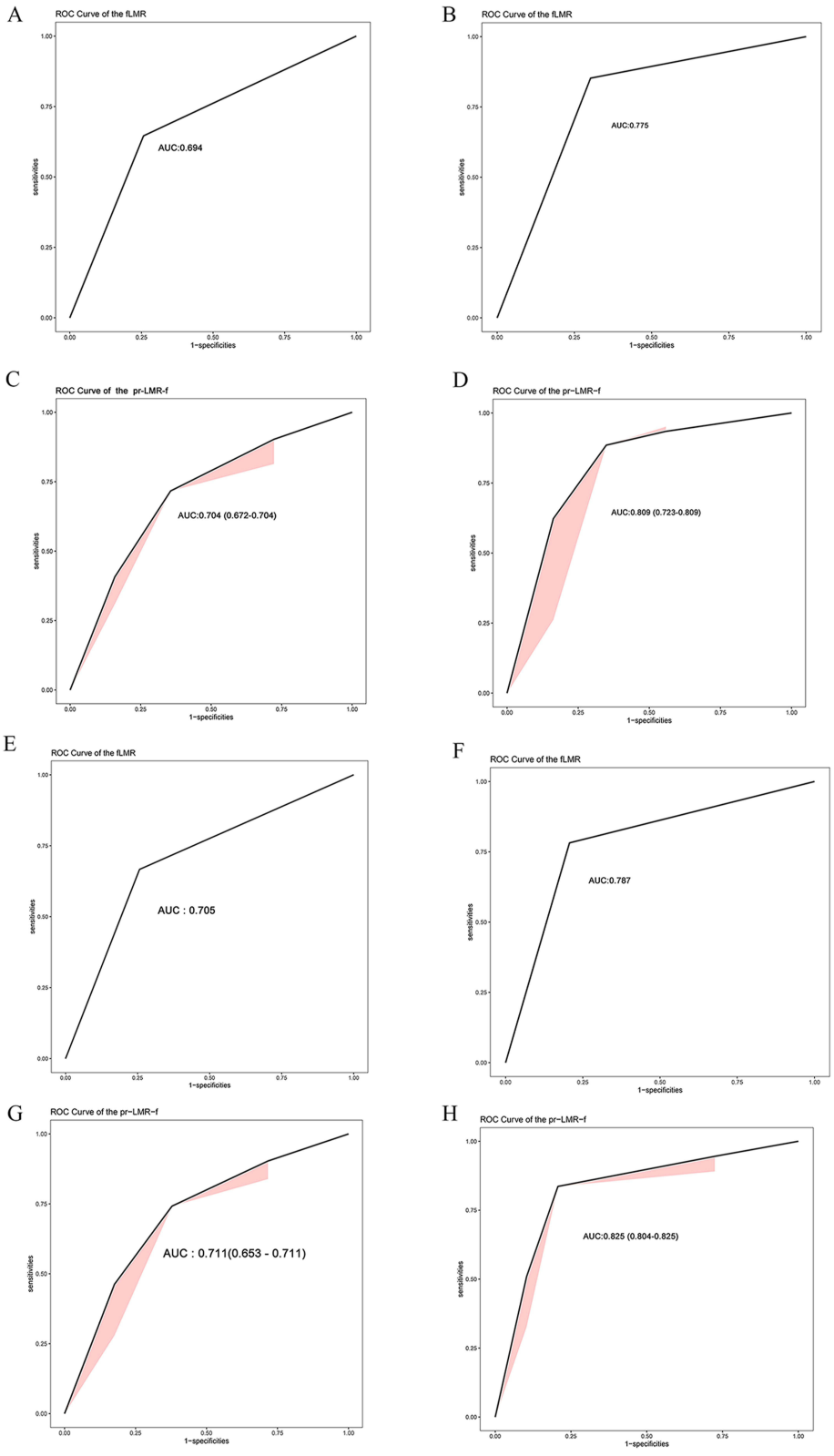

Figure 3 ROC curve analysis results in the total cohort and the adjuvant therapy group.
Notes: (**A** and **C**) ROC curves of f-LMR and pr-LMR-f in the total cohort. (**B** and **D**) ROC curves of f-LMR and pr-LMR-f in Fn-positive patients in the total cohort. (**E** and **G**) ROC curves of f-LMR and pr-LMR-f in the adjuvant therapy group. (**F** and **H**) ROC curves of f-LMR and pr-LMR-f in the adjuvant therapy group Fn-positive patients. f-LMR, lymphocyte-monocyte ratio at the end of follow-up; pr-LMR-f, pre-treatment-post-treatment-The end of follow-up changes in lymphocyte-monocyte ratio; ROC, receiver operating characteristic curve; CI, confidence interval, Fn, Fusobacterium nucleatum.

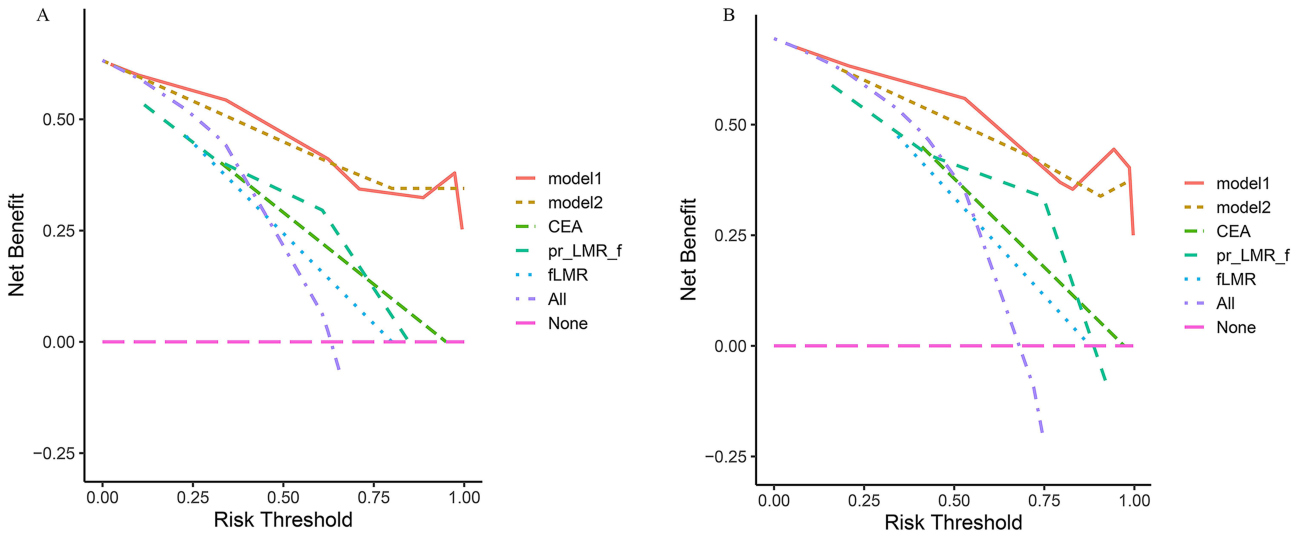

Figure 4 Decision curve analysis (DCA) results of the predictive model.
Notes: (**A**) DCA results of the predictive model in the total Fn-positive colorectal cancer patients' cohort. (**B**) DCA results of the predictive model in the adjuvant therapy group of Fn-positive colorectal cancer patients. model1, CEA + pr-LMR-f; model2, CEA + f-LMR.

clinical net benefit than Model 2. In conclusion, in terms of diagnostic performance, Model 1 performed better than Model 2 in the adjuvant therapy group cohort.

Discussion

This study evaluated the potential value of longitudinal dynamic changes in pr-LMR-f for predicting postoperative recurrence in Fn-positive CRC patients and pr-LMR-po as a prognostic factor for postoperative outcomes in Fn-positive CRC. We found that the dynamic changes in LMR are significantly associated with recurrence in Fn-positive CRC patients and have the potential to serve as a biomarker for monitoring recurrence. It is the first study to report the association between longitudinal dynamic changes in LMR and recurrence in Fn-positive CRC.

The main objective of this study was to evaluate the potential value of pr-LMR-f as a biomarker for postoperative recurrence in Fn-positive CRC. Before this study, research on LMR primarily focused on its role as a prognostic marker in CRC. Still, understanding LMR as a biomarker for CRC recurrence, especially in Fn-positive CRC, was not well-established. Firstly, we evaluated the abundance of Fn in CRC tumor tissues. In this study, Fn was found to be widely present in CRC lesions, predominantly in the right colon, accompanied by LNM, consistent with previous research findings.[23]

Additionally, in both the total cohort and the adjuvant therapy group cohort, compared to Fn-negative CRC patients, Fn-positive CRC patients had a worse prognosis, which is consistent with previous research findings.[22] However, in the surgery-only group cohort, there was no significant difference in the RFS between Fn-positive CRC patients and Fn-negative CRC patients, which may be attributed to the role transition of Fn in CRC development and progression. Previous studies have indicated that a cautious diet rich in whole grains and dietary fiber is associated with a reduced risk of Fn-positive CRC, but not Fn-negative CRC, with an HR of 0.43 (95% CI: 0.25–0.72, p=0.003).[24] Additionally, Sadia et al found that Fn can metabolize dietary fiber to produce butyrate, which inhibits the proliferation of Caco-2 cell lines.[25] This suggests that the composition of host nutritional components may play a significant role in determining the transition of Fn from an opportunistic pathogen to an early pathogenic role in the disease. Therefore, the prognosis of Fn-positive CRC patients is influenced by multiple factors.

Fn-positive CRC patients have higher immune levels, and some studies have found that the Fn DNA levels in tumor tissues are associated with high microsatellite instability (OR=5.22, 95% CI: 2.86–9.55).[26] Fn can selectively recruit immune cells and modulate the quantity and phenotype of immune cells in the TME, reshaping the TME.[7] T cell-mediated adaptive immunity can inhibit tumor progression. Patients with a higher density of $CD3^+$ T cells in CRC tumor

tissues and T cell subsets (CD8, CD45RO, and FOXP3 T cells) have better prognosis.[27] However, in Fn-positive tumor tissues, there is a decrease in $CD3^+$ T cells and $CD8^+$ T cells and an increase in MDSCs[14,28]. At the same time, the proportion of circulating immune cells may indirectly reflect changes in the immune status in the TME. Studies have shown that in sarcoma tissues, the LMR positively correlates with the CD3/CD68 ratio in tumor tissues (R=0.959).[29] Furthermore, in liver cancer patients who underwent liver transplantation, the CD3/CD68 ratio in the low LMR group was significantly reduced.[30] In CRC, the $CD3^+$ T cell and $CD8^+$ T cell density in tumor tissues positively correlate with the circulating $CD3^+$ T cell, $CD4^+$ T cell, and $CD8^+$ T cell levels.[18,19] Moreover, decreased peripheral blood monocytes are associated with increased $CD3^+$ T cells in tumors (p=0.009).[31] Circulating monocytes are also positively correlated with tumor-associated macrophages.[20] Previous studies have shown that pre-operative LMR or the dynamic changes in LMR from pre-operative to postoperative can be prognostic factors for CRC prognosis.[32,33]

In our study, pr-LMR-po was a prognostic factor for CRC in all patient cohorts, including the total, adjuvant therapy, and surgery-only cohorts. This approach avoids the differences that may arise when using pre-LMR or pos-LMR separately in different cohorts. Furthermore, we observed that pr-LMR-po can better identify individuals at high risk of recurrence. These results are consistent with previous research findings.[32,34] In Fn-positive CRC, we found that pr-LMR-po is only associated with CRC recurrence in the total and adjuvant therapy group cohorts, serving as a prognostic factor. The performance of pr-LMR-po in the surgery-only group cohort may be associated with fewer Fn-positive samples. Additionally, through Kaplan-Meier curves, we found that pr-LMR-po can stratify Fn-positive patients and identify individuals at high risk of recurrence, which is beneficial for the post-treatment management of CRC patients. However, in Fn-negative CRC, we found that pr-LMR-po and pre-LMR were not associated with CRC prognosis in the total, adjuvant therapy, and surgery-only cohorts. Only pos-LMR showed statistical significance in the total cohort but was not a prognostic factor in the adjuvant therapy and surgery group cohorts.

Fn can promote CRC metastasis through various pathways, including activating autophagy,[35] activating the NF-κB pathway,[36] and activating the TLR4/MYD88/miR21 axis.[37] Furthermore, Susan et al found that the same strain of Fn exists in both metastatic and primary tumor tissues, suggesting that Fn may co-migrate with CRC tumor cells and regulate the microbiota of the metastatic tissue, promoting the development of metastatic tumor tissue.[10] Fn can potentially be a biomarker for early diagnosis of CRC (AUC: 0.85)[38] and synchronous adenoma (AUC: 0.73).[39] However, the difficulty in obtaining tissue samples hinders the potential of Fn as a biomarker for monitoring CRC recurrence. But pr-LMR-f indirectly reflects the tumor immune microenvironment. This study found that pr-LMR-f was a risk factor for CRC recurrence in both the total and adjuvant therapy cohorts. However, its prognostic performance is not ideal, with relatively low specificity (64.4% vs 62.2%). Previous studies have shown that the dynamic changes of LMR have the potential to serve as biomarkers for monitoring CRC recurrence, which is consistent with the results of this study.[21]

Interestingly, in Fn-positive CRC, the diagnostic performance of pr-LMR-f is significantly improved. Particularly in the adjuvant therapy group cohort, the AUC is 0.825 (95% CI: 0.804–0.8251), sensitivity is 83.6%, and specificity is 79.3%. AUC and DCA analysis indicate that the diagnostic performance of pr-LMR-f is superior to CEA. Meanwhile, in the surgery-only group cohort, the value of pr-LMR-f cannot be thoroughly analyzed due to the limited number of Fn-positive patients. However, the performance of fLMR is still acceptable, with an AUC of 0.801, sensitivity of 83.3%, and specificity of 76.9%. Additionally, we found that in Fn-negative CRC, fLMR and pr-LMR-f are statistically significant only in the total cohort and not in the adjuvant therapy group cohort or surgery-only group cohort. However, in the total cohort, pr-LMR-f has low sensitivity (36.5%), an AUC of 0.642, and poor diagnostic performance. Therefore, pr-LMR-f can serve as a dynamic monitoring indicator for postoperative changes in disease status in Fn-positive CRC patients.

In Fn-positive CRC, we constructed Model 1 by combining pr-LMR-f and CEA. We found that in the total cohort, Model 1 had relatively low specificity (59.4%). However, in the adjuvant therapy group cohort, Model 1 had a C-statistic of 0.9295 (95% CI: 0.8841–0.9295), sensitivity of 94%, and specificity of 72.7%. The combination of pr-LMR-f and CEA overcame the limitations of low sensitivity when using CEA alone.[40] Additionally, both biomarkers showed convenience, speed, affordability, and good reproducibility in testing, making them ideal for dynamically monitoring disease progression.

Our study has limitations. Evidence suggests Fn can be a biomarker for metachronous adenomas,[39] and inflammatory biomarkers predict colorectal adenoma recurrence.[41] However, we did not assess how adenoma occurrence in Fn-positive CRC impacts LMR changes. In the adjuvant therapy group of Fn-positive CRC, pr-LMR-po changes were linked to

specific treatments. Still, the number receiving chemotherapy or radiation was small, limiting the evaluation of adjuvant treatment effects on LMR changes in Fn-positive CRC.

Moreover, dietary composition impacts Fn pathogenicity, but the patient's dietary status was not assessed, hindering the evaluation of changes in LMR in Fn-positive CRC under varied nutrition. With a small sample size in the Fn-positive surgery-only group, the diagnostic value of pr-LMR-f was evaluated and limited. Clinical data from a single center lacked external validation, necessitating further prospective studies for result validation.

Conclusion

In summary, pr-LMR-po is a significant prognostic factor for recurrence in Fn-positive CRC. Meanwhile, pr-LMR-f has the potential to monitor recurrence in Fn-positive CRC. Additionally, the combination of pr-LMR-f and CEA can improve predictive performance, surpassing that of single indicators.

Acknowledgments

The authors acknowledge the assistance of their colleagues at the Affiliated Hospital of Jiangnan University. This study was supported by the National Natural Science Foundation of China (81600152), Jiangsu Province Natural Science Foundation (BK20160194), Jiangsu Province Young Medical Talents (QNRC2016155), Taihu Talent Program (HB2020048) and Jiangsu Research Hospital Association Lean Medication - Special Research Fund Project (JY202107).

Disclosure

The authors report no conflicts of interest in this work.

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