

An increased number of negative lymph nodes is associated with a higher immune response and longer survival in colon cancer patients

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Background: The purpose of the present study was to examine the relationship among the number of negative lymph nodes (LNs), the local and systemic immune response, and survival in patients with colon cancer.

Patients and methods: One thousand one hundred and fifty-seven patients with colon cancer who underwent surgery at Sun Yat-sen University Cancer Center between 2009 and 2014 were included. We examined negative LNs in relation to the local and systemic immune response, including percentage carcinoma, neutrophil and lymphocyte infiltration, Crohn's-like reaction, neutrophil to lymphocyte ratio, platelets, and C-reactive protein (CRP). Disease-free survival and overall survival were also examined. We performed subgroup analysis based on the distribution of negative LNs.

Results: An increased number of negative LNs was associated with greater neutrophil invasion ($p=0.001$), more lymphocyte invasion ($p=0.001$), and more Crohn's-like reaction ($p=0.001$). No significant correlation was observed between negative LNs and the neutrophil to lymphocyte ratio. More than 12 negative LNs were associated with increased platelets and CRP levels. A higher number of negative LNs was independently associated with longer disease-free survival in stage I-II patients ($p=0.004$) and stage III patients ($p=0.015$), while negative LNs were also independent prognostic factors in stage IV patients ($p=0.007$).

Conclusion: Our study suggests that negative LNs are indicators of the immune response and are associated with a better prognosis in patients with colon cancer.

Keywords: colon cancer, negative lymph nodes, immune response, survival

Introduction

Lymph node (LN) metastasis has important prognostic implications in patients with cancer.¹ Recently, the number of negative LNs has also attracted attention for its prognostic value. It has been reported that an increased number of negative LNs was associated with longer survival in breast cancer,^{2,3} lung cancer,⁴ gastric cancer,^{5,6} esophageal cancer,⁷ cervical cancer,⁸ and colorectal cancer.⁹⁻¹² The mechanism underlying the relationship between the number of negative LNs and the patient survival remains elusive. One possible reason is that increased numbers of negative LNs indicate accurate staging and higher care quality.⁹ However, Zhuo et al reported that negative LNs are also prognostic factors in metastatic gastric cancer,⁵ which cannot be explained by accurate staging or surgical intervention.

Another possible explanation for the prognostic value of negative LNs is that the increased negative LN number indicates higher tumor and host immune interaction. Until now, little evidence has been reported to support this hypothesis.¹² Therefore,

we performed this study to evaluate the relationship between negative LNs and the host immune response, including both the local and the systemic tumor environment. We also studied the prognostic value of negative LNs in patients with colon cancer. Moreover, we performed subgroup analysis based on tumor stage in order to minimize its impact on our assessment of patients' survival.

Patients and methods

Patient selection

The data set of Sun Yat-sen University Cancer Center was built prospectively. The records were retrospectively analyzed. Patients who met the following criteria were enrolled: 1) diagnosed with colon cancer between 2009 and 2014; 2) underwent primary tumor resection; 3) available records of routine blood tests before any treatment; 4) available paraffin-embedded tissue blocks; and 5) available follow-up information. The exclusion criteria were patients with infection, hyperpyrexia, hematological disease, enterobrosis, or intestinal obstruction at the time of first diagnosis. Written informed consent was obtained from all patients. The study was approved by the ethics review board in Sun Yat-sen University Cancer Center (GZR2015-034).

Measurement of neutrophil, lymphocytes, platelets (PLT), CRP, and carcinoembryonic antigen (CEA)

Neutrophil, lymphocytes, and PLT were measured using routine blood tests. These tests were conducted using the Sysmex XE-5000™ Automated Hematology System (Shanghai, People's Republic of China). CRP was measured using the Hitachi Ltd. Automatic Analyzer 7600-020 (Tokyo, Japan). CEA was evaluated using electrochemiluminescence with the Hoffman-La Roche Ltd. Elecsys 2010 Chemistry Analyzer (Basel, Switzerland). The neutrophil to lymphocyte ratio (NLR) was categorized into two groups (>3 and ≤ 3).

Assessment of mismatch repair (MMR) status

Immunohistochemistry for the four most common MMR proteins was performed with the standard Envision (Dako Denmark A/S, Glostrup, Denmark) two-step procedure. The slides were dried overnight at 37°C, dewaxed in xylene, rehydrated through graded alcohol, and immersed in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activities. They were then pretreated in antigen retrieval buffer (EDTA buffer, pH 8.0, 100°C, 2 minutes in a

pressure cooker), and incubated with 10% normal goat serum at room temperature for 10 minutes to reduce nonspecific binding. Subsequently, the slides were incubated overnight at 4°C using the following antibodies: MLH1 (1:50; Beijing Zhong Shan Golden Bridge Biological Technology, Beijing, People's Republic of China), PMS2 (1:50; Beijing Zhong Shan Golden Bridge Biological Technology), MSH2 (1:50; Beijing Zhong Shan Golden Bridge Biological Technology), and MSH6 (1:50; Beijing Zhong Shan Golden Bridge Biological Technology). After rinsing five times with 0.01 mol/L PBS (pH=7.4) for 10 minutes, detection of the primary antibody was performed using a secondary antibody (Envision) for 1 hour at room temperature and stained with diaminobenzidine (DAB) after washing in PBS again. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. PBS was used instead of the primary antibody in the negative control. Nonneoplastic colonic mucosa, stromal cells, and infiltrating lymphocytes or the centers of lymphoid follicles were used as internal positive controls. Known MMR-deficient (dMMR) colon carcinomas served as external negative controls. Two experienced pathologists evaluated the staining results independently and without any prior knowledge of the patients' clinical data. Normal expression was defined as nuclear staining within tumor cells, whereas negative protein expression was defined as the complete absence of nuclear staining within tumor cells with concurrent internal positive controls. Tumors with the loss of MLH1/PMS2/MSH2/MSH6 proteins, as visualized using light microscopy, were classified as MLH1/PMS2/MSH2/MSH6 negative. If internal nonneoplastic tissues showed invalid negative staining, the procedure was repeated. When the opinions of the two pathologists were different, an agreement was reached by careful discussion.

Assessment of local tumor microenvironment

The carcinoma percentage, neutrophil infiltration (both in the central region and at the invasive margin), lymphocyte infiltration (both central region and invasive margin), and Crohn's-like reaction were assessed as previously reported¹³⁻¹⁵ by two pathologists who were blinded to the clinical data. Inflammatory cell reactions (neutrophil and lymphocytes) were estimated in H&E-stained sections by studying the central areas of the tumor and at the invasive margin. Four to six sections were routinely evaluated per tumor. We evaluated all of the sections and those with the most invasive part of the primary tumor were chosen to avoid bias. Dr Wan-Ming Hu and Dr Hui-Zhong Zhang assessed these markers. When the

opinions of the two pathologists were different, agreement was reached by careful discussion.

Patient follow-up and statistical analysis

Disease-free survival (DFS), defined as the time from diagnosis to the time of the first event (loco-regional recurrence, metastasis, or death), and overall survival (OS), defined as the time from diagnosis to the date of death or the date of last follow-up, were examined in this study. The date of last follow-up was December 31, 2016.

All statistical analyses were performed using SPSS version 22. Frequencies and descriptive statistics were used to compare patient characteristics. Chi-square test was used to detect difference. Survival curves were calculated with the Kaplan–Meier method, and the differences were compared using the log-rank test. Multivariate analysis using a Cox proportional hazards model was used to test independent significance by backward elimination of insignificant explanatory variables. A p -value of <0.05 was considered significant.

Results

Patient characteristics

Seven hundred and thirteen men and 444 women were included. The median age was 59 (range from 19 to 87) years. Four hundred and thirty-five (37.6%) patients had right-sided colon cancer, and 703 (60.8%) had left-sided colon cancer. One hundred and six patients (9.2%) had stage I disease, 504 (43.6%) had stage II, 264 (22.8%) had stage III, and 283 (24.4%) had stage IV. The mean total LN counts was 15.9. The mean negative LN counts was 14.5. The distributions of clinicopathological features and their relationship with negative LNs are shown in Table 1. Increased negative LNs were associated with right-sided colon cancer ($p=0.001$), well or moderately differentiated disease ($p=0.005$), a lower CEA level ($p=0.001$), dMMR status ($p=0.005$), less neural invasion ($p=0.001$), and venous invasion ($p<0.001$).

Negative LNs, the local tumor environment, and systemic inflammatory response

The relationship between negative LNs, the local tumor microenvironment, and the systemic inflammatory response is shown in Table 2. An increased number of negative LNs was associated with greater neutrophil invasion ($p=0.001$), higher-grade lymphocyte invasion ($p=0.001$), and more Crohn's-like reactions ($p=0.001$). For the systemic inflammatory response, no significant correlation was observed

between negative LNs and the NLR. More than 12 negative LNs were associated with increased PLT and CRP levels.

Impact of negative LNs on recurrence and survival

Since only 106 patients had stage I disease, we grouped them together with the 504 patients with stage II disease. Among the patients with stage I and stage II disease, 58 experienced recurrence or distant metastasis during the follow-up time. Seven of 35 (20.0%) patients with 0–3 negative LNs, 7/50 (14.0%) patients with 4–6 negative LNs, 20/167 (12.0%) patients with 7–12 negative LNs, and 24/358 (6.7%) patients with ≥ 13 negative LNs experienced recurrence or distant metastasis. As shown in Figure 1, an increased number of negative LNs was associated with longer DFS ($p=0.036$). In multivariable analyses, the impact of negative LNs ($p=0.004$) was independent of age, sex, primary tumor location, T stage, differentiation, venous invasion, neural invasion, MMR status, neutrophil infiltration, lymphocyte infiltration, CEA level, CRP level, and the NLR (Table S1). Among patients with stage III disease, 6/14 (42.9%) patients with 0–3 negative LNs, 17/36 (47.2%) patients with 4–6 negative LNs, 22/85 (25.9%) patients with 7–12 negative LNs, and 21/129 (16.3%) patients with ≥ 13 negative LNs experienced recurrence or distant metastasis. As shown in Figure 1, an increased number of negative LNs was a predictor of longer DFS ($p<0.001$). In multivariable analyses, the impact of negative LNs ($p=0.015$) was also independent of age, primary tumor location, T stage, differentiation, venous invasion, neural invasion, MMR status, neutrophil infiltration, lymphocyte infiltration, CEA level, CRP level, and the NLR (Table S2).

For patients with stage IV disease, the median OS was 19.8 months for those with 0–3 negative LNs, 23.2 months for those with 4–6 negative LNs, 23.2 months for those with 7–12 negative LNs, and 27.1 months for those with ≥ 13 negative LNs. Similarly, an increased number of negative LNs was associated with longer OS ($p<0.001$). In multivariable analyses, the impact of negative LNs ($p=0.007$) was an independent factor for OS (Table S3).

Subgroup analysis by location of LN

Previous studies reported that the location of positive LNs has an important prognostic value.¹⁶ However, the impact of the distribution of negative LNs has not been fully studied. Thus, we performed subgroup analysis by grouping LNs into pericolic nodes, intermediate nodes, and nodes at the origin of the inferior mesenteric artery. Associations were observed between negative LNs and the inflammatory response, even

Table 1 Correlations between the number of negative lymph nodes and patients' characteristics

Variable	Total	Number of negative lymph nodes				p*
		0–3	4–6	7–12	≥13	
Number of patients	1157	117 (10.1%)	133 (11.5%)	336 (29.0%)	571 (49.4%)	
Age						0.003
≤50 years	313	29 (9.3%)	33 (10.5%)	66 (21.1%)	185 (59.6%)	
51–65 years	518	55 (10.6%)	57 (11%)	161 (31.1%)	245 (47.2%)	
>65 years	326	33 (10.1%)	43 (13.2%)	109 (33.4%)	141 (43.3%)	
Sex						0.764
Male	713	74 (10.4%)	84 (11.8%)	212 (29.7%)	343 (48.1%)	
Female	444	43 (9.7%)	49 (11.0%)	124 (27.9%)	228 (51.4%)	
Primary tumor						0.001
Right-side colon	435	27 (6.2%)	26 (6.0%)	83 (19.1%)	299 (68.7%)	
Left-side colon	703	88 (12.5%)	106 (15.1%)	248 (35.3%)	261 (37.1%)	
Multiple	19	2 (10.5%)	1 (5.3%)	5 (26.3%)	11 (57.9%)	
T stage						<0.001
I	30	10 (33.3%)	3 (10%)	7 (23.4%)	10 (33.3%)	
2	108	12 (11.1%)	13 (12.1%)	41 (37.9%)	42 (38.9%)	
3	788	47 (6.0%)	83 (10.5%)	234 (29.7%)	424 (53.8%)	
4	231	48 (20.8%)	34 (14.7%)	54 (23.4%)	95 (41.1%)	
N stage						<0.001
0	698	51 (7.3%)	65 (9.3%)	195 (27.9%)	387 (55.5%)	
I	296	30 (10.1%)	42 (14.2%)	93 (31.4%)	131 (44.3%)	
2	163	36 (22.1%)	26 (16.0%)	48 (29.4%)	53 (32.5%)	
Stage						<0.001
I	106	19 (17.9%)	9 (8.5%)	39 (36.8%)	39 (36.8%)	
II	504	16 (3.2%)	41 (8.1%)	128 (25.4%)	319 (63.3%)	
III	264	14 (5.3%)	36 (13.6%)	85 (32.2%)	129 (48.9%)	
IV	283	68 (24.0%)	47 (16.6%)	84 (29.7%)	84 (29.7%)	
Differentiation						0.005
Well/moderate	875	71 (8.1%)	98 (11.2%)	265 (30.3%)	441 (50.4%)	
Poor	282	46 (16.3%)	35 (12.4%)	71 (25.2%)	130 (46.1%)	
Mucinous histology						0.114
Yes	65	12 (18.5%)	6 (9.2%)	16 (24.6%)	31 (47.7%)	
No	1088	102 (9.4%)	127 (11.7%)	319 (29.3%)	540 (49.6%)	
Neural invasion						0.001
Absent	916	83 (9.1%)	94 (10.4%)	264 (28.8%)	475 (51.9%)	
Present	241	34 (14.1%)	39 (16.2%)	72 (29.9%)	96 (39.8%)	
Venous invasion						<0.001
Absent	830	63 (7.6%)	87 (10.5%)	243 (29.3%)	437 (52.6%)	
Present	327	54 (16.5%)	46 (14.1%)	93 (28.4%)	134 (41.0%)	
CEA#						0.001
≤5 ng/mL	594	52 (8.6%)	51 (8.6%)	173 (29.1%)	318 (53.7%)	
>5 ng/mL	562	65 (11.6%)	82 (14.6%)	163 (29.0%)	252 (44.8%)	
Tumor size						0.001
≤2 cm	96	24 (25.0%)	16 (16.7%)	40 (41.6%)	16 (16.7%)	
2–5 cm	773	76 (9.9%)	94 (12.1%)	241 (31.2%)	362 (46.8%)	
>5 cm	284	16 (5.6%)	23 (8.1%)	54 (19%)	191 (67.3%)	
MMR status						0.005
dMMR	114	5 (4.5%)	12 (10.7%)	23 (20.5%)	72 (64.3%)	
pMMR	1045	112 (10.7%)	121 (11.6%)	313 (30.0%)	499 (47.7%)	

Notes: #One patient had missing CEA values. *Chi-square test was used to compare patient characteristics, and a p -value <0.05 was considered significant.

Abbreviations: CEA, carcinoembryonic antigen; MMR, mismatch repair; dMMR, MMR-deficient; pMMR, MMR-proficient.

when the distribution of negative LNs was taken into account (Tables S4–S9). However, no association was observed between age and the number of negative pericolic nodes ($p=0.351$) nor was any association observed between the NLR

and the number of negative intermediate nodes ($p=0.196$). We found that the number of negative pericolic nodes had no prognostic value, whereas an increased number of negative intermediate nodes were associated with better survival in

Table 2 Correlation between mismatch repair status, the local tumor microenvironment, and the systemic inflammatory response

Tumor microenvironment	Total	Number of negative lymph nodes				p*
		0–3	4–6	7–12	≥13	
Local tumor microenvironment						
Carcinoma percentage						0.031
Low	359	50 (13.9%)	42 (11.7%)	103 (28.7%)	164 (45.7%)	
High	797	67 (8.4%)	91 (11.4%)	233 (29.2%)	406 (51.0%)	
Neutrophil – central region						0.001
Low grade	483	64 (13.3%)	67 (13.9%)	147 (30.4%)	205 (42.4%)	
High grade	674	53 (7.9%)	66 (9.8%)	189 (28%)	366 (54.3%)	
Neutrophil – invasive margin						0.001
Low grade	786	100 (12.7%)	105 (13.4%)	234 (29.8%)	347 (44.1%)	
High grade	370	16 (4.3%)	28 (7.6%)	102 (27.6%)	224 (60.5%)	
Lymphocytes – central region						0.001
Low grade	538	71 (13.2%)	74 (13.8%)	153 (28.4%)	240 (44.6%)	
High grade	619	46 (7.4%)	59 (9.5%)	183 (29.6%)	331 (53.5%)	
Lymphocytes – invasive margin						0.001
Low grade	645	84 (13.0%)	92 (14.3%)	187 (29%)	282 (43.7%)	
High grade	512	33 (6.4%)	41 (8.0%)	149 (29.1%)	289 (56.5%)	
Crohn's-like reaction						0.001
Yes	95	5 (5.3%)	3 (3.2%)	15 (15.8%)	72 (75.7%)	
No	1059	110 (10.4%)	130 (12.3%)	320 (30.2%)	499 (47.1%)	
Systemic inflammatory response						
NLR						0.074
≤3	782	71 (9.1%)	88 (11.3%)	244 (31.2%)	379 (48.4%)	
>3	375	46 (12.3%)	45 (12%)	92 (24.5%)	192 (51.2%)	
PLT						0.004
Normal	835	85 (10.2%)	98 (11.7%)	265 (31.7%)	387 (46.4%)	
Elevated	322	32 (9.9%)	35 (10.9%)	71 (22.1%)	184 (57.1%)	
CRP						0.044
Normal	784	76 (9.7%)	89 (11.4%)	248 (31.6%)	371 (47.3%)	
Elevated	373	41 (11.0%)	44 (11.8%)	88 (23.6%)	200 (53.6%)	

Note: *Chi-square test was used to compare patient characteristics, and a p -value <0.05 was considered significant.

Abbreviations: NLR, neutrophil to lymphocyte ratio; PLT, platelets.

patients with all stages of colon cancer (Figure S1). Increased numbers of negative nodes at the origin of the inferior mesenteric artery were only associated with longer survival in patients with stage IV colon cancer ($p=0.006$, Figure S1).

Discussion

Negative LNs have been reported as prognostic factors in colon cancer, gastric cancer, breast cancer, esophageal cancer, and cervical cancer.^{2–4,6–9,11,12} In this study, we confirmed a significant correlation between the number of negative LNs and the survival of patients with colon cancer, even in metastatic colon cancer. The correlation was independent of tumor stage and other routine clinicopathological features. More importantly, we have provided clear evidence that an increased number of negative LNs was also an indicator of greater neutrophil and lymphocyte infiltration, both in the invasive margin and in the central region of tumor microenvironment. Negative LNs were also a predictor of a Crohn's-like reaction in patients with colon cancer.

The mechanism underlying the relationship between the number of negative LNs and the survival remains unclear. Several hypotheses have been proposed. One possible reason is that assessing the numbers of negative LNs helps to reduce the likelihood of misclassification of stage III disease as stage II.⁹ Furthermore, increased numbers of negative LNs might be an indicator of better therapy, including both surgical treatment and pathological assessment. However, the survival advantage associated with negative LNs in patients with metastatic colon cancer cannot be explained by these reasons.

An alternative explanation is that an increased number of negative LNs indicates a stronger immune reaction to the tumor, which is a well-known predictor of a better prognosis.¹² Once the immune system detects a tumor, the local LNs become enlarged in response. Enlarged LNs can be more easily detected surgically and pathologically. However, little evidence to date has supported this hypothesis. By analyzing the tumor microenvironment, we found that negative LNs are correlated with local neutrophil and lymphocyte infiltration,

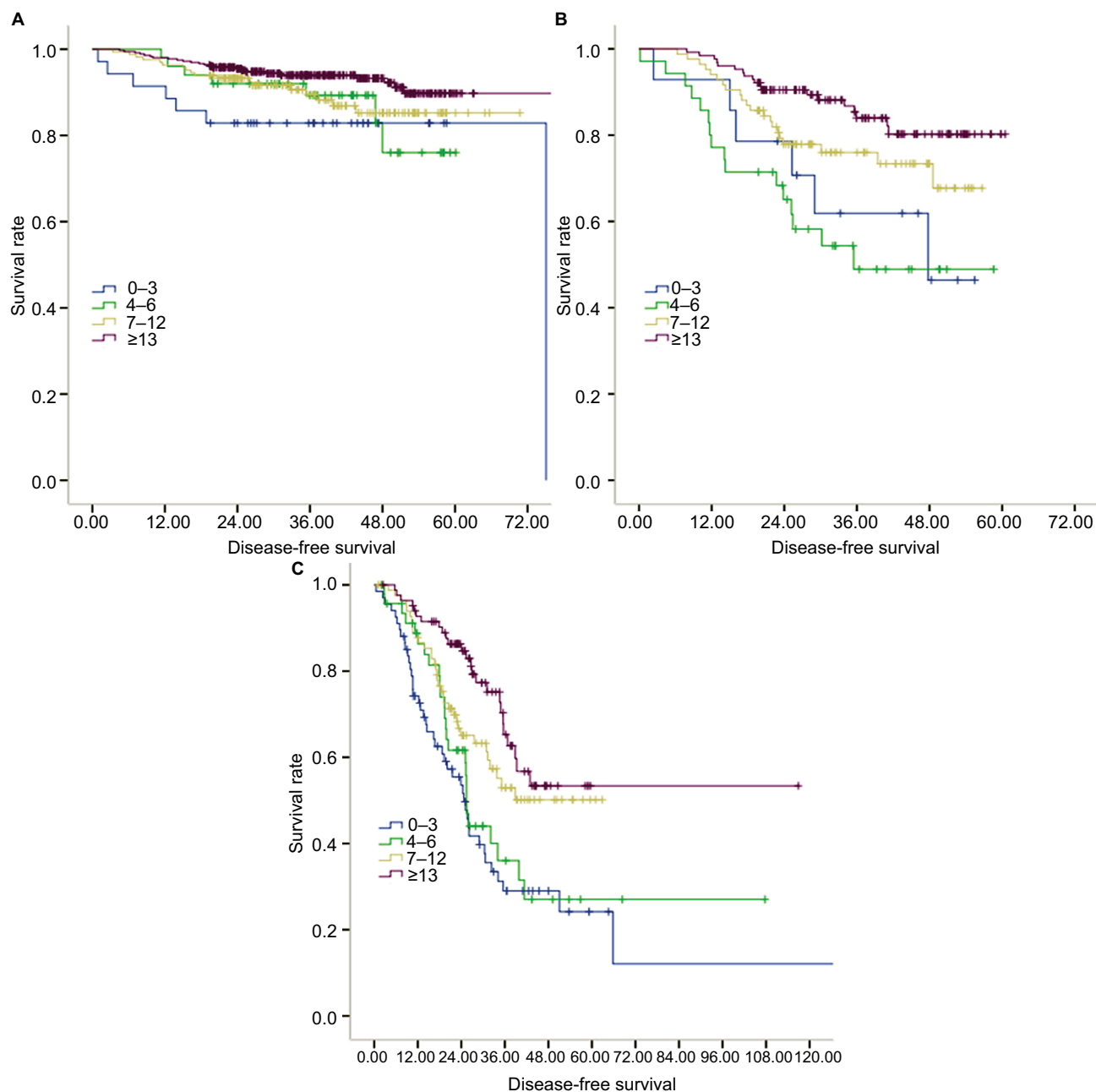


Figure 1 Disease-free survival.

Notes: Disease-free survival of patients with stage I-II colon cancer (**A**) and stage III colon cancer (**B**) grouped by negative lymph nodes (LNs); overall survival of patients with stage IV colon cancer (**C**) grouped by negative LNs.

which supports this hypothesis. Furthermore, patients with increased numbers of negative LNs are more likely to be young, have colon cancer on the right side, have a larger primary tumor, and have dMMR status. These characteristics are associated with a higher lymphocytic reaction to the tumor. A better understanding of this interaction would lead to improved risk stratification and therapeutic intervention.

Recently, the concept of negative LNs has attracted increased attention in various types of cancer.^{17–19} Ahmadi et al observed that LN yield and negative LNs were influenced

by patient age, site of disease, and T stage in patients with colorectal cancer.²⁰ By analyzing 1,167 patients with colorectal cancer, Tsai et al found that age, tumor size, and higher T stage were independent factors affecting the examinations of LN.²¹ Zhang et al reported a higher predicted accuracy of survival through the incorporation of negative LN into American Joint Committee on Cancer stages.²² Others presumed the immune response and the node count are interrelated, since antitumor immune response may lead to the enlargement of LNs and facilitate detection.²³ Those studies, together with

our finding, highlight the importance of negative LNs in patients with colorectal cancer.

Guinney et al classified most colon cancers into four consensus molecular subtypes (CMSs).^{24,25} The CMS1 group, including most dMMR colon cancers, is characterized by right-sided lesions, a higher histopathological grade, enrichment of *BRAF* mutations and strong immune cell infiltration, particularly T lymphocytes.²⁴ Notably, patients with ≥ 13 negative LNs also have these characteristics. Ogino et al also observed a positive correlation between *BRAF* mutations and increased numbers of negative LNs.¹² Although we did not classify patients into different CMS types, it is possible that patients with CMS1 colon cancer have more negative LNs, indicating that immune classification is associated with tumor molecular subtypes.

Li et al reported that the negative to positive LN ratio was a superior prognostic factor in patients with stage III colorectal cancer.¹⁰ In this study, we did not evaluate the ratio since it was also influenced by the number of positive LNs. Our study suggests that LNs could provide more prognostic information besides N stage and require more attention in clinical practice.

This study has several limitations. First, it is a retrospective study in a single institute; thus, the results should be interpreted with caution. Second, we determined the MMR status using only immunohistochemistry, and we did not use PCR. Data regarding several important molecular features, including *RAS* and *BRAF* mutations, were also absent since these factors are not routinely tested in our institute. Third, only DFS was analyzed in patients with stages I, II, and III disease since OS had not been reached yet. Fourth, due to the retrospective nature of this study, we do not know the name of the Dr who harvested the LNs. The size of mesentery was also unavailable. Those factors could lead to bias in our data.

In conclusion, our study suggests that negative LNs are indicators of immune response and are associated with a better prognosis in patients with colon cancer.

Availability of data

The data of the study were deposited in the Research Data Deposit system of Sun Yat-sen University Cancer (RDDA2018000547).

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Disclosure

The authors report no conflicts of interest in this work.

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