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Original Article

Peripheral low-density granulocytes after colorectal cancer surgery in predicting recurrence

Yurie Futoh, Yuko Kumagai, Hideyo Miyato, Hideyuki Ozawa, Rihito Kanamaru, Ai Sadatomo, Yasuharu Ohnishi, Koji Koinuma, Hisanaga Horie, Hironori Yamaguchi, Alan Kawarai Lefor, Naohiro Sata and Joji Kitayama*

Department of Gastrointestinal Surgery, Jichi Medical University, Shimotsuke, Japan

*Correspondence to: Joji Kitayama, Department of Gastrointestinal Surgery, Jichi Medical University, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan (e-mail: kitayama@jichi.ac.jp)

Abstract

Background: Low-density granulocytes (LDGs) have been shown to be increased in the peripheral blood of patients with inflammatory and malignant diseases. This study evaluated LDGs in patients who underwent radical surgery for colorectal cancer (CRC) and their impact on survival.

Methods: Patients who underwent radical colectomy between 2017 to 2021 were screened for enrolment in the study. Peripheral blood was obtained in the operating room before and after surgery and cells were recovered from the mononuclear layer after density gradient preparations. The ratio of CD66b(+) LDG to CD45(+) leukocytes was determined with flow cytometry, and the association of the ratios with patient outcomes was examined. The main outcome of interest was recurrence-free survival (RFS).

Results: Out of 228 patients treated, 176 were enrolled, including 108 colonic and 68 rectal cancers. Overall, 38 patients were stage I, 30 were stage II, 72 were stage 3, and 36 were stage IV. The number of LDGs was markedly increased immediately after surgery and the proportion of LDGs correlated positively with operating time ($r = 0.2806$, $P < 0.001$) and intraoperative blood loss ($r = 0.1838$, $P = 0.014$). Purified LDGs produced high amounts of neutrophil extracellular traps after short-term culture and efficiently trapped tumour cells *in vitro*. The proportion of postoperative LDGs was significantly higher in 13 patients who developed recurrence (median 9 (range 1.63–47.0)) per cent *versus* median 2.93 ((range 0.035–59.45) per cent, $P = 0.013$). When cut-off values were set at 4.9 per cent, a higher proportion of LDGs was strongly and independently associated with decreased RFS ($P = 0.005$). In patients with stage III disease, adjuvant chemotherapy significantly improved RFS of patients with high ratios of LDGs, but not low LDGs.

Conclusion: LDGs are recruited to circulating blood by surgical stress early in the postoperative interval after colectomy for colonic cancer and their postoperative proportion is correlated with recurrence.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and leading causes of cancer-related death worldwide[1,2]. Surgery is the standard treatment for locally advanced non-metastatic CRC; however, approximately 30 per cent of patients with stage II and III CRC still develop recurrence after curative surgery[3–6]. To date, the benefits of adjuvant treatment is noted to be approximately in 5 per cent in stage II patients[7,8], and therefore, it would be beneficial to identify high-risk patients who will benefit most from postoperative chemotherapy using biomarkers.

It is well known that neutrophils are the most abundant leucocyte population and play crucial roles as the first line of defence against pathogens. Recent studies have shown that there is heterogeneity among neutrophilic granulocytes with regard to their morphology, phenotype, and function[9–11]. Low-density granulocytes (LDGs) are recovered from the fraction of peripheral blood mononuclear cells (PBMCs) after density gradient separation and have biological characteristics distinct from conventional normal-density granulocytes[12,13]. Morphologically, LDGs are divided into two distinct subpopulations, mature granulocytes with multilobular nuclei and relatively immature neutrophils with less-segmented nuclei, which exert either proinflammatory or immunosuppressive properties[14]. Previous studies have shown that LDGs are significantly increased in the peripheral blood of patients with various inflammatory diseases, such as systemic lupus erythematosus[12,15], asthma[16], and sepsis[17–19] as well as patients with cancer[14,20–23]; however, the impact of LDGs on the pathophysiology of each disease state has not yet been fully clarified.

In a previous study, the frequency of LDGs was examined in the peripheral blood of patients who underwent abdominal surgery and found that the proportion of immunosuppressive LDGs with an immature phenotype is markedly elevated immediately after surgery[24]. The LDGs efficiently suppresses the proliferation of T cells and cytotoxicity of activated T cells *in vitro*[24]; however, the correlation with patient outcome was unclear. This study aims to examine the impact of the proportion of the LDGs on the outcome of patients who underwent colectomy with curative intent. The primary aim of this study was to evaluate the correlation of LDGs with recurrence-free survival (RFS); the secondary aim was to investigate their correlation with clinical/operating variables.

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Methods

Patients

All consecutive patients treated with surgical resection with curative intent for CRC in the Department of Gastrointestinal Surgery, Jichi Medical University, from June 2017 to October 2021, were screened for inclusion for this study, independently from tumour location and stage. Exclusion criteria were no previous chemotherapy or radiotherapy, and no background inflammatory diseases. Clinical, laboratory, pathological, and operating data collected included preoperative blood tests, pathological findings on tumour invasion, and lymphatic metastasis, and recurrence. For the analyses CRCs were categorized as right-sided and left-sided (including left CRC and rectal cancers). In all patients, follow-up was conducted with blood tests and/or radiological examination using CT at least every 3 months for 2 years after surgery. Written informed consent was obtained from all included patients and the study protocol was approved by the Ethics Committee of Jichi Medical University (RIN-A21–004).

Sample acquisition

For LDGs, peripheral blood (5 ml) was obtained from patients in the operating room just before the start of surgery and after the end of the surgical procedures. After dextran sedimentation, leucocyte-enriched plasma was overlaid on Ficoll-Hypaque solution (Pharmacia Biotech, Piscataway, NJ, USA) and centrifuged at 3000 r.p.m. for 15 min. The intermediate layers were taken as mononuclear cells (PMBCs), washed twice with PBS + 0.02 per cent EDTA, and the proportion of neutrophils included in these cells was determined with flow cytometry.

Other samples for determination of inflammatory markers (neutrophil–lymphocyte ratio (NLR), C-reactive protein (CRP), and onco-markers (carcinoembryonic antigen (CEA)) were obtained at admission (1–5 days before surgery).

Flow cytometry

Cells recovered from the PBMC layer were suspended in 100 ml PBS + 0.02 per cent EDTA, incubated with 10 ml Fc-blocker for 20 min, and then incubated with FITC-conjugated anti-CD66b and PE-conjugated anti-CD45 monoclonal antibodies (Becton-Dickinson, San Jose, CA, USA) for 30 min at 4°C. After washing, 7-amino-actinomycin D (7-AAD; Becton-Dickinson, San Jose, CA, USA) was added to a final concentration of 0.25 mg/ml. Cells positive for CD66b were considered as LDGs and their proportion against the whole live leukocytes determined as CD45(+) cells in 7-AAD (–) fraction was calculated.

Fluorescence microscopy

Neutrophil extracellular traps (NETs) and adhesion of tumour cells to NETs were examined as described previously to examine the function of LDGs[24]. After density gradient separation of blood obtained postoperatively, LDG and normal-density granulocytes were purified from PBMCs and polymorphonuclear cell (PMN) fractions respectively, using a MACS separation kit for neutrophils (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells (1×10^6) were cultured on poly-l-lysine-coated six-well plates for 2 h without stimulation and extracellular DNA visualized with 50 nM SYTOX orange under a fluorescence microscope (BZ8000, Keyence, Osaka, Japan). DLD-1 cells, a human colonic cancer cell line, were stained green by PKH-67 (Sigma-Aldrich Japan, Osaka, Japan) and 1×10^6 cells resuspended in 1 ml RPMI 1640/1 ml were added to the LDGs. After 5 min of co-incubation, the wells were gently washed with warmed medium three times and NETs and attached tumour cells were observed using appropriate wavelength filters for SYTOX orange and PHK-67 respectively.

Outcomes of interest

The outcomes of interest were the correlation of LDG with patient survival and clinical factors. RFS was defined as the length from surgery to the date when recurrence was confirmed with CT. Overall survival (OS) was defined as the length from surgery to death. A sub-analysis focused on LDGs and adjuvant treatment in stage III patients was conducted aiming to examine the impact of adjuvant chemotherapy on their outcome.

Statistical analysis

The proportion of LDGs was expressed as the median (range) and statistical differences in clinical and pathological factors were evaluated using Fisher's exact test or the Mann–Whitney *U* test. Correlations were analysed with Spearman rank regression analysis. RFS and OS were calculated using the Kaplan–Meier method and differences were evaluated using the log rank test. Independence of variables for RFS or OS was examined with Cox regression analysis including pathological T category and CEA level. Receiver operating curve (ROC) analysis was performed to determine the cut-off point of the proportion of postoperative LDGs for recurrence.

All tests were performed with Graph Pad Prism 8 Software (San Diego, CA, USA), and the standard for a significant difference was set as $P < 0.050$.

Results

Patients

Out of 228 patients screened, 176 patients who underwent colectomy with curative intent (108 patients for colonic cancer and 68 patients for rectal cancer) were enrolled in this study. The majority were male and more than half were less than 65 years old. Pathological stage was 1 or less in 51, 2 in 63, and 3 in 62 patients, and serum CEA level was elevated in 47 patients (*Table 1*).

LDGs and clinical-pathological features

The proportion of LDGs in circulating blood before and after surgery in 176 patients is shown in *Fig. 1*. The proportion of LDGs in blood obtained before surgery was generally low (median 0.95 (range 0.019–31.96) per cent); however, the proportion of LDGs was markedly elevated in blood obtained immediately after surgery (median 3.3 (range 0.035–59.45) per cent, $P < 0.001$). *Table 1* shows the correlation between the proportion of LDGs and clinical factors. The ratios of preoperative LDGs did not correlate significantly with age, sex, location, or other pathological factors as well as serum tumour markers; however, the ratio was significantly higher in patients with NLR in blood obtained at admission higher than 4.0. The ratio of postoperative LDGs did not correlate with those factors except NLR in blood obtained on admission but showed significant correlations with intraoperative blood loss ($r = 0.1838$, $P = 0.014$) and operating time ($r = 0.2806$, $P = 0.001$) as well as the NLR in blood obtained the next day after surgery (*Fig. 2*).

LDGs and NETs

The LDGs were purified from blood obtained immediately after surgery and examined for the ability to form NETs *in vitro*. After

Table 1 Ratio of low density granulocytes in circulating blood before and after surgery in 176 patients

Variables	*n*	Preoperative samples Median (range)	*P*	Postoperative samples Median (range)	*P*
Age					
<65	76	0.78% (0.025%–31.96%)	0.445	3.33% (0.059%–40.53%)	0.879
≥65	100	1.04% (0.020%–22.64%)		3.30% (0.035%–59.45%)	
Gender					
Male	104	0.86% (0.025%–31.96%)	0.903	3.15% (0.035%–51.20%)	0.590
Female	72	1.09% (0.020%–22.64%)		3.51% (0.170%–59.45%)	
Location*					
Right	60	0.82% (0.025%–21.08%)	0.207	2.51% (0.035%–59.45%)	0.208
Left	115	1.00% (0.020%–31.96%)		3.42% (0.121%–51.20%)	
cT category					
≤1	38	0.94% (0.025%–14.00%)	0.508	3.36% (0.059%–59.45%)	0.838
2	30	1.18% (0.120%–21.10%)		2.55% (0.382%–51.20%)	
3	72	0.78% (0.020%–22.64%)		2.73% (0.035%–37.26%)	
4	36	1.51% (0.062%–31.96%)		5.17% (0.121%–39.90%)	
cN category					
0	113	1.16% (0.020%–31.96%)	0.226	3.45% (0.035%–59.45%)	0.740
≥1	63	0.78% (0.065%–21.07%)		3.12% (0.121%–43.72%)	
cStage					
≤I	51	1.08% (0.025%–21.10%)	0.596	3.42% (0.059%–59.45%)	0.913
II	63	1.19% (0.020%–31.96%)		3.63% (0.035%–39.90%)	
III	62	0.82% (0.065%–21.07%)		3.21% (0.120%–43.72%)	
NLR†					
<4	148	0.82% (0.025%–22.64%)	0.010	2.56% (0.059%–59.45%)	0.006
≥4	28	3.52% (0.019%–31.96%)		9.88% (0.035%–47.40%)	
CEA‡					
Normal	129	0.92% (0.025%–22.64%)	0.610	2.62% (0.059%–59.45%)	0.320
Elevated	47	1.29% (0.020%–31.96%)		4.98% (0.035%–47.40%)	
CRP§					
Normal	130	0.98% (0.020%–31.96%)	0.178	2.48% (0.059%–59.45%)	0.150
Elevated	43	0.88% (0.062%–12.85%)		5.43% (0.035%–39.90%)	

All continuous variables are expressed as median (range). *Right side: caecum, ascending and transverse colon, Left side: descending, sigmoid and rectum. One patient had two lesions both in right and left colon. †NLR: neutrophil lymphocyte ratio. ‡CEA: serum carcinoembryonic antigen, elevated is > 5.0 ng/mL. §CRP: serum C-reactive protein, elevated is >0.3 mg/dl. Data are missing for three patients.

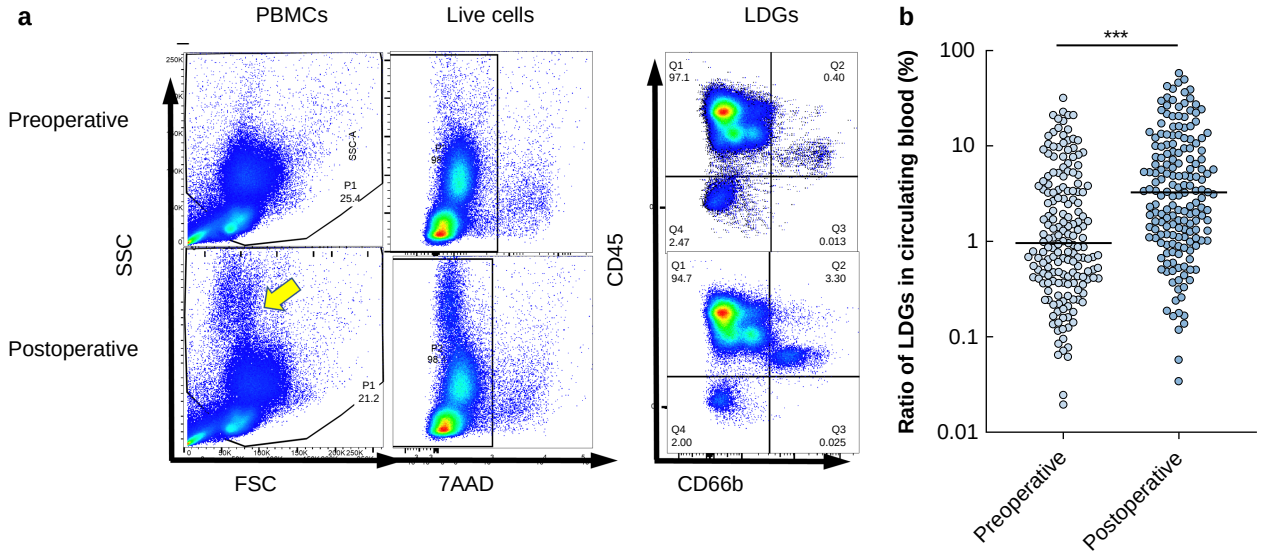

Fig. 1 Detection of low-density granulocytes using flowcytometry in blood from a representative patient

a Cells recovered from the intermediate layers after density gradient centrifugation (peripheral blood mononuclear cell (PBMC) fraction) were immunostained with phycoerythrin (PE)-conjugated anti-CD45 monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated anti-CD66b monoclonal antibody, and the ratio of CD45(+)CD66b(+) cells to total CD45(+) cells was calculated in 7-amino-actinomycin D (7-AAD) negative gated area. **b** The ratios of LDGs in blood obtained before and after surgery. The *y* axis was plotted with log scale, and bars show median values of 176 patients. ***$P < 0.001$. FSC, forward scatter; SCC, side scatter; LDG, low-density granulocyte.

2 h culture of LDGs, large amounts of NETs were detected by SYTOX staining even without stimulation (*Fig. 3*). NET structure was rarely observed in normal-density granulocytes purified from the same blood samples. When DLD-1, human colonic cancer cells, were added to the LDGs and co-incubated for 5 min, many tumour cells selectively attached to the NETs after washing.

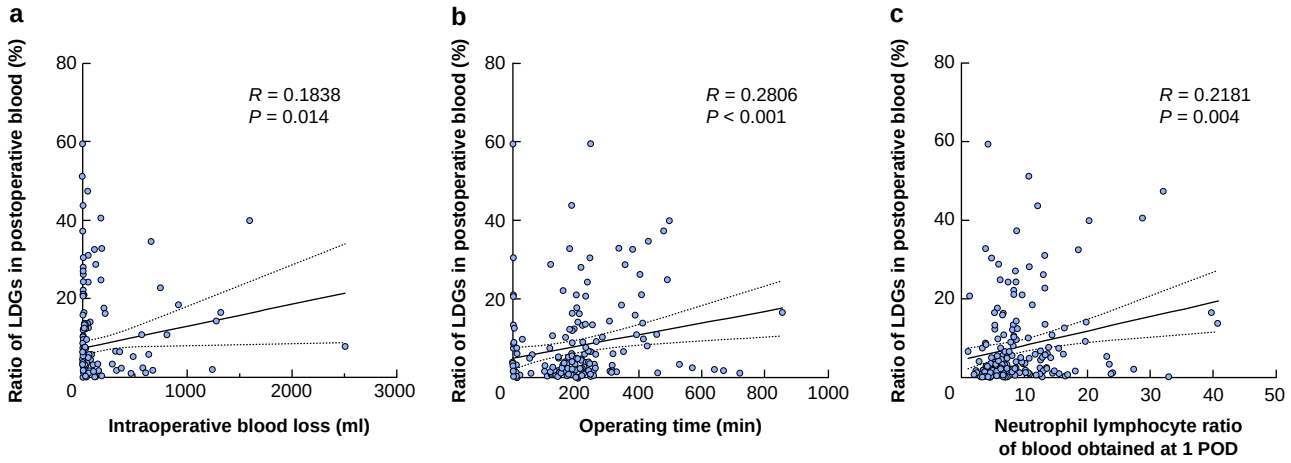

Fig. 2 Correlation between the ratios of low-density granulocytes (LDGs) examined in blood obtained the next day after surgery

a Intraoperative blood loss. **b** Operating time. **c** Neutrophil–lymphocyte ratio. *R* and *P* values were calculated with Spearman's rank regression analysis. POD, postoperative day.

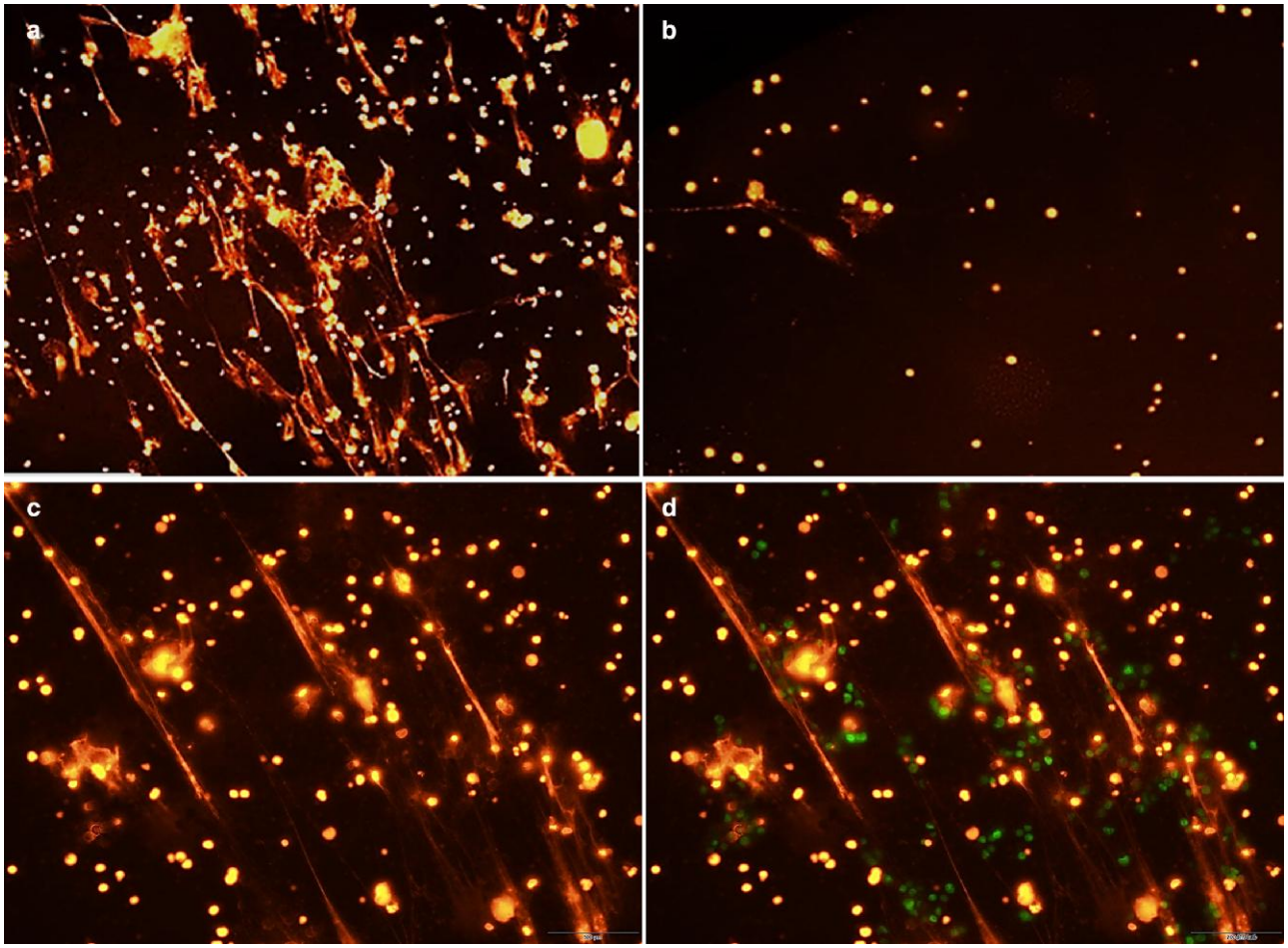

Fig. 3 Granulocytes observed under microscopy under different treatments

a,**c** Low-density granulocytes were purified from postoperative blood samples using MACS separation kit and cultured on poly-l-lysine coated six-well plates. After 2 h, SYTOX orange was added and observed with a fluorescence microscope. **b** Normal-density granulocytes were purified from bottom layer after density gradient centrifugation (polymorphonuclear cell fraction) of the same sample and similarly observed for neutrophil extracellular trap (NET) formation. **d** DLD-1 stained with PKH-67 were added on cells from **c** and wells were gently washed after 5 min co-incubation. NETs and attached tumour cells were observed using appropriate wavelength filters for SYTOX orange and PKH67 respectively, and two photos were merged.

Table 2 Clinical and pathological factors in patients with or without recurrence

Variables	Recurrence (+) $n = 13$	Recurrence (–) $n = 163$	P
Age (years), median (range)	69 (47–83)	67 (25–88)	0.775
Sex ratio (M : F)	6 : 7	98 : 65	0.385
Location (right : left)	5 : 8	55 : 107	0.767
pT (1,2 *versus* 3,4)	2 : 11	66 : 97	0.084
pN (negative *versus* positive)	6 : 7	107 : 56	0.228
p stage (I : II : III)	2 : 4 : 6	49 : 59 : 56	0.474
CEA, median (range)	5.8 (0.9–104.9)	2.7 (0.6–136.9)	0.200
CRP, median (range)	0.09 (0.02–1.98)	0.085 (0.01–11.41)	0.940
NLR, median (range)	3.02 (1.49–10.07)	2.48 (0.70–10.24)	0.066
Adjuvant chemotherapy (+ : –)	4 : 9	51 : 110	1.000
Operating time (min), median (range)	244 (143–524)	215 (110–857)	0.146
Intraoperative blood loss (ml), median (range)	50 (0–2510)	0 (0–2510)	0.013
Ratio of preoperative LDGs (%), median (range)	1.82 (0.019–18.30)	0.94 (0.025–31.96)	0.348
Ratio of postoperative LDGs (%), median (range)	7.90 (1.63–47.40)	2.93 (0.035–59.45)	0.004

Values are *n* unless otherwise indicated. CEA, carcinoembryonic antigen; CRP, C-reactive protein; NLR, neutrophil–lymphocyte ratio. *P* values were calculated with Mann–Whitney *U* test or Fisher's exact test.

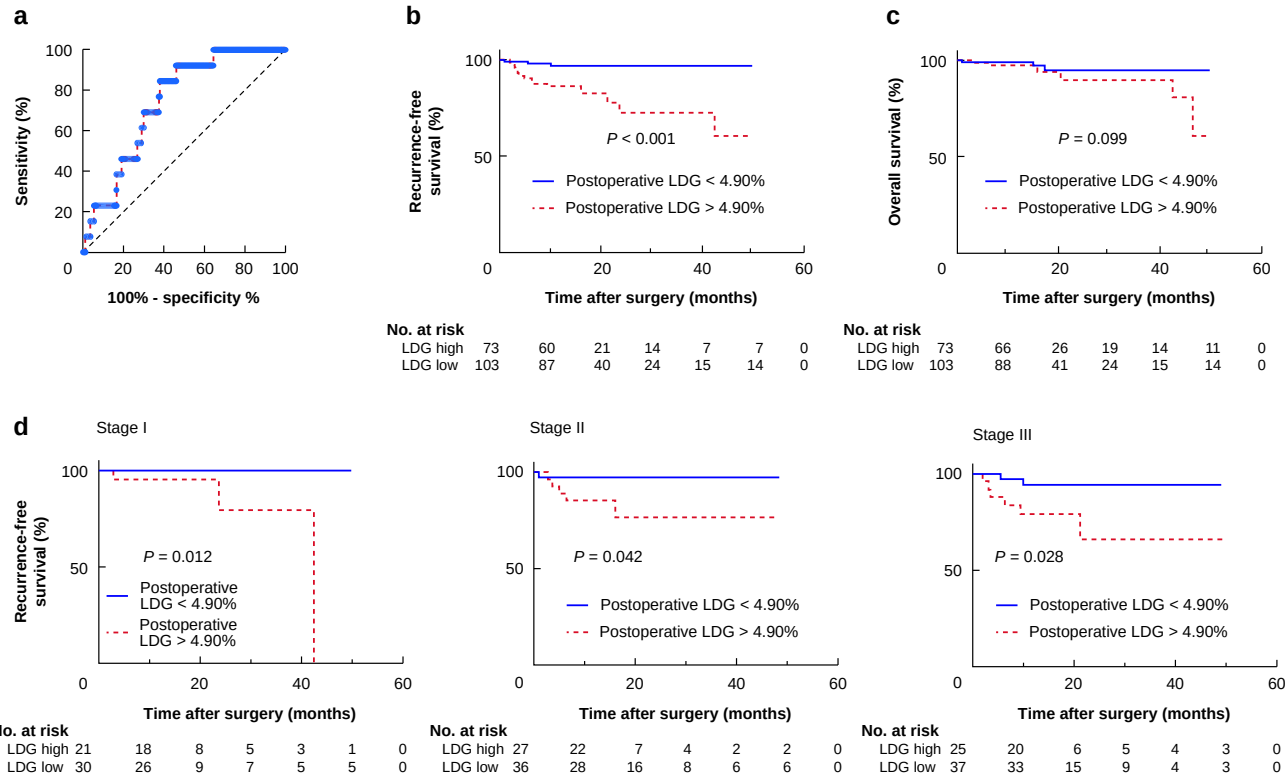

Fig. 4 Impact of postoperative low-density granulocytes (LDGs) on postoperative outcome of the patients with colorectal cancer
a Receiver operating characteristic curve for the ratio of postoperative LDGs in tumour recurrence. **b** Recurrence-free survival (RFS). **c** Overall survival. **d** RFS according to tumour stages. *P* values were calculated with a log rank test. LDN, low-density neutrophil.

LDGs and survival

Median follow-up was 18 months. Thirteen patients developed recurrences, including seven in the liver, three in the lung, two local/peritoneal, and one in retroperitoneal lymph nodes. Mean time to recurrence was 310 days. One patient was stage I, five were stage 2, and seven were stage 3. Four patients received adjuvant chemotherapy.

As shown in *Table 2*, intraoperative blood loss was significantly more in patients with recurrence compared with those without recurrence ($P = 0.013$). In addition, the recurrence tended to be higher in patients with high pT3 and pT4 ($P = 0.084$), high serum CEA levels ($P = 0.200$), high NLR ($P = 0.066$), and longer operating time ($P = 0.146$), although the difference was not statistically significant. The proportion of LDGs in blood obtained before surgery were not different in patients who did and did not develop recurrence ($P = 0.348$). However, the proportion of LDGs in blood obtained after surgery were significantly higher in patients who developed recurrence compared with those who did not ($P = 0.004$).

Table 3 Univariate and multivariate Cox regression analysis on the impact of clinical factors for recurrence-free survival

Variables	Univariate HR (95% c.i.)	*P*	Multivariate HR (95% c.i.)	*P*
Age (≥65 *versus* <65 years)	1.538 (0.570–4.165)	0.397		
Sex ratio (M : F)	1.283 (0.495–3.327)	0.608		
Location (right side *versus* left side)	1.253 (0.455–3.452)	0.663		
pT (3,4 *versus* 1,2)	3.156 (0.906–10.998)	0.071	2.112 (0.568–7.858)	0.264
pN (positive *versus* negative)	1.630 (0.629–4.428)	0.315		
CEA (elevated *versus* normal)	3.779 (1.451–9.829)	0.007	2.437 (0.884–6.719)	0.085
CRP (elevated *versus* normal)	1.020 (0.328–3.169)	0.973		
Preoperative NLR (high *versus* low)	2.394 (0.685–8.361)	0.171		
Ratio of postoperative LDGs (high *versus* low)	7.276 (2.088–25.359)	0.002	6.036 (1.709–21.328)	0.005

HR, hazard ratio; CEA, carcinoembryonic antigen (elevated is more than 5.0 ng/ml); CRP, C-reactive protein (elevated is more than 0.3 mg/dl); NLR, neutrophil to lymphocyte ratio (high is more than 2.1); LDG, low-density granulocyte (high is more than 4.9 per cent). Data are missing in three patients.

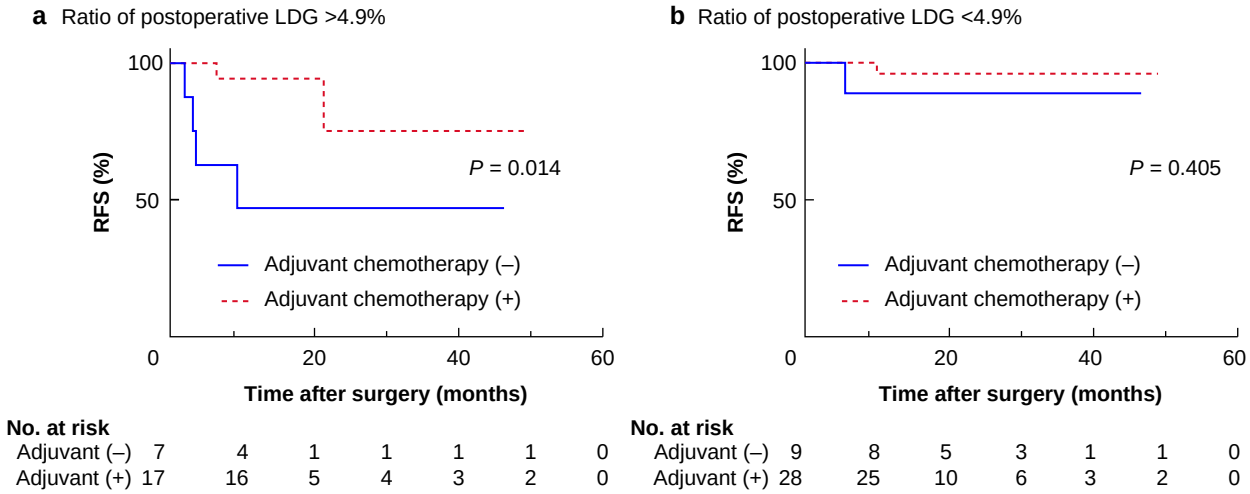

Fig. 5 Recurrence-free survival (RFS) of patients with stage III colorectal cancer with and without adjuvant chemotherapy

a High postoperative low-density granulocyte (LDG) values. **b** Low postoperative LDG values. *P* values were calculated with the log rank test. –, without adjuvant chemotherapy; +, with adjuvant chemotherapy.

The authors examined the association between the proportion of postoperative LDGs and patient outcome. As shown in *Fig. 4*, with a cut-off value of 4.9 per cent determined by ROC analysis, RFS was significantly shorter in patients with a proportion of LDGs above 4.9 per cent ($P < 0.001$). The same trend was observed in patients with tumours of all stages ($P = 0.012$ for stage I, $P = 0.042$ for stage II, and $P = 0.028$ for stage III). OS also tended to be shorter in patients with a high proportion of LDGs compared with those with a low proportion ($P = 0.099$).

Univariate Cox regression analysis revealed that a high proportion of LDGs in blood obtained after surgery predicted shorter RFS (HR 7.267, $P = 0.002$). With multivariate analysis including pathological T categories, high CEA levels, and a high proportion of LDGs in blood obtained after surgery correlated independently with shorter RFS (HR 6.036, $P = 0.005$) (*Table 3*).

LDG and adjuvant chemotherapy in stage III patients

This analysis was conducted using samples obtained immediately after surgery. Among 62 patients with stage III disease, 45 patients received postoperative adjuvant chemotherapy with a capecitabine or capecitabine + oxaliplatin (CapeOX) regimen for 3 to 6 months, whereas 17 patients did not receive adjuvant treatment. As shown in *Fig. 5*, 25 patients had a proportion of LDGs higher than 4.9 per cent, and within this subgroup, those treated with adjuvant chemotherapy had prolonged RFS ($P = 0.014$); however, among 37 patients with a low proportion of LDGs, only two developed recurrence, and adjuvant therapy did not have a significant effect on their outcome ($P = 0.405$).

Discussion

The results of the present study show that the proportion of LDGs in the peripheral blood of patients with CRC was markedly increased immediately after surgery. The proportion of LDGs in blood obtained after surgery is positively associated with operating time and intraoperative blood loss. Previous studies have shown that number of LDGs with immunosuppressive properties are significantly increased in the peripheral blood of patients with severe sepsis[17–19] and traumatic injuries[25] as well as healthy volunteers challenged with endotoxin[26], and that LDGs can suppress T cell proliferation[18,25,26]. As major surgical procedures induce a systemic inflammatory response, which causes not only the activation of circulating mature neutrophils but also enhances mobilization of immature neutrophils in the peripheral circulation[27], the increased proportion of LDGs in blood obtained after surgery is considered to be the same type as the LDGs found in previously studied conditions. Indeed, in a previous study, the LDGs showed an immature phenotype and potently inhibited the proliferation of T cells stimulated with anti-CD3 monoclonal antibodies and their cytotoxicity for tumour cells[24].

More importantly, the proportion of LDGs in blood samples obtained after surgery was strongly associated with clinical outcomes. The ratio of LDGs was significantly higher in patients who recurred after colectomy with curative intent, and a proportion higher than 4.9 per cent was a strong and independent predictor for shorter RFS in those patients. The results suggest that the LDGs may locally or systemically suppress T cell-mediated anti-tumour immunity, allowing the survival of remnant tumour cells in the host, which would facilitate the development of recurrence after surgery. In addition, the LDGs in blood obtained after surgery robustly produced NETs with attachment of many tumour cells *in vitro*. Recent studies have shown that NETs in hepatic sinusoids have been shown to capture circulating tumour cells, which results in the augmentation of hepatic metastases in murine models[28,29]. From these findings, it is speculated that the increased proportion of LDGs can effectively mediate the lodging of circulating tumour cells through NETs formed in target organs, which may be involved in the higher rate of recurrence in distant organs in these patients.

Based on the results of randomized clinical trials[4–6], adjuvant chemotherapy for 3–6 months is now considered the standard of care for patients with stage III CRC; however, approximately half of these patients are cured by surgery alone and 20 per cent experience disease recurrence despite adjuvant treatment and thus the patients who actually benefit from adjuvant treatment are estimated to be 30 per cent at most[30]. Therefore, it is necessary to improve assessment of the risk of recurrence for each individual patient. The results of this study demonstrate that a high proportion of LDGs after surgery is a predictor of postoperative recurrence and that the effect of adjuvant therapy for patients with stage III disease is significant in those with a high proportion of LDGs, while being less clear in patients with a low proportion of LDGs. Although the number of patients in this study is insufficient to draw definite conclusions, these results suggest that the proportion of postoperative LDGs is a useful biomarker to select the specific subgroup of patients most likely to benefit from adjuvant chemotherapy among patients with stage III CRC.

Although many studies have suggested that surgical stress promotes tumour recurrence[31,32], the mechanisms are fully understood[33,34]. The results of this study are highly suggestive that LDGs are recruited in the circulating blood by surgical stress early in the postoperative interval as 'emergency granulopoiesis' and play supportive roles in the development of metastases formation. The proportion of postoperative LDGs may be useful to select patients who should receive adjuvant treatment. Functional blockade of the LDGs during the perioperative interval may be effective to improve the outcomes of patients with CRC.

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Disclosure

The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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