Original articles

Title

Proposal for novel histological findings of colorectal liver metastases with preoperative chemotherapy

Short running title

Histology of colorectal liver metastasis

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Abstract

This study aimed to clarify the histological characteristics related to preoperative chemotherapy for colorectal liver metastases (CRLM). Sixty-three patients with CRLM were divided into two groups: CRLM with chemotherapy (41 cases, group A) and CRLM without chemotherapy (22 cases; surgical treatment alone, group S) to identify the histological differences associated with chemotherapy. In addition, we investigated the effects of combination chemotherapy on the histology of metastatic lesions. Infarct-like necrosis (ILN), three-zonal changes, and cholesterol clefts were more frequent in group A than in group S ($P < 0.05$). ILN and three-zonal changes were more common in the FOLFOX or FOLFIRI with or without additional bevacizumab groups than in group S ($P < 0.05$). Cholesterol clefts in the FOLFOX or FOLFIRI with bevacizumab group and foamy macrophages in the FOLFOX or FOLFIRI group were more common than in group S ($P < 0.05$). Cases with more than three of the four histological findings–i.e., ILN, three-zonal changes, cholesterol clefts, and foamy macrophages–were more frequent in the FOLFOX or FOLFIRI with or without additional bevacizumab groups than in group S ($P < 0.05$). We showed histological findings for every representative chemotherapy regimen for CRLM to clarify the effects of preoperative chemotherapy.

Key words

colorectal liver metastases; preoperative chemotherapy; histological findings; infarct-like necrosis; three-zonal changes; cholesterol clefts; foamy macrophages; chemotherapy regimen

Introduction

Colorectal carcinoma is one of the most common cancers in the world. It has an estimated incidence of 43.7 per 100,000 with over 136,000 estimated new cases expected in the United States in 2014, as reported by the Centers for Disease Control.[1] Liver metastasis is the most common complication of colorectal cancer, and approximately 50% of patients develop colorectal liver metastases (CRLM) at some point during the course of their disease.[2, 3] Patients who are candidates for surgical resection of their liver metastases can expect a prolonged survival or even a cure.[4, 5] However, the resectability rate of metastases at the time of diagnosis is low, accounting for the low proportion of patients who may benefit from a surgical approach.[6] Preoperative chemotherapy provides the potential for unresectable tumors to become resectable if they become smaller in response to treatment.[7-9] The efficacy of preoperative chemotherapy is generally assessed by radiological evaluation. The radiological response according to the Response Evaluation Criteria in Solid Tumors (RECIST) corresponds to the reduction in the number and size of metastases, essentially a tumor shrinkage.[9, 10] However, preoperative radiology has been shown to overestimate the downstaging of the tumor, and histology remains the best way of assessing residual tumor viability.[11]

Previously, the standard treatment for advanced colorectal cancer was 5-fluorouracil (5-FU)-based chemotherapy with or without leucovorin. Recently, new therapeutic approaches have predominated. Oxaliplatin (trans-1-diaminocyclohexane oxalatoplatinum) or irinotecan-based neoadjuvant chemotherapy alters the natural history of unresectable CRLM by downstaging the disease, allowing resection and prolonging survival in some patients.[9, 12-15] Additionally, bevacizumab (Avastin), a monoclonal humanized antibody directed against vascular endothelial growth factor (VEGF), has been shown to extend overall survival in patients treated with 5-FU-based chemotherapy.[16-19]

Recent studies have shown that the pathological tumor response to chemotherapy is an important factor in patients treated with preoperative chemotherapy for CRLM. Moreover, grading of the histologic response of tumors to preoperative chemotherapy correlates with postoperative disease-free and overall survival.[17, 20, 21] Despite the increasing importance of and opportunity for histological evaluation of preoperative chemotherapy, detailed histological findings for evaluating its effectiveness, and differences associated with various treatment regimens have not yet been proposed. The aim of this study was to identify and standardize the histological findings related to preoperative chemotherapy for CRLM.

Materials and Methods

Ethical approval

The study was approved by the institutional review board of Iwate Medical University.

Patient population

All patients (n=63) with colorectal carcinoma who underwent a first hepatic resection for liver metastases at the Iwate Medical University Hospital from 2008 to 2012 were examined. There were 41 men and 22 women, with ages ranging from 41 to 87 years (mean, 66 years). Eleven patients had a synchronous liver metastasis with colon cancer, and 52 patients had metachronous metastases. In patients undergoing multiple resections for metastatic lesions, only the first resection was included in this study. We included all cases, irrespective of whether they had received preoperative chemotherapy. Surgical resection was the only treatment in 22 cases. Systemic chemotherapy was administered before hepatic surgery in 41 cases. Among the chemotherapeutic agents used were 5-FU, 5-FU with leucovorin and oxaliplatin (FOLFOX), 5-FU with leucovorin and irinotecan (FOLFIRI), and FOLFOX or FOLFIRI with bevacizumab (BV). Several

different protocols were used: 11 patients received 5-FU only (5-FU group); 9 patients received FOLFOX or FOLFIRI (FOLFOX or FOLFIRI group); and 21 patients received FOLFOX or FOLFIRI with BV (FOLFOX or FOLFIRI with BV group).

Tissues and pathological assessment

All archival slides of CRLM, which were originally prepared from formalin-fixed, paraffin-embedded tissue, were reviewed. Histological examination of the hepatectomy specimens was made according to our routine hospital process. In patients with multiple metastases, sections of the lesion with the maximum diameter were examined and samples were systematically taken for histology from the whole selected section. Histopathological examination was performed using hematoxylin and eosin staining. Slides were independently examined by two experienced pathologists (K.I. and N.U.), who were unaware of the subject's clinical data; specifically, no information was available on the administration and the regimen of preoperative chemotherapy. In some cases for which the evaluation provided different results, a consensus interpretation was reached after re-examination.

The following histological features were evaluated: usual necrosis (UN), infarct-like necrosis (ILN), three-zonal changes, dangerous halos, cholesterol clefts, foamy macrophages and calcification. UN was defined as containing nuclear debris in a patchy distribution, with the necrosis admixed and bordered by viable cells. In contrast, ILN was defined as being composed of large confluent areas of eosinophilic cytoplasmic remnants located centrally within a lesion with absent or minimal admixed nuclear debris.[22] Three-zonal changes were recognized as a central zone of necrosis, a mid zone of fibrosis and an outer zone of residual tumor.[23] Dangerous halos showed that viable tumor cells appeared to infiltrate the surrounding liver parenchyma without a fibroinflammatory reaction (Fig. 1).[24] UN and ILN were defined positive when they occupied more than 5% of the tumor area. Cholesterol clefts, foamy macrophages and calcification were regarded as clearly existing cases with positive judging from low to middle power magnification (Fig. 2).

The presence of residual tumor cells was scored for each CRLM according to the modified tumor regression grade (mTRG), which is similar to the tumor regression grade (TRG), with the exception that ILN was considered equivalent to fibrosis.[21, 22] TRG1 corresponded to the absence of tumor cells replaced by abundant fibrosis; TRG2 corresponded to rare residual tumor cells scattered

throughout abundant fibrosis; TRG3 corresponded to more residual tumor cells throughout the predominant fibrosis; TRG4 corresponded to a large amount of tumor cells predominating over fibrosis; and TRG5 corresponded to the almost exclusive presence of tumor cells without fibrosis. The mTRG score was categorized into three groups using previously published guidelines.[21] mTRG1 and mTRG2 were categorized as having complete or major histological tumor response (MjHR); mTRG3 was categorized as having partial histological tumor response (PHR); and mTRG4 and mTRG5 were categorized as having no histological tumor regression or response (NHR).

Statistical analysis

The χ^2-test was used to correlate the different groups of patients according to the use of preoperative treatment and the type of chemotherapy versus the presence of histological findings. A result was considered statistically significant if $P<0.05$.

Results

1. Histological changes in samples of CRLM with chemotherapy versus samples of CRLM without chemotherapy

Sixty-three patients with CRLM were divided into two groups: CRLM with preoperative chemotherapy (41 cases,) and CRLM without preoperative chemotherapy (22 cases, surgery-alone group) in order to identify histological differences associated with chemotherapy. Clinicopathological characteristics of patients treated with surgery alone or with preoperative chemotherapy for CRLM are summarized in Table 1. There was a significant difference in the number of liver metastases between the surgery-alone group and the FOLFOX or FOLFIRI with BV group ($P < 0.01$).

Histological findings of the surgery-alone and preoperative chemotherapy groups are summarized in Table 2. There were low frequencies of ILN, three-zonal changes, and cholesterol clefts in the surgery-alone group (1/22 [4.5%], 4/22 [18.2%], and 5/22 [22.7%], respectively), while the frequencies of these findings in the chemotherapy group (18/22 [43.9%], 20/22 [48.8%], and 20/22 [48.8%], respectively) were significantly higher ($P < 0.05$). However, there were no differences in the frequency of UN, dangerous halos, foamy macrophages or calcification between the two groups.

2. Histological changes in CRLM following combination chemotherapy

Histological findings by type of preoperative chemotherapy for CRLM are shown in Table 3. The frequencies of ILN and three-zonal changes between the FOLFOX or FOLFIRI group (4/9 [44.4%], 5/9 [55.6%], respectively) and the FOLFOX or FOLFIRI with BV group (12/21 [57.1%], 11/21 [52.4%], respectively) were significantly higher than those of the surgery-alone group ([1/22 [4.5%], 4/22 [18.2%], respectively] ($P < 0.05$). Cholesterol clefts were more common in the FOLFOX or FOLFIRI with BV group (13/21 [61.9%]) than in the surgery-alone group (5/22 [22.7%]) ($P < 0.01$). Foamy macrophages were more common in the FOLFOX or FOLFIRI group (8/9 [88.9%]) than in the surgery-alone group (11/22 [50%]) ($P < 0.05$). In contrast, the frequency of UN in the FOLFOX or FOLFIRI with BV group (16/21 [76.2%]) was significantly lower than in the surgery-alone group (22/22 [100%]) ($P < 0.05$). No significant differences in dangerous halos and calcification were found between the surgery-alone group and the three preoperative chemotherapy groups.

3. Histological assessment of the effects of combination chemotherapy in CRLM

Correlations between the numbers of histological findings and the type of preoperative chemotherapy for CRLM are summarized in Table 4. We examined

how many histological findings (ILN, three-zonal changes, cholesterol clefts, and foamy macrophages) were found in the surgery alone, 5-FU, FOLFOX or FOLFIRI, and FOLFOX or FOLFIRI with BV groups. Cases with all four histological findings were significantly more common in the FOLFOX or FOLFIRI with BV group than in the surgery-alone group ($P < 0.001$) and the 5-FU group ($P < 0.05$). The FOLFOX or FOLFIRI group and the FOLFOX or FOLFIRI with BV group were significantly different from the surgery-alone group in satisfying more than three of the four histological findings ($P < 0.01$). The numbers of cases that had less than or equal to two pathological findings did not differ among the groups.

In the preoperative chemotherapy group, we examined the number of histological findings for CRLM according to tumor regression grade (Table 5). The frequency of MjHR (mTRG1 and mTRG2) and PHR (mTRG3) for cases with more than three histological findings was 57.9% (1/19 [5.3%] and 10/19 [52.6%], respectively). In contrast, MjHR or PHR for cases with less than or equal to two pathological findings was present in only 27.2% of cases (3/22 [13.6%] and 3/22 [13.6%], respectively; $P < 0.05$).

Discussion

The use of surgery for CRLM has expanded with the progress of preoperative chemotherapy, and opportunities are increasing to evaluate the histologic response of tumors to preoperative chemotherapy.[7-9, 12, 25] Previous histologic grades for preoperative chemotherapy were evaluated by the ratio of fibrosis for tumors that disappeared, and tumors that remained were evaluated on the basis of tumor regression grade (TRG) and Dworak grading.[21, 26] However, it was noted that it was difficult to determine whether the pathological findings resulted from preoperative chemotherapy. This is the first report presenting an objective judgment of histological findings after preoperative chemotherapy.

ILN, three-zonal changes and cholesterol clefts were among seven histological findings that appeared most often in the preoperative chemotherapy groups in comparison with the surgery-alone group and were regarded as the most important findings that reflected an effect of the preoperative chemotherapy on CRLM. Aloysius et al. reported that Dworak grading of a group that received FOLFOX4 before hepatectomy for CRLM was significantly higher, and the treatment was histologically effective, compared to that of a control group that underwent hepatectomy only.[23] It was shown that the histological findings of three-zonal changes appeared in FOLFOX4-treated liver nodules, but they were not described with regard to the frequency of appearance and the difference

between groups in that report. Fibrosis does not necessarily show where a tumor disappeared, and it might occur with permeation of the tumor. In our study, when we performed a pathological evaluation of CRLM, in addition to determining whether fibrosis is present, it was important that we paid attention to fibrosis between viable tumor cells and necrosis. It was thought that we could evaluate the histological effect of appropriate chemotherapy by judging the three levels of structure to be formed from viable tumor cells, fibrosis and necrosis.

Chemotherapy regimens for CRLM have changed and have progressed from 5-FU alone to FOLFOX and FOLFIRI.[14, 15] Furthermore, it was recently reported that a greater antitumor effect was provided by adding BV, which is an anti-VEGF antibody and panitumumab, which is a human anti-epidermal growth factor receptor (EGFR) monoclonal antibody.[19, 27] However, antitumor effects vary according to the regimen, whereas few reports clarified how histological findings vary. In our results, ILN and three-zonal changes significantly appeared in the FOLFOX or FOLFIRI group and the FOLFOX or FOLFIRI with BV group in comparison with the surgery-alone group. It was speculated that ILN represents cell death due to the cytotoxic effects of chemotherapy and it was noted that the efficacy of BV may be related to the appearance of ILN, because BV was given to half of the cases in which ILN appeared.[22] However, as for the incidence of ILN in

this study, a difference was not seen between the FOLFOX or FOLFIRI group and the FOLFOX or FOLFIRI with BV group. This result suggested that ILN was not changed specifically following treatment with BV. On the other hand, the incidence of UN decreased only in the FOLFOX or FOLFIRI with BV group in this study. UN is an ongoing process that arises within areas of hypoxia as a tumor enlarges and outgrows its vascular supply.[28] Our results suggested that the anti-VEGF action affects the enlargement and outgrowth of CRLM.

In this examination, it is possible that in some cases the histological findings of the chemotherapy group were similar to those of the surgery-alone group. This demonstrates the risk of performing histologic effect measurement of chemotherapy only for a single finding. A significant difference was seen in the incidence of pathological findings between the surgery-alone group and the FOLFOX or FOLFIRI group and the FOLFOX or FOLFIRI with BV group when more than three findings were seen among ILN, three-zonal changes, cholesterol clefts and foamy macrophages. This result indicated that cases with preoperative chemotherapy using a more effective regimen had more complex histological findings in CRLM. The pathological evaluation of preoperative chemotherapy should include analysis of various histological findings.

Previous studies have evaluated pathologic assessment of tumor regression to preoperative chemotherapy according to the ratio of residual tumor cells in CRLM.[20, 21, 23] In this study, when we classified histological tumor regression as MjHR (mTRG1 and mTRG2), PHR (mTRG3), or NHR (mTRG4 and mTRG5) according to previously published guidelines,[17, 21, 22] there was significantly more MjHR and PHR in cases with three or four histological findings than cases with two or fewer findings. MjHR was categorized as having major or complete histological response, and PHR was categorized as having partial histological tumor response. On the other hand, NHR was categorized as having no histological tumor regression or response. These results suggested that the existence of three or more of these four pathological features was correlated with the effects of preoperative chemotherapy for CRLM. Therefore, the histological influence of chemotherapy on the tumor tissue may provide useful information for the patient's oncologist. In addition, it is expected that histological findings become an index for treatment choice in the case of recurrence after CRLM excision to determine whether the patient responded to preoperative chemotherapy and should use the same regimen.

We were able to show histological findings for every representative chemotherapy regimen for CRLM to clarify the effects of preoperative

chemotherapy. The presentation of histological findings in contrast to the chemotherapy regimen is needed from a pathological perspective in the future.

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Disclosure statement

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. Representative photomicrographs of CRLM demonstrating (a) usual necrosis containing nuclear debris and surrounded by viable tumor cells, (b) infarct-like necrosis including a confluent eosinophilic area with absent or minimal admixed nuclear debris, (c) three-zonal changes recognized in the central zone of necrosis*, the mid zone of fibrosis** and the outer zone of the residual tumor, and (d) dangerous halo showing that viable tumor cells appear to infiltrate the surrounding liver parenchyma without a fibroinflammatory reaction. Hematoxylin & eosin-stained section (a,b, x40; c,d, x20).

Figure 2. Representative photomicrographs of CRLM demonstrating (a) a cholesterol cleft, (b) a foamy macrophage-like mass and (c) calcification. Hematoxylin & eosin-stained section (a, x200; b,c, x100).

Table 1. Clinicopathological characteristics of patients treated with surgery alone or preoperative chemotherapy for colorectal liver metastases

Parameter	Surgery alone	Preoperative chemotherapy (n = 41)		
		5-FU	FOLFOX or FOLFIRI	FOLFOX or FOLFIRI with BV
Number of cases	22	11	9	21
Median age (range), years	67.5 (53-87)	70.0 (48-82)	61.0 (58-78)	64.0 (41-77)
Male:female ratio	15 : 7	4 : 7	8 : 1	14 : 7
Synchronous:Metachronous metastases	6 : 16	0 : 11	0 : 9	5 : 16
Median number of metastases (range)	1 (1-4)	1 (1-2)	2 (1-5)	4 (1-11) *
Median diameter of largest metastasis (range), mm	26.5 (14-160)	24.0 (20-65)	23.0 (9-67)	23.0 (7-155)

5-FU, 5-fluorouracil; FOLFOX, 5-fluorouracil with leucovorin and oxaliplatin; FOLFIRI, 5-fluorouracil with leucovorin and irinotecan; BV, bevacizumab.

*Significantly different from the surgery-alone group ($P < 0.05$).

Table 2. Histological findings of the surgery-alone and the preoperative chemotherapy groups

Histological finding	Surgery alone (n = 22)	Preoperative chemotherapy (n = 41)	P value
Usual necrosis	22 (100%)	36 (87.8%)	$P > 0.05$
Infarct-like necrosis	1 (4.5%)	18 (43.9%)*	$P = 0.0012$
Three-zonal changes	4 (18.2%)	20 (48.8%)*	$P = 0.017$
Dangerous halos	13 (59.1%)	30 (73.1%)	$P > 0.05$
Cholesterol clefts	5 (22.7%)	20 (48.8%)*	$P = 0.044$
Foamy macrophages	11 (50.0%)	30 (73.2%)	$P > 0.05$
Calcification	8 (36.4%)	17 (41.5%)	$P > 0.05$

*Significantly different from the surgery-alone group ($P < 0.05$).

Table 3. Histological findings by type of preoperative chemotherapy for colorectal liver metastases

Histological finding	Surgery alone (n = 22)	Preoperative chemotherapy (n = 41)			P value
		5-FU (n = 11)	FOLFOX or FOLFIRI (n = 9)	FOLFOX or FOLFIRI with BV (n = 21)	
Usual necrosis	22 (100%)	11 (100%)	9 (100%)	16 (76.2%)**	**P = 0.015
Infarct-like necrosis	1 (4.5%)	2 (18.2%)	4 (44.4%)*	12 (57.1%)**	*P = 0.0061 **P = 0.0002
Three-zonal changes	4 (18.2%)	4 (36.4%)	5 (55.6%)*	11 (52.4%)**	*P = 0.037 **P = 0.019
Dangerous halos	13 (59.1%)	8 (72.7%)	8 (88.9%)	14 (66.7%)	P > 0.05
Cholesterol clefts	5 (22.7%)	3 (27.3%)	4 (44.4%)	13 (61.9%)**	**P = 0.0092
Foamy macrophages	11 (50.0%)	7 (63.6%)	8 (88.9%)*	15 (71.4%)	*P = 0.044
Calcification	8 (36.4%)	5 (45.5%)	6 (66.7%)	6 (28.6%)	P > 0.05

5-FU, 5-fluorouracil; FOLFOX, 5-fluorouracil with leucovorin and oxaliplatin; FOLFIRI, 5-fluorouracil with leucovorin and irinotecan; BV, bevacizumab.

*, **Significantly different from the surgery-alone group (P < 0.05).

Table 4. Correlations between the numbers of histological findings and the type of preoperative chemotherapy for colorectal liver metastases

Number of histological findings†	Surgery alone (n = 22)	Preoperative chemotherapy (n = 41)			P value
		5-FU (n = 11)	FOLFOX or FOLFIRI (n = 9)	FOLFOX or FOLFIRI with BV (n = 21)	
4	1 (4.5%)	1 (9%)	2 (22.2%)	11 (52.4%)**,***	**P = 0.0005 ***P = 0.016
More than or equal to 3	1 (4.5%)	3 (27.3%)	5 (55.6%)*	11 (52.4%)**	*P = 0.0005 **P = 0.0011
More than or equal to 2	6 (27.3%)	5 (45.5%)	6 (66.7%)	14 (66.7%)	P > 0.05
1	13 (59.1%)	7 (63.6%)	8 (88.9%)	15 (71.4%)	P > 0.05

5-FU, 5-fluorouracil; FOLFOX, 5-fluorouracil with leucovorin and oxaliplatin; FOLFIRI, 5-fluorouracil with leucovorin and irinotecan; BV, bevacizumab.

†The four histological findings were infarct-like necrosis, three-zonal changes, cholesterol clefts, and foamy macrophages.

*, **Significantly different from the surgery-alone group (P < 0.05).

***Significantly different from the 5-fluorouracil group (P < 0.05).

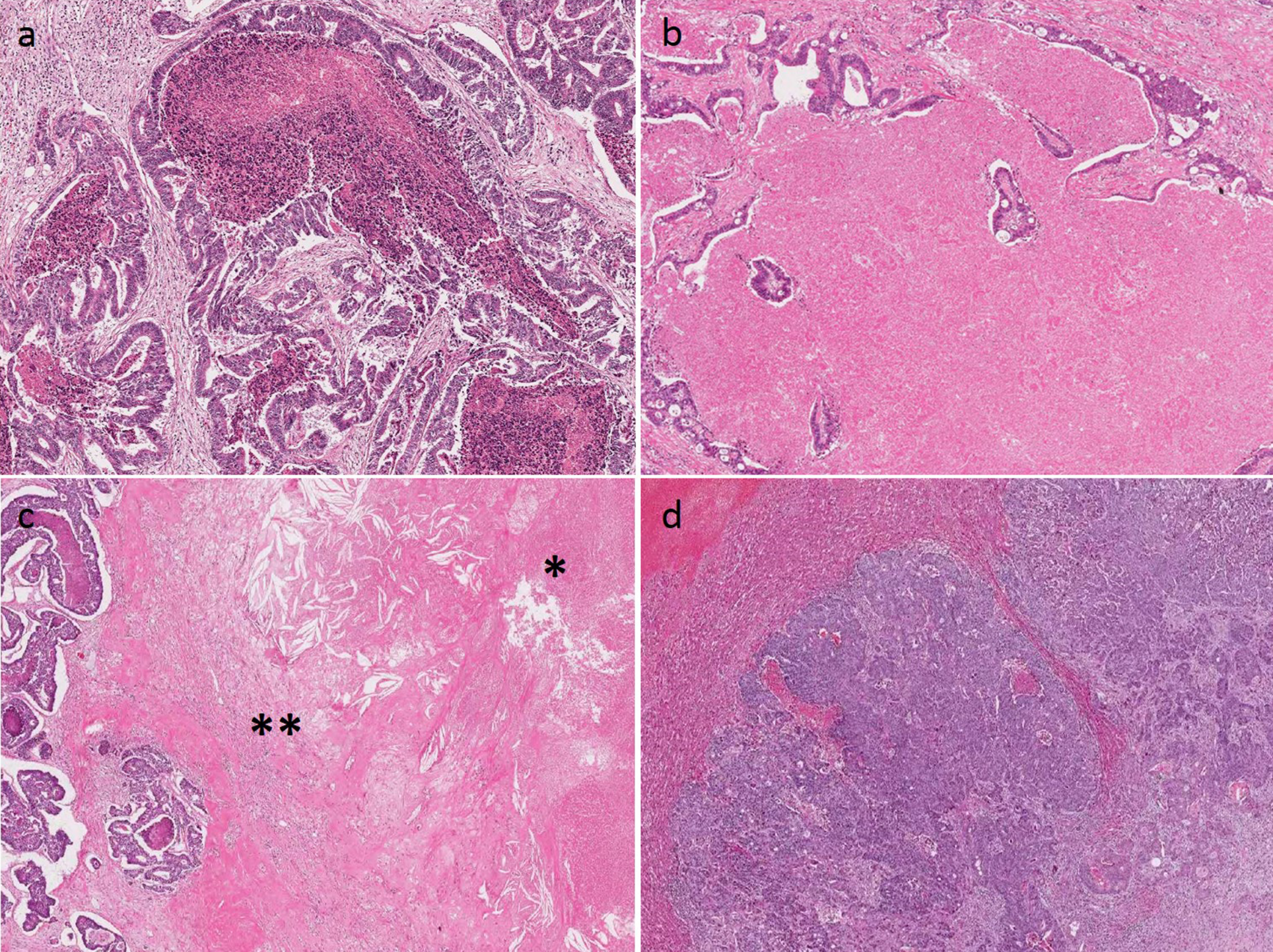
Table 5. Correlations between the numbers of histological findings and the modified tumor regression grade for colorectal liver metastases

Number of histological findings	MjHR (mTRG1, 2)	PHR (mTRG3)	NHR (mTRG4, 5)
More than or equal to 3 (*n*=19)	1 (5.3%)	10 (52.6%)	8 (42.1%)
Less than or equal to 2 (*n*=22)	3 (13.6%)	3 (13.6%)	16 (72.7%)

P=0.0266

MjHR, major histological tumor response; PHR, partial histological tumor response; NHR, no histological tumor response; mTRG, modified tumor regression grade.

†The 4 histological findings were infarct-like necrosis, three-zonal changes, cholesterol clefts and foamy macrophages.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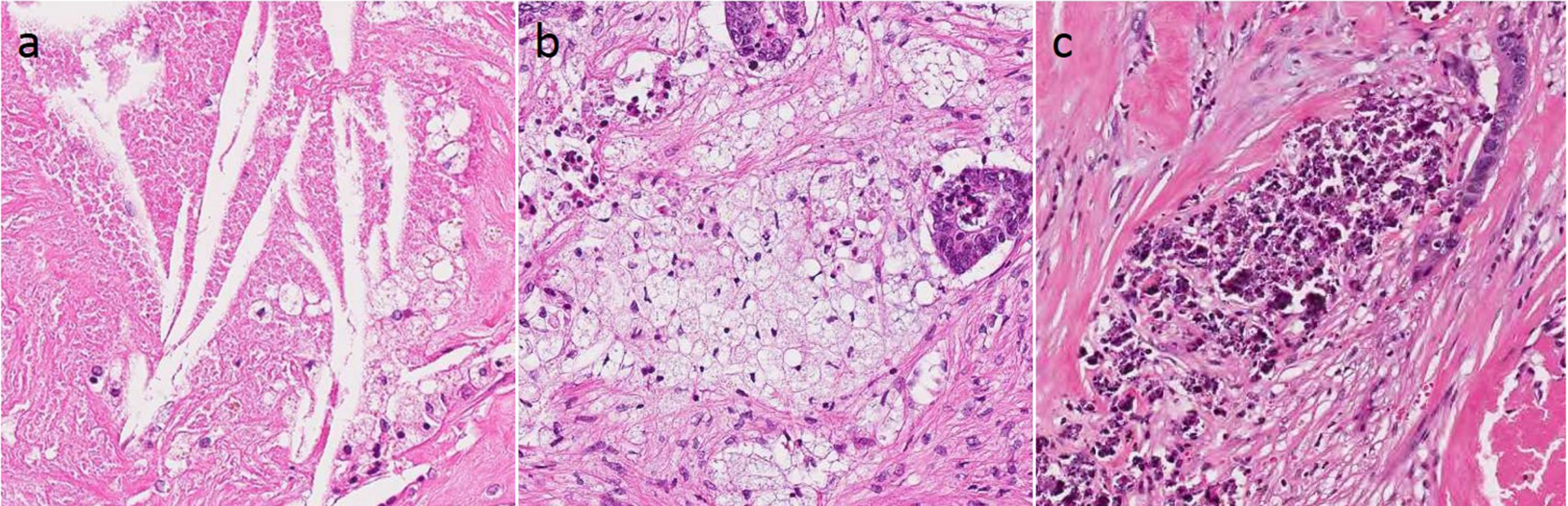
**
d


a
b
c