Tumor-Infiltrating Lymphocytes and Perforation in Colon Cancer Predict Positive Response to 5-Fluorouracil Chemotherapy

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Abstract

Purpose: The major pathologic markers of prognosis in colorectal cancer include vascular invasion by tumor cells, invasion of adjacent lymph nodes, and perforation of the serosal wall. Recent work suggests that a high density of tumor-infiltrating lymphocytes (TIL) is associated with good outcome independently of these established prognostic markers. The aim of the present study was to investigate the prognostic significance of TILs and other routinely reported pathologic features in colon cancer, particularly in relation to the use of adjuvant chemotherapy.

Experimental Design: Pathologic markers, disease-specific survival, and the use of adjuvant chemotherapy were recorded in a retrospective, population-based series of 1,156 stage III colon cancer patients with a median follow-up time of 52 months.

Results: In patients treated by surgery alone ($n = 851$), markers with significant prognostic value included poor histologic grade, T4 stage, N2 nodal status, vascular invasion, and perforation, but not the presence of TILs. In patients treated with 5-fluorouracil–based chemotherapy ($n = 305$), TILs were associated with significantly improved survival [hazard ratio (HR), 0.52; 95% confidence interval, 0.30-0.91; $P = 0.02$] and perforation with a trend for improved survival (HR, 0.67; 95% confidence interval, 0.27-1.05; $P = 0.16$). Patients with TILs or perforation seemed to gain more survival benefit from chemotherapy (HR, 0.22 and 0.21, respectively) than patients without these features (HR, 0.84 and 0.82, respectively).

Conclusion: The apparent survival advantage from 5-fluorouracil associated with TILs and perforation requires confirmation in prospective studies. Because the presence of TILs reflects an adaptive immune response and perforation is associated with inflammatory response, these results suggest that there may be interactions between the immune system and chemotherapy leading to improved survival of colon cancer patients.

Colorectal cancers (CRC) are commonly infiltrated by immune cells along the invasive margin. The most frequent among these are T and B lymphocytes; however, natural killer cells, dendritic cells, macrophages, and neutrophils are also present (1). The survival advantage associated with pronounced tumor-infiltrating lymphocytes (TIL) in CRC has been widely documented (2–9). The density of TILs is highest in stage I and II CRC and lowest in stage III to IV CRC; however, the favorable prognosis associated with this feature seems to be independent of tumor stage (3, 4, 6, 7). Naito et al. (4) reported that cytotoxic $CD8^+$ cells located within tumor cell nests were a strong prognostic factor and this became more apparent after longer patient follow-up times. Menon et al. (6) found that $CD8^+$ and $CD57^+$ stromal cells at the advancing tumor margin were independent prognostic factors. The excellent prognosis associated with infiltrating lymphocytes was particularly evident in tumors displaying the microsatellite instability phenotype (5, 7).

The above studies support the notion that immune surveillance plays a significant role in determining CRC prognosis. $CD8^+$ cells and other activated T lymphocytes might suppress the metastasis rather than the growth of these tumors. High levels of infiltrating memory $CD45RO^+$ cells were shown recently in CRC that showed no signs of early metastatic invasion (8). A follow-up study by the same group found that quantitative analysis of the adaptive immune response ($CD3^+$, $CD8^+$, and $CD45RO^+$) could provide prognostic information that was superior to and independent of the International Union Against Cancer tumor-node-metastasis classification system (9). High densities of adaptive immune cells correlated with a highly favorable prognosis regardless of the extent of tumor invasion through the bowel wall or the involvement of regional lymph nodes.

Robust prognostic markers would be especially useful for the management of stage II CRC due to the uncertainty surrounding the overall benefit of chemotherapy for patients with this stage of disease (10). The ability to identify stage II CRC patients with very good prognosis would allow them to be spared the toxicity and inconvenience of adjuvant treatments. Established tumor markers for poor prognosis are extramural vascular invasion, peritoneal involvement, extent of extramural

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spread, incomplete resection, and perforation (10). It remains to be determined whether the presence of TILs provides prognostic information that is independent of these established markers in the routine clinical setting. Whether the adaptive immune response has value in the prediction of survival benefit from chemotherapy is also unknown.

In the present study, we investigated the prognostic value of routinely reported clinicopathologic features in a large, population-based series of stage III colon cancer with long patient follow-up and known adjuvant treatment status. In contrast to most of the studies published to date, these features were analyzed separately for patients treated by surgery alone and for those who received 5-fluorouracil (5-FU)-based chemotherapy. This has allowed us to evaluate the prognostic significance of various markers, including TILs and perforation, in the absence of possible confounding effects of chemotherapy on survival.

Materials and Methods

Study population. Pathology records from the four major hospitals in Western Australia (Royal Perth, St John of God, Fremantle, and Sir Charles Gairdner) were used to identify patients diagnosed with colon carcinoma during the period 1994 to 2001 inclusive. The pathology services located within these hospitals also process tumor specimens from minor district and country hospitals. By conducting cross-checks with the Cancer Registry of Western Australia, we ascertained that >90% of all colon cancer patients who underwent surgical resection in this state were included in the study. Tumor stage was classified according to the current American Joint Committee on Cancer guidelines (American Joint Committee on Cancer Staging Manual, 6th ed., 2002). Colonic cancers were classified as being proximal or distal to, and including, the splenic flexure. All cases showed clear margins (R0 resections). Information on the pathologic features shown in Table 1 was obtained from the histopathology report for each case. From 1998 onwards, a proforma system was introduced in Western Australia for the standardized reporting of pathologic variables in CRC, including the presence of TILs and pathologic evidence of perforation. Tumors were considered positive for TILs if there was documented evidence of lymphocytic response on the pathology report. No formal evaluation of immune cell density or type was done.

Perforation was also considered to be present if noted by the surgeon at operation. The clinical relevance of perforated cancers was assessed by linking patient records with the morbidity database maintained by the Health Department of Western Australia. This database records the morbidity codes (International Classification of Diseases 10) for all patients admitted to hospital in the state. Linkage of the 97 patients with perforation revealed that 10 (10.3%) presented as clinical emergencies with free perforations and peritonitis. All 10 patients proceeded to emergency surgery and the mean length of hospital stay for this group was 24.2 ± 19.3 days. The remaining patients (n = 87) underwent elective surgery and were identified as showing histologic evidence of perforation. They were considered to have subclinical perforations and their mean length of hospital stay was 13.3 ± 13.9 days. The percentage of patients receiving 5-FU chemotherapy did not differ significantly between patients with (28 of 97, 29%) or without (277 of 1,059, 26%) perforation.

Ethics approval for the project was obtained from the Human Research Ethics Committees of the University of Western Australia, the Confidentiality of Health Information, and the four major hospitals.

Adjuvant chemotherapy and survival information. Procedure codes from the morbidity database of the Data Linkage Unit, Health Department of Western Australia, were used to identify patients who began chemotherapy within 120 days of surgery. The hospital records of each case were individually reviewed and only those patients who completed at least four to six cycles with the Mayo regimen of 5-FU/leucovorin were included in the study (n = 305). Patients who initiated chemotherapy but did not complete at least four cycles were excluded (n = 156) because this was considered to represent subtherapeutic management (11, 12). A total of 851 patients were treated by surgery alone. Mortality data were obtained from the Death Registry of the Health Department of Western Australia. Death reports were reviewed individually and classified as death due to colon cancer or from other causes. At the end of the study period, 159 (12.0%) patients died from unrelated causes and 662 (49.8%) from recurrence of colonic cancer. The perioperative mortality rate defined as death within 30 days of operative resection was 4.7% (n = 63). The mean follow-up time for patients was 52 months (median, 37 months).

Statistical analysis. Survival analysis was conducted using Kaplan-Meier analysis and Cox proportional hazards regression. The log-rank test was used to determine significance for Kaplan-Meier analysis. A Cox proportional hazards regression model was developed for survival in which each variable was adjusted for all others. The prognostic and predictive significance of each clinicopathologic variable was examined by multivariate analysis of patient groups treated by surgery alone or with chemotherapy. Statistical significance was deemed as $P < 0.05$.

Results

Prognostic significance. The prognostic significance of each clinical and pathologic feature was determined separately in

Table 1. Prognostic significance of clinicopathologic variables in stage III colon cancer treated by surgery alone or 5-FU chemotherapy

Variable (N1, N2)	Surgery alone (n = 851)			5-FU chemotherapy (n = 305)		
	N1, N2	HR (95% CI)	P	N1, N2	HR (95% CI)	P
Age (≤65 vs >65 y)	242, 609	0.65 (0.52-0.83)	<0.001	182, 123	0.92 (0.62-1.37)	0.47
Female vs male	451, 400	0.86 (0.71-1.05)	0.23	143, 162	0.67 (0.46-0.98)	0.04
Distal site vs proximal site	381, 470	0.84 (0.68-1.03)	0.08	161, 144	0.76 (0.51-1.13)	0.22
Poor grade vs well/moderate grade	200, 651	1.21 (0.96-1.52)	0.09	67, 238	1.27 (0.81-1.99)	0.20
T4 vs T1-T3	214, 637	1.38 (1.08-1.77)	0.01	68, 237	0.93 (0.54-1.62)	0.99
N2 vs N1	291, 560	1.65 (1.35-2.03)	<0.001	100, 205	1.69 (1.13-2.51)	0.01
Mucinous vs nonmucinous	223, 628	1.09 (0.88-1.36)	0.56	81, 224	1.03 (0.68-1.58)	0.81
Vascular invasion vs none	258, 593	1.28 (1.02-1.60)	0.03	91, 214	0.99 (0.64-1.53)	0.90
TILs present vs TILs absent	114, 737	0.98 (0.73-1.31)	0.77	55, 250	0.52 (0.30-0.91)	0.02
Perforation vs none	69, 782	1.30 (0.90-1.89)	0.23	28, 277	0.67 (0.27-1.05)	0.23

Abbreviation: 95% CI, 95% confidence interval.

patients treated by surgery alone (n = 851) or with adjuvant 5-FU chemotherapy (n = 305). As expected, the pathologic features of poor histologic grade, T4 stage, N2 nodal status, vascular invasion, and perforation were associated with worse outcome in multivariate analysis, with hazard ratios (HR) ranging from 1.21 to 1.65 (Table 1). The presence of TILs showed no prognostic significance in patients treated by surgery alone.

The same variables were evaluated for prognostic significance in patients treated with 5-FU (Table 1). Only N2 remained as a significant marker of worse prognosis. Interestingly, female gender and the presence of TILs were associated with significantly better survival in patients treated with 5-FU. This contrasts with their lack of prognostic value in patients treated by surgery alone. Perforation was associated with worse outcome in patients treated by surgery alone (HR, 1.30) but was prognostic for improved survival in patients treated with 5-FU (HR, 0.67).

Predictive significance for response to chemotherapy. One explanation for the observation that TILs and perforation are associated with improved survival in patients treated with 5-FU but not those treated by surgery alone is because these markers are predictive for response to chemotherapy. To test this possibility, we compared the survival of patient subgroups treated with or without 5-FU in a multivariate analysis (Table 2). The survival advantage associated with 5-FU chemotherapy in the overall cohort of stage III colon cancer patients was 24%. The features of older age, female gender, T4 stage, mucinous phenotype, and vascular invasion were each associated with a relatively greater survival benefit from chemotherapy. However, the greatest apparent survival benefit from chemotherapy was observed in patients with TILs (Fig. 1) or patients who had a perforation (Fig. 2). The HRs associated with the use of chemotherapy in these patients were 0.22 and 0.21, respectively (Table 2). Patients with TILs or perforation showed considerably more survival benefit compared with all other clinical or pathologic subgroups, with the exception of T4 stage (HR, 0.46). However, almost half (28 of 62, 45%) of the patients with T4 stage tumors and who were treated with 5-FU also showed evidence of perforation.

Table 2. Predictive significance of clinicopathologic variables in stage III colon cancer patients

Feature	N1, N2*	HR† (95% CI)	*P*
Total	851, 305	0.76 (0.61-0.96)	0.02
Age (y)			
≤65	242, 182	0.85 (0.62-1.17)	0.63
>65	609, 123	0.68 (0.47-0.97)	0.03
Sex			
Male	400, 162	0.81 (0.60-1.10)	0.31
Female	451, 143	0.71 (0.50-1.00)	0.048
Site			
Proximal	476, 144	0.71 (0.56-1.05)	0.13
Distal	380, 156	0.70 (0.50-0.98)	0.04
Grade			
Well/moderate	659, 237	0.74 (0.57-0.96)	0.02
Poor	197, 63	0.84 (0.52-1.34)	0.76
T stage			
T1-T3	639, 243	0.87 (0.67-1.13)	0.35
T4	212, 62	0.46 (0.28-0.74)	0.002
Nodal involvement			
N1	562, 209	0.74 (0.55-0.99)	0.04
N2	289, 96	0.70 (0.49-1.01)	0.13
Mucinous			
Yes	223, 81	0.64 (0.42-0.98)	0.04
No	628, 224	0.88 (0.67-1.16)	0.36
Vascular invasion			
Yes	600, 216	0.62 (0.42-0.93)	0.02
No	256, 84	0.80 (0.60-1.05)	0.20
TILs			
Yes	114, 55	0.22 (0.10-0.46)	<0.001
No	737, 250	0.84 (0.66-1.07)	0.29
Perforation			
Yes	69, 28	0.21 (0.07-0.61)	0.004
No	782, 277	0.82 (0.65-1.04)	0.13

*N1 = surgery alone; N2 = 5-FU chemotherapy.
†Survival of patients treated with 5-FU chemotherapy versus treatment by surgery alone.

Discussion

The ability to individually tailor a patient's treatment for cancer is a major goal for oncology clinicians and researchers. Central to this objective is the identification of independent prognostic and predictive markers. The former provide information on patient outcome that can be used to guide therapeutic decisions, whereas the latter provide information as to the likely response to treatment regimens. The identification of good prognostic markers is especially important for the management of stage II colon cancer in which the potential survival benefits from chemotherapy must be weighed against the toxicity and expense of this treatment. Because the majority of stage III colon cancer patients now receive adjuvant chemotherapy as standard practice, there are less compelling reasons to find prognostic markers for this group. In the present study, we found that older patient age, a higher burden of nodal involvement (N2), T4 stage, and vascular invasion were independent markers of poor outcome in stage III colon cancer patients treated by surgery alone (Table 1). The two latter features were also recently highlighted as major prognostic factors for stage II colon cancer (10, 13).

The ability to predict which colon cancer patients will gain a survival benefit from 5-FU–based adjuvant chemotherapy regimens has proven much more difficult than finding reliable and robust prognostic markers. Several candidate molecular predictive markers have been proposed, including *TP53* mutation, microsatellite instability, CpG island methylation, and thymidylate synthase expression. To date, however, none of these molecular-based markers has been validated in prospective trials with an appropriately powered study design. Current American Society of Clinical Oncology (14) and European (15) guidelines for the treatment of colon cancer state that there is insufficient evidence to recommend any molecular marker to guide routine clinical practice in the use of 5-FU.

Evaluation of the prognostic significance of pathologic markers in stage III colon cancer patients treated with 5-FU in the present study (Table 1) suggested that TILs and perforation may be predictive factors for good response to 5-FU. Both features were associated with better survival in patients treated by 5-FU (HR, 0.52 and 0.67, respectively) but not in patients treated by surgery alone (HR, 0.98 and 1.30, respectively). These findings show the importance of studying patient cohorts

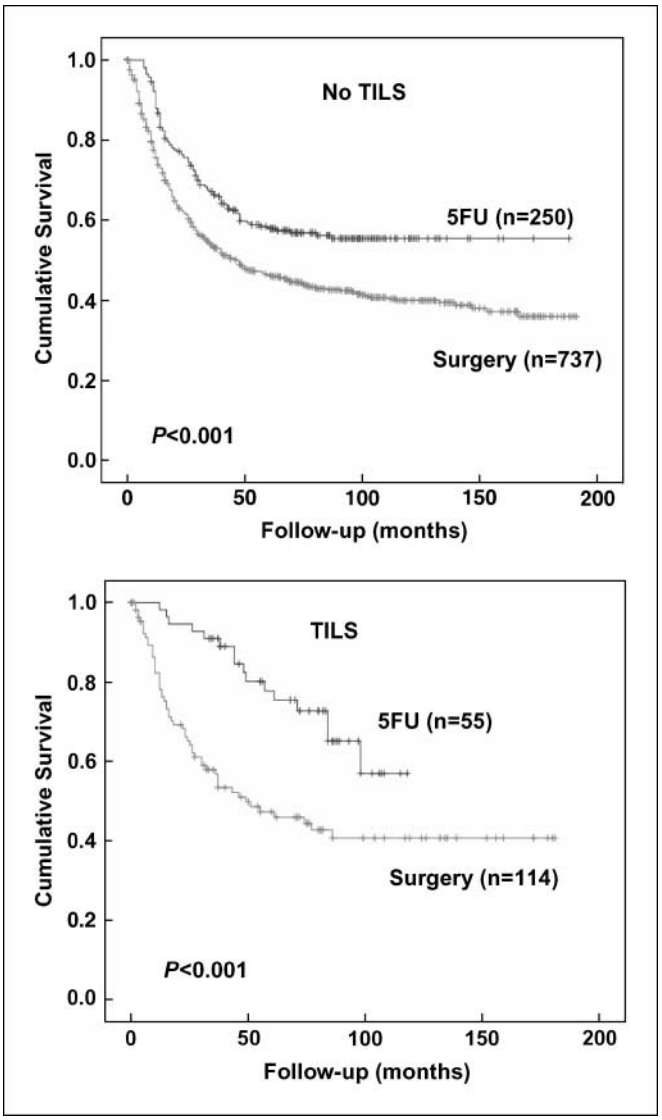

Fig. 1. Kaplan-Meier survival analysis for stage III colon cancer patients with or without the presence of TILs and treated by surgery alone or with adjuvant 5-FU chemotherapy. Patients with TILs seem to gain more survival benefit from 5-FU.

that have the same adjuvant treatment status when evaluating the prognostic significance of pathologic and molecular markers. The most obvious explanation for the above results is that TILs and perforation are predictive factors for response to 5-FU.

To investigate this possibility further, we compared the survival of patients treated with or without chemotherapy (Table 2). In the overall patient cohort, 5-FU reduced the HR by 24% compared with patients treated by surgery alone. This compares with reductions of 27% (16) and 34% (17) reported in previous observational studies of elderly stage III colon cancer patients. For the subgroup of older (>65 years) patients, the improved survival with 5-FU (32%) was comparable with these earlier studies (Table 2). The largest reductions in HR associated with 5-FU were observed for patients with TILs or perforation (Table 2; Figs. 1 and 2). These patients gained ~4-fold more survival benefit from 5-FU than patients without TILs or perforation.

The results of this work suggest that TILs and perforation have predictive value for positive response to 5-FU in stage III colon cancer. Although requiring confirmation in prospective studies, it is interesting to note that both of these features involve the immune response. Several previous studies have shown that TILs are associated with better prognosis in CRC (2–9). This was also observed here for stage III colon cancer patients treated with 5-FU but not for those treated by surgery alone (Table 1). We have recently shown that TILs had no prognostic significance in a large cohort of 1,306 stage II colon cancers treated by surgery alone (13). TILs might therefore be predictive for a good response to 5-FU rather than being a marker of a less aggressive phenotype in colon cancer. Unfortunately, none of the earlier studies (2–9) reported separate analyses for the prognostic value of TILs in stage-specific patient cohorts treated by surgery alone or with 5-FU. In agreement

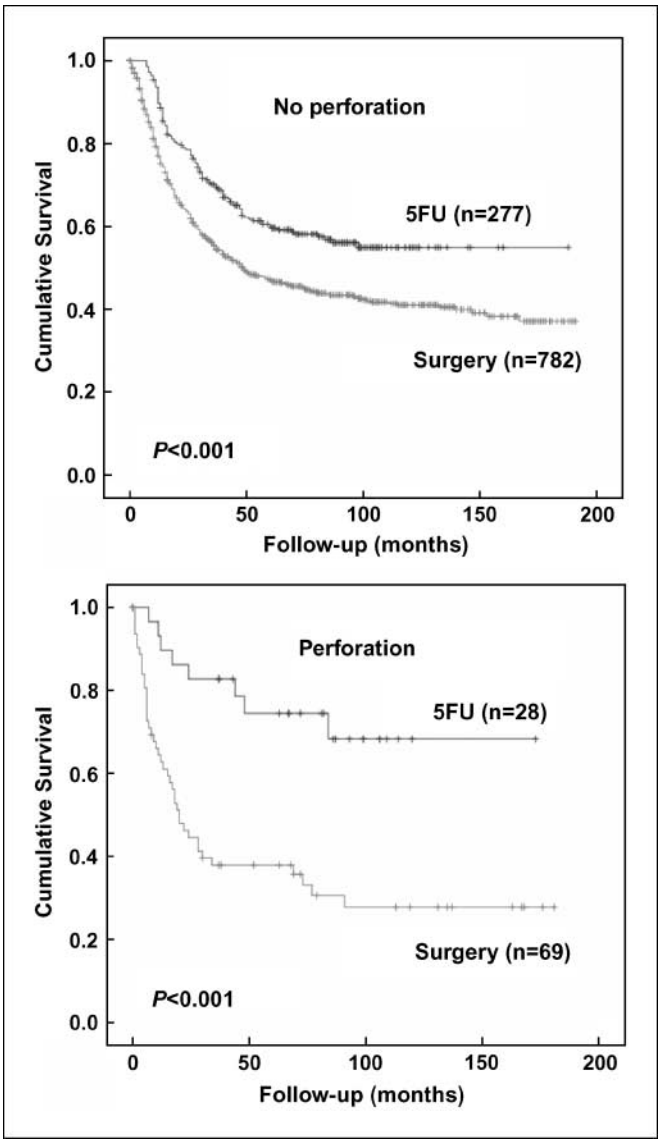

Fig. 2. Kaplan-Meier survival analysis for stage III colon cancer patients with or without perforation and treated by surgery alone or with adjuvant 5-FU chemotherapy. Patients with perforation seem to gain more survival benefit from 5-FU.

with the present results, Prall et al. (7) found that stage III CRC patients with high tumor densities of $CD8^+$ cells showed excellent survival when treated with 5-FU compared with those with low densities. Although these authors did not examine the prognostic value of $CD8^+$ densities in patients treated by surgery alone, they did suggest that $CD8^+$ cell density could serve as a predictive factor for benefit from 5-FU chemotherapy.

One of the major limitations of the present retrospective study was that tumors with TILs were identified from pathology reports. Although a proforma reporting system was used for the majority of the study cohort, significant variation among pathologists might still be expected for the reporting of TILs. Further work is required to determine both the prognostic and predictive values of lymphocyte type, density, and distribution in CRC.

Tumor perforation is often characterized by a systemic inflammatory response culminating in septic shock. In contrast to TILs, perforation has generally been associated with poor outcome in CRC (10, 13, 18). The adjusted HR associated with this feature was estimated to be as high as 2.93 in a study of 39 curatively resected colon cancers diagnosed before the widespread introduction of 5-FU chemotherapy (18). Our group previously reported a HR for perforation of 1.21 in stage II colon cancer treated by surgery alone (13). In the present study, the HR for perforation in stage III colon cancer patients treated by surgery alone was 1.30 (Table 1). An unexpected and novel finding of this study was the excellent survival of 28 patients with perforation who subsequently received 5-FU (Table 1; Fig. 2). Similar to TILs, we propose that perforation may be a predictive marker for good response to 5-FU chemotherapy.

The significance of clinical and subclinical perforations remains to be explored further. All such perforations will expose the tumor cells to a relatively new immune environment in the peritoneal cavity that is primed to react aggressively to any breach in gut integrity (19). This is in stark contrast to the immune mechanisms in the gut that are far more tolerant to the presence of bacteria. The initiation of an intense peritoneal inflammatory response may enhance the immune capacity of the body to respond to malignant cells. Similarly, this priming may improve responsiveness to adjuvant chemotherapy.

We hypothesize that postoperative administration of 5-FU chemotherapy to colon cancer patients with TILs or perforation preferentially activates the immune system against micrometastases compared with patients without these features, thus leading to improved survival. Conventional cytotoxic chemotherapy is a potent activator of antitumor immune responses (20, 21). The effects of chemotherapy in this process are thought to be multifactorial and could include the creation of a wave of dead or dying tumor cells that enters the antigen presentation pathway. Chemotherapy could also create a milieu during the recovery phase, particularly from lymphopenia, in which the immune system is receptive to the breaking of tolerance and thereby allows tumor cells to be recognized and eliminated. Finally, chemotherapy might transiently reduce the number and functional activity of T regulatory cells with suppressive properties. Whatever the underlying mechanism(s), the present results suggest that 5-FU chemotherapy is more effective at destroying micrometastatic tumor deposits in patients with TILs or perforation compared with patients without these features.

The current observations made in a retrospective, population-based cohort require confirmation in independent clinical data sets that include both quantitative and qualitative assessment of TILs. Comparison of the immune status between colon cancer patients with or without TILs and with or without perforation is also warranted, particularly at times before, during, and after the administration of 5-FU chemotherapy. Ultimately, such studies may lead to more effective combinations of chemotherapy and immunotherapy for the treatment of colon cancer.

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