Immunoprofiling for Prognostic Assessment of Colon Cancer: a Novel Complement to Ultrastaging

Simon Lavotshkin,

Department of Surgical Oncology, The John Wayne Cancer Institute at Providence Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

John R. Jalas,

Department of Pathology, Providence Saint John's Health Center, Santa Monica, CA, USA

Hitoe Torisu-Itakura,

Department of Surgical Oncology, The John Wayne Cancer Institute at Providence Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

Junko Ozao-Choy,

Department of Surgical Oncology, The John Wayne Cancer Institute at Providence Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

Ji Hey Lee,

Department of Surgical Oncology, The John Wayne Cancer Institute at Providence Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

Myung Shin Sim,

UCLA School of Medicine, Santa Monica, CA, USA

Alexander Stojadinovic,

Uniformed Services University of Health and Sciences, Bethesda, MD, USA

Zev Wainberg,

UCLA School of Medicine, Santa Monica, CA, USA

Carlo B. Bifulco,

Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center, Portland, OR, USA

Bernard A. Fox, and

Department of Molecular Microbiology and Immunology, and Knight Cancer Institute, Oregon Health and Science University, Portland, OR, USA

Anton J. Bilchik

Department of Surgical Oncology, The John Wayne Cancer Institute at Providence Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA; UCLA School of Medicine, Santa Monica, CA, USA; California Oncology Research Institute, Culver City, CA, USA

A. J. Bilchik bilchka@jwci.org.

Abstract

Background—Although AJCC/TNM staging remains the gold standard for prognostic assessment of colon cancer, stage-specific outcomes vary. We therefore prospectively evaluated the prognostic role of immunoprofiling.

Methods—Our cohort included 35 patients from an ongoing prospective trial of ultrastaging for colon cancer. Specimens were analyzed for T cell markers (CD3, CD4, CD8, and FoxP3). The number of tumor-infiltrating lymphocytes was analyzed at the tumor's margin and center and correlated with AJCC/TNM stage, clinicopathologic variables, and disease-free survival.

Results—There was a significant inverse association between number of $CD3^+$ cells in the tumor center and tumor stage ($P=0.05$). The tumor center/margin ratio of $CD3^+$ cells also showed an inverse but non-significant relationship with nodal involvement ($P=0.07$). Body mass index was inversely associated with numbers of $CD3^+$($P=0.04$) and $CD8^+$($P=0.02$) cells. Longer disease-free survival was correlated with higher CD8+ counts ($P=0.07$), lower $CD4^+/CD8^+$ ratios ($P=0.008$), and higher $CD8^+/FoxP3^+$ ratios ($P=0.02$).

Conclusions—This is the first prospective validation of immunoprofiling in patients whose colon cancer is staged with strict surgical and pathology quality measures. The apparent correlation between immunophenotypic response and clinical outcome warrants evaluation in a larger prospective trial.

Keywords

Colon cancer; Immunoprofiling; Recurrence; Staging

Introduction

The adaptive immune system plays an integral role in the defense against cancers such as melanoma,[1,2] CNS tumors,[3] renal carcinomas,[4] gynecologic malignancies,[5,6] lung cancer,[7] breast cancer,[8] and gastrointestinal tumors.[9–13] However, despite the increasing success of targeted therapies and other immune-based approaches to malignancy, the prognostic potential and assessment of the immune response are not well understood.

Galon et al. proposed that standardized scoring of the immune response to colorectal cancer may be a better prognostic tool than staging based on American Joint Commission on Cancer (AJCC) guidelines. Their immunoscore quantifies two specific lymphocyte populations in the tumor center (CT) and its invasive margin (IM).[11] In a series of seminal papers, they demonstrated a strong correlation between immunoscore (*high* in the setting of a brisk tumor immune response and *low* in its paucity) and outcome of patients with colorectal cancer. While these data were consistent across a number of recent publications, the findings have not been prospectively validated.[11,12,14,15]Additionally, while surgical and pathological quality measures have been shown to impact disease outcomes,[16,17]specifically a minimum of 12 lymph node specimen count, these measures were not considered in earlier immunoscore publications.

In this study, we analyzed prospectively collected data to determine whether the immunoprofile of a primary colon cancer was prognostically significant for patients treated in a clinical trial that used rigorously controlled surgical and pathological quality measures.

Materials and Methods

Our cohort was selected from patients enrolled in an ongoing prospective trial of ultrastaging for colon cancer (NCI trial #00949312). The statistician randomly identified 35 patients with non-metastatic disease (AJCC/TNM Stage I–III), adequate tissue, and complete follow-up.[32] Immunohistochemistry was performed on 4-μm formalin-fixed, paraffin-embedded colon cancer slides using the following antibodies: rabbit anti-human CD3 clone 2GV6, rabbit anti-human CD4 clone SP35, rabbit anti-human CD8 clone SP57 (Ventana Medical Systems Tucson, AZ USA), and mouse anti-human FoxP3 clone 236A/E7 (eBiosciences San Diego, CA USA). In brief, sections were deparaffinized in xylene (3 changes), rehydrated through graded alcohols (3 changes 100 % ethanol, 3 changes 95 % ethanol), and rinsed in distilled water. Heat-induced epitope retrieval was performed in a 1200-W microwave oven at 100 % power in 10 mM sodium citrate buffer, pH 6.0. CD3 and CD8 were retrieved for 10 min. CD4 and FoxP3 were retrieved for 20 min. Sections were allowed to cool for 30 min and then rinsed in distilled water. Antibody incubation and detection were carried out on a NexES or Discovery Instrument (Ventana Medical Systems Tucson, AZ USA) using Ventana's reagent buffer and detection kits unless otherwise noted. Endogenous peroxidase activity was blocked with hydrogen peroxide. FoxP3 was diluted 1:75 in Dulbecco's phosphate buffered saline and incubated for 12 h at room temperature. CD3, CD4, and CD8 were applied without dilution. All antibodies were detected with Ventana's biotinylated goat anti-mouse/goat anti-rabbit cocktail followed by application of streptavidin-horseradish-peroxidase conjugate. The complex was visualized with 3,3-diaminobenzidine and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with synthetic permanent media. Appropriate positive and negative controls were included with the study sections.

All colorectal carcinoma slides were reviewed by a pathologist blinded to all clinical data. Tissue sections were evaluated to ensure the presence of invasive adenocarcinoma, and all immunohistochemical stains were reviewed at scanning magnification (20×) to evaluate the density of staining at both the invasive margin (IM) and tumor center (CT). Areas of greatest density were then evaluated on higher power (100×) to ensure that tumor-infiltrating lymphocytes (TILs) were present, rather than innate lymphoid aggregates and/or germinal centers. If the presence of TILs was confirmed, the area was image captured for digital analysis. In order to standardize this process, image analysis was carried out using ImageJ Java-based image processing program. ImageJ analysis and threshold determination were carried out by a single author for all 35 patients. The median cell count was determined for each marker (CD3=791.5, CD4=792, CD8=569.5, FoxP3=315), and each sample was then classified as "high" if it was above the median and "low" if it was below the median.

Group comparisons were done by Student t test (or Wilcoxon rank sum) or ANOVA (or Kruskal-Wallis test) for continuous variables and chi-square test or Fisher's exact test for

categorical variables. The association of continuous variables was examined by Pearson (or Spearman rank) correlation coefficients. The test of agreement was done using McNemar's test. Survival estimates were plotted using Kaplan Meier methods and compared using log-rank test. All analyses were done by SAS 9.2 (NC, Cary), and *P* value <0.05 was considered statistically significant.

Results

The median follow-up time for this study was 46.8 months from time of surgery. Overall and stage-specific patient demographics and tumor characteristics are shown in Table 1. The patient population was 57 % male, with a median age of 74 years. The majority of patients had T3 tumors, and the mean lymph node count was 17.7, with only small variation between stages.

Figure 1 shows a representative immunostain for the pan T cell marker CD3, the helper T cell marker CD4, the cytotoxic T cell marker CD8, and the regulatory T cell marker FoxP3.

Mean number of CD3$^+$TILs in the CT was inversely correlated with tumor stage (Fig. 2a, *P*=0.05); this association was not observed for CD3$^+$TILs in the IM (Fig. 2b). The mean CD3$^+$CT/IM cell ratio also was inversely correlated with stage (Fig. 2c, *P*=0.05); likewise, the mean CD8$^+$ cell count in the CT followed this pattern, although the relationship was not significant (Fig. 2d).

The mean CD3$^+$ CT/IM cell ratio was inversely related to the number of tumor-involved nodes (Fig. 3a). Interestingly, there also was a statistically significant inverse association between body mass index (BMI) and number of CD3$^+$TILs (IM+CT; *P*=0.04) and CD8$^+$TILs (IM+TC; *P*=0.02) (Fig. 3b), suggesting that obesity attenuates the local tumor immune response.

Disease-free survival (DFS) tended to be longer among patients with higher numbers of CD8$^+$ cytotoxic T cells (Fig. 4a) or lower CD4$^+$/CD3$^+$ ratios (Fig. 4b). DFS was significantly longer among patients with lower CD4$^+$/CD8$^+$ ratios (Fig. 4c, *P*=0.0082). DFS also was significantly longer among patients with higher CD8$^+$/FoxP3$^+$ ratios (Fig. 4d, *P*=0.0282).

Discussion

Although AJCC staging remains standard for prognostic assessment of colon cancer,[32] outcomes vary widely among patients with the same stage of disease. For example, up to 25 % of patients with stage I–II tumors will develop recurrence after colectomy. Unfortunately, there is no accurate tool for identifying these high-risk patients. At present, adjuvant chemotherapy for stage II disease is associated with only a 3.6 % absolute improvement in survival[18] and is usually limited to those patients with "high-risk features" (poorly differentiated tumors, lymphovascular and/or perineural invasion, perforated tumors, inadequate lymph node sample).[19] Conversely, although adjuvant chemotherapy is the standard of care for node-positive (stage III) colon cancer, there may be a low-risk group for whom surgery alone is curative.[20]

The less than optimal correlation between AJCC stage and clinical outcome mandates a more accurate method of classifying the primary tumor. Proposed techniques include molecular classifications based on tumorigenic pathways, such as analysis for microsatellite instability testing,[21] and the analysis of gene expression profiles.[22–24] Other classification schemes have included sorting based on morphology (e.g., mucinous histopathology), classifying by mutational analysis, and based on assessment of tumor's cell of origin. However, these methods fail to account for the body's immune response to the tumor, and for the tumor's interaction with its microenvironment.[25]

The immunoscore quantifies the anti-tumor immune response of two lymphocyte populations in two distinct tumor regions. Its prognostic value has been consistently demonstrated in retrospective cohorts, most of which were in Europe. Furthermore, while the immunoscore is now the subject of a worldwide taskforce dedicated toward its international and prospective validation,[26] few studies in colon cancer have analyzed its prognostic utility based on prospectively collected data and those that have utilized older colorectal databases that did not control for surgical and pathologic variables.[27]

In two recent international multicenter prospective trials, our group has demonstrated that surgical quality and nodal ultrastaging impacts survival in colon cancer. Patients with stage II colon cancer having 12 lymph nodes evaluated and found to be negative for micrometastases (IHC negative for pan-cytokeratin) had significantly better DFS than patients with less than the minimum 12 lymph node count evaluated (recurrence rates at 3-year follow-up were 3 vs 18 %, respectively) (P=0.0015).[16] Given the prospect of the immunoscore's prognostic power in colon cancer, the aim of the current study was to address whether the TIL immunophenotype would remain an accurate prognosticator even when evaluated prospectively, in a patient cohort in whom quality of surgery and pathologic evaluation was strictly controlled. Additionally, the bulk of previous immunoscore population studies were conducted in European cohorts, and this is the first study to combine prospectively collected data, with rigorous quality measures, in a North American population of patients.

To assess the correlation between TIL density and AJCC/TNM stage, the common T cell marker, CD3, was analyzed first. Mean $CD3^+$ values in the CT were significantly smaller in higher tumor stages. While this pattern was not detected in the IM, upon closer examination, the average proportion of the two regions ($CD3^+$ CT/IM) analyzed by stage did indeed reveal a statistically significant decrease with disease progression. These results suggest that not only is the depletion of the lymphocytic response in the CT associated with advancing stage, but that as the tumor progresses, $CD3^+$ lymphocytes may be trapped in the IM, limiting their contribution to the anti-tumor response.

Next, cytotoxic T cells, as represented by $CD8^+$ cells, were examined. We chose to study this marker because the increased presence of $CD8^+$ cells has been implicated with better clinical outcome, and along with CD3 positivity, has been studied in previous immunoscore publications. Consistent with earlier studies, we found that these T cell effectors also followed a down-trending pattern with disease progression, and though not reaching statistical significance, a non-significant association was recognizable in the CT.

Additionally, to capture and quantify the remaining T cells populations in the tumor environment, we chose to analyze CD4 staining, which represents the memory T cells, and their subpopulation of FoxP3+ cells, representing the regulatory T cell population.

This pilot study has a number of important limitations. Due to its limited sample size, it was not designed to generate far-reaching conclusions, but rather to be a feasibility platform for the validation of immunoprofiling as an adjunct to AJCC/TNM staging. Additionally, since T3 tumors made up the majority, this may have skewed some of the data presented. Another limitation is that while 19/35 (54 %) of patients received adjuvant treatment (primarily 5FU based), the study due to its limited numbers did not address whether the immunoprofile correlates with response to treatment. Yet, despite the limitations in statistical power, the cornerstone of this study was its prospective adherence to strict quality measures. Since tumors were routinely ultrastaged, undergoing not only standard H&E, but also IHC analysis for pancytokeratin, the finding of increased nodal positivity with decreasing CD3 lymphocyte numbers is novel and gives added credence to immunoprofiling.

Furthermore, a unique and unexpected finding was the significant inverse correlation between BMI and TIL count. Nosho et al. found no association between BMI (dichotomized value of 30) and TILs,[27] but our results show that when BMI was analyzed as a continuous variable and as a sum of both tumor regions (IM+TC), increases in BMI were associated with lower CD3$^+$ (P=0.04) and lower CD8$^+$ lymphocytes (P=0.02). These preliminary findings are intriguing, particularly since obesity is increasingly linked with worse outcome in colon cancer. This suggestion that obesity attenuates the immune response, if confirmed in larger patient samples, may explain its putative role in the pathogenesis of colon cancer.

Consistent with previous results,[12–15] these findings show that a higher level of infiltrating CD8$^+$ cells correlates with improved DFS. Conversely, a higher ratio of CD4$^+$/CD8$^+$ cells has been shown to correlate with a lesser probability of DFS.[28] Since high numbers of tumor-infiltrating FoxP3$^+$ T regulatory cells have been shown to correlate with a poor prognosis in multiple cancer types,[8,29,30] our finding of better DFS associated with lower CD4$^+$/CD8$^+$ ratio can perhaps be accounted for by the down-regulation of T cell effector cells by the FoxP3$^+$ subpopulation of CD4$^+$ TILs. This interpretation is in agreement with the reported inverse correlation between CD8$^+$/FoxP3$^+$ ratio and survival in colon cancer.[31] Indeed, we found a significantly improved DFS in patients with a higher CD8$^+$/FoxP3+ ratio.

Conclusion

This is the first evaluation of immunophenotyping of TILs in colon cancer using a prospectively collected patient cohort, in which surgical and pathological quality measures were strictly adhered to. Taken together, the findings in this study are consistent with the mounting evidence in the literature that distinct subpopulations of TILs are associated with disease prognosis. Further investigation will focus on the complex interplay between these distinct T cell populations and their anti-tumor activity. Results can be used to design clinical trials of prognostic tools that quantify TIL populations in patients with colon cancer.

Acknowledgments

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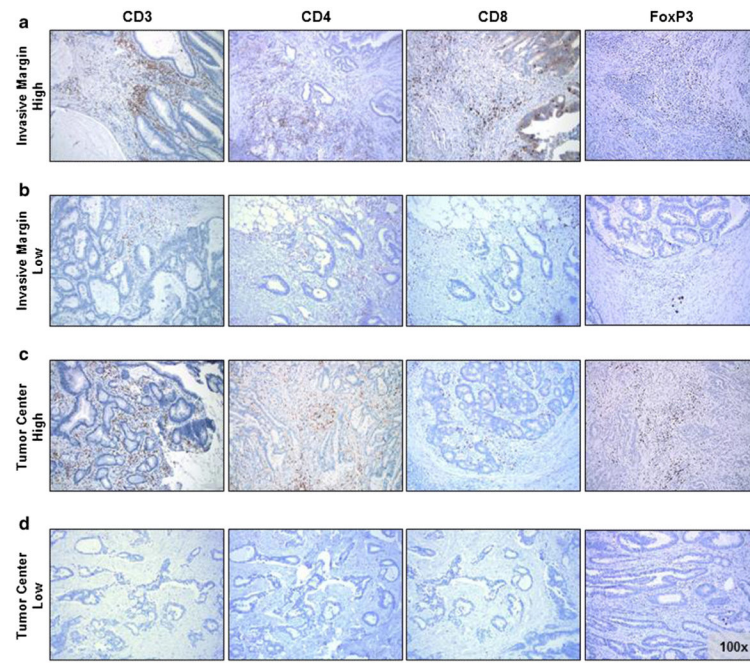
Fig. 1.
Representative images of immunostaining (CD3 *left*, CD4 *middle left*, CD8 *middle right*, FoxP3 *right*) at the IM (**a** and **b**) and CT (**c** and **d**); **a** and **c** show images classified as "high"; **b** and **d** were classified as "low"
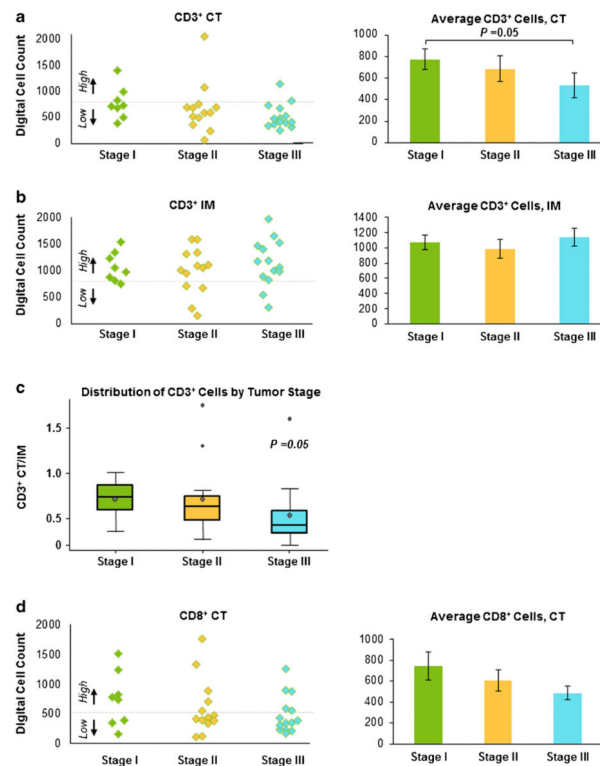
Fig. 2.
Tumor-infiltrating lymphocytes distributed by tumor stage. **a** CD3+ cells in the CT (*left*, individual cell counts by patient; *right*, average cell count); **b** CD3+ cells in the IM (*left*, individual cell counts by patient; *right*, average cell count); **c** ratio of CD3+ cells in the CT to the IM; **d** CD8+ cells in the CT (*left*, individual cell counts by patient; *right*, average cell count); **a, b, d** *Gray line* indicates median cell count per immune marker; above median classified as "high", below median classified as "low"
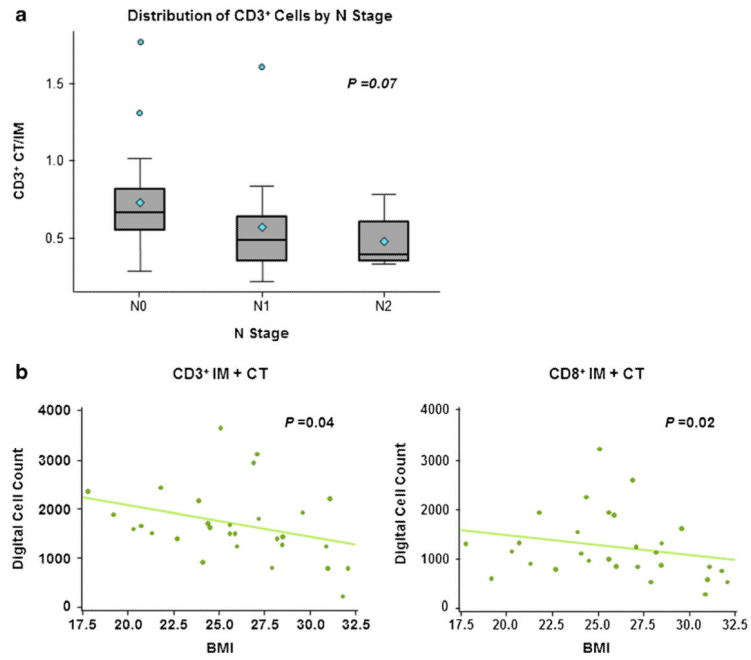
Fig. 3.
Number of tumor-infiltrating lymphocytes showed an inverse correlation with nodal stage (**a** CD3^{+} CT/IM) and body mass index (**b** CD3^{+} IM+CT *left*, CD8^{+} IM+CT *right*)
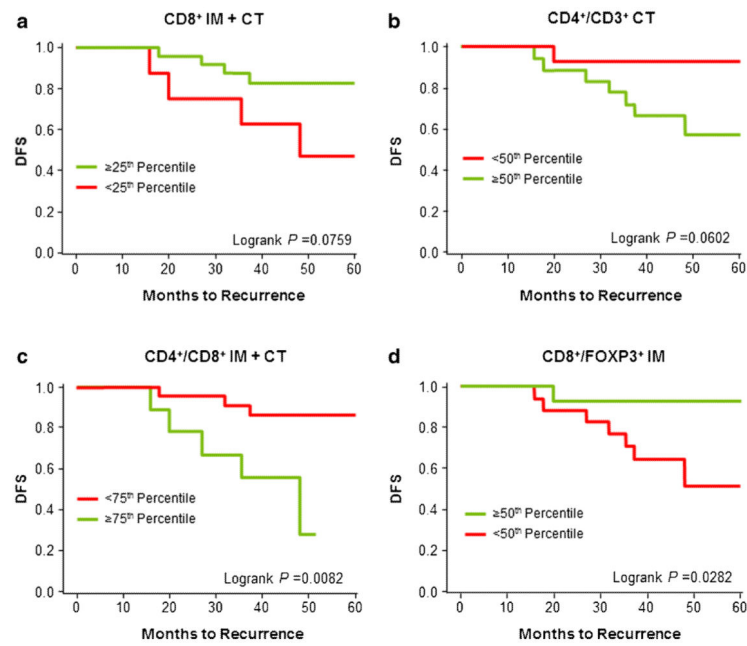
Fig. 4.
Kapan-Meier curves for disease-free survival (DFS) of patients grouped by percentage of tumor-infiltrating lymphocytes. **a** Patients with CD8^{+}IM+CT counts in the top 25th percentile vs below the 25th percentile; **b** patients with CD4^{+}/CD3^{+}CT ratios below the 50th percentile vs in 50th percentile or above; **c** patients with CD4^{+}/CD8^{+}IM+CT ratios below the 75th percentile vs in 75th percentile or above; **d** patients with CD8^{+}/FoxP3^{+}IM ratios in the top 50th percentile vs below the 50th percentile

Table 1

Patient demographics and tumor characteristics

Parameter	Overall *n*=35	Stage I *n*=8	Stage II *n*=13	Stage III *n*=14
Male, *n*	20	3	7	10
Female, *n*	15	5	6	4
Age, median	74	75	68	71
BMI, mean (±SD)	25.9 (±3.7)	25.9 (±3.2)	25.5 (±3.9)	26.3 (±4.0)
Average tumor size, cm	4.6	2.0	5.6	5.5
Depth of invasion, (T stage)	T1=5	T1=5	–	–
	T2=4	T2=3	–	T2=1
	T3=25	–	T3=12	T3=13
	T4=1	–	T4=1	–
Node count, mean (±SD)	17.7 (±11.3)	11.8 (±4.5)	21.5 (±14.9)	17.6 (±9.1)
No. of positive LNs, *n*	–	–	–	10
1–3				3
4–6				1
7				
Lymphovascular invasion (LVI), *n*	4	0	3	1
Adjuvant chemotherapy, *n*	19	0	5	14