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ARTICLE

Intratumoral budding and automated CD8-positive T-cell density in pretreatment biopsies can predict response to neoadjuvant therapy in rectal adenocarcinoma

Lama Farchoukh[1] · Douglas J. Hartman[1] · Changqing Ma[1] · James Celebrezze[2] · David Medich[2] · Nathan Bahary[3] · Madison Frank[1] · Liron Pantanowitz[1] · Reetesh K. Pai[1]

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Abstract

Tumor budding and CD8-positive (+) T-cells are recognized as prognostic factors in colorectal adenocarcinoma. We assessed CD8+ T-cell density and intratumoral budding in pretreatment rectal cancer biopsies to determine if they are predictive biomarkers for response to neoadjuvant therapy and survival. Pretreatment biopsies of locally advanced rectal adenocarcinoma from 117 patients were evaluated for CD8+ T-cell density using automated quantitative digital image analysis and for intratumoral budding and correlated with clinicopathological variables on postneoadjuvant surgical resection specimens, response to neoadjuvant therapy, and survival. Patients with high CD8+ T-cell density (≥157 per mm^2) on biopsy were significantly more likely to exhibit complete/near complete response to neoadjuvant therapy (66% vs. 33%, $p = 0.001$) and low tumor stage (0 or I) on resection (62% vs. 30%, $p = 0.001$) compared with patients with low CD8+ T-cell density. High CD8+ T-cell density was an independent predictor of response to neoadjuvant therapy with a 2.63 higher likelihood of complete response (95% CI 1.04–6.65, $p = 0.04$) and a 3.66 higher likelihood of complete/near complete response (95% CI 1.60–8.38, $p = 0.002$). The presence of intratumoral budding on biopsy was significantly associated with a reduced likelihood of achieving complete/near complete response to neoadjuvant therapy (odds ratio 0.36, 95% CI 0.13–0.97, $p = 0.048$). Patients with intratumoral budding on biopsy had a significantly reduced disease-free survival compared with patients without intratumoral budding (5-year survival 39% vs 87%, $p < 0.001$). In the multivariable model, the presence of intratumoral budding on biopsy was associated with a 3.35-fold increased risk of tumor recurrence (95% CI 1.25–8.99, $p = 0.02$). In conclusion, CD8+ T-cell density and intratumoral budding in pretreatment biopsies of rectal adenocarcinoma are independent predictive biomarkers of response to neoadjuvant therapy and intratumoral budding associates with patient survival. These biomarkers may be helpful in selecting patients who will respond to neoadjuvant therapy and identifying patients at risk for recurrence.

Introduction

Colorectal carcinoma is the third leading cause of cancer deaths in the United States, and an estimated 43,340 individuals are diagnosed specifically with rectal adenocarcinoma each year [1]. Treatment decisions of rectal adenocarcinoma are primarily determined by TNM staging and include local excision, neoadjuvant therapy, and radical surgical resection [2]. For patients with locally advanced rectal cancer, neoadjuvant therapy is the recommended treatment. However, there remains significant variability in response to neoadjuvant therapy ranging from patients with complete response to patients with tumor progression during therapy [3–5]. For patients with locally advanced rectal cancer, treatment strategies following neoadjuvant therapy

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✉ Reetesh K. Pai
pair@upmc.edu

[1] Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

[2] Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

[3] Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

also vary widely and include surgical resection for patients with significant residual tumor, organ-preserving treatments such as transanal local excision for limited tumor, or a watch-and-wait approach for patients with a complete clinical response [2]. Identification of predictive biomarkers for response to neoadjuvant therapy would help in guiding appropriate personalized therapy for patients with rectal adenocarcinoma. To date, clinicopathologic features that can predict response to neoadjuvant therapy in rectal adenocarcinoma are lacking.

Immune cell infiltration and tumor budding are being increasingly recognized as important prognostic features in colorectal carcinoma [6, 7]. A limited number of studies have evaluated immune cell infiltration and response to neoadjuvant therapy in patients with rectal cancer and found conflicting results [8–11]. Tumor budding within the tumor center, rather than at the invasive front, is termed intratumoral budding [6], and it can be assessed in biopsy specimens of colorectal adenocarcinoma [12–14]. Intratumoral budding has been proposed as a predictive biomarker of response to neoadjuvant therapy and patient survival in rectal adenocarcinoma [14]; however, to our knowledge, these results have not been validated.

The aim of this study was to assess quantitative CD8+ T-cell density and intratumoral budding in pretreatment biopsies of locally advanced rectal adenocarcinoma as predictive biomarkers for response to neoadjuvant therapy and patient survival. Quantitative CD8+ T-cell density was performed using a previously validated automated digital image analysis platform [15, 16]. Our data demonstrate that (1) CD8+ T-cell density and intratumoral budding are independent predictive biomarkers of response to neoadjuvant therapy and (2) that intratumoral budding is associated with disease-free survival. Evaluation of quantitative CD8+ T-cell density and intratumoral budding in biopsies can aid in the identification of patients who are at risk for non-response to current neoadjuvant therapy approaches and may benefit from alternative multimodal treatment regimens.

Methods

Study group

Patients diagnosed with primary invasive rectal adenocarcinoma on pretreatment biopsy accessioned at the Department of Pathology, University of Pittsburgh Medical Center from 2010 through 2019 were retrospectively identified by review of an institutional database. Patients with locally advanced rectal adenocarcinoma involving the mid or distal rectum and who underwent neoadjuvant therapy were included. Patients received one of two neoadjuvant therapy protocols: neoadjuvant chemoradiation versus total neoadjuvant therapy. Neoadjuvant chemoradiation involved preoperative radiotherapy (50.4 Gray) and concurrent 5-fluorouracil chemotherapy. Total neoadjuvant therapy involved preoperative systemic chemotherapy with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) followed by preoperative radiotherapy with concurrent 5-fluorouracil. Patients without available pretreatment tumor biopsy for analysis or with distant metastases at the time of diagnosis were specifically excluded. The study was approved by the University of Pittsburgh Institutional Review Board/Ethical Board (IRB# PR016040136).

Immunohistochemistry and quantitative digital image analysis of CD8+ T-cell density

CD8 immunohistochemistry (clone CD8/144B, DAKO, Carpinteria, CA) was performed on pretreatment biopsies of rectal adenocarcinoma. The CD8 slides were digitized using an Aperio AT2 scanner (Leica Biosystems, Buffalo Grove, IL) at ×40 magnification. The Aperio nuclear v9 algorithm was used to count the CD8+ stained cells based on different classes of staining intensity (0, 1+, 2+, and 3+) as previously described [16] (Fig. 1). This algorithm has been previously optimized compared with a manual count of CD8+ T-cells by immunohistochemistry and cross-validated with fluorescence-based CD8+ T-cell quantification [16]. The algorithm is a component of the commercially available Leica/Aperio image analysis platform. The CD8+ T-cell density was determined by dividing the number of CD8+ T-cells including cells stained with 3+, 2+, and 1+ intensity by the area in mm^2 examined. One pathologist (RKP) manually annotated the hematoxylin and eosin-stained slide and corresponding CD8 immunostained slide for each case to outline the areas of invasive adenocarcinoma. The annotations were then reviewed by an additional pathologist (DJH). Any differences in the annotations between the two pathologists were reviewed jointly to achieve consensus, as needed. Nonneoplastic rectal mucosa, precursor adenoma, and areas of necrosis were specifically excluded from the area of analysis. For 60 of 117 cases (51%), all biopsy fragments represented invasive adenocarcinoma, and all fragments were included in the analysis. For the remaining 57 (49%) cases, areas of the biopsy fragments contained precursor adenoma and/or nonneoplastic mucosa unassociated with invasive adenocarcinoma; these areas were excluded from the analysis. The CD8+ T-cell density analysis was performed blinded to patient outcomes and histopathologic variables.

DNA mismatch repair (MMR) protein immunohistochemistry was performed using primary monoclonal antibodies against MLH1 (clone M1, Ventana), MSH2 (clone G219–1129, Ventana), MSH6 (clone 44, Ventana),

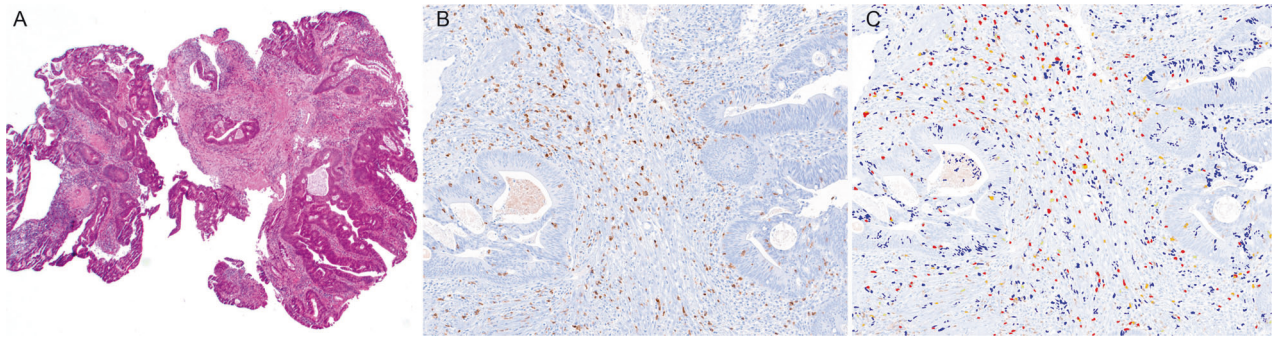

Fig. 1 Quantitative CD8+ T-cell analysis using the Aperio algorithm. a Pretreatment biopsy of a rectal mass demonstrating invasive moderately differentiated adenocarcinoma (×100 magnification). **b** CD8 immunohistochemistry identifying CD8+ T-cells within the biopsy. **c** Automated CD8 image analysis algorithm identifying CD8+ T-cells of varying intensity (3+ intensity, red; 2+ intensity, orange; 1+ intensity, yellow). A subset of non-lymphocyte nuclei is labeled in blue color. The CD8+ T-cell density was determined by dividing the number of CD8+ T-cells including cells stained with 3+, 2+, and 1+ intensity by the entire area in mm^2 examined. Importantly, the entire area of the invasive adenocarcinoma was used to determine the CD8+ T-cell density. Analysis limited to "hot spot" regions of the tumor was not performed given the variability introduced by selection bias.

and PMS2 (clone EPR3947, Cell Marque, Rocklin, CA) on whole sections, as previously described [17].

Pathologic evaluation and assessment of intratumoral budding

Histologic examination of all pathology slides of pretreatment biopsies of rectal adenocarcinoma from all 117 patients was performed by two pathologists (LF and RKP) blinded to outcome variables. The median number of histologic sections of the pretreatment biopsy per patient was two sections with an interquartile range of 5.0. The following histologic features were recorded for each case: histologic grade, angiolymphatic invasion, mucinous histology, intratumoral budding, chronic inflammatory reaction by visual assessment of H&E stains, and tumor infiltrating lymphocytes by visual assessment of H&E stains. Tumor grade was assessed for all tumors, including those with mucinous differentiation, using WHO 5th edition criteria with high-grade defined as <50% gland formation, regardless of MMR status [18]. Intratumoral budding was assessed using a modification of the methods described by Rogers et al. and the hotspot method detailed by the International Tumor Budding Consensus Conference (ITBCC) [6, 14]. Tumor buds were defined as isolated cancer cells or a cluster of <5 neoplastic cells. All biopsy fragments were assessed at a scanning (×10 objective) magnification for intratumoral buds. In the focus with maximal intratumoral buds, the number of tumor buds was determined in a 0.785 mm^2 area. Intratumoral budding was classified as present if ≥2 tumor buds were identified per 0.785 mm^2 and absent if <2 tumor buds were identified per 0.785 mm^2. Visual assessment of the chronic inflammatory reaction on H&E stained sections was performed by applying the four-tier scoring scheme proposed by Klintrup et al. to biopsy specimens [19]. The biopsies were grouped into two categories as proposed by Klintrup et al: moderate to severe chronic inflammatory reaction versus absent to weak chronic inflammatory reaction [19]. The presence of tumor infiltrating lymphocytes within tumor epithelium was visually assessed on H&E stained sections using the criteria outlined by Williams et al. [20]. Briefly, each histologic section of tumor was assessed at low-power (×4) objective magnification to identify the "hotspot" area with the most tumor infiltrating lymphocytes within tumor epithelium. In the "hotspot" area, the number of tumor infiltrating lymphocytes within tumor epithelium was counted in five consecutive high-power (×40 objective) fields. If on average ≥2 lymphocytes per high-power field were identified within the tumor, the tumor was scored as positive for tumor infiltrating lymphocytes within tumor epithelium. All cases with discrepancies between the two study pathologists in the histologic assessment of the pretreatment biopsies were reviewed at a multiheaded microscope to achieve consensus.

Histologic examination of all pathology slides from surgical resection specimens were reviewed by one pathologist (RKP). The following histologic features were recorded for each case: grade, stage, angiolymphatic invasion, perineural invasion, venous invasion, extracellular mucin, tumor budding at the invasive front, and tumor regression score. For each rectal adenocarcinoma, the entire tumor bed was submitted for histologic examination. Tumor budding was assessed using the method advocated by the ITBCC [6] and adopted by the College of American Pathologists colorectal carcinoma protocol. Tumor regression score in the resection specimen was assessed according to the College of American Pathologists guidelines [21, 22] using a four-point system similar to that of Ryan et al. [23] defined as follows: tumor regression score 0 (complete

response), no viable cancer cells; (1) (near complete response), single cells or rare small groups of cancer cells; (2) (partial response), residual cancer outgrown by fibrosis; and (3) (poor or no response), extensive residual cancer with no evident tumor regression. For each patient, the tumor regression score was assessed by at least two pathologists, including the original sign-out pathologist at the time of initial pathologic evaluation and one of the authors (RKP). For some of the subsequent analyses, patients were grouped into tumor regression score 0–1 versus tumor regression score 2–3. Given that the distinction between tumor regression score 1 and tumor regression score 2 can be subjective, all cases assigned a tumor regression score 1 or tumor regression score 2 were reviewed by two of the authors (LF and RKP). All cases with discrepancies between any of the pathologists in the assessment of tumor regression were reviewed at a multi-headed microscope to achieve consensus between the authors (LF and RKP).

Statistical analysis

Comparisons of CD8+ T-cell density and categorical variables were performed using the Mann–Whitney test. Chi-square or Fisher exact tests were used to characterize the relationship between categorical variables, as appropriate.

Tumor regression score and disease-free survival were the primary endpoints. The discriminative accuracy of the CD8+ T-cell density was evaluated using area under the receiver operating characteristic curve (AUROC) with calculation of sensitivity and specificity. The optimal cutoff for CD8+T-cell density using the Youden Index of the AUROC was chosen to separate patients into groups using complete/near complete tumor regression score as the anchor. Logistic regression modeling was performed for response to neoadjuvant therapy. Univariate modeling was performed and covariates with significance of $p < 0.05$ on univariate modeling were included in the multivariate model. Data from univariate and multivariate logistic regression analyses were reported as odds ratios with 95% confidence intervals (CI). All statistics were assessed using two-sided tests with p values < 0.05 considered statistically significant.

Disease-free survival was defined as the time (measured in months) from the date of initial diagnosis to the date of disease recurrence, either local recurrence or distant systemic metastasis (i.e. tumor involving the peritoneum and/or other organs/sites) and censored at the date of last clinical follow-up. Tumor recurrence was established by either biopsy confirmation or radiographic evidence without biopsy confirmation. Survival rates were determined by the Kaplan–Meier method and differences between groups were evaluated by log-rank test. Hazard ratios were calculated from a Cox proportional hazard model to identify individual predictors of survival. Multivariate analysis of significant individual risk factors ($p < 0.05$) was performed using Cox proportional hazard regression to identify independent risk factors for survival. Data from univariate and multivariate analyses were reported as hazard ratios with 95% CI. All statistics were assessed using two-sided tests with p values < 0.05 considered statistically significant. Statistical analyses were performed using SPSS (for Windows 23, IBM, Armonk, NY).

Results

CD8+ T-cell density and intratumoral budding on pretreatment biopsy correlate with clinicopathologic features

The clinicopathologic features of the 117 patients with pretreatment biopsies of rectal adenocarcinoma treated with neoadjuvant therapy are summarized in Table 1. Of the 117 patients, 105 patients had surgical resection following neoadjuvant therapy and 12 patients had a complete clinical response by endoscopic and radiographic imaging studies. Patients with complete clinical response were included as stage 0 and tumor regression score 0 in the subsequent analyses. Of the 117 patients, 64 (55%) were located in the distal rectum and 53 (45%) in the mid rectum. Most patients received neoadjuvant chemoradiotherapy (81%) with fewer treated with total neoadjuvant therapy (19%). The vast majority of tumors showed proficient MMR protein expression (114/117, 97%) with three patients demonstrating MMR deficiency (all three with confirmed Lynch syndrome, two with germline *MSH2* mutation and one with germline *MSH6* mutation). Most tumors were low grade (93%) and were conventional adenocarcinoma without mucinous differentiation on biopsy (93%).

CD8+ T-cell density in pretreatment biopsies correlated with clinicopathologic features of rectal adenocarcinomas. Higher CD8+ T-cell density was more often identified in distal rectal tumors compared with mid rectal tumors ($p = 0.005$) and in MMR deficient tumors compared with MMR proficient tumors ($p = 0.01$). Higher CD8+ T-cell density in pretreatment biopsies was observed in patients with tumor regression score of 0 or 1 ($p = 0.006$) and lower tumor stage (0 or 1) ($p = 0.004$) on subsequent resection and follow-up. Tumors with venous invasion on subsequent resection exhibited lower CD8+ T-cell density on the pretreatment biopsy ($p = 0.03$). There was no association between CD8+ T-cell density and tumor grade, intratumoral budding on biopsy, tumor budding at the invasive front on resection, mucinous differentiation, and perineural invasion.

Table 1 Correlation of CD8+ T-cell density in pretreatment biopsy of rectal adenocarcinoma as a continuous variable with clinicopathologic variables.

Clinicopathologic feature	*N* (%)	Median CD8/mm^2 on biopsy (range)	*p* value
Location in rectum			
Mid	53 (45)	97 (9–448)	0.005
Distal	64 (55)	152 (33–613)	
Type of neoadjuvant therapy			
Chemoradiotherapy	95 (81)	136 (18–409)	0.01
Total neoadjuvant therapy	22 (19)	219 (9–613)	
Stage after neoadjuvant therapy			
0–I[a]	49 (42)	170 (9–613)	0.004
II–III	68 (58)	112 (18–416)	
Tumor Regression Score (TRS)			
TRS 0 or 1[a]	53 (45)	177 (9–613)	0.006
TRS 2 or 3	64 (55)	110 (18–450)	
Tumor grade in biopsy			
Low	109 (93)	134 (9–613)	0.7
High	8 (7)	169 (46–351)	
Intratumoral budding in biopsy			
Absent	91 (78)	141 (9–613)	0.5
Present (two or more)	26 (22)	108 (40–409)	
Mucinous differentiation in biopsy			
Absent	108 (92)	141 (18–613)	0.2
Present	9 (8)	93 (9–351)	
Tumor Budding per 0.785 mm^2 in resection			
Low/Intermediate (0–9)	98 (93)	139 (9–450)	0.1
High (ten or more)	7 (7)	73 (47–155)	
Venous invasion in resection			
Absent	90 (86)	142 (9–450)	0.03
Present	15 (14)	87 (32–409)	
Lymphatic invasion in resection			
Absent	81 (77)	139 (9–450)	0.5
Present	24 (23)	94 (26–409)	
Perineural invasion in resection			
Absent	90 (86)	133 (9–450)	1.0
Present	15 (14)	117 (45–409)	
MMR status			
MMR proficient	114 (97)	134 (9–613)	0.01
MMR deficient	3 (3)	327 (296–417)	

[a]12 patients with complete clinical response by endoscopic and magnetic resonance imaging without surgical resection are included in the stage 0 and tumor regression score 0 categories. Histopathologic features on resection are not available for these 12 patients.

Receiver operating curves were generated for CD8+ T-cell density using tumor regression score 0 or 1 as the anchor (Supplemental Fig.). The AUROC for CD8+ T-cell density was 0.65 (0.54–0.75, 95% CI, $p = 0.007$) with an optimal cutoff of 157/mm^2 achieving a 65% sensitivity and 78% specificity for tumor regression score 0 or 1. Patients were separated into high (≥157/mm^2) and low (<157/mm^2) CD8+ T-cell density in pretreatment biopsies of rectal adenocarcinoma (Figs. 2 and 3).

High CD8+ T-cell density in pretreatment biopsies was identified in 44 patients (38%) (Table 2). Compared with patients with low CD8+ T-cell density on biopsy, patients with high CD8+ T-cell density were more often men (73% vs. 51%, $p = 0.02$), had tumor centered in the distal rectum (68% vs. 47%, $p = 0.02$), and had MMR deficient tumors (7% vs. 0%, $p = 0.02$). In addition, compared with tumors with low CD8+ T-cell density, tumors with high CD8+ T-cell density more often demonstrated moderate to severe chronic inflammatory reaction by visual assessment of H&E stained sections using the criteria of Klintrup et al. [19] ($p = 0.008$) and positive tumor infiltrating lymphocytes in tumor epithelium by visual assessment of H&E stained sections using the criteria of Williams et al. [20] ($p < 0.001$). CD8+ T-cell density on the pretreatment biopsy correlated with a number of histopathologic variables on subsequent clinical follow-up and resection. Patients with high CD8+ T-cell density on pretreatment biopsy more often had tumor regression score 0 or 1 (66% vs. 33%, $p = 0.001$) and low tumor stage (0 or 1) (62% vs. 30%, $p = 0.001$). Patients with high CD8+ T-cell density on pretreatment biopsy less frequently demonstrated high tumor budding at the invasive front on surgical resection (0% vs. 10%, $p = 0.04$).

Intratumoral budding in pretreatment biopsies was identified in 26 (22%) patients (Table 2 and Fig. 4). The detection of intratumoral budding was not influenced by the number of histologic sections evaluated of the pretreatment biopsies. A median of two histologic sections was evaluated for both cases with and without intratumoral budding identified on pretreatment biopsy ($p = 0.5$). Compared with patients without intratumoral budding on pretreatment biopsy, patients with intratumoral budding more often exhibited high tumor grade using WHO criteria on biopsy (27% vs. 1%, $p < 0.001$). However, using WHO grading criteria, 73% of patients with intratumoral budding on biopsy had low-grade adenocarcinoma and 27% patients with intratumoral budding had high-grade adenocarcinoma. Compared with patients without intratumoral budding on pretreatment biopsy, patients with intratumoral budding less often had tumor regression score 0 or 1 (27% vs. 50%; $p = 0.03$) and less often had low tumor stage (0 or 1) (27% vs. 46%), although this was not significant ($p = 0.08$). Lastly, the presence of intratumoral budding in the pretreatment biopsy significantly correlated with the tumor budding score at the invasive margin on subsequent surgical resection. All patients with a high tumor budding score at the invasive margin on surgical resection demonstrated intratumoral budding on the pretreatment biopsy (7/7, 100%) while only 19% (19/98) of patients with low/intermediate tumor

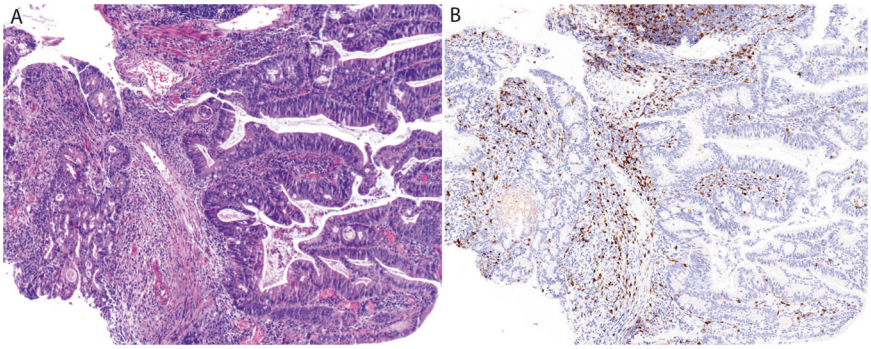

Fig. 2 High CD8+ T-cell density in pre-treatment biopsy of rectal adenocarcinoma. a This patient with a pretreatment biopsy of a rectal mass demonstrating invasive moderately differentiated adenocarcinoma (×200 magnification). **b** CD8 immunohistochemistry demonstrated a high CD8+ T-cell density (×200 magnification).

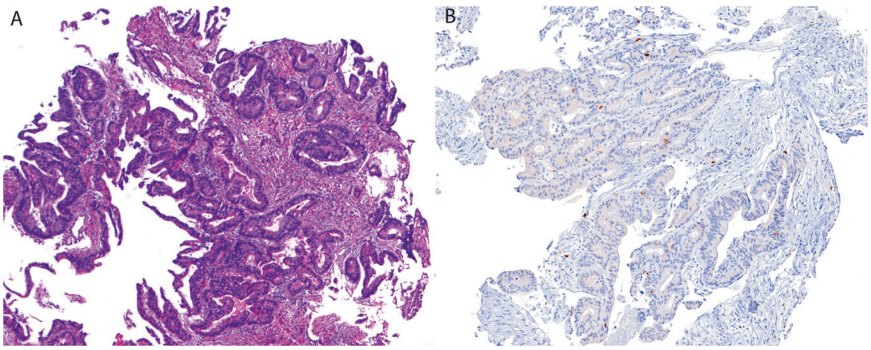

Fig. 3 Low CD8+ T-cell density in pre-treatment biopsy of rectal adenocarcinoma. a This patient with pretreatment biopsy of a rectal mass demonstrating invasive moderately differentiated adenocarcinoma (×200 magnification). **b** CD8 immunohistochemistry demonstrated a low CD8+ T-cell density (×200 magnification).

budding score at the invasive margin on surgical resection demonstrated intratumoral budding on the pretreatment biopsy ($p < 0.001$). Thus, intratumoral budding on pretreatment biopsy has a 100% sensitivity and 81% specificity for high tumor budding at the invasive margin on subsequent surgical resection.

CD8+ T-cell density and intratumoral budding are independent predictors of response to neoadjuvant therapy

Univariate and multivariate logistic regression analysis was performed to identify features associated with response to neoadjuvant therapy, including the category of complete response (tumor regression score 0) and the combined categories of complete/near complete response (tumor regression score 0 or 1) (Table 3). In the multivariate regression analysis, high CD8+ T-cell density on pretreatment biopsy was an independent predictor of response to neoadjuvant therapy. Patients with a high CD8+ T-cell density on biopsy had a 2.63 higher likelihood of achieving complete response (tumor regression score 0) to neoadjuvant therapy (95% CI, 1.04–6.65, $p = 0.04$) and a 3.66 higher likelihood of achieving either complete or near complete response (tumor regression score 0 or 1) to neoadjuvant therapy (95% CI 1.60–8.38, $p = 0.002$). The only other independent predictor of complete response to neoadjuvant therapy was the use of total neoadjuvant therapy that was associated with a 4.08 higher likelihood of complete response compared with chemoradiotherapy alone (95% CI 1.49–11.39, $p = 0.007$).

Intratumoral budding was also associated with response to neoadjuvant therapy (Table 3). In the multivariable model of predictors of response to neoadjuvant therapy, the presence of intratumoral budding on pretreatment biopsy was associated with a reduced likelihood of complete/near complete response to neoadjuvant therapy (multivariate odds ratio 0.36, 95% CI 0.13–0.97, $p = 0.048$). However, the presence of intratumoral budding on pretreatment biopsy was not associated with achieving complete response.

Patient age, tumor location in the rectum, and other histopathologic variables in the pretreatment biopsy (tumor grade, mucinous differentiation, and angiolymphatic invasion) were not predictors of response to neoadjuvant therapy (all with $p > 0.05$). In addition, visual estimation of chronic inflammatory reaction on H&E stained sections using the scheme reported by Klintrup et al. [19] and visual estimation of tumor infiltrating lymphocytes in tumor epithelium on H&E stained sections using the criteria reported by Williams et al. [20] were not associated with response to neoadjuvant therapy.

To evaluate the association of CD8+ T-cell density with tumor response to neoadjuvant therapy, we performed a subanalysis stratified by tumor location in the rectum (distal rectum versus mid rectum). However, the analysis was

Table 2 Correlation of CD8+ T-cell density and intratumoral budding with clinicopathologic variables.

Clinicopathologic feature	Low (<157/mm^2) CD8 + T-cell density in biopsy (%)	High (≥157/mm^2) CD8 + T-cell density in biopsy (%)	*p* value	Intratumoral budding present in biopsy (%)	Intratumoral budding absent in biopsy (%)	*p* value
Number of patients (%)	73 (62)	44 (38)	NA	26 (22)	91 (78)	NA
Number with surgical resection	68 (93)	37 (84)	NA	24 (92)	81 (89)	NA
Median age in years (IQR)	60 (17)	62 (17)	0.4	65 (20)	60 (17)	0.4
Gender, male/female	37 (51)/36 (49)	32 (73)/12 (27)	0.02	17 (65)/9 (35)	52 (57)/39 (43)	0.5
Location						
Mid rectum	39 (53)	14 (32)	0.02	10 (38)	43 (47)	0.4
Distal rectum	34 (47)	30 (68)		16 (62)	48 (53)	
Median tumor size in cm (IQR)	5.0 (3.5)	5.0 (3.0)	0.4	5.4 (4.8)	5.0 (2.7)	0.3
Neoadjuvant therapy						
Chemoradiotherapy	64 (88)	31 (70)	0.02	23 (88)	72 (79)	0.3
Total neoadjuvant therapy	9 (12)	13 (30)		3 (12)	19 (21)	
MMR status						
MMR deficient	0	3 (7)	0.02	0	3 (3)	0.3
MMR proficient	73 (100)	41 (93)		26 (100)	88 (97)	
Intratumoral budding present in biopsy	17 (23)	9 (20)	0.7	NA	NA	NA
Angiolymphatic invasion present in biopsy	2 (3)	1 (2)	0.9	2 (8)	1 (1)	0.06
Mucinous differentiation on biopsy	7 (10)	2 (5)	0.3	4 (15)	5 (5)	0.1
High tumor grade on biopsy	4 (5)	4 (9)	0.5	7 (27)	1 (1)	<0.001
Moderate or severe chronic inflammatory reaction on visual assessment of H&E	28 (38)	28 (64)	0.008	12 (46)	44 (48)	0.8
Positive for tumor infiltrating lymphocytes on visual assessment of H&E	3 (4)	14 (32)	<0.001	1 (4)	16 (18)	0.08
Tumor regression score						
0 (including complete CR)	11 (15)	16 (36)	0.001*	4 (15)	23 (25)	0.03*
1	13 (18)	13 (30)		3 (12)	23 (25)	
2	32 (44)	7 (16)		12 (46)	27 (30)	
3	17 (23)	8 (18)		7 (27)	18 (20)	
Stage						
0	13 (18)	14 (32)	0.001**	5 (19)	22 (24)	0.08**
1	9 (12)	13 (30)		2 (8)	20 (22)	
2	24 (33)	6 (14)		5 (19)	25 (27)	
3	27 (37)	11 (25)		14 (54)	24 (26)	
Venous invasion on resection	13 (19)	2 (5)	0.06	5 (21)	10 (12)	0.3
Perineural invasion on resection	11 (16)	4 (11)	0.5	8 (33)	7 (9)	0.002
Lymphatic invasion on resection	17 (25)	7 (19)	0.5	11 (46)	13 (16)	0.002
High tumor budding (ten or more per 0.785 mm^2) in resection	7 (10)	0	0.04	7 (29)	0	<0.001
Positive circumferential margin	7 (10)	1 (3)	0.2	1 (4)	7 (9)	0.5
Extracellular mucin in resection	12 (18)	7 (19)	0.9	6 (25)	13 (16)	0.3
Recurrence pattern						
No recurrence	60 (82)	40 (91)	0.4	17 (65)	83 (91)	0.003
Local recurrence	3 (4)	0		1 (4)	2 (2)	
Systemic recurrence	10 (14)	4 (9)		8 (31)	6 (7)	

NA not applicable, *IQR* interquartile range, *CR* clinical response.

**p* value is for tumor regression score 0 or 1 vs. 2 or 3.

***p* value is for stage 0 or 1 vs. 2 or 3.

somewhat limited by the smaller number of patients in each category. In the distal rectum, the presence of high CD8+ T-cell density in the pretreatment biopsies was associated with a 5.98-fold higher likelihood of complete response (tumor regression score 0) (95% CI 1.48–24.22, $p = 0.01$) and a 3.67-fold higher likelihood of complete/near complete response (tumor regression score 0 or 1) (95% CI 1.30–10.32, $p = 0.01$). In the mid rectum, the presence of high CD8+ T-cell density in the pretreatment biopsies was associated with a 4.05-fold higher likelihood of complete/near complete response (tumor regression score 0 or 1) (95% CI 1.12–14.68, $p = 0.03$). However, in the mid

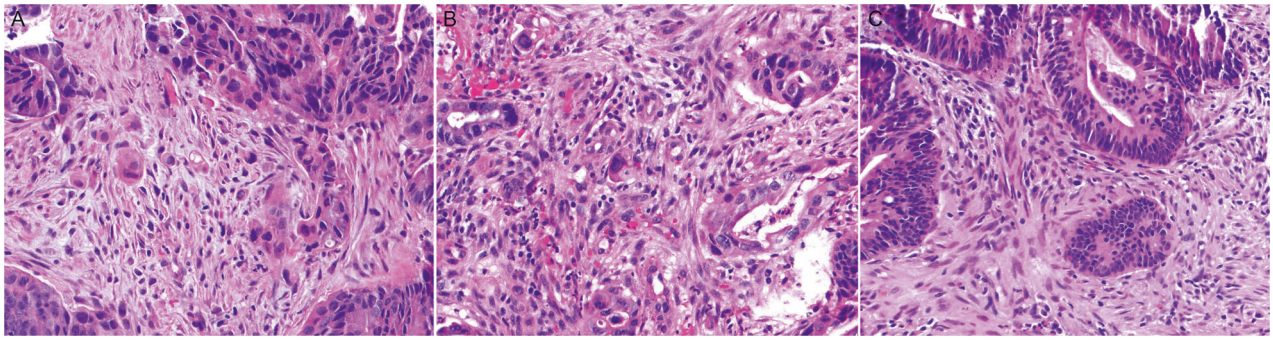

Fig. 4 Intratumoral budding in pretreatment biopsies of rectal adenocarcinoma. a Pretreatment biopsy demonstrating high-grade rectal adenocarcinoma that had intratumoral budding. **b** Pretreatment biopsy demonstrating low-grade rectal adenocarcinoma that had intratumoral budding (two tumor buds per 0.785mm^2). **c** Pretreatment biopsy demonstrating low-grade rectal adenocarcinoma with no intratumoral budding. All images were taken at ×200 magnification.

rectum, the presence of high CD8+ T-cell density in their pretreatment biopsies was not associated with achieving complete response (tumor regression score 0) alone.

Intratumoral budding in pretreatment biopsies associates with tumor recurrence

Clinical follow-up was available for all 117 patients with a median follow-up interval of 29 months (range: 3–106 months) from the time of initial diagnosis. There were 17 patients with tumor recurrence including 14 patients who developed systemic metastatic disease (either to the lungs, liver, bone, or non-regional lymph nodes) and 3 patients who developed local recurrence. Of the 17 patients with tumor recurrence, 14 patients had biopsy confirmation of tumor recurrence, and three patients had radiographic evidence of tumor recurrence without biopsy confirmation.

Using Kaplan–Meier survival functions, patients with intratumoral budding in their pretreatment biopsy had significantly reduced disease-free survival compared with patients without intratumoral budding (5-year disease-free survival 39% vs 87%, $p < 0.001$) (Fig. 5). Patients with low CD8+ T-cell density in their pretreatment biopsy also had reduced disease-free survival compared with patients with high CD8+ T-cell density (5-year disease-free survival 74% vs. 83%), although this did not reach statistical significance ($p = 0.1$) (Fig. 5).

Variables chosen for Cox proportional hazards regression analysis for disease-free survival were optimized to reduce multicollinearity. High tumor budding at the invasive front in the resection specimen significantly correlated with intratumoral budding on biopsy (Pearson correlation coefficient of 1.0). Given the strong correlation between these two variables, to reduce multicollinearity in the Cox proportional hazards model, only intratumoral budding on the pretreatment biopsy was included as a variable in the analysis of disease-free survival. In the Cox proportional hazards model, variables significantly associated with disease-free survival were intratumoral budding in the pretreatment biopsy (hazard ratio 4.34, 95% CI 1.67–11.31) and tumor regression score 2 or 3 (hazard ratio 3.92, 95% CI 1.13–13.63, $p = 0.03$) (Table 4). In the multivariable model, only intratumoral budding on pretreatment biopsy was associated tumor recurrence (hazard ratio 3.35, 95% CI 1.25–8.99, $p = 0.02$).

Discussion

Neoadjuvant therapy for rectal adenocarcinoma is the standard of care for patients with locally advanced rectal adenocarcinoma [2]. However, there remains significant variability in response to treatment with some patients achieving complete response while other patients experience progression of disease during treatment. Response to neoadjuvant therapy is as an important prognostic biomarker for locally advanced rectal adenocarcinoma [24–26]. To date, clinicopathologic features that can predict tumor response to neoadjuvant therapy have been lacking. Recent studies suggest that increased T-cell infiltration in the tumor microenvironment has a favorable prognostic effect in colonic and rectal adenocarcinoma [7, 8] with some, but not all, studies suggesting the immune cells within tumor immune environment may affect response to neoadjuvant therapy [8–10]. The presence of intratumoral budding in pretreatment biopsies has also been proposed as a histopathologic predictor of response to neoadjuvant therapy [14]. In this study, we demonstrated that automated quantitative CD8+ T-cell analysis of pretreatment biopsies of rectal adenocarcinoma can predict tumor response to neoadjuvant therapy. In our analysis, high CD8+ T-cell density was an independent predictor of complete response to neoadjuvant therapy in rectal adenocarcinoma. The absence of intratumoral budding was also an independent predictor of complete/near complete response to

Table 3 Logistic regression of clinicopathologic variables in predicting response to neoadjuvant chemoradiotherapy in rectal adenocarcinoma.

Variable	Complete response (TRS 0)				Complete/Near complete response (TRS 0–1)			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value
Age	0.98 (0.95–1.02)	0.3	–	–	1.01 (0.98–1.04)	0.4	–	–
Distal rectum (vs. mid rectum)	0.86 (0.36–2.04)	0.7	–	–	1.52 (0.73–3.18)	0.3	–	–
High CD8+ T-cell density in biopsy	3.22 (1.33–7.83)	0.01	2.63 (1.04–6.65)	0.04	3.94 (1.78–8.71)	0.001	3.66 (1.60–8.38)	0.002
Moderate or severe chronic inflammatory reaction on visual assessment of H&E (vs. absent or weak)	1.23 (0.52–2.91)	0.6	–	–	1.09 (0.53–2.26)	0.8	–	–
Positive for tumor infiltrating lymphocytes on visual assessment of H&E (vs. negative)	2.80 (0.91–8.27)	0.08	–	–	1.89 (0.67–5.38)	0.2	–	–
Intratumoral budding present (two or more per 0.785 mm^2 hotspot field) in biopsy	0.54 (0.17–1.72)	0.5	–	–	0.36 (0.14–0.94)	0.04	0.36 (0.13–0.97)	0.048
High tumor grade in biopsy	2.13 (0.47–9.54)	0.3	–	–	0.71 (0.16–3.11)	0.7	–	–
Angiolymphatic invasion present in biopsy	1.69 (0.15–19.46)	0.7	–	–	0.60 (0.05–6.76)	0.7	–	–
Mucinous differentiation present in biopsy	0.39 (0.05–3.30)	0.4	–	–	0.96 (0.25–3.78)	1.0	–	–
MMR deficient (vs. MMR proficient)	1.07 (0.12–22.35)	1.0	–	–	0.60 (0.05–6.76)	0.7	–	–
Total neoadjuvant therapy (vs. chemoradiotherapy)	4.94 (1.83–13.34)	0.002	4.08 (1.49–11.39)	0.007	3.21 (1.20–8.62)	0.02	2.35 (0.81–6.81)	0.1

TRS College of American Pathologist (CAP) tumor regression score, *CI* confidence interval, *H&E* hematoxylin and eosin.

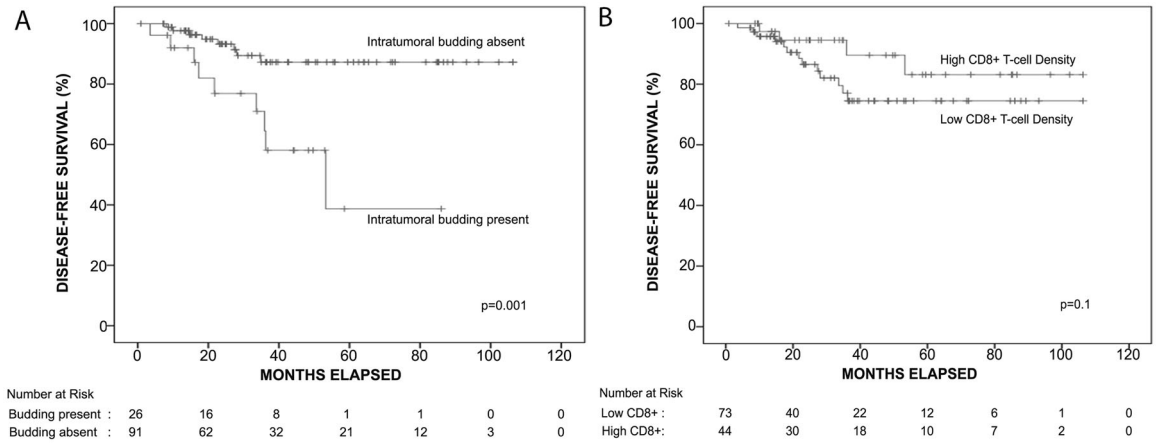

Fig. 5 Kaplan-Meier disease-free survival analysis. a Kaplan–Meier disease-free survival function of the entire cohort of patients stratified by intratumoral budding on pretreatment biopsy. Patients with intratumoral budding present on pretreatment biopsy had a significantly reduced disease-free survival compared with patients with absent intratumoral budding ($p = 0.001$). **b** Kaplan–Meier disease-free survival function of the entire cohort of patients stratified by CD8+ T-cell density on pretreatment biopsy. Patients with a high CD8+ T-cell density on pretreatment biopsy had an improved disease-free survival compared with patients with low CD8+ T-cell density, although this did not reach statistical significance ($p = 0.1$).

Table 4 Univariate and multivariate Cox regression analysis of disease-free survival in patients with rectal adenocarcinoma treated with neoadjuvant therapy.

Clinicopathologic variable	Univariate		Multivariate	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Age	1.01 (0.97–1.05)	0.6	–	–
Distal rectum (vs. mid rectum)	1.05 (0.40–2.77)	0.9	–	–
High CD8+ T-cell density in biopsy	0.48 (0.16–1.49)	0.1	–	–
Intratumoral budding present in biopsy	4.34 (1.67–11.31)	0.003	3.35 (1.25–8.99)	0.02
Moderate or severe chronic inflammatory reaction on visual assessment of biopsy H&E (vs. absent or weak)	1.35 (0.51–3.55)	0.5	–	–
Positive tumor infiltrating lymphocytes on visual assessment of biopsy H&E	0.04 (0.01–11.07)	0.3	–	–
Stage III (vs. all other stages)	1.68 (0.65–4.36)	0.3	–	–
Tumor regression score 2–3 (vs. tumor regression score 0–1)	3.92 (1.13–13.63)	0.03	2.85 (0.79–10.31)	0.1
Total neoadjuvant therapy (vs. chemoradiotherapy)	2.27 (0.61–8.47)	0.2	–	–
High tumor grade in resection	0.41 (0.04–18.70)	0.4	–	–
Venous invasion in resection	1.93 (0.62–5.99)	0.3	–	–
Perineural invasion in resection	1.53 (0.44–5.37)	0.5	–	–
Extracellular mucin in resection	0.67 (0.15–2.94)	0.7	–	–
Positive circumferential resection margin	1.52 (0.35–6.72)	0.6	–	–
Adjuvant chemotherapy given (vs. not given)	0.95 (0.37–2.47)	0.9		

CI confidence interval, *H&E* hematoxylin and eosin.

neoadjuvant therapy but did not independently predict complete response. Our data indicate that assessment of CD8+ T-cell density and intratumoral budding on pretreatment biopsies of rectal adenocarcinoma can help anticipate tumor response to neoadjuvant therapy.

Immune cell infiltration in the tumor microenvironment has been extensively evaluated in colon cancer with many studies supporting its prognostic relevance [7, 27–30]. However, few studies have evaluated the impact of immune cell infiltration as a predictive biomarker for therapy,

particularly in the setting of locally advanced rectal adenocarcinoma. In an early study using the Immunoscore, Anitei et al. analyzed pretreatment biopsies of rectal adenocarcinoma in 55 patients and demonstrated that high T-cell infiltration was identified in 72% of patients with complete/partial response to neoadjuvant therapy and in only 28% of patients with no response to neoadjuvant therapy [8]. However, Anitei et al. did not perform a multivariate analysis of factors predicting response to therapy. Using manual quantitation of CD8+ T-cells in 48 pretreatment biopsies of rectal adenocarcinoma, Yasuda et al. demonstrated that CD8+ T-cell density was the only independent predictor of complete response to neoadjuvant therapy in a multivariate analysis [11]. To our knowledge, only two prior studies have assessed automated quantitative CD8+ T-cell digital imaging analysis of pretreatment biopsies of rectal adenocarcinoma as an independent predictor of response to therapy. In an analysis of 106 patients, McCoy et al. did not identify T-cell density in pretreatment biopsies as predictive of response to neoadjuvant therapy [10]. In contrast, Akiyoshi et al. demonstrated that CD8+ tumor infiltrating T-cells were an independent predictor of complete/near complete response to neoadjuvant therapy; however, CD8+ stromal T-cells were not predictive in their analysis [9]. The reason for these different results is not entirely clear but may relate to the how CD8+ T-cell quantitation was performed in each study. In our study, we evaluated all areas of invasive adenocarcinoma excluding precursor adenoma, nonneoplastic mucosa, and areas of necrosis. In contrast, McCoy et al. assessed T-cell density across all biopsy fragments and did not specifically exclude noninvasive and nonneoplastic areas. Akioyoshi et al. limited their quantitative analysis to only two 0.6 mm^2 regions, did not evaluate all areas of invasive adenocarcinoma within the biopsies, and performed a separate analysis of tumor and stromal areas. Given the variability introduced by selection bias related to how tumor subcompartments are determined and annotated, we did not perform a subanalysis of CD8+ T-cell density within tumor epithelium and tumor stroma within the biopsy fragments.

A major impediment to the routine evaluation of infiltrating lymphocytes is the availability of a reproducible and reliable method to quantify the amount of lymphocytes within tissue. A number of different techniques have been employed to score lymphocyte density with many studies using manual counts or semiquantitative visual assessment of either H&E stained sections or immunohistochemically stained tissue sections. In a study of 1265 patients, Williams et al. demonstrated that high tumor infiltrating lymphocytes (defined as ≥2 tumor infiltrating lymphocytes within tumor epithelium per high-power field by manual count on routine H&E stained sections) correlated with patient outcome independent of mismatch repair protein status and tumor stage [20]. Similarly, Klintrup et al. also identified visual estimation of the inflammatory reaction on H&E stained sections is a prognostic factor [19]. Neither Williams et al. or Klintrup et al. evaluated the association of inflammatory cells in rectal adenocarcinoma or in pretreatment biopsy specimens. In our study, biopsies with a high CD8+ T-cell density were more likely to have positive tumor infiltrating lymphocytes within tumor epithelium and to have a moderate to severe chronic inflammatory reaction by visual assessment of H&E stained sections. However, neither of these visual assessment parameters were predictive of response to neoadjuvant therapy. These results suggest that automated quantitative CD8 T-cell analysis is better than visual assessment of H&E sections at predicting response to neoadjuvant therapy in rectal adenocarcinoma.

Tumor budding at the invasive margin has been shown to be strongly associated with lymph node metastasis in colorectal carcinoma and is an independent predictor of survival in early stage colorectal cancer [6]. Intratumoral budding differs from tumor budding at the invasive margin and refers to tumor buds within the tumor center. Assessment of intratumoral budding can be performed on biopsy specimens from tumors [6, 12]. The 22% rate of intratumoral budding on pretreatment biopsies identified in our study is very similar to the 20% rate reported by Rogers et al. [14]. Similar to the results of our study, Rogers et al. demonstrated that no patient with intratumoral budding achieved complete response to neoadjuvant therapy although the association of intratumoral budding and response to neoadjuvant therapy was only of borderline significance ($p = 0.06$) [14]. We demonstrated that intratumoral budding is an independent predictor of complete/near complete response to neoadjuvant therapy and is only rarely identified in patients who achieve complete response. In both our analysis and that of Rogers et al., intratumoral budding in biopsies was scored as absent versus present. Our data indicate that the presence of two or more intratumoral buds in biopsies is predictive of the eventual tumor budding score and has a 100% sensitivity of detecting a high tumor budding score at the invasive front on subsequent surgical resection.

The underlying reasons for the association of tumor immune cell infiltration and intratumoral budding with response to neoadjuvant therapy in rectal adenocarcinoma are still unknown. In a recent analysis of molecular alterations and immune cell infiltration of 17 patients with rectal adenocarcinoma treated with neoadjuvant therapy, Kamran et al. identified that patients without response to neoadjuvant therapy more often had reduced CD8+ T-cells in pretreatment biopsies, similar to the results of our study [31]. Interestingly, the authors also found that patients with no response to neoadjuvant therapy more often had rectal tumors harboring *TP53* mutations. Mutations in *TP53* have

been associated with epithelial-to-mesenchymal transition in numerous studies [32, 33], while intratumoral and peritumoral budding are thought to be morphologic manifestations of the epithelial-to-mesenchymal transition. The presence of *TP53* mutations in rectal adenocarcinoma may induce an epithelial-to-mesenchymal transition that manifests as intratumoral budding and local immune escape of the tumor with an associated poor response to neoadjuvant therapy. Additional larger patient populations with rectal adenocarcinoma with molecular characterization and extended follow-up are needed to further determine the predictive impact of CD8+ T-cell density and intratumoral budding.

Lastly, we also assessed the prognostic effect of CD8+ T-cell density and intratumoral budding on pretreatment biopsies on disease-free survival. Our results indicate that intratumoral budding in pretreatment biopsies may help identify patients with a more aggressive clinical course. Similarly, Rogers et al. did identify intratumoral budding on pretreatment biopsy as an independent prognostic biomarker for reduced disease-free survival with a reported hazard ratio 3.51 [14]. Only a limited number of studies have evaluated the prognostic effect CD8+ T-cell density in pretreatment biopsies of rectal adenocarcinoma. In one study using visual estimation [34], CD8+ T-cell density was not an independent predictor of patient survival. However, in a study using quantitative digital assessment, CD8+ T-cell density was an independent predictor of disease-free survival [9]. Additional, larger patient populations with rectal adenocarcinoma with extended follow-up are needed to assess the prognostic effect of pretreatment tumor characteristics on patient survival.

The strengths of our study include the use of an internally validated automated quantitative digital image analysis platform and rigorous histopathologic analysis of factors associated with prognosis in rectal adenocarcinoma. Our analysis has limitations including the retrospective design and inclusion of patients treated at a large academic medical center with its inherent referral bias. Lastly, further validation of CD8+ T-cell density and intratumoral budding in pretreatment biopsies as predictive biomarkers of response to neoadjuvant therapy in rectal adenocarcinoma in large, well-characterized, and prospective patient cohorts is needed.

In conclusion, we demonstrated that automated quantitative CD8+ T-cell analysis and intratumoral budding in pretreatment biopsies of rectal adenocarcinoma can predict tumor response to neoadjuvant therapy. In our analysis, high CD8+ T-cell density was an independent predictor of complete response to neoadjuvant therapy in rectal adenocarcinoma. Intratumoral budding was also an independent predictor of complete/near complete response to neoadjuvant therapy. Patients with intratumoral budding on their pretreatment biopsy also had reduced disease-free survival following neoadjuvant therapy. Our results suggest that assessment of CD8+ T-cell density and intratumoral budding on pretreatment biopsies of rectal adenocarcinoma may be useful as part of routine comprehensive pathologic risk assessment of rectal adenocarcinoma to allow for a more patient-centered approach when selecting multimodal neoadjuvant treatment regimens and for identifying patients at increased risk for tumor recurrence.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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