Lymph Node Tumor Burden Correlates With Tumor Budding and Poorly Differentiated Clusters: A New Prognostic Factor in Colorectal Carcinoma?

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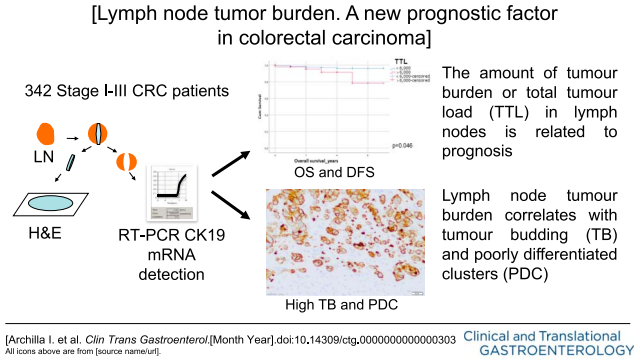
INTRODUCTION: **Molecular lymph node (LN) staging in early colorectal cancer (CRC) has demonstrated to be more precise than conventional histopathology pN staging. Tumor budding (TB) and poorly differentiated clusters (PDCs) are associated with LN metastases, recurrences, and lower survival in CRC. We evaluated the correlation between the total tumor load (TTL) in LNs from CRC surgical specimens with patient outcome, TB, and PDC.**

METHODS: **In this retrospective multicentre study, 5,931 LNs from 342 stage I–III CRC were analyzed by both hematoxylin and eosin and molecular detection of tumor cytokeratin 19 mRNA by one-step nucleic acid amplification. TB and PDC were evaluated by hematoxylin and eosin and cytokeratin 19 immunohistochemistry.**

RESULTS: **One-step nucleic acid was positive in 38.3% patients (n = 131). Tumor Budding was low in 45% cases, intermediate in 25%, and high in 30%. Poorly Differentiated Clusters were low-grade G1 in 53%, G2 in 32%, and G3 in 15%. TB and PDC correlated with TTL, high-grade, lymphovascular and perineural invasion, pT, pN and stage ($P < 0.001$). TB, PDC, and TTL ≥ 6,000 copies/μL were associated with worse overall survival ($P = 0.002$, $P = 0.013$, and $P = 0.046$) and disease-free survival ($P < 0.001$).**

DISCUSSION: **The implementation of more sensitive molecular methods to assess LN status is a promising alternative approach to pN staging, which could be integrated to other factors to help risk stratification and management of patients with early-stage CRC. This study demonstrates the correlation of the amount of LN tumor burden with TB and PDCs. TTL is related to the outcome and could be used as a new prognostic factor in CRC (see Visual Abstract, Supplementary Digital Content 2, http://links.lww.com/CTG/A512).**



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COLON

INTRODUCTION

Colorectal cancer (CRC) is the third carcinoma in incidence, with almost 1.8 million new cases in 2018, being the second cause of cancer-related death worldwide, accounting for 800,000 deaths in 2018 (1,2). CRC screening programs in average-risk population have achieved a significant mortality reduction because of an increased diagnosis at early stages of the disease (3). In addition, up to 70% CRC diagnosed in the context of screening programs are stages I–II, which could be cured after surgical excision. Nevertheless, 10%–15% of stage II patients recur within 5 years of curative-intended surgery (4,5). These stage II high-risk patients are difficult to identify with current strategies.

Lymph node (LN) status determines prognosis and the need of adjuvant therapy (6). In addition, the current pathology-based LN staging performed with the gold standard hematoxylin and eosin (H&E) does not guarantee a reliable pN0 because of its low sensitivity to detect LN micrometastases (7). A recent metanalysis showed that patients with LN micrometastases not detected by H&E had worse prognosis and impaired survival rates and would benefit from adjuvant therapy (7). Therefore, there is an unmet need for the incorporation of more sensitive methods of LN staging for early-stage CRC (5,7). In parallel, highly sensitive molecular methods have been recently incorporated into pathology diagnosis. New molecular techniques, such as the one-step nucleic acid assay (OSNA; Sysmex, Kobe, Japan), make it possible to detect the presence of LN tumor burden in 11.5%–50% of patients with CRC staged as pN0 by H&E (8–14). Therefore, molecular LN analysis could help to narrow down undetected patients with stage II CRC (8–10). Tumor burden in LNs is determined by the OSNA assay as the total tumor load (TTL), defined as the amount of tumor cytokeratin 19 (CK19) mRNA/μL copies present in LNs of CRC surgical specimens. We and other authors have recently demonstrated the correlation of the TTL with other CRC risk factors, such as pN, pT, tumor grade, male sex, tumor size and lymphovascular invasion (LVI), poor prognosis, and worse disease-free survival (DFS) (8–11,13,15,16).

Tumor budding (TB) and poorly differentiated clusters (PDCs) are morphologic manifestations of the epithelial-to-mesenchymal transition phenotype, a physiological process that allows epithelial cells to acquire mesenchymal properties and the potential for migration and stromal invasion, which is essential for developing metastases. TB is defined as the presence of single tumor cells or cell clusters formed by 4 cells or less at the tumor invasive front (17). PDCs consist of aggregates of at least 5 neoplastic cells in the tumor stroma that do not form glandular structures. Both TB and PDCs are independent prognostic factors in stage II colon cancer (17–22), associated with LN metastases, high pT stage, tumor grade, perineural invasion (PNI), LVI, extramural vascular invasion (EMVI), and distant metastases (22–28).

This study demonstrates the correlation of the TTL, a sensitive method to detect LN metastases, with patient outcome, TB, and PDC, considered risk factors for LN metastasis in early-stage CRC. The incorporation of molecular methods to assess LN status, together with other pathological risk factors, could help to improve risk stratification and management of patients with early-stage CRC.

METHODS

Study samples

This is a retrospective multicenter observational study from 3 hospitals, which includes all patients with stage I–III CRC who underwent LN analysis with both the OSNA assay and H&E between 2010 and 2018. Inclusion criteria were patients older than 18 years old with histologically confirmed primary CRC and positive CK19 immunoreaction. Exclusion criteria included synchronous CRC or presence of other malignant neoplasms, metastatic disease, neoadjuvant therapy, familial adenomatous polyposis, carcinomas on inflammatory bowel disease, and the presence of intraluminal stent-type devices. The study was presented and approved by the Scientific and Ethics Committee of each institution.

Study procedure

Fresh LN dissection and sample processing. All freshly dissected LNs from the mesocolon or mesorectal fat were analyzed by 2 methods, H&E and OSNA, as previously described (8,9). Briefly, all LNs were sectioned, submitting part of the LN for conventional formalin-fixation, paraffin-embedding, and H&E staining. The rest of the LN was put into PCR tubes and stored at −80°C until OSNA analysis was performed. After fresh LN procurement, the fat was fixed in 10% neutral buffered formalin, and a second-look dissection was performed for remaining LNs, which were processed only by conventional histopathological analysis (8,9).

OSNA assay. The OSNA assay is a standardized, quantitative, objective, and reproducible method of evaluation based on a type of QRT-PCR called reverse transcription loop-mediated isothermal amplification (RT-LAMP), which amplifies CK19 mRNA. It has been validated for LN analysis of breast and CRC. The OSNA assay was performed at each institution using the protocol described by Tsujimoto et al. (29) and following the manufacturer's instructions. Briefly, each PCR tube containing LNs was homogenized with the lysis buffer Lynorhag (Sysmex, Hyogo, Japan) for mRNA stabilization and genomic DNA precipitation, centrifugated and amplified using the RT-LAMP method with the RD-100i automated gene-amplification system (Sysmex). As a result, the number of tumor CK19 mRNA copies/μL present in all LNs of the surgical specimen, defined as the TTL, was obtained. An OSNA result of ≥250 copies/μL was considered positive, as established and validated in previous studies (11,12).

Pathology report and LN staging. Pathology reports including LN staging were performed based on H&E analysis according to the AJCC/UICC TNM, 8th edition (30). Both pathologists and clinicians were blinded to the OSNA results.

TB and PDC assessment. Histopathological H&E-stained slides were reviewed by a pathology fellow and a gastrointestinal pathologist (I.A., and M.C.). Discordant cases were revisited together and a consensus about the definite score was reached. All slides containing tumor were first scanned at low-power magnification to identify the area with the highest density of TB and PDCs on H&E. Then, the selected slide or slides were immunostained with CK19.

Table 1. Demographic, clinical, and pathological characteristics

Gender	
Male	207 (60.5%)
Female	135 (39.5%)
Age (yr, mean ± SD)	68.8 ± 11.5
Tumor location	
Right colon	115 (33.6%)
Transverse colon	45 (13.2%)
Left colon	130 (38%)
Rectosigmoid colon	36 (10.5%)
Rectum	16 (4.7%)
Tumor size (cm, mean ± SD)	3.3 ± 1.8
Macroscopic configuration	
Polypoid	174 (50.9%)
Ulcerated	168 (49.1%)
Grade	
Low	189 (55.3%)
High	153 (44.7%)
Invasive front	
Pushing border	165 (55.2%)
Infiltrative margin	101 (33.8%)
Mixed	33 (11%)
PNI	
Absent	298 (87.1%)
Present	44 (12.9%)
LVI	
Absent	246 (71.9%)
Present	96 (28.1%)
EMVI	
Absent	324 (94.7%)
Present	18 (5.3%)
pT stage	
pT1	66 (19.3%)
pT2	85 (24.8%)
pT3	148 (43.3%)
pT4a	42 (12.3%)
pT4b	1 (0.3%)
pN stage	
pN0	269 (78.7%)
pN1a	32 (9.4%)
pN1b	24 (7%)
pN2a	8 (2.3%)
pN2b	7 (2%)
pN1c	2 (0.6%)
Stage	
I	139 (40.7%)
II	130 (38%)
III	73 (21.3%)

EMVI, extramural vascular invasion; LVI, lymphovascular invasion; PNI, perineural invasion.

The number of TB was evaluated at the invasive front of the tumor corresponding to the hotspot area of the selected region. TB was counted on one field of 0.785 mm^2 using a 20× objective lens and normalized according to the International Consortium on TB Recommendations (17). TB was graded as Bd1/low (0–4 buds), Bd2/intermediate (5–9 buds) and Bd3/high (≥10 buds). PDCs were evaluated at 20× either at the invasive front or the center of the tumor and graded as G1 (0–4 clusters), G2 (5–9 clusters), and G3 (≥10 clusters) (31). In mucinous carcinomas, tumor cells within mucin pools were not classified as TB, only considering tumor cells infiltrating the stroma with minimal extracellular mucin. Contrarily, PDCs were evaluated within mucin lakes, as proposed by Barresi et al. (32).

CK19 immunohistochemistry. Immunohistochemistry (IHC) stainings were performed to all primary CRC using the CK19 antibody (A53-B/A2, 760–4,281, Roche). IHC was performed on consecutive sections of the tumor where TB and PDC were assessed with 2 purposes: first, to enable the comparison of TB and PDC counts with H&E and IHC and second, to assess the positivity of the tumor for CK19 and ensure reliable negative molecular CK19 mRNA OSNA results. Positive immunostaining was defined as ≥10% membranous staining with or without cytoplasm staining of tumor cells.

Statistical analysis

Clinicopathological characteristics collected for each patient were reviewed, and cases with incomplete data were excluded. The statistical analysis was performed in accordance with the study protocol. The primary endpoint was the concordance between TTL with TB, PDC, and patient outcome. Secondary endpoints were the correlations of TTL with TB and PDC assessed by H&E or IHC and the correlation of TB and PDCs with other clinicopathological factors. The χ^2 test, Fisher exact test, and Spearman correlation coefficient were used for testing the association between categorical or numerical variables, respectively. The Mann-Whitney and Kruskal-Wallis tests were used to compare group distributions. A $P < 0.05$ was considered statistically significant. A Kaplan–Meier survival analysis with logrank tests was performed for the variables TB, PDC, and TTL. A multivariate logistic regression analysis was used to predict the TTL outcome (cutoff point = 6,000 copies/μL) including the following variables: TB, LVI, PNI, pT, tumor size, gross tumor configuration, histological grade, and peripheral growth pattern. All analyses were performed using SPSS v25 statistician package (IMB, Chicago, IL).

RESULTS

Patients and pathological data

The study included 429 stages I–III CRC with LN analysis by OSNA and H&E. Forty-nine patients with incomplete data and 37 *in situ* carcinomas and one case with synchronous carcinomas were excluded. Finally, the study was performed on 342 patients recruited in 3 hospitals between June 2012 and July 2018. Seventy-three patients (21.3%) had LN metastasis on H&E examination, of which only 14 cases (19.18%) were diagnosed as cN positive by preoperative computed tomography. The patient's follow-up was between 40 days and 6.5 years. Demographic, clinical, and pathological characteristics are summarized in Table 1.

LN dissection

A total of 6,785 LNs were dissected, with a mean of 19 LNs per case. Of those, 5,931 LNs (87.5%) were freshly dissected (17 LNs per case) and analyzed by both H&E and OSNA and 854 (12.5%) LNs were procured on a second look after formalin fixation and analyzed by H&E. A weak positive correlation between the number of fresh LNs sampled and the amount of TTL was observed ($r = 0.12$, $P = 0.027$), showing no differences between the amount of LNs between OSNA negative patients (16.7 LNs/case) and OSNA positive cases (18.4 LNs/case) ($P = 0.089$).

TB and PDC assessment by H&E and IHC

All primary tumors included in the study were positive for CK19 IHC. TB distribution assessed on H&E was: 204 (59.6%) Bd1, 79 (23.1%) Bd2, and 59 (17.3%) Bd3. TB distribution performed on IHC was as follows: 154 (45%) Bd1, 85 (25%) Bd2, and 103 (30%) Bd3. Regarding PDCs, assessment with H&E resulted in 207 (60.5%) G1, 94 (27.5%) G2, and 41 (12%) G3, whereas assessment by IHC resulted in 180 (53%) G1, 111 (32%) G2, and 51 (15%) G3. Cases were similarly distributed with both methods of evaluation, with a strong correlation between H&E and CK19 IHC, being higher for PDC assessment than for TB ($r = 0.879$, $P < 0.001$ for PDC and $r = 0.76$, $P < 0.001$ for TB). When the evaluation was performed with IHC, more TB cases were classified as Bd3 and to a much lesser extent as G3-PDC (Table 2).

TTL positivity correlates with TB and PDC

OSNA positivity was found in 38.3% of the cases (131/342) with a mean TTL of 36,662 copies/μL among positive cases. We observed a positive correlation between the amount of tumor burden present in the LNs (TTL) assessed by the OSNA assay, with both TB ($r = 0.249$ by IHC; $r = 0.243$ by H&E) and PDC ($r = 0.266$ by IHC; $r = 0.257$ by H&E) ($P < 0.001$). In mucinous carcinomas ($n = 34$), we observed a trend between PDC grades within mucin pools and TTL, although it was not statistically significant (TTL in G1 = 127 copies/μL; G2 = 921 copies/μL; G3 = 10,714 copies/μL; $P = 0.26$).

The mean TTL was similar for low and intermediate TB (Bd1: 3,292 copies/μL and Bd2: 18,002 copies/μL), with no significant differences between both groups ($P = 0.154$). The mean TTL of high-Bd3 TB was 45,331 copies/μL, with significant differences with Bd1 and Bd2. Similarly, there were no significant differences when comparing the mean TTL of PDC G1, with 4,962 copies/μL and G2, with 13,146 copies/μL ($P = 0.068$), although PDC G3 had 61,108 copies/μL, with significant differences between low and intermediate grades. Thus, we grouped low and intermediated grades of TB and PDC into one category, obtaining 2 groups with significant differences for both TB and PDC ($P < 0.001$) as well (Figure 1).

Correlation of TB, PDCs, and TTL with other histopathologic features

TB and PDC were highly correlated among them when the evaluation was performed with either IHC and H&E, being higher for IHC ($r = 0.69$; $P < 0.001$ by H&E and $r = 0.773$; $P < 0.001$ by IHC). TB and PDCs were both significantly associated with each other ($P < 0.001$) and with LVI, PNI, EMVI, infiltrative type of invasion at the invasive front, ulcerative gross configuration, pT, pN, and stage ($P < 0.001$) Regarding the WHO grade, TB did not show a significant association ($P = 0.054$), whereas PDCs were associated with grade ($P < 0.001$). We did not find any association for TB and PDC with tumor location, age, sex, nor size of the tumor (Table 3). A similar analysis was conducted concerning TTL, showing that it was also associated to a higher tumor grade, infiltrative type of tumor invasion, LVI, PNI, EMVI, pT, pN, and stage ($P < 0.001$), whereas it was not related to sex, age, tumor location, size of the tumor, nor macroscopic configuration.

Table 2. Correlation between TB and PDCs grades assessed by H&E and CK19 IHC

CK19 IHC	Bd1 (n = 154, 45%)	Bd2 (n = 85, 25%)	Bd3 (n = 103, 30%)	Rho spearman	*P* value
H&E staining					
Bd1 (n = 204, 59.6%)	148	43	13	0.76	0.001
Bd2 (n = 79, 23.1%)	6	40	33		
Bd3 (n = 59, 17.3%)	0	2	57		
CK19 IHC	**PDC G1 (n = 180, 52.6%)**	**PDC G2 (n = 111, 32.5%)**	**PDC G3 (n = 51, 14.9%)**	**Rho spearman**	***P* value**
H&E staining					
PDC G1 (n = 207, 60.5%)	179	28	0	0.879	0.001
PDC G2 (n = 94, 27.5%)	1	81	12		
PDC G3 (n = 41, 12%)	0	2	39		

Bd1, Bd1, Bd2, and Bd3, budding 1, 2, and 3; CK19, cytokeratin 19; H&E, hematoxylin and eosin; IHC, immunohistochemistry; PDC, poorly differentiated cluster; PDC G1, PDC G2, and PDC G3, grades 1, 2, and 3; PNI, perineural invasion; TB, tumor budding.

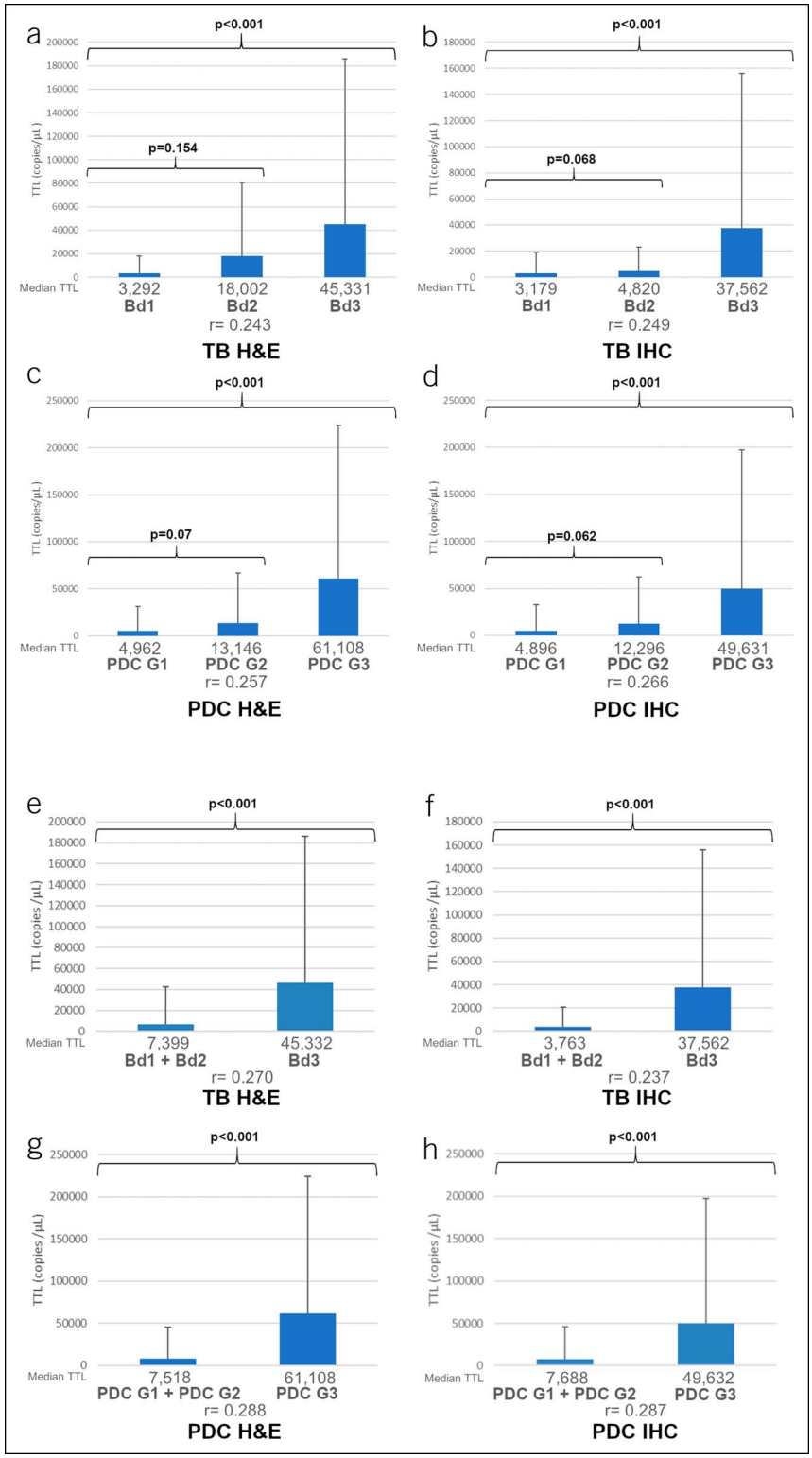

Figure 1. Correlation between TB and PDCs with TTL: (**a**) Correlation between TTL with Bd1, Bd2, and Bd3 TB by H&E and; (**b**) by IHC; (**c**) correlation between TTL with PDC G1, G2, and G3 by H&E; (**d**) and by IHC. (**e**) Correlation between TTL with 2-tier TB grading by H&E and (**f**) IHC. Correlation of TTL with 2-tier PDC (**g**) by H&E; (**h**) and IHC. H&E, haematoxylin and eosin; IHC, immunohistochemistry; PDC, poorly differentiated cluster; TB, tumor budding; TTL, total tumor load.

Regarding CRC stages, most stage I cases had low-Bd1 TB (76.3%, n = 190), low-grade G1 PDC (77.7%, n = 108), and lower TTL, with a mean of 294 copies/µL compared with stage II: 3,358 copies/µL and stage III cases: 58,781 copies/µL ($P < 0.001$), being also significant when comparing stage II and stage III ($P < 0.001$). Similarly, the TTL progressively increased with pT and pN stages,

Table 3. Correlation of TB and PDCs with histopathological features

	TB H&E				PDC H&E			
	Bd1	Bd2	Bd3	*P* value	PDC G1	PDC G2	PDC G3	*P* value
Gender				0.4491				0.2201
Male	127 (61.3%)	43 (20.8%)	37 (17.9%)		132 (63.8%)	50 (24.2%)	25 (12.1%)	
Female	77 (57%)	36 (26.7%)	22 (16.3%)		75 (55.6%)	44 (32.6%)	16 (11.9%)	
Age (yr, mean ± SD)	67.9 ± 10.9	69.5 ± 11.5	72 ± 13	0.182	68.4 ± 11.1	69.1 ± 11.9	71.4 ± 12.5	0.1962
Tumor location				0.3161				0.2691
Right colon	68 (59.1%)	28 (24.3%)	19 (16.5%)		63 (54.8%)	38 (33%)	14 (12.2%)	
Transverse colon	25 (55.6%)	8 (17.8%)	12 (26.7%)		23 (51.1%)	17 (37.8%)	5 (11.1%)	
Left colon	82 (63.1%)	25 (19.2%)	24 (17.7%)		90 (69.2%)	25 (19.2%)	15 (11.5%)	
Rectosigmoid colon	20 (55.6%)	13 (36.1%)	3 (8.3%)		22 (61.1%)	10 (27.8%)	4 (11.1%)	
Rectum	9 (56.3%)	5 (31.3%)	2 (12.5%)		9 (56.3%)	4 (25%)	3 (18.8%)	
Tumor size (cm. Mean ± SD)	3.3 ± 2	3.4 ± 1.8	3.6 ± 1.7	0.4332	3.3 ± 2	3.5 ± 1.7	3.7 ± 1.7	0.1232
Macroscopic configuration				**0.0001**				**0.002**
Polypoid	126 (72.4%)	31 (17.8%)	17 (9.8%)		120 (69%)	41 (23.5%)	13 (7.5%)	
Ulcerated	78 (46.4%)	48 (28.6%)	42 (25%)		87 (51.8%)	53 (31.5%)	28 (16.7%)	
Grade				0.054				**0.0001**
Low	122 (64.6%)	42 (22.2%)	25 (13.2%)		133 (70.4%)	39 (20.6%)	17 (9%)	
High	82 (53.6%)	37 (24.2%)	34 (22.2%)		74 (48.4%)	55 (35.9%)	24 (15.7%)	
Invasive front				**0.0001**				**0.0001**
Pushing border	139 (84.2%)	12 (11.5%)	7 (4.2%)		130 (78.8%)	30 (18.2%)	5 (3%)	
Infiltrative margin	33 (32.7%)	30 (29.7%)	38 (37.6%)		38 (37.6%)	35 (34.7%)	28 (27.7%)	
Mixed	15 (45.5%)	12 (36.4%)	6 (18.2%)		17 (51.5%)	11 (33.3%)	5 (15.2%)	
PNI				**0.0001**				**0.0001**
Absent	124 (65.1%)	69 (23.2%)	35 (11.7%)		193 (64.8%)	81 (27.2%)	24 (8.1%)	
Present	10 (22.7%)	10 (22.7%)	24 (54.5%)		14 (31.8%)	13 (29.5%)	17 (38.6%)	
LVI				**0.0001**				**0.0001**
Absent	168 (68.3%)	57 (23.2%)	21 (8.5%)		173 (70.3%)	61 (24.8%)	12 (4.9%)	
Present	36 (37.5%)	22 (22.9%)	38 (39.6%)		34 (35.4%)	33 (34.4%)	29 (30.2%)	
EMVI				**0.0001**				**0.0001**
Absent	200 (61.7%)	74 (22.8%)	50 (15.4%)		204 (63%)	87 (26.9%)	33 (10.2%)	
Present	4 (22.2%)	5 (27.8%)	9 (50%)		3 (16.7%)	7 (38.9%)	8 (44.4%)	
pT stage				**0.0001**				**0.0001**
pT1	59 (89.4%)	4 (6.1%)	3 (4.5%)		57 (86.4%)	7 (10.6%)	2 (3%)	
pT2	54 (63.5%)	23 (27.1%)	8 (9.4%)		57 (67.1%)	23 (27.1%)	5 (5.9%)	
pT3	76 (51.4%)	44 (29.7%)	28 (18.9%)		82 (55.4%)	47 (31.8%)	19 (12.8%)	
pT4a	15 (35.7%)	8 (19%)	19 (45.2%)		11 (26.2%)	17 (40.5%)	14 (33.3%)	
pT4b	0 (0%)	0 (0%)	1 (100%)		0 (0%)	0 (0%)	1 (100%)	
pN stage				**0.0001**				**0.0001**
pN0	180 (66.9%)	61 (22.7%)	28 (10.4%)		183 (68%)	69 (25.7%)	17 (6.3%)	
pN1a	15 (46.9%)	5 (15.6%)	12 (37.5%)		14 (43.8%)	10 (31.3%)	8 (25%)	
pN1b	5 (21.7%)	8 (34.8%)	10 (43.5%)		6 (26.1%)	7 (30.4%)	10 (43.5%)	
pN2a	1 (14.3%)	2 (28.6%)	4 (57.1%)		0 (0%)	4 (57.1%)	3 (42.9%)	
pN2b	2 (28.6%)	1 (14.3%)	4 (57.1%)		2 (28.6%)	3 (42.9%)	2 (28.6%)	

Table 3. (*continued*)

	TB H&E				PDC H&E			
	Bd1	Bd2	Bd3	*P* value	PDC G1	PDC G2	PDC G3	*P* value
pN1c	1 (50%)	0 (0%)	1 (50%)		1 (50%)	1 (50%)	0 (0%)	
Stage				**0.0001**				**0.0001**
I	106 (76.3%)	22 (15.8%)	11 (7.9%)		108 (77.7%)	25 (18%)	6 (4.3%)	
II	74 (56.9%)	39 (30%)	17 (13.1%)		75 (57.7%)	44 (33.8%)	11 (8.5%)	
III	24 (32.9%)	18 (24.6%)	31 (42.5%)		24 (32.9%)	25 (34.2%)	24 (32.9%)	
TTL (Copies/μL. Mean ± SD)	3,292 ± 14,543	18,002 ± 62,747	45,331 ± 140,709	**0.0002**	4,962 ± 26,527	13,146 ± 53,823	61,108 ± 162,682	**0.0002**

Bd1, Bd2, and Bd3, budding 1, 2, and 3; EMVI, extramural vascular invasion; H&E: hematoxylin and eosin; LVI, lymphovascular invasion; PDC, poorly differentiated cluster; PDC G1, PDC G2, and PDC G3, grades 1, 2, and 3; PNI, perineural invasion; TB, tumor budding; TTL, total tumor load. Numbers in bold highlight significant *P* values.

with 750 copies/μL in pT1, 2,622 copies/μL in pT2, 24,797 copies/μL in pT3, and 19,131 copies/μL in pT4. As for pN, a mean of 1,775 copies/μL was found in pN0, 49,413 copies/μL in pN1 and 95,000 copies/μL in pN2 cases ($P < 0.001$), with a trend but no statistical differences between pN1 and pN2 ($P = 0.27$). The logistic regression analysis showed the variables LVI ($P < 0.001$) and T stage ($P = 0.016$) as the only predictive factors of TTL ≥ 6,000 copies/μL.

Survival analysis

Kaplan-Meier survival analysis using the logrank test among all cases was performed, which showed that TB, PDC, and TTL were significantly associated with overall survival ($P = 0.002$; $P = 0.013$ and $P = 0.046$, respectively) (Figure 2) and DFS ($P < 0.001$). When the survival analysis was conducted in stage I and II patients, only PDC ($P = 0.026$), but not TB nor TTL, was associated with DFS (see Supplemental Figure, Supplemental Digital Content 1, http://links.lww.com/CTG/A511, which shows DFS curves in stage I and II cases).

DISCUSSION

Pathological H&E assessment is the gold standard for the evaluation of the LN status or pN staging. Nevertheless, its interpretation is rather subjective and it yields some false negative results, mainly because of tumor allocation bias within LNs

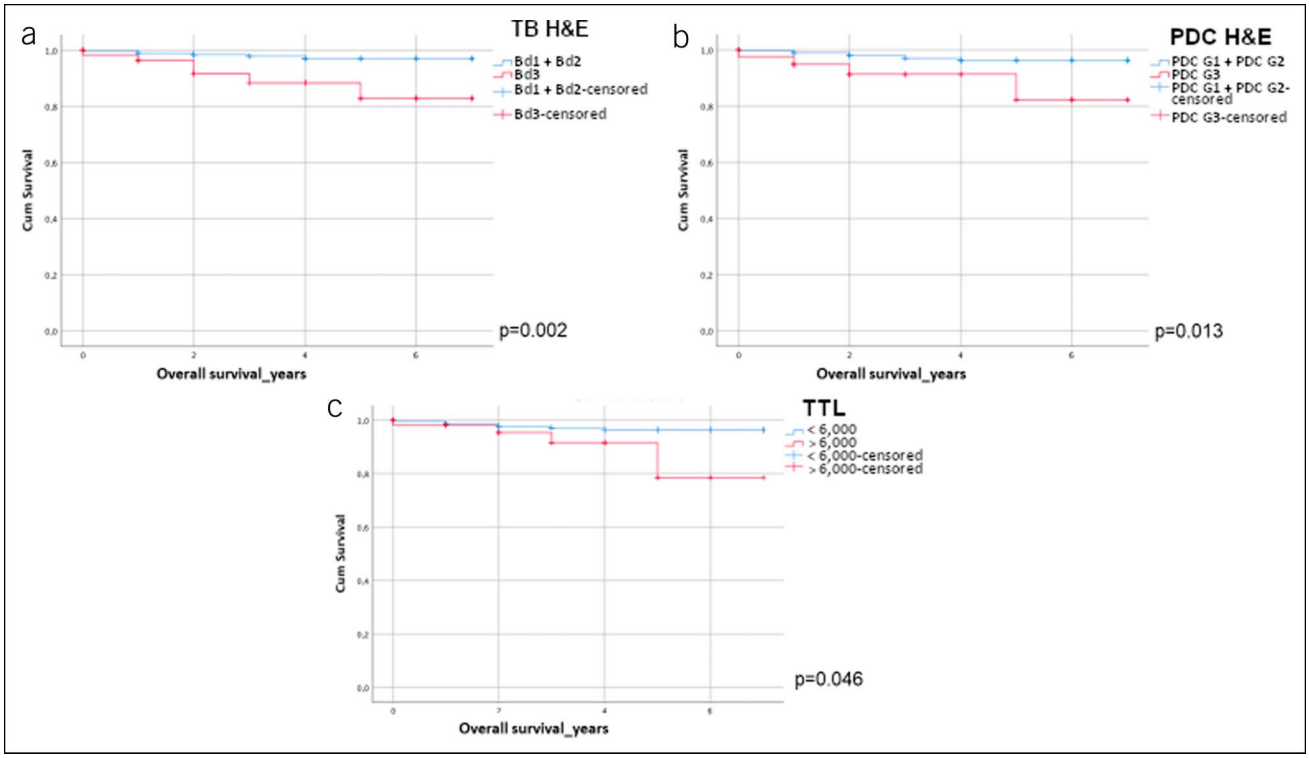

Figure 2. OS curves showing (**a**) worse OS for patients with Bd3 than for patients with Bd1 or Bd2, (**b**) worse OS for patients with PDC G3 than for patients with PDC G1 or PDC G2, (**c**) worse OS for patients with TTL ≥ 6,000 copies/μL. Cum survival, cumulative survival; H&E, hematoxylin and eosin; OS, overall survival; PDC, poorly differentiated cluster; TB, tumor budding; TTL, total tumor load.

(7,8,15). In fact, up to 15% of patients with histological stage II CRC recur within 5 years after curative-intended surgery (4,5). Moreover, there is evidence that molecular detection of LN micrometastases in stage II CRC is associated with a higher risk of recurrence, worse prognosis, and lower survival rates (7,9,16). We aimed at demonstrating the importance of the incorporation of LN molecular staging in the diagnosis and LN staging of CRC by demonstrating its correlation with patient outcome, TB, and PDC as factors related to LN metastasis and prognosis.

The OSNA assay is a molecular method for the detection of tumor CK19 mRNA within the LNs. It is quantitative and objective, providing data on the amount of tumor burden present in the LNs. Its sensitivity and specificity in CRC have been reported to be 86.2% and 96.5%, respectively, with a concordance rate between H&E slides and the OSNA results of 96.5% (11). It is a feasible method which makes it possible to reduce interobserver variability and being used in routine diagnosis for molecular LN staging. We believe that it could be an alternative tool to H&E pN staging in the setting of early-stage CRC (8,11,15,33). We and other authors have previously reported the correlation of OSNA positiveness with clinicopathologic risk factors such as male sex, pT, pN, high histological grade, mucinous/signet ring histological types, and tumor size (8,9,11).

We analyzed 5,931 LNs from 342 stage I–III CRC using the OSNA assay. We have shown that a TTL $\geq$ 6,000 copies/μL is associated to worse DFS and overall survival, and it is also correlated with TB and PDCs, being both factors related to outcome in CRC, implying that TTL could be used as an additional risk factor in patients with CRC. Besides, the correlation of TTL with other clinical and pathological CRC risk factors is further demonstrated here with additional factors such as stage and LVI. Nevertheless, when the survival analysis was conducted among stages I and II, only PDC, but not TB nor TTL, was associated to DFS, which could be explained because of the low number of recurrent early stages in our cohort (15/269) and to the low frequency of recurrences among stage I CRC. Therefore, stage II patients with TTL $\geq$ 6,000 copies/μL could be individually assessed for therapeutic decision with all other risk factors taken together. TB and PDC are both manifestations of the epithelial-to-mesenchymal transition process, which have been widely reported to have a strong relationship with LN metastasis and are prognostic factors in CRC (17,21–26,34–38). LN involvement is a crucial prognostic factor in patients with early-stage CRC, and the most significant predictor of 5-year cancer specific and DFS (7,15,16,39); thus, it should be a priority to know the amount of tumor present in the LN compartment. In fact, the TTL quantifies the amount of tumor cells or tumor burden present in the LNs of a given patient, which has been proven in breast cancer to be a better prognostic predictor, and reflects better the aggressiveness of the tumor than the number of positive axillary LNs (40–42).

We evaluated TB and PDC with both H&E and IHC. We observed that more cases were classified as high-Bd3 TB when TB analysis was performed with IHC, suggesting that IHC could be more useful for risk stratification. Although the international consortium on TB recommends evaluating TB on H&E stained slides, and it may seem obvious that the use of IHC for TB assessment reduces subjectivity and enhances quantification, a recent study has shown that IHC is not especially helpful in the assessment of individual TB and also facilitates the visualization of TB mimickers, which may impact on the budding count (43,44).

Regarding LN tumor burden, we found similar TTL values in cases with Bd1 or Bd2 TB, and there were no differences among TTL values for PDC G1 and G2, suggesting that intermediate TB and PDC G2 may behave as low grade with respect to the amount of tumor present in the LNs. Therefore, in predicting the risk of LN metastasis related to the grade of both TB and PDC, a dichotomous system of evaluation (low grade vs high grade) might be more accurate in the clinical setting of early-stage CRC, than using a three-tier system. This observation endorses other authors' observations that have also suggested to better stratify patients' risk, by combining TB and PDC grades into a dual grading system (high-grade: Bd3+G2/G3, Bd2+G3; low-grade: other combinations) (22,45). Regarding stage, our results are encouraging because the TTL shows differences between stage II and III, which reflects the clinical implication of its value regarding CRC management. Taken together, the TTL could be used as an alternative method to H&E pN staging to better stage patients because it is able to identify real stage II or III patients; thus, selecting those who are candidates for adjuvant therapy.

In agreement with previous reports, we did not find any correlation between WHO grade and TB, whereas both PDCs and TTL showed an association with the WHO grade. In fact, it has been shown that PDCs is a better prognostic factor, having a higher reproducibility and correlation with patient outcome than the conventional WHO grading system (27,28,31,35). A possible explanation is the fact that the WHO grade is based on the degree of tumor differentiation and the proportion of gland formation by the tumor, which is a method with low interobserver concordance (27,28,31,32). The evidence that the PDC grading system successfully stratifies patients with CRC by survival outcome may generate some doubts on the value of the WHO grading system in this type of tumor. Our results suggest that TB and PDCs may reflect different features of the tumor; although TB might be more closely related to LN metastasis, PDCs may better reflect the grade and aggressiveness of the tumor.

To our knowledge, this is the largest OSNA study on CRC, and our results are aligned with those observed in previous publications. One limitation of all OSNA studies performed in CRC, including ours, is that the analysis has been performed using only a part of the LNs while using the rest of the LN tissue for conventional histological analysis and pN staging. Despite that, our results are very promising and have demonstrated good correlations with many CRC risk factors and patient outcome as a new prognostic value. As it is performed in breast cancer sentinel LNs, complete molecular LN analysis should be performed in CRC to be able to establish more accurate TTL values.

To conclude, the implementation of more sensitive molecular methods of LN staging makes it possible to better detect and quantify the amount of tumor burden present in regional LNs. The combination of the TTL as a new prognostic factor, together with other clinicopathologic risk factors such as TB and PDC, could help to better stratify and manage patients with early-stage CRC at risk of recurrence.

CONFLICTS OF INTEREST

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Potential competing interests: None to report.

Study Highlights

WHAT IS KNOWN

- ✓ Molecular lymph node (LN) staging in colorectal cancer (CRC) is more sensitive than conventional pathology pN staging.
- ✓ Tumor budding (TB) and poorly differentiated clusters (PDCs) are prognostic factors in CRC.
- ✓ LN tumor burden has proven to be risk factor in CRC.

WHAT IS NEW HERE

- ✓ LN tumor burden is correlated with TB and PDCs and is related to prognosis.

TRANSLATIONAL IMPACT

- ✓ The implementation of molecular LN staging will allow to use LN tumor burden as a new prognostic factor, which would improve CRC patients' staging and management.

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