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ORIGINAL ARTICLE

Analysis of tumor microenvironmental features to refine prognosis by T, N risk group in patients with stage III colon cancer (NCCTG N0147) (Alliance)

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Background: Tumor-infiltrating lymphocytes (TILs), tumor budding, and micropapillary architecture may influence tumor growth and metastatic potential, thereby enhancing prognostic stratification. We analyzed these features and their relative contribution to overall outcome and in low (T1—3 N1) and high (T4 and/or N2) risk groups that are used to inform the duration of adjuvant chemotherapy in patients with resected stage III colon cancers.

Patients and methods: Among 1532 patients treated in a phase III adjuvant trial of FOLFOX-based therapy, intraepithelial TIL densities, tumor budding, and micropapillary features were analyzed and quantified in routine histopathological sections with light microscopy. Optimal cut-points were determined in association with disease-free survival (DFS) in training and validation sets. Associations or relative contributions of individual features or combined variables with DFS were determined using multivariable Cox regression models.

Results: TILs, tumor budding, and micropapillary features were shown to differ significantly by T, N risk groups and by mismatch repair (MMR) status. Low TILs, high budding, and their combined variable [hazard ratio = 2.07 (95% CI, 1.50% to 2.88%); $P_{adj} < 0.0001$], but not micropapillary features, were each significantly associated with poorer DFS in a training data set and confirmed in a validation set. TILs were prognostic in proficient mismatch repair (pMMR) and deficient mismatch repair (dMMR) tumors; budding was prognostic only in pMMR tumors. The percentage relative contribution of budding/TILs to DFS was second only to nodal status overall, was second (24.4%) after *KRAS* in low-risk patients, and was the most important contributor (45.4%) in high-risk patients.

Conclusions: TIL density and tumor budding were each validated as significant prognostic variables and their combined variable provided robust prognostic stratification by T, N risk groups, being the strongest predictor of DFS among high-risk stage III patients.

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Key words: colon cancer, prognosis, risk group, tumor budding, tumor-infiltrating lymphocytes

INTRODUCTION

In patients with resected colon cancer, postoperative adjuvant chemotherapy has been shown to significantly reduce tumor relapse, yet nearly 30% of stage III patients will have recurrence and most of these will die of their disease.[1] Given the significant heterogeneity in prognosis that is observed among stage III patients, analysis of the tumor microenvironment (TME) may further refine patient prognosis and enable personalization of adjuvant chemotherapy. Recent evidence underscores the importance of features of the TME in determining metastatic potential.[2] Tumor-infiltrating lymphocytes (TILs) reflect the host anti-tumor immune response and their density and localization was shown to be prognostic in patients with colorectal cancer (CRC).[3,4] TILs are enriched in tumors with deficient DNA mismatch repair (dMMR) in response to hypermutation and abundant mutation-specific neopeptides.[5] Tumor budding, defined as single or a cluster of tumor cells (four or fewer) at the tumor's invasive margin (IM),[6] has been linked to epithelial—mesenchymal transition (EMT) and is intimately associated with stromal fibroblasts.[7] Tumor budding predicted lymph node metastasis in pT1 CRC and was associated with survival in patients with stage II CRC.[6] Therefore, tumor budding may represent a highly invasive subset of tumor cells, especially if they have the ability to evade the host immune response.[8] Furthermore, the interaction of tumor budding with the intratumoral immune response, indicated by TILs, may be a critical factor for the tumor's potential to metastasize.

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Micropapillary architecture is defined as small clusters (five or more) of malignant epithelial cells with reversed polarity, abundant eosinophilic cytoplasm, and retraction artifact creating lacunae-like spaces around the micropapillae.[9] Micropapillary features may represent a continuum of tumor budding,[10] have been linked with EMT, and are associated with an increased frequency of mutations in *KRAS* and $BRAF^{V600E}$ and a poorer prognosis.[11,12] To date, however, studies of the association of these TME features with patient outcomes have typically been limited to patient case series of pooled tumor stages with variable treatment and follow-up.

The International Duration Evaluation of Adjuvant Therapy (IDEA) study was designed to determine whether 3 months of adjuvant fluoropyrimidine plus oxaliplatin is non-inferior to the standard 6 months in relation to 3-year disease-free survival (DFS) in patients with resected stage III colon cancer.[13] In a post hoc analysis whereby patients were categorized into low-risk (T1−3 N1) and high-risk (T4 and/or N2) groups,[14] 3 months of therapy was non-inferior for low-risk patients but not high-risk patients where 6 months of chemotherapy is recommended.[13] We hypothesized that evaluation of TILs, tumor budding, and micropapillary features overall and within T and N risk groups may allow for a more precise and accurate prognostic stratification in patients with stage III colon cancer. Such findings may have implications for the recommendation of duration of adjuvant chemotherapy. Our study population consisted of patients with resected stage III colon cancer who were participants in a phase III trial of FOLFOX-based adjuvant chemotherapy [North Central Cancer Treatment Group (NCCTG) N0147].

PATIENTS AND METHODS

Study population

Patients with surgically resected stage III colon adenocarcinomas ($N = 1532$) were participants in a phase III clinical trial of adjuvant mFOLFOX6 chemotherapy ± cetuximab (NCCTG N0147).[1] Since the addition of cetuximab was not associated with a statistically significant difference in the primary endpoint of DFS, data from both treatment arms were pooled for translational studies. Median patient follow-up on the clinical trial was 83 months. The N0147 trial was approved by the Mayo Clinic Institutional Review Board (IRB) and conducted by the NCCTG (now part of Alliance for Clinical Trials in Oncology). Each participant signed an IRB-approved, protocol-specific informed consent document. Data quality was ensured by review of the Alliance Statistics and Data Center.

Histologic examination of TILs, tumor budding, and micropapillary features

A representative hematoxylin- and eosin-stained tumor section from each patient was reviewed for TIL density, tumor budding, and micropapillary features. The tumor was scanned at low power to identify the areas with the most intraepithelial TILs, and once identified, five consecutive ×40 fields were counted and the mean TIL per high power field (HPF) was calculated by dividing the total number of TILs by five. The number of tumor buds was counted in one hotspot (in a field measuring 0.785 mm^2) at the invasive margin, as proposed in a consensus statement published in 2016.[6] To ensure accurate discrimination from tumor budding, only clusters of more than four tumor cells surrounded by a thin lacunar space and separated by dense fibrous stroma[11] were considered micropapillary features whose proportion of the entire tumor was recorded as a percentage (%) in each case. Micropapillary features were dichotomized as present or absent based on optimized cut-offs determined for their association with DFS. All cases were scored independently by two gastrointestinal pathologists (HEL, TCS), who were blinded to all clinical and molecular data related to the study cohort. A test set of 100 total cases was scored together for TILs, tumor budding, and micropapillary features and each pathologist scored 50% of the remaining cases with periodic crossover review of 50 case-sets to monitor inter-observer reproducibility. In case of discrepant scoring, the section was re-examined and a consensus interpretation was achieved.

MMR status, *KRAS*, and *BRAF* mutation analysis

Biospecimens were prospectively collected and analyzed for DNA MMR status by analysis of MLH1, MSH2, and MSH6 proteins by immunohistochemistry (IHC).[15] Tumors with dMMR were defined as those with absent expression of one or more MMR proteins. Testing for the *BRAF* (c.1799T>A V600E) mutation in exon 15 and *KRAS* (c.35G>C G12A, c.35G>A G12D, c.34G>C G12R, c.34G>T G12C, c.34 G>A G12S, c.35 G>T G12V, and c.38 G>A G13D) mutation in exon 2, and codons 12 and 13 mutation status was performed as previously described.[16]

Statistical analysis

The study cohort was randomly and equally divided into training ($n = 766$) and validation ($n = 766$) sets. Optimized cut-points were identified based on the Contal and O'Quigley method[17] in the training set. Cut-offs for TIL densities, tumor budding, and micropapillary features were determined based on their association with DFS: low (≤3/HPF) versus high TIL (>3/HPF), low (≤3/0.785 mm^2) versus high budding (>3/0.785 mm^2), and present versus absent micropapillary features. DFS was defined as time from randomization to recurrence or death, whichever occurred first. The association of the dichotomized TIL density, tumor budding, and micropapillary features with clinicopathological covariates and molecular features was evaluated using chi-square or Wilcoxon rank-sum tests. Associations with the primary outcome of DFS and overall survival (OS) were evaluated by Kaplan−Meier methodology and Cox proportional hazards models. Models were adjusted for patient age, T/N stage, primary tumor sidedness, histologic grade, *KRAS*/*BRAF*, DNA MMR, performance status (PS), and treatment. Two-sided *P* values are reported; values <0.05

were considered statistically significant and were not adjusted for multiple comparisons. Analyses were performed using SAS version 9.4 (SAS Institute Inc., NC) using the study database. Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center. The relative contribution of each variable to DFS was calculated using χ^2 from Harrell's rms R package (version 3.2.3; http://biostat.mc.vanderbilt.edu/rms) based on multivariable Cox regression models.

RESULTS

Training set and validation set

Intratumoral features of budding/TILs and micropapillary architecture in representative colon carcinomas are shown in Figure 1A–C. Optimized cut-offs for each TME feature were determined in a randomly selected training set ($n = 766$) and then confirmed in an internal validation set ($n = 766$). Patients with stage III colon cancer whose tumors had low versus high TIL densities had significantly poorer DFS in the training set [adjusted hazard ratio (HR_{adj}) = 2.01; 95% CI, 1.41% to 2.85%; $P < 0.0001$] that was confirmed in the validation set ($HR_{adj} = 1.53$; 95% CI, 1.06% to 2.21%; $P = 0.0196$). High versus low tumor budding was significantly associated with poorer DFS in the training set ($HR_{adj} = 1.56$; 95% CI, 1.19% to 2.05%; $P = 0.0002$) and then confirmed in the validation set ($HR_{adj} = 1.24$; 95% CI, 0.94 to 1.64; $P = 0.0307$). While tumor micropapillary features (present versus absent) were prognostic in the training set ($HR_{adj} = 1.43$; 95% CI, 1.08% to 1.91%; $P = 0.0158$), results were not confirmed in the validation set ($HR_{adj} = 0.94$; 95% CI, 0.68% to 1.31%; $P = 0.7232$).

Association of TIL density, tumor budding, and micropapillary features with patient characteristics

Patient clinicopathologic characteristics and their associations with TILs, tumor budding, and micropapillary features are shown in Table 1. Among 1532 patient tumors, 1132 (74%) had low TILs (≤3/HPF) and 400 (26%) had high TILs (>3/HPF). Compared with those with high TILs, patients with low TILs were significantly more likely to have a higher T ($P = 0.0003$) and N stage ($P = 0.0001$), poor or undifferentiated histologic grade ($P = 0.0001$), left-sided primary location ($P < 0.0001$), non-mutated $BRAF^{V600E}$ ($P < 0.0001$), and pMMR ($P < 0.0001$) (Table 1). Among all patients, 699 (46%) tumors had low budding and 833 (54%) had high budding. Patients with high versus low budding were significantly more likely to have a higher T ($P = 0.0002$) and N stage ($P < 0.0001$) and pMMR status ($P < 0.0001$). Micropapillary features were present in 326 (21%) tumors and patients whose tumors had this feature were more likely to have higher T ($P = 0.0329$) and N stages ($P = 0.0010$) and pMMR ($P < 0.0001$) compared with tumors lacking this feature.

Within the study population, 10.1% of tumors showed dMMR. Low TIL density was found in 37% (56/153) of tumors with dMMR versus 78% (1060/1357) with pMMR. High budding was found in 37% (56/153) of tumors with dMMR versus 57% (768/1357) with pMMR. Micropapillary features were present in 14% (21/153) of tumors with dMMR versus 22% (303/1377) with pMMR. *KRAS* status and treatment arm were not associated with TIL density, tumor budding, or micropapillary features (Table 1). Mutant *BRAF* was associated with high TILs, which was explained by enrichment of $BRAF^{V600E}$ in dMMR tumors; $BRAF^{V600E}$ was not associated with tumor budding or micropapillary features (Table 1). Analysis of patient characteristics with the combined variable of budding/TILs revealed similar results as seen for TILs and budding individually with the exception that mutant *KRAS* was more frequently detected in tumors with high budding/low TILs ($P = 0.0499$).

Associations of TME features with prognosis in the overall cohort

In the overall study cohort, patients whose tumors had low versus high TILs had significantly poorer DFS ($HR_{adj} = 1.74$;

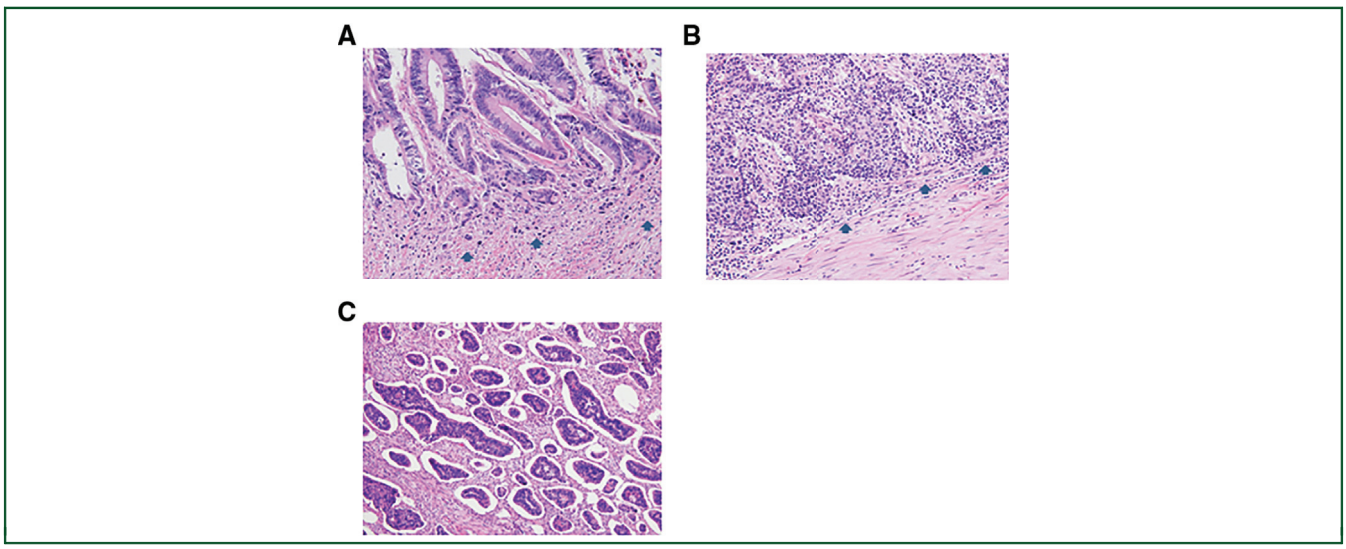

Figure 1. Representative colon adenocarcinomas with invasive margin showing (A) high budding/low TILs or (B) low budding/high TILs, per definitions provided in *Methods*. (C) Representative colon carcinoma with micropapillary architecture (see Methods). Images display hematoxylin- and eosin-stained tissue sections at ×200 magnification (A, B) and ×100 magnification (C).

Table 1. Patient characteristics by TIL density, tumor budding and micropapillary features

	Low TILs (*N* = 1132)	High TILs (*N* = 400)	*P* value	Low budding (*N* = 699)	High budding (*N* = 833)	*P* value	Micropap absent (*N* = 1205)	Micropap present (*N* = 326)	*P* value
Age (years)			0.0775[a]			0.6057[a]			0.3520[a]
Mean (SD)	58.0 (10.8)	58.8 (11.8)		58.0 (11.3)	58.4 (10.9)		58.3 (11.3)	57.9 (10.4)	
Median	58.0	60.0		59.0	59.0		59.0	58.0	
Range	(23.0–85.0)	(19.0–86.0)		(19.0–86.0)	(23.0–85.0)		(19.0–86.0)	(31.0–5.0)	
Sex			0.4937[b]			0.8362[b]			0.7201[b]
Female	535 (47.3%)	197 (49.3%)		336 (48.1%)	396 (47.5%)		579 (48.0%)	153 (46.9%)	
Male	597 (52.7%)	203 (50.8%)		363 (51.9%)	437 (52.5%)		626 (52.0%)	173 (53.1%)	
Performance status			0.0435[b]			0.8363[b]			0.3079[b]
0	843 (74.5%)	318 (79.5%)		528 (75.5%)	633 (76.0%)		906 (75.2%)	254 (77.9%)	
1/2	289 (25.5%)	82 (20.5%)		171 (24.5%)	200 (24.0%)		299 (24.8%)	72 (22.1%)	
T stage			0.0003[b]			0.0002[b]			0.0329[b]
T1 or T2	145 (12.8%)	81 (20.3%)		129 (18.5%)	97 (11.6%)		190 (15.8%)	36 (11.0%)	
T3 or T4	987 (87.2%)	319 (79.8%)		570 (81.5%)	736 (88.4%)		1015 (84.2%)	290 (89.0%)	
N stage			0.0001[b]			<0.0001[b]			0.0010[b]
N1 (1–3 nodes)	636 (56.2%)	270 (67.5%)		460 (65.8%)	446 (53.5%)		739 (61.3%)	167 (51.2%)	
N2 (≥4 nodes)	496 (43.8%)	130 (32.5%)		239 (34.2%)	387 (46.5%)		466 (38.7%)	159 (48.8%)	
Tumor site			<0.0001[b]			0.4489[b]			0.3057[b]
Right	534 (47.8%)	249 (62.9%)		349 (50.7%)	434 (52.6%)		624 (52.4%)	159 (49.2%)	
Left	584 (52.2%)	147 (37.1%)		340 (49.3%)	391 (47.4%)		566 (47.6%)	164 (50.8%)	
Histologic grade			0.0001[b]			0.6028[b]			0.1786[b]
Poor/undifferentiated	880 (77.7%)	272 (68.0%)		530 (75.8%)	622 (74.7%)		916 (76.0%)	236 (72.4%)	
Well/moderate	252 (22.3%)	128 (32.0%)		169 (24.2%)	211 (25.3%)		289 (24.0%)	90 (27.6%)	
KRAS			0.5117[b]			0.0749[b]			0.5306[b]
Mutant	402 (36.1%)	134 (34.3%)		228 (33.2%)	308 (37.7%)		427 (36.1%)	109 (34.2%)	
Wild type	711 (63.9%)	257 (65.7%)		458 (66.8%)	510 (62.3%)		757 (63.9%)	210 (65.8%)	
BRAF			<0.0001[b]			0.7374[b]			0.3299[b]
Mutant (V600E)	109 (10.0%)	89 (23.7%)		88 (13.2%)	110 (13.8%)		151(13.1%)	47 (15.3%)	
Wild type	976 (90.0%)	286 (76.3 %)		577 (86.8%)	685 (86.2%)		1000 (86.9%)	261 (84.7%)	
MMR			<0.0001[b]			<0.0001[b]			<0.0001[b]
dMMR	56 (5.0%)	97 (24.6%)		97 (14.1%)	56 (6.8%)		132 (11.1%)	21 (6.5%)	
pMMR	1060 (95.0%)	297 (75.4%)		589 (85.9%)	768 (93.2%)		1054 (88.9%)	303 (93.5%)	
Treatment arm			0.2043[b]			0.1358[b]			0.3558[b]
FOLFOX	615 (54.3%)	232 (58.0%)		372 (53.2%)	475 (57.0%)		674 (55.9%)	173 (53.1%)	
FOLFOX + Cetuximab	517 (45.7%)	168 (42.0%)		327 (46.8%)	358 (43.0%)		531 (44.1%)	153 (46.9%)	

dMMR, deficient mismatch repair; micropap, micropapillary features; pMMR, proficient mismatch repair; SD, standard deviation.

[a] Wilcoxon test.

[b] Chi-square test.

95% CI, 1.35% to 2.24%; $P < 0.0001$) (Figure 2A). Importantly, low versus high TILs were shown to prognostically stratify patients with dMMR ($HR_{adj} = 2.14$; 95% CI, 1.14% to 4.01%; $P = 0.0173$) and pMMR ($HR_{adj} = 1.65$; 95% CI, 1.25% to 2.16%; $P = 0.0002$) tumors. Patient tumors with high versus low tumor budding had significantly poorer DFS ($HR_{adj} = 1.37$; 95% CI, 1.13% to 1.67%; $P = 0.0011$) (Figure 2B). The presence versus absence of micropapillary

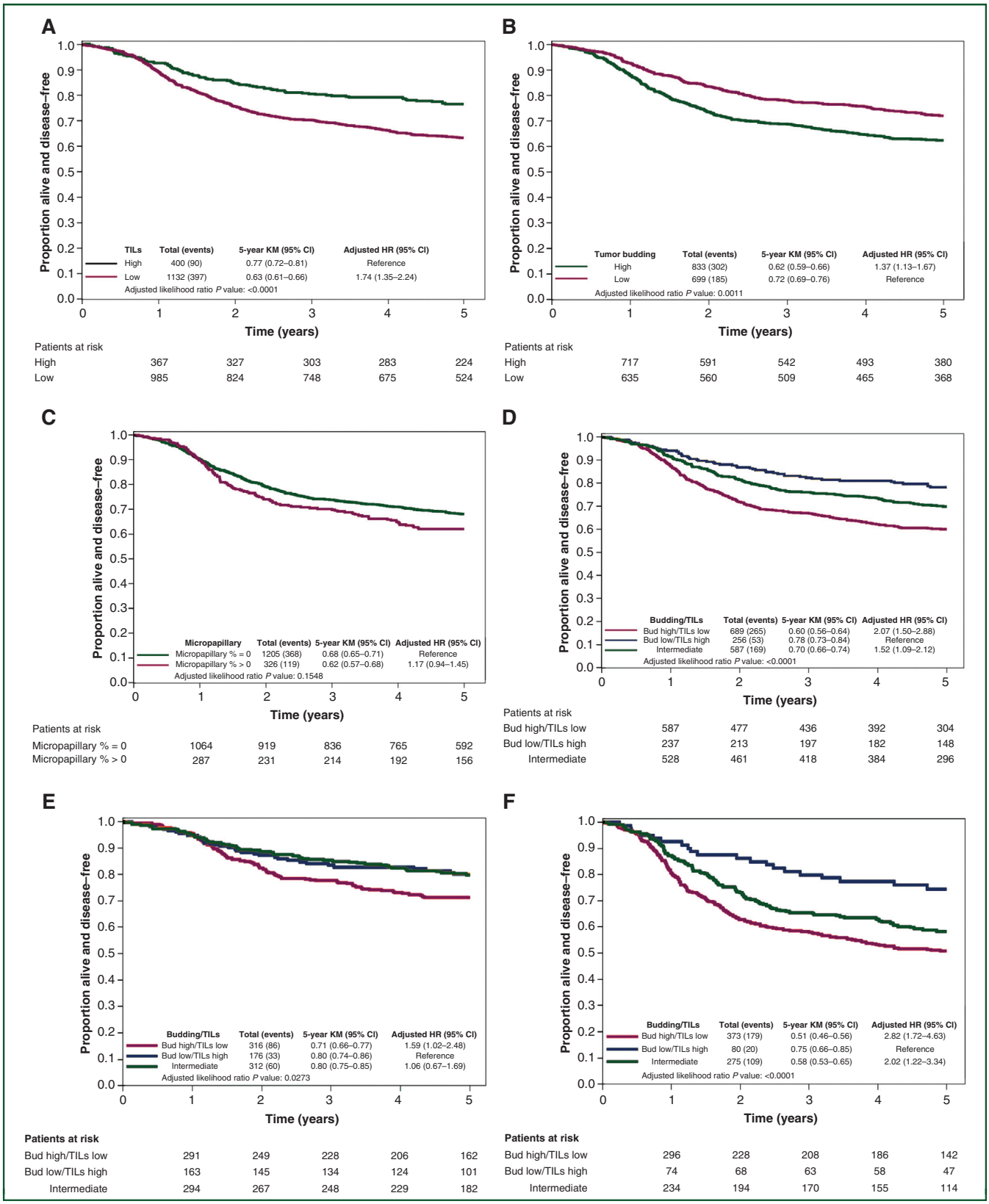

Figure 2. Kaplan–Meier plots of disease-free survival (DFS) in the overall cohort by (A) tumor-infiltrating lymphocytes (TILs), (B) tumor budding, and (C) micropapillary architecture. Also shown is (D), the combined variable of tumor budding/TILs, where the intermediate group includes high budding/high TILs and low budding/low TILs. The tumor budding/TILs combined variable was also analyzed in patients with (E) low-risk (T1–3 N1) and (F) high-risk (T4 and/or N2) stage III tumors.

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features was not significantly associated with DFS (HR_{adj} = 1.17; 95% CI, 0.94% to 1.45%; $P = 0.1548$) (Figure 2C).

Association of combined variable (tumor budding/TILs) with overall patient prognosis and by T and N stage risk groups

Because TILs and tumor budding were individually prognostic, we examined the association of their combined variable with patient DFS. When categorized into three subgroups of low budding/high TILs, intermediate (see legend for Figure 2D), and high budding/low TILs, we found that the combined variable was shown to significantly stratify patients for prognosis with 5-year DFS rates of 78%, 70% and 60%, respectively ($P < 0.0001$). These data for the combined variable were more robust for prediction of DFS compared with the individual variables (Figure 2D).

We then examined the combined variable in patients within low- (T1−3 N1) and high-risk (T4 and/or N2) groups as defined by the IDEA adjuvant study.[13] Compared with the low-risk group, tumor budding/TILs better stratified high-risk patients for DFS (Figure 2E and F). Among low-risk patients, 5-year DFS rates for low budding/high TILs, intermediate, and high budding/low TILs were 80%, 80%, and 71%, respectively ($P = 0.0273$) (Figure 2E); among high-risk patients, DFS rates were 75%, 58% and 51%, respectively ($P < 0.0001$) (Figure 2F). In both risk groups, patients with high-budding/low-TILs tumors had the poorest DFS (Figure 2E and F). Importantly, patients with 5-year DFS rates in low-risk tumors with high budding/low TILs were lower than rates in high-risk tumors with low budding/high TILs (71% versus 75%; Figure 2E and F).

Relative contribution of variables to predict DFS in the overall cohort and in T and N stage risk groups

In the overall study cohort, we analyzed the relative contribution (%) of each tumor variable to DFS in multivariable (MV) models. In the MV model with TILs, the top three contributors to DFS were N stage (44.3%), TILs (14.8%) and *KRAS* (13.8%) (Figure 3A). In the MV model with tumor budding, budding ranked fourth (8.3%) behind N stage (47.6%), T stage (14.3%) and *KRAS* (13.6%) (Figure 3B). Moreover, the contribution of the budding/TILs combined variable to DFS ranked second (17.7%) behind N stage (42.1%), and whose contribution also exceeded that of *KRAS* (13.1%) and T stage (11.7%) (Figure 3C).

We then examined the relative contribution of the tumor budding/TILs combined variable to DFS in patients with low- and high-risk T and N stage groups. In the low-risk group, the combined variable ranked second (24.4%) behind *KRAS* (45.5%) and ahead of treatment (7.2%) and MMR status (6.1%) (Figure 3D). In the high-risk group, the combined variable contributed the most to DFS (45.4%), followed by primary tumor site (13.0%), PS (12.0%) and MMR (10.4%) (Figure 3E). Furthermore, the contribution of the combined variable to DFS in the high-risk group was increased two-fold compared with the low-risk group.

DISCUSSION

Low intraepithelial TILs, high budding, and micropapillary features were each significantly associated with higher T stage, N stage, and pMMR but not with *KRAS* or $BRAF^{V600E}$ status. Furthermore, TIL density and tumor budding, but not micropapillary features, were each significantly associated with patient DFS after adjustment for covariates, and their prognostic significance was confirmed in an internal validation cohort. Our finding that low TIL densities were significantly and independently associated with poorer DFS in the overall cohort is consistent with previous studies of TILs in CRC, including a population-based cohort,[18] case series of pooled tumor stages and variable treatment,[4] and data for $CD3^+$ and $CD8^+$ T cell densities and Immunoscore®.[4,5] We made the novel observation that TIL densities

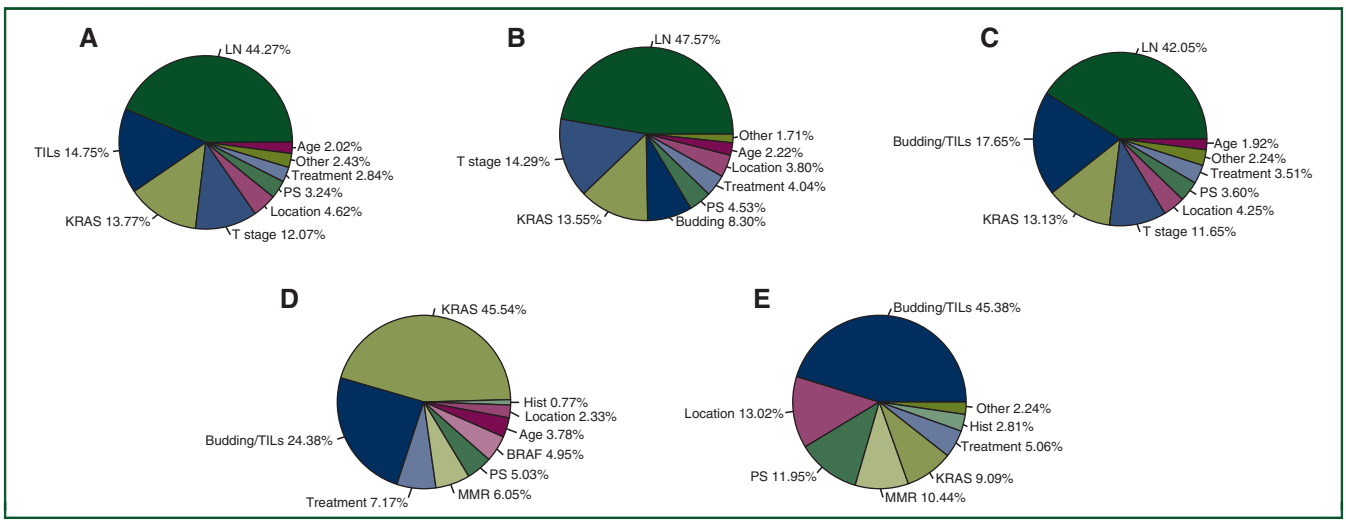

Figure 3. Relative contribution (percent) of (A) tumor-infiltrating lymphocytes (TILs), (B) tumor budding, and (C) the budding/TILs combined variable to disease-free survival (DFS) in the overall cohort of patients with stage III colon cancer. If the relative contribution was less than 1.9% for a given variable, results were pooled into an 'Other' category consisting of (A, B, C) MMR, *BRAF*, and histologic grade. Among low- and high-risk T, N groups, the relative contribution of the tumor budding/TILs combined variable to patient DFS is shown for (D) low-risk (T1−3 N1) or (E) high-risk (T4 and/or N2) patients. 'Other' includes age and *BRAF*.

were able to prognostically stratify both dMMR and pMMR tumors. In a previous report that included the N0147 cohort, no association of *BRAF* or *KRAS* mutations with prognosis was observed in patients with dMMR tumors.[19] These data demonstrate the ability of TILs to prognosticate dMMR tumors, and may have implications for predicting responsiveness to or outcome of treatment with immune checkpoint inhibitors.[20]

High versus low tumor budding was independently associated with shorter patient DFS in stage III tumors. Previous studies have evaluated budding in all stages of CRC,[21] including a meta-analysis where, although the definition of budding varied widely, it was significantly associated with lymph node metastasis, disease recurrence, and cancer-related death.[22] The International Tumor Budding Consensus Conference (ITBCC) scoring system was developed in stage I and II CRCs where it enabled risk stratification of pT1 and stage II CRCs.[6] However, only limited data exist for tumor budding in stage III tumors where ITBCC scoring was not evaluated.[23,24] This three-tiered scoring system (low budding: 0—4 buds; intermediate budding: 5—9 buds; and high budding: 10 or more buds)[6] differs from that used in our study where tumor budding was dichotomized as low ($\leq$3 tumor buds) versus high ($>$3 tumor buds) based upon optimized cut-offs determined for their association with DFS. Importantly, we found that our two-tiered versus ITBCC three-tiered scoring system provided better prognostic stratification in patients with stage III colon cancers as shown by a C-statistic MV model for DFS (data not shown). We emphasize that our results found in the training set were confirmed in a validation set.

Tumor budding has been linked to EMT and CRCs with high budding at the invasive margin contain tumor cells that overexpress EMT-related genes, such as *ZEB1*, *ZEB2*, *DES*, *TGFβ3*, and *VIM*, involved in cell migration and extracellular matrix remodeling.[25,26] We found that high tumor budding was significantly less common in dMMR versus pMMR cancers. An association between a higher number of tumor buds and the mesenchymal subtype (CMS4) of CRC has been documented using transcriptomic-based tumor profiling.[27,28] CMS4 shows upregulation of genes involved in EMT and signatures associated with the transforming growth factor β (TGF-β) signaling and matrix remodeling pathways.[27] In human colon cancers, CMS4 was associated with the poorest relapse-free and OS rates among the four identified CMS subtypes.[27] Further study is needed to determine whether high tumor budding could potentially be used to identify CRCs of the CMS4 subtype. Micropapillary features in CRCs have also been linked to EMT, an increased frequency of *p53* and *KRAS* mutations, lymph node metastasis, and a poor prognosis.[11,12] Nevertheless, our data in stage III tumors did not find an association of micropapillary features with *KRAS* or with DFS.

We found that the combined variable of budding/TILs showed a stronger association with patient prognosis than the individual variables. Overall, tumors with high budding/low TILs had the poorest DFS whereas the best DFS was seen for tumors with low budding/high TILs (5-year DFS rate of 60% versus 78%). In a multivariable analysis of the relative contribution of variables for the prediction of DFS, we found that the contribution of TILs to DFS exceeded that of tumor budding, and the ability of the budding/TILs combined variable to predict DFS was superior to either variable alone, with its contribution to DFS being second only to N stage. Among patients with low-risk tumors, the contribution of budding/TILs to DFS was second after *KRAS*, and was the most important contributor in high-risk patients with an almost doubling of its percent contribution (24.4% versus 45.4%). Interestingly, budding/TILs and *KRAS* contributed differentially to DFS by risk group, wherein *KRAS* was the major contributor to DFS among low-risk groups but ranked fifth among high-risk groups. We emphasize that the budding/TILs variable is a key predictor of patient survival in both low- and high-risk groups and can further refine prognostic stratification within these groups. These data have implications for the recommended duration of adjuvant chemotherapy in stage III tumors.

Strengths of our study include the large number of same-stage patients with uniform treatment in a clinical trial and with long-term follow-up. In addition to TILs, intratumoral $CD3^+$ and $CD8^+$ T-cells[5] or the Immunoscore® (which uses a complex proprietary algorithm[4]) has been shown to provide prognostic information in patients with colon cancer. However, analysis of TILs, as shown here, can be performed in routine histological tumor sections with concurrent determination of tumor budding. Our study is the first to report on the combined variable of budding/TILs that was shown to provide robust prognostic stratification and was the strongest variable among the high-risk T, N group. Limitations of our study include the lack of characterization of TIL composition within the TME. Since all patients in our study received adjuvant chemotherapy, we were not able to address the predictive utility of the studied biomarkers.

In conclusion, TILs and tumor budding were each validated as significant prognostic variables in stage III colon cancers, and their combination provided a more precise stratification for prognosis, especially among low and high T and N stage risk groups. These data suggest that budding/TILs can improve anatomic tumor staging and warrant evaluation in patients who received 3 versus 6 months of adjuvant chemotherapy in a clinical trial. Such data have the potential to inform recommendations for the duration of adjuvant chemotherapy in stage III disease. Since all histologic parameters were determined in routine histologic tissue sections, our results are directly applicable to routine clinical practice.

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DISCLOSURE

The authors have declared no conflicts of interest.

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