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Influence of Microsatellite Instability and *KRAS* and *BRAF* Mutations on Lymph Node Harvest in Stage I–III Colon Cancers

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Lymph node (LN) harvest is influenced by several factors, including tumor genetics. Microsatellite instability (MSI) is associated with improved node harvest, but the association to other genetic factors is largely unknown. Research methods included a prospective series of stage I–III colon cancer patients undergoing *ex vivo* sentinel-node sampling. The presence of MSI, *KRAS* mutations in codons 12 and 13, and *BRAF* V600E mutations was analyzed. Uni- and multivariate regression models for node sampling were adjusted for clinical, pathological and molecular features. Of 204 patients, 67% had an adequate harvest (≥12 nodes). Adequate harvest was highest in patients whose tumors exhibited MSI (79%; odds ratio (OR) 2.5, 95% confidence interval (CI) 1.2–4.9; $P = 0.007$) or were located in the proximal colon (73%; 2.8, 1.5–5.3; $P = 0.002$). In multiple linear regression, MSI was a significant predictor of the total LN count ($P = 0.02$). Total node count was highest for cancers with MSI and no *KRAS*/*BRAF* mutations. The independent association between MSI and a high LN count persisted for stage I and II cancers ($P = 0.04$). Tumor location in the proximal colon was the only significant predictor of an adequate LN harvest (adjusted OR 2.4, 95% CI 1.2–4.9; $P = 0.01$). An increase in the total number of nodes harvested was not associated with an increase in nodal metastasis. In conclusion, number of nodes harvested is highest for cancers of the proximal colon and with MSI. The nodal harvest associated with MSI is influenced by *BRAF* and *KRAS* genotypes, even for cancers of proximal location. Mechanisms behind the molecular diversity and node yield should be further explored.

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INTRODUCTION

In colon cancer, lymph node (LN) status is a strong prognosticator that usually guides adjuvant chemotherapy when regional LN metastasis (pN+) is found in the resected specimen. Obtaining an adequate LN harvest is therefore essential for guiding postoperative treatment. The LN harvest has become an extensively investigated quality standard for both surgeons and pathologists (1–4), and the number of LNs examined is associated with survival (2). The recommended number of nodes for appropriate staging is a minimum of 12 (5), although this goal has been achieved in as low as 50% of the patients (6). Factors associated with either a poor (<12 nodes) or adequate (≥12 nodes) LN harvest is the subject of extensive debate (7–10). Notably, few studies have investigated the molecular pathways in colorectal carcinogenesis and their potential relevance for the LN harvest. However, in a previous cohort, we found that microsatellite instability (MSI) was associated with the total number of LNs harvested (11), which has been confirmed by others (12,13). The aim of the present study was to further investigate MSI and *KRAS* and *BRAF* mutations and their relationship to the number of harvested LNs in colon cancer patients.

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MATERIALS AND METHODS

Study Population

The study population comprised a prospective patient series with stage I–III colon cancer enrolled in a sentinel node

project, as previously described (14). Briefly, all the patients underwent elective surgery with a curative intent at the Department of Surgery of Stavanger University Hospital in Stavanger, Norway, from June 2003 to February 2010. The patients were prospectively included after providing informed consent.

The Norwegian health system covers all medical expenses for diagnosis, management and surveillance, indicating no systematic selection bias in the cohort presented. Stavanger University Hospital provides all surgical services to a primarily Western population catchment area of approximately 330,000 inhabitants. Accordingly, the results should be representative for other Western populations.

Patients were excluded from the study cohort if any of the following occurred: noninvasive tumors (T0 and Tis), evidence of distant metastases (M^+) at the time of surgery or on pre-op staging, preoperative chemotherapy, preoperative treatment with a self-expanding metal stent because of acute colon obstruction, missing tumor biopsies for DNA retrieval, missing or incomplete histopathology report (for node status, number and pN), or deviations from the SLN mapping protocol.

Radical surgical resection was applied to the specific tumor-bearing segment of the colon by using either an open or a laparoscopic technique following general surgical oncological principles. Tumors located proximal to the left flexure were defined as right-sided and tumors from the left flexure through the sigmoid colon as left-sided.

Tissue and LN Sampling

Ex vivo sentinel LN mapping was performed on resected specimens from all patients, as previously described (14). In the current study, the total numbers of histologically verified LNs were analyzed without separating the nodes into "sentinel" and "nonsentinel" categories.

The resected colonic segment was evaluated by gross and microscopic histopathological examination, including regional LN harvesting, following an in-

Table 1. Characteristics and the associated LN count.

Feature	n (%)	Number of nodes harvested (median (range))	P^a	Number of nodes with metastasis (median (range))	P^a
Sex			0.037		0.086
Female	116 (57)	14 (4–39)		0 (0–10)	
Male	88 (43)	12 (5–43)		0 (0–17)	
Age (years)			0.329		0.447
<75	88 (43)	13 (5–43)		0 (0–8)	
≥75	116 (57)	14 (4–35)		0 (0–17)	
Stage			0.519[b]		NA
I	40 (20)	13 (4–35)		—	
II	103 (50)	13 (5–39)		—	
III	61 (30)	14 (6–43)		2 (1–17)	
Histological type			0.653		0.007
Non-mucinous	175 (85)	14 (4–43)		0 (0–8)	
Mucinous type	29 (15)	14 (8–32)		0.5 (0–17)	
Histological grade[c]			0.071		0.005
High	147 (72)	13 (4–43)		0 (0–8)	
Low	57 (28)	16 (7–32)		0 (0–17)	
Tumor invasion			0.248		0.003
pT1-2	47 (23)	13 (4–35)		0 (0–5)	
pT3-4	157 (77)	14 (5–43)		1 (0–17)	
MSI			0.002		0.693
MSS	137 (67)	14 (4–43)		0 (0–17)	
MSI	67 (33)	17 (7–39)		0 (0–10)	
KRAS mutation			0.864		0.979
Wild-type	129 (63)	14 (5–35)		0 (0–17)	
Mutated	75 (37)	14 (4–28)		0 (0–17)	
BRAF mutation[d]			0.137		0.639
Wild-type	146 (72)	13 (0–43)		0 (0–17)	
Mutated	57 (28)	14 (7–39)		0 (0–10)	
Location in colon			0.001		0.938
Proximal	145 (71)	14 (0–43)		0 (0–10)	
Distal	59 (29)	11 (4–35)		0 (0–17)	
Tumor size (cm)			0.030		0.261
<5	85 (41)	12 (4–39)		0 (0–6)	
≥5	119 (59)	14 (5–43)		0 (0–17)	

[a]Mann-Whitney *U* test.
[b]Comparison of stage I and II versus stage III.
[c]High = G3/G4, low = G1/G2.
[d]*BRAF* mutation status missing for one patient.

Table 2. Prevalence of MSI, *KRAS* and *BRAF* and associated LN yield rate.

	n	MSI		*KRAS* codon 12 + 13		*BRAF* V600E	
		Prevalence	Node yield[a]	Prevalence	Node yield[a]	Prevalence[b]	Node yield[a]
All	204	67 (33)	53 (79)	75 (37)	51 (68)	57 (28)	41 (72)
Stage I	40	17 (43)	12 (71)	17 (43)	8 (47)	12 (30)	8 (67)
Stage II	103	32 (31)	26 (81)	37 (36)	26 (70)	27 (27)	21 (78)
Stage III	61	18 (30)	15 (83)	21 (34)	17 (81)	18 (30)	12 (67)

Data are n (%) or n.
[a]Node yield, denotes the rate of patients with ≥12 nodes sampled after surgery.
[b]Missing data in one patient for *BRAF* mutation status.

stitutional template established several years before the study (15) to determine the number of LNs present and the disease stage according to the tumor, node, metastasis (TNM) staging system of the Union for International Cancer Control. The extent of node metastasis was defined as pN0 for node-negative specimens, pN1 for one to three metastatic LNs and pN2 for three or more metastatic LNs in the specimen.

Outcome

The primary outcome of this study was the number of LNs harvested, reported either as the total number observed (that is, both sentinels and non-sentinels, with or without metastasis), and, alternatively, as the rate of appropriately harvested cases (that is, patients with ≥12 nodes per specimen, defined as an adequate harvest). Secondary outcomes were limited to number of metastatic LNs (pN+) and to the survival outcome at follow-up. Cancer-specific death was used as an endpoint. Patients alive at the end of follow-up were censored. Patients who died of other causes than cancer were censored at time of death. Follow-up was performed blinded to the patients' clinical characteristics or molecular profile in the study.

Genetic Analyses

A sample from the tumor and the normal surrounding mucosa was collected from the resected colonic segment and was instantly frozen in liquid nitrogen. DNA was extracted from the tissue by using either a combination of the RNeasy Mini Kit and the DNeasy Mini Kit or the AllPrep DNA/RNA Mini Kit (all manufactured by Qiagen, Hilden, Germany) according to the manufacturer's protocols.

The MSI status (microsatellite stable [MSS]; low frequency [MSI-L], or high frequency [MSI-H]) was determined by using Bethesda markers (BAT25, BAT26, D2S123, D5S346 and D17S250), as described (16). For MSI analyses, DNA from tumor and corresponding normal tissue were analyzed and compared. Analyses were run twice, and two people evaluated all results independently. Presence of two or more instable markers was defined as high-frequency MSI (MSI-H) and only one single unstable marker as low-frequency MSI (MSI-L). MSI-L was coded as MSS for the regression analysis in the current study, as per convention. Thus, MSI denotes MSI-H if not otherwise indicated. Mutations in codons 12 and 13 of the *KRAS* gene were detected by peptide nucleic acid clamp polymerase chain reaction (PCR) (17,18). The hot-spot mutation V600E in exon 15 of the *BRAF* gene was identified by using PCR and Sanger sequencing, as previously described (19).

For *BRAF* mutation analysis, all PCR products were sequenced in the 5′ direction, and electropherograms were scored both manually and semiautomatically by using Sequencing Analysis (version 5.3.1) and SeqScape software (version 2.5), respectively. The resulting sequence was compared with reference sequence NM_004333 (GenBank). For samples in which a mutation was found, a confirmatory sequencing reaction was performed in the 3′ direction.

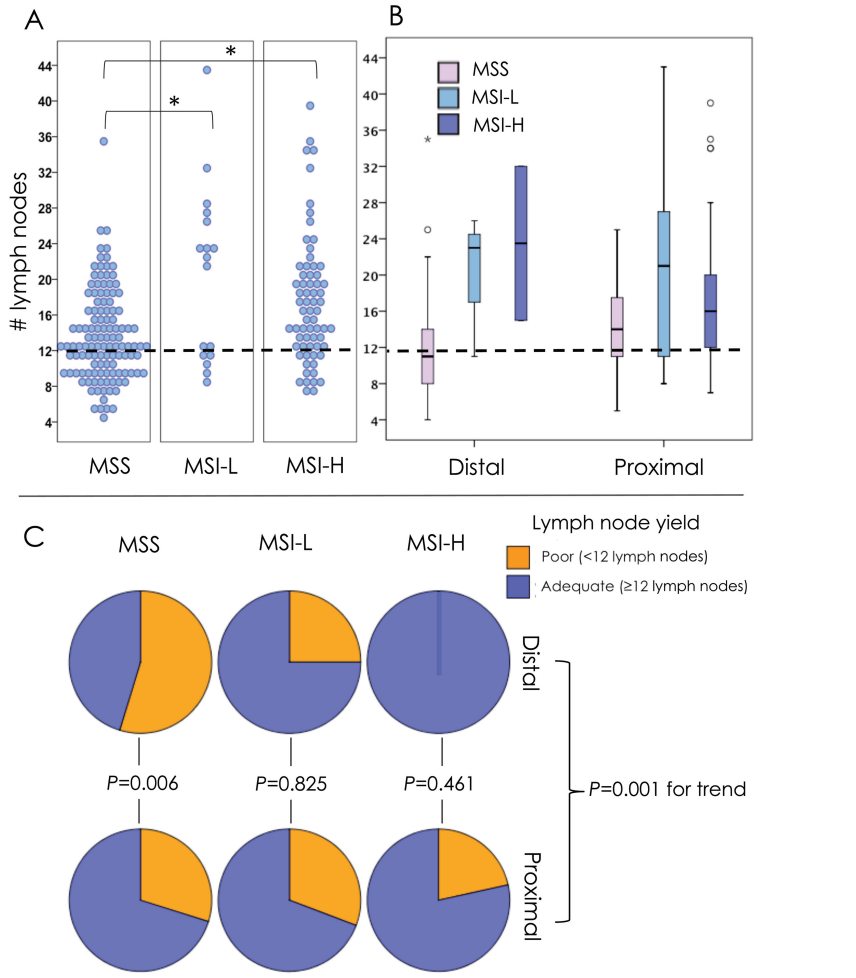

Figure 1. LN counts for MSI status and tumor location in the colon. (A) Total node counts according to MSI status (MSS, MSI-L or MSI-H). The asterisks indicate statistical significance. (B) The total LN counts for MSI status according to location in either the proximal or distal colon. The box-and-whiskers plot shows the medians, interquartile ranges and 95% CIs, and the circles/asterisks indicate outliers. (C) Adequate LN harvest improved significantly within the MSS cancers for proximal cancers. Adequate harvest proportion increased significantly with addition of MSI to either location ($P = 0.001$ for trend).

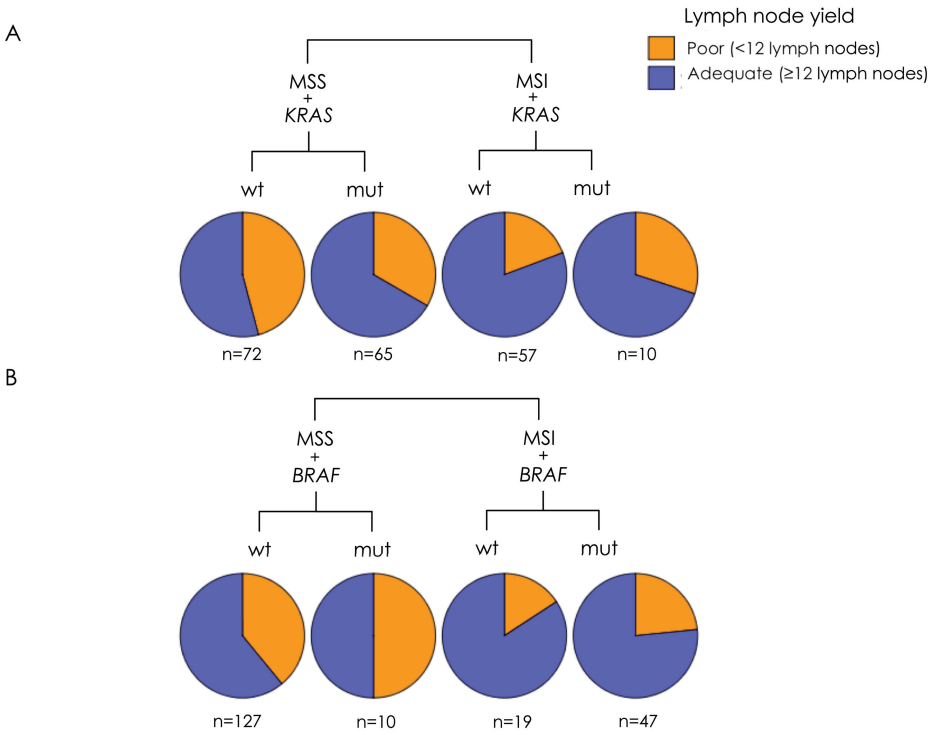

Figure 2. Proportion of patients with adequate node harvest according to MSI and *KRAS/BRAF* genotypes. The rates of adequate LN sampling are shown for MSI/MSS tumors with or without *KRAS* mutations (A) and MSS/MSI tumors with or without *BRAF* V600E mutations (B).

Study Ethics

Written, informed consent was obtained from all the study subjects. This research project was approved by the Regional Committee for Medical Research Ethics (REK Vest 197.04, Biobank 15-10) according to national legislation.

Statistical Analyses

All statistical analyses were performed by using the software package IBM SPSS Statistics, version 20 (SPSS, Chicago, IL, USA). Descriptive data are presented as medians and ranges (or interquartile ranges) or as proportions, as appropriate. Dichotomous variables were tested by χ^2 or Fisher exact test, as appropriate. Continuous variables were analyzed by using a nonparametric Mann-Whitney *U* test or the Kruskal-Wallis test for more than two groups. For variables associated with the number of LNs as a continuous outcome variable, a multiple linear regression adjusted for age and sex in a hierarchical mode was performed, as previously recommended (20,21). The variables were analyzed for normality, colinearity and interactions. The factors found to be associated with a sufficient (≥12 LNs) harvest were investigated by using univariate and multivariable analyses and are presented with odds ratios (ORs) and 95% confidence intervals (95% CIs). The multivariable model was adjusted for age and sex and included variables with $P < 0.2$. Goodness of fit was assessed with the Hosmer-Lemeshow test. Survival analyses were performed with the log-rank method by using Kaplan-Meier curves. All the tests were two-tailed, and the statistical significance was set at $P < 0.05$.

RESULTS

Patient and Tumor Characteristics

A total of 204 patients with stage I–III colon cancer and a median age of 76 years (range 21–93 years) were included. A sufficient number of harvested LNs (defined as ≥12) was observed in 136 patients (67%). Clinicopathological characteristics and molecular features (MSI, *KRAS* and *BRAF*) related to the LN harvest are given in Table 1. The prevalence of MSI and *KRAS* and *BRAF* mutations for each stage and the associated number of adequate node harvest is presented in Table 2.

Total Number of LNs Harvested

Among the 3,068 LNs collected, metastases (pN+) were detected in 173 (5.6%) nodes. The median number of harvested nodes was 14 (range 4–43; interquartile range 11–19), with significantly more nodes found in women, in proximal located tumors, in MSI genotype and larger (≥5 cm) tumors (Table 1). The total nodes sampled for each frequency stratum of MSI is presented in Figures 1A–C, with and without tumor location.

The total number of harvested LNs was investigated as an outcome variable by using a multiple linear regression adjusted for age, sex, MSI status, *BRAF* mutation status, tumor location and tumor size. According to the model, the only significant predictor of LN harvest size was MSI, with a β coefficient of 0.224 ($P = 0.02$) and a model R^2 of 0.07 ($F = 2,834$; $P = 0.01$). Although MSI was the only factor that was significantly associated with an increased number of harvested LNs, the R^2 value indicates that only 7% of the variance in the LN number was explained by MSI. However, the model demonstrated a good fit. In addition, MSI had a significant effect on LN number in stage I–II cancers only ($P = 0.04$) after controlling for age, sex and tumor location. The addition of *BRAF* status to MSI in the analysis produced an effect of LN harvest numbers that had borderline significance ($P = 0.05$); there was a slight increase in R^2 to 0.09, indicating a 9% contribution to the variance in the model. For stage III cancers, proximal location was the only factor determined to be significantly associated with an elevated LN count ($P = 0.04$).

Characteristics Associated with Adequate LN Harvest

A sufficient LN harvested LNs (defined as ≥12) was observed in 136 patients

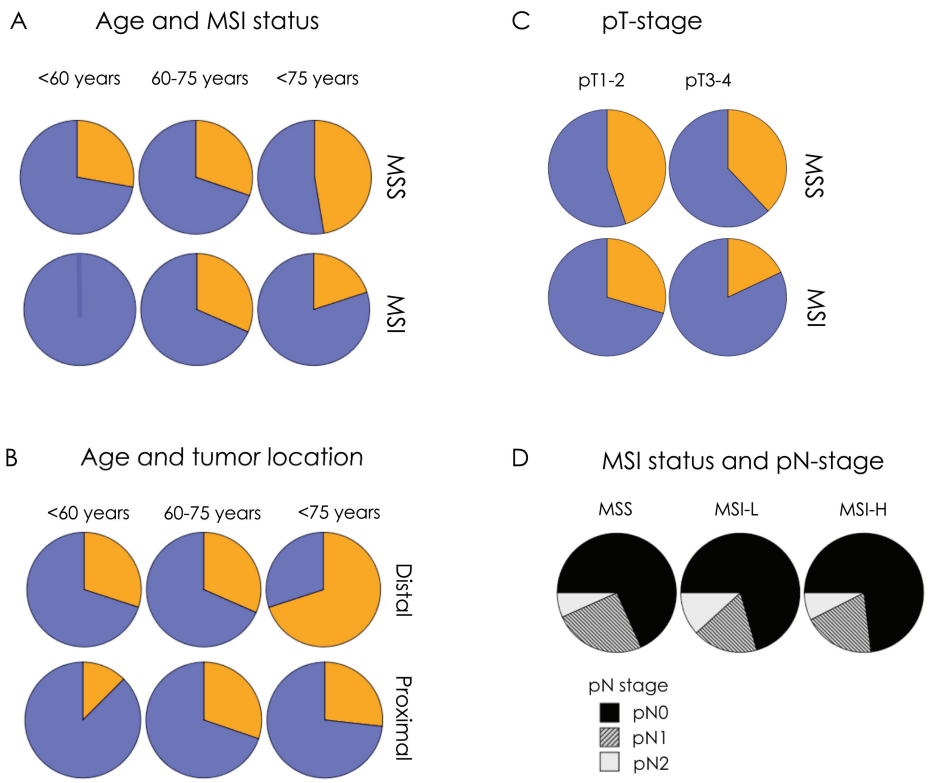

Figure 3. Proportion of adequate node harvest according to age, location and pT-stage. (A) Proportion of adequate LN harvest according to age-groups, stratified for MSI status ($P < 0.05$ for trend). (B) Proportion of adequate LN harvest according to age and tumor location ($P < 0.05$ for trend). (C) Proportion of adequate LN harvest according to pT-stage and MSI, $P < 0.05$ for trend. (D) Distribution of pN categories according to MSI status.

(67%) of the entire cohort. In patients with ≥12 nodes sampled, 39% (n = 53) exhibited MSI ($P = 0.007$), and 79% had tumors located in the proximal colon ($P = 0.002$). Inversely, among the patients with MSI (n = 67), 79% had ≥12 nodes harvested, and 73% (106 of 145) of the patients with proximal tumors had ≥12 nodes harvested.

The proportion of patients with adequate node harvest varied according to the presence of MSI, *BRAF* mutations and *KRAS* mutations (Table 2), including the coexistence of two or more of these factors (Figure 2). The adequate harvest proportions (Figure 3) also differed according to MSI combined with tumor location, age and pT-stage, but neither the number of metastatic nodes nor the pN+ stage distribution varied according to MSI status (Figure 3D).

The results of the univariate analysis of the clinicopathological factors predicting a sufficient LN harvest are summarized in Table 3. The multivariate logistic regression analysis of MSI status, tumor location, tumor size and *BRAF* status, which controlled for sex and age, revealed that a proximal tumor location was the only significant predictor of an LN harvest ≥12, with an adjusted OR of 2.4 (95% CI 1.2–4.9; $P = 0.01$), a change in R^2 between 0.09 (Cox and Snell) and 0.13 (Nagelkerke) and an appropriate model estimate (Hosmer-Lemeshow goodness of fit, $P = 0.7$).

The same model used to analyze the data from the stage I–II patients (n = 143) produced no significant predictors of a sufficient LN harvest. However, it revealed that a proximal location was a predictor of a sufficient LN harvest in the smaller group of stage III patients, with an OR of 5.7 (95% CI 1.2–27.3; $P = 0.03$), a good model fit (Hosmer-Lemeshow $P =$ 0.8) and a corresponding R^2 change between 0.15 and 0.22.

At the time of diagnosis, 88 patients (43%) were <75 years of age. In this subset, 72% of patients had a sufficient number of harvested LNs, although the median number was lower (13, range 0–43) than that for patients ≥75 years of age (14, range 4–35). In the subset of patients <75 years of age, the multivariate regression analysis revealed no significant factors associated with a sufficient LN harvest. However, for the patients ≥75 years of age (n = 116), the model revealed that a proximal location was a predictor of sufficient LN sampling (OR 5.0, 95% CI 1.9–13.6; $P = 0.001$), with an R^2 change between 0.18 and 0.24 (Hosmer-Lemeshow goodness of fit $P = 0.6$).

Survival at End of Follow-up

At the end of follow-up (median 3.5 years, interquartile range 2.0–5.4 years), a total of 55 patients died (27%), of which 18 (8.8%) died of colon cancer. Stage (Figure 4) predicted cancer-specific survival at the end of follow-up (stages I–II, 97% alive, versus stage III, 78% alive; $P < 0.001$; hazards ratio 7.0, 95% CI 2.5–19.8) with a nonsignificantly better prognosis for MSI (Figure 4). The combined negative genetic feature of *BRAF* mutated with MSS was significantly prognostic (Figure 4C). None of the other clinical variables, neither tumor variables nor the molecular features, influenced cancer-specific survival. Also, there was no difference in cancer-specific survival for patients with <12 LN versus those with ≥12 LN. This was consistent when stratified for features such as tumor location and genetic subtypes.

DISCUSSION

In this prospective study, an adequate node harvest (≥12) was achieved in a high proportion of patients (67%; n = 136), with the best node harvest seen in patients with MSI tumors (79% had ≥12 nodes) and those with tumors in the proximal colon (73% had ≥12 nodes). In total, MSI was demonstrated in 33% of the patient samples. This is in line with

Table 3. Univariate and multivariable analysis of factors associated with sufficient LN sampling (≥12 nodes).

	LN <12 (n (%))	LN ≥12 (n (%))	OR (95% CI)	P^a	Adjusted OR (95% CI)[b]	P^a
Sex			0.6 (0.3–1.1)	0.089	0.3 (0.7–2.6)	0.329
Female	33 (28)	83 (72)				
Male	35 (40)	53 (60)				
Age (years)			0.6 (0.3–1.1)	0.110	0.6 (0.3–1.1)	0.061
<75	24 (27)	64 (73)				
≥75	44 (38)	72 (62)				
Stage			1.4 (0.7–2.8)	0.280	—	NA
I and II	51 (36)	92 (64)				
III	17 (28)	44 (72)				
Histology grade			1.4 (0.7–2.8)	0.279	—	NA
High	53 (36)	94 (64)				
Low	16 (28)	41 (72)				
Tumor size (cm)[c]			1.8 (1.0–3.3)	0.045	1.6 (0.7–3.5)	0.099
<5	35 (41)	50 (59)				
≥5	33 (28)	86 (72)				
Tumor invasion			1.5 (0.7–2.9)	0.343	—	NA
pT1-2	18 (39)	28 (61)				
pT3-4	50 (32)	108 (68)				
MSI status			2.5 (1.2–4.9)	0.008	2.3 (0.9–6.3)	0.223
MSS	54 (39)	83 (61)				
MSI	14 (21)	53 (79)				
KRAS status			1.1 (0.6–2.0)	0.758	—	NA
Wild-type	44 (34)	85 (66)				
Mutated	24 (32)	51 (68)				
BRAF status			1.4 (0.7–2.8)	0.306	—	NA
Wild-type	52 (36)	94 (64)				
Mutated	16 (28)	41 (72)				
Location in colon			2.9 (1.5–5.5)	0.001	2.4 (1.2–4.9)	0.014
Proximal	39 (27)	106 (73)				
Distal	30 (51)	29 (49)				

[a]χ^2 two-sided test, df = 1.
[b]Multiple logistic regression.
[c]Largest diameter of tumor.

existing literature reporting on MSI for the different segments of the colon (22).

Tumor location appeared to consistently predict a sufficient number (≥12) of nodes, which is in line with recent reports (22,23). However, when the factors affecting the total number of harvested LNs were assessed and adjusted for age and sex, MSI was the only significant factor, explaining 11% of the change in the regression model. Furthermore, a specific genotype combination (that is, MSI with wild-type *KRAS*/*BRAF*) was associated with a higher rate of adequate LN harvest (>83%), whereas the presence of mutations in both *KRAS* and *BRAF* was associated with lower rates of adequate node sampling, even for tumors within the proximal colon. The importance of combining genetic features within the colon may point to the previous concept of a continuum diversity within the colon rather than the blunt dichotomized difference in proximal or distal location (24). Furthermore, the associated findings of the molecular features to the LN harvest in this study mirrors the previous knowledge that MSI cancers are associated with a favorable prognosis (25), whereas *BRAF* mutations, which predicted the smallest number of LN harvest in this study, are known to be associated with a poor prognosis (26).

The current results substantiate previous findings by our group (11) and others (12,13) demonstrating an effect of MSI in determining the actual LN counts after surgery. Both MSI and proximal tumor location appear to be associated with a higher LN harvest, both independently and in combination. This relationship has been demonstrated in different populations with the use of various cutoffs to determine the appropriate nodal count (11–13,23). In contrast to these studies, a Canadian study found no significant difference between the effects of MSI and MSS on LN harvest in 168 selected stage III colon cancer patients (27). However, the study used the median and mean node counts as a cutoff, rather than the guideline of ≥12 nodes suggested for clinical decision-making. In contrast, the relationship between MSI and a larger number of harvested nodes has been demonstrated in study populations in Norway (11), France (13), the Netherlands (12), and the U.S. (23).

The association between a proximal tumor location and an increased MSI prevalence is well established for colorectal cancer. The correlation between tumor location and node yield may be confounded by higher-quality surgery performed for right-sided colon cancers, or it may indicate anatomical differences, for example, in the lymphatic system between the right and left sides of the colon. However, while we do not necessarily disagree with this, nor can we rule out the potential confounding effect of surgery and anatomical differences in the tumor-bearing segments of the colon, the finding of molecularly mixed genotypes and an association with LN count, even within the proximal colon tumors, argues against a purely anatomical and/or surgical explanation. Although cancers with MSI had a higher LN yield, this benefit was reduced in the presence of *BRAF* mutations, a genetic feature associated with a poor outcome. Furthermore, the arbitrarily dichotomized locations of "proximal" and "distal" may be too approximate of an estimate for exploring the subsegmental continuum difference

observed in the colon, which affects both the molecular profile and the LN numbers (28). Also, the overall node yield in the current study was high (over 67% had adequate node sampling), as a proxy indicating proper surgery performed for the majority of patients, and all had R0 resections performed ("R0" denotes a surgical resection with free margins proven on histopathology).

Notably, MSI tumors exhibit a characteristic Crohn's-like lymphoid reaction at the tumor border (29), which is believed to be a tumor-targeted immunogenic activation of specific lymphocytes (30) and may explain the favorable prognostic profiles of MSI cancers. Although the current study was not designed to provide a tumor-biological mechanistic explanation, the consistent findings of more nodes in tumors with MSI may suggest immunogenic mechanisms that favorably influence tumor biology and, subsequently, prognosis (31,32). Why more nodes are found with MSI, yet are reduced when associated with *KRAS* or *BRAF* mutations, remains an intriguing observation and warrants further investigation.

CONCLUSION

A higher LN yield is found for proximal tumor location, and the total number of nodes is influenced by the prevalence of MSI, as well as *KRAS* and *BRAF* mutations. Higher yield was not associated with an increased number of metastatic nodes in the current study, which is in agreement with recent large cohorts (33,34). Each factor likely influences the LN sample to a modest, yet significant, degree, as demonstrated by the multivariable modeling in this series. This observation is in accordance with a previous study, in which four factors collectively explained 19% of the overall variation in the prediction model (35). Genetic features should be considered together with other clinicopathological factors when assessing node count after surgery for colon cancer. The information obtained in this study adds to the evolving knowledge about diversity in colon cancer features and points to associations between the location of the tumor, the underlying genetic features and the node count. These factors are associated with the individual demographic features and eventually may explain observed variance in clinical outcomes.

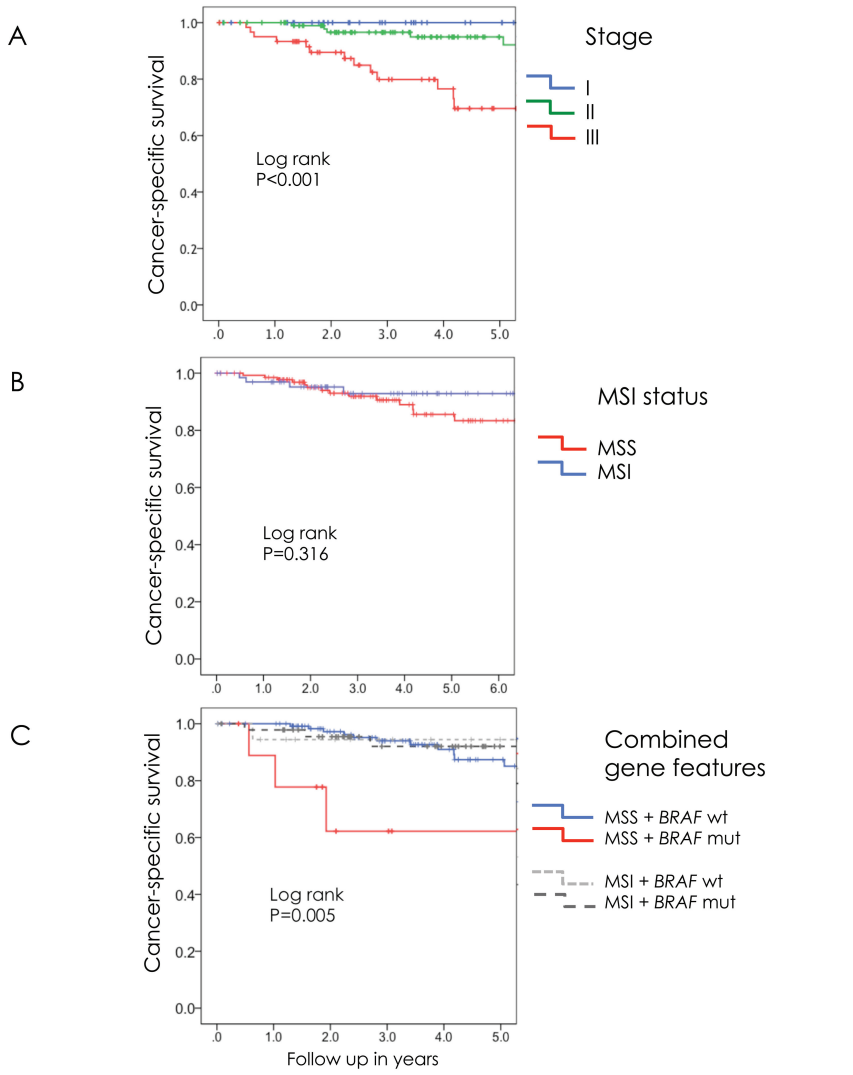

Figure 4. Cancer-specific survival for stage (A), MSI and MSS (B) and MSI/MSS and *BRAF* wt/mut genotypes (C). Hashes on the survival curves denote censored patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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