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Negative Lymph Node Count Is Associated With Survival of Colorectal Cancer Patients, Independent of Tumoral Molecular Alterations and Lymphocytic Reaction

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Abstract

OBJECTIVES—The number of recovered lymph nodes is associated with good prognosis among colon cancer patients undergoing surgical resection. However, little has been known on prognostic significance of lymph node count after adjusting for host immune response to tumor and tumoral molecular alterations, both of which are associated with the lymph node count and patient survival.

METHODS—Among 716 colorectal cancers (stages 1–4) in two independent prospective cohorts, we examined patient survival in relation to the negative lymph node count and lymph node ratio (LNR; positive to total lymph node counts). Cox proportional hazard models were used to compute hazard ratio of deaths, adjusted for patient, specimen, and tumoral characteristics, including lymphocytic reactions, *KRAS* and *BRAF* mutations, p53 expression, microsatellite instability (MSI), the CpG island methylator phenotype (CIMP), and LINE-1 methylation.

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RESULTS—Compared with patients with 0–3 negative lymph nodes, patients with 7–12 and ≥ 13 negative nodes experienced a significant reduction in cancer-specific and overall mortality in Kaplan–Meier analysis (log-rank $P < 0.0001$), univariate Cox regression ($P_{\text{trend}} < 0.0001$), and multivariate analysis ($P_{\text{trend}} < 0.0003$), independent of potential confounders examined. The benefit associated with the negative node count was apparent across all stages, although the effect was significantly greater in stages 1–2 than stages 3–4 ($P_{\text{interaction}} = 0.002$). In both stage 3 and stage 4, smaller LNR was associated with improved survival (log-rank $P < 0.0001$).

CONCLUSIONS—The negative lymph node count is associated with improved survival of colorectal cancer patients, independent of lymphocytic reactions to tumor and tumoral molecular features including MSI, CIMP, LINE-1 hypomethylation and *BRAF* mutation.

INTRODUCTION

The presence of lymph node metastasis has important prognostic implications for colorectal cancer patients. Observational studies indicate that the number of lymph nodes assessed by pathologic examination is positively associated with longer survival of patients with colorectal cancer (1–11). Other studies have shown that an increasing number of negative lymph nodes assessed (12) or a diminished lymph node ratio (LNR, the ratio of positive to total lymph node counts) (13–19) similarly predicts patient survival. However, the optimal number of lymph nodes that must be assessed remains controversial (2–12). In addition, the mechanisms underlying the relationship between the lymph node count and survival remain uncertain. Several hypotheses have been related to patient care, including accurate tumor staging, more efficacious surgical intervention, and superior quality of pathology service. Some investigators have raised the possibility of an underlying biologic mechanism of action, including a greater host immune response among patients with a larger negative lymph node count, or other underlying molecular/biological characteristics of tumor [e.g., microsatellite instability (MSI) or the CpG island methylator phenotype (CIMP)]. To assess a prognostic role of the lymph node count independent of host immune response and tumoral molecular features, it is necessary to examine host immune response and tumoral molecular features.

We therefore examined the prognostic significance of the negative lymph node count in relation to patient survival among 716 stages 1–4 colorectal cancer patients identified in two independent prospective cohort studies. As we concurrently assessed pathologic characteristics (including lymphocytic reaction as a surrogate of host immune response) as well as tumoral molecular variables including MSI, CIMP, and *BRAF* mutation, we could evaluate the effect of the number of negative lymph nodes, independent of these potential confounders.

METHODS

Study population

We used the databases of two independent prospective cohort studies; the Nurses' Health Study ($N = 121,701$ women followed since 1976) and the Health Professionals Follow-Up Study ($N = 51,529$ men followed since 1986) (20). For most participants who developed colorectal cancer, study physicians reviewed all records related to colorectal cancer, and recorded TNM stage, tumor location, the length of colon that was resected for a primary colorectal cancer, the number of positive and negative lymph nodes, and the presence or absence of extranodal involvement. We collected paraffin-embedded tissue blocks from hospitals where patients underwent tumor resections (20). We excluded cases preoperatively treated with radiation and / or chemotherapy. On the basis of availability of lymph node and tissue data, we included a total of 716 stages 1–4 colorectal cancer cases diagnosed up to 2003. Patients were followed until death or June 2006, whichever came first. The cause of death was assigned by physicians blinded to information on lifestyle exposures and molecular changes in colorectal

cancer. Written informed consent was obtained from all study subjects. This study was approved by the Human Subjects Committees at Brigham and Women's Hospital and the Harvard School of Public Health.

Histopathologic evaluations

Tissue sections from all colorectal cancer cases were reviewed by the pathologist (S.O.). Tumor grade was categorized as high (≤ 50 % glandular area) or low (> 50 % glandular area). A proportion of mucinous component and signet ring cell component were recorded, and mucinous or signet ring cell tumor was defined as 50 % or greater mucinous or signet ring cell component, respectively. Tumors with both ≥ 50 % mucinous and signet ring cell components were classified as signet ring cell tumors. Lymphocytic reactions, i.e., Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, and tumor infiltrating lymphocytes were examined as described earlier (21), and scored as 0 (no/minimal), 1 (mild), 2 (moderate), or 3 (marked). The overall lymphocytic reaction score (0–9) was calculated as the sum of scores of Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, and tumor infiltrating lymphocytes. Relations of each reaction pattern and patient survival are shown in Supplementary Figure 1 online.

Pyrosequencing of *KRAS* and *BRAF*, and microsatellite instability analysis

DNA from paraffin-embedded tissue was extracted, and polymerase chain reaction (PCR) and pyrosequencing targeted for *KRAS* codons 12–13 (22) and *BRAF* codon 600 (23) were performed. MSI status was determined using D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487 (24). MSI high was defined as the presence of instability in ≥ 30 % of the markers, MSI low as the presence of instability in 1–30 % of the markers, and microsatellite stability as no unstable marker.

Real-time PCR (MethyLight) for CpG island methylation, and pyrosequencing to measure LINE-1 methylation

Sodium bisulfite treatment on tumor DNA and subsequent real-time PCR (MethyLight) assays were validated and performed as described earlier (25). We quantified promoter methylation in eight CIMP-specific markers (CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and *SOCS1*) (24,26,27). CIMP high was defined as $\geq 6/8$ methylated markers, CIMP low/0 as 0–5 methylated markers, according to the previously established criteria (26). To accurately quantify relatively high LINE-1 methylation levels, we used pyrosequencing (28).

Immunohistochemistry for p53

Tissue microarrays were constructed (29), and p53 immunohistochemistry was performed as described earlier (29). p53 positivity was defined as the presence of moderate/strong unequivocal staining in ≥ 50 % of tumor cells, and p53 negativity as no staining, at most weak staining in any fraction of tumor cells, or moderate/strong staining in $< 50\%$ of tumor cells, based on the earlier study that compared p53 immunohistochemistry with *TP53* mutation detection analysis (30). Appropriate positive and negative controls were included in each assay run. All slides were interpreted by a pathologist (S.O.) unaware of other data. A random sample of 118 tumors were re-examined by a second observer (K.N.) unaware of other data, and the concordance between the two observers was substantial ($K = 0.75$, $P < 0.0001$).

Statistical analysis

All analyses used SAS version 9.1 (SAS Institute, Cary, NC) and all P values were two-sided. The Kaplan–Meier method was used to describe the distribution of colorectal cancer-specific and overall survival time, and the log-rank test was performed. The χ^2 test was used to examine

an association between categorical variables. The analysis of variance was performed to compare mean age and mean LINE-1 methylation level across the lymph node categories.

To assess independent effect of the negative lymph node count on mortality, we used stage-matched (stratified) Cox proportional hazard models, and calculated hazard ratios (HRs) of death, adjusted for sex, age at diagnosis (continuous), year of diagnosis (continuous), body mass index (BMI) (≥ 30 vs. $< 30 \text{ kg/m}^2$), family history of colorectal cancer in any first-degree relative (present vs. absent), tumor location (proximal vs. distal colon vs. rectum), length of resected colorectum (≥ 20 vs. $< 20 \text{ cm}$), tumor grade (high vs. low), lymphocytic reactions (score ≤ 4 vs. ≥ 5), MSI (high vs. low/microsatellite stability), CIMP (high vs. low/0), LINE-1 methylation (continuous), *KRAS*, *BRAF*, and p53 (positive vs. negative). Tumor stages [1, 2A, 2B, 3A, 3B, 3C, 4, unknown (4.9 %)] were used as a matching (stratifying) variable to avoid residual confounding, using the “strata” option in SAS “phreg” command. For analyses of colorectal cancer-specific mortality, death as a result of colorectal cancer was the primary end point and deaths as a result of other causes were censored. In the main analysis, we categorized cases according to the number of negative lymph nodes (0–3 vs. 4–6 vs. 7–12 vs. ≥ 13). We also examined the negative lymph node categories as a continuous variable, to assess significance of a linear trend. In Cox regression analyses, we did not use the raw number of lymph node count to avoid influential data points. We included the squared term and/or the cubic term of the ordinal negative node variable, and confirmed that inclusion of any or all of those terms did not significantly improve the model fit ($P > 0.31$ by likelihood ratio tests). In addition, the linearity assumption seemed to be generally satisfied by non-parametric restricted cubic spline plots (see below). The proportionality of hazards assumption was verified by evaluating time-dependent variables, which were the cross product of the ordinal lymph node variable and survival time ($P = 0.31$ for colorectal cancer-specific mortality; $P = 0.45$ for overall mortality). For cases with missing information in any of the categorical covariates [including BMI (3.8 % missing), tumor location (0.7%), length of colorectum (8.9%), tumor grade (4.6%), lymphocytic reactions (3.4%), MSI (1.7%), CIMP (3.0%), *KRAS* (1.3%), *BRAF* (3.4%), p53 (2.1%)], we included those cases in a majority category, to minimize the number of covariates and avoid overfitting. For cases missing LINE-1 data (5.0 %), we assigned the median LINE-1 methylation level. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). An interaction was assessed by the Wald test on the cross product of the ordinal lymph node variable and another variable of interest in a multivariate Cox model. P values were conservatively interpreted, considering multiple hypothesis testing. To assess an interaction with tumor stage, we used stage as an ordinal variable (1,2,3,4) and as a binary variable (1–2 vs. 3–4).

For stages 3–4 cases, we calculated an LNR (LNR = the number of positive lymph nodes / the total number of lymph nodes) as described earlier (13–19). We used LNR as a continuous variable, and as a categorical variable (< 0.20 vs. $0.20\text{--}0.39$ vs. ≥ 0.40), similar to the earlier described classification (13).

We examined the possibility of non-linear relations between the number of lymph nodes (or LNR) and mortality, non-parametrically with restricted cubic splines (31). This flexible method allowed us to examine the relations with mortality without any categorization of the negative lymph node count (or LNR), or without the assumption of linear relationship of the number of negative lymph nodes (or LNR) with mortality. In addition, this method avoided the possibility of selecting cut points that could maximize the associations between the number of negative lymph nodes and outcome.

RESULTS

Number of negative lymph nodes in colorectal cancer patients

Among 716 patients with stages 1–4 colorectal cancer in the two prospective cohorts, we recorded the numbers of positive lymph nodes and negative lymph nodes that were identified in resected colorectal specimens. Distribution of the total lymph node count was as follows: mean 11.9, median 10, 25th percentile 6, 75th percentile 15, range 0–55. A categorized distribution of the total lymph node count is shown in Table 1 (in boldface). For this study, we examined the effect of the negative lymph node count, because the total lymph node count included positive lymph nodes, which determined tumor stage and influenced patient mortality. In contrast, the negative lymph node count was a variable independent of tumor stage. Thus, the prognostic effect of the negative lymph node count (independent of tumor stage) could be measured more accurately than that of the total lymph node count. In fact, when the total lymph node count was used, its prognostic effect was attenuated in stages 3–4 (data not shown). For these reasons, we examined the effect of the negative lymph node count in further analyses. We used the positive lymph node count for tumor staging and the LNR (positive lymph node count/total lymph node count) in analysis of stages 3–4 patients (see below). We categorized all patients into 4 groups according to the number of negative lymph nodes (0–3, 4–6, 7–12, and ≥ 13). Table 1 shows clinical, pathological, and molecular features of colorectal cancer according to the number of negative lymph node category. Table 2 shows pathologic features of colorectal cancer according to the number of lymph node involved by tumor.

Negative lymph node count and patient survival

During follow-up, there were a total of 304 deaths, including 171 colorectal cancer–specific deaths. We assessed colorectal cancer–specific and overall survival according to the number of negative lymph nodes (Figure 1). Both five-year colorectal cancer–specific survival and overall survival were significantly higher with an increasing number of negative nodes (log-rank $P < 0.0001$). When analysis was limited to stage 2 cases, the negative node count was associated with improved survival (log-rank $P = 0.0002$ for colorectal cancer–specific survival; log-rank $P = 0.029$ for overall survival) (Figure 1b).

In univariate Cox regression analysis, compared to patients with 0–3 negative lymph nodes, patients with 7–12 and ≥ 13 negative lymph nodes experienced significantly low colorectal cancer–specific mortality (HR 0.42; 95 % confidence interval (CI), 0.28–0.62; and HR 0.28; 95 % CI, 0.18–0.44, respectively; $P_{\text{trend}} < 0.0001$) (Table 3). We examined the effect of the number of negative lymph nodes after adjusting for potential predictors of patient survival, including age, sex, BMI, family history of colorectal cancer, year of diagnosis, tumor location, stage, grade, length of large bowel resected, host lymphocytic reactions, and tumoral molecular features. Compared to patients with 0–3 negative lymph nodes, patients with 7–12 and ≥ 13 negative lymph nodes experienced an improved colorectal cancer–specific mortality (multivariate HRs 0.56 (95 % CI, 0.36–0.87) and 0.43 (95 % CI, 0.27–0.71), respectively; $P_{\text{trend}} = 0.0002$) (Table 3). The attenuation of the effect of the negative lymph node count in multivariate analysis was mainly the result of adjusting for tumor stage. When we simply adjusted for tumor stage, the HR for colorectal cancer–specific mortality was 0.54 (95 % CI, 0.36–0.81) for patients with 7–12 negative lymph nodes, and 0.38 (95 % CI, 0.24–0.60) for patients with ≥ 13 negative lymph nodes. No other major confounder was observed. When overall mortality was used as an end point, results were similar (Table 3).

Effect of negative lymph node count on mortality in strata of tumor stage

We examined the effect of the number of negative lymph nodes across tumor stages 1–4 (Table 4). A large number of negative lymph nodes appeared to be consistently associated with low colorectal cancer–specific mortality, and the effect was much stronger in stages 1–2 than stages

3–4 ($P_{\text{interaction}} = 0.002$). When we used overall mortality as an end point, such modifying effect of tumor stage was not apparent ($P_{\text{interaction}} = 0.36$), possibly because of non-cancer-related deaths.

Continuous evaluation of negative lymph node count in relation to mortality using smoothing splines

We analyzed patient mortality non-parametrically using restricted cubic splines (Figure 2) (31). This flexible method allowed us to examine mortality without any categorization of the negative lymph node count, or without the assumption of a linear relation between the negative lymph node count and mortality. Compared with patients with 0 negative lymph nodes, the negative lymph node count was inversely associated with cancer-specific and overall mortality without a clear plateau (Figure 2a). When we limited the analysis to patients with stages 1, 2, or 3 disease, the effect of the negative lymph node count on mortality was still evident (Figure 2b, c). Among patients with stage 4 cancer, a similar trend toward the inverse relation between the negative lymph node count and mortality was observed, although statistical power was limited (Figure 2d).

Lymph node ratio and mortality in stages 3–4 colorectal cancer

Among patients with stage 3 or 4 disease, we examined mortality according to the LNR (the positive lymph node count divided by the total lymph node count), as described earlier (13–19). In both stages 3 and 4 patients, decreasing LNR was associated with low cancer-specific and overall mortality (Figure 3a, b). When we categorized stage 3 cancer patients into three groups according to LNR (<0.20 vs. $0.20\text{--}0.39$ vs. ≥ 0.40), the three groups were associated with significantly different survival in Kaplan–Meier analysis (log-rank $P < 0.0001$) (Figure 3c).

Extranodal involvement and prognosis in stage 3 colorectal cancer

We examined the prognostic effect of extranodal involvement in stage 3 colorectal cancer. The presence of extranodal involvement was associated with inferior survival (log-rank $P = 0.0018$ for colorectal cancer–specific survival; log-rank $P = 0.019$ for overall survival) (Figure 4).

Stratified analysis of negative lymph node count and mortality

Finally, we examined the influence of the negative lymph node count on colorectal cancer–specific and overall mortality across strata of other potential predictors of patient survival (Figure 5). The beneficial effect of the negative lymph node count appeared to be stronger in patients < 65 -year old compared to patients ≥ 65 -year old ($P_{\text{interaction}} = 0.02$), though multiple hypothesis testing should be considered. The effect of negative lymph node count did not significantly differ across any other strata ($P_{\text{interaction}} \geq 0.10$). Notably, the effect of the negative lymph node count did not significantly differ between the two independent cohort studies ($P_{\text{interaction}} = 0.10$).

DISCUSSION

We examined the prognostic significance of the number of negative lymph nodes in population of stages 1–4 colorectal cancer patients who were concurrently assessed for other clinical and molecular predictors of patient outcome. We observed a significant relation between negative lymph node count and survival, independent of patient characteristics and other related molecular variables including p53, *KRAS*, *BRAF*, MSI, the CIMP, and LINE-1 hypomethylation. The effect of the negative lymph node count was apparent in all stages of disease, although the benefit was significantly greater among patients with earlier pathologic stage.

Examining molecular features or clinical outcome is important in colon cancer research (32–39). In previous studies (1–11), the number of recovered lymph nodes or the number of negative lymph nodes has consistently been associated with longer survival in colorectal cancer. Nonetheless, the benefit associated with a higher number of negative lymph nodes may simply reflect the host lymphocytic reaction to tumor (which is associated with lymph node count (10)), as lymphocytic reaction to tumor has been associated with longer survival in colorectal cancer (40,41). Moreover, greater lymphocytic reaction has been associated with MSI high (42,43), which, in turn, is associated with longer patient survival (44). Recent studies have further shown that MSI is associated with the CIMP, *BRAF* mutation (26,45), and LINE-1 methylation level (28), and all of these factors (MSI, CIMP, *BRAF* mutation, and LINE-1 methylation) have been independently related with survival of colon cancer patients (46–48). Therefore, numerous pathologic and molecular features (the lymph node count, lymphocytic reactions, MSI, CIMP, *BRAF* mutation, and LINE-1 methylation) could account for the effect of the negative lymph node count. However, none of the previous studies of the number of lymph nodes and patient survival examined the aforementioned molecular features (MSI, CIMP, *BRAF* mutation, and LINE-1 methylation) in colorectal cancer. In our analysis, the benefit associated with higher negative lymph node count remained significant after adjusting the various pathologic and molecular features.

The mechanism underlying the survival advantage associated with the negative lymph node count remains uncertain. Examining more lymph nodes may more accurately identify a metastatic focus and thus, avoiding misclassification of pathologic stage. Although this hypothesis can explain the effect of the negative lymph node count in patients with stages 1–2 cancers, it does not adequately account for the beneficial effect observed in stage 3 or 4 patients. In addition, a greater number of recovered lymph nodes may be an indicator of quality of surgical care or pathology (11,49). However, a recent study has shown that hospitals that examine more lymph nodes in colon resection specimens are not associated with superior patient survival, raising a question on the value of lymph node counts as measure of quality of care (50).

Alternatively, the number of recovered lymph nodes may be an indicator of host immune response to tumor cells (12,51) or reflect some other specific tumoral molecular alteration associated with indolent tumor behavior. In this study, the negative lymph node count was associated with MSI high and CIMP high, both of which have been shown to be independently associated with longer patient survival (44,47,48). Nonetheless, the effect of negative lymph node number on survival was not substantially altered by adjusting for lymphocytic reactions to tumor or tumoral molecular alterations including MSI and CIMP, although other unidentified tumoral feature may still account for the negative lymph node effect.

A location of a lymph node that shows a metastatic tumor may be important in predicting patient outcome. Metastasis to apical lymph node may imply worse outcome. Metastasis to non-regional lymph nodes, such as external iliac and para-aortic nodes, should be classified as distant metastasis (stage 4). However, a study with detailed record of locations of all recovered lymph nodes is difficult to conduct, and needs a close collaboration between surgeons and pathologists. Such collaborative large-scale studies are necessary in this area.

There are limitations in this study. For example, data on cancer treatment were limited. Nonetheless, it is unlikely that chemotherapy use differed substantially according to the number of negative lymph nodes, as such data were not typically used for treatment decision making during the conduct of the study. In addition, beyond cause of mortality, data on cancer recurrences were not available in these cohorts. Nonetheless, given the median survival for metastatic colorectal cancer was approximately 10–12 months during much of the time period

of this study (46), colorectal cancer–specific survival should be a reasonable surrogate for cancer-specific outcomes.

Most patients in this study developed colorectal cancers in 1990s and late 1980s, when the recommendation to obtain at least 12 lymph nodes was not present or widespread. The number of recovered lymph nodes in some stage 2 patients was indeed low; 30 % of stage 2 cases had less than seven lymph nodes examined. Nonetheless, we were indeed able to show that those stage 2 cases with fewer number of nodes experienced a significantly reduced survival, and this relationship between the node count and survival was independent of year of diagnosis, and other clinical, pathologic, and molecular features examined.

There are advantages in using the database of the two independent prospective cohort studies, the Nurses' Health Study and Health Professionals Follow-Up Study to examine prognostic significance of the lymph node count and its interactions with tumoral and host factors. Anthropometric measurements, family history of cancer, other clinical information, pathologic and tumor staging data, and tumoral molecular features were collected prospectively, and entered into the database blinded to patient outcome. Cohort participants who developed colorectal cancer were treated at hospitals throughout the United States, and thus more representative of colorectal cancers in the general population, than studies based on a single to few hospitals. Tumor specimen procurement rate has been 60–70 %, and there were no demographic difference between cases with tumor tissue analyzed and those without tumor tissue analyzed (20). In addition, our rich tumor database enabled us to simultaneously assess pathologic and molecular features of tumor and control for confounding by tumoral variables to some extent. None of the previous studies on lymph node count and patient outcome has examined as many molecular variables as we did in this study.

In summary, our large cohort study suggests that the number of negative lymph nodes is associated with longer survival of colorectal cancer patients, independent of patient, pathologic and molecular characteristics. Future studies are needed to elucidate exact mechanisms by which the lymph node count affects clinical outcome of colorectal cancer.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The lymph node count is associated with survival among colorectal cancer patients.
- ✓ The node count is associated with lymphocytic reaction to colorectal cancer and tumoral microsatellite instability (MSI).
- ✓ MSI and lymphocytic reaction are associated with patient survival.
- ✓ The node count is influenced by other factors including length of resected colorectum.

WHAT IS NEW HERE

- ✓ The negative lymph node count is associated with patient survival, independent of MSI, lymphocytic reaction, and length of colorectum.
- ✓ Lymph node ratio (i.e., positive to total node count ratio) is inversely associated with survival among stage 3 as well as stage 4 patients.
- ✓ There is a stronger beneficial effect of the negative node count in stage 1–2 patients than in stage 3–4 patients.
- ✓ There is no threshold of the negative node count above which its beneficial effect reaches plateau.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Kaplan-Meier survival according to the number of recovered negative lymph nodes in colorectal cancer in all cases (**a**) and stage 2 cases (**b**).

Figure 2.

Smoothing spline plot of unadjusted hazard ratios for colorectal cancer-specific (left panel) and overall mortality (right panel) according to the number of negative lymph nodes: all stages (a), stages 1-2 (b), stage 3 (c), and stage 4 (d). Patients with 0 negative lymph nodes were set as a referent; 95 % confidence intervals are indicated by hatched lines.

Figure 3.

Lymph node ratio (LNR, the number of positive lymph nodes divided by the total number of lymph nodes) and survival of colorectal cancer patients. Smoothing spline plot of unadjusted hazard ratios for colorectal cancer-specific (left panel) and overall mortality (right panel) according to LNR (continuous) among stage 3 (a) and stage 4 (b) patients. Patients with LNR of 1 were set as a referent; 95 % confidence intervals are indicated by hatched lines. Kaplan-Meier curves for colorectal cancer-specific survival (left panel) and overall survival (right panel) in stage 3 colorectal cancer, according to LNR (c). LNR is defined as the number of positive lymph nodes divided by the total number of lymph nodes.

Figure 4.
Extranodal involvement and survival of stage 3 colorectal cancer patients.

Figure 5.

Negative lymph node count and overall mortality in various strata. Adjusted HR with 95 % CI was calculated using the lymph node categorical variable (0–3 vs. 4–6 vs. 7–12 vs. ≥ 13 negative lymph nodes) as an ordinal continuous variable. The HR was calculated across from the bottom category (0–3, referent) to the top category (≥ 13 negative lymph nodes). BMI, body mass index; CI, confidence interval; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-Up Study; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study.

Table 1

Features of colorectal cancer according to the number of negative lymph nodes

Clinical or molecular feature	All cases	No. of negative lymph nodes				P value
		0-3	4-6	7-12	≥13	
Total, <i>n</i>	716	139	128	233	216	
Mean age (years)±s.d.	66.5 ± 8.2	65.9 ± 7.5	66.0 ± 8.2	66.5 ± 8.3	67.4 ± 8.3	0.059
Sex						0.45
Male (HPFS)	333 (47%)	61 (44%)	53 (41%)	113 (48%)	106 (49%)	
Female (NHS)	383 (53%)	78 (56%)	75 (59%)	120 (52%)	110 (51%)	
Body mass index						0.78
< 30 kg/m ²	572 (83%)	113 (84%)	104 (84%)	187 (84%)	168 (81%)	
≥ 30 kg/m ²	117 (17%)	22 (16%)	20 (16%)	35 (16%)	40 (19%)	
Family history of colorectal cancer in first-degree relative(s)						0.004
(-)	541 (76%)	116 (83%)	95 (74%)	159 (68%)	171 (79%)	
(+)	175 (24%)	23 (17%)	33 (26%)	74 (32%)	45 (21%)	
Year of diagnosis						0.019
Before 1995	341 (48%)	79 (57%)	51 (40%)	117 (50%)	94 (44%)	
1995-2002	375 (52%)	60 (43%)	77 (60%)	116 (50%)	122 (56%)	
Tumor location						<0.0001
Proximal colon	331 (47%)	41 (29%)	51 (40%)	103 (45%)	136 (63%)	
Distal colon	237 (33%)	58 (42%)	49 (39%)	78 (34%)	52 (24%)	
Rectum	143 (20%)	40 (29%)	27 (21%)	49 (21%)	27 (13%)	
Length of resected colorectum						<0.0001
< 20 cm	275 (42%)	61 (53%)	61 (54%)	101 (46%)	52 (25%)	
20-39 cm	323 (50%)	49 (43%)	42 (38%)	102 (47%)	130 (63%)	
≥ 40 cm	54 (8.3%)	4 (3.5%)	9 (8.0%)	16 (7.3%)	25 (12%)	
Total no. of lymph nodes						<0.0001
0-3	86 (12%)	86 (62%)	0	0	0	
4-6	107 (15%)	22 (16%)	85 (66%)	0	0	
7-12	257 (36%)	22 (16%)	37 (29%)	198 (85%)	0	
≥ 13	266 (37%)	9 (6.5%)	6 (4.7%)	35 (15%)	216 (100%)	

Clinical or molecular feature	All cases	No. of negative lymph nodes				P value	
		0–3	4–6	7–12	≥13		
No. of positive lymph nodes							
0	446 (62%)	76 (55%)	69 (54%)	150 (64%)	151 (70%)	<0.0001	
1–3	166 (23%)	24 (17%)	37 (29%)	58 (25%)	47 (22%)		
4–6	58 (8.1%)	18 (13%)	13 (10%)	17 (7.3%)	10 (4.6%)		
≥ 7	46 (6.4%)	21 (15%)	9 (7.0%)	8 (3.4%)	8 (3.7%)		
Depth of invasion							
T1	66 (9.7%)	22 (17%)	18 (15%)	16 (7.2%)	10 (4.8%)	0.0015	
T2	150 (22%)	34 (26%)	29 (25%)	48 (22%)	39 (19%)		
T3	427 (63%)	67 (51%)	64 (54%)	149 (67%)	147 (71%)		
T4	36 (5.3%)	8 (6.1%)	7 (5.9%)	10 (4.5%)	11 (5.3%)		
AJCC tumor stage							
I	172 (24%)	43 (31%)	36 (28%)	51 (22%)	42 (19%)	<0.0001	
II	219 (31%)	18 (13%)	22 (17%)	84 (36%)	95 (44%)		
III	212 (30%)	46 (33%)	44 (34%)	68 (29%)	54 (25%)		
IV	81 (11%)	22 (16%)	16 (13%)	24 (10%)	19 (8.8%)		
Unknown	32 (4.5%)	10 (7.2%)	10 (7.8%)	6 (2.6%)	6 (2.8%)	<0.0001	
Tumor size							
≤ 2 cm	75 (11%)	24 (19%)	20 (16%)	22 (10%)	9 (4.3%)		
2–5 cm	412 (61%)	84 (66%)	79 (65%)	136 (62%)	113 (54%)		
>5 cm	193 (28%)	20 (16%)	23 (19%)	62 (28%)	88 (42%)	0.39	
Tumor grade							
Low	624 (91%)	121 (95%)	109 (90%)	208 (92%)	186 (89%)		
High	59 (8.6%)	7 (5.5%)	12 (9.9%)	18 (8.0%)	22 (11%)		
Tumor type							
Signet ring cell	9 (1.3%)	3 (2.2%)	0	4 (1.7%)	2 (0.9%)	0.0035	
Mucinous	88 (12%)	13 (9.4%)	5 (3.9%)	29 (12%)	41 (19%)		
Undifferentiated	16 (2.2%)	1 (0.7%)	5 (3.9%)	5 (2.2%)	5 (2.3%)		
Adenocarcinoma, no special type	603 (84%)	122 (88%)	118 (92%)	195 (84%)	168 (78%)		
Mucinous component							
Absent (0%)	465 (65%)	96 (69%)	101 (79%)	147 (63%)	121 (56%)	0.0004	

Clinical or molecular feature	All cases	No. of negative lymph nodes				P value
		0-3	4-6	7-12	≥13	
1-49%	154 (22%)	27 (19%)	22 (17%)	53 (23%)	52 (24%)	0.55
≥ 50%	97 (14%)	16 (12%)	5 (3.9%)	33 (14%)	43 (20%)	
<i>Signet ring cell component</i>						
Absent (0%)	671 (94%)	129 (93%)	119 (93%)	217 (93%)	206 (95%)	0.31
1-49%	36 (5.0%)	7 (5.0%)	9 (7.0%)	12 (5.2%)	8 (3.7%)	
≥ 50%	9 (1.3%)	3 (2.2%)	0	4 (1.7%)	2 (0.9%)	
<i>Tumor necrosis (extraglandular)</i>						0.0003
Absent	632 (89%)	123 (89%)	119 (94%)	204 (88%)	186 (88%)	
Present	77 (11%)	15 (11%)	8 (6.3%)	29 (12%)	25 (12%)	
<i>Crohn's-like reaction</i>						0.022
Absent/minimal (0)	503 (72%)	115 (85%)	99 (78%)	159 (69%)	130 (62%)	
Mild (1)	144 (21%)	16 (12%)	20 (16%)	53 (23%)	55 (26%)	
Moderate/marked (2-3)	55 (7.8%)	5 (3.7%)	8 (6.3%)	17 (7.4%)	25 (12%)	0.0022
<i>Peritumoral reaction</i>						
Absent/minimal (0)	34 (4.8%)	11 (8.1%)	5 (3.9%)	9 (3.9%)	9 (4.3%)	
Mild (1)	588 (84%)	118 (87%)	110 (87%)	194 (85%)	166 (79%)	0.0001
Moderate/marked (2-3)	80 (11%)	7 (5.2%)	12 (9.5%)	26 (11%)	35 (17%)	
<i>Tumor infiltrating lymphocytes</i>						
Absent/minimal (0)	518 (74%)	109 (80%)	101 (80%)	174 (76%)	134 (64%)	0.0001
Mild (1)	105 (15%)	20 (15%)	16 (13%)	30 (13%)	39 (19%)	
Moderate/marked (2-3)	79 (11%)	7 (5.2%)	10 (7.9%)	25 (11%)	37 (18%)	
<i>Lymphocytic reactions^a</i>						<0.0001
Absent/mild (score ≤3)	452 (64%)	104 (76%)	93 (73%)	143 (62%)	112 (53%)	
Moderate (score 4-5)	150 (21%)	21 (15%)	20 (16%)	56 (24%)	53 (25%)	
Strong (score ≥6)	100 (14%)	11 (8.1%)	14 (11%)	30 (13%)	45 (21%)	<0.0001
<i>MSI</i>						
MSI low/MSS	594 (84%)	132 (95%)	118 (94%)	194 (85%)	150 (71%)	

Clinical or molecular feature	All cases	No. of negative lymph nodes				P value
		0-3	4-6	7-12	≥13	
MSI high	110 (16%)	7 (5.0%)	8 (6.4%)	35 (15%)	60 (29%)	
CIMP						<0.0001
CIMP low/0	581 (84%)	123 (94%)	112 (91%)	193 (84%)	153 (73%)	
CIMP high	113 (16%)	8 (6.1%)	11 (8.9%)	36 (16%)	58 (27%)	
Mean LINE-1 methylation (%) ± s.d.	61.4 ± 9.5	61.7 ± 10.9	60.3 ± 9.9	60.9 ± 9.2	62.5 ± 8.5	0.30
p53 expression						0.017
(-)	403 (57%)	66 (49%)	64 (50%)	140 (61%)	133 (63%)	
(+)	298 (43%)	68 (51%)	63 (50%)	88 (39%)	79 (37%)	
BRAF mutation						0.038
(-)	594 (86%)	119 (88%)	112 (90%)	199 (88%)	164 (80%)	
(+)	98 (14%)	17 (12%)	13 (10%)	27 (12%)	41 (20%)	
KRAS mutation						0.72
(-)	453 (64%)	90 (65%)	82 (65%)	153 (66%)	128 (61%)	
(+)	254 (36%)	48 (35%)	45 (35%)	79 (34%)	82 (39%)	

AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-Up Study; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study.

(%) Indicates the proportion of tumors with a specific clinical, pathologic, or molecular feature in patients with a specific category of negative lymph nodes.

^a Lymphocytic reaction score is based on the sum of scores for Crohn's-like reaction (0-3), peritumoral reaction (0-3), and tumor infiltrating lymphocytes (0-3).

Table 2
Pathologic features of colorectal cancer according to the number of positive lymph nodes

Clinical or molecular feature	All cases	No. of positive lymph nodes				P value
		0	1–3	4–6	≥7	
Total, n	716	446	166	58	46	
<i>Tumor type</i>						0.065
Signet ring cell	9 (1.3%)	4 (0.9%)	3 (1.8%)	0	2 (4.3%)	
Mucinous	88 (12%)	63 (14%)	17 (10%)	2 (3.4%)	6 (13%)	
Undifferentiated	16 (2.2%)	8 (1.8%)	4 (2.4%)	1 (1.7%)	3 (6.5%)	
Adenocarcinoma, no special type	603 (84%)	371 (83%)	142 (86%)	55 (95%)	35 (76%)	
<i>Depth of invasion</i>						< 0.0001
T1	66 (9.7%)	56 (13%)	9 (5.7%)	1 (1.9%)	0	
T2	150 (22%)	120 (28%)	24 (15%)	4 (7.6%)	2 (4.7%)	
T3	427 (63%)	228 (54%)	120 (75%)	43 (81%)	36 (84%)	
T4	36 (5.3%)	20 (4.7%)	6 (3.8%)	5 (9.4%)	5 (12%)	
<i>Tumor size</i>						0.18
≤2cm	75 (11%)	53 (13%)	18 (11%)	2 (3.6%)	2 (4.4%)	
2–5cm	412 (61%)	245 (59%)	100 (63%)	40 (71%)	27 (59%)	
>5cm	193 (28%)	120 (29%)	42 (26%)	14 (25%)	17 (37%)	
<i>Tumor grade</i>						0.0005
Low	624 (91%)	403 (94%)	139 (90%)	51 (88%)	31 (76%)	
High	59 (8.6%)	26 (6.1%)	16 (10%)	7 (12%)	10 (24%)	
<i>Mucinous component</i>						0.27
Absent (0%)	465 (65%)	281 (63%)	112 (67%)	44 (76%)	28 (61%)	
1–49%	154 (22%)	98 (22%)	34 (20%)	12 (21%)	10 (22%)	
≥50%	97 (14%)	67 (15%)	20 (12%)	2 (3.5%)	8 (17%)	
<i>Signet ring cell component</i>						0.012
Absent (0%)	671 (94%)	423 (95%)	155 (93%)	56 (97%)	37 (80%)	
1–49%	36 (5.0%)	19 (4.3%)	8 (4.8%)	2 (3.5%)	7 (15%)	
≥50%	9 (1.3%)	4 (0.9%)	3 (1.8%)	0	2 (4.4%)	
<i>Tumor necrosis (extraglandular)</i>						0.16
Absent	632 (89%)	397 (90%)	149 (90%)	48 (83%)	38 (83%)	

Clinical or molecular feature	All cases	No. of positive lymph nodes				P value
		0	1-3	4-6	≥7	
Present	77 (11%)	43 (9.8%)	16 (9.7%)	10 (17%)	8 (17%)	
<i>Crohn's-like reaction</i>						0.59
Absent/minimal (0)	503 (72%)	316 (72%)	113 (70%)	40 (69%)	34 (74%)	
Mild (1)	144 (21%)	83 (19%)	35 (22%)	15 (26%)	11 (24%)	
Moderate/marked (2-3)	55 (7.8%)	38 (8.7%)	13 (8.1%)	3 (5.2%)	1 (2.2%)	
<i>Peritumoral reaction</i>						0.23
Absent/minimal (0)	34 (4.8%)	15 (3.4%)	13 (8.1%)	4 (6.9%)	2 (4.4%)	
Mild (1)	588 (84%)	367 (84%)	131 (81%)	49 (84%)	41 (89%)	
Moderate/marked (2-3)	80 (11%)	55 (13%)	17 (11%)	5 (8.6%)	3 (6.5%)	
<i>Tumor infiltrating lymphocytes</i>						0.18
Absent/minimal (0)	518 (74%)	320 (73%)	119 (74%)	47 (81%)	32 (70%)	
Mild (1)	105 (15%)	62 (14%)	24 (15%)	11 (19%)	8 (17%)	
Moderate/marked (2-3)	79 (11%)	55 (13%)	18 (11%)	0	6 (13%)	

(%) Indicates the proportion of tumors with a specific clinical, pathologic or molecular feature in patients with a specific category of positive lymph nodes.

Table 3

Number of recovered negative lymph nodes in stages 1–4 colorectal cancer and patient mortality

Number of negative lymph nodes	Total, n	Colorectal cancer-specific mortality				Overall mortality		
		Deaths/ person-years	Univariate HR (95% CI)	Stage- matched HR (95% CI)	Multivariate HR (95% CI)	Deaths/ person-years	Univariate HR (95% CI)	Stage- matched HR (95% CI)
0–3	139 (19%)	56/987	1 (referent)	1 (referent)	1 (referent)	82/987	1 (referent)	1 (referent)
4–6	128 (18%)	39/949	0.69 (0.46–1.04)	0.81 (0.53–1.23)	0.87 (0.56–1.34)	63/949	0.79 (0.57–1.10)	0.86 (0.61–1.21)
7–12	233 (33%)	46/2,111	0.42 (0.28–0.62)	0.54 (0.36–0.81)	0.56 (0.36–0.87)	92/2,111	0.53 (0.39–0.71)	0.60 (0.44–0.82)
≥13	216 (30%)	30/1,948	0.28 (0.18–0.44)	0.38 (0.24–0.60)	0.43 (0.27–0.71)	67/1,948	0.41 (0.30–0.57)	0.48 (0.34–0.67)
P for trend ^a			<0.0001	<0.0001	0.0002		<0.0001	<0.0001

CI, confidence interval; HR, hazard ratio.

The multivariate, stage-matched (stratified) Cox regression model included age, year of diagnosis, sex, family history of colorectal cancer, tumor location, length of resected colorectal cancer, tumor grade, lymphocytic reactions, KRAS, BRAF, p53, LINE-1 methylation, microsatellite instability (MSI), and CpG island methylator phenotype (CIMP).

^aThe lymph node categorical variable was used as an ordinal continuous variable.

Table 4
Stage-specific analysis of the number of negative lymph nodes and colorectal cancer mortality

AJCC tumor stage	No. of cases	HR for colorectal cancer-specific mortality (the number of negative lymph nodes, ≥ 13 vs. 0–3) ^a		HR for overall mortality (the number of negative lymph nodes, ≥ 13 vs. 0–3) ^a	
		Univariate HR (95% CI)	Multivariate HR (95% CI)	Univariate HR (95% CI)	Multivariate HR (95% CI)
Stage 1	172	0.11 (0.023–0.58) <i>P</i> _{trend} =0.009	0.096 (0.019–0.49) <i>P</i> _{trend} =0.005	0.42 (0.19–0.93) <i>P</i> _{trend} =0.033	0.34 (0.15–0.77) <i>P</i> _{trend} =0.009
Stage 2	219	0.16 (0.046–0.55) <i>P</i> _{trend} =0.004	0.19 (0.055–0.65) <i>P</i> _{trend} =0.008	0.43 (0.21–0.87) <i>P</i> _{trend} =0.018	0.36 (0.18–0.74) <i>P</i> _{trend} =0.005
Stage 3	212	0.26 (0.13–0.53) <i>P</i> _{trend} =0.0002	0.31 (0.15–0.65) <i>P</i> _{trend} =0.002	0.30 (0.17–0.51) <i>P</i> _{trend} <0.0001	0.33 (0.19–0.58) <i>P</i> _{trend} =0.0001
Stage 4	81	0.48 (0.26–0.89) <i>P</i> _{trend} =0.021	0.57 (0.28–1.13) <i>P</i> _{trend} =0.11	0.54 (0.29–1.01) <i>P</i> _{trend} =0.054	0.49 (0.25–0.94) <i>P</i> _{trend} =0.031
<i>P</i> for interaction (negative lymph nodes and stage) ^b		0.003	0.002	0.64	0.36

AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio.

The multivariate Cox regression model included the lymph node variable stratified by tumor stages (1–4), age, year of diagnosis, sex, family history of colorectal cancer, tumor location, length of colorectum, grade, lymphocytic reactions, KRAS, BRAF, p53, LINE-1 methylation, microsatellite instability (MSI), and CpG island methylator phenotype (CIMP).

^aThe lymph node ordinal categorical variable (0–3 vs. 4–6 vs. 7–12 vs. ≥ 13) was used as a continuous variable, and HR shown was calculated across from the bottom category (0–3, referent) to the top category (≥ 13 negative lymph nodes).

^bBoth the lymph node and tumor stages (1–4) categories were used as ordinal continuous variables.