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RESEARCH ARTICLE

Tumor Markers and Signatures

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qRT-PCR analysis of CEACAM5, KLK6, SLC35D3, MUC2 and POSTN in colon cancer lymph nodes—An improved method for assessment of tumor stage and prognosis

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Abstract

One fourth of colorectal cancer patients having curative surgery will relapse of which the majority will die. Lymph node (LN) metastasis is the single most important prognostic factor and a key factor when deciding on postoperative treatment. Presently, LN metastases are identified by histopathological examination, a subjective method analyzing only a small LN volume and giving no information on tumor aggressiveness. To better identify patients at risk of relapse we constructed a qRT-PCR test, ColoNode, that determines levels of CEACAM5, KLK6, SLC35D3, MUC2 and POSTN mRNAs. Combined these biomarkers estimate the tumor cell load and aggressiveness allocating patients to risk categories with low (0, −1), medium (1), high (2) and very high (3) risk of recurrence. Here we present result of a prospective, national multicenter study including 196 colon cancer patients from 8 hospitals. On average, 21 LNs/patient, totally 4698 LNs, were examined by both histopathology and ColoNode. At 3-year follow-up, 36 patients had died from colon cancer or lived with recurrence. ColoNode identified all patients that were identified by histopathology and in addition 9 patients who were undetected by histopathology. Thus, 25% of the patients who recurred were identified by ColoNode only. Multivariate Cox regression analysis proved ColoNode (1, 2, 3 vs 0, −1) as a highly significant risk factor with HR 4.24 [95% confidence interval, 1.42-12.69, $P = .01$], while pTN-stage (III vs I/II) lost its univariate significance. In conclusion, ColoNode surpassed histopathology by

Abbreviations: ACT, adjuvant chemotherapy treatment; CC, colon cancer; H&E, hematoxylin and eosin; LN, lymph node; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; TD, tumor deposit.

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identifying a significantly larger number of patients with future relapse and will be a valuable tool for decisions on postoperative treatment.

KEYWORDS
colon cancer, ColoNode, lymph nodes, prognosis, tumor markers

What's new?

In colorectal cancer (CRC), lymph node metastases are a critical prognostic factor following curative surgery. Current approaches to lymph node analysis, however, remain suboptimal for assessing recurrence risk. Here, the authors examined the utility of ColoNode, a novel test for mRNA biomarker detection in lymph nodes, for assessing CRC recurrence risk. ColoNode successfully identified CRC patients at risk of recurrence, doing so with significantly higher accuracy than standard histopathology. ColoNode further allocated patients to different risk groups after curative surgery. The findings suggest that ColoNode can help guide CRC treatment decisions, potentially improving patient quality of life and outcome.

1 | INTRODUCTION

Colorectal cancer is globally one of the most prevalent cancers and a common cause of cancer-related mortality.[1] Histopathological staging of surgically resected localized colon cancer (CC) specimens remains mainly based on the depth of invasion in the intestinal wall (pT) and the number of regional lymph node (LN) metastases (pN). Many studies have been performed to improve the tumor classification system, but the pTN-stage, with various modifications over the years, still forms the basis for risk stratification and decisions on adjuvant chemotherapy treatment (ACT). The Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) updated tumor staging manuals for TNM classification and comments are described by Weiser.[2] European Society for Medical Oncology (ESMO) and American Society of Clinical Oncology (ASCO) guideline updates for diagnosis, treatment and follow-up after curative surgery are presented by Argilés et al[3] and Baxter et al.[4]

LN metastasis is the single most important prognostic risk factor and LNs have long been a focus of efforts to develop the staging system. For example, the number of harvested LNs should be at least 12 per resected specimen to estimate the accurate pN-stage.[5-8] However, there is a substantial risk of missing significant tumor cell dissemination to LNs in stage II CC when only one or a few thin sections are used for staining and light microscopy. Patients with undetected occult tumor cell dissemination will not receive ACT and, thus, be undertreated. Indeed, approximately 25% of patients with negative LNs recure.[9,10] Moreover, if LN metastases are detected in the resected specimen in stage III, the higher the ratio of LN metastases to the total number of harvested LNs, the higher the risk of tumor spread.[11] On the other hand, there will be a considerable number of stage III patients with low LN ratios where the selection of patients to ACT is not obvious, as LNs then also may contain tumor cells with a low-risk profile for distant metastases. In such cases, ACT is most likely of no advantage since the patient may be cured by surgery alone.

Several reports have been published on additional histopathology markers, for example, the importance of primary tumor grade, pT4, lymphovascular invasion (tumor cells in lymphatics or blood vessels), perineural invasion, and tumor deposits (TDs).[12-21] Of these markers, only TDs have emerged as an independent risk factor, which has been incorporated into the TNM AJCC 8th edition as N1c.[2,22] Commonly, the number of positive histopathology markers is included in evaluating the risk of recurrence, where a higher number of risk factors correlates with a higher risk of tumor metastases. It has been suggested that combining the presence of TDs with the number of LN metastases improves the prognostic accuracy of stage III CC.[14,21] Many attempts have been made to analyze LNs for small clusters of tumor cells, that is, isolated tumor cells consisting of up to 20 cells and <0.2 mm. Micrometastases measure ≥0.2 to <2.0 mm in diameter. Adding immunohistochemistry, usually for CEACAM5/CEA and cytokeratins, has not improved their role in prognostication. However, molecular analysis of such cells, usually using reverse transcriptase-polymerase chain reaction (RT-PCR), has improved prognosis.[7,23] A systematic review of 39 studies revealed that molecular tumor-cell detection by RT-PCR in LNs was associated with poor overall survival, disease-specific survival, and disease-free survival.[23] Real-time quantitative RT-PCR analysis is a most useful method for assessment of biomarker mRNA expression. Important advantages compared to histopathology are that the technique is objective and that large tissue volumes, up to the entire LN, can be analyzed. The CEACAM5 mRNA level has proven to be a particularly good proxy for tumor cells in LNs. CEACAM5 mRNA is expressed at high levels in tumor cells, not detected in immune cells, and notably identified all LNs with metastases and/or micrometastases as LNs with high CEACAM5 mRNA levels in a side-by-side comparison between microscopic examination of H&E-stained sections and determination of the CEACAM5 level in an RNA extract from the tissue juxtaposed to the microscopic section.[24-26] However, not all tumor cells have propensity to develop distant metastases. Hence, we argued that markers for aggressiveness were needed to improve prediction of outcome. Genome-wide gene

expression screening of metastases-positive LNs and primary tumors of CC patients identified KLK6, SLC35D3 and POSTN mRNAs as indicators of risk of recurrence.[27,28] Additionally, we found that a high MUC2:CEACAM5 ratio in LNs is a sign of good prognosis.[29] Analysis of the five biomarkers in combination was shown to identify patients at risk of recurrence with higher sensitivity than histopathology, and combined in a formula, these biomarkers allowed allocation of colorectal cancer patients to categories with different risk of recurrence.[28]

The objective of the current study was to validate the findings that detection and characterization of tumor cell aggressiveness in LNs by determination of the expression signature of mRNA for the five biomarkers CEACAM5, KLK6, SLC35D3, POSTN and MUC2 can identify patients who are at risk of recurrence with higher sensitivity than histopathological examination, and, in addition, allows allocation of CC patients to categories with different risk with respect to recurrence in a new, larger clinical material. Here we report the results from a Swedish multicenter study in which LN-by-LN of patients operated for CC were examined both for the signature of the five biomarkers using the ColoNode kit and for presence of metastases by standard histopathology. The capacity of the two methods to identify patients at risk of recurrence was evaluated at 3-year follow-up.

2 | MATERIALS AND METHODS

2.1 | Study design

LNs were collected from patients in whom a locally radical tumor resection for CC was carried out. Eight Swedish hospitals (Umeå University Hospital; Skellefteå Hospital; Blekinge Hospital, Karlskrona; Mälarsjukhuset, Eskilstuna; the County Hospital in Sundsvall; Örnsköldsvik Hospital; Gävle Hospital; Vrinnevisjukhuset, Norrköping) participated in the study. Samples were collected from November 2017 to January 2021. All LNs examined for the presence of disseminated tumor cells by routine histopathology were also analyzed by ColoNode in a double-blinded manner. Compilation of data from the two methods was performed by different researchers (MLH and GL). Codes were not broken until results from both methods were completed to ascertain that the results from one method did not influence the interpretation of the other. LNs were divided into two halves and were given an individual code at the clinical pathology laboratories serving the respective hospital. One half of the LN was used in the clinic for routine histopathology, and results were recorded in a COLONODE-study histopathology form. The other half was sent to the laboratory of the PI of the COLONODE-study at Umeå University, recorded in a biobank, given a new code, and the RNA was extracted and analyzed by ColoNode. Some LNs were too small to be bisected. In these cases, all material that could be spared was cut into sections from paraffin blocks and processed for ColoNode analysis. Samples were stored at −80°C until RNA extraction. Patients received postoperative ACT according to present guidelines in which pN-stage is based on histopathology. ColoNode results were not known to the attending physicians.

2.2 | Patients

Elective surgery for CC was carried out in 196 patients (88 men, 108 women, median age 75.0 years; interquartile range [IQR]: 68.4-80.3; range: 38.1-91.7 years). Patients with other cancers within 3 years before the diagnosis of CC, except for skin cancers other than malign melanoma, were excluded. Two CC patients had a synchronous tumor in the rectum. A locally radical tumor resection was carried out in all patients. The sample size was determined with regards to detecting a difference between ColoNode and histopathology in the sensitivity to predict cancer death or recurrence within 3 years based on the findings in the previous study[28] (with a target power of 80% and a significance level of 5%; McNemar's-test). Fifty-eight tumors were located in the left colon (descending/sigmoid colon) and 138 in the right colon (cecum/appendix/transverse colon). Primary tumor stages were: 12 pT1, 34 pT2, 114 pT3 and 36 pT4. Characterization of tumors into the two categories, high grade (poorly differentiated) and low grade (moderately/well differentiated), whether the tumor was mucinous or not, occurrence of invasion into blood and lymphatic vessels, including extramural venous invasion as well as perineural invasion, was performed as part of the clinical histopathological evaluation according to the recommendations of the Gastrointestinal Pathology-Colon and Rectum—committee,[30] and the Royal College of Pathologists' Dataset for histopathological reporting of colorectal cancer.[31] Ten patients had preoperatively unknown, synchronous distant metastases (M1). They were either found at the abdominal exploration or did small, unspecific nodular changes observed at the preoperative CT scan of the thorax and abdomen shortly afterwards developed into typical lung or liver metastases. The median follow-up time was 35 (IQR: 29-40; range: 7-61) months. No patient was lost to follow-up. Fifty-three patients received postoperative ACT of whom 3 had distant metastases.

2.3 | Lymph nodes

In total, 4698 LNs with a median of 21 LNs/patient (IQR: 16-29; range: 6-77 LNs/patient) were harvested from the resected specimens. Of these, 2450 LNs (median: 12 [IQR: 11-13; range: 3-30] LNs/patient) were given individual codes by the pathologist, bisected, and handled for pairwise comparison of ColoNode analysis of RNA extract from half the LN and routine histopathology analysis of the other half. These bisected LNs are referred to as half-LNs from now on. Another 44 samples were handled in the same way but turned out not to be LNs but TDs (n = 10), adipose tissue or vessels. Residual LN samples contained more than one LN and/or tissue volumes less than half of a LN, and they were mainly samples of sectioned paraffin blocks containing embedded small LNs. Comparisons between the capacity of ColoNode analysis and that of histopathology for the detection of tumor cells in LNs were done on half-LNs with an individual code, while comparisons of the capacity to identify patients at risk of recurrence were based on analyses of all LN samples. Results were based exclusively on investigations of LNs available for analyses with both methods.

Half-LNs were either collected directly after fixation in 10% buffered formalin (n = 967), after formalin-fixation and GEWF-treatment (n = 801), after formalin-fixation and paraffin-embedding (n = 656), and formalin-fixation, GEWF-treatment, and paraffin-embedding (n = 170). Sections were from formalin-fixed only (n = 169) and formalin-fixed, GEWF-treated (n = 455) paraffin-embedded LNs. The weight of half-LNs with individual code was on average 46 mg with a wide range of 1.5 to 1183 mg (IQR: 22-102 mg). The average weight of formalin-fixed paraffin-embedded LNs was lower than that of LNs that had not been paraffin-embedded (median 21 and 68 mg, respectively; $P < .0001$), which most likely is the consequence of the dehydration in the paraffin-embedding procedure.

2.4 | Primary CC tumors

Primary tumor tissue pieces were obtained from 171 patients. Ten of the tumors were stage pT1, 25 pT2, 101 pT3 and 35 pT4. Ninety-five samples were formalin-fixed only, 43 were formalin-fixed and GEWF-treated, and 33 were formalin-fixed and paraffin-embedded.

2.5 | RNA preparation

RNA was extracted using High Pure FFPET RNA Isolation Kit (Lifescience, Roche, Basel, Switzerland; Cat. No. 06650775001). Half-LNs and primary tumor tissue samples were homogenized before RNA extraction. Paraffin was removed from paraffin-embedded samples before homogenization. The same procedure was used for formalin-fixed only and formalin-fixed GEWF-treated tissues. See Supplementary Material and Methods for details of the homogenization and RNA extraction procedure. Concentrations and purity of RNA samples were determined by measuring optical density at 260 nm (OD260), OD280 and OD230 using a NanoDrop ND-2000 spectrophotometer V1.4.1 (Thermo-Fisher-Scientific, Waltham, Massachusetts). There was a 2-fold difference in yield of total-RNA per mg tissue between formalin-fixed, paraffin-embedded half-LNs and half-LN samples that were formalin-fixed only. The median yield was 1734 (IQR: 1027-2534) ng RNA/mg tissue for formalin-fixed paraffin-embedded samples and 871 (IQR: 453-1485) ng RNA/mg tissue for LN samples that had not been paraffin-embedded before RNA extraction. This difference was compatible with the overall lower weight of formalin-fixed paraffin-embedded samples. Primary tumor tissue displayed the same pattern, with a median yield of 3224 (IQR: 2665-4435) ng total-RNA/mg tissue from biopsies that had been paraffin-embedded and a median of 1925 (IQR: 1373-2263) from biopsies that had not been paraffin-embedded. The purity, determined as OD260/OD280 and OD260/OD230, was generally high and similar for all the LN sample types (Table S1). The OD260/OD280 value for all LN sample RNA extracts was on average 2.00 (IQR: 1.96-2.03) and the OD260/OD230 value was 2.07 (IQR: 1.86-2.18). The OD260/OD280 and OD260/OD230 values of tumor samples were 2.01 (IQR: 1.98-2.04) and 2.20 (IQR: 2.15-2.24). RNA was stored at −80°C in RNase-free water containing RNasin ribonuclease inhibitor (Promega, Madison, Wisconsin).

2.6 | Gene expression analysis by real-time qRT-PCR

Quantification of 18S rRNA and mRNAs for CEACAM5, KLK6, SLC35D3, POSTN and MUC2 was done in total-RNA extracts using a ColoNode two-triplex qRT-PCR kit (Cat. No. CN201604, HiloProbe, Umeå, Sweden). Design and technical performance of the ColoNode assay are described in Supplementary Material and Methods. RNA extracts with concentrations <1000 ng total-RNA/µL were analyzed undiluted. Samples with higher RNA concentrations were diluted 1:10 with RNase-free water. Emission from reporter dyes released from the specific probes was measured by a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts). qRT-PCR results are delivered as mean concentration of RNA copies/µL. CEACAM5 mRNA levels were calculated by normalization to the 18S rRNA content in the sample. Risk group value was calculated for all samples with CEACAM5 mRNA levels above background (1×10^{-8} CEACAM5 mRNA copies/18S rRNA copy) according to the formula: SLC35D3 (expressed = 1/not expressed = 0) + KLK6 (expressed = 1/not expressed = 0) + POSTN/18S rRNA (level above cut-off = 1/level below cut-off = 0) − MUC2/CEACAM5 (level above cut-off = 1/level below cut-off = 0) which gives either of the values −1 and 0 = Low risk, 1 = Medium risk, 2 = High risk and 3 = Very high risk.[28] Based on 18S rRNA concentrations, the quality of the extracted LN samples was generally high. Ninety-eight percent of the samples (3180/3246) had 18S rRNA concentrations ≥10^8 copies/µL. The average yield of 18S rRNA was 1.8×10^7 copies/ng total-RNA with a median of 1.5×10^7 (IQR: 7.2×10^6-2.6×10^7; n = 1772), 2.8×10^7 (IQR: 1.3×10^7-5.8×10^7; n = 826) and 1.2×10^7 (IQR: 4.4×10^6-2.9×10^7; n = 630) 18S rRNA copies/ng total RNA in formalin-fixed half-LNs, formalin-fixed and paraffin-embedded half-LNs, and sections of formalin-fixed paraffin-embedded samples, respectively.

2.7 | Histopathological examination

Standard histopathology examination was done by light microscopic inspection of one to three 4 µm H&E-stained sections of formalin-fixed, paraffin-embedded LNs. These sections were used in routine histopathology for determination of pN-stage of the patients at the Clinical Pathology units of six Swedish hospitals serving the hospitals where surgery was performed (Umeå University Hospital, Sundsvall Hospital, Linköping University Hospital, Blekinge Hospital, Gävle Hospital, and Unilabs in Eskilstuna). Stained sections from all LNs fulfilled the criteria for identifying tumor cells in LNs, as described in the evaluation protocols for CC tumor resections given in the Swedish National Health Care Program for Colon Cancer.[30-32] Analyses were performed in a double-blinded manner relative to ColoNode analyses.

In addition to the 4698 LNs analyzed by both H&E and ColoNode, 267 LNs (5.4%) were analyzed by H&E only. They were from 91 patients with an average of 2 (IQR: 1-4) LNs/patient. The results from these additional analyses were disregarded here since they did not follow the study protocol and it is unknown which features these LNs would have shown in the ColoNode analysis.

2.8 | Statistical analysis

GraphPad Prism 6 (GraphPad Software, San Diego, California) was used for statistical analysis of differences in CEACAM5 mRNA levels and RNA yields by Mann-Whitney *U* test and for cross-sectional comparisons between CEACAM5 levels and H&E result as well as identification of CC patients who had recurrence. The ColoNode risk group was compared to H&E by two-tailed Chi-square test. SPSS version 27 (IBM Corporation, Armonk, New York) was used for statistical analyses of differences in disease-free survival and risk of recurrent disease after surgery between patient groups. Calculations were performed according to the Kaplan-Meier survival model in combination with the Mantel-Cox log rank test and univariate Cox's regression analysis. The risk group low (0, −1) was used as baseline in analyses of ColoNode results. Variables such as covariates of survival were evaluated using the backward elimination model in multivariate Cox's regression analysis. Variables were selected by two strategies: (1) variables that had a hazard ratio >2 and a *P*-value <.05 in univariate analysis and (2) the panel of variables examined in clinical routine examination plus age and gender. The criterion for transfer from one level of multivariate analysis to the next was a hazard ratio >2 and a *P*-value <.1. Missing values for variables was <2.3%. Patients who died from causes other than CC were considered disease-free. Descriptive values of risk and survival time are given as mean and 95% confidence interval (CI). Other descriptive values are given as median and IQR. Two-tailed analysis was used throughout. A *P*-value <.05 was considered statistically significant.

3 | RESULTS

The ColoNode assay determines: (1) if the patient's regional LNs harbor tumor cells derived from the large intestine or not, and (2) if the tumor cells in the LNs are aggressive, that is, could cause relapse, or not. A CEACAM5 mRNA level >1 × 10^{-8} copies/18S rRNA copy indicates the presence of tumor cells.[26,29] Tumor aggressiveness is

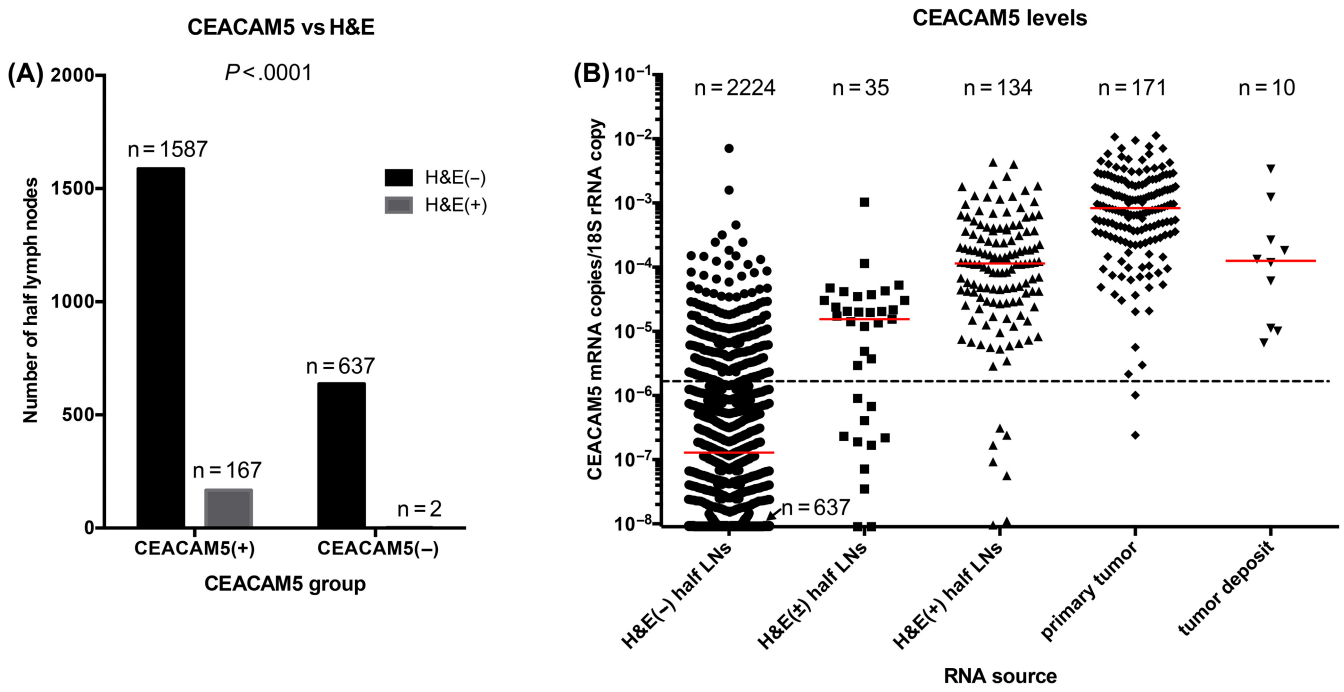


FIGURE 1 Comparison between CEACAM5 positivity and H&E positivity in bisected lymph nodes from colon cancer patients. (A) 2393 lymph nodes from 196 colon cancer patients were analyzed by ColoNode and histopathology. One half of each node was homogenized, RNA-extracted, and an aliquot was analyzed by the ColoNode-assay. The other half of the node was subjected to histopathology. The cut-off value for the ColoNode assay was 1.0 × 10^{-8} mRNA copies/18S rRNA copy. Black bars indicate H&E-negative lymph nodes, gray bars indicate H&E-positive lymph nodes. The *P*-value for comparison between ColoNode and histopathology was determined by two-sided Chi-square test. (B) CEACAM5 levels in lymph nodes, the primary tumor, and tumor deposits from colon cancer patients. The number of lymph nodes in each category is indicated in the figure. Note that 637 half lymph nodes were judged to be negative by ColoNode. Horizontal bars indicate median CEACAM5 levels. Hatched line indicates the previous "clinical cut-off" for CEACAM5. CEACAM5(+), CEACAM5 level ≥1.0 × 10^{-8} RNA copies/18S rRNA copy; CEACAM5(−), CEACAM5 level <1.0 × 10^{-8} mRNA copies/18S rRNA copy; H&E(+), metastasis detected by histopathology; H&E(±), micrometastasis detected by histopathology; H&E(−), no metastasis or micrometastasis detected.

determined by the expression levels of four biomarker mRNAs combined in a formula.[28] Each individual LN from a patient is assigned a risk factor, where (−1) and (0) combined = low risk; (1) = medium risk; (2) = high risk; and (3) = very high risk. The patient is then assigned a risk group based on the highest risk group found among the LNs of the patient.

3.1 | Sensitivity of tumor cell detection in LNs from CC patients

The results obtained by ColoNode were compared to those obtained by routine histopathology. For comparison between the two methods, only LNs that had been bisected were included. A total of 2393 LNs from 196 CC patients were compared. Figure 1A shows that ColoNode is much more sensitive than histopathology in detecting tumor cells. Thus, tumor cells were detected in 73.3% (1754/2393) of the LNs by ColoNode compared to 7.1% (169/2393) by histopathology. The difference is highly significant ($P < .0001$). Whether in fact *all* regional LNs contain tumor cells but in very low numbers cannot be determined because the lower limit for detection is set by the highest CEACAM5 mRNA level in LNs from control patients.[26,29] The CEACAM5 mRNA levels in half-LNs are distributed over a very wide range from 1×10^{-8} to 8×10^{-2} mRNA copies/18S rRNA copy with a median value of 1.2×10^{-7} (Figure 1B). Note that the CEACAM5 levels of the LNs in which metastases were detected by histopathology had high CEACAM5 levels in almost all cases (median 1.1×10^{-4} CEACAM5 mRNA copies/18S rRNA copy). Moreover, a large fraction of the histopathology-negative LNs (353/1754 = 20.3%) had CEACAM5 levels above the "clinical cut-off"[29] (dashed line in Figure 1B), and were undetected with by histopathology.

3.2 | Risk group analysis of CC patients

The distribution of risk factors in LNs varies considerably from one patient to another. Figure 2 shows the patterns of four patients with very high risk and one patient with low risk. LNs identified as metastatic by histopathology are indicated. One risk factor (3) node, one risk factor (2) node and 11 risk factor (1) nodes were *not* detected by histopathology in the four high risk patients. Note that in almost all patients there were a few LNs that lacked tumor cells as determined

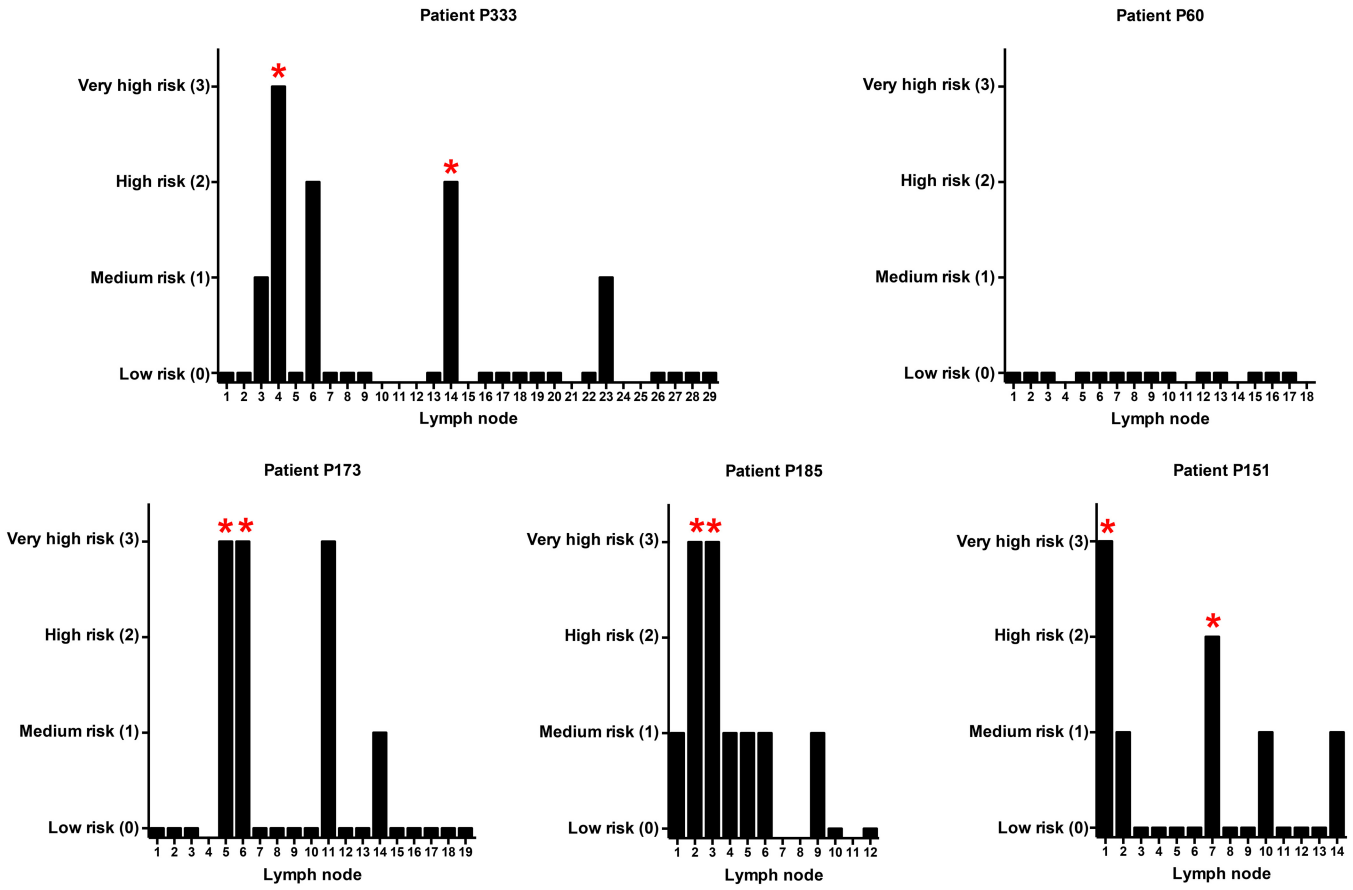
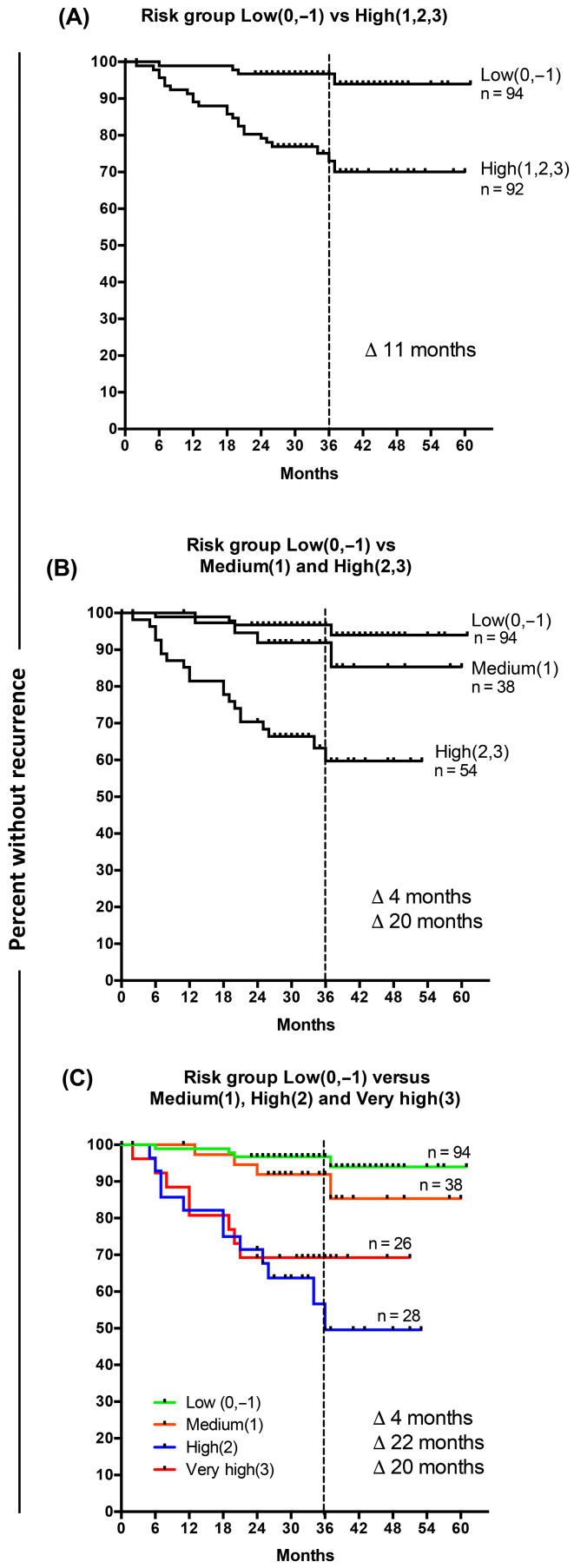


FIGURE 2 Risk group classification of colon cancer patients—five examples. Four patients were classified as group (3) and one patient as group (0) based on the value of the highest lymph node risk group. The risk group value of individual lymph nodes is shown as black bars. Lymph nodes identified by histopathology are indicated by a star above the bar. Lack of a black bar indicates that the lymph node lacked tumor cells as detected by ColoNode.



by the CEACAM5 mRNA level. Ninety-six patients (49.0%) were group (0, −1), 42 patients (21.4%) group (1), 30 patients (15.3%) group (2) and 28 patients (14.3%) group (3).

Using relapse of cancer or cancer-specific death after on average 3 years as the primary outcome measure, we compared ColoNode with histopathology for ability to identify patients at risk of relapse. A patient was judged to be at risk of relapse if the risk group was (1) or higher while patients with risk group (0, −1) were assessed as likely cured by surgery alone. Figure 3A shows cumulative recurrence curves for patients without distant metastasis at surgery according to Kaplan-Meier using risk groups (1-3) as the *positive group* and risk group (0, −1) as the *negative group*. At 36 months, 97% of the risk group (0, −1) patients had no detectable recurrence while 30% of risk group (1-3) patients had tumor recurrence. This difference is statistically highly significant ($P < .0001$) with a hazard ratio of 6.84 (95% CI, 2.37-19.71) (Table 1). The mean survival difference was 11 months ($P < .001$).

In comparison to histopathology, ColoNode was more sensitive in detecting patients who recurred, as it identified 9 patients who relapsed that were undetected by histopathology. Four of these patients were risk group (2) and 5 were risk group (1). Histopathology did not identify any patient who relapsed that was not identified by ColoNode. Twenty-one patients were detected by both methods, of which almost all were risk group (2) or (3). Six patients (3.1%) had recurrence even though no aggressive tumor cells were detected by ColoNode, and the patients hence were allocated to risk group (0, −1). None of these patients were judged to have metastatic LNs by histopathology either.

Univariate analysis for covariation to survival showed that both the ColoNode-positive risk group and TNM-stage III were strong indicators of poor prognosis (Table 1). However, multivariate analysis including factors that are examined during histopathological examination, that is, primary tumor stage, pTN-stage, mucinous primary tumor, TDs, lymphovascular invasion and perineural invasion, revealed that ColoNode is the strongest prognostic factor of all the variables (Table S2) as well as among those that were significant in univariate analysis (Table 1). Thus, ColoNode turned out to be superior to pTN-staging giving a *P*-value of .01 with a hazard ratio of 4.24 (95% CI,

FIGURE 3 Cumulative recurrence curves according to Kaplan-Meier for colon cancer patients belonging to ColoNode low-risk group (0, −1) and the medium and high-risk groups (1), (2) or (3). All colon cancer patients except 10 TNM stage IV patients were subjected to analysis (n = 186). (A) Comparison between the low-risk group (0, −1) and risk groups (1, 2, 3). The difference between the two groups had a *P*-value of <.001 by the log rank test. Hazard ratio was 6.84 (95% CI, 2.37-19.71). (B) Comparison between low-risk group (0, −1), medium-risk group (1) and combined high and very high-risk group (2, 3). (C) Comparison between all four risk groups. The numbers next to the curves indicate the number of patients in the risk group. The difference between mean survival time without recurrence between the risk groups and the low (0, −1) group are given as Δ-values. The dashed line indicates 3 years of observation.

TABLE 1 Cox proportional hazard regression analysis of demographic, clinical and prognostic factors for patients with colon cancer.

	Univariate analysis			Multivariate analysis 1[a]			Multivariate analysis 2[b]		
Variable	n	Hazard ratio (95% CI)	*P*-value	n	Hazard ratio (95% CI)	*P*-value	n	Hazard ratio (95% CI)	*P*-value
ColoNode risk group Medium-very high (1, 2, 3) vs low (0, −1)	92/94	6.84 (2.37-19.71)	.0001	91/92	3.24 (0.94-11.20)	.06	91/92	4.24 (1.42-12.69)	.01
pTN-stage[c] (III vs I/II)	51/135	5.84 (2.68-12.69)	.0001	51/132	1.54 (0.55-4.29)	.41			
Lymphovascular invasion (yes vs no)	74/112	4.86 (2.12-11.12)	.0001	73/110	2.25 (0.88-5.73)	.09	73/110	2.66 (1.10-6.46)	.03
Perineural invasion (yes vs no)	24/160	4.52 (2.08-9.83)	.0001	24/159	2.29 (0.96-5.49)	.06	24/159	2.52 (1.09-5.80)	.03
Primary tumor stage (T4 vs T1, T2, T3)	33/153	3.05 (1.40-6.61)	.005	33/150	1.18 (0.50-2.78)	.70	33/150	1.32 (0.56-3.09)	.52
Tumor deposit (yes vs no)	15/169	3.14 (1.27-7.74)	.013	14/169	1.68 (0.66-4.27)	.28			
Age (≤75 vs >75 years)	93/93	0.48 (0.22-1.05)	.07						
Mucinous primary tumor (yes vs no)	36/148	1.77 (0.78-4.02)	.17						
Site of lesion (left colon vs right colon)	52/134	1.51 (0.70-3.27)	.30						
Gender (male vs female)	80/106	1.45 (0.69-3.06)	.33						
Primary tumor grade (high vs low)	53/129	1.16 (0.53-2.57)	.71						

[a]Criterion for selection for multivariate analysis was a hazard ratio >2 and a *P*-value <.05 in univariate analysis.
[b]Criterion for selection for follow-up multivariate analysis was a hazard ratio >2 and a *P*-value <.1. Primary tumor stage was selected as representative of nonsignificant parameter for estimation of hazard.
[c]pTN-stage is based on presence (III) or absence (I/II) of lymph node metastasis.

1.42-12.69; Table 1). Multivariate analysis further revealed that lymphovascular invasion and perineural invasion of the primary tumor were independent risk factors while primary tumor stage (pT4 vs pT1-pT3) was not (Table 1).

3.3 | Micrometastases

Seventeen CC patients had one or more LNs with micrometastases as detected by histopathology. The total number was 42 of 3153 investigated LNs (1.3%). Does the presence of micrometastases affect the risk group classification by ColoNode? It did not upgrade the risk group in 15 of 17 patients because one or more LNs from the patient had the same or higher risk group. However, in two patients the LN with micrometastases did upgrade the risk group of the patients. In both cases the risk group was upgraded from (1) to (2).

3.4 | Tumor deposits

A total of 10 TDs from 8 patients were detected by histopathology among samples marked with individual code. The median CEACAM5 level in the TDs was almost identical to that of the LNs judged metastases-positive by histopathology (median: 1.24×10^{-4} and 1.14×10^{-4} mRNA copies/18S rRNA copy, respectively) but 7 times lower than that of the primary tumors (median: 8.32×10^{-4} mRNA copies/18S rRNA copy) (Figure 1B). The difference between CEACAM5 levels in TD and primary tumor was significant ($P = .0036$). In four patients the risk group was the same for TD and the highest LN, in two patients the risk group was higher in LNs than in TDs and in two patients lower. Thus, TDs show great similarity to metastatic LNs.

3.5 | Postoperative adjuvant treatment

Figure 3B,C show the same dataset as in Figure 3A now divided into individual risk groups. In Figure 3B, risk group (2) and (3) are combined showing the expected pattern with the two high-risk groups having a worse prognosis with shorter disease-free survival time and higher hazard ratio compared to the medium-risk group (Figure 3B and Table S3). Unexpectedly, further division showed that risk group (3) patients had a lower frequency of recurrence than risk group (2) patients (Figure 3C and Table S3)!

Figure 4 shows the result when patients who had received ACT (n = 50) and those who had not (n = 136) were analyzed separately. Both groups showed a significant difference in disease-free survival time between patients with low risk (0, −1) and patients with higher risk, that is, groups (1), (2) and (3) (*P*-values .013 and .011 for ACT treated and untreated patients, respectively).The difference in recurrence between risk group (2) and (3) was only marginal in the

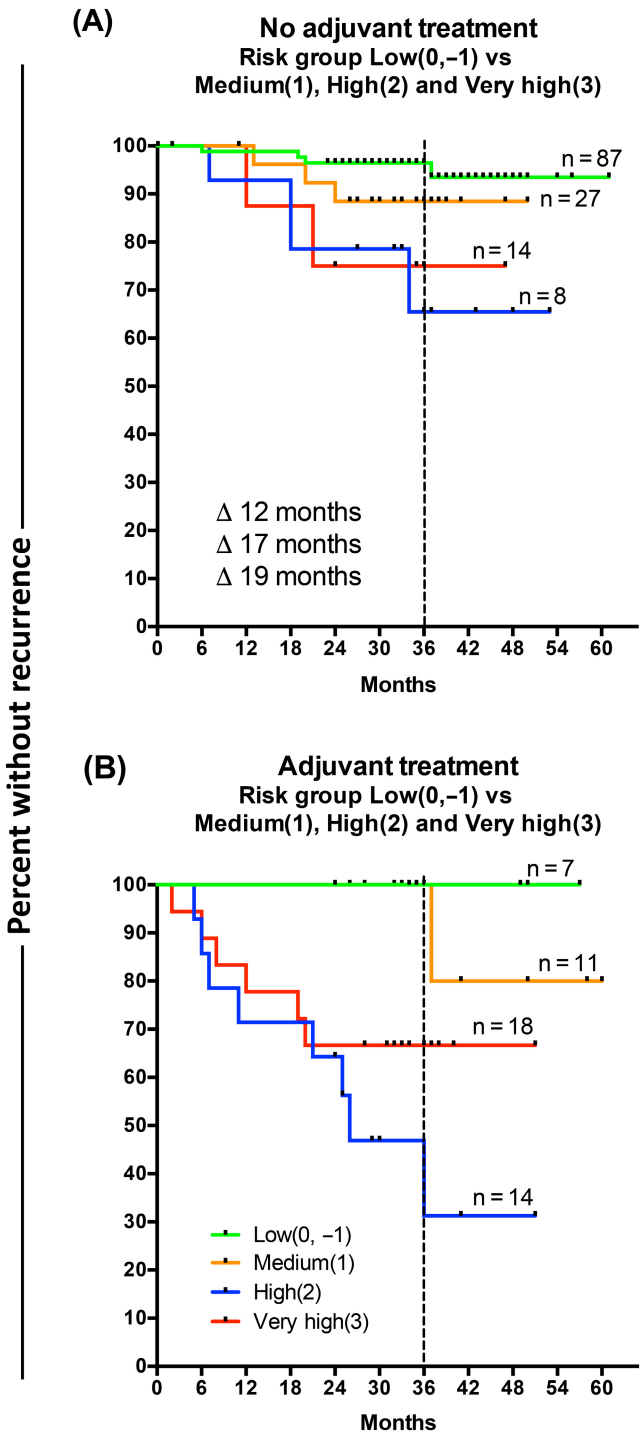


FIGURE 4 Cumulative recurrence curves according to Kaplan-Meier of colon cancer patients who did or did not receive postoperative adjuvant chemotherapy. (A) Colon cancer patients who did *not* receive adjuvant chemotherapy and who were not in TNM stage IV were analyzed (n = 136). Note that the severity grading by ColoNode is as expected. (B) Colon cancer patients that had received adjuvant chemotherapy and who were not in stage IV were analyzed (n = 50). Note that risk group (3) patients appear to have been more successfully treated than risk group (2) patients. The difference between mean survival time without recurrence between the risk groups and the low (0, −1) group are given as Δ-values in (A). The number of patients in the risk groups of those who had received ACT were too small to allow calculations of disease-free survival time according to the Kaplan-Meier model. See legend to Figure 3 for further information.

untreated group (Figure 4A) while risk group (2) had a worse prognosis compared to risk group (3) in the ACT treated group (Figure 4B). Unfortunately, the clinical material was not large enough to allow statistical analysis of risk groups (1), (2) and (3) separately. The observed unexpected pattern could be a consequence of ACT treatment or that patients selected for ACT treatment have specific features.

3.6 | Risk group classification of the primary tumor in comparison to classification of LNs

It could be argued that it would be enough to perform the ColoNode risk group classification of the patient's primary tumor. As shown in Figure 5, there is no correlation between the risk group of the patient's primary tumor and the risk group of the highest LN. Usually the risk group of the primary tumor is high while it varies considerably in LNs.

4 | DISCUSSION

The most important finding in our study is that classification of LNs by ColoNode risk group surpassed pTN-stage by histopathology in identification of patients at risk of recurrence. The main reasons for this are that ColoNode is a highly sensitive and reliable test to determine (1) if tumor cells are present in the patient's LNs, and (2) if the tumor is prone to give rise to distant metastases. ColoNode detected approximately 10 times as many LNs harboring tumor cells compared to histopathology due to the volume factor and the high sensitivity of the CEACAM5 mRNA assay. The higher sensitivity of ColoNode compared to histopathology is underscored by the finding that as much as 20% of the H&E(−) LNs had the same or higher CEACAM5 levels

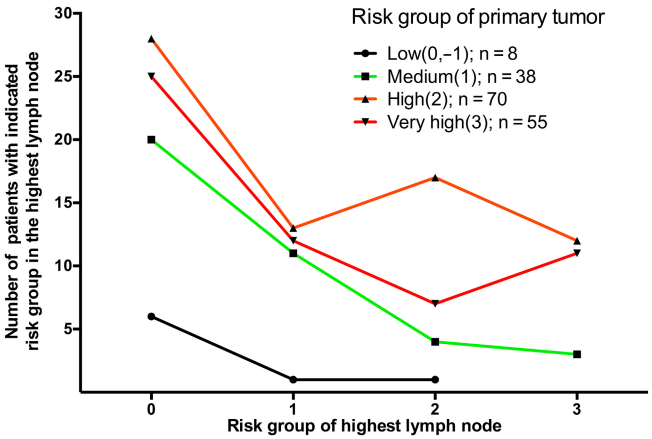


FIGURE 5 Comparison between patient's risk group of the primary tumor and the risk group of the lymph nodes. One hundred and seventy-one primary tumors from the group of 196 colon cancer patients included in the study group were available for analysis. There is no correlation between the risk groups determined in the two tumor cell sources. Analysis of primary tumors by the ColoNode aggressiveness factors cannot substitute for lymph node analysis.

as those in LNs where metastases were observed ($P < .0001$). ColoNode characterizes the tumor cells in the LN plus the tumor cell environment with respect to propensity to form distant metastases. Tumor-cell-derived KLK6 and SLC35D3 and fibroblast-derived POSTN promote formation of distant metastases while MUC2 probably has the opposite effect.[26-28] These biomarker mRNAs provide another quality to LN-analysis, namely measurement of functional properties such as ability to leave the intestine and the regional LNs and go to secondary sites like the liver and lungs to multiply there.

Ideally, one should homogenize the entire LN to avoid that one or more metastasis/micrometastasis/tumor cell cluster is missed because it is in the half that is not subjected to analysis. However, analysis of half a node goes a long way and can suffice in comparative studies. Homogenates can be stored in a frozen state for a long time allowing for reanalysis if needed. A drawback of the technique is that homogenization and RNA extraction is relatively labor intense and requires qualified laboratory personnel. We experienced that the workload is less if extraction is initiated directly after harvest from the formalin-fixed specimen, that is, before dehydration and paraffin embedding. Automation of RNA extraction would be desirable for large scale implementation of ColoNode analysis.

In a situation where only micrometastases are detected the clinician faces a demanding situation: should this patient be recommended ACT or not? ColoNode results may be very helpful here. Even relatively few cells may be of the aggressive type giving rise to distant metastases. These cells will eventually be identified as risk group (1), (2) or (3) and hence classifying the patient as a risk patient.

The finding that TDs show great resemblance to metastatic LNs suggests that TDs could be handled by ColoNode risk group classification without need of discriminating between TDs and LNs. However, the number of TDs studied here is limited and further investigations are needed.

ColoNode, like histopathology, did not detect 6 patients that relapsed within the 3-year follow-up period. In five of the six patients, LNs with suspected metastases on preoperative abdominal CT scan were described by the radiologist (cN1-2), but no such positive LNs could be identified in the resected specimens and were therefore not available for analysis by ColoNode and histopathology. Four of the six patients had lymphovascular invasion known to be a risk factor for CC.[13,18] It is possible that in these cases aggressive tumor cells had bypassed the regional LNs by hematogenic dissemination, causing relapse at secondary sites. However, this explanation seems less likely because the frequency of patients with lymphovascular invasion was the same as in the entire clinical material (66%). Finally, LNs with aggressiveness factors may have been undetected because of lack of CEACAM5 mRNA in the tumor cells. This cannot be the case here. All six patients had CEACAM5 mRNA in the majority of the analyzed LNs and three of them had LNs with very high levels. It is a possibility that other biomarkers indicating risk of recurrence are expressed in tumor cells in these LNs, for instance the stem cell marker LGR5 or the chemokine CXCL16.[33,34] Further studies may shed light on this question.

A patient without distant metastasis at surgery with risk group (2) or (3) is at very high risk of recurrence (frequency: 37%) while a patient with risk group (0, −1) has a low risk of recurrence (4.3%) (Table S3). Thus, ColoNode analysis proved to have high efficiency in identifying patients with both types of outcomes. The frequency of recurrency in risk group (1) was 11%. It would be desirable to increase the accuracy in identifying risk patients in this risk group. This could possibly be achieved by taking the number of LNs with risk factor 1 into consideration. However, frequency of recurrence in all three risk groups are likely to increase with longer follow-up time. Obviously, ACT treatment also impacts on these frequencies.

In conclusion, we anticipate that ColoNode will be used as an important complement to histopathology for LN analysis, possibly replacing the latter in the clinical routine.

AUTHOR CONTRIBUTIONS

Conceptualization: Marie-Louise Hammarström, Gudrun Lindmark, Sten Hammarström, Lina Olsson, Anne Israelsson, Basel Sitohy, Mattias Söderholm, Thorbjörn Sakari, George Dafnis, Chih-Han Kung, Martin Rutegård, Måns Muhrbeck, Annamaria Turi, Pehr Forsberg, Michiel Dooper, and Johanna Nordmyr. *Design of the study*: Marie-Louise Hammarström, Gudrun Lindmark, and Sten Hammarström. *Recruitment of patients, collection of clinical data and follow-up*: Mattias Söderholm, Thorbjörn Sakari, George Dafnis, Susanne Skovsted, Maria Walldén, Chih-Han Kung, Martin Rutegård, and Måns Muhrbeck. *Histopathological evaluation*: Annamaria Turi, Pehr Forsberg, Michiel Dooper, and Johanna Nordmyr. *Experimental work and data compilation*: Anne Israelsson, Joel Blomqvist, Sara Kero, Tamer Roshdy, and Jessica Isaksson. *Data curation*: Marie-Louise Hammarström, Gudrun Lindmark, Anne Israelsson, and Basel Sitohy. *Project administration*: Marie-Louise Hammarström, Anne Israelsson, and Lina Olsson. *Validation*: Marie-Louise Hammarström, Gudrun Lindmark, and Sten Hammarström. *Visualization*: Marie-Louise Hammarström and Sten Hammarström. *Funding acquisition*: Marie-Louise Hammarström, Gudrun Lindmark, Sten Hammarström, Lina Olsson, and Basel Sitohy. *Writing of original draft*: Marie-Louise Hammarström, Sten Hammarström, and Gudrun Lindmark. The work reported in the paper has been performed by the authors, unless clearly specified in the text. All authors reviewed the manuscript and approved that the final version was submitted.

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CONFLICT OF INTEREST STATEMENT

Lina Olsson is cofounder, minority shareholder and since October 2018 fully employed by HiloProbe AB. Anne Israelsson and Gudrun Lindmark are cofounders, minority shareholders and partly employed by HiloProbe AB as of October 2018. Marie-Louise Hammarström is cofounder, minority shareholder and is partially (20%) supported by HiloProbe AB as of October 2018. Sten Hammarström is cofounder, minority shareholder and previously partly employed by HiloProbe AB. Joel Blomqvist and Sara Kero are fully employed by HiloProbe AB since March and June 2022, respectively. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures involving human participants were performed in accordance with the ethical standards of the institutional research committee and with the Helsinki Declaration. LNs and biopsies of the primary tumor were collected after patients gave written, informed consent. The study was approved by the Regional Ethics Review Board in Umeå and the Swedish Ethical Review Authority. Registration numbers: Dnr 2017-71-31M, main, date of approval 7 April 2017; Additional hospital approvals: Dnr 2017-226-32M, Dnr 2018-201-32M, Dnr 2018-499-32M, Dnr 2019-03038 and Dnr 2019-01601.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209-249.
2. Weiser MR. AJCC 8th edition: colorectal cancer. *Ann Surg Oncol*. 2018;25:1454-1455.
3. Argilés G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2020;31:1291-1305.
4. Baxter NN, Kennedy EB, Bergsland E, et al. Adjuvant therapy for stage II colon cancer: ASCO guideline update. *J Clin Oncol*. 2020;40:892-910.
5. Chang GJ, Rodriguez-Bigas MA, Skibber JM, Moyer VA. Lymph node evaluation and survival after curative resection of colon cancer: systematic review. *J Natl Cancer Inst*. 2007;99:433-441.
6. Iddings D, Bilchik A. The biologic significance of micrometastatic disease and sentinel lymph node technology on colorectal cancer. *J Surg Oncol*. 2007;96:671-677.
7. Nicastri DG, Doucette JT, Godfrey TE, Hughes SJ. Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature. *J Mol Diagn*. 2007;9:563-571.

8. Tsai HL, Huang CW, Yeh YS, et al. Factors affecting number of lymph nodes harvested and the impact of examining a minimum of 12 lymph nodes in stage I-III colorectal cancer patients: a retrospective single institution cohort study of 1167 consecutive patients. *BMC Surg*. 2016;16:17.
9. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics. *CA Cancer J Clin*. 2007;57:43-66.
10. Böckelman C, Engelmann BE, Kaprio T, Hansen TF, Glimelius B. Risk of recurrence in patients with colon cancer stage II and III: a systematic review and meta-analysis of recent literature. *Acta Oncol*. 2015;54:5-16.
11. Nguyen AT, Luu M, Nguyen VP, et al. Quantitative nodal burden and mortality across solidB cancers. *J Natl Cancer Inst*. 2022;114:1003-1011.
12. Huang A, Yang Y, Shi JY, et al. Mucinous adenocarcinoma: a unique clinicopathological subtype in colorectal cancer. *World J Gastrointest Surg*. 2021;13:1567-1583.
13. Leijssen LGJ, Dinaux AM, Amri R, et al. Impact of intramural and extramural vascular invasion on stage II-III colon cancer outcomes. *J Surg Oncol*. 2019;119:749-757.
14. Brouwer NPM, Nagtegaal ID. Tumor deposits improve staging in colon cancer: what are the next steps? *Ann Oncol*. 2021;32:1209-1211.
15. Kataoka M, Hirano Y, Ishii T, et al. Impact of lymphovascular invasion in patients with stage II colorectal cancer: a propensity score-matched study. *In Vivo*. 2021;35:525-531.
16. McClelland D, Murray GI. A comprehensive study of extramural venous invasion in colorectal cancer. *PloS One*. 2015;10:e0144987.
17. Gao Z, Cao H, Xu X, Wang Q, Wu Y, Lu Q. Prognostic value of lymphovascular invasion in stage II colorectal cancer patients with an inadequate examination of lymph nodes. *World J Surg Oncol*. 2021;19:125.
18. Huh JW, Lee WY, Shin JK, et al. A novel histologic grading system based on lymphovascular invasion, perineural invasion, and tumor budding in colorectal cancer. *J Cancer Res Clin Oncol*. 2019;145:471-477.
19. Pricolo VE, Steingrimsson J, McDuffie TJ, McHale JM, McMillen B, Shparber M. Tumor deposits in stage III colon cancer: correlation with other histopathologic variables, prognostic value, and risk stratification-time to consider "N2c". *Am J Clin Oncol*. 2020;43:133-138.
20. Wang X, Cheng W, Dou X, et al. The new 'coN' staging system combining lymph node metastasis and tumour deposit provides a more accurate prognosis for TNM stage III colon cancer. *Cancer Med*. 2023;12:2538-2550.
21. Cohen R, Shi Q, Meyers J, et al. Combining tumor deposits with the number of lymph node metastases to improve the prognostic accuracy in stage III colon cancer: a post hoc analysis of the CALGB/SWOG 80702 phase III study (Alliance). *Ann Oncol*. 2021;32:1267-1275.
22. Amin MB, Greene FL, Edge SB, et al. The eighth edition AJCC cancer staging manual: continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin*. 2017;67:93-99.
23. Rahbari NN, Bork U, Motschall E, et al. Molecular detection of tumor cells in regional lymph nodes is associated with disease recurrence and poor survival in node-negative colorectal cancer: a systematic review and meta-analysis. *J Clin Oncol*. 2012;30:60-70.
24. Öberg ÅN, Lindmark GE, Israelsson AC, Hammarström SG, Hammarström ML. Detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for CEA and CK20 mRNAs. *Int J Cancer*. 2004;111:101-110.
25. Ohlsson L, Hammarström ML, Israelsson A, et al. Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. *Br J Cancer*. 2006;95:218-225.
26. Olsson LM, Lindmark GE, Israelsson ACE, Korkocic D, Hammarström SG, Hammarström MLKC. CEACAM5, KLK6, SLC35D3, POSTN, and MUC2 mRNA analysis improves detection and allows characterization of tumor cells in lymph nodes of patients who have colon cancer. *Dis Colon Rectum*. 2021;64:1354-1363.
27. Ohlsson L, Lindmark G, Israelsson A, et al. Lymph node tissue kallikrein-related peptidase 6 mRNA: a progression marker for colorectal cancer. *Br J Cancer*. 2012;107:150-157.
28. Olsson L, Hammarström ML, Israelsson A, Lindmark G, Hammarström S. Allocating colorectal cancer patients to different risk categories by using a five-biomarker mRNA combination in lymph node analysis. *PloS One*. 2020;15:e0229007.
29. Ohlsson L, Israelsson A, Öberg Å, et al. Lymph node CEA and MUC2 mRNA as useful predictors of outcome in colorectal cancer. *Int J Cancer*. 2012;130:1833-1843.
30. Palmqvist R, Miklos C, Fred Å, Nobin Gustafsson H, Sahibzada J. Kolon och rektum—Tumörer och tumörlika förändringar. Document name: KVAST-CRC-2022. https://medlem.foreningssupport.se/foreningar/uploads/L15178/kvast/gastro/KVAST-CRC-2022.pdf. Accessed 2022.
31. Loughrey MB, Quirke P, Shepherd NA. Dataset for histopathological reporting of colorectal cancer. Document number G049. In: *Standards and Datasets for Reporting Cancers*. www.rcpath.org/uploads/assets/c8b61ba0-ae3f-43f1-85ffd3ab9f17cfe6/G049-Dataset-for-histopathological-reporting-of-colorectal-cancer. Accessed 2018.
32. *Regional Cancer Centra (RCC) Knowledge Bank* (cancercentrum.se). National Health Care Program for Colon and Rectal Cancer version. https://kunskapsbanken.cancercentrum.se/diagnoser/tjock-och-andtarmscancer/vardprogram.pdf. Version 1.0 Accessed February 24, 2016; Version 3.1. Accessed February 3, 2023.
33. AbdelMageed M, Ismail HTH, Olsson L, et al. Clinical significance of stem cell biomarkers EpCAM, LGR5 and LGR4 mRNA levels in lymph nodes of colon cancer patients. *Int J Mol Sci*. 2021;23:403.
34. AbdelMageed M, Ali H, Olsson L, et al. The chemokine CXCL16 is a new biomarker for lymph node analysis of colon cancer outcome. *Int J Mol Sci*. 2019;20:5793.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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