Molecular testing for lymph node metastases as a
determinant of colon cancer recurrence: results from a
retrospective multicenter study

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Running Title: GCC Lymph Node Ratio and Colon Cancer Recurrence
Summary

Background: Recurrence risk assessment to make treatment decisions for early stage colon cancer patients is a major unmet medical need. The aim of this retrospective multicenter study was to evaluate the clinical utility of guanylyl cyclase C (GCC) mRNA levels in lymph nodes on colon cancer recurrence.

Methods: The proportion of lymph nodes (LN) with GCC positive mRNA (LNR) was evaluated in 463 untreated T3N0 patients, blinded to clinical outcomes. One site’s (n=97) tissue grossing method precluded appropriate LN assessment resulting in post hoc exclusion. Cox regression models tested the relationship between GCC and the primary endpoint of time to recurrence (TTR). Assay methods, primary analyses, and cut-points were all pre-specified.

Findings: Final data set contained 366 patients, 38 (10%) of whom had recurrence. Presence of 4 or more GCC-positive LNs was significantly associated with risk of recurrence (HR=2.46, 95% CI: 1.07-5.69, p=0.035), while binary GCC LNR risk class (HR=1.87, 95% CI: 0.99-3.54, p=0.054) and MMR status (HR=0.77, 95% CI: 0.36-1.62, p=0.49) were not. In a secondary analysis using a 3-level GCC LNR risk group classification of high (LNR>0.20), intermediate (0.10<LNR≤0.20) and low (LNR≤0.10), high risk patients had a 2.5 times higher recurrence risk compared to low risk patients (HR=2.53, 95% CI: 1.24-5.17, p=0.011).

Interpretation: GCC status is a promising prognostic factor independent of traditional histopathology risk factors in a contemporary population of stage IIa colon cancer patients not treated with adjuvant therapy, but GCC determination must be performed with methodology adapted to the tissue procurement and fixation technique.
Keywords: colorectal cancer; lymph nodes; guanylyl cyclase-C receptor; molecular staging; prognosis; recurrence

Statement of translational relevance: This multicenter prospectively specified retrospective study provides evidence that the expression level of GCC in lymph nodes is a promising determinant of recurrence in low risk stage IIa colon cancer patients, independent of other traditional risk factors. Tumor burden in the lymph nodes has become more widely recognized by treating physicians as a key prognostic factor to determine the risk of recurrence of cancer patients, and hence, to determine which patients might benefit most from adjuvant chemotherapy and which could be safely managed without chemotherapy.
Introduction

For patients with colon cancer, prognosis after surgical resection is directly related to the status of regional lymph nodes. However, recurrence risk discrepancy exists among node-negative (pN0) colon cancer patients and the identification of a sensitive and specific prognostic marker is needed to aid the management of this heterogeneous population.

About 42% of patients undergoing potentially curative surgery for colon cancer will have pN0 nodal status, however approximately 15% to 20% of stage II patients will experience disease recurrence within 5 years.\(^1\)-\(^4\) This phenomenon is likely due, at least in part, to the presence of undetected nodal metastasis during the initial pathologic examination, which predisposes colon cancer patients to a higher risk of disease recurrence. The prognostic value of the molecular detection of occult disease in regional nodes has been supported in numerous studies involving pN0 colon cancer patients.\(^5\)-\(^7\) Despite the importance of nodal status, there is no consensus on whether molecular detection of lymph node metastases is of clinical significance among stage IIa colon cancer patients.

The application of improved molecular methods to detect occult disease in regional nodes could enable better risk stratification between stage IIa colon cancer patients who could be safely managed without adjuvant chemotherapy and those at higher risk of recurrence who potentially may benefit from further treatment.

It is well known that current histopathologic examination in lymph node negative colon cancer patients is suboptimal to accurately identify patients at higher risk of disease recurrence. One way to overcome standard practice limitations is to increase sampling of the specimen and to identify clinically relevant lymph node metastases that may not have been observed by manual microscopic examination. As such, molecular detection of
Guanylyl Cyclase C (GCC) may be a particularly sensitive and specific method for the detection of colorectal tumor cells in extra-intestinal tissues and could identify pN0 colon cancer patients at increased recurrence risk.(8-10) GCC is a human receptor for the gastrointestinal hormones guanylin and uroguanylin normally found in the luminal aspect of intestinal epithelium and whose expression is preserved in primary and metastatic colorectal cancer cells.(11) Preliminary studies have suggested that the presence of GCC mRNA expression in lymph nodes increases the likelihood of colon cancer recurrence, independently of traditional high risk features.(12-14)

Tumor markers and gene signatures have also been evaluated to identify patients with a higher risk of recurrence. Deficiency of the mismatch repair (MMR) genes is of particular relevance in stage II colon cancer as these cancers have specific clinicopathologic features and better prognosis.(15, 16) For patients with stage II colon cancer, dMMR tumors have lower recurrence risk and do not appear to benefit from fluorouracil-based adjuvant chemotherapy.(17, 18)

The purpose of this study is to determine whether the ratio of GCC expressing lymph nodes to the total number of lymph nodes examined (LNR) is a more powerful predictor of outcome than current risk factors such as lymphovascular invasion (LVI), MMR or tumor grade. Our findings provide further insight into the clinical utility of molecular staging for predicting the risk of recurrence in lymph node-negative invasive colon cancer.

**Methods**

**Patients Selection**
Patients from five US, one Canadian, and two European sites were screened for inclusion into the study. Eligibility criteria required patients with histologically confirmed stage IIa (pT3N0) colon adenocarcinoma who had undergone curative surgical resection, were less than 80 years old at time of surgery, had negative surgical margins, 12 or more regional lymph nodes assessed and a minimum of 3 years of follow-up data obtained by the treating physician or until the first occurring event of death or local or distant recurrence. Patients were ineligible if they had been treated with adjuvant chemotherapy or had rectal cancer. In total, 478 patients were identified whose surgical resection dated from 1999 to 2008, lymph node tissue blocks and follow-up information were available from 471 patients; 8 cases were subsequently excluded as they did not meet the inclusion criteria (Figure 1A). Of the resulting 463 eligible patients, microscopic examination of the H&E stained slide was performed on 10,728 individual lymph nodes using standardized laboratory procedures to ensure absence of surrounding fat and connective tissue that might have contained bowel tissue, as these tissues could impact the quality of the RNA extraction and yield false positive results. Following histological review, one site that provided 97 evaluable patients (3,367 LNs) was excluded after the primary analysis; due to the fact that the tissue grossing method used precluded appropriate LN assessment by current GCC quantification methods. The study was approved by the local institutional review board of each participating institution.

**Tissue Processing, RNA extraction and GCC Status Determination**

For each evaluable patient, hematoxylin and eosin (H&E) staining was prepared for all formalin-fixed, paraffin-embedded (FFPE) lymph node tissue blocks and verified centrally by qualified technicians with expertise in gastrointestinal pathology and blinded
to clinical outcomes to confirm lymph node count and histology. Each lymph node retrieved from FFPE blocks was bisected, independently homogenized and RNA was extracted as previously described.(19, 20) First strand cDNA was synthesised using gene-specific reverse primer 5’-CCAAAAACTTCCAGCTGAGATCA-3’ for GCC (NM_004963) and 5’-ACTCTCGTCGGTGACTGTTCAG-3’ for β-glucuronidase (GUSB; NM_000181) as described by Sargent et al.(21) Subsequently, cDNA products were used to perform quantitative real-time PCR and establish a cycle to threshold (Ct) value for GCC and GUSB using specific probes (5’-6FAM-CAGAATTGAGCTACCCC-MGBNFQ-3’ and 5’-VIC-TTTTGCCGATTTCATG-MGBNFQ-3’ respectively). As a measure of RNA integrity, specimens with a GUSB Ct level higher than 31 were considered non-informative and were excluded from further analysis. Individual LN status was determined by relative quantification of GCC and GUSB using the delta Ct methods (ΔCt = Ct GUSB - Ct GCC) with a validated cut-off (-5.9).(21) Analytical number of GCC positive lymph node as well as the ratio of number of GCC positive LNs over the total number of informative LNs (LNR) were evaluated for association with recurrence risk. For the primary GCC LNR risk stratification, patients were classified as low risk if LNR ≤0.1, and high risk if LNR >0.1. Alternatively, a 3-level risk categorization was applied to delineate the subset of pN0 patients thought to be at higher risk of recurrence using a LNR >0.2 to define these high risk patients.

**MMR status Determination**

All tumors were reviewed and MMR status was evaluated centrally by a certified pathologist (T.T.W) without knowledge of the clinical outcome. MMR status was assessed using immunohistochemistry analysis of MLH1, MSH2, MSH6, and PMS2
protein expression as previously described. Protein expression was defined as abnormal when nuclear staining of tumor cells was absent in the presence of positive staining in surrounding cells.

**Statistical Analysis**

TTR, defined as time from surgery to first event of recurrence (local or distant), or death related to cancer, was the pre-specified primary endpoint. Additional clinical endpoints examined include disease-free survival (DFS) defined as the time from surgery to first event of recurrence, new primary, or death due to any cause and overall survival (OS) defined time from surgery to death due to any cause. The distributions of TTR, DFS and OS were estimated by the Kaplan-Meier method. Stratified Cox models (univariate and multivariate) were used to estimate unadjusted and adjusted HR, comparing the risk of recurrence and/or death between risk groups defined by GCC LNR values. The attained 366 patients and 38 events provides 80% power to detect a Hazard Ratio (HR) of at least 2.5 for the primary endpoint of time to recurrence (TTR) when 33% of patients are classified as ‘high risk’, using a two-sided stratified log-rank test at level 0.05 (assuming 5 year recurrence rate of 27% in the high risk group). Statistical analyses were performed using Linux SAS software, version 9.2 (SAS Institute, Cary, NC). P-values <0.05 were considered statistically significant.

**Results**

**Patient Characteristics**

A total of 478 patients diagnosed as having histopathologically confirmed stage IIa colon cancer were identified before applying the exclusion criteria. Lymph node tissue blocks
and follow-up information were retrieved from 471 of these patients and 463 were
deemed eligible (Figure 1a). Histological review of blocks revealed that the tissue
grossing method used at one site (n=97) did not fully separate lymph node tissue from
normal and/or tumoral bowel tissue before RNA extraction, resulting in a higher rate of
false positive results due to presence of bowel tissue regardless of patient’s outcome
(Figure 1b). We therefore performed all subsequent analyses excluding this site.

Demographic and clinicopathologic data of the 366 patients included in the final
evaluable data set are listed in Table 1. The final analytic cohort contained 366 patients,
38 (10.4%) had disease recurrence. Median follow-up in all patients was 57.5 months
(range 0.16-135.5) and 61.3 months in patients alive at last follow-up. Overall, 69% of
patients (252/366) had at least one GCC-positive LN and about 1/3 of these patients
(82/252) had 4 or more GCC-positive LNs. A total of 358 patients also had tumor tissues
available for MMR analysis with 101 patients (28%) identified with dMMR tumors
(Table 1).

**Association between GCC and Clinicopathological Factors**

Of the 366 patients, 222 (61%) had GCC LNR value of 0.1 or less and were grouped into
the low risk category. Patients in the low risk group were more likely to have a high
histology grade (G3-G4) tumor (14.2% vs 6.9%; p=0.033), and more likely to have a
dMMR tumor than patients with high GCC LNR value (32.7% vs 21.3%; p=0.019).

**GCC Classification and Recurrence Risk Prediction**

Univariate and multivariate results for each factor included in the Cox proportional
hazards regression are shown in Table 2. Based on the prospectively specified binary
GCC LNR classification, a non-significant trend toward increased risk of recurrence was
found for patients with a GCC LNR value above 0.1 (HR=1.87, 95% CI: 0.99-3.54, p=0.054, Table 2). Patients with lower GCC LNR values (LNR ≤0.1) had significantly better DFS compared to patients in the high risk group (LNR > 0.1, 84% vs. 66%; HR=1.61, 95% CI:1.05-2.47, p=0.030, Figure 2).

When the alternative 3-level risk classification was used to stratify the GCC LNR values, a significant association with the risk of recurrence was observed for GCC high-risk group versus low-risk (HR=2.59, 95% CI:1.31-5.15, p=0.007). The prognostic value of the 3-level risk classification remained significant after adjustment for covariates, including tumor grade, number of LNs examined, MMR status and LVI (HR=2.53, 95% CI: 1.24-5.17, p=0.011). Furthermore, patients in the low risk group (LNR ≤0.1) had significantly improved OS and DFS than patients in the high risk group (LNR >0.2) (Supplementary Table 1).

In multivariate analysis, the risk of recurrence was also significantly associated with the presence of 4 or more GCC-positive nodes (versus 0 positive nodes, HR=2.89, 95% CI:1.20-6.97, p=0.02 and the continuous GCC LNR (HR=1.22, 95% CI: 1.04-1.43, p=0.018). However, MMR status alone was not significantly associated with TTR (HR=0.77, 95% CI: 0.36-1.62, p=0.49). Additionally, MMR status did not affected TTR in patients classified in GCC LNR high- or low-risk groups (Supplementary Table 2).

Discussion

Although presence of lymph node metastases remains the strongest prognostic predictor in non-metastatic colon cancer, prognostic stratification of low risk stage IIa colon cancer patients remains a clinically important and controversial issue. Recently, multiple studies
have demonstrated that there is an increased risk of recurrence associated with occult metastases in lymph node-negative colon cancer.\cite{Sargent2011, Rahbari2012, Bilchik2014} In a systematic review with a cumulative sample size of 4,087 patients, Rahbari et al reported that molecular detection of occult disease in regional nodes is associated with an increased risk of disease recurrence and poor survival in pN0 patients.\cite{Rahbari2012} Similarly, Bilchik et al.\cite{Bilchik2014} reported a significantly increased recurrence rate of 22% in patients with micrometastases versus 6% without micrometastases, and Fearden et al\cite{Fearden2015} reported a 5 year recurrence rate of 23% in patients with micrometastases compared to 7% without micrometastases respectively.

The prognostic value of molecular detection of occult disease was further demonstrated in a prospective study of 257 patients in which node-negative colorectal cancer patients harboring molecular-positive metastases behaved similarly to lymph node-positive patients in terms of recurrence and molecular features.\cite{Sargent2011}

The analytical validity of the GCC RT-qPCR assay and its application to FFPE tissue samples was previously demonstrated by Haince et al in 2010\cite{Haince2010} and confirmed by Sargent et al in 2011.\cite{Sargent2011} In the first study involving 123 colon cancer patients who had undergone curative surgical resection, patients with pN0 disease whose lymph nodes were GCC-positive were more than twice as likely to relapse when compared to patients with GCC negative nodes (HR=3.54; \textit{p}=0.008) and had recurrence rates similar to those observed with stage III colon cancer.\cite{Haince2010} The second study included 241 untreated stage II colon cancer patients with at least ten LNs examined and showed that the GCC LNR status significantly predicted higher recurrence risk for 84 patients (34.9%) classified as high risk (HR=2.38; \textit{p}=0.02).\cite{Sargent2011} In the subset of 181 patients with traditionally favorable prognostic factors, that is, an invasive T3 tumor and 12 or more lymph nodes
Sargent et al.

examined, the high risk group had a 5 times greater likelihood of recurrence than the low risk group (HR=5.06; p=0.003).

In the present study, molecular staging identified that 69% of patients had at least one GCC positive node and 39% had a GCC LNR value >0.1 and were thus grouped into the high risk category. Strengths of the study include a modern cohort of untreated low risk stage IIa colon cancer patients, long-term follow-up, high nodal sampling, and protocol specification of all primary analyses. Although the exclusion of 97 patients from a single site was post-hoc, this was deemed necessary due to the incompatibility with the established GCC testing methodology. The previously demonstrated 2-level risk classification was significantly associated with outcome only for disease-free survival.

The prognostic value of the 3-level GCC LNR risk stratification was also evaluated as a post hoc analysis in the context of a comparison with the analytical number of GCC positive LN. Final analysis demonstrated that in addition to the analytical number of GCC positive lymph nodes, the 3-level GCC LNR risk classification significantly predicted poorer outcomes in both univariate and multivariate analysis. It is noteworthy that LVI and tumor grade were not significantly associated with recurrence risk while these risk factors have previously been shown to have prognostic impact in stage II colon cancer.(4)

Advances in genomic evaluation of tumor tissues have also brought new opportunities for identifying prognostic and predictive markers. As reported in a recent pooled analysis of patients with stage II and stage III disease, MMR status has emerged as a relevant marker to select patients who have improved disease-free survival when treated with surgery alone.(16) Surprisingly, MMR status was not significantly associated with recurrence risk
in this cohort. Potential reasons for this result, which differs from most recent MMR-related publications, include that the present study was performed in an older patient population, with a greater number of nodes sampled, and in a cohort with an overall lower recurrence rate when compared to clinical-trial based patient cohorts.

During the last few years, gene signatures have been developed from FFPE tumor samples to identify stage II colon cancer patients who are at higher risk of disease recurrence. (28-32) The study by Gray et al. (28) was designed as a validation study based on prospectively specified retrospective analyses of the QUASAR (QUick And Simple And Reliable) trial. The Oncotype Dx Colon Cancer (Genomic Health; Redwood City, CA) multigene algorithm identified a 5-year risk of recurrence of 12% and 22% between low- and high-risk patients respectively. However, the generalizability of the QUASAR study validation to current stage II patients may be limited by differences in lymph node sampling, as a median of only 6 lymph nodes was examined in the Gray et al study.

Although using slightly different Recurrence Score (RS) cut points than initially defined in QUASAR, the Venook et al. validation study of the 12-gene RS in CALGB 9581 confirmed the risk discrimination of Oncotype Dx in stage II patients (risk ranged 9% to 26%). (30) Based on a different prognostic assay, Kennedy et al. (29) reported that the 634-probes signature used in the ColDx test (Almac Diagnostics, Souderton, PA) also had the ability to discriminate risk of recurrence in stage II colon cancer patients (HR=2.53; p=0.003). However, these findings did not account for the MMR characterization, and the sample size of the validation set was limited and enriched for recurrence. (29) The relative merit of enhanced nodal characterization versus genomic profiling of the primary tumor is a critical question for future study.
In practice, adjuvant therapy is generally considered for lymph node negative colon cancer patients believed to have higher recurrence risk, based on the expectation that high risk patients may derive larger absolute benefits. The current study provides strong evidence that molecular detection of GCC in lymph nodes offers quantitative information about individual recurrence risk which has not been available with conventional methods. Nevertheless, molecular staging based on the determination of the GCC LNR status in stage IIa colon cancer patients who have had adequate nodal sampling must be performed with methodology adapted to the tissue procurement and fixation technique before being considered for clinical application. Measuring GCC mRNA expression in lymph nodes offers a practical approach to the individualization of recurrence risk assessment that could improve staging of node-negative colon cancer and may help to further reduce the use of unnecessary adjuvant chemotherapy in low risk patients who have little likelihood to benefit from such treatment.

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Conflict of Interest Statements

Employment or Leadership Position: Guillaume Beaudry, DiagnoCure; Jean-Francois Haince, DiagnoCure; Yves Fradet, DiagnoCure.

Consultant or Advisory Role: Daniel J. Sargent, DiagnoCure.

Stock Ownership: Yves Fradet, DiagnoCure.

Research Funding: Daniel J. Sargent, DiagnoCure; Sharlene Gill, DiagnoCure; Christophe Louvet, DiagnoCure; Richard B. Everson, DiagnoCure; Udo Kellner, DiagnoCure; Thomas E. Clancy, DiagnoCure; J. Marc Pipas, DiagnoCure; Murray B. Resnick, DiagnoCure; Michael O. Meyers, DiagnoCure; Tsung-Teh Wu, DiagnoCure.

Patents Applications: Guillaume Beaudry, (US20110306055); Jean-Francois Haince, (US20110306055); Yves Fradet, (US20110306055).

Role of the funding source

DiagnoCure provided support for data collection, processing of the samples and data analyses. The sponsor of the study had no role in study design, data analysis or data interpretation and they were blinded to clinical outcomes. DJS, QS and ESP had full access to all the data. GB, JFH and YF had only access to the GCC lymph node status raw data. DiagnoCure authors and clinical investigators jointly participate in writing the manuscript. DJS and clinical investigators had final responsibility for the decision to submit for publication.

Authors Contributions

Conception and design: Daniel J. Sargent and Qian Shi
**Collection and assembly of data:** Sharlene Gill, Christophe Louvet, Richard B. Everson, Udo Kellner, Thomas E. Clancy, J. Marc Pipas, Murray B. Resnick, Michael O. Meyers, Tsung-Teh Wu, David Huntsman, Pierre Validire, Umar Farooq, Emily S. Pavey, Guillaume Beaudry, Jean-Francois Haince and Yves Fradet

**Data analysis and interpretation:** Daniel J. Sargent, Qian Shi, Emily S. Pavey

**Manuscript writing:** All authors

**Final approval of manuscript:** All authors
References


### Table 1: Patient demographics and clinicopathologic factors stratified by GCC LNR status

<table>
<thead>
<tr>
<th>GCC LNR risk groups</th>
<th>No of Patient</th>
<th>No of Events</th>
<th>Low Risk LNR ≤ 0.1</th>
<th>High risk LNR &gt; 0.1</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at Surgery</strong>, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤80, n (%)</td>
<td>366 (100%)</td>
<td>38</td>
<td>222</td>
<td>144</td>
<td>0.22(^3)</td>
</tr>
<tr>
<td>Median</td>
<td>68</td>
<td>67</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18-80</td>
<td>18-80</td>
<td>18-80</td>
<td>37-80</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong>, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11(^1)</td>
</tr>
<tr>
<td>Female</td>
<td>169 (46.2%)</td>
<td>19</td>
<td>110 (49.5%)</td>
<td>59 (41.0%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>197 (53.8%)</td>
<td>19</td>
<td>112 (50.5%)</td>
<td>85 (59.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Surgery Year Group</strong>, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.75(^1)</td>
</tr>
<tr>
<td>1999-2003</td>
<td>123 (33.6%)</td>
<td>9</td>
<td>76 (34.2%)</td>
<td>47 (32.6%)</td>
<td></td>
</tr>
<tr>
<td>2004-2008</td>
<td>243 (66.4%)</td>
<td>29</td>
<td>146 (65.8%)</td>
<td>97 (67.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor Grade</strong>, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.033(^1)</td>
</tr>
<tr>
<td>Low (G1+G2)</td>
<td>321 (88.7%)</td>
<td>32</td>
<td>187 (85.8%)</td>
<td>134 (93.1%)</td>
<td></td>
</tr>
<tr>
<td>High (G3+G4)</td>
<td>41 (11.3%)</td>
<td>6</td>
<td>31 (14.2%)</td>
<td>10 (6.9%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>-</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor Location</strong>, n (%) *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19(^1)</td>
</tr>
<tr>
<td>Right Colon</td>
<td>192 (52.6%)</td>
<td>21</td>
<td>120 (54.1%)</td>
<td>72 (50.3%)</td>
<td></td>
</tr>
<tr>
<td>Left Colon</td>
<td>122 (33.4%)</td>
<td>12</td>
<td>67 (30.2%)</td>
<td>55 (38.5%)</td>
<td></td>
</tr>
<tr>
<td>Transverse Colon</td>
<td>51 (14.0%)</td>
<td>5</td>
<td>35 (15.8%)</td>
<td>16 (11.2%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphovascular Invasion</strong>, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.97(^1)</td>
</tr>
<tr>
<td>Absent</td>
<td>331 (93.0%)</td>
<td>35</td>
<td>200 (93.0%)</td>
<td>131 (92.9%)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>25 (7.0%)</td>
<td>3</td>
<td>15 (7.0%)</td>
<td>10 (7.1%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>10</td>
<td>-</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>LN Assessed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.79(^2)</td>
</tr>
<tr>
<td>≥ 12 LNs, n (%)</td>
<td>366 (100%)</td>
<td>38</td>
<td>222</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>12-66</td>
<td>12-66</td>
<td>12-66</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMR Status</strong>, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.019)^(^1)</td>
</tr>
<tr>
<td>pMMR</td>
<td>257 (71.8%)</td>
<td>29</td>
<td>146 (67.3%)</td>
<td>111 (78.7%)</td>
<td></td>
</tr>
<tr>
<td>dMMR</td>
<td>101 (28.2%)</td>
<td>9</td>
<td>71 (32.7%)</td>
<td>30 (21.3%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>8</td>
<td>-</td>
<td>5</td>
<td>3</td>
<td></td>
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</tbody>
</table>

*Chi-Square test  \(^\text{1}\)Wilcoxon Rank Sum test  \(^\text{2}\)Two sample t-test

*One patient excluded due to having both Right and Transverse tumor location, this patient was a high risk (LNR > 0.1).
Table 2: Unadjusted and Adjusted Association between Prognostic Factors and Time to Recurrence

<table>
<thead>
<tr>
<th></th>
<th>Univariate (n=366)</th>
<th></th>
<th></th>
<th>Multivariate(^\d) (n=344**)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Event</td>
<td>No. Cases</td>
<td>HR (95% CI)</td>
<td>p value</td>
<td>No. Event</td>
<td>No. Cases</td>
</tr>
<tr>
<td>Age, continuous (per year)</td>
<td>38</td>
<td>366</td>
<td>1.03 (1.00* - 1.07)</td>
<td>0.057</td>
<td>38</td>
<td>344</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Female</td>
<td>19</td>
<td>169</td>
<td>1.08 (0.57 - 2.04)</td>
<td>0.81</td>
<td>19</td>
<td>162</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>197</td>
<td>ref</td>
<td></td>
<td>19</td>
<td>182</td>
</tr>
<tr>
<td>Tumor Grade</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>41</td>
<td>1.52 (0.64 - 3.64)</td>
<td>0.34</td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td>Low</td>
<td>32</td>
<td>321</td>
<td>ref</td>
<td></td>
<td>32</td>
<td>303</td>
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<tr>
<td>Lymphovascular Invasion</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3</td>
<td>25</td>
<td>1.21 (0.37 - 3.94)</td>
<td>0.75</td>
<td>3</td>
<td>25</td>
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<tr>
<td>Absent</td>
<td>35</td>
<td>331</td>
<td>ref</td>
<td></td>
<td>35</td>
<td>319</td>
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<tr>
<td>MMR Status, n (%)</td>
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<tr>
<td>dMMR</td>
<td>9</td>
<td>101</td>
<td>0.77 (0.36, 1.62)</td>
<td>0.49</td>
<td>9</td>
<td>99</td>
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<tr>
<td>pMMR</td>
<td>29</td>
<td>257</td>
<td>ref</td>
<td></td>
<td>29</td>
<td>245</td>
</tr>
<tr>
<td>No of Nodes Examined, continuous (per node)</td>
<td>38</td>
<td>366</td>
<td>0.97 (0.93 - 1.02)</td>
<td>0.24</td>
<td>38</td>
<td>344</td>
</tr>
<tr>
<td>Number of GCC Positive Nodes</td>
<td></td>
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<tr>
<td>4+</td>
<td>14</td>
<td>82</td>
<td>2.46 (1.07 - 5.69)</td>
<td><strong>0.035</strong></td>
<td>14</td>
<td>82</td>
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<tr>
<td>1-3</td>
<td>15</td>
<td>170</td>
<td>1.16 (0.51 - 2.65)</td>
<td>0.72</td>
<td>15</td>
<td>170</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>114</td>
<td>ref</td>
<td></td>
<td>9</td>
<td>114</td>
</tr>
<tr>
<td>GCC LNR, continuous (per 0.1 unit)</td>
<td>38</td>
<td>366</td>
<td>1.21 (1.04 - 1.41)</td>
<td><strong>0.016</strong></td>
<td>38</td>
<td>344</td>
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<tr>
<td>GCC LNR, 2-level Risk group</td>
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<tr>
<td>High</td>
<td>20</td>
<td>144</td>
<td>1.87 (0.99 - 3.54)</td>
<td>0.054</td>
<td>20</td>
<td>138</td>
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<tr>
<td>Low</td>
<td>18</td>
<td>222</td>
<td>ref</td>
<td></td>
<td>18</td>
<td>206</td>
</tr>
<tr>
<td>GCC LNR, 3-level Risk group</td>
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<td></td>
</tr>
<tr>
<td>High</td>
<td>15</td>
<td>80</td>
<td>2.59 (1.31 - 5.15)</td>
<td><strong>0.007</strong></td>
<td>15</td>
<td>77</td>
</tr>
<tr>
<td>Intermediate</td>
<td>5</td>
<td>64</td>
<td>1.02 (0.38 - 2.75)</td>
<td>0.97</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>Low</td>
<td>18</td>
<td>222</td>
<td>ref</td>
<td></td>
<td>18</td>
<td>206</td>
</tr>
</tbody>
</table>

*Unrounded value >.99 and <1.0 **344 out of 366 patients have complete data on all covariates §Prognostic factors adjusted for continuous GCC LNR in multivariate model.
Figure Legends

**Figure 1.** (A) Flowchart showing patient selection for GCC mRNA expression analysis by RT-qPCR. (B) Representative H&E sections from two different sites. Upper panel: typical H&E section of LN tissues. Lower panel: section from excluded site.

**Figure 2.** Kaplan-Meier estimates at 5 years for (A) Recurrence-free survival and (B) Disease-free survival using the prospectively specified binary GCC LNR classification. Multivariate analysis adjusted for age, gender, grade, number of LNs examined, MMR status, presence of lymphovascular invasion and continuous GCC LNR values. CI=Confidence Interval, p=P-value
Figure 1a

Patient enrolled (n=478)
Stage IIa (pT3N0) colon cancer

7 Patients excluded
Blocks or follow-up information not obtained

Patient sample processed (n=471)

8 Patients excluded
Did not meet inclusion criteria

Patient meeting criteria (n=463)
T3N0 colon cancer with at least
12 LNs examined, negative margins,
≤ 80 years and minimum 36 mo follow-up

97 Patients excluded (post-hoc)
Tissue grossing method impeded molecular
staging using PCR methods

Final study population (n=366)
Figure 2a

<table>
<thead>
<tr>
<th></th>
<th>No. of Patients (%)</th>
<th>Event-free rate at 5 years (95% CI)</th>
<th>Multivariate Hazard Ratio (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Risk (LNR ≤ 0.1)</td>
<td>222 (61%)</td>
<td>92% (88-96)</td>
<td>ref</td>
<td>0.07</td>
</tr>
<tr>
<td>High Risk (LNR &gt; 0.1)</td>
<td>144 (39%)</td>
<td>84% (78-91)</td>
<td>1.82 (0.94 -3.51)</td>
<td></td>
</tr>
</tbody>
</table>

Low Risk: 222 208 192 179 152 99 61
High Risk: 144 128 111 103 76 53 39
Figure 2b

- Event-free rate at 5 years (95% CI):
  - Low Risk (LNR ≤ 0.1): 84% (79-89)
  - High Risk (LNR > 0.1): 66% (58-75)

- Multivariate Hazard Ratio (95% CI):
  - Low Risk: 1.61 (1.05 - 2.47) (p = 0.03)

- No. of Patients (%):
  - Low Risk (LNR ≤ 0.1): 222 (61%)
  - High Risk (LNR > 0.1): 144 (39%)
Molecular testing for lymph node metastases as a determinant of colon cancer recurrence: results from a retrospective multicenter study

Daniel J. Sargent, Qian Shi, Sharlene Gill, et al.

Clin Cancer Res Published OnlineFirst June 11, 2014.

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