Molecular Testing for Lymph Node Metastases as a Determinant of Colon Cancer Recurrence: Results from a Retrospective Multicenter Study

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Abstract

Purpose: Recurrence risk assessment is an important treatment decision for early-stage colon cancer patients. 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 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 lymph node assessment resulting in post hoc exclusion. Cox regression models tested the relationship between GCC and the primary endpoint of time to recurrence. Assay methods, primary analyses, and cut points were all prespecified.

Results: Final dataset contained 366 patients, 38 (10%) of whom had recurrence. Presence of four or more GCC-positive lymph nodes was significantly associated with risk of recurrence [hazard ratio (HR) = 2.46, 95% confidence interval (CI), 1.07–5.69, P = 0.035], whereas binary GCC LNR risk class (HR = 1.87, 95% CI, 0.99–3.54, P = 0.054) and mismatch repair (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 with low-risk patients (HR = 2.53, 95% CI, 1.24–5.17, P = 0.011).

Conclusions: GCC status is a promising prognostic factor independent of traditional histopathology risk factors in a contemporary population of patients with stage IIa colon cancer not treated with adjuvant therapy, but GCC determination must be performed with methodology adapted to the tissue procurement and fixation technique. Clin Cancer Res; 20(16); 4361–9. ©2014 AACR.

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 patients with colon cancer 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 patients with stage IIa colon cancer. The application of improved molecular methods to detect occult disease in regional nodes could enable better risk stratification between...
patients with stage IIa colon cancer 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 extraintestinal 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 seem 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 (LNRR) is a more powerful predictor of outcome than current risk factors such as lymphovascular invasion (LVI), MMR, or tumor grade. Our findings provide a new perspective for predicting the risk of recurrence in lymph node-negative invasive colon cancer.

Materials and Methods

Patient selection

Patients from five United States, 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; eight cases were subsequently excluded as they did not meet the inclusion criteria (Fig. 1A). Of the resulting 463 eligible patients, microscopic examination of the hematoxylin and eosin (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 histologic review, one site that provided 97 evaluable patients (3,367 lymph nodes) was excluded after the primary analysis; due to the fact that the tissue grossing method used precluded appropriate lymph node 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, 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 synthesized using gene-specific reverse primer 5′-CCAAAATCTCCAGCTGAGATCA-3′ for GCC (NM_004963) and 5′-ACTCTCGGTGACTGTCAG-3′ for β-glucuronidase (GUSB; NM_000181) as described by Sargent and colleagues (21). Subsequently, cDNA products were used to perform qRT-PCR and establish a cycle to threshold (Ct) value for GCC and GUSB using specific probes (5′-6FAM-CAGAATGGACTGCC-MGBNFQ-3′ and 5′-VIC-TTCTTGGCGATATGC-MGBNFQ-3′, respectively). As a measure of RNA integrity, specimens with a GUSB Ct level higher than 31 were considered noninformative and were excluded from further analysis. Individual lymph node status was determined by relative quantification of GCC and GUSB using the delta Ct methods (ΔCt = CtgusB − Ctgcc) with a validated cutoff (−5.9; ref. 21). Analytical numbers of GCC-positive

Translational Relevance

This multicenter prospectively specified retrospective study provides evidence that the expression level of guanylyl cyclase C 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 patients with cancer, and hence, to determine which patients might benefit most from adjuvant chemotherapy and which could be safely managed without chemotherapy.
lymph node as well as the ratio of number of GCC-positive lymph nodes over the total number of informative lymph nodes (LNR) were evaluated for association with recurrence risk. For the primary GCC LNR risk stratification, patients were classified as low risk if $\text{LNR} \leq 0.1$, and high risk if $\text{LNR} > 0.1$. 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. Wu) without knowledge of the clinical outcome. MMR status was assessed using IHC analysis of MLH1, MSH2, MSH6, and PMS2 protein expression as previously described (22, 23). 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**

Time to recurrence (TTR), defined as time from surgery to first event of recurrence (local or distant), or death related to cancer, was the prespecified 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 provide 80% power to detect a HR of at least 2.5 for the primary endpoint of 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). $P$ 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 (Fig. 1A). Histologic 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 the presence of bowel tissue regardless of patient outcome (Fig. 1B). We therefore performed all subsequent analyses excluding this site. Demographic and clinicopathologic data of the 366 patients included in the final evaluable dataset 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 (range 0.16–135.5) and 61.3 months in patients alive at last follow-up. Overall, 69% of patients...
had at least one GCC-positive lymph node and about one-third of these patients (82/252) had four or more GCC-positive lymph nodes. 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. On the basis of the prospectively specified binary GCC LNR classification, a nonsignificant trend toward increased risk of recurrence was found for patients with a GCC LNR value above 0.1 [HR = 1.87, 95% confidence interval (CI), 0.99–3.54, P = 0.054; Table 2]. Patients with lower GCC LNR values (LNR ≤ 0.1) had significantly better DFS compared with patients in the high-risk group (LNR > 0.1, 84% vs. 66%; HR = 1.61, 95% CI, 1.05–2.47, P = 0.030; Fig. 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.
Table 2. Unadjusted and adjusted association between prognostic factors and TTR

<table>
<thead>
<tr>
<th></th>
<th>No. of events</th>
<th>No. of cases</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>169</td>
<td>1.08 (0.57–2.04)</td>
<td>0.81</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>197</td>
<td>Ref.</td>
<td>—</td>
</tr>
<tr>
<td>Tumor grade</td>
<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>
</tr>
<tr>
<td>Low</td>
<td>32</td>
<td>321</td>
<td>Ref.</td>
<td>—</td>
</tr>
<tr>
<td>LVI</td>
<td></td>
<td></td>
<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>
</tr>
<tr>
<td>Absent</td>
<td>35</td>
<td>331</td>
<td>Ref.</td>
<td>—</td>
</tr>
<tr>
<td>MMR status, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dMMR</td>
<td>9</td>
<td>101</td>
<td>0.77 (0.36–1.62)</td>
<td>0.49</td>
</tr>
<tr>
<td>pMMR</td>
<td>29</td>
<td>257</td>
<td>Ref.</td>
<td>—</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>
</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>0.016</td>
</tr>
<tr>
<td>GCC LNR, 2-level risk group</td>
<td>20</td>
<td>144</td>
<td>1.87 (0.99–3.54)</td>
<td>0.054</td>
</tr>
<tr>
<td>Low</td>
<td>18</td>
<td>222</td>
<td>Ref.</td>
<td>—</td>
</tr>
<tr>
<td>GCC LNR, 3-level risk group</td>
<td>15</td>
<td>80</td>
<td>2.59 (1.31–5.15)</td>
<td>0.007</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>64</td>
<td>1.02 (0.38–2.75)</td>
<td>0.97</td>
</tr>
<tr>
<td>Intermediate</td>
<td>18</td>
<td>222</td>
<td>Ref.</td>
<td>—</td>
</tr>
<tr>
<td>Low</td>
<td>18</td>
<td>206</td>
<td>Ref.</td>
<td>—</td>
</tr>
</tbody>
</table>

**NOTE:** Bold P-values show statistical significance at 0.05 level.

*aPrognostic factors adjusted for continuous GCC LNR in multivariate model.

*b344 of 366 patients have complete data on all covariates.

cUnrounded value > 0.99 and < 1.0.
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 lymph nodes 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 \( \leq 0.1 \)) had significantly improved OS and DFS than patients in the high-risk group (LNR > 0.2; Supplementary Table S1).

In multivariate analysis, the risk of recurrence was also significantly associated with the presence of four or more GCC-positive nodes (vs. 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, \( P = 0.49 \)). In addition, MMR status did not affect TTR in patients classified in GCC LNR high- or low-risk groups (Supplementary Table S2).

**Discussion**

Although presence of lymph node metastases remains the strongest prognostic predictor in nonmetastatic 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 (7, 24, 25). In a systematic review with a cumulative sample size of 4,087 patients, Rahbari and colleagues 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 (25). Similarly, Bilchik and colleagues (26) reported a significantly increased recurrence rate of 22% in patients with micrometastases versus 6% without micrometastases, and Faerden and colleagues (27) reported a 5-year recurrence rate of 23% in patients with micrometastases compared with 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 (14).

The analytical validity of the GCC qRT-PCR assay and its application to FFPE tissue samples was previously demonstrated by Haince and colleagues in 2010 (19) and confirmed by Sargent and colleagues in 2011 (21). In the first study involving 123 patients with colon cancer 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 with patients with GCC-negative nodes (HR = 3.54; P = 0.008) and had recurrence rates similar to those observed with stage III colon cancer (19). The second study included 241 untreated patients with stage II colon cancer with at least ten lymph nodes 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; P = 0.02; ref. 21). In the subset of 181 patients with traditionally favorable prognostic factors, that is, an invasive T3 tumor and 12 or more lymph nodes examined, the high-risk group had a five 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 DFS. 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 lymph nodes. 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 DFS 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 with clinical trial-based patient cohorts.

During the last few years, gene signatures have been developed from FFPE tumor samples to identify patients with stage II colon cancer who are at higher risk of disease recurrence (28–32). The study by Gray and colleagues (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) 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 six lymph nodes was examined in the Gray and colleagues study. Although using slightly different Recurrence Score (RS) cut points than initially defined in QUASAR, the Venook and colleagues validation study of the 12-gene RS in CALGB 9581 confirmed the risk discrimination of Oncotype Dx in stage II patients (risk ranged 9%–26%; ref. 30). On the basis of a different prognostic assay, Kennedy and colleagues (29) reported that the 634-probes signature used in the ColoDx test (Almac Diagnostics) also had the ability to discriminate risk of recurrence in patients with stage II colon cancer (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 patients with stage IIA colon cancer 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.

Disclosure of Potential Conflicts of Interest

R.B. Everson is an employee of Precision Staging and reports receiving a commercial research grant from Diagnoscore. P. Validire reports receiving a commercial research grant from Diagnoscore. No potential conflicts of interest were disclosed by the other authors.

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References


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