METASTASIS OF TUMOR CELLS to regional lymph nodes is the single most important prognostic factor in patients with colorectal cancer. Recurrence rates increase from approximately 25% in patients with lymph nodes free of tumor cells by histopathology (pN0) to approximately 50% in patients with 4 or more lymph nodes harboring metastases. Adjuvant chemotherapy improves disease-free and overall survival in patients with histopathologically evident lymph node metastases, but its role in patients with pN0 colorectal cancer remains unclear.

Given the established relationship between lymph node metastasis and prognosis, recurrence in a substantial fraction of patients with pN0 colorectal cancer suggests the presence of occult metastases in regional lymph nodes that escape histopathological detection. Conversely, patients with pN0 colorectal cancer who are free of lymph node metastases may be at lowest risk for developing recurrent disease. Thus, a more accurate assessment of occult metastases in regional lymph nodes could improve risk stratification in this clinically heterogeneous population. Precise evaluation of lymph node metastases may also identify patients with pN0 colorectal cancer who could benefit from adjuvant chemotherapy.

Context The established relationship between lymph node metastasis and prognosis in colorectal cancer suggests that recurrence in 25% of patients with lymph nodes free of tumor cells by histopathology (pN0) reflects the presence of occult metastases. Guanylyl cyclase 2C (GUCY2C) is a marker expressed by colorectal tumors that could reveal occult metastases in lymph nodes and better estimate recurrence risk.

Objective To examine the association of occult lymph node metastases detected by quantifying GUCY2C messenger RNA, using the reverse transcriptase–polymerase chain reaction, with recurrence and survival in patients with colorectal cancer.

Design, Setting, and Participants Prospective study of 257 patients with pN0 colorectal cancer enrolled between March 2002 and June 2007 at 9 US and Canadian centers (7 academic medical centers and 2 community hospitals) provided 2570 fresh lymph nodes measuring 5 mm or larger for histopathology and GUCY2C messenger RNA analysis. Patients were followed up for a median of 24 months (range, 2-63 months) for disease recurrence or death.

Main Outcome Measures Time to recurrence (primary outcome) and disease-free survival (secondary outcome) relative to expression of GUCY2C in lymph nodes.

Results Thirty-two patients (12.5%) had lymph nodes negative for GUCY2C (pN0 [mol−]), and all but 2 remained free of disease during follow-up (recurrence rate, 6.3%; 95% confidence interval [CI], 0.8%-20.8%). Conversely, 225 patients (87.5%) had lymph nodes positive for GUCY2C (pN0 [mol+]), and 47 developed recurrent disease (20.9%; 95% CI, 15.8%-26.8%) (P = .006). Multivariate analyses revealed that GUCY2C in lymph nodes was an independent marker of prognosis. Patients who were pN0 (mol+) exhibited earlier time to recurrence (adjusted hazard ratio, 4.66; 95% CI, 1.11-19.57; P = .04) and reduced disease-free survival (adjusted hazard ratio, 3.27; 95% CI, 1.15-9.29; P = .03).

Conclusion Expression of GUCY2C in histologically negative lymph nodes appears to be independently associated with time to recurrence and disease-free survival in patients with pN0 colorectal cancer.
Guanylyl cyclase 2C (GUCY2C), an intestinal tumor suppressor, is the receptor for the paracrine hormones guanylin and uroguanylin, which are gene products frequently lost early in colon carcinogenesis.\textsuperscript{10,11} Loss of hormone expression with dysregulated GUCY2C signaling contributes to neoplastic transformation through unrestricted proliferation, crypt hypertrophy, metabolic remodeling, and genomic instability.\textsuperscript{11} Selective expression by intestinal epithelial cells normally, and overexpression by intestinal tumor cells,\textsuperscript{12-14} reflecting cells normally, and overexpression by intestinal epithelial cells, suggests that GUCY2C is a specific molecular marker for metastatic colorectal cancer.\textsuperscript{15-17}

In a previous retrospective study, GUCY2C expression quantified by the reverse transcriptase–polymerase chain reaction (RT-PCR) was associated with disease recurrence.\textsuperscript{15} The current study prospectively examined the utility of GUCY2C quantitative RT-PCR in patients with pN0 colorectal cancer to identify occult metastases and to define the risk of developing recurrent disease after surgical treatment.

**METHODS**

**Study Design**

This study was a prospective observational investigation. Investigators and clinical personnel were blinded to the results of the molecular analyses, while laboratory personnel and analysts were blinded to patient and clinical information. To have at least 80% power to detect a hazard ratio (HR) of 1.6 (2-sided P≤.05),\textsuperscript{18} 225 patients with pN0 colorectal cancer were included. Thus, GUCY2C expression in tumors was below background levels in 14 patients who were excluded from further analysis.\textsuperscript{13} There were no differences in the clinicopathologic characteristics of patients with and without available tumors. Moreover, analysis of the 2656 lymph nodes available from the remaining 259 patients with pN0 colorectal cancer yielded 86 lymph nodes with RNA of insufficient integrity by β-actin quantitative RT-PCR, excluding 2 additional patients (see online supplemental information at http://www.jama.com).\textsuperscript{13}

Overall, the 257 patients with pN0 colorectal cancer who met eligibility criteria provided 6699 lymph nodes (range, 2-139 lymph nodes/patient; median, 21 lymph nodes/patient) for histopathologic examination, of which 2570 lymph nodes (range, 1-33 lymph nodes/patient; median, 8 lymph nodes/patient) were eligible for analysis by quantitative RT-PCR. The greater number of lymph nodes available for histopathology compared with molecular analysis from patients with pN0 colorectal cancer includes those collected after formalin fixation or smaller than 5 mm in diameter, which is smaller than the limit of bisection.

**Patients and Tissues**

Between March 2002 and June 2007, we enrolled 273 patients with stage 0 to II pN0 and 87 stage III pN1 colorectal cancer who provided informed consent in writing prior to surgery at 1 of 7 academic medical centers and 2 community hospitals in the United States and Canada (Figure 1). The study protocol was approved by the institutional review board of each participating hospital. Patients were ineligible if they had a previous history of cancer, metachronous extraintestinal cancer, or perioperative mortality associated with primary resection. For all eligible patients, preoperative and perioperative examinations revealed no evidence of metastatic disease. lymph nodes, and when available tumor specimens (51%), were dissected from colon and rectum resections and frozen at −80°C within 1 hour to minimize warm ischemia. Half of each resected lymph node was fixed with formalin and embedded in paraffin for histopathological examination. Specimens from patients with pN0 colorectal cancer were subjected to molecular analysis if (1) tumor samples, where available, expressed GUCY2C messenger RNA above background levels in disease-free lymph nodes (≥30 copies) and (2) at least 1 lymph node was provided that yielded RNA of sufficient integrity for analysis.\textsuperscript{13} This study was a prospective observational investigation. Investigators and clinical personnel were blinded to the results of the molecular analyses, while laboratory personnel and analysts were blinded to patient and clinical information. To have at least 80% power to detect a hazard ratio (HR) of 1.6 (2-sided P≤.05),\textsuperscript{18} 225 patients with pN0 colorectal cancer were included. Thus, GUCY2C expression in tumors was below background levels in 14 patients who were excluded from further analysis.\textsuperscript{13} There were no differences in the clinicopathologic characteristics of patients with and without available tumors. Moreover, analysis of the 2656 lymph nodes available from the remaining 259 patients with pN0 colorectal cancer yielded 86 lymph nodes with RNA of insufficient integrity by β-actin quantitative RT-PCR, excluding 2 additional patients (see online supplemental information at http://www.jama.com).\textsuperscript{13}

**Disease status, obtained during routine follow-up by the treating physicians, was provided for all patients through December 7, 2007.**

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**Figure 1. Patient Selection for GUCY2C Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis**

- 360 Patients enrolled with pN0–pN1 colorectal cancer
- 87 Patients had stage III pN1 colorectal cancer
- 273 Patients had stage II (pN0) colorectal cancer with ≥1 lymph node available (2773 lymph nodes)
- 14 Patients excluded (primary tumors had inadequate GUCY2C expression) (117 lymph nodes)
- 259 Patients expressing GUCY2C in tumors (2056 lymph nodes)
- 2 Patients excluded (all lymph nodes had inadequate RNA integrity)
- 257 Patients with tumor RNA of adequate integrity (2570 lymph nodes) included in analysis
- 87 Patients included in analysis

GUCY2C indicates guanylyl cyclase 2C.
RNA Isolation
The RNA was extracted from tissues by a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method.\textsuperscript{15,16} Briefly, individual tissues were pulverized in 1.0 mL of TRI Reagent (Molecular Research Center, Cincinnati, Ohio) with 12 to 14 sterile 2.5-mm zirconium beads in a bead mill (Biospec, Bartlesville, Oklahoma) for 1 to 2 minutes. Phase separation was performed with 0.1 mL of bichloropropane, and the aqueous phase reextracted with 0.5 mL of chloroform. The RNA was precipitated with 50% isopropanol and washed with 70% ethanol. The air-dried RNA was dissolved in water, the concentration was determined by spectrophotometry, and it was stored at −80°C.

Reverse Transcriptase–Polymerase Chain Reaction
The GUCY2C messenger RNA was quantified by RT-PCR using an established analytically validated assay.\textsuperscript{13} The E\textsuperscript{2} RT-PCR kit (Applied Biosystems, Foster City, California) was used to amplify GUCY2C messenger RNA from total RNA in a 50-µL reaction. Optical strip tubes were used for all reactions, which were conducted in an ABI 7000 Sequence Detection System (Applied Biosystems). In addition to the kit components (50 mM of bicine [pH, 8.2], 115 mM of potassium acetate, 10 µM of EDTA, 60 nM of ROX dye, 8% glycerol, 3 mM of magnesium acetate, 300 µM each of deoxyadenosine triphosphate, deoxyctydine triphosphate, and deoxyguanosine triphosphate, 600 µM of 2’-deoxyuridine 5’-triphosphate, 0.5 U of uracil N-glycosylase, and 5 U of DNA polymerase), the reaction master mix contained 900 nM each of forward (ATTCTAGTGATCTTCTTTCATGACCA) and reverse primers (CTGTCAGAAACAGGACATTTTTT), a 200-nM Taqman probe (FAM-TACCTGAGGAGCATGTCACG-GCCCTG-TAMRA), and 1 µg of RNA template. The housekeeping gene β-actin was amplified using similar conditions except that forward (CCACACTG TGCCCATCTACG) and reverse (AG-}

GATCTTCATGAGGTAGTCAGTCA GAG) primers were 300 nM each, while the Taqman probe (FAM-ATGCCC-X[TAMRA]-CCCCCATGCCAT CCTGCGT) was 200 nM. The thermocycler program used for reverse transcriptase included 50°C × 2 minutes, 60°C × 30 minutes, and 95°C × 5 minutes; and for polymerase chain reaction: 45 cycles of 94°C × 20 seconds and 62°C × 1 minute. Reactions were performed at least in duplicate and the results were averaged.

Statistical Methods
The GUCY2C and β-actin messenger RNA were estimated by logistic regression analyses of amplification profiles from individual RT-PCR reactions, providing an efficiency-adjusted relative quantification based on parameter estimates from the fitted models, which reduces bias and error (see online supplemental information for details).\textsuperscript{19} The distribution of relative GUCY2C expression for all lymph nodes was quantified and the overall median computed.

In the absence of established methods to define optimal cut points for molecular markers from variable lymph node collections from individual patients, it was established a priori that lymph nodes in which relative GUCY2C messenger RNA was greater than or equal to the overall median would be considered positive while those less than the median would be considered negative (see online supplemental information for details; eFigure 1). Patients were considered pN0 (mol−) if 1 or more lymph nodes were positive.

The primary clinical end point was time to recurrence, which was measured from the date of surgery to the time of the last follow-up, recurrence event, or death.\textsuperscript{20} Disease-free survival, defined as time from surgery to any event regardless of cause, was a secondary outcome.\textsuperscript{20} Date of recurrence was established by radiographic studies, laboratory studies, physical examination, and/or histopathology. The 95% confidence intervals (CIs) for raw survival rates were computed by the method of Clopper-Pearson.\textsuperscript{21} Survival distributions for patients with and without occult metastases were compared using the likelihood ratio test. While Kaplan-Meier plots display censored survival at 36 months to ensure availability of at least 20% of patients at all time points, analyses incorporated all events up to the date of the last follow-up visit.\textsuperscript{22} The association of pN0 (mol−) with categorical patient characteristics was quantified using χ² tests or the Fisher exact test in cases of small sample sizes.

Simultaneous prognostic associations with different parameters were estimated using Cox regression analysis. Established prognostic variables in the Cox model for recurrence included T stage, grade, tumor location, lymphovascular invasion, chemotherapy, total lymph nodes harvested, and pN0 molecular status.\textsuperscript{23} The multivariate model for each outcome included all of the established prognostic measures regardless of significance to establish the additional independent prognostic association with molecular status (eTable 1 and eTable 2). Multivariate analyses using total number of lymph nodes harvested as a categorical variable also was performed. A global test of proportional hazards for each of the Cox models was completed according to the method by Hosmer and Lemeshow.\textsuperscript{24} All tests were 2-sided and P < .05 was considered statistically significant. All analyses were performed with SAS version 9.1 (SAS Institute Inc, Cary, North Carolina) and Stata version 8.0 (StataCorp, College Station, Texas).

RESULTS
Patient Characteristics
The 257 patients with pN0 colorectal cancer whose lymph nodes were subjected to quantitative RT-PCR had a mean age of 68 years at diagnosis and 44.8% were female (TABLE). Clinico-pathologic features, including depth of tumor penetration (T1/2, T3, T4), and tumor anatomical location (right, left, sigmoid colon) were similar to national experience.\textsuperscript{3,4,23} The patients with colon cancer represented 87.4% of the study population, while those with rectal tumors represented 13.6%.

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There were no statistically significant differences in baseline characteristics of patients included or excluded from quantitative RT-PCR analysis, and in those with and without occult metastases, with the exception of tumor grade and total number of lymph nodes harvested (Table). Patients exhibited the well-established direct relationships between time to recurrence, disease-free survival, and disease stage (eFigure 2 and eFigure 3). Adjuvant 5-fluorouracil–based chemotherapy, delivered at the discretion of treating physicians, was received by 22.2% of patients with pN0 colorectal cancer and 71.3% of patients with stage III pN1 disease (Table).

### Occult Metastases and Disease Recurrence

GUCY2C expression, presumably indicating the presence of occult metastases, was detected in at least 1 lymph node from 225 patients (87.5%) with pN0 colorectal cancer. With a median follow-up of 24.0 months (range, 1.8-62.7 months) for patients with pN0 (mol−), 20.9% (95% CI, 15.8%-26.8%) of patients with, but only 6.3% (95% CI, 0.8%-20.8%) without, occult metastases developed recurrent disease ($P= .006$; Figure 2). Both patients negative for GUCY2C who developed recurrent disease provided 2 or less lymph nodes for analysis by quantitative RT-PCR, perhaps reflecting the requirement by any staging technique for adequate lymph node sampling. Subgroup analyses suggested that GUCY2C expression was associated with a worse time to recurrence among patients with American Joint Committee on Cancer stage 0/I and II and those with colon and rectal cancer (eFigure 4). Moreover, GUCY2C-positive lymph nodes were associated with reduced disease-free survival (eFigure 5). Like time to recurrence, subgroup analyses suggested that occult metastases were associated with reduced disease-free survival in patients with tumors of different stages and lo-
GUCY2C as a Prognostic Variable

Univariate and multivariate analyses using Cox proportional hazards models (Figure 3 and Figure 4) revealed that grade, tumor location, lymphovascular invasion, therapy, and total number of lymph nodes harvested contributed little as prognostic factors in this cohort of patients with pN0 colorectal cancer. Whether lymph nodes serve as a categorical or continuous variable had minimal impact on these results (see online supplemental information; eTable 1 and eTable 2). In that context, the global test of nonproportional hazards for time to recurrence ($\chi^2_p = 8.67; \ P = .73$) and disease-free survival ($\chi^2_p = 10.31; \ P = .59$) indicated that there were no significant departures from the proportional hazards assumptions of these models. T stage was a weak prognostic variable, reflecting the disproportionate number of T3 tumors (52.9%) compared with T4 tumors (7.4%) in the pN0 colorectal cancer cohort and the established relationship between tumor size, depth of penetration, and prognosis.2,9,21 However, GUCY2C expression in lymph nodes provided independent prognostic information and patients with pN0 (mol+) exhibited earlier time to recurrence (absolute event rates: pN0 [mol−], 6.3%; pN0 [mol+], 20.9%; adjusted HR, 4.66 [95% CI, 1.11-19.57]; $\ P = .04$; Figure 3) and reduced disease-free survival (absolute event rates: pN0 [mol−], 12.5%; pN0 [mol+], 26.2%; adjusted HR, 3.27 [95% CI, 1.15-9.29]; $\ P = .03$; Figure 4).

COMMENT

A near-universal principle of cancer staging recognizes the established relationship between regional lymph node metastases and prognostic risk.2,3 In colon and rectal cancer, lymph node metastasis is the single most important prognostic characteristic, representing pathological evidence of dissemination of tumor cells beyond their primary location. Clinically, approximately 50% of patients with stage III disease will experience disease recurrence.1,2,9,23,25-27 Because up to 25% of patients without histological evidence of nodal involvement also experience recurrent disease, it is presumed that many such patients harbor occult metastases not identified at the time of primary resection.1,2 Understaging by conventional methods reflects the

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**Figure 3.** Cox Proportional Hazards Analyses of Time to Recurrence in Patients With pN0 Colorectal Cancer Undergoing Molecular Staging

<table>
<thead>
<tr>
<th>T stage</th>
<th>No. of Events</th>
<th>No. of Patients</th>
<th>Univariate HR (95% CI)</th>
<th>$P$ Value</th>
<th>Multivariate HR (95% CI)</th>
<th>Time to Recurrence Better</th>
<th>Time to Recurrence Worse</th>
<th>Multivariate $P$ Value</th>
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<tr>
<td>1/2</td>
<td>14</td>
<td>102</td>
<td>1 [Reference]</td>
<td>.09</td>
<td>1 [Reference]</td>
<td>■</td>
<td>■</td>
<td>.11</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>136</td>
<td>1.73 (0.92-3.25)</td>
<td>.09</td>
<td>1.75 (0.89-3.43)</td>
<td>■</td>
<td>■</td>
<td>.19</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>19</td>
<td>1.64 (0.54-4.99)</td>
<td>.38</td>
<td>2.35 (0.67-8.28)</td>
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<td>■</td>
<td>.84</td>
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Grade

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<thead>
<tr>
<th>Location</th>
<th>No. of Events</th>
<th>No. of Patients</th>
<th>Univariate HR (95% CI)</th>
<th>$P$ Value</th>
<th>Multivariate HR (95% CI)</th>
<th>Time to Recurrence Better</th>
<th>Time to Recurrence Worse</th>
<th>Multivariate $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal</td>
<td>6</td>
<td>35</td>
<td>1 [Reference]</td>
<td>.07</td>
<td>1 [Reference]</td>
<td>■</td>
<td>■</td>
<td>.86</td>
</tr>
<tr>
<td>Right</td>
<td>17</td>
<td>108</td>
<td>0.95 (0.38-2.42)</td>
<td>.92</td>
<td>1.09 (0.40-3.03)</td>
<td>■</td>
<td>■</td>
<td>.54</td>
</tr>
<tr>
<td>Left</td>
<td>4</td>
<td>17</td>
<td>1.63 (0.46-5.80)</td>
<td>.45</td>
<td>1.52 (0.40-5.86)</td>
<td>■</td>
<td>■</td>
<td>.22</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>22</td>
<td>97</td>
<td>1.55 (0.63-3.83)</td>
<td>.34</td>
<td>1.81 (0.71-4.60)</td>
<td>■</td>
<td>■</td>
<td>.17</td>
</tr>
</tbody>
</table>

Lymphovascular invasion

<table>
<thead>
<tr>
<th>No of lymph nodes harvested</th>
<th>No. of Events</th>
<th>No. of Patients</th>
<th>Univariate HR (95% CI)</th>
<th>$P$ Value</th>
<th>Multivariate HR (95% CI)</th>
<th>Time to Recurrence Better</th>
<th>Time to Recurrence Worse</th>
<th>Multivariate $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;12</td>
<td>13</td>
<td>45</td>
<td>1 [Reference]</td>
<td>.36</td>
<td>0.61 (0.20-1.32)</td>
<td>■</td>
<td>■</td>
<td>.16</td>
</tr>
<tr>
<td>≥12</td>
<td>36</td>
<td>212</td>
<td>1.30 (0.70-2.41)</td>
<td>.42</td>
<td>0.61 (0.31-1.21)</td>
<td>■</td>
<td>■</td>
<td>.16</td>
</tr>
</tbody>
</table>

Treatment

<table>
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<tr>
<th>No of lymph nodes harvested</th>
<th>No. of Events</th>
<th>No. of Patients</th>
<th>Univariate HR (95% CI)</th>
<th>$P$ Value</th>
<th>Multivariate HR (95% CI)</th>
<th>Time to Recurrence Better</th>
<th>Time to Recurrence Worse</th>
<th>Multivariate $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>35</td>
<td>200</td>
<td>1 [Reference]</td>
<td>.09</td>
<td>1 [Reference]</td>
<td>■</td>
<td>■</td>
<td>.57</td>
</tr>
<tr>
<td>Surgery and chemotherapy</td>
<td>14</td>
<td>57</td>
<td>0.58 (0.31-1.09)</td>
<td>.09</td>
<td>1.22 (0.61-2.41)</td>
<td>■</td>
<td>■</td>
<td>.57</td>
</tr>
</tbody>
</table>

Occult metastases

<table>
<thead>
<tr>
<th>No of lymph nodes harvested</th>
<th>No. of Events</th>
<th>No. of Patients</th>
<th>Univariate HR (95% CI)</th>
<th>$P$ Value</th>
<th>Multivariate HR (95% CI)</th>
<th>Time to Recurrence Better</th>
<th>Time to Recurrence Worse</th>
<th>Multivariate $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN0 (mol−)</td>
<td>2</td>
<td>32</td>
<td>1 [Reference]</td>
<td>.05</td>
<td>1 [Reference]</td>
<td>■</td>
<td>■</td>
<td>.04</td>
</tr>
<tr>
<td>pN0 (mol+)</td>
<td>47</td>
<td>225</td>
<td>4.09 (0.99-16.85)</td>
<td>.05</td>
<td>4.66 (1.11-19.57)</td>
<td>■</td>
<td>■</td>
<td>.04</td>
</tr>
</tbody>
</table>

Error bars indicate 95% confidence intervals (CIs). The $P$ values for univariate and multivariate analyses describe interactions between prognostic characteristics and time to recurrence. GUCY2C indicates guanylyl cyclase 2C; HR, hazard ratio; pN0 (mol−), lymph nodes negative for GUCY2C; pN0 (mol+), lymph nodes positive for GUCY2C (occult metastases).
combination of insufficient numbers of lymph nodes for review, the analysis of only small volumes of individual lymph node tissue missing metastatic tumor cells,28 and the sensitivity of histopathology, which reliably detects only 1 cancer cell in 200 normal cells.29 Molecular staging could overcome limitations in the detection of occult lymph node metastases by incorporating all available tissue into analyses and increasing detection sensitivity through quantifiable disease-specific molecular markers,1,10 which nominally identify a single cancer cell in 1 million normal cells.30

In this study, prospective detection of occult metastases by GUCY2C quantitative RT-PCR appeared to be an independent prognostic marker of risk. Molecular staging revealed that about 13% of patients with pN0 colorectal cancer were free of tumor cells, while about 87% had GUCY2C results that suggested occult metastases. Even in the context of shorter follow-up, which could introduce a bias against the utility of GUCY2C in this setting, patients with pN0 (mol+) exhibited a significantly greater risk of earlier disease recurrence and reduced disease-free survival, the primary and secondary outcomes of the study, compared with patients with pN0 (mol–). While enrollment was sufficient to satisfy requirements for these outcomes, the 95% CIs around estimates in multivariate analyses were broad. Future studies with greater numbers of patients should provide more precise estimates of the prognostic utility of GUCY2C quantitative RT-PCR.

Although a high proportion of patients with pN0 colorectal cancer exhibit GUCY2C expression, indicating occult metastases, most patients with pN0 colorectal cancer will not recur.3,22 Similarly, not all patients with stage III pN1 disease who have histopathologically detectable lymph node metastases ultimately develop recurrent disease.3,22 Reconciliation of this apparent inconsistency relies on the recognition that nodal metastases, regardless of the methods used to detect them, do not ensure recurrence but, rather, indicate risk. In support of this concept, our study suggests recurrence rates for patients with pN0 (mol+) with occult metastases that are nearly identical to those for patients with stage III pN1,3 the lowest stage in which all patients have histopathologically detectable metastases (Figure 2 and eFigure 5).3,4

There is a well-established relationship between burden of disease, quantified as the number of lymph nodes harboring tumor cells by histopathology and prognostic risk in patients with
colorectal cancer. Assuming there are adequate numbers of lymph nodes to review, patients with stage III pN1 disease with 4 or more involved lymph nodes exhibit a recurrence rate that is approximately 50% to 100% greater than those with 3 or less involved lymph nodes. As in histologically based analyses, one limitation of our study is the variable number of lymph nodes available for molecular staging from individual patients, reflecting the requirement for fresh dissection of surgical specimens. Additionally, lymph nodes smaller than 5 mm were excluded from molecular analyses, reflecting size limits for tissue bisec-tion, although they are a particularly rich source of tumor metastases. These considerations suggest that the precision of staging by molecular analyses will benefit from optimum lymph node sampling to incorporate tumor burden into prognostic risk stratification.

An analysis of the subset of patients with pN0 colorectal cancer providing 12 or more lymph nodes for GUCY2C quantitative RT-PCR, applying standard American Joint Committee on Cancer definitions for pN1 and pN2, revealed that those with 0 to 3 involved lymph nodes exhibited a prognostic risk similar to patients with pN0 (5.9% vs 8.3%, respectively; eFigure 7). Conversely, those with 4 or more involved lymph nodes exhibited a risk (≥3 lymph nodes vs ≥4 lymph nodes; P = .03) identical to patients with stage III pN1 disease (eFigure 4). Improved prognostic risk stratification by integrating detection of occult metastases and estimates of tumor burden underscores the essential importance of adequate lymph node sampling for optimum molecular, as well as histopathological staging of patients with colorectal cancer. Beyond the number of involved lymph nodes, there is an evolving relationship between the volume of cancer cells in individual lymph nodes, disease burden, and prognostic risk. While metastatic foci of 0.2 mm or larger are associated with increased disease recurrence, the relationship between individual tumor cells or nests smaller than 0.2 mm and prognostic risk remains undefined. The emergence of quantitative RT-PCR provides an unprecedented opportunity for cancer cell enumeration in tissues. However, the superior sensitivity of RT-PCR, with its optimum tissue sampling and capacity for single cell discrimination, could identify occult cancer cells in lymph nodes below the threshold of prognostic risk, limiting the specificity of molecular staging. In that context, the current study was not designed to identify the quantitative threshold defining risk. Indeed, one limitation of this study was the requirement to define a priori the diagnostic limit of GUCY2C. In future studies, it will be essential to more precisely define the quantitative relationship between marker expression and disease risk that incorporates tumor burden to optimize prognostic sensitivity and specificity.

The presence of tumor cells in regional lymph nodes also directs therapy in patients with colon cancer. While adjuvant chemotherapy provides a survival benefit to patients with stage III disease, its utility in patients with pN0 colorectal cancer remains uncertain, with marginal survival benefits in patients with stage II disease in some, but not all, clinical trials. This uncertainty of treatment benefit in patients with stage II disease is echoed in the dynamic evolution of treatment guidelines, in which adjuvant therapy has become discretionary in patients with stage II disease with clinicopathologic features of poor prognostic risk, including T4 stage, intestinal obstruction, and intestinal perforation. The heterogeneous responses to therapy in patients with pN0 colorectal cancer may reflect, in part, heterogeneity with respect to occult nodal metastases. Moreover, standard of care includes adjuvant chemotherapy for patients with stage III pN1 disease, a cohort with a recurrence rate identical to patients with pN0 (mol+) (Figure 2 and eFigure 5). These considerations highlight the importance of advancing beyond the present study to refine the prognostic specificity of molecular staging using GUCY2C quantitative RT-PCR to more precisely stratify risk in patients with pN0 colorectal cancer and better inform the use of adjuvant chemotherapy.

Molecular staging represents one component of a comprehensive diagnostic, prognostic, and predictive strategy to personalize management strategies for individual patients. It provides adjunctive clinicopathological information that supplements, but does not replace, complimentary anatomical, microscopic, and morphological staging modalities. Beyond enhancing these current approaches, molecular staging offers a unique opportunity to prioritize future complex resource-intensive analyses of primary tumors that will optimize patient management. In that context, analyses of primary tumors to define mutations, gene expression and epigenetic profiles, and proteomic signatures to stratify risk, predict responses to chemotherapy, and to personalize interventions may best be applied to patients with pN0 (mol+) rather than pN0 (mol−). These considerations underscore the present and future importance of integrating molecular approaches incorporating specific markers of disease, like GUCY2C, and powerful detection methods like quantitative RT-PCR, into analytical strategies directing the management of patients with colorectal cancer.

Author Contributions: Dr Waldman had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Waldman, Hyslop, Weinberg.
Acquisition of data: Waldman, Hyslop, Schulz, Barkun, Nielsen, Haaf, Bonaccorso, Li, Weinberg.
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