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Association of GUCY2C expression in lymph nodes with time to recurrence and disease-free survival in pN0 colorectal cancer.

Scott A Waldman

Thomas Jefferson University, scott.waldman@jefferson.edu

Terry Hyslop

Thomas Jefferson University

Stephanie Schulz

Thomas Jefferson University

Alan Barkun

McGill University


Karl Nielsen

Thomas Jefferson University

See next page for additional authors

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Authors

Scott A Waldman, Terry Hyslop, Stephanie Schulz, Alan Barkun, Karl Nielsen, Janis Haaf, Christine Bonaccorso, Yanyan Li, and David S Weinberg

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Scott A. Waldman, M.D., Ph.D., Terry Hyslop, Ph.D., Stephanie Schulz, Ph.D., Alan
Barkun, M.D., Karl Nielsen, Janis Haaf, Christine Bonaccorso, Yanyan Li, and David S.
Weinberg, M.D., M.Sc.

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ABSTRACT

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.

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 mRNA, employing the reverse transcriptase-polymerase chain reaction, with recurrence and survival in patients with colorectal cancer.

Design, Setting, and Participants. Prospective enrollment of 257 patients with pN0 colorectal cancer enrolled between March 2002 and June 2007 at 9 centers provided 2,570 fresh lymph nodes ≥ 5 mm for histopathology and GUCY2C mRNA analysis. Patients were followed for a median of 24 months (range: 2-63) 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 (12.5%) patients had lymph nodes negative for GUCY2C [pN0(mol-)], and all but two remained free of disease during follow-up (recurrence rate 6.3% [95%CI 0.8-20.8%]). Conversely, 225 (87.5%) patients had lymph nodes positive for GUCY2C [pN0(mol+)], and 47 (20.9% [15.8-26.8%]) developed recurrent disease (p=0.006). Multivariable 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 [1.11-19.57]; p=0.035) and reduced disease-free survival (adjusted hazard ratio 3.27 [1.15-9.29]; p=0.026).

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.

AFFILIATIONS

Affiliations are listed at the end of the article.

Corresponding Author: Scott A. Waldman M.D., Ph.D., F.C.P., Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, 132 South 10th Street, 1170 Main, Philadelphia, PA 19107; Tel: (215) 955-6086; Fax: (215) 955-5681; E-mail: Scott.Waldman@jefferson.edu

Metastasis of tumor cells to regional lymph nodes is the single most important prognostic factor in patients with colorectal cancer.^{1, 2} 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 lymph nodes harboring metastases.^{3, 4} Adjuvant chemotherapy improves disease-free and overall survival in patients with histopathologically evident lymph node metastases, but its role in pN0 patients remains unclear.⁵⁻⁹

Given the established relationship between lymph node metastasis and prognosis, recurrence in a substantial fraction of pN0 patients suggests the presence of occult metastases [pN0(mol+)³] in regional lymph nodes that escape histopathological detection.^{1, 2} Conversely, pN0 patients 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 in pN0 patients could improve risk stratification in this clinically heterogeneous population. Precise evaluation of lymph node metastases may also identify pN0 patients who could benefit from adjuvant chemotherapy.

GUCY2C (guanylyl cyclase C), an intestinal tumor suppressor, is the receptor for the paracrine hormones guanylin and uroguanylin, gene products frequently lost early in colon carcinogenesis.^{10, 11} Loss of hormone expression, with dysregulated GUCY2C signaling contributes to neoplastic transformation through unrestricted proliferation, crypt hypertrophy, metabolic remodeling and genomic instability.¹¹ Selective expression by intestinal epithelial cells normally, and over-expression by intestinal tumor cells¹²⁻¹⁴ reflecting receptor supersensitization in the context of ligand deprivation, suggest that

GUCY2C is a specific molecular marker for metastatic colorectal cancer.¹⁵⁻¹⁷ In a previous retrospective study, GUCY2C expression quantified by the reverse transcriptase-polymerase chain reaction (RT-PCR) was associated with disease recurrence.¹⁵ The current study prospectively examined the utility of GUCY2C quantitative (q) 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 trial. Investigators and clinical personnel were blinded to results of 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 of 1.6 ($P \leq 0.05$, 2-sided)¹⁸, 225 pN0 patients were required.

PATIENTS AND TISSUES

Between March 2002 and June 2007, we enrolled 273 stage 0-II pN0 and 87 stage III pN1 colorectal cancer patients who provided informed consent in writing prior to surgery at one of 7 academic medical centers and 2 community hospitals in the U.S. and Canada (Fig. 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 extra-intestinal 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 one hour to minimize warm ischemia. Half of each resected lymph node was fixed with formalin and embedded in paraffin for histopathological examination. Specimens from pN0 patients were subjected to molecular analysis if (1) tumor samples, where available, expressed GUCY2C mRNA above background levels in disease-free lymph nodes (≥ 30 copies) and (2) at least one lymph node was provided which yielded RNA of sufficient integrity for analysis.¹³ Thus, GUCY2C expression in tumors was below background levels in 14 patients who were excluded from further analysis.¹³ It is noteworthy that there were no differences in the clinicopathologic characteristics of patients with and without available tumors. Moreover, analysis of the 2,656 lymph nodes available from the remaining 259 pN0 patients revealed 86 yielding RNA of insufficient integrity by β -actin qRT-PCR, excluding two additional patients (see Supplemental Information).¹³

Overall, the 257 pN0 patients who met eligibility criteria provided 6,699 lymph nodes (range 2-159, median 21 lymph nodes/patient) for histopathologic examination, of which 2,570 nodes (range 1-33, median 8 lymph nodes/patient) were eligible for analysis by qRT-PCR. The greater number of lymph nodes available for histopathology compared to molecular analysis from pN0 patients includes those collected after formalin fixation or <5 mm in diameter, smaller than the limit of bisection.

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

RNA ISOLATION

RNA was extracted from tissues by a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method.^{15, 16} Briefly, individual tissues were pulverized in 1.0 mL Tri-Reagent (Molecular Research Center, Cincinnati, OH) with 12-14 sterile 2.5 mm zirconium beads in a bead mill (Biospec, Bartlesville, OK) for 1-2 min. Phase separation was performed with 0.1 mL dichloropropane, and the aqueous phase re-extracted with 0.5 mL chloroform. RNA was precipitated with 50% isopropanol and washed with 70% ethanol. Air-dried RNA was dissolved in water, concentration determined by spectrophotometry, and stored at -80 °C.

RT-PCR

GUCY2C mRNA was quantified by RT-PCR employing an established analytically validated assay.¹³ The EZ RT-PCR kit (Applied Biosystems, Foster City, CA) was employed to amplify GUCY2C mRNA 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, Foster City, CA). In addition to the kit components [50 mM Bicine (pH 8.2), 115 mM KOAc, 10 µM EDTA, 60 nM ROX, 8% glycerol, 3 mM Mg(OAc)₂, 300 µM each dATP, dCTP, and dGTP, 600 µM dUTP, 0.5 U uracil N-glycosylase, and 5 U rTth DNA polymerase], the reaction master mix contained 900 nM each of forward (ATTCTAGTGGATCTTTTCAATGACCA) and reverse primers (CGTCAGAACAAGGACATTTTTCAT), 200 nM Taqman probe (FAM-TACTTGGAGG-ACAATGTCACAGCCCCTG-TAMRA), and 1 µg RNA template. The housekeeping gene β-actin was amplified employing similar conditions except that forward (CCCACTGTG-CCCATCTACG) and reverse (AGGATCTTCATGAGGTAGTCAGTCAG) primers were

300 nM each, while the Taqman probe (FAM-ATGCCC-X(TAMRA)-CCCCCATGCCAT-CCTGCGTp) was 200 nM. The thermocycler program employed for RT included: 50° x 2 min, 60° x 30 min, 95° x 5 min; and for PCR: 45 cycles of 94° x 20 sec, 62° x 1 min. Reactions were performed at least in duplicate and results averaged.

STATISTICAL METHODS

GUCY2C and β -actin mRNA 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 Supplementary Information for details).¹⁹ The distribution of relative GUCY2C expression for all lymph nodes was quantified and the overall median computed. In the absence of established methodologies to define optimal cutpoints for molecular markers from variable lymph node collections from individual patients, it was established *a priori* that nodes in which relative GUCY2C mRNA was greater than or equal to the overall median would be considered positive while those less than the median would be considered negative, (see Supplementary Information for details). Patients were considered pN0(mol+) if 1 or more nodes were positive.

The primary clinical endpoint was time to recurrence, measured from the date of surgery to the time of the last follow-up, recurrence event or death.²⁰ Disease-free survival, defined as time from surgery to any event regardless of cause, was a secondary outcome.²⁰ Date of recurrence was established by radiographic studies, laboratory studies, physical exam, and/or histopathology. Confidence intervals for raw survival rates were computed by the method of Clopper-Pearson.²¹ Survival distributions for patients with and without occult metastases were compared employing 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 last follow-up.²² The association of pN0(mol+) with categorical patient characteristics was quantified using chi-square tests or the Fisher's exact test in cases of small sample sizes. Simultaneous prognostic effects of different parameters were estimated employing 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.²³ The multivariable model for each outcome included all of the established prognostic measures regardless of significance in order to establish the additional independent prognostic effect of molecular status. A global test of proportional hazards for each of the Cox models was completed according to Hosmer and Lemeshow.²⁴ All tests were two-sided, and $p < 0.05$ was considered statistically significant. All analyses were performed with SAS v9.1 and Stata v8.0.

RESULTS

PATIENT CHARACTERISTICS

The 257 pN0 patients whose lymph nodes were subjected to qRT-PCR had a mean age of 68 years at diagnosis and 44.8% were female (Table 1). Clinicopathologic features, including depth of tumor penetration (T1/2, T3, T4), and tumor anatomical location (right, left, sigmoid colon) were similar to national experience.^{3, 4, 23} Patients with colon cancer represented 87.4%, while those with rectal tumors were 13.6%. There were no statistically significant differences in baseline characteristics of patients included or excluded from qRT-PCR analysis, and in those with and without occult metastases, with

the exception of tumor grade and total lymph nodes harvested (Table 1). Patients exhibited the well-established direct relationships between time to recurrence, disease-free survival and stage (Supplemental Figs. 1, 2).^{3, 4, 23} Adjuvant 5-fluorouracil-based chemotherapy, delivered at the discretion of treating physicians, was received by 22.2% of pN0, and 71.3% of stage III pN1, patients (Table 1).

OCCULT METASTASES AND DISEASE RECURRENCE

GUCY2C expression, presumably indicating the presence of occult metastases, was detected in at least one lymph node from 225 (87.5%) patients with pN0 colorectal cancer. With a median follow-up of 24.0 months (range 1.8 to 62.7) for pN0(mol+) patients and 35.9 months (range, 2.5 to 62.1) for pN0(mol-) patients, 20.9% (CI, 15.8-26.8%) of patients with, but only 6.3% (CI, 0.8-20.8%) without, occult metastases developed recurrent disease ($p=0.006$; Fig. 2). Both GUCY2C-negative patients who developed recurrent disease provided ≤ 2 lymph nodes for analysis by qRT-PCR, perhaps reflecting the requirement, by any staging technique, for adequate lymph node sampling.^{3, 23, 25, 26} Subgroup analyses suggested that GUCY2C expression conferred a worse time to recurrence among patients with AJCC stage 0/I and II and those with colon and rectal cancer (Supplemental Fig. 3). Moreover, GUCY2C-positive lymph nodes were associated with reduced disease-free survival (Supplemental Fig. 4). 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 locations (Supplemental Fig. 5). Time to recurrence (Fig. 2) and disease-free survival (Supplemental Fig. 4) in pN0(mol+) patients were comparable to that of patients

with stage III pN1 (stage IIIA + IIIB) disease, all of whom have histopathologically-detectable metastases in regional lymph nodes.

GUCY2C AS A PROGNOSTIC VARIABLE

Univariable and multivariable analyses employing Cox proportional-hazards models (Figs. 3, 4) revealed that grade, tumor location, lymphovascular invasion, therapy, and total lymph nodes harvested contributed little as prognostic factors in this cohort of patients with pN0 colorectal cancer. In that context, the global test of non-proportional hazards for time to recurrence (chi-square, 8.67; 12 df; p=0.73) and disease-free survival (chi-square, 10.31; 12 df; p=0.59) 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 (52.9%), compared to T4 (7.4%), tumors in the pN0 cohort and the established relationship between tumor size, depth of penetration and prognosis.^{3, 4, 9, 23} However, GUCY2C expression in lymph nodes provided independent prognostic information and patients who were pN0(mol+) exhibited earlier time to recurrence (absolute event rates: pN0(mol-) 6.3%, pN0(mol+) 20.9%; adjusted hazard ratio 4.66 [1.11-19.57]; p=0.035; Fig. 3) and reduced disease-free survival (absolute event rates: pN0(mol-) 12.5%, pN0(mol+) 26.2%; adjusted hazard ratio 3.27 [1.15-9.29]; p=0.026; Fig. 4).

COMMENT

A near-universal principle of cancer staging recognizes the established relationship between regional lymph node metastases and prognostic risk.^{4, 23} In colon and rectal cancer, lymph node metastasis is the single most important prognostic characteristic, representing pathologic evidence of dissemination of tumor cells beyond their primary

location. Clinically, approximately 50% of stage III patients will suffer disease recurrence.^{1, 2, 4, 9, 23, 25-27} Because up to 25% of patients without histological evidence of nodal involvement also suffer 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 combination of insufficient numbers of nodes for review, the analysis of only small volumes of individual lymph node tissue missing metastatic tumor cells²⁸, and the sensitivity of histopathology, which reliably detects only 1 cancer cell in 200 normal cells²⁹. 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 cells³⁰.

In this study, prospective detection of occult metastases by GUCY2C qRT-PCR appeared to be an independent prognostic marker of risk. Molecular staging revealed that about 13% of pN0 patients 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, pN0(mol+) patients exhibited a significantly greater risk of earlier disease recurrence and reduced disease-free survival, the primary and secondary outcomes of the study, compared to pN0(mol-) patients. **While enrollment was sufficient to satisfy requirements for these outcomes, confidence intervals around estimates in multivariable analyses were broad. Future studies with greater numbers of patients should provide more precise estimates of the prognostic utility of GUCY2C qRT-PCR.**

Although a high proportion of pN0 patients exhibit GUCY2C expression, indicating occult metastases, most pN0 patients will not recur.^{3, 23} Similarly, not all stage III patients who have histopathologically-detectable lymph node metastases ultimately develop recurrent disease.^{3, 23} Reconciliation of this apparent inconsistency relies on the recognition that nodal metastases, regardless of methods used to detect them, do not assure recurrence but, rather, indicate risk. In support of this concept, our study suggests recurrence rates for pN0(mol+) patients with occult metastases that are nearly identical to those for stage III pN1 patients³, the lowest stage in which all patients have histopathologically-detectable metastases (see Fig. 2, Supplemental Fig. 4).^{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 colorectal cancer patients. Assuming there are adequate numbers of nodes to review^{3, 23, 25, 26}, stage III patients with ≥ 4 involved lymph nodes exhibit a recurrence rate that is approximately 50-100% greater than those with ≤ 3 involved nodes.^{3, 23} As in histology-based analyses, one limitation of the present 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 < 5 mm were excluded from molecular analyses, reflecting size limits for tissue bisection, although they are a particularly rich source of tumor metastases.^{31, 32} 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.^{1, 2, 27} An analysis of the subset of pN0 patients providing ≥ 12 lymph nodes for GUCY2C qRT-PCR, applying standard AJCC definitions for pN1 and pN2^{3, 23}, revealed that those with

0-3 involved nodes exhibited a prognostic risk similar to pN0(mol-) patients (5.9% v 8.3%, respectively; Supplemental Fig. 6). Conversely, those with ≥ 4 involved nodes exhibited a risk (≤ 3 versus ≥ 4 , $p=0.027$) identical to patients with stage III pN1 disease (Supplemental Fig. 3). 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^{1, 2, 27}, as well as histopathological^{3, 23, 25, 26}, 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 nodes, disease burden, and prognostic risk.^{3, 28} While metastatic foci ≥ 0.2 mm are associated with increased disease recurrence, the relationship between individual tumor cells or nests smaller than 0.2 mm and prognostic risk remains undefined.³ The emergence of qRT-PCR provides an unprecedented opportunity for cancer cell enumeration in tissues. However, the superior sensitivity of RT-PCR³⁰, with its optimum tissue sampling and capacity for single cell discrimination, could identify occult cancer cells in lymph nodes below the threshold of prognostic risk³, 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 colon cancer remains uncertain, with marginal survival benefits in stage II patients in some, but not all, clinical trials.^{3, 5-9, 23, 33,}

³⁴ This uncertainty of treatment benefit in stage II patients is echoed in the dynamic evolution of treatment guidelines, in which adjuvant therapy has become discretionary in stage II patients with clinicopathologic features of poor prognostic risk, including T4 stage, intestinal obstruction, and intestinal perforation.^{9, 33, 35, 36} It is tempting to speculate that heterogeneous responses to therapy in pN0 patients reflect, in part, heterogeneity with respect to occult nodal metastases. Moreover, standard of care includes adjuvant chemotherapy for stage III pN1 patients, a cohort with a recurrence rate identical to pN0(mol+) patients (see Fig. 2, Supplemental Fig. 4). **These considerations highlight the importance of advancing beyond the present study to refine the prognostic specificity of molecular staging employing GUCY2C qRT-PCR to more precisely stratify risk in pN0 patients and better inform the use of adjuvant chemotherapy.**

Molecular staging represents one component of a comprehensive diagnostic, prognostic and predictive paradigm to personalize management strategies for individual patients.^{37,}

³⁸ 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 personalize interventions, may

best be applied to pN0(mol+), rather than pN0(mol-), patients.³⁹⁻⁴³ These considerations underscore the present and future importance of integrating molecular approaches incorporating specific markers of disease, like GUCY2C, and powerful detection methods like qRT-PCR, into analytical paradigms directing the management of patients with colorectal cancer.

AUTHOR AFFILIATIONS: From the Department of Pharmacology and Experimental Therapeutics (S.A.W., T.H., S.S., K.N., J.H., C.B., Y.L.), Thomas Jefferson University, Philadelphia, PA, U.S.A.; the Division of Gastroenterology, Department of Medicine (A.B.), McGill University, Montreal, Canada; and the Department of Medicine (D.S.W.), Fox Chase Cancer Center, Philadelphia, PA, U.S.A.

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: Hyslop, Waldman, Weinberg.

Acquisition of data: Bonaccorso, Haaf, Nielsen, Schulz.

Analysis and interpretation of data: Hyslop, Waldman.

Drafting of the manuscript: Barkun, Hyslop, Schulz, Waldman, Weinberg.

Critical revision of the manuscript for important intellectual content: Barkun, Hyslop, Schulz, Waldman, Weinberg.

Statistical analysis: Hyslop, Li

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Administrative, technical, or material support: Bonaccorso, Haaf, Nielsen.

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PARTICIPATING GROUPS:

Thomas Jefferson University, Philadelphia, Pennsylvania (Thomas Jefferson Hospital)-Coordinating Center. Scott A. Waldman, M.D., Ph.D. Project PI; Stephanie Schulz, Ph.D., Analytical PI; Terry Hyslop, Ph.D. Statistical PI; Janis Haaf, R.N. and Christine Bonaccorso, R.N., Project Coordinators; Karl Nielsen, Analytical Coordinator; Juan Palazzo, M.D., Pathologist; Kathleen Ackerman, Pathology Assistant; Robert Fry, M.D., Pauline Park, M.D., Scott Goldstein, M.D., Gerald Isenberg, M.D., Surgeons.

McGill University, Montreal, Canada (Montreal General Hospital, Royal Victoria Hospital, Montreal Jewish Hospital)-Study Site. Alan Barkun, M.D., Site PI; Victoria Marcus, M.D., Leslie Alpert, M.D., Esther Lamoureux, M.D., Pathologists; Gabriel Gabriel, Ehab Yaney, Pathology Assistants; Vicky Thiffault, Laura Kor, Kellee Durnin, Joyce Strazzulla, Clinical Coordinators.

Virtua Health System, Voorhees and Mt. Holly, New Jersey-Study Sites. Stephen Pilipshen, M.D., Site PI; William Cody, M.D., David Schaffzin, M.D., and Gary Siemons, M.D., Surgeons; Sergiy Koval, ASCP, Pathology Assistant; Judy Neuman, Teri Grand, Patricia Harris, Joyce Guy, Raina Moyer, Clinical Coordinators.

University of Florida, Gainesville, Florida (University Hospital, Gainesville VA Medical Center)-Study Site. Rodwige J. Desnoyers, M.D., Site PI; Scott Schell, M.D., Ph.D., Surgeon; James Crawford, M.D., Ph.D. Pathology; Valerie Paget-Wilkes, R.N., Clinical Coordinator.

Fox Chase Cancer Center, Philadelphia, Pennsylvania-Study Site. David Weinberg, M.D., Site PI and Project co-I, Harry Cooper, M.D., Pathologist; Elin Sigurdson, M.D., Natalie Joseph, M.D., James Watson, M.D., Surgeons; Paul Engstrom, M.D., Medical Oncologist; Eileen Keenan, Clinical Coordinator.

Data Safety and Monitoring Committee. Bruce Boman, Department of Medicine; Neal Flomenberg, M.D., Ph.D., Department of Medical Oncology; Walter Curran, M.D., Department of Radiation Oncology, Thomas Jefferson University.

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FIGURE LEGENDS

Figure 1. Patient selection for GUCY2C qRT-PCR Analysis.

Figure 2. Time to Recurrence in Patients with pN0 Colorectal Cancer Stratified by Occult Lymph Node Metastases. Time to recurrence in 87 patients with stage III pN1 (stage IIIA + IIIB) disease is presented for comparison.

Figure 3. Cox Proportional-Hazards Analyses of Time to Recurrence in Patients with pN0 Colon Cancer Undergoing Molecular Staging. Hazard ratios (circles) with 95% confidence intervals (horizontal lines) and P values for univariable and multivariable analyses describe interactions between prognostic characteristics (Parameter) and time to recurrence. Parameters that are significantly prognostic ($P < 0.05$) are highlighted in red.

Figure 4. Cox Proportional-Hazards Analyses of Disease-Free Survival in Patients with pN0 Colon Cancer Undergoing Molecular Staging. Hazard ratios (circles) with 95% confidence intervals (horizontal lines) and P values for univariable and multivariable analyses describe interactions between prognostic characteristics (Parameter) and disease-free survival. Parameters that are significantly prognostic ($P < 0.05$) are highlighted in red.

Table 1. Characteristics of Enrolled Patients with Colorectal Cancer.

	pN0(mol-)		pN0(mol+)		P	Stage III pN1	
	N	%	N	%		N	%
Totals	32	12.5	225	87.5		87	
Age, years					0.25		
<50	3	9.4	18	8.0		10	11.5
50-75	24	75.0	140	62.2		50	57.5
>75	5	15.6	67	29.8		27	31.0
Sex					0.38		
Male	20	62.5	122	54.2		43	49.4
Female	12	37.5	103	45.8		44	50.6
T Stage					0.32		
T1/T2	14	43.8	88	39.1		16	18.4
T3	14	43.7	122	54.2		50	57.5
T4	4	12.5	15	6.7		21	24.1
Grade					0.04		
Well	2	6.3	17	7.6		6	7.0
Moderate	20	62.5	178	79.1		61	70.1
Poor/unknown	10	31.3	30	13.3		20	22.9
Chemotherapy					0.68		
Yes	8	23.5	49	21.6		62	71.3
No	24	75.0	176	78.2		25	28.7
Tumor Site					0.84		
Left Colon	3	9.4	14	6.2		9	10.3
Right Colon	12	37.5	96	42.7		31	35.6
Sigmoid Colon	13	40.6	84	37.3		37	42.5
Rectum	4	12.5	31	13.8		10	11.5
Nodes Harvested					0.007		
<12	11	34.4	34	15.1		20	23.0
≥12	21	65.6	191	84.9		67	77.0