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Molecular staging estimates occult tumor burden in colorectal cancer

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Table of Contents

Section	Page
1. Introduction	4
2. Staging colorectal cancer	4
3. Adjuvant therapy in colon cancer	5
4. Staging and molecular diagnostics	6
5. Guanylyl cyclase C (GUCY2C), a biomarker for colorectal cancer	6
6. GUCY2C as a biomarker for occult colorectal metastases	7
6.1. Evolution of molecular diagnostics supporting prospective biomarker validation	8
6.2. GUCY2C qRT-PCR to stage colorectal cancer patients	10
6.3. Results from prospective clinical trial	12
7. Future Considerations	17
8. Summary	20

Abstract

Tumor cells in regional lymph nodes are a key prognostic marker of survival and predictive marker of response to adjuvant chemotherapy in colorectal cancer. However, clinicopathologic techniques to detect lymph node metastases remain imperfect, and ~30% of patients with lymph nodes negative by histology (pN0) develop recurrent disease, reflecting occult metastases that escape detection. These observations underscore an unmet clinical need for accurate approaches to identify occult nodal metastases in colorectal cancer patients. GUCY2C is a receptor whose expression normally is restricted to intestinal epithelial cells, but is universally over-expressed by colorectal cancer cells. A prospective, multicenter, blinded clinical trial established the prognostic utility of GUCY2C qRT-PCR to detect occult nodal metastases in pN0 colorectal cancer patients. Molecular staging revealed that ~13% of pN0 patients were free of cancer cells, while ~87% had GUCY2C results that suggested occult metastases. The presence of occult nodal metastases was the most powerful independent predictor of time to recurrence and disease-free survival. These observations establish the utility of molecular detection of occult nodal metastases for assessing prognostic risk in pN0 colorectal cancer patients. Advancing GUCY2C into staging paradigms in clinical laboratories will require validation in independent patient populations, definition of the relationship between the quantity of occult tumor metastases and risk, and determination of the utility of

GUCY2C qRT-PCR to identify pN0 patients who might benefit from adjuvant chemotherapy.

1. Introduction

Colorectal cancer continues to be the 4th most frequent tumor, with ~140,000 new cases annually in the U.S., and the 2nd leading cause of cancer-related mortality (1). Colorectal cancer causes ~10% of cancer-related deaths in the U.S., with a mortality rate approaching ~50% (1-3). Mortality reflects metastases: ~20% of colorectal cancer patients have unresectable disease at presentation (stage IV) and >30% will develop metastases during the course of their disease (2-5). Surgery continues to have the greatest impact on survival. However, while "curative" surgery removes all detectable tumor and is most successful in early-stage disease, occult metastases result in relapse (1-3, 6-9). Recurrence rates range from ~10% for disease limited to mucosa (stage I) to >60% for tumors metastatic to lymph nodes (stage III) (1-3, 6-19).

2. Staging colorectal cancer

Historically, the single most important prognostic determinant of clinical outcomes in colorectal cancer is tumor cells in regional lymph nodes (1-6, 9, 20-24). The importance of cancer cells in lymph nodes is underscored by the discovery that the biology of nodal and hematogenous metastases are identical (25), and tumor cells in lymph nodes offer a unique diagnostic window for prognostic and predictive risk stratification with respect to distant metastases that define outcomes. Although histopathology remains the standard paradigm, staging imprecision by conventional microscopy reflects methodological limitations (2, 5, 24). Microscopic visualization is insensitive, with

a lower limit for detection of ~1 cancer cell in 200 normal cells (26). Also, there is an inherent sampling error and typically less than 0.1% of available lymph node tissue is examined by microscopy (4, 5, 26). These limitations are highlighted by the frequency of post-surgical disease recurrence. In stage I and II (pN0) patients, who represent nearly 50% of all colorectal cancer patients, tumors are limited to the bowel wall without histological evidence of lymph node metastases or dissemination beyond intestine and should be cured by surgery. However, recurrence rates as high as 30% in stage I and 50% in stage II have been observed (2, 3, 5, 24). In stage III, where all obvious tumor, including lymph nodes harboring metastases, is removed, recurrence rates >70% have been described (2, 10, 12-15, 17-19, 27, 28). In pN0 patients, recurrences reflect a mixture of true pN0 lesions and occult stage III or IV lesions undetected by histopathology (2, 4, 5, 12, 21, 29, 30).

3. Adjuvant therapy in colon cancer

Beyond prognosis, stage identifies patients that receive adjuvant therapy. Chemotherapy administered after surgery to patients with stage III colon cancer enhances survival, increasing time-to-recurrence by 40% and overall survival by 30% (6, 20, 31-37). Also, introduction of molecularly targeted therapeutics increases 5 year median and overall survival in stage IV patients, from ~7% to >30% (38). In contrast, the benefit of adjuvant chemotherapy in pN0 colon cancer patients is unclear, with only small benefits in stage II patients in some, but not all, studies (2, 3, 6, 7, 20, 22, 23, 39). This uncertainty of therapeutic

benefit is reflected in the evolution of treatment guidelines, in which adjuvant therapy is optional in pN0 patients with clinicopathologic characteristics suggesting poor prognostic risk (9, 40-42). Heterogeneous responses to therapy in pN0 patients reflect, in part, the variability of occult lymph node metastases (4, 5, 21, 24, 43-45). Consequently, there is an unmet clinical need for better methods that detect prognostic occult nodal metastases, to identify pN0 patients who could benefit from adjuvant therapy (6, 38) and who are candidates for pharmacogenomic testing to identify critical mutations defining responses to molecular targeted agents (46).

4. Staging and molecular diagnostics

Histology remains the clinical standard for staging, reflecting the prognostic and predictive relationship between tumor cells in lymph nodes and outcomes (1-6, 9, 20-23). However, this approach underestimates metastases. In lymph nodes burdened with metastases, ~70% contain metastases that are <0.5 cm which often escape detection by standard clinicopathology approaches reflecting their size (2, 3, 5, 24). In contrast, evolving technologies including qRT-PCR, may offer the most sensitive and specific evaluation of nodal metastases (5, 24). Advantages of molecular staging include the ability to sample the entire specimen and to detect one tumor cell in ~10^7 normal cells (5, 24). While staging by RT-PCR has yielded inconsistent results, reflecting inadequate population size without appropriate clinical follow-up and variable analytic techniques, meta-analyses suggest the prognostic value of occult nodal

metastases detected by RT-PCR in pN0 colorectal cancer patients (4, 5, 21, 30, 45, 47).

5. Guanylyl cyclase C (GUCY2C), a biomarker for colorectal cancer

GUCY2C, one member of a family of receptor-enzyme proteins synthesizing guanosine 3′ 5′ cyclic monophosphate (cyclic GMP; cGMP), is specifically expressed by intestinal epithelial cells (48-57). GUCY2C is the cognate receptor for the paracrine hormones guanylin and uroguanylin, which interact with the extracellular domain, activating the cytoplasmic catalytic domain, inducing cGMP accumulation (53, 56, 58-64). GUCY2C regulates the dynamic progression of cells along the crypt-villus and crypt-surface axis, coordinating homeostatic processes including proliferation, DNA repair, metabolic programming, lineage-specific cell fate, and epithelial-mesenchymal interactions organizing that axis (65-77). Further, guanylin and uroguanylin are gene products universally lost early in colorectal neoplasia (78-82). Moreover, eliminating GUCY2C expression increases the burden of tumors in mouse models of intestinal cancer induced by inherited germline mutations or chemical carcinogenesis, reflecting dysregulation of the cell cycle and DNA repair (68). These observations suggest that GUCY2C is a tumor suppressor regulating homeostasis whose silencing reflecting the loss of paracrine hormones contributes to neoplasia (66-68, 73, 83). Of significance, GUCY2C was detected in all samples of normal intestine, but not in any extra-gastrointestinal specimens (43, 47, 49, 50, 58). Also, GUCY2C protein or mRNA was detected near-

universally (>95%) in all primary and metastatic human colorectal tumors regardless of anatomical location or grade, but not in tumors arising outside the GI tract (43, 47, 49, 50, 58, 81, 84-87). Further, GUCY2C mRNA and protein are over-expressed by >80% of colorectal cancers (84, 88, 89). Restriction of expression normally to intestinal epithelial cells, but universal over-expression by colorectal cancer cells highlights the use of GUCY2C as a biomarker for metastatic colorectal cancer (45).

6. GUCY2C as a biomarker for occult colorectal metastases

Early retrospective studies suggested that in colorectal cancer patients GUCY2C mRNA detected by RT-PCR predicted risk of disease recurrence (47). These initial observations supported an adequately powered, prospective, blinded clinical trial of the use of GUCY2C qRT-PCR to identify prognostically important occult nodal metastases using an analytically validated assay. This trial provided level 1 evidence (90) of the utility of RT-PCR for identifying prognostic lymph node metastases in colorectal cancer patients. This study (a) compared staging of colorectal cancer patients by GUCY2C RT-PCR with histopathology; (b) compared the predictive utility of staging by GUCY2C qRT-PCR or histopathology for recurrent colorectal cancer; and (3) developed a predictive model for disease recurrence employing GUCY2C qRT-PCR as an independent biomarker of risk.

6.1. Evolution of molecular diagnostics supporting prospective biomarker validation. Validation of GUCY2C as a biomarker for staging colorectal cancer

patients presented unexpected challenges reflecting the untested character of quantitative (q)RT-PCR to detect clinically significant biomarkers in clinical trials involving substantial numbers of patients. These studies depended on an analytically validated assay platform to quantify GUCY2C mRNA reliably across >5,000 specimens. Moreover, the validity of transcript quantification by qRT-PCR relies on the equivalence of reaction efficiencies in individual incubations, a characteristic that remarkably varies reflecting differences between patients, specimens, and reaction conditions. To compare GUCY2C mRNA quantities in ~20,000 qRT-PCR reactions, a platform was needed that incorporated adjustments to correct for variations in individual reaction efficiencies.

- **Validation of qRT-PCR assay for GUCY2C** (88). Analytic performance characteristics of the qRT-PCR assay for GUCY2C were defined employing GUCY2C complimentary (c)RNA standards. Analysis using linear mixed models of the relationship between GUCY2C cRNA concentrations and threshold cycles produced in the PCR phase of the reaction yielded a mean intercept of 42.36 (95% CI: 41.94, 42.79), mean slope of -3.53 (95% CI: -3.62, -3.44), and an average amplification slope efficiency of 92%. This assay exhibited a broad dynamic range, with linearity from 2.5×10^1 to 2×10^6 copies, and high sensitivity, with a limit of quantification of 25 copies. The assay was robust, with plate-to-plate variability (CV) of 1% and within-plate variability of <5% across all cRNA concentrations. These performance characteristics applied across various biological matrices, including human

lymph nodes. Clinicopathologic characteristics were established using total RNA extracted from lymph nodes with metastases identified by histology (true positives, 15 nodes) and from patients without colon cancer (true negatives; 164 nodes). Negative nodes exhibited median GUCY2C copy numbers $\leq$50 while positive nodes exhibited median copy numbers >1,000. Evaluation of these performance characteristics using receiver-operator curve analysis revealed a sensitivity of 93% and specificity of 97%. These robust performance characteristics suggest the suitability of GUCY2C qRT-PCR for examining the utility of that marker for staging colorectal cancer patients.

- **Relative qRT-PCR incorporating efficiency adjustments.** In PCR, DNA templates are enzymatically replicated at each cycle and copies created in each cycle emit a fluorescence signal proportional to the number of templates. For each PCR reaction, the fluorescence signal is measured after each cycle. With the cycle number, fluorescence measures constitute a kinetic PCR amplification history for each reaction. Ideal reactions are described by an exponential (base 2) growth model. In reality, not all templates are duplicated in a reaction cycle and the proportion of templates that are duplicated at each cycle is the amplification efficiency. This is a key issue in PCR quantification because many reactions do not have ideal or similar efficiencies, while comparisons of results between reactions presume equal efficiencies. Thus, variations in estimating GUCY2C expression reflecting heterogeneity of efficiencies between reactions could hide true

differences reflecting the presence of metastatic tumor cells. We developed a four-parameter logistic model which provides a method for efficiency-adjusted relative RT-PCR quantification based on estimates from the parameterized logistic model fitted to the full kinetic data from each RT-PCR reaction (45, 91). The efficiency-adjusted relative RT-PCR quantification using the parameterized logistic model fitted to the full kinetic data provides more accurate and precise estimates of individual PCR reaction efficiencies than traditional efficiency estimates based on exponential growth models (88). Thus, traditional exponential growth models were characterized by up to 5-fold greater variability and 6-fold greater bias in normalized estimates of GUCY2C expression, compared to the parameterized logistic model. Further, ~80% of individual RT-PCR reactions for GUCY2C or the reference gene β-actin provided insufficient exponential growth phase (<4 cycles) to apply traditional models for efficiency adjustments, suggesting that most reactions would be uninformative using traditional approaches. This new method for efficiency-adjusted relative qRT-PCR based on logistic models minimizes bias and variability, maximizes precision and accuracy, and preserves the integrity of information available from all reactions (45, 91-93). Of significance, this approach accommodates estimation of target analyte expression relative to reference genes using replicate reactions. Given these advantages, this technique was applied to analyze GUCY2C expression to detect occult metastases in lymph nodes of colorectal cancer patients.

6.2. GUCY2C qRT-PCR to stage colorectal cancer patients

- **Study design.** This was a prospective multicenter clinical trial in which investigators and clinical personnel were blinded to results of qRT-PCR analyses while laboratory personnel and analysts were blinded to clinicopathology information (45). To have at least 80% power to detect a hazard ratio of 1.6 ($P \leq 0.05$, 2-sided), an established threshold for stage-specific risk stratification(94), 225 pN0 patients were required.
- **Study population.** Between March 2002 and June 2007, 273 stage 0-II pN0 and 87 stage III pN1 colorectal cancer patients were enrolled at one of 9 hospitals in the U.S. and Canada (45). Patients were ineligible if they had a previous history of cancer, metachronous extra-intestinal cancer, or perioperative mortality associated with tumor resection.
- **Analytic approaches**

Pathology. Lymph nodes, and tumor specimens when available (51%), were 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 histological examination. Specimens from pN0 patients were subjected to qRT-PCR if (1) tumors, where available, expressed $\geq$30 copies GUCY2C mRNA, the baseline amount expressed in normal lymph nodes, and (2) at least one lymph node yielding RNA of sufficient integrity was available (88). GUCY2C in tumors was lower than background in 14 patients who were excluded from analysis (88). 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 (88). Overall, the 257 pN0 patients who were eligible 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. Greater numbers of lymph nodes available for histology compared to molecular analysis from pN0 patients includes those collected after formalin fixation or <5 mm in diameter, below the limit for accurate bisection of fresh tissue.

RT-PCR. GUCY2C and β-actin mRNA was quantified using qRT-PCR by an analytically validated assay (88) employing logistic regression of amplification profiles from individual RT-PCR reactions, providing an efficiency-adjusted relative quantification (91).

Statistics. In the absence of established methodologies to define optimal cutpoints for molecular markers from incomplete and variable collections of lymph nodes, it was established a priori that nodes in which relative GUCY2C mRNA was higher than or equal to the overall median would be considered pN0(mol+) while those lower than the median would be considered pN0(mol-) (45). Patients were considered categorically pN0(mol+) if ≥1 lymph nodes were positive. The primary clinical endpoint was time to recurrence, measured from date of surgery to time of last follow-up, recurrence event or death (95). The secondary clinical outcome was disease-free survival, defined as time from surgery to any event regardless of cause (95). Date of recurrence was

established by radiography, laboratory studies, physical exam, and/or histology. Simultaneous prognostic effects of parameters, including T stage, grade, tumor location, lymphovascular invasion, chemotherapy, total lymph nodes harvested, and pN0 molecular status (3), were estimated employing Cox regression analysis. The multivariable model for each outcome included all prognostic measures, to establish the additional independent prognostic effect of molecular status.

6.3. Results from prospective clinical trial

- **Occult metastases and disease recurrence (45).** GUCY2C expression, reflecting occult metastases, was detected in at least one lymph node from 225 (87.5%) patients with pN0 colorectal cancer (45). These data suggest that, unexpectedly, most patients staged as node-negative by traditional histopathology harbor occult metastases. The working hypothesis suggests that staging based on GUCY2C qRT-PCR should better predict colorectal cancer recurrence than histology. Thus, patients who are pN0(mol+) by GUCY2C qRT-PCR are at greater risk for recurrent disease than patients who are pN0(mol-). 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) (45). Both GUCY2C-negative patients who developed recurrent disease provided $\leq$2 lymph nodes for analysis by qRT-PCR, perhaps reflecting the requirement, by

any staging technique, for adequate lymph node sampling (2, 3, 96-102). Further, GUCY2C mRNA conferred a worse prognosis among stage I and II patients and those with colon and rectal cancer. Moreover, occult metastases were associated with reduced disease-free survival in patients with tumors of different stages and locations. Time to recurrence and disease-free survival in pN0(mol+) patients were comparable to that of patients with stage III pN1 (stage IIIA + IIIB) disease, all of whom have histologically detectable nodal metastases (45).

- **GUCY2C is an independent prognostic variable (45).** Occult lymph node metastases detected using GUCY2C qRT-PCR should enhance multivariable analyses incorporating known prognostic indicators to improve identification of patients with increased prognostic risk. Cox proportional-hazards analyses revealed that the established clinicopathologic parameters, including T stage, grade, tumor location, lymphovascular invasion, therapy, and total lymph nodes harvested, did not contribute substantially to prognosis. However, GUCY2C qRT-PCR provided the most powerful independent prognostic information, and patients who were pN0(mol+) experienced earlier time to recurrence (absolute event rates: pN0(mol-) 6.3%, pN0(mol+) 20.9%; hazard ratio 4.66 (1.11-19.57); p=0.035) and reduced disease-free survival (absolute event rates: pN0(mol-) 12.5%, pN0(mol+) 26.2%; hazard ratio 3.27 (1.15-9.29); p=0.026) (45).

Occult metastases detected by GUCY2C qRT-PCR for categorical risk stratification in pN0 colorectal cancer. Prospective detection of occult metastases by GUCY2C qRT-PCR was an independent prognostic marker of risk in pN0 colorectal cancer patients. Molecular staging revealed that ~13% of pN0 patients were free of tumor cells, while ~87% harbored occult metastases by GUCY2C qRT-PCR. Interestingly, while a high proportion of pN0 patients harbored occult metastases by GUCY2C, ~70% of pN0 patients will not recur (2, 3). Similarly, by comparison, only ~50% of stage III patients ultimately develop recurrent disease, although all have histology-detectable lymph node metastases (2, 3). Reconciliation of this apparent inconsistency requires the realization that nodal metastases, regardless of methods used to detect them, do not assure recurrence but, rather, are a marker of risk. Analyses using GUCY2C qRT-PCR suggests recurrence rates for pN0(mol+) patients with occult metastases that are nearly identical to those for stage III pN1 patients (2), the earliest stage in which all patients have microscopy-detectable metastases (1, 2). This analysis is the first to demonstrate the utility of molecular analysis to detect prognostic occult metastases in lymph nodes in an adequately powered, prospective trial with sufficient longitudinal follow-up employing analytically validated assays. Indeed, the absence of this level of evidence has been one limitation to the translation of these paradigms to patient management (4, 5). These considerations underscore the importance of future

validation with independent cohorts to confirm the prognostic utility of GUCY2C qRT-PCR in colorectal cancer.

There is an established relationship between tumor burden, quantified as the number of lymph nodes harboring tumor cells by microscopy, and prognostic risk in colorectal cancer patients. Assuming that adequate numbers of nodes are available for review (2, 3, 96-102), stage III patients with $\geq$4 lymph nodes harboring metastases exhibit a recurrence rate that is ~50-100% greater than those with $\leq$3 involved nodes (2, 3). As in histology-based analyses, one limitation of our prospective trial was the variable number of lymph nodes available for qRT-PCR from individual patients. Additionally, lymph nodes <5 mm were excluded, reflecting size limits for fresh tissue bisection, although they are a rich source of tumor metastases (103, 104). 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. (4, 5, 21). Our working hypothesis suggested that there is an inverse relationship between the number of lymph nodes that contain occult metastases and risk. Specifically, it was hypothesized that patients with more lymph nodes containing occult metastases will have a greater prognostic risk compared to patients with fewer involved lymph nodes. In an exploratory analysis, we examined the subset of pN0 patients who provided $\geq$12 lymph nodes for molecular analysis, then applied standard AJCC definitions for pN1 and pN2 (2, 3). This analysis revealed that individuals with 0-3 involved nodes exhibited a prognostic risk

similar to pN0(mol-) patients (5.9% versus 8.3%) (45). Conversely, those with ≥4 involved nodes exhibited a risk (≤3 versus ≥4, p=0.027) identical to patients with stage III pN1 disease (45). Improved risk stratification by integrating occult metastases and estimates of tumor burden underscores the importance of adequate lymph node sampling for optimum molecular (4, 5, 21), as well as histological (2, 3, 99, 100), staging in colorectal cancer. Moreover, the issue of adequacy of lymph node sampling in the context of the evolving prognostic and predictive significance of molecular staging is highlighted by the emergence of limited access surgical techniques for colon cancer like laparoscopic-assisted colectomy (105). Indeed, the success of these novel surgical approaches, with their inherent restricted opportunities for diagnostic tissue collection (105), will be informed substantially by the co-evolution of molecular staging and the requirements for adequate lymph node collections to provide the richest source of prognostic and predictive information for patient management.

Beyond the number of lymph nodes harboring metastases, there is an emerging relationship between the volume of cancer cells in individual nodes, tumor burden, and prognostic risk (2, 106). Metastatic foci ≥0.2 mm are associated with increased disease recurrence (2). However, the relationship between individual tumor cells or nests <0.2 mm and risk is unknown (2). The emergence of qRT-PCR provides an unprecedented opportunity for quantification of metastatic burden in tissues. The enhanced sensitivity of qRT-PCR (107), with

optimum sampling of tissue volumes and capability for single cell discrimination, may identify occult cancer cells in lymph nodes below the threshold of prognostic risk (2), limiting the specificity of molecular staging (45). Our prospective study was not designed to identify a quantitative threshold defining risk. Indeed, one limitation of that study was the requirement to define *a priori* the diagnostic threshold for GUCY2C. In the future, it will be essential to define the quantitative relationship between marker expression and disease risk that incorporates estimates of tumor burden to optimize prognostic sensitivity and specificity (45). Indeed, the potential for qRT-PCR to quantify occult metastases across all lymph nodes harvested, providing an integrated correlation of tumor burden and risk, further reinforces the central importance of empirically defining the number of lymph nodes required to provide optimum prognostic and predictive information to improve patient management.

7. Future Considerations

The most significant prognostic marker of survival and predictive marker of response to adjuvant chemotherapy in colorectal cancer is the histologic detection of metastatic tumor cells in lymph nodes (1-6, 9, 20-23). Despite its significance, approaches that evaluate lymph node metastases are inadequate and ~30% of pN0 patients develop disease recurrence, reflecting occult metastases that evade identification by established approaches (2-5, 21, 24, 43, 44, 108). These observations reinforce the clinical need for new approaches to more accurately evaluate occult nodal metastases in colorectal

cancer patients. We have completed a prospective, multicenter, blinded clinical trial that for the first time demonstrated the utility of molecular staging by GUCY2C qRT-PCR lymph node assessment to predict prognostic risk (45). Occult nodal metastases defined by GUCY2C qRT-PCR was the most powerful independent indicator of prognostic risk in pN0 patients, providing the first level 1 evidence that supports the association of prognostic risk and occult nodal metastases (90). These observations underscore the utility of molecular biomarker platforms generally, and GUCY2C qRT-PCR specifically, for staging patients with pN0 colorectal cancer. Translation of these preliminary studies into clinically applicable staging algorithms will require several essential analyses over the next several years.

- The prognostic utility of GUCY2C qRT-PCR for categorical identification (yes/no) of occult metastases as a marker of disease recurrence will require validation in an independent patient cohort. This approach conforms to the emerging learn-confirm paradigm in the translation of molecular biomarkers, in which their integration into clinical practice requires validation in independent populations (109-116).
- The enhanced sensitivity of qRT-PCR (107), with its advantageous tissue volume sampling and ability to discriminate single cells, may identify occult tumor deposits in lymph nodes below the threshold of prognostic risk (2), limiting the specificity of molecular staging (45, 93). This is reflected in the detection of occult metastases in 87% pN0 patients, most of whom will not

develop recurrent disease (2). There is an emerging paradigm that goes beyond the categorical (yes/no) presence of tumor cells, to quantify metastatic tumor burden (how much) to more accurately stratify risk (93). In that context, qRT-PCR provides a unique opportunity to quantify occult tumor burden across the regional lymph node network to establish prognostic risk in pN0 patients.

- Beyond prognosis, there is an established relationship between nodal metastases and therapeutic benefit in colon cancer patients. While stage III patients treated with adjuvant therapy exhibit better survival outcomes, there continues to be ambiguity about the application of adjuvant therapy to pN0 patients (2, 3, 6, 9, 20, 22, 23). Indeed, the heterogeneity of therapeutic benefit in pN0 patients may reflect a contribution of inaccurate staging (4, 5, 21, 24, 43-45). In our prospective trial (45), GUCY2C qRT-PCR identified a subset of pN0 patients whose clinical outcomes matched that of stage III patients, staged by established criteria. Typically, those patients receive adjuvant therapy suggesting that if pN0 patients at similar risk could be identified, they too could benefit from adjuvant chemotherapy. In the future, studies will define whether occult lymph node metastases detected by GUCY2C qRT-PCR is a predictive marker of chemotherapeutic benefit (93). These studies will determine if, among patients with occult lymph node metastases, those who receive chemotherapy have better clinical outcomes than those who do not.

- Most (2, 3, 96-102) studies support the critical relationship between the number of lymph nodes collected at staging colectomy and prognostic risk, although the precise number required for optimum patient management is not yet defined (102). In contrast, the emergence of limited access procedures like laparoscopy-assisted colectomy restricts the collection of lymph nodes for staging (105). The development of molecular staging, providing a rich source of prognostic and predictive information, underscores the importance of defining the number of lymph nodes required to optimize these new analyses. In turn, these molecular innovations in staging will inform the co-evolution of advancements in surgical management, driving the technical specifications of limited access surgery to optimize lymph node yields, producing the best surgical and staging solutions for patients.
- Molecular staging offers a unique opportunity to prioritize emerging complex resource-intensive analyses of primary tumors to optimize cost-effective patient management (45). 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 individualize targeted biological interventions, will best be applied to patients harboring occult nodal metastases, rather than to those free of disease (117-121). Thus, future studies will examine the utility of a sequential diagnostic algorithm, in which all pN0 patients first are staged using GUCY2C qRT-PCR, to determine <u>if</u> they have clinically significant nodal metastases, followed by

pharmacogenomic testing only of those patients at risk, to identify therapeutic interventions best matched to the biology of their tumors (46).

- Preliminary studies are compelling that molecular staging by comprehensive GUCY2C qRT-PCR lymph node analysis identifies pN0 patients at increased risk of developing recurrent disease. However, qRT-PCR is an emerging molecular platform that has not yet found broad dissemination to primary and secondary medical centers, raising a question of the limitations to implementation of molecular staging as a clinical standard of practice. In that context, molecular diagnostics is a burgeoning $14 billion dollar industry, growing at more than 10% each year (122, 123). The number of esoteric molecular diagnostic tests approved by the FDA annually is increasing exponentially, from 72 in 2006 to 134 in 2009 (124). Further, the number of laboratory-developed ("home brew") molecular diagnostic tests exceeded 1,400 in 2009 (125). In that context, it is anticipated that, like the vast majority of these esoteric molecular diagnostic tests, which include qRT-PCR, staging by GUCY2C lymph node analysis will be broadly available to practitioners through central reference laboratories providing established expertise and validated analytic platforms that conform to prevailing regulatory and CMS reimbursement requirements.

8. Summary

Traditional paradigms for staging patients with colorectal cancer incorporating standard histopathological assessment of regional lymph nodes underestimate

the extent of metastatic disease, reflected by 25-30% of pN0 patients developing recurrent disease (93). Limitations of traditional staging paradigms, including volume of tissue assessed and analytic sensitivity, can be eliminated by employing disease-specific markers and a powerful molecular amplification technology such as qRT-PCR (45, 93). GUCY2C identifies metastatic colorectal cancer cells in extra-intestinal tissues, and occult lymph node metastases detected by GUCY2C qRT-PCR is an independent prognostic indicator for risk of disease recurrence in pN0 colorectal cancer patients (45, 93).

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