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Molecular staging individualizing cancer management

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ABSTRACT

Although the most important prognostic and predictive marker in colorectal cancer is tumor cells in lymph nodes, ~30% of patients who are node-negative die from occult metastases. Molecular staging employing specific markers and sensitive detection technologies has emerged as a powerful platform to assess prognosis in node-negative colon cancer. Integrating molecular staging into algorithms that individualize patient management will require validation and the definition of relationships between occult tumor cells, prognosis, and responses to chemotherapy.

INTRODUCTION

Clinicopathological staging remains the most important prognostic marker of survival and predictive marker of therapeutic response for most cancer patients. Despite its importance, clinicopathological staging remains imperfect, and identification of patients at greatest risk for disease recurrence or deriving optimum benefit from therapy has eluded definition for most tumors. Emergence of platform technologies to interrogate genomic and post-genomic structure and function has provided an explosion of new diagnostic markers and therapeutic targets with the potential to individualize cancer prevention, detection and cure. Despite these exponential scientific advances, clinical translation has substantially lagged, in part, reflecting the absence of the evidence base positioning these new technologies in diagnostic and therapeutic management algorithms.

Employing colon cancer as a clinical model, this review will explore the application of molecular staging to prognosis and prediction, to individualize patient management. Specifically, the ability of molecular staging to quantify occult metastases in regional lymph nodes, predict disease recurrence, and identify patients who could benefit from adjuvant chemotherapy will be examined. A conceptual framework for integrating molecular staging of lymph nodes into a reflex diagnostic paradigm incorporating standard clinicopathological indices and molecular signatures from primary tumors that optimizes individualization of patient management will be discussed. The

objective of this review is to demonstrate for the clinician the potential power of emerging molecular technologies for the diagnostic and therapeutic management of patients with cancer. It is anticipated that this review will provide practicing physicians with an appreciation of those molecular technologies, their emerging role in diagnostic and therapeutic algorithms, and the evidence supporting their utility in patient-centric management algorithms.

COLORECTAL CANCER

Cancer of the colorectum is the 4th most common malignancy, with ~150,000 new cases annually, and the 2nd most common cause of cancer-related death (1). Colorectal cancer causes ~10% of cancer-related deaths in the U.S., and mortality approaches ~50% (1-3). Death from colorectal cancer reflects metastatic disease: ~20% of patients have unresectable metastases at the time of initial evaluation while more than 30% of patients will develop metastatic disease during the course of their illness (2-5). Surgery continues to be the mainstay of treatment, with the greatest influence on survival. However, while presumptively curative surgery excises all obvious tumor, occult metastases conspire to produce disease recurrence (1-3,6-9). Rates of disease recurrence nominally extend from 10% for tumors confined to mucosa (stage I) to more than 50% for tumors with metastases to regional lymph nodes (stage III) (1-3,6-19).

A. Staging as a prognostic marker. The most significant prognostic marker of colorectal cancer survival is tumor cells in regional lymph nodes (1-6,9,20-24).

Although staging by histology remains the standard, imprecision reflects limitations inherent to the method (2,5,24). Microscopy has restricted sensitivity, with detection limits of 1 cancer cell in about 200 normal cells (25). Also, histology typically reviews less than 0.1% of biopsied tissue, producing sampling error, since more than 99.9% of available tissue is not examined and cancer cells do not distribute homogeneously (4,5,25). These restrictions imposed by microscopy are brought into specific relief by considering the rate of post-operative cancer recurrence. Stage I and II (node-negative) disease, limited to the bowel wall without microscopic detection of metastases in lymph nodes, should be completely cured by surgical resection. Yet, up to 30% of stage I and 50% of stage II patients develop recurrent disease (2,3,5,24). Stage III patients, in whom all obvious cancer, including that metastasized to regional lymph nodes, is excised, exhibit recurrence rates of up to 70% (2,10,12-15,17-19,26,27). Differences in reported recurrence rates in patients with node-negative disease likely reflect the combination of patients who are truly node-negative and those with stage III or IV disease that escape identification by histology (2,4,5,12,21,28,29).

B. Staging as a predictive marker. Disease stage in colorectal cancer not only determines patient prognosis, but also predicts which patients will derive benefit from adjuvant therapy. Chemotherapy administered after presumptively curative surgery to stage III colon cancer patients improves survival, enhancing time-to-recurrence up to 40% and overall survival up to 30% (6,20,30-36).

Moreover, the recent introduction of biologically targeted individualized therapies, such as monoclonal antibodies aimed at key signaling molecules including VEGF and EGF receptors, has further increased 5 year median and overall survival in patients with widely metastatic disease, from 7% to more than 30% (37). In striking contrast, the activity of adjuvant chemotherapies in patients with node-negative colon cancer is unclear, with only minimal impact on survival in some clinical trials (2,3,6,9,20,22,23,38). This indeterminate therapeutic benefit is reflected in the evolution of treatment guidelines, some of which advocate adjuvant chemotherapy for patients with poor clinicopathologic features including lymphovascular invasion, deep penetration into the bowel wall, or extension to surrounding structures (9,39-41). In that context, unpredictable responses to adjuvant therapy in node-negative patients may reflect heterogeneity of occult lymph node metastases (4,5,21,24,42-44). Thus, methods that detect occult metastases in lymph nodes may better identify node-negative patients who would best benefit from adjuvant therapy (6,37).

MOLECULAR STAGING

Histology remains the most important procedure for staging patients with colon cancer, reflecting the relationship between tumor cells in regional lymph nodes and patient prognosis and prediction (1-6,9,20-23). However, microscopy underestimates the presence of metastases in tissues and about 70% of regional lymph nodes that contain metastases have nests of cells below <0.5 cm which escape observation (2,3,5,24). Beyond histopathology, more recently

developed molecular staging approaches, including coupling disease-specific markers with a powerful detection technology like quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), offer a sensitive detection system for metastases (Fig. 1) (5,24). Molecular staging can interrogate the whole specimen, avoiding sampling errors, and detect one tumor cell in about one million normal cells, overcoming sensitivity limitations (5,24). Until recently, the utility of these molecular approaches for staging patients has been unclear, because studies have been burdened by insufficient patient sample size, deficient longitudinal follow-up, and heterogeneous analytic approaches. Meta-analyses suggest that these molecular approaches offer a diagnostic advantage for staging patients with colorectal cancer (4,5,21,29,44,45).

A. GUCY2C: A unique paradigm for molecular staging in colon cancer.

GUCY2C is one member of a family of enzyme receptors synthesizing guanosine 3', 5' cyclic monophosphate (cyclic GMP; cGMP) from GTP. This protein is specifically expressed by intestinal cells, but not by extra-intestinal tissues (46-55). GUCY2C is the receptor for the paracrine hormones uroguanylin and guanylin produced locally in the small intestine and colon, respectively. Their interaction with the extracellular ligand binding domain of GUCY2C activates the intracellular catalytic domain, initiating cGMP synthesis (51,54,56-62). GUCY2C regulates epithelial cell cycle kinetics, DNA damage sensing and repair, metabolic signaling, and epithelial-mesenchymal cross-talk directing homeostasis along the dynamic crypt-surface axis (63-75). Of significance,

guanylin and uroguanylin expression is universally eliminated early in neoplastic transformation (76-80). In close agreement, silencing GUCY2C signaling increases tumors in mouse models of genetic and carcinogen-based tumorigenesis, reflecting dysregulation of proliferation and chromosomal instability (66). Indeed, GUCY2C is a tumor suppressor organizing the crypt-surface axis whose dysregulation reflecting loss of paracrine hormone expression contributes to intestinal neoplasia (64-67,71).

Beyond its role as a tumor suppressor, GUCY2C exhibits expression characteristics that make it uniquely suitable as a molecular marker of colorectal cancer metastases in extra-intestinal tissues. GUCY2C has been identified in all samples of normal intestine, but not in any extra-gastrointestinal tissues (42,45,47,48,56). Further, GUCY2C has been identified in nearly all human colorectal tumors, independent of anatomical location or grade, but not in extra-gastrointestinal malignancies (42,45,47,48,56,79,81-84). Moreover, GUCY2C expression is amplified in most colorectal tumors, compared to normal intestinal tissues (81,85,86). Thus, expression restricted to intestinal epithelial cells in normal physiology, but global excess expression by metastatic cancer cells, highlights the potential applicability of GUCY2C to identify occult metastases in lymph nodes of patients undergoing staging for colorectal cancer (44).

B. Detection of occult metastases using GUCY2C. Analyses conducted retrospectively suggested that GUCY2C RT-PCR detected occult metastases in lymph nodes related to disease recurrence in patients with colorectal cancer

(45). These preliminary studies served as the foundation for an appropriately powered prospective multicenter analysis of the applicability of GUCY2C, detected by qRT-PCR, to detect clinically important occult lymph node metastases. Thus, 257 stage 0-II pN0 colorectal cancer patients were enrolled at one of 7 academic medical centers and 2 community hospitals in the U.S. and Canada (44). To have at least 80% power to detect a clinically relevant difference in outcomes, a minimum of 225 pN0 patients were needed for this analysis. In this study, lymph nodes were dissected from fresh colon and rectum specimens, and half of each was used for histopathology, while the other half was subjected to molecular analysis by GUCY2C qRT-PCR. Indeed, more than 85% of patients with histologically node-negative disease harbored occult metastases by molecular staging (44). Thus, surprisingly, most patients staged as free of metastatic disease by histopathology have minimum residual disease in regional lymph nodes. Further, 20.9% (CI, 15.8-26.8%) of patients with, but only 6.3% (CI, 0.8-20.8%) without, occult metastases in regional lymph nodes developed recurrent disease ($p=0.006$) (44). Indeed, occult metastases detected by molecular staging were associated with poorer prognosis and reduced disease-free survival in both stage I and II patients and in patients with colon and rectal cancers. Importantly, time to recurrence and disease-free survival in patients with occult metastases were nearly identical to those of patients with stages IIIA and IIIB disease, highlighting the ability of these molecular approaches to upstage patients (44). Moreover, multivariate analyses

demonstrated that molecular staging provided the most powerful independent risk marker and patients who harbored occult metastases experienced shorter times to recurrence and reduced disease-free survival (44).

C. Prognostic utility of molecular staging to individualize risk assessment.

Occult metastases identified by molecular staging were an independent marker of risk of recurrent disease. In these analyses, the vast majority of patients who were node-negative by histopathology were molecularly positive, suggesting that standard approaches under-estimate the incidence of metastases to regional lymph nodes in patients with colon cancer. Interestingly, although a substantial fraction of node-negative patients harbored occult metastases by molecular staging, most of these patients did not develop recurrent disease (2,3). To provide context, only about 50% of patients with stage III disease ultimately develop recurrent disease, and all have lymph node metastases identified by histology (2,3). Resolving this apparent inconsistency relies on the concept that metastases in lymph nodes, independent of approaches employed for their detection, do not guarantee disease recurrence in any individual. Rather, they help to stratify risk. This study is the first to provide level 1 evidence for the application of molecular staging of lymph nodes to individualize prognostic risk in cancer, employing an adequately powered, blinded, prospective multicenter clinical trial design. Absence of data using this stringent study design has been one critical barrier limiting the translation of

molecular diagnostics into patient-centric management paradigms that individualize prediction of risk and therapeutic response. (4,5).

There is an established association between histologic tumor burden, assessed as the number of regional lymph nodes containing cancer cells, and risk of recurrent colorectal cancer (2,3,87-93). In the context of sufficient numbers of lymph nodes for analysis, stage III patients in whom ≥ 4 lymph nodes harbor histologic metastases have rates of disease recurrence that are as much as 100% greater than those patients with ≤ 3 lymph nodes containing tumor cells (2,3). By extension, the precision of molecular staging also should benefit from appropriate lymph node collections, to most accurately incorporate an assessment of tumor burden into stratification of prognostic risk (4,5,21). There is a presumption of an inverse relationship between the quantity of regional lymph nodes harboring molecularly-detected occult metastases and risk of recurrent disease. In the cohort of histologically node-negative patients who provided ≥ 12 lymph nodes (2,3) for molecular staging, patients with 0-3 nodes harboring occult metastases experienced minimum risk of developing recurrent disease (44). In striking contrast, patients who had ≥ 4 lymph nodes infiltrated with occult metastases detected by molecular staging exhibited a prognostic risk that was identical to patients with stage III N1 colorectal cancer (44). These considerations support the central importance of adequate regional lymph node collection to optimize molecular (4,5,21), as well as histological (2,3,90,91),

detection of metastases to estimate tumor burden and improve risk stratification in colorectal cancer staging.

While the exact number of lymph nodes necessary to optimize patient management has not been precisely clarified, the importance of sufficient lymph node collections to maximize the accuracy of staging and optimize outcomes for patient survival is a mainstay of patient management algorithms in colorectal cancer (2,3,87-93). There is an emerging clinical paradigm involving the application of laparoscopy-assisted colectomy to manage patients with colon cancer (94). It is noteworthy that this evolution in technique, which strives to reduce surgical morbidity and mortality, restricts collections of lymph nodes for staging (94). While these innovations in surgical approaches improve intra-operative management and post-operative recovery, the impact of reduced lymph node sampling on staging accuracy and, ultimately, patient survival has not been precisely quantified. The emergence of molecular staging, offering an unprecedented opportunity to accurately evaluate patient prognosis and predict responses to chemotherapy, highlights the value of defining best practices for lymph node collection that optimize patient outcomes. Defining the optimum number of nodes for molecular staging, in turn, will provide a rich source of data to inform the evolution of advances in surgical management. It is envisioned that optimizing tissue requirements for molecular staging will drive restricted access surgical techniques to refine lymph node collections,

producing integrated algorithms to evolve best management solutions for patients.

In addition to the number of lymph nodes harboring cancer cells, there is an apparent association between the quantity of cancer cells in each lymph node, the burden of tumor metastases, and risk of disease recurrence (2,95). Thus, metastatic foci of cancer cells in lymph nodes ≥ 0.2 mm are associated with increased risk of disease recurrence, while the association between individual tumor cells or nests < 0.2 mm and prognosis is unclear (2). The evolution of molecular staging using qRT-PCR provides a unique paradigm to specifically quantify metastases in tissues, including regional lymph nodes. In that context, qRT-PCR offers unparalleled sensitivity for detection, with the capability of single cell identification in conjunction with analysis of optimum volumes of tissue to avoid sampling errors (96). However, that improved sensitivity may translate into identification of occult tumor cells in regional lymph nodes that are below the limit for increased prognostic risk (2), restricting the specificity of molecular staging (44). Future studies will need to identify the quantitative relationship between biomarker levels and prognostic risk, to assess the impact of tumor burden on optimizing prognostic sensitivity and specificity of molecular staging paradigms in cancer (44).

PERSPECTIVE

To date, the most powerful indicator of prognosis and response to adjuvant

chemotherapy in colorectal cancer is the identification of cancer cells in lymph nodes by histopathology (1-6,9,20-23). Despite its central position in all staging paradigms, approaches to detecting lymph node metastases are inadequate. Up to 30% of patients with node-negative colon cancer succumb to disease recurrence, associated with occult metastases in lymph nodes undetected by conventional methods (2-5,21,24,42,43,97). There is an unmet clinical need for new approaches to more precisely evaluate tumor metastases in regional lymph nodes in colon cancer patients. Recently, a blinded, multicenter, prospective study demonstrated the utility of molecular staging to detect occult tumor metastases in regional lymph nodes to predict risk of disease recurrence (44). Occult tumor metastases, defined by molecular staging, was the most powerful independent marker of risk of disease recurrence (44). This represents the first level 1 evidence supporting the importance of occult metastases in regional lymph nodes in defining prognostic risk in patients with colon cancer (98). These data establish a framework for the application of molecular staging in lymph nodes for individualizing prognostic risk assessment in patients with cancer.

While these observations are a beginning, their translation into useful staging tools in cancer will require considerable analyses in the future. These results will require confirmation in an independent cohort of patients with colorectal cancer, consistent with the emerging learn-confirm paradigm in biomarker translation, wherein integration into patient management algorithms require

validation in independent populations (99-106). Also, the exquisite sensitivity of qRT-PCR (96), reflecting optimum tissue sampling and ability to discriminate single cells, may reveal occult cancer cells in lymph nodes below the limit for clinical risk (2), restricting the specificity of molecular staging (44). This is exemplified by the identification of occult metastases in the majority of patients, most of whom will remain free of disease (2). The next step in the evolution of molecular staging will require a move beyond the simple presence of tumor cells to a standard that integrates the quantity of tumor burden across metastatic sites, including lymph nodes. Molecular staging, specifically the application of qRT-PCR, provides a remarkable platform to quantify occult cancer burden across all regional lymph nodes, and perhaps to more accurately stratify risk and predict therapeutic responses.

Beyond prognosis, there is an established association between metastases in regional lymph node and the efficacy of chemotherapy in patients with colorectal cancer. While adjuvant chemotherapy improves clinical outcomes in stage III patients, its impact on survival in patients that are node-negative by histology remains unclear (2,3,6,9,20,22,23,38). This heterogeneity of therapeutic benefit in node-negative patients may, in part, reflect the inherent inaccuracy of staging by histopathology (4,5,21,24,42-44). In contrast, molecular staging identified node-negative patients with a prognostic risk profile that closely matched stage III patients, a cohort that derives benefit from adjuvant chemotherapy (2,3). These observations suggest that node-negative patients

who harbor occult metastases detected by molecular staging also could benefit from adjuvant chemotherapy. In the future, studies will examine whether occult lymph node metastases defined by molecular staging predicts chemotherapeutic efficacy. These studies will assess if, in patients with occult metastases in regional lymph nodes identified by molecular staging, those treated with chemotherapy have improved clinical outcomes compared to those who are followed without treatment.

SUMMARY

Standard algorithms for staging colon cancer patients are largely based on a combination of histological evaluation of primary tumor and regional lymph nodes. However, this gold standard underestimates the extent of disease, and 25-30% of node-negative patients ultimately die of disease recurrence (107). Inadequacies of accepted optical staging algorithms, including tissue sampling and detection limits, can be overcome by molecular staging (44,107). The molecular detection of occult lymph node metastases is a powerful independent indicator of prognostic risk of colorectal cancer recurrence (44,107). Early prospective trials strongly suggest that molecular staging through comprehensive lymph node analysis quantifies tumor burden that identifies node-negative patients at increased risk of developing recurrent disease who might be candidates for adjuvant chemotherapy.

Beyond lymph node analyses, evolving genomic platforms provide a rich source of prognostic and predictive information about primary tumors that can enhance staging algorithms optimizing outcomes that drive patient management. Analyses of primary tumors to define gene expression and epigenetic profiles, disease-associated mutations in oncogenes or tumor suppressors, and metabolomic and proteomic signatures that individualize assessments of recurrence risk, responses to adjuvant chemotherapy, and biologically-targeted treatments are enhancing the prognostic and predictive management of cancer patients (108-112). However, defining the prognostic and predictive character of primary tumors by molecular analyses may be most relevant in the context of whether tumors have metastasized. A primary tumor with a molecular signature suggesting a poor prognosis might represent less risk to the patient if that tumor was completely resected at the time of surgery, before metastases occurred. Thus, emerging technology platforms defining prognosis and prediction for clinical management employing molecular analyses of primary tumors might produce the greatest benefit when applied to patients harboring occult nodal metastases, rather than to those free of metastatic disease. Here, molecular staging offers a unique opportunity to prioritize complex and expensive molecular analyses of primary tumors to optimize cost-effective patient management (44). In the future, trials will examine the applicability of reflexed analytical paradigms in which all histologically node-negative patients undergo molecular staging, to determine

whether there is clinically important occult lymph node metastases, followed by further molecular testing of primary tumors only for patients at increased prognostic risk, to identify therapies personalized to the biology of their individual malignancies (113).

It is important to consider that qRT-PCR is an evolving technical platform that primarily remains the domain of centralized specialty laboratories, and has not yet been broadly distributed to most academic and community medical centers. These realities raise the important question concerning limitations to implementation of molecular staging as a clinical standard central to practice guidelines. In that regard, molecular diagnostics is an emerging \$14 billion dollar business, that is increasing at a rate exceeding 10% annually (114,115). Indeed, the number of esoteric molecular diagnostic tests approved by the FDA each year is growing aggressively, from 72 in 2006 to 134 in 2009 (116). Additionally, the number of home brew molecular diagnostic tests, developed in individual laboratories, was in excess of 1,400 in 2009 (117). These considerations suggest that molecular diagnostic tests, including molecular staging, available to clinicians and patients will grow. In the near term, central laboratory performance sites provide the depth of experience and validated technology platforms that align with requirements for FDA regulatory performance and CMS reimbursement. They will ultimately support the most informative approaches to incorporate molecular staging paradigms into patient-centered algorithms for disease management.

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Figure Legends

Figure 1. Identification of occult tumor metastases in lymph nodes employing marker-specific quantitative RT-PCR. At the time of colectomy, regional lymph nodes are harvested from tumor-associated mesenteric structures for staging. In the canonical paradigm, these lymph nodes are formalin-fixed and paraffin-embedded and thin sections are reviewed by standard histopathology to identify metastatic tumors cells. This approach is associated with limitations in sampling, in which only a small portion of the available tissue is subject to review. Also, there is a limitation in sensitivity, in which the pathologist can reliably identify only one tumor cell in 200 normal cells in a lymph node specimen. Together, these limitations result in under-staging of patients, in which lymph nodes apparently free of disease by standard histopathology (pN0) actually harbor occult metastases (pN0(mol+)). One approach to identify lymph nodes harboring occult metastases that escape detection by the standard paradigm is to couple a sensitive and specific tumor marker, like GCC, to a powerful amplification technology, like RT-PCR. This molecular approach overcomes the sampling limitation of standard histopathology, since mRNA from the entire available specimen is extracted and sampled for expression of the tumor-associated marker. Moreover, RT-PCR is exquisitely sensitive and can detect one tumor cell in one million normal cells, unlike standard histopathology.