Published in final edited form as:

Curr Colorectal Cancer Rep. 2012 September ; 8(3): 225–231. doi:10.1007/s11888-012-0132-7.

Gene Signatures in Stage II Colon Cancer: A Clinical Review

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Abstract

Understanding the biology of cancer is the key to understanding its behavior. Stage II colon cancers represent a unique treatment challenge for medical oncologists because they contain a very heterogeneous group of tumors with a wide range of recurrence risks after resection. Defining these differences in biology can help to explain differences in behavior. To this end, gene signatures have been developed to define various prognostic groups beyond the clinicopathologic features alone. Adjuvant chemotherapy for stage II colon cancers as a group has not shown survival advantage in clinical trials. Future research to develop gene signatures to predict a group that will benefit from adjuvant chemotherapy will be helpful in the clinical decision-making process. The purpose of this review is to present the prognostic gene signatures currently available for use, those in development, and their utility in stratifying recurrence risk in stage II colon cancer patients.

Keywords

Colon cancer; stage II; gene signatures; prognostic score; recurrence score; predictive score; *Oncotype* DX RS; ColoPrint; ColDx; MSI; guanylyl cyclase C; Adjuvant! Online; numeracy

Introduction

Approximately 103,170 new cases of colon cancer are expected in the United States in 2012 [1]. Thirty-nine percent of these will be diagnosed with early-stage disease. The relative five-year survival rates reported from the SEER database between 1998 and 2000 for stage IIA (T3N0), IIB (T4aN0M0), and IIC (T4bN0M0) were 87.5%, 79.6%, and 58.4% respectively, which is the basis of the updated stage II sub-staging in the 7th edition of the AJCC Cancer Staging Manual published in 2010 [2, 3]. In an attempt to better subcategorize patients with T4 lesions based on survival, T4 is now subdivided into T4a, in which the tumor perforates the surface of the visceral peritoneum and T4b, in which, in addition to the above, the tumor directly invades or shows histologic adherence to other organs and structures thereby creating a new substage IIC. This represents a significant change from the AJCC Cancer Staging Manual, 6th edition, which grouped stage II only into IIA and IIB with an 84.7% and 72.2% relative five-year survival, respectively [4, 5]. Clinical trials for stage II/III colon cancer have shown no survival advantage with the addition of adjuvant chemotherapy with 5-fluorouracil (5-FU) in stage II patients. The absolute risk reduction for

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Disclosure

No potential conflicts of interest relevant to this article were reported.

relapse seen in stage II patients is only 3–5% at 5 years. There appears to be no additional benefit to adding oxaliplatin to unselected stage II patients [6–8]. Argument has been made that stage II patients have represented only 40% of the total population in the MOSAIC trial and only 30% in the NSABP C-07 trial, and were therefore underpowered to detect a benefit from the addition of oxaliplatin to 5-FU. An exploratory analysis was performed on the results of the MOSAIC trial looking at absolute chemotherapy benefit on a subset of stage II patients with high-risk features and showed a 7% absolute benefit in disease-free survival (DFS) and a 2% survival benefit, which failed to meet statistical significance [7]. The QUick and Simple and Reliable (QUASAR) trial randomly assigned 2,800 stage II patients after surgery to observation versus 5-FU/LV without oxaliplatin, unlike MOSAIC and NSABP C-07, and showed a 3.6% absolute survival benefit ($P=0.05$) when compared to those who did not receive any adjuvant chemotherapy [6]. A pooled analysis of 2,966 stage II/III patients from NSABP C-01 through C-05 shows that stage II (1,255) patients derive benefit in terms of recurrence-free survival (RFI), disease-free survival (DFS), and overall survival (OS) from receiving adjuvant 5-FU/LV based chemotherapy when compared to surgery alone [9•].

Current NCCN Guidelines and ASCO Recommendations for Risk Stratification of Stage II Patients

Clinical and pathological high-risk features as defined in the NCCN guidelines are used to offer chemotherapy to stage II patients, which include <12 nodes analyzed after surgery, and poor prognostic features defined as T4 tumor, obstruction, perforation, peritumoral lymphovascular invasion (LVI), perineural invasion (PNI), poorly differentiated histology (signet) or grade 3 tumors [10]. A retrospective review of the SEER-linked Medicare database from 1992–2005 failed to show a survival benefit of adjuvant chemotherapy given to stage II patients older than 65 years of age with either none or at least one clinicopathologic high-risk feature [11•]. Current ASCO guidelines recommend a discussion with patients about the small absolute benefit in survival offered by adjuvant chemotherapy in stage II patients before coming to a mutual decision about adjuvant treatment [12]. Stage II patients who have low-risk clinicopathologic features also relapse, which argues for an urgent need for developing more reliable predictors of aggressive biology for risk stratification and response to therapy.

Web-Based Tools Calculating Recurrence Risk Based on Clinicopathologic Factors

There are two web-based tools available for risk stratification of patients with resected colon cancer based on clinicopathologic criteria. Adjuvant! Online was developed using prognostic estimates from US SEER registry-reported outcomes and takes into account the age, gender, co-morbidities, depth of invasion, number of lymph nodes examined, number of positive lymph nodes, and histologic grade [13]. Numeracy was developed at the Mayo Clinic from pooled results from a meta-analysis of seven randomized clinical trials and takes into account in its estimate calculations the age, grade of the tumor, T stage, and number of positive lymph nodes [14]. Both assist not only in calculating relative risk of recurrence but also provide the relative benefit from giving adjuvant chemotherapy. Neither takes into account perforation, LVI, or PNI.

Role of Defective Mismatch Repair Genes and Guanylyl Cyclase C in Risk Stratification of Stage II Patients

Mismatch repair (MMR) genes are responsible for correcting the ubiquitous nucleotide base mispairings and small insertions or deletions that occur during DNA replication. Several of these genes exist and include *hMLH1* and *hMSH2*. Loss of expression of MMR genes leads to defective DNA repair and subsequent accumulation of abnormalities in short sequences of nucleotide bases that repeat multiple times called microsatellites. This leads to frame shift

mutations in these genes controlling cell growth called microsatellite instability (MSI). High degree of MSI (MSI-H) occurs in about 15% of sporadic colon tumors, which have been shown to have a good prognosis and do not appear to benefit from fluoropyrimidine adjuvant therapy [15•]. Testing tumors for MSI is currently a tool recommended for use as part of the decision-making process in determining adjuvant therapy in stage II disease only and is part of the NCCN guidelines [10].

Guanylyl cyclase C (GCC) is normally only expressed in the intestinal mucosa and its presence in the lymph node has been shown to be a marker for metastatic disease [16]. Expression of guanylyl cyclase C (GCC) in histologically-negative lymph nodes detected with reverse transcriptase polymerase chain reaction (RT-PCR) is the basis of the Previstage Assay [17••]. This test requires formalin fixed paraffin embedded (FFPE) tissue. The preliminary work published by Sargent et al shows that the recurrence risk in stage II patients with GCC-positive but histologically-negative lymph nodes is similar to node-positive stage III tumors when compared to histologically-negative, GCC-negative lymph nodes. This has not yet been validated but may have utility in upstaging patients with histologically-negative lymph nodes. The training set for development for this assay had 241 patients with 36 months follow-up. The primary endpoint used was five-year recurrence free survival (RFS). Currently a multi-institutional validation study (VITAR) is underway. This assay is not commercially available for clinical use outside the setting of a clinical trial.

Gene Signatures in Stage II Colon Cancer

The ultimate goal of personalized medicine is to limit chemotherapy intervention to those at high risk for recurrence who will derive maximum benefit from it and to only observe those at low risk. We are moving forward from an era of risk assessment of stage II colon cancers based on clinicopathological features into a new era of using gene signatures to better risk stratify these patients. Over the last few years, research has focused on the impact of molecular factors on the prognosis and response to chemotherapy including 18q deletion, MSI, thymidylate synthetase overexpression, *TP53*, *KRAS*, and *BRAF* mutations [15•, 18–24]. Tumors with *BRAF* mutations have a poor prognosis. Those that are MSI-H have a good prognosis but account for only 15% of stage II colon cancers and receive no improvement in DFS with 5-FU/LV chemotherapy [15•, 25–28]. Research efforts have been directed towards developing gene signatures useful for prognosis of stage II colon cancers over the last few years but some of these signatures contain relatively small patient sample sizes, thereby limiting their validity [29–32]. Here we review three multi-gene prognostic signatures developed within the last couple of years from larger patient sample sizes, which hold promise to move research in this area forwards (Table 1).

Oncotype DX® Recurrence Score (RS) developed by Genomic Health is a quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay on RNA extracted from FFPE tumor tissue to assess risk of recurrence in stage II colon cancer patients at three years after surgery [33••]. The RS consists of 12 genes that include seven prognostic genes and five reference genes. It was developed in 761 candidate genes studied from 578 stage II/III patients from NSABP C-01, C-02, and C-04 treated with surgery alone and with surgery plus 5-FU/LV respectively and a second confirmatory study done in a subset of 375 genes studied from 1,273 stage II/III patients from Cleveland Clinic and NSABP C-06 treated with surgery alone and surgery plus 5-FU/LV respectively. The combined analysis identified 48 genes associated with a risk of recurrence. Seven recurrence-risk genes and five reference genes were selected and an algorithm was developed to identify groups of patients with low, intermediate, and high likelihood of recurrence. The higher expression of cell cycle genes was associated with increased relapse-free interval (RFI). Interestingly, the association of stromal gene expression in the RS (a poor prognostic feature) is consistent with Dukes'

observation that tumor invasion into the bowel wall is a critical characteristic that should be used in staging of colon cancer [34, 35]. The genes studied indicated a marked similarity of expression in stage II and stage III colon cancers, however, there could be differences in genes not profiled, which therefore does not help solve conclusively whether there is a difference in the expression of genes between stage II and III cancers. The prognostic gene signature has been clinically validated on 1,436 patient samples from the QUASAR study and was confirmed in a 690-patient CALGB 9581 trial [36••]. The RS separates the recurrence risk of patients at three years into low, intermediate, and high risk groups at 12%, 18%, and 22%, respectively. Although the range of recurrence risk defined by the RS is relatively small between low, intermediate, and high risk, it does provide prognostic value in addition to T stage (HR, 1.94; $P<.001$) and MMR status (HR, 0.31; $P<.001$) can be used independently of these histopathological prognostic features, and does improve the ability to estimate recurrence risk. This assay is available for clinical use.

In parallel with the development of the RS, 66 genes were identified as being significantly associated with 5-FU/LV benefit of which six were selected, along with five reference genes and an 11-gene *Oncotype* DX treatment score (TS) was developed to identify groups of patients who will receive benefit from 5-FU/LV. Some genes identified that predict benefit from 5-FU were not unexpected, however, the significance of expression of some genes associated with gap junction communication and notch signaling pathways were unclear. Also very interestingly, the quantitative expression of genes related to 5-FU activity (*TS*, *DPD*, *TP*) or markers associated with MMR (*hMLH1*, and *hMSH2*) [37–43], were not associated with differential 5-FU benefit in this study and therefore were not included in the genes selected for the development of the TS. *Oncotype* DX TS did not realize its promise during the validation phase to deliver estimates of benefits from 5-FU chemotherapy to stage II cancer patients in QUASAR and CALGB 9581 trials, and therefore, is not available for use in clinical practice.

The 12-gene *Oncotype* DX RS has been further validated on 892 stage II/III colon cancer patients from the NSABP C-07 study treated with surgery plus bolus 5-FU/LV without or with oxaliplatin (FLOX) [44••]. The RS was found to be highly prognostic in patients receiving 5-FU/LV. The RS predicted DFS ($P<0.001$) and OS ($P<0.001$). Pre-specified high-risk groups (RS ≥ 41 , 26% patients) had a higher recurrence risk than did low-risk groups (RS ≤ 30 , 39% patients) (HR=2.11, $P<0.001$). The RS captures the underlying biology of the tumor and provides risk information independent of T and N stage, MMR, nodes examined, grade, and treatment. The proportional benefit derived from chemotherapy was similar across all risk groups but high-risk patients demonstrated a greater absolute benefit from chemotherapy utilizing FLOX. The RS is not predictive of relative benefit of oxaliplatin added to 5-FU/LV but enables better discrimination of absolute oxaliplatin benefit as a function of risk. These results are being further validated in approximately 1000 patients from the MOSAIC study.

ColoPrint® developed by Agendia is an 18-gene prognostic classifier developed using RT-PCR on fresh frozen tumor tissue obtained during surgery to identify five-year metastasis-free survival [45••]. Tissue from 188 patients with stage I–IV disease was analyzed using Agilent 44K oligonucleotide assay. Median follow-up time on these patients when first reported was 65.1 months and the majority (83.6%) did not receive adjuvant chemotherapy. The goal was to identify a set of genes for their association with five-year distant metastasis-free survival. This signature was validated in a group of 206 samples of patients with stage I, II, and III disease. This signature classified 63.2% patients as low risk for recurrence and 36.8% as high risk for recurrence with a five-year relapse-free survival (RFS) rate of 87.6% and 67.2%, respectively. Tumors were classified as ColoPrint low versus high risk and there is no intermediate risk category. Authors of this assay conclude that the ColoPrint

significantly improves on the ASCO criteria in assessing the risk of cancer recurrence without prescreening for MSI. ColoPrint uses the same technology, methods, and quality control as the FDA-approved MammaPrint[®] assay (Agendia, Huntington Beach, CA) used for breast cancer. Updated results of the clinical and technical validation were presented at ASCO GI 2012 Jan 20–22 by Tabernero et al. during an oral presentation with a total 320 patients and 70-month median follow up. ColoPrint identified two-thirds of patients as low risk (209/320) with a three-year and five-year RFS of 91% and 88% for low risk and 74% and 71% for high risk patients, respectively [46••]. On a univariate analysis of three-year RFS, the ColoPrint was the only parameter that reached statistical significance with a HR 2.9 (95% CI 1.55–5.39, $P=0.001$). Clinical parameters in the univariate analysis using T4, perforation, grade, and <12 nodes examined did not help in identification of high-risk patients. Among 67 patients classified as MSI-H, only 53 were classified as low-risk patients identified by ColoPrint. ColoPrint appears to have not only high precision and accuracy to identify low-risk patients with stage II disease but also identifies low-risk patients *beyond* MSI-H status who can be managed without adjuvant chemotherapy. It appears to be better at identifying high-risk patients than any clinical risk factor alone. It complements the current clinicopathological factors for better treatment decisions. This is a prognostic signature and claims to have no predictive value to benefit of chemotherapy [46••]. A prospective clinical validation study in stage II and III patients is underway at MD Anderson Cancer Institute, and an international Prospective Assessment of Risk Stratification of ColoPrint (PARSC) Study of 575 patients is underway in the US, Asia, and Europe in stage II and III patients. ColoPrint is not a commercially available assay for use outside of the clinical trial setting.

Colorectal Cancer DSA (ColDx)[®] developed by Almac is the most recently reported DNA-based 634 transcript gene microarray signature developed on FFPE tumor tissue that identifies patients at a higher risk for recurrence five years after surgery for stage II colon cancer [47••]. ColDx was developed using a balanced set of 73 patients with recurrent disease (high risk) and 142 patients with no recurrence (low risk) within 5 years of surgery. The training set consists of 215 patients as mentioned above. In an independent validation set of 144 patients this signature identifies a high-risk cohort of patients with a HR of 2.53 ($P<0.001$) for recurrence and a HR of 2.21 ($P=0.0084$) for cancer-related death. This signature performed independently from known prognostic factors. High- and low-risk patients can be identified using this signature. Functional analysis of the gene signature revealed that the *IGF-1*, *TGF- β* and *HMGB1* pathways were the most significant pathways identified. All of these have been previously reported to confer a poor prognosis in colon cancer through promoting tumor growth, invasion, metastasis, and preventing apoptosis [48–50]. Utilizing a DNA microarray platform on FFPE tissue increases the number of detectable mRNA transcripts and biologic processes relative to quantitative PCR. Formalin fixation results in degradation of mRNA transcripts through cross linking of RNA to protein. This degradation is mostly immediate although some develops over time. The DNA microarray platform was refined on another set of colon cancer specimens to prevent using probe sets that detected degraded transcripts, thereby eliminating detection of unstable or differentially stable mRNA transcripts from being detected as part of the signature. No MSI data was included because that information was not available for the majority of patients. This can be a potential concern given the fact that the transcriptomes differ between MSI-H and stable tumors [51]. A prospective validation of the prognostic signature in a large cohort of stage II colon cancer samples collected as part of a completed clinical trial is being planned. This assay is not available for commercial use outside the setting of a clinical trial.

Discussion

Current paradigms for selection of patients with stage II disease for adjuvant chemotherapy based on clinicopathologic features are far from perfect. It is clear that there is minimal

benefit of adjuvant chemotherapy for unselected stage II colon cancer patients. The goal of any future successful research is to help identify patients who are cured without adjuvant therapy and to identify those who can be offered a significant survival benefit from adjuvant chemotherapy. For this field to move forward, gene signature development needs to be able to improve upon the current risk stratification achieved from either the high-risk clinicopathologic features or the current AJCC staging criteria. Where are we now in risk-stratifying patients with stage II colon cancer with higher risk of relapse? And how close are we to predicting chemotherapy benefit from the addition of 5-FU/LV and oxaliplatin?

The *Oncotype DX RS* is the only externally validated gene signature available commercially for clinical use that can be used to risk-stratify stage II colon cancer patients beyond clinicopathologic risk factors. The RS is best interpreted in combination with T stage and MMR status given that patients with T4 and microsatellite stable (MSS) tumors derive a 4–7% DFS benefit from therapy. The strength of the RS includes the large sample size of over 1,800 patients to develop the algorithm of 12 prognostic genes and its external validation completed in approximately 1,400 patients. However, only 35% of patients in the training set in the development of these scores had more than 12 nodes examined, which does, therefore, represent a historically predefined high-risk group. An important question answered affirmatively by the validation study in NSABP C-07 patients whether the absolute benefit from chemotherapy is really dependent on baseline risk. The data from the clinical validation study of the *Oncotype DX* in QUASAR samples with 5-FU/LV also showed an absolute risk reduction in low- versus high-risk patients of 3.1% versus 5.7%, respectively, which translates into a 20% relative risk reduction and an absolute risk reduction similar to that seen in stage III patients in the MOSAIC, NSABP C-07, and ACCENT [52] data sets. If we can identify a group of stage II patients with recurrence risks of similar magnitude as stage III colon cancer, which can be accomplished with the RS (high-risk recurrence risk 18%), oncologists will be justified in recommending adjuvant chemotherapy for a 20% absolute risk reduction. In summary, what matters the most is the absolute benefit at each risk level and this signature helps determine that risk level.

ColoPrint and ColDx are newest to emerge on the horizon. Development and validity testing of both of these assays lag behind that of *Oncotype DX*. Both are being developed as prognostic signatures and neither is currently available for use outside the clinical trial setting. The technology for ColoPrint is an FDA-approved assay MammaPrint used to determine prognosis in women with breast cancer [53, 54]. Unlike *Oncotype DX* and ColDx, ColoPrint requires fresh tissue for analysis, which may limit its practical utility when it becomes available for clinical use. Paradoxically, the DNA mismatch repair (dMMR) status was not prognostic in univariate analysis during the development of ColoPrint for reasons unknown. Once validated, ColoPrint has the potential to identify a much larger group of low-risk patients beyond dMMR status who can safely be observed without chemotherapy. ColDx makes no attempt to evaluate their gene signature against dMMR status because this information was not available in the parent samples. This lack of dMMR status in the multivariate analysis alongside other clinicopathologic factors is concerning because the relative contribution of MSI-H cases to the performance of this signature is unknown. Approximately 35% of the patients in the validation set had adjuvant chemotherapy, which likely made the validation set a better prognostic group. There was a high discordance with the clinicopathologic risk factors. Only 6 lymph nodes were required for eligibility, and no treatment information was available on the patients whose samples were used to develop this signature which brings into questions its prognostic capability since this does not appear to be a ‘clean’ patient population. Low- and high-risk groups were defined retrospectively by recurrence status rather than as a prospective assessment of the assay. Both ColoPrint and ColDx are prognostic assays and claim to provide information independent of clinicopathologic risk criteria. However, neither is available yet for commercial use.

Conclusions

The process of gene profiling to develop signatures for prognosis is beginning to show promise. Incorporating the *Oncotype*DX RS in the correct clinical context may better inform adjuvant therapy decisions for patients with stage II colon cancer. We now have preliminary evidence from the *Oncotype*DX RS validation study in NSABP C-07 that this signature is prognostic of outcome in patients treated with 5-FU/LV or FLOX and this is dependent upon each patient's baseline risk. Patients with high recurrence risk do achieve a higher absolute benefit from adjuvant therapy. It appears that the benefit of addition of oxaliplatin to 5-FU/LV is a function of risk. Therefore, what matters most in clinical practice is to be able to estimate the recurrence risk and then the benefit at each risk level from chemotherapy. There is currently no gene signature predictive of benefit from oxaliplatin per se. The *Oncotype*DX RS does not replace the need for a predictive oxaliplatin signature independent of specific chemotherapy regimens, but it is a step in the right direction. At this point in time we believe it is reasonable for physicians to consider using *Oncotype*DX RS for risk stratification of their stage II patients.

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Table 1

Overview of status of the three gene signatures reviewed in this article

Assay	Characteristics and requirements	Recurrence score (RS)	Treatment score (TS)	Clinico-pathologic Features	Availability
OncotypeDX ^{*,#}	12 gene RT-qPCR Formalin fixed paraffin embedded tissue	Yes 1. 3-yr RFS 2. Validated, retrospectively 3. Prognostic of outcome with 5-FU/LV or FLOX Chemotherapy	No Retrospective validation failed	Independent	Commercially Available
ColoPrint ^{,\$&}	18 gene RT-PCR Fresh frozen Tissue	Yes 1. 3- and 5-yr RFS 2. Validated retrospectively 3. Prospective validation ongoing	Not available	Independent	Research setting only
Colon Cancer DSA (ColDx) [^]	634 gene Microarray analysis of DNA Formalin fixed paraffin embedded tissue	Yes 1. 5-yr RFS 2. Retrospective validation pending	Not available	Independent	Research setting only

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RFS, recurrence-free survival