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Identification of a biomarker profile associated with resistance to neoadjuvant chemoradiation therapy in rectal cancer

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Abstract

Objective—To identify a biomarker profile associated with tumor response to chemoradiation (CRT) in locally advanced rectal cancer.

Background—Rectal cancer response to neoadjuvant CRT is variable. While some patients have a minimal response, others achieve a pathologic compete response (pCR) and have no viable cancer cells in their surgical specimens. Identifying biomarkers of response will help select patients more likely to benefit from CRT.

Methods—This study includes 132 patients with locally advanced rectal cancer treated with neoadjuvant CRT followed by surgery. Tumor DNA from pre-treatment tumor biopsies and control DNA from paired normal surgical specimens was screened for mutations and polymorphisms in 23 genes. Genetic biomarkers were correlated with tumor response to CRT (pCR versus non-pCR), and the association of single or combined biomarkers with tumor response was determined.

Results—Thirty-three out of 132 (25%) patients achieved a pCR and 99 (75%) patients had non-pCR. Three individual markers were associated with non-pCR; *KRAS* mutation (p = 0.0145), *CCND1* G870A (AA) polymorphism (p = 0.0138), and *MTHFR* C677T (TT) polymorphism (p = 0.0120). Analysis of biomarker combinations revealed that none of the 27 patients with both *p53* and *KRAS* mutations had a pCR. Further, in patients with both *p53* and *KRAS* mutations or the *CCND1* G870A (AA) polymorphism or the *MTHFR* C677T (TT) polymorphism (n = 52) the

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Study Description: Rectal cancer response to neoadjuvant chemoradiation (CRT) is variable. Some patients have a minimal response while others have a pathologic complete response (pCR) and have no viable cancer cells in their surgical specimens. Identifying biomarkers of response will help select patients more likely to benefit from CRT. Here, we identify a biomarker profile strongly associated with non-pCR to CRT in locally advanced rectal cancer.

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association with non-pCR was further strengthened; 51 out of 52 (98%) of patients were non-pCR. These biomarker combinations had a validity of >70% and a positive predictive value of 97%–100%, predicting that patients harboring these mutation/polymorphism profiles will not achieve a pCR.

Conclusions—A specific biomarker profile is strongly associated with non-pCR to CRT and could be used to select optimal oncologic therapy in rectal cancer patients.

Introduction

In recent years combined chemotherapy and radiation therapy (CRT) before total mesorectal excision (TME) has become the standard treatment for patients with locally advanced rectal cancer. This approach provides excellent tumor control and long-term survival[1–4] but it is associated with measurable mortality, significant morbidity, and long-lasting sequelae that may permanently impair quality of life.[5–8] It is now evident that preoperative CRT is not equally beneficial for all rectal cancer patients. Some patients have a minimal response to CRT, whereas others have no detectable cancer cells in the primary tumor location or in regional lymph nodes in the surgical specimen. Patients with such a pathologic complete response (pCR) have a better prognosis compared to non-pCR patients.[9–15] If tumor response could be predicted before surgery, patients with resistant tumors could be spared CRT-related toxicity and expense. Furthermore, patients likely to achieve a pCR could potentially avoid the morbidity and functional consequences of TME. The benefit for these patients in terms of quality of life would be significant. Unfortunately, identifying responders and non-responders to CRT before surgery remains a challenge.

While tumor response to CRT depends on treatment-related factors, such as radiation dose and the type of chemotherapy administered, tumor biology appears to play the most important role in governing rectal cancer response to CRT.[16,17] The search for molecular predictors of rectal cancer response to CRT has been an active area of research because such biomarkers could profoundly affect the clinical management of rectal cancer patients and influence the use of organ-preserving treatment strategies such as local excision or observation. Many studies have reported biomarkers of response to CRT, focusing on gene expression, mutations, and polymorphisms;[16–22] and although select genes or gene combinations have been identified as potential surrogates of response, none have been validated and incorporated into clinical practice.

In this study we screened a series of 132 patients who were treated in a prospective rectal cancer trial for mutations and polymorphisms in 23 genes with previously reported roles in the pathogenesis of colorectal cancer. Our objective was to determine whether these molecular alterations alone or in combination were associated with response to CRT.

Patients and methods

Patient eligibility

Patients with clinical American Joint Committee on Cancer (AJCC)[23] stage II (T3-4, N0) or stage III (any T, N1-2) invasive adenocarcinoma of the rectum with a distal tumor border within 12 cm of the anal verge were enrolled in the Timing of Rectal Cancer Response to Chemoradiation study, a multi-institutional prospective clinical trial investigating the effect of increasing the CRT-to-surgery time interval, and adding modified FOLFOX-6 chemotherapy (mFOLFOX-6) during the interval period (ClinicalTrials.org Identifier: NCT00335816). This trial was designed as a series of sequential Phase II trials or study groups (SGs), each with a progressively longer CRT-to-surgery interval and increasing cycles of preoperative mFOLFOX-6. This study was approved by an Institutional Review

Board (IRB) at each participating institution as well as a central IRB, and informed written consent was obtained from each patient prior to enrollment in the trial. Patients included in the present study were pooled from SG1 (n = 52), SG2 (n = 58) and SG3 (n = 22). Further details of patient eligibility for this trial are presented elsewhere.[24]

Treatment protocol

Patients in all SGs were treated with CRT; 5-Fluorouracil (FU) 225 mg/m^2/day for 7 days in continuous infusion, and a total of 50.4Gy radiation. Patients in SG1 underwent TME an average of 6 weeks after completing CRT (standard of care). Following CRT, patients in SG2 and SG3 with signs of stable disease or disease progression compared with baseline staging had surgery without further delay. All other patients received 2 and 4 cycles of additional chemotherapy (mFOLFOX-6), respectively; leucovorin 200 mg/m^2 or 400 mg/m^2 plus oxaliplatin 85 mg/m^2 by 2h infusion, followed by bolus of 5-FU 400 mg/m^2 and a 46h infusion of 5-FU 2,400 mg/m^2. Patients in SG2 and SG3 underwent TME an average of 11 and 16 weeks after completing CRT. The clinical outcomes for these patients are presented elsewhere.[24]

Tumor response to CRT

Pathologic complete response was defined as the complete absence of tumor cells from the rectal wall and regional lymph nodes by hematoxylin and eosin staining under microscopy. Tumor pathology was assessed by two independent pathologists and graded according to the recommendations of the AJCC.[23] For the purposes of the study, response was classified as either pCR or non-pCR based on the above criteria.

Sample preparation and molecular analysis

Tumor DNA from pre-treatment tumor biopsies and control DNA from paired normal surgical specimens for all patients was extracted as follows: 10–20 slides per patient sample from formalin-fixed paraffin-embedded (FFPE) tumor biopsies and normal tissues were de-paraffinized, hydrated, and stained with 0.2% methylene blue. A 27.5 gauge needle was then used to manually micro-dissect cells under inverted microscopy. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions with the following modifications; an extension of digestion time at 56°C from 1 hour to 48 hours and the addition of three 20 μl aliquots of Proteinase-K at 4, 20, and 28 hours during digestion. DNA was then quantified by measuring absorbance at 260nm.

PCR analysis and Sanger sequencing

Gene mutations and polymorphisms were screened by standard polymerase chain reaction (PCR) followed by Sanger sequencing (primer sequences shown in Supplementary Table 1). PCR reactions consisted of 10 mM Tris-HCl (pH: 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM primers, 200 mM dNTP, 0.1 μg/ml BSA, 0.5 U Amplitaq Gold (Applied Biosystems, Foster City, CA) plus 2 μl of tumor or control DNA in a total volume of 25 μl. Cycling conditions were 94°C for 30 seconds, annealing for 30 seconds (specific temperatures shown in Supplementary Table 1), and 72°C for 1 minute, for a total of 40–45 cycles. An initial denaturation at 94°C for 10 minutes and a final extension at 72°C for 7 minutes were used. Two independently extracted DNA samples for each patient biopsy or surgical specimen were simultaneously amplified with a negative control (H_20). 2 μl of each PCR reaction was analyzed in a 2% agarose gel to verify the presence of the expected amplified product. All sequencing reactions were carried out in both sense and antisense directions with PCR primers by Sanger sequencing and all mutations and polymorphisms were confirmed by

sequencing two independently-derived PCR products. Somatic mutations and polymorphisms were verified by comparison to paired normal surgical-specimen controls.

Statistical analysis

Patient characteristics—To determine differences in clinical and pathological features between pCR and non-pCR patients, the Mann-Whitney U test was used for comparing means of continuous variables between groups and the two sided Fisher's Exact test and chi-square test were used for testing the significance of differences in the distributions of categorical variables.

Characterizing biomarkers—For genes which could be classified as wild-type or mutant, 2 × 2 analysis tables were constructed and genes were tested for association with tumor response using Fisher's Exact test. For genes with multiple polymorphisms, each polymorphism was partitioned into three separate binary variables comparing a single allelic variation to the remaining combined alleles.

An exhaustive combinatorial analysis was performed to determine the association of each marker or combinations of markers with tumor response. All logical combinations were used (*i.e.*, logical operator AND, logical operator OR) among the binary variables including 1, 2, 3 or 4 markers at a time. Over 3×10^6 combinations were tested. This combinatorial approach allowed us to determine whether patients fail to respond if they carry at least one biomarker (OR) or whether the presence of numerous simultaneous biomarkers (AND/OR) are required to predict non-pCR.

False discovery rate (FDR) was used to control for multiple comparisons.[25] Markers that remained significant after FDR adjustment were internally cross-validated using 100 iterations, randomly selecting 70% of the subjects for "training" and 30% of the subjects for "testing". The Monte Carlo-based *CVLR* in SAS cross-validation algorithm was used.[26] Finally the 2 × 2 tables and logistic regression scores were used to calculate the area under the curve (AUC) of the receiver operating characteristic (ROC) curve, and the sensitivity, specificity, and positive and negative predictive values of the molecular markers.

Results

Patient characteristics and tumor response

A total of 132 patients (58 in SG1, 52 in SG2, and 22 in SG3) were included in the analysis. Patient demographics stratified by pCR status for all patients are shown in Table 1. Overall, 33 out of 132 (25%) patients achieved a pCR. The 99 remaining non-pCR patients (75%) showed either a pathologic partial response (pPR) or had stable disease. No patients had disease progression. Seventeen (29%) patients in SG1 achieved a pCR, 10 (19%) in SG2 and 6 (27%) in SG3 (Table 1). There were no significant differences in tumor response between SGs, and there were no significant differences in clinical or pathological factors between pCR and non-pCR patients, or between SGs.

Individual biomarker analysis

Tumor DNA from pre-treatment tumor biopsies and control DNA from paired normal surgical specimens for all patients was screened for mutations and polymorphisms in 23 genes (Supplementary Table 1). Following screening and statistical analysis, 3 biomarkers were found to associate individually with non-pCR (Table 2). v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations were more common in non-pCR patients compared to patients with a pCR (49% versus 24%, p = 0.0145), while the cyclin D1 (*CCND1*) G870A and methylenetetrahydrofolate reductase (NAD(P)H) (*MTHFR*) C677T

polymorphisms were also associated with non-pCR. Specifically, patients with the AA polymorphism at the 870 locus of the *CCND1* gene were significantly less likely to achieve a pCR compared to patients who carried either the GA or GG alleles at the same location ($p = 0.0138$). Of the total number of patients who carried the AA polymorphism in our patient population (19 out of 132), 18 of 19 (95%) did not achieve a pCR. None of the patients that carried the TT polymorphism at the 677 locus of the *MTHFR* gene (14 out of 132) achieved a pCR ($p = 0.0120$).

Of interest, mutations in tumor protein p53 (*p53*), v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), catenin (cadherin-associated protein), beta 1, 88kDa (*CTNNB1*) and phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*), each previously reported to play a role in the pathogenesis of colorectal cancer, were not significantly associated with pCR in our patient population as stand-alone biomarkers (Table 2). No other single gene mutation or polymorphism was significantly associated with tumor response (Supplementary Table 2).

Combination biomarker analysis

Next, mutation/polymorphism combinations for all molecular alterations were tested up to the order of 4 simultaneous markers. Approximately 3-million different combinations were tested and screened with FDR correction to the p-value. Following analysis, 6 different biomarker combinations were identified as both sufficiently prevalent in our patient population for meaningful validation and statistically associated with tumor response (pCR versus non-pCR) in a synergistic fashion (Table 3). The majority of these biomarker combinations included mutations in both *p53* and *KRAS* (5 out of 6). The significance of this molecular profile is reflected in our patient population, where 27 out of 132 patients had concurrent *KRAS* and *p53* mutations and none had a pCR to CRT. Further, in patients with both *p53* and *KRAS* mutations or the *CCND1* G870A (AA) polymorphism (n = 43) or the *MTHFR* C677T (TT) polymorphism (n = 52) the association with non-pCR was further strengthened (Table 3). When either polymorphism was added to the molecular profile, 51 out of 52 (98%) patients failed to achieve a pCR to CRT.

Validation of predictive biomarker profiles

The four most prevalent biomarkers (*KRAS* mutation; *p53* mutation; *CCND1* G870A [AA] polymorphism; *MHTFR* C677T [TT] polymorphism) based on a significant association with non-pCR individually or in combination were chosen for further validation to determine their value for predicting non-pCR. Following internal validation, each biomarker combination analyzed had a validity of over 70% (Table 4). All combination sets had similar specificity and positive predictive values (97%–100%). The combination of all four markers - *p53* and *KRAS* mutation, or the *CCND1* G870A (AA) polymorphism, or the *MTHFR* C677T (TT) polymorphism - resulted in the highest AUC of the ROC curve (AUC = 0.74) and the highest sensitivity (52%) predicting that patients with this mutation/polymorphism profile will not achieve a pCR to CRT.

Discussion

In the current study we identified gene mutations and polymorphisms that are individually associated with failure to achieve a pCR to CRT. We also found that when combined, these mutations and polymorphisms synergistically identify a subset of rectal cancer patients who do not develop a pCR in response to CRT with a high degree of accuracy.

Our results have immediate clinical relevance given that achieving a complete clinical response to CRT may be followed by an organ preservation approach such as local excision

or observation in select patients with rectal cancer. Indeed a number of collaborative groups are already exploring the feasibility of these approaches. The American College of Surgeons Oncology Group (ACOSOG) recently completed a trial of CRT before local excision for T2N0 rectal cancer patients[27] and a wait-and watch approach has been attempted at a small number of institutions.[28] The deferral of surgery for rectal cancer patients who develop a clinical complete response (cCR) to CRT is also being studied prospectively by the National Cancer Research Network in collaboration with the Pelican Cancer Foundation in the United Kingdom. However, a cCR does not always correlate with a pCR and patients with a cCR after CRT may still have cancer cells in their surgical specimens. Our results show that biomarker profiling could help identify a subset of patients highly unlikely to develop a pCR to CRT and consequently help direct these patients away from organ preservation treatment strategies and towards clinically beneficial therapies such as TME.

KRAS is a key component of the mitogen activated kinase (MAPK) pathway that is activated by cell surface receptors such as epidermal growth factor receptor (EGFR). These signals are then transduced to the nucleus where they phosphorylate and activate transcription factors leading to changes in gene expression.[29,30] Mutant variants in the *KRAS* gene result in constitutive activation of its encoded protein, resulting in persistent activation of the MAPK pathway.[29,30] *KRAS* is mutated in over a third of colorectal cancers and experimental evidence has shown that *KRAS* mutations can be found in the earliest tumor stage, and that once acquired, these mutations are preserved throughout the natural history of the tumor.[31,32] Although previous studies on the prognostic value of *KRAS* mutation in patients with colorectal cancer have reported different results,[33] the recent discovery that *KRAS* mutation is a strong predictor of colorectal cancer response to the anti-EGFR monoclonal antibodies Cetuximab and Panitumumab[34,35] has further established this gene as an important biomarker in colorectal cancer. Indeed *KRAS* mutation status is now routinely checked in every rectal cancer patient for potential candidacy of anti-EGFR therapy.[36]

Few studies have evaluated *KRAS* as a biomarker for tumor response in rectal cancer patients treated with CRT and TME. Luna-Perez, et al. described a series of rectal cancer patients treated with preoperative CRT and reported that tumors with wild-type *KRAS* were more likely to respond to CRT than tumors with mutant *KRAS*.[37] However, these results should be interpreted with caution due to their small sample size, low rate of pCR, sub-standard radiotherapy, and the use of radiated cancer tissue for *KRAS* analysis. Bengala, et al. also found that tumors with wild-type *KRAS* were more likely to respond to CRT compared to mutant *KRAS* (37% versus 11%) in 39 patients treated with Cetuximab and CRT.[38] Our data confirm these observations indicating that rectal cancers with wild-type *KRAS* are more likely to develop a pCR to 5-FU based CRT compared to tumors with mutant *KRAS*. Mutant *KRAS* may therefore be a biomarker for non-pCR in rectal cancer patients treated with CRT, with or without EGFR-inhibitors. However, other studies have reported contradictory results.[39,40] Erben, et al. found no correlation between *KRAS* mutation and tumor down-staging in 57 rectal cancer patients treated with Cetuximab, Irinotecan and Capecitabine in combination with pelvic irradiation.[39] Similarly, Gaedcke, et al. found no correlation between *KRAS* mutation and tumor down-staging in rectal cancer patients treated with 5-FU and Oxaliplatin during radiation.[40] However, these were smaller series, utilizing different radio-sensitizing drugs, and using different definitions of tumor response.

The *CCND1* gene encodes the cyclin D1 protein which is a key regulator of the cell cycle, promoting the transition from G1 to S phase and committing the cell to division and proliferation.[41] *CCND1* expression is elevated in many types of cancers and its expression is regulated at multiple levels including transcription, translation and protein stability and degradation.[41,42] Polymorphisms within *CCND1* contribute to its regulation and possibly to

its oncogenic potential. Of over 100 reported *CCND1* polymorphisms, the GA polymorphism at locus 870 has received the most attention. This polymorphism is located at the exon 4/intron 4 boundary and has been linked to alternative gene splicing. The G allele codes for the optimal splicing which produces the canonical form, termed cyclin D1a, while the A allele constrains exon 4 excision and allows translation into intron 4 resulting in a truncated cyclin D1b transcript that lacks the sequences required for degradation. The increase in the half-life in this variant form of *CCND1* is consistent with an increase in cell proliferation, and the A allele has been associated with increased risk and advanced tumor stage in colorectal cancer, and a poor prognosis in a variety of cancers.[43] Li, et al. recently reported that cyclin D1a is also important to elicit the DNA damage response (DDR) that may result in DNA repair.[44] Our findings, that patients homozygous for the A allele are less likely to respond to CRT, are consistent with the increased cell proliferation and reduced contribution to DDR associated with cyclin D1b. However, other series have reported contradictory results. Ho-Pun-Cheung, et al. reported better tumor response and lower risk of local recurrence associated with the AA allele among 65 patients with rectal cancer treated with preoperative radiotherapy.[45] However in this smaller series, patients did not receive sensitizing chemotherapy, and response was based on histological tumor regression rather than on pCR.

Exposure of cells to DNA-damaging agents, such as ionizing radiation and chemotherapeutic drugs, elicits a complex set of acute cellular responses that involve the coupling of cell cycle arrest, DNA repair, and apoptosis. The central component of these responses is the product of the *p53* gene, which modulates transcription of responsive genes involved in temporary or permanent growth arrest or apoptosis. Inactivation of *p53* contributes to cellular resistance to DNA-damaging agents *in vitro* and *in vivo*.[46,47] Over 50% of colorectal cancers harbor *p53* mutations. Most of them are missense mutations, leading to the synthesis of a stable but inactive protein that accumulates in the nucleus of tumor cells. A number of studies have evaluated *p53* mutations as predictors of response in rectal cancer patients treated with neoadjuvant therapy.[48–50] While some studies found an association between mutant *p53* and tumor response to radiation,[48,49] other studies have not confirmed this association.[50] Our results concur with those series which found that mutant *p53* alone is not associated with tumor response to CRT. However, we found that mutant *p53* becomes a predictive biomarker of non-pCR when present in cancer cells harboring a *KRAS* mutation. In these cells, the combined mutation/polymorphism profile of these biomarkers may synergistically promote increased proliferation (*KRAS* mutation) coupled with tumor resistance to radiation and chemotherapy (*p53* mutation) resulting in non-pCR.

The *MTHFR* gene codes for an enzyme that catalyzes conversion of 5,10-MTHF to 5-MTHF, the dominant circulating form of folate. 5,10-MTHF is used for the thymidylase synthase (TS)-catalyzed conversion of deoxyuridylate to deoxythymidylate, important for DNA synthesis. A C-to-T transition in codon 677 of the *MTHFR* gene results in a genotypic variant associated with decreased enzyme activity.[51] Reduced *MTHFR* activity may increase the amount of 5,10-MTHF available for the TS enzyme, and increase the effect of TS inhibitors such as 5-FU, in individuals carrying this polymorphism. While there are mixed results from studies investigating the effect of the *MTHFR* C677T polymorphism on colorectal cancer response to 5-FU,[52–54] two studies suggest this polymorphism does affect rectal cancer response.[53,54] Terrazzino, et al. found that the likelihood of response for 122 patients treated with fluoropyrimidine-based CRT and surgery was higher among patients homozygous for the C allele compared to carriers of the T allele.[53] Similarly, Cecchin, et al. found that the T allele was associated with lower response rates in a series of 238 rectal cancer patients treated with fluoropyrimidine-based chemotherapy.[54] Our study found similar resistance to CRT associated with *MTHFR* C677T. Despite methodological differences between these two studies and our trial (both used radio-sensitizers in addition to

fluoropyrimidines, defined response using tumor regression grade (TRG) rather than pCR, and used different CRT-to-surgery intervals), their combined results suggest that the *MTHFR* C677T (TT) polymorphism is associated with non-pCR to CRT.

There are a number of limitations to our study that warrant consideration. First, the sample size is relatively small and the study endpoint, pCR, occurred in only 25% of patients. Furthermore, while we used a common statistical cross-validation method, which has been applied extensively to biomarker-validation studies,[55] a larger independent series with more pCR and non-pCR patients will be important to validate these results. To address this we are continuing to collect specimens from additional patients in SG1–SG3 to further validate our results as well as extending our studies to an independent patient cohort. Second, although this is a prospective study with a homogenous patient population, the treatment regimen varied between the three SGs in both the use of adjuvant chemotherapy and the CRT-to-surgery interval. Third, the tissue used to extract normal control DNA was obtained from the proximal resection margin of the surgical specimens. This tissue is usually outside the radiation field and it is unlikely that it received the full dose of radiation, but it was exposed to chemotherapy. While we have recently shown that mutations in *KRAS* and *p53* remain largely unchanged in rectal cancer after CRT,[56] the possibility of mutations arising due to treatment can not be totally excluded.

In conclusion, we have identified a biomarker profile that is associated with tumor resistance to CRT, evidenced by a lack of pCR. These findings are important because they help identify a subset of patients with rectal cancers who most likely will not respond to CRT and therefore should not be considered as candidates for organ preservation following CRT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Patient demographics and tumor characteristics overall and stratified by study group

Demographic/Characteristic	pCR (n=33)	Non-pCR (n=99)
Age, years *	56 (32–80)	57 (26–87)
Female	13 (39%)	42 (42%)
Race		
Asian/Pacific Islander	3 (9%)	4 (4%)
Black	2 (6%)	2 (2%)
White	26 (79%)	85 (86%)
Unknown	2 (6%)	8 (8%)
Clinical T stage		
T2	5 (15%)	5 (5%)
T3	27 (82%)	90 (91%)
T4	0 (0%)	2 (2%)
Tx	1 (3%)	2 (2%)
Clinical N stage		
N0	4 (12%)	24 (24%)
N1	26 (79%)	66 (67%)
N2	1 (3%)	6 (6%)
Nx	2 (6%)	3 (3%)
Clinical AJCC stage		
II	4 (12%)	24 (24%)
III	27 (82%)	73 (74%)
Unknown	2 (6%)	2 (2%)
Study group (SG) †		
SG1 (n=58)	17 (29%)	41 (71%)
SG2 (n=52)	10 (19%)	42 (81%)
SG3 (n=22)	6 (27%)	16 (73%)

* Median (Range).

† SG1 had surgery 6 weeks after the completion of CRT (standard of care); SG2 had surgery 11 weeks after the completion of CRT, and had 2 cycles of modified FOLFOX-6 during the waiting period; SG3 had surgery 16 weeks after the completion of CRT, and had 4 cycles of modified FOLFOX-6 during the waiting period. Abbreviations: pCR – Pathologic complete response; AJCC – American Joint Committee on Cancer; SG – Study group.

Table 2

Gene mutations and polymorphisms individually associated with non-PCR

Mutation/Polymorphism	Prevalence in patient population (n=132)	pCR (n=33)	Non-pCR (n=99)	p-value
KRAS	57 (43%)	8 (24%)	49 (49%)	0.0145 *
BRAF	1 (1%)	1 (3%)	0 (0%)	0.2500
p53	75 (57%)	16 (48%)	59 (60%)	0.3124
PIK3CA	40 (30%)	12 (36%)	28 (28%)	0.3898
CTNNB1	1 (1%)	0 (0%)	1 (1%)	1.0000
CCND1 G870A				0.0138 *
AA	19 (14%)	1 (3%)	18 (18%)	
GA	68 (52%)	23 (70%)	45 (45%)	
GG	45 (34%)	9 (27%)	36 (36%)	
MTHFR C677T				0.0120 *
CC	60 (45%)	18 (55%)	42 (42%)	
TC	58 (44%)	15 (45%)	43 (43%)	
TT	14 (11%)	0 (0%)	14 (14%)	

* Statistically significant at 0.05. Abbreviations: pCR – Pathologic complete response.

Table 3

Significant biomarker combinations associated with non-pCR

Biomarker combination	Prevalence in patient population (n=132)	pCR (n=33)	Non-pCR (n=99)	p-value	FDR
KRAS mutation and *p53* mutation	27 (20%)	0 (0%)	27 (27%)	0.0003	0.0303
KRAS mutation and *CCND1* = AA	71 (54%)	9 (27%)	62 (63%)	0.0005	0.0575
CCND1 = AA and *MTHFR* = TT	32 (24%)	1 (3%)	31 (31%)	0.0007	0.0661
KRAS mutation and *p53* mutation, or *CCND1* = AA	43 (33%)	1 (3%)	42 (42%)	< 0.0001	0.0010
KRAS mutation and *p53* mutation, or *MTHFR* = TT	37 (28%)	0 (0%)	37 (37%)	< 0.0001	0.0010
Any 1 or more of: *KRAS* mutation and *p53* mutation or *CCND1* = AA or *MTHFR* = TT	52 (39%)	1 (3%)	51 (52%)	< 0.0001	0.0005

Abbreviations: pCR – Pathologic complete response; FDR – False discovery rate.

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Table 4

Cross-validation for biomarker combinations associated with non-pCR

Characteristic	***KRAS* mutation and *p53* mutation**	***KRAS* mutation and *p53* mutation, or *CCND1* = AA**	**Any 1 or more of: *KRAS* mutation and *p53* mutation, or *CCND1* = AA, or *MTHFR* = TT**
Prevalence (n=132)	27 (20%)	43 (33%)	52 (39%)
pCR	0	3%	3%
Fisher's Exact p-value	0.0003	< 0.0001	< 0.0001
ROC curve AUC	0.63	0.70	0.74
Validity	75% ± 0.6%	76% ± 0.6%	75% ± 0.5%
Sensitivity	27/99 (27%)	42/99 (42%)	51/99 (52%)
Specificity	33/33 (100%)	32/33 (97%)	32/33 (97%)
PPV	27/27 (100%)	42/43 (98%)	51/52 (98%)
NPV	33/105 (31%)	32/89 (36%)	32/80 (40%)

Abbreviations: pCR – Pathologic complete response; ROC curve – Receiver operating characteristic; AUC – Area under the curve; PPV – Positive predictive value; NPV – Negative predictive value.