Mutation Profiling and Microsatellite Instability in Stage II and III Colon Cancer: An Assessment of their Prognostic and Oxaliplatin Predictive Value

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ABSTRACT

Purpose: The purpose of this study was to examine the prognostic and oxaliplatin predictive value of mismatch repair (MMR) status and common hotspot mutations which we previously identified in stage II and III colon cancer.

Experimental Design: Mutations in BRAF, KRAS, NRAS, MET, and PIK3CA were profiled in 2299 stage II and III colon tumors from NSABP clinical trials C-07 (N=1836) and C-08 (N=463) with Typlex chemistry and Mass Spectrometry. C-07 tested the worth of adding oxaliplatin to 5-fluorouracil plus leucovorin and C-08 tested the worth of adding bevacizumab to FOLFOX. Cox proportional hazard models were used to assess prognostic or oxaliplatin predictive value of mutations for tumor recurrence, overall survival (OS), and survival after recurrence (SAR).

Results: BRAF mutations were associated with MMR deficient tumors (P<0.0001), poor OS (HR=1.46, 95%CI 1.20; 1.79, p≤.0002) and poor SAR (HR=2.31, 95%CI 1.83, 2.95, p<.0001). Mutations in KRAS, NRAS, MET and PIK3CA were not associated with recurrence, OS or SAR. MMR deficient tumors were associated with an improved prognosis based on recurrence (HR=0.48, 95%CI 0.33, 0.70 p<0.0001). Mutations and MMR status were not predictive for oxaliplatin benefit.

Conclusions: This study shows that BRAF mutations profiled from stage II and III colon cancer tumors were associated with poor SAR and validates and explains at least in part previous observations associating it with poor OS. Profiling of all of these mutations is warranted for future clinical trial testing new targeted therapies which block relevant signaling pathways. Such clinical trials are under development at NSABP.
Translational Relevance

The prognostic and oxaliplatin predictive value of mismatch repair (MMR) and common hot spot mutations in stage II and III colon cancer utilizing NSABP clinical trials C-07 (N=1836) and C-08 (N=463) were assessed. The findings of several other studies demonstrating that \textit{BRAF} mutations were associated with MMR and poor overall survival (OS) were validated and show that this is at least in part due to \textit{BRAF}'s association with poor survival after recurrence (SAR). \textit{KRAS}, \textit{PIK3CA}, \textit{NRAS}, and \textit{MET} mutations were not significantly associated with prognosis (N=2299). None of the mutations were shown to be predictive for oxaliplatin benefit. Future novel clinical trials targeting \textit{BRAF} are warranted due to its association with poor prognosis and resistance to anti-EGFR therapies and profiling of all of these mutations will be necessary in future clinical trials utilizing targeted therapies which inhibit RAS-RAF and AKT pathways.
INTRODUCTION

Mutation profiling has proven to be a useful tool for predicting response to targeted therapies in colon cancer but the clinical impact of mutation profiling for prognosis and for prediction of standard chemotherapies has yielded little clinical utility. PIK3CA mutations have been shown to be associated with a poor prognosis in stage II and III colon cancer but these results have not been confirmed in a randomized clinical trial.1-3 BRAF mutations have been shown to be associated with poor prognosis in many colon cancer studies including randomized clinical trials,4-9 but not in all studies.10,11 Likewise, results from studies evaluating KRAS mutations in early stage colon cancer have been contradictory.6,10,12-16

As a first step in identifying mutations as potential biomarkers, we identified the common colon cancer mutations by profiling 187 different cancer mutations in 19 genes in a cohort of 235 cases from NSABP C-07 trial, using Type Plex chemistry, mass spectrometry, and the OncoCarta™ Panel v1.0 (Sequenom, San Diego, CA). Twenty-four hot spot mutations in 5 genes (BRAF, KRAS, MET, NRAS, PIK3CA) were found to occur in more than 1% of stage II and III colon cancer. These mutations were re-plexed into 6 new pools referred to as ColoCarta (Supplementary Table 1). This technology provided a cost effective and sensitive method for the detection of hot spot mutations in routinely processed formalin-fixed paraffin-embedded samples.17

MMR status and mutations in BRAF, KRAS, MET, NRAS or PIK3CA, in C0-7 and C-08 samples were tested for their prognostic value in stage II and III colon cancer. The predictive value of mutations and MMR status for oxaliplatin benefit in C-07 was also tested.
PATIENTS and METHODS

Patient Selection Clinical Samples

The NSABP C-07 trial demonstrated that the addition of oxaliplatin to 5-fluorouracil + leucovorin (5-FU+LV) improved survival of resected stage II and III colon cancer patients. Patients who participated in that study provided written informed consent to participate, and the study was approved by institutional review boards at participating NSABP sites. All cases with available tumor blocks, consented for research, and with follow-up information from NSABP C-07 (N=1836) were used in the current study (REMARK Fig 1). In addition we performed gene mutation profiling on a representative cohort of 500 cases from NSABP C-08 trial. This cohort was selected using stratified random sampling with strata for treatment and N-stage (N0, N1, N2) so that the proportion of patients in each stratum of the sample was the same as the proportion of eligible patients with tissue in each stratum of the parent C-08 trial. C-08 tested the worth of adding bevacizumab to (5-FU + LV) and oxaliplatin (mFOLFOX6) for treatment of patients with resectable stage II and III colon cancer. The original purpose of profiling C-08 was to identify predictive markers for bevacizumab benefit. However, there was no benefit from bevacizumab overall so we elected to add the mutation profiles from C-08 to the C-07 cases to add to the prognostic power of our study.

This correlative science study was approved by an Institutional Review Board and written informed consent was obtained from each patient for biomedical research.

DNA Isolation and Mutation Detection

Preparation of DNA from formalin-fixed paraffin-embedded tumor blocks were prepared with the method using an Ambion Recover-All kit or with the E.Z.N.A.® FFPE DNA Isolation Kit from Omega Bio-Tek (Norcross, GA) in a semi-automated method utilizing the King Fisher Flex instrument (ThermoFisher,
Burlington, ON).\textsuperscript{17} DNAs were quantified with fluorescence, using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA) and the InfiniteF200 fluorometer (Tecan, Mannedorf, Switzerland).

**Mass Spec Type Plex Technology and Mutation Panels**

Mutation profiling with OncoCarta and ColoCarta and the running of samples on the MassSpec platform have been described previously\textsuperscript{17}. All mutations were reviewed independently by 3 investigators and all inconsistencies were reexamined and a consensus was reached. Reactions with >20% unextended primer were excluded from analysis. Mutation at any position was considered evidence of mutation for that gene. A failed reaction resulted in missing data for that gene only if the other reactions were wild type (wt). OncoCarta\textsuperscript{TM} interrogated 187 mutations in 19 genes and was used to interrogate 235 C-07 cases.\textsuperscript{17} Twenty-four colon cancer mutations in *BRAF*, *NRAS*, *KRAS*, *MET*, and *PIK3CA* were reconfigured into ColoCarta, which was used to profile all of the remaining C-07 (N=1601) cases and the cases from the C-08 (N=463) randomly selected cohort. Based on the Catalogue of Somatic Mutation in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/) (COSMIC) data base, the ColoCarta panel included assays that captured 99%, 98%, and 79% of the known colon cancer mutations in *BRAF*, *KRAS*, and *PIK3CA*, respectively. COSMIC mutation frequencies are based on the sequence of 4628, 858, and 203 samples for *BRAF*, *KRAS*, and *PIK3CA*, respectively. Roughly 20% of the *PIK3CA* mutations in the COSMIC data base were the result of 8 mutations in *PIK3CA* that occurred only once. These 8 mutations were not represented in either the OncoCarta or ColoCarta assays.

**Immunohistochemistry**

Antibodies for immunohistochemistry included anti-MSH2 (clone FE-11; 1:200; Calbiochem, Darmstadt, Germany) and anti-MLH1 (clone G168-15; 1:30; BD Biosciences, San Jose, CA, USA). Sections were incubated with normal goat serum for 30 minutes and then overnight at 4°C with specific mouse monoclonal antibodies and stained with DAKO EnVision\textsuperscript{TM} + system Kit (DAKO) according to the...
manufacturer’s instructions. Mismatch repair (MMR) status was determined by staining results of MSH2 and MLH. Tumors that showed no staining with anti-MSH2 or anti-MLH1 or both in the tumor cells were considered MMR deficient or unstable. Only tumors that showed staining for both were considered MMR proficient or stable. Positive staining in non-tumor cells was used as a control for assay success.

**Statistical Analysis**

Time to recurrence and time to death (overall survival, OS) were measured from random assignment. Survival after recurrence (SAR) was the time from recurrence to death in the set of patients with recurrence. For all three endpoints, patients without an event were censored at last follow-up.

Mutations in \textit{BRAF}, \textit{KRAS}, \textit{KRASG12V}, \textit{NRAS}, \textit{MET}, and \textit{PIK3CA} were analyzed as dichotomous variables in which all mutations for a particular gene were combined so that each gene within each case was scored as 0 to indicate no mutation and 1 to indicate the presence of a mutation. In addition, the \textit{BRAFV600E} mutation was tested as a categorical variable using the frequency data for alleles. In this case, \textit{BRAFV600E} mutations were split into two groups at a median cut with a frequency of mutant alleles of 0.16. Thus, cases were split into 3 groups: \textit{BRAFV600E} wild-type, \textit{BRAFV600E} low frequency allele, and \textit{BRAFV600E} high frequency allele.

Mutations were tested for their association with age (<65, \geq 65), gender, MMR status, T-stage (T1 or T2, T3, T4), and nodal status (N0=0, N1=1-3, and N2=4 or more invaded lymph nodes). Associations were evaluated by Fisher’s exact test for tables with less than 5 patients in any category and the Chi square test for other covariates. Two-sided P-values less than 0.05 were considered significant.

The association of mutations and MMR status with the endpoints of recurrence, OS, and SAR were tested in Cox proportional hazard models controlling for treatment and the combination of nodal status and T-Stage (8 categories: N0 T3, N0 T4, N1 T1 or T2, N1 T3, N1 T4, N2 T1 or T2, N2 T3, and N2 T4). To control
for multiple testing of 7 explanatory variables and 3 endpoints, a significant p-value was considered less than 0.05 / 21 = 0.00238 according to the method of Bonferroni.

Data from C-07 and C-08 were combined for all analyses with the exception of interaction treatment tests for the prediction of oxaliplatin benefit, which was restricted to cases from C-07. The mutation-by-treatment interaction or the MMR-by-treatment interaction was tested by adding a cross-product term of indicator variables for oxaliplatin treatment and mutation status to the Cox models described in the previous paragraph. Statistical significance was corrected for multiple tests via the method of Bonferroni.

Our study had more than 80% power to detect a prognostic effect of mutant KRAS (35% in population) and mutant BRAF (12% in population) using recurrence as the end point and assuming a significant level of 0.05 if the corresponding hazard ratio (HR) was more than 1.4. For rare mutations such as NRAS (3% in population), our study had more than 70% power to detect an HR of more than 1.8. The estimated power to detect an HR of 1.5 for each mutation and each endpoint is summarized in Supplementary Table 2.

Our study for oxaliplatin prediction had more than 80% power to detect an interaction effect of KRAS mutations and oxaliplatin treatment for recurrence if the corresponding HR was more than 2.0. However, for NRAS mutations there was only a 30% power to detect a predictive effect if the corresponding HR was more than 2.

RESULTS

Frequency and Association of Mutations with Each Other

The frequencies for the well-known colon mutations were 38.11% for KRAS, 20.18% for PIK3CA, and 14.20% for BRAF, and were mostly similar to those seen in the COSMIC data base (35.3% for KRAS, 12.4% for PIK3CA, and 14.3% for BRAF). Other infrequent mutations including NRAS (2.9%) and MET (3.7%) have also been described previously (Supplementary Table 3). While the sequence alterations in MET were listed as somatic mutations in COSMIC, they are actually single nucleotide polymorphisms. Sequence alterations
responsible for MET R970 and T992I were present in both normal and tumor tissues and are listed in the SNP data base (data not shown).

The pairwise distribution of the multiple mutations in tumors differed from what would be expected under statistical independence for several pairs of mutations in our study. Similar to previous results we found that \textit{BRAF} and \textit{KRAS} mutations were almost mutually exclusive ($p<0.0001$). Only 4 cases of 2226 had mutations in both \textit{KRAS} and \textit{BRAF}. In Figure 2 only 3 cases overlap because this figure only includes cases for which all mutations for all 4 genes were available. One of the cases with both \textit{NRAS} and \textit{BRAF} mutations had an assay failure for one of the other mutation and was eliminated from the figure. Likewise, only one tumor with a \textit{NRAS} mutation had a \textit{BRAF} mutation, which is similar to other reports that have found them to be mutually exclusive. Conversely, \textit{PIK3CA} mutations were significantly associated with \textit{KRAS} mutations ($p<0.0001$) but \textit{PIK3CA} did not show any significant association with \textit{BRAF}. The overlap of the mutations in these 4 different genes is shown in Figure 2.

\textbf{Association of Mutations and MMR Status with Clinical Variables}

In agreement with previous observations, \textit{BRAF} mutations were significantly more prevalent in older-aged individuals ($p<0.0001$), in females ($p<0.0001$), and in MMR deficient tumors ($p<0.0001$) (Table 1). MMR status was associated with \textit{KRAS} ($p=0.001$) and \textit{KRASG12V} ($p<0.0001$), as expected, since \textit{BRAF} and \textit{KRAS} were mutually exclusive. No mutations were associated with T-stage or nodal status when corrected for multiple comparisons via Bonferroni but \textit{BRAF} mutations showed a trend toward significance for association with higher T stage ($p=0.01$) and nodal status ($p=0.02$). MMR deficient tumors were significantly associated with higher T-stage ($p=0.0005$) (tumors with a greater depth of invasion) and with lower N stage ($p<0.0001$), as has been previously been observed. (Table 1).
Association of Mutations and MMR status with Prognosis

MMR status and mutations in BRAF, KRAS, KRASG12V, MET, NRAS, and PIK3CA were examined for prognostic value by evaluation of time to recurrence, OS, and SAR (Fig 3). MMR deficient tumors were positively associated with good prognosis for recurrence (HR=0.48 [95%CI: 0.33, 0.70], p<0.0001) and showed a trend for association with OS (HR=0.63, [95%CI: 0.46, 0.89] p=0.0084) and for SAR (HR=1.60, [95%CI: 1.07, 2.41], p=0.02) but the trend for SAR was in the opposite direction. Conversely, BRAF mutations were associated with poor OS (HR=1.46 [95% CI: 1.20, 1.79], p=0.0002) and SAR (HR=2.3 [95% CI: 1.83, 2.95, p<.0001]) but not for recurrence (HR=1.02, [95%CI: 0.82, 1.28], p=0.86). BRAF's association with poor SAR may help explain why BRAF mutations were not prognostic for recurrence but were for OS. The Kaplan Meier plot in Fig 4A clearly demonstrates that patients with BRAF mutations have a much shorter survival time after recurrence than do patients whose tumors are wild type for BRAF. KRAS, KRASG12V, MET, NRAS, and PIK3CA mutations were not associated with recurrence, OS, or SAR. NRAS mutations showed a trend for an association with recurrence but was not significant when corrected for multiple comparisons (HR=1.53 [95%CI: 1.01 2.31], p=0.04).

Association of combined effect of MMR and BRAF with Prognosis

It seems counter intuitive that BRAF mutations were associated with poor OS, SAR, and with MMR deficient tumors, even though deficient MMR tumors were associated with a good prognosis based on recurrence and OS. To determine whether MMR status and BRAF mutation effects were additive or interactive, KM plots for each subgroup were generated (Figure 4B). Patients with tumors that were MMR deficient and BRAF wt had the best prognosis (HR=0.55, [95%CI: 0.35, 0.88], p=0.011) compared to the patients with MMR proficient tumor and BRAF wt tumors, with a 5-year survival of 89.7%. Patients with MMR proficient and BRAF mutant tumors had the worst prognosis (HR=1.58, [95%CI: 1.22, 2.03], p=0.0005) compared to those with MMR
proficient tumors and \textit{BRAF} wt), with a 5-year survival of 69.1%. Patients with \textit{BRAF} wt, MMR proficient or with \textit{BRAF} mutant, MMR-deficient tumors had intermediate survival (p=0.27 between those two groups) with a 5-year survival of 82.1% and 84%, respectively (Fig 4B). The increased HR for OS for patients with \textit{BRAF} mutations was similar in MMR-proficient tumors (HR=1.58, [95\%CI: 1.23, 2.04], p=0.0004) and MMR-deficient tumors (HR=1.76, [95\%CI: 0.88, 3.49], p=0.11) but this effect was only significant in the MMR proficient group. These results demonstrate that \textit{BRAF} and MMR status are prognostic and their effect is additive.

The Sequenom platform allows for the sensitive quantification of mutant alleles\textsuperscript{17,30,31}. The sensitivity is critical because colon tumors are heterogeneous, such that not all tumor cells have the same mutations and tumor samples invariably contain some normal fibroblastic and lymphocytic cells. Therefore, as part of an exploratory analysis, we analyzed \textit{BRAF} mutations to determine whether the sensitivity of mutation detection provided a potentially meaningful clinical assessment of tumors, \textit{BRAF} mutations were re-analyzed based on the amount of mutant \textit{BRAFV600E} alleles within a tumor. All C-07 and C-08 cases were split into 3 groups: \textit{BRAFV600E} wt, \textit{BRAFV600E}-low frequency allele, and \textit{BRAFV600E}-high frequency allele. (The split for low- and high-frequency mutant alleles was at 0.16, which represented the median cut for tumors with \textit{BRAF} mutations). Patients with \textit{BRAFV600E} mutant tumors with less than 0.16 \textit{BRAF} mutant alleles were significantly associated with SAR (HR=1.25, [95\%CI: 1.60; 3.18], p<.0001) and were marginally associated with OS (HR=1.4, [95\% CI: 1.4; 1.87], p=0.022). Thus, if \textit{BRAF} mutations are used as a prognostic tool or to determine the appropriateness of a particular treatment, the sensitivity of the assay may be important.

\textbf{Association of Mutations and MMR with Oxaliplatin Benefit}

Mutations have been identified as sensitivity and resistance markers to cancer therapies. Therefore, we tested the six mutations and MMR status for interaction with oxaliplatin benefit in C-07 on the three outcomes (recurrence, OS, and SAR). No significant interactions were seen between mutations (\textit{BRAF}, \textit{KRAS},...
KRASG12V, MET, NRAS, and PIK3CA) and MMR status with oxaliplatin treatment in C-07 (Table 2).

Mutations and MMR status did not come close to reaching significance for interaction with oxaliplatin. The power to detect an interaction HR of 2.0 for mutations in BRAF, KRAS, and PIK3CA was 71%, 96%, and 87%, respectively, for recurrence and OS. We found no significant interaction for these mutations and MMR status in this analysis and conclude that these common mutations are not good markers for predicting response to oxaliplatin therapy.

DISCUSSION
We conducted this study to evaluate the potential prognostic value and oxaliplatin predictive value of common hot spot mutations in stage II and III colon cancer patients in a large cohort consisting of all available and consented tissues from NSABP clinical trial C-07 and an additional 463 cases from a representative cohort from NSABP clinical trial C-08.

This study represents one of the largest analyses of common hot spot mutations of prospective randomized clinical trial samples of stage II and III colon cancer. We utilized Type Plex chemistry and Mass Spectrometry, which we have previously shown to be sensitive, reproducible, and cost effective for mutation detection of DNAs isolated from FFPE. The mutation frequencies in this study for KRAS 38.1%, PIK3CA 20.2%, NRAS 2.9% and BRAF 14.2% were comparable to other studies for stage II and III colon cancer. Lower frequencies for PIK3CA (14%) and BRAF (7%) mutations have been reported previously. This could be due to differences in the population or to the greater sensitivity of the Mass Spec platform which has been shown to be more sensitive than the methods used in the other studies.

No significant interactions were seen between mutations (BRAF, KRAS, KRASG12V, MET, NRAS, and PIK3CA) or MMR status with oxaliplatin treatment in C-07. We can conclude that these common mutations...
and MMR do not render tumors resistant to benefit from oxaliplatin within the limitations of detection based on the sample size.

FOLFOX has become the standard of care for resected stage III and for most stage II colon cancer patients based on NSABP trial C-07 and the MOSAIC trial.\textsuperscript{19,33,34} However, in some studies 5-FU has been shown to render no benefit to MMR deficient tumors, but because no clinical trial has demonstrated that there was no benefit from treatment of this group with FOLFOX, FOLFOX remains the standard of care.\textsuperscript{34} However, mechanistic arguments suggest that oxaliplatin would be particularly beneficial to patients with MMR deficient tumors because oxaliplatin forms platinum adducts with DNA, which cannot be repaired in MMR deficient tumors. One study has shown that stage III patients with deficient MMR had improved outcomes with FOLFOX over 5-FU, however, these were not randomized controlled trials and we are unaware of any data that supports this mechanistic argument.\textsuperscript{35} In our study oxaliplatin did not interact with MMR status.

As in previous studies we showed that MMR-deficient tumors and \textit{BRAF} mutations were associated with each other and with prognosis.\textsuperscript{4,6,8} MMR-deficient tumors were associated with a longer recurrence-free interval but showed a trend for poor survival after recurrence. The seeming reversal of MMR as a good prognostic factor based on recurrence to a bad prognostic factor based on SAR maybe the result of the association of \textit{BRAF} mutations with MMR tumors because \textit{BRAF}-mutant tumors were associated with a shorter SAR. These observations seem confusing but can be explained by speculating that MMR-deficient tumors are held in check by a strong immunological reaction associated with MMR-deficient tumors but is overcome by \textit{BRAF} mutations, which may allow for immune evasion as discussed below.\textsuperscript{36}

We and others have previously reported that \textit{BRAF} and \textit{KRAS} were largely mutually exclusive and \textit{PIK3CA} mutations were significantly associated with \textit{KRAS} mutations.\textsuperscript{17,24,37} The overrepresentation of \textit{PIK3CA} mutations in \textit{KRAS} mutant tumors suggests that \textit{PIK3CA} mutations may occur after \textit{KRAS} mutations and therefore later in the etiology of the tumor, which has been discussed previously.\textsuperscript{17}
Early research utilizing institution-based studies and meta-analyses have reported that KRAS and KRASG12V mutations were prognostic for colon cancer, respectively.\textsuperscript{12-14, 16,38} In agreement with these early studies are the results from two large clinical trials: the Quasar study (n=1583) and the MRC study, both of which reported significant association with KRAS for recurrence and OS, respectively. However, not all of these early studies found KRAS mutations to be prognostic.\textsuperscript{15} More recently, the PETACC trial, another large study utilizing tissue collected as part of a randomized clinical trial of stage II and III colon cancer, also did not find any prognostic significance for KRAS mutations when relapse-free survival and OS were used as end points.\textsuperscript{6} What accounts for these differences in the results of these well-powered clinical trials? One possible explanation is that the MRC focus study evaluated metastatic tumors and it is possible that KRAS mutations have a different impact on the survival of metastatic patients than on stage II and III patients, which has been reported previously.\textsuperscript{6,39} The Quasar study consisted largely of stage II colon cancer patients (91%) and included rectal cancers (28%) unlike our study and the PETACC study, which included a substantial number of stage III patients and did not include rectal cancers.\textsuperscript{16, 6} Thus it seems likely that KRAS mutations are not prognostic in stage II and III colon cancer. However, it will be of interest to determine whether KRAS mutations are associated with prognosis in rectal cancer.

We also did not find any prognostic value for PIK3CA mutations in stage II and III colon cancer in contrast to findings in other earlier studies.\textsuperscript{1-3} Reasons for the differences in the results between our study and these other studies are differences in the patient population, covariates included in the multivariate Cox model, and differences in the methodologies used to detect mutations. In addition, our study included mutations in PIK3CA in exons 1, 7, and 13, which were not included in the other studies and constituted 14.4% of the total PIK3CA mutations. A study published during the review of this paper has reported that concomitant PIK3CA mutations in exon 9 and 20 were associated with bad prognosis but this analysis, as the author points out, should be viewed with caution because there were only 7 patients with this genotype\textsuperscript{40} out of a total of more than 1170
colorectal cancers. We did not detect any double mutations of exon 9 and 20 in PIK3CA in our 2299 samples of colon cancer. The distinction between these two samples sets may be the presence of rectal cancer in the tumors in the Liao et al study whereas our study included only colon cancer. It should not be surprising that the mutation spectrum varies between colon and rectal cancers because the frequency of BRAF mutations varies within the colon depending whether the tumor is on the right or left side of the colon.

This study validates the results from previous studies that associated BRAF mutations with OS4-9 and shows that BRAF is associated with poor SAR in patients first diagnosed as stage II or III and is at least in part responsible for poor OS. The association of BRAF mutations with poor SAR is consistent with previous observations that have associated it with poor OS in metastatic patients.39 Further confirmation of BRAF’s association with OS and SAR were recently published during the review of this manuscript.8,41 BRAF tumors showed no significant relationship with recurrence even though they were associated with OS and SAR. Thus we could conclude that BRAF mutant tumors do not readily metastasize but once they have metastasized they rapidly lead to the death of the patient. This may help to explain the differences observed in the frequency of BRAF mutations found in metastatic patients compared to our study. Patients who have BRAF-mutant tumors have such a short survival after recurrence that few of these patients survive long enough to be recruited into metastatic studies. This would help explain the lower frequency of BRAF mutations (4.7%) in the De Rook study, which profiled tumors from chemo-refractory metastatic patients.23 However, if BRAF mutant tumors are unlikely to become metastatic what accounts for the very poor SAR of these tumors? BRAF mutations may associate with poor SAR due to resistance to systemic anti EGFR therapies that may have been given after recurrence. However, we would have anticipated that we would have also detected poor SAR for mutant KRAS tumors but we did not. Unfortunately, we are unable to test this possibility because post-recurrence-treatment information was not collected in C-07 or C-08. The older age of patients with BRAF mutant tumors could also have contributed to poor SAR because older patients may not have received as aggressive treatment as did
younger patients. We feel that this is unlikely to be the case because we did not find that age was a confounder for the association between *BRAF* and any of the endpoints. The estimates adjusted for age were very similar to those without adjustment (non-adjusted HR=2.31, adjusted HR=2.29). Another speculative possibility is that *BRAF* mutant tumors may have an altered immune response. MMR deficient tumors have been shown to be associated with a high density of cytotoxic (CD8+) T and memory (CD45RO+) T cells and may be responsible for the good prognosis associated with MMR deficient tumors.42-44 One of the roles of the activated BRAF/MAPK pathway is immune evasion by the suppression of immunosuppressive factors and inflammatory cytokines, such as IL-10.45 IL-10 has been shown to be required for optimal promotion and sustainment of T cell memory in a mouse model with IL-10 knock-out mice.42,45 However, if *BRAF*-mutant tumors have an altered interaction with memory T cell, it is not likely to be the result of a decrease in the density of these cells in the primary tumor because higher density of memory T cells (CD45R0) are also associated with *BRAF*-mutant tumors.43

All of the mutations screened in this study are possible resistance or sensitivity biomarkers that could aid in the selection of patients for clinically-approved targeted therapies. Recent evidence suggests that *KRAS*, *BRAF*, *PIK3CA*, and *NRAS* may render tumors non-responsive to anti-EGFR therapies. Currently, metastatic colon cancer patients with *KRAS* or *BRAF*-mutant tumors are not recommended for treatment with cetuximab due to their inherent resistance.23 In melanoma, *BRAF* mutations appear to be sensitivity markers for the *BRAF* inhibitor PLX4032, which has been shown to give a significant survival benefit in untreated *BRAF*V600E mutation-positive metastatic melanoma patients but results in colon cancer have not been as promising. However, given that *BRAF* mutations are associated with poor post-recurrence survival, and may confer resistance to EGFR targeted agents, there is a strong rationale to design colon cancer clinical trials that use new *BRAF* targeted therapies.46
New clinical trials are being developed to correlate the mutation status of tumors and their response to targeted therapies, in an effort to develop biomarkers that will identify those tumors that are sensitive or resistant to treatment. It is important to remember that approximately 35% to 40% of all stage II and III colon tumors did not have mutations in any of the 5 genes profiled here and may be good candidates for targeted therapies such as cetuximab. Conversely, mutations in these genes may also act as sensitivity markers for targeted therapies such as vemurafinib, PLX4032, and others that block the signaling pathways for which these genes are a part. Therefore, at a minimum, the mutation status of \textit{BRAF}, \textit{KRAS}, \textit{NRAS}, and \textit{PIK3CA} will need to be determined in clinical trials assessing targeted therapies that block the RAS-RAF-MAPK and PI3K-PTEN-AKT pathways. Such clinical trials are under development at NSABP.
Here are the corrected KPG refs for the C-07/9 Mutations paper

REFERENCES


32. Li WQ, Kawakami K, Ruszkiewicz A, Bennett G, Moore J, Iacopetta B. BRAF mutations are associated with distinctive clinical, pathological and molecular features of colorectal cancer independently of microsatellite instability status. Mol Cancer 2006; 5: 2.


### Table 1. Association of Mutations and MMR status with Clinical Variables

<table>
<thead>
<tr>
<th>Gene</th>
<th>T-Stage</th>
<th>Gender</th>
<th>Age</th>
<th>Nodal Status</th>
<th>MisMatch RepairStatus</th>
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<td>WT (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mutant (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<td>3.7%</td>
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*Abstr=250/250; Txt=4447/5000; Refs=46; Tbls=2; Figs=4; Supl Tbs=3*
*Probability calculated with Fisher's exact test. Unknown cases for MMR status were excluded from contingency tables. N0, N1, and N2 are tumors with 0, 1-3, and 4 or more positive lymph nodes, respectively.

<table>
<thead>
<tr>
<th>MMR Status</th>
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<th>0.79</th>
<th>0.81</th>
<th>0.15</th>
<th>0.06*</th>
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<td>0.25</td>
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<td>0.47</td>
<td>0.003</td>
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<td>0.06</td>
<td>0.47</td>
<td>0.003</td>
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<table>
<thead>
<tr>
<th>PIK3CA</th>
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<th>0.87*</th>
<th>0.79</th>
<th>0.81</th>
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<th>0.06*</th>
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<td>162</td>
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<td>118</td>
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<td>346</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
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<td>20.2%</td>
<td>24.4%</td>
<td>16.1%</td>
<td>20.4%</td>
<td>22.4%</td>
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</tr>
<tr>
<td>p</td>
<td>0.51</td>
<td>0.06</td>
<td></td>
<td>0.47</td>
<td></td>
<td>0.003</td>
</tr>
</tbody>
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Table 2. Interaction of Mutations and MMR Status with Oxaliplatin Treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Recurrence</th>
<th>Overall Survival</th>
<th>Survival after Recurrence</th>
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<tr>
<td></td>
<td>logHR</td>
<td>stder</td>
<td>p</td>
</tr>
<tr>
<td>BRAF mutated</td>
<td>0.260</td>
<td>0.247</td>
<td>0.292</td>
</tr>
<tr>
<td>KRASG12V mutated</td>
<td>0.094</td>
<td>0.300</td>
<td>0.755</td>
</tr>
<tr>
<td>KRAS mutated</td>
<td>0.040</td>
<td>0.191</td>
<td>0.832</td>
</tr>
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<td>MET mutated</td>
<td>-1.086</td>
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<td>MMR status</td>
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<td>0.424</td>
<td>0.971</td>
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<tr>
<td>NRAS mutated</td>
<td>0.054</td>
<td>0.486</td>
<td>0.912</td>
</tr>
<tr>
<td>PIK3CA mutated</td>
<td>0.099</td>
<td>0.244</td>
<td>0.685</td>
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</tbody>
</table>
Figure Legends

Figure 1. REMARK Diagram of Mutation Profiling of NSABP trials C-07 and C-08

Figure 2. Overlap of mutations in the BRAF, KRAS, NRAS and PIK3CA are shown. Each circle is drawn roughly to scale with the total number of cases for that specific mutation indicated n=. The size of the overlap between mutations is approximated with the specific numbers indicating the number of cases within the designated overlap. The total number of cases includes only cases for which data were available for all of the mutations.

Figure 3. Forest plot of mutations and MMR status and their association with recurrence, overall survival (OS) and survival after recurrence (SAR). Hazard rate >1 indicates detriment.

Figure 4. Kaplan Meier plots of BRAF and MMR status cases
A. Kaplan Meier plot of survival after recurrence of patients who have had a recurrence. Blue line = BRAF wt; black line = BRAF mutant.
B. Overall Survival of patients segregated by MMR and BRAF status.
   Key:
   MMR proficient BRAF wt = blue solid line
   MMR proficient BRAF mutant = black solid line
   MMR deficient t BRAF wt = blue dashed line
   MMR deficient BRAF mutant = black dashed line
Figure 1

**NSABP C-07**
Randomly assigned
N=2492

Group 1
5-FU + Leucovorin

Group 2
5-FU + Leucovorin + Oxaliplatin

Ineligible or no consent
no follow up
n=122

Clinically eligible with
follow-up
(n=2370)

No block or unsuccessful DNA preparation
n=534

With tumor blocks and DNA isolation
n=1836

Mutation profiling
with OncoCarta n= 235
with ColoCarta n=1601

**NSABP C-08**
Randomly assigned
N=2710

Group 1
mFLOXOX6

Group 2
Bevacizumab + mFLOXOX6

Ineligible or no consent
no follow up
n=278

Clinically eligible with
follow-up
(n=2432)

Defined representative cohort
n=500

Mutation profiling
with ColoCarta n=463

Missing blocks
or unsuccessful DNA isolation
n=47
KRAS mutated
n=750

PIK3CA mutated
n=371

BRAF mutated
n=281

NRAS mutated
n=73

Figure 2
Figure 3

**TIME TO RECURRENCE**

<table>
<thead>
<tr>
<th>Mutations</th>
<th>P</th>
<th>Hazard Ratio</th>
<th>95% Lower</th>
<th>95% Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>0.86</td>
<td>1.02</td>
<td>0.82</td>
<td>1.28</td>
</tr>
<tr>
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<td>1.60</td>
</tr>
<tr>
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<td>0.21</td>
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<td>0.94</td>
<td>1.32</td>
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<td>0.64</td>
<td>1.11</td>
<td>0.73</td>
<td>1.68</td>
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<td>NRAS</td>
<td>0.04</td>
<td>1.53</td>
<td>1.01</td>
<td>2.31</td>
</tr>
<tr>
<td>PIK3CA</td>
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<td>0.88</td>
<td>0.71</td>
<td>1.09</td>
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<tr>
<td>MMR status</td>
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<td>0.48</td>
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<td>0.70</td>
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**OVERALL SURVIVAL**

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<th>95% Upper</th>
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</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>0.0002</td>
<td>1.46</td>
<td>1.20</td>
<td>1.79</td>
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<tr>
<td>KRAS_G12V</td>
<td>0.49</td>
<td>1.11</td>
<td>0.83</td>
<td>1.47</td>
</tr>
<tr>
<td>KRAS</td>
<td>0.33</td>
<td>1.09</td>
<td>0.92</td>
<td>1.29</td>
</tr>
<tr>
<td>MET</td>
<td>0.40</td>
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<tr>
<td>NRAS</td>
<td>0.34</td>
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<tr>
<td>PIK3CA</td>
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<tr>
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**SURVIVAL AFTER RECURRENCE**

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<th>Hazard Ratio</th>
<th>95% Lower</th>
<th>95% Upper</th>
</tr>
</thead>
<tbody>
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<td>&lt;.0001</td>
<td>2.31</td>
<td>1.83</td>
<td>2.95</td>
</tr>
<tr>
<td>KRAS_G12V</td>
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<td>0.91</td>
<td>0.66</td>
<td>1.25</td>
</tr>
<tr>
<td>KRAS</td>
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<td>1.11</td>
<td>0.92</td>
<td>1.34</td>
</tr>
<tr>
<td>MET</td>
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<td>0.81</td>
<td>2.12</td>
</tr>
<tr>
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<td>0.40</td>
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</tr>
<tr>
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<td>1.23</td>
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</table>
Survival Probability

OS Time Yrs.

No. at risk

- MMR-p, BRAFwt: 1358, 1271, 1126, 830, 626, 100
- MMR-d, BRAFwt: 130, 123, 114, 88, 67, 15
- MMR-p, BRAFmut: 176, 145, 121, 93, 76, 11
- MMR-d, BRAFmut: 71, 61, 53, 45, 35, 3
Mutation Profiling and Microsatellite Instability in Stage II and III Colon Cancer: An Assessment of their Prognostic and Oxaliplatin Predictive Value


Clin Cancer Res  Published OnlineFirst October 8, 2012.

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