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

Patrick G. Gavin[1], **Linda H. Colangelo**[1,2,3], **Debora Fumagalli**[1], **Noriko Tanaka**[1,2,3], **Matthew Y. Remillard**[1], **Greg Yothers**[1,2,3], **Chungyeul Kim**[1], **Yusuke Taniyama**[1], **Seung Il Kim**[1], **Hyun Joo Choi**[1], **Nicole L. Blackmon**[1], **Corey Lipchik**[1], **Nicholas J. Petrelli**[1,5], **Michael J. O'Connell**[1], **Norman Wolmark**[1,4], **Soonmyung Paik**[1], and **Kay L. Pogue-Geile**[1]

[1]National Surgical Adjuvant Breast and Bowel Project (NSABP)

[2]NSABP Biostatistical Center

[3]Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh

[4]Allegheny Cancer Center at Allegheny General Hospital, Pittsburgh, Pennsylvania

[5]Helen F. Graham Cancer Center at Christiana Care, Newark, Delaware

Abstract

Purpose—The purpose of this study was to examine the prognostic and oxaliplatin predictive value of mismatch repair (MMR) status and common hot spot 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 2,299 stage II and III colon tumors from National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trials C-07 (n = 1,836) and C-08 (n = 463) with Type Plex 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% confidence interval (CI), 1.20–1.79; P S: 0.0002], and poor SAR (HR, 2.31; 95% CI, 1.83–2.95; P < 0.0001). 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 trials testing new targeted therapies that block relevant signaling pathways. Such clinical trials are under development at NSABP.

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 use. 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 bio-markers, we identified the common colon cancer mutations by profiling 187 different cancer mutations in 19 genes in a cohort of 235 cases from the NSABP C-07 trial, using Type Plex chemistry, mass spectrometry, and the OncoCarta Panel v1.0 (Sequenom). Twenty-four hot spot mutations in 5 genes (BRAF, KRAS, MET, NRAS, and PIK3CA) were found to occur in more than 1% of stage II and III colon cancer. These mutations were replexed into 6 new pools referred to as ColoCarta (Supplementary Table S1). This technology provided a cost-effective and sensitive method for the detection of hot spot mutations in routinely processed formalin-fixed, paraffin-embedded (FFPE) 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 were also tested.

Patients and Methods

Patient selection clinical samples

The National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 trial showed that the addition of oxaliplatin to 5-fluorouracil+ leucovorin (5-FU + LV) improved survival of patients with resected stage II and III colon cancer (18, 19). 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 the NSABP C-07 (n = 1,836) were used in the current study (REMARK Fig. 1). In addition, we conducted gene mutation profiling on a representative cohort of 500 cases from the 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) in the treatment of patients with resectable stage II and III colon cancer (20). 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

DNA from FFPE tumor blocks was prepared using an Ambion Recover-All kit or with the E.Z.N.A. FFPE DNA Isolation Kit from Omega Bio-Tek in a semi-automated method using the King Fisher Flex Instrument (Thermo-Fisher; [17]). DNAs were quantified with fluorescence, using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and the InfiniteF200 fluorometer (Tecan).

MassSpec 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 (17). All mutations were reviewed independently by 3 investigators, and all inconsistencies were reexamined and a consensus was reached. Reactions with more than 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 interrogated 187 mutations in 19 genes and was used to interrogate 235 C-07 cases (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 = 1,601) cases and the cases from the C-08 (n = 463) randomly selected cohort. On the basis of the Catalogue of Somatic Mutation in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/; COS-MIC) database, 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 4,628, 858, and 203 samples for BRAF, KRAS, and PIK3CA, respectively.

Roughly 20% of the PIK3CA mutations in the COSMIC database were the result of 8 mutations in PIK3CA that occurred only once. These 8 mutations were not represented in either the OncoCarta or the ColoCarta assays.

Immunohistochemistry

Antibodies for immunohistochemistry included anti-MSH2 (clone FE-11; 1:200; Calbiochem) and anti-MLH1 (clone G168-15; 1:30; BD Biosciences). 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 EnVisionþ System Kit (DAKO) according to the manufacturer's instructions. Mismatch repair (MMR) status was determined by staining results of MSH2 and MLH1. 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 nontumor 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 3 endpoints, patients without an event were censored at last follow-up.

Mutations in BRAF, KRAS, KRASG12V, NRAS, MET, and 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 BRAFV600E mutation was tested as a categorical variable using the frequency data for alleles. In this case, BRAFV600E mutations were split into 2 groups at a median cutoff with a frequency of mutant alleles of 0.16. Thus, cases were split into 3 groups: BRAFV600E wild-type, BRAFV600E low-frequency allele, and BRAFV600E high-frequency allele.

Mutations were tested for their association with age (<65, 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 exact test for tables with less than 5 patients in any category and the c^2 test for other covariates. Two-sided P values <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 endpoint and assuming a significance level of 0.05 if the corresponding 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 S2.

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 database (35.3% for KRAS, 12.4% for PIK3CA, and 14.3% for BRAF; ref. 21). Other infrequent mutations including NRAS (2.9%) and MET (3.7%) have also been described previously (Supplementary Table S3). 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 database (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 BRAF and KRAS mutations were almost mutually exclusive (P <0.0001; [22, 23]). Only 4 cases of 2,226 had mutations in both KRAS and BRAF. In Fig. 2, only 3 cases overlap because this figure includes only cases for which all mutations for all 4 genes were available. One of the cases with both NRAS and BRAF mutations had an assay failure for one of the other mutations and was eliminated from the figure. Likewise, only one tumor with an NRAS mutation had a BRAF mutation, which is similar to other reports that have found them to be mutually exclusive (24). Conversely, PIK3CA mutations were significantly associated with KRAS mutations (P < 0.0001) but PIK3CA did not show any significant association with BRAF. The overlap of the mutations in these 4 different genes is shown in Fig. 2.

Association of mutations and MMR status with clinical variables

In agreement with previous observations, 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; [6, 25–28]). MMR status was associated with KRAS (P = 0.001) and KRASG12V (P < 0.0001), as expected, as BRAF and KRAS were mutually

exclusive (6, 26). No mutations were associated with T stage or nodal status when corrected for multiple comparisons via Bonferroni, but 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[29]).

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% confidence interval (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 < 0.0001) but not for recurrence (HR, 1.02; 95% CI, 0.82–1.28; P = 0.86). The association of BRAF 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 shows 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 were 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 counterintuitive that BRAF mutations were associated with poor OS, SAR, and MMR-deficient tumors, even though MMR-deficient 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, Kaplan-Meier plots for each subgroup were generated (Fig. 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 with the patients with MMR-proficient tumor and BRAF-wt tumors, with a 5-year survival rate 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 with those with MMR-proficient tumors and BRAF-wt, with a 5-year survival rate of 69.1%. Patients with BRAF-wt, MMR-proficient or BRAF-mutant, MMR-deficient tumors had intermediate survival (P = 0.27 between those 2 groups) with 5-year survival rates of 82.1% and 84%, respectively (Fig. 4B). The increased HR for OS for patients with 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 show that BRAF and MMR status are prognostic and their effect is additive.

The Sequenom platform allows for the sensitive quantification of mutant alleles (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 BRAF mutations to determine whether the sensitivity of mutation detection provided a potentially meaningful clinical assessment of tumors. BRAF mutations were re-analyzed based on the amount of mutant BRAFV600E alleles within a tumor. All C-07 and C-08 cases were split into 3 groups: BRAFV600E wt, BRAFV600E-low-frequency allele, and BRAFV600E high-frequency allele. (The split for low- and high-frequency mutant alleles was at 0.16, which represented the median cutoff for tumors with BRAF mutations.) Patients with BRAFV600E-mutant tumors with less than 0.16 BRAF-mutant alleles were significantly associated with SAR (HR, 1.25; 95% CI, 1.60–3.18; $P < 0.0001$) and were marginally associated with OS (HR, 1.4; 95% CI, 1.4–1.87; $P = 0.022$). Thus, if 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.

Association of mutations and MMR with oxaliplatin benefit

Mutations have been identified as sensitivity and resistance markers to cancer therapies. Therefore, we tested the 6 mutations and MMR status for interaction with oxaliplatin benefit in C-07 on the 3 outcomes (recurrence, OS, and SAR). No significant interactions were seen between mutations (BRAF, 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 patients with stage II and III colon cancer in a large cohort consisting of all available and consented tissues from NSABP clinical trial C-07 and an are 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 used 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 (17). The mutation frequencies in this study (KRAS, 38.1%; PIK3CA, 20.2%; NRAS, 2.9%; and BRAF, 14.2%) were comparable with other studies for stage II and III colon cancer (1, 6, 10, 21, 32). Lower frequencies for PIK3CA (14%) and BRAF (7%) mutations have been reported previously (6, 21). 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 (30, 31, 32).

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 sample size.

FOLFOX has become the standard of care for patients with resected stage III and most stage II colon cancer based on NSABP trial C-07 and the MOSAIC trial (19, 33, 34). However, in some studies, 5-fluorouracil has been shown to render no benefit to MMR-deficient tumors, but because no clinical trial has shown that there was no benefit from treatment of this group with FOLFOX, FOLFOX remains the standard of care (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-fluorouracil; however, these were not randomized controlled trials, and we are unaware of any data that support this mechanistic argument (35). In our study, oxaliplatin did not interact with MMR status.

As in previous studies, we showed that MMR-deficient tumors and BRAF mutations were associated with each other and with prognosis (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 BRAF mutations with MMR tumors because 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 immunologic reaction associated with MMR-deficient tumors but are overcome by BRAF mutations, which may allow for immune evasion as discussed below (36).

We and others have previously reported that BRAF and KRAS were largely mutually exclusive and PIK3CA mutations were significantly associated with KRAS mutations (17, 24, 37). The overrepresentation of PIK3CA mutations in KRAS-mutant tumors suggests that PIK3CA mutations may occur after KRAS mutations and therefore later in the etiology of the tumor, which has been discussed previously (17).

Early research using institution-based studies and meta-analyses have reported that KRAS and KRASG12V mutations were prognostic for colon cancer, respectively (12–14, 16, 38). In agreement with these early studies are the results from 2 large clinical trials: the Quasar study (n = 1,583) 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 (15). More recently, the PETACC trial, another large study using 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 endpoints (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 (6, 39). The Quasar study consisted largely of patients with stage II colon cancer (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 (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 (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 article has reported that concomitant PIK3CA mutations in exons 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 (40) of a total of more than 1,170 colorectal cancers. We did not detect any double mutations of exons 9 and 20 in PIK3CA in our 2,299 samples of colon cancer. The distinction between these 2 samples sets may be the presence of rectal cancer in the tumors in the study of Liao and colleagues, 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 OS (4–9) 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 the association of BRAF with OS and SAR was recently published during the review of the 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 explain the differences observed in the frequency of BRAF mutations found in metastatic patients compared with 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 chemorefractory 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 (nonadjusted 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 interleukin (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 knockout 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 nonresponsive to anti-EGFR therapies. Currently, patients with metastatic colon cancer 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 BRAFV600E mutation-positive patients with metastatic melanoma, 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 vemurafenib, PLX4032, and others that block the signaling pathways for which these genes are a part. Therefore, at a minimum, the mutation status of BRAF, KRAS, NRAS, and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Ogino S, Nosho K, Kirkner GJ, Shima K, Irahara N, Kure S, et al. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. J Clin Oncol. 2009; 27:1477–1484. [PubMed: 19237633]

2. Fariña Sarasqueta A, Zeestraten E, van Wezel T, van Lijnschoten G, van Eijk R, Dekker J, et al. PIK3CA kinase domain mutation identifies a subgroup of stage III colon cancer patients with poor prognosis. Cell Oncol. 2001; 34:523–531.

3. Kato S, Iida S, Higuchi T, Ishikawa T, Takagi Y, Yasuno M, et al. PIK3CA mutation is predictive of poor survival in patients with colorectal cancer. Int J Cancer. 2007; 121:1771–1778. [PubMed: 17590872]

4. Samowitz WS, Sweeney C, Herrick J, Albertsen H, Levin TR, Murtaugh MA, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res. 2005; 65:6063–6069. [PubMed: 16024606]

5. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut. 2009; 58:90–96. [PubMed: 18832519]

6. Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol. 2010; 28:466–474. [PubMed: 20008640]

7. Fariña-Sarasqueta A, van Lijnschoten G, Moerland E, Creemers G-J, Lemmens VEPP, Rutten HJT, et al. The BRAF V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. Ann Oncol. 2010; 21:2396–2402. [PubMed: 20501503]

8. Ogino S, Shima K, Meyerhardt JA, McCleary NJ, Ng K, Hollis D, et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from Intergroup Trial CALGB 89803. Clin Cancer Res. 2012; 18:890–900. [PubMed: 22147942]

9. Saridaki Z, Papadatos-Pastos D, Tzardi M, Mavroudis D, Bairaktari E, Arvanity H, et al. BRAF mutations, microsatellite instability status and cyclin D1 expression predict metastatic colorectal patients' outcome. Br J Cancer. 2010; 102:1762–1768. [PubMed: 20485284]

10. Barault L, Charon-Barra C, Jooste V, de la Vega MF, Martin L, Roignot P, et al. Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. Cancer Res. 2008; 68:8541–8546. [PubMed: 18922929]

11. French AJ, Sargent DJ, Burgart LJ, Foster NR, Kabat BF, Goldberg R, et al. Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. Clin Cancer Res. 2008; 14:3408–3415. [PubMed: 18519771]

12. Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer. 2001; 85:692–696. [PubMed: 11531254]

13. Conlin A, Smith G, Carey FA, Wolf CR, Steele RJC. The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. Gut. 2005; 54:1283–1286. [PubMed: 15843421]

14. Lièvre A, Bachet J-B, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol. 2008; 26:374–379. [PubMed: 18202412]

15. Zauber NP, Wang C, Lee PS, Redondo TC, Bishop DT, Goel A. Ki-ras gene mutations, LOH of the APC and DCC genes, and microsatellite instability in primary colorectal carcinoma are not associated with micrometastases in pericolonic lymph nodes or with patients' survival. J Clin Pathol. 2004; 57:938–942. [PubMed: 15333653]

16. Hutchins G, Southward K, Handley K, Magill L, Beaumont C, Stahlschmidt J, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol. 2001; 29:1261–1270. [PubMed: 21383284]

17. Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer. 2010; 10:101. [PubMed: 20233444]

18. Kuebler JP, Wieand HS, O'Connell MJ, Smith RE, Colangelo LH, Yothers G, et al. Oxaliplatin combined with weekly bolus fluorouracil and leu- covorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. J Clin Oncol. 2007; 25:2198–2204. [PubMed: 17470851]

19. Yothers G, O'Connell MJ, Allegra CJ, Kuebler JP, Colangelo LH, Petrelli NJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. J Clin Oncol. 2011; 29:3768–3774. [PubMed: 21859995]

20. Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Colangelo LH, et al. Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP Protocol C-08. J Clin Oncol. 2011; 29:11–16. [PubMed: 20940184]

21. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, Clements J, et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet. 2008; Chapter 10(Unit 10):11. [PubMed: 18428421]

22. Lambrechts D, De Roock W, Prenen H, De Schutter J, Jacobs B, Biesmans B, et al. The role of KRAS, BRAF, NRAS, and PIK3CA mutations as markers of resistance to cetuximab in chemorefractory metastatic colorectal cancer. J Clin Oncol (Meeting Abstracts). 2009; 27(suppl): 15s. abstr 4020.

23. Simi L, Pratesi N, Vignoli M, Sestini R, Cianchi F, Valanzano R, et al. High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. Am J Clin Pathol. 2008; 130:247–253. [PubMed: 18628094]

24. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010; 11:753–762. [PubMed: 20619739]

25. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006; 38:787–793. [PubMed: 16804544]

26. Rozek LS, Herron CM, Greenson JK, Moreno V, Capella G, Rennert G, et al. Smoking, gender, and ethnicity predict somatic BRAF mutations in colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2010; 19:838–843. [PubMed: 20200438]

27. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology. 2005; 129:837–845. [PubMed: 16143123]

28. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature. 2002; 418:934. [PubMed: 12198537]

29. Elsaleh H, Cserni G, Iacopetta B. Extent of nodal involvement in stage III colorectal carcinoma: relationship to clinicopathologic variables and genetic alterations. Dis Colon Rectum. 2002; 45:1218–1222. [PubMed: 12352240]

30. Basu P, Chandna P, Bamezai RNK, Siddiqi M, Saranath D, Lear A, et al. MassARRAY spectrometry is more sensitive than pretect HPV-proofer and consensus PCR for type-specific

detection of high-risk oncogenic human papillomavirus genotypes in cervical cancer. J Clin Microbiol. 2011; 49:3537–3544. [PubMed: 21813716]

31. Querings S, Altmüller J, Anse'n S, Zander T, Seidel D, Gabler F, et al. Benchmarking of mutation diagnostics in clinical lung cancer specimens. PLoS One. 2011; 6:e19601. [PubMed: 21573178]

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 micro-satellite instability status. Mol Cancer. 2006; 5:2. [PubMed: 16403224]

33. Andrè T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med. 2004; 350:2343–2351. [PubMed: 15175436]

34. Andrè T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol. 2009; 27:3109–3116. [PubMed: 19451431]

35. Ng K, Schrag D. Microsatellite instability and adjuvant fluorouracil chemotherapy: a mismatch? J Clin Oncol. 2010; 28:3207–3210. [PubMed: 20498398]

36. Loddenkemper C, Nagorsen D, Zeitz M. Foxp3 and microsatellite stability phenotype in colorectal cancer. Gut. 2008; 57:725–726. [PubMed: 18477674]

37. Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, et al. Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res. 2002; 62:6451–6455. [PubMed: 12438234]

38. Barault L, Veyrie N, Jooste V, Lecorre D, Chapusot C, Ferraz J-M, et al. Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. Int J Cancer. 2008; 122:2255–2259. [PubMed: 18224685]

39. Richman SD, Seymour MT, Chambers P, Elliott F, Daly CL, Meade AM, et al. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. J Clin Oncol. 2009; 27:5931–5937. [PubMed: 19884549]

40. Liao X, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. Clin Cancer Res. 2012; 18:2257–2268. [PubMed: 22357840]

41. Popovici V, Budinska E, Tejpar S, Weinrich S, Estrella H, Hodgson G, et al. Identification of a poor-prognosis BRAF-mutant-like population of patients with colon cancer. J Clin Oncol. 2011; 30:1288–1295. [PubMed: 22393095]

42. Drescher K, Sharma P, Watson P, Gatalica Z, Thibodeau S, Lynch H. Lymphocyte recruitment into the tumor site is altered in patients with MSI-H colon cancer. Fam Cancer. 2009; 8:231–239. [PubMed: 19165625]

43. Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. J Pathol. 2010; 222:350–366. [PubMed: 20927778]

44. Mlecnik B, Bindea G, Pages F, Galon J. Tumor immunosurveillance in human cancers. Cancer Metastasis Rev. 2011; 30:5–12. [PubMed: 21249426]

45. Foulds KE, Rotte MJ, Seder RA. IL-10 is required for optimal CD8 T cell memory following listeria monocytogenes infection. J Immunol. 2006; 177:2565–2574. [PubMed: 16888018]

46. Flaherty KT, Hodi FS, Bastian BC. Mutation-driven drug development in melanoma. Curr Opin Oncol. 2010; 22:178–173. [PubMed: 20401974]

Translational Relevance

The prognostic and oxaliplatin predictive value of mismatch repair (MMR) and common hot spot mutations in stage II and III colon cancer using National Surgical Adjuvant Breast and Bowel Project clinical trials C-07 (n = 1,836) and C-08 (n = 463) was assessed. The findings of several other studies showing that BRAF mutations were associated with MMR and poor overall survival were validated and show that this is at least in part due to the association of BRAF with poor survival after recurrence. KRAS, PIK3CA, NRAS, and MET mutations were not significantly associated with prognosis (N = 2,299). None of the mutations was shown to be predictive for oxaliplatin benefit. Future novel clinical trials targeting BRAF are warranted because of its association with poor prognosis and resistance to anti-EGF receptor therapies, and profiling of all of these mutations will be necessary in future clinical trials using targeted therapies that inhibit RAS/RAF and AKT pathways.
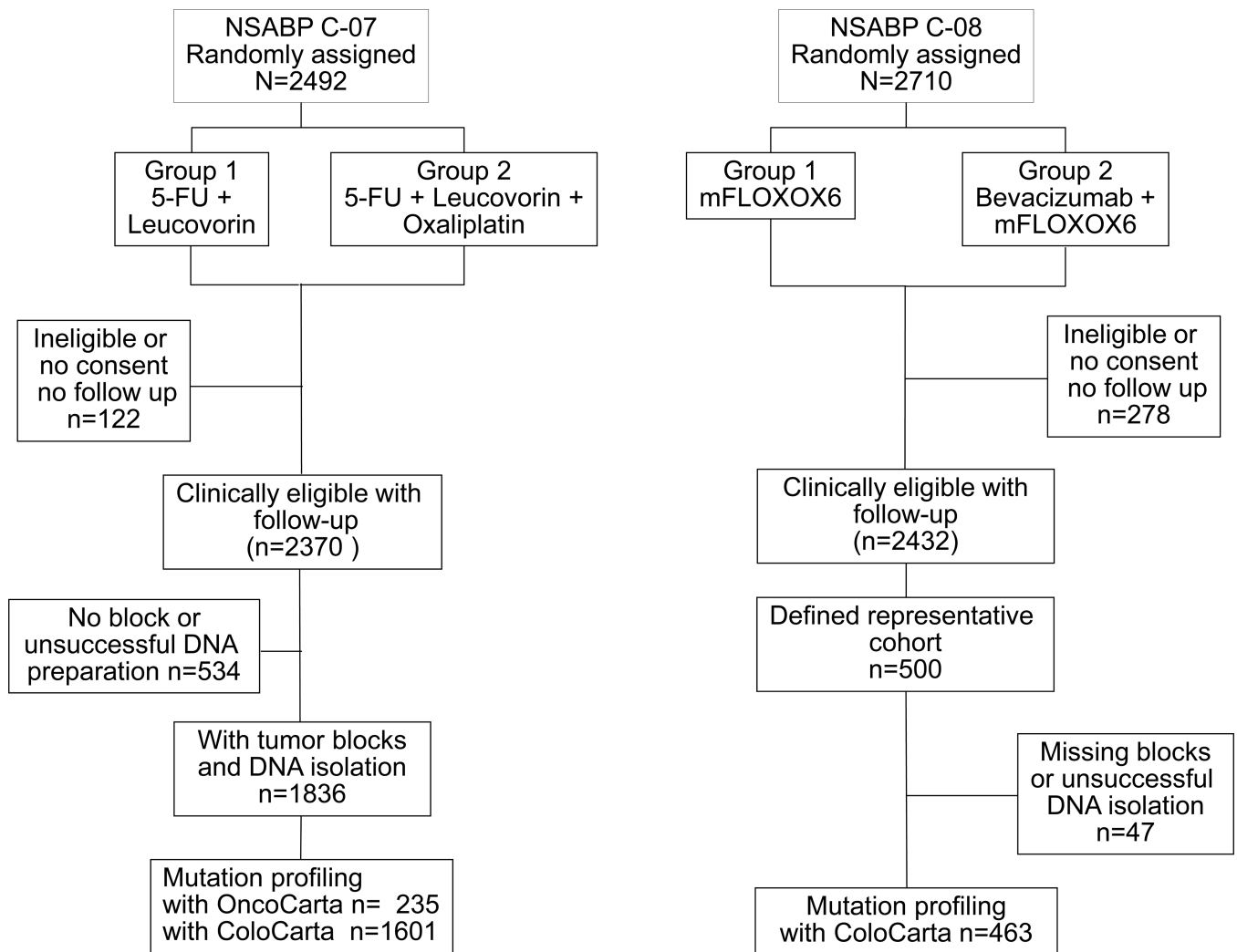
Figure 1.
REMARK diagram of mutation profiling of NSABP trials C-07 and C-08.
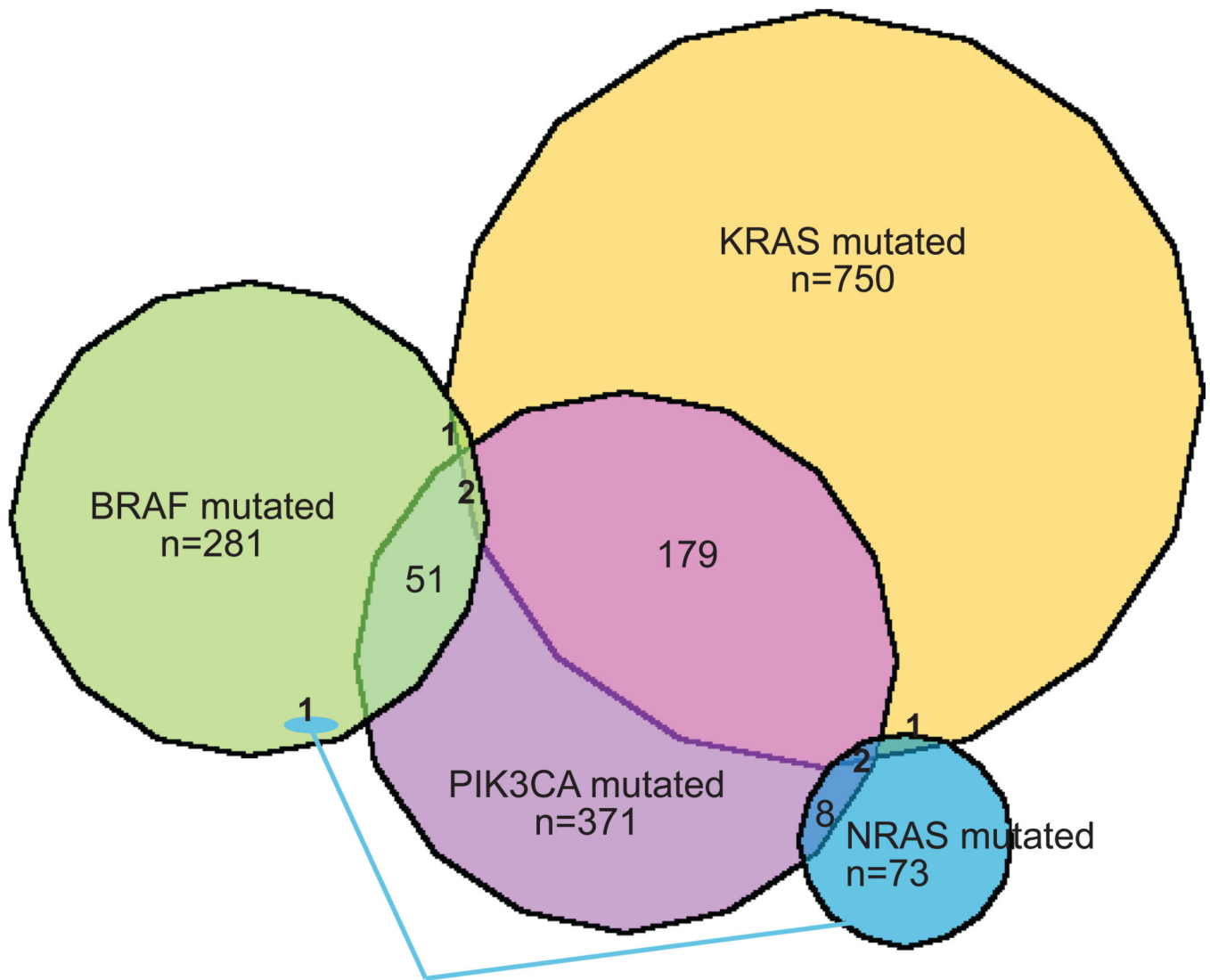
Figure 2.
Overlap of mutations in the BRAF, KRAS, NRAS, and PIK3CA are shown. Each circle is drawn roughly to scale with the total number of cases for that specific mutation indicated as n. The size of the overlap between mutations is approximated with the specific numbers indicating the number of cases within the designated overlap. The total number of cases includes only cases for which data were available for all of the mutations.

	P	Hazard Ratio	95% Lower	95% Upper	
TIME TO RECURRENCE					
BRAF	0.86	1.02	0.82	1.28	
KRAS_G12V	0.16	1.22	0.93	1.60	
KRAS	0.21	1.12	0.94	1.32	
MET	0.64	1.11	0.73	1.68	
NRAS	0.04	1.53	1.01	2.31	
PIK3CA	0.25	0.88	0.71	1.09	
MMR status	0.0001	0.48	0.33	0.70	
OVERALL SURVIVAL					
BRAF	0.0002	1.46	1.20	1.79	
KRAS_G12V	0.49	1.11	0.83	1.47	
KRAS	0.33	1.09	0.92	1.29	
MET	0.40	1.19	0.79	1.80	
NRAS	0.34	1.25	0.80	1.95	
PIK3CA	0.32	0.90	0.72	1.11	
MMR status	0.0084	0.64	0.46	0.89	
SURVIVAL AFTER RECURRENCE					
BRAF	<.0001	2.31	1.83	2.95	
KRAS_G12V	0.55	0.91	0.66	1.25	
KRAS	0.30	1.11	0.92	1.34	
MET	0.27	1.31	0.81	2.12	
NRAS	0.11	0.67	0.40	1.10	
PIK3CA	0.10	1.23	0.96	1.57	
MMR status	0.02	1.60	1.07	2.41	

Figure 3.
Forest plot of mutations and MMR status and their association with recurrence, OS, and SAR. HR > 1 indicates detriment.
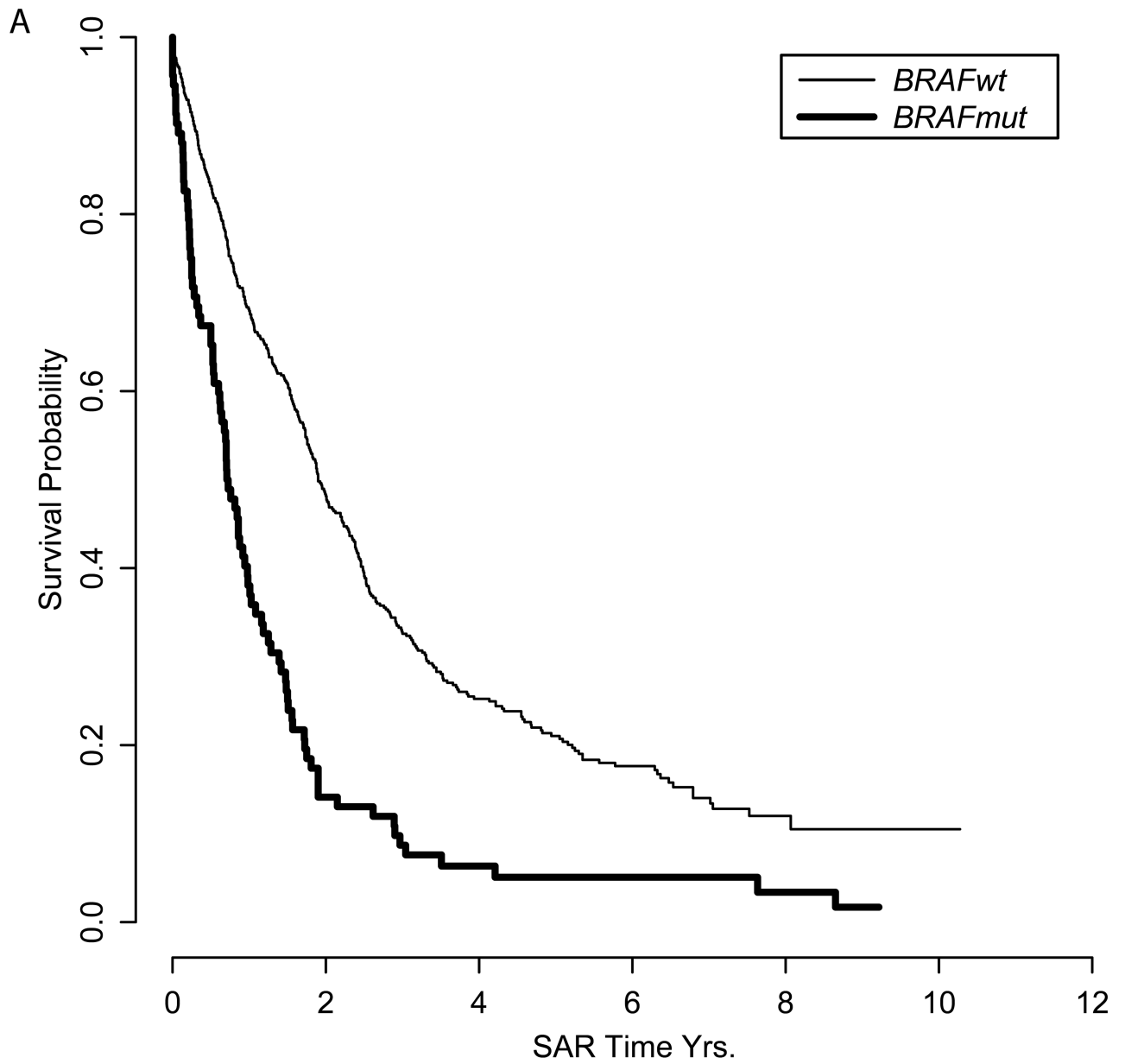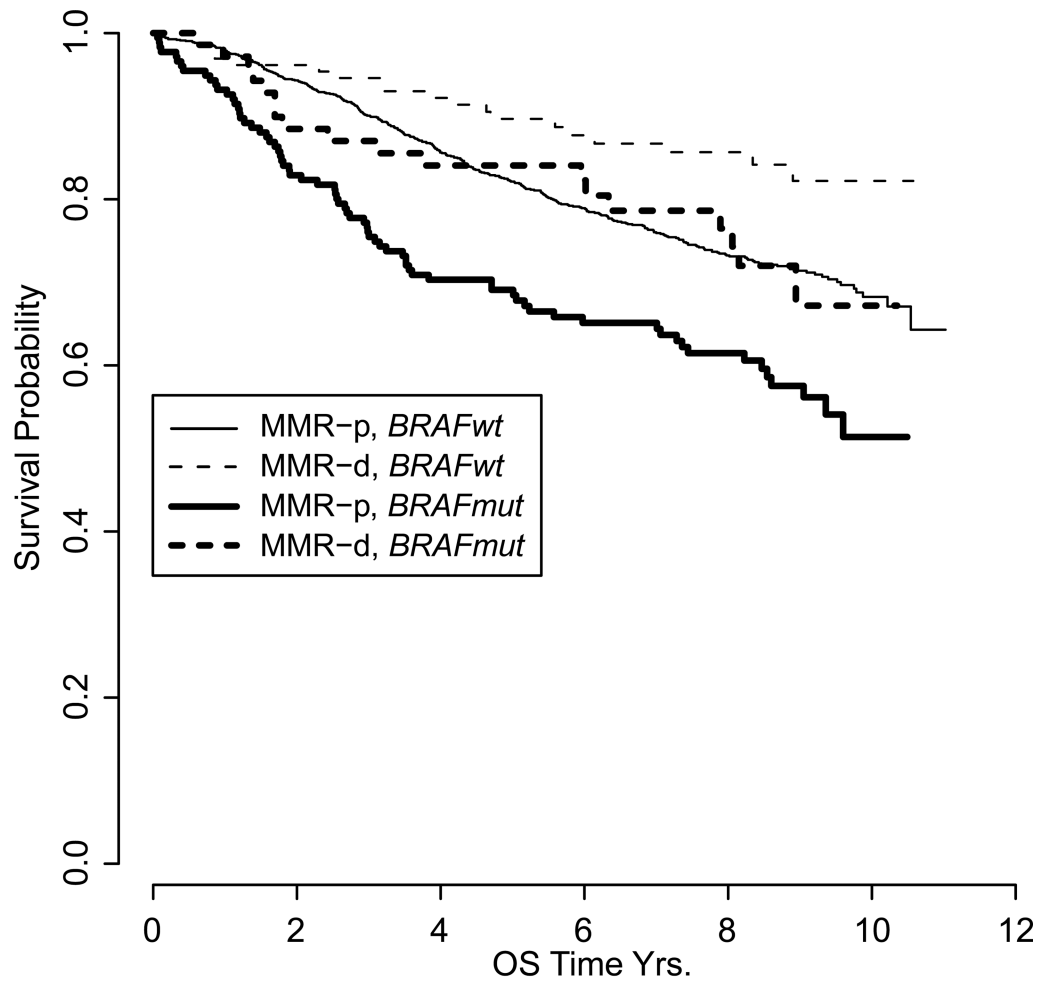
A

B

Figure 4.
Kaplan-Meier plots of BRAF and MMR status cases. A, Kaplan-Meier plot of SAR of patients who have had a recurrence. Thin line, BRAF-wt; thick line, BRAF-mutant. B, OS of patients segregated by MMR and BRAF status. Thin solid line, MMR-proficient (p) BRAF-wt; thick solid line, MMR-proficient BRAF-mutant; thin dashed line, MMR-deficient (d) BRAF-wt; thick dashed line, MMR-deficient BRAF-mutant.

Table 1

Association of mutations and MMR status with clinical variables

		T-Stage					Gender		Age		Nodal Status			MisMatch Repair Status		
	Total	T1	T2	T3	T4	Tx	female	male	<65	65	N0	N1	N2	unknown	Proficient	Deficient
BRAF																
WT (n)	1910	41	188	1552	128	1	812	1098	1372	538	534	894	482	422	1358	130
Mutant (n)	316	6	15	263	32	0	180	136	144	172	81	132	103	69	176	71
%	14.2%	12.8%	7.4%	14.5%	20.0%		18.1%	11.0%	9.5%	24.2%	13.2%	12.9%	17.6%		11.5%	35.3%
P				0.01			0.0001		<0.0001		0.02				<0.0001	
KRAS-G12V																
WT (n)	1976	39	181	1614	141	1	881	1095	1337	639	552	901	523	434	1354	188
Mutant (n)	182	4	17	145	16	0	74	108	129	53	51	90	41	41	138	3
%	8.4%	9.3%	8.6%	8.2%	10.2%		7.7%	9.0%	8.8%	7.7%	8.5%	9.1%	7.3%		9.2%	1.6%
P				0.80*			0.31		0.3736		0.47				0.0001[a]	
KRAS																
WT (n)	1288	26	125	1051	85	1	575	713	866	422	366	585	337	284	869	135
Mutant (n)	793	16	64	647	66	0	346	447	550	243	219	371	203	168	575	50
%	38.1%	38.1%	33.9%	38.1%	43.7%		37.6%	38.5%	38.8%	36.5%	37.4%	38.8%	37.6%		39.8%	27.0%
P				0.39			0.65		0.31		0.83				0.001	
MET																
WT (n)	2004	40	184	1635	144	1	883	1121	1363	641	571	914	519	435	1390	179
Mutant (n)	77	2	7	62	6	0	35	42	51	26	16	39	22	16	56	5
%	3.7%	4.8%	3.7%	3.7%	4.0%		3.8%	3.6%	3.6%	3.9%	2.7%	4.1%	4.1%		3.9%	2.7%
P				0.90[a]			0.81		0.74		0.33				0.54[a]	
NRAS																
WT (n)	1986	42	181	1617	145	1	875	1111	1351	635	560	912	514	426	1377	183
Mutant (n)	59	0	5	49	5	0	27	32	41	18	10	30	19	13	45	1
%	2.9%	0.0%	2.7%	2.9%	3.3%		3.0%	2.8%	2.9%	2.8%	1.8%	3.2%	3.6%		3.2%	0.5%
P				0.87[a]			0.79		0.81		0.15				0.06[a]	
PIK3CA																

	Total	T-Stage					Gender		Age		Nodal Status			MisMatch Repair Status		
		T1	T2	T3	T4	Tx	female	male	<65	65	N0	N1	N2	unknown	Proficient	Deficient
WT (n)	1669	34	162	1354	118	1	722	947	1128	541	444	770	455	379	1156	134
Mutant (n)	422	11	31	346	34	0	204	218	293	129	142	192	88	67	303	52
%	20.2%	24.4%	16.1%	20.4%	22.4%		22.0%	18.7%	20.6%	19.3%	24.2%	20.0%	16.2%		20.8%	28.0%
P		0.51					0.06		0.47		0.003			0.03		
MMR Status																
unknown	503.0	15	45	395	47	1	225	278	358	145	128	237	138			
Stable	1589.0	30	159	1309	91	0	698	891	1074	515	420	750	419			
Unstable	207.0	3	8	172	24	0	100	107	131	76	93	69	45			
%	11.5%	9.1%	4.8%	11.6%	20.9%		12.5%	10.7%	10.9%	12.9%	18.1%	8.4%	9.7%			
P		0.0005[a]					0.26		0.25		0.0001					

NOTE: 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.

[a] Probablity calculated with Fisher exact test.

Table 2

Interaction of mutations and MMR status with oxaliplatin treatment

Variable	Recurrence			OS			SAR		
	logHR	stder	P	logHR	stder	P	logHR	stder	P
BRAF mutated	0.260	0.247	0.292	0.376	0.220	0.088	−0.172	0.256	0.503
KRASG12V mutated	0.094	0.300	0.755	−0.098	0.305	0.748	−0.126	0.347	0.716
KRAS mutated	0.040	0.191	0.832	−0.060	0.185	0.745	0.086	0.213	0.685
MET mutated	−1.086	0.653	0.096	0.103	0.483	0.831	0.914	0.675	0.175
MMR status	0.015	0.424	0.971	0.071	0.369	0.848	0.041	0.477	0.931
NRAS mutated	0.054	0.486	0.912	0.153	0.486	0.753	−0.344	0.548	0.531
PIK3CA mutated	0.099	0.244	0.685	0.075	0.236	0.752	0.206	0.276	0.457