NIH Public Access
Author Manuscript
Clin Cancer Res. Author manuscript; available in PMC 2009 June 1.

Published in final edited form as:
Clin Cancer Res. 2008 June 1; 14(11): 3408–3415. doi:10.1158/1078-0432.CCR-07-1489.

Prognostic Significance of Defective Mismatch Repair and BRAF-V600E in Patients with Colon Cancer

Amy J. French[1], **Daniel J. Sargent**[2], **Lawrence J. Burgart**[3], **Nathan R. Foster**[2], **Brian F. Kabat**[2], **Richard Goldberg**[4], **Lois Shepherd**[5], **Harold E. Windschitl**[6], and **Stephen N. Thibodeau**[1]

1 *Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, Minnesota USA*

2 *Department of Health Sciences Research, Mayo Clinic College of Medicine, Rochester, Minnesota USA*

3 *Department of Pathology, Abbott Northwestern Hospital/Virginia Piper Cancer Institute, Minneapolis, Minnesota USA*

4 *Division of Hematology and Oncology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina USA*

5 *National Cancer Institute of Canada Clinical Trials Group, Queen's University, Kingston, Ontario, Canada*

6 *CentraCare Clinic, St. Cloud, Minnesota USA*

Abstract

Purpose—Colon tumors with defective DNA mismatch repair (dMMR) have a well characterized phenotype and accounts for ~15–20% of sporadic colon cancer (CC) as well as those colon cancer patients with Lynch Syndrome. Although the presence of dMMR appears to be a favorable prognostic marker, data suggests that these patients do not respond as well to adjuvant chemotherapy.

Experimental Design—In this study, we examined the prognostic significance of tumor MMR deficiency and the presence of a specific mutation in BRAF (V600E) in a group of patients (n=533) who participated in a randomized prospective clinical trial through the North Central Cancer Treatment Group (NCCTG).

Results—Tumors with dMMR were found to be associated with higher tumor grade (p=0.001), proximal location (p<0.0001), and improved overall and disease-free survival (p=0.049 and 0.04, respectively). Among all cases examined, evaluation of the BRAF V600E mutation status revealed no statistically significant differences in either disease-free or overall survival. Patients were then grouped in 4 categories for further analysis; dMMR/BRAF(−), dMMR/BRAF(+), pMMR/BRAF(−), and pMMR/BRAF(+). The dMMR/BRAF(−) group had a significantly improved overall survival (5-year overall survival of 100% vs. 73%, p=0.002) compared to all others. The remaining 3 groups had very similar survival outcomes. An additional cohort of tumors previously classified as having dMMR were also tested for the BRAF V600E alteration. Results remained significant (p=0.006) when the two groups were combined for analysis.

Conclusions—Overall, these data suggest that the underlying molecular etiology of those tumors having dMMR may influence the disease outcome in these patients.

Address correspondence to: Stephen N. Thibodeau, Ph.D., Mayo Clinic College of Medicine, 200 First Street SW, 920 Hilton Building, Rochester, MN 55905, Phone: 507-284-9185 Fax: 507-284-0670, E-mail: E-mail: sthibodeau@mayo.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Keywords

microsatellite instability; BRAF; defective DNA mismatch repair

Introduction

Colon cancer (CC) is one of the leading causes of cancer deaths worldwide. It is estimated that greater than 148,000 new cases of CC were reportedin the United States in 2006 (1). Rather than assuming that CC are homogenous, an assessment of prognosis based on features of the resected tumor would permit treating physicians to qualify the benefit of adjuvant chemotherapy to individual patients. Currently, anatomical and pathologic staging is still the most accurate predictor of patient outcome. It would be valuable to supplement standard clinical and pathologic staging using molecular markers to more precisely define the subset of patients at highest or lowest risk of relapse following colon cancer surgery. This would facilitate better selection of high-risk patients -- those who would benefit most from adjuvant therapy, and of low risk patients -- those with a lower likelihood of a treatment benefit who would instead potentially benefit from avoiding the toxicity, expense, inconvenience, and risks of adjuvant therapy. One of the most promising molecular markers investigated in CC to date is the presence of tumor microsatellite instability (2).

Based on the presence or absence of functional DNA mismatch repair (MMR), CC is generally divided into two broad categories (3,4). Tumors with defective MMR (dMMR) are characterized by the presence of a particular tumor phenotype, termed microsatellite instability (MSI), and by the absence of protein expression for any one of a number of genes involved in DNA mismatch repair, including *hMLH1, hMSH2, hMSH6, or PMS2* (5,6). Tumors with dMMR have been identified in approximately 15–20% of sporadic CC and in patients with Lynch Syndrome, a subset of hereditary non-polyposis colon cancer (7). In sporadic CC, three distinct MSI phenotypes have been described: MSS (none of the examined loci demonstrate instability), MSI-L (MSI at <30% of loci examined) and MSI-H (MSI at ≥30% of loci examined) (8). The MSI-H phenotype is associated with distinct clinicopathologic features, including proximal tumor site, high grade, early stage and diploidy (9–13). Importantly, this phenotype has been associated with a more favorable outcome (2). Among sporadic CC, the majority of MSI-H cases are due to inactivation of *hMLH1* (~95%), with *hMSH2* and *hMSH6* accounting for a smaller percentage, ~5% and <1% respectively (14). Germ-line mutations in these same mismatch repair genes are responsible for Lynch Syndrome, with *hMLH1* and *hMSH2* accounting for the majority of cases (approximately 40% each) and *hMSH6* and *PMS2* again accounting for a smaller percentage, ~10% and 5% respectively (15,16). Among all cases involving *hMSH2* and *hMSH6* (sporadic or inherited), the presence of a germline mutation appears to be the most common mechanism of gene inactivation. For *hMLH1*, however, current data suggests that the most common mechanism (~90% of cases) of gene inactivation among unselected cases is promoter hypermethylation and, less frequently, by mutations in the gene itself (14,17,18). Thus, the molecular etiology of those tumors involving dMMR is very heterogeneous, involving several different genes and numerous mechanisms of gene inactivation, including epigenetic, somatic and germline alterations.

The *Ras/Raf/MEK/MAP* kinase cascade is an essential component of intracellular signaling from activated cell-surface receptors to transcription factors in the cell nucleus. Mutations of the *Raf* activator *Ras* are present in 30% of human cancers (19,20) and their transforming potential is dependent on *Raf* (21). *BRAF* is one of three known *Raf* genes thought to have arisen from gene duplication (the other two are ARAF1 and CRAF). Davies et al. reported the presence of *BRAF* somatic mutations in 66% of malignant melanomas and at a lower frequency in a wide range of other human cancers, including colon cancers (22). *BRAF* mutations in CC

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

were then reported to occur more frequently in those cases characterized by the presence of dMMR (23). Although the etiology is still ill-defined, *BRAF* mutations were found in subsequent studies to occur almost exclusively in tumors demonstrating the involvement of *hMLH1* due to promoter hypermethylation. Current studies suggest that the *BRAF* V600E alteration occurs in ~10–15% of tumors that are proficient in the MMR pathway (MSS/MSI-L) and in ~70% of tumors that have dMMR (MSI-H) due to promoter hypermethylation of *hMLH1*. *BRAF* mutations rarely, if ever, occur in tumors with dMMR due to the presence of germline mutations (24,25). Thus, BRAF V600E is tightly associated with dMMR due to *hMLH1* promoter hypermethylation but not with dMMR due to germline alterations.

Given that the survival advantage reported in dMMR tumors are represented mostly by tumors with *hMLH1* promoter hypermethylation, the V600E mutation may provide additional predictive value regarding disease outcome. In this study we sought to determine if the *BRAF*-V600E alteration, in addition to the MMR status of the tumor, was able to better define patient outcome. For this analysis, we examined 533 tumors from patients classified as either high risk Stage II or Stage III colon cancer who participated in a randomized prospective clinical trial. The MMR status was defined by both immunohistochemistry staining of paraffin tissue coupled with testing for microsatellite instability. Tumor was then examined for the V600E mutation within the BRAF gene.

Materials and Methods

Patient population

Paraffin-embedded tissue was obtained from patients enrolled onto a Phase III randomized trial (Intergroup 0135/NCCTG 91-46-53/NCIC CTG CO.9) which evaluated high vs. standard dose levamisole when combined with 5-flouruoracil (5-FU) and leucovorin as adjuvant therapy following en bloc resection of colon cancer. To be enrolled in the clinical trial, patients were required to be at high risk for tumor recurrence following surgery, as indicated by one or more of the following features of the primary colon cancer: 1) regional lymph node metastases; or 2) transmural tumor involvement through the serosa; 3) pericolonic fat invasion and either tumor perforation, adherence to or invasion of adjacent organ(s); or 4) central laboratory flow cytometry determination of either non-diploid or proliferation index (%s-phase +%g2m) >0.20 (26).. Patients with Stage II lesions were eligible if associated with any of the two criteria noted above. Patients with distant metastases were excluded. A total of 878 patients were enrolled onto this trial. At the time the molecular study was conducted, tissue blocks were available from 562 patients, 533 (95%) of which had a sufficient amount of tumor for analysis. This sample represents a non-random subset of the population from which it was drawn. This molecular correlative study was reviewed and approved by local Institutional Review Boards.

In an independent validation set, paraffin-embedded tumors that had previously been characterized as having defective mismatch repair were obtained for further analysis (27). This prior study included patients from seven different NCCTG Phase III clinical trials investigating surgical adjuvant chemotherapy in Stage II and Stage III patients having either en bloc resection of colon or rectal carcinoma as previously described. For details of treatments used and the follow-up protocol, see Halling et al. (27).

Tissue selection and DNA extraction

Paraffin blocks were serially cut into 5 or 10 micron thick sections. Slide 6 (of 20) was stained with hematoxilyn and eosin and areas of neoplastic (>50%) and normal tissue were identified by a pathologist. The areas containing marked normal and tumor tissue from slides one through five were scraped and placed into separate tubes for DNA extraction using the QIAamp Tissue Kit (QIAgen, Valencia, California) according to the manufacturers instructions.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Immunohistochemistry (IHC)

Slides seven through nine were selected for immunohistochemical staining with antibodies to hMLH1, hMSH2, and hMSH6 as previously described (28). When needed, additional testing for PMS2 protein expression was performed as previously described (29).

Microsatellite Instability (MSI) Testing

PCR for the various microsatellite markers was carried out on matched tumor and normal DNA for each of the patients studied. Standard PCR conditions were used and included 10x buffer type II, taq gold, and dNTP's. Primers were custom ordered with various fluorescent dyes from Applied Biosystems (Applied Biosystems, Foster City, California). PCR product was analyzed on an ABI 3100 (Applied Biosystems, Foster City, California).

Mismatch Repair Definition

Defective mismatch repair (dMMR) was defined by the presence of microsatellite instability at the marker BAT 26 coupled with absence of protein expression for *hMLH1*, *hMSH2*, or *hMSH6*. For patients that were stable at BAT 26 and showed normal protein expression for all 3 proteins, 16 additional microsatellite markers (myc-L, D8S262, D8S1742, D8S261, D8S254, D8S133, D8S136, D8S560, D8S1055, D8S1820, D8S255, ANK1, D8S1760, D8S1720, D8S1842, D8S1925) were used to test for MSI. If >30% of the markers demonstrated MSI, then that tumor was classified as MSI-H and additional IHC testing was performed with antibody for PMS2. These were also defined as having dMMR.

BRAF testing

Testing for the *BRAF* – V600E mutation in exon 15 was performed by conformation sensitive gel electrophoresis (CSGE) analysis (30), DNA sequencing, or both for the 533 tumors that were entered onto this study, regardless of the MMR status. PCR primers for this assay (available upon request) were custom ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, Iowa). Standard PCR conditions were used and included 10x buffer type II, taq gold, and dNTP's from Applied Biosystems (Applied Biosystems, Foster City, California). For the CSGE analysis, PCR product was denatured at 95 C for 5 minutes and cooled to 65 C over 30 minutes. The reannealed product was mixed with loading dye (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol FF) and then loaded onto a CSGE gel consisting of 15% acrylamide/1,4-bis(acrollyl)piperazine (19:1), 0.5XTTE buffer (44.4mM TRIS, 14.25 mM Taurine, and 0.1 mMEDTA, pH 9.0), 15% formamide, and 10% ethylene glycol. The gel was run at 30W for 5 hours. DNA sequence analysis was performed on all samples that demonstrated an abnormally migrating fragment by CSGE, all ambiguous CSGE results, and all cases that were classified as having dMMR tumors by MSI and IHC testing of tumor tissue.

Statistical Methods

Overall Survival (OS; censored at 8 years) was calculated as the number of days from random assignment to the date of death or last contact. Disease-Free Survival (DFS; censored at 5 yr) was calculated as the number of days from random assignment to the date of disease recurrence or death. The distributions of OS and DFS were estimated using Kaplan-Meier (31) methodology. Univariate and multivariate Cox proportional hazards models were used to explore the association of clinical and laboratory parameters with OS and DFS (32). The Score statistic was used to test for significance in univariate models. The Likelihood-ratio test was used to test for the significance of a single covariate in the presence of, or adjusting for, other covariate(s) in multivariate models.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Laboratory parameters (e.g., MMR status, BRAF) were correlated with clinical characteristics (e.g., stage, grade). Consistent with previous practice (27), MSI-L and MSS patients were grouped together and classified as pMMR for the purposes of this analysis. Pooling of this data was confirmed in this dataset as there were no statistically significant differences between the MSI-L and MSS patient groups for OS or DFS. Summary statistics (e.g., mean, median) and frequency tables were used to describe the distributions of parameters investigated. Appropriate statistical tests were used to test for differences in the distributions of continuous and categorical variables (e.g., t-test, Wilcoxon, Chi-square, Fisher's Exact tests). All statistical tests were 2-sided and a p-value of ≤ 0.05 was considered statistically significant. P-values were not adjusted for multiple comparisons. Statistical analyses were performed via SAS software (SAS Institute, Cary, North Carolina).

Results

Of the 562 patients enrolled onto this study, 17 were eliminated due to the absence of usable tumor in available tissue. An additional 12 patients had tumor present, but the tumor percentage was lower than the established threshold for reliable MSI testing. The remaining 533 patients were evaluated by IHC for the presence or absence of hMLH1, hMSH2, and hMSH6 and for MSI with the mononucleotide repeat marker BAT 26. Fifty-seven cases showed both an absence of protein expression for at least one of the MMR genes and demonstrated MSI at BAT 26, and these were classified as having defective mismatch repair (dMMR). Of the 57 cases with dMMR, 44 had an absence of protein expression for hMLH1, 3 for hMLH1/hMSH6, 9 for hMSH2/hMSH6 and 1 for hMSH6 alone. Four additional cases with dMMR were also identified. Two of these each had an absence of protein expression for one of the MMR genes (one for hMLH1 and one for hMSH2/hSMH6), but due to poor quality DNA in the extracted specimen or a lack of sufficient tumor area for microdissection, no MSI testing was possible. These two patients were used for the MMR analyses, but were not included in the *BRAF* portion of this study as no DNA was available. In a third case, tumor was positive for instability at BAT 26, but had normal expression for all three proteins. After additional testing, this case was shown to have an absence of PMS2 staining. The final case demonstrated normal protein expression for all four MMR proteins tested, was negative for BAT 26 instability, but subsequently demonstrated instability at 15 of 16 (94%) microsatellite markers that were additionally tested. Thus, 61 cases (11%) were classified as having dMMR and 472 (89%) were classified as having proficient MMR (pMMR).

Patient and tumor characteristics with respect to the MMR status are shown in Table 1. Overall, tumors with dMMR were more likely to be high grade (P=0.001) and more frequently located on the proximal side of the colon (P<0.0001) (Table 1). There were no statistically significant differences in age, gender or stage.

Testing for the V600E mutation in the *BRAF* gene was performed on tumor DNA from 533 patients but informative results were obtained for only 490. The DNA from the missing 43 samples was not of sufficient quality for the CSGE and DNA sequence analysis. Patient and tumor characteristics with respect to both the MMR and *BRAF*-V600E status are shown in Table 2. Of the 490 cases where both the MMR and *BRAF* mutation status was defined, 23 (4.7%) cases with dMMR were *BRAF*(−) and 35 (7.1%) were *BRAF*(+), while 390 (79.6%) cases with pMMR were *BRAF*(−) and 42 (8.6%) were *BRAF*(+). Among these four groups, significant differences were observed for Age (*BRAF*(−)/dMMR cases more likely to have a younger age of diagnosis, p=0.0015), Gender (*BRAF*(+)/dMMR cases more likely to be female, p=0.0204), Grade (*BRAF*(−)/pMMR cases more likely to have lower grade disease, p<0.0001) and Site (*BRAF*(−) and *BRAF*(+)/dMMR cases more likely to be located in the proximal colon, p<0.0001). Overall, no differences were noted in the stage distribution among these four groups. The fraction of Stage II cases in all groups was ~25%.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

In univariate survival analysis (Table 3), significantly improved DFS ($p<0.001$) and OS ($p<0.001$) were observed for Stage (II vs. III). In addition, dMMR patients were found to have significantly improved DFS ($p=0.04$) and OS ($p=0.049$) compared to pMMR patients. For the MMR status, the DFS and OS rates were 82% and 87%, respectively, in patients whose tumors exhibited dMMR, compared to 67% and 72%, respectively, in patients with pMMR tumors. This improved DFS and OS did not depend on stage or grade (interaction p-values ≥ 0.50). Finally, no significant differences were found with respect to *BRAF* mutations for either DFS or OS when the entire population was evaluated ($p\geq 0.31$; Table 3).

Additional analyses were then performed taking into account both the MMR and *BRAF* status as shown in Table 2. Of the four groups examined, patients with dMMR/*BRAF*(−) tumors had significantly improved OS (*P*=0.002; Figure 1B, Table 3), but not DFS (P=0.06; Figure 1A, Table 3) compared to the other 3 patient groups combined. In addition, within the subgroup of patients with dMMR tumors, the 23 patients with BRAF(−) tumors had significantly better OS than the 35 BRAF(+) patients (100% vs. 77%, p=0.001); no difference was observed by BRAF status in the 432 pMMR patients for DFS or OS ($p\geq 0.57$). Thus, the *BRAF* mutation status appears to be important in the group of cases having dMMR but not in the group of patients whose tumors had pMMR. The lack of deaths in the dMMR/BRAF(−) group prevented any multivariate modeling to adjust for other covariates, including age. However, we completed a sensitivity analysis for OS and found that the results remained significant for dMMR/BRAF negative patients vs. all others and vs. only the dMMR/BRAF positive patients, even after we used a model that presumed up to 3 hypothetical patient deaths in this cohort; both in univariate models (p=0.042) and in limited multivariate models adjusting individually for age (p=0.03), stage (p=0.01), and tumor grade (p=0.01). Although we did not censor deaths as part of the statistical analysis, a review of the medical records was performed to determine cause of death: 8 subjects died of cancer, 4 subjects died of other causes (no evidence of disease) and in 1 case the cause of death was not documented.

Overall, our results suggest that the BRAF mutation status may provide additional prognostic information among those cases that have dMMR. Because of the limited sample size in the initial analysis, however, *BRAF* testing was performed on a second set of cases (n=76) previously identified with dMMR (27). Of the 76 patients, 48 were found to be positive for the *BRAF* – V600E alteration. Although no statistically significant differences in DFS and OS were observed between tumors with and without BRAF mutations individually in this validation cohort, the results demonstrated a trend toward improved survival for the BRAF negative group, similar to that found in the initial analysis. When both groups of patients were combined (n=134), a significant difference in OS was retained (*P*=0.006), with patients with dMMR tumors without a BRAF mutation demonstrating improved survival compared to those with a BRAF mutation (Figure 2B, Table 4).

Discussion

The presence of defective DNA mismatch repair, as assessed by the presence of tumor microsatellite instability (specifically the MSI-H phenotype), continues to be one of the most consistent and promising molecular markers of prognosis for colon cancer (2). In this study, we show that dMMR (MSI-H phenotype) provides prognostic information with a magnitude of effect consistent with that previously observed (approximately 15% increase in 5 year DFS and OS, p=0.04 and 0.049 respectively) (2,27,33). Furthermore, this phenotype may also be important in predicting treatment effects (33). Ribic et al. (33) suggested that patients whose tumors are characterized by the presence of dMMR may be adversely effected when treated with a 5-FU based regimen. However, given that the molecular etiology of those tumors involving dMMR is very heterogeneous (different genes and different mechanisms of gene

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

inactivation-epigenetic, somatic and germline alterations), it is very likely to be the case that not all of these tumors will behave in a similar fashion.

The presence of tumor heterogeneity led us to explore the utility of additional markers, specifically, the presence or absence of the V600E alteration in the *BRAF* proto-oncogene. As noted earlier, *BRAF* V600E is tightly associated with dMMR due to *hMLH1* promoter hypermethylation but not with dMMR due to germline alterations. The *BRAF* V600E alteration occurs in ~10% of tumors that are MSS/MSI-L and in ~70% of tumors that are MSI-H with hMLH1 promoter hypermethylation. *BRAF* mutations have not been found in tumors that are MSI-H due to the presence of germline mutations in *hMLH1*, *hMSH2* or *hMSH6* (24,25). Additionally, both *hMLH1* methylation-associated microsatellite instability and the presence of the *BRAF* V600E alteration has also been strongly associated with tumors that have the CpG Island Methylator Phenotype (CIMP) phenotype (24,25,34,35). Since *hMLH1* methylation-associated microsatellite instability generally does not occur among sporadic cases outside the context of CIMP, it appears that the underlying basis for mismatch repair deficiency among this select group of sporadic colon cancer is a broader epigenetic control defect that affects *hMLH1* in some, but not all CIMP tumors. Furthermore, the CIMP phenotype appears to also underlie nearly all tumors characterized by the presence of the *BRAF* V600E alteration. Cumulatively, these data highlight the heterogeneity of CCs. Understanding these differences will be critical for optimal patient care and the BRAFV600E may provide a useful marker representing a unique subset of CC.

With this information in mind, the clinical significance of the *BRAF* V600E alteration was examined across the entire group of patients and among subsets defined by the MMR status **(**Table 2). Among the groups examined, *BRAF* V600E provided additional prognostic information only for the subset of patients having dMMR. Among patients with dMMR, BRAF (−) cases had a significantly improved OS compared to those that were BRAF(+) (p=0.001). There were no differences in DFS or OS among cases whose tumors were proficient in MMR (p=0.57 and 0.59, respectively). When all four groups were compared, patients whose tumors were BRAF(−)/dMMR continued to show significantly improved OS (p=0.002).

Because the sample size among the various subsets with dMMR was small, we identified an additional cohort of patients with known dMMR status as a second validation set (27). When examined, the results of the BRAF testing showed a similar trend in overall survival, but the results were not statistically significant. There may be a number of reasons for this difference. First, the average age of patients with dMMR within the validation cohort is older (median age 67.5, range 39–86) compared to the original cohort (median age 63.0, range 30–85), suggesting a possible difference in the proportion of germline versus sporadic cases within each group. Second, the validation cohort of patients was obtained from several different clinical trials that accrued from 1978 to 1989. The treatment and care of patients has improved over the years, potentially affecting our ability to observe a BRAF effect in earlier trials. Surgical techniques have also improved over the last 20 years, which might impact survival; descriptively comparing outcomes of stage matched patients, our more recent cohort had improved OS compared to the previous cohort (5 yr OS was 87% and 69% for stage II and stage III patients, respectively, compared to rates of 81% and 58%, respectively, in the previous cohort). The most likely explanation, however, is that our original group of patients with dMMR likely over-represent cases for improved survival and the previous group might under-represent cases for improved survival. Upon combining the two studies, the BRAF status among patients with dMMR was still significantly associated with overall survival, with improved survival in the dMMR/BRAF(−) group (p=0.006).

The clinical significance of *BRAF* V600E in colon cancer has been examined in only one other study (36). In contrast to our current study, Samowitz et al. demonstrated that microsatellite-

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

unstable tumors (dMMR) were associated with an excellent 5-year survival regardless of the V600E status. However, these investigators also report that the V600E mutation was associated with poor survival among the microsatellite stable tumors (pMMR) (36). We found no difference in this subset of patients. Although the discrepancies between these two studies are perplexing, there are some differences in the two study populations. Our study was confined to high-risk Stage II and Stage III disease, while all four stages were represented in the study by Samowitz et al. The number of *BRAF* positive cases among the pMMR cases in the series reported by Samowitz was approximately half of what was observed in this study (40/803, 5% vs. 42/432, 10%, respectively). This apparent difference may again be due to the stage differences between the 2 studies. In the study reported by Samowitz et al (36), *BRAF* V600E was found to be highly correlated with stage, with higher frequency of this mutation found in cases with higher stage. The presence of Stage I and low-risk Stage II cases in the study by Samowitz et al. would dilute the overall number of cases showing the *BRAF* alteration (they report only 7 mutations among 400 cases in these two groups). Finally, participants of the study by Samowitz et al. represented different racial groups and they were not in a controlled clinical trial setting. Since there are differences between these studies, it is difficult to compare results directly. Clearly, additional studies with larger patient groups are warranted.

Among our cases with BRAF(−)/dMMR tumors (n=23), the age at diagnosis was nearly 10 years younger than the other 3 groups (median age of 52, range 30–72, p=0.0015). This data suggest that this group is composed of a higher fraction of germline cases. This is consistent with both the fact that germline cases (Lynch Syndrome) have a younger age of onset (37) and that BRAF mutations are associated with hypermethylation of the hMLH1 promoter but not germline derived dMMR (24,25,34,38,39). Of the ten patients that had an absence of protein expression for either hMSH2 (n=9) or hMSH6 (n=1), all were negative for a BRAF mutation. Absence of hMSH2 or hMSH6 is almost always associated with a germline event. Of the 12 that have an absence of protein expression for hMLH1 in the BRAF(−)/dMMR group, some of these are also likely to be germline. Given the young age at diagnosis for a substantial fraction of the BRAF(−)/dMMR cases, the role of age in our findings must be considered. Survival may be different between the two groups for a variety of reasons. Older subjects may have more co-morbidity and may be more likely to die from unrelated causes. Additionally, it may be that the adverse effects chemotherapy described by Ribic et al (ref) may apply to subjects with sporadic MSI-H CRC but not HNPCC (which may benefit from chemotherapy). At this point, there is essentially no data in the literature that helps to address these issues. Fortunately for the patients but unfortunately for our analysis, the complete absence of any deaths in this group of patients prohibited multivariate modeling to adjust for age. However, our sensitivity analyses, where 3 hypothetical events were added to the BRAF negative/dMMR patient group, suggests that these results would be maintained were a true multivariate analysis possible.

Colon cancer with defective DNA MMR (MSI-H) is recognized to be heterogeneous. Thus, even for this rather defined group, it would still be valuable to identify additional prognostic markers. Given that *BRAF* V600E mutation occurs at a high frequency in this group, and has the ability to help distinguish between inherited from sporadic cases, this marker provides a potential approach to more precisely define the subset of patients at highest or lowest risk of relapse following colon cancer surgery. Overall, our study showed that the concomitant evaluation of both the MMR status and BRAF mutation status in colon cancer provides useful prognostic information beyond evaluation of either factor alone. Patients in whose tumors are BRAF(−)/dMMR have a significantly improved overall survival. However, given the difficulty in obtaining a sufficiently large number of patients with this tumor phenotype, additional confirmatory studies pooling specimens and data from multiple trials will be required to confirm these findings. It appears likely that over time, with further research, our understanding of the taxonomy of colon cancer will become clearer and more complex as we unravel the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

importance molecular markers of tumor heterogeneity and the relevance of these markers to patient management.

Acknowledgements

This study was supported by grants from the Public Health Service, National Institutes of Health (CA 25224 and CA 60117).

Bibliography

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30. [PubMed: 16514137]
2. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609–18. [PubMed: 15659508]
3. Lengauer C, Kinzler K, Vogelstein B. Genetic instability in colorectal cancers. Nature 1997;386:623–7. [PubMed: 9121588]
4. Loeb LA. Microsatellite instability: Marker of a mutator phenotype in cancer. Cancer Res 1994;54:5059–63. [PubMed: 7923117]
5. Peltomaki P. Deficient DNA mismatch repair: A common etiologic factor for colon cancer. Hum Mol Genet 2001;10:735–40. [PubMed: 11257106]
6. Aaltonen LA, Salovaara R, Kristo P, et al. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med 1998;338:1481–7. [PubMed: 9593786]
7. Marra G, Boland CR. Hereditary nonpolyposis colorectal cancer: The syndrome, the genes, and historical perspectives. J Natl Cancer Inst 1995;87:1114–25. [PubMed: 7674315]
8. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: Development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–57. [PubMed: 9823339]
9. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Rishel R, Ruschoff J. Diagnostic microsatellite instability: Definition and correlation with mismatch repair protein expression. Cancer Res 1997;57:4749–56. [PubMed: 9354436]
10. Kim H, Jen J, Vogelstein B, Hamilton SR. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. Am J Pathol 1994;145:148–56. [PubMed: 8030745]
11. Ruschoff J, Dietmaier W, Luttges J, et al. Poorly differentiated colonic adenocarcinoma, medullary type: Clinical, phenotypic, and molecular characteristics. Am J Pathol 1997;150:1815–25. [PubMed: 9137104]
12. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science 1993;260:816–9. [PubMed: 8484122]
13. Hoyer JD, Wick MJ, Thibodeau SN, Kechteiger KS, Cook JD, Fairbanks VF. Hb Silver Springs [beta 131(H9)Gln to His], a new hemoglobin variant found in six African-Americans. Hemoglobin 1998;22:37–44. [PubMed: 9494046]
14. Cunningham JM, Kim CY, Christensen ER, et al. The frequency of hereditary defective mismatch repair in a prospective series of unselected colorectal carcinomas. Am J Hum Genet 2001;69:780–90. [PubMed: 11524701]
15. Leach FS, Nicolaides NC, Papadopoulos N, et al. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 1993;75:1215–25. [PubMed: 8261515]
16. Peltomaki P. Genetic basis of hereditary nonpolyposis colorectal carcinoma. Ann Med 1994;26:215–9. [PubMed: 8074840]
17. Cunningham JM, Christensen ER, Tester DJ, et al. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 1998;58:3455–60. [PubMed: 9699680]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

18. Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 1997;57:808–11. [PubMed: 9041175]
19. Abrams SI, Hand PH, Tsang KY, Schlom J. Mutant ras epitopes as targets for cancer vaccines. Semin Oncol 1996;23:118–34. [PubMed: 8607022]
20. Khosravi-Far R, Der CJ. The Ras signal transduction pathway. Cancer Metastasis Rev 1994;13:67–89. [PubMed: 8143346]
21. Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 1991;349:426–8. [PubMed: 1992343]
22. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54. [PubMed: 12068308]
23. 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]
24. Wang L, Cunningham JM, Winters JL, et al. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res 2003;63:5209–12. [PubMed: 14500346]
25. Domingo E, Laiho P, Ollikainen M, et al. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. J Med Genet 2004;41:664–8. [PubMed: 15342696]
26. Witzig T, Loprinzi C, Gonchoroff N, et al. DNA ploidy and cell kinetic measurements as predictors of recurrence and survival in stages B2 and C colorectal adenocarcinoma. Cancer 1991;68:879–88. [PubMed: 1855188]
27. Halling KC, French AJ, McDonnell SK, et al. Microsatellite instability and 8p allelic imbalance in stage B2 and C colorectal cancers. J Natl Cancer Inst 1999;91:1295–1303. [PubMed: 10433618]
28. Lindor NM, Burgart LJ, Leontovich O, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 2002;20:1043–8. [PubMed: 11844828]
29. Gill S, Lindor N, Burgart L, et al. Isolated loss of PMS2 expression in colorectal cancers: Frequency, patient age, and familial aggregation. Clin Cancer Res 2005;11:6466–71. [PubMed: 16166421]
30. Ganguly A. An update on conformation sensitive gel electrophoresis. Hum Mutat 2002;19:334–42. [PubMed: 11933188]
31. Kaplan E, Meier P. Nonparametric estimation for incomplete observations. JASA 1958;53:457–81.
32. Cox DR. Regression models and life-tables. J Roy Stat Soc (Series B) 1972;34:187–220.
33. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003;349:247–57. [PubMed: 12867608]
34. Deng G, Bell I, Crawley S, et al. BRAF mutation is frequently present in sporadic colorectal cancer with methylated hMLH1 but not in hereditary nonpolyposis colorectal cancer. Clin Cancer Res 2004;10:191–5. [PubMed: 14734469]
35. Weisenberger D, Siegmund K, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006;38:787–93. [PubMed: 16804544]
36. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005;65:6063–9. [PubMed: 16024606]
37. Chung DC, Rustgi AK. The hereditary nonpolyposis colorectal cancer syndrome: Genetics and clinical implications. Ann Intern Med 2003;138:560–70. [PubMed: 12667026]
38. McGivern A, Wynter CV, Whitehall VL, et al. Promoter hypermethylation frequency and BRAF mutations distinguish hereditary non-polyposis colon cancer from sporadic MSI-H colon cancer. Fam Cancer 2004;3:101–7. [PubMed: 15340260]
39. Miyaki M, Iijima T, Yamaguchi T, et al. Both BRAF and KRAS mutations are rare in colorectal carcinomas from patients with hereditary nonpolyposis colorectal cancer. Cancer Lett 2004;211:105–9. [PubMed: 15194222]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Figure 1A

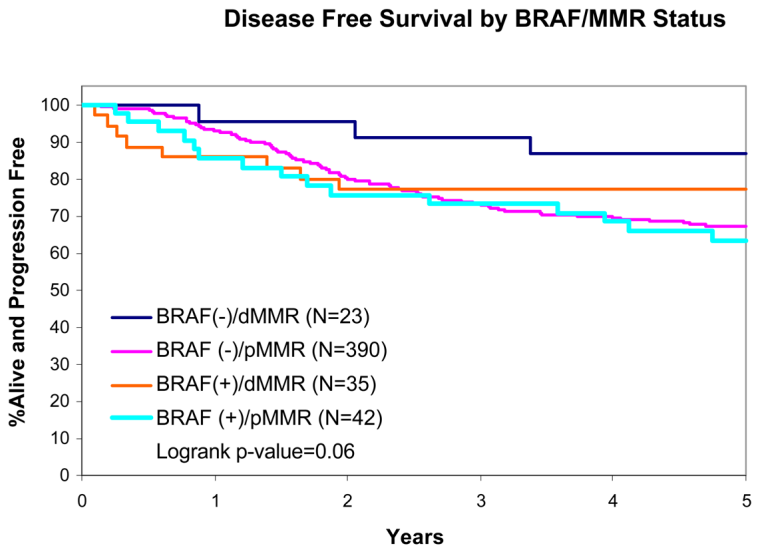

Figure 1B

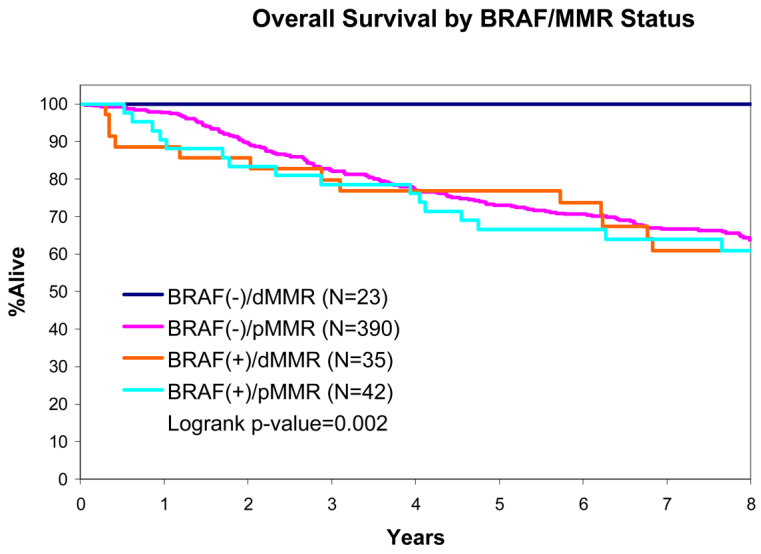

Figure 1.
Kaplan-Meier plots for the four BRAF/MMR subgroups. The Logrank p-value is derived from comparing DFS (1A) or OS (1B) for all 4 patient groups.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Figure 2A

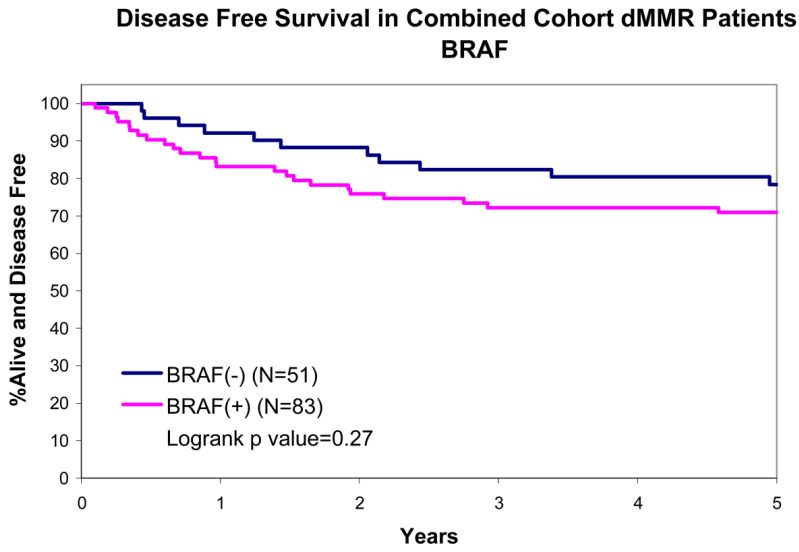

Figure 2B

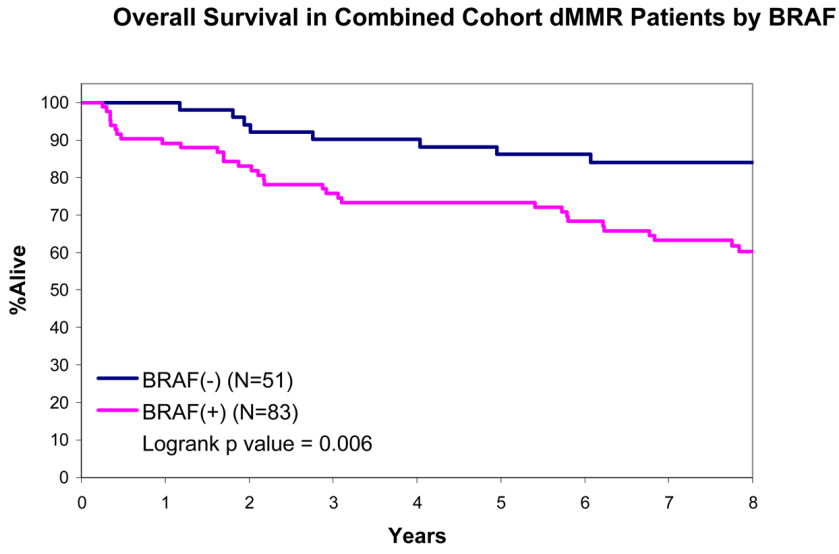

Figure 2.
Kaplan-Meier plots for the combined cohort of dMMR patients by BRAF. The Logrank p-value is derived from comparing DFS (2A) or OS (2B) for the 2 patient groups.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Patient Characteristics by MMR Status

	pMMR (N=472, 88.6%)	dMMR (N=61, 11.4%)	Total (N=533)	p-value
Age				0.8403[b]
Mean (SD)	61.5 (10.21)	60.9 (11.77)	61.5 (10.39)	
Median	63.0	64.0	63.0	
Range	(31.0–85.0)	(30.0–78.0)	(30.0–85.0)	
Gender				0.1132[a]
f	220 (46.6%)	35 (57.4%)	255 (47.8%)	
m	252 (53.4%)	26 (42.6%)	278 (52.2%)	
Grade				0.001[a]
Grade 1,2	344 (72.9%)	32 (52.5%)	376 (70.5%)	
Grade 3,4	128 (27.1%)	29 (47.5%)	157 (29.5%)	
Site				<0.0001[a]
Proximal	205 (43.6%)	53 (86.9%)	258 (48.6%)	
Distal	265 (56.4%)	8 (13.1%)	273 (51.4%)	
Stage				0.7519[a]
Stage II	125 (26.5%)	15 (24.6%)	140 (26.3%)	
Stage III	347 (73.5%)	46 (75.4%)	393 (73.7%)	

[a] Chi-Square p-value

[b] Wilcoxon Rank Sum p-value

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 2

Patient Characteristics by BRAF/MMR

	BRAF(−)/dMMR (N=23, 4.7%)	BRAF(−)/pMMR (N=390, 79.6%)	BRAF(+)/dMMR (N=35, 7.1%)	BRAF(+)/pMMR (N=42, 8.6%)	Total (N=490)	p-value
Age						0.0015 [b]
Mean (SD)	53.8 (12.69)	61.5 (10.12)	65.9 (7.29)	60.5 (11.49)	61.4 (10.38)	
Median	52.0	63.0	67.0	59.5	63.0	
Range	(30.0–72.0)	(31.0–85.0)	(50.0–78.0)	(33.0–83.0)	(30.0–85.0)	
Gender						0.0204 [a]
	8 (34.8%)	185 (47.4%)	25 (71.4%)	18 (42.9%)	236 (48.2%)	
	15 (65.2%)	205 (52.6%)	10 (28.6%)	24 (57.1%)	254 (51.8%)	
Grade						<0.0001[a]
Grade 1,2	12 (52.2%)	299 (76.7%)	17 (48.6%)	23 (54.8%)	351 (71.6%)	
Grade 3,4	11 (47.8%)	91 (23.3%)	18 (51.4%)	19 (45.2%)	139 (28.4%)	
Site						<0.0001[a]
Proximal	17 (73.9%)	162 (41.8%)	33 (94.3%)	28 (66.7%)	240 (49.2%)	
Distal	6 (26.1%)	226 (58.2%)	2 (5.7%)	14 (33.3%)	248 (50.8%)	
Stage						0.9367[a]
Stage II	6 (26.1%)	108 (27.7%)	8 (22.9%)	11 (26.2%)	133 (27.1%)	
Stage III	17 (73.9%)	282 (72.3%)	27 (77.1%)	31 (73.8%)	357 (72.9%)	

[a] Chi-Square p-value

[b] Kruskal-Wallace Rank Sum p-value

Clin Cancer Res. Author manuscript; Available in PMC 2009 June 1.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 3

Five-Year Survival Rates & Univariate Associations with Survival

Factor	N 533	5-Year Disease Free Survival (DFS)	HR (95% CI)	p-value[a]	5-Year Overall Survival (OS)	HR (95% CI)	p-value[a]
Age							
10 yr increase	533		1.008 (.9 – 1.2)	.92		1.1 (.98 – 1.3)	.09
Stage							
Stage II	140	83%		**<.001**	87%		**<.001**
Stage III	393	64%	2.4 (1.5 – 3.7)		69%	2.2 (1.4 – 3.2)	
Grade							
Grades 1 & 2	376	70%		.28	77%		.06
Grades 3 & 4	157	66%	1.2 (.9 – 1.7)		68%	1.3 (1.0 – 1.8)	
Site							
Proximal	258	69%	1.0 (.7 – 1.3)	.90	72%	1.1 (.8 – 1.5)	.51
Distal	273	68%			76%		
Gender							
Female	255	71%		.25	76%		.26
Male	278	67%	1.2 (.9 – 1.6)		72%	1.2 (.9 – 1.6)	
MMR							
pMMR	472	67%		**.04**	72%		**.049**
dMMR	61	82%	.5 (.3 – 1.0)		87%	.6 (.3 – 1.0)	
BRAF							
Negative	413	68%		.97	75%		.31
Positive	77	70%	1.0 (.6 – 1.6)		71%	1.2 (.8 – 1.8)	
pMMR							

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Factor	N 533	5-Year Disease Free Survival (DFS)	HR (95% CI)	p-value[a]	5-Year Overall Survival (OS)	HR (95% CI)	p-value[a]
BRAF(−)	390	67%		.57	73%		.59
BRAF(+)	42	64%	1.2 (.7 – 2.0)		67%	1.2 (.7 – 1.9)	
dMMR							
BRAF(−)	23	87%		.30	100%	Cox model inappropriate due to lack of events	**.001**[b]
BRAF(+)	35	77%	2.0 (.5 – 7.5)		77%		
BRAF & MMR							
BRAF(−)/dMMR							
Other	23	87%	.4 (.1 – 1.1)	**.06**	100%	Cox model inappropriate due to lack of events	**.002**[b]
	467	68%			73%		

[a] Univariate Score statistic

[b] Logrank p-value – no hazard ratio due to shortage of events

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 4

Univariate analysis in combined cohort

Factor	N 134*	5-Year Disease Free Survival	HR (95% CI)	p-value	5-Year Overall Survival	HR (95% CI)	p-value
dMMR Tumors							
BRAF(−)	51	78%		.27	86%		.006
BRAF(+)	83	71%	1.5 (.7 – 3.1)		73%	2.9 (1.3 – 6.4)	

Score p-value computed after stratifying by study

*Three patients are missing BRAF information