Microsatellite Instability and \textit{BRAF} Mutation Testing in Colorectal Cancer Prognostication

Paul Lochhead, Aya Kuchiba, Yu Imamura, Xiaoyun Liao, Mai Yamauchi, Reiko Nishihara, Zhi Rong Qian, Teppei Morikawa, Jeanne Shen, Jeffrey A. Meyerhardt, Charles S. Fuchs, Shuji Ogino

Manuscript received November 14, 2012; revised March 9, 2013; accepted May 30, 2013.

Correspondence to: Shuji Ogino, MD, PhD, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 450 Brookline Ave, Rm M422, Boston, MA 02215 (e-mail: shuji_ogino@dfci.harvard.edu).

\textit{BRAF} mutation in colorectal cancer is associated with microsatellite instability (MSI) through its relationship with high-level CpG island methylator phenotype (CIMP) and MLH1 promoter methylation. MSI and \textit{BRAF} mutation analyses are routinely used for familial cancer risk assessment. To clarify clinical outcome associations of combined MSI/\textit{BRAF} subgroups, we investigated survival in 1253 rectal and colon cancer patients within the Nurses’ Health Study and Health Professionals Follow-up Study with available data on clinical and other molecular features, including CIMP, LINE-1 hypomethylation, and \textit{KRAS} and \textit{PIK3CA} mutations. Compared with the majority subtype of microsatellite stable (MSS)/\textit{BRAF}-wild-type, MSS/\textit{BRAF}-mutant, MSI-high/\textit{BRAF}-mutant, and MSI-high/\textit{BRAF}-wild-type subtypes showed multivariable colorectal cancer-specific mortality hazard ratios of 1.60 (95% confidence interval [CI] = 1.12 to 2.28; \textit{P} = .009), 0.48 (95% CI = 0.27 to 0.87; \textit{P} = .02), and 0.25 (95% CI = 0.12 to 0.52; \textit{P} < .001), respectively. No evidence existed for a differential prognostic role of \textit{BRAF} mutation by MSI status (\textit{P} \text{interaction} > .50). Combined \textit{BRAF}/MSI status in colorectal cancer is a tumor molecular biomarker for prognostic risk stratification.


High-level microsatellite instability (MSI-high) is present in approximately 15% of colorectal cancers and is associated with superior survival (1–9). \textit{BRAF} mutation, present in 10% to 20% of colorectal cancers, is associated with MSI-high through its relationship to high-level CpG island methylator phenotype (CIMP) (10–14) and is generally associated with inferior prognosis (15–28). Because the presence of \textit{BRAF} mutation in MSI-high colorectal cancer decreases the likelihood of Lynch syndrome, MSI and \textit{BRAF} analyses have an established clinical utility (29–31). Clinicians are therefore increasingly aware of MSI/\textit{BRAF} status in colorectal cancer (29–31); however, outcomes for combined MSI/\textit{BRAF} subgroups have not been clearly defined. It remains uncertain whether the prognostic role of \textit{BRAF} mutation depends on MSI status (15–18).

Using the database of two US nationwide prospective cohort studies, the Nurses’ Health Study and the Health Professionals Follow-up Study (32–34), we tested the hypothesis that combined MSI/\textit{BRAF} status could serve as a prognostic molecular biomarker.

Rectal and colon cancer cases were identified through reporting by participants or next-of-kin and by searching the National Death Index for unreported lethal cases. The National Death Index was used to ascertain deaths (32–34). Cause of death was determined by study physicians. Informed consent was obtained from all study subjects. This study was approved by the Human Subjects Committees of Harvard School of Public Health and Brigham and Women’s Hospital.

DNA was extracted from formalin-fixed paraffin-embedded specimens, collected from hospitals across the United States where participants had undergone tumor resection or diagnostic biopsy (33). No statistically significant demographic differences existed between case subjects with and without available tissue (33). Tumor molecular biomarkers (including MSI, CIMP, LINE-1 hypomethylation, and \textit{KRAS}, \textit{BRAF}, and \textit{PIK3CA} mutations) were analyzed as previously described (35–41) (details provided in Supplementary Methods, available online).

All statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). All statistical tests were two-sided. Survival time was assessed using the Kaplan–Meier and log-rank methods. Cox proportional hazards models were used to estimate mortality hazard ratios (HRs), adjusting for potential confounders (details provided in Supplementary Methods).

Characteristics of 1253 colorectal cancer case subjects are summarized in Supplementary Table 1 (available online). During follow-up (median = 8.2 years; interquartile range = 3.5–13.1 years), there were 608 deaths, including 361 colorectal cancer–specific deaths. We first analyzed \textit{BRAF} mutation and MSI status as independent variables in survival analyses (Supplementary Figures 1 and 2, Supplementary Table 2, available online). In multivariable analyses, \textit{BRAF} mutation was associated with statistically significantly higher colorectal cancer–specific mortality (multivariable HR = 1.64, 95% confidence interval [CI] = 1.18 to 2.27; \textit{P} = .003). MSI-high was associated with statistically significantly lower colorectal cancer–specific mortality (multivariable HR = 0.28, 95% CI = 0.17 to 0.46; \textit{P} < .001). MSI status was a confounder for \textit{BRAF} mutation; when we simply adjusted for MSI status, the colorectal cancer–specific hazard ratio for \textit{BRAF} mutation was 2.05 (compared with univariate HR estimate of 1.14).

Increased colorectal cancer–specific mortality appeared to be associated with \textit{BRAF} mutation in both MSS (multivariable HR = 1.60, 95% CI = 1.12 to 2.28; \textit{P} = .009) and MSI-high tumor strata (multivariable HR = 1.90, 95% CI = 0.79 to 4.57; \textit{P} = .15) (Supplementary Table 3, available online). Lower colorectal cancer–specific mortality
was associated with MSI-high in both \textit{BRAF–wild-type} (multivariable HR = 0.25, 95\% CI = 0.12 to 0.52; \textit{P} < .001) and \textit{BRAF–mutant} strata (multivariable HR = 0.30, 95\% CI = 0.16 to 0.58; \textit{P} < .001).

For combined MSI/\textit{BRAF} subgroups, 5-year colorectal cancer–specific survival was 46\% for MSS/\textit{BRAF–mutant}, 65\% for MSS/\textit{BRAF–wild-type}, 73\% for MSI-high/\textit{BRAF–mutant}, and 79\% for MSI-high/\textit{BRAF–wild-type} (log-rank \textit{P} < .001) (Figure 1). In multivariable analyses (Table 1), compared with the majority subtype of MSS/\textit{BRAF–wild-type}, MSS/\textit{BRAF–mutant}, MSI-high/\textit{BRAF–mutant} and MSI-high/\textit{BRAF–wild-type} subtypes showed colorectal cancer–specific mortality hazard ratios of 1.60 (95\% CI = 1.12 to 2.28; \textit{P} = .009), 0.48 (95\% CI = 0.27 to 0.87; \textit{P} = .02), and 0.25 (95\% CI = 0.12 to 0.52; \textit{P} < .001), respectively. We found no evidence of interaction between MSI and \textit{BRAF} status in survival models (all \textit{P}_{interaction} > .50).

Tumor molecular classification has become crucial for clinical, translational, and epidemiologic research (42–49) because of uniqueness of each tumor and the continuum of colorectal biogeography influencing tumor characteristics (50–52). Despite their frequent coexistence as a result of their associations with high-level CIMP (CIMP–high) (53–58), we found MSI-high and \textit{BRAF} mutation in colorectal cancer to have divergent associations with patient survival. Our findings are compatible with previous

Figure 1. Kaplan–Meier survival plots for colorectal cancer according to combined MSI/\textit{BRAF} subgroup. A) Colorectal cancer–specific survival. B) Overall survival. Multi-group log-rank \textit{P} values demonstrate statistically significant deviation of any one of the survival curves from the null hypothesis. MSI = microsatellite instability; MSS = microsatellite stable.
Table 1. Colorectal cancer–specific and overall mortality according to combined microsatellite instability (MSI)/BRAF subgroup*

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>No. of cases (%)</th>
<th>No. of events</th>
<th>Colorectal cancer–specific mortality</th>
<th>Overall mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Univariate HR (95% CI)</td>
<td>Multivariable HR (95% CI)</td>
</tr>
<tr>
<td>MSS/BRAF–wild-type</td>
<td>979 (78)</td>
<td>299</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>MSS/BRAF–mutant</td>
<td>81 (6.5)</td>
<td>40</td>
<td>2.10 (1.51 to 2.93)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>MSI-high/BRAF–mutant</td>
<td>101 (8.1)</td>
<td>14</td>
<td>0.44 (0.26 to 0.79)</td>
<td>.003</td>
</tr>
<tr>
<td>MSI-high BRAF–wild-type</td>
<td>92 (73)</td>
<td>8</td>
<td>0.26 (0.13 to 0.52)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

* The multivariable Cox regression models were stage-stratified. In addition to MSV/BRAF subgroup, covariables initially included: age at diagnosis (continuous), sex, year of diagnosis (continuous), body mass index (≥30 vs <30 kg/m²), tumor location (proximal vs distal colorectum), tumor differentiation (poor vs well–moderately differentiated), family history of colorectal cancer in any first degree relative (present vs absent), CIMP status (CIMP-high vs CIMP-low/0), LINE-1 methylation (continuous), and KRAS and PIK3CA mutations (present vs absent). A backward elimination with threshold of P equal to .10 was used to select covariables. Age, year of diagnosis, body mass index, tumor differentiation, and LINE-1 methylation remained in the colorectal cancer–specific survival model. The same covariables, with the exception of LINE-1 methylation, remained in the overall survival model. CI = confidence interval; HR = hazard ratio; MSS = microsatellite stable.
studies that have found MSI-high to be associated with favorable outcome (2–8,15,17) and BRAF mutation to be associated with poor survival (16–28) [except for (59)]. MSI status is an established prognostic biomarker and is associated with host–tumor immune response (60–65).

Concordant with several other studies (16–20,66,67) [except for (15)], MSS/BRAF-mutant tumors were associated with the highest mortality. Patients with MSI-high/BRAF–wild-type tumors experienced the lowest mortality, consistent with a number of previous reports (15–20,67). Although we found MSI-high/BRAF-mutant tumors to be associated with favorable prognosis (vs MSS/BRAF–wild-type), confirmation in other populations is required.

Although some studies (17–19,68) suggest that the adverse prognostic association of BRAF mutation is limited to MSS tumors, other studies (15,16) and our analysis suggest that BRAF mutation remains prognostic among MSI-high cancers. We found no evidence for a differential prognostic role of BRAF mutation according to MSI status, consistent with a large population-based study (18). Taking into account existing literature, our data justify stratifying patients into poor (MSS/BRAF–mutant), intermediate (MSS/BRAF–wild-type), and favorable (MSI-high/BRAF–wild-type) prognostic groups (Supplementary Figure 4, available online).

Limitations of our study include its observational nature and lack of treatment data, and thus unknown bias, including differential treatment assignment, might confound results. Nevertheless, our regression analyses were adjusted for disease stage, on which treatment decisions are largely based, and our findings are consistent with data from independent clinical trials of colon cancer patients (15,16).

Strengths of our study include use of a molecular pathological epidemiology (69–79) database containing more than 1200 colorectal cancer cases characterized for key tumor molecular features. MSI-high and BRAF–mutant tumors represent a minority of colorectal cancers. The size and comprehensiveness of this population-based, molecular pathological epidemiology database enabled us to estimate an effect size for each tumor subtype while controlling for multiple potential confounders, including disease stage, age at diagnosis, body mass index, tumor differentiation, and tumor LINE-1 methylation level.

In conclusion, our data support a prognostic role for combined MSI/BRAF testing in colorectal cancer. Future studies should examine the predictive role of MSI/BRAF classification for response to therapeutic and lifestyle interventions.

References
mutation is a powerful prognostic factor in ad
tus on outcomes from the phase III AGITG MAX trial of capecitabine alone or in combi
38. Ogino S, Kawasaki T, Brahmann M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methyla
42. Wang D, Dubois RN. Associations between obesity and cancer: the role of fatty acid cy
47. Rex DK, Ahn en DJ, Baron JA, et al. Serrated lesions of the colorectum: review and recom
50. Ogino S, Fuchs CS, Giovannucci E. How many molecular subtypes? Implications of the unique tumor principle in person
52. Yamauchi M, Morikawa T, Kuchiba A, et al. Assessment of colorectal cancer molecular features along bowel subsites challenges the concep
55. Beggs AD, Jones A, El-Bahwary M, Abulafi M, Hodgson SV, Tomlinson IP. Whole
57. Bae JM, Kim JH, Kang GH. Epigenetic alter
65. Ogino S, Nosho K, Irahara N, et al. Lymphocytic reaction to colorectal cancer is asso
carcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucin

jnci.oxfordjournals.org JNCI | Brief Communication 1155


**Funding**

This work was supported by the US National Institutes of Health (P01 CA87969 to S.E. Hankinson; P01 CA55075 and U01 CA167552 to W.C. Willett; P50 CA127903 to CSF; and R01 CA151993 to SO); by the Bennett Family Fund and the Entertainment Industry Foundation through the National Colorectal Cancer Research Alliance; by the Frank Knox Memorial Fellowship at Harvard University (to PL); by a fellowship from the Chief Scientist Office of the Scottish Government (to PL); and by a fellowship from the Japan Society for Promotion of Science (to TM).

**Notes**

P. Lochhead, A. Kuchiba, Y. Imamura, and X. Liao contributed equally. C.S. Fuchs and S. Ogino contributed equally.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We would like to thank the participants and staff of the Nurses’ Health Study and the Health Professionals Follow-up Study for their valuable contributions as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY.

**Affiliations of authors:** Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA (PL, AK, YI, XL, MY, RN, ZRQ, TM, JAM, CSF, SO); Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK (PL); Department of Pathology (US, SO) and Channing Division of Network Medicine, Department of Medicine (CSF), Brigham and Women’s Hospital and Harvard Medical School, Boston, MA; Department of Epidemiology, Harvard School of Public Health, Boston, MA (SO).