Clinical Cancer Research

Mismatch Repair Status and *BRAF* Mutation Status in Metastatic Colorectal Cancer Patients: A Pooled Analysis of the CAIRO, CAIRO2, COIN, and FOCUS Studies

Sabine Venderbosch[1,2], Iris D. Nagtegaal[1], Tim S. Maughan[3], Christopher G. Smith[4], Jeremy P. Cheadle[4], David Fisher[5], Richard Kaplan[5], Philip Quirke[6], Matthew T. Seymour[7], Susan D. Richman[6], Gerrit A. Meijer[8], Bauke Ylstra[8], Danielle A.M. Heideman[8], Anton F.J. de Haan[9], Cornelis J.A. Punt[2], and Miriam Koopman[10]

Abstract

Purpose: To determine the prevalence and prognostic value of mismatch repair (MMR) status and its relation to *BRAF* mutation ($BRAF^{MT}$) status in metastatic colorectal cancer (mCRC).

Experimental Design: A pooled analysis of four phase III studies in first-line treatment of mCRC (CAIRO, CAIRO2, COIN, and FOCUS) was performed. Primary outcome parameter was the hazard ratio (HR) for median progression-free survival (PFS) and overall survival (OS) in relation to MMR and *BRAF*. For the pooled analysis, Cox regression analysis was performed on individual patient data.

Results: The primary tumors of 3,063 patients were analyzed, of which 153 (5.0%) exhibited deficient MMR (dMMR) and 250 (8.2%) a $BRAF^{MT}$. $BRAF^{MT}$ was observed in 53 (34.6%) of patients with dMMR tumors compared with 197 (6.8%) of patients with proficient MMR (pMMR) tumors ($P < 0.001$). In the pooled dataset, median PFS and OS were significantly worse for patients with dMMR compared with pMMR tumors [HR, 1.33; 95% confidence interval (CI), 1.12–1.57 and HR, 1.35; 95% CI, 1.13–1.61, respectively), and for patients with $BRAF^{MT}$ compared with *BRAF* wild-type ($BRAF^{WT}$) tumors (HR, 1.34; 95% CI, 1.17–1.54 and HR, 1.91; 95% CI, 1.66–2.19, respectively). PFS and OS were significantly decreased for patients with $BRAF^{MT}$ within the group of patients with pMMR, but not for *BRAF* status within dMMR, or MMR status within $BRAF^{WT}$ or $BRAF^{MT}$.

Conclusions: Prevalence of dMMR and $BRAF^{MT}$ in patients with mCRC is low and both biomarkers confer an inferior prognosis. Our data suggest that the poor prognosis of dMMR is driven by the $BRAF^{MT}$ status. *Clin Cancer Res; 20(20); 5322–30. ©2014 AACR.*

Introduction

Colorectal cancer is a heterogeneous disease arising through different pathways (1, 2). Three molecular pathways are well known to be involved in the multistep process of colorectal carcinogenesis, including the chromosomal instability (CIN) pathway, the mutator pathway [microsatellite instability (MSI)], and the epigenetic instability pathway or CpG island methylator phenotype (CIMP), the latter of which has substantial overlap with the other two.

MSI is the result of a deficient DNA mismatch repair (dMMR) system. A germline mutation in one of the MMR genes, most often *MLH1* or *MSH2*, is the cause of dMMR in patients with Lynch syndrome, which comprises 0.8% to 5% of all colorectal cancers (3). dMMR is also observed in 10% to 20% of patients with sporadic colorectal cancer, of which the majority of dMMR tumors are due to inactivation of *MLH1* (~95%), caused by hypermethylation of the gene promoter, with *MSH2* and *MSH6* accounting for a smaller percentage (3–5). These dMMR tumors have distinct features, such as origin in the proximal colon, prominent lymphocytic infiltrate, poorly differentiated morphology, mucinous or signet ring differentiation (6), and association with a favorable prognosis in early-stage colorectal cancer (7). In metastatic colorectal cancer (mCRC), the prevalence of dMMR is low (3.5%; refs. 8, 9). This supports the hypothesis that dMMR tumors have a reduced metastatic

[1]Department of Pathology, Radboud University Medical Center, Nijmegen, the Netherlands. [2]Department of Medical Oncology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands. [3]Department of Oncology, Gray Institute for Radiation Oncology, Biology University of Oxford, Oxford, United Kingdom. [4]Institute of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, United Kingdom. [5]MRC Clinical Trials Unit, Aviation House, London, United Kingdom. [6]Department of Pathology, Anatomy and Tumor Biology, Leeds Institute of Cancer and Pathology, St James's University Hospital, Leeds, United Kingdom. [7]Department of Medical Oncology, Leeds Institute of Cancer and Pathology, St James's University Hospital, University of Leeds, Leeds, United Kingdom. [8]Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands. [9]Department for Health Evidence, Radboud University Medical Center, Nijmegen, the Netherlands. [10]Department of Medical Oncology, University Medical Center Utrecht, Utrecht, the Netherlands.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Miriam Koopman, Department of Medical Oncology, University Medical Centre Utrecht, P.O. Box 85500-3508 GA, Utrecht, the Netherlands. Phone: 31-88-7556265; Fax: 31-30-2523741; E-mail: M.Koopman-6@umcutrecht.nl

doi: 10.1158/1078-0432.CCR-14-0332

Translational Relevance

This is the first pooled analysis on individual patient data to assess the role of the mismatch repair (MMR) status in relation to the *BRAF* mutation ($BRAF^{MT}$) status in respect to prevalence and outcome in patients with metastatic colorectal cancer (mCRC). These patients participated in four large randomized prospective phase III studies, namely the CAIRO, CAIRO2, COIN, and FOCUS studies. We show that the prevalence of deficient MMR (dMMR) and $BRAF^{MT}$ is low in patients with mCRC. Both biomarkers confer an inferior prognosis. We observed a higher incidence of $BRAF^{MT}$ in dMMR tumors than reported for patients with early-stage dMMR colorectal cancer, and our data suggest that the poor prognosis of dMMR is driven by $BRAF^{MT}$ status.

potential (10, 11). Because of its lower frequency, the prognostic role of dMMR in mCRC has not been properly evaluated thus far.

The presence of a *BRAF* mutation ($BRAF^{MT}$) in a dMMR tumor indicates a sporadic origin, and essentially excludes a diagnosis of Lynch syndrome (12, 13). In colorectal cancer, the overall prevalence of $BRAF^{MT}$ is approximately 10% (14). $BRAF^{MT}$ has a negative prognostic impact, although this may be restricted to patients with proficient MMR (pMMR) tumors (15, 16). Data on the role of *BRAF* in relation to MMR status in mCRC are scarce and are derived from small subsets of selected patients.

The current study was initiated to assess the role of MMR in relation to the $BRAF^{MT}$ status in respect to prevalence and outcome in patients with mCRC who participated in four large prospective phase III studies: CAIRO (17), CAIRO2 (18), COIN (19, 20), and FOCUS (21).

Materials and Methods

Patients and treatment

Data were derived from patients with mCRC included in four large phase III studies in first-line treatment: CAIRO (ClinicalStudys.gov; NCT00312000), CAIRO2 (ClinicalStudys.gov; NCT00208546), COIN (ISRCTN; 27286448), and FOCUS (ISRCTN; 79877428), of which the results have been published previously (17–21). Collection of formalin-fixed paraffin-embedded material (FFPE) of the primary tumor was part of the initial protocol in all four studies.

MMR status

For samples of both CAIRO studies, immunohistochemistry (IHC) was performed on FFPE tissue with antibodies against MMR proteins hMLH1, hMSH2, hMSH6, and hPMS2. In addition, MSI analysis was performed where there was an absence of MMR protein expression or equivocal IHC results. dMMR status was determined using two microsatellite markers (BAT 25 and BAT 26). If only one of these markers showed instability, the analysis was extended with four additional markers (BAT 40, D2S123, D5S346, and D17S250). A tumor was defined as dMMR if at least two of the six markers showed instability or pMMR if none of the markers showed instability. Tumors with only one of the markers showing instability were defined as dMMR-low and included in the pMMR category. For samples from the COIN study, dMMR status was assessed using two microsatellite markers (BAT25 and BAT26). If only one of these markers showed instability, the tumor was defined as dMMR, and as pMMR if no instability was observed. For samples from the FOCUS study, dMMR status was based on loss of MLH1 and MSH2 protein expression, assessed by IHC. If either protein showed loss of expression, the tumor was defined as dMMR, and pMMR if no loss of expression was observed.

Hypermethylation status of the *MLH1* gene promoter

Hypermethylation of the *MLH1* gene promoter in patients with a dMMR tumor was analyzed in samples from the CAIRO and CAIRO2 studies only and therefore not included in the pooled analysis. The DNA methylation status of the *MLH1* promoter region was determined after bisulfite treatment of the DNA using the EZ DNA Methylation Kit (ZYMO Research), as described previously (8).

$BRAF^{MT}$ status

The *BRAF* V600E mutation status was assessed in duplicate by high-resolution melting (HRM) sequencing analysis for tumor material in the CAIRO study (22) and by direct sequencing analysis in the CAIRO2 study (23). For samples of the COIN and FOCUS studies, the *BRAF* V600E mutation status was determined by Pyrosequencing (and Sequenom in COIN), and verified by Sanger sequencing as described previously (19, 24). Non-V600E $BRAF^{MT}$ detected by these assays ($n = 19$) were not included in the current analyses on outcome.

Statistical methods

Individual patient data were included in the pooled analysis. Progression-free survival (PFS) was defined as the time from the date of randomization to first progression or death, whichever came first. Overall survival (OS) was defined as the time from randomization to the date of death. The primary outcome measure was the hazard ratio (HR) for PFS and OS in relation to MMR and $BRAF^{MT}$ status. For PFS and OS, all studies were included in a Cox regression model (proportional hazard model) by using the study as a factor in the model. In this way, dependence of the hazard on study could be modeled. The HR was corrected for study effect. Survival curves were plotted and log-rank tests were performed to compare survival for the different groups defined. A statistical interaction analysis for survival data of dMMR and *BRAF* status was performed. All analyses were conducted using the SAS system version 9.2; $P < 0.05$ was considered statistically significant.

Results

Study population and MMR/$BRAF^{MT}$ status

Tumor and normal samples from 3,063 out of 6,155 randomized mCRC patients were available and suitable

Table 1. Prevalence of MMR and *BRAF*^{MT} status in patients with mCRC subdivided by study

	dMMR	pMMR	Total	*BRAF*^{MT}	*BRAF*^{WT}	Total
CAIRO	18 (5.6%)	304 (94.4%)	322	25 (7.8%)	297 (92.2%)	322
CAIRO2	29 (5.6%)	487 (94.4%)	516	45 (8.7%)	471 (91.3%)	516
COIN	65 (4.4%)	1,396 (95.6%)	1,461	120 (8.2%)	1,341 (91.8%)	1,461
FOCUS	41 (5.4%)	723 (94.6%)	764	60 (7.9%)	704 (92.1%)	764
Pooled dataset	153 (5.0%)	2,910 (95.0%)	3,063	250 (8.2%)	2,813 (91.8%)	3,063
P			0.614			0.943

NOTE: *P* values represent heterogeneity between the four studies.
Abbreviations: mt, mutant tumors; wt, wild-type tumors.

for analysis of both MMR and *BRAF*^{MT} status. Of these 3,063 patients, 322 patients participated in the CAIRO study, 516 patients in the CAIRO2 study, 1,461 patients in the COIN study, and 764 patients in the FOCUS study.

The prevalence of MMR status and *BRAF*^{MT} status and their correlation are presented in Tables 1 and 2, respectively. dMMR was found in tumors of 153 (5.0%) patients and 250 (8.2%) patients had a *BRAF*^{MT} (Table 1). There was no evidence of heterogeneity for the prevalence of dMMR and *BRAF*^{MT} in the four studies; $P = 0.614$ and $P = 0.943$, respectively (Table 1). A *BRAF*^{MT} was observed in 53 (34.6%) of patients with dMMR tumors compared with 197 (6.8%) of patients with pMMR tumors ($P < 0.001$; Table 2). There was heterogeneity for the prevalence of combined MMR and *BRAF*^{MT} status between the four studies. In the CAIRO study, there were significantly more patients with a combined dMMR and *BRAF*^{MT} (dMMR/*BRAF*^{MT}) tumor compared with the other three studies ($P = 0.002$; Table 2).

Patient and tumor characteristics (sex, age, location of the primary tumor, performance status, and number of metastatic sites involved) for the different subgroups defined by the combined MMR and *BRAF*^{MT} status are summarized in Supplementary Table S1. Hypermethylation of *MLH1* was the main cause of dMMR in both CAIRO and CAIRO2 studies (30 out of 45 patients), this was associated with a high frequency of *BRAF*^{MT} (73%) compared with tumors without *MLH1* hypermethylation (7%).

Survival data

The survival data of the individual studies, the pooled dataset, and the pooled analysis for patients with dMMR, pMMR, *BRAF*^{MT}, and *BRAF* wild-type (*BRAF*^{WT}) tumors are presented in Table 3. The median PFS and OS were significantly worse for patients with dMMR compared with pMMR tumors [PFS: 6.2 vs. 7.6 months, respectively; HR, 1.33; 95% confidence interval (CI) 1.12–1.57; $P = 0.001$; OS: 13.6 vs. 16.8 months, respectively; HR, 1.35; 95% CI, 1.13–1.61; $P = 0.001$). Median PFS and OS were also significantly worse for patients with *BRAF*^{MT} compared with *BRAF*^{WT} tumors (PFS: 6.2 vs. 7.7 months, respectively; HR, 1.34; 95% CI, 1.17–1.54; $P < 0.001$; OS: 11.4 vs. 17.2 months, respectively; HR, 1.91; 95% CI, 1.66–2.19; $P < 0.001$).

To determine a possible interaction between MMR and *BRAF* status, with respect to the survival, a Cox regression was performed by using the study as a factor in the model. For PFS and OS, all studies were included in a Cox regression model

Table 2. Prevalence of *BRAF*^{MT} status stratified for MMR status, and MMR status stratified for *BRAF* status in mCRC patients subdivided by study

	BRAF^{MT}			*BRAF*^{WT}			dMMR			pMMR		
	dMMR	pMMR	Total	dMMR	pMMR	Total	*BRAF*^{MT}	*BRAF*^{WT}	Total	*BRAF*^{MT}	*BRAF*^{WT}	Total
CAIRO	12 (48.0%)	13 (52.0%)	25	6 (2.0%)	291 (98.0%)	297	12 (66.7%)	6 (33.3%)	18	13 (4.3%)	291 (95.7%)	304
CAIRO2	12 (26.7%)	33 (73.3%)	45	17 (3.6%)	454 (96.4%)	471	12 (41.4%)	17 (58.6%)	29	33 (6.8%)	454 (93.2%)	487
COIN	20 (16.7%)	100 (83.3%)	120	45 (3.4%)	1,296 (96.6%)	1,341	20 (30.8%)	45 (69.2%)	65	100 (7.2%)	1,296 (92.8%)	1,396
FOCUS	9 (15.0%)	51 (85.0%)	60	32 (4.5%)	672 (95.5%)	704	9 (22.0%)	32 (78.0%)	41	51 (7.1%)	672 (92.9%)	723
Pooled dataset	53 (21.2%)	197 (78.8%)	250	100 (3.6%)	2,713 (96.4%)	2,813	53 (34.6%)	100 (65.4%)	153	197 (6.8%)	2,713 (93.2%)	2,910
P			**0.002**			0.239			**0.007**			0.330

NOTE: Statistically significant results are set in bold. *P* values represent heterogeneity between the four studies.
Abbreviations: mt, mutant tumors; wt, wild-type tumors.

Table 3. Individual study data, pooled dataset, and pooled analysis of survival data in relation to MMR and $BRAF^{MT}$ status

		dMMR	pMMR	$BRAF^{MT}$	$BRAF^{WT}$
CAIRO					
	Number of patients	18	304	25	297
PFS	mo. (95% CI)	5.7 (4.2–8.8)	6.9 (6.2–7.9)	5.1 (4.1–7.7)	7.0 (6.3–8.2)
	HR (95% CI)	1.34 (0.81–2.22)		**1.57 (1.03–2.38)**	
OS	mo. (95% CI)	14.8 (12.0–26.0)	17.9 (16.1–19.2)	11.3 (8.3–15.0)	18.1 (16.2–19.4)
	HR (95% CI)	1.26 (0.74–2.16)		**2.20 (1.43–3.38)**	
CAIRO2					
	Number of patients	29	487	45	471
PFS	mo. (95% CI)	7.5 (6.4–10.5)	10.5 (9.6–11.4)	6.9 (6.2–8.5)	10.6 (9.7–11.8)
	HR (95% CI)	**1.66 (1.13–2.45)**		**2.03 (1.48–2.79)**	
OS	mo. (95% CI)	15.6 (12.9–22.3)	22.0 (20.3–24.1)	13.1 (10.7–16.5)	22.4 (21.0–24.9)
	HR (95% CI)	**1.60 (1.07–2.40)**		**2.30 (1.65–3.20)**	
COIN					
	Number of patients	65	1,396	120	1,341
PFS	mo. (95% CI)	5.7 (5.4–6.1)	6.5 (6.2–6.8)	5.8 (5.6–6.2)	6.5 (6.3–6.9)
	HR (95% CI)	**1.56 (1.20–2.02)**		**1.38 (1.14–1.68)**	
OS	mo. (95% CI)	10.7 (9.3–13.0)	16.0 (15.0–16.9)	10.2 (9.0–11.7)	16.5 (15.3–17.1)
	HR (95% CI)	**1.80 (1.37–2.37)**		**2.02 (1.65–2.48)**	
FOCUS					
	Number of patients	41	723	60	704
PFS	mo. (95% CI)	8.1 (6.5–9.1)	8.0 (7.4–8.3)	8.1 (6.8–8.9)	8.0 (7.4–8.3)
	HR (95% CI)	0.98 (0.71–1.35)		0.98 (0.74–1.28)	
OS	mo. (95% CI)	16.6 (13.6–21.7)	15.5 (14.5–16.6)	12.3 (10.5–14.8)	15.7 (14.8–17.0)
	HR (95% CI)	0.90 (0.64–1.27)		**1.52 (1.15–2.00)**	
Pooled dataset					
	Number of patients	153	2,910	250	2,813
PFS	mo. (95% CI)	6.2 (5.9–7.0)	7.6 (7.3–8.0)	6.2 (6.0–6.8)	7.7 (7.4–8.0)
	HR (95% CI)	**1.33 (1.12–1.57)**		**1.34 (1.17–1.54)**	
OS	mo. (95% CI)	13.6 (12.4–15.6)	16.8 (16.3–17.5)	11.4 (10.5–12.4)	17.2 (16.7–18.0)
	HR (95% CI)	**1.35 (1.13–1.61)**		**1.91 (1.66–2.19)**	

NOTE: Statistically significant results are shown in bold.

Abbreviations: mo., median PFS and OS time in months; mt, mutant tumor; wt, wild-type tumor.

(proportional hazard model) by using the study as a factor in the model. Results are presented for MMR status in a $BRAF^{MT}$ and $BRAF^{WT}$ background, and vice versa for *BRAF* status in a dMMR and pMMR background in Table 4. Survival curves, as estimated by the Cox regression, are presented in Fig. 1. In $BRAF^{MT}$ tumors stratified by MMR status, there was no significant survival difference for patients with dMMR compared with pMMR tumors (PFS: 6.1 vs. 6.2 months, respectively; HR, 0.95; 95% CI, 0.62–1.46; $P = 1.000$; OS: 11.7 vs. 11.3 months, respectively; HR, 1.05; 95% CI, 0.68–1.63; $P = 1.000$). Also in $BRAF^{WT}$ tumors stratified by MMR status, there was no significant survival difference for patients with dMMR compared with pMMR tumors (PFS: 6.3 vs. 7.8 months, respectively; HR, 1.32; 95% CI, 1.00–1.75; $P = 0.051$; OS: 15.0 vs. 17.3 months, respectively; HR, 1.22; 95% CI, 0.91–1.65; $P = 0.463$). In dMMR tumors stratified by *BRAF* status, there was no significant survival difference for patients with $BRAF^{MT}$ compared with $BRAF^{WT}$ tumors (PFS: 6.1 vs. 6.3 months, respectively; HR, 1.07; 95% CI, 0.67–1.70; $P = 1.000$; OS: 11.7 vs. 15.0 months, respectively; HR, 1.51;

Table 4. Individual study data, pooled dataset, and pooled analysis of survival data and association between MMR and *BRAF*^{MT} status

		BRAF^{MT}		*BRAF*^{WT}		dMMR		pMMR	
		dMMR	pMMR	dMMR	pMMR	*BRAF*^{MT}	*BRAF*^{WT}	*BRAF*^{MT}	*BRAF*^{WT}
CAIRO									
	Number of patients	12	13	6	291	12	6	13	291
PFS	mo. (95% CI)	6.6 (4.6–12.6)	4.1 (2.4–6.4)	3.6 (2.0–16.4)	7.1 (6.4–8.2)	6.6 (4.6–12.6)	3.6 (2.0–16.4)	4.1 (2.4–6.4)	7.1 (6.4–8.2)
	HR (95% CI)	2.38 (0.80–7.09)		3.19 (0.96–10.63)		0.34 (0.08–1.44)		**2.60 (1.21–5.55)**	
OS	mo. (95% CI)	13.2 (10.1–28.6)	8.3 (5.9–13.6)	18.6 (12.4–53.5)	18.2 (16.2–19.4)	13.2 (10.1–28.6)	18.6 (12.4–53.5)	8.3 (5.9–13.6)	18.2 (16.2–19.4)
	HR (95% CI)	2.14 (0.70–6.54)		0.97 (0.25–3.57)		1.63 (0.34–7.77)		**3.29 (1.53–7.04)**	
CAIRO2									
	Number of patients	12	33	17	454	12	17	33	454
PFS	mo. (95% CI)	5.7 (4.4–8.5)	7.5 (6.4–9.7)	9.3 (7.2–14.8)	10.7 (9.9–12.1)	5.7 (4.4–8.5)	9.3 (7.2–14.8)	7.5 (6.4–9.7)	10.7 (9.9–12.1)
	HR (95% CI)	0.54 (0.22–1.34)		1.25 (0.62–2.49)		2.65 (0.95–7.41)		**1.79 (1.09–2.93)**	
OS	mo. (95% CI)	10.4 (7.8–17.2)	14.1 (11.5–19.4)	21.0 (15.2–36.4)	22.4 (21.1–25.0)	10.4 (7.8–17.2)	21.0 (15.2–36.4)	14.1 (11.5–19.4)	22.4 (21.1–25.0)
	HR (95% CI)	0.61 (0.25–1.52)		1.14 (0.54–2.40)		**2.94 (1.02–8.52)**		**2.04 (1.21–3.44)**	
COIN									
	Number of patients	20	100	45	1,296	20	45	100	1,296
PFS	mo. (95% CI)	5.9 (5.4–8.5)	5.8 (5.6–6.2)	5.6 (5.1–6.1)	6.6 (6.3–7.0)	5.9 (5.4–8.5)	5.6 (5.1–6.1)	5.8 (5.6–6.2)	6.6 (6.3–7.0)
	HR (95% CI)	1.05 (0.53–2.11)		**1.72 (1.14–2.60)**		0.78 (0.37–1.66)		**1.42 (1.07–1.88)**	
OS	mo. (95% CI)	10.5 (8.2–16.3)	10.2 (8.9–11.8)	10.8 (9.2–14.0)	16.7 (15.6–17.5)	10.5 (8.2–16.3)	10.8 (9.2–14.0)	10.2 (8.9–11.8)	16.7 (15.6–17.5)
	HR (95% CI)	1.04 (0.52–2.10)		**1.85 (1.19–2.88)**		1.08 (0.50–2.34)		**2.07 (1.54–2.79)**	
FOCUS									
	Number of patients	9	51	32	672	9	32	51	672
PFS	mo. (95% CI)	6.8 (4.9–12.0)	8.3 (6.8–9.1)	8.3 (6.8–9.6)	8.0 (7.4–8.3)	6.8 (4.9–12.0)	8.3 (6.8–9.6)	8.3 (6.8–9.1)	8.0 (7.4–8.3)
	HR (95% CI)	0.75 (0.28–2.00)		0.91 (0.56–1.49)		1.37 (0.49–3.79)		0.93 (0.63–1.39)	
OS	mo. (95% CI)	13.5 (9.8–28.7)	12.2 (10.2–14.8)	17.5 (14.0–24.0)	15.6 (14.7–16.9)	13.5 (9.8–28.7)	17.5 (14.0–24.0)	12.2 (10.2–14.8)	15.6 (14.7–16.9)
	HR (95% CI)	1.21 (0.44–3.36)		0.85 (0.50–1.44)		1.51 (0.52–4.42)		**1.55 (1.03–2.33)**	
Pooled dataset									
	Number of patients	53	197	100	2,713	53	100	197	2,713
PFS	mo. (95% CI)	6.1 (5.6–7.7)	6.2 (6.0–6.9)	6.3 (5.8–7.4)	7.8 (7.4–8.1)	6.1 (5.6–7.7)	6.3 (5.8–7.4)	6.2 (6.0–6.9)	7.8 (7.4–8.1)
	HR (95% CI)	0.95 (0.62–1.46)		1.32 (1.00–1.75)		1.07 (0.67–1.70)		**1.34 (1.10–1.64)**	
OS	mo. (95% CI)	11.7 (9.9–14.4)	11.3 (10.3–12.5)	15.0 (13.1–18.0)	17.3 (16.7–18.1)	11.7 (9.9–14.4)	15.0 (13.1–18.0)	11.3 (10.3–12.5)	17.3 (16.7–18.1)
	HR (95% CI)	1.05 (0.68–1.63)		1.22 (0.91–1.65)		1.51 (0.93–2.46)		**1.94 (1.57–2.40)**	

NOTE: Statistically significant results are shown in bold.

Abbreviations: mo., median PFS or OS time in months; mt, mutant tumor; wt, wild-type tumor.

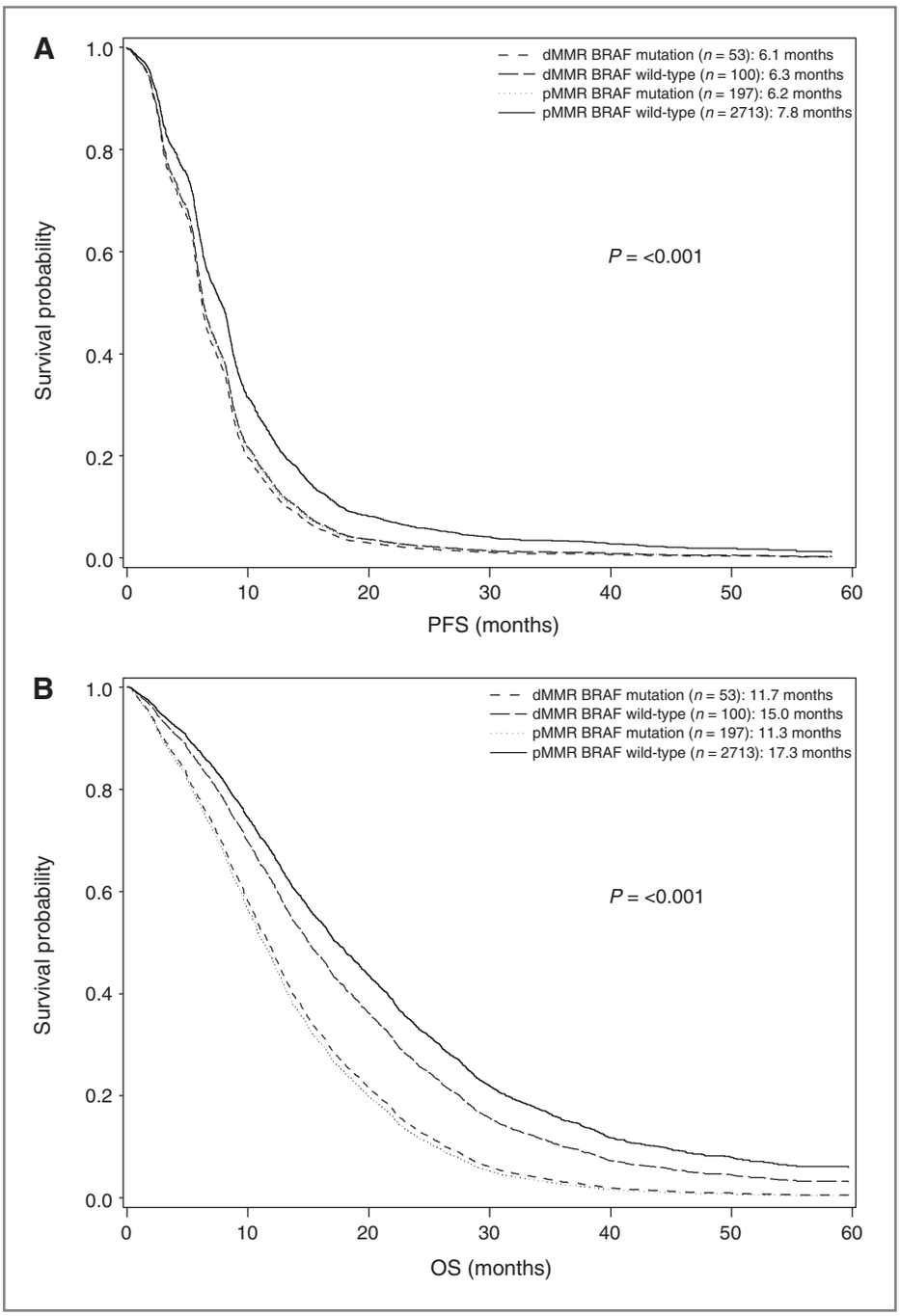

Figure 1. PFS (A) and OS (B) curves of all patients included in the pooled dataset comparing patients with dMMR/*BRAF*^{MT} tumors, dMMR/*BRAF*^{WT} tumors, pMMR/*BRAF*^{MT} tumors, and pMMR/*BRAF*^{WT} tumors.

95% CI, 0.93–2.46; $P = 0.155$). In pMMR tumors stratified by *BRAF* status, there was a significantly decreased median PFS and OS for patients with *BRAF*^{MT} compared with *BRAF*^{WT} tumors (PFS: 6.2 vs. 7.8 months, respectively; HR, 1.34; 95% CI, 1.10–1.64; $P < 0.001$; OS: 11.3 vs. 17.3 months, respectively; HR, 1.94; 95% CI, 1.57–2.40; $P < 0.001$) The test for interaction between dMMR and *BRAF*^{MT} was statistically not significant (PFS: HR, 0.79; 95% CI, 0.54–1.16; $P = 0.234$; OS: HR, 0.78; 95% CI, 0.52–1.15; $P = 0.211$).

Discussion

This study presents the largest dataset on the role of tumor MMR status and *BRAF*^{MT} status in respect to prevalence and outcome in a population of patients ($n = 3{,}063$) with mCRC who participated in four prospective phase III studies. We found that dMMR and *BRAF*^{MT} in mCRC each have a low prevalence (5% and 8.2%, respectively), and that both biomarkers indicate a

poor prognosis. Given the absence of a statistically significant interaction between $BRAF^{MT}$ and dMMR, our data suggest that the poor prognostic value of dMMR is driven by the $BRAF^{MT}$ status.

Several aspects of our study warrant further discussion. In this pooled analysis, different methods for detecting dMMR were applied, which, however, have all been validated for the detection of dMMR in colorectal cancer. In both CAIRO studies, an approach based on test methods described in the Bethesda criteria, used for standard clinical practice for patients suspected for Lynch syndrome, has been applied (25). The COIN study analyzed the BAT25 and BAT26 mononucleotide markers, which have a high sensitivity (94%) and specificity (98%), and the use of these two markers alone identifies 97% of MSI tumors (26). The FOCUS study evaluated MLH1 and MSH2 protein expression by IHC, which is a sensitive (92.3%) and specific (100%) method for screening for dMMR (27).

We acknowledge that the difference in MMR detection methods represents a weakness of our study; however, the comparable prevalence of the dMMR status among the four studies in this pooled analysis, ranging from 4.4% to 5.6%, argues against this. The results from the individual studies show that the patient population with dMMR tumors is heterogeneous. The observed difference in the prevalence of a $BRAF^{MT}$ in dMMR tumors suggests a possible difference in the origin of dMMR, sporadic versus hereditary. Unfortunately, data on the hypermethylation status of the *MLH1* gene promoter, which could differentiate between these two groups, are not available of all four studies.

Furthermore, different methods for detecting the *BRAF* V600E mutation were applied. HRM sequencing, Sanger sequencing, and Pyrosequencing have all shown to be reliable methods (22, 28). Data from systematic studies to assess the test accuracy or reproducibility of the different techniques used for $BRAF^{MT}$ testing are not available.

Another issue is the difference in availability of tumor samples among the trials. This is partly caused by nonavailability of an extra paraffin-embedded block for DNA analysis, and partly due to nonresected primary tumors in patients with synchronous disease. In these patients, often only a diagnostic biopsy was performed, which does not provide sufficient material for further molecular analysis for research purposes. This is an important, underexposed issue that may introduce a sample/case bias not only in our analysis, but in other translational studies in mCRC as well.

The low prevalence of dMMR in mCRC can be explained by the reduced potential of stage I–III dMMR tumors to metastasize (10, 11). However, the underlying mechanisms of this low metastatic potential are yet to be elucidated. It has been suggested that a greater immunoreactivity of dMMR tumors (29, 30) or decreased tumor cell viability due to excessive DNA damage (31) may play a role. In mCRC, data about the prevalence of $BRAF^{MT}$ in dMMR tumors are scarce, but in line with our results (32, 33). The strong inter-relationship between $BRAF^{MT}$ and dMMR is well established in early-stage colorectal cancer (14, 34); however, the etiology of both alterations still needs to be elucidated.

We observed a higher prevalence of $BRAF^{MT}$ in mCRC dMMR tumors (34.6%) than reported for early-stage dMMR colorectal cancer tumors (24%; 16). Patients with early-stage dMMR in general have a better prognosis compared with patient with early-stage pMMR; however, within the group of dMMR, patients with $BRAF^{MT}$ tumors have a worse prognosis (35). Subsequently, this may lead to a shift in the dMMR/$BRAF^{MT}$ ratio in patients with mCRC. There is increasing evidence identifying $BRAF^{MT}$ as a significant poor prognostic factor in early stage and mCRC (18, 36–38). *BRAF* is an oncogene and it is known that the mutations constitutively activate the MAPK pathway for cell growth, in the absence of extracellular stimuli. However, by itself *BRAF* is not sufficient for cancer and must cooperate with other processes to induce the fully cancerous state (39). Another explanation for the inferior prognosis of $BRAF^{MT}$ tumors might be their distinct pattern of metastatic spread. Previous studies have demonstrated a significantly increased rate of peritoneal and distant lymph node metastases and a decreased rate of lung metastases compared with $BRAF^{WT}$ tumors (9, 40).

It has been speculated that the worse prognostic value of dMMR tumors in mCRC may be related to a difference in metastatic spread. Earlier studies showed a reduced rate of liver metastases for dMMR tumors in mCRC (40), and a higher incidence of peritoneal metastases; these factors are known to be related to prognosis (41, 42). This was confirmed by a previous analysis of the COIN study (9), but these data are not available from the other studies of our analysis.

Finally, due to the different treatment regimens among the four studies of this pooled analysis, the predictive role of dMMR and $BRAF^{MT}$ in mCRC could not be addressed.

In conclusion, dMMR and $BRAF^{MT}$ each have a low prevalence in mCRC, and both biomarkers confer a poor prognosis. Our data suggest that the poor prognosis of dMMR is driven by the $BRAF^{MT}$ status. However, we caution against a firm conclusion on this issue because our study was not sufficiently powered to test this interaction.

Disclosure of Potential Conflicts of Interest

J.P. Cheadle reports receiving a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Venderbosch, T.S. Maughan, C.J.A. Punt, M. Koopman

Development of methodology: S. Venderbosch, I.D. Nagtegaal, C.J.A. Punt, M. Koopman

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I.D. Nagtegaal, T.S. Maughan, C.G. Smith, J.P. Cheadle, R. Kaplan, P. Quirke, M.T. Seymour, S.D. Richman, G.A. Meijer, B. Ylstra, D.A.M. Heideman, M. Koopman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Venderbosch, I.D. Nagtegaal, C.G. Smith, J.P. Cheadle, D. Fisher, P. Quirke, M.T. Seymour, A.F.J. de Haan, C.J.A. Punt, M. Koopman

Writing, review, and/or revision of the manuscript: S. Venderbosch, I.D. Nagtegaal, T.S. Maughan, C.G. Smith, J.P. Cheadle, D. Fisher, R. Kaplan,

P. Quirke, M.T. Seymour, S.D. Richman, G.A. Meijer, B. Ylstra, D.A.M. Heideman, A.F.J. de Haan, C.J.A. Punt, M. Koopman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Kaplan, D.A.M. Heideman, M. Koopman

Study supervision: P. Quirke, C.J.A. Punt

Other (laboratory analyses and interpretation of FOCUS data): P. Quirke

Acknowledgments

The authors thank the patients and their families who participated in all the studies and gave their consent for this research, and the investigators and pathologists who submitted samples for assessment. The CAIRO studies were conducted with the support of the network of the Dutch Colorectal Cancer Group (DCCG). The COIN and FOCUS studies were conducted with the support of Cancer Research UK, the UK Medical Research Council (MRC) and the National Cancer Research Networks.

Grant Support

CAIRO: This study was supported by the Dutch Colorectal Cancer Group (DCCG), a grant support from the Commissie Klinisch Toegepast Onderzoek (CKTO) of the Dutch Cancer Foundation (KWF), unrestricted scientific grants from Roche, Aventis, Sanofi, and Pfizer, the Cornelis Visser Foundation and the framework of CTMM, the Center for Translational Molecular Medicine, DeCoDe project (grant 03O-101).

CAIRO2: This study was supported by the DCCG, and grants for data management and analysis from the CKTO of the Dutch Cancer Foundation and unrestricted research grants from Roche, Merck Serono, Sanofi-Aventis. DxS.

COIN: This study was supported by the Bobby Moore Fund from Cancer Research UK, Cancer Research Wales, and the NISCHR Cancer Genetics Biomedical Research Unit. The COIN trial was funded by Cancer Research UK and the MRC. An unrestricted educational grant from Merck Serono provided additional support to this work and the trial.

FOCUS: This study was supported by Merck KGgA, Yorkshire Cancer Research, Leeds Experimental Cancer Medicine Center, and the Leeds CRUK Center.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 8, 2014; revised June 28, 2014; accepted August 6, 2014; published OnlineFirst August 19, 2014.

References

1. Hermsen M, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, et al. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology 2002;123:1109–19.
2. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:113–30.
3. Cunningham JM, Kim CY, Christensen ER, Tester DJ, Parc Y, Burgart LJ, 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.
4. Cunningham JM, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, et al. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 1998;58:3455–60.
5. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, 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.
6. Jass JR, Do KA, Simms LA, Iino H, Wynter C, Pillay SP, et al. Morphology of sporadic colorectal cancer with DNA replication errors. Gut 1998;42:673–9.
7. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609–18.
8. Koopman M, Kortman GA, Mekenkamp L, Ligtenberg MJ, Hoogerbrugge N, Antonini NF, et al. Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. Br J Cancer 2009;100:266–73.
9. Smith CG, Fisher D, Claes B, Maughan TS, Idziaszczyk S, Peuteman G, et al.Somatic profiling of the epidermal growth factor receptor pathway in tumors from patients with advanced colorectal cancer treated with chemotherapy {+/-} cetuximab. Clin Cancer Res 2013;19:4104–13.
10. Malesci A, Laghi L, Bianchi P, Delconte G, Randolph A, Torri V, et al. Reduced likelihood of metastases in patients with microsatellite-unstable colorectal cancer. Clin Cancer Res 2007;13:3831–9.
11. Buckowitz A, Knaebel HP, Benner A, Blaker H, Gebert J, Kienle P, et al. Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases. Br J Cancer 2005;92:1746–53.
12. Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, et al. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res 2003;63:5209–12.
13. Domingo E, Niessen RC, Oliveira C, Alhopuro P, Moutinho C, Espin E, et al. BRAF-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional MLH1 and MSH2 genes. Oncogene 2005;24:3995–8.
14. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002;418:934.
15. 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;65:6063–9.
16. 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;28:466–74.
17. Koopman M, Antonini NF, Douma J, Wals J, Honkoop AH, Erdkamp FL, et al. Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO): a phase III randomised controlled trial. Lancet 2007;370:135–42.
18. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 2009;360:563–72.
19. Maughan TS, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, et al. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. Lancet 2011;377:2103–14.
20. Adams RA, Meade AM, Seymour MT, Wilson RH, Madi A, Fisher D, et al. Intermittent versus continuous oxaliplatin and fluoropyrimidine combination chemotherapy for first-line treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. Lancet Oncol 2011;12:642–53.
21. Seymour MT, Maughan TS, Ledermann JA, Topham C, James R, Gwyther SJ, et al. Different strategies of sequential and combination chemotherapy for patients with poor prognosis advanced colorectal cancer (MRC FOCUS): a randomised controlled trial. Lancet 2007;370:143–52.
22. Heideman DA, Lurkin I, Doeleman M, Smit EF, Verheul HM, Meijer GA, et al. KRAS and BRAF mutation analysis in routine molecular diagnostics: comparison of three testing methods on formalin-fixed, paraffin-embedded tumor-derived DNA. J Mol Diagn 2012;14:247–55.
23. Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, et al. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. Eur J Cancer 2010;46:1997–2009.
24. 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;27:5931–7.
25. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, 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.
26. Cicek MS, Lindor NM, Gallinger S, Bapat B, Hopper JL, Jenkins MA, et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the Colon Cancer Family Registry. J Mol Diagn 2011;13:271–81.
27. Lindor NM, Burgart LJ, Leontovich O, Goldberg RM, Cunningham JM, Sargent DJ, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 2002; 20:1043–8.
28. Curry JL, Torres-Cabala CA, Tetzlaff MT, Bowman C, Prieto VG. Molecular platforms utilized to detect BRAF V600E mutation in melanoma. Semin Cutan Med Surg 2012;31:267–73.
29. Ishikawa T, Fujita T, Suzuki Y, Okabe S, Yuasa Y, Iwai T, et al. Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. Cancer Res 2003;63: 5564–72.
30. Kloor M, Becker C, Benner A, Woerner SM, Gebert J, Ferrone S, et al. Immunoselective pressure and human leukocyte antigen class I antigen machinery defects in microsatellite unstable colorectal cancers. Cancer Res 2005;65:6418–24.
31. Peltomaki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol 2003;21:1174–9.
32. 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;102:1762–8.
33. Tie J, Gibbs P, Lipton L, Christie M, Jorissen RN, Burgess AW, et al. Optimizing targeted therapeutic development: analysis of a colorectal cancer patient population with the BRAF(V600E) mutation. Int J Cancer 2011;128:2075–84.
34. Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004;53:1137–44.
35. Lochhead P, Kuchiba A, Imamura Y, Liao X, Yamauchi M, Nishihara R, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J Natl Cancer Inst 2013;105:1151–6.
36. Farina-Sarasqueta A, van Lijnschoten G, Moerland E, Creemers GJ, Lemmens VE, Rutten HJ, 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;21:2396–403.
37. 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.
38. Price TJ, Hardingham JE, Lee CK, Weickhardt A, Townsend AR, Wrin JW, et al. Impact of KRAS and BRAF gene mutation status on outcomes from the phase III AGITG MAX trial of capecitabine alone or in combination with bevacizumab and mitomycin in advanced colorectal cancer. J Clin Oncol 2011;29:2675–82.
39. Dhomen N, Marais R. New insight into BRAF mutations in cancer. Curr Opin Genet Dev 2007;17:31–9.
40. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 2011;117:4623–32.
41. Catalano V, Loupakis F, Graziano F, Torresi U, Bisonni R, Mari D, et al. Mucinous histology predicts for poor response rate and overall survival of patients with colorectal cancer and treated with first-line oxaliplatin- and/or irinotecan-based chemotherapy. Br J Cancer 2009;100:881–7.
42. Franko J, Shi Q, Goldman CD, Pockaj BA, Nelson GD, Goldberg RM, et al. Treatment of colorectal peritoneal carcinomatosis with systemic chemotherapy: a pooled analysis of north central cancer treatment group phase III trials N9741 and N9841. J Clin Oncol 2012;30:263–7.