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ORIGINAL ARTICLE

CMS-dependent prognostic impact of *KRAS* and *BRAF*^{V600E} mutations in primary colorectal cancer

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Background: The prognostic impact of *KRAS* and *BRAF*^{V600E} mutations in primary colorectal cancer (CRC) varies with microsatellite instability (MSI) status. The gene expression–based consensus molecular subtypes (CMSs) of CRC define molecularly and clinically distinct subgroups, and represent a novel stratification framework in biomarker analysis. We investigated the prognostic value of these mutations within the CMS groups.

Patients and methods: Totally 1197 primary tumors from a Norwegian series of CRC stage I–IV were analyzed for MSI and mutation status in hotspots in *KRAS* (codons 12, 13 and 61) and *BRAF* (codon 600). A subset was analyzed for gene expression and confident CMS classification was obtained for 317 samples. This cohort was expanded with clinical and molecular data, including CMS classification, from 514 patients in the publically available dataset GSE39582. Gene expression signatures associated with *KRAS* and *BRAF*^{V600E} mutations were used to evaluate differential impact of mutations on gene expression among the CMS groups.

Results: *BRAF*^{V600E} and *KRAS* mutations were both associated with inferior 5-year overall survival (OS) exclusively in MSS tumors (*BRAF*^{V600E} mutation versus *KRAS*/*BRAF* wild-type: Hazard ratio (HR) 2.85, $P < 0.001$; *KRAS* mutation versus *KRAS*/*BRAF* wild-type: HR 1.30, $P = 0.013$). *BRAF*^{V600E}-mutated MSS tumors were strongly enriched and associated with metastatic disease in CMS1, leading to negative prognostic impact in this subtype (OS: *BRAF*^{V600E} mutation versus wild-type: HR 7.73, $P = 0.001$). In contrast, the poor prognosis of *KRAS* mutations was limited to MSS tumors with CMS2/CMS3 epithelial-like gene expression profiles (OS: *KRAS* mutation versus wild-type: HR 1.51, $P = 0.011$). The subtype-specific prognostic associations were substantiated by differential effects of *BRAF*^{V600E} and *KRAS* mutations on gene expression signatures according to the MSI status and CMS group.

Conclusions: *BRAF*^{V600E} mutations are enriched and associated with metastatic disease in CMS1 MSS tumors, leading to poor prognosis in this subtype. *KRAS* mutations are associated with adverse outcome in epithelial (CMS2/CMS3) MSS tumors.

Key words: colorectal cancer, consensus molecular subtypes (CMSs), BRAF mutation, KRAS mutation, microsatellite instability, prognosis

Introduction

Despite major efforts to identify molecular prognostic biomarkers in colorectal cancer (CRC), the TNM staging system remains the mainstay in prognostication and decision of initial patient management. However, disease stage alone cannot predict which patients will benefit from adjuvant chemotherapy, as 50% of stage III patients receiving adjuvant chemotherapy are cured by surgery alone [1].

The only biomarkers recommended for routine clinical use due to their prognostic properties in CRC are DNA mismatch repair (MMR) status and *BRAF*^{V600E} mutation [2]. Deficient MMR or microsatellite instability (MSI) is associated with a lower relapse rate

and possibly also with resistance to 5-fluorouracil monotherapy [3], and thus limited benefit of adjuvant chemotherapy. *BRAF*^{V600E} mutations are associated with poorer overall survival (OS) across stages, with the negative prognostic impact being most prominent in microsatellite stable (MSS) and left-sided tumors [4–8].

KRAS mutations are negative predictors of anti-EGFR therapy efficacy in metastatic CRC [9], while the evidence of a prognostic impact is more ambiguous. Studies on stages I–III disease have shown inconsistent associations with survival [10–12] but a recent study indicated a negative prognostic impact after relapse [4]. Stratification according to primary tumor site and MSI status in stages I–III suggests the negative prognostic effect of *KRAS* mutations to be distinct for left-sided tumors [4, 13] and MSS tumors [7, 14].

The classification of primary CRCs according to the gene expression–based consensus molecular subtypes (CMSs) defines four molecularly and clinically distinct subgroups, and represents a biological stratification framework with great potential in biomarker development [15]. CMS2 and CMS3 display epithelial-like gene expression profiles, whereas CMS1 is associated with immune-infiltration and CMS4 with epithelial-to-mesenchymal transition and both exhibit low expression of genes associated with colonic epithelial differentiation [15, 16]. Tumors with MSI and *BRAF*^{V600E} mutations are enriched in the CMS1-immune subtype and *KRAS* mutations in the CMS3 epithelial-metabolic subtype. This may indicate diverging oncogenic dependencies between the CMS groups and subtype-specific prognostic significance of the mutations.

Here, we report the distribution and prognostic impact of mutations in the cancer-critical genes *KRAS* and *BRAF*^{V600E} according to clinicopathological and molecular variables, including the CMS groups, in a population-based series of primary CRC.

Materials and methods

Patient material

Totally 1197 primary tumor samples from a consecutive series (Oslo-series) of patients treated surgically for stages I–IV CRC at Oslo University Hospital, Norway between 1993 and 2014 were analyzed (supplementary Table S1, available at *Annals of Oncology* online). Formalin-fixed paraffin-embedded tumor tissue was available from patients operated between 1993 and 2003 ($n = 761$), while fresh frozen samples were available from patients operated between 2005 and 2014 ($n = 436$). For analysis of CMS-associations, publically available data from a French multi-centre cohort of stages I–IV primary colon cancer ($n = 514$) was included (Gene Expression Omnibus accession number GSE39582) [17] (supplementary Table S1, available at *Annals of Oncology* online). A total of 831 patients with confident CMS classification from both cohorts were analyzed.

Mutation analyses

DNA extraction, determination of MSI status, and Sanger sequencing of mutation hotspots in *KRAS* (exon 2: codons 12 and 13, exon 3: codon 61) and *BRAF* (codon 600) were performed as previously described [7, 8, 18–20]. The majority of sequencing data was previously published in the referenced papers.

Gene expression analyses and CMS classification

From fresh frozen tumor samples, RNA was extracted and analyzed for gene expression using Affymetrix exon-level microarrays ($n = 409$), and tumors were classified according to CMS using the classifyCMS.RF-function in the R package CMSclassifier [15] (supplementary Data, available at *Annals of Oncology* online). Confident CMS classification was obtained for 317 (78%) of the tumors (supplementary Table S1, available at *Annals of Oncology* online).

For patients in the GSE39582 dataset, CMS assignments were available for 514 patients and downloaded from the Colorectal Cancer Subtyping Consortium website at SAGE Synapse (https://www.synapse.org/#!Synapse:syn2623706/wiki/67246). Gene expression data, MSI status, *BRAF*^{V600E} and *KRAS* mutation status, and clinical data were downloaded from the GEO accession number.

Sample-wise gene set expression enrichment scores for genes previously found to be upregulated in *KRAS*-mutated CRCs ($n = 13$ genes) [21], a *BRAF*^{V600E} mutation signature ($n = 163$ genes) [22, 23] and a colonic differentiation signature ($n = 165$ genes) [24] were calculated using the R package GSVA [25].

Statistical analyses

Statistical analysis was performed using SPSS 21.0 software (SPSS Inc.) (supplementary Data, available at *Annals of Oncology* online). Five-year OS and relapse-free survival were defined according to the guidelines by Punt et al. [26].

Results

Clinicopathological and molecular associations of *KRAS* and *BRAF*^{V600E} mutations

Among the 1197 patients with stages I–IV primary CRC in the Oslo-series, *KRAS* and *BRAF*^{V600E} mutations were mutually exclusive, with mutation rates of 31% and 16%, respectively (supplementary Table S1, available at *Annals of Oncology* online). Previously described clinicopathological and molecular associations were confirmed, including frequent *BRAF*^{V600E} mutations in MSI, right-sided and poorly differentiated tumors, as well as in females and elderly patients (Table 1). The strong association with MSI was also found on the transcriptional level, based on single-sample enrichment scores of a *BRAF*-mutant gene expression signature [22, 23], and MSI tumors were highly '*BRAF*-like' compared with MSS tumors (supplementary Figure S1A, available at *Annals of Oncology* online). In contrast, when comparing the '*BRAF*-like' activation level between *BRAF*^{V600E}-mutated and wild-type tumors, we found that the effect of mutations on the transcriptional activity was larger in MSS tumors than in MSI tumors, which was validated in the French cohort (supplementary Figure S1B and C, available at *Annals of Oncology* online).

BRAF^{V600E} mutations were enriched in the CMS1 subtype in both MSI and MSS tumors in both patient series (total $n = 737$; supplementary Figure S2, available at *Annals of Oncology* online). Among MSS tumors in general, the mutation frequency of *BRAF*^{V600E} across the two datasets was 4%. However, MSS tumors with the CMS1 phenotype had a mutation frequency of 34% [odds ratio = 21; 95% confidence interval (CI) 8.7–50.4, $P < 0.001$; supplementary Table S2, available at *Annals of Oncology* online].

KRAS mutations were most frequent in MSS tumors (Table 1), and single-sample enrichment analysis showed transcriptional upregulation of a *KRAS* mutant gene signature [21] in MSS tumors compared with MSI tumors (supplementary Figure S3A,

Table 1. Distribution of mutations according to clinicopathological and molecular characteristics (Oslo-series, n = 1197)

Characteristic[a]	Total	*KRAS* (*n* = 1097)		*BRAF* (*n* = 1185)	
	n	mut (%)	*P*	mut (%)	*P*
Total	1197				
Age (years)					
≤70	493	28	0.098	13	**0.025**
>70	704	33		18	
Gender					
Male	563	33	0.102	8	**<0.001**
Female	634	29		23	
MSI status					
MSS	993	35	**<0.001**	7	**<0.001**
MSI	184	10		68	
CMS					
CMS1	63	14	**<0.001**	71	**<0.001**
CMS2	138	30		1	
CMS3	54	52		17	
CMS4	62	29		10	
Location					
Right	493	33	0.512	32	**<0.001**
Left	369	29		6	
Rectum	312	29		3	
Synchronous	23	35		22	
Stage[b]					
I	195	27	**0.043**	9	0.125
II	475	29		19	
III	327	35		14	
IV	198	33		20	
pT[b]					
1	46	34	0.65	9	**0.001**
2	193	27		9	
3	840	32		18	
4	118	30		20	
pN[b]					
0	723	28	**0.022**	16	0.844
1	316	34		14	
2	148	36		20	
Differentiation					
High	72	30	0.591	14	**<0.001**
Medium	932	31		13	
Low	154	27		38	
Mucinous	10	23		40	
Other/NA	29	42		7	
KRAS					
wt	758			24	**<0.001**
mut	339			0	
BRAF					
wt	993	37	**<0.001**		
mut	192	0			

[a]*P* values according to Fisher's exact test unless otherwise stated.
[b]Spearman correlation test.
mut, mutation; wt, wild-type. Statistically significant *P* values in bold.

available at *Annals of Oncology* online). Similarly to $BRAF^{V600E}$, a comparison of the *KRAS* mutant expression signature between mutated and wild-type tumors showed that the transcriptional effects of *KRAS* mutations were higher in MSS tumors than in MSI tumors (supplementary Figures S3B and C, available at *Annals of Oncology* online). Furthermore, *KRAS* mutations were most frequent in CMS3, also when analyzing MSS tumors exclusively (supplementary Table S2 and Figure S4A and B, available at *Annals of Oncology* online). However, a comparison of the *KRAS* mutant gene expression signature between mutated and wild-type MSS tumors revealed the effect of *KRAS* mutations to be largest in CMS2 in both patient cohorts (supplementary Figure S4C and D, available at *Annals of Oncology* online).

Prognostic impact of *KRAS* and $BRAF^{V600E}$ mutations according to standard clinicopathological and molecular variables

In multivariable analysis in the Oslo-series, patients with $BRAF^{V600E}$ mutation had significantly worse OS (HR 1.61; 95% CI 1.15–2.23; $P = 0.005$, Table 2, supplementary Table S3, available at *Annals of Oncology* online) compared with *KRAS/BRAF* wild-type. However, the negative prognostic impact was highly specific to the MSS phenotype (MSS: HR 2.85; 95% CI 2.07–3.92; $P < 0.001$ versus MSI: HR 0.93; 95% CI 0.49–1.77; $P = 0.8$, $P_{interaction} = 0.002$, Figure 1, supplementary Table S4 and Figure S5, available at *Annals of Oncology* online), reinforcing a clinical relevance of the stronger transcriptional effect of the mutations in this population. In MSS tumors, inferior prognosis for patients with $BRAF^{V600E}$ mutation was found both in stages I–III and metastatic disease (supplementary Figure S6, available at *Annals of Oncology* online), but was distinct for left-sided tumors in multivariable analysis (HR 2.75; 95% CI 1.41–5.38; $P = 0.003$, supplementary Table S5, available at *Annals of Oncology* online).

Patients with tumors harboring *KRAS* mutations exhibited significantly worse OS compared with patients with *KRAS/BRAF* wild-type tumors in univariable analysis of the Oslo-series (HR 1.28; 95% CI 1.05–1.56; $P = 0.016$), while statistical significance was lost in multivariable analysis (Table 2). Stratification according to clinicopathological and molecular variables revealed the negative prognostic impact to be clearly distinct for the MSS subgroup, again reinforcing a clinical relevance of the stronger transcriptional effect of *KRAS* mutations in this subgroup (MSS: HR 1.30; 95% CI 1.06–1.59; $P = 0.013$ versus MSI: HR 0.84; 95% CI 0.30–2.38; $P = 0.742$, Figure 1 and supplementary Table S4, available at *Annals of Oncology* online). In subsequent multivariable analysis limited to MSS tumors, the inferior prognostic association of *KRAS* mutation was seen only in left-sided tumors (HR 1.41; 95% CI 0.99–2.02; $P = 0.055$) and stage IV disease (HR 1.56; 95% CI 1.06–2.29; $P = 0.025$, supplementary Table S6, available at *Annals of Oncology* online).

Poor prognostic value of $BRAF^{V600E}$ mutations in MSS tumors is reinforced in CMS1

Analyzing both patient series combined, the poor prognostic impact of $BRAF^{V600E}$ mutations in MSS tumors was found only in CMS1, likely due to the strong mutation enrichment in this subtype (supplementary Figure S7A, available at *Annals of Oncology*

Table 2. Univariable and multivariable analyses of prognostic impact (5-year overall survival) of clinicopathological and molecular variables

		Univariable analysis		Multivariable analysis[a]	
Variable	**Patients, *n* (%)**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Total	1197 (100)				
Gender					
Male	563 (47)	1		1	
Female	634 (53)	1.03 (0.87–1.23)	0.713	0.91 (0.75–1.11)	0.362
Age					
≤70	493 (41)	1		1	
>70	704 (59)	1.57 (1.31–1.89)	**<0.001**	2.00 (1.63–2.44)	**<0.001**
MSI status					
MSS	993 (84)	1		1	
MSI	184 (16)	0.66 (0.50–0.86)	**0.002**	0.52 (0.36–0.77)	**0.001**
Location					
Right	493 (41)	1		1	
Left	369 (31)	1.06 (0.87–1.29)	0.574	1.02 (0.81–1.29)	0.877
Rectum	312 (26)	0.82 (0.66–1.02)	0.078	0.96 (0.74–1.25)	0.751
Stage					
I	195 (16)	1		1	
II	475 (40)	1.49 (1.07–2.08)		1.37 (0.95–1.99)	
III	327 (27)	2.54 (1.82–3.54)		2.52 (1.75–3.63)	
IV	198 (17)	10.17 (7.30–14.16)	**<0.001**	10.34 (7.18–14.90)	**<0.001**
Differentiation					
High	72 (6)	1		1	
Medium	932 (80)	0.97 (0.68–1.40)		1.07 (0.70–1.62)	
Low[b]	164 (14)	1.66 (1.11–2.47)	**<0.001**	1.87 (1.17–3.0)	**<0.001**
KRAS and/or *BRAF*[c]					
Both wt	570 (52)	1		1	
KRAS mut	339 (31)	1.28 (1.05–1.56)	**0.016**	1.21 (0.98–1.49)	0.08
BRAF mut	192 (17)	1.29 (1.01–1.64)	**0.043**	1.61 (1.15–2.23)	**0.005**

See supplementary Table S3, available at *Annals of Oncology* online, for analyses of relapse-free survival.
[a]Includes all variables in the table. $n = 1037$, 160 cases dropped due to missing variables.
[b]Includes mucinous.
[c]Includes only patients with conclusive wild type status in both genes or conclusive mutation in one gene.
mut, mutation; wt, wild-type. Statistically significant *P* values in bold.

online). Here, patients with $BRAF^{V600E}$ mutations ($n = 12$) had an OS rate of 22%, significantly lower than the corresponding survival rate of 81% for patients with $BRAF^{V600E}$ wild-type tumors ($n = 23$; $P = 0.001$; Figure 2A). This subtype-specific prognostic impact was stronger than for MSS tumors in general (supplementary Figure S7C, available at *Annals of Oncology* online), and irrespective of tumor location ($P_{interaction} = 0.8$). The poor prognostic association in CMS1 was found in both patient series separately (supplementary Figure S8, available at *Annals of Oncology* online). However, stratification into early- and late stage disease revealed this association to be mainly driven by an enrichment of metastatic disease in $BRAF^{V600E}$-mutated CMS1 MSS tumors (supplementary Figure S7D, available at *Annals of Oncology* online). A similar propensity for metastatic disease of $BRAF^{V600E}$-mutated tumors was not evident in the other CMS subgroups (supplementary Table S7, available at *Annals of Oncology* online). Among MSI tumors, no prognostic association for this mutation was seen within any of the CMS subtypes (supplementary Figure S7B, available at *Annals of Oncology* online). Consequently, the prognostic impact of $BRAF^{V600E}$ mutations was highly dependent on MSI status within CMS1 ($P_{interaction} = 0.007$).

KRAS mutations are associated with adverse outcome for patients with epithelial (CMS2/3) MSS tumors

KRAS mutations were found to have strongest prognostic associations in epithelial (CMS2/3) MSS tumors, with statistical significance only in CMS2 (supplementary Figure S9, available at *Annals of Oncology* online). Patients with *KRAS*-mutated CMS2 and MSS tumors ($n = 108$) had an OS rate of 59%, significantly lower than the corresponding 75% survival rate for patients wild-type for *KRAS* ($n = 233$; $P = 0.004$; Figure 2B). A nonsignificant trend was retained in multivariable analysis (HR 1.32; 95% CI 0.83–2.10; $P = 0.249$). The prognostic association in CMS2 was similar for left- and right-sided MSS tumors ($P_{interaction} = 0.326$, supplementary Figure S10, available at *Annals of Oncology* online) and limited to stages I–III (OS: HR 2.09; 95% CI 1.29–3.38;

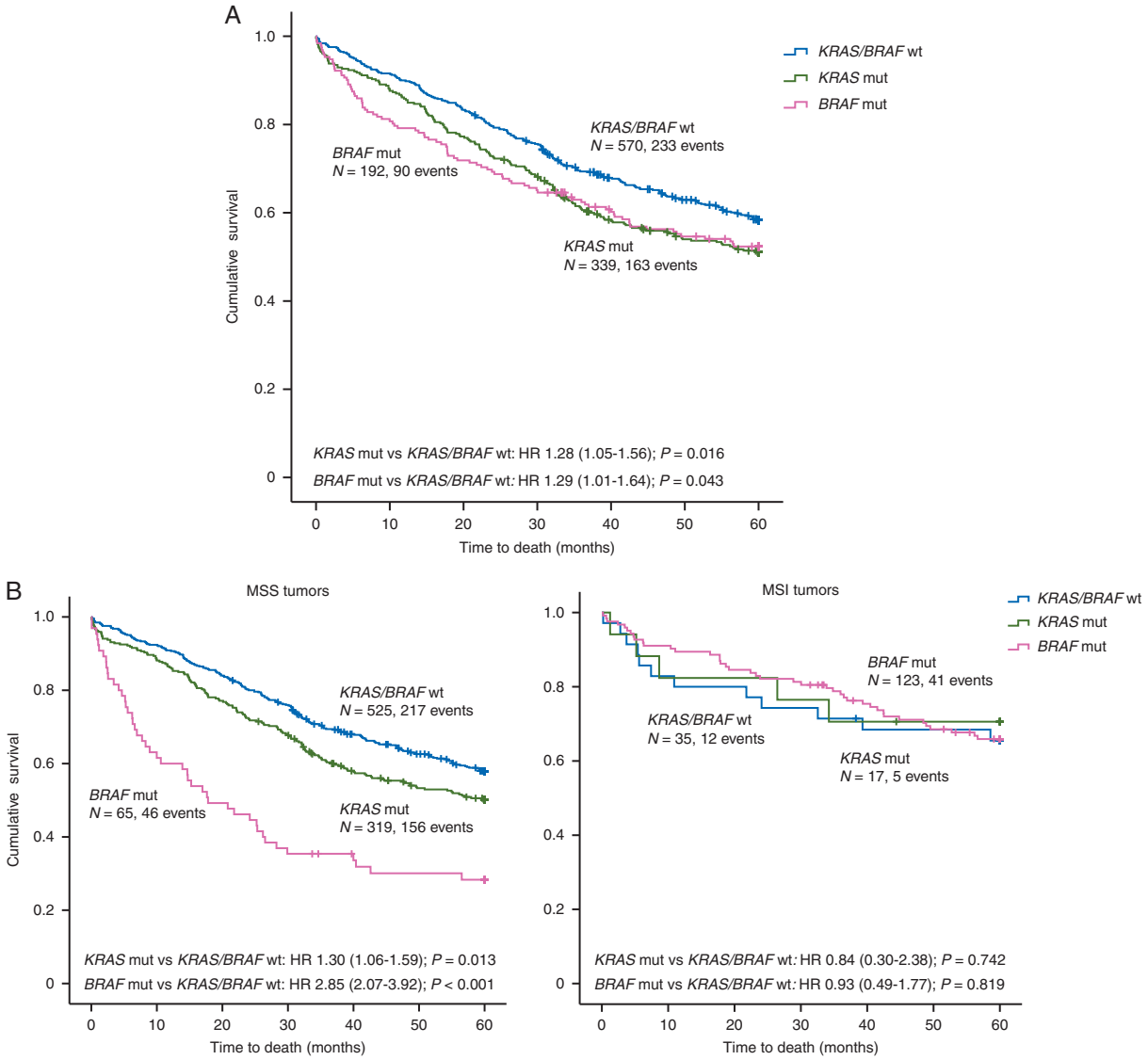

Figure 1. Prognostic impact of *KRAS* and *BRAF*^{V600E} mutations in unstratified Oslo-series and according to MSI status. Kaplan–Meier survival curves showing 5-year overall survival (OS) for tumors with *KRAS* and *BRAF*^{V600E} mutations versus *KRAS/BRAF* wild-type in (A) the unstratified Oslo-series and (B) stratified according to MSI status. See supplementary Figure S5, available at *Annals of Oncology* online for analyses of 5-year relapse-free survival.

$P = 0.003$, supplementary Figure S11, available at *Annals of Oncology* online).

Based on single-sample enrichment scores of a colonic differentiation signature, CMS1/4 and CMS2/3 were confirmed to display undifferentiated and epithelial-like gene expression profiles, respectively (supplementary Figure S12, available at *Annals of Oncology* online). *KRAS* mutations were weakly associated with poor survival also in CMS3 MSS tumors (OS: HR 3.77; 95% CI 0.87–16.34; $P = 0.076$), and there was a clear difference in the prognostic impact between epithelial CMS2/3 cancers and undifferentiated CMS1/4 cancers (Figure 2C).

Discussion

In a large single-hospital series of primary CRCs, we confirm previous findings that the prognostic impact of *KRAS* and *BRAF*^{V600E} mutations is specific to MSS tumors [4–8, 10, 14, 27], and show that this is associated with a greater transcriptional effect of both mutations in the MSS subgroup. Integration with CMS classification reveals that the poor prognostic associations of *BRAF*^{V600E} mutations in MSS are strengthened among CMS1 tumors. This is likely due to strong mutation enrichment in this subtype and a propensity for metastatic disease among the mutated tumors. For *KRAS* mutations, the negative prognostic impact is limited to epithelial (CMS2/3) tumors. These novel context-dependent prognostic associations are irrespective of primary tumor location and for *KRAS* mutations, biologically substantiated by the varying transcriptional effect of the mutations according to the CMS group.

Preclinical studies have shown *KRAS* oncogenic dependency to be strongly linked to epithelial differentiation [28]. Our finding that *KRAS* mutations have specific negative prognostic impact within CMS2 and CMS3, translates these observations into a

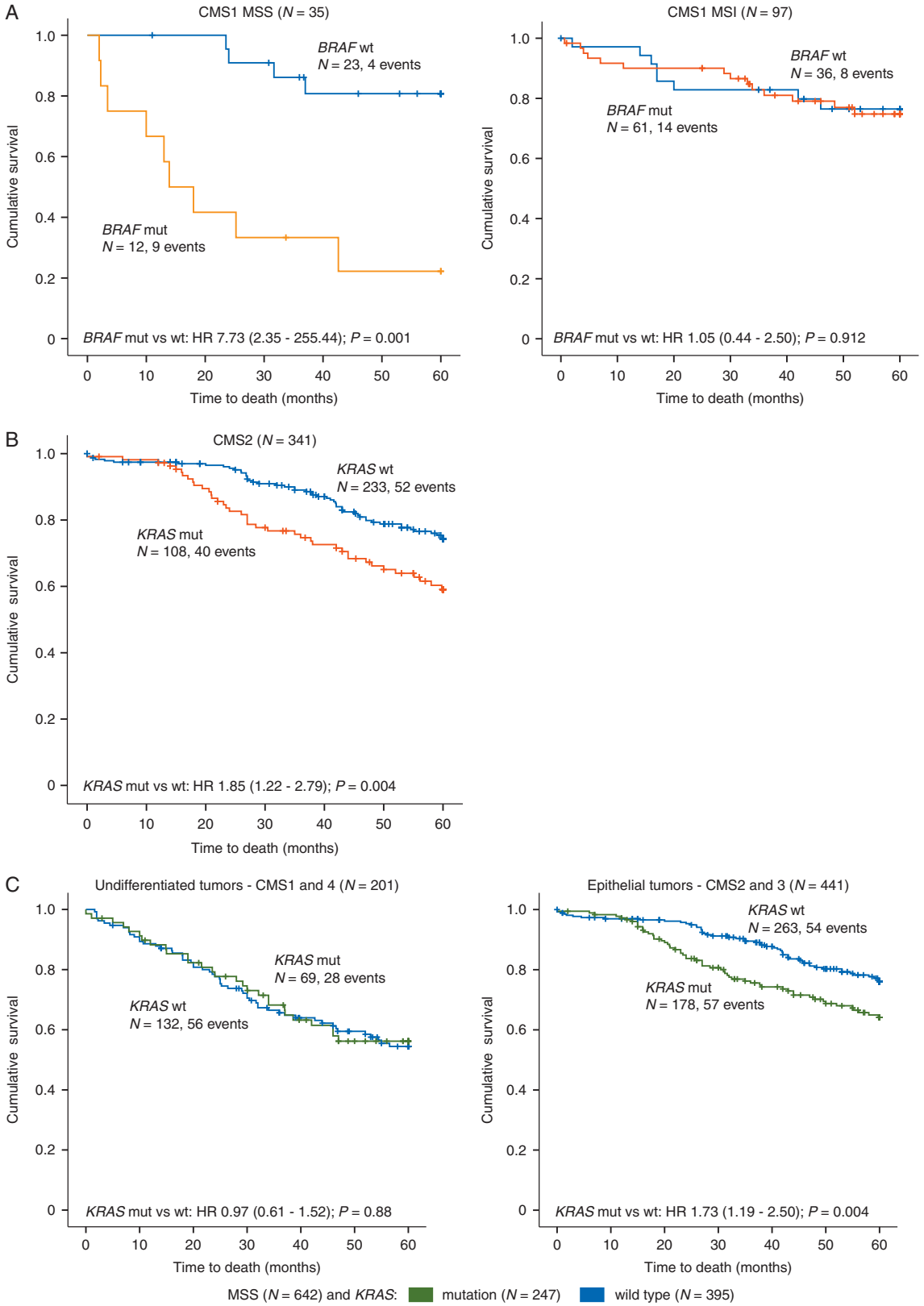

Figure 2. *BRAF*^{V600E} and *KRAS* mutations are associated with poor patient prognosis in specific CMS groups. (A) In 737 patients with stages I–IV CRC from two independent series (Oslo-series and GSE39582), 35 (5%) had MSS tumors of the CMS1 subtype. Among these patients, *BRAF*^{V600E} mutations were associated with a poor OS (left panel). No prognostic impact of *BRAF*^{V600E} mutations was seen in 97 patients with MSI tumors of the CMS1 subtype. (B) In the same set of patients, 341 (46%) had MSS tumors of the CMS2 subtype. Here, *KRAS* mutations were associated with a poor survival. (C) Analyzing undifferentiated (CMS1 and 4) and epithelial (CMS2 and 3) tumors within the MSS phenotype revealed *KRAS* mutations to have poor prognostic impact limited to epithelial tumors.

clinical setting. The biological mechanism for *KRAS* mutations having stronger prognostic significance in CMS2 than CMS3 is unclear, and may partially be explained by the limited number of patients in the MSS CMS3 subgroup. We hypothesize that *KRAS* dependency is a hallmark of most CMS3 tumors regardless of mutation status, as indicated by the lesser effect of *KRAS* mutations on its corresponding gene expression signature in CMS3 compared with CMS2. Furthermore, mutations outside the known hotspots analyzed in this study, in addition to in *NRAS* and *HRAS*, may be differentially distributed across the CMS groups and could influence the survival analysis. However, the CMS2 subgroup is particularly sensitive to EGFR blockade in preclinical models [29], indicating that this subtype is particularly susceptible to alterations in this signaling pathway. Negative prognostic value of *KRAS* mutations in CMS2 may also be explained by recent results showing reduced immune reactivity in this patient subgroup [30].

The efficacy of targeting the MAP kinase pathway in $BRAF^{V600E}$ mutant CRC has remained inferior to results in malignant melanoma [31]. Still, such treatment is active in a subset of CRC patients, in particular when combined with EGFR and/or MEK inhibition, but no molecular characteristics predicting treatment efficacy have been identified. Our finding of $BRAF^{V600E}$ mutations potentially having varying prognostic effect according to the CMS and MSI status possibly reflects phenotype-dependent oncogenic impact, and could point to a biological association with predictive relevance.

For both *KRAS* and $BRAF^{V600E}$ mutations, the magnitude of the prognostic effect is gradually increased as analysis is performed within the biologically relevant subgroups, emphasizing the clinical relevance of integrated molecular models for prognostic assessment. $BRAF^{V600E}$ mutations have stronger prognostic effect than *KRAS* mutations, and this study clearly indicated $BRAF^{V600E}$ mutations to be a more crucial oncogenic driver with pronounced transcriptomic and prognostic consequences, when analyzed within the correct phenotype. However, a caveat of performing biomarker analysis within increasingly stratified subgroups is the corresponding reduction in sample size and statistical power. Notably, multiple testing is not corrected for in our study. $BRAF^{V600E}$ mutations are infrequent among MSS tumors, and the lacking prognostic impact of $BRAF^{V600E}$ mutations in CMS2-4 could be due to the low numbers of mutated tumors within these subtypes. The differential prognostic impact of $BRAF^{V600E}$ mutations according to MSI status within CMS1 is more convincing. This supports the notion that the prognostic effect of these mutations depends more on the mutator phenotype than the extent of immune infiltration.

Conclusion

In conclusion, by incorporation of CMS classification, novel subtype-specific prognostic associations of the extensively studied *KRAS* and $BRAF^{V600E}$ mutations in primary CRC were indicated. However, due to the small sample sizes within certain subgroups, the results must be interpreted with caution. If validated, these findings could have clinical implications, and suggest relevance of interpreting the prognostic and predictive value of molecular aberrations within the context of gene expression–based subtypes in biomarker research.

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Disclosure

The authors have declared no conflicts of interest.

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