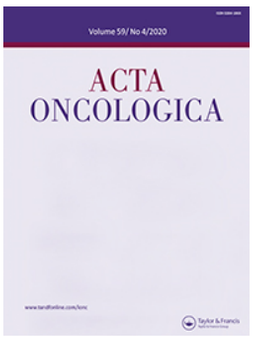

Acta Oncologica

ISSN: 0284-186X (Print) 1651-226X (Online) Journal homepage: https://www.tandfonline.com/loi/ionc20

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To cite this article: Luís Nunes, Kristine Aasebø, Lucy Mathot, Viktor Ljungström, Per-Henrik Edqvist, Magnus Sundström, Anca Dragomir, Per Pfeiffer, Adam Ameur, Fredrik Ponten, Artur Mezheyeuski, Halfdan Sorbye, Tobias Sjöblom & Bengt Glimelius (2020) Molecular characterization of a large unselected cohort of metastatic colorectal cancers in relation to primary tumor location, rare metastatic sites and prognosis, Acta Oncologica, 59:4, 417-426, DOI: 10.1080/0284186X.2019.1711169

To link to this article: https://doi.org/10.1080/0284186X.2019.1711169

ACTA ONCOLOGICA
2020, VOL. 59, NO. 4, 417–426
https://doi.org/10.1080/0284186X.2019.1711169

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ORIGINAL ARTICLE

Molecular characterization of a large unselected cohort of metastatic colorectal cancers in relation to primary tumor location, rare metastatic sites and prognosis

Luís Nunes[a], Kristine Aasebø[b], Lucy Mathot, Viktor Ljungström[a], Per-Henrik Edqvist[a], Magnus Sundström[a], Anca Dragomir[a,c], Per Pfeiffer[d], Adam Ameur[a], Fredrik Ponten[a], Artur Mezheyeuski[a], Halfdan Sorbye[b,e], Tobias Sjöblom[a] and Bengt Glimelius[a]

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ABSTRACT

Background: We have reported that *BRAF* V600E mutations and microsatellite instability-high (MSI-H) are more prevalent in a population-based cohort of metastatic colorectal cancer (mCRC) patients than has been reported from clinical trials or hospital-based patient groups. The aim was to explore if other mutations in mCRC differ in prevalence between these cohorts in relation to mismatch repair status and primary tumor location and if presence of bone or brain metastases is associated with any mutations.

Material and methods: A population-based cohort of 798 mCRC patients from three regions in Scandinavia was used. Forty-four cancer related genes were investigated in a custom designed Ampliseq hotspot panel. Differences in survival were analyzed using the Kaplan–Meier estimator and the Cox regression analysis.

Results: Determination of mutations was possible in 449/501 patients for 40/44 genes. Besides *BRAF* V600E, seen in 19% of the tumors, none of the other mutations appeared more prevalent than in trial cohorts. *BRAF* V600E and MSI-H, seen in 8%, were associated with poor prognosis as was right-sided primary tumor location (39%) when compared to left-sided and rectum together; however, in a multivariable regression, only the *BRAF* mutation retained its statistical significance. No other mutations were associated with poor prognosis. ERBB2 alterations were more common if bone metastases were present at diagnosis (17% vs. 4%, $p = .011$). No association was found for brain metastases. Fifty-two percent had an alteration that is treatable with an FDA-approved targeted therapy, chiefly by EGFR-inhibitor for RAS wild-type and a check-point inhibitor for MSI-H tumors.

Conclusions: Right-sided tumor location, *BRAF* V600E mutations, but no other investigated mutation, and MSI-H are more commonly seen in an unselected cohort than is reported from clinical patient cohorts, likely because they indicate poor prognosis. Half of the patients have a tumor that is treatable with an already FDA-approved targeted drug for mCRC.

Abbreviations: CRC: colorectal cancer; mCRC: metastatic colorectal cancer; MSI-H: microsatellite instability-high; EGFR: epidermal growth factor receptor; MSI: microsatellite instability; TMA: tissue microarray; IHC: immunohistochemistry; PCR: polymerase chain reaction; MSS: microsatellite stability; MSI-L: microsatellite instability-low; SISH: silver-enhanced *in situ* hybridization; CEP17: INFORM Chromosome 17; OS: overall survival; VAF: variant allele frequency

ARTICLE HISTORY
Received 6 September 2019
Accepted 26 December 2019

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer globally [1]. Previous studies have sought to discover prognostic and predictive somatic mutations for novel treatments in CRC by genomic sequencing [2,3]. This is particularly important for metastatic CRC (mCRC) patients that have the poorest survival, median between 10 and 12 months in the general population and up to 30 months in selected patient groups [4,5]. It is important that exploratory studies not only focus on the fittest patients, i.e., those suitable for trial inclusion, but instead look at the entire disease population.

We have shown that *BRAF* V600E mutations [6] and microsatellite instability-high (MSI-H) [7] are more common in an unselected population of Scandinavian patients with mCRC

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Supplemental data for this article can be accessed here.

than in patient groups derived from clinical trials or specialized hospitals (21% *BRAF* [6] mutated in the population vs. 5–12% [8–10]; and 7% MSI-H [7] vs. 3–4% [10,11]). A likely reason for this is that patients with tumors harboring *BRAF* mutations or MSI-H have a poor prognosis with short survival [12,13]; they often fail to be included in trials or are not referred to specialized hospitals. For decades, the generally held view was that the primary tumor location was not important beyond separating colon from rectum. However, this has recently emerged as a prognostic factor in mCRC and as a predictive factor for treatment with epidermal growth factor receptor (*EGFR*) inhibitors [14]. In a meta-analysis of 14 first-line studies, the proportion of right-sided tumors varied between 18 and 36% [15]. Several reports claim that right-sided tumors have a worse prognosis and require different treatment upfront, beyond the information provided by investigation of RAS, *BRAF* and microsatellite instability (MSI). However, most of this evidence has been obtained from trial patients and real-world evidence is limited [16].

The primary purpose of this study was to explore whether mutation prevalence, in known cancer genes in an unselected population differs from that reported in trial populations, and if the location of the primary tumor is prognostic in a population-based cohort. Further, the unselected material allows exploration of mutations related to two uncommon metastatic sites in mCRC, bone and brain, which are frequently underrepresented in clinical trials. Finally, we wanted to examine how frequent other molecular changes of potential interest for targeted therapy are in a population-based material.

Materials and methods

Patient cohort

The cohort represents an unselected population of all non-resectable mCRC patients diagnosed in three regions in Scandinavia (Uppsala, Sweden; Odense, Denmark; Bergen, Norway), with an mCRC diagnosis between October 2003 and August 2006 [17]. An informed consent was signed by all patients expect for 49 patients identified from regional cancer registries [6]. All information was prospectively collected from the clinical records by clinicians and research nurses, subsequently anonymized and de-identified before analysis. Regional ethical committees in Norway, Sweden and Denmark approved the study as well permission to include patients not prospectively identified to make the cohort truly population-based.

Tissue retrieval, tissue microarray generation and DNA extraction

Hematoxylin–eosin stained slides from primary tumors or metastases were examined so representative tumor parts could be selected from the corresponding tissue blocks, and non-necrotic tumor areas with few other cells admixed marked. Tissue microarray (TMA) generation and DNA extraction were performed using 1 mm tissue cores from the original primary tumor block except in six patients that were from metastatic lesions. The Beecher Instruments Manual Tissue Arrayer MTA-1 was used to generate TMAs. DNA was recovered from 505 (63%) tissue cores by Recoverall Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA). The remaining cases had either no remaining cancer tissue or not enough material to take cores for research; these were all diagnosed using small colorectal biopsies or needle biopsies of metastatic lesions.

Microsatellite instability analysis

MSI status was determined via a combination of immunohistochemistry (IHC) and polymerase chain reaction (PCR) techniques, see Supplementary Methods.

ERBB2/HER2 *IHC and dual-color silver-enhanced* in situ *hybridization (SISH)*

Tumors were stained with a monoclonal antibody against human HER2, clone CL0268 (mouse), dilution 1:250 (Atlas Antibodies, Stockholm, Sweden). Protein expression was ranked from 1+ (weak intensity) to 3+ (strong intensity). Bright-field dual-color SISH analysis was performed for all TMAs using an automatic SISH staining device BenchMark ULTRA, according to manufacturer's instructions for INFORM HER2 DNA and INFORM Chromosome 17 (CEP17) probes (Ventana Medical Systems, Tucson, AZ, USA). HER2/CEP17 SISH signals were counted for all 2+ and 3+ IHC scored samples according to the guidelines for staining of gastric cancers. All samples with a HER2/CEP17 ratio $\geq$2.0 were considered to have amplified ERBB2 expression. Adjacent benign cells were used as controls. HER2 status was also determined accordingly to HERACLES diagnostic criteria for CRC [18].

Targeted sequencing and data analysis

A custom designed Ampliseq hotspot panel (Thermo-Fisher Scientific, Waltham, MA, USA) covering 194 amplicons from 44 cancer related genes was designed using Ion AmpliSeq Designer (Supplementary Table 1). The AmpliSeq panel was designed using specific settings customized for FFPE samples. Sequencing libraries were prepared from 10 ng of genomic DNA according to the Ion AmpliSeq Library Kit 2.0 user guide and quantified using the Agilent Bioanalyzer instrument and the Agilent High Sensitivity DNA kit. Emulsion PCR, enrichment and chip loading were performed on the Ion Chef system using the Ion PI Chef Kit (Thermo Fisher Scientific, Waltham, MA, USA). The samples were sequenced on the Ion Proton System using an Ion PI chip and 200 bp chemistry. Data analysis and variant calling were performed as described in Supplementary Methods. Somatic alterations were defined in tiers according to OncoKB levels of evidence [19] and presented in a figure made with Oncoprinter [20,21].

Statistical analyses

Fisher's exact test was performed for group comparisons and p value $< .05$ was considered statistically significant. Overall survival (OS) was the time between the dates of diagnosis of metastatic disease and death or censored for patients alive in February 2014. The Kaplan–Meier estimator and the Cox multiple regression were used for OS analysis. Statistical analyses were performed using R software, version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Cohort and mutation characterization

The study included 798 patients, of which 701 patients had surgical specimens with invasive cancer. Tissue cores could be generated from 505 patients (Supplementary Figure 1). The sequenced patients were representative of the entire cohort, by age and sex distribution, and primary tumor location (Supplementary Table 2). Sequencing data were obtained from 501 tumors at a median amplicon coverage per tumor of 4,801. *EZH2* had the highest amplicon coverage (median 19,084) while *PTEN* had the lowest coverage (median 2,194) (Supplementary Table 3). Due to the high overall coverage, we used a stringent cutoff of 1,000-fold average coverage; 95% (476/501) of the samples met this criterion. After applying the filters described in Supplementary Methods, 411/449 (92%) patients carried at least one mutation in 40/44 genes sequenced (*EZH2*, *HNF1A*, *MPL* and *SRC* had no mutations). In total, 1,249 somatic nonsynonymous single-nucleotide variants and indels were identified (40 frameshift deletions, 33 frameshift insertions, four non-frameshift deletions, 984 missense and 188 nonsense point mutations), corresponding to 437 unique and 142 recurrent mutations within the set. The most recurrent hotspot mutation was *BRAF* V600E (86 cases) followed by *KRAS* G12D and G12V with 60 and 40 cases, respectively. From the gene panel, 6/44 genes were mutated in more than 10% of the patients, namely *TP53* (242/449; 54%), *KRAS* (201/449; 45%), *APC* (155/449; 35%), *BRAF* (93/449; 21%), *PIK3CA* (84/449; 19%) and *SMAD4* (56/449; 12%) (Figure 1). To identify significantly mutated genes, we fit a linear regression model using the total number of sequenced base pairs and total number of mutations for each gene. Ten genes, *KRAS*, *BRAF*, *TP53*, *APC*, *CTNNB1*, *PIK3CA*, *AKT1*, *NRAS*, *SMAD4* and *FBXW7* had a higher mutation rate than expected by chance (Supplementary Figure 2).

Microsatellite instability

By combining the data from IHC and PCR genotyping, we divided the cohort in MSI-H (36/449; 8%) and MSS/MSI-L phenotypes (413/449; 92%), referred to as MSS. As expected, MSI-H patients were predominately females with poorly or undifferentiated tumors located in the right colon (Table 1) [11]. They differed in metastatic pattern from MSS tumors with fewer liver (36% vs. 66%, $p < .001$) and lung (6% vs. 26%, $p = .004$) metastases but more lymph node metastases (53% vs. 28%, $p = .004$). Overall, 64% of the patients received chemotherapy with no significant difference between MSI-H and MSS tumors. OS was shorter in patients with MSI-H tumors (6 vs. 13 months, $p = .006$), even when only considering patients receiving chemotherapy (11 vs. 19 months, $p = .013$, Figure 2(A,B)). Complete or partial response to first-line treatment was higher for the MSS cases (43% vs. 5%, $p < .001$, Table 1). As expected, the MSI-H patients had a higher *BRAF* mutation prevalence (75% vs. 16%, $p < .001$, Supplementary Table 4) and OS was shorter if the tumor was *BRAF* mutated (Figure 2(C,D)). From the significantly mutated genes, only *APC* (37% vs. 11%, $p = .002$) and *KRAS* (48% vs. 11%, $p < .001$) had significantly higher mutation prevalence in MSS compared to MSI-H tumors (Supplementary Table 4). Aside from *BRAF*, no other significantly mutated gene was associated with poor prognosis even when considering first-line treated patients only (Supplementary Figure 3).

Gene alterations according to primary tumor location, age and selected metastatic sites

In the cohort of 449 patients with sequenced tumors, 38% were right-sided (right colon and transversum), 35% left-sided (left colon and sigmoideum) and 25% were rectal tumors. The patients with right-sided tumors were more likely to be older, female and have poorly differentiated tumors with lymph node and peritoneal metastases (Table 2). The MSI-H phenotype was more frequent in right-sided tumors (18% vs. 1% and 3% for left-sided and rectal tumors, respectively, $p < .001$). Similarly, *BRAF* mutation frequency was higher in right-sided tumors but decreased throughout the left colon and rectum (38% vs. 14% vs. 5%, $p < .001$). Also, the *PIK3CA* mutation frequency was highest in the right colon, followed by rectal tumors and lowest in the left colon (24% vs. 17% vs. 13%, $p = .044$). No other gene mutation prevalence differed significantly by primary tumor location. No gene mutation prevalence, aside from *AKT1*, was influenced by age (Supplementary Table 5). In all patients (and in patients treated with chemotherapy, for choice of chemotherapy, see Supplementary Figure 4), no significant differences could be detected for OS according to tumor sidedness based on MSI expression and *BRAF* mutation status (Figure 2(E,F); Table 2). When right-sided colon tumors were compared with left-sided colon and rectum tumors together, median OS was 10 months vs. 14 months for all patients ($p = .046$) and 15 months vs. 18.5 months ($p = .270$) for patients that received first-line treatment. In a multiple Cox regression analysis, including MSI status, *BRAF* mutation status, primary tumor location (right colon vs. left colon and rectum together) and whether the patient received first-line treatment, only *BRAF* mutation was significantly associated with reduced OS, while receiving first-line treatment was associated with an increased OS (Supplementary Figure 5).

Patients with bone metastases at time of diagnosis of metastatic disease ($n = 30$, 7%) more often had a rectal primary (54%) than a colon primary (29% right-sided and 18% left-sided, $p = .004$), and more often lung ($p = .049$) and multiple site metastases ($p < .001$), but less often liver metastases

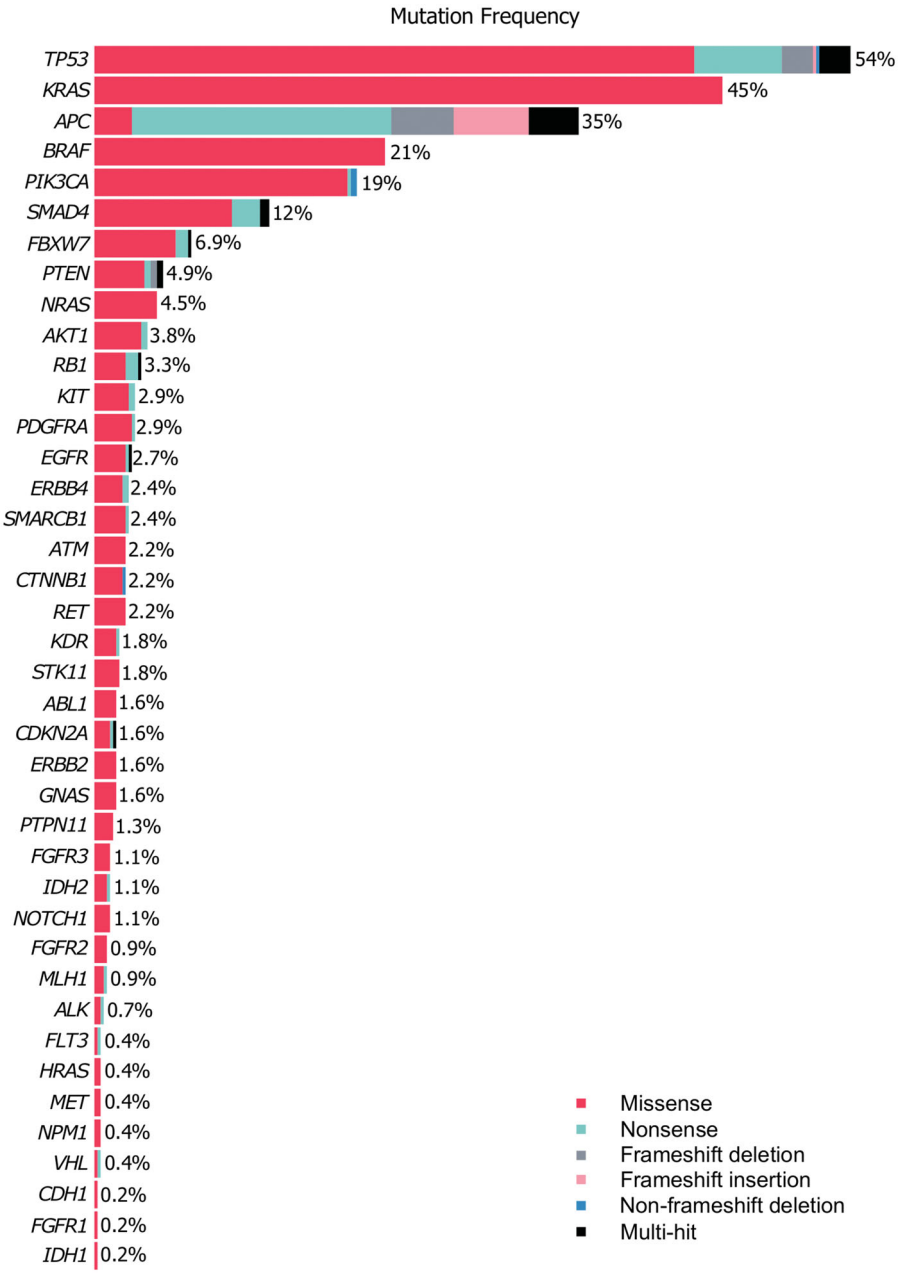

Figure 1. Frequency of altered genes in a Scandinavian unselected cohort of metastatic colorectal cancer by type of mutation.

($p = .009$). They also received radiotherapy more often than patients without simultaneous bone metastases (57% vs. 7%, $p < .001$). Patients with bone metastases had poorer performance status and their OS was significantly shorter (8 vs. 12 months, $p = .015$). None of the patients with simultaneous bone metastases had MSI-H tumors, but two (6%) patients with MSI-H tumors later developed bone metastases. Thirty-nine (10%) patients with MSS tumors without simultaneous bone metastases later developed bone metastases. Furthermore, ERBB2 alteration was more often seen in patients with simultaneous bone metastasis (17% vs. 4%, $p = .011$, Supplementary Table 6).

No patient had brain metastases at the time of diagnosis of their primary tumor. Of the 26 patients who developed brain metastases after a median of 20 months (range 1–139), one (3%) developed in a patient with an MSI-H tumor and 25 (6%, $p = .711$) in patients with MSS tumors. Lung metastases were more common for the patients with brain metastases (54% vs. 23%, $p = .001$) and no gene alteration frequency differed between these groups (Supplementary Table 7).

Clinically actionable alterations

KRAS was the most altered oncogene with 45% of the tumors having at least one oncogenic alteration. Five MSS patients had two co-occurring *KRAS* mutations, where one of them had two known oncogenic mutations (G13D and A18T with variant allele frequency (VAF) of 12%, Supplementary Figure 6A). *NRAS* was altered in 4.5% of the cases. *BRAF* was

Table 1. Comparison of patient and tumor characteristics between patients with MSS and MSI-H tumors.

Characteristics	MSS patients ($n = 413$) *n* (%)	MSI-H patients ($n = 36$) *n* (%)	*p* Value	Missing
Age (years)				
Mean + S.D.	68.4 + 12.3	71.0 + 14.0		
Median (range)	68 (24–96)	74.5 (22–85)		
>75 years	132 (32)	17 (47)	ns (.067)	
Sex				
Male	214 (52)	11 (31)	.015	
Female	199 (48)	25 (69)		
Primary tumor location				
Right colon	141 (34)	30 (83)	<.001	
Left colon	155 (38)	2 (6)		
Rectum	111 (27)	3 (8)		
Multiple[a]	6 (1)	1 (3)		
Synchronous metastases	213 (52)	24 (67)	ns (.116)	
Tumor grade				
Grade 1–2: well-medium differentiated	295 (82)	12 (36)	<.001	55
Grade 3–4: poorly-undifferentiated	66 (18)	21 (64)		
Primary tumor resected	377 (91)	33 (92)	ns (1.000)	
Metastases[b]				
Liver	273 (66)	13 (36)	<.001	
Lymph node	115 (28)	19 (53)	.004	
Lung	108 (26)	2 (6)	.004	
Peritoneum	78 (19)	6 (17)	ns (1.000)	
Abdominal mass	31 (8)	2 (6)	ns (1.000)	
Bone	30 (7)	0 (0)	ns (.156)	
Skin	8 (2)	1 (3)	ns (.532)	
Local relapse	24 (6)	5 (14)	ns (.072)	
Other soft tissue	12 (3)	2 (6)	ns (.311)	
Performance status				
0–1	283 (69)	21 (58)	ns (.264)	1
2–4	129 (31)	15 (42)		
Weight loss >5%	155 (41)	21 (62)	.019	33
Best supportive care	120 (29)	14 (39)	ns (.255)	1
1st-line chemotherapy	269 (65)	19 (53)	ns (.150)	
5-FU monotherapy	50 (12)	6 (17)	ns (.429)	
Combination chemotherapy[c]	211 (51)	13 (36)	ns (.117)	
2nd-line chemotherapy	159 (39)	7 (19)	.030	1
3rd-line chemotherapy[d]	76 (18)	1 (3)	.011	2
1st-line response rate				
CR/PR	103 (43)	1 (5)	<.001	193
SD	94 (40)	10 (53)		
PD	40 (17)	8 (42)		
Development of brain metastases	25 (6)	1 (3)	ns (.711)	1
Development of bone metastases[e]	59 (14)	2 (6)	ns (.204)	2
Last status				
Alive	18 (4)	1 (3)	ns (1.000)	
Dead	395 (96)	35 (97)		
Median overall survival (95% CI) all patients	13 m (11.3–14.7)	6 m (3.1–8.9)	.006	
Received 1st-line treatment	19 m (16.3–21.7)	11 m (6.8–15.2)	.013	

[a]Multiple: rectum and colon locations.
[b]Metastases: at time of diagnosis of metastatic disease.
[c]Combination chemotherapy: irinotecan or oxaliplatin with a fluoropyrimidine (FU).
[d]Patients that received 3rd-line chemotherapy or more (until 5th-line).
[e]30/59 patients had bone metastasis already at diagnosis of metastatic disease.
ns: not significant; S.D.: standard deviation; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; *p* value: Fisher's exact test for dichotomous and nominal variables and the log-rank test for survival times.

mutated in 21% of the cohort and the most common *BRAF* alteration was V600E, observed in 19% of tumors. One MSS patient had a complex substitution (c.1798_1799GT > AG) in *BRAF* that led to a rare V600R alteration. Non-V600 *BRAF* mutations were present in six cases and further classified according to [22]; G469R, a class 2 mutation leading to intermediate kinase activity; G466E (two cases), D594G and D594N, class 3 alterations leading to no kinase activity; and V590I of undefined non-V600 class described in one previous salivary gland tumor [23]. Two of the class 3 alterations, observed in colon cancers were co-mutated with *KRAS*, while two were present in rectal cancers without *KRAS* co-mutation. Two patients had two co-occurring *BRAF* mutations, where one of them presented two neighboring oncogenic mutations (V600E and K601N with VAF of 33% and 26%, respectively, Supplementary Figure 6B). PIK3CA was altered in 84 (19%) patients. In total, 15 (3.3%) patients were scored 2+ or 3+ by IHC and had a positive SISH analysis for ERBB2 according to the breast and gastric cancer criteria, while 12 (2.6%) had this when considering HERACLES CRC diagnostic criteria. One-third of the patients had a co-occurring *KRAS* mutation irrespective of what criteria was used (Supplementary Table 8). *ERBB2* mutation was found in seven additional cases.

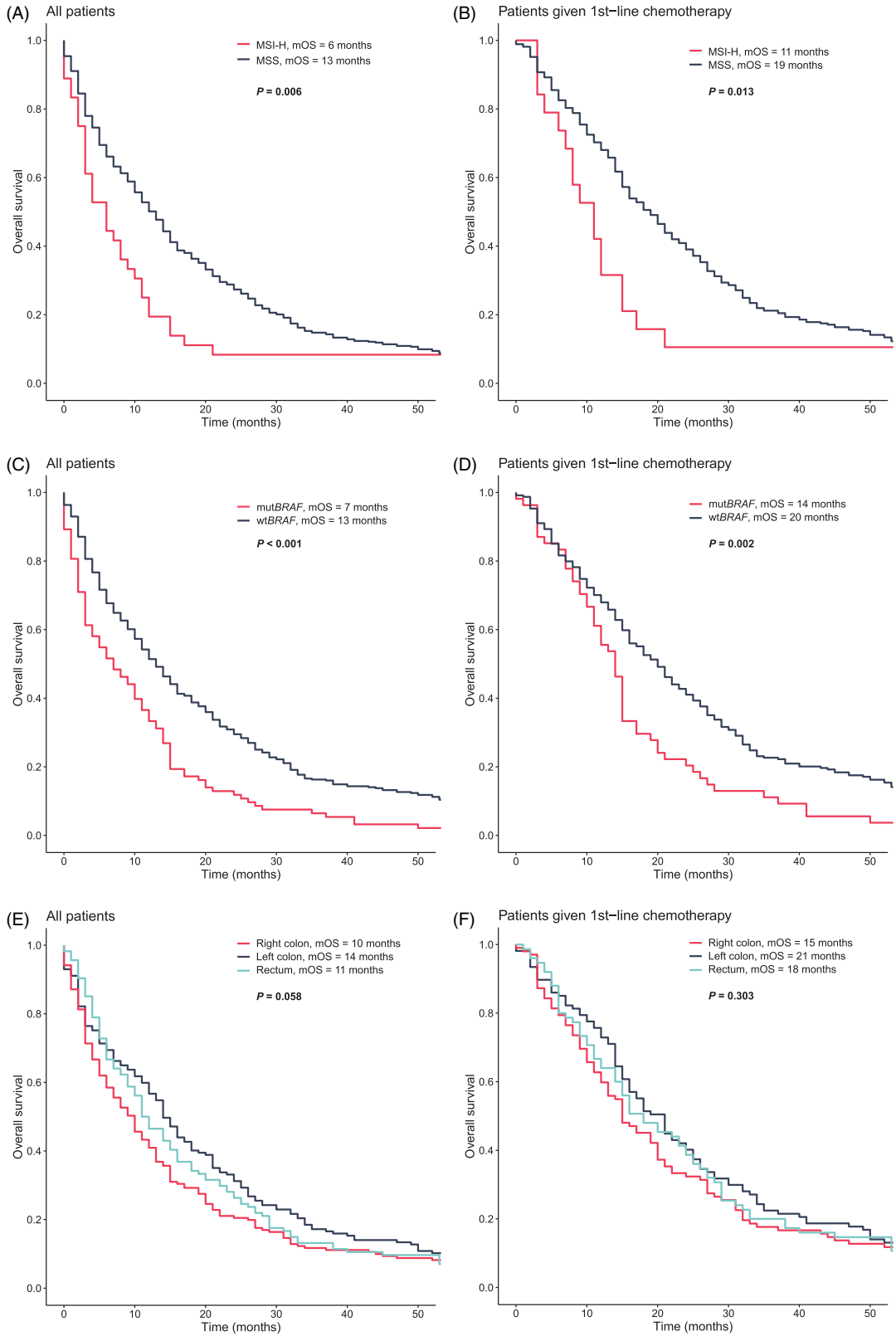

Figure 2. The Kaplan–Meier analysis of overall survival (OS) for all patients and for patients given 1st-line chemotherapy treatment according to MSI status, *BRAF* mutation and primary tumor location. *p* value was calculated with log-rank test. (A) OS for all patients and (B) for patients given 1st-line chemotherapy by MSI status. (C) OS for all patients and (D) for patients given 1st-line chemotherapy by *BRAF* mutation status. (E) OS for all patients and (F) for patients given 1st-line chemotherapy by primary tumor location.

Table 2. Comparison of patient characteristics and altered genes by primary tumor location (seven patients had multiple sites).

Characteristics	Right colon ($n = 171$) *n* (%)	Left colon ($n = 157$) *n* (%)	Rectum ($n = 114$) *n* (%)	*p* Value
Age (years)				
Mean + S.D.	71.9 + 10.2	65.9 + 13.2	67.1 + 12.9	
Median (range)	73 (47–96)	65 (24–92)	68 (22–93)	
>75 years	70 (41)	39 (25)	37 (32)	.008
Sex				
Female	100 (58)	78 (50)	42 (37)	.002
MSI status				
MSS	141 (82)	155 (99)	111 (97)	<.001
MSI-H	30 (18)	2 (1)	3 (3)	
Synchronous metastases	93 (54)	100 (64)	40 (35)	<.001
Tumor grade[a]				
Grade 1–2: well-medium differentiated	104 (66)	117 (84)	81 (89)	<.001
Grade 3–4: poorly undifferentiated	53 (34)	23 (16)	10 (11)	
Metastases[b]				
Liver	99 (58)	118 (75)	63 (55)	<.001
Lymph node	63 (37)	44 (28)	26 (23)	.033
Lung	31 (18)	43 (27)	35 (31)	.031
Peritoneum	46 (27)	23 (15)	12 (11)	.001
Abdominal mass	15 (9)	12 (8)	5 (4)	ns (.363)
Bone	8 (5)	5 (3)	15 (13)	.004
Skin	6 (4)	1 (1)	2 (2)	ns (.242)
Local relapse	7 (4)	7 (4)	15 (13)	.008
1st-line treatment	102 (60)	107 (68)	75 (66)	ns (.259)
Combination chemotherapy[c]	70 (41)	90 (57)	61 (54)	.008
Median overall survival (95% CI) all patients[d]	10 m (7.6–12.4)	14 m (11.5–16.5)	11 m (8.2–13.8)	ns (.058)
Received 1st-line treatment[e]	15 m (10.8–19.2)	21 m (17.3–24.7)	18 m (11.4–24.6)	ns (.303)
MSI-H	4 m (1.0–7.2)	2 m	9 m (0–23.4)	ns (.609)
MSS	10 m (7.5–12.5)	15 m (12.8–17.1)	12 m (9.1–14.9)	ns (.163)
BRAF mutated	6 m (2.6–9.4)	14 m (8.4–19.7)	2 m (1–3.1)	ns (.122)
Mutations (significantly mutated genes)				
KRAS	76 (44)	67 (43)	27 (47)	ns (.818)
BRAF	65 (38)	22 (14)	6 (5)	<.001
TP53	93 (54)	83 (53)	62 (54)	ns (.962)
APC	57 (33)	50 (32)	45 (40)	ns (.395)
CTNNB1	3 (2)	4 (3)	3 (3)	ns (.850)
PIK3CA	41 (24)	21 (13)	19 (17)	.044
AKT1	8 (5)	7 (5)	2 (2)	ns (.443)
NRAS	4 (2)	10 (6)	6 (5)	ns (.171)
SMAD4	22 (13)	18 (12)	14 (12)	ns (.932)
FBXW7	12 (7)	8 (5)	11 (10)	ns (.350)
ERBB2 alterations				
Mutation	1 (1)	1 (1)	5 (4)	.030
Mutation + amplification	7 (4)	5 (3)	10 (9)	ns (.106)
BRAF non-V600 mutations[f]	2 (1)	1 (1)	2 (2)	ns (.852)

[a]Missing for 54 patients.
[b]Metastases: at time of diagnosis of metastatic disease.
[c]Combination chemotherapy: irinotecan or oxaliplatin with a fluoropyrimidine.
[d]If right-sided colon cancers were compared with left-sided colon and rectum together, median survival was 10 months (7–12) vs. 14 months (12–16), $p = .046$.
[e]If right-sided colon cancers were compared with left-sided colon and rectum together, median survival was 15 months (13–20) vs. 18.5 months (16–23), $p = .270$.
[f]*BRAF* non-V600 mutations with defined classification.
ns: not significant; S.D.: standard deviation; *p* value: Fisher's exact test for dichotomous and nominal variables and the log-rank test for survival times.

Discussion

Numerous studies have explored the genomic and phenotypical heterogeneity in mCRC, but almost exclusively from selected trial or hospital-based patient series not representative of the general population. We evaluated genomic properties in almost 450 mCRC tumors from an unselected population-based cohort where all diagnosed individuals with mCRC not immediately possible to resect were identified. We could substantiate that *BRAF* V600E mutations and MSI-H are about twice as common as in previously reported datasets. While other studies commonly report about 6–8% (range 5–12%) *BRAF* V600E mutations [24] and 3–4% MSI-H tumors [11], this study revealed 19% *BRAF* V600E mutations and 8% MSI-H. The likely explanation for this is the poor prognosis of these patient groups; therefore, they are underrepresented in patient materials from clinical trials or hospital-based cohorts where patients must fulfill certain inclusion. The lower than expected mutation frequency in two genes, *APC* and *TP53*, 35% vs. expected 80% and 54% vs. expected 65%, respectively can likely be explained by incomplete coverage of these genes in the gene panel design where only the main mutation driver cluster regions were covered as previously published by Overman et al. [25].

None of the other known somatic mutations of potential interest in mCRC, including *PIK3CA* mutations and ERBB2 expression, were more prevalent in this patient group compared to other cohorts, indicating that the prognosis for those groups is not particularly poor. Since both *BRAF* V600E mutation and MSI-H phenotype are indicators of poor

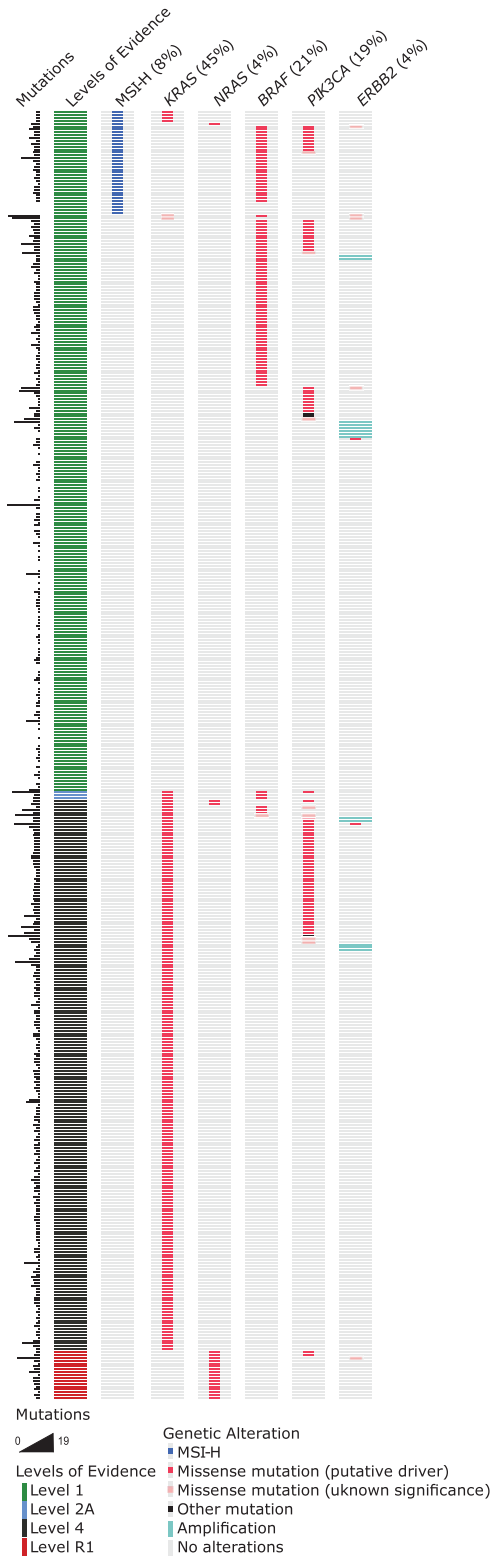

Figure 3. Distribution of patients with an FDA-approved (237/449, 53%) or potentially actionable alteration in an unselected cohort of metastatic colorectal cancers. Total amount of mutations, OncoKB levels of evidence, MSI status and mutation status of selected genes are represented in different columns for all patients. Each row represents one patient. Color coding indicates the type of event in each column. Patients in rows indicated with level 1 evidence have at least one FDA-approved biomarker that responds to an FDA-approved drug (RAS wild-type, EGFR-inhibitor; MSI-H, checkpoint inhibitor); patients in level 2A have at least one standard of care biomarker predicted to respond to an FDA-approved drug (*BRAF* V600E, BRAF-inhibitor); those in level 4 have a biomarker with predictive compelling biological evidence to respond to a drug (*KRAS* mutation, MEK-inhibitor) and level those in R1 have a standard care biomarker that is resistant to an FDA-approved drug (*NRAS* mutation, EGFR-inhibitor).

prognosis and poor response to conventional chemotherapy, there is an urgent need to determine these properties at the time of diagnosis. The very poor prognosis for patients with MSI-H tumors is likely a combination of a more aggressive tumor and a poorer response to therapy. The very poor response to first-line treatment in patients with MSI-H tumors reported here, underlines the importance of evaluating immunotherapy upfront [26].

In contrast to most recent studies in mCRC, this study could not substantiate worse survival for right-sided compared to left-sided tumors [27,28]. In this study and a Canadian population study [16], we find that about 40% of patients with mCRC have a right-sided tumor. The two mCRC subgroups with the worst prognosis, MSI-H and *BRAF*-mutated tumors are all more common in right-sided tumors. Whether the poor prognosis of right-sided tumors is solely caused by these molecular properties is still an unresolved question, but it has been suggested that even if there is no overactivation of the MAPK signaling pathway, right-sided tumors should not be treated with EGFR-inhibitors [14]. The Canadian study also noticed poorer prognosis for treated patients (47% received chemotherapy) with right-sided tumors but did not report any molecular analyses. Interestingly, in this cohort, *BRAF* and *PIK3CA* were the only genes with significantly higher mutation prevalence in right-sided tumors, as previously reported [27,29]. However, we did not observe that any other gene was associated with sidedness, which could be due to the unselected nature of this cohort.

Wild-type *KRAS* and *NRAS* are FDA-approved biomarkers for administration of anti-EGFR antibodies [30–32], and 51% (230/449) of the cases in this study were wild-type for both genes. Further, *BRAF* and *PIK3CA* mutations or ERBB2 amplifications can also result in resistance to EGFR-inhibition [33] meaning that only about half (126/230) of the RAS wild-type patients are ideal candidates for this treatment. ERK and MEK inhibitors are studied as targeted drugs for RAS oncogenic mutations [34], as well as AMG 510 (NCT03600883), a novel small molecule *KRAS* G12C inhibitor potentially suitable for 2% (8/449) of our cohort. In mCRC, there is compelling clinical evidence for the use of encorafenib and cetuximab with binimetinib in patients with *BRAF* V600E mutation, meaning this could be a possible therapy for 19% (87/449) of this cohort [35]. *In vitro* evidence supports the use of PLX8394 for *BRAF* non-V600 alterations [36], present in five (1%) patients in this study. ERBB2 amplification assessment is routinely done in breast and esophagogastric cancers as these tumors can be targeted by FDA-approved drugs. We could detect ERBB2 amplification with SISH analysis in 3% (15/449) of the patients, translating to the percentage of mCRC patients that could benefit from targeted therapy. Increasing evidence has led to FDA approval of the use of immune checkpoint inhibitors in patients with MSI-H tumors, representing 8% (36/449) of our population [37]. To summarize, 53% (237/449) of our cohort could have potential benefit from an FDA-approved targeted therapy in mCRC, mainly based on the absence of overactive RAS-MAPK signaling (Figure 3).

In conclusion, aside from MSI-H phenotype and *BRAF* V600E mutations, no other molecular change was more, or less, prevalent than in selected cohorts. Both MSI-H and *BRAF* mutation are associated with poor prognosis, potentially explaining why these features are more prevalent than has previously been reported [8–11]. This may also explain why there is no difference in mutation prevalence for other genes included in this study, as no other mutation was associated with poor prognosis. However, the limited numbers for most of those mutations prevent us from making firm conclusions. Besides alterations in ERBB2, seen more frequently if bone metastases were present, we could not identify alterations associated with the rarer metastatic sites of bone or brain. Similar to previous studies, a rectal primary was more often associated with bone metastatic sites than colon cancer [38]. Finally, and opposed to a generally held view [27,28], we could not substantiate that right-sided tumors that do not harbor a *BRAF* mutation or an MSI-H phenotype have a worse outcome than left-sided tumors, whether receiving chemotherapy or not.

The patient material collected from 2003 to 2006 and the comparatively short OS could make one question the relevance of using this study to draw conclusions that are applicable to today's mCRC diagnosis and prognosis. However, the tumor panorama has not changed, and the OS reflects the population-based nature of the material. As such, it is likely unique. The median OS of 9 months (7.8–10.2) is heavily influenced by the non-actively treated patients (43% of all patients), due to their old age, presence of severe co-morbidities or very aggressive disease with poor performance status. The comparatively low median OS of 15 months (13.4–16.6) in the chemotherapy group can be explained by (i) the 22% that only received single fluoropyrimidine, (ii) the inclusion of patients with co-morbidities and laboratory abnormalities that disqualify them from trial participation and (iii) the exclusion of patients with upfront resectable metastatic disease. For patients eligible for trial participation, all three active cytotoxic drugs, bevacizumab and the EGFR-inhibitor cetuximab were available and used, resulting in a median OS of about 24 months [17], in line with what has been reported from clinical trials including mCRC patients [30,31,39]. The up to 30 months median OS reported in recent trials [4] reflects molecular selection and the inclusion of patients having resectable metastatic disease. Thus, we believe our prognostic associations are relevant for today's patients. Taken together, we present unbiased mutation frequencies in mCRC and estimate the true percentage of patients potentially eligible for targeted treatment in an unselected western-world mCRC cohort.

Disclosure statement

The authors declare no conflicts of interest.

Funding

This work was supported by the Swedish Cancer Society under Grant CAN 2016/447 and CAN 2018/1165.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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