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Correlation of the *KRAS* gene's copy number variation and the results of targeted therapy for colorectal cancer

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ABSTRACT *BACKGROUND: to find predictive value of KRAS gene's copy number variation (CNV_KRAS) to anti-EGFR therapy.*
PATIENTS AND METHODS: a prospective cohort single-center study included 150 patients, 103 patients with colorectal cancer (CRC) and wild-type RAS/BRAF, 39 patients with colorectal cancer with somatic mutations in the KRAS gene, as well as 8 non-oncological patients (as normal controls). CNV_KRAS was determined using digital droplet PCR.
RESULTS: the clinically significant CNV_KRAS level of ⩾ 9 copies established for a refusal of targeted anti-EGFR therapy. The incidence of clinically significant CNV_KRAS level in patients with wild-type RAS/BRAF was 17% (the first group of patients). Incidence of clinically significant CNV_KRAS level in patients with mutations in the KRAS gene was 3% (the second group of patients). At the I stage of CRC clinically significant CNV_KRAS was not detected in either the first or second group; at the stage II of CRC in the first group — in 14% of patients (3/22), and in the second group — not detected; at the stage III of CRC in the first group — in 21% of patients (8/39), and in the second group of patients — not detected; at the stage IV of CRC in the first group — in 17% (6/35) of patients, and in the second group of patients — in 5% (1/20). Tumor DNA was analyzed in 10 patients with the stage IV CRC from the first group who received anti-EGFR therapy to find out the clinically significant level of CNV_KRAS. Disease control was achieved in 7 out of 10 patients. The median CNV_KRAS score in the remaining three patients was higher than in the disease control group, 9.2 (9.05, 10.10) and 5.38 (4.77, 7.35) (p = 0.017).
CONCLUSIONS: detection of CNV_KRAS level of ⩾ 9 copies in a malignant colon tumor is a contraindication to targeted therapy. This phenomenon occurs significantly more often in patients without somatic mutations in the RAS genes (KRAS, NRAS) and BRAF, than in patients with point mutations in the KRAS gene (p = 0.02).

KEYWORDS: *colorectal cancer, copy number variation (CNV), KRAS gene, targeted therapy, anti-EGFR therapy, resistance to targeted therapy*

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BACKGROUND

Mutational status of the RAS family genes (*KRAS* (exons 2, 3, 4), *NRAS* (exons 2 and 3) and *BRAF* (exon 15), as well as *HER2-neu* amplification, is a prerequisite for anti-EGFR therapy in patients with stage IV colorectal cancer (CRC) [1,2]. Point mutations of the RAS family genes, the *BRAF* gene and amplification of the *HER2-neu* gene activate the MAPK-kinase pathway, which leads to uncontrolled cell division, impaired apoptosis, proliferation, malignant transformation and activation of the EGFR signaling receptor [3,4]. However, about 20% of patients with CRC, in whose tumors there are no mutations and amplification in these genes, do not respond to blocking the EGFR receptor with monoclonal antibodies [5]. There is probably an alternative mechanism for activating the

MAPK-kinase pathway. Xiong Q. et al. describe a clinical case in which a patient with stage IV CRC has got cetuximab due to the absence of point mutations in the RAS and *BRAF* genes. Before therapy, the number of copies of the *KRAS* gene in circulating tumor DNA was normal. After two cycles of therapy, positive dynamics were noted, but subsequently progression of the disease with liver metastases was found, while an increase in the number of copies of the *KRAS* gene by more than 3 times was detected in circulating tumor DNA [6]. Bontoux C. et al. studied the change in the number of gene copies in lung cancer and CRC using different methods of molecular genetic diagnostics: it was shown that some patients with colorectal cancer have a high value of the number of copies of the *KRAS* gene (CNV_ *KRAS*, from English "copy number variation") [7].

Thus, the hypothesis of the study is that high CNV_ *KRAS* value results in lack of response to anti-EGFR therapy.

PATIENTS AND METHODS

The single-center cohort study included 150 patients: 142 patients with stage I-IV colorectal cancer and 8 non-oncological patients. Informed consent was obtained from all patients included in the study. The study was approved by the local ethics committee. Patients with colorectal cancer were divided in 2 groups depending on the presence/absence of mutations in the RAS/*BRAF* genes (Table 1).

Isolation of DNA and measurement of its concentration. All tumors were morphologically selected and blocks with a tumor cell content of at least 50% were included in the study. DNA was isolated from the tumor sample using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA was isolated from blood leukocytes using the MagNa pure compact automatic station, using the MagNa Pure Compact Nucleic Acid Isolation Kit (Roche, Switzerland). DNA concentration was measured using the Denovix device, using the Qubit HS assay kit (ThermoFisher, Latvia). Samples with a concentration of ⩾ 5 ng/μl were included in the study.

Table 1. *The 142 patients with colorectal cancer studied*

Characteristic	1 group N = 103[1]	2 group N = 39[1]
Age	58 (45, 66)	59 (48, 68)
Gender		
F	50 (49%)	19 (49%)
M	53 (51%)	20 (51%)
Mutations RAS/*BRAF*		
WT	103 (100%)	0
KRASmut	0	39 (100%)*
Tumor site		
Right	21 (20%)	7 (18%)
Left	41 (40%)	16 (41%)
Rectum	41 (40%)	16 (41%)
T		
1,2	12 (12%)	0
3	60 (58%)	23 (59%)
4a	19 (18%)	10 (26%)
4b	12 (12%)	6 (15%)
N		
0	37 (36%)	12 (31%)
1a-c	42 (41%)	11 (28%)
2 a,b	24 (23%)	16 (41%)
M		
0	68 (66%)	19 (49%)
1a-c	35 (34%)	20 (51%)
Tumor stage		
I	7 (7%)	0
II	22 (21%)	10 (26%)
III	39 (38%)	9 (23%)
IV	35 (34%)	20 (51%)
[1]Median (IQR); *n* (%)		

** — c.35G > A (p.Gly12Asp) — 13 (33%), c.34G > T (p.Gly12Cys) — 8 (21%), c.35G > C (p.Gly12Ala) — 4 (10%), c.35G > T (p.Gly12Val) — 2 (5.1%), c.34G > A (p.Gly12Ser) — 2 (5.1%), c.436G > A (p.Ala146Thr) — 2 (5.1%), c.182A > G (p.Gln61Arg) — 2 (5.1%), c.181C > A (p.Gln61Lys) — 1 (2.6%), c.182A > C (p.Gln61Pro) — 1 (2.6%), c.183A > C (p.Gln61His) — 1 (2.6%), c.34G > C (p.Gly12Arg) — 1 (2.6%), c.351A > T (p.Lys117Asn) — 1 (2.6%), c.351A > T (p.Lys117Asn) — 1 (2.6%).*

Determination of CNV_KRAS was performed by the digital droplet PCR method (ddPCR) using the ddPCR CNV assay KRAS, Hsa (FAM) (BioRad, USA) and ddPCR CNV assay EIF2C1, Hsa (HEX) (BioRad, USA) (control gene) kits. Before ddPCR, all DNA

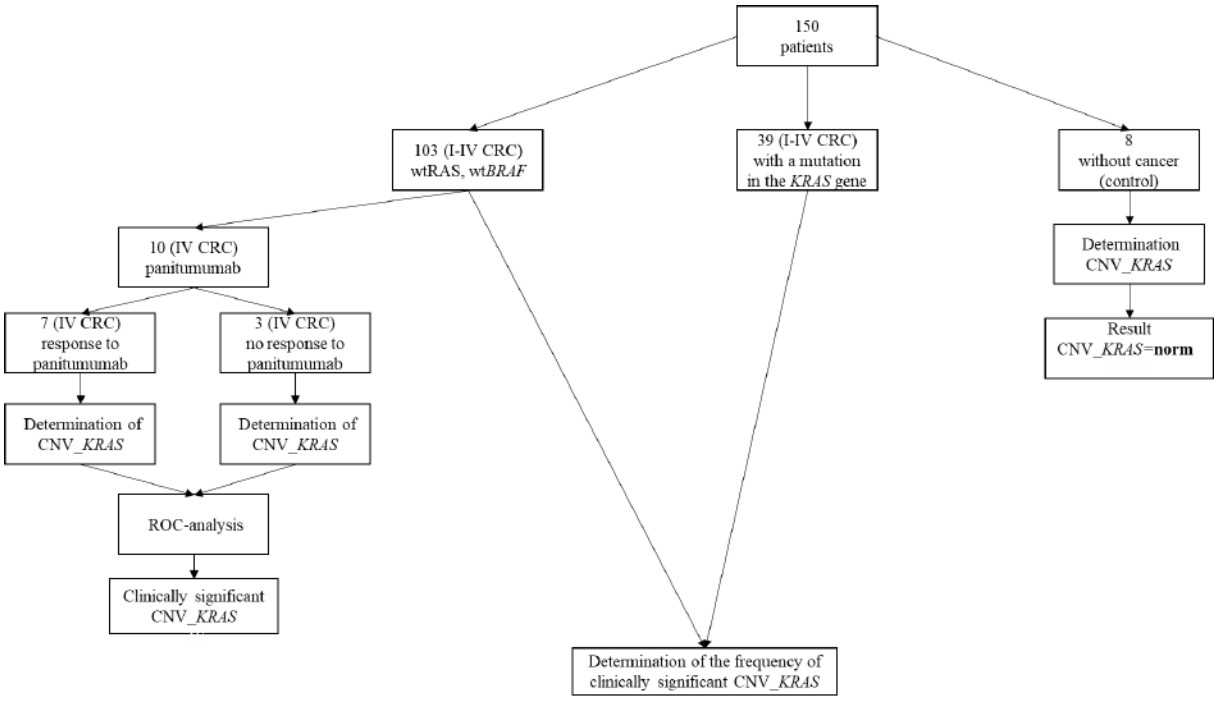

Figure 1. *The study design*

samples were treated with HindIII restriction enzyme (Thermo Scientific, Latvia) for 16 hours according to the instructions. To form droplets, a reader was used, into which a cartridge with the mixture was placed (10 µl Mix; 1 µl primer labeled with FAM dye (*KRAS*); 1 µl primer labeled with HEX dye (*EIF2C1*); 3 µl DNA; 5 µl bidistilled water and oil (BioRad, USA). After droplet formation, the mixture was transferred to a plate and placed in a T100 amplifier (Biorad, USA). Amplification conditions: 95°C — 10 min.; 40 cycles: 94°C — 30 sec., 60°C — 1 min.; 98°C — 10 min; 4°C — ∞. After amplification, the plate with samples was placed in a QX200 device (Biorad, USA) for droplet analysis.

The results were analyzed using Biorad software (BioRad, USA). CNV calculation: CNV = (target gene (copies/µl) / reference gene (copies/µl)) x2.

Statistics

Quantitative variables were presented as medians (Me), the 1st, and the 3rd quartiles (Q1; Q3), since no test for normality of distribution of values was performed. Statistical analysis was performed using Rstudio software (version 2023.12.0). Two-sided Fisher's exact test was used to compare two groups. Other specific tests are described in the figure legends.

RESULTS

The design of the study shown in Figure 1.

To develop the method for determining the variation in the number of copies of the *KRAS* gene (CNV_*KRAS*), DNA from 8 non-oncological patients was studied. The median CNV_*KRAS* values were 1.95 (1.91, 2.01), which corresponds to the norm.

To determine the clinically significant level of CNV_*KRAS*, tumor DNA from 10 patients with stage IV CRC from the first group who received anti-EGFR therapy was analyzed. Disease control

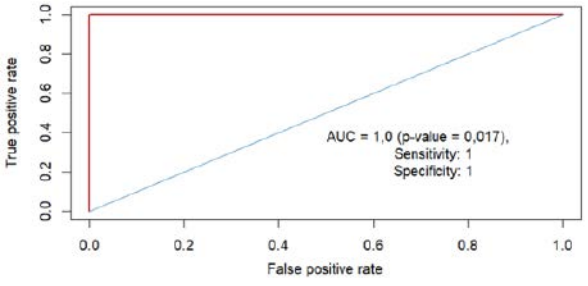

Figure 2. *ROC-curve demonstrating the relationship between CNV_KRAS values and anti-EGFR therapy*

Table 2. *Characteristics of patients who received panitumumab*

Characteristic	No response, N = 3[1]	Response, N = 7[1]	*p*-value[2]
Age	34 (33, 45)	71 (62, 73)	**0.017**
Gender			> 0.9
F	1 (33%)	4 (57%)	
M	2 (67%)	3 (43%)	
Localization of the tumor			0.7
Right side	2 (67%)	2 (29%)	
Left side	0 (0%)	3 (43%)	
Rectum	1 (33%)	2 (29%)	
T			0.8
1,2	1 (33%)	0 (0%)	
3	1 (33%)	2 (29%)	
4a	1 (33%)	4 (57%)	
4b	0 (0%)	1 (14%)	
N			0.4
0	2 (67%)	1 (14%)	
1a-c	1 (33%)	3 (43%)	
2a,b	0 (0%)	3 (43%)	
M			
1a-c	3 (100%)	7 (100%)	
RAS/*BRAF*			
WT	3 (100%)	7 (100%)	
MSI			
MSS	3 (100%)	7 (100%)	
CNV_*KRAS*	9.20 (9.05, 10.10)	5.38 (4.77, 7.35)	**0.017**

[1]Median (IQR); *n* (%)

[2]Wilcoxon rank sum exact test; Fisher's exact test

was achieved in 7 patients out of 10. The median CNV_*KRAS* value in the remaining three patients was higher than in the group of patients with disease control, 9.2 (9.05, 10.10) and 5.38 (4.77, 7.35) ($p = 0.017$ (Wilcoxon rank sum exact test; Fisher's exact test)) (Table 2).

To determine the relationship between the CNV_*KRAS* value and anti-EGFR therapy, ROC analysis was performed. It was shown that if CNV_*KRAS* ⩾ 8.55 (9) copies were detected in the tumor, then no response to anti-EGFR therapy occurred (area under the ROC curve = 1, $p = 0.017$) (Figure 2).

Next, it was decided to establish the frequency of CNV_*KRAS* in patients with colorectal cancer. Two groups of patients were studied: the first group included patients with the wild type of RAS/*BRAF*, the second one — patients with the mutated type (mutation in the *KRAS* gene) (Table 1). In the first group, the frequency of CNV_*KRAS* ⩾ 9 copies were 17% (17/103), in the second group — 3% (1/39) ($p = 0.02$) (Figure 3).

Subsequently, the frequency of clinically significant CNV_*KRAS* in groups 1 and 2 according to the CRC stage was assessed. CNV_*KRAS* ⩾ 9 was not detected in either the first or second group at the stage I; at the stage II in the first group — detected in 14% (3/22), and not detected in the second group; at the stage III in the first group — detected in 21% (8/39), and not detected in the second group; at the stage IV in the first group — detectedin17% (6/35), and in the second group — detected in 5% (1/20) (Figure 4).One patient had a CNV_*KRAS* value above the cutoff of 9 copies in the group of patients with point mutations in the *KRAS* gene.

DISCUSSION

Copy number variation is a general term used to describe a molecular event in which genome sequences are repeated and the number of repeats varies among individuals of the same species [8]. Copy number variation in a gene can affect various

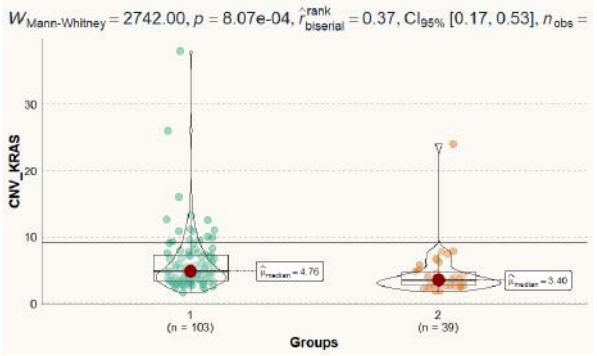

Figure 3. *CNV_KRAS values in the groups. The black horizontal line is the cutoff of 9 copies. The first group includes patients without mutations in the wtKRAS, wtNRAS, and wtBRAF genes; the second group includes patients with point mutations in the KRAS gene.*

biological processes in human body, such as metabolism, playing an important biological role, and also be of medical importance as a marker of certain pathological process or environmental exposure, such as radiation [9]. In a normal state, the number of gene copies in a somatic cell is 2, due to the diploid set of chromosomes. However, if there is a decrease in the number of gene copies due to the deletion of one of the alleles or an increase (insertion of one or more gene regions) in one, then we can talk about the presence of CNV. Data on the presence of CNV can be obtained by various methods: FISH (for fluorescence *in-situ* hybridization) analysis, real-time PCR, digital droplet PCR, high-throughput sequencing. At the same time, CNV rates can vary significantly. In a retrospective study, Bontoux S. et al. showed that the CNV value of a number of genes ⩾ 6 copies can be considered positive for patients with colorectal cancer when determined by digital droplet PCR (ddPCR), and 2.4 copies — when determined with NGS. In addition, the authors emphasize that for a qualitative determination of CNV, it is recommended to use material containing at least 40% tumor cells [7]. However, when comparing different methods for detecting and determining a significant level of CNV, the authors did not consider the relationship with treatment. If we apply the cutoff of ⩾ 6 copies obtained by them to our results, the frequency of CNV_*KRAS* in the first group will be 34% (35/103), and in the second — 18% (7/39). The obtained frequencies are significantly higher than those found in other studies (presented on the website https://www.cbioportal.org/), which vary from 0.4% to 4.2% [10–13].

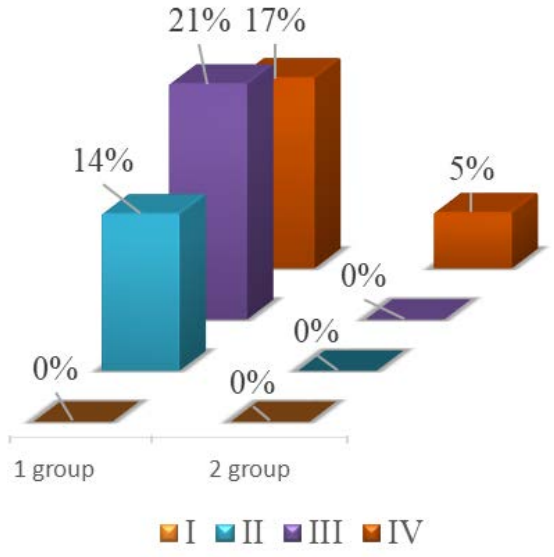

Figure 4. *CNV_KRAS ⩾ 9 at different stages of CRC. Group 1 — patients with wild type RAS/BRAF genes, group 2 — patients with mutations in the KRAS gene*

We have performed a retrospective analysis of 10 patients with colorectal cancer who received anti-EGFR therapy. It was shown that the CNV_*KRAS* level ⩾ 9 is associated with resistance to panitumumab therapy. It is important to note that the patients who did not respond to therapy were young, 34 (33,45) years old (Table 2). Next, we determined the frequency of the CNV_*KRAS* marker based on our data — with a cutoff of 9 copies. The frequency in the first group became 17%, and in the second — 3%. The frequencies obtained with CNV_*KRAS* level ⩾ 9 are also significantly higher than the results from the above studies ($p < 0.05$) [10–13]. This example clearly demonstrates how much the use of different cutoff levels can affect the frequency of occurrence of the potential CNV_*KRAS* marker among patients with CRC. At the same time, in the works where the CNV_*KRAS* frequency is in the range from 0.4% to 4.2%, the cutoff level was not specified, and all studies were performed using NGS. Probably for this reason, the data can vary so much.

Since anti-EGFR therapy is prescribed to patients with wild-type RAS/*BRAF* at stage IV of the disease, we calculated the frequency of CNV_*KRAS* ⩾ 9 at this stage. It turned out that the frequency of CNV_*KRAS* ⩾ 9 at stage IV of the disease (17%) does not differ from the overall frequency at all stages (17%). It is worth noting that at stage I CNV_*KRAS* ⩾ 9 was not detected at all (Figure 4).

CONCLUSION

Detection of CNV_*KRAS* ⩾ 9 copies in patients with colorectal cancer is a clinically significant predictive marker of ineffectiveness of targeted therapy.

In patients without somatic mutations in the RAS genes (*KRAS*, *NRAS*) and *BRAF*, this marker is significantly more common than in patients with point mutations in the *KRAS* gene ($p = 0.02$).

AUTHORS CONTRIBUTION

Concept and study design: *Vitaly P. Shubin, Aleksey S. Tsukanov*

Material collection and processing: *Vitaly P. Shubin, Anna I. Arzamastseva, Aleksey A. Barinov*

Statistical processing: *Aleksey A. Ponomarenko, Vitaly P. Shubin*

Writing of the text: *Vitaly P. Shubin, Aleksey S. Tsukanov, Aleksey A. Ponomarenko*

Editing: *Sergey I. Achkasov, Yuri A. Shelygin, Anna N. Loginova*

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REFERENCES

1. Morris VK, Kennedy EB, Baxter NN, et al. Treatment of Metastatic Colorectal Cancer: ASCO Guideline. *J Clin Oncol*. 2022;41:678–700. doi: 10.1200/JCO.22

2. Fedyanin M.Yu., Gladkov O.A., Gordeev S.S., et al. Practical recommendations for drug treatment of cancer of the colon, rectosigmoid junction and rectum. *Malignant tumors*. 2022;12(3s2–1):401–454. (in Russ.). doi: 10.18027/2224-5057-2022-12-3s2-401-454

3. Shubin V.P., Pospekhova N.I., Tsukanov A.S., et al. Frequency and spectrum of mutations in the *KRAS* gene in colon cancer of different localizations and anal canal cancer. *Medical genetics*. 2014;5(13):31–35.(in Russ.).

4. Huang W, Chen Y, Chang W, et al. HER2 positivity as a biomarker for poor prognosis and unresponsiveness to anti-EGFR therapy in colorectal cancer. *Journal of Cancer Research and Clinical Oncology*. 2022;148(4):993–1002. doi: 10.1007/s00432-021-03655-x

5. Watanabe J, Muro K, Shitara K, et al. Panitumumab vs Bevacizumab Added to Standard First-line Chemotherapy and Overall Survival among Patients with RAS Wild-type, Left-Sided Metastatic Colorectal Cancer: A Randomized Clinical Trial. *Journal of the American Medical Association*. 2023;329(15):1271–1282. doi: 10.1001/jama.2023.4428

6. Xiong Q, Zeng Z, Yang, Y, et al. *KRAS* Gene Copy Number as a Negative Predictive Biomarker for the Treatment of Metastatic Rectal Cancer With Cetuximab: A Case Report. *Frontiers in Oncology*. 2022;12:872630. doi: 10.3389/fonc.2022.872630

7. Bontoux C, Guyard A, Lupo A, et al. Detection of Nine Oncogenes Amplification in Lung and Colorectal Cancer Formalin-Fixed Paraffin-Embedded Tissue Samples using Combined Next-Generation Sequencing-Based Script and Digital Droplet Polymerase Chain Reaction. *Cancer Control*. 2023;30:10732748231167257. doi: 10.1177/10732748231167257

8. Pös O, Radvanszky J, Buglyó G, et al. DNA copy number variation: Main characteristics, evolutionary significance, and pathological aspects. *Biomedical Journal*. 2021;44(5):548–559). doi: 10.1016/j.bj.2021.02.003

9. Arlt MF, Rajendran S, Birkelan SR. Copy number variants are produced in response to low-dose ionizing radiation in cultured cells. *Environmental and Molecular Mutagenesis*. 2014;55(2):103–113. doi: 10.1002/em.21840

10. Cercek A, Chatila WK, Yaeger R, et al. A Comprehensive Comparison of Early-Onset and Average-Onset Colorectal Cancers. *Journal of the National Cancer Institute*. 2021;113(12):1683–1692. doi: 10.1093/jnci/djab124

11. Muzny DM, Bainbridge MN, Chang K, et al. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012. 487(7407):330–337. doi: 10.1038/nature11252

12. Chatila WK, Walch H, Hechtman JF, et al. Integrated clinical and genomic analysis identifies driver events and molecular evolution of colitis-associated cancers. *Nature Communications*. 2023;14(1). doi: 10.1038/s41467-022-35592-9

13. Mondaca S, Walch H, Nandakumar S. Specific Mutations in APC, but Not Alterations in DNA Damage Response, Associate With Outcomes of Patients With Metastatic Colorectal Cancer. *Gastroenterology*. 2020;159(5):1975–1978.e4. doi: 10.1053/j.gastro.2020.07.041