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Genetic and phenotypic characteristics of Russian patients with BRAF-mutated colorectal cancer

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Colorectal cancer (CRC) is one of the most common malignancies in the world. It's estimated about 1.8 M new CRC cases worldwide per year. A somatic mutation in the BRAF gene in the tumor is a negative prognostic factor. This work is aimed at studying the clinical and genetic characteristics of Russian CRC patients with the BRAF mutation. The BRAF mutations were studied by Sanger sequencing and digital droplet PCR in 489 patients and found in 34 (7%) cases. The most common mutation was p.V600E (82%). Also, rare variants were found: p.K601E, p.N581I, p.G596R, and p.D594N. All the patients with rare mutations were characterized by an unfavorable prognosis of the disease. The clinical features of the patients with BRAF mutations in the study include the predominant primary tumor site in the rectum, in addition to the right colon. Then, most of the cases were diagnosed in the advanced stages of the disease and were represented by high-grade adenocarcinomas. This article demonstrates the feasibility of analysis of the entire exon 15 of BRAF gene in CRC patients regardless of tumor localization.

Key words: colorectal cancer, BRAF gene, somatic mutations, digital droplet PCR

Colorectal cancer (CRC) is one of the most frequently diagnosed tumors in both sexes with an increasing incidence rate all over the world [1]. In Russian Federation, more than 77,200 new cases of CRC were detected in 2020 [2]. The tendency of the last decades is the increasing incidence of CRC as well as mortality among young people. In most cases, it can be explained by late diagnosis of relatively rare expected disease in younger individuals. On the other hand, the onset of CRC at a young age is more likely associated with microsatellite instability, location of primary tumors in the distal colon, and less common *BRAF* gene mutations compared to patients aged over 50 years [3].

There is a wide range of molecular and biological features of colorectal tumors (DNA methylation, microsatellite instability (MSI), mutations in the *BRAF, KRAS, NRAS* genes, molecular subtypes (CMS), etc.) that are prognostic or predictive factors [4]. Several cancer groups are distinguished depending on these markers.

Thus, a mutation in the *BRAF* gene accounts for approximately 10% of all cases of CRC [5, 6] and *BRAF* mutated CRC is more likely associated with elderly age, female gender, proximal tumor location, as well as a low tumor differentiation [4, 7, 8].

It should be noted that the *BRAF* mutations play an important role in determining the prognosis of the disease: the survival rate in such patients in the presence of a mutation is 10–16 months less, hence, the *BRAF* mutation is a negative prognostic factor. In addition, the presence of a mutation in the *BRAF* gene in patients with CRC is a contraindication to the use of anti-EGFR therapy [4, 9, 10].

Despite the fact that the most common mutation in the *BRAF* gene is p.V600E, there are also rare mutations that may have a better prognosis, such as p.D594G or p.G596N [11, 12]. Compared to p.V600E, these mutations are more often detected in rectal cancer. Rare mutations in the *BRAF* gene found in codons 597/601 are similar in prognosis to p.V600E [9, 10, 13]. In this regard, the following classification of mutations in the *BRAF* gene was proposed: class 1 – p. V600E, 2 – codons 597/601 with a similar prognosis, and class 3 – codons 581/594/596 with a significantly better prognosis [14].

Currently, there are no clearly defined treatment standards for the patients with mutations in the *BRAF* gene, and it is

still not possible to achieve an increase in their survival rate [4, 15]. It is worth noting that the incidence of mutations in the *BRAF* gene differs in patients from different populations. For example, in the USA mutation were detected in 28.4% of cases, while in Iran only in 7% [16, 17].

A crucial point is a fact that tumors in patients with Lynch syndrome do not have mutations in the *BRAF* gene, thus identifying it in a patient allows excluding this syndrome [18].

Since the characteristics of patients with *BRAF*-mutated tumors vary widely in different populations [15–17], in this work, we studied the clinical and genetic features of Russian probands with CRC taking into account their *BRAF* status.

Patients and methods

Four hundred eighty-nine patients (250 females) aged between 18–89 years with histologically confirmed CRC were included in the study. These patients underwent treatment from January 2018 to August 2020 at the Ryzhykh National Medical Research Center of Coloproctology. All patients underwent a complex preoperative examination including CT, MRI, colonoscopy, gastroscopy, and their family history was collected. The diagnosis of colorectal cancer was morphologically verified. Informed consent was obtained from all the patients involved in this study.

The DNA was isolated from the removed specimen of the tumor or paraffin-fixed bio-samples using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's protocol. The experiments were performed on one sample from each patient. In the case of synchronous tumors, at least 2 samples were investigated.

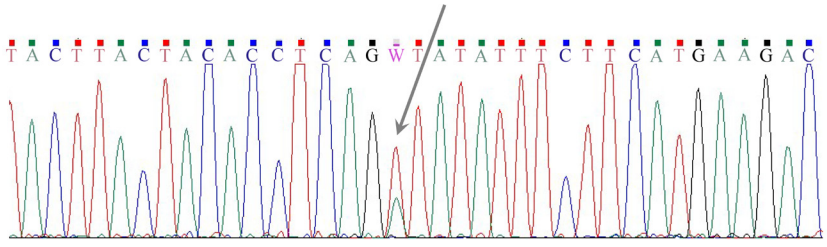

Figure 1. Sequence fragment of a patient with the p.N581I mutation in the BRAF gene.

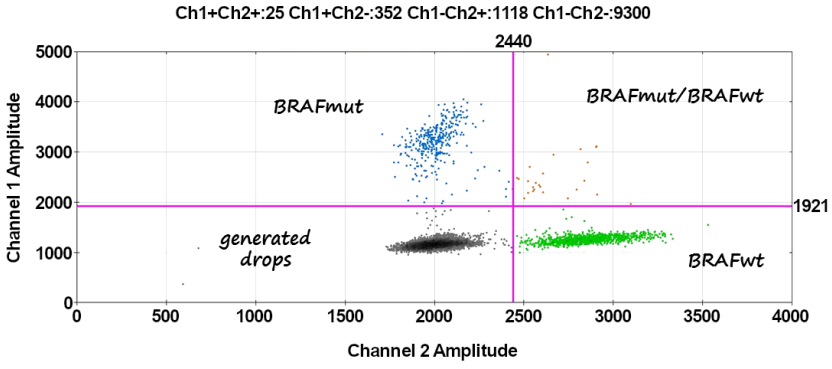

Figure 2. 2D scatter plot of a ddPCR for detection p.V600E BRAF mutation. The y-axis shows the fluorescence amplitude of the FAM probe, only mutant allele (blue droplets). The x-axis shows the fluorescence amplitude of the HEX probe, only wild type allele (green droplets). Droplets including mutant and wild type allele simultaneously are shown in orange. Droplets without amplifications PCR fragment are shown in grey.

The search for mutations in the *BRAF* gene (RefSeq NM 004333) (15 exon) was performed using a polymerase chain reaction (PCR) on a programmable thermal cycler TP4-PCR-01-"Tertsik" ("DNA technology", Russia), using original oligonucleotide primers (F-5'-ata-atgcttgctctgatagga-3'; R-5'-gccaaaaatt-taatcagtgga-3'). The composition of the reaction mixture: 0.1–1.0 ng of DNA; 0.25 mM of each original oligoprimer; 200 mM of each nucleoside triphosphate; 1 unit of Taq-polymerase; PCR buffer (500 mM Tris, 500 mM KCl, pH 8.74), 2.5 ml $MgCl_2$ (25 mM); deionized water. The amplified fragments were sequenced using the ABI PRISM 3500 automatic sequencer (Applied Biosystems, the USA).

Mutation detection and analysis were performed using software from Applied Biosystems. To confirm the identified variants, a digital droplet polymerase chain reaction (ddPCR) was used on a QX200 device from BioRad; ddPCR was performed using the original UTP master mix and primer probes. Before amplification, droplets were formed using the QX200 droplet Generator (BioRad) (the recommended number of generated droplets is at least 10,000). The amplification software: 95 °C – 10 s; 40 cycles: 94 °C – 30 s, 55 °C – 1 min; 98 °C – 10 min. The results were analyzed using the QuantaSoft Version 1.6.6.0320 (BioRad) software.

Results

Somatic mutations in the *BRAF* gene were detected in 34 (7%) of the 489 examined patients. Searching for mutations was performed by the Sanger sequencing (Figure 1). We used ddPCR as an additional method (Figure 2).

Mutation p.V600E in the *BRAF* gene was the most common and found in 28 (82.4%) of 34 tumors (Tables 1, 2). Of the other 6 mutations, there were two mutations p.K601E and two p.N581I. In addition, one case of p.G596R and one of p.D594N was detected (Figure 3).

Female patients were predominant, with male:female ratio of 1:2. The age of patients with *BRAF*-mutated tumors varied from 29 to 85 years (Tables 1, 2).

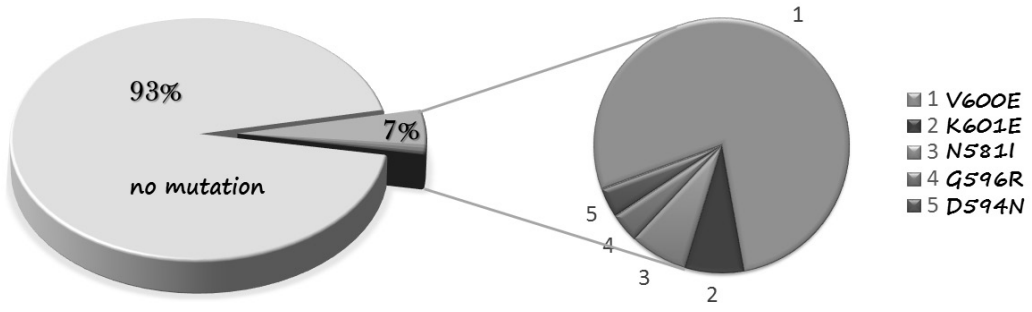

Figure 3. Frequency and spectrum of mutations in the BRAF gene in Russian patients with CRC.

Table 1. Patients and *BRAF* mutations.

#	Age	Sex	Mutation in *BRAF*	TNM	Stage	Grade	Location
1	55	M	V600E	pT4aN1bM0	III	G3	Rectum
2	51	F	**N581I**	pT4aN2bM0	III	G2	Rectum
3	73	F	V600E	pT4aN0M0	II	G2	Right colon
4	77	F	V600E	pT4bN1aM0	III	G2	Rectum
5	63	F	V600E	pT4aN2bM1b	IV	G3	Right colon
6	55	M	V600E	pT4N0M1	IV	n/d	Left colon
7	85	M	**N581I**	pT4bN2bM1c	IV	G3	Multiply tumors
8	78	F	V600E	pT3N0M0	II	G3	Right colon
9	56	F	V600E	pT3N2bM0	III	G3	Right colon
10	64	F	**G596R**	pT4aN2bM0	III	G2	Rectum
11	63	F	V600E	pT4aN2bM1c	IV	G3	Right colon
12	77	F	V600E	pT4aN2bM1a	IV	G3	Right colon
13	61	F	V600E	pT4aN2bM1	IV	G3	Right colon
14	29	M	**K601E**	pT4aN2bM0	III	G3	Left colon
15	56	F	V600E	pT4aN2bM1b	IV	G3	Right colon
16	65	F	V600E	pT4bN2bM0	III	G3	Right colon
17	70	M	**D594N**	pT4aN2bM1a	IV	G2	Left colon
18	64	M	V600E	pT1N0M0	I	G2	Transverse colon
19	49	M	V600E	pT4aN0M0	II	G2	Left colon
20	74	F	V600E	pT4aN2bM1b	IV	G2	Right colon
21	58	M	V600E	pT4aN2bM1b	IV	n/d	Left colon
22	84	F	V600E	pT4aN2bM1a	IV	G3	Right colon
23	84	M	V600E	pT3N0M0	II	G3	Right colon
24	63	F	V600E	pT4bN0M0	II	n/d	Right colon
25	70	F	V600E	pT4aN2aM1a	IV	G3	Right colon
26	36	F	V600E	pT3N0M1a	IV	G3	Rectum
27	53	M	**K601E**	pT3N0M1b	IV	n/d	Rectum
28	46	F	V600E	pT3N2bM0	III	G2	Right colon
29	59	F	V600E	pT4aN1bM0	III	G3	Left colon
30	80	F	V600E	pT3N0M0	II	G3	Right colon
31	56	F	V600E	pT4aN0M0	II	G2	Right colon
32	76	F	V600E	pT4bN1bM0	III	G3	Right colon
33	69	M	V600E	pT3N0M0	II	G3	Transverse colon
34	60	F	V600E	pT3N1aM1b	IV	n/d	Right colon

Table 2. Summary data of patients.

Characteristics	N (%)
No of patients	34
Median age (range)	63.5 (29–85)
Sex	
Male	11 (32.4)
Female	23 (67.6)
Mutation	
V600E	28 (82.4)
other	6 (17.6)
Tumor location	
Right colon	21 (61.8)
Left colon	6 (17.6)
Rectum	6 (17.6)
Multiple	1 (2.9)
Stage	
I–II	9 (26.5)
III	10 (29.4)
IV	15 (44.1)
Tumor grade	
G2	10 (29.4)
G3	19 (55.9)
No data	5 (14.7)

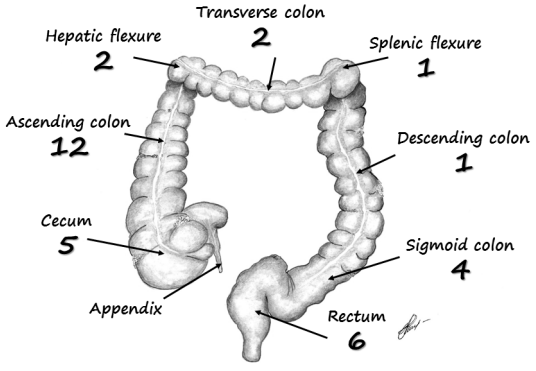

Figure 4. Location of the primary tumor in Russian patients with mutations in the BRAF.

In 19 patients (55.9%), the tumor had a histological grade G3; in 10 patients – G2 (29.4%); in 5 patients (14.7%), there was no available data (Tables 1, 2).

In 21 (63.6%) patients with the *BRAF* gene mutation, the primary tumor was located in the right colon: 5 – in the caecum, 12 – in the ascending colon, 2 – in the right flexure, and 2 – in the transverse colon. Six had left colon cancer and another 6 patients had rectal cancer (Figure 4). In addition, one patient had several dozen colorectal tumors.

Discussion

The study of the mutational status of the primary CRC tumor showed that in this cohort of patients, somatic mutations in the *BRAF* gene were detected in 7% of cases (34/489) (Table 3). Obtained results demonstrated significantly lower ($p<0.05$) frequency of *BRAF* mutations as compared with CRC patients from the United States – 28.4% (44/155) [16]. In addition, it is quite lower, but not significantly, than the data obtained from the patients in Germany – 8% (160/1,995) and Japan – 8.7% (41/472) ($p>0.05$; Table 3) [15, 19]. These differences can be explained by the fact that life expectancy in these countries is higher than in Russia, as it is well known that *BRAF* mutations are mainly found in elderly patients [5, 8].

Most of the mutations found in the *BRAF* gene in the Russian CRC patients (82%) are represented by the variant p.V600E, which is significantly lower than the results obtained in patients from the other populations – 95% [11, 19–21]. In addition to the p.V600E mutation, mutations p.K601E and p.N581I were detected in 2 cases each, p.G596R and p.D594N – in one case each.

According to the previously proposed classification of variants with different prognoses [11, 14], the mutations p.N581I, p.G596R, and p.D594N belong to class 3 and should have a more favorable prognosis. However, all 6 patients with rare mutations (including variants of p.N581I, p.G596R, and p.D594N) already had stage 3 or 4 of the disease at the time of diagnosis, in this way their prognosis seems rather unfavorable. Thus, the feasibility of isolating a favorable class of mutations in the *BRAF* gene in the presented group of patients was not confirmed.

As an illustration of the unfavorable prognosis of the disease in Russian patients with rare mutations in the *BRAF* gene, we consider it important to discuss more the 2 patients with somatic *BRAF* mutations p.K601E and p.N581I.

The first patient had the p.K601E mutation (Table 1 No.14) and the cancer was diagnosed at the age of 29 years as well as a classical form of familial adenomatous polyposis (the patient had a germinal mutation in the *APC* gene – p.1572_1599ins28) [22]. This is an extremely unfavorable prognostic factor. Such an observation is very rare in the literature [23].

The second patient, 85 years old, had the p.N581I mutation (Table 1 No.7) and was diagnosed with several dozen primary malignant tumors located in all parts of the large intestine (Figure 5). Three neoplasms were selected for the molecular genetic study; and a somatic mutation of p.N581I in the *BRAF* gene was detected in all the 3 tumors of the patient, which definitely indicates its role in the development of such a severe disease.

Overall, the poor prognosis of the disease in carriers of any pathogenic mutations in the *BRAF* gene is also confirmed by the fact that 25 (73.5%) of 34 patients already had metastases in the lymph nodes and/or in the distant organs at the time of diagnosis (Table 1).

It is known that clinical features of CRC patients with mutations in the *BRAF* gene are predominantly female gender and older age [4, 5, 7, 8]. In our study, the females also prevail, while the number of male patients was twice as small (Table 2).

Table 3. Data on patients with *BRAF* mutations in different countries.

Country	*mBRAF*	Median age	Male/female	Tumor localization proximal/distal colon/rectum	G2:G3	Stage II/III/IV
Russia [presented study]	7% (34/489)	63.5	11/23	21/6/6	10/19	8/10/15
France [29]	5.5% (269/1,735)	74.7	109/160	186/39/14	84/68	n/s
Germany [15]	8% (159/1,995)	~71	105/54	134/14/10	n/s	65/48/24
Japan [19]	8.7% (41/472)	~65	20/21	31/10/0	23/18	16/25/0
USA [16]	8.4% (44/155)	70	30/14	35/8/1	n/s	n/s
China [30]	3.1% (34/1,110)	61.4	18/16	21/6/7	21/11	8/15/4
Mexico [24]	7%	63.4	10/8	6/7/0	6/1	0/3/8
India [25]	21% (12/57)	61	4/8	8/0/4	8/3	1/7/4
Sweden [26]	20.6% (92/446)	66	34/58	53/12/3	n/s	22/17 (III+IV)
Taiwan [31]	8.6% (11/59)	28.5	7/4	4/7/0	7/4	2/9 (III+IV)
Iran [17]	7% (7/100)	69	4/3	1/2/4	4/3	2/3/2

Note: n/s - not specified

The median age of onset of the disease in the included patients was 63.5 years, which corresponds to the data from other populations [23–26]. The majority (61.7%) of the patients were aged over 60 years, and only 2 (5.9%) patients were younger than 45 years old (Table 2).

It seems an interesting fact that in the other countries more than half of the patients have tumors with histological Grade 2, whereas in Russia most cancers (19/29) are characterized by Grade 3, which is a distinctive feature of the analyzed cohort (Table 3).

In patients from other countries, the primary tumor is mainly localized in the right colon, and the presence of a tumor in the rectum is extremely rare and occurs with an incidence rate of 0–2% of cases [27, 28]. In this regard, it is important that rectal cancer was detected in 6 (17.6%) out of 34 cases among the Russian patients with *BRAF*-mutated tumors (Table 1, Figure 4). The rate of rectal lesions is significantly higher than the data obtained from the patients in Germany (6.3%; 10/158), United States (2.3%; 1/44), and France (5.8%; 14/239) [15, 16, 29] (Table 2). One explanation for this high incidence of rectal cancer here may be the fact that 3 out of 6 patients had not a mutation in the 600 codons of the *BRAF* gene. Meanwhile, a routine diagnostic test is the study of exactly the 600 codons. Therefore, in some patients with other mutations, they may not be detected due to the limitations of the genetic method used. Accordingly, this approach to sequencing all the codons of the 15 exons of the *BRAF* gene is more informative.

In conclusion, the data presented in this work has demonstrated a number of clinical and genetic features in Russian patients with CRC. Thus, a significant proportion of the patients (18%) had not a mutation in the 600 codons of the *BRAF* gene, which indicates the need for an extended search in the entire 15 exons in the absence of the p.V600 mutation. At the same time, none of the rare mutations was characterized by a favorable prognosis for the patient, which suggests that any mutation found in the *BRAF* gene in the Russian patients should be assessed as unfavorable. In most patients, tumors are represented by high-grade adenocarcinomas and are detected at the advanced stage of the disease. At the same time, one of the most common cancer sites, in addition to the right colon, is the rectum, which indicates the need for molecular genetic study of the *BRAF* gene in all Russian patients, regardless of the tumor location.

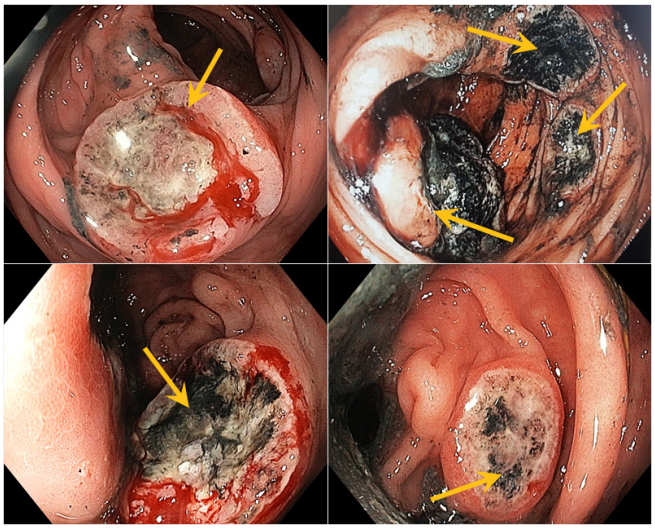

Figure 5. Endoscopic image of multiple tumors in the patient 85 years old with the somatic mutation p.N581I in the BRAF gene, (the arrows indicate malignant neoplasms).

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