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KRAS, NRAS, BRAF, PIK3CA, and AKT1 signatures in colorectal cancer patients in south-eastern Romania

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Abstract

Somatic mutations in the oncogenes of the epidermal growth factor receptor signaling pathway play vital roles in colorectal carcinogenesis and have been closely linked with clinical resistance to monoclonal therapy. In this study, we have analyzed the mutation frequencies of 5 genes and compared the genetic findings with clinicopathological variables in order to determine diagnostically relevant alterations and compare these findings with those of other studies In our Sanger sequencings, KRAS (exons 2, 3, and 4), NRAS (exons 2, 3, and 4), PIK3CA (exons 9 and 20), BRAF (exon 15), AKT1 (exon 2) genes, and microsatellite instability (MSI) status were analyzed using an ABI 3500 analyzer in a cohort of 58 Romanian colorectal cancer (CRC) patients who underwent surgical resection at Emergency County Clinical Hospital in Constanța, Romania. In our series, mutation rates of KRAS, BRAF, PIK3CA, and AKT1 genes were 39.63%, 8.62%, 6.88%, and 3.44%, respectively. By contrast, we did not find any tumor harboring mutation in the NRAS gene. Notably, the KRAS and PIK3CA mutations were not mutually exclusive, 1 patient harbored 2 mutations in exon2, codon 12 (Gly12Val) of KRAS and exon 20, codon 1047 (His1047Arg) of PIK3CA. The finding of our study are generally consistent with data found in the literature. Regarding to clinicopathological variables, mutation of KRAS was associated with distant metastasis at the time of diagnosis, while mutation of BRAF was significantly associated with MSI-H in contrast with MSI-L/MSS tumors. Moreover, PIK3CA mutation tends to be located in the proximal segment of the colon and to be well/moderately differentiated compared to wild-type tumors. In conclusion, the assessment of these mutations suggests that CRC patients from southeast Romania exhibit a mutation profile similar to other populations. These results could contribute to creating a better method of qualifying patients for molecularly targeted therapies and obtaining better screening strategies.

Abbreviations: AKT1 = v-akt murine thymoma viral oncogene, BRAF = v-RAF murine sarcoma viral oncogene homolog B1, CRC = colorectal cancer, EGFR = epidermal growth factor receptor, KRAS = Kirsten rat sarcoma viral oncogene homolog, MSI = microsatellite instability, NRAS = neuroblastoma rat sarcoma viral oncogene homolog, PCR = polymerase chain reaction, PIK3CA = Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

Keywords: AKT, BRAF, colorectal cancer, KRAS, NRAS, PIK3CA

1. Introduction

Colorectal cancer (CRC), which includes malignancies of the colon, rectum, and appendix, is the third most prevalent and deadly cancer worldwide.[1] Romania has seen an increase in CRC over recent decades, consistent with its rapid socio-economic development and Westernization of living standards and dietary habits. As a result, CRC is now the second most common cancer in Romania after lung cancer in men and breast cancer in women.[2]

From a pathophysiological and molecular point of view, CRC is a multifactorial/polygenic neoplastic disease, the consequence of successive accumulations of genetic and epigenetic alterations, including somatic mutations, gene fusions, genetic deletions/amplifications, and epigenetic modifications.[3,4] Accordingly, somatic mutations in key oncogenes including KRAS, NRAS, BRAF, PIK3CA, and AKT1 genes activate multiple signaling pathways downstream of the epidermal growth factor receptor (EGFR). These pathways include RAS/RAF/MAPK signaling, which leads to unrestricted cell growth, and/or the PI3K/PTEN/

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article [and its supplementary information files].

The study was carried out in accordance with the Helsinki Declaration and was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies.

The study was mainly performed at the Research Center for the Morphological and Genetic Study in Malignant Pathology – CEDMOG from Ovidius University.

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AKT/mTOR axis, which plays a major role in increased cell motility and resistance to apoptosis.[5]

The assessment of mutations in pathogenetic genes in gastrointestinal neoplasia is now standard clinical practice. Assessment and understanding the prognostic (i.e., the natural course of the disease) and predictive (i.e., response to therapy) value of these mutations has revolutionized the treatment of CRC patients. For example, chemotherapy and molecularly targeted therapies are given as first-line treatment for metastatic CRCs with wild-type but not mutated KRAS/NRAS.[6] Similarly, anti-EGFR therapies are only effective in a subset of patients with CRC, suggesting these patients harbor mutations in other genes that act downstream of or parallel to the EGFR axis.[7]

To date, numerous studies found KRAS (Kirsten rat sarcoma viral oncogene homolog) and NRAS (neuroblastoma rat sarcoma viral oncogene homolog) are the most commonly altered genes of the RAS oncogene family, occurring in about 20% to 30% of all human cancers. Furthermore, mutations of KRAS and NRAS in CRCs are also frequently, found in 35% to 45% and 1% to 6% of the cases, respectively. Nearly 90% of driver mutations are detected in codons 12 or 13 of exon 2 and to lesser magnitude in codons 59 and 61 of exon 3 or codons 117 and 146 of exon 4.[5,8]

The activation of the BRAF (v-RAF murine sarcoma viral oncogene homolog B1), another constitutive gene of the EGFR pathway, is recognized as a strong predictor of resistance to monoclonal antibody therapy. Additionally in CRC, BRAF gene status has been found to be mutually exclusive from RAS mutations, being persistent in up to 8% to 10% of all CRCs and 90% of these mutations involve substitution to glutamic acid (V600E) from exon 15 (codon T1799A). The mutations result in the constitutively active forms of the protein that lead to a transformation of normal epithelia into serrated adenomas at an early stage of tumorigenesis.[9]

Likewise, mutations in the PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) have been associated with a lack of response to anti-EGFR therapy, suggesting that 10% of CRC patients harboring PIK3CA activating mutations, commonly clustered in the 2 hotspot regions (exons 9 and 20).[10] The AKT1 (v-akt murine thymoma viral oncogene) gene represents another component in PI3K signaling pathway. The somatic mutation of this serine-threonine kinase appears in CRC with frequency at 0.7% to 6.0% in exon 2 (E17K), which leads to abnormal activation of AKT1.[11]

The mutations frequency of the aforementioned genes presents wide geographical, racial, and ethnic differences in this type of cancer. Furthermore, most of the studies were carried out in Western countries, and data available for South-East European countries are limited.[12–14] In the same manner, the South-East area of Romania is known as a heterogeneous population with ethnic diversity, where the prevalence of these genetic alterations, especially in patients with CRC has not been explored in detail and often was limited to a few genes and a subset of patients.

In concordance with the background, in this study, we aimed to determine the molecular spectrum of KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations, and MSI (microsatellite instability) in a series of patients with CRC from the Black Sea coast geographical area of Romania. We compared the genetic findings with clinical and pathological variables in order to determine diagnostically relevant alterations and compare these findings with those of other studies.

2. Materials and Methods

2.1. Case selection

We have conducted an observational study that included 63 fresh tumor samples collected from patients who previously underwent elective surgery or endoscopic colonoscopy for curative or diagnostic purposes at the Emergency County Clinical Hospital in Constanța, Romania. Patients who had poor or insufficient DNA quality of the tumor specimens (n = 5) were excluded. All tissue samples were collected from patients which signed informed consent and preserved in DNA/RNA Shield (Zymo Research, USA) until the total DNA was extracted. The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (06/15.03.2021).

Specimens were processed and evaluated by 2 experienced pathologists according to standard protocols from the Department of Pathology. The tumor staging of the cancer was classified using the tumor-node-metastasis staging system of the American Joint Committee on Cancer guidelines.[15] The individual characteristics of the CRC patients were abstracted from the medical observation sheets and pathology reports, including the following variables: age, sex, tumor location, histological type, degree of histological differentiation, depth of tumor invasion, lymph node involvement, and distant metastasis.

2.2. DNA extraction and quantification

Genomic DNA was extracted and purified from tissue samples by using QAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

The quality and yield of the DNA solutions were assessed by measuring the optical density at 260 and 280 nm wavelengths using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), where an absorbance ratio A_{260}/A_{280} = 1.8–2.0, and A_{260}/A_{230} > 2 was considered acceptable. Furthermore, the concentration of solutions was measured by fluorescence-based with Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) using the Qubit DNA HR (High-Range) Assay Kit.

2.3. Sanger sequencing of KRAS, NRAS, BRAF, PIK3CA, and AKT1

The MSI status was evaluated using fluorescently labeled polymerase chain reaction (PCR) primers for 5 microsatellite mononucleotide markers (BAT25, BAT26, NR-21, NR-24, and MONO-27) and 2 pentanucleotide markers (Penta D and Penta C). The main protocol was described in detail previously.[16]

Bidirectional sequencing and PCR amplification were conducted in the coding sequences of the following genes: KRAS (exons 2, 3, and 4), NRAS (exons 2, 3, and 4), BRAF (exon 15) PIK3CA (exons 9 and 20), and AKT1 (exon 2). Sequences of primers used in PCR reactions are detailed in Table 1. In the first PCR run, 10 to 15 ng of DNA was amplified using Platinum II Taq Hot-Start DNA Polymerase (Invitrogen). Each reaction consists of 10 µL of Platinum II Taq Hot-Start Master Mix (2×), 0.8 µL of pooled PCR primers (0.2 µM each), 5 µL DNA template, and 4.2 µL water nuclease-free. The following PCR conditions were used: initial denaturation at 94°C for 2 minutes, then 35 cycles for denaturing at 94°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 68°C for 15 seconds, and a cooled at 4°C. For optimal results, primers and unincorporated nucleotides from the PCR templates were purified with ExoSAP-IT Express, being incubated for 5 minutes at 37°C and 1 minute at 80°C.

Sequencing reactions consisted of 3 µL purified PCR product and 1 µL of 3.2 µM forward primer or 1 µL 3.2 µM reverse primer, 2 µL of Big Dye Terminator v3.1 reaction mix, 1 µL of 5× Sequencing Buffer, and 3 µL nuclease-free water. The PCR sequencing plate was conducted on a Biometra thermocycler with the following parameters: 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, and hold at 4°C until purify. Sequencing products obtained were purified prior to sequencing in order to remove unincorporated

Table 1

Detailed information of primers used in Sanger Sequencing analysis.

Nr	Gene		Primer sequences	Length (bp)
1	KRAS (exon 2)	Forward	GAGTGAACATCATGGACCCTGACA	559
		Revers	TTAAGCGTCGATGGAGGAGTTG	
2	KRAS (exon 3)	Forward	CCCACCTATAATGGTGAATATCTTCAAATGAT	232
		Revers	AGTAAAAGGTGCACTGTAATAATCCAGAC	
3	KRAS (exon 4)	Forward	CAGATCTGTATTTATTTCAGTGTTACTTACCT	168
		Revers	GACTCTGAAGATGTACCTATGGTCCTA	
4	NRAS (exon 2)	Forward	TACAGAATATGGGTAAAGATGATCCGACA	246
		Revers	CTGTAGATGTGGCTCGCCAA	
5	NRAS (exon 3)	Forward	ACTTGCTATTATTGATGGCAAATACACAGA	256
		Revers	TAGATGCTTATTTAACCTTGGCAATAGCA	
6	NRAS (exon 4)	Forward	CTCTACCAGAGTTAATCAACTGATGCAA	274
		Revers	ACCCAGCCTAATCTTGTTTTTCTTATGT	
7	PIK3CA (exon 20)	Forward	TGTAAAACGACGGCCAGTCAGAGTAACAGACTAGCTAGAGACAATGA	272
		Revers	CAGGAAACAGCTATGACCGCACTTACCTGTGACTCCATAGAAA	
8	PIK3CA (exon 9)	Forward	TGTAAAACGACGGCCAGTTATTCGACAGCATGCCAATCTCTT	136
		Revers	CAGGAAACAGCTATGACCTCCAGAGTGAGCTTTCATTTTCTCAG	
9	BRAF (exon 15)	Forward	TGTAAAACGACGGCCAGTGTTGAGACCTTCAATGACTTTCTAGT	513
		Revers	CAGGAAACAGCTATGACCTCTGGGCCTACATTGCTAAAATCTA	
10	AKT1 (exon 2)	Forward	TTGTTGCTTGCCAGCCCAGG	501
		Revers	AGCCCGTTTTCAGACACAGCTC	

dye terminators and dNTPs, using BigDye XTerminator Kit, and subsequently, capillary electrophoresis was run on a 3500 Genetic Analyzer (Thermo Fisher Scientific). Electropherograms were analyzed using Sequencing Analysis Software v5.4 (Foster City, CA) and SnapGene® v5.3.2 (San Diego, CA). The electropherogram of each amplicon sequenced was independently read manually by 2 researchers.

2.4. Statistical analysis

Data obtained were analyzed using MedCalc version 19.0.3 software (MedCalc, Ostend, Belgium). The proportions between KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutation status and clinicopathological variables were evaluated using an appropriate chi-square test (χ 2) or Fisher's exact test. The *P* value was considered to be significant if it was less than .05.

3. Results

3.1. Clinical and pathological features of CRC patients

In this study, a total of 58 patients were included, representing the major ethnic groups from the southeast part of Romania. The prevalence of CRC rates was higher in males 53.4% (31/58) compared to females 46.6% (27/58). The mean age of the patient cohort was 68 years (range 45–89). The primary locations of tumors included the rectum, the left side of the colon (respectively sigmoid colon and splenic flexure), and the right side of the colon (cecum, hepatic flexure, and transverse colon). Tumors were significantly higher in the colon (85.6%) where the tumors were equally distributed in the right and left colon, accounting for each 41.4% (24/58) compared to the rectum with 17.2% (10/58).

Regarding the histological subtypes, 89.7% (52/58) of tumors were common adenocarcinomas and 10.3% (6/58) was mucinous carcinoma. In accordance with World Health Organization criteria for Classification of Tumors of the Digestive System, the tumors were graded as follows: 8.6% (5/58) were well-differentiated, 81.1% (47/58) were moderately differentiated, and 10.3% (6/58) were poorly differentiated. As regarding of depth of tumor invasion in the layers of the colon or rectum, T3 represented 65.5% of all cases, followed by T4 and T2 with 17.2% each. The N0, N1, and N2 stages of lymph node involvement had percentages of 39.7%, 36.2%, and 24.1%, respectively. Based on the clinical inspection and medical electronic sheets, 31% of patients had distant metastasis and 69% displayed no metastasis, respectively. Metastases were located in the lungs in 9 patients (50% of patients with metastases), the liver in 6 patients (33.3% of patients with metastases), and other organs in 3 patients (16.7% of patients with metastases). Additionally MSI status was observed in 8.62% (5/58) of CRC patients comparatively with 5.18% (3/58) who display MSI-L and 86.20 (50/58) MSS.

3.2. The KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations distribution in CRC patients

The prevalences of KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations in the Romanian CRC patients are summarized in Table 2. Overall, KRAS mutations were detected in 39.63% of cases (23/58), PIK3CA mutations in 6.88% of cases (4/58), BRAF mutations in 8.62% of cases (5/58), and AKT1 in 3.44% of cases (2/58). NRAS mutations were not identified in any of the cases. Altogether, we found 32 patients with driver mutations and 26 patients with the absences of any of the examined mutations. All mutations identified were in the heterozygous state.

Table 2

Aminoacid, base changes, and prevalence of KRAS, NRAS, PIK3CA, BRAF, and AKT1.

Gene	Aminoacid change	Base change	Count	Prevalence (%)
KRAS	G12D	35G > A	5	8.62
	G12V	35G > T	5	8.62
	G13D	38G > A	5	8.62
	G12S	34G > A	3	5.17
	G12R	34G > C	1	1.72
	G12C	34G > T	1	1.72
	G13C	37G > T	1	1.72
	Q61L	182A > T	1	1.72
	K117R	350A > G	1	1.72
NRAS	-	-	-	-
PIK3CA	E545D	1635G > T	1	1.72
	H1047R	3140A > G	2	3.44
	M1040L	3118A > T	1	1.72
BRAF	V600E	1799T > A	5	8.62
AKT1	E17K	49G > A	2	3.44

Regarding KRAS, mutations were detected in 91.3% of cases in exon 2 (21/23), 4.35% in exon 3 (1/23), and 4.35% in exon 4 (1/23). Within KRAS exon 2, 71.5% of the mutations were detected in codon 12, and 28.5% were identified in codon 13. As regards exons 3 and 4 of the KRAS, mutations were detected in codons 61 and 117, respectively. All KRAS mutations are comprised of missense substitution.

The 4 most frequent mutations in exon 2 were the substitution of glycine with aspartate, valine, or serine at codon 12 and the substitution of glycine with aspartate at codon 13. These 4 mutations, G12D, G12V, G12S, and G13D, occur due to substitution at c.35G > A, c.35G > T, c.34G > A, and c.38G > A, which appear in 21.74% (5/23), 21.74% (5/23), 13.03% (3/23), and 21.74% (5/23) of the cases. Other common mutations detected in exon 2 were G12R, G12C, and G13C (Fig. 1A–H). The missense mutations Q61L and K117R have been detected in exons 3 and 4 of KRAS, which occur due to substitution at c.182A > T and c.350 A > G, respectively. Each mutation was noted in a single case, excepting 1 patient who carried 2 mutations, in exons 2 and 4 of the gene.

Mutations in the PIK3CA gene were identified in 75% of the cases in exon 20 (3/4) and 25% in exon 9 (1/4). The mutations detected in exon 20 was the substitution of histidine with arginine at codon 1047 and methionine with leucine at codon 1040. These occur due to transitions of A > G at c.3140 and transversion of A > T at c.3118 (Fig. 1I–J). In exon 9 was noted the substitution of glutamic acid with aspartate in codon 545, as a result of the transversion of G > T at c.1635 (Fig. 1K). The KRAS and PIK3CA mutations were not mutually exclusive, 1 patient harbored 2 mutations in exon2, codon 12 (G12V) of KRAS and exon 20, codon 1047 (H1047R) of PIK3CA.

Reference to the BRAF gene, all 5 mutations detected in this study were represented by the missense substitution of valine with glutamic acid at position 600 (V600E), which appear due to transversion of T > A at the level of c.1799 (Fig. 1L–M). All BRAF mutations were mutually exclusive from KRAS mutations.

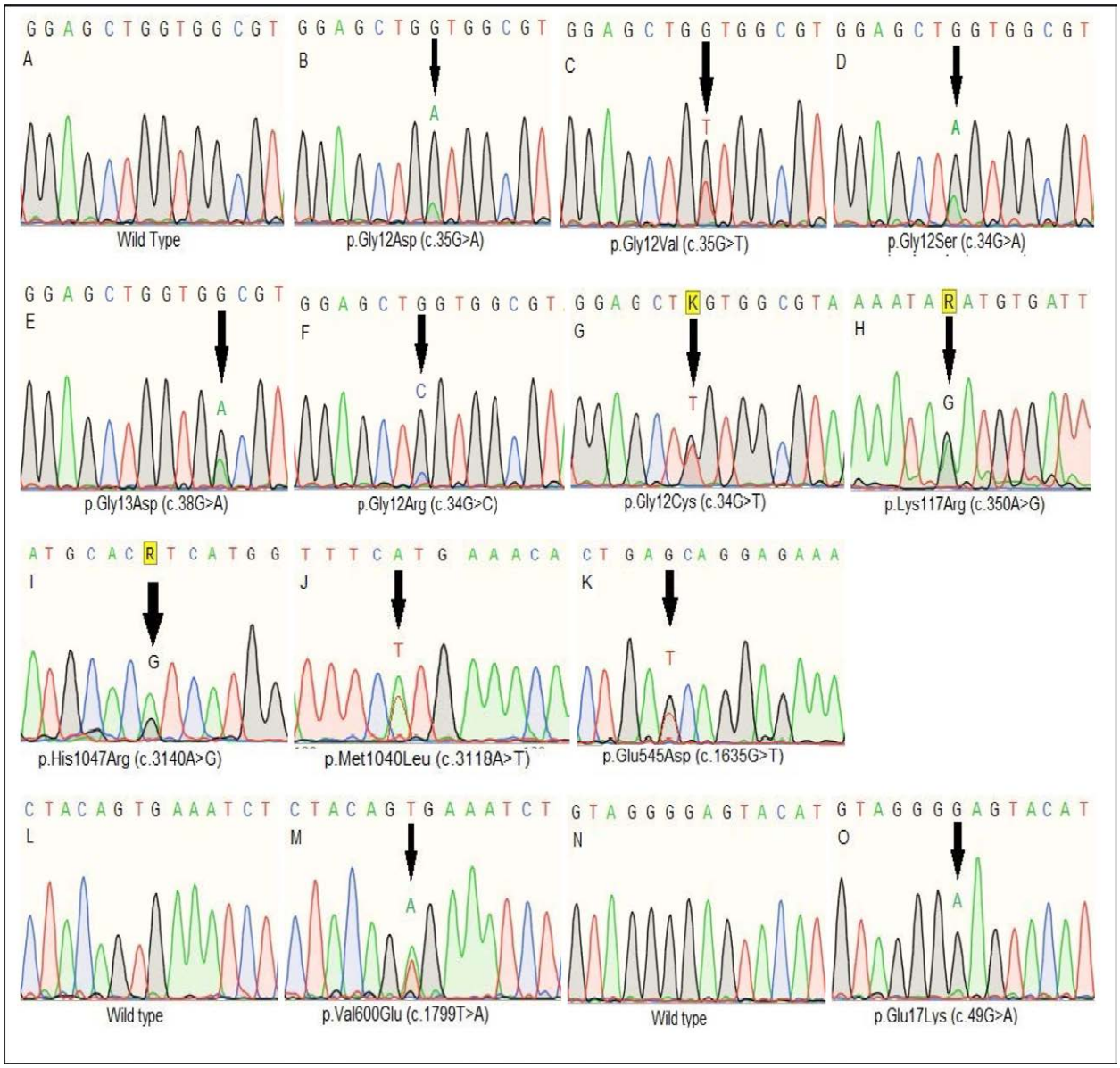

Figure 1. Representative Sanger electropherograms detected in our cohort. (A–K) Wild-type and mutations detected in exons 2, 3, and 4 of KRAS gene. (L–K) Mutations of PIK3CA gene detected in exons 20 and 9. (L–M) Wild-type and mutation detected in exon 15 of BRAF gene. (N–O) Wild-type and mutation detected in exon 2 of AKT1 gene.

The substitution of glutamate with lysine in the AKT1 gene was the single mutation detected across our CRC patients (Fig. 1N–O). Furthermore, all tumors with the AKT1 E17K mutation are found to be negative for KRAS, NRAS, BRAF, or PIK3CA.

3.3. Correlation of gene mutations with clinical and pathological variables

The mutations analysis has been evaluated in relation to several clinical and pathological parameters. No significant correlation was found between the mutational status of any of the 5 genes and gender, age, invasion depth, and lymph node metastasis (Table 3). As an exception, tumors with PIK3CA mutation tend to be located in the proximal segment of the colon ($P = .04$) and to be well and moderately differentiated compared to wild-type tumors ($P = .001$). Moreover, BRAF mutation was significantly associated with MSI-H tumors in contrast with MSI-L/MSS tumors ($P = .001$). The KRAS mutation was significantly correlated with distant metastasis among our cohort ($P = .01$).

4. Discussion

CRC is triggered by activations of intracellular signaling pathways downstream of EGFR, including the RAS-RAF-MAPK and PI3K-PTEN-AKT. The orchestrating of these pathways is achieved by a series of exchange proteins, adaptor proteins, kinases, and phosphatases upheld under tumor suppressors genes and oncogenes regulation.[17] Mutations assessment in the EGFR cascade genes is used not only for selecting the adequate treatment of patients with advanced and recurrent CRC but also for identifying subjects with an unfavorable prognosis. It has been found that patients with mutations in the extended RAS family are non-responsive to therapy with anti-EGFR monoclonal antibodies. Moreover, over 40% of CRC subjects who exhibit wild-type RAS are known to be resistant to monoclonal therapy, thereby highlighting the need for additional biomarkers.[6] A possible explanation for this resistance could be the presence of mutations in other genes of the EGFR cascade, such as BRAF, PIK3CA, or AKT1. While several studies have reported the mutational status of the above-mentioned genes in CRC patients from Western countries, there continues to be a paucity of genotyping data from CRC subjects from the South-East of Europe.[12–14,18] Consequently, the mutation rate of these genetic alterations in the context of Romanian patients was characterized in the present study, after which their prevalence was compared with clinical and pathological variables along with other similar studies.

Our Sanger sequencing reports have indicated that alterations in the KRAS, BRAF, PIK3CA, and AKT1 genes are common events in colorectal tumorigenesis, given that they are mutated in 55.18% (32/58) of the CRC cases.

The KRAS mutations distribution observed in our study was 39.63%, which is in accordance with the average level of KRAS mutations observed in Turkish, Italian, Greek, and Moroccan patients (33.2%–39.5%), but lower than mutation rates observed in the Sloven and Spanish cohorts (46.2%–48%).[12,13,18–21] This evidence suggests that genotyping data can be influenced by a variety of factors such as genetic background, environmental conditions, dietary lifestyle, heterogeneity of populations (different ethnicities), the number of patients enrolled, or methodology of the assay. Furthermore, no substantial differences were observed in the distribution of these mutations among KRAS gene exons in comparison to other studies carried out in the same geographical area. Indeed, the

Table 3

Correlation between KRAS, BRAF, PIK3CA and AKT1 mutations and clinicopathological variables in CRC.

Clinicopathological variables		No. of patients (%)	KRAS status			BRAF status			PIK3CA status			AKT1 status		
			Mutation	Wild-type	P; χ^2	Mutation	Wild-type	P; χ^2	Mutation	Wild-type	P; χ^2	Mutation	Wild-type	P; χ^2
Gender	Male	31 (53.4)	13 (22.4)	18 (31)	.48; 0.48	1 (1.7)	30 (51.7)	1.20	2 (3.4)	29 (50)	.70; 0.14	1 (1.7)	30 (51.7)	.53; 0.38
	Female	27 (46.6)	8 (13.8)	19 (32.8)		4 (6.9)	23 (39.7)		2 (3.4)	25 (43.1)		1 (1.7)	26 (44.8)	
Age	< 60	14 (24.1)	4 (6.9)	10 (17.2)	.71; 1.13	1 (1.7)	13 (22.4)	.74; 0.10	1 (1.7)	13 (22.4)	.57; 0.31	1 (1.7)	13 (22.4)	.97; 0.001
	≥ 60	44 (75.9)	17 (29.3)	27 (46.6)		4 (6.9)	40 (69)		3 (5.2)	41 (70.7)		1 (1.7)	43 (74.1)	
Tumor site	Right	24 (41.4)	9 (15.5)	15 (25.9)	.90; 0.20	3 (5.2)	21 (36.2)	.58; 1.08	4 (6.9)	20 (34.5)	**.04**; 6.08	NC	24 (41.4)	.23; 2.93
	Left	24 (41.4)	9 (15.5)	15 (25.9)		1 (1.7)	23 (39.7)		NC	24 (41.4)		2 (3.4)	22 (37.9)	
	Rectum	10 (17.2)	3 (5.2)	7 (12.1)		1 (1.7)	9 (15.5)		NC	10 (17.2)		NC	10 (17.2)	
Histological type	Adenocarcinoma	52 (89.7)	19 (32.8)	33 (56.9)	.76; 0.08	3 (5.2)	49 (84.5)	.13; 2.27	4 (6.9)	48 (82.8)	.88; 0.02	2 (3.4)	50 (86.2)	.48; 0.48
	Muc carcinoma	6 (10.3)	2 (3.4)	4 (6.9)		2 (3.4)	4 (6.9)		NC	6 (10.3)		NC	6 (10.3)	
Grading	G1	5 (8.6)	1 (1.7)	4 (6.9)	.38; 1.92	NC	5 (8.6)	.06; 5.42	3 (5.2)	2 (3.4)	**.001**; 24.06	NC	5 (8.6)	.78; 0.48
	G2	47 (81.1)	19 (32.8)	28 (48.3)		3 (5.2)	44 (75.9)		1 (1.7)	46 (79.3)		2 (3.4)	45 (77.6)	
	G3	6 (10.3)	1 (4.75)	5 (8.6)		2 (3.4)	4 (6.9)		NC	6 (10.3)		NC	6 (10.3)	
Depth of invasion	T2	10 (17.2)	3 (5.2)	7 (12.1)	.22; 2.97	NC	10 (17.2)	.27; 2.61	1 (1.7)	9 (15.5)	.62; 0.95	1 (1.7)	9 (15.5)	.42; 1.72
	T3	38 (65.5)	12 (20.7)	26 (44.8)		3 (5.2)	35 (60.3)		3 (5.2)	35 (60.3)		1 (1.7)	37 (63.8)	
	T4	10 (17.2)	6 (10.3)	4 (6.9)		2 (3.4)	8 (13.8)		NC	10 (17.2)		NC	10 (17.2)	
No. of lymph nodes	N0 (0)	23 (39.7)	6 (10.3)	17 (29.3)	.33; 2.20	1 (1.7)	22 (37.9)	.56; 1.12	2 (3.4)	21 (36.2)	.87; 0.26	2 (3.4)	21 (36.2)	.20; 3.15
	N1 (1-3)	21 (36.2)	8 (13.8)	13 (22.4)		2 (3.4)	19 (32.8)		1 (1.7)	20 (34.5)		NC	21 (36.2)	
	N2 (>3)	14 (24.1)	7 (12.1)	7 (12.1)		2 (3.4)	12 (20.7)		1 (1.7)	13 (22.4)		NC	14 (24.1)	
Metasstasis	M0 (No)	40 (69)	10 (17.2)	30 (51.7)	**.01**; 5.52	3 (5.2)	37 (63.8)	.95; 0.003	4 (6.9)	36 (62.1)	.40; 0.69	2 (3.4)	38 (65.5)	.85; 0.03
	M1 (Yes)	18 (31)	11 (19)	7 (12.1)		2 (3.4)	16 (27.6)		NC	18 (31)		NC	18 (31)	
MSI status	MSS/MSI-L	53 (91.38)	21 (36.2)	32 (55.2)	.20; 1.62	1 (1.7)	52 (89.7)	**.001;** 26.11	5 (8.6)	49 (84.5)	.77; 0.08	2 (3.4)	51 (87.9)	.40: 0.70
	MSI-H	5 (8.62)	NC	5 (8.6)		4 (6.9)	1 (1.7)		NC	4 (6.9)		NC	5 (8.69)	

CRC = colorectal cancer, MSI = microsatellite instability, NC = not calculable.

4 most frequent mutations observed in the present study were in codons 12 and 13 of exon 2, in particular G12D, G12V, G12S, and G13D (21.74%, 21.74%, 13.04% and 21.74%), similar to those reported by Greece (29.4%, 19.3%, 11.27%, and 19.3%).[19] Other common mutations identified were found to occur with a less magnitude in codons 61 and 117 of exons 3 and 4, Q61L, and K117R (4.35% each). This indicates that geographical distribution is not a significant factor in how such mutations are positioned in the codons. Moreover, we found 2 patients with more than 1 mutation, 1 patient carried 2 KRAS mutations (G13D and K117R), whereas the other one had a double point mutation, in exon 20 of PIK3CA (H1047R) and exon 2 of KRAS (G12V). However, no significant associations were found between PIK3CA and KRAS mutations. The coexistence of KRAS mutation with different other gene mutations could be linked to the heterogeneity of the tumor. Likewise, KRAS mutation may influence the activity of kinases from other pathways, suggesting these pathways are synergistically interconnected.[22]

Some studies showed that missense mutations in codon 12 are associated with the mucinous phenotype, while mutations in codon 13 are characterized by a non-mucinous phenotype, localization in the proximal colon, tumor aggressiveness, and increased metastatic potential.[23,24] In this study, no association was observed between histological type and codons 12/13 mutations of KRAS. However, we observed that KRAS mutations tend to be more frequent in males than females (61.9% vs 38.1%) and to be located in the distal and proximal colon compared to the rectum (42.8% vs 14.4%), which is in accordance with studies of Kawazoe et al, but unfortunately with no statistical evidence.[25] Instead, a significant difference was noted in the KRAS mutation frequency with respect to distant metastasis (52% vs 48%; $P = .01$), suggesting that KRAS mutations are associated with a higher incidence of metastatic disease at diagnosis time.[26]

In the framework of the EGFR signaling pathway, alteration of the BRAF gene has important implications in the growth, proliferation, apoptosis, differentiation, and survival of cells in an independent way from the EGFR pathway.[9] Mutations of the BRAF gene affect several sites located in the kinase domain, and over 70% of these alterations imply the V600E codon. Structural studies have shown that valine from position 600 of the kinase domain is essential to keep the Braf protein in an inactive conformation in the absence of the Kras-Braf interaction.[27]

In the present study, BRAF mutation was identified in 8.62% of patients (5/58), nearby value to other studies, namely those conducted in Turkey, Germany, Slovenia, Spain, and UK (5.1%–7.9%), but smaller than identified in Greek patients (14.4%).[13,14,18,19,21,28] The V600E mutation was noted in all BRAF mutant samples. Some reports suggested that BRAF mutations are associated with distinct clinical and pathological features, such as the increased frequency in women, right tumor location, mucinous histology, positivity of Keratin-7, and MSI.[29] In our study, except for the significant association of BRAF mutation with the MSI-H phenotype compared to MSI-L/MSS ($P < .001$), we could not establish a significant association of BRAF with the parameters mentioned above, probably due to the modest number of patients and reduced frequency of these mutations. This finding is consistent with the other data from the literature, which suggests that BRAF mutation, MSI-H status, along with a high CD3 rate, and absence of p53 nuclear expression with Mapsin cytoplasmatic predominance represent the best prognosis for CRC patients. In contrast, in patients who exhibit BRAF mutation, MSS phenotype, the low score of CD3 and p53 (>50%) with Mapsin nuclear predominance, have the worst prognosis.[30]

Activating mutations of the PIK3CA gene are noticed in 7% to 32% of CRC patients.[31] In our study, the frequency of PIK3CA was 6.88%, lower than average reported in Italy and France (13.3%–17.8%), rather closer to rates reported in Singapore and Poland (2.2%–9.6%).[10,12,17] Furthermore, we observed the predominance of mutations at exon 20 compared with exon 9 (5.16% vs 1.72%), which is concordance with Chinese studies, but rather different from the results from Westerns countries.[10] De Roock et al suggest that mutations in exon 20 of PIK3CA might be associated with a low response rate to therapy and outcome.[17] Interestingly, some studies demonstrated that tumors with PIK3CA mutations are characterized by location in the proximal colonic and low grade of histological differentiation.[10] In accordance, PIK3CA mutations in our subjects were exclusively found in proximal colon tumors relative to the distal colon or rectum ($P = .04$). Likewise, PIK3CA mutations were more likely to exhibit well and moderately differentiation ($P = .01$).

AKT1 is an active core of the PI3KCA/AKT/mTOR signaling pathway, controlling diverse cellular processes, including cell survival, proliferation, invasion, and metabolism.[32] To date, AKT1 E17K mutations have been reported in several tumors (colon, lung, and breast) ranging between 1% and 3%.[33] In this study, the sequencing analysis of exon 2 of the AKT1 showed the point mutation of G to A at nucleotide 49 (E17K) in 2 patients (3.44%), which is in concordance with other studies.[34] This missense mutation substitutes glutamic acid with lysine at amino acid position 17, a mutation that affects the Pleckstrin homology domain of the enzyme that is no longer dependent on activation of upstream components of the pathway.[11] Many studies revealed that tumors harboring the AKT1 E17K mutation are generally found to be negative for KRAS and BRAF, which is in concordance with our study. Nevertheless, AKT1 E17K mutations in diverse cancers play a dual role (antitumor functions and oncogenic). The antitumor effect could appear through negative feedback of the AKT pathway, while the oncogenic effect may enhance the migration and metastatic potential of tumor cells.[33]

Particularly, our study has some limitations. First, the relatively small sample size might not provide enough statistical data to explore the relationship between genotyping and clinical and pathological features. Secondly, because our patients were diagnosed recently, follow-up information such as recurrence, and the therapeutic response were not available. Third, hotspot mutations in other exons of the above-mentioned genes were not screened due to financing limitations. Fourthly, MSI-PCR based on PCR amplification of MS regions followed by capillary electrophoresis does not provide indications about MMR genes and requires at least the presence of 20% tumor cells in the sample.

5. Conclusion

In conclusion, our study showed that the occurrence of mutations in the KRAS, BRAF, PIK3CA, and AKT1 genes are common events in CRC patients from the Black Sea coast geographical area of Romania. The results of this study are in concordance with other studies conducted in the same geographical area. These findings have important implications for the personalized treatment of Romanian CRC patients, thereby providing an opportunity to improve healthcare efficiency and resource use in these patients.

Author contributions

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