K-RAS mutations in colorectal cancer in patients from Podlaskie region

Chomczyk M.[1 A,B,C*], Czajka P.[2 D,E,F]

1. II Department of General and Gastroenterological Surgery, Medical University of Bialystok, Poland
2. Department of Pathomorphology, Medical University of Bialystok, Poland

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ABSTRACT

Introduction: In Poland, colorectal cancer is the second leading cause of death. The incidence of colorectal cancer increases with age and early onset indicates and increased likelihood for genetic predisposition for this disease. The somatic genetics of tumor development in relation to patients age, gender, sex and morphological factors are unknown in Podlaskie region, Poland**.**

Materials and methods: We investigated seventy five patients (43 men and 32 women) who underwent surgery for cancer of the colorectal in the II Department of General and Gastroenterological Surgery, Medical University of Białystok in 2002-2007. The average age of patients was 64.8 years (the average age of women 66.7, men 63.1). All patients for the study of molecular research (absence or presence of K-*RAS* mutations) had histopathology confirmed adenocarcinoma.

Results: There was no correlation presence or absence of mutations in *K-RAS* of the following clinical and morphological factors: gender, age, location, degree of tumor differentiation, tumor size and metastases to lymph nodes and other organs The gene encoding the K-Ras protein is mutated in 20-50% of cases of colorectal cancer. Such a difference of results is influenced by several factors: differences of the techniques used for detecting mutations, differences in codon of the gene that is considered codon 12 and /or 13 and / or 61 and differences in the selection and study population.

Conclusions: These data suggest the clinical and morphological factors in patients with colorectal cancer have no effect on the presence of *K-RAS*. mutation.

Key words: colorectal cancer, *K-RAS* mutation, Ras protein, Podlaskie region

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***Corresponding author:**
II Department of General and Gastroenterological Surgery
Medical University of Białystok, Poland
e-mail: m.chomczyk@wp.pl

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INTRODUCTION

The incidence of colorectal cancer has increased in the western country including Poland during the last year. Each year, approximately 3.2 million Europeans are diagnosed with cancer [1]. Cancer is the second leading cause of death in the European Union. Lung cancer, cervical and colorectal cancer are the most common causes of death in the European Union. It is estimated that in 2006 the colorectal cancer deaths in Europe 68,000 women, 78,000 men, while new cases reported of 140,000 women and 170,000 men. In Poland, colorectal cancer is the second leading cause of death. It is recognizable in 12 000 Poles year [2]. It is rare in people under 40 years of age, most cases falls on the eighth decade of life. Morbidity and mortality increased in both sexes with age, the growth is faster in men. The population of men diagnosed with colorectal cancer accounts for 62% of cases after 65 years, women 66% (deaths respectively 70 and 77%). The increasing trend of mortality has been above 65 years of age for men, women mortality rate of growth is inhibited. Most cases of colon cancer at all ages reported in the Polish western provinces and in women in the Opole province, Wielkopolska and Silesia. Index incidence/mortality is higher in women. Indicator 5-year survival of patients with colon cancer is 30-33% [1]. In terms of the effects of treatment of colorectal cancer, Poland is one of the last places in Europe [by. WHO]

Ras proteins first described in murine sarcoma virus, Harvey and Kirsten (Kirsten rat sarcoma oncogene). Ras proteins are small G proteins that belong to a superfamily of proteins which are capable of binding and hydrolysis of GTP. Regulate cell growth, proliferation and differentiation. Various forms of Ras: H-ras, N-ras and K-ras transmit signals independently of the interaction with different effectors and promoters [3]. They are activated by guanine nucleotide exchange. Many Ras effectors have been identified - the most important is GEFs. By initiating EGFR are transported from the cytosol to the plasma membrane or Golgi apparatus, where the effectors are located along the Ras protein. Active GTP-bound Ras interact with many effector proteins: Raf kinase, PI3K, and NORE/MST1 RasGEFS [4].

Ras genes encode a family of proteins with a molecular weight of 21 kDa, composed of 188 amino acids. The structure of Ras protein is distinguished by two main catalytic domain domain-domain G and C.

There are 3 different protoncogenes Ras located on different chromosomes (the gene for the protein K-Ras is located on the short arm of chromosome 12 locus 12.1, which is shown in Figure 1 [5]).

The gene consists of 6 exons. The first exon - exon 0, is not translated - UTR region. *K-RAS* gene has two alternative forms of exon 4-4A and 4B, which makes the expressed protein produced two forms of K-RAS - 4A K-ras and K-ras 4B. 90-95% of the resulting transcripts in tissues and cells of the K-ras isoform 4B. Gene promoter is located upstream of the transcription initiation site and is rich in GC. There is no sequence TATA and CCAAT, characterized housekeeping genes. Within the promoter is polypurine element which is sensitive to nuclease NHE (nuclease hipersensitive element), which plays an important role in transcription. Scientific reports indicate that this is the place structurally polymorphic. Under certain conditions, this place can take the form of G-quadroduplex, leading to inhibition of transcription [6]. Presented by Coagi et al.[7] results of research on the place polypurine can contribute to the creation of new opportunities for inhibiting expression of *K-RAS* in cancer cells [7].

K-Ras acts as a "molecular switch" from outside the cell to the nucleus. Under normal conditions, the protein can be "turned on" and "off" in order to transmit signals that control the cell growth. The mutant protein is still active, resulting in uncontrolled cell growth and inhibit apoptosis. Until now it was believed that K-Ras its function only if it is permanently connected to the cell membrane. K-ras protein is also found on the surface of the mitochondrium (involved in the regulation of apoptosis). The location may be regulated by protein kinase C. In vitro were positive function of Ras proteins in the initiation of cell death [6]. Proteins belonging to the family of Ras have two main domains: the catalytic domain of G (located at the N-terminus) and domain C (localized at the C-protein).

G domain (amino acids 1-165) are highly conserved, the first 164 amino acids are homologous of 4 proteins of this family. II-row G forms a domain structure consisting of a core β-bellows, which are connected by hydrophilic chains 5 α-helices and loops G. 5 α-helices play a key role G loops that bind GDP/GTP and are responsible for protein-protein interaction. Structural studies of Ras family proteins distinguished switches that are in the domain G. Within the switches are major structural differences. Switch and associated regions of proteins Ras effectors, switch II binds to guanine, phosphate groups and / or magnesium ions [6].

Proteins belonging to the family of Ras molecules are produced as precursors in the cytoplasm. Possible biological functions are combined to cytosolic membrane surface. Change is highly variable post-translational protein HVR area, completed by the CAAX motif (C-cysteine, A-aliphatic amino acid, X-another amino acid). This place is to modification by the action of enzymes. The first step is lipidation catalyzed by farnesyl transferase and geranylgeranyl (the enzyme

determines the last amino acid in the sequence CAAX) [8]. The attachment means 15 prenylation-carbon farnesyl pyrophosphate, farnesyl transferase when it operates, or 20 carbon geranylogeranyl pyrophosphate (under the influence of geranylgeranyl transferase) to the cysteine in the sequence CAAX. The prenylation facilitates the combination of RAS proteins from the endoplasmic reticulum membrane. The endoplasmic reticulum occur further changes - proteolysis and methylation. Ras prenylation is exposed to the RCE endopeptidase 1 (Ras converting enzyme), which removes part of the sequence CAAX AAX. Then, at the C-terminal of Ras acts ICMTA methyltransferase (carboxymethyl cysteine isoprenyl transferase), which joins a methyl group to the α-carboxyl group prenylocystein. In the hydrophilic C-terminal hydrophobic protein domain created Ras, which gives the opportunity to bind the protein to the cell membrane [9,10].

The races are effectors of c-Raf-1, A-Raf, B-Raf, p110α, p110 β, p110δ, p110γ, Ral GDS, RGL, RGL2 / Rif, RG13, RASSF1A, RASSF2, RASSF4, RASSF5/NORE1, acting in a cell the following function:

- NORE1-is associated with RASSF1A and can induce Ras-dependent apoptosis
- RASSF1A-can induce apoptosis or inhibit cell cycle
- RASFF2 - is an effector for *H-RAS* initiates apoptosis (described in the lungs), is often governed by promoter methylation during tumor formation.

The signal from the cell is transferred via the Ras kinase phosphorylation RAF kinase and MAP, ERK. Signal from the cell membrane to the cell nucleus results in the activation of transcription [11-15].

Proteins belonging to the family of Ras cytolozol are located on the cell surface. The proteins undergo post-translational modification C-terminus. The K-RAS4A attachable the rest of palmitic and are transported through the Golgi apparatus to the so-called classical path, lipid rafts, and the K-RAS4B sequence rich in lysine (transport mechanism is unrecognized). In normal cell, they are inactive.

Activity gain by connecting a ligand to the extracellular portion of the receptor membrane. An important role in the activation of adapter proteins play GRB, SOS, which do not have an enzymatic activity. Adapter proteins interact with the tyrosine residues of the activated receptor. One of the residues is the point of attachment of the adapter protein SHC which then undergoes phosphorylation. Recognized and bound by a protein tyrosine GRB SHC protein complexing with protein SOS. Protein mSOS1 guanidine nucleotide exchange catalyzed in the active center of protein G. This results in Ras protein activity through a combination of GTP through intrinsic activity of GTP-ase comes to the hydrolysis of GTP to GDP. The signal is passed to the raf [16]. In Western countries, about 50% of colorectal cancers observed abnormalities in the signal path RAS / RAF / MEK / ERK pathway [17].

Modern targeted therapies bavazicumab and cetuximab in combination with chemotherapy increase survival of patients. Mutational status of *K-RAS* gene is a biomarker for targeted therapies. Modern research is intended to optimize the anti-EGFR therapy in colorectal cancer [18-24]. Molecular and pharmacogenetic studies in cancer is to determine the relationship between genotype and phenotype of gene expression in individual responses, drug resistance and toxicity. Pharmacogenetics describes genes involved in drug metabolism. Genetic polymorphism represents more than 1.5% of the population. Somatic mutations and variations in copy gene may be responsible for a variety of pharmacokinetic and pharmacodynamic responses in drug metabolism and activation. In the treatment of colon cancer, as well as other cancers, it was observed that the same drugs cause a different response in patients [18,16,25-27]. Genetic differences in the targeted therapies can have a great impact on the efficacy of the treatment, as well as toxicity. EGFR belongs to the erbB family of receptor tyrosine kinases, which also includes [15,28,29] ErbB1 (EGFR, HER1), ErbB2 (HER2/neu), ErbB3 (HER 3) and ErbB4 (HER 4).

Family of receptors initiates a signal transduction two ways, which are involved in cellular processes such as proliferation, signal transduction and cell cycle. On the one hand location on the cell membrane lipid kinase PIK3CA interacts with PTEN as a consequence there is an activation phosphorylation of AKT1, on the other hand, activated *BRAF*, *KRAS* (activation pathway described earlier). Two monoclonal antibodies (moAbs) against EGFR: IgG2a moAb - pamitumumab and IgG1 moAb cetuximab have been used in the clinic practice of colon cancer. Both antibodies bind to domain of EGFR and inhibit two pathways. Patients who were excluded if the surgical treatment of EGFR expression by immunohistochemistry. In 2005, Moroni et al study, said larger response in patients with *K-RAS* WT (Wild Type) [30]. These studies have shown longer survival in patients without the mutation in the tumor. Summarize, it can be concluded that *K-RAS* mutation is a predictive factor for the treatment of anti-EGFR therapy and is associated with a worse prognosis and shorter survival. For the family of serine-threonine kinases include c-RAF RAF1, Araf, BRAF. As effectors of Ras proteins have RBD domain, through which interact with Ras protein. *BRAF* is the second element of the signal transduction in the above-described path. Single substitution causes mutations in codon 600, resulting in cell transformation (V600E-is the most common mutation in BRAF and comprises about 80% of all

mutations) [31]. In colorectal cancer, there are about 10% of the mutations in the *BRAF* gene. The development of modern pharmacogenetics and personalized therapies has led researchers to study the effects of mutations in the BRAF gene in the treatment of patients. Running experiments must be confirmed clinically. Perhaps the mutation in the gene next to the mutational status of *K-RAS* will be a predictive factor for targeted therapy.

The gene encoding K-Ras protein is mutated in 20-50% of cases of colorectal cancer (incidence of mutations in K-ras gene in colon cancer cells in various research centers are shown in Table 1). Such a difference of results is influenced by several factors: differences of the techniques used for detecting mutations, differences in codon of the gene that is considered codon 12 and / or 13 and / or 61 and differences in the selection and study population.

Table 1. The frequency of *K-RAS* mutations in colorectal cancer cells in research centers

Research Team	n	*K-RAS* mutations (%)
Moroni et al.	31	32
Livere et al.	30	43
Livere et al.	89	27
De Rock et al.	66	40

MATERIALS AND METHODS

Seventy five patients (43 men and 32 women) who underwent surgery for cancer of the colon in the II Department of General and Gastroenterological Surgery, Medical University of Bialystok in 2002-2007. The average age of patients was 64.8 years (the average age of women 66.7, men 63.1). All patients enrolled for the study of molecular histopathology confirmed adenocarcinoma. Research were approved by local Ethics Committee (Medical University of Bialystok).

In order to optimize the course of the implementation of current research in the planned working conditions - including the minimization of errors associated with the metrology and test the process was divided into eight stages:

1. Preparation of a clinical test
2. DNA isolation
3. PCR
4. Electrophoresis
5. Purification
6. PCR sequencing
7. Removing terminator sequencing reactions
8. Sequencing codon 12 and 13.

The material consisted of tumor fragments obtained from surgically excised sections of the colon. The material for the pathological diagnosis were prepared by downloading snippets obtained from the tumor of the colon, which were fixed in 10% buffered formalin at 4 ° C for 24 hours and adjusted to a routine paraffin. Paraffin blocks were cut on a microtome into sections, then stained with hematoxylin and eosin. Microscopic examination identified the tumor histological type of colorectal cancer. These procedures were perform like part of routine post-operative diagnostic. Isolation of DNA from formalin were settled in the Qiagen kit after deparaffinized samples. Duplication of DNA chains were made by polymerase chain reaction (PCR Polymerase Chain Reaction) in Eppendorf using appropriate primers. After the PCR reaction was performed by agarose gel electrophoresis to verify the presence of products and for the isolation of DNA from the gel. Polymerase chain reaction was carried out to obtain the desired DNA sequences (containing codon 12 and 13 of the *K-RAS* gene) using the designed primers, and the Big Dye Terminator, Applied Biosystem's buffer and water. The last step was sequenced genet containing the *K-RAS* codon 12 and 13 The principle of the method developed by Sanger used, the chain termination method. The procedure was performed on an automatic ABI PRISM 377 sequencer.

Statistical analysis was performed in the Department of Statistics and Informatics, Medical University of Bialystok. The results were used to determine the arithmetic mean, standard deviation and median individual measurable characteristics. They were subjected to statistical analysis using STATISTICA software for Windows 7.1 StatSoft.

RESULTS

The study consisted of 75 patients treated surgically for colorectal cancer. 20 patients were have *K – RAS* mutations. This represents 27% of the patients. In the remaining 73% of patients (55 patients), there were no mutations in *K - RAS*. The incidence of *K–RAS mutation* in the study population are shown in Figure 1 and types of mutations in Figure 2.

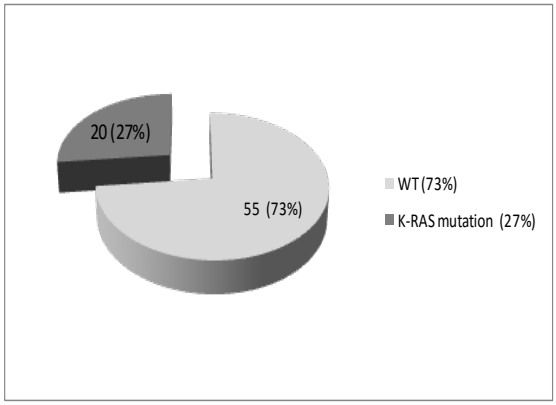

Figure 1. The incidence of *K–RAS mutation* in the study population

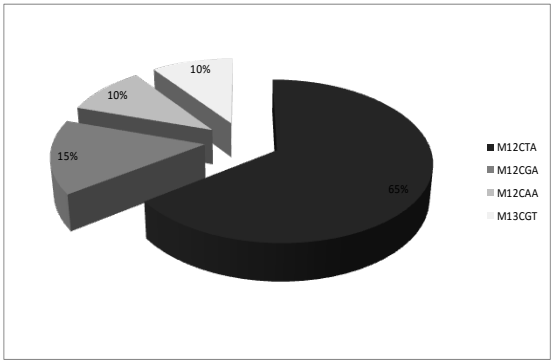

Figure 2. Types of mutations

There was no correlation presence or absence of mutations in *K-RAS* of the following clinical and morphological factors: gender, age, location, degree of tumor differentiation, tumor size and metastases to lymph nodes and other organs. There was no evidence of any significant association of the presence or absence of mutations in *K-RAS* gene from the above analyzed clinical and morphological factors. Patients with distant metastases showed a higher percentage of mutations in *K-RAS*, however, no signs of statistical significance.

DISCUSSION

Understanding and describing genes involved in the development of colorectal cancer has contributed to significant progress in the diagnosis and treatment of this disease. The increasing incidence of cancer implies the need to search for new prognostic and predictive factors, and knowledge of the prevalence of mutations in *K-RAS* and its correlation with clinical and morphological factors.

The frequency of mutations in the *K-RAS* in colorectal cancer by a variety of scientific studies ranges from 20% - 50% [1,3,5,6]. The individual selected countries, this value is at the following levels: Taiwan - 20.7%, Morocco - 29%, South Korea - 20.7, India 23%, Australia - 48.4% France 27%, Germany 31.6%, -31.1 Czech Republic, Sweden 39%, Spain 34%, Romania 44%, United Kingdom - 30%, and the U.S. - 32%. The results of the incidence of mutations in *K - RAS* in the population in patients with colorectal cancer in Podlaskie have significantly lower interest income, which was only 27%. Analyzing the results can be seen that in the Podlaskie proportion of patients with colorectal cancer who were found to have mutations in the lower end of the range (20% - 50%). The detected frequency of mutations in *K-RAS* in patients with colorectal cancer in Podlaskie is much lower than in a study conducted in Poland [32]. The Polish study in the incidence of mutations in the *K-RAS* patients diagnosed with colorectal cancer was performed at the Medical University in Krakow. In the group of 163 patients with histologically proven adenocarcinoma of the colon revealed the presence of mutations in 35.6% of patients [33]. In the group of 51 patients with the most frequently mutated were reported in codon 12 and 13 (respectively, at codon 12 - 66% and at codon 13 -22%) [32].

Impact on the observed differences in the results obtained by different research centers may include a variety of techniques used to detect mutations (SSCP, DPHLC, PCR-RFLP and direct sequencing). The development of the use of genetic methods in the study of cancer necessitates the use of more effective and more modern research methods. In an academic paper, validation of selected molecular techniques for determining a mutation at codon 12 and 13 of the *K-RAS* gene carried out in five centers of Polish scientific research [33] and published in Oncology in Clinical Practice [Volume 4, No. 6, 232 - 244], a target to develop a standard procedure for determining the status of the *K-RAS* gene and to validate the selected molecular techniques for determining mutations in five centers in Poland, which is the treatment of patients with colorectal cancer. Using the principles of good laboratory practice, it is recommended that one of the signs carried were performed using direct sequencing methods at least one thread - in addition to the PCR/RFLP, SSCP and DHPLC. The PCR / RFPL allowed only to detect mutations in codon 12, and the other three methods permit the detection of both mutations in codon 12 and 13 Sequencing method used is the technique preferably materialized mutations in *K-RAS* mutation at codon 12 and 13 with regard to the variant nucleotide mutation. Please note that the products obtained after amplification (material from paraffin blocks) should not exceed 200 bp. due to the fact that the quality of the paraffin may be low. The result of poor quality paraffin may be the lack of amplification or inability to sequence analysis because of the very high and non-specific background. Also keep in mind that cell clones with mutations in codon 12 and 13 may be present in small quantities, which can be seen as small peaks in the sequence. Sequencing of both strands the sense and antisense is necessary when the cell clone containing the mutation is small. In conclusion belong to emphasize the fact that sequencing is a technique used to great effect to identify mutations in the *K-RAS* [34].

Differences in the frequency of mutations in the *K-RAS* gene can also be caused by a number of respondents codon-codon 12 and / or 13 and / or 61 and / or others. The majority (over 90%) of N codon 12 mutations (82%) and codon 13 (17%) and codon 61 (1%) [6]. Less likely to be acting mutation in codon 59 and 63. Taking into account only the two most frequently mutated codons 12 and 13 and frequency of their occurrence in the literature is respectively 70% and 30%. The results of their

study, these values were 90% and 10%. Scientific publications also describe mutations in codons 10,11,15,18, 19 and 22 - but their biological significance is not known so far. Mutations affecting codon 146 were observed in 4% of mutations in patients with colorectal cancer, suggesting that this change is more important in colon cancer than mutation affecting codon 61 [6]. The research carried out in many centers in the world, it was found that the most common mutation in codons 12 and 13 is a transition of guanine to adenine and guanine to thymine transversion (except for residents of the former Yugoslavia, who had dominated transversion of guanine to thymine). This is also confirmed its own findings that point mutations in codons 12 and 13 result in altered amino acid sequence of the protein. Most states change of glycine to valine. GGT triplet codon GGC codon 12 and 13 are responsible for the incorporation of glycine. Glycine at position 12 is in spatial proximity to the loop that connects the GAP protein. Each mutation at this position results in the inclusion of an amino acid having a side chain. This results in an inability to move inactive.

Differences in the selection and number of test group can also affect the incidence of mutations in *K-RAS*. The study carried out in the framework of our work included patients operated in the II Department of General and Gastroenterological Surgery, Medical University of Bialystok in the years 2002-2007. In the study group, 43% were women, and the remaining 57% were men. A similar distribution of interest in terms of gender were obtained in a study conducted in the United States by Ogino [22]. The proportion of both sexes was similar - women accounted for 46% of the study group and 54% of men. However, the incidence of mutations was 32%.

In our study, the average age of the patients was 64.8 ± 11.7 years. Among women it was 66.7 ± 10.8 years and males, respectively: 63.1 ± 12.1 years. In a study conducted in the United States, the average age of all patients was 59.8 ± 11.5 years, and in patients with mutations 59.1 ± 11.4 (in our study, 65.0 ± 10.6). The average age of women who are found to have mutations was 71.2 ± 7.0 years and 64.9 respectively ± 11.7 years-in patients who did not have the mutation. In the group of men were somewhat younger age, which was 59.8 ± 10.5 years, respectively, mutated, and 64.2 ± 12.5 years - in patients who did not have mutations. The median age of women who have mutations in the 72 years (67 years - in patients who did not have the mutation), while among men is 58 years (64 years - in patients who show no evidence of mutations) together 66 years ± 11.7 [30]. In summary we selected a group of patients did not differ significantly from the groups selected in other centers. Comparisons of the results obtained by us with the results carried out in the United States for clinical factors in terms of gender and age have a similar effect [22].

Our results based on *K-RAS* mutation from the tumor morphological factors such as location, severity and degree of differentiation and presence of metastases to regional lymph nodes showed no significant differences in both the presence and absence of mutations ($p > 0.05$).

Similar studies that showed no correlation between the presence of mutations in *K-RAS*, and various factors obtained morphological and clinical factors [22].

U.S. studies that focused on the correlation between *K-RAS* mutations as gender, age, location and severity of the tumor included a group of 508 patients diagnosed with colorectal cancer [32]. There was no evidence of any correlation between mutations in the gene *K-RAS* and gender (54% men and 46% women), age (mean age 59.8), tumor location (the location because of the right and left part of the colon) and the severity (T2 -T4) (all $P > 0.23$) [35]. Similar studies conducted in India, where there was no correlation between clinical factors such as age and sex, as well as the location of the tumor (colon and rectum) and tumor stage [35].

CONCLUSIONS

These data support the conclusion that the clinical and morphological factors in patients with colorectal cancer have no effect on the presence of *K-RAS* mutation. This is reflected in their test results obtained, which are similar to those obtained in other research centers in the world.

In the available literature there are no studies proving the correlation between mutations in the gene *K-RAS* and clinical and morphological factors. A pooled analysis of the results indicates that colon cancer is not a homogeneous disease, which could be determined only by mutations in the gene *K-RAS*. Of all gastrointestinal cancers molecular diagnosis of colorectal cancer is of paramount importance and is now at the highest level. It is necessary to conduct further research to early diagnosis and therefore earlier start to treatment of patients with colorectal cancer targeted therapy. Designation status of *K-RAS* gene as a predictive factor for the treatment of patients with colorectal cancer is the greatest achievement in modern studies conducted in connection with this condition. Research also suggests the potential role of other markers, such as the *BRAF* gene mutation, mutations in the path of drug resistance PI3K/AKT in patients with colorectal cancer. The need for research on the other markers to help better qualify patients to targeted therapy, which in turn will improve the effectiveness of treatment.

Conflicts of interest

The authors declare that they have no conflicts interests.

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