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The predictive value of clinical genetic and immunohistochemical characteristics of colorectal cancer

Abstract. *Colorectal cancer is a global problem not only in the Republic of Kazakhstan but also in the world. The annual incidence of colorectal cancer reaches 1 million cases, and the annual mortality rate exceeds 500,000. Colorectal cancer ranks second in mortality from malignant neoplasms among men and women.*

In Kazakhstan, colorectal cancer occupies a leading position. The incidence of colorectal cancer on average per 100 thousand populations from January 2011 to December 2015 amounted to 17.02‰. The absolute number of cases in the Republic of Kazakhstan is 14511 of them: 6937 men, 7574 women. In recent years, the treatment tactics of colorectal cancer, in particular, metastatic cancer has undergone significant changes. To date, the treatment strategy is based not only on the use of classical cytostatics but also targeted therapy. This article provides an overview of the possibilities of using modern targeted drugs, depending on the presence or absence of points of application.

Purpose of the study – *to carry out an analysis of modern literature on the topic of drug therapy based on the study of molecular genetics and immunohistochemical features of the tumor.*

Keywords: *colorectal cancer (CRC), metastatic CRC, PCR, IHC, RAS mutation.*

Angiogenesis is one of the main conditions for tumor growth and metastasis in colon cancer. Experimental studies show the dependence of colon cancer recurrence frequency from the primary tumor vascular density [1]. Vascular endothelial growth factor (VEGF) is one of the main factors for angiogenesis. VEGF induces proliferation and migration of vascular endothelial cells and leads to the formation of new capillaries; it increases vascular wall permeability, thus creating the necessary conditions for better access of oxygen and nutrients to the tumor cells [2]. VEGF activates the relevant receptors (VEGFR-1, -2 and -3), and their signal passes through various signaling pathways including Akt (protein kinase B) and ERK [3]. Therefore, VEGF signaling pathway does not only promotes angiogenesis but also plays an important role in such cellular processes as reproduction, migration, tumor cells invasion and the inhibition of apoptosis [4].

A number of studies conducted in the 1990s have shown a prognostic meaning of the level of VEGF in the serum and tumor tissue of colon cancer patients. Higher VEGF expression was related to a worse prognosis [5, 6].

Today, Kazakhstan has approved three targeted drugs that affect angiogenesis: Bevacizumab, sunitinib, and sorafenib.

Inhibitors of vascular endothelial growth factor (VEGF)

Bevacizumab. Bevacizumab blocks VEGF A what results in a rapid decrease of microvascular bed density. It also normalizes the structure and function of the altered vessels to improve the penetration of chemotherapeutic drugs into the tumor and inhibits its neovascularization [2, 7, 8]. The importance of Bevacizumab in first-line chemotherapy for metastatic colorectal cancer has been shown in four key randomized studies.

In the first study, Bevacizumab has supplemented chemotherapy with irinotecan and 5-fluorouracil/Leucovorin in the IFL regimen. It has significantly increased the frequency of objective response, the time to progression, and the median life expectancy from 15.6 to 20.3 months [9].

In the study NO16966, the patients received a combination of FOLFOX (Oxaliplatin + 5-fluorouracil (5-FU) + calcium folinate (Leucovorin)) or XELOX (Capecitabine + Oxaliplatin) with or without the addition of Bevacizumab. The combination with Bevacizumab has reliably increased the time to progression [10]. The subgroups analysis has shown a significant gain only from the addition of Bevacizumab to the XELOX regimen vs. no statistically significant difference in time to progression in patients treated with FOLFOX. It was noted that half of the patients had to terminate therapy for reasons not related to the disease progression. Among patients who managed to complete treatment before progression, the addition of Bevacizumab has increased the median time to progression regardless of the treatment regimen [11].

Then, the efficiency studies of Bevacizumab in the adjuvant treatment of colon cancer were initiated. The NSABP (National Surgical Adjuvant Breast and Bowel Project) C-08 study included more than 2,600 patients with stage II–III colon cancer. The regimens "FOLFOX ± Bevacizumab" were compared. During the median follow-up of 36 months, no reliable improvement in survival without signs of disease was noted (75.5% in the non-Bevacizumab group vs. 77.4% in the Bevacizumab group; p = 0.15; risk ratio (RR) = 0.85) [12]. Similar results were obtained in AVANT (Randomized, three-arm multinational Phase III

study to investigate with XELOX or FOLFOX4 vs. FOLFOX4 alone as adjuvant treatment for colon cancer) study with a similar design [13].

Three main conclusions can be drawn from the results of Bevacizumab clinical studies. First, sooner or later the patients develop tumor resistance to conducted treatment; moreover, some tumors are apparently not sensitive to Bevacizumab. Considering Bevacizumab as a targeted drug requires finding biomarkers for it. Still, the available clinical signs do not allow predicting who of the patients has or will develop resistance to the drug [14]. Some of the mentioned clinical signs in retrospective and often non-randomized studies could be effectively used to predict the response to adding Bevacizumab to chemotherapy. The most interesting signs include some blood plasma markers (VEGF, soluble VEGF receptors), markers in the tumor tissue (VEGF D), arterial hypertension, and lactate dehydrogenase (LDH) level. However, prior to the practical application of these markers, their significance shall be confirmed in prospective and randomized studies. Therefore, today it is not possible to recommend using any factors to select patients for Bevacizumab therapy. Secondly, monotherapy with Bevacizumab is not efficient enough. It requires its combined use with cytostatics while it does not seem to matter with which chemotherapy drug it should be combined. Thirdly, the inclusion of Bevacizumab in the adjuvant therapy of patients with colon cancer has proved to be inefficient. It might be due to the lack of a substrate for Bevacizumab action, an altered vascular network in micrometastases, in patients who received adjuvant treatment.

Aflibercept. Unlike Bevacizumab, Aflibercept is a "trap" for several growth factors – all VEGF isoforms (A and B), as well as a placenta growth factor (PlGF).

In 2011, the results of a randomized Phase III placebo-controlled study to on assessment of the efficacy of Aflibercept 4 mg/m^2 in second-line treatment of patients with metastatic colon cancer in combination with FOLFIRI (irinotecan + fluorouracil + Leucovorin) were presented. The addition of an anti-angiogenic drug was shown to improve the median life expectancy (13.50 months vs. 12.06 months; RR = 0.817; p = 0.0032), the median time to progression (6.90 months vs. 4.67 months; RR = 0.758; p = 0.00007), as well as to increase the frequency of achieving an objective response (19.8% vs. 11.1%; p = 0.0001) in comparison with the "FOLFIRI + placebo" combination. Diarrhea, asthenia, stomatitis, arterial hypertension, proteinuria, and neutropenia were the most often adverse effects caused by treatment. The subgroup analysis has shown the greatest gain in overall survival in patients with ECOG 0 status (RR = 0.768), in patients younger than 65 years (RR = 0.796), in patients with a history of arterial hypertension (RR = 0.714), and in patients with isolated liver damage (RR = 0.649) [15]. Interestingly, the drug was effective in patients regardless of whether they had received Bevacizumab in the first-line chemotherapy. The median life expectancy among patients who had previously received Bevacizumab was 12.5 months in the group receiving Aflibercept vs. 11.7 months in the group receiving placebo (RR = 0.862) [16]. Thus, a randomized study is required to determine optimal tactics for this category of patients – a continuation of Bevacizumab therapy after progression or a transfer to Aflibercept. Such a study is expected to be registered shortly.

Regorafenib. This drug binds and inhibits 2nd and 3rd type VEGFR tyrosine kinases, c-kit, PDGFR, and Raf-kinase leading to the depression of the tumor angiogenesis and the termination of the tumor cells proliferation.

In February 2012, the results of a Phase III comparative study of Regorafenib and placebo were presented in 760 patients with metastatic colon cancer refractory to standard therapy. Regorafenib has shown a statistically significant increase in the median life expectancy compared with placebo: 6.4 months vs. 5.0 months (calculated RR = 0.773; 95% CI 0.635–0.941; p = 0.0051). The median time to progression in the Regorafenib group was 1.9 months vs. 1.7 months in the placebo group (calculated RR = 0.493; 95% CI 0.418–0.581; p <0.000001). The objective response was 1.6% and 0.4%, respectively. Disease control was achieved in 44% of Regorafenib cases and 15% of placebo cases (p <0.000001). Such complications as the hand-foot syndrome (17%), asthenia (15%), diarrhea (15%), hyperbilirubinemia (8%) and arterial hypertension (7%) were more frequent in the Regorafenib group [17].

The use of other receptor tyrosine kinase inhibitors to VEGF did not increase the life expectancy of patients.

Ramucirumab. Ramucirumab is a human monoclonal antibody IgG-1 that targets the extracellular domain VEGFR-2 which is the main mediator of the VEGF pathway. By binding to VEGFR-2, Ramucirumab prevents all VEGF ligands from binding to VEGFR-2 and inhibits the VEGF pathway. Unlike Aflibercept and Bevacizumab, Ramucirumab prevents VEGF from interacting with the receptor by binding to VEGFR-2 rather than VEGF [18, 19].

Phase II study with the FOLFOX regimen and Phase III study with the FOLFIRI regimen in patients with metastatic colon cancer are still ongoing. In addition, despite the negative results of joint use of angiogenesis inhibitors and anti-EGFR drugs, a Phase II study of a combination of Cetuximab and Ramucirumab was initiated [20]. IMC-18F1 is a monoclonal antibody that selectively blocks VEGFR-1. VEGF is thought to bind directly to VEGFR-2, while VEGFR-1 plays an important role in regulating the activity of VEGFR-2 [21].

A Phase II study is currently underway with IMC-18F1 drug in patients with metastatic colon cancer. Even if its results turn out to be negative, an assessment of clinical efficacy of such drugs as Aflibercept, Ramucirumab, and IMC-18F1 is very important. Since their clinical effect is likely to vary, it will help to understand the role of various components of the VEGF system in colon cancer pathogenesis.

Thus, antibodies to VEGF are most efficient in blocking angiogenesis in patients with metastatic colon cancer, while the inhibitors of receptor tyrosine kinases to VEGF have proven ineffective. An exception is the encouraging results of the study on the use of Regorafenib.

Inhibitors of the signaling pathway from the epidermal growth factor receptor (EGFR)

EGFR hyper-expression is observed in 60–92% of colon cancer cases. After stimulation of the receptor, the signal from the epidermal growth factor is transmitted through a number of intracellular protein molecules, including RAS-RAF-MEK-ERK and PI3K-Akt-mTOR, to the cell's genome and affects cellular processes such as differentiation, proliferation, migration, angiogenesis, and apoptosis [22, 23]. This receptor can be blocked by monoclonal antibodies, two of them, Cetuximab and Panitumumab, being already used in clinical practice. However, a mutation in the KRAS protein gene disrupts this pathway, and the use of monoclonal antibodies to EGFR becomes ineffective. Therefore, the effect of EGFR inhibitors is observed only in patients without such a mutation. The KRAS gene mutation frequency is 40% [24-26].

Cetuximab is a chimeric monoclonal antibody to the outer EGFR domain. In contrast, Panitumumab is a fully human immunoglobulin. The inhibition of EGF receptors not only in the tumor, but also in the normal tissues of the body, determines the development of a number of specific adverse effects during therapy with EGFR inhibitors such as skin toxicity, diarrhea, and hypomagnesemia. A clear correlation has been established between the severity of skin toxicity and the efficacy of anti-EGFR antibody therapy. Patients receiving Panitumumab who developed degree 3-4 skin toxicity had a longer time to progression vs. the patients who developed degree 1-2 skin toxicity and the patients who received only chemotherapy (11.3, 6.1, and 8.7 months, respectively) [27].

In the CRYSTAL study (Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer – Cetuximab in combination with irinotecan as a first-line therapy for metastatic colorectal cancer), in the absence of a KRAS mutation the use of Cetuximab has increased the time to progression by 1.5 months, the overall survival rate – by 3.5 months, and the frequency of achieving the objective response – by almost 1.5 times [28]. In the OPUS study (Oxaliplatin and Cetuximab in First-Line Treatment of mCRC - Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer), in the group of patients with wild type KRAS protein the addition of Cetuximab o the FOLFOX regimen has increased the time to progression, but not the life expectancy [29]. A meta-analysis of these CRYSTAL and OPUS studies (845 patients with the wild KRAS phenotype) has revealed that the addition of Cetuximab increased the probability of achieving an objective response by more than twice in comparison with the patients who received only chemotherapy ($p < 0.0001$) [30].

In the COIN (COntinuous or INtermittent) study conducted by the Medical Research Council (MRC), in contrast to the results of previous studies, the use of a combination of Cetuximab with Oxaliplatin-containing chemotherapy regimens did not lead to an increase in survival without progression and the overall survival, although the objective response was significantly higher (64% vs. 57%, $p = 0.049$) [29]. The negative COIN data has contributed to the emergence of the hypothesis that Oxaliplatin-based regimens were not optimal for co-administration with Cetuximab. The subsequent analysis of that study has shown a lack of gain from Cetuximab only in the XELOX group. This was due to higher toxicity which resulted in a greater reduction of doses of chemotherapeutic drugs and, consequently, reduced the regimen efficacy.

No such inverse correlation was noted in the "FOLFOX + Cetuximab" group. The results of the Scandinavian randomized study NORDIC VII (Phase III trial of Cetuximab with continuous or intermittent fluorouracil, Leucovorin, and Oxaliplatin (Nordic FLOX) vs. FLOX alone in the first-line treatment of metastatic colorectal cancer) in which Cetuximab was studied in combination with the 5-fluorouracil stream regimen in the first-line therapy has made additional intrigue [31]. The addition of Cetuximab did not improve the survival rates but was not associated with higher toxicity.

Another randomized trial PRIME (Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy) has compared the combination of "FOLFOX + Panitumumab" with the FOLFOX regimen as the first-line treatment of patients with metastatic colon cancer. In patients without KRAS mutation, the addition of Panitumumab has reliably increased the median time to progression from 8.0 to 9.6 months ($p = 0.02$) and the frequency of objective response from 48% to 57% ($p = 0.02$) [32]. The life expectancy tended to increase from 19.7 to 23.9 months ($p = 0.07$).

Based on the results of these studies with Cetuximab and Panitumumab, the best chemotherapeutic "partners" for them are the regimens which include irinotecan or FOLFOX, but not XELOX or Nordic FLOX (5-fluorouracil + folinate + Oxaliplatin (Eloxatin®)).

Joint inhibition of the VEGFR and EGFR pathways

Combined blockade of several key receptors (the combined use of Bevacizumab and Cetuximab/Panitumumab) has not been successful. In two randomized Phase III studies, the combination of two targeted drugs has statistically significantly reduced the median time to progression. Among patients with the wild KRAS gene type, the median time to progression and life expectancy did not differ. It shows a negative influence of the combination of EGFR inhibitors with Bevacizumab; its mechanism is still unknown [33, 34].

HER2-inhibitors

The human epidermal growth factor receptor 2 (HER2) is an oncogenic factor and a well-known therapeutic target in breast and stomach cancer. The functional and genomic analysis of xenografts obtained from the patients has shown that a subgroup of approximately 5% of metastatic colorectal cancer (CRC) tumors is due to HER2 amplification or mutation. HER2 amplification is considered as an oncogenic factor, prognostic biomarker and a clinically acceptable target in CRC, taking into account the specifics of HER2 testing in this type of tumor. Although the role of HER2 as a biomarker for prediction in CRC remains uncertain, its importance as a therapeutic goal has been established. Indeed, independent studies have confirmed a substantial clinical benefit in patients receiving therapy fo-

cused on biomarkers targeting HER2, with an impact on the response rates and duration that was favorably distinguished from immunotherapy and other examples of precision oncology. HER2-oriented therapeutic strategies could change the treatment paradigm for the clinically significant subgroup of patients with metastatic CRC.

HER2 is the only member of the EGFR family that does not bind ligands; it is activated by heterodimerization with other ligand-bound receptors [35], with the strongest mitogenic signals generated by HER2-HER3 heterodimers. HER2 overexpression usually caused by gene amplification allows activating HER2 even in the absence of a ligand associated with other partners [36]. Overexpression or amplification of HER2 has been observed in 13–20% of breast cancer cases [37], in 7–34% of stomach cancer cases [37], and in 1.9–14.3% of lung cancer cases [38]. Different levels of HER2 hyper-expression have been recorded in CRC, at that, the rate of membrane expression varied from 2% to 11% [39]. Those factors could be explained by a number of factors including small populations under study, various antibodies for immunohistochemistry (IHC), the analysis of individual subgroups of patients with heterogeneous clinic-pathological characteristics of CRC, and the use of various assessment systems [40]. More recent studies have consistently shown that HER2 overexpression amounts to approximately 2% of all mCRC [40] increasing up to 5% at stage III [9] or IV of KRAS exon 2 wild-type tumors.

The predictive role of HER2 in CRC remains uncertain. Earlier studies proposed a negative prognostic effect of HER2 hyperexpression [41] but later no correlation between HER2 amplification and the outcome has been established. However, in one of the largest studied cohorts (1,645 patients with stages I-IV CRC), OS tended to worsen in 26 patients (1.6%) with HER2-positive disease compared with patients with HER2-negative disease [37]. HER2 was also identified as a poor prognostic indicator in the PETACC-8 study among patients with stage III colon cancer. HER2 changes were present in 66/1,689 patients (3.9%), and both NGS, FISH, and HER2 mutational status determined by NGS were associated with a shorter time to relapse [Hazard Ratio (HR) 1.9, 95% CI 1.1–3.2; P = 0.03] and a shorter overall survival (HR 1.7, 95% CI 0.9 –3.2; P = 0.045). Such predictive value remained after correction for age, treatment, RAS mutation, histological extent, tumor location, pT and pN condition, obstruction or bowel perforation, as well as vascular or lymphatic invasion.

The assessment of a potential prognostic effect of HER2 amplification in CRC is hindered by a low frequency of such changes what could also potentially explain the controversial research findings on that issue. However, according to the available data, a negative prognostic effect of HER2 is less pronounced than in other changes, such as the BRAF mutation.

HER2 amplification is a clinically significant genetic change in mCRC as confirmed by the HERACLES and MyPathway studies. This biomarker can be screened using established diagnostic tools. It is found in a significant 5% of patients with wild-type KRAS mRDC and can potentially be a predictor of the lack of effect of monoclonal antibodies against EGFR. HER2-targeted therapy favorably differs from new therapeutic strategies for mCRC, such as BRAF-directed therapy and immunotherapy with control point inhibitors. HER2 amplification shows a frequency similar to the frequency of tumors with a high level of MSI (MSI-H) (5%) [40] and lower than in BRAF mutations (10%); however, compared with BRAF-targeted combinations (ORR 16–21%; median PFS 4.2 months [40]), the responses achieved so far in clinical studies with HER2-directed therapy are higher (ORR 30–38%) and longer (median PFS 5.2 months [6]), resembling the results obtained with control points inhibitors for MSI-H tumors. The toxicity of HER2-directed combinations is also less than BRAF- or MSI-H-directed therapeutic agents [42]. Thus, HER2-directed therapy appears to combine the advantages of precision medicine (rapid and deep induction of tumor contraction) with immunotherapy (long-term response and better tolerance). Although no data from Phase III studies with HER2-targeted drugs is available, randomized studies will require a long time to achieve results in such a selected population [42]. A strong underlying biological rationale [42], consistent action at a therapeutic level [42], and a favorable comparison with other approaches of precision medicine confirm the need for conditional approval of drugs targeting HER2 for their clinical use by regulatory authorities.

BRAF inhibitors

BRAF-targeted treatment of mCRC has changed rapidly over the past 4 years, with the new opportunities emerging at a high speed. New drugs like Wnt or cyclin-dependent kinase inhibitors in combination with BRAF inhibitors are currently being evaluated in Phase I and II studies. The introduction of targeted therapy at an early stage of the disease, with or without chemotherapy, is also under assessment. Changes in the BRAF-mt treatment scenario are expected in the coming years. The results of using the new drugs targeted at the MAPK pathway are also expected. E.g., new molecules specifically targeting both BRAF, and C-RAF or ERK (downline mediators of the MAPK pathway) have proven their safety and efficacy in preclinical models, and the development of Phase I has begun (NCT02607813; NCT02711345).

Nevertheless, the resistance to targeted therapy is developing almost systematically, and its characterization and overcoming are the subjects of ongoing studies. A pre-clinical study published by Oddo et al. [43] has shown reactivation of the MAPK pathway in resistant cells: the mutations or amplifications of KRAS, EGFR, MAP2K1 or BRAF were reported. KRAS mutations were also observed in circulating DNA plasma in a resistant patient report. A group of authors has also tested in vitro combinations in those resistant cells to show that the combined inhibitors of ERK, BRAF and EGFR could overcome the acquired resistance. Clinical trials are currently required to study such combinations in an attempt to improve the results of treatment of CKPP BRAF-mt, but safety profiles can be a problem. Similarly, Ahronian et al. have reported the emergence of KRAS amplification, BRAF amplification, and MEK1 mutation in the matched post-treatment biopsies of patients receiving targeted BRAF therapy [44]. These

changes lead to a resistance that can be reversed with ERK inhibitors. MET amplification has also been described as a mechanism of resistance to BRAF inhibition which could be reversed by double inhibition of BRAF and MET (Vemurafenib and Crizotinib) [45].

BRAF-mt tumors have played a major role in the era of the CRC molecular classification based on gene expression profiling. CRC consortium has proposed four different subtypes based on gene expression signatures. BRAF mutations have been observed in all four subtypes [46]. However, there is a clear enrichment of CMS1 (MSI immune subtype) characterized by greater immune infiltration and worse survival after relapse, similar to the classic CRC BRAF-mt. Prior to this classification, a 64-gene expression signature with 96% sensitivity and 86% specificity was identified for BRAF-mt CRC [47]. However, the same signature has been found in the BRAF-WT CRC subpopulation which also had a worse survival rate similar to the classic BRAF-mt CRC. Consequently, all tumors with this genetic signature have been identified as BRAF-like CRC. There are many ongoing studies to confirm this and propose new treatments. Vecchione et al. [48] have recently reported that RANBP2 (a gene encoding a protein that regulates the organization of microtubules in the mitotic spindle) is necessary for the survival in these tumors. *In vitro* and *in vivo* studies have shown a possible significant efficacy of Vinorelbine, as a microtubule disruptor, in these tumors. A prospective study to prove this hypothesis will start soon in the framework of the European MoTriColor project.

On the other hand, these objects appear to be heterogeneous if we go further in the transcriptome classification of colorectal BRAF-mt tumors [49]. The analysis of the gene profile BM1 and BM2 has allowed identifying two subgroups from uncontrolled clusterization. BM1 shows the activation of the KRAS/AKT pathway, mTOR deregulation and epithelial-mesenchymal transition, while BM2 is characterized by cell cycle deregulation (high level of CDK1 and low level of cyclin D1). BM1 has a worse prognosis, and a different treatment approach is recommended compared to BM2. For example, inhibition of BRAF/MEK/PI3K can be of great benefit to BM1 as compared to inhibition of CDK1, which can be of great benefit to BM2. These results may offer a new perspective for CRC BRAF-mt allowing to classify and select the best treatment beyond the BRAF/EGFR blockade.

Finally, partial overlapping of mutations between MSI and BRAF is common in this population. In the era of cancer immunotherapy, anti-PD1 drugs have been approved for MSI tumors including mCRP. However, the role of these antibodies in mCRC MSI BRAF-mt remains unclear. Therefore, the best sequence (target therapy or checkpoint inhibitors) may be studied in the future.

Conclusion. All the foregoing necessitates the research aimed at a comprehensive study and immunohistochemical characterization of colorectal cancer. Some mCRC patients can achieve tumor regression and improve their long-term survival prospects thanks to more active use of modern cytostatic regimens in conjunction with targeted drugs.

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