Copy number variation and clinical response to chemotherapy and bevacizumab in the Czech metastatic colorectal cancer patients

Variabilita počtu kopií genů a léčebná odpověď na chemoterapii a bevacizumab u českých pacientů s metastatickým karcinomem kolorekta

Stránská J.[1,2], Bartáková K.[1], Rožánková Z.[1], Kotková L.[1], Vrbková J.[1], Trojanec R.[1], Flodr P.[3], Jurtíková H.[1,2], Líznerová B.[1,2], Drábek J.[1,2]

[1] Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic
[2] University Hospital Olomouc, Czech Republic
[3] Institute of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic

Summary

Background: Despite bevacizumab being the first biological agent approved for the treatment of metastatic colorectal cancer (mCRC), there is not any established DNA biomarker to improve its efficacy and personalize the treatment. ***Materials and methods:*** Thirty patients with mCRC on bevacizumab therapy (15 with a good response and 15 with a poor response) from the University Hospital Olomouc were followed. Formalin-fixed paraffin-embedded (FFPE) samples were used for copy number variation (CNV) analysis using the OncoScan FFPE Assay Kit in order to capture approx. 900 tumor genes. ***Results:*** In the group of good responding patients, 102 genes (classified as ATPases, type AAA, neuronal signal transmission, regulation of transcription, and superior domain PH type), potentially significant positive predictive tumor biomarkers of bevacizumab treatment, were found. In the poorly responding group, 74 potentially negative predictive genes (classified as galectines, Jak-STAT signalling pathway, MAPK cascade, differentiation, and F-box associated domain) were identified. ***Conclusion:*** In the pilot study, we found promising copy number variation biomarkers of bevacizumab response in FFPE samples of mCRC patients. The validation phase should be focused especially on the genes associated with angiogenesis (*AGRN, MAPK8, ARHGAP22, LGALS13, LGALS4, ZFP36,* and *MYC*), tumorigenesis (DVL1), and tumor proliferation (*IFNL1, IFNL2, IFNL3, MAP3K10,* and *MAP4K1*).

Key words

bevacizumab – colorectal carcinoma – structural genetic variation

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Prof. Jiří Drábek, PhD
Ústav molekulární a translační medicíny
Hněvotínská 1333
779 00 Olomouc
Czech Republic
e-mail: jiri_drabek@seznam.cz

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Souhrn

Východiska: Přestože je bevacizumab prvním biologickým léčivem schváleným pro léčbu metastatického kolorektálního karcinomu (mCRC), neexistuje žádný zavedený DNA biomarker, který by zlepšil jeho účinnost a personalizoval léčbu. ***Materiál a metody:*** Sledováno bylo 30 pacientů s mCRC na terapii bevacizumabem (15 s dobrou odpovědí a 15 se špatnou odpovědí) z Fakultní nemocnice Olomouc. Pro analýzu variací v počtu kopií genů (copy number variation – CNV) byly použity vzorky FFPE a OncoScan FFPE Assay Kit, který zachycuje přibližně 900 nádorových genů. ***Výsledky:*** Ve skupině dobře reagujících pacientů bylo jako potenciálně významné pozitivní prediktivní nádorové biomarkery léčby bevacizumabem identifikováno 102 genů (klasifikovaných jako ATPázy, typ AAA, neuronální přenos signálu, regulace transkripce a domény typu PH superior). Ve špatně reagující skupině bylo identifikováno 74 potenciálně negativních prediktivních genů (klasifikovaných jako galektiny, signální dráha Jak-STAT, kaskáda MAPK, diferenciace a doména asociovaná s F-boxem). ***Závěr:*** V pilotní studii jsme našli slibné biomarkery variace počtu kopií odpovědi na bevacizumab v FFPE vzorcích nádorů pacientů s mCRC. Validační fáze by měla být zaměřena zejména na geny spojené s angiogenezí (*AGRN, MAPK8, ARHGAP22, LGALS13, LGALS4, ZFP36* a *MYC*), tumorigenezí (DVL1) a proliferací tumoru (*IFNL1, IFNL2, IFNL3, MAP3K10 a MAP4K1*).

Klíčová slova

bevacizumab – kolorektální karcinom – variabilita počtu kopií segmentů DNA

Introduction

Bevacizumab (Avastin®, F. Hoffman-La Roche AG, Basel, Switzerland) is a recombinant humanized monoclonal antibody that binds extracellularly to vascular endothelial growth factor A (VEGF-A), preventing its interaction with VEGF receptors (VEGFR) on the surface of endothelial cells. Bevacizumab inhibits the angiogenic activity of VEGF-A, thus limiting the formation of new blood vessels (antiangiogenic effect) and reducing the density of existing vasculature (antivascular effect) [1,2]. Bevacizumab used to be the mainstay of targeted biological colorectal cancer (CRC) therapy for patients with mutations in *RAS* genes (40–50% cases) [3] in whom cetuximab or panitumumab cannot be used [4]. According to Institute of Biostatistics and Analyses, Brno, Czech Republic, bevacizumab is usually part of the FOLFOX or XELOX combination treatment for CRC in Czech cancer centers. Compared to cetuximab, bevacizumab prolongs overall survival and progression-free survival (PFS) in right-sided RAS wildtype and BRAF wildtype and BRAF mutant tumors [5,6].

Despite bevacizumab being the first biological agent approved for metastatic colorectal cancer (mCRC), there is not any established DNA biomarker to improve its efficacy [7]. It does not mean that such a marker was not searched for: somatic mutations in *NRAS*, *BRAF*, and/or *PIK3CA* gene were suggested as a negative prognostic biomarker [8]. Even mutation in *KRAS* that disqualifies patients for cetuximab and panitumumab treatment and originally steered patient to bevacizumab treatment is suggested as a negative prognostic factor of bevacizumab [8,9]. Also, many single nucleotide polymorphisms (germ-line variants) in genes are involved in inflammation, immune system, and RAS signalling [10–13]. However, none of these markers was confirmed in independent studies or used clinically.

We hypothesize that somatic DNA variants, biomarkers of bevacizumab treatment, exist but were for a long time overlooked because of technical limitations – because they pose copy number variants (CNVs). Such type of variation now became accessible for testing using technology of molecular inversion probes.

Material and methods

Clinical records of 142 patients with metastatic colorectal cancer treated at University Hospital Olomouc were used to select 15 + 15 patients according to their clinical response to bevacizumab treatment. The tumor tissue was examined by an experienced pathologist and the percentage of tumor cells was determined. Formalin-fixed paraffin-embedded (FFPE) sections were used to isolate genomic DNA using the Cobas DNA Sample Preparation Kit (Roche). DNA quantification was performed by qPCR and was related to the concentration of the operational gene *GAPDH* in the sample compared to the standard [14].

Tab. 1. Patients' cohort parameters.

	Poor responding patients	Good responding patients
sex	8 female / 7 male	7 female / 8 male
age	42–76 years (median 62 years)	45–70 years (median 62 years)
tumor tissue	10 primary carcinomas / 5 metastasis	7 primary carcinomas / 8 metastasis
colon	3 right / 12 left	3 right / 12 left
therapy length	median 168 days	median 236 days

Tab. 2. Patients' characteristics in both cohorts.

	Sex	Age at diagnosis	Tumor tissue	Tissue origin	Colon	Therapy length (days)	PFS (months)
Poor responding patients							
patient 1	M	66	meta	rectosigmoid junction	left	98	3
patient 2	F	64	prim	rectum	left	108	4
patient 3	M	63	prim	sigmoid colon	left	154	5
patient 4	F	60	meta	caecum	right	245	8
patient 5	M	64	prim	ascending colon	right	161	8
patient 6	M	52	prim	ascending colon	right	111	9
patient 7	M	61	prim	rectum	left	161	5
patient 8	F	64	meta	rectum	left	120	5
patient 9	F	57	prim	rectosigmoid junction	left	168	6
patient 10	F	56	prim	sigmoid colon	left	181	6
patient 11	F	62	meta	rectum	left	177	6
patient 12	F	42	prim	sigmoid colon	left	189	6
patient 13	M	49	prim	sigmoid colon	left	180	7
patient 14	M	69	prim	rectum	left	236	7
patient 15	F	76	meta	splenic flexure	left	184	6
Good responding patients							
patient 16	M	62	prim	large intestine	left	301	10
patient 17	M	65	prim	rectum	left	154	13
patient 18	F	68	meta	sigmoid colon	left	877	14
patient 19	M	59	meta	caecum	right	236	14
patient 20	F	70	meta	caecum	right	739	16
patient 21	F	65	prim	sigmoid colon	left	113	10
patient 22	M	68	prim	hepatic flexure	right	245	11
patient 23	F	49	meta	rectum	left	159	12
patient 24	M	54	prim	rectum	left	499	12
patient 25	F	52	prim	rectum	left	351	18
patient 26	M	45	prim	sigmoid colon	left	238	18
patient 27	F	65	meta	rectum	left	168	19
patient 28	M	61	meta	sigmoid colon	left	132	23
patient 29	M	51	meta	sigmoid colon	left	109	94
patient 30	F	69	meta	sigmoid colon	left	145	11

meta – metastases, PFS – progression-free survival, prim – primary

Analysis of DNA segment copy number variation (CNV) was performed using the OncoScan FFPE Assay Kit 1.0 (Thermo Fisher Scientific), which uses molecular inversion probe (MIP) technology and is designed primarily for analysis in limited amounts of degraded FFPE-derived DNA. An input amount of 80 ng of DNA is sufficient. The probes are designed for more than 220,000 single nucleotide polymorphisms (SNPs) located every 50–120 kb in approx. 900 tumor genes. The OncoScan FFPE Assay protocol was performed according to the manufacturer's instructions.

After scanning the arrays, raw data were obtained in the form of CELL files, which were analyzed using OncoScan

Console 1.3 (Thermo Fisher Scientific). Quality control of MAPD and ndSNPQC parameters was also performed. Subsequently, the data were analyzed in R software [15] using the rCGH package [16]. The resulting segmentation data obtained by the segmentCGH function were normalized by the EMnormalize function with default parameter settings, except for the mergeVal parameter set to 0. This normalized segmentation file was divided into two subsets according to the experimental groups. Both these segmentation subsets were further analyzed with the GISTIC 2 tool [17] to identify significant tumor targets in the genome. The threshold log2 ratio value for the section with loss of DNA segments (deletion section) was set to −0.3 (19% decrease) and for the section with increased DNA segment copy number (amplified section) to 0.3 (23% increase). This resulted in the identification of sites in the genome with a demonstrably higher or lower copy number in each experimental group compared to the normal population and the genes located in these sections. The DAVID database version 6.8 [18] and its Functional Annotation Chart tool were used to annotate genes.

Results

The combination of chemotherapy and biological therapy (first-line FOLFOX and bevacizumab), mutated *KRAS* gene, wildtype *BRAF* gene, and PFS were taken into account in the selection process, which divided the 30 patients into a poor responding group (PFS ≤ 9 months) and a well responding group (PFS ≥10 months). The experimental groups consisted of 15 men and 15 women aged 42 to 76 years at the time of the initiation of bevacizumab treatment (with a median age of 62 years), from whom 13 metastases (meta) and 17 primary tumors (prim) were collected (Tab. 1, 2). Tissue samples from study patients were obtained with informed consent and the study was approved by the ethical committee of University Hospital Olomouc (NV15-31230A).

In the group of good responding patients, 102 genes, potentially significant positive predictive tumor biomarkers of bevacizumab treatment, were found. In the poor responding group, 74 potentially negative predictive genes were identified.

In the group of good responders, only the 18p11.32 region, where 14 genes were located, was significantly amplified, while multiple regions were significantly deleted: 1p36.33 (61 genes), 8p11.22 (2 genes), 10q11.23 (21 genes), 14q32.33 (2 genes), 16p13.3 (1 gene), and 20p12.1 (1 gene) (Fig. 1 A, B, on the left). In the poor responding group, regions with increased copy number of segments 8q24.21 (1 gene), 14q12 (1 gene) and 19q13.2 (72 genes) were found (Fig. 1A, on the right), but no deletion exceeded the threshold of 0.25 (Fig. 1B, on the right).

Using the Functional Annotation Chart function, the DAVID gene ontology tool version 6.8 divided the genes identified in the group of good responders into four groups at the 5% significance level. Almost all genes were lying within regions with a loss of DNA segments, except for the *THOC1* gene, and accounted for no more than 5% of the total number of genes in this group (identified in this group of patients). The first group consisted of ATPase enzymes (*ATAD3A*, *ATAD3B*, and *ATAD3C*) occurring in the 1p36.33 region. The next group consisted of genes involved in neuronal signal transduction (*AGRN*, *MAPK8*, *DVL1*, *CHAT*, and *SLC18A3*) from 1p36.33 and 10q11.23. *ERCC6* from 10q11.23 and *THOC1* from 18p11.32 were included in the group involved in transcription regulation. A group of genes from the 1p36.33 and 10q11.23 regions encode proteins with the PH domain involved in signal transduction processes. Some of them have GTPase activity, while others bind to phospholipids (*AGAP4*, *ACAP3*, *ARHGAP22*, *WDFY4*, and *PLEKHN1*).

In the group of patients with poor response to bevacizumab treatment, five groups of amplified genes were generated at a 5% significance level after analysis in the DAVID tool. The genes accounted for a maximum of 11% of the total number of genes in this group and are almost exclusively found in the 19q13.2 region. The *CLC*, *LGALS13*, *LGALS17A*, *LGALS14*, *LGALS16*, *LGALS4*, *LGALS7*, and *LGALS7B* genes belong to the carbohydrate-binding galectins involved in apoptotic and hydrolytic processes.

The *IFNL1*, *IFNL2*, and *IFNL3* genes are involved in the positive regulation of the immune response and contribute to the Jak-STAT signalling pathway. The *MAP3K10*, *MAP4K1*, *ZFP36*, *PSMC4*, *PSMD8*, *MYC*, and *RASGRP4* genes are part of the MAPK signalling pathway. The *EID2*, *EID2B*, *SIRT2*, *CATSPERG*, *DLL3*, and *GGN* genes are associated with differentiation. The *FBXO17*, *FBXO27*, and *NCCRP1* genes encode F-box proteins involved in protein degradation (Tab. 3).

Discussion

We performed the analysis on a cohort of 30 patients that was divided into two subcohorts depending on their good or poor response to bevacizumab treatment. We discovered several groups of genes that may be related to cancer initiation, development, and spread.

In the good responding patients' group, we found three genes belonging to the AAA-ATPase family (namely *ATAD3A*, *ATAD3B*, and *ATAD3C*) in the DNA segment loss region. Of this group, the *ATAD3A* gene is most frequently found in patients with lung adenocarcinoma. Overproduction of ATAD3A is associated with increased resistance to therapy and poorer prognosis [19,20]. Similar findings also apply to astrocytomas, in which the production of ATAD3B may be involved in chemoresistance. In contrast, oligodendrogliomas do not produce ATAD3 protein at all and are among the treatment-sensitive gliomas [21]. ATAD3B somatic mutation is associated with shorter overall survival and appeared only in metastatic patients [22]. Treatment resistance has also been confirmed for these genes in patients with breast and prostate cancer [23, 24].

Furthermore, a group of genes involved in neuronal signal transmission was created in the annotation tool for this group of patients, including *AGRN*, *MAPK8*, *DVL1*, *CHAT,* and *SLC18A3* genes, which were also located in the region with loss of DNA segments. Agrin (AGRN), a heparan sulphate polysaccharide found both on the cell surface and

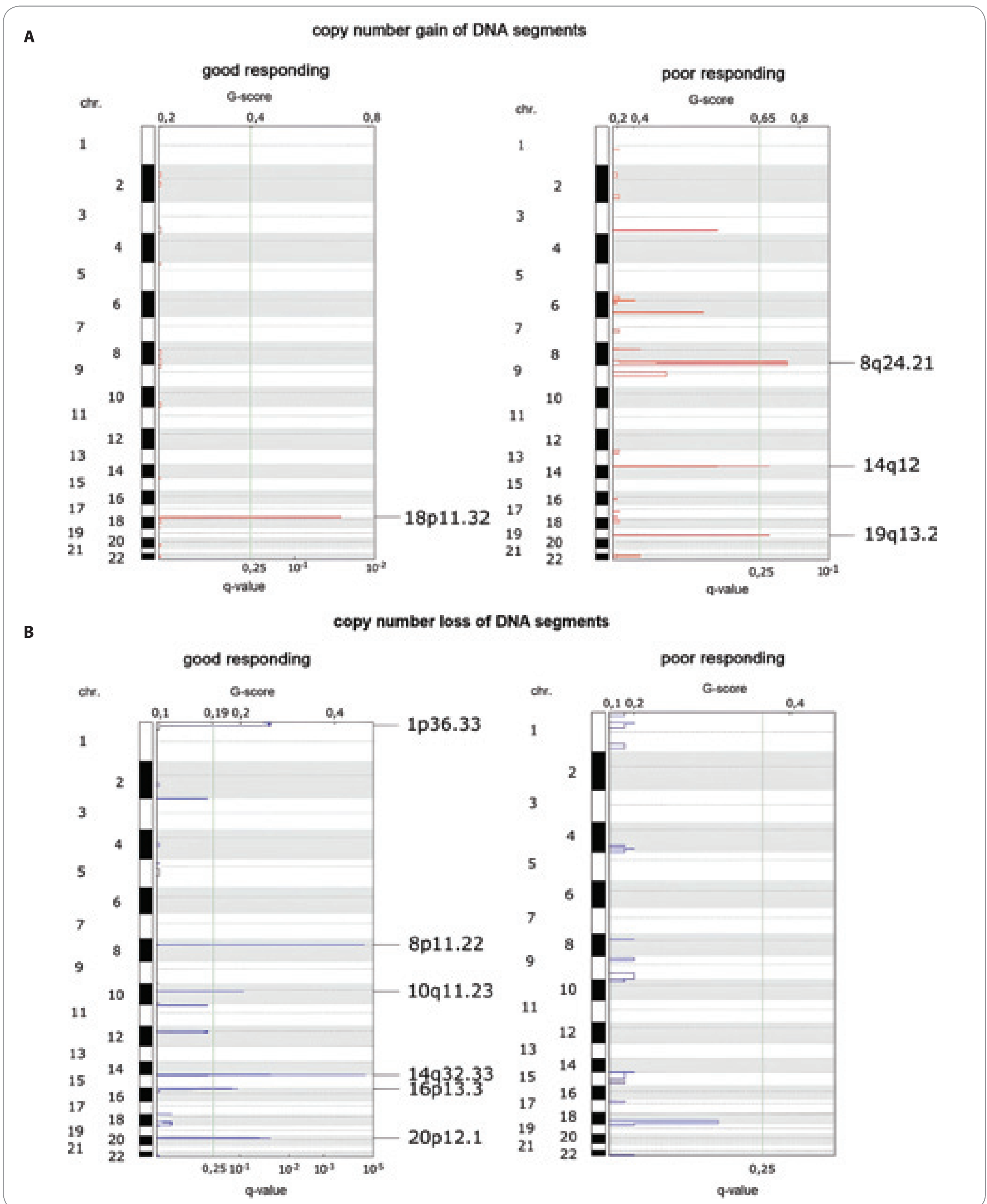

Fig. 1. An amplification/gain plot (A) and deletion/loss plot (B) generated by GISTIC 2 that identifies significant tumour targets in the genome by analysing all features with increased copy numbers of DNA segments within selected regions. The G-score value takes into account the intensity of the aberration as well as the frequency of its occurrence across samples. The q-value = 0.25, illustrated by the green line, represents the significance threshold.

Tab. 3. Significant functional groups of genes overview.

	Altered area	Genes in the area	Frequency of signal
Good responding patients			
ATPases, type AAA	1p36.33	*ATAD3A, ATAD3B,* and *ATAD3C*	7/15
neuronal signal transmission	1p36.33 10q11.23	*AGRN* and *DVL1* *MAPK8, CHAT,* and *SLC18A3*	7/15 2/15
regulation of transcription	10q11.23 18p11.32	*ERCC6* *THOC1*	2/15 3/15
superior domain PH type	1p36.33 10q11.23	*ACAP3* and *PLEKHN1* *AGAP4, ARHGAP22,* and *WDFY4*	7/15 2/15
Poor responding patients			
galectines	19q13.2	*CLC, LGALS13, LGALS17A, LGALS14, LGALS16, LGALS4, LGALS7,* and *LGALS7B*	8/15
Jak-STAT signalling pathway	19q13.2	*IFNL1, IFNL2,* and *IFNL3*	8/15
MAPK cascade	19q13.2 8q24.21	*MAP3K10, MAP4K1, ZFP36, PSMC4, PSMD8,* and *RASGRP4* *MYC*	8/15 12/15
differentiation	19q13.2	*EID2, EID2B, SIRT2, CATSPERG, DLL3,* and *GGN*	8/15
F-box associated domain	19q13.2	*FBXO17, FBXO27,* and *NCCRP1*	8/15

intracellularly, is important in angiogenesis [25,26]. This protein has been produced in increased amounts by bile duct and liver cancer cells as well as squamous cell carcinoma cells, where it has been involved in cell migration, cell adhesion and resistance to treatment [27,28].

Mitogen-activated protein kinases (*MAPK8*, formerly *JNK1*) are involved in many functions in the body – cell proliferation, differentiation, survival, stress response, apoptosis, and cell transformation by activated oncogenes in many cell types [29]. The overproduction of MAPK8 protein in healthy organisms promotes invasiveness and angiogenesis and is involved in tumor progression and resistance to cytostatic drugs [30–32]).

The *DVL1* gene is part of the Wnt signalling pathway, which has a function in embryogenesis and tumorigenesis. Increased DVL1 production accelerates breast tumor growth and in CRC, it is associated with the perineural spread of tumor and liver metastasis [33–35]. Increased choline acetyltransferase (CHAT) production has been observed in patients with squamous cell carcinoma of the lung [36]. Increased *SLC18A3* gene activity has been reported in CNS metastases of papillary thyroid carcinoma [37].

Another group of genes regulating transcription consisted of the *ERCC6* gene from the region with a loss of DNA segments and the *THOC1* gene from the region with an increased number of DNA segments. The ERCC6 protein, a DNA protection factor, is involved in cell hypertrophy, but when it is knocked out of function, cell proliferation is reduced, and apoptosis is triggered. B cells are also more sensitive to commonly used chemotherapeutic drugs [38]. THOC1, a subunit of the THO complex involved in the formation of mRNA ribonucleoprotein complexes, is produced to a greater extent in lung and ovarian tumors, but less so in the skin and testicular tumors [39]. In general, more THOC1 is formed in tumor cells, and in CRC patients, the expression level can distinguish patients with an aggressive phenotype and associated poor prognosis [40,41].

The last group of genes in patients with a good response to treatment consisted of genes functionally superior to the PH-type domain, namely *ACAP3*, *PLEKHN1*, *AGAP4*, *ARHGAP22*, and *WDFY4*. Of this group of genes, according to the literature, only the product of the *ARHGAP22* gene is related to angiogenesis and has been linked to the development of diabetic retinopathy in patients with diabetes [42].

In patients with a poor response to treatment, the first identified group was a family of abundantly represented galectins, which find their applications in diverse cellular processes such as embryonic development, wound healing, apoptosis, intercellular junction formation, cell migration, immune response and, last but not least, malignant proliferation [43–45]. Representatives of this group were specifically the *CLC* gene, *LGALS13*, *LGALS17A*, *LGALS14*, *LGALS16*, *LGALS4*, *LGALS7*, and *LGALS7B*. The *CLC* gene was more highly expressed in patients with early CRC compared to those with late-onset disease [46]. The amount of protein corresponds to the number of eosinophils at the site of inflammation [47]. A group of genes on chromosome 19 encoding placental galectins (*LGALS13*, *LGALS14*, and *LGALS16*) trigger

T cell apoptosis and are involved in immune tolerance [48]. During pregnancy, the product (PP13) of the *LGALS13* gene is involved in the vasodilatation of maternal blood vessels required to increase blood flow to the fetus [49]. The *LGALS4* gene has been reported to be less expressed in the tissue of CRC patients and was essentially absent in patients with the invasive form of the disease. The product of this gene is involved in cell cycle arrest and slowing cell migration and motility. *LGALS4* expression reduces the production of proteins of the Wnt signalling pathway. The Wnt signalling pathway is important in many biological processes, such as cell differentiation, migration, and polarity. Dysregulation of this signalling pathway is a hallmark of CRC [50]. However, another research group found elevated levels of galectin 4 in the serum of CRC patients examined, and levels were even higher in metastatic disease [51,52]. Elevated levels of circulating galectins 2, 4, and 8 in the blood induce the secretion of cytokines and chemokines from the vascular endothelium, which promotes the formation of endothelial adhesion molecules and endothelial ducts as a part of angiogenesis [53].

The second group consisted of lambda interferons (*IFNL1*, *IFNL2,* and *IFNL3*), functionally classified as a group of genes associated with the Jak-STAT signalling pathway that triggers an immune response and antiviral, antiproliferative, and antitumor responses [54,55].

The third group was represented by the *MAP3K10*, *MAP4K1*, *ZFP36*, *PSMC4*, *PSMD8*, *MYC,* and *RASGRP4* genes and functionally aligned to the MAPK cascade. In ductal adenocarcinoma of the pancreas, *MAP3K10* and *MAP4K1* genes have opposing effects. Loss of *MAP3K10* expression decreases cell proliferation, whereas the absence of *MAP4K1* is associated with the progression and development of invasive disease [56,57]. The opposite situation occurs when MAP4K1 is knocked out of function in colorectal cancer, and when tumor cell invasiveness is suppressed [58]. The *ZFP36* gene has a tumor suppressor function. It suppresses the growth of colorectal cancer cells by regulating the expression of *VEGF* and *COX-2*. Its production is reduced in cancer cells, but it is produced in high amounts in healthy mucosa [59–61]. The *PSMC4* gene promotes cell death caused by stress factors and increases intracellular protein ubiquitination [62]. The *MYC* (c-Myc) gene influences the regulation of angiogenesis, and an increased copy number of this gene indicates a worse disease prognosis for patients in clinical stage II and III disease [63,64]. Other genes are not well described in the literature.

The fourth group of genes was associated with differentiation and includes the following genes: *EID2*, *EID2B*, *SIRT2*, *CATSPERG*, *DLL3,* and *GGN*. The *SIRT2* gene plays an important role in the cellular response to hypoxia through the regulation of the hypoxia-inducible factor (HIF-1α) [65]. The remaining genes have not been described at all or only marginally.

The last group consisted of genes from the F-box associated domain. These were the *FBXO17*, *FBXO27,* and *NCCRP1* genes. None of these genes has been described in the literature concerning angiogenesis or cancer.

Increased expression of the *DVL1* gene may be important in breast cancer carcinogenesis due to disruption of the Wnt signalling pathway [33,34]. It may be also involved in the development of cervical cancer, again through disruption of the Wnt signalling pathway [66]. The protein is formed to a greater extent in prostate cancer and this may be related to progression through the Wnt/beta-catenin pathway [67].

The hERG1 and aHIF-2α genes were found to be independent prognostic factors for a positive response to bevacizumab [68]. It appears that hERG1 is upstream of HIF-2α and the entire proangiogenic signalling pathway, confirming what has been previously shown in CRC cells. Indeed, in the latter model, hERG1 activity positively regulates HIF-2α expression and subsequently VEGF-A secretion, suggesting that hERG1-positive patients who have more aHIF-2α and greater VEGF-A secretion would benefit from VEGF-A blockade via bevacizumab treatment, as shown in the reported survival analysis. The importance of hERG1 and its downstream pathway is also suggested by the finding that hERG1-positive patients with KRAS mutations have longer disease PFS than patients with the same mutation but negative for hERG1 [68, 69].

Conclusion

In the pilot study of FFPE samples from mCRC patients (15 good responders and 15 poor responders to bevacizumab), using OncoScan method, we found promising copy number variation biomarkers. This small cohort of 30 patients can serve as a learning set, ready for validation using method amenable for a low number of targets but a higher throughput (such as PCR methods) in a larger cohort of patients. The validation phase should be focused especially on genes associated with angiogenesis (*AGRN*, *MAPK8*, *ARHGAP22*, *LGALS13*, *LGALS4*, *ZFP36,* and *MYC*), tumorigenesis (*DVL1*), and tumor proliferation (*IFNL1*, *IFNL2*, *IFNL3*, *MAP3K10*, and *MAP4K1*).

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Availability of data and materials

The data used in this study are available from the corresponding author upon request.

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