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FIRST LINE 5-FU-BASED CHEMOTHERAPY WITH/ WITHOUT BEVACIZUMAB FOR METASTATIC COLORECTAL CANCER: TISSUE BIOMARKER CANDIDATES

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ABSTRACT

Purpose: Colorectal cancer is the second leading cause of cancer mortality in the USA. According to Bulgarian National Statistics Institute, 2370 colon and 1664 rectal cancer cases were diagnosed in 2012 with total number of patients 29995. Adding bevacizumab to chemotherapy in patients with metastatic disease improves progression-free survival (PFS) but no predictive markers have been proven in the clinical practice. In our study we examined two tissue biomarkers that may correlate with response to bevacizumab-containing chemotherapy in patients with metastatic colorectal cancer.

Patients and Methods: 54 patients with metastatic colorectal cancer were assigned to first line 5-Fu-based chemotherapy with/without bevacizumab. The primary end point was PFS, with additional determination of response and toxicity. Paraffin-embedded samples from primary tumors were collected from all 54 patients. Expression levels of two tumor biomarkers VEGFR-2 and Neuropilin 1 (NP-1) were evaluated with immunohistochemistry.

Results: The median PFS for the group treated with CT/Bev was 8.8 months, compared with 5.4 months for the group with chemotherapy alone (95% CI, log-rank test *P* =0.003). The corresponding overall response rates were 19.3% and 10.2% respectively ($P < 0.05$ for CT/Bev *vs* CT). Patients with low NP-1 had statistically significant prolongation of PFS as compared to those with high NP-1 (95% CI, log rank test p= 0.017). Patients with low NP-1 appeared to experience a larger bevacizumab treatment effect in terms of PFS (p=0,049,HR 0.333, 95% CI, 0.111 to 0.995) than patients with high NP-1.

Conclusion: The addition of bevacizumab to 5-Fu based chemotherapy improves PFS for patients with metastatic colorectal cancer. Expression of tumor NP-1 is a potential biomarker candidate for prediction of clinical outcome in patients with metastatic colorectal cancer, treated with first line chemotherapy plus bevacizumab.

Keywords: colorectal, bevacizumab, VEGFR-2, biomarkers, neuropillin-1,

INTRODUCTION

The development of new blood vessels, termed angiogenesis, is a typical hallmark of cancer development. Four decades ago, angiogenesis was recognized as a therapeutic target for blocking cancer growth and antiangiogenic therapy showed broad clinical activity. (1) The most important signaling molecule is the vascular endothelial growth factor or VEGF – it plays a central role in angiogenesis and is frequently highly expressed in cancers. Thus clinical efforts to develop antiangiogenic therapies have largely focused on inhibiting VEGF. (2) However not all patients benefit from antiangiogenic therapy; the magnitude of response to this treatment also varies among patients, which makes identification of potential predictive biomarkers a crucial point in clinical practice. (3) Identifying which tumors are most sensitive to anti-VEGF therapy would improve therapeutic outcome of patients and could provide insights into the mechanism of resistance to anti-VEGF therapy.

Multiple VEGF receptors are expressed on endothelial cells, including signaling receptor tyrosine kinases (VEGFR-1 and VEGFR-2) and the non signaling co-receptor Neuropilin-1. It is considered that the proangiogenic effect of VEGF is mediated predominantly via VEGFR-2. (4-6) Some reports found significant correlations between VEGF and VEGFR-2 genes with survival after bevacizumab treatment in metastatic colorectal cancer. (7) VEGF interaction with VEGFR-2 and NRP-1 in cancer cells may be critical for the growth of tumors that depend on this pathway for survival and, through indirect mechanisms, to angiogenesis in tumors. (8) Neuropilin-1(NP-1) binds only the isoform of VEGF responsible for pathological angiogenesis (VEGF165), regulating its activity (9); thus it is a potential target for inhibiting VEGF signaling.

In our single center study we compared the efficacy of *bevacizumab* (anti-VEGF antibody) plus chemotherapy versus only chemotherapy as first line-treatment for patients with metastatic colorectal cancer (mCRC). We tried to improve our understanding of the complexity of tumor angiogenesis with the evaluation of two tissue biomarkers in the primary tumors: VEGFR-2 and NP-1.

PATIENT SELECTION

We conducted a prospective non-experimental clinical study of 54 patients with histologically confirmed metastatic colorectal adenocarcinoma stage IV as per AJCC, 7th ed. All patients underwent surgery of the primary tumor; they had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) (10) and were eligible for bevacizumab-containing 5-Fu based chemotherapy regimens as first line treatment. Their ECOG performance status was <2. Chemotherapy was performed at the University Hospital "Sveta Marina" and patients were subsequently followed for a period of up to 2 years. Prior to inclusion in our study we obtained ICF for collection of biological material (tumor sample) from all patients willing to participate.

Prespecified tissue biomarkers included protein expression of NP-1 and VEGFR-2. Tumor samples from paraffin-embedded blocks from the primary tumor were collected at baseline.

VEGFR-2 and Neuropilin-1 evaluation procedure

Tissue biomarkers were analyzed centrally at the General and Clinical Pathology Department at University Hospital "St. Marina", Varna. Immunohistochemistry (IHC) was performed on 4-mkm sections of paraffin-embedded tissue. Sections were deparaffinized with xyline, dehydrated in graded series of ethanol and incubated with 3 % hydrogen peroxide. Antigen retrieval was performed into the preheated EnVision FLEX Target Retrieval Solution (working solution) in PT Link tanks and incubated for 20 minutes at 97°C. After cooling, the slides were placed in diluted room temperature FLEX Wash Buffer (20x) for 1-5 minutes. Sections were stained using FLEX protocol in Dako Autostiner/ Autostainer Plus. Samples were tested with recombinant monoclonal Rabbit antibody [EPR3113] to Neuropilin-1, Abcam's RabMAb® technology and rabbit monoclonal antibody [Flk-1/KDR/VEGFR2 Ab-1, Thermo Scientific] to VEGFR-2 according to the manufacturer's instructions. Primary antibodies (anti-VEGFR2, diluted 1:50, Neuropilin-1, diluted 1:100) were incubated for 20 minutes. Detection of expression levels of VEGFR-2 and Neuropilin-1 was achieved using the ultra vision system anti-polyvalent HRP/DAB. Finally, the reaction was visualized by the appropriate substrate-chromogen (DAB, Diaminobenzidine) reagent. Counterstaining was done using Mayer's haematoxylin.

VEGFR-2 and Neuropilin-1 expression levels interpretation

Positive staining was identified when the cytoplasm showed brown staining. Both percentage (P) and intensity (I) of staining of VEGFR-2 and Neuropilin-1 positive tumor cells were considered in a semi-quantitative assessment. Percentage of VEGFR-2 and Neuropilin-1 positive cells (P) was scored as 0 (less than 10 % positive cells), 1 (10-49 % positive cells), 2 (50-74% positive cells) or 3 (more than 75% positive cells). The intensity was scored as follows: 0 (colourless), 1(light yellow), 2 (brown), 3 (tan).

The scores for the percentage of stained cells (P) and staining intensity (I) were added together. The sum of both (P) and (I) was evaluated for each case and a final score was assigned 0 (negative), 1-2 (weak expression), 3 (moderate expression) and 4-6 (strong expression). Tissue scores were dichotomized by median value to high and low expression levels for both biomarkers.

To assess tissue biomarker expression, a median was calculated for each sample. Biomarker levels were dichotomized according to the sample median (ie, greater than the median were denoted as high expression levels, and below or equal to the median denoted low expression levels). H-score was defined as the percentage of cells with weak stain intensity plus two times the percentage of cells with moderate stain intensity plus three times the percentage of cells with strong stain intensity.

CLINICAL AND PATHOLOGIC FEATURES

We collected the following clinical data: demographical data (age at initial staging, sex, etc), date of surgery, extent of surgery, tumor localization and TNM classification, sites of metastatic dissemination, ECOG performance status.

We collected the following pathologic data: tumor characteristics – histology, grade of differentiation and TNM classification, RAS- status determination.

Clinical and pathologic baseline patient characteristics are summarized in Table 1.

Table 1. Clinical and pathologic baseline patient characteristics.

Characteristic	5-FU-based CT + Bev (n = 31)	5-FU-based CT (n = 23)
Age at diagnosis		
Median	62.5	64.8
Range	37-81	59-81
Distribution by sex, %	females 45.1 males 54.9	females 65.2 males 34.8
Performance status, %		
0	41.9	49.2
1	49.9	44.0
2	8.2	5.8
Disease site		
Liver, %	70.9	65.4
Lung, %	19.4	16.3

Mutational status of KRAS, %	KRAS WT	35.4	KRAS WT	59.7
	KRAS M+	48.3	KRAS M+	26.08
	Inadequate for genetic testing	16.3	Inadequate for genetic testing	14.2

Abbreviations: CT – chemotherapy; bev – bevacizumab

TREATMENT CHOICE AND DURATION

Patients received a minimum of 3 months of treatment. Chemotherapy regimens used are summarized in Table 2.

Table 2. Treatment Regimens

Arm	Dosage	Administration	Schedule
FOLFOX4 (± Bevacizumab*)*		Every 14 days	
Oxaliplatin	85 mg/m^2	IV 120 minutes	Day 1
Leucovorin	200 mg/m^2	IV 120 minutes	Days 1 + 2
Fluorouracil	400 mg/m^2	IV bolus, followed by	
Fluorouracil	600 mg/m^2	IV over 22 hours	Days 1 + 2
± Bevacizumab	10 mg/kg	30-90 minutes	Day 1
FOLFIRI (± Bevacizumab*)*		Every 14 days	
Irinotecan	180 mg/m^2	IV 30-90 minutes	Day 1
Leucovorin	200 mg/m^2	IV 120 minutes	Days 1 + 2
Fluorouracil	400 mg/m^2	IV bolus, followed by	
Fluorouracil	600 mg/m^2	IV over 22 hours	Days 1 + 2
± Bevacizumab	10 mg/kg	30-90 minutes	Day 1
XELOX (± Bevacizumab*)*		Every 21 days	
Oxaliplatin	85 mg/m^2	IV 120 minutes	Day 1
Capecitabine	2000-2500 mg/m^2	p.o.	Day 1 - 14
± Bevacizumab	15 mg/kg	30-90 minutes	Day 1
Capecitabine ± Bevacizumab		Every 21 days	
Capecitabine	2000-2500 mg/m^2	p.o.	Day 1 - 14
± Bevacizumab	15 mg/kg	30-90 minutes	Day 1

Abbreviations: FOLFOX - oxaliplatin, fluorouracil, and leucovorin; FOLFIRI – irinotecan, fluorouracil, and leucovorin; XELOX – oxaliplatin, capecitabine; IV – intravenous; p.o. – per os.

IMAGING ASSESSMENT, RESPONSE PATTERNS AND END POINTS DETERMINATION

Imaging the disease was performed at baseline and tumor response was assessed at regular intervals - every 4-6 cycles (3 months) for all cycles of CT/Bev till EOT or upon clinical symptoms. Imaging consisted of either CT of thorax, abdomen (and other areas if needed for additional lesion assessment) or PET/CT. Evaluation was performed using RECIST 1.1. During systemic treatment, disease free survival and response rate were assessed. Response was defined as either complete response (CR), partial response (PR) or stable disease (SD). Patients were followed for up to 2 years after start of first line treatment.

STATISTICAL DESIGN AND ANALYSIS

Descriptive statistics was used. Categorical features were summarized with frequencies and percentages. Our statistical analysis included 54 patients treated with CT alone or CT/Bev. Our aim was to evaluate PFS and potential correlations and identification of good responders to bevacizumab-containing treatment. PFS was defined as the time from assignment of treatment until progression. Survival curves were estimated by the Kaplan-Meier method, (11) with differences assessed by the log-rank test. (12) Although our study was not powered enough to compare different subgroups, hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated by Cox regression models. Two-tailed p-values (<0.05) were considered as significant.

RESULTS

Efficacy

Our experience confirms that the addition of bevacizumab to chemotherapy resulted in improvement in

progression-free survival which remains a good surrogate for measurement of overall survival in patients with colorectal cancer. Our study demonstrate a significant improvement in PFS with the addition of bevacizumab to chemotherapy (95% CI, log-rank test P =.003). Median PFS was 5.4 months (3,44-6,55) with CT versus 8.8 months (5,84-10,15) with CT/Bev.

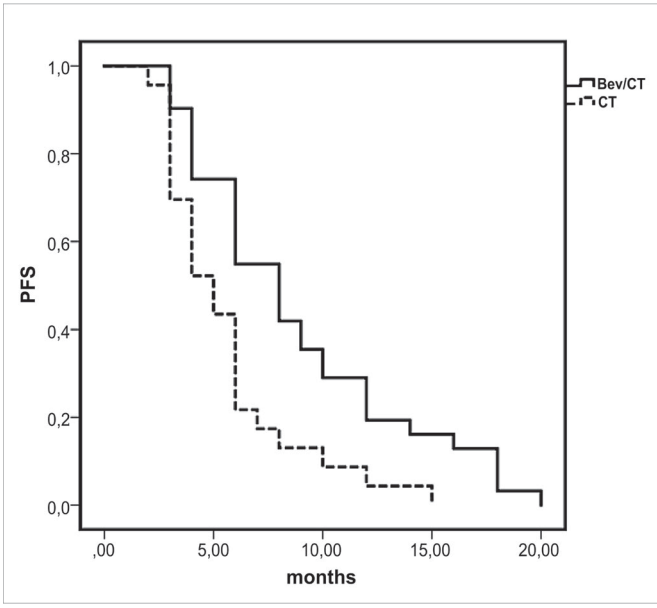

Fig. 1. Kaplan-Meier estimates of progression-free survival (PFS). The median PFS for the group treated with CT/Bev was 8.8 months, as compared with 5.4 months for the group treated with CT alone (95% CI, log-rank test P = .003).

Using the RECIST 1.1 criteria (10) for response, 19.3% of patients treated with CT/Bev achieved a confirmed response to therapy (PR+SD) compared with 10.5% of patients treated with CT alone. Our results – PFS, response rates and toxicity profile of bevacizumab are consistent with that documented in previous trials and the literature. (13-15)

Tissue biomarker levels at baseline and association with PFS

Patients received first line bevacizumab containing chemotherapy was summarized in Table 2. They were divided dichotomously into two groups according to expression levels of every tissue biomarker – high and low expression levels (VEGFR-2 or NP-1).

Patients with high expression levels of VEGFR-2 in the primary tumor had no significant difference in PFS as compared to those with low expression (Figure 2).

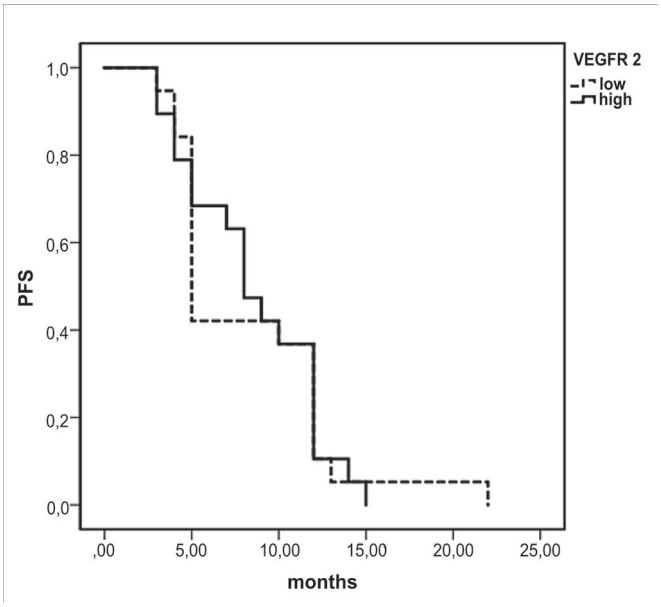

Fig. 2. Kaplan-Meier estimates of progression-free survival by baseline primary tumor VEGFR-2 levels (dichotomized by median value to high and low expression levels). There was no difference in the median PFS between both groups with high and low expression levels VEGFR-2.

Patients with low expression levels of NP-1 in the primary tumor treated with bevacizumab had significant difference in PFS as compared to those with high expression levels (log rank test p= 0.017). Patients with low NP-1 expression levels at baseline appeared to experience a larger bevacizumab treatment effect in terms of PFS (HR 0.333, 95% CI, 0.111 to 0.995) than patients with high NP-1 expression levels (Figure 3).

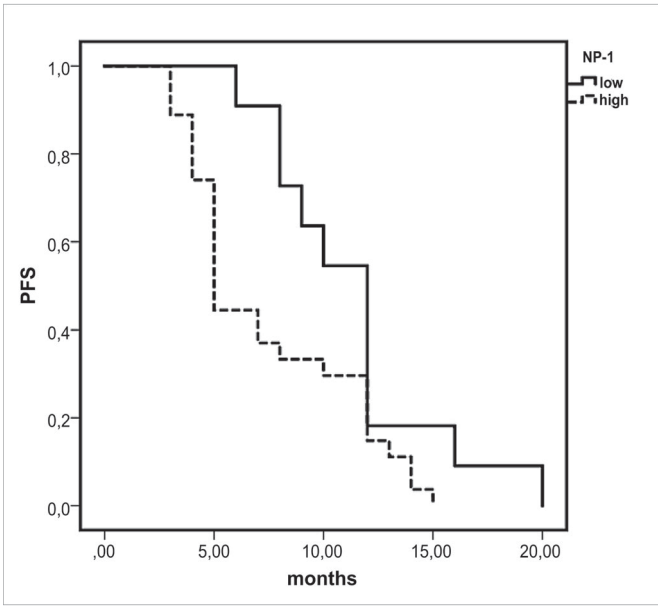

Fig. 3. Kaplan-Meier estimates of progression-free survival by baseline tissue NP1 expression levels (dichotomized by median value to high and low expression levels). There was statistical difference in the median PFS

between groups with high and low expression levels NP-1 (95% CI, log rank test p= .017).

Toxicity

The toxicity profile of bevacizumab was consistent with that documented in previous trials. (13-15) As we expected all grade adverse events were registered at higher frequency in the group of patients, treated with CT/Bev as compared to the only chemotherapy group. Most frequent AE as expected were nausea and vomiting, asthenia, neuropathy, neutropenia and thrombocytopenia. The occurrence of any grade 3 adverse event was greater for the individuals treated with the combination CT/Bev compared with patients treated with chemotherapy alone (49% *vs* 37%) with neuropathy, hypertension, bleeding, and vomiting. No AEs grade 4 were registered in our groups of patients.

DISCUSSION

The goal of our study was to determine tumor tissue angiogenic biomarkers such as VEGFR-2 and NP-1 which may have predictive value for bevacizumab efficacy in patients with colorectal cancer.

Antiangiogenic therapy with bevacizumab in combination with chemotherapy prolongs survival and PFS for patients with metastatic colorectal cancer as this has been previously reported in clinical trials.(16, 17) Improvements in clinical outcome do not appear to be limited to a single chemotherapy regimen.

There are currently no validated surrogate markers of biological activity for anti-VEGF therapy. The reported mechanism of action of bevacizumab and the potential for delayed efficacy had led to the speculation that PFS or overall survival may be more relevant measures of activity than objective response rate. Interestingly, improvements in objective response rate and PFS translated into better overall survival in patients with metastatic colorectal cancer receiving first-line chemotherapy plus bevacizumab.(13, 14)

Neuropilins are transmembrane glycoproteins, but the molecular mechanism for their antiangiogenic signaling remains unknown. There are clinical and preclinical data, showing that NP-1 levels are significantly decreased compared to the levels in the extraneoplastic tissue.(18) Some research showed that high expression levels of NP-1 in colon cancer patients correlate with a better prognosis as compared to cases with decreased NP-1 expression. (18)

Our results suggest that patients with colorectal cancer and low expression levels of NP-1 appear to derive more benefit from bevacizumab therapy than patients with higher levels of expression in terms of progression-free survival. Other studies, such as NO16966 reported that patients with colon cancer and low expression levels of NP-1 appeared to derive more benefit from bevacizumab therapy than patients with higher levels of expression in terms of overall survival and response rate. (18) A possible explanation for its predictive potential in our study is that low NP-1 expression may reflect greater dependence on VEGF ligand binding without providing an alternative pathway for VEGFR activation, making the tumor more susceptible to bevacizumab therapy.

To the best of our knowledge, this is the first clinical study in Bulgaria, evaluating the efficacy of bevacizumab with a tissue biomarker analysis. We demonstrate baseline tumor expression of NP-1 is candidate biomarkers of bevacizumab efficacy in patients with advanced colorectal cancer.

In **conclusion,** tumor NP-1 is biomarker candidate with potential to predict clinical outcome in patients with advanced colorectal cancer treated with first line chemotherapy plus bevacizumab. Prospective studies are required to further characterize these markers. Further research is warranted to clarify the predictive value of these tissue markers.

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