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Microvascular density assessed by CD31 predicts clinical benefit upon bevacizumab treatment in metastatic colorectal cancer: results of the PassionATE study, a translational prospective Phase II study of capecitabine and irinotecan plus bevacizumab followed by capecitabine and oxaliplatin plus bevacizumab or the reverse sequence in patients in mCRC

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Abstract

Background: Targeted therapies offer novel opportunities to explore biomarkers based on their mode of action. Taking this into consideration, we evaluated six angiogenesis-related proteins as potential predictive biomarkers, which expression might predict the benefit of bevacizumab treatment in patients with metastatic colorectal cancer (mCRC).

Methods: This was a phase II multicenter, two-armed, randomized study, in which patients with mCRC were treated with XELIRI (capecitabine and irinotecan) plus bevacizumab followed by XELOX (capecitabine and oxaliplatin) plus bevacizumab (Arm A) or the reverse sequence (Arm B). Tissue expression level of six prespecified candidates [microvessel density assessed by CD31, PTEN, αV integrin, CD98hc, uPAR and NRP-1] was analyzed *via* immunohistochemistry. The prognostic impact on survival was quantified using the Cox regression model. The predictive potential for benefit from Arm A *versus* Arm B treatment was investigated by fitting an interaction between the biomarkers and treatment assignment within a multivariable Cox model.

Results: In total, 74 out of 126 patients were included in the analysis. The expression of PTEN, αV integrin, uPAR and NRP-1 was not associated with progression-free survival (PFS) or overall survival (OS). For the first time, we identified that patients with tumors expressing CD98hc had a longer PFS than patients without CD98hc-expression ($p = 0.032$). More importantly, and in accordance with previous studies, low microvessel density was found to be associated with a reduced PFS [adjusted HR per doubling of CD31-expression ($p = 0.53$, 95% confidence interval: 0.30–0.95, $p = 0.034$)].

Conclusions: These results can contribute to the development of a personalized strategy for the treatment of mCRC with bevacizumab.

Keywords: angiogenesis, bevacizumab, biomarker, CD31, colorectal cancer

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Introduction

Despite being the third most common cancer type and the second leading cause of cancer related deaths worldwide, colorectal cancer (CRC) might be one of the best examples of scientific progress in terms of improvement on cancer patient's outcome.[1] Incidence and death rates for CRC are decreasing at least in the USA and the European Union, partly because of improved public awareness of smoking associated risks, early cancer detection, and new cancer treatments.[2,3] These developments are also reflected in an improved clinical outcome in patients with metastatic CRC (mCRC), whose overall survival (OS) has increased from an initial median OS of 12 months three decades ago to a median OS of around 30 months at present, especially due to the introduction of targeted therapies such as bevacizumab [an anti-vascular endothelial growth factor (VEGF) monoclonal antibody] and the establishment of tumor molecular profiling as the new standard-of-care for this disease.[4,5]

Back in 2004, bevacizumab was the first antiangiogenic compound approved by the US Food and Drug Administration (FDA) as an anticancer treatment and its approval was granted based on the results of a phase III clinical trial which showed that this monoclonal antibody prolonged median OS by about 5 months when given in combination with a standard backbone chemotherapy in patients with untreated mCRC.[6] Preclinical models have evinced that this compound is able to bind to extracellular VEGF-A, the major pro-angiogenic growth factor; thus, leading to tumor vessel growth inhibition by hindering the interaction between VEGF-A and its receptor.[7] Since its approval in 2004, bevacizumab has been approved for the treatment of several other types of cancers in combination with different cytotoxic chemotherapeutic therapies.[8] However, despite these medical advances, there are primordial issues that still have to be elucidated in order to further improve the outcome of mCRC, like the identification of a preferred backbone of targeted therapies, optimized therapy sequences and biomarkers for targeted-treatments.

In this context, several clinical trials aiming to address these issues have been conducted.[9–12] Due to the proposed mode of action of bevacizumab, angiogenesis-related molecules may be useful as predictors of response to bevacizumab. Thus, in the present study, our objective was to analyze if the tumor expression of six prespecified angiogenic-related proteins could predict the efficacy of bevacizumab in a phase II prospective clinical trial that aimed to determine the efficacy of modified XELIRI (capecitabine and irinotecan) plus bevacizumab followed by XELOX (capecitabine and oxaliplatin) plus bevacizumab at progression in comparison with the reverse sequence, based on the duration of disease control in patients with mCRC. The targets analyzed by immunohistochemistry (IHC) were: vascular density based on the expression of CD31 and expression of neuropilin-1 (NRP1), urokinase receptor (uPAR), αV integrin, CD98 heavy chain (CD98hc), and phosphatase and tensin homolog (PTEN). These biomarker candidates were selected on the proposed mode of action of bevacizumab and on previously published research works. With the exception of αV integrin, we hypothesized that the higher the expression of the candidate biomarker, the greater a patient could benefit from the treatment with bevacizumab.

Methods

Characteristics of study population

The PASSION study was a phase II multicenter, open label, two-armed, randomized pilot study (ML25153, EUDRACT Number: 2011-002191-16, Supplemental List 1), in which the primary endpoint was to assess the efficacy and safety of XELIRI plus bevacizumab followed by XELOX plus bevacizumab or the reverse sequence in patients with mCRC. A total of 126 patients were randomized into one of two treatment groups: Arm A (XELIRI plus bevacizumab followed by XELOX plus bevacizumab) or Arm B (XELOX plus bevacizumab followed by XELIRI plus bevacizumab). The length of the study was 64 months. All patients were older than 18 years and did not receive any prior systemic treatment for mCRC. Patients had to provide an expressed informed consent to be included in the study. The informed consent for the translational study included the immunostaining of the tumor tissue presented herein. The protocols of the clinical and translational study were approved by the ethics committees of each treating institution (Supplemental List 1) and were carried out in accordance with the Declaration of Helsinki. The present study was performed according to the REMARK guidelines. Patients had to sign both informed consents to participate in the present study. Treatment details and primary results regarding OS and PFS have not been published yet.

Immunohistochemistry

Immunostaining was performed according to standard protocols on paraffin-embedded tissue as we have described before.[13] Tumor tissue was immunostained using antibodies against PTEN, αV integrin, CD98hc, uPAR, and NRP-1. Further details on the antibodies used and method are described in Supplemental Methods. Staining was scored by adding the distribution score (0 = no staining; 1+ = staining of <33% of cells; 2+ = between 33% and 66% of cells; and 3+ = staining of >66% of cells) to the intensity score (0 = no staining; 1+ = weak; 2+ = moderate; 3 = strong). The average number of microvessels was assessed in five higher-power fields (HPF) per sections stained for CD31. All immunostainings were evaluated by an expert pathologist who was blind to the clinical and treatment data. Representative stained samples were scanned using the Pannoramic 250 Scanner (3DHISTECH) at 20× objective magnification.

Statistics

All statistical analyses were performed with Stata 15.0 (Stata Corp., Houston, TX, USA) by FP. Continuous variables were reported as medians (25th–75th percentile), whereas count data were summarized as absolute frequencies (%). Missing data were reported as absolute counts (%), and a complete case analysis was performed. The distribution of baseline covariables between the two treatment arms was compared with χ^2-tests, Fisher's exact tests, and rank-sum tests, respectively. Co-primary endpoints were first-line PFS and OS. PFS and OS were defined as the time from randomization to (a) the date of disease progression, death-from-any-cause, or censoring alive, whatever came first, and (b) the date of death-from-any-cause or censoring alive, whatever came first, respectively. PFS and OS functions were estimated with Kaplan–Meier estimators and compared between groups using log-rank tests. The prognostic impact of IHC biomarker variables on PFS and OS was quantified with univariable and multivariable Cox regression. Treatment assignment to Arm A *versus* Arm B was pre-specified as a fixed covariable in all multivariable analyses. Moreover, we selected each non-biomarker covariable with a p-value for association with the outcome of <0.10 for multivariable analysis (Table 2, relaxed threshold due to low sample size). Multivariable analyses were only performed in case a biomarker was associated with the outcome in the univariable setting. Thus, multivariable Cox modeling included (a) the biomarker under study, (b) treatment assignment to Arm A *versus* Arm B, and (c) all covariables with a p of association with the outcome <0.10. To gauge whether biomarker expression may modulate the benefit from a certain treatment sequence, we also fitted interactions between the biomarkers and treatment assignment within these multivariable Cox models (thus additionally including an interaction term between biomarker expression and treatment assignment to Arm A *versus* Arm B). One patient had a PFS event at the day of study inclusion and was thus not assessable for the PFS analysis.

Results

Cohort characteristics

A total of 126 patients were enrolled in the clinical study and underwent random assignment to a treatment group. Twenty of these patients underwent surgery in external hospitals and therefore tumor tissue was not available for the proposed analysis. In six cases, there was not enough tumor tissue material to perform the immunostainings. A total of 26 patients did not sign the additional informed consent for the present study. Thus, 74 (59%) out of these 126 patients were included in the current translational study of whom $n = 36$ patients (49%) and $n = 38$ patients (51%) were randomized to treatment arms A and B, respectively. Except for the presence of lung and liver metastases, baseline characteristics were well balanced between the two treatment groups (Table 1). During a median follow-up of 37.2 months, we observed 64 PFS events during first-line therapy, and 53 patients eventually died. This corresponded to median first-line PFS and OS estimates of 7.8 months [95% confidence interval (CI): 6.96–11.28, Supplemental Figure S1] and 19.44 months (16.08–29.88, Supplemental Figure S2), respectively. Univariable predictors of PFS and OS are reported in Table 2. Both PFS and OS did not differ by treatment assignment (Supplemental Figure S3).

High CD31 expression predicts a better PFS, independently of the sequence treatment

A total of 73 tissue samples were available for examination. CD31 immuno-reactive vascular structures were found in all tumor samples with a median of 11.2 microvessels per HPF (range: 1.8–21.4). Representative immunostainings are shown in Figure 1. A higher number of CD31+

Table 1. Baseline characteristics of the study cohort ($n = 74$).

Variable	*n* (% miss.)	Overall ($n = 74$)	Arm A ($n = 36$)	Arm B ($n = 38$)	*p*
Demographic variables					
Age (years)	74 (0%)	66 (58–72)	66 (58–71)	66 (56–73)	0.871
Female sex	74 (0%)	20 (27%)	9 (25%)	11 (29%)	0.702
ECOG ⩾1 point	73 (1%)	17 (23%)	9 (25%)	8 (22%)	0.733
Caucasian ethnicity	74 (0%)	74 (100%)	36 (100%)	38 (100%)	0.999
Cancer variables					
Stage IV at time of tumor diagnosis	74 (0%)	61 (82%)	30 (83%)	31 (82%)	0.843
Liver metastasis	74 (0%)	66 (89%)	29 (81%)	37 (97%)	0.020
Lung metastasis	70 (5%)	32 (46%)	21 (62%)	11 (31%)	0.009
KRAS mutation	66 (11%)	32 (48%)	15 (44%)	17 (53%)	0.464
IHC variables					
Origin of tissue: Primary tumor	74 (0%)	59 (80%)	27 (75%)	32 (84%)	0.325
CD31 (microvessels/HPF)	73 (1%)	11.2 (9.0–13.0)	11.2 (9.2–13.0)	11.1 (9.0–13.4)	0.925
PTEN IHC positive	72 (3%)	2 (3%)	2 (6%)	0 (0%)	0.493
uPAR IHC positive	71 (4%)	30 (42%)	18 (50%)	12 (34%)	0.180
NRP-1 expression	72 (3%)	2 (0–4)	2 (0–4)	2 (0–4)	0.866
αV-integrin expression	67 (9%)	4 (2–5)	4 (2–5)	4 (2–4)	0.832
CD98he IHC positive	70 (5%)	28 (40%)	15 (44%)	13 (36%)	0.494

ECOG, Eastern Cooperative Oncology Group Performance Status; HPF, higher-power fields; IHC, immunohistochemistry; PTEN, phosphatase and tensin homolog; uPAR, urokinase receptor.

microvessels per HPF was significantly associated with a better PFS experience [Hazard ratio (HR) per doubling of CD31+ microvessels/HPF = 0.52, 95% CI: 0.29–0.92, $p = 0.024$]. In detail, median PFS estimates were 11.28 and 4.92 months in patients with CD31+ microvessels per HPF ⩾ and < the 25th percentile of this variable's distribution, respectively (HR = 3.07, 1.60–5.87, $p = 0.001$; log-rank $p = 0.0004$, Figure 2). The association between low CD31+ expression and worse PFS prevailed in multivariable analysis adjusting for stage IV at initial diagnosis and treatment assignment (adjusted HR per doubling of CD31 expression = 0.53, 95% CI: 0.30–0.95, $p = 0.034$). However, low CD31 expression did not emerge as a predictive biomarker for benefit from a certain treatment sequence (Interaction *p*-value between CD31 expression and treatment arms A and B = 0.814), confirming it as a prognostic but not predictive biomarker (Supplemental Figure S4). Moreover, low CD31 expression appeared to be associated with worse OS (log-rank $p = 0.038$, Supplemental Figure S5), but this was not the case when considering CD31 expression as a continuous variable (Table 2).

uPAR, αV integrin, NRP1, and PTEN expression did not predict survival outcome

Protein expressions of uPAR, NRP1, αV integrin, and PTEN were examined by immunohistochemistry in all available samples (Table 1). Representative micrographs of the immunostainings are shown in Supplemental Figure S6.

Table 2. Predictors of first-line PFS and OS in the study cohort. Univariable Cox models.

Endpoint	PFS			OS		
Variable	**Hazard ratio**	**95% CI**	***p***	**Hazard ratio**	**95% CI**	***p***
Demographic variables						
Age (per 5 years increase)	0.95	0.83–1.09	0.496	1.02	0.88–1.17	0.826
Female sex	1.05	0.59–1.86	0.876	1.33	0.72–2.44	0.360
ECOG ⩾1 point	0.85	0.46–1.57	0.595	1.77	0.96–3.27	0.070
Caucasian ethnicity	N/A	N/A	N/A	N/A	N/A	N/A
Cancer variables						
Stage IV at time of tumor diagnosis	1.96	0.90–4.26	0.089	1.82	0.78–4.29	0.168
Liver metastasis	1.16	0.52–2.57	0.720	1.42	0.60–3.33	0.421
Lung metastasis	1.44	0.86–2.41	0.164	0.98	0.56–1.72	0.947
KRAS mutation	0.87	0.51–1.50	0.628	1.34	0.75–2.41	0.326
IHC variables						
CD31 (microvessels/HPF, per doubling)	0.52	0.29–0.92	0.024	1.21	0.70–2.10	0.491
PTEN IHC positive	N/A	N/A	N/A	N/A	N/A	N/A
Any uPAR expression	0.71	0.42–1.20	0.204	0.76	0.43–1.34	0.339
NRP-1 expression (per 1-point increase)	0.99	0.86–1.14	0.869	1.07	0.92–1.25	0.380
αV-integrin expression (per 1-point increase)	0.97	0.85–1.12	0.697	1.01	0.86–1.18	0.949
CD98he positivity	0.55	0.31–0.96	0.035	0.68	0.37–1.22	0.192
Treatment variables						
Randomization to Arm B	0.66	0.40–1.09	0.102	1.29	0.75–2.22	0.360

ECOG, ; HPF, higher-power fields; IHC, ; OS, overall survival; PFS, progression-free survival; PTEN, phosphatase and tensin homolog; uPAR, urokinase receptor.

Cytoplasmic uPAR expression was observed in tumor cells in 30 samples. Of note, immunopositivity for uPAR was also detected in goblet cells surrounding the tumor in 13 samples (Supplemental Figure S6). This was considered as an unspecific staining. Membrane and cytoplasm staining of αV integrin and cytoplasm expression of NRP1 were detected in tumor cells and in some cases, immunostaining was also observed in stroma cells (Supplemental Figure S6). PTEN expression in tumor cells was only observed in two cases, whereas 18 cases showed stromal expression of PTEN (Supplemental Figure S6). We did not observe any evidence for an association between uPAR, NRP1, and αV integrin expression and any of the survival variables under study (Table 2).

Patients with CD98hc positive tumor cells exhibited a better PFS, independently of treatment arm

CD98hc expression was positive in 28 cases, with positivity defined as any CD98hc expression (Figure 3a). In some cases, CD98hc was observed

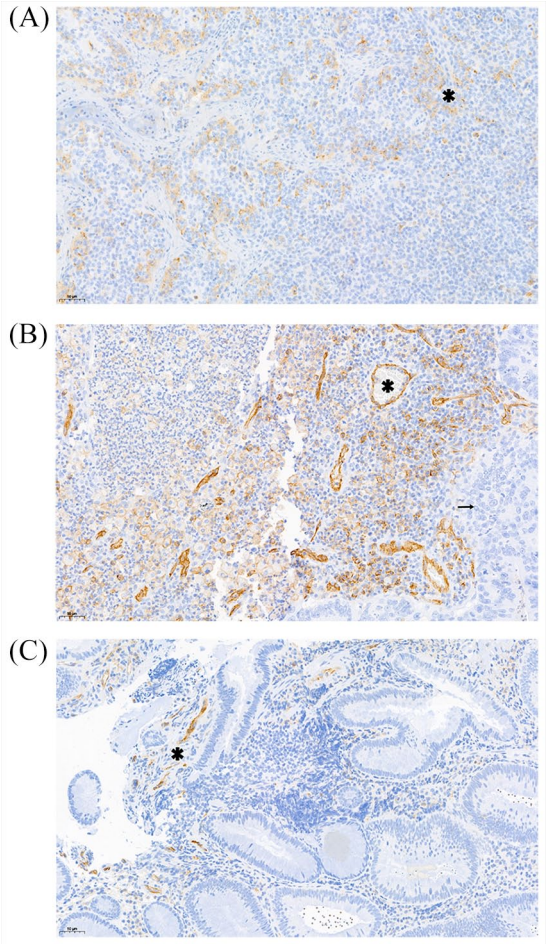

Figure 1. Representative immunostaining of CD31. (a) CD31 positive vascular proliferates (asterisk) in palatine tonsil tissue (positive control). (b) High number (>5/HPF) of CD31 positive vascular proliferates (asterisk) between tumor cells of primary colonic cancer (arrow). (c) Low number (<5/HPF) of CD31 positive vascular proliferates (asterisk) between tumor cells of primary colonic cancer.
HPF, higher-power field.

in stroma cells (Figure 3b) but this was not considered as positivity. Patients with CD98hc+ tumors experienced a more favorable PFS experience than patients with CD98hc- tumors, respectively (log-rank $p = 0.032$, Table 2, Figure 4). This association prevailed in multivariable analysis adjusting for stage IV at initial diagnosis (adjusted HR for CD98hc positivity = 0.49, 0.27–0.91, $p = 0.023$). Moreover, CD98hc positivity was associated with numerically better OS (Supplemental Figure S7), although this did not reach statistical significance with the number of patients we had (Table 2). Although the beneficial association between CD98hc positivity and a more favorable PFS experienced appeared to be stronger in treatment arm A, CD98hc positivity did not statistically significantly emerge as a predictive biomarker for treatment sequence with the numbers of patients and events we had (p-value for interaction between CD98hc status and treatment assignment within a PFS analysis = 0.363, Supplemental Figure S8).

Discussion

The proposed mode of action of bevacizumab has opened a myriad of possibilities to investigate putative biomarkers that could predict response to this anti-angiogenic monoclonal antibody (reviewed in[14]). Several studies with focuses on different types of candidates have been conducted, such as circulating and tissue proteins, tissue mRNAs, or genetic variants. However, none of these candidates have reached clinical use yet. In the present study, we aimed to assess predictive tissue markers in patients treated with XELIRI and XELOX in combination with bevacizumab. The six candidates for this prospective study were pre-selected based on previous research. NRP1 is a transmembrane protein expressed by endothelial and tumor cells which promotes angiogenesis by interacting with VEGF.[15] It was shown that NRP1 expression could predict the responsiveness of treatments in different types of cancers, including treatment with bevacizumab.[16] For instance, the AVAGAST trial has shown that there was an inverse correlation between NRP1 protein expression and OS benefit upon bevacizumab treatment in gastric cancer.[9] Up to now, the correlation between NRP1 expression level and treatment benefit in patients with mCRC was only assessed in the BOND-2 study by Saltz *et al.* and in the present study by our group. The BOND-2 study has shown *via* RT-PCR that NRP1-mRNA expression level in tumor tissue was associated with a longer OS for the treatment of cetuximab plus bevacizumab with or without irinotecan.[17] The discrepancy between both studies might be grounded in the fact that one study has focused on mRNA, whereas our study has focused on the protein level. This discordance between protein and mRNA level is widely accepted and has been reported before.[13] Furthermore, the findings of the BOND-2 study were based on patients with CRC who received a different chemotherapy setting than our patients. Therefore, this could also explain the discrepancies in the correlation between NRP1 and treatment outcome.

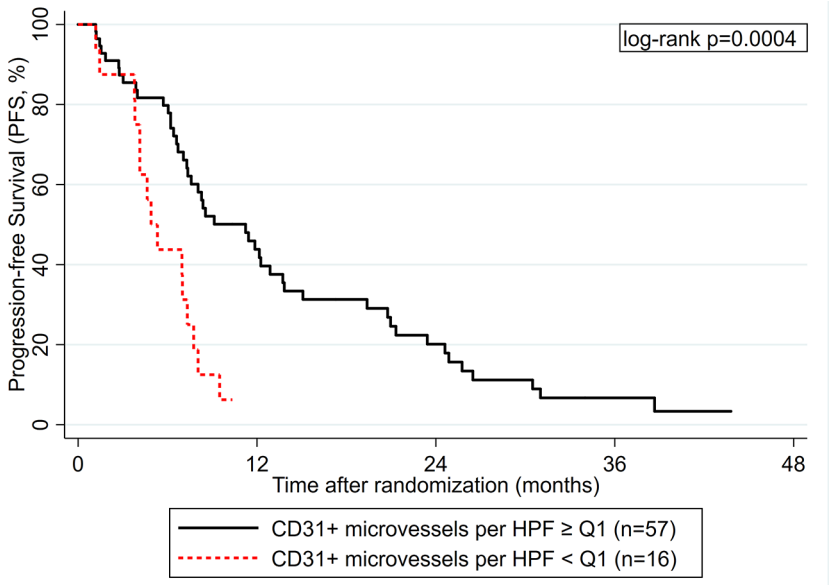

Figure 2. Progression-free survival (PFS) experience according to CD31+ microvessel density.
HPF, higher-power field.

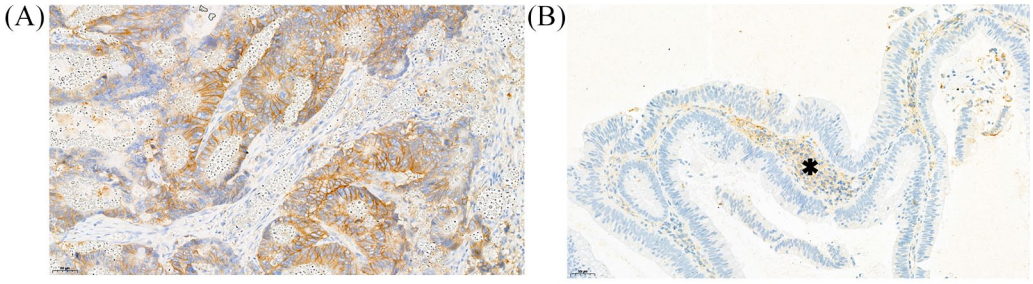

Figure 3. (a) Moderate to high expression of CD98hc in primary colonic cancer. (b) Expression of CD98hc in in tumor surrounding stromal cells (asterisk). Tumor cells showed no expression of CD98hc.

αV integrins are expressed by different types of cells, including tumor-associated endothelial cells and neoplastic cells, and are involved in different pathophysiological processes, such as tumor angiogenesis.[18] Studies have shown that some integrins might be involved in bevacizumab resistance and therefore, we hypothesized that high tumor expression levels of αV integrin might predict a poor clinical benefit of bevacizumab treatment, which has not been previously evaluated.[19] However, our study has demonstrated that αV integrin expression was not associated with PFS or OS in patients with mCRC. We could not confirm our hypothesis in the case of uPAR tissue expression either. The urokinase-type plasminogen activation (uPA)/uPAR system is involved in physiological and tumor angiogenesis, tumor cell migration, and invasion.[20,21] In CRC, uPAR tissue expression was shown to inversely correlate with OS and soluble levels of uPAR were shown to inversely correlate with bevacizumab-based first-line treatment response.[22,23] However, in the present study, uPAR tissue expression did not correlate with any patient's outcome.

PTEN is a tumor suppressor gene which is involved in angiogenesis and its loss of expression in neoplastic cells has already been reported in CRC.[24] Furthermore, it has been suggested that expression of PTEN might be associated with a beneficial response to cetuximab.[25] In our study, only two patients retained PTEN expression and therefore, due to the small sample size, we could not assess any association between PTEN expression and PFS or OS.

CD98hc is a surface protein which is involved in crucial pathological and physiological processes,

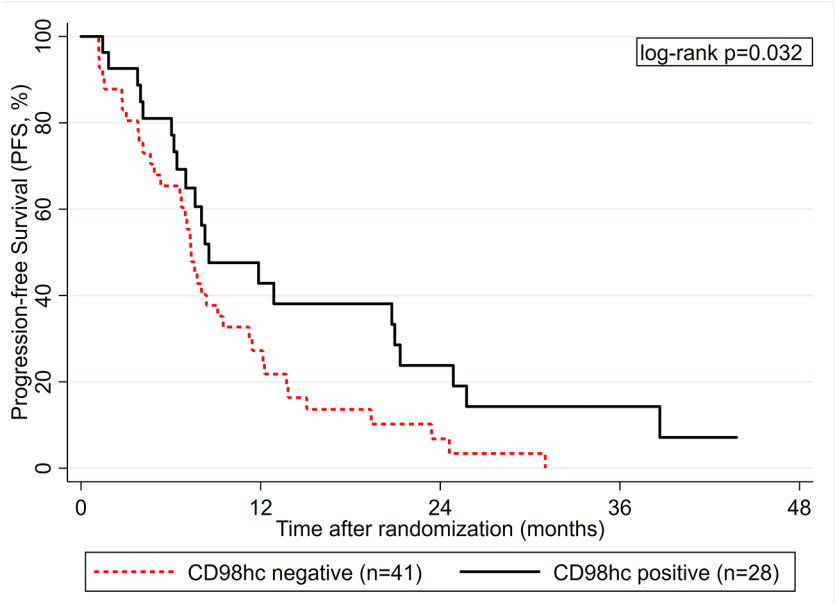

Figure 4. Progression-free survival (PFS) experience according to CD98hc status ($n = 69$).

such as modulation of integrin signaling and adaptive immunity, amino acid transport, angiogenesis, and tumor growth.[26–28] Previously, we have shown that CD98hc expression was expressed in renal cancer and that its expression correlated with the grade of malignancy.[29] Moreover, we have observed that patients with no expression of CD98hc in tumor tissue or stroma cells surrounding pancreatic tumors exhibited a longer OS in comparison with patients with CD98hc immunopositivity (data unpublished). Our findings were confirmed by Ying *et al.*, who showed that CD98hc was upregulated in up to ten different types of cancers, including CRC.[30] In line with our previous findings, this group has demonstrated that CD98hc expression predicted a poor outcome in patients with resectable CRC.[30] Although our present study has not confirmed these findings, there is no inconsistency between the results presented herein and those mentioned above. Here, we have demonstrated that expression of CD98hc predicted a better PFS in mCRC when patients were treated with bevacizumab, independently of the treatment arm. As initially hypothesized, CD98hc expression might predict a clinical benefit of a bevacizumab treatment.

Different studies have shown that the assessment of microvessel density measured by immunostaining might predict OS or disease recurrence in primary CRC.[31–33] A meta-analysis reported that high microvessel density assessed by CD31, CD34 and/or factor VIII predicted poor disease recurrence and OS.[34,35] The present study revealed that high microvessel density, assessed by CD31 staining, predicted a longer PFS for the treatment of XELOX or XELIRI plus bevacizumab. These findings are in line with a retrospective study performed by Bais *et al.*, which included 980 patients with ovarian cancer treated with chemotherapy plus bevacizumab.[36] In mCRC, Jubb *et al.* have shown that microvessel density measured by CD34 staining was not associated with clinical benefit upon bevacizumab treatment.[37] No correlation was found either by Zygoń *et al.* when microvessel density was measured using CD34 antibodies.[38] Thus, the discrepancy might lie in the selected protein for microvessel density assessment. Of note, this year, the FDA approved some bevacizumab biosimilars for the treatment of five cancer types and some are being studied for the treatment of mCRC.[39,40] As the name implies, biosimilars are highly similar (but not identical) to the approved reference products and, notwithstanding of some minor differences, they do not have clinically meaningful differences from the originator molecules in terms of safety and effectiveness.[41] However, some structural differences, such as glycosylation patterns, do exist and this can have an impact in several properties as reviewed here.[42] Therefore, these and previous findings should also be re-evaluated when patients are treated with these biosimilars.

In conclusion, our analysis has shown that microvessel density measured by CD31 staining predicted a longer PFS in patients with mCRC treated with XELIRI or XELOX plus bevacizumab. As previously described, this finding is in line with previous reports and might pave the way for a tailored treatment for patients with mCRC. In addition, we have demonstrated for the first time that CD98hc expression in tumor tissue might predict a better PFS in patients with mCRC treated with the evaluated chemotherapy strategy. Additional studies are urgently needed to confirm our findings and ensure that they can be translated into the clinical use as rapidly as possible.

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Conflict of interest

MK received travel support from Merck, Bayer, Bristol-Myers Squibb, and Roche and has participated in advisory board meetings from Bayer. All other authors declare no conflict of interest.

Disclaimer

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Supplemental material

Supplemental material for this article is available online.

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