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Methylated free-circulating *HPP1* DNA is an early response marker in patients with metastatic colorectal cancer

Andreas Herbst[1,3], Nikolay Vdovin[1], Sanja Gacesa[2], Alexander Philipp[1], Dorothea Nagel[3], Lesca M. Holdt[3], Mark op den Winkel[1], Volker Heinemann[4,5,6], Petra Stieber[3], Ullrich Graeven[7], Anke Reinacher-Schick[8], Dirk Arnold[9], Ingrid Ricard[2], Ulrich Mansmann[2,5,6], Susanna Hegewisch-Becker[10] and Frank T Kolligs[1,5,6,11]

[1] Department of Medicine II, University of Munich, Munich, Germany
[2] Institute for Medical Informatics, Biometry and Epidemiology, University of Munich, Munich, Germany
[3] Institute of Laboratory Medicine, University of Munich, Munich, Germany
[4] Department of Medicine III and The Comprehensive Cancer Center, University of Munich, Munich, Germany
[5] German Cancer Consortium (DKTK), Heidelberg, Germany
[6] German Cancer Research Center (DKFZ), Heidelberg, Germany
[7] Medizinische Klinik I, Kliniken Maria-Hilf GmbH, Mönchengladbach, Germany
[8] Department of Hematology and Oncology, Ruhr-University of Bochum, St. Josef Hospital, Bochum, Germany
[9] Instituto CUF de Oncologia (I.C.O.), Lisbon, Portugal
[10] HOPE - Practice for Oncology, Hamburg, Germany
[11] HELIOS Klinikum Berlin-Buch, Berlin, Germany

Detection of methylated free-circulating DNA (mfcDNA) for hyperplastic polyposis 1 (*HPP1)* in blood is correlated with a poor prognosis for patients with metastatic colorectal cancers (mCRC). Here, we analyzed the plasma levels of *HPP1* mfcDNA in mCRC patients treated with a combination therapy containing a fluoropyrimidine, oxaliplatin and bevacizumab to test whether *HPP1* mfcDNA is a suitable prognostic and response biomarker. From 467 patients of the prospective clinical study AIO-KRK-0207, mfcDNA was isolated from plasma samples at different time points and bisulfite-treated mfcDNA was quantified using methylation specific PCR. About 337 of 467 patients had detectable levels for *HPP1* mfcDNA before start of treatment. The detection was significantly correlated with poorer overall survival (OS) (HR = 1.86; 95%CI 1.37–2.53). About 2–3 weeks after the first administration of combination chemotherapy, *HPP1* mfcDNA was reduced to non-detectable levels in 167 of 337 patients. These patients showed a better OS compared with patients with continued detection of *HPP1* mfcDNA (HR *HPP1*(sample 1: pos/ sample 2: neg) *vs.* *HPP1*(neg/neg) = 1.41; 95%CI 1.00–2.01, *HPP1*(neg,pos/pos) *vs.* *HPP1*(neg/neg) = 2.60; 95%CI 1.86–3.64). Receiver operating characteristic analysis demonstrated that *HPP1* mfcDNA discriminates well between patients who do (not) respond to therapy according to the radiological staging after 12 or 24 weeks (AUC = 0.77 or 0.71, respectively). Detection of *HPP1* mfcDNA can be used as a prognostic marker *and* an early marker for response (as early as 3–4 weeks after start of treatment compared with radiological staging after 12 or 24 weeks) to identify patients who will likely benefit from a combination chemo-therapy with bevacizumab.

Introduction

The introduction of chemotherapeutic combination regimen including fluoropyrimidines, oxaliplatin and irinotecan, as well as monoclonal antibodies (like cetuximab, panitumumab and bevacizumab) to first- and further line treatment regimen have improved the overall survival of patients with mCRC. However, long-term survival rates of patients with mCRC are still low, with only few patients being eventually cured.[1–4]

Key words: colorectal cancer, prognosis, response, free-circulating DNA, HPP1

Conflict of Interest

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ARS: Honoraria: Amgen, Roche, Sanofi-Aventis, Merck-Serono, Celgene, Baxalta. Consulting or Advisory Board: Amgen, Roche, Pfizer, Sanofi-Aventis, Merck-Serono, Celgene, Baxalta. Studies sponsored by: Roche, Sanofi-Aventis, Celgene.
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Correspondence to: Andreas Herbst, Inst. of Laboratory Medicine, Marchioninistr. 15, University of Munich, 81377 Munich, Germany, Tel.: +49 89 4400-73207, Fax: +49 89 4400-78888, E-mail: Andreas.Herbst@med.uni-muenchen.de

What's new?

Tumor cells of primary tumors and metastases are constantly turned over with their DNA being released into blood circulation. Here the authors quantified circulating DNA of the hyperplastic polyposis 1 (HPP1) gene, which is frequently methylated in colorectal tumors, in a prospective clinical study of combination chemotherapy in patients with metastatic colorectal cancer. They confirmed their previous finding that HPP1 methylated free-circulating DNA (mfcDNA) is a prognostic marker for progression-free and overall survival in these patients. In addition, HPP1 mfcDNA served as a marker differentiating between chemotherapy responders and non-responders, underscoring the usefulness of DNA-based biomarkers in cancer treatment.

Currently, only the pathohistological tumor staging (TNM) is used as prognostic parameter in the clinic. Analysis of the RAS mutational status is the only predictive marker for the treatment of CRC patients with the anti-EGFR antibodies cetuximab and panitumumab.[1–4] For this reason, additional tissue or blood biomarkers are urgently needed to improve prediction and to guide therapies.

So far, several tissue-based biomarkers with the potential to be used in the clinic have been described to classify subtypes of colorectal tumors, for example, microsatellite instability, CpG island methylator phenotype (CIMP), mutations of DNA repair genes[5] and *RAS*.[1] However, the detection of these markers depends on the analysis of a biopsy or tumor tissue after resection. In addition, because of intratumoral heterogeneity as well as heterogeneity between metastases, the analysis of one biopsy might not necessarily represent the whole tumor burden of a given patient.[6] However, analysis of multiple biopsies is not feasible in the clinical routine. Potent blood-derived biomarkers might overcome this problem.[7]

CEA and CA19-9 were the first blood-based biomarkers for colorectal cancer. CEA has been described as an independent prognostic tumor marker for the overall survival of patients with curatively resectable or metastasized CRC.[8–13] New studies revealed that high CEA serum levels at the beginning of a combination therapy including bevacizumab are correlated with a poor prognosis.[14] Furthermore, increasing CEA levels during a treatment with oxaliplatin-based chemotherapy with bevacizumab indicate tumor progression.[15] Nevertheless, CEA is not an established response marker. Instead, response evaluation criteria in solid tumors (RECIST-1) are commonly used to define the degree of tumor response to chemotherapies.[16] Treatment of patients with tumors should result in tumor shrinkage that can be monitored using radiological screening.[17]

Tumor cells of primary tumors and metastases are constantly turned over with their DNA being released into circulation. Free-circulating tumor DNA can be isolated and analyzed for mutations and methylation patterns derived from tumor cells.[7] In contrast to repeated biopsies, repeated sampling and analysis of blood-derived markers is feasible. Next to the detection of mutations in proto-oncogenes and tumor suppressor genes in free-circulating DNA,[18–20] detection of methylated, free-circulating tumor DNA has been intensively studied in recent years.[21–24]

Increased methylation of regulatory gene sequences, so called CpG islands in the promoter region of genes, is a hallmark of tumor cells[25–27] and can be used to differentiate normal and tumor cells.[28,29] It has been shown that the detection of gene methylation for individual genes in blood samples, like *CDKN2A*, *MYOD1*, *ID4* and *HPP1*,[30–33] as well as the methylation of a panel of genes was correlated with a poor prognosis of patients with colorectal carcinomas at late stages.[34,35]

The gene *HPP1* (hyperplastic polyposis 1/transmembrane protein containing epidermal growth factor and follistatin domains) encodes a transmembrane protein and is frequently methylated in colorectal tumors.[36,37] Previously, HPP1 has been shown to activate STAT1 signaling for its function as a tumor suppressor, however, *Hpp1* mutant mice did not show an increased tumor burden.[38,39] We have demonstrated that detection of methylated free-circulating *HPP1* DNA in blood samples is a prognostic factor for patients with mCRC.[33,40–42]

In the prospective study AIO-KRK-0207, different strategies for maintenance treatments were examined, following a 24 week combination chemotherapy with a fluoropyrimidine (5-FU or capecitabine), oxaliplatin plus bevacizumab.[43] Blood samples were collected during the combination chemotherapy for translational projects. Here, the levels of *HPP1* mfcDNA and CEA in blood samples before and 2–3 weeks after the start of the chemotherapy were determined and correlated with OS and response (radiological staging after 12 and 24 weeks, respectively), to evaluate whether *HPP1* mfcDNA and CEA are suitable markers for prognosis and early response to therapy.

Material and Methods

Study design and patients

The clinical study AIO-KRK-0207 (NCT00973609; https://clinicaltrials.gov/ct2/show/record/NCT00973609) is a randomized three arm phase III trial with different maintenance strategies after a 24-weeks combination chemotherapy, consisting of a treatment with a fluoropyrimidine, oxaliplatin and bevacizumab. Investigators assessed tumor response by CT or MRI scans at weeks 12 and 24 according to RECIST version 1.0 (see Ref. 43 for details regarding the protocols). Blood samples were drawn prior to the treatment start (Day 1; "BS1") and after 15 to 22 days (corresponding to the first administration of FOLFOX or CAPOX regime, respectively; "BS2"). Plasma was used to quantify *HPP1* mfcDNA; CEA was measured as a reference using serum. Characteristics of the patient cohort are shown in Table 1. Out of the 825 patients that were included in the clinical study AIO-KRK-0207,

Table 1. Patient characteristics

	N (%)
All patients	467 (100)
Age	
<70 years	310 (66.4)
≥70 years	157 (33.6)
Gender	
Female	168 (36.0)
Male	299 (64.0)
ECOG	
0	258 (55.2)
1 + 2	193 (41.3)
Unknown	16 (3.4)
Primary tumor site	
Colon	298 (63.8)
Rectum	169 (36.2)
Number of metastatic sites	
1	198 (42.4)
>1	267 (57.2)
Unknown	2 (0.4)
Synchronous/metachronous metastasis	
Synchronous	391 (83.7)
Metachronous	76 (16.3)
Induction therapy[1]	
CAPOX	19 (4.1)
FOLFOX4	145 (31.0)
FOLFOX4MOD	107 (22.9)
FOLFOX4SIMPLE	46 (9.9)
FOLFOX6	84 (18.0)
FOLFOX7MOD	5 (1.1)
XELOX	59 (12.6)
Unknown	2 (0.4)
Radiological staging (12 weeks)	
CR	3 (0.6)
PR	210 (45.0)
SD	175 (37.5)
PD	32 (6.9)
ND	47 (10.1)
Radiological staging (24 weeks)	
CR	10 (2.1)
PR	222 (47.5)
SD	140 (30.0)
PD	79 (16.9)
ND	16 (3.4)

Blood samples were available for 467 patients.
[1]All patients received bevacizumab; ND not determined.

blood samples were available for 467 patients. The 467 patients did not differ from the 358 patients that have been excluded from this study due to missing blood samples with respect to several variables (Supporting Information Table 1).

Blood samples

Blood samples underwent standardized pre analytical procedures. Serum: Blood was drawn using serum monovettes (Sarstedt, Nürnbrecht, Germany), incubated at room temperature for 60 min and centrifuged for 10 min at 2,000*g*. The supernatant was decanted and used for CEA measurements. Plasma: Blood was drawn using EDTA monovettes (Sarstedt) and centrifuged for 10 min at 2,000*g*. The supernatant was transferred to a new tube and used for DNA isolation and bisulfite conversion.

DNA isolation and bisulfite conversion

The frozen plasma samples were thawed at room temperature and homogenized by smoothly flicking the tube. Genomic DNA from 200 μL of each plasma sample was isolated using the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and eluted in 50 μL of Elution Buffer. Sodium bisulfite conversion of DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's protocol.

Analysis of DNA methylation

Bisulfite-treated DNA was analyzed by a fluorescence-based, real-time PCR assay, described previously as Methy-Light.[44] Dispersed Alu repeats were used to control for DNA amplification and to normalize for input DNA. Primer and probe sequences for *HPP1* and *Alu* have been described previously.[33] PCRs were performed in a reaction volume of 20 μL containing 1× PCR buffer (Qiagen, Hilden, Germany), 4 mmol/L $MgCl_2$, 250 μmol/L desoxynucleotide triphosphate mixture, 2 μL bisulfite-treated DNA, 0.05 units/μL Taq DNA polymerase (HotStar Taq, Qiagen) along with *HPP1* specific primers and probe as described previously[33] (see also Supporting Information Fig. 1). PCRs were conducted in a Mastercycler ep realplex[4] (Eppendorf, Hamburg, Germany) using the following conditions: 95°C for 900 sec followed by 50 cycles of 95°C for 30 sec, 60°C for 120 sec and 84°C for 20 sec. The specificity of all reactions for methylated DNA was confirmed by separately amplifying completely methylated and unmethylated human control DNA (Chemicon, Temecula, CA) with each set of primers and probes. Samples were analyzed in triplicates and the average amount of *HPP1* or Alu, respectively, was used for the calculations. The percentage of fully methylated reference (PMR) at a specific locus was calculated as described previously[44] by dividing the gene/Alu ratio of a sample by the gene/Alu ratio of fully methylated, bisulfite-treated DNA (CpGenomeTM Universal Methylated DNA, Millipore, Billerica, MA). A gene was considered methylated if

the percentage of the fully methylated reference value was greater than 0.

Quantification of CEA

CEA was quantified using a micro particle immuno enzymometric assay (AxSYM, Abbott Laboratories, Chicago, IL).

Statistical analysis

The event time data on OS and PFS were analyzed by Kaplan–Meier estimates, log rank test, and the Cox proportional hazards model. We defined the time to event starting at the date of the first biomarker measurement. These analyses also were performed in the subgroup of randomized patients stratified for treatment arms. Stratification allows taking into account non-proportional treatment specific baseline hazards by studying effects of factors across the treatment arms (Supporting Information Tables 3 and 4). In addition, multivariate Cox regression models including established prognostic factors together with CEA and HPP1 were analyzed and compared by means of the Akaike information criterion (AIC). In the sense of the AIC a model is better with respect to the comparator if the AIC of the comparator is larger (Supporting Information Table 5). Small AICs indicate better models. Robustness of the preferred final model was investigated in 200 bootstrap samples. How biomarker values or changes are able to discriminate response to therapy was analyzed using receiver-operator curves (ROC) curves and area under the curves (AUC) as described previously.[45] The analyses were performed with SAS V9.4 and R Version 2.13.2 using the packages *survival* [Version 2.36-9, Terry Therneau, 2011] and *ROCR* [Version 1.0-4, Tobias Sing, Oliver Sander, Niko Beerenwinkel, Thomas Lengauer, 2009].

Results

Correlation of the *HPP1* methylation status with overall survival

Blood samples were drawn at defined time points before treatment ("BS1") and after one administration of the combination chemotherapy treatment (Day 15 or 22; "BS2"). Radiological staging of the tumor was performed 12 ("RS1") and 24 weeks ("RS2") after the start of the combination chemotherapy as indicated by arrows (Fig. 1*a*). Out of the 825 patients that were eligible to participate in the study AIO-KRK-0207 and received combination chemotherapy, blood samples BS1 *and* BS2 were available for 467 patients (Fig. 1*b* and Table 1).

Of the 467 mCRC patients, 337 (72%) had detectable *HPP1* mfcDNA levels before therapy. Patients with detectable levels of *HPP1* mfcDNA in the first blood sample had a lower overall survival compared with patients with non-detectable levels of *HPP1* mfcDNA (HR = 1.86; 95% CI 1.37–2.53) (Fig. 2*a*, Table 2). Likewise, patients with CEA levels above the median of 56.4 ng/mL ("CEA high") in the first blood sample had a lower overall survival compared with patients with CEA levels below the median ("CEA low") (HR = 1.82; 95% CI 1.41–2.35) (Fig. 2*b*, Table 2). In the *second* blood sample, the respective hazard ratio was higher for *HPP1* mfcDNA compared with CEA ("*HPP1* BS2": HR = 2.13; 95% CI 1.65–2.74 *vs.* "CEA BS2": HR = 1.75; 95% CI 1.36–2.25) (Figs. 2*c* and 2*d*, Table 2).

Treatment of the patients resulted in a statistically significant change of *HPP1* mfcDNA and CEA levels in the second blood sample compared with the first one ($p < 0.0001$ for both variables, Supporting Information Fig. 2). After the first administration of combination chemotherapy, *HPP1* mfcDNA levels were reduced to non-detectable levels in 167 out of 337 patients. The 167 patients with non-detectable *HPP1* mfcDNA levels in the second blood sample ("pos/neg") showed a better OS compared with the 176 patients showing detectable *HPP1* mfcDNA levels in the second blood sample ("neg,pos/pos," including 6 patients with nondetectable levels in the first and detectable levels in the second sample) and a lower OS compared with patients with nondetectable HPP1 mfcDNA in both samples (HR "pos/neg" *vs.* "neg/neg" = 1.41; 95% CI 1.00–2.01, HR "pos/pos" *vs.* "neg/neg" = 2.60; 95% CI 1.86–3.64) (Table 2). In contrast, only 34 (of 234) patients switched from CEA levels above the cut-off value ("CEA BS1," "high") to CEA levels below the cut-off value ("CEA BS1/BS2," "high/low"). The median overall survival of these patients ("CEA high/low") was 26.5 months compared with 19.5 months of the 207 patients that had CEA levels above the cut-off value after treatment ("CEA low,high/high," including 7 patients switching from CEA low to CEA high) (Fig. 1*f*, Table 2). These data indicate that *HPP1* mfcDNA and CEA levels in the first and the second blood sample are prognostic markers for OS in the univariate analysis. In addition, non-detectable *HPP1* mfcDNA levels in the second blood sample of patients with initially detectable *HPP1* mfcDNA levels might indicate a response to therapy. For progression-free survival respective results are shown in Supporting Information Table 2. The different treatment arms had neither an effect on OS nor PFS, since the hazard ratios after stratification are comparable to the hazard ratios without stratification (compare Table 2 and Supporting Information Table 3 for OS; Supporting Information Table 2 and Supporting Information Table 4 for PFS; see also Supporting Information Fig. 3).

Different Cox models were calculated and compared by the Akaike information criterion (AIC) to find a suitable Cox model that includes established clinical parameters, like the mutational status, grading, ECOG, and tumor load (Supporting Information Table 5). These clinical parameters were analyzed either alone or in combination with the *HPP1* mfcDNA and/or CEA levels in blood sample 1 (BS1) or 2 (BS2) as well as the change of *HPP1* mfcDNA or CEA levels between BS1 and BS2 ("change"). Interactions between *HPP1* and CEA were investigated but did not improve any of the models. Since the mutational status of *RAS* and *BRAF* was only available for 85% of the patients, we also calculated the AIC without the mutational status to show that results in principle do not differ for a greater number of cases. It is important to note, however, that AIC values can directly be compared only for analyses within the same group of cases

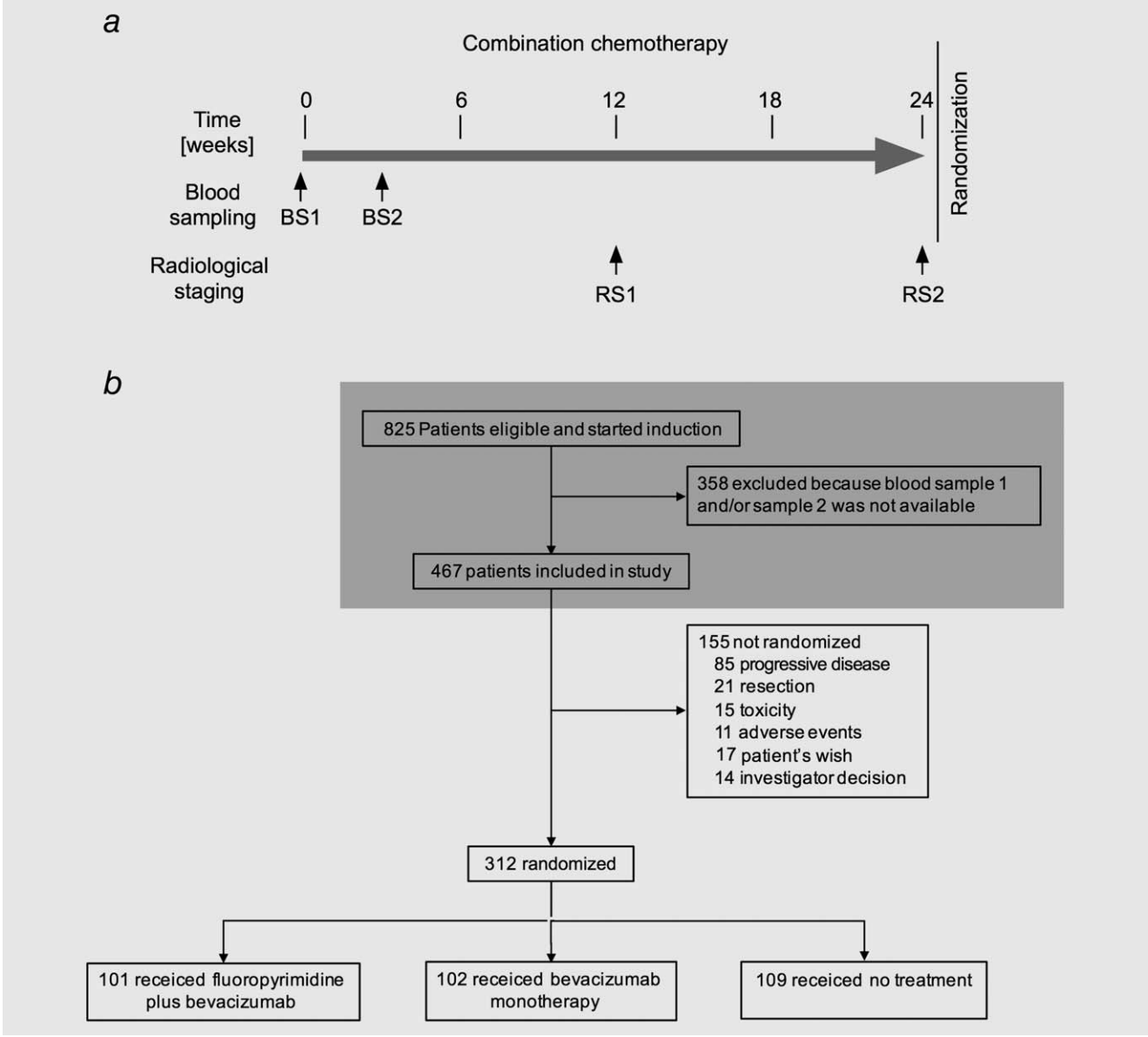

Figure 1. Schematic diagram of the combination chemotherapy and CONSORT diagram. (*a*) The combination chemotherapy of the prospective study AIO-KRK-0207 lasted 24 weeks. Blood was drawn before the start ("BS1") and after one administration ("BS2") of the combination chemotherapy. Radiological staging was performed after 12 ("RS1") and 24 weeks ("RS2"). (*b*) 825 mCRC patients were eligible to participate in the study AIO-KRK-0207 and were treated with the combination chemotherapy. Blood samples 1 (BS1) *and* 2 (BS2) were available for 467 patients and were analyzed in this study. This part is highlighted by a gray box.

and events. Comparison of the models for OS including mutational status, grading, ECOG, and tumor load yielded an AIC of 1,913 for the model including only these clinical variables and AICs ranging from 1,891 to 1,905 for models including additionally CEA and *HPP1*. Thus, all models including also CEA and/or *HPP1* were better with respect to AIC, the best models being clinical variables together with *HPP1* BS2 and CEA BS2 or *HPP1* change and CEA change, respectively (AIC = 1,891, Supporting Information Table 5). However, models including only *HPP1* BS2 or *HPP1* change were nearly as good (AIC = 1,892). Of these "best" models based on the AIC calculation, the least complex one including clinical parameters and *HPP1* mfcDNA levels at BS2 is shown (Table 3). According to this model, *HPP1* mfcDNA levels in the second blood sample represent an independent prognostic factor for OS next to the BRAF or RAS mutational status, grading, ECOG and the number of metastatic sites, respectively ("*HPP1* BS2": HR = 2.08; 95% CI 1.31–1.53, Table 3). This result for *HPP1* was confirmed in a bootstrap analysis: The average hazard ratio for *HPP1* mfcDNA in BS2 in 200 bootstrap samples was 2.16 (range 1.36–3.58, $p < 0.05$ for 199/200 samples). Comparison of the models for

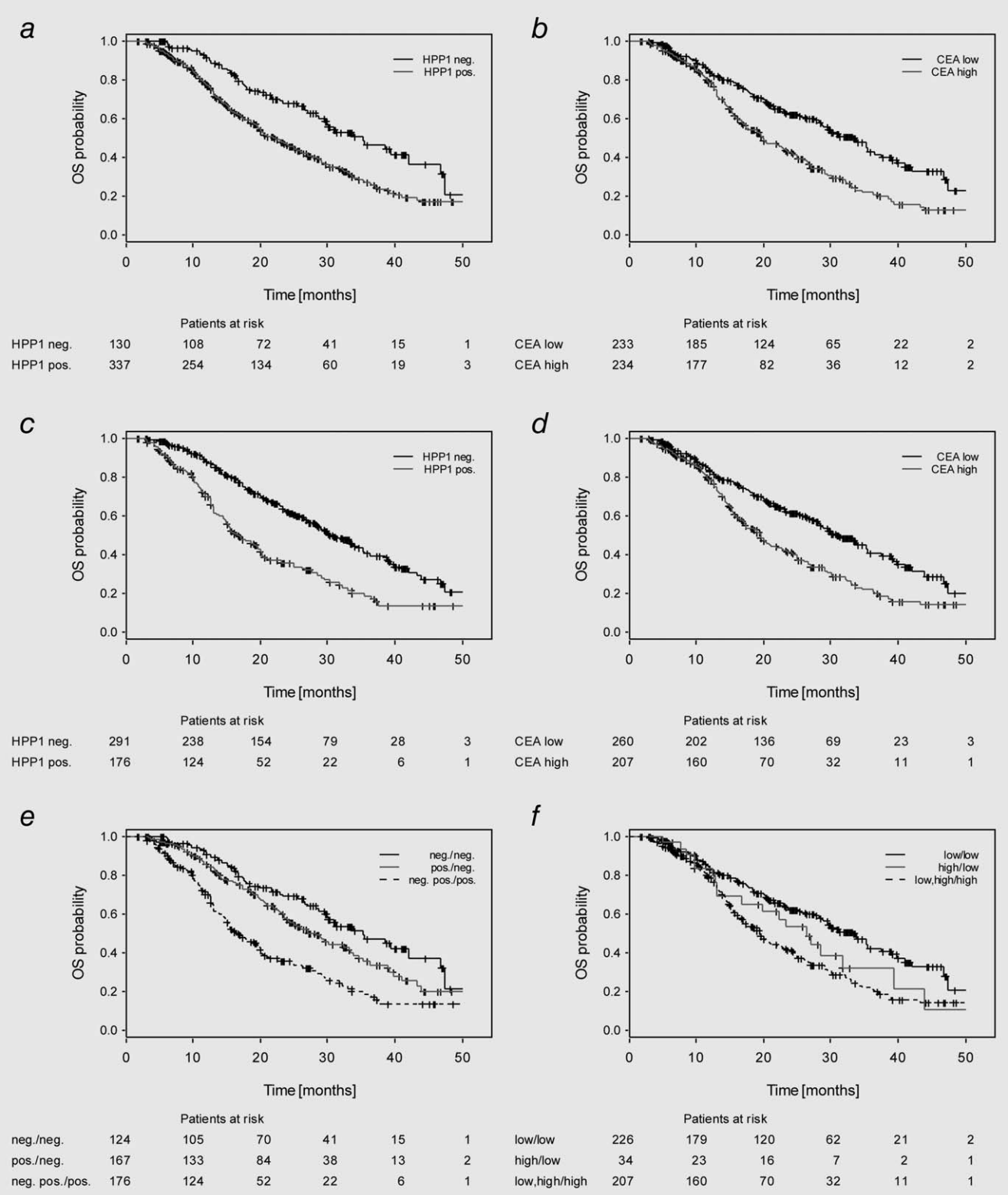

Figure 2. Kaplan-Meier plots showing overall survival. mCRC patients with undetectable/detectable *HPP1* mfcDNA levels were classified as "neg" or "pos," respectively. Patients with CEA levels below/above the cut-off value were classified as "low" or "high," respectively. (*a*) Overall survival is shown for all 467 mCRC patients according to the levels of *HPP1* mfcDNA in the first blood sample (BS1) (*b*) Kaplan–Meier plot based on the CEA level in BS1. (*c*) and (*d*) The corresponding Kaplan–Meier plots based on the levels of *HPP1* mfcDNA or CEA levels in the second blood sample (BS2). (*e*) Kaplan–Meier plot showing the combined information regarding the *HPP1* mfcDNA levels before and after the first administration of treatment (HPP1 BS1/BS2). (*f*) Kaplan–Meier plot based on the combined information of CEA serum levels before and after the first administration of treatment (CEA BS1/BS2).

Table 2. Median overall survival (OS) and hazard ratios were calculated for various clinical parameters, *HPP1* mfcDNA or CEA levels

Parameter	Value	Events/cases	Median OS [months]	CI	*p*	HR	HRCI
Gender	Female	93/168	21.9	(17.9–28.3)	0.0657	.	
	Male	153/299	28.2	(23.4–32.9)		0.79	(0.61–1.02)
Age	<70	166/310	27.0	(22.8–30.2)	0.9143	.	
	≥70	80/157	24.9	(20.5–30.3)		0.99	(0.75–1.29)
ECOG	0	127/258	29.5	(25.3–34.6)	0.0002	.	
	1 + 2	118/193	19.7	(16.7–26.7)		1.61	(1.25–2.07)
Site	Colon	164/298	23.4	(20.7–27.0)	0.0564	.	
	Rectum	82/169	30.0	(26.5–35.2)		0.77	(0.59–1.01)
Grading	1 + 2	141/312	30.2	(27.0–34.6)	<0.0001	.	
	3 + 4	81/124	16.7	(14.9–22.3)		2.02	(1.53–2.65)
Metastasis	metachron	35/76	31.3	(27.0– .)	0.0332	.	
	synchron	211/391	24.2	(20.9–28.2)		1.47	(1.03–2.11)
No. of metastatic sites	1	90/198	29.5	(24.9–36.2)	0.0086	1.41	(1.09–1.84)
	>1	155/267	23.4	(19.5–28.2)		.	
Mutation	Wild type	75/160	30.1	(26.2–38.5)	0.0001	.	
	NRAS/KRAS	115/205	24.2	(20.0–29.5)		1.33	(0.99–1.78)
	BRAF	20/33	10.7	(9.8– .)		2.84	(1.72–4.66)
HPP1 BS1	neg.	53/130	35.2	(29.5– .)	<0.0001	.	
	pos.	193/337	21.9	(19.5–25.9)		1.86	(1.37–2.53)
HPP1 BS2	neg.	134/291	30.2	(28.2–35.2)	<0.0001	.	
	pos.	112/176	16.6	(14.9–20.2)		2.13	(1.65–2.74)
HPP1 BS1/BS2	neg./neg.	51/124	35.2	(30.0– .)	<0.0001	.	
	pos./neg.	83/167	28.2	(23.9–33.9)		1.41	(1.00–2.01)
	neg.,pos./pos.	112/176	16.6	(14.9–20.2)		2.60	(1.86–3.64)
CEA BS1	Low	102/233	33.4	(28.7–37.5)	<0.0001	.	
	High	144/234	19.7	(17.3–24.4)		1.82	(1.41–2.35)
CEA BS2	Low	117/260	31.3	(28.3–35.5)	<0.0001	.	
	High	129/207	19.5	(17.1–23.6)		1.75	(1.36–2.25)
CEA BS1/BS2	Low/low	98/226	33.4	(28.8–39.1)	<0.0001	.	
	High/low	19/34	26.5	(19.7– .)		1.46	(0.89–2.39)
	Low,high/high	129/207	19.5	(17.1–23.6)		1.84	(1.41–2.40)
Rad Staging 12 weeks	CR + PR + SD	199/388	28.3	(25.3–31.3)	<0.0001	.	
	PD	27/32	10.1	(6.1–13.4)		6.91	(4.52–10.6)
Rad Staging 24 weeks	CR + PR + SD	174/372	30.1	(28.2–33.7)	<0.0001	.	
	PD	61/79	11.5	(10.0–13.4)		4.57	(3.38–6.18)

Patients were grouped according to the levels of *HPP1* mfcDNA in the first (BS1) or the second blood sample (BS2) into the categories "HPP1 detectable" (pos.) or "HPP1 non-detectable" (neg.). Similarly, patients with CEA levels below/above the cut-off CEA level were defined as "CEA low" or "CEA high," respectively. For the combined analysis of *HPP1* (and *CEA)* levels in BS1 and BS2, the groups "neg./pos." ("low/high") and "pos./pos." ("high/high") were combined to generate the group "neg.,pos./pos"("low,high/high"), since the group "neg./pos." was small and clinically not relevant. The number of events (deaths) and cases are listed. For each parameter the median OS was calculated. *p* values indicate if the median OS is statistically significant different between matching values. Hazard ratios (HR) and the 95% confidence interval (HRCI) indicate the risk associated with a given parameter. The mean OS was 27.2 months for the patient cohort, the median OS was 25.9 months (95% CI: 22.6–29.9).

PFS yielded similar results with *HPP1* mfcDNA levels as an independent prognostic factor (Supporting Information Table 6). These data indicate that *HPP1* mfcDNA levels in the first and the second blood sample are independent prognostic markers of clinical variables and CEA. In comparison, CEA levels in the first and second blood sample are independent prognostic markers of clinical variables, but only CEA in the first blood sample is also independent of *HPP1* levels.

Table 3. Multivariate analysis regarding the overall survival (OS)

Parameter	Value	HR	95% CI	*p*
HPP1 BS2	Positive *vs*. negative	2.08	(1.54–2.80)	<0.0001
Mutation	BRAF *vs*. wild-type	2.63	(1.58–4.38)	0.0002
	RAS *vs*. wild-type	1.31	(0.96–1.78)	0.0889
Grading	G3 + G4 *vs*. G1 + G2	1.85	(1.36–2.51)	<0.0001
ECOG	>0 *vs*. 0	1.53	(1.15–2.05)	0.0041
No. of metastatic sites	>1 *vs*. 1	1.42	(1.05–1.92)	0.0211

Based on the calculation and comparison of AIC values for different Cox models (Supporting Information Table 5), we decided to use a Cox model including clinical parameters and the *HPP1* mfcDNA levels at BS2 to perform a multivariate analysis for OS. The corresponding hazard ratios (HR) and confidence intervals (95% CI) of this model are presented for each parameter. *p* values indicate statistical significant differences between values.

HPP1 mfcDNA is a response marker

About 12 and 24 weeks after the start of treatment, radiographic evaluation based on RECIST criteria was done. Patients showing a complete remission (CR), partial remission (PR) or stable disease (SD) were classified as "responders," whereas patients who showed a progressive disease (PD) were defined as "non-responders." According to the staging, 388 (12 weeks, RS1) and 372 patients (24 weeks, RS2), respectively, responded to the combination chemotherapy (Table 1). To test the suitability of *HPP1* mfcDNA as a marker for response, we performed an ROC analysis using *HPP1* mfcDNA levels in the second blood sample to discriminate between responders (CR, PR, SD) and non-responders (PD) according to the results of the radiological staging after 12 and 24 weeks (Table 1). The area under the curve (AUC) was 0.77 (radiological staging after 12 weeks) and 0.71 (24 weeks), respectively (Figs. 3*a* and 3*b*). The negative predictive value (NPV) of *HPP1* mfcDNA in BS2 was 97.7 (95.1–99.2) at RS1 and 94.4 (90.9–96.8) at RS2. In contrast, CEA serum levels in the second blood sample did not discriminate between these two groups of mCRC patients (12 weeks: AUC = 0.49; 24 weeks: AUC = 0.52) (Figs. 3*c* and 3*d*). According to these data, *HPP1* mfcDNA levels after the first administration of the combination chemotherapy can be used as a marker for response to identify CRC patients that go into remission or show a stable tumor size. Moreover, information regarding the *HPP1* mfcDNA levels was available 2–3 weeks after the start of the treatment, whereas the radiological staging took place after 12 and 24 weeks, respectively.

Discussion

Detection of free-circulating tumor DNA in blood samples represents a minimally invasive approach to biologically represent the tumor of a patient and offers the chance to monitor the response to treatment by measuring the levels of fcDNA throughout the administration of a therapy.[7,46,47]

We have demonstrated in retrospective studies that detection of methylated free-circulating *HPP1* DNA is a prognostic factor for CRC patients UICC stage IV.[33,40–42] The results of this prospective study utilizing a homogenously treated cohort of mCRC patients confirmed our previous results: Detection of *HPP1* mfcDNA is an independent prognostic factor for lower overall survival. Furthermore, a reduction of *HPP1* mfcDNA to non-detectable levels after the first administration of treatment was correlated with reduced risk of progression compared with patients who still had detectable *HPP1* mfcDNA levels. In addition, the level of *HPP1* mfcDNA after the first administration of treatment was able to discriminate between patients with a "response or stable" (CR, PR, SD) or a "progressive" (PD) course of disease.

The results of this study show that CEA serum levels have a prognostic value. In contrast to CEA, detection of *HPP1* mfcDNA in the second plasma sample has the additional advantage of being a marker for response. Whereas CEA is not able to distinguish between mCRC patients with a response or stable versus a progressive course of disease, *HPP1* mfcDNA offers the possibility to identify patients who benefit from the therapy. Apart from patients that respond to the treatment with a reduction of *HPP1* mfcDNA to non-detectable levels, it is also important to identify patients who do not respond to a given therapy. This information offers the opportunity to identify progressors earlier and to potentially switch these patients to a different treatment regimen. We have not intended in our study to give any advice in terms of which alternative therapy should be used for patients progressing during induction therapy. This question remains open and needs to be answered by future studies. Taken together, *HPP1* mfcDNA has the potential to monitor response to therapy at an earlier time point than the radiological imaging.

To our knowledge, this is the first report demonstrating the suitability of detection of methylated, free-circulating DNA as a response marker for patients with metastatic colorectal cancer. In a different study, circulating *KRAS* G13D tumor DNA (ctDNA) was used as an early marker for the response of patients with metastatic colorectal cancers to treatment with oxaliplatin or irinotecan (with or without bevacizumab). There, ctDNA levels decreased in 41 of 48 patients and correlated with response to therapy measured by radiologic staging, whereas no significant changes in CEA levels were observed.[48] In another study circulating miR-126 was identified as a potential biomarker for response to a therapy consisting of chemotherapy and bevacizumab in 68 patients with mCRCs.[49] Another group identified a marker panel for circulating tumor cells in 50 RAS-BRAF

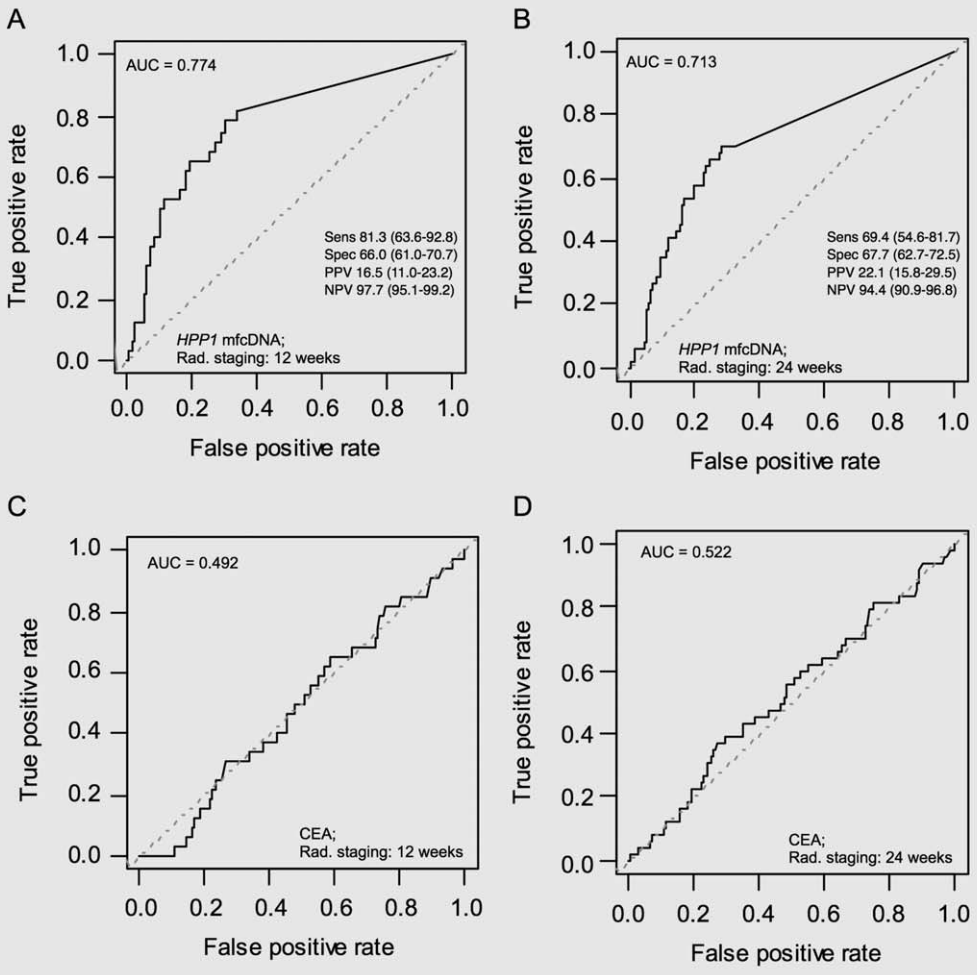

Figure 3. Response curves for second blood HPP1 mfcDNA and CEA levels. (*a, b*) Response curves were generated by receiver-operator-analysis (ROC) analysis and the area under the curve (AUC) was determined to find out if *HPP1* mfcDNA in the second blood sample discriminate between CRC patients with "response or stable" (CR, PR, SD) *versus* "progressive" (PD) disease, respectively, according to the radiological staging 12 weeks (*a*) or 24 weeks (*b*) after start of treatment. The sensitivity ("Sens."), specificity ("Spez."), positive predictive value ("PPV") and negative predictive value ("NPV") are given for both analyses. (*c, d*) These ROC analyses were repeated for CEA levels in the second blood sample based on the radiological staging 12 (*c*) or 24 weeks (*d*) after start of treatment. The AUC for each analysis is given in the upper left corner of the graph area.

wild-type mCRC patients and were able to identify patients that did (not) respond to a therapy including cetuximab or panitumumab.[50] These studies are all characterized by a limited number of patient samples and retrospective data analysis. In comparison, we studied a homogeneous collection of 467 mCRC patients that have all been treated with a fluoropyrimidine, oxaliplatin *and* bevacizumab in a prospective clinical study. Based on our results, detection of methylated, free-circulating *HPP1* DNA is a prognostic as well as a response biomarker for mCRC patients. For this reason, *HPP1* mfcDNA might become a biomarker that could be used for monitoring response to first-line therapy and switching therapy protocols earlier than indicated by radiological staging. However, this potential use needs to be confirmed by prospective studies guiding therapy depending on the response of the biomarker. Furthermore, a study is desirable that tests the various above-mentioned biomarkers and potentially other markers head-to-head. Interestingly, in our as well as another study,[48] CEA was not suitable as an early marker to indicate a response to therapy.

Our study benefits from the prospective collection of samples, the large number of available patient samples and the homogenous treatment of the mCRC patients included. Nevertheless, the treatment protocol only included patients receiving a fluoropyrimidine, oxaliplatin and bevacizumab; other relevant chemotherapeutics or biologicals, like irinotecan or anti-EGFR antibodies, have not been included in this study. The patients included in this study were not pretreated. Hence, results using samples from pretreated patients might be different. Due to the implemented protocol, blood samples were only taken before and after the first administration of the combination chemotherapy. Repeated blood sampling during the chemotherapy was not performed. Therefore, we do not know whether even earlier time points after the first administration of the chemotherapy would be feasible for the analysis of *HPP1* mfcDNA. In the current study, there is (apart from CEA) no

comparison with other biomarkers with potential clinical relevance, like *RAS* mutational status or lactate dehydrogenase (LDH) levels. However, we compared the suitability of *HPP1* mfcDNA, CEA and LDH as prognostic biomarkers in a previous study.[42] Similarly, the correlation of the *HPP1* methylation status in tumor and blood samples of the same patients was not part of this study but has been reported earlier by us.[33]

In conclusion, detection of *HPP1* mfcDNA has the potential to become a clinically relevant biomarker. In CRC patients with metastatic diseases, analysis of *HPP1* mfcDNA is a suitable prognostic biomarker. In addition, detection of *HPP1* mfcDNA could be used as a marker to monitor response to therapy and help to identify mCRC patients who most likely benefit from a combination therapy containing a fluoropyrimidine, oxaliplatin and bevacizumab. However, further studies are needed to establish the detection of *HPP1* mfcDNA as a prognostic and response marker for clinical use and to guide therapeutic decisions.

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