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Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer

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Abstract

Background: Hypermethylation of DNA is an epigenetic alteration commonly found in colorectal cancer (CRC) and can also be detected in blood samples of cancer patients. Methylation of the genes helicase-like transcription factor (*HLTF*) and hyperplastic polyposis 1 (*HPP1*) have been proposed as prognostic, and neurogenin 1 (*NEUROG1*) as diagnostic biomarker. However the underlying mechanisms leading to the release of these genes are unclear. This study aimed at examining the possible correlation of the presence of methylated genes *NEUROG1*, *HLTF* and *HPP1* in serum with tissue breakdown as a possible mechanism using serum lactate dehydrogenase (LDH) as a surrogate marker. Additionally the prognostic impact of these markers was examined.

Methods: Pretherapeutic serum samples from 259 patients from all cancer stages were analyzed. Presence of hypermethylation of the genes *HLTF*, *HPP1*, and *NEUROG1* was examined using methylation-specific quantitative PCR (MethyLight). LDH was determined using an UV kinetic test.

Results: Hypermethylation of *HLTF* and *HPP1* was detected significantly more often in patients with elevated LDH levels (32% vs. 12% [$p = 0.0005$], and 68% vs. 11% [$p < 0.0001$], respectively). Also, higher LDH values correlated with a higher percentage of a fully methylated reference in a linear fashion (Spearman correlation coefficient 0.18 for *HLTF* [$p = 0.004$]; 0.49 [$p < .0001$] for *HPP1*). No correlation between methylation of *NEUROG1* and LDH was found in this study. Concerning the clinical characteristics, high levels of LDH as well as methylation of *HLTF* and *HPP1* were significantly associated with larger and more advanced stages of CRC. Accordingly, these three markers were correlated with significantly shorter survival in the overall population. Moreover, all three identified patients with a worse prognosis in the subgroup of stage IV patients.

Conclusions: We were able to provide evidence that methylation of *HLTF* and especially *HPP1* detected in serum is strongly correlated with cell death in CRC using LDH as surrogate marker. Additionally, we found that prognostic information is given by both *HLTF* and *HPP1* as well as LDH. In sum, determining the methylation of *HLTF* and *HPP1* in serum might be useful in order to identify patients with more aggressive tumors.

Keywords: Colorectal cancer, Dna methylation, Hltf, Hpp1, Neurog1, Ldh

Background

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of death from cancer worldwide with about 1.2 million cases and about 633,000 deaths in 2008 [1]. Despite significant advances in the last decades, especially patients with metastatic disease suffer from poor prognosis [2]. In addition to new therapeutic options, biomarkers are needed that allow the identification of different subgroups of patients potentially benefitting from different treatment regimens and intensity.

In many human cancers aberrant hypermethylation of CpG islands is a common epigenetic DNA modification leading to transcriptional silencing of genes that is already detectable in early stages of carcinogenesis [3]. Genes found hypermethylated in colorectal cancer have many functions, including mismatch repair, cell-cycle regulation and cell differentiation [4]. Methylated tumor DNA cannot only be found in primary colorectal cancer

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tissue, but can also be detected in remote media like serum or stool and potentially be used as biomarkers for various purposes [5-7]. We have previously described methylation of the genes *neurogenin 1 (NEUROG1)* in serum and *HIC1* in stool as diagnostic markers [8,9] and *helicase-like transcription factor (HLTF)* and *hyperplastic polyposis 1 (HPP1)*, also known as *transmembrane protein with EGF-like and two follistatin-like domains 2 (TMEFF2)*, as prognostic serum markers [10,11].

NEUROG1 is a basic helix-loop-helix transcription factor which has been identified as one of the main players in neurosensory evolution and development, especially of the inner ear [12]. Moreover *NEUROG1* has been described to be frequently hypermethylated in colorectal cancers and has been proposed as a marker to classify the CpG-island methylator phenotype in colorectal cancers [13,14].

HLTF is a transcription factor and a member of the SWI/SNF family of chromatin-remodeling factors [15]. The physiological function of *HLTF* has not yet been fully understood, but evidence for its association with genesis and progression of cancer exists [16]. Recently *HLTF* deficiency has been reported to significantly increase the formation of small intestinal adenocarcinoma and colon cancer in mice on a $Apc^{min/+}$ mutant background and to be associated with chromosomal instability [15]. Hypermethylation of *HLTF* can commonly be found in all stages of CRC as well as in adenomas and is associated with tumor size, stage and poor prognosis [17-20]. Besides its occurrence in serum, methylated *HLTF* has also been detected in stool samples of CRC patients [21,22].

HPP1 encodes a transmembrane protein containing epidermal growth factor and follistatin domains. While reported to function as a tumor suppressor related to the STAT1 pathway earlier [23], a recently published study failed to identify tumors in *HPP1* mutant mice [24]. Hypermethylation of *HPP1* can be detected already early in colorectal carcinogenesis [25-27]. Hyperplastic polyps and ulcerative colitis associated dysplasias as well as a several other tumor entities, including Barrett's-associated esophageal adenocarcinoma, gastric adenocarcinoma, bladder cancer, non-small cell lung cancer and others, frequently showed *HPP1* methylation [26-32].

Lactate dehydrogenase (LDH) is essential for anaerobic glycolysis and reversably converts pyruvate to lactate. Its expression has been shown to be related to the hypoxia inducible factor HIF-1 [33-36]. Activation of the HIF pathway is a common finding in cancers [37,38]. LDH in serum is a frequently used parameter in clinical routine and is released upon cell membrane disintegration. Thus, it is an unspecific marker for tissue damage, e.g. caused by necrosis. Elevated LDH levels can be found in numerous diseases including myocardial infarction, hemolysis and malignancies [39]. Additionally LDH has been reported to be associated with more aggressive tumors and shorter survival [40-43] in CRC. In other cancer entities like testicular cancer [44,45] and aggressive non-hodgkin lymphoma [46] elevated LDH levels are used as prognostic biomarkers. Recently, LDH has been discussed as a predictive biomarker for anti-angiogenic therapies in colorectal cancer [43,47,48].

Cell death, especially necrosis, is considered to be the source of circulating cell-free DNA (cfDNA) in cancer patients [49,50]. However, the exact mechanisms leading to the release of the tumor markers discussed here with prognostic (*HLTF* and *HPP1*) or diagnostic (*NEUROG1*) information have not been examined so far. This study aimed at investigating a possible correlation of the presence of the methylated genes *NEUROG1*, *HLTF* and *HPP1* in serum with tissue breakdown as a possible release mechanism using serum lactate dehydrogenase (LDH) as a surrogate marker. Additionally, the prognostic information given by these markers was examined.

Methods

Patients and serum samples

Pretherapeutic serum samples from 259 patients with colorectal cancer were included in the study. For these cases clinicopathologic and follow-up data as well as pretherapeutic lactate dehydrogenase values were available. Characteristics of the cohort are shown in Table 1. All measurements were performed blinded to patient data.

Table 1 Clinical features of the patient population

Clinical feature	Number of patients (%)	Clinical feature	Number of patients (%)
Total number of patients 259			
Age[a]		**Metastatic disease**	
≤ 65 years	129 (50)	M0	170 (66)
> 65 years	130 (50)	M1	89 (34)
Sex		**Tumor grade**[d]	
Male	145 (56)	G1 & G2	132 (51)
Female	114 (44)	G3 & G4	117 (45)
Tumor size[b]		**Localization**	
T1	15 (6)	Colon	122 (47)
T2	48 (19)	Sigmoid	47 (18)
T3	153 (59)	Rectum	90 (35)
T4	42 (16)	**UICC stage**	
Nodal status[c]		I	51 (20)
N0	137 (53)	II	68 (26)
N1	66 (25)	III	51 (20)
N2	50 (19)	IV	89 (34)

[a]Mean age: 64.8 years.
[b]Tumor size was unknown in 1 case.
[c]Nodal status was unknown in 6 cases.
[d]Tumor grade was unknown in 10 cases.

Blood samples were obtained pretherapeutically and underwent the following standardized preanalytical procedure: All specimens were transported by a shock absorbed tube mailing system within 15 to 30 minutes after blood drawing to the central laboratory, followed by centrifugation at 2,000 g at 4°C for 10 minutes. The supernatant serum was transferred into polypropylene cryotubes and stored frozen at −80°C. In each case, DNA methylation and lactate dehydrogenase levels were determined in the same blood sample. The study was approved by the ethical committee of the Medical Faculty of the University of Munich.

DNA isolation and bisulfite conversion

The frozen serum samples were thawed at room temperature and homogenized by smoothly flipping the tube containing the serum. Genomic DNA from 200 μL of each serum 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. Bisulfite conversion was performed as described previously [11].

Analysis of DNA methylation

Bisulfite-treated DNA was analyzed by a fluorescence-based, real-time PCR assay, described previously as MethyLight [51]. Dispersed *Alu* repeats were used to control for DNA amplification and to normalize for input DNA. Primer and probe sequences are listed in Additional file 1: Table S1. PCRs were done in 20 μL volumes containing 1x PCR buffer (Qiagen, Hilden, Germany), 4 mmol/L MgCl2, 250 μmol/L deoxynucleotide triphosphate mixture, 4 μL bisulfite-treated DNA, 0.05 units/μL Taq DNA polymerase (HotStar Taq, Qiagen) along with a pair of primers and probes according to Additional file 1: Table S1. PCRs were conducted in a Mastercycler® ep realplex^4 (Eppendorf, Hamburg, Germany) using the following conditions: 95°C for 900 s followed by 50 cycles of 95°C for 30 s, 60°C for 120 s, and 84°C for 20 s. 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. The percentage of fully methylated reference *(PMR)* at a specific locus was calculated as described previously [51] by dividing the gene/Alu ratio of a sample by the gene/Alu ratio of fully methylated, bisulfite-treated DNA (CpGenome™ Universal Methylated DNA, Millipore, Billerica, MA) and multiplying by 100. A gene was considered methylated if the percentage of the fully methylated reference value was > 0.

Determination of LDH

LDH values were determined by a UV kinetic test using the Beckman Coulter AU 2700 analyser (Beckman Coulter GmbH, Krefeld, Germany) by the central laboratory of the university hospital of Munich. The upper limit of normal for this assay applied in everyday clinical routine is 250 U/l in our hospital. LDH levels above this value were defined as elevated in this study.

Statistical analysis

All statistical analysis was done using SAS 9.3 (SAS Institute Inc., Cary, NC). Pearson's χ^2 test was used to explore associations between clinicopathologic features and categorized variables. Associations between categorized and continuous variables were tested by means of the Wilcoxon-Mann–Whitney test and correlations between continuous variables were examined using Spearman Correlation Coefficients. For evaluation of simultaneous influence of clinicopathologic features and methylation markers on LDH values a multivariate logistic regression model was developed. Overall survival was calculated from the date of diagnosis of the primary tumor to the date of death or end of follow-up. Univariate analysis of overall survival according to gene methylation status and LDH values was performed using the Kaplan–Meier method and log-rank tests.

Results

Clinicopathologic features and DNA methylation in serum

A total number of 259 serum samples were analyzed. An overview of the clinocopathologic characteristics is shown in Table 1. Methylation of *HLTF* was detected in 41 cases (16%), methylation of *HPP1* in 57 cases (22%) and methylation of *NEUROG1* in 66 cases (25%). The distribution of PMR values is demonstrated in Additional file 2: Table S2. *HLTF* methylation in the serum was significantly correlated with metastatic diseases (p = 0.013) and advanced tumor stages (p = 0.0489) as well as T4 tumors (T1-3 vs. T4, p = 0.046). A non-significant trend towards spread to lymph nodes was observed (N0 vs. N1-2, p = 0.050). *HPP1* methylation in serum was significantly correlated with larger tumor size (p < 0.001), positive nodal status (p < 0.0001), metastatic disease (p < 0.0001), tumor stage (p < .0001) as well as higher tumor grades (p = 0.0002). No significant correlation between *NEUROG1* methylation and clinicopathologic features existed. The complete distribution of the markers among the clinicopathologic features is presented in Table 2.

LDH values ranged from 100 to 1730 U/l with a mean value of 238 U/l (standard deviation 202 U/l) and a median value of 185 U/l. A cutoff of 250 U/l, representing the upper limit of normal of the assay used, was chosen, resulting in 50 patients (19%) with elevated LDH levels. These patients suffered more frequently from T4 tumors (T1-3 vs. T4, p = 0.038), nodal and distant metastases (p = 0.0006 and p < 0.0001, respectively) as well as higher tumor stages (p < 0.0001). Additionally, a non-

Table 2 Distribution of LDH and methylation of *HLTF*, *HPP1* and *NEUROG1* among clinicopathologic features

Clinical feature	LDH ≥ 250 U/l		HLTF methylation		HPP1 methylation		NEUROG1 methylation	
	n (%)	P	n (%)	P	n (%)	P	n (%)	p
Total positive	50 (19)		41 (16)		57 (22)		66 (25)	
Age[a]								
≤ 65 years	31 (24)		18 (14)		31 (24)		36 (28)	
> 65 years	19 (15)	0.055	23 (18)	0.410	26 (20)	0.434	30 (23)	0.372
Sex								
Male	26 (18)		22 (15)		34 (23)		34 (23)	
Female	24 (21)	0.528	19 (17)	0.744	23 (20)	0.528	32 (28)	0.397
Tumor size[a]								
T1	0 (0)		2 (13)		1 (7)		4 (27)	
T2	9 (19)		3 (6)		3 (6)		12 (25)	
T3	28 (18)		25 (16)		32 (21)		39 (25)	
T4	13 (31)	0.062	11 (27)	0.080	20 (48)	<.0001	11 (26)	0.999
Nodal status[b]								
N0	14 (10)		16 (12)		13 (9)		37 (27)	
N1	19 (29)		13 (20)		23 (35)		13 (20)	
N2	15 (30)	0.0006	11 (22)	0.139	18 (36)	<.0001	16 (32)	0.307
Metastatic disease								
M0	13 (8)		20 (12)		10 (6)		48 (28)	
M1	37 (42)	<.0001	21 (24)	0.013	47 (53)	<.0001	18 (20)	0.160
Localization								
Colon	25 (20)		22 (18)		33 (27)		38 (31)	
Sigmoid	9 (19)		10 (21)		8 (17)		8 (17)	
Rectum	9 (19)	0.884	9 (10)	0.151	16 (18)	0.180	20 (22)	0.114
Tumor grade[c]								
G1 & G2	22 (17)		16 (12)		16 (12)		37 (28)	
G3 & G4	25 (21)	0.344	23 (20)	0.102	37 (32)	0.0002	27 (23)	0.372
UICC stage								
I	6 (12)		4 (8)		2 (4)		16 (31)	
II	4 (6)		11 (16)		4 (6)		19 (28)	
III	3 (6)		5 (10)		4 (8)		13 (25)	
IV	37 (42)	<.0001	21 (24)	0.049	47 (53)	<.0001	18 (20)	0.486

[a]Tumor size was unknown in 1 case.
[b]Nodal status was unknown in 6 cases.
[c]Tumor grade was unknown in 10 cases.

significant trend towards higher LDH levels in younger patients was found (p = 0.055).

Correlation between LDH and DNA methylation in serum

First we analyzed the correlation of methylation of *HLTF, HPP1* and *NEUROG1* with LDH in a binary way. For this purpose we used a cutoff of LDH at 250 U/l as mentioned above. For the methylation markers we considered a PMR > 0 as methylation positive which has been shown previously to be reasonable for serum methylation analysis by our and other groups [10,52,53]. In the 50 samples with elevated LDH levels, methylation of *HLTF, HPP1,* or *NEUROG1* was detected in 16 (32%), 34 (68%), or 12 cases (24%), respectively, compared to 25 (12%), 23 (11%), or 54 (26%) in those 209 samples with normal LDH levels. Patients with elevated LDH levels revealed significantly more often methylation of *HLTF* or *HPP1* (p = 0.0005 or p < 0.0001, respectively), whereas no correlation between *NEUROG1* methylation and elevated LDH was found.

We also examined the relation of the methylation markers between each other. Methylation of *HLTF* was

found significantly more often in *HPP1* positive samples (51% vs. 17%, $p < 0.0001$). No significant difference in the frequency of either *HLTF* or *HPP1* methylation was observed between *NEUROG1* positive and *NEUROG1* negative cases (32% vs. 24% and 26% vs. 25%, respectively).

In a second step, correlations were analyzed using LDH as a continous variable without cutoff. In *HPP1* positive samples significantly higher LDH levels were measured (median 298 U/l vs. 173 U/l, $p < 0.0001$). Patients with methylation of *HLTF* had slightly, but still significantly higher LDH levels (median 208 U/l vs. 180 U/l, $p = 0.0050$), while no difference was found in LDH levels between *NEUROG1* positive and negative samples (median 187 U/L vs. 184 U/l, $p = 0.95$). Figure 1 provides a more detailed view on the distribution of LDH levels among the three methylation markers.

Additionally, we tested *HLTF*, *HPP1* and *NEUROG1* as continuous variables without cutoff using the PMR values and calculated univariate Spearman correlation coefficients. As in the analyses before, *HLTF* and *HPP1* showed significant correlation with LDH, while *NEUROG1* did not. All linear correlation coefficients and p-values are presented in Table 3.

Multivariate model

Next, a multivariate model was developed using logistic regression analysis with LDH values higher than 250 U/l as target variable. *HPP1* and *HLTF* methylation as binary parameters, i.e. with a PMR > 0, as well as clinicopathological features were entered as independent variables. Only presence of distant metastases and *HPP1* correlated significantly and independently with elevated LDH levels higher than 250 U/l. The odds ratios were 3.1 for metastatic disease (95% CI 1.3-7.2, $p = 0.009$) and 9.5 for *HPP1* methylation (95% CI 4.2-21.9, $p < 0.0001$).

Survival analysis

We earlier reported methylation of *HLTF* and *HPP1* to be independent prognostic markers in metastastatic colorectal cancer [11]. On the other hand, elevated LDH levels have been described to be linked to shorter survival [54]. Thus we compared methylation of *HLTF* and *HPP1* with LDH as prognostic factors in our patient population.

As reported earlier [11] methylation of *HLTF* and *HPP1* was associated with a higher mortality. In the current study, the median survival was 6.4 years (95% CI 4.9-9.0) and 8.0 years (95% CI 6.1-11.2) for *HLTF*- and *HPP1*-negative cases compared to 3.7 years (95% CI 1.1-5.2) and 1.2 years (95% CI 0.9-1.9) in case of positivity for *HLTF* or *HPP1* methylation ($p = 0.0008$ and $p < 0.0001$), respectively (Figure 2A, 2B). LDH levels above a cutoff of 250 U/l were associated with shorter overall survival (median survival 1.1 years, 95% CI 0.9-2.0) compared to low LDH levels (median survival 7.2 years, 95% CI 5.6-9.6) ($p < 0.0001$) (Figure 2C).

Next, we evaluated the prognostic significance stratified by tumor stage. For patients with UICC stage I-III no significant difference in overall survival, neither for LDH ($p = 0.41$) nor for *HLTF* and *HPP1* ($p = 0.41$ and $p = 0.08$, respectively), was found. However, in stage IV *HLTF* methylation positive patients showed a median survival of 0.86 years (95% CI 0.5-1.2) versus 1.6 years (95% CI 1.2-2.3) for *HLTF* negative cases ($p = 0.0081$; Figure 2D). For *HPP1* positive and negative cases the median survival was 1.0 years (95% CI 0.6-1.4) and

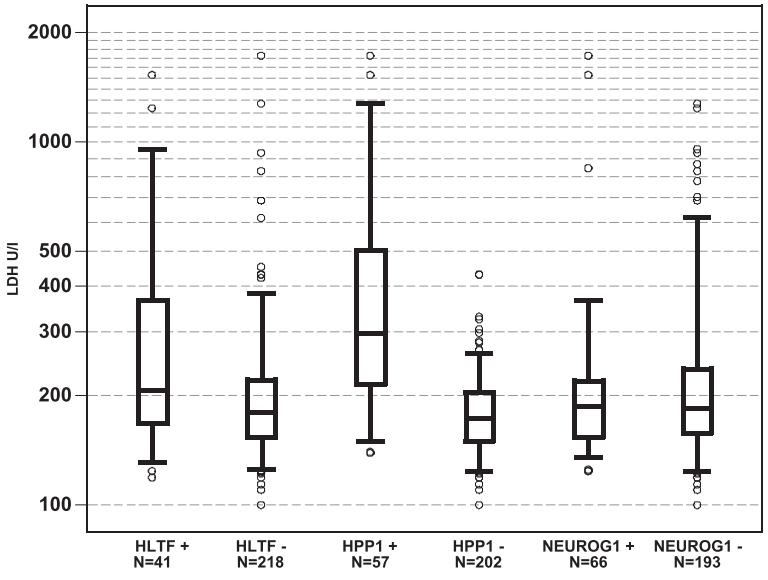

Figure 1 LDH values and methylation status of *HLTF*, *HPP1* and *NEUROG1* (as binary variables, cutoff PMR > 0).

Table 3 Linear Spearman correlation coefficients for the percentage of fully methylated reference (PMR) of *HLTF*, *HPP1* and *NEUROG1*, and LDH levels among each other

	PMR HLTF	PMR HPP1	PMR NEUROG1	LDH
PMR HLTF	1.0	-	-	-
PMR HPP1	0.32 (p < .0001)	1.0	-	-
PMR NEUROG1	0.05 (p = 0.41)	−0.00 (p = 0.97)	1.0	-
LDH	0.18 (p = 0.004)	0.49 (p < .0001)	0.01 (p = 0.85)	1.0

1.8 years (95% CI 1.2-3.3), respectively (p = 0.0005; Figure 2E). For LDH, elevated levels > 250 U/l were associated with shorter median survival (1.0 years, 95% CI 0.6-1.2, vs. 1.8 years, 95% CI 1.3-2.5; p = 0.0002; Figure 2F).

Discussion

In this study we examined the correlation between cell damage using LDH as a surrogate marker and the methylation status of three genes which have previously been proposed as prognostic (*HLTF, HPP1*) [10,11] or diagnostic (*NEUROG1*) [8] biomarkers for patients with CRC.

Our data confirm our previous findings that methylation of *HLTF* or *HPP1* in serum is found more often in

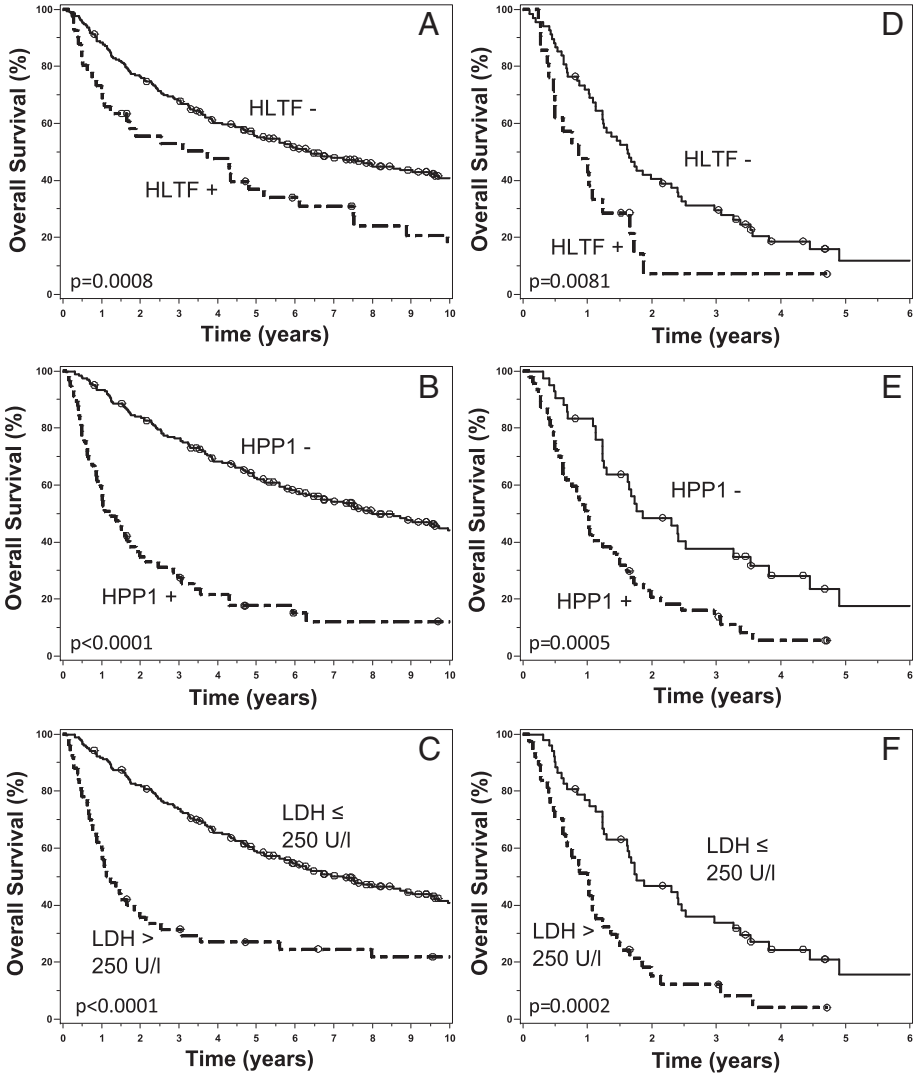

Figure 2 Kaplan-Meier plots of overall survival. A-C: Overall survival for all patients according to methylation status of *HLTF* **(A)**, *HPP1* **(B)** and high LDH levels > 250 U/l **(C)**, respectively. **D-F**: Overall survival for stage IV patients patients according to methylation status of *HLTF* **(D)**, *HPP1* **(E)** and high LDH levels > 250 U/l **(F)**, respectively.

patients with advanced stages of colorectal cancer, especially in those with distant metastases, whereas no correlation between methylation of *NEUROG1* and any clinicopathologic data was found. While methylation of *HLTF* was only correlated with metastastatic disease, methylation of *HPP1* was also associated with local tumor extent and nodal status as well as tumor grade with high statistic significance.

It is well known that patients with elevated serum levels of LDH tend to have more aggressive tumors and a shorter survival time [40-43]. Consistent with the literature high LDH levels in our data were significantly correlated with advanced tumor stages as well as nodal and distant metastases. Trends towards larger tumor size and younger age were observed but did not reach statistical significance.

Cell death associated mechanisms like apoptosis or, especially in cancer, necrosis have been suggested as main sources for cell-free DNA (cfDNA) in the blood, but other mechanisms like physiological active release have been described as well (for reviews see refs. [49,50]). In this study we found methylation of *HLTF* and, even to a higher degree, *HPP1* to be correlated with elevated LDH levels. This finding was robust, as it was confirmed by different statistical methods. Given that elevated LDH indicates cell membrane damage, this observation might be a hint that methylated *HLTF* and *HPP1* DNA is released by tumor cells undergoing cell death. The fact that necrosis tends to be found more often in larger, more aggressive tumours and advanced cancer stages [55,56], which was likewise the case for LDH as well as methylated *HLTF* and *HPP1* in our data, also suggests an interrelation.

For *NEUROG1*, on the other hand, hypermethylation in serum was detectable independently of LDH levels and tumor stage. This is consistent with earlier analyses revealing methylation of *NEUROG1* in primary tissue not to be associated with tumor stage (A.P. and F.K., data not published). Hence the observed correlation between DNA methylation in serum and LDH seems not to be linked to global methylation levels and cell death alone. Besides the methylation status of distinct genes, other parameters influencing this observation might include DNA integrity and stability of the respective segments as well as still unknown factors. Therefore it seems likely that tumor cell death might not be the only mechanism by which methylated tumor DNA is released to the blood.

In addition to the correlation analysis we examined the prognostic significance of the methylation markers *HPP1* and *HLTF* as well as of LDH. All three markers were significantly associated with worse overall survival. This could be attributed to the fact that all three markers are found more frequently in advanced cancer stages. However, earlier analyses [11] as well as the survival data presented here furthermore divide patients with already metastasized disease into two subgroups with better or worse prognosis, respectively.

Conclusion

In conclusion we were able to provide evidence that methylation of *HLTF* and especially *HPP1* detected in serum is strongly correlated with cell death in colorectal cancer using LDH as surrogate marker. However, this finding was specific for those two genes and did not occur for *NEUROG1*, suggesting that mechanisms other than release by membrane disintegration could be responsible for the occurrence of cell-free DNA in blood of CRC patients. Additionally, we found that prognostic information is given by both *HLTF* and *HPP1* as well as LDH. In sum, determining the methylation of *HLTF* and *HPP1* in serum might be useful in order to identify patients with more aggressive tumors. Future research needs to further clarify the underlying biological mechanisms and to validate methylated cell-free circulating DNA as a biomarker for colorectal cancer.

Additional files

Additional file 1: MethyLight Reaction Details.

Additional file 2: Distribution of the percentage of fully methylated reference (PMR) of *HLTF, HPP1* and *NEUROG1*.

Abbreviations

cfDNA: Cell-free deoxyribonucleic acid; CI: Confidence interval; CIMP: CpG island methylator phenotype; CRC: Colorectal cancer; HIF: Hypoxia inducible factor; HLTF: Helicase-like transcription factor; HPP1: Hyperplastic polyposis; LDH: Lactate dehydrogenase; NEUROG1: Neurogenin 1; PCR: Polymerase chain reaction; PMR: Percentage of fully methylated reference; UICC: Union for international cancer control; UV: Ultraviolet.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

Sample collection and experiments: AP, IT, PS, and RL; data analysis and interpretation: AP, DN, PS, and FK; study design and preparation of the manuscript: AP, AH, and FK. All authors read and approved the final manuscript.

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