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Frei zirkulierende methylierte DNA als Tumormarker des kolorektalen Karzinoms

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Meiner Familie

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1 Einleitung

1.1 Das kolorektale Karzinom

Das kolorektale Karzinom (KRK) war im Jahr 2012 weltweit mit über 1,3 Millionen Erkrankungsfällen die dritthäufigste Krebsart und die vierthäufigste krebsbezogene Todesursache (Ferlay et al. 2013). Hierbei entfielen 746.000 Fälle auf Männer und 614.000 Fälle auf Frauen. Die Inzidenz variiert je nach Entwicklungsstatus des Landes und geographischer Region stark. Eine deutliche Häufung findet sich insbesondere in den sogenannten entwickelten Ländern (Ferlay et al. 2013). In Deutschland betrug die alterstandardisierte Erkrankungsrate im Jahr 2010 57,8 pro 100.000 Männer bzw. 36,8 pro 100.000 Frauen (Kaatsch et al. 2013).

Mit 694.000 Todesfällen durch das KRK im Jahr 2012 belegt es den vierten Platz in der weltweiten Mortalitätsstatistik der Krebsarten. Die Todesraten weisen im Vergleich zur Inzidenz eine geringere geographische Schwankungsbreite auf (Ferlay et al. 2013), das heißt, die Letalität ist in Industrienationen geringer. In Deutschland war das kolorektale Karzinom im Jahr 2010 die Krebsart mit der zweithöchsten Mortalität mit einer altersstandardisierten Sterberate von 22,3 pro 100.000 Männer bzw. 13,9 pro 100.000 Frauen (Kaatsch et al. 2013). Wie diese Zahlen belegen, handelt es sich also beim KRK trotz vergleichsweise besserer Überlebenschancen in den sogenannten entwickelten Ländern um eine weit verbreitete Erkrankung mit zahlreichen Todesfällen.

Zu Beginn der 1980er Jahre betrug die relative 5-Jahres-Überlebensrate des Darmkrebs ab dem Zeitpunkt der Diagnosestellung in Deutschland noch ca. 45% (Bertz et al. 2010).

Durch Früherkennungsprogramme sowie signifikante therapeutische Verbesserungen in den letzten Jahren und Jahrzehnten konnte eine Verbesserung der relativen 5-Jahres-Überlebensraten auf ca. 66 % erreicht werden (Heinemann et al. 2013). Dennoch ist die Prognose für Patienten mit primär metastasiertem kolorektalem Karzinom weiterhin ungünstig. Hier liegen die berichteten 5-Jahres-Überlebensraten bei 8-15% (O'Connell, Maggard und Ko 2004; Heinemann et al. 2013).

Risikofaktoren für die Entwicklung eines kolorektalen Karzinoms sind neben familiärer Vorbelastung insbesondere ein fortgeschrittenes Alter sowie ein ungünstiger Lebensstil. Hierzu zählen geringe körperliche Aktivität, falsche Ernährung, Übergewicht und Alkoholkonsum (Kaatsch et al. 2013; Chan und Giovannucci 2010).

Die kolorektale Karzinogenese ist ein mehrstufiger Prozess, der durch verschiedene genetische und epigenetische Veränderungen über mehrere Jahre von gesunder Dickdarmschleimhaut über Adenome zu Karzinomen führt. Diese Abfolge wurde erstmals in den 1920er Jahren beschrieben (Stewart 1931; Dukes 1932) und erstmals 1951 mit dem heute gängigen Titel „Adenom-Karzinom-Sequenz" versehen (Jackman und Mayo 1951). In den darauf folgenden Jahrzehnten wurde diese durch intensive pathologische Forschung auf eine solide Datenbasis gestellt (Morson 1966; Muto, Bussey und Morson 1975). Einen wichtigen Meilenstein stellt das von Fearon und Vogelstein entwickelte Modell dar, das typische genetische Mutationen den einzelnen Schritten der Karzinogenese zuordnet (Fearon und Vogelstein 1990).

Auf dieser Grundlage konnten in den folgenden beiden Jahrzehnten weitere, darüber hinaus gehende Varianten der kolorektalen Karzinogenese auf molekularbiologischer Ebene identifiziert werden, so dass aktuell drei wesentliche, zum Teil in Kombination auftretende, molekulare Mechanismen bekannt und allgemein akzeptiert sind: chromosomale Instabilität (CIN) und Mikrosatelliteninstabilität (MSI) auf genetischer und der CpG-Methylierungs-Phänotyp (CIMP) auf epigenetischer Ebene (Markowitz und Bertagnolli 2009; Jass 2007; Al-Sohaily et al. 2012).

Letzterer wurde unter diesem Namen erstmals 1999 von Toyota und Kollegen als Untergruppe des KRK mit einem hohen Anteil an Hypermethylierung von CpG-Inseln in Promotorregionen beschrieben (Toyota et al. 1999). Diese führt zu einem transkriptionellen „Silencing“ der entsprechenden Gene, was durch die verminderte Expression von Tumorsuppressor- und DNA-Reparaturgenen einen essentiellen Teil der Karzinogenese beim KRK und diversen weiteren Tumorentitäten darstellen kann (Jones und Baylin 2007). CIMP findet sich in bis zu 20 % der kolorektalen Karzinome, geht mit typischen klinischen und pathologischen Charakteristika einher und ist mit BRAF-Mutationen und dem serratierten Karzinogeneseweg assoziiert (Leggett und Whitehall 2010; Jass 2007; Weisenberger et al. 2006). Auch wenn die Existenz des CIMP-Subtyps beim KRK nach anfänglichen Diskussionen mittlerweile unbestritten ist, gibt es bis zum heutigen Tag keine Übereinkunft über einen methodischen Goldstandard zu dessen Definition und Detektion (Hughes et al. 2012).

1.2 Biomarker des kolorektalen Karzinoms

1.2.1 CEA als etablierter Tumormarker des KRK

Das carcinoembryonale Antigen (CEA) beschreibt eine Gruppe von Glykoproteinen aus der Immunglobulinsuperfamilie und beinhaltet 29 Gene auf Chromosom 19q (Duffy 2001). Es wurde erstmals 1965 von Gold und Freedman als Antigen beschrieben, das sowohl im fetalen Kolon als auch in Adenokarzinomen des Kolon nachgewiesen werden konnte (Gold und Freedman 1965). In späteren Studien konnte CEA zwar auch in gesundem Gewebe gefunden werden, allerdings in deutlich geringeren Konzentrationen als in Tumoren (Boucher et al. 1989).

Da CEA oft auch im Serum von Patienten mit gastrointestinalen Tumoren nachgewiesen werden kann, hat es sich als am häufigsten genutzer Tumormarker für diese Entitäten etabliert. Die CEA-Konzentration im Serum hängt u.a. von Tumorstadium, Grading, Le-

berfunktion, Tumorlokalisation im Kolon, Nikotinabusus und Darmobstruktion ab (Duffy 2001). In zahlreichen großen klinischen Studien konnte gezeigt werden, dass hohe CEA-Spiegel bei Diagnosestellung mit einer schlechten Prognose einhergehen (siehe z.B. Duffy 2001; Park et al. 1999; Thirunavukarasu et al. 2011; Sun et al. 2009).

Darüber hinaus scheint CEA als Verlaufsmarker im Rahmen eines intensiven *follow-ups* notwendig zu sein, um eine signifikante Verbesserung des Überlebens zu erreichen (Bruinvels et al. 1994; Figueredo et al. 2003; Tjandra und Chan 2007). Die *European Group on Tumour Markers* (*EGTM*) empfiehlt CEA als einzigen Tumormarker beim kolorektalen Karzinom insbesondere zur postoperativen Überwachung nach kurativer Resektion, aber auch mit geringerer Evidenz zur Prognoseabschätzung und zum Therapiemonitoring (Duffy et al. 2007; Duffy et al. 2014). Die kolorektale Arbeitsgruppe des *American Joint Committee on Cancer* (*AJCC*) empfiehlt bereits seit Längerem CEA als Tumormarker im Serum zum etablierten TNM-Staging-System hinzuzufügen (Compton et al. 2000). Auch wenn dies bislang nicht umgesetzt wurde, so stellt das CEA den Goldstandard dar, an dem sich jeder neue prognostische Tumormarker für das kolorektale Karzinom messen muss.

1.2.2 Lactatdehydrogenase (LDH)

Das Enzym Laktatdehydrogenase (LDH) bewirkt die reversible Umwandlung von Pyruvat und Laktat und ist somit ein essentielles Element der anaeroben Glykolyse. Die Expression von LDH wird durch den *hypoxia inducible factor* HIF-1 beeinflusst (Semenza et al. 1994; Firth et al. 1994; Firth, Ebert und Ratcliffe 1995; Weidemann und Johnson 2008), einem Bestandteil des HIF-Signalwegs, welcher häufig aktiviert in Krebszellen gefunden wird (Maxwell, Pugh und Ratcliffe 2001; Keith, Johnson und Simon 2012).

Die im Serum gemessene LDH-Aktivität ist ein gängiger Parameter im klinischen Alltag. Die Freisetzung geschieht im Rahmen von Zerfall oder Auflösung der Zellmembran und ist somit ein unspezifischer Marker für Gewebeschäden, z.B. durch Nekrose.

Erhöhte LDH-Spiegel finden sich bei vielen Erkrankungen wie Herzinfarkt, Hämolyse oder Malignomen (Huijgen et al. 1997). Bei Hodenkrebs oder aggressiven Non-Hodgkin-Lymphomen werden erhöhte LDH-Spiegel als prognostische Biomarker verwendet (International Germ Cell Cancer Collaborative Group. 1997; Krege et al. 2008; Anon 1993).

Auch beim KRK wurden hohe LDH-Spiegel im Serum mit aggressiveren Tumoren und kürzerem Überleben in Verbindung gebracht (Mekenkamp et al. 2010; de Gramont et al. 2000; Wu, Ma und Wang 2010; Scartozzi et al. 2012). In den letzten Jahren wurde auch über LDH als möglicher Prädiktor des Ansprechens auf eine antiangiogenetische Therapie diskutiert (Hecht et al. 2011; Van Cutsem et al. 2011; Scartozzi et al. 2012).

1.2.3 Helicase-like transcription factor (HLTF)

Das Protein *Helicase-like transcription factor* (HLTF) gehört zur SWI/SNF-Familie (SWItch /Sucrose NonFermentable) und ist neben SHPRH eines der beiden humanen Homologe von Rad5 in *Saccharomyces cerevisiae* (Unk et al. 2010). Es konnte gezeigt werden, dass HLTF funktionell durch eine doppelsträngige DNA-Translokase-Aktivität ein Remodeling der Replikationsgabel bewirken und diese dadurch rückgängig machen kann (Blastyák et al. 2010). Des Weiteren wurde beschrieben, dass HLTF als Ubiquitin-Ligase fungiert und die Polyubiquitinilierung von PCNA (proliferating cell nuclear antigen) vermittelt, einem essentiellen Bestandteil der Replikationsmaschinerie (Unk et al. 2008; Motegi et al. 2008). Darüber hinaus kann HLTF als Transkriptionsfaktor mit DNA-Zielsequenzen interagieren und die Expression von Zielgenen beeinflussen (Debauve et al. 2008). In Vorarbeiten zur vorliegenden Dissertation wurde Methylierung von HLTF im Serum von Patienten mit KRK als unabhängiger prognostischer Marker und als Prädiktor für Krankheitsrezidive beschrieben (Wallner et al. 2006; Herbst et al. 2009).

1.2.4 Hyperplastic polyposis 1 (HPP1)

Das Gen HPP1 codiert ein Transmembran-Protein, welches epidermal growth factor- sowie Follistatin-Domänen enthält und ist auch unter den Bezeichnungen TPEF (transmembrane protein containing epidermal growth factor and follistatin domain) oder TMEFF2 (transmembrane protein with EGF-like and two follistatin-like domains 2) bekannt. Die Expression von HPP1 kann regelhaft in gesunder Kolonmucosa festgestellt werden (Young et al. 2001). Die Hypermethylierung des Gens, assoziert mit einer Minderexpression, kann bereits frühzeitig in der kolorektalen Karzinogenese nachgewiesen werden und wurde in hyperplastischen Polypen sowie Colitis-ulcerosa-assoziierten Dysplasien festgestellt (Young et al. 2001; Sato et al. 2002; Saito et al. 2011). Auch in anderen Tumorentitäten wie Ösophagus(-Barrett-)- und Magenadenokarzinom sowie Blasen- und nichtkleinzelligem Lungenkarzinom wurde eine Hypermethylierung von HPP1 beschrieben (Eads, Lord et al. 2000; Ivanauskas et al. 2008; Hellwinkel et al. 2008; Lee, Park und Kim 2012).

Für HPP1 wurde eine Tumorsupressorfunktion beschrieben, die durch die Aktivierung des STAT1-Signalwegs vermittelt wird (Elahi et al. 2008), allerdings konnte in HPP1-mutierten Mäusen kein gehäuftes Auftreten von Tumoren beobachtet werden (Chen et al. 2012). In den Vorarbeiten für die vorliegende Arbeit konnte gezeigt werden, dass auch Methylierung von HPP1 ein unabhängiger prognostischer Marker für das KRK ist (Wallner et al. 2006).

1.2.5 Neurogenin 1 (NEUROG1)

NEUROG1 ist ein Helix-loop-helix-Transkriptionsfaktor, welcher bei der neurosensorischen Entwicklung, insbesondere des Innenohrs, eine wichtige Rolle spielt (Pan et al. 2012). Beim kolorektalen Karzinom findet sich häufig eine Hypermethylierung von NEUROG1 (Ogino, Cantor et al. 2006). Darüber hinaus wurde es von Weisenberger und Kollegen als Teil eines Markerpanels zur Klassifizierung des CpG-Insel-Methylierung-

Phänotyps (*CpG island methylator phenotype, CIMP*) vorgeschlagen (Weisenberger et al. 2006). Zudem wurde der Nachweis von NEUROG1-Methylierung der frei im Blut zirkulierenden DNA als potentieller Screeningmarker vorgeschlagen (Herbst et al. 2011).

1.3 Zielsetzungen

1.3.1 Prognose des Krankheitsverlaufes

Ziel der vorliegenden Studien war es, frei zirkulierende methylierte DNA als Biomarker des kolorektalen Karzinoms zu untersuchen, insbesondere in Hinblick auf ihre prognostische Bedeutung. Ein besonderer Schwerpunkt wurde hierbei auf die Marker HLTF und HPP1 gelegt, die bereits in einer kleineren Pilotstudie als prognostische Marker des KRK identifiziert wurden. In einem größeren Patientenkollektiv sollten nun die Korrelationen mit klinisch-pathologischen Parametern sowie insbesondere die prognostische Aussagekraft validiert werden. Darüber hinaus wurde mit CEA der einzig relevante vorbeschriebene Blutbiomarker des KRK als Vergleichsparameter in die Auswertungen eingeschlossen. Des Weiteren war es Ziel, Subgruppen zu identifizieren, in denen die untersuchten Marker eine besonders hohe prognostische Aussagekraft haben, was erstmals durch die hohe Patientenzahl ermöglicht wurde.

1.3.2 Charakterisierung der Marker

Ein weiteres Ziel war es, durch Analyse von Gewebeproben aus dem Primärtumor in einer Subgruppe des Kollektivs die Herkunft der frei zirkulierenden methylierten DNA aus dem Tumor zu verifizieren. Des Weiteren war hierdurch die Untersuchung der Korrelation mit dem CpG-Insel-Methylierungsphänotyp (CIMP) als molekularbiologische Eigenschaft des Tumors möglich.

In weiteren Auswertungen wurde ein Schwerpunkt auf einen möglichen Zusammenhang von frei zirkulierender methylierter DNA und Tumorzerfall als möglichem Frei-

setzungsmechanismus gelegt. Auch wenn Zelluntergang, insbesondere durch Nekrose, als wahrscheinliche Quelle der frei zirkulierenden DNA angesehen wird, sind die genauen Mechanismen aktuell noch unklar (Jung, Fleischhacker und Rabien 2010; Schwarzenbach, Hoon und Pantel 2011). In einer Substudie wurde die Korrelationen der Marker HLTF, HPP1 und NEUROG1 mit LDH als Surrogatmarker für Zellzerfall untersucht.

1.4 Material und Methodik

1.4.1 Patientenkollektiv

Es wurden insgesamt Serumproben von 311 Patienten mit kolorektalem Karzinom analysiert, die am Klinikum München-Großhadern untersucht und behandelt wurden. Alle Analysen wurden verblindet und ohne Kenntnis der Patientendaten durchgeführt. Die Verwendung der Proben wurde durch die Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität München genehmigt. Im Kollektiv befanden sich Patienten aller Tumorstadien. Die genaue Verteilung der klinisch-pathologischen Parameter ist in den angehängten Publikationen detailliert dargestellt. Von 54 der 103 Patienten mit KRK im Stadium IV wurden zudem Gewebeproben aus dem Primärtumor untersucht.

1.4.2 Tumormarkeranalysen

Material

Sämtliche 311 Blutproben wurden vor Therapiebeginn gewonnen und mit einem stoßgedämpften Rohrpostsystem in das Zentrallabor transportiert. Dort erfolgte eine Weiterverarbeitung nach standardisiertem Verfahren mit anschließender Lagerung des Serums bei -80° C. Von 54 der 103 Patienten mit metastasiertem kolorektalem Karzinom lagen formalin-fixierte, in Paraffin eingebettete Gewebeproben vor, die zur Analyse zur Verfügung standen und von denen Schnittserien erstellt wurden. Auf einem mit Hämatoxylin und Eosin gefärbten Schnitt (HE-Färbung) wurde von einem Facharzt für Pathologie

die Tumorregion markiert. Von einem deparaffinierten benachbarten Schnitt wurde das Tumorgewebe dann abgekratzt.

DNA-Aufreinigung und Bisulfit-Behandlung

Aus den Serumproben wurde mittels des High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim) gemäß Protokoll des Herstellers DNA extrahiert. Diese wurde in einem standardisierten Verfahren mit Bisulfit behandelt, um eine Umwandlung von unmethyliertem Cytosin in Uracil und somit eine Unterscheidbarkeit von methylierten und unmethylierten DNA-Sequenzen zu erreichen. Im Anschluss erfolgte eine Aufreinigung der DNA unter Verwendung des Wizard DNA Clean-up System (Promega, Mannheim) gemäß Instruktionen des Herstellers.

Die DNA aus den Tumorgewebeproben wurde mit Hilfe des QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden) nach einer leicht modifizierten Version des Herstellerprotokolls extrahiert. Die Bisufitbehandlung erfolgte im Falle der Gewebeproben mit dem EZ DNA Methylation-Gold Kit (Zymo Research, Freiburg) gemäß den Instruktionen des Herstellers.

Analyse der DNA-Methylierung von HLTF, HPP1 und NEUROG1

Die bisulfitbehandelte DNA wurde mittels eines erstmalig von Eads und Kollegen beschriebenen MethyLight-Assays untersucht, der auf fluoreszenz-basierter quantitativer real-time Polymerase-Kettenreaktion (qPCR) beruht (Eads, Danenberg et al. 2000). Kurz zusammengefasst erfolgt der Nachweis der Methylierung der Zielgene (HLTF, HPP1, NEUROG1) durch methylierungsspezifische Primer und Sonden. Durch Messung von methylierungsunabhängigen Alu-Sequenzen, die hochrepetitiv im gesamten menschlichen Genomen vorkommen (Batzer und Deininger 2002), erfolgte eine Kontrolle der DNA-Amplifikation und eine Normalisierung auf die Menge der eingesetzten DNA (Weisenberger et al. 2005). Als Positivkontrolle wurde vollmethylierte DNA (Chemicon, Temecula,

CA) verwendet.

Für jede Probe und jedes Zielgen wurde die *percentage of fully methylated reference (PMR)* berechnet, indem das Gen-Alu-Verhältnis einer Probe durch jenes der vollmethylierten, bisulfitbehandelten DNA dividiert und anschließend mit 100 multipliziert wurde (Eads, Danenberg et al. 2000). Bei den Serumproben wurde eine PMR > 0 als methylierungspositiv gewertet. Für die Gewebeproben wurde eine Grenz-PMR von 4 gewählt, für die die beste Unterscheidung zwischen normalem und Tumorgewebe beschrieben wurde (Eads, Lord et al. 2000; Ogino, Kawasaki et al. 2006).

Weitere Messparameter (CEA, LDH, CIMP)

Die Konzentration des carcinoembryonale Antigens (CEA) wurde mittels eines immunoenzymometrischen Mikropartikel-Assays bestimmt (AxSYM, Abbott Laboratories, Chicago, IL). Die Aktivität der Laktatdehydrogenase (LDH) wurde mittels eines UV-kinetischen Tests auf dem Beckman Coulter AU 2700 analyser (Beckman Coulter GmbH, Krefeld) bestimmt.

In den oben beschriebenen Gewebeproben wurde der CpG-Insel-Methylierungs-Phänotyp (*CIMP, CpG island methylator phenotype*) mit dem von Weisenberger et. al vorgeschlagenem Markerpanel bestimmt (Weisenberger et al. 2006). Hierzu wurde der Methylierungsstatus der Zielgene CACNA1G, IGF2, NEUROG1, RUNX3 und SOCS1 mit dem bereits beschriebenen MethyLight-Assay bestimmt. Wenn mindestens drei der Marker methylierungspositiv waren, wurden die entsprechenden Tumoren als CIMP-positiv klassifiziert.

1.4.3 Statistik

Alle statistischen Auswertungen wurden mit Hilfe der Software SAS 9.2 bzw 9.3 durchgeführt (SAS Institute Inc., Cary, NC). Korrelationen zwischen klinisch-pathologischen Parametern und kategorialen Messwerten wurden mit Pearson's $\chi 2$-Test bestimmt. Für

Assoziationen zwischen kategorialen und kontinuierlichen Variablen wurde der Wilcoxon-Mann–Whitney-Test, für solche zwischen kontinuierlichen Variablen wurden Spearman Korrelationskoeffizienten verwendet.

Das Gesamtüberleben wurde als Differenz zwischen Datum der Erstdiagnose bis zum Zeitpunkt des Todes oder des Endes des *follow-ups* berechnet. Die Überlebenskurven wurden mittels Kaplan-Meier-Methode erstellt. Unterschiede zwischen den Kurven wurden mittels log-rank-Test ermittelt. Bei allen Analysen wurden p-Werte $< 0{,}05$ als signifikant erachtet.

1.5 Ergebnisse

Insgesamt konnte Methylierung von HLTF, HPP1 und NEUROG1 in je 48, 64 und 81 der insgesamt 311 Proben nachgewiesen werden. Für HLTF und HPP1 zeigte sich ein Zusammenhang mit größeren Tumoren, Tumorstadium, Tumorgrad und Fernmetastasierung. HPP1-Methylierung korrelierte darüber hinaus mit Lymphknotenfiliae. Für NEUROG1 ergaben sich keine Korrelationen mit klinischen oder pathologischen Parametern.

Patienten mit Nachweis von HLTF-Methylierung zeigten ein medianes Überleben von 36,3 Monaten im Vergleich zu 80,2 Monaten für HLTF-negative Fälle. Für HPP1 ergibt sich mit 12,6 Monaten bei positiven und 104,7 Monaten bei negativen Fällen ein noch deutlicherer Unterschied. In beiden Fällen war der Unterschied zwischen den Gruppen hochsignifikant ($p=0{,}0001$ bzw. $p<0{,}0001$). Keine Korrelation bestand dagegen zwischen NEUROG1-Methylierung und dem Überleben. Wie erwartet gingen auch hohe CEA-Spiegel mit einer schlechteren Prognose einher (151,9 vs. 42,3 Monate medianes Überleben bei Cutoff 2,5 ng/ml).

In der Subgruppenanalyse zeigte sich, dass HLTF- und HPP1-Methylierung im Stadium IV mit deutlich schlechterer Prognose einhergehen (19,7 Monate für HLTF+, 10,0 Monate für HLTF-, $p=0{,}0005$. 10,5 Monate für HPP1+, 23,2 Monate für HPP1-, $p=0{,}0003$), während dies für die Stadien I bis III nicht der Fall war. Im metastasierten Stadium zeigte

sich bei Verwendung des üblichen Cutoffs von 2,5 ng/ml für CEA kein Unterschied zwischen den beiden Gruppen. Erst bei Verwendung von höheren Cutoff-Werten konnte ein signifikanter Unterschied im Gesamtüberleben erreicht werden (bei Cutoff am Median [27 ng/ml] 26,8 Monate für CEA $\leq$ 27 ng/ml, 12,9 Monate für CEA $>$ 27 ng/ml, p=0,002). Durch eine multivariate Analyse konnte gezeigt werden, dass HLTF und HPP1 auch unabhängig voneinander und unabhängig von CEA prognostische Marker sind (HLTF: Hazard Ratio 1.8 [95% Konfidenzintervall 1.0–3.0], p = 0,0438; HPP1 Hazard Ratio 1.6 (95% Konfidenzintervall 1.0–2.7), p=0,0495].

In der Analyse der Gewebeproben aus Primärtumor von 54 der Patienten mit metastiertem KRK konnte HLTF- bzw. HPP1-Methylierung in 24 bzw. 50 Fällen nachgewiesen werden. Bei allen Patienten mit Nachweis von HPP1-Methylierung im Serum konnte diese auch im Primärtumor nachgewiesen. Für HLTF war das Bild bis auf eine Ausnahme, bei der Methylierung nur im Serum, aber nicht im Primärtumor nachgewiesen werden konnte, ähnlich. Zwischen CIMP-Status, der in fünf Proben nachgewiesen werden konnte, bestand kein signifikanter Zusammenhang mit den untersuchten Markern.

In die Korrelationsanalyse mit LDH konnten 259 Sera eingeschlossen werden. In dieser wurde Methylierung sowohl von HLTF als auch von HPP1 signifikant häufiger in Sera von Patienten mit erhöhten LDH-Spiegeln gefunden (32% vs. 12% [p = 0.0005] bzw. 68% vs. 11% [p $<$ 0.0001]). Zudem korrelierten auch höhere Methylierungsgrade, gemessen durch den Prozentsatz einer vollmethylierten Referenz (*percentage of a fully methylated reference*, PMR), signifikant mit höherer LDH-Aktivität (Spearman-Korrelationskoeffizient 0.18 für HLTF [p = 0.004]; 0.49 [p $<$ .0001] für HPP1). Im untersuchten Kollektiv konnte kein Zusammenhang zwischen LDH und Methylierung von NEUROG1 gefunden werden.

2 Zusammenfassung

Ziel der Studien war die Untersuchung der prognostischen Aussagekraft der methylierten Gene HLTF, HPP1 und NEUROG1. Bislang wird eine Prognoseabschätzung hauptsächlich über radiologische und pathologische Kriterien erreicht. Im Blut bestimmte Marker haben den Vorteil einer relativ unaufwändigen, nichtinvasiven Gewinnung und könnten eine wertvolle Ergänzung der etablierten Faktoren darstellen. Als Vergleichsmarker wurde mit dem CEA der einzige für das KRK relevante Tumormarker, für den eine prognostische Wertigkeit beschrieben wurde, gewählt.

Anhand der vorliegenden Daten konnte gezeigt werden, dass HLTF- und HPP1-Methylierung am häufigsten im Serum von Patienten mit fortgeschrittenen, insbesondere metastasierten, Erkrankungen zu finden sind und Marker für eine deutlich schlechtere Prognose sind. Dieser hochsignifikante Effekt zeigte sich insbesondere bei den Patienten mit Metastasen, bei denen jeweils eine Subgruppe mit einer deutlich schlechteren Prognose identifiziert werden konnte. Im Vergleich mit CEA zeigten HLTF und HPP1 eine mindestens gleichwertige prognostische Bedeutung im vorliegenden Kollektiv. Auch in der multivariaten Analyse blieben HLTF, HPP1 und CEA als voneinander unabhängige prognostische Faktoren im Stadium IV bestehen, wobei der Vorteil von HLTF und HPP1 darin liegt, dass diese weiter als binäre Parameter verwendet werden können, während für CEA erst ein passender Grenzwert innerhalb der Population definiert werden muss.

Das Vorliegen von korrespondierenden Gewebeproben zu den untersuchten Blutproben ermöglichte die erstmalige Untersuchung der Korrelation von Methylierung von HLTF,

HPP1 und NEUROG1 in Serum und Primärtumor. Alle positiven Serumproben zeigten bis auf eine Ausnahme auch Methylierung der entsprechenden Gene im Gewebe. Damit konnte diese Untersuchung die angenommene Herkunft der frei zirkulierenden methylierenden DNA aus dem Tumor bestätigen. Ein Zusammenhang mit dem Methylierungsphänotyp CIMP ergab sich im Kollektiv nicht.

In einer weiteren Untersuchung wurde der Zusammenhang der drei Zielparameter mit LDH im Blut als Surrogatmarker für einen hohen Zellzerfall untersucht. Die hohe Korrelation von HLTF und HPP1 mit erhöhten LDH-Spiegeln legt den Zerfall der Tumorzellen als möglichen Mechanismus der Freisetzung der Tumor-DNA in die Blutbahn nahe. Auf der anderen Seite bestand kein Zusammenhang von LDH und Methylierung von NEUROG1. Somit müssen neben tumorassoziiertem Zelltod weitere Mechanismen bei der Freisetzung von methylierter Tumor-DNA eine Rolle spielen, die aktuell noch ungeklärt sind.

Zusammenfassend wurde frei zirkulierende methylierte HLTF- und HPP1-DNA als unabhängiger prognostischer Marker des metastasierten kolorektalen Karzinoms untersucht und charakterisiert. Diese vielversprechenden Ergebnisse stellen wertvolle Ansatzpunkte für die weitere Erforschung der Marker in Folgestudien dar, um klinische Anwendungsgebiete zu evaluieren, beispielsweise in der prätherapeutischen Risikostratifizierung, im Therapiemonitoring oder auch zur Prädiktion des Ansprechens auf spezifische Tumortherapien.

3 Summary

The aim of these studies was to evaluate the prognostic value of methylated genes HLTF, HPP1, and NEUROG1. To date, the assessment of prognosis was typically done via radiological or pathological criteria. The significant advantage of blood-based markers lies in the relatively easy, non-invasive retrieval and could therefore prove a useful addition to the established markers. CEA was chosen as a comparative tumor marker for being, until now, the only one with a proven prognostic relevance for colorectal carcinoma.

The conducted measurements demonstrated that methylated DNA of HLTF and HPP1 genes was found more frequently in blood samples of patients with advanced, and specifically, metastasized colorectal cancer (CRC) and is a marker for worse prognosis. This highly significant effect appeared specifically among the group of patients with metastases, within which a subgroup with a notably worse prognosis could be identified. Compared with CEA, the prognostic relevance of HLTF and HPP1 was at least equal in the collective studied. Likewise, in multivariate analysis HLTF, HPP1 and CEA remained independent prognostic factors in stage IV, the advantage of HLTF and HPP1 being that these markers can continue to be used as binary parameters, whereas a suitable cutoff value for CEA within the population needs to be defined first.

The availability of tissue samples corresponding to the examined blood samples allowed for the first study of the correlation between methylation of HLTF, HPP1 and NEUROG1 in serum and primary tumor. All positive serum samples, with one exception, also showed methylation of the respective genes in the tissue samples. Hence this study was able to

confirm the hypothesized provenance of circulating cell-free methylated DNA from the tumor. An interrelation with methylation phenotype CIMP was not evident in the study population.

In a subsequent study the correlation of the three target parameters with LDH in blood as a surrogate marker for cell disintegration was examined. The high correlation of HLTF and HPP1 with elevated LDH levels suggests the decomposition of tumor cells as a possible mechanism by which tumor DNA is released into the bloodstream. On the other hand, no correlation between LDH and methylation of NEUROG1 existed. Therefore mechanisms other than tumor associated cell death have to play a role in the release of methylated tumor DNA, which are unexplained yet.

In summary, methylated circulating cell-free HLTF and HPP1 DNA was analyzed and characterized as an independent prognostic marker for metastasized CRC. The promising results provide valuable groundwork for further examination of these markers in subsequent studies in order to evaluate potential clinical use for example in pretherapeutic risk stratification, therapy monitoring or prediction of response to specific tumor therapies.

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Originalarbeiten

Publikation 1: Prognostic role of methylated free circulating DNA in colorectal cancer.

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Prognostic role of methylated free circulating DNA in colorectal cancer

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DNA hypermethylation is frequently found in colorectal cancer (CRC). Methylation of helicase-like transcription factor (HLTF) and hyperplastic polyposis 1 (HPP1) are potential and carcinoembryonic antigen (CEA) is an established prognostic factor in serum of patients with CRC. The aim of this study was to perform a direct comparison of the prognostic roles of these markers. Methylation status of HLTF and HPP1 was examined in pretherapeutic sera of 311 patients with CRC and matched primary tissues of 54 stage IV patients using methylation-specific quantitative PCR. CEA was determined using an immunoenzymometric assay. Methylation of HLTF and HPP1 DNA in serum significantly correlated with tumor size, stage, grade and metastatic disease. HPP1 methylation correlated with nodal status. Overall survival was shortened in case of methylation of HLTF or HPP1 or elevated levels of CEA ($p < 0.0001$ for all). In stage IV, patients survival was impaired if HLTF ($p = 0.0005$) or HPP1 ($p = 0.0003$) were methylated or CEA was above the median of 27 ng/ml ($p = 0.002$). Multivariate analysis revealed that methylation of HLTF [hazard ratio (HR) 1.8, $p = 0.0438$], HPP1 (HR 1.6, $p = 0.0495$) and CEA >27 ng/ml (HR 1.7, $p = 0.0317$) were independent prognostic factors in stage IV. The combination of any two or all three of these factors outperformed each marker on its own. In conclusion, the presence of methylated DNA of the genes HLTF or HPP1 in serum are independent prognostic factors in metastasized CRC. Prospective validation is required to determine their usefulness in clinical routine along with the established marker CEA.

Introduction

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 633,000 deaths in 2008.[1] Five-year survival rates vary from approximately 93% for patients with UICC (Union for International Cancer Control) stage I disease to 8% for patients with stage IV CRC.[2] Although important advances in treating metastatic CRC have been made in the last decade and survival rates are rising,[3] there is still need for prognostic and predictive markers to optimize therapeutic decisions.

Aberrant hypermethylation of CpG islands is a common epigenetic DNA modification in human cancers leading to transcriptional silencing and can already be detected in early stages of carcinogenesis.[4] Genes found hypermethylated in CRC have functions in mismatch repair, cell-cycle regulation and cell differentiation.[5] A subset of colorectal tumors which exhibits an exceptionally high frequency of hypermethylated genes is referred to as CpG island methylator phenotype (CIMP),[6] of which two types with different grades of hypermethylation have been described.[7–9] Both, CIMP1 (or CIMP-high) and CIMP2 (or CIMP-low) cancers have been reported to have distinct clinicopathologic, morphological and molecular features and can be used for the classification of CRCs into five distinct subtypes.[10] Different marker panels for the detection of CIMP have been proposed,[6,11] but so far no consensus regarding the optimal panel has been found.

Methylated tumor DNA cannot only be found in primary CRC tissue, but can also be detected in remote media like serum or stool.[12,13] For example, several serum methylation markers have been described as potential screening markers for early stages of CRCs in asymptomatic patients.[14–16] Other markers have been linked to clinicopathologic features, and

Key words: colorectal cancer, prognosis, HLTF, HPP1, CEA
Abbreviations: AIC: Akaike Information Criterion; CEA: Carcinoembryonic antigen; CI: Confidence interval; CIMP: CpG island methylator phenotype; CRC: Colorectal cancer; fcDNA: free circulating DNA; HLTF: Helicase-like transcription factor; HPP1: Hyperplastic polyposis 1; HR: Hazard ratio; PMR: percentage of fully methylated reference; UICC: Union for International Cancer Control
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prognostic significance has been described when found methylated in serum. We previously reported helicase-like transcription factor (HLTF) and hyperplastic polyposis 1/transmembrane protein containing epidermal growth factor and follistatin domains (HPP1/TPEF) to be found only in serum samples of patients with CRC, not in healthy controls, and to be significantly correlated with tumor size, metastatic disease and tumor stage.[17] CRC patients with serum methylation of *HLTF* and/or *HPP1* had an unfavorable prognosis in this study.[17] Furthermore, *HLTF* hypermethylation in serum is an independent predictor of disease recurrence.[18]

HLTF is a transcription factor and a member of the SWI/SNF family of chromatin-remodeling factors. Few data are available on the normal function of *HLTF*; however, it seems to be linked to the genesis and progression of cancer.[19] *HLTF* is commonly hypermethylated in all stages of CRC as well as in adenomas.[20,21] Methylation of *HLTF* has also been detected in stool samples of patients with colorectal carcinomas.[22,23] *HPP1* encodes a TPEF whose function is largely unknown. *HPP1* methylation has been described to occur relatively early in colorectal carcinogenesis [24,25] as well as in hyperplastic polyps.[25]

Carcinoembryonic antigen (CEA) is the only serum marker that has been recommended to be added to the established tumor-node-metastasis (TNM) staging system.[26] Still, CEA is used for postoperative surveillance and therapy monitoring rather than pretherapeutic determination of prognosis or treatment planning.[27,28] This study aimed at validating the significance of methylation of *HLTF* and *HPP1* DNA in serum regarding correlation with clinicopathologic features and patients' prognosis in a large cohort. In addition, we sought to correlate methylation of these genes with CEA serum levels and to compare their prognostic significance. A subset of cases was further validated for the presence of methylation in the primary tumors.

Materials and Methods

Patients and serum samples

Serum samples from 311 patients with CRC drawn before initiation of therapy were selected by availability of clinicopathologic and long term follow-up data. Characteristics of the cohort are shown in Table 1. All analyses of the serum samples were performed blinded to patient data. Blood samples were obtained pre-therapeutically and were transported by a shock absorbed tube mailing system within 15–30 min after blood drawing to the central laboratory. All specimens were centrifuged at 2,000*g* at 4°C for 10 min. The supernatant was transferred into polypropylene cryotubes and stored frozen at −80°C. The study was approved by the ethical committee of the Medical Faculty of the University of Munich.

DNA isolation and bisulfite conversion of serum samples

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. As a carrier to the eluted DNA, 2 μg of salmon sperm DNA (Agilent Technologies, Waldbronn, Germany) was added. DNA was denatured by 0.2 mol/l NaOH for 15 min at 37°C. A total of 30 μl of 10 mmol/l hydroquinone (Sigma-Aldrich, Munich, Germany) and 520 μl of 3 mol/l sodium bisulfite (Sigma-Aldrich, Munich, Germany) at pH 5.1 were added, and the samples were incubated for 16 hr at 55°C. After bisulfite treatment, DNA was purified using the Wizard DNA Clean-up System (Promega, Mannheim, Germany) following the manufacturer's protocol, and incubated for 5 min at room temperature with 0.3 mol/l NaOH. Then, DNA was ethanol-precipitated and resuspended in 30 μl of Tris-HCl [1 mmol/l (pH 8.0)].

DNA isolation and bisulfite conversion of tissue samples

Of the 103 patients with UICC stage IV disease, formalin fixed and paraffin-embedded (FFPE) samples were available in 54 cases. Serial sections were performed of these tissue samples. One slide was stained with hematoxylin and eosin (H&E), which was then inspected by a pathologist (JN) who marked the tumor region. Tumor tissue was scraped from a deparaffinized, adjacent slide using the H&E stained slide as blueprint. DNA was purified using the QIAamp DNA FFPE Tissue Kit (Qiagen). Proteinase K incubation at 56°C was done overnight. The following incubation step at 90°C was omitted. Subsequent steps were performed following the manufacturer's protocol. 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 MethyLight.[17,29,30] Dispersed *Alu* repeats were used to control for DNA amplification and to normalize for input DNA. Primer and probe sequences are listed in Supporting Information Table S1. For the analysis of serum samples, PCRs were done in 20 μl volumes containing 1× PCR buffer (Qiagen, Hilden, Germany), 4 mmol/l $MgCl_2$, 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 Supporting Information 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. For the analysis of tissue samples, only 1 μl of bisulfite-treated DNA was used for each reaction and the annealing step was shortened to 60 s. For *CACNA1G*, *IGF2*, *RUNX3* and *SOCS1*, Q-Solution (Qiagen) was added to the reaction mixture according to

Table 1. Clinical features of the patient population and frequency of methylated genes and high CEA levels according to clinicopathologic data

			HLTF			HPP1			CEA > 2.5 ng/ml		
Clinical feature	**No. patients**	**Percentage**	**No. positive**	**% Positive**	p^1	**No. positive**	**% Positive**	p^1	**No. positive**	**% Positive**	p^1
Total number of patients	311	100	38	12	–	64	21	–	177	57	–
Age[2]											
≤63 years	134	43	21	16		30	22		76	57	
>63 years	177	57	27	15	0.920	34	19	0.492	101	57	0.951
Sex											
Male	171	55	26	15		39	23		97	57	
Female	140	45	22	16	0.902	25	18	0.283	80	57	0.941
Localization											
Colon	147	47	28	19		37	25		86	59	
Sigmoid	58	19	10	17		10	17		36	62	
Rectum	106	34	10	10	0.103	17	16	0.163	55	52	0.392
Tumor Size[3]											
T1	18	6	2	11		1	6		1	6	
T2	64	21	4	6		4	6		24	38	
T3	184	59	30	16		37	20		116	63	
T4	44	14	12	27	0.027	21	48	<0.0001	35	80	<0.0001
Nodal status[4]											
N0	164	53	19	12		16	10		77	47	
N1	80	26	16	20		26	33		47	59	
N2	60	19	11	18	0.169	18	33	<0.0001	47	78	0.0001
Metastatic disease											
M0	208	67	23	11		11	5		91	44	
M1	103	33	25	24	0.002	53	51	<0.0001	86	84	<0.0001
UICC stage											
I	64	21	5	8		2	3		15	23	
II	78	25	12	15		4	5		41	53	
III	66	21	6	9		5	8		35	53	
IV	103	33	25	24	0.012	53	51	<0.0001	86	83	<0.0001
Tumor grade[5]											
G1 and G2	162	52	18	11		19	12		81	50	
G3 and G4	136	44	27	20	0.036	39	29	0.0002	87	64	0.015
CEA											
CEA ≤ 2.5 ng/ml	134	43	17	13		12	9				
CEA > 2.5 ng/ml	177	57	31	18	0.243	52	29	<0.0001			
CEA ≤ 27 ng/ml	246	79	31	13		28	11				
CEA >27 ng/ml	65	21	17	26	0.007	36	55	<0.0001			

HLTF: Helicase-like transcription factor. HPP1: Hyperplastic polyposis 1. CEA: Carcinoembryonic antigen.
[1]*p*-Values were calculated by means of the χ^2-test. [2]Mean age: 64.5 years ± 10.9 years. [3]Tumor size was unknown in one case. [4]Nodal status was unknown in seven cases. [5]Tumor grade was unknown in 13 cases.

the manufacturer's instructions. For *CACNA1G* and *RUNX3* the annealing temperature was 64°C. 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 [29] by dividing the gene/Alu ratio of a sample by

the gene/Alu ratio of fully methylated, bisulfite-treated DNA (CpGenome^{TM} 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 CEA

CEA was quantified using a microparticle immunoenzymometric assay (AxSYM, Abbott Laboratories, Chicago, IL).

Statistical analysis

All statistical analysis was done using SAS 9.2 (SAS Institute, Cary, NC). Pearson's χ^2-test was used to explore associations between clinicopathologic features. Overall survival was calculated from the date of diagnosis of the primary tumor to the date of death or end of follow-up. Overall survival curves were calculated with the Kaplan–Meier method. Univariate analysis of overall survival according to clinicopathologic data and gene methylation status was performed using the Kaplan–Meier method and log-rank tests. Hazard ratios (HRs) were estimated using Cox's regression model. We used the Akaike information criterion (AIC) [31] to compare the viability of different models.

Results

Correlation of HLTF and HPP1 methylation status in serum samples with clinicopathologic data

The methylation status of *HLTF* and *HPP1* was analyzed in the sera of 311 patients with CRC. An overview of the clinicopathologic characteristics can be found in Table 1. Methylation of *HLTF* was found in 48 samples (15.4%), methylation of HPP1 in 64 samples (20.6%). All samples with a PMR > 0 were considered as methylation positive. The mean PMR values of the positive cases were 19.2 for *HLTF* and 14.75 for *HPP1*. Serum methylation of *HPP1* was significantly correlated with serum methylation of *HLTF* ($p < 0.0001$). The methylation status of *HLTF* and *HPP1* was analyzed for association with clinicopathologic data (Table 1). No correlation with methylation status of these genes and age or sex was found. Methylation of *HLTF* was detected significantly more often in patients with colon cancer than in patients with rectal cancer ($p = 0.0352$), whereas no correlation between localization and methylation status of *HPP1* was found. Methylation of *HLTF* and *HPP1* significantly correlated with tumor size ($p = 0.0267$ and $p < 0.0001$, respectively) and presence of distant metastases ($p = 0.0024$ and $p < 0.0001$, respectively). Also, methylation of both genes correlated with high tumor grade ($p = 0.0358$ and $p = 0.0002$, respectively). Only methylation of *HPP1* was detected more frequently in nodal positive patients ($p < 0.0001$). In accordance with these findings, methylation of *HLTF* and *HPP1* was found significantly more often in patients with advanced UICC stages ($p = 0.0115$ and $p < 0.0001$, respectively). High CEA levels above a cutoff value of 2.5 ng/ml significantly correlated with depth of tumor infiltration ($p < 0.0001$), positive nodal status ($p = 0.0001$), metastasized disease ($p < 0.0001$), advanced tumor stage ($p < 0.0001$) and high tumor grade ($p = 0.015$).

Analysis of prognostic significance of DNA methylation in serum

The association of clinicopathologic data and serum methylation status of the genes *HLTF* and *HPP1* with overall patient survival was analyzed in all 311 patients. Statistical analysis revealed prognostic significance of tumor size, the presence of nodal or distant metastases, tumor grade and higher UICC stages ($p < 0.0001$ for all parameters; Table 2 and Fig. 1*a*). Similarly, presence of methylation of *HLTF* or *HPP1* was significantly correlated with poorer prognosis ($p < 0.0001$ for both; Figs. 1*b* and *c* and Table 2). Additionally, the prognostic significance of elevated levels of CEA in serum was tested using two different cutoff values. The lower cutoff value (2.5 ng/ml) marks the 95th percentile of healthy individuals based on the assay used in our study, whereas the higher cutoff value (27 ng/ml) is based on the median of UICC stage IV cases in our cohort. In both cases, values above the respective threshold were associated with shorter overall survival ($p < 0.0001$ for both; Fig. 1*d* and Table 2) when analyzing all tumor stages together.

In patients with UICC stage I disease, *HLTF* methylation in serum correlated with shorter survival ($p = 0.0007$), whereas no significant difference was found for *HPP1* in UICC stage I ($p = 0.2629$). No prognostic relevance of the serum methylation status of *HLTF* and *HPP1* was found in UICC stages II and III (*HLTF*: $p = 0.7415$ and $p = 0.6742$, and *HPP1*: $p = 0.8687$ and $p = 0.9258$, respectively). Similarly, in the combined stages I–III *HLTF* and *HPP1* did not provide prognostic information. In stages I–III CEA was prognostically significant when the cutoff value was set above 2.5 ng/ml ($p = 0.001$; Table 2). However, this was not the case when analyzing each stage by itself ($p = 0.114$, $p = 0.629$ and $p = 0.107$, respectively) or when the median of stage IV patients was used as a cutoff value.

A notable difference in median overall survival was found in the UICC IV subset for *HLTF* as well as for *HPP1* methylation. Patients in this subgroup showed a median survival of 10.0 months (95% CI 5.9–12.9) if serum methylation of *HLTF* was found, compared to 19.7 months (95% CI 14.8–26.8) if no methylation of *HLTF* could be detected ($p = 0.0005$; Fig. 2*a* and Table 2). If *HPP1* methylation in serum was detected, the median overall survival in the UICC stage IV subgroup was 10.5 months (95% CI 7.5–14.8) compared to 23.2 months (95% CI 15.5–30.3) if the *HPP1* methylation status was negative ($p = 0.0003$; Fig. 2*b* and Table 2). High CEA levels turned out to be a prognostic factor in the UICC stage IV patient subset only when using the median of 27 ng/ml (P = 0.002; Fig. 2*d* and Table 2). Therefore, CEA cutoff concentrations between >0 and 100 ng/ml were tested. Cutoff values from 14 to 21 ng/ml revealed statistically highly significant prognostic information (all with $p < 0.001$). No

Table 2. Univariate analysis of overall survival

Variable	UICC stage IV			UICC stages I–III			All stages		
	No. patients who died/ total no.	Median survival in months (95% CI)	p^1	No. patients who died/ total no.	Median survival in months (95% CI)	p^1	No. patients who died/ total no.	Median survival in months (95% CI)	p^1
Age									
≤63 years	40/51	14.8 (10.0–25.7)		33/83	n.d. (104.5–n.d.)		73/134	77.0 (45.7–n.d.)	
>63 years	44/52	16.8 (12.8–20.7)	0.948	73/125	116.7 (83.1–137.9)	0.015	117/177	68.9 (49.3–96.0)	0.214
Sex									
Male	47/57	19.3 (12.8–22.2)		59/114	115.0 (83.1–n.d.)		106/171	62.3 (46.3–95.8)	
Female	37/46	14.6 (10.0–22.5)	0.679	47/94	140.8 (102.4–n.d.)	0.691	84/140	75.4 (52.8–120.4)	0.721
Localization									
Colon	45/55	13.5 (9.3–16.4)		44/92	141.1 (83.1–n.d.)		89/147	56.4 (39.4–114.7)	
Sigmoid	15/17	11.5 (5.1–19.7)		20/41	137.9 (102.4–n.d.)		35/58	102.4 (52.1–137.9)	
Rectum	24/31	25.7 (19.4–35.6)	0.045	42/75	106.5 (76.9–181.5)	0.633	66/106	76.9 (45.7–95.9)	0.752
Tumor Size									
T1–T3	55/71	19.4 (13.6–22.4)		95/195	137.3 (106.5–n.d.)		147/248	76.9 (59.4–108.2)	
T4	28/31	12.0 (6.0–19.8)	0.070	11/13	68.9 (20.3–137.9)	0.005	39/44	19.3 (10.4–28.8)	<0.0001
Nodal Status									
N0	14/22	30.2 (7.4–n.d.)		63/142	145.4 (120.4–n.d.)		77/164	137.3 (108.2–n.d.)	
N1 and N2	64/74	14.8 (12.2–19.7)	0.038	43/66	75.3 (51.6–114.8)	0.004	107/140	29.5 (23.4–43.2)	<0.0001
Grade									
Low (G1 and G2)	23/31	22.4 (13.4–40.4)		63/131	141.1 (90.2–n.d.)		86/162	95.8 (70.4–181.4)	
High (G3 and G4)	54/63	14.6 (10.6–17.4)	0.046	40/73	131.5 (104.5–151.9)	0.597	94/136	39.4 (27.4–83.0)	<0.0001
CEA									
CEA ≤ 27 ng/ml	36/50	26.8 (14.2–39.1)		97/196	137.5 (104.7–n.d.)		133/246	104.7 (77.0–137.5)	
CEA > 27 ng/ml	48/53	12.9 (10.0–18.1)	0.002	9/12	88.7 (18.8–181.7)	0.071	57/65	16.4 (12.3–19.9)	< 0.0001
CEA ≤ 2.5 ng/ml	14/17	23.2 (7.1–45.8)		47/117	n.d. (134.9–n.d.)		61/134	151.9 (106.5–n.d.)	
CEA > 2.5 ng/ml	70/86	15.0 (12.3–20.1)	0.411	59/91	86.9 (70.4–116.7)	0.001	129/177	42.3 (27.9–55.9)	< 0.0001
HLTF									
Unmethylated	62/78	19.7 (14.8–26.8)		90/185	130.0 (108.4–n.d.)		152/263	80.2 (62.5–114.9)	
Methylated	22/25	10.0 (5.9–12.9)	0.0005	16/23	90.3 (52.1–120.6)	0.139	38/48	36.3 (12.3–57.7)	0.0001
HPP1									
Unmethylated	36/50	23.2 (15.5–30.3)		99/197	135.1 (104.7–n.d.)		135/247	104.7 (77.0–137.5)	
Methylated	48/53	10.5 (7.5–14.8)	0.0003	7/11	75.6 (51.6–181.7)	0.466	55/64	12.6 (10.0–20.7)	< 0.0001

Table 2. Univariate analysis of overall survival (Continued)

Variable	No. patients who died/ total no.	UICC stage IV Median survival in months (95% CI)	p^1	No. patients who died/ total no.	UICC stages I–III Median survival in months (95% CI)	p^1	All stages No. patients who died/ total no.	All stages Median survival in months (95% CI)	p^1
HLTF ± HPP1[2,3]									
HLTF– and HPP1–	34/47	26.8 (15.5–39.1)		86/177	138.0 (108.4–n.d.)		120/224	108.4 (75.4–138.0)	
HLTF+ and/or HPP1+	50/56	11.0 (8.0–14.8)	0.0002	20/31	90.3 (62.3–162.5)	0.210	70/87	23.8 (12.9–37.1)	<0.0001
HLTF ± CEA[2,4]									
HLTF– and CEA–	29/41	28.0 (15.0–42.7)		81/174	141.3 (114.9–n.d.)		110/215	116.8 (80.3–145.6)	
HLTF+ and/or CEA+	55/62	12.6 (10.0–16.4)	0.0003	25/34	90.3 (51.6–119.4)	0.009	80/96	20.7 (14.8–29.5)	<0.0001
HPP1 ± CEA[3,4]									
HPP1– and CEA–	22/32	28.7 (20.1–45.8)		91/186	138.0 (104.7–n.d.)		113/218	116.8 (90.3–145.6)	
HPP1+ and/or CEA+	62/71	12.4 (10.0–163.9)	0.0008	15/22	75.6 (34.1–181.7)	0.059	77/93	18.1 (12.4–22.5)	<0.0001

CEA: Carcinoembryonic antigen. HLTF: Helicase-like transcription factor. HPP1: Hyperplastic polyposis 1. n.d.: upper limit of 95% CI could not be calculated due to insufficient number of events in this group.
[1]*p*-Values were calculated by the log-rank test. [2]HLTF+ indicates methylation of HLTF, HLTF– indicates no methylation. [3]HPP1+ indicates methylation of HPP1, HPP1– indicates no methylation.
[4]CEA+ indicates CEA values higher than the median of all UICC IV patients (27 ng/ml), CEA– indicates CEA values lower than the median.

significant difference was seen when using the low threshold value of 2.5 ng/ml ($p = 0.4109$; Fig. 2*c* and Table 2). Furthermore, the combination of *HLTF* and/or *HPP1* methylation as well as the combination of *HLTF* methylation and high CEA values (> 27 ng/ml) and the combination of *HPP1* methylation and high CEA values showed prognostic significance in the UICC stage IV group ($p = 0.0002$, $p = 0.0003$ and $p = 0.0008$, respectively).

In correlation analysis, we found serum methylation of *HPP1* in the UICC stage IV subgroup to be significantly correlated with serum methylation of *HLTF* ($p < 0.0001$) and high CEA values (>27 ng/ml; $p = 0.0023$). Likewise, the presence of methylation of *HLTF* and/or *HPP1* correlated with high CEA values ($p = 0.0012$), whereas methylation of *HLTF* alone showed no correlation with high CEA values ($p = 0.1493$). Therefore, we performed a multivariate analysis to test if there are any dependencies between these three parameters in UICC stage IV. In this analysis, the methylation of *HLTF* [HR 1.8 (95% CI 1.0–3.0); $p = 0.0438$] and the methylation of *HPP1* [HR 1.6 (95% CI 1.0–2.7); $p = 0.0495$] as well as high CEA values [HR 1.7 (95% CI 1.1–2.7); $p = 0.0317$] appeared as independent prognostic factors. Using the AIC, we evaluated the performance of this model compared to models either combining any two of the three parameters or testing parameters alone (Supporting Information Table S2). The combination of any two of the three markers yielded a better AIC than any marker alone. The best AIC of 648 was reached when all three markers were combined. AIC-ranking revealed HPP1 (AIC = 652) as the best single marker compared to either HLTF or CEA (both AIC = 655).

Comparison of methylation status in tissue and serum

To find out whether the *HLTF* and *HPP1* methylation status of the serum matched the *HLTF* and *HPP1* methylation status of the primary colorectal carcinoma, tissue samples of 54 primary CRCs of patients from the UICC stage IV subgroup were analyzed. The PMR values were dichotomized at a threshold value of 4%, which has been described to give the best discrimination between normal and malignant tissues.[32,33] DNA methylation of *HLTF* was found in 24 cases (44%) of which ten also showed serum methylation of *HLTF* (42%). *HPP1* methylation was observed in 50 tumors (93%) which could also be detected in 28 sera (56%). In one case, *HLTF* methylation could only be found in serum, but not in tissue. In none of the cases, *HPP1* methylation in serum without methylation in the matched tumor tissue was found (Fig. 3). Serum methylation of *HLTF* ($p = 0.001$) and *HPP1* ($p = 0.031$) significantly correlated with tissue methylation of the respective genes. There was no significant correlation between tissue methylation of *HLTF* or *HPP1* and tumor size, nodal status, tumor grade, localization, age or CEA values above 27 ng/ml (Table 3). Tissue methylation of *HLTF* but not of *HPP1* was found significantly more often in men ($p = 0.024$). Although all *HLTF* positive tissues also showed

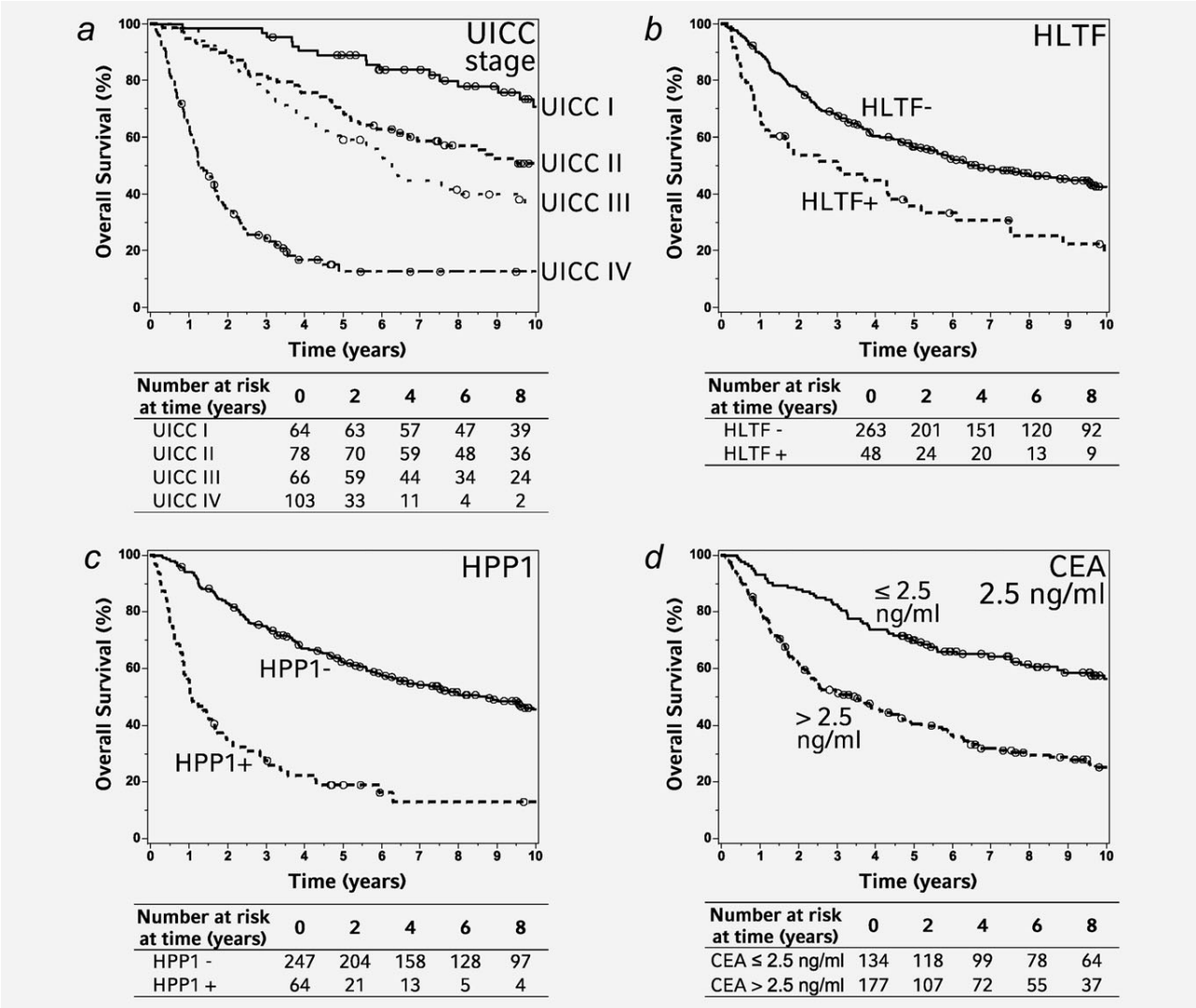

Figure 1. Kaplan–Maier plots of overall survival according to (*a*) UICC stage ($p < 0.0001$) as well as methylation status of (*b*) *HLTF* ($p = 0.0001$) and (*c*) *HPP1* ($p < 0.0001$) and (*d*) CEA values above 2.5 ng/ml ($p < 0.0001$). All *p*-values were calculated using the log-rank test.

HPP1 methylation, the correlation between *HLTF* and *HPP1* was not significant.

In addition, we determined the CIMP status of this 54 tumor samples using the marker panel proposed by Weisenberger *et al.*[11] to examine the relationship between *HLTF* and *HPP1* methylation and CIMP. Tumors were deemed CIMP positive if at least three of the five markers CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1 were hypermethylated. In our cohort, five of 54 stage IV tumors (9%) showed CIMP. However, neither serum methylation nor tissue methylation of *HLTF* or *HPP1* showed a significant correlation with CIMP.

There was no significant difference in overall survival between the patients with tissue methylation of *HLTF* (median survival 17.1 months) and those without (median survival 16.1 months; $p = 0.9284$). Because of the small number of *HPP1* methylation negative cases the difference in overall survival for *HPP1* (median survival 27.7 for negative vs. 14.9 month) was not significant ($p = 0.3291$). Furthermore, the prognostic influence of the combination of serum and tissue methylation was analyzed. Patients with *HLTF* methylation in tissue and serum (median survival 11.6 months) showed shorter survival than patients with methylation only in tissue (median survival 24.3 months) or no methylation at all (median survival 17.4 months; $p = 0.019$). The combination of tissue and serum methylation of *HPP1* was not analyzed due to the small number of tissue-negative cases. To test whether availability of tissue samples could introduce potential bias, we also analyzed overall survival according to only the serum parameters. Methylation of *HLTF* ($p = 0.006$) and *HPP1* ($p = 0.0118$) significantly correlated with worse prognosis similarly as in the full UICC stage IV population; however,

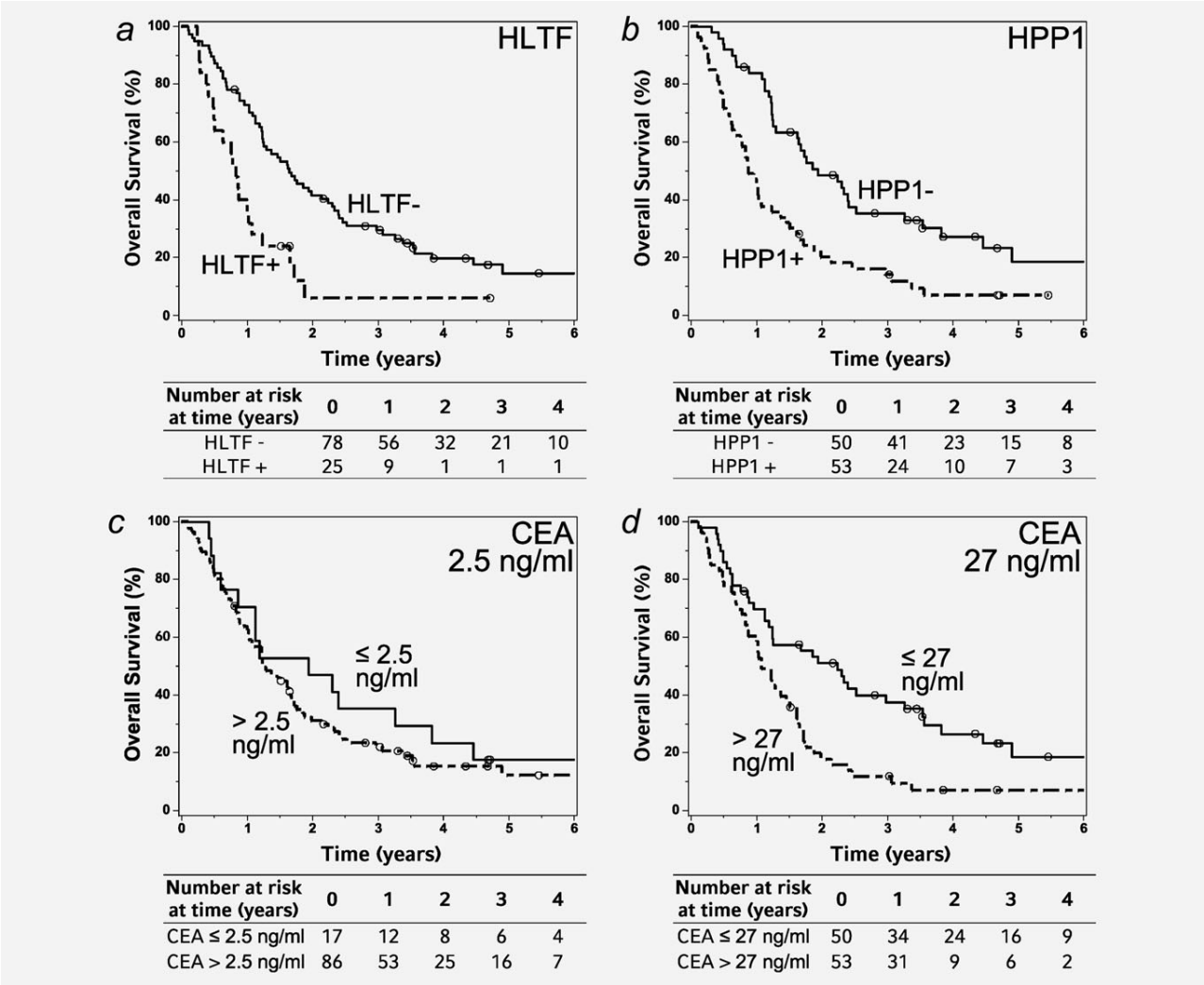

Figure 2. Kaplan–Maier plots of overall survival in UICC stage IV according to methylation status of (*a*) HLTF ($p = 0.0005$) and (*b*) HPP1 ($p = 0.0003$) as well as according to CEA values above (*c*) 2.5 ng/ml ($p = 0.4109$) and (*d*) 27 ng/ml ($p = 0.0020$). All *p*-values were calculated using the log-rank test.

high CEA values above 27 ng/ml did not reach the required level of significance in this subset of cases.

Discussion

DNA hypermethylation in serum of patients has been reported to have prognostic and predictive value in many different types of cancer.[34] As we previously reported, methylation of *HLTF* and *HPP1* is found most frequently in serum of patients with advanced CRC.[17,18] Moreover, we demonstrated *HLTF* and *HPP1* to be independent prognostic factors for survival [17] and *HLTF* to be an independent prognostic factor for disease recurrence.[18] We conducted this study to validate these findings in a larger patient population. As expected, we were able to confirm that serum methylation of *HLTF* and/or *HPP1* DNA is a marker for a worse prognosis when examining all tumor stages. Subgroup analysis revealed their prognostic role being mainly limited to UICC stage IV. The median overall survival in stage IV was substantially shorter in case of HLTF or HPP1 methylation in serum. To our knowledge, this is the first study to systematically evaluate prognostic significance of *HLTF* and *HPP1* methylation status in serum of patients with metastasized CRC and to correlate as well as compare the methylation status with serum CEA levels and tissue methylation status of *HLTF* and *HPP1*.

The phenomenon of free circulating DNA (fcDNA) in plasma or serum samples of cancer patients has been an established fact for several decades [35] and its tumorous origin has been substantiated through detection of cancer specific alterations in the fcDNA.[36,37] Still, the underlying mechanisms are not yet fully understood whereas involvement of active DNA release as well as apoptotic and necrotic processes have been reported.[38] To ascertain that the DNA methylation detected in serum derives from CRC cells, we

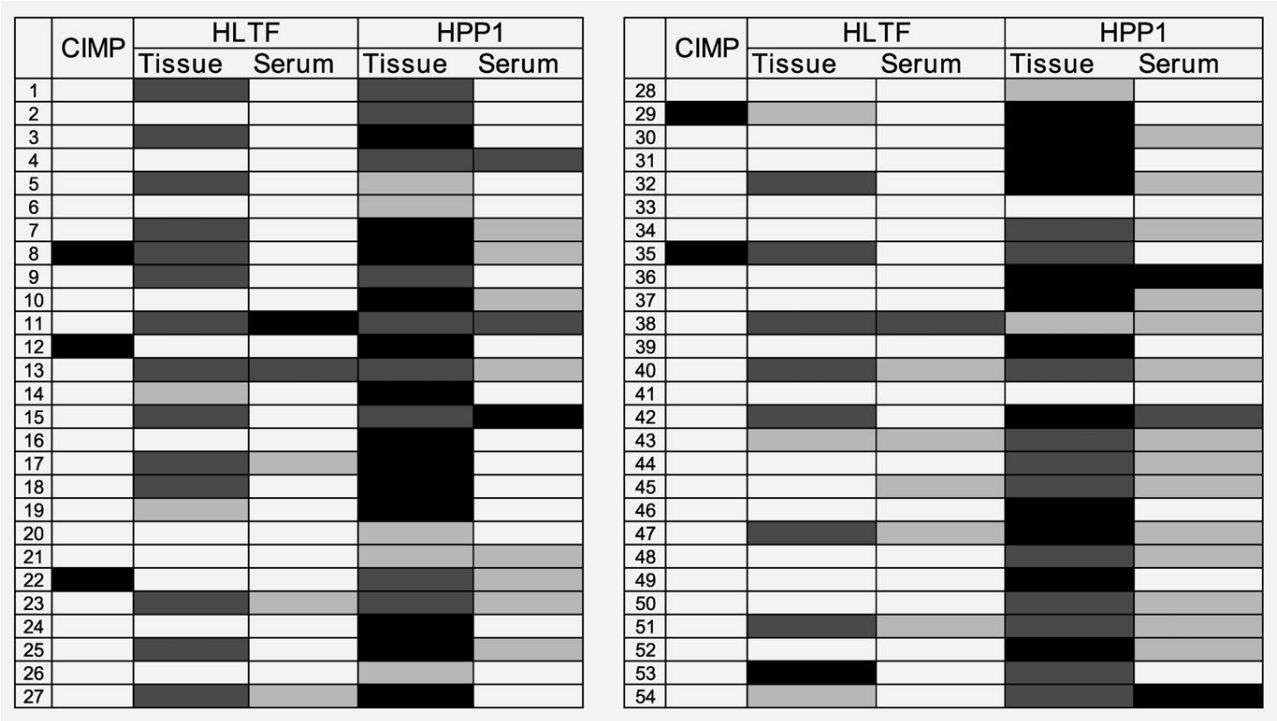

Figure 3. Methylation status of *HLTF* and *HPP1* and CIMP status in 54 matched tissue and serum samples of UICC stage IV patients. The color of the box indicates the PMR according to the following code: white PMR = 0, light gray PMR > 0 and < 10, dark gray PMR ≥ 10 and < 50, black ≥ 50.

determined the methylation status of *HLTF* and *HPP1* in 54 matched primary tissue samples. Tissue DNA methylation was found in 44% of the samples for *HLTF* and 93% for *HPP1* which is similar to published results from other groups.[20,21,24,25] With one exception, all cases with positive serum methylation status of *HLTF* or *HPP1* also showed corresponding tissue methylation status. The reasons for this mismatch showing only a low frequency of methylated *HLTF* DNA in the serum samples can only be speculated about. It might be caused by a heterogeneous distribution of methylation within the tumor itself – like it has been described for allelic loss by Lindforss *et al.*[39]– provoking false results when analyzing a fraction of the tumor only. In 14 and 22 cases, respectively, methylated *HLTF* or *HPP1* DNA was only detectable in cancer tissue, not in serum, which might be explained by low DNA turnover or little vascularization limiting the amount of tumor DNA in the blood. Independently of serum methylation status, we could not identify any relevant correlation between tissue methylation of *HLTF* or *HPP1* and clinicopathologic features or prognosis.

As no information about the relation between *HLTF* or *HPP1* and the CpG island methylator phenotype in CRC has been published, we additionally determined the CIMP status in these tissue samples using a marker panel first established by Weisenberger *et al.*[11] Although a correlation between *HLTF* methylation and CIMP has been described in gastric cancer,[40] in our cohort of stage IV tumors *HLTF* or *HPP1* methylation neither in tissue nor in serum was correlated with CIMP.

The current gold standard for determining prognosis in patients with CRC is the extent of disease at time of diagnosis as defined by the TNM staging systems.[27] Recently, many different molecular markers in tissue, blood and other media have been reported, *e.g.*, loss of heterozygosity on chromosome 18q [41] or microsatellite instability[42] but no recommendations for routine use of these parameters exist yet.[27,28] Thus far, while many protein serum markers have been studied, the only serum marker of prognostic significance suggested to be added to the established staging systems is CEA.[26] We earlier reported methylation of *HPP1* but not methylation of *HLTF* to be correlated with high CEA values.[17] This data was reproduced in our current study. Furthermore, we analyzed the prognostic value of CEA in patients with metastatic CRC. In this tumor stage, various cutoff values for CEA have been described in the past [26] but – at least partly due to different measurement methods – so far no consensus has been reached nor has any cutoff value been defined. First, we tested all concentrations between 0 and 100 ng/ml for their prognostic significance when used as cutoff value. Additionally, we used a cutoff value of 2.5 ng/ml, which marks the 95th percentile in a healthy population for the test method applied and is commonly used in clinical routine. However, no significant difference in overall survival was found with this cutoff value for patients with

Table 3. Frequency of methylated genes in primary tissue according to clinicopathologic data

		HLTF			HPP1		
	No. patients	No. positive	% Positive	p^1	No. positive	% Positive	p^1
Age							
≤63 years	20	7	35		18	90	
>63 years	34	17	50	0.284	32	94	0.577
Sex							
Male	29	17	59		28	97	
Female	25	7	28	0.024	22	88	0.232
Localization							
Colon	17	8	47		16	94	
Sigmoid	13	9	69		13	100	
Rectum	24	7	29	0.062	21	88	0.367
Tumor size							
T2	2	1	50		2	100	
T3	34	12	35		30	88	
T4	17	11	65	0.137	17	100	0.299
Nodal status							
N0	10	4	40		9	90	
N1	18	7	39		16	89	
N2	23	12	52	0.654	22	96	0.698
Tumor grade							
G1 and G2	16	5	31		15	94	
G3 and G4	38	19	50	0.206	35	92	0.833
Serum methylation							
HLTF−	43	14	33		39	91	
HLTF+	11	10	91	0.001	11	100	0.293
HPP1−	26	9	35		22	85	
HPP1+	28	15	54	0.161	28	100	0.031
CEA							
CEA ≤ 27 ng/ml	27	11	41		25	93	
CEA > 27 ng/ml	27	13	48	0.584	25	93	1.000

HLTF: Helicase-like transcription factor. HPP1: Hyperplastic polyposis 1. CEA: Carcinoembryonic antigen.
[1] p-Values were calculated by means of the χ^2-test.

metastasized disease. When using higher cutoff values, – in our case the median of the UICC stage IV subgroup (27 ng/ml) – the prognosis for the CEA positive cases was similarly unfavorable as when *HLTF* and/or *HPP1* methylation was detected.

Multivariate analysis revealed all three markers to be prognostic markers independent of each other with similar HRs. Additionally, the comparison of different models by its AIC value revealed that adding the methylation markers *HLTF* and *HPP1* to a model improves its goodness of fit. Hence, *HLTF* and *HPP1* methylation contribute independently to the determination of patients' prognosis and could supplement the current standard CEA as a prognostic serum marker in stage IV CRC. However, as it has been shown that the prognostic significance of CEA corresponds to the logarithmic function of its serum concentration,[43,44] defining a single cutoff value for all patients is at least difficult, if not even useless for personalized treatment decisions. In contrast, the mere detection of methylated *HLTF* or *HPP1* DNA in serum already indicated worse prognosis in our population of patients with distant metastases. Further studies in large-sized populations have to clarify, if patient groups with specific characteristics can be stratified using a combination of markers, *e.g.*, through a prognostic score.

Beyond the prognostic significance of *HLTF* methylation in the serum of patients with metastatic CRCs, patients with very early disease, UICC stage I, showed shorter survival when *HLTF* methylation could be detected in serum.

Potentially, *HLTF* methylation in serum might indicate more aggressive tumors or even the presence of micrometastasis. However, it is confusing that *HLTF* methylation does not also identify high risk groups in the UICC II and III subgroups. The reason for this remains obscure and needs further evaluation in larger cohorts of stage UICC I to III patients.

Determination of DNA methylation in serum might have advantages over tissue-based prognostic factors, as serum can easily be gained in a noninvasive way. For our measurements only, 200 μl of serum were necessary to detect even small amounts of methylated DNA. Moreover, patients with distant metastases frequently suffer from unresectable disease and gaining tumor biopsies is usually burdensome. As biopsies are usually only gained from the primary tumor or a metastatic lesion they only provide information about the biopsied tissue. Serum-based markers, in contrast, represent a "cross section" of all tumor sites.

In conclusion, the present study demonstrates that methylation of the genes *HLTF* and *HPP1* is frequently detected in serum of patients with metastatic CRC and that both of them are significant markers for shorter survival in this stage, showing at least equal significance to that of the current standard prognostic marker *CEA*. Moreover, analysis of primary tissue revealed that methylation of *HLTF* and *HPP1* indicates prognosis independently of the CIMP status of the primary tumor. Prospective studies have to further evaluate the role of serum methylation of *HLTF* and *HPP1* alone and in combination with *CEA* in the clinical management of patients.

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Publikation 2: Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer.

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RESEARCH ARTICLE Open Access

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

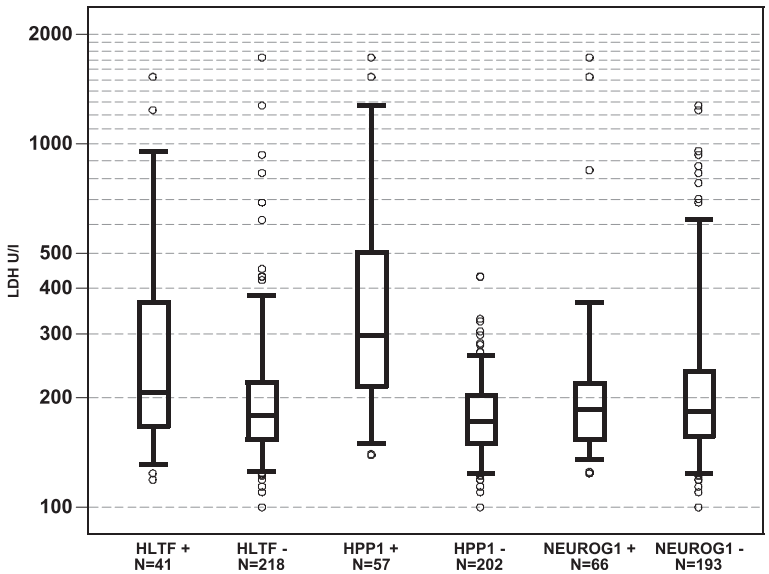

Figure 1 LDH values and methylation status of *HLTF*, *HPP1* and *NEUROG1* (as binary variables, cutoff PMR > 0).

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

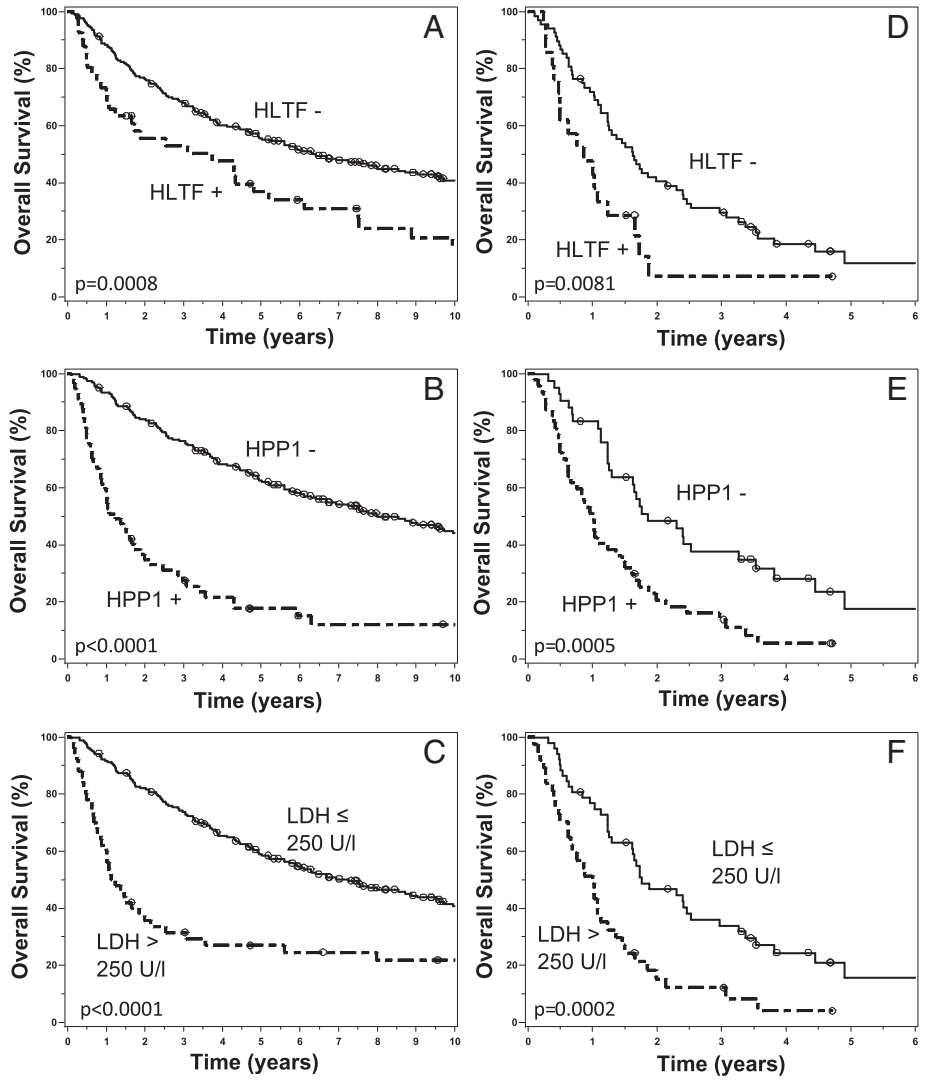

Figure 2 Kaplan-Meier plots of overall survival. A-C: Overall survival for all patients according to methylation status of *HLTF* **(A)**, *HPP1* **(B)** and high LDH levels > 250 U/l **(C)**, respectively. **D**-**F**: Overall survival for stage IV patients patients according to methylation status of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