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Abkürzungsverzeichnis

ALX4	Aristaless-like Homeobox-4
APC	Adenomatous Polyposis Coli
bp	Basenpaare
CEA	Carcinoembryonales Antigen
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
EGRF	Epidermal Growth Factor Receptor
fcDNA	Free Circulating DNA
HLTF	Helicase-like transcription factor
HPP1	Hyperplastic polyposis 1
iFOBT	immunologischer fäkaler Okkultbluttest
LINE	Long Interspersed Elements
MSI	Microsatellite Instability
OPG	Osteoprotegerin
PCR	Polymerase Chain Reaction
RANKL	Receptor Activator of NF-κB Ligand
SINE	Short Interspersed Element
TNF	Tumornekrosefaktor
TRAIL	TNF-related Apoptosis-inducing Ligand
UICC	Union for International Cancer Control

Publikationsliste

Bestandteil dieser kumulativen Dissertation sind folgende Veröffentlichungen:

Publikation I:

E.N. Toni, D. Nagel, A.B. Philipp, A. Herbst, I. Thalhammer, J. Mayerle, H.-P. Torok, L. Brandl and F.T. Kolligs, **Correlation Between Baseline Osteoprotegerin Serum Levels and Prognosis of Advanced-Stage Colorectal Cancer Patients**, *Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology* **45** (2018), 605–613.

Publikation II:

I. Anzinger, D. Nagel, E.N. Toni, A. Ofner, A.B. Philipp, L.M. Holdt, D. Teupser, F.T. Kolligs and A. Herbst, **Cell-free circulating ALU repeats in serum have a prognostic value for colorectal cancer patients**, *Cancer Biomarkers* **37** (2023), 237-248.

Weitere Veröffentlichungen mit Co-Autorschaft:

A.B. Philipp, D. Nagel, P. Stieber, R. Lamerz, I. Thalhammer, A. Herbst and F.T. Kolligs, **Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer**, *BMC cancer* **14** (2014), 245.

C. Goßler, M. May, S. Weikert, S. Lenart, A. Ponholzer, C. Dreissig, G. Stojanoski, I. Anzinger, J. Riester, M. Burger, C. Gilfrich, R. Mayr, J. Bründl, **Long-Term Follow-Up of Peritoneal Interposition Flap in Symptomatic Lymphocele Reduction following Robot-assisted Radical Prostatectomy: Insights from the PIANOFORTE Trail**, *Cancers 16* (2024), 1932

1 Beitrag zu den Veröffentlichungen

1.1 Beitrag zu Publikation I

Bei Publikation I bestand der Eigenanteil der Autorin zunächst in der Mitarbeit an der Studienkonzeption und Formulierung der Forschungsfragen. Sie wirkte bei der Etablierung und Planung der Laborversuche mit. Ferner unterstützte sie die Autoren intensiv bei der Literaturrecherche und brachte sich bei der Interpretation der Daten ein. Sie beteiligte sich an einer kritischen Diskussion der Studienergebnisse. Schließlich war sie am Aufbau, der Verfassung und Überarbeitung des Manuskripts beteiligt.

1.2 Beitrag zu Publikation II

Die Autorin wirkte an sämtlichen Phasen der Entstehung der vorliegenden Veröffentlichung mit. Der Eigenanteil umfasst neben der Erarbeitung des Konzepts, Formulierung der wissenschaftlichen Fragestellung und ausführlicher Literaturrecherche die Verwaltung der Proben, Planung und Durchführung der Vorversuche, Prozessierung der Serumproben, Durchführung der Messungen und Erhebung sämtlicher Daten sowie Mitwirkung an der wissenschaftlichen und statistischen Auswertung. Anschließend wurden die Ergebnisse mit den Co-Autoren kritisch diskutiert sowie das Manuskript selbstständig verfasst. Im Rahmen des Peer-Review-Prozesses wurde das Manuskript dann in Absprache mit den Co-Autoren bis zur akzeptierten Version überarbeitet.

2 Einleitung

2.1 Die Bedeutung von Prognosemarkern für das kolorektale Karzinom

Kolorektale Karzinome stellten in Europa im Jahr 2020 die zweithäufigste Krebsart dar und sind verantwortlich für die zweithäufigsten krebsassoziierten Sterbefälle (Dyba et al., 2021). Die Einführung von Darmkrebsvorsorgeuntersuchungen, insbesondere iFOBT (immunologischer fäkaler Okkultbluttest) und Koloskopie, hat in hoch entwickelten Ländern dazu geführt, dass Darmkrebs häufig schon in frühen Stadien erkannt bzw. bereits präkanzeröse Läsionen entfernt werden können. Durch diese Maßnahmen, welche Patienten ab 50 Jahren angeboten werden, kann das Risiko von Darmkrebserkrankungen bzw. darmkrebsassoziierter Todesfälle gesenkt werden (Bretthauer et al., 2022). Jedoch führen gerade in diesen Ländern fortschreitende Lebensstilveränderungen, speziell der Konsum von Alkohol, bestimmte Ernährungsgewohnheiten (geringer Verzehr von Obst und Gemüse bei hohem Verzehr von rotem/verarbeitetem Fleisch), Übergewicht, mangelnde körperliche Aktivität und Rauchen zu hohen Inzidenzen (Arnold et al., 2017).

Trotz der angebotenen Früherkennungsmaßnahmen erhält rund ein Fünftel der Patienten die Diagnose eines kolorektalen Karzinoms erst im metastasierten Stadium (Adam et al., 2015). Dies ist vor allem der fehlenden Beteiligung an den erwähnten Früherkennungsmaßnahmen wie auch der Tatsache, dass die Erkrankung je nach Lage des Tumors zunächst auch sehr lange asymptomatisch verlaufen kann, zuzuschreiben. Obwohl kontinuierlich Fortschritte bei der Behandlung von Patienten mit kolorektalen Karzinomen erzielt werden können, bleibt die Therapie im metastasierten Erkrankungsstadium eine medizinische Herausforderung mit insgesamt schlechter Prognose. Im Rahmen ihrer retrospektiven Studie stellten Sudo et al. eine 5-Jahres-Überlebensrate von nur 19,1 % fest (Sudo et al., 2019). Eine vollständige Heilung mittels einer Kombination aus Operationen und systemischen Therapien kann nur selten erzielt werden.

Beim kolorektalen Karzinom akkumulieren im Rahmen des Prozesses der Karzinogenese zahlreiche Mutationen in Tumorsuppressorgenen und Onkogenen. Die meisten Karzinome entstehen aus Adenomen über die Adenom-Karzinom-Sequenz durch sequenzielle Mutationen in verschiedenen, sich teils überschneidenden Signalwegen wie dem Chromosomal Instability (CIN)- oder dem Microsatellite Instability (MSI)-Signalweg (Nguyen et al., 2020). Im CIN-Signalweg, welcher in ca. zwei Drittel der Fälle vorliegt, führt der Verlust von Chromosomen oder Chromosomenteilen häufig zu einer Aneuploidie oder zum Verlust der Heterozygotie. Zugleich kommt es zu einer Anhäufung von

Mutationen in charakteristischen Genen, insbesondere Tumorsuppressorgenen wie APC (Adenomatous Polyposis Coli) oder TP53. Da das mutierte APC-Gen im Proteinkomplex den Abbau von ß-Catenin verhindert, kommt es zu einer Aktivierung des Wnt/ß-Catenin-Signalwegs (Parker and Neufeld, 2020). Ob die CIN im Allgemeinen selbst Mutationen begünstigt oder umgekehrt, ist bislang ungeklärt (Pino and Chung, 2010). MSI wird bei ca. 15 % der kolorektalen Karzinome festgestellt. Im Gegensatz zur CIN kommt es bei der MSI zu Basenpaarmutationen innerhalb einzelner Gene. Instabilität in Mikrosatellitenregionen führt zu Fehlern bei der Erkennung und vor allem Reparatur von Mutationen. In der Folge häufen sich die Mutationen und begünstigen damit die weitere Tumorentwicklung (Boland and Goel, 2010). Der dritte bedeutende Signalweg der Karzinogenese kolorektaler Karzinome ist der CIMP (CpG Island Methylator Phenotype)-Signalweg. Weitere Signalwege spielen eher eine untergeordnete Rolle. Beim CIMP-Signalweg werden im Wesentlichen Tumorsuppressorgene durch die Hypermethylierung von CpG-reichen Promotorstellen (sog. CpG-Inseln) durch ausbleibende Transkription inaktiviert (Nazemalhosseini Mojarad et al., 2013). Diese CpG-Inseln sind in der Regel vor Methylierung geschützt, in Tumoren kann es jedoch zu einer abnormen Methylierung und damit zu einer unkontrollierten Zellteilung kommen. Es handelt sich im Gegensatz zu den anderen Signalwegen also um eine epigenetische Inaktivierung.

Durch mehr Wissen über die genetischen und epigenetischen Veränderungen, die in den verschiedenen Signalwegen der Karzinogenese kolorektaler Karzinome stattfinden, können neue zielgerichtete Therapien, sog. „targeted therapies“, entwickelt werden. Diese konnten in den letzten Jahren bereits das Gesamtüberleben von Patienten mit metastasiertem kolorektalem Karzinom verbessern (Biller and Schrag, 2021). Die Therapien werden individuell auf die molekularen und pathologischen Eigenschaften des Tumors zugeschnitten und greifen direkt in die Signalwege ein (Piawah and Venook, 2019). Ziel ist es hierbei, den dominanten Signalweg auszuschalten. Vorteil dieser Behandlung ist ein Gleichgewicht zwischen einerseits Abtötung von Tumorzellen und andererseits Verhinderung von Nebenwirkungen der Standard-Chemotherapien wie Knochenmarksdepression oder Schädigung von Epithelzellen und den damit verbundenen Konsequenzen für die Patienten wie etwa Leukopenien, Anämien und Verdauungsstörungen. Am Beispiel des KRAS-Tumorstatus zeigt sich, dass nur Patienten mit KRAS-Wildtyp von einer zusätzlichen Therapie mit monoklonalen Antikörpern gegen den epidermalen Wachstumsfaktorrezeptor (EGFR) profitieren. Bei Mutationen im KRAS-Onkogen ist diese Therapie nicht vorteilhafter als die Chemotherapie nach Standardschema (Van Cutsem et al., 2009).

Ferner lassen sich durch Kenntnisse der Karzinogenese auch molekulare Marker, sog. Biomarker, ableiten. Mit ihrer Hilfe können Patienten, die von bestimmten Therapien profitieren, identifiziert, die Prognose eingeschätzt und insbesondere Übertherapien verhindert werden. Biomarker sind definiert als genau und reproduktiv messbare Indikatoren des Gesundheitszustands, welche im Blut, in Körperflüssigkeiten oder im Gewebe vorkommen (Schwarzenbach et al., 2011). Mutationen in bestimmten Genen, welche in den oben genannten Signalwegen eine Rolle spielen, beispielsweise KRAS oder TP53, konnten in einigen Studien prognostische Hinweise liefern. Bei insgesamt jedoch uneinheitlichen Ergebnissen werden sie derzeit nicht als Prognosemarker eingesetzt (Pino and Chung, 2010).

Auch die im Rahmen der Tumorgenese eine Rolle spielende Hypermethylierung verschiedener Gene wurde auf ihre Tauglichkeit als epigenetischer Biomarker untersucht. Hierbei konnten sowohl Methylierungsmarker mit diagnostischem als auch prognostischem Potenzial identifiziert werden (deVos et al., 2009, Wallner et al., 2006). In einem Kollektiv aus Gesunden, Patienten mit Kolonadenomen und –karzinomen sowie weiteren Tumorentitäten wie Ösophagus- oder Leberzellkarzinomen konnte beispielsweise eine Methylierung von Aristaless-like Homeobox-4 (ALX4) im Blut von Patienten mit kolorektalen Karzinomen häufiger festgestellt werden als bei Patienten ohne Tumordiagnose ($P < 0.0001$) (Ebert et al., 2006).

Ein im peripheren Blut bestimmbarer Biomarker, welcher sich durch seine einfache und reproduzierbare Bestimmung im klinischen Alltag etabliert hat, ist das Carcinoembryonale Antigen (CEA). CEA erwies sich in verschiedenen Studien als unabhängiger Prognosemarker für das kolorektale Karzinom, insbesondere im UICC (Union for International Cancer Control) Stadium II. Leitlinien empfehlen daher die Bestimmung von CEA präoperativ zur Prognosebestimmung, zur Überwachung von Patienten nach einer kurativen Therapie und zum Therapiemonitoring in fortgeschrittenen Erkrankungsstadien (Duffy et al., 2014). Jedoch weist die Bestimmung von CEA als Tumormarker einige Limitationen auf. Einerseits ist CEA weder hochsensitiv noch hochspezifisch und kann daher bei verschiedenen Tumorentitäten wie z.B. Schilddrüsenkarzinomen, Magenkarzinomen oder auch Leberkarzinomen erhöht sein. Andererseits kann eine Erhöhung auch durch Lebensgewohnheiten sowie nicht-tumoröse Erkrankungen bedingt sein, beispielsweise bei Rauchern, chronisch entzündlichen Erkrankungen oder Pankreatitis (Lakemeyer et al., 2021, Tan et al., 2009). Daher sind die CEA-Konzentrationen interindividuell grundsätzlich nur sehr eingeschränkt vergleichbar. Für jeden Patienten muss also im Therapieverlauf ein individuelles Ausgangsniveau gefunden werden. Aufgrund dieser Limitationen sowie der genannten Vorteile der „targeted therapy“

besteht weiterhin großer Bedarf an neuen prognostischen Biomarkern bzw. Kombinationen verschiedener Marker für eine höhere prognostische Aussagekraft.

2.2 OPG als Prognosemarker des kolorektalen Karzinoms

Osteoprotegerin (OPG) ist ein lösliches Glycoprotein und spielt als Decoy-Rezeptor der Tumornekrosefaktor-(TNF-)Rezeptor-Superfamilie durch Bindung an Receptor Activator of NF-κB Ligand (RANKL) unter anderem eine Rolle in der Regulation des Knochenstoffwechsels durch Hemmung der Osteoklastogenese (Theoleyre et al., 2004). Weiterhin ist jedoch auch bekannt, dass OPG durch Bindung an das Protein *TNF-related apoptosis-inducing ligand* (TRAIL) Apoptose verhindern und hierdurch zur Tumorentstehung beitragen kann (Reid and Holen, 2009, Baud'huin et al., 2013). Erhöhte Serumspiegel von OPG konnten bereits bei einigen Krebsentitäten wie Prostata-, Mamma- oder Pankreaskarzinomen festgestellt werden (Eaton et al., 2004, Rachner et al., 2019, Brand et al., 2011) und zeigten somit ihr Potenzial als prognostischer und diagnostischer Biomarker. Daher stellte sich die Frage, ob OPG als Marker auch für das kolorektale Karzinom eine Relevanz haben könnte.

In früheren Versuchen gelang uns bereits der Nachweis, dass OPG durch den Wnt/ß-Catenin-Signalweg reguliert wird. Ferner konnten wir eine höhere Expression von OPG in Kolonkarzinomzellen im Vergleich zu normalen Epithelzellen feststellen (De Toni et al., 2008). Der Wnt/ß-Catenin-Signalweg spielt eine entscheidende Rolle für die Entstehung kolorektaler Karzinome. Auslösend ist hierbei oftmals eine Mutation im APC-Gen. Eine Unterdrückung der ß-Catenin-Expression in Tumorzellen ging in unseren Versuchen mit einer ebenfalls verminderten Expression von ß-Catenin-Zielgenen wie auch einer verminderten Expression von OPG einher. Es kam zu einer messbaren Abnahme von OPG in den Überständen der Zellen. Darüber hinaus konnte die TRAIL-assoziierte Apoptose in mehreren Zelllinien durch Zugabe von OPG gehemmt werden. Bei unterdrückter ß-Catenin-Expression kam es ebenfalls zu einer erhöhten Empfindlichkeit gegenüber TRAIL-induzierter Apoptose, was die regulatorischen Eigenschaften von ß-Catenin auf OPG und die TRAIL-induzierte Apoptose bestätigte. Zuletzt konnte gezeigt werden, dass die Serumspiegel von OPG im Blut von Patienten im metastasierten Stadium einer Darmkrebserkrankung wie auch im lokal fortgeschrittenen, nicht metastasierten Erkrankungsstadium im Vergleich zu Gesunden signifikant erhöht waren, wobei wiederum auch ein signifikanter Unterschied der OPG-Spiegel im metastasierten Stadium im Vergleich zum lokal fortgeschrittenen Stadium (UICC Stadium IV vs. III) feststellbar war (De Toni et al., 2008). Weitere Studien konnten diese Ergebnisse auch bei Brustkrebspatientinnen bestätigen. So fand sich unter anderem eine Korrelation der

Serumspiegel von OPG mit der Tumorgröße im metastasierten Stadium, wodurch metastasierte von nicht-metastasierten Erkrankungen unterschieden werden konnten (Shaker and Elbaz, 2020). Meltzer et al. bestimmten die Konzentration von OPG im Serum von Patienten mit Rektumkarzinomen im fortgeschrittenen Erkrankungsstadium. Hierbei konnte einerseits beobachtet werden, dass ein erhöhter OPG-Spiegel im Serum mit einem schlechteren progressionsfreien Überleben einherging. Andererseits wiesen steigende OPG-Konzentrationen nach induktiver Chemotherapie auf ein verbessertes Überleben hin, was beispielsweise eine Art Gegensteuerung auf die krankheitsbedingt erhöhte Osteoklasten-Funktion darstellen könnte, alternativ könnte die erhöhte Konzentration auch auf eine körpereigene, systemische Immunmodulation hinweisen bzw. diese selbst vermitteln (Meltzer et al., 2016). Basierend auf diesen interessanten Beobachtungen war es unser Bestreben, in einer Population von Patienten mit fortgeschrittenen kolorektalen Karzinomen die prognostische Bedeutung von OPG als Serummarker weiter zu untersuchen, um gegebenenfalls hieraus therapeutische Strategien entwickeln zu können.

2.3 fcDNA als Prognosemarker des kolorektalen Karzinoms

Bei zellfreier zirkulierender DNA (fcDNA – free circulating DNA) handelt es sich um DNA-Moleküle, welche in Körperflüssigkeiten wie Blut, Liquor oder Sputum in unterschiedlichen Konzentrationen vorkommen (Szilagyi et al., 2020). Hier zeigen sie, vermutlich bedingt durch verminderten enzymatischen Abbau, eine hohe Stabilität und sind beispielsweise mittels quantitativer Realtime-PCR (Polymerase Chain Reaction) einfach und reproduzierbar nachweisbar.

Im Blut von Tumorpatienten lassen sich höhere Mengen frei zirkulierender DNA als im Blut Gesunder feststellen (Alix-Panabières et al., 2012, van der Vaart and Pretorius, 2008, Goebel et al., 2005).

Eine Frage, die bislang noch nicht vollständig geklärt werden konnte, betrifft die genauen Freisetzungsmechanismen von fcDNA aus dem Tumor. Es wird sowohl eine passive Freisetzung im Rahmen von Apoptose- und/oder Nekroseprozessen als auch eine aktive Sekretion durch insbesondere Lymphozyten diskutiert (González-Masiá et al., 2013). Im Rahmen einer Apoptose werden typischerweise gleichmäßige DNA-Fragmente von ca. 185 bis 200 Basenpaaren (bp) Länge generiert, wohingegen die Fragmente aus soliden Tumoren, welche gehäuft durch Nekrose entstehen, größer und ungleichmäßig lang sind. Durch die Bestimmung der Längen bzw. des Verhältnisses der Längen der Fragmente zueinander können Rückschlüsse auf die Herkunft der fcDNA gezogen werden (Marzese et al., 2013). Für einige Krebsentitäten konnte dieser sogenannten DNA-

Integrität bereits eine mögliche klinische Bedeutung als prognostischer bzw. diagnostischer Marker zugesprochen werden (Eskander et al., 2022, Vizza et al., 2018, Elhelaly et al., 2022).

Aber auch die Konzentration der fcDNA ist für verschiedene klinische Anwendungen von wissenschaftlichem Interesse. Durch Quantifizierung der DNA-Fragmente aus dem Blut oder anderen Körperflüssigkeiten könnten diese als *„liquid biopsy“* zur Diagnosestellung herangezogen werden. Im Vergleich hierzu ist die übliche Diagnosesicherung mittels Tumorbiopsie oft aufwendiger, teurer und deutlich komplikationsbehafteter, gerade bei schwer erreichbaren Befunden. Zudem kann die gewonnene Biopsie durch die Heterogenität des Tumors in ihrer Aussagekraft verfälscht sein (Kustanovich et al., 2019). Ferner erhofft man sich durch die Bestimmung der fcDNA-Menge Aussagen zur Prognose oder zur Beurteilung des Therapieerfolgs. Durch Analyse der im Einzelfall vorliegenden Mutationen oder epigenetischen Veränderungen wie Methylierungen von Genen in der fcDNA können gezielte Therapien ausgewählt werden (Szilagyi et al., 2020).

Zur Quantifizierung der fcDNA im Blut eignen sich besonders nicht-kodiererende, repetitive Sequenzen, welche über 50 % des gesamten Genoms ausmachen und daher auch bei geringen DNA-Mengen sicher bestimmt werden können. Hierzu zählen die Non-LTR-Retrotransposons, welche die kürzeren, etwa 300 bp langen SINEs (*Short Interspersed Elements*) und die längeren LINEs (*Long Interspersed Elements*) enthalten. Den SINEs sind wiederum die ALU-Sequenzen zuzuordnen (Gezer et al., 2022).

Ferner ist auch die mitochondriale DNA (mtDNA) durch ihre hohe Kopienzahl sowie die einfache Struktur sehr gut als Biomarker geeignet.

Anhand von fcDNA-Markern aus den genannten Klassen in Kombination mit CEA als klinisch etabliertem Marker konnten in einer Studie von Mead et al. gesunde Patienten von Patienten mit Colonadenomen bzw. -karzinomen unterschieden werden (Mead et al., 2011).

In einer früheren Studie konnten wir bereits in einem Kollektiv von Kolonkarzinompatienten aller Erkrankungsstadien zeigen, dass die Methylierung von *Helicase-like Transcription Factor* (HLTF) und *Hyperplastic Polyposis 1* (HPP1) in frei zirkulierender DNA einen unabhängigen Prognosemarker darstellt. Kombinationen der Marker untereinander bzw. mit CEA erhöhten jeweils die prognostische Aussagekraft. Im Vergleich waren die Methylierungsmarker mindestens so aussagekräftig wie CEA als klinisch gebräuchlicher Marker (Philipp et al., 2012). Da die Mechanismen der Freisetzung dieser Marker ins Blut weitestgehend unklar sind, führten wir eine weitere Studie durch. Diese konnte belegen, dass die Methylierung von HLTF und HPP1 mit dem Zellzerfall

korreliert und daher zur Identifikation besonders aggressiver Tumore dienen bzw. prognostische Informationen liefern kann (Philipp et al., 2014).

Unser gegenständliches Forschungsvorhaben hatte nun das Ziel, die von Mead et al. untersuchten fcDNA-Marker (ALU115, ALU247, LINE1-79, LINE1-300 und ND1-mt) auf ihre prognostische Relevanz in unserem rein aus Karzinomen der UICC-Stadien I bis IV bestehenden Kollektiv zu testen bzw. mit CEA und unseren bereits erprobten Methylierungsmarkern zu kombinieren. Perspektivisch stellt sich die Frage, ob diese Marker dann auch bei der Therapieentscheidung sowie zum Therapiemonitoring eingesetzt werden können.

3 Zusammenfassung

Aufgrund der hohen Inzidenz sowie der hohen tumorbedingten Sterblichkeit beim kolorektalen Karzinom bedarf es neben flächendeckender Früherkennungsmaßnahmen und kontinuierlicher Fortschritte bei der Behandlung prognostischer Marker, um Patienten optimal und ihrem individuellen Risiko entsprechend zu behandeln.

Ziel der vorliegenden Promotionsarbeit war es, Biomarker aus dem Serum auf ihre prognostische Relevanz bei Patienten mit kolorektalen Karzinomen, insbesondere auch im fortgeschrittenen Erkrankungsstadium, zu untersuchen. Wir verwendeten hierfür einerseits Marker mit vielversprechenden Ergebnissen aus unseren eigenen Studien und andererseits Marker, welche in anderen Tumorentitäten bzw. Studien prognostische Relevanz gezeigt haben, und kombinierten diese mit CEA.

Zunächst bestimmten wir im Rahmen von Studie I (De Toni et al.) die Konzentrationen von OPG und CEA im Serum von 81 Patienten mit kolorektalen Karzinomen im UICC Stadium IV. In der Überlebensanalyse zeigte sich, dass klinische und pathologische Variablen nicht mit dem Überleben korrelierten, jedoch zeigten erhöhte Konzentrationen von OPG und CEA im Serum eine signifikante Korrelation. Die Grenzwerte wurden hierbei durch die jeweiligen Mediane definiert. Mit einer OPG-Konzentration über dem Grenzwert sank das mittlere Gesamtüberleben von 1.8 Jahre [1.3 – 3.0] auf 1.0 Jahre [0.7 – 1.2]; P = 0.013. Auch erhöhte CEA-Werte korrelierten mit einem kürzeren Überleben (2.2 Jahre [1.1 – 3.3] vs. 1.2 Jahre [0.9 – 1.6]; P = 0.014). Die multivariate Analyse bestätigte sowohl OPG (HR und 95 % Konfidenzintervall 1.69 [1.03 – 2.79]) als auch CEA (1.68 [1.03 – 2.75]) als unabhängigen Prognosemarker.

Ferner zeigte sich, dass die Kombination von OPG und CEA im Vergleich zur Betrachtung der einzelnen Marker die prognostische Aussagekraft noch verstärkt. Waren beide Marker über dem Grenzwert, war das Überleben schlechter als mit beiden Markern unterhalb des Grenzwertes. Die Überlebensraten in 1,3 und 5 Jahren betrugen 78.9 % vs. 50 %, 46.5 % vs. 10 % und 13 % vs. 10 %; P = 0.015.

In einer Untergruppe von 33 Patienten führten wir zusätzlich immunhistochemische Untersuchungen auf OPG am Tumorgewebe selbst durch. Die Färbung wurde nach einem Punktesystem von 0 bis 3+ bewertet. Für die Färbungen mit hoher Punktzahl (2+ und 3+) ergab sich im Vergleich zu den schwachen Färbungen (0 und 1+) ein Trend hin zum schlechteren Überleben. Zwischen OPG-Expression im Gewebe und Serumkonzentration von OPG zeigte sich hingegen keine Korrelation. Dies führen wir auf die Tatsache zurück, dass OPG nicht nur von Tumorzellen, sondern auch von Zellen aus der

Tumormikroumgebung exprimiert wird, wie auch auf Schwankungen der Expression im Zuge der Tumorheterogenität, besonders bei fortgeschrittenen Tumoren.

In unserer zweiten Studie (Anzinger et al.) wählten wir, ergänzend zu zwei Methylierungsmarkern, welche im vorliegenden Studienkollektiv von 268 Patienten mit kolorektalen Karzinomen aller Tumorstadien in einer früheren Arbeit bereits ihren prognostischen Wert unter Beweis gestellt haben, fünf verschiedene fcDNA-Fragmente (ALU115, ALU247, LINE1-79, LINE1-300 und ND1-mt), bestimmten die Konzentrationen im Serum und analysierten die Überlebensdaten auf prognostische Relevanz.

Zunächst zeigten sich einige teils hochsignifikante Korrelationen der fcDNA-Marker mit einzelnen klinischen respektive pathologischen Parametern wie z.B. Tumorgröße, Lymphknotenstatus oder UICC-Stadium. Ferner wiesen insbesondere die Fragmente ALU115 und ALU247 einen signifikanten Zusammenhang mit CEA als gebräuchlichen Marker auf, bei einigen fcDNA-Fragmenten bestanden auch teils hochsignifikante Korrelationen mit den Methylierungsmarkern HLTF und HPP1. Eine Erhöhung von ALU115, ALU247, L1-79 und L1-300 Konzentrationen im Serum korrelierte im Rahmen der Kaplan-Meier-Überlebensanalyse signifikant mit einem kürzeren Überleben in allen Tumorstadien wie auch in der Untergruppe der metastasierten kolorektalen Karzinome (UICC IV).

In der multivariaten Analyse aller Marker erwiesen sich ALU115 (HR = 2.754; 95 % Cl 1.662 - 4.565; $P < 0.001$) und HPP1 (HR = 1.925; 95 % Cl 1.187 - 3.121; $P = 0.008$) als unabhängige Prognosemarker für das Überleben. Die Kombination beider Marker zeigte eine hochsignifikante Korrelation mit der Überlebenszeit ($P < 0.001$). Dieses sank von 2.4 Jahren mit beiden Markern unterhalb auf 0.7 Jahre mit beiden Marken oberhalb des festgelegten Grenzwertes.

Zusammenfassend gelang es uns, mit OPG und ALU115 zwei unabhängige Prognosemarker, insbesondere für Patienten mit fortgeschrittenen kolorektalen Karzinomen, zu identifizieren. Ferner konnten wir zeigen, dass Kombinationen von Markern, insbesondere OPG und CEA bzw. ALU 115 und HPP1, die prognostische Aussagekraft sogar noch erhöhen können.

Biomarker im Blut als sog. “*liquid biopsy*” sind dabei nicht nur einfach und reproduzierbar zu gewinnen und bilden dabei die Tumorlast ab, sondern können auch Informationen über den Primärtumor auf molekularbiologischer Ebene liefern, welche wiederum auch für die Auswahl einer Therapie im Sinne der “*targeted therapy*” eine entscheidende Rolle spielen können. Auch für die Vermeidung von Über- und Untertherapien sowie das Therapiemonitoring liefern Prognosemarker wichtige Erkenntnisse. Daher bieten unsere Ergebnisse wertvolle Ansatzpunkte für die Etablierung von

Serummarkern sowie für weitere Studien, die den sinnvollen und effektiven Einsatz dieser Marker im klinischen Alltag prüfen können.

4 Abstract (English)

Due to the high incidence and high tumor-related mortality of colorectal carcinoma, in addition to comprehensive screening tests and continuous progress in treatment, there is a need for prognostic markers in order to optimally treat patients according to their individual risk.

The aim of this doctoral thesis was to investigate the prognostic value of serum biomarkers in patients with colorectal carcinoma, particularly in the advanced stage of the disease. For this purpose, we used markers with promising results from our own studies as well as markers that have shown prognostic relevance in other tumor entities or studies and combined these with CEA.

In study I (De Toni et al.), we determined the concentrations of OPG and CEA in the serum of 81 patients with UICC stage IV colorectal cancer. The survival analysis revealed that clinicopathological parameters did not correlate with survival, but increased concentrations of OPG and CEA in serum showed a significant correlation. The cutoff values were defined by the respective median concentrations. With an OPG level above the cutoff value, median overall survival decreased from 1.8 years [1.3 - 3.0] to 1.0 years [0.7 - 1.2]; P = 0.013. Increased concentrations of CEA also correlated with shorter survival (2.2 years [1.1 - 3.3] vs. 1.2 years [0.9 - 1.6]; P = 0.014). The multivariate analysis confirmed both OPG (HR and 95 % confidence interval 1.69 [1.03 - 2.79]) and CEA (1.68 [1.03 - 2.75]) as independent prognostic markers.

Furthermore, the combination of OPG and CEA was shown to increase the prognostic value compared to considering the individual markers. If both markers were above the cutoff value, survival was worse than if both markers were below the cutoff value. Survival rates at 1, 3 and 5 years were 78.9 % vs. 50 %, 46.5 % vs. 10 % and 13 % vs. 10 %, respectively; P = 0.015.

In a subgroup of 33 patients, we additionally performed immunohistochemical analysis for OPG on the tumor tissue itself. The staining was scored according to a 0 - 3+ point system. For the high staining intensities (2+ and 3+), there was a trend toward poorer survival compared to the weak stainings (0 and 1+). However, there was no correlation between OPG expression in tissue and its serum concentration. We attribute this to the fact that OPG is expressed not only by tumor cells but also by cells from the tumor microenvironment, as well as to fluctuations in expression due to tumor heterogeneity, especially in advanced tumors.

In our second study (Anzinger et al.), we selected five different fcDNA fragments (ALU115, ALU247, LINE1-79, LINE1-300 and ND1-mt) in addition to two methylation

markers, which have already proven their prognostic value in the present study population of 268 colorectal cancer patients of all tumor stages in a previous work, determined the concentrations in serum and analyzed the survival data for prognostic relevance.

First, some highly significant correlations were found between fcDNA markers and individual clinicopathological parameters such as tumor size, lymph node status or UICC stage. Furthermore, especially the fragments ALU115 and ALU247 showed a significant correlation with CEA as a common marker and for some fcDNA fragments, there were also highly significant correlations with the methylation markers HLTF and HPP1. An increase of ALU115, ALU247, L1-79 and L1-300 concentrations in serum correlated significantly with shorter survival in all tumor stages as well as in the subgroup of metastatic colorectal carcinomas (UICC IV) in the Kaplan-Meier survival analysis.

In multivariate analysis of all markers, ALU115 (HR = 2.754; 95 % Cl 1.662 - 4.565; $P < 0.001$) and HPP1 (HR = 1.925; 95 % Cl 1.187 - 3.121; $P = 0.008$) were found to be independent prognostic markers for survival. The combination of both markers showed a highly significant correlation with survival ($P < 0.001$). Survival decreased from 2.4 years with both markers below to 0.7 years with both markers above the defined cutoff value.

In summary, with OPG and ALU115 we have identified two independent prognostic markers, particularly for patients with advanced colorectal cancer. Furthermore, we were able to show that combinations of markers, especially OPG and CEA or ALU 115 and HPP1, can even increase the prognostic value. Biomarkers in blood as a so-called "liquid biopsy" are not only easy and reproducible to obtain and thereby map the tumor burden but can also provide information about the primary tumor at the molecular biological level, which in turn can also play a decisive role in the selection of a therapy in the sense of "targeted therapy". Prognostic markers also provide important information for the avoidance of over- and under-treatment as well as for therapy monitoring. Therefore, our results offer valuable starting points for the establishment of serum markers as well as for further studies on the meaningful and effective use of these markers in clinical practice.

5 Publikation I

E.N. Toni, D. Nagel, A.B. Philipp, A. Herbst, I. Thalhammer, J. Mayerle, H.-P. Torok, L. Brandl and F.T. Kolligs, **Correlation Between Baseline Osteoprotegerin Serum Levels and Prognosis of Advanced-Stage Colorectal Cancer Patients**, *Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology* **45** (2018), 605–613

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Original Paper

Correlation Between Baseline Osteoprotegerin Serum Levels and Prognosis of Advanced-Stage Colorectal Cancer Patients

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Key Words
Mcrc • OPG • TRAIL

Abstract
Background/Aims: Osteoprotegerin (OPG) is a soluble receptor of the pro-apoptotic cytokine TRAIL which is thought to contribute to tumour development by inhibiting apoptosis or affecting other aspects of tumour biology, including cell proliferation and immune response. Although immunohistochemical studies suggest that OPG correlates with survival in metastatic colorectal cancer (mCRC), only scarce data are available on serum OPG in CRC patients. ***Methods:*** In this pilot study, we assessed the prognostic significance of serum OPG and CEA (Carcinoembryonic antigen) in 81 patients with UICC (Union for International Cancer Control) stage-IV mCRC. OPG was additionally assessed by immunohistochemistry in primary tissue samples from 33 patients of the same cohort. ***Results:*** Baseline serum OPG correlated with CEA ($r=0.36$, $p=0.0011$), but independently predicted survival of mCRC patients. Life expectancy was poorer in patients with OPG levels above the median concentration of 51ng/ml (median overall survival [95% confidence interval] 1.8 years [1.3-3.0] vs. 1.0 [0.7-1.2] $p=0.013$). Patients with high levels of both OPG and CEA had an even poorer life expectancy vs. low-OPG/low-CEA patients (0.9 years [0.6-1.5] vs. 3 years [1.2-4.4], $p=0.015$), indicating that CEA and OPG have additive prognostic significance. Immunohistochemical analysis of OPG failed to show a correlation between OPG staining and survival ($p=0.055$) or OPG concentration from matched serum samples. ***Conclusions:*** This pilot study provides evidence of independent prognostic significance of serum OPG in patients with advanced mCRC and warrants its further prospective validation.

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Introduction

Apoptosis mediated by tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) receptors represents a well-established mechanism of immune-mediated tumour surveillance [1]. *In vivo* investigation has shown that the TRAIL-system plays a role in the clearance of metastatic cells [2] and clinical data from human specimens have consistently shown a correlation between TRAIL-Receptor (TRAIL-R) loss and patients' survival across different tumour entities [3-5]. Besides the downregulation of TRAIL-R1 and TRAIL-R2, apoptosis resistance can be caused by overexpression of decoy receptors for TRAIL (such as the membrane receptors TRAIL-R3 and TRAIL-R4 which competitively bind to TRAIL without inducing apoptotic signalling [1]) or by osteoprotegerin (OPG), a third, soluble form of the decoy receptor for TRAIL initially identified as a regulator of bone tissue modelling [1]. According to the proposed role of the TRAIL-system in oncogenesis, OPG is thought to contribute to the development of several tumour entities comprising breast, prostate and gastric cancer [6-8]. More recently, however, it has been proposed that OPG also affects other mechanisms of tumour formation, including enhancement of cell proliferation and paracrine mechanisms influencing tumour microenvironment [9].

We previously provided the first report showing that OPG is a transcriptional target of β-catenin in colorectal cancer, and that its concentration is increased in serum of late-stage mCRC patients [10]. Subsequently, basing on mRNA expression analysis of immunohistochemical samples, other authors independently confirmed that OPG is associated with an aggressive phenotype and metastasis formation in colorectal cancer patients [11]. Very recently, by using a protein screening array, Melzer and colleagues [12] independently observed an increase in OPG serum concentration during neo-adjuvant treatment of rectal tumours. These authors reported a trend towards a poorer survival in CRC patients with high baseline-OPG; on the other hand, an increase of OPG during the neoadjuvant treatment was associated to a better progression-free survival. The concept that OPG favours tumour development has been questioned also by recent data showing that lower immunoreactivity for OPG in tissue samples from CRC is associated to a poorer outcome [13]. These data suggest that OPG plays different roles in different stages of tumour development or in different therapeutic settings. However, in spite of conflicting reports from different immunohistochemical analyses of OPG in colorectal cancer specimens [11, 13], to our knowledge serum OPG has been thus far assessed only in the patients' cohort with rectal carcinoma assessed my Meltzer and colleagues [12]. Following up on these results from the neoadjuvant treatment setting, we contribute to the elucidation of the role of OPG by assessing a cohort of patients with colonic or rectal carcinoma in advanced stage.

Materials and Methods

Patients and serum samples

Sera from patients diagnosed with metastatic colorectal cancer between 1987 to 2006 were obtained before initiation of therapy and were selected by availability of clinicopathologic and long term follow-up data. A subset of 33 patients, selected according to availability of archival pathological material at the Institute of Pathology of our institution was used for immunohistochemical staining of OPG. Blood samples were delivered to the central laboratory through the internal tube mailing system of our institution within 30 min after blood drawing. All specimens were centrifuged at 2, 000g 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. Analyses of serum samples were performed blinded to patient data.

Determination of CEA and of OPG

CEA was quantified using a microparticle immunoenzymometric assay (AxSYM, Abbott Laboratories, Chicago, IL). OPG concentrations in serum of patients with colorectal cancer were assayed by ELISA (Raybiotech) according to the manufacturer's instructions as previously reported [10].

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Immunohistochemistry

Immunohistochemical staining was performed on 5 µm sections of tumor tissue. As primary antibody, osteoprotegerin monoclonal rabbit antibody (Abcam, Cat.No. ab124820, dilution 1:220, Cambridge, United Kingdom) was used. Pre-treatment for antigen retrieval was performed by microwaving for 2 x 15 min at 750 W in Enhancer (Linaris, Cat.No. E7000, Dossenheim, Germany). Detection was performed using ImmPress Reagent Kit Anti-Rabbit Ig (Fa.Vector, Cat.No. MP-7401). AEC+ (Dako, Cat.No. K3468, Hamburg, Germany) was used as a chromogen. Finally, slides were counterstained with Hematoxylin Gill`s Formula (Vector Laboratories, Cat. No. H-3401, Eching, Germany).

Immunohistochemical analysis

Evaluation of immunohistochemical staining was performed by assigning cytoplasmic OPG protein level scores ranging from 0 to 3+ for increasing signal intensities. Samples exhibiting a staining intensity score of 0 (no OPG detectable) or 1+ were referred to as "low staining" samples; "high staining" was defined upon detection of staining scores of 2+ and 3+.

Statistical analysis

All statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC). Spearman Correlation test was used to assess the correlation between OPG and CEA. Wilcoxon-Mann-Whitney test was used to explore the relationship between clinicopathological features and OPG and CEA levels. Overall survival was calculated from the date of diagnosis of the primary tumour 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 was performed using the Kaplan–Meier method and log-rank tests. Hazard ratios (HRs) were estimated using Cox's regression model.

Results

Patient characteristics

Altogether, 81 serum samples of patients with colorectal cancer in stage IV treated between 1987 and 2006 at the Hospital of the University of Munich could be retrieved and considered for analysis in this study. By the end of follow-up, 67/81 (82.7%) of all patients had died. Overall median survival was 1.4 years (95% CI 1.1-1.7). The 1-, 3-, and 5-year OS rates were 66.6%, 24.1% and 11.2%, respectively. Altogether, the demographic and clinical-pathological features of this patients collective are in line with the expected characteristics of colorectal cancer patients in Germany. The main characteristics are summarized in Table 1.

OPG-serum concentrations directly correlate with CEA but independently predict the outcome of stage IV mCRC patients

Since CEA is an established tumour marker of colorectal cancer, CEA serum levels were first compared to those of OPG: as assessed by the Spearman correlation coefficient, a positive correlation between the serum concentration of these two se-

Table 1. Patients' characteristics

Characteristic	Frequency	%	Cumulative frequency	Cumulative %
Gender				
Male	46	56.79	46	56.79
Female	35	43.21	81	100.00
Localization				
Sigma	12	14.81	12	14.81
Rectum	23	28.40	35	43.21
Colon	46	56.79	81	100.00
Histology				
Adenocarcinoma	65	89.04	65	89.04
Mucinous Adenocarcinoma	6	8.22	71	97.26
Squamous cell carcinoma	1	1.37	72	98.63
Signet ring cell carcinoma	1	1.37	73	100.00
T-Stage				
2	5	6.25	5	6.25
3	53	66.25	58	72.50
4	22	27.50	80	100.00
N-Stage				
0	16	21.62	16	21.62
1	33	44.59	49	66.22
2	25	33.78	74	100.00
Grading				
2	22	30.56	22	30.56
3	50	69.44	72	100.00

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De Toni et al.: OPG in Colorectal Cancer

Table 2. Multivariate analysis of survival comprising serum OPG and CEA concentration and clinical and pathological variables

	OPG pg/ml			CEA ng/ml		
	Median	Range	p	Median	Range	p
Age						
<65	46.6	19.0 - 112.6	0.132	29.6	1.1 - 3945.0	0.365
≥65	54.7	29.4 - 135.4		19.9	1.0 - 3471.0	
Gender						
M	47.1	20.5 - 135.4	0.257	14.4	1.0 - 3945.0	0.082
F	56.1	19.0 - 112.6		31.5	1.2 - 2298.0	
T-Stage						
T2/T3	48.3	19.0 - 106.3	0.046	26.3	1.0 - 3945.0	0.543
T4	55.6	29.4 - 135.4		13.5	1.1 - 2778.0	
N-Stage						
N0	43.9	20.5 - 106.3	0.208	9.2	2.4 - 2298.0	0.609
N1/2	52.3	19.0 - 135.4		28.2	1.0 - 3945.0	
Grading						
G2	47.0	25.9 - 106.3	0.085	21.4	1.2 - 203.0	0.249
G3	55.2	19.0 - 135.4		29.0	1.0 - 3945.0	

Table 3. Long Rank test of different clinical and pathological variables

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
Age				
<65	34/44	1.7	1.0-2.5	0.134
≥65	33/37	1.2	1.0-1.6	
Gender				
M	38/46	1.6	1.0-2.1	0.541
F	29/35	1.2	0.9-1.9	.
T-Stage				
T2/T3	47/58	1.5	1.1-1.8	0.535
T4	19/22	1.2	0.5-2.5	.
N-Stage				
N0	10/16	2.5	0.5	0.093
N1/2	51/58	1.3	1.1-1.7	.
Grading				
G2	17/22	1.9	1.0-3.6	0.106
G3	43/50	1.2	1.0-1.6	.

rum markers was found (r=0.36, p=0.001).

Subsequently, a survival analysis according to different clinical-pathological variables as well as OPG and CEA serum levels and the respective median concentrations as stratification

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factor was conducted. While no clinical and pathological variables significantly correlated with patients' survival (Table 2 and Table 3), outcome was poorer in patients with serum OPG levels above the collective's median concentration of 51 pg/ml (median survival in years and confidence interval: 1.8 [1.3-3.0] vs. 1.0 [0.7-1.2] p=0.013) and in patients with CEA levels above the median concentration of 27ng/ml (2.2 years [1.1-3.3] vs. 1.2 [0.9-1.6] p = 0.014 – Table 4, Fig. 1). A multivariate analysis of survival comprising serum OPG and CEA concentration and clinical and pathological variables confirmed that OPG and CEA have independent prognostic relevance in determining patients' outcome (HR and 95% confidence interval for CEA and OPG were respectively 1.69 [1.03-2.79] and 1.68 [1.03-2.75] - Table 5).

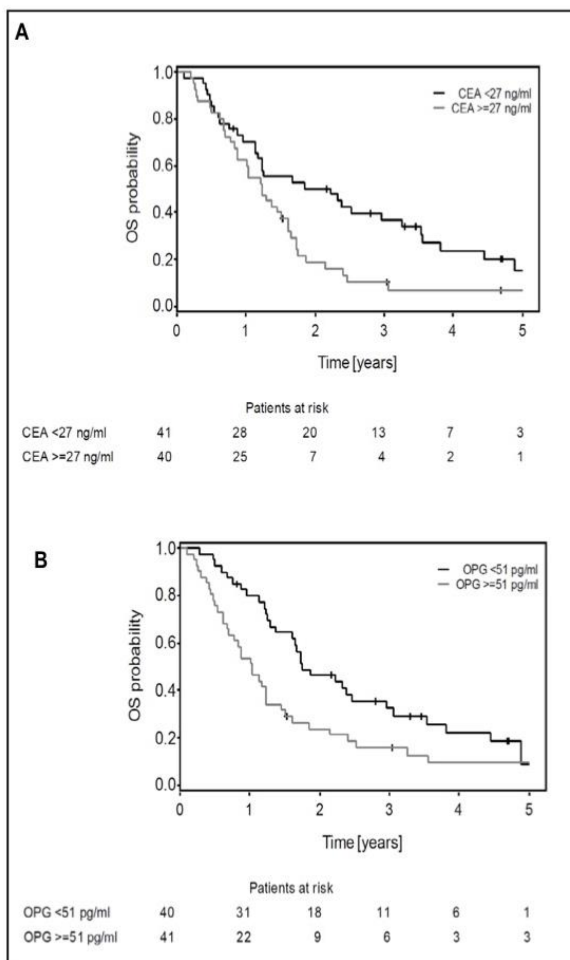

Fig. 1. OPG correlates with patients' survival. Survival curves showing overall survival according to median values of CEA (A) and OPG (B) concentrations. In graphs, censored cases are indicated by a cross.

Combined assessment of CEA and OPG defines a patients' population with poor outcome

Due to the independent prognostic effect of CEA and OPG, a further analysis was conducted to assess patients' outcome according to the combined assessment of these both biomarkers. Patients with both CEA and OPG concentrations above the cut-off levels defined by the respective median values showed, as expected, a poorer prognosis in comparison to patients with both low CEA and OPG concentrations. Survival rates at 1, 3, and 5 years in these two groups were 78.4 vs. 50%, 46.5 vs. 10% and 13 vs. 10% respectively (p=0.015 – Fig. 2, Table 4). In line with the results of the multivariate analysis showing an independent prognostic value of OPG and CEA, these data show that the combined assessment of CEA and OPG enhances the prognostic significance of each biomarker considered individually.

Immunohistochemical staining shows a trend toward increased survival in tumour specimens with high OPG-immunoreactivity

To assess whether the effect of OPG serum concentrations on survival reflects a tumour-derived increased synthesis of OPG, OPG immunoreactivity was assessed in a subgroup of

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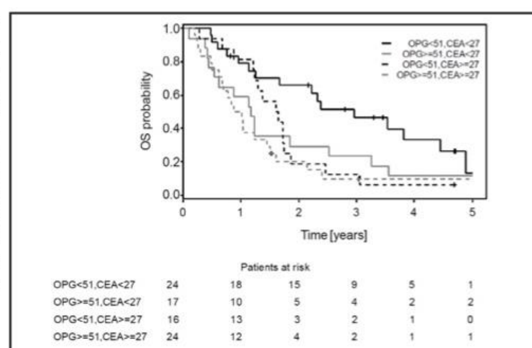

Fig. 2. Patients' stratification by combined assessment of CEA and OPG defines a patients' population with poor outcome. Survival of patients according to serum levels of both OPG and CEA. Kaplan-Meier curves represent overall survival according to: both OPG and CEA "high" serum levels, low-OPG and high-CEA, low-CEA and high-OPG and both OPG and CEA "low" serum levels. In graphs, censored cases are indicated by a cross.

33 tissue specimens from primary tumours of the same patients' collective. Immunohistochemical evaluation was performed by assigning OPG staining scores ranging from 0 to 3+ (Fig. 3A-D). A trend towards a poorer outcome was observed in patients with high OPG-staining (2+ and 3+) in comparison to patients with low staining intensity (0 and 1+, p=0.055) (Fig. 3E, Table 6). However, no correlation was found between serum OPG and OPG-immunoreactivity in matched histological specimens (p=0.47).

Table 4. Survival of stage IV patients after stratification acc. to OPG and CEA median concentration

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
OPG (ng/ml)				
<51	31/40	1.8	1.3-3.0	0.013
≥51	36/41	1.0	0.7-1.2	
CEA (ng/ml)				
<27	31/41	2.2	1.1-3.3	0.014
≥27	36/40	1.2	0.9-1.6	
OPG/CEA				
<51/<27	16/24	3.0	1.2-4.4	0.015
<51/≥27	15/17	1.2	0.4-2.5	
≥51/<27	15/16	1.6	1.2-1.8	
≥51/≥27	21/24	0.9	0.6-1.5	

Table 5. Multivariate analysis of survival according to CEA and OPG serum levels

		HR	95% CI	P
OPG	≥51 vs <51	1.68	1.03-2.75	0.0385
CEA	≥27 vs <27	1.69	1.03-2.79	0.0397

Discussion

Our assessment of a cohort of patients with metastatic colorectal cancer shows for the first time that high serum OPG has a prognostic significance in mCRC patients which is independent of the well-established prognostic value of CEA. Our data are in agreement with previous immunohistochemical findings provided by Tsukamoto and colleagues [11], who found that OPG staining was increased in tumours of patients with metastasic disease and was associated with poorer prognosis. Our results are also in keep with the Tromsø study, a large Norwegian study which prospectively investigated a large population cohort showing that serum OPG is associated with increased risk of developing cancers of gastrointestinal origin and that OPG predicts cancer-related mortality [14].

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This data also confirm the very recent findings by Meltzer et al. showing that high baseline OPG tends to correlate with poor survival in the neoadjuvant treatment setting of rectal cancer [12].

Our data are instead inconsistent with the observations reported by Kim and colleagues [13] who found that low immunohistochemical staining intensity for OPG correlated with hepatic metastasis formation and poor outcome. Such results were corroborated by the high degree of methylation found in the promoter region of OPG in cancer cells and by *in vitro* experiments showing decreased MMP-2 and VEGF-A in response to incubation with recombinant OPG. These data show that beyond the postulated role of OPG in apoptosis resistance, OPG might play different roles yet to be defined e.g. in cell proliferation and angiogenesis. In addition, these data suggest that hypermethylation is a mechanism contributing to OPG regulation in addition to the beta-catenin-driven transcription previously reported by us [10].

Independently of possible additional roles of OPG in tumour biology, however, the discrepancies between the observations by Kim et al [13]. and ours on the effect of OPG on patients' survival may be attributable to differences in size and characteristics of the investigated collective and to the different methods used, and in particular to the utilization of immunohistochemistry to assess OPG in tissue specimens vs. ELISA-based assessment of OPG in serum. OPG has been shown to be expressed not only by cancer cells

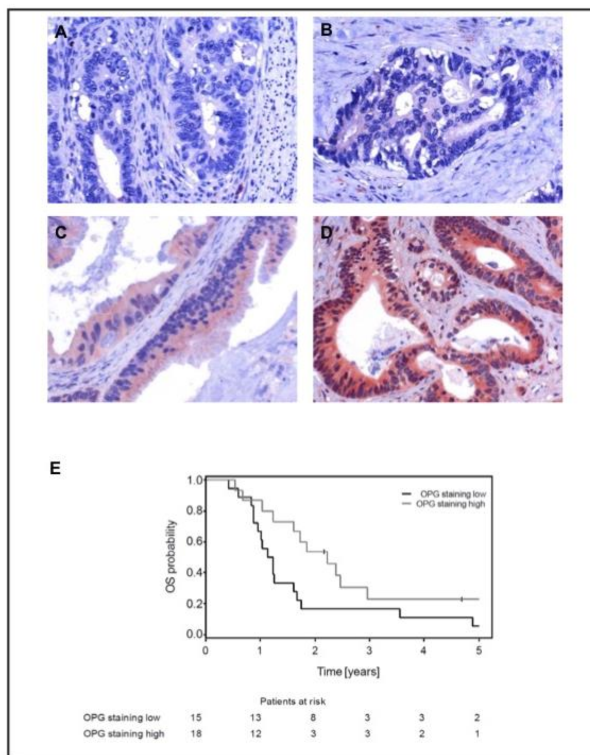

Fig. 3. Immunohistochemical staining of OPG shows a trend toward an increased survival in OPG-high tumors. Representative negative staining of OPG in tumor tissue (A) and (B-D) of increasing staining intensity of OPG (1 to 3+). Original magnification ×400. (E) Survival of patients according OPG staining as defined by high vs. low staining intensities.

Table 6. Survival according to immunohistochemical staining of OPG

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
OPG staining intensity				
low	11/15	2.2	1.0-3.0	0.055
high	17/18	1.2	0.9-1.6	

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but also by cells of the tumor microenvironment, ([15, 16] and reviewed by Goswami and Sharma-Walia [9]); assessment of serum OPG has therefore the advantage of accounting for OPG deriving also from other sources than the tumour cells (e.g. blood vessels and immune cells [9]). Furthermore, measurement of OPG in serum is less influenced by the investigator-related variability of immunohistochemical investigation, and is likely more representative than immunohistochemical assessment of OPG in biopsies from single tumour lesions, which can be influenced by clonal effects and the tumour heterogeneity typical of late-stage tumours. The lack of correlation between serum OPG and immunohistochemical staining of OPG from the subset of matched tissues samples in our cohort might reflect these factors.

Our data therefore reinforce the notion of OPG as marker of poor survival in late-stage colorectal cancer patients. Our report is consistent with the proposed role of the TRAIL-system in carcinogenesis [3-5], with previous observations from different tumour entities [6-8], with the recent report on pre-therapeutic baseline levels of OPG in rectal carcinoma patients [12], and with data from a large prospective epidemiological Norwegian study showing that OPG in serum correlates with cancer-related mortality [14].

The additional recent finding by Meltzer et al. that increasing OPG levels during treatment correlate with a favourable prognosis [12] suggests that OPG may have properties which deserve to be further investigated. In particular, additional studies should assess whether changes in OPG concentration during therapy play a functional role in determining response to treatment or rather reflect increased release of OPG from tumours responding to chemotherapy or radiation-treatment.

Confirming a biological significance of OPG in the development of colorectal cancer could open potential therapeutic perspectives: the discovery of a different role of OPG within the OPG–RANKL–RANK system led to the development of denosumab, which is employed to prevent the consequences of bone fragility in patients with bone metastases [17]. In a similar way, antibodies targeting OPG might be used as cancer treatment in tumours overexpressing OPG.

Conclusion

In summary, our paper has some limitations due to the fact that immunohistochemical and genetic characterization and treatment data could not be retrieved for all individuals of this cohort. However, our pilot study is to our knowledge the first report on the prognostic effect of OPG in pre-therapeutic sera of metastatic colorectal cancer patients and warrant prospective investigation of OPG in serum of patients in different tumour stages and therapeutic settings.

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Disclosure Statement

The authors have no conflicts of interest to declare

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6 Publikation II

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Cell-free circulating ALU repeats in serum have a prognostic value for colorectal cancer patients

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Abstract.
BACKGROUND: Carcinoembryonic antigen (CEA) is the only established serum biomarker for colorectal cancer (CRC). To facilitate therapy decisions and improve the overall survival of CRC patients, prognostic biomarkers are required.
OBJECTIVE: We studied the prognostic value of five different cell free circulating DNA (fcDNA) fragments. The potential markers were ALU115, ALU247, LINE1-79, LINE1-300 and ND1-mt.
METHODS: The copy numbers of the DNA fragments were measured in the peripheral blood serum of 268 CRC patients using qPCR, the results were compared to common and previously described markers.
RESULTS: We found that ALU115 and ALU247 fcDNA levels correlate significantly with several clinicopathological parameters. An increased amount of ALU115 and ALU247 fcDNA fragments coincides with methylation of HPP1 ($P < 0.001$; $P < 0.01$), which proved to be a prognostic marker itself in former studies and also with increased CEA level (both $P < 0.001$). ALU115 and ALU247 can define patients with poor survival in UICC stage IV (ALU115: HR = 2.9; 95% Cl 1.8–4.8, $P < 0.001$; ALU247: HR = 2.2; 95% Cl 1.3–3.6; $P = 0.001$). Combining ALU115 and HPP1, the prognostic value in UICC stage IV is highly significant ($P < 0.001$).
CONCLUSIONS: This study shows that an increased level of ALU fcDNA is an independent prognostic biomarker for advanced colorectal cancer disease.

Keywords: Colorectal cancer, biomarkers, fcDNA, prognosis, ALU repeats

Abbreviations

CEA	Carcinoembryonic antigen
CRC	Colorectal cancer
fcDNA	cell free circulating DNA
UICC	Union for International Cancer Control
TNM	Tumor site and size, Lymph node involvement, Metastatic spread
APC	Adenomatous polyposis coli gene
CIN	chromosomal instability
EGFR	epidermal growth factor receptor
TGFB	transforming growth factor beta
qPCR	real-time quantitative polymerase chain reaction
HLTF	Helicase-like transcription factor
HPP1	Hyperplastic polyposis 1
ALU	Arthrobacter luteus
LINE	long interspersed elements

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1. Introduction

Colorectal cancer (CRC) is the second most common cancer in Europe with almost 500,000 new cases in 2018 and is responsible for the second most cases of cancer-related deaths in men and women [13]. This high incidence, particularly in western Europe, may reflect changes in lifestyle such as an increased prevalence of obesity and great variability in diet and physical activity across all European countries [3]. Although great efforts have been undertaken to improve cancer treatment and new therapy options find their way into clinical practice, survival of patients is still poor, especially in metastatic cases [22].

CRC carcinogenesis is characterized by genetic and epigenetic changes, which can be attributed to different signaling pathways. Basically, development of colorectal cancer through mutation in or loss of the adenomatous polyposis coli gene (APC) follows the adenoma-carcinoma sequence. Subsequently, different pathways lead to cancer development. Amongst others, the most frequent pathway in 65–70% of the cases is the Chromosomal instability (CIN) – pathway, where mutations of P53 and KRAS activate different pathways in turn, such as Wnt, MYC, EGFR (epidermal growth factor receptor), TGFB (transforming growth factor beta) and Akt signaling [1,37].

Currently, the most valuable prognostic tool for cancer patients is the UICC (Union for International Cancer Control) TNM (Tumor site and size, Lymph node involvement, Metastatic spread) staging classification [6]. Although it is the only valid marker in predicting patient outcome [4], the TNM system is surgically oriented and neither integrates tumor biology nor differentiates the metastatic stage [32]. For this reason, TNM system is not able to specify the individual risk of a given patient [38]. Biopsies taken from the tumor also cannot reflect tumor biology properly due to genetic heterogeneity of the tumor tissue [55]. Nomograms based on TNM classification, combined with different clinicopathological parameters such as age and race, were created to predict survival of CRC patients [30].

A prognostic marker for CRC, widely used in clinical practice, is carcinoembryonic antigen (CEA). CEA proved to be an independent prognostic marker in various studies and guidelines recommend measuring preoperative CEA serum levels to determine prognosis, keep patients under surveillance after curative surgery and monitor therapy in advanced diseases [11]. CEA levels vary between individuals. Therefore, CEA baseline levels must be established for each patient. Although CEA determination is also recommended in the metastatic disease [10], Wanebo et al. reported that there was no correlation between CEA level and survival in metastatic disease [56].

Cell-free DNA is circulating in the blood stream in different concentrations. Compared to normal individuals, the amount of cell free circulating DNA (fcDNA) is higher in the blood of cancer patients [2] and is also increased in metastasized disease compared to nonmetastatic patients [21,28]. Tumor-related nucleic acids as potential biomarkers for malignancies have been intensively studied recently [16,24]. Circulatory microRNAs are also the subject of current research and have even been demonstrated to distinguish between HCV associated fibrosis, cirrhosis, and hepatocellular carcinoma [15,58]. Sequencing of plasma DNA revealed that fcDNA represents the complete tumor genome as it can be released from either the primary tumor, metastasis, or apoptotic circulating tumor cells. Apoptosis, necrosis and even active secretion have been discussed as possible sources of tumor DNA, but the mechanism of release of fcDNA is still incompletely understood [54].

fcDNA fragments can be measured using real-time quantitative polymerase chain reaction (qPCR), which offers a quick, cost-effective, and robust method for quantification of the fcDNA fragments of interest. Alternative approaches compared are microarray-based linear amplification or Illumina sequencing of fcDNA [14,47].

Notably, epigenetic alterations proved to be a promising predictive and prognostic tool. We previously described the prognostic value of methylation of various genes in free circulating DNA, namely helicase-like transcription factor (HLTF) and hyperplastic polyposis 1 (HPP1) [40]. It could be demonstrated that HLTF/HPP1 methylation status corresponds with shorter survival and therefore turned out to be a prognostic marker in UICC stage IV.

In addition to DNA methylation, DNA integrity has already been discussed as diagnostic and prognostic marker for colorectal cancer [57] based on the idea that the length of nucleic acids released from cancer cells differs from the DNA of non-malignant apoptotic cells [33].

Non-LTR (long terminal repeats) retrotransposons, which include short interspersed elements (SINEs) like the ALU repetitive sequence and long interspersed elements (LINEs), represent almost one third of the whole genome [8]. ALU elements are characterized by having a cleavage site for the Arthrobacter luteus (ALU)

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restriction endonuclease [19]. SINEs are shorter than LINEs and contain about 300bp. These non-coding DNA elements have function in regulating transcription and gene expression [51].

European population is yet not well considered in studies concerning fcDNA as a biomarker for cancer. The prognostic and diagnostic value of ALU repeats as biomarkers has been shown for different types of cancer, such as breast cancer, non-small-cell lung cancer and colorectal cancer, and across different populations, such as American and Egyptian [12,46,52,53].

LINE1 has lately been discussed not only as a biomarker but also therapeutic drug target as it seems to have a regulatory impact on gene expression in cancer cells using different pathways of carcinogenesis. Furthermore, promoter methylation, transcription, translation, and retro transposition are different activities LINE1 is said to perform in cancer cells [26,43]. LINE1 as a prognostic and diagnostic biomarker has been investigated in different types of cancer such as colorectal cancer, gastric cancer, and breast cancer, in addition to the Japanese mostly in the American population [9,25,49].

Human cells contain about 200 mitochondria, which possess their own genomic material. Due to its molecular characteristics such as an inefficient DNA repair system, mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than nuclear DNA. Thus, DNA mutations accumulate easily and lead to mitochondrial dysfunction, which is associated with tumorigenesis. Through activation of the Toll-like 9 receptor (TLR9) pathway, inflammatory processes are initiated, which provide a condition for cancer development [29,36].

Furthermore, copy numbers of circulating mtDNA have been discussed as a biomarker for different types of cancer such as hepatocellular carcinoma in an Egyptian population [18], lung cancer in a Chinese population [7] or head and neck squamous cell cancer in Northeast India [27].

Using mtDNA with its simple structure and a higher copy number than nuclear DNA allows a sensitive detection and exact quantification even of very low concentrations of circulating DNA in serum and other body fluids. Thus, mtDNA is ideally suited as biomarker.

The aim of this study was to evaluate the prognostic value of cell free circulating DNA fragments in colorectal cancer patients with metastasized and non-metastasized disease. We selected five potential markers, namely ALU115, ALU247, LINE1-70, LINE1-300 and ND1-mt, which were able to discriminate between CRC patients with benign and malignant tumors and healthy individuals in blood samples of a British patient population [34] and compared the results with common tumor markers as CEA and other predictive tools, e.g. the HLTF and HPP1 methylation status [40].

2. Methods

2.1. *Patients and serum samples*

Male and female patients over the age of 18 years were recruited from the Medical Department 2, Ludwig-Maximilians-University Munich and were diagnosed by histopathological examination of biopsies taken during colonoscopy or sigmoidoscopy using the valid WHO-classification at that time. Blood serum samples from 268 patients were taken before any therapeutic interventions and processed blinded to patient data in the central laboratory using routine procedures. All subjects underwent medical treatment at the Ludwig-Maximilians-University of Munich. Exclusion criteria were pretreatment of the current cancer disease, clinical history of any other cancer and simultaneous tumors. Staging was based on postoperative pathology findings or, for inoperable patients, on diagnostic imaging, namely CT of thorax and abdomen.

All samples were centrifuged at 2,000 g at 4°C for 10 minutes. Aliquots were stored at −80°C. We previously described the prognostic value of the HLTF/HPP1 methylation status in an almost identical sample set [40]. The study was approved by the ethical committee of the Medical Faculty of the University of Munich (project number 22-0873).

2.2. *DNA isolation*

After thawing the serum aliquots at room temperature, genomic DNA was isolated from 100 μl of each serum sample using the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Product No. 11858874001, Mannheim, Germany) according to the manufacturer's instructions. The isolated DNA was eluted in 50 μl of elution buffer, diluted 1:40 and stored at 4°C until quantification of DNA. Peripheral blood leucocytes DNA from the blood of a healthy volunteer was isolated [35]. DNA concentration was determined by measuring absorbance at 260 nm and 280 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

2.3. *Quantitative polymerase chain reaction*

Two different fluorescence-based real-time qPCR assays were performed. The primer sequences for all

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markers are shown in Table S1 [9,29,49,53]. We confirmed *in silico* that these primers form neither unwanted secondary structures nor primer dimers using the software Primer Express v2.0 (Applied Biosystems). Focusing on the amplification of ALU115 and L1-79 DNA fragments, we determined an intraassay coefficient of variation (CoV) of 3.2% and the interassay CoV was 3.0% for ALU115. The corresponding values for L1-79 were 5.3% (intraassay CoV) and 2.8% (interassay CoV).

To quantify the ALU repeats, the following reaction mixture was used: 1 × Phusion buffer, 1 × Q-Solution, 4 μl genomic DNA, 200 μM deoxynucleotide triphosphate mixture, 2 mM $MgCl_2$, 0.5 μM forward Primer, 0.5 μM reverse Primer and 0.02 U/μl Phusion® High-Fidelity DNA Polymerase. Sybr Green was used as DNA binding dye. Quantitative PCR was done in a Mastercycler ep realplex[4] (Eppendorf, Hamburg, Germany) with precycling heat activation of the polymerase at 98°C for 30 s, followed by 40 cycles of 98°C for 30 s, 62°C for 10s and 72°C for 15 s. The reaction mixture for LINE1-79, LINE1-300 and ND1-mt consisted of 1 × PCR buffer, 1 × Q-solution, 4 μl genomic DNA, 200 μM deoxynucleotide triphosphate mixture, 0.5 μM forward Primer, 0.5 μM reverse Primer, 0.02 U/μl Paq5000 DNA Polymerase.

Real-time PCR was performed using the following conditions: 95°C for 120 s, followed by 40 cycles of 95°C for 20 s, 62°C for 20 s and 72°C for 15 s.

For each fcDNA of interest, a standard curve was generated using leucocyte DNA. A single standardised solution of leucocyte DNA was used as a standard curve reference in each qPCR run. Mean values across triplicates were used for further analysis. For each serum sample, the number of fcDNA copies was normalized to the sample volume.

2.4. Statistical analysis

Comparisons of marker values between different clinico-pathological groups were done using the Wilcoxon-Mann-Whitney test in case of two groups and the Jonckheere-Terpstra test in case of three or more groups.

Survival analysis was done with Kaplan-Meier survival curves with UICC stage 1 to 3 and UICC stage 4 being analyzed separately. For calculation of overall survival, the period between the date of diagnosis of the primary tumor and the date of death respectively end of follow-up was included. Hazard ratios (HRs) for univariate and multivariate analysis of overall survival were estimated using Cox's regression model. All statistical analyses were performed with SAS 9.3 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Correlation of DNA markers with clinicopathological parameters and DNA methylation status

The characterization of our study population is shown in Table 1. The population consists of 142 female and 126 male patients with cancer in cecum, ascending, transverse and descending colon (46%), sigmoid colon (20%) and rectum (34%). 45% of the patients had positive regional lymph nodes. Distant metastases were present in 83 patients. UICC stage I tumors was 21%, UICC stage II 26%, UICC stage II 22% and UICC stage IV 31% of our patient population.

First, we quantified the copy numbers of each marker using qPCR. The median concentrations are shown in Table S2. Common clinicopathological parameters were analyzed for a relationship with the copy numbers of the candidate biomarkers (Table 1). The amount of ALU115 and ALU247 fcDNA showed a significant correlation with tumor size ($P = 0.050$ and $P = 0.038$; respectively Fig. 1a–b) and also with nodal status ($P < 0.001$ and $P = 0.008$; respectively Fig. 1c–d). In metastasized colorectal cancer, the copy numbers of ALU115 and ALU247 were significantly higher than in non-metastasized disease ($P = 0.005$ and $P < 0.001$; respectively Fig. 1e–f). Furthermore, UICC stage correlated highly significantly with the copy number of both ALU fragments ($P < 0.001$; respectively Fig. 1g–h). Related to tumor grade, the amount of L1-79 was significantly higher in grade 3 and 4 than in grade 1 and 2 ($P = 0.022$). L1-79 and ND1-mt fragments significantly increased in patients above age 65 years ($P = 0.036$ and $P = 0.023$).

Besides the association with clinical parameters, we also analyzed the correlation of our markers with carcinoembryonic antigen (CEA) as a widely used tumor marker and the methylation status of HLTF and HPP1, which have already been demonstrated to be prognostic markers for CRC [40]. Carcinoembryonic antigen is more efficient in discriminating between good and poor prognosis when individualized cutoff values are used [23]. In this study, two different CEA levels served as cutoff values: The lower concentration (2.5 ng/ml) represents the 95th percentile of healthy

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Table 1
Correlation between copy numbers/CEA concentration/methylation status and clinical features of our study population

Clinicopathological variables	Alu115			Alu247			L1-79			L1-300			ND1-mt		
	Median no. of copies	IQR	p-values	Median no. of copies	IQR	p-values	Median no. of copies	IQR	p-values	Median no. of copies	IQR	p-values	Median no. of copies	IQR	p-values
Sex															
Male ($n = 142$, 53%)	640	1,588		635	1,267		324	602		165	278		480	693	
Female ($n = 126$, 47%)	729	1,693	n.s.[a]	722	1,687	n.s.[a]	359	616	n.s.[a]	194	379	n.s.[a]	366	617	n.s.[a]
Age															
< 65 years ($n = 135$, 50%)	624	1,270		505	1,238		280	578		162	290		497	732	
⩾ 65 years ($n = 133$, 50%)	771	1,896	n.s.[a]	839	1,635	n.s.[a]	437	650	0.036	199	315	n.s.[a]	440	504	0.023
Localisations															
Colon ($n = 124$, 46%)	618	1,695		604	1,373		295	573		180	337		492	669	
Sigmond ($n = 52$, 20%)	689	1,321		895	1,687		353	735		150	252		321	559	
Rectum ($n = 92$, 34%)	712	1,612	n.s.[a]	581	1,168	n.s.[a]	374	553	n.s.[a]	181	269	n.s.[a]	409	667	n.s.[a]
Tumor size															
T1 ($n = 15$, 5%)	685	1,001		562	1,183		228	650		192	372		467	736	
T2 ($n = 59$, 22%)	432	657		505	942		295	426		141	276		388	721	
T3 ($n = 160$, 60%)	761	1,936		716	1,684		334	583		166	311		442	711	
T4 ($n = 34$, 13%)	958	2,527	0.050	774	3,269	0.038	574	807	n.s.[a]	249	496	n.s.[a]	487	467	n.s.[a]
Nodal status															
N0 ($n = 146$, 55%)	491	845		522	1,045		288	650		164	276		478	727	
N1 ($n = 69$, 26%)	1,078	2,729		847	1,772		417	604		212	381		383	652	
N2 ($n = 49$, 19%)	878	2,624	< 0.001	568	3,732	0.008	333	389	n.s.[a]	165	296	n.s.[a]	386	534	n.s.[a]
Metastasis															
M0 ($n = 185$, 69%)	613	942		515	983		296	592		167	302		477	727	
M1 ($n = 83$, 31%)	1,199	1,199	0.005	1,294	1,294	< 0.001	363	637	n.s.[a]	196	334	n.s.[a]	325	494	n.s.[a]
UICC stage															
I ($n = 57$, 21%)	478	639		562	996		281	485		185	282		414	772	
II ($n = 69$, 26%)	382	819		462	916		241	723		163	301		494	736	
III ($n = 59$, 22%)	917	2,531		665	1,288		394	527		203	311		450	675	
IV ($n = 83$, 31%)	1,199	2,796	< 0.001	1,294	3,945	< 0.001	363	637	n.s.[a]	196	334	n.s.[a]	325	494	n.s.[a]
Tumor grade															
G1 and G2 ($n = 142$, 55%)	626	1,277		581	1,230		293	546		171	291		446	665	
G3 and G4 ($n = 115$, 45%)	752	1,665	n.s.[a]	716	1,918	n.s.[a]	398	605	0.022	163	310	n.s.[a]	405	686	n.s.[a]
CEA															
< 2.5 ng/ml ($n = 116$, 43%)	508	980		482	1,086		298	543		154	288		447	802	
⩾ 2.5 ng/ml ($n = 152$, 57%)	836	2,313	< 0.001	884	2,157	< 0.001	371	655	n.s.[a]	198	324	n.s.[a]	429	617	n.s.[a]
< 27 ng/ml ($n = 217$, 81%)	613	1,181		562	1,192		312	590		165	288		460	666	
⩾ 27 ng/ml ($n = 51$, 19%)	1,349	2,897	0.003	1,401	3,823	< 0.001	538	725	0.008	234	544	n.s.[a]	325	642	n.s.[a]
HLTF															
Negative ($n = 227$, 85%)	628	1,435		597	1,311		316	576		162	292		423	677	
Positive ($n = 41$, 15%)	935	2,688	n.s.[a]	1,122	3,125	0.014	637	1,087	0.032	237	435	0.027	490	620	n.s.[a]
HPP1															
Negative ($n = 218$, 81%)	613	1,057		585	1,186		323	576		165	290		442	681	
Positive ($n = 50$, 19%)	2,261	4,241	< 0.001	1,413	3,755	0.002	477	895	n.s.[a]	229	457	n.s.[a]	400	686	n.s.[a]

[a] p-value not significant.

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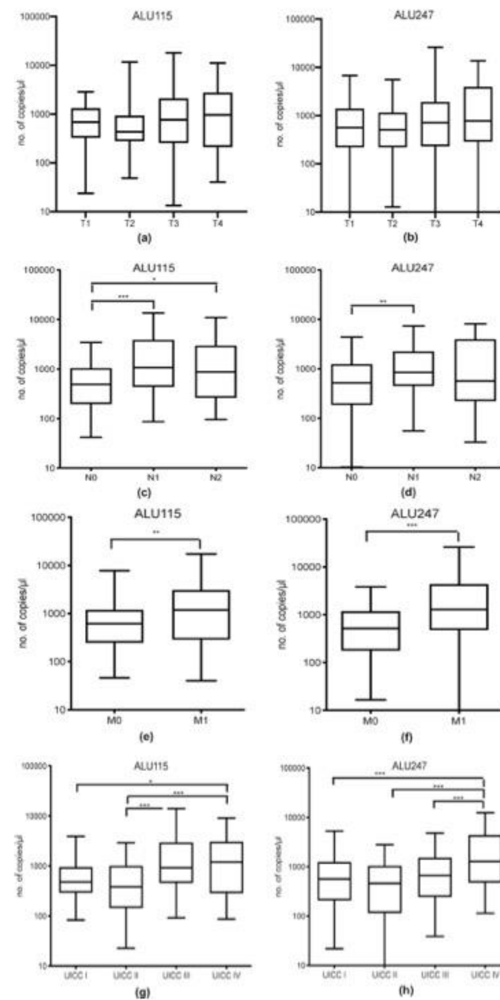

Fig. 1. Correlation of copy numbers with clinicopathologigal parameters.

persons in the study population, the higher cutoff value (27 ng/ml) is based on the median CEA-concentration of UICC stage IV cases. Copy numbers of ALU115 and ALU247 showed a highly significant correlation with CEA levels above 2.5 ng/dl (both $P < 0.001$). The amount of ALU115, ALU247 and L1-79 fragments correlated significantly with CEA levels above 27 ng/ml. Three markers correlated with the methylation status of HLTF: ALU247, L1-79 and L1-300 showed significantly higher copy numbers when HLTF was methy-

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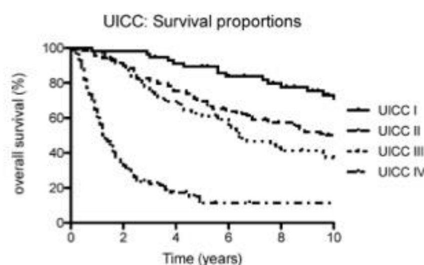

Fig. 2. Overall survival in association with UICC stage

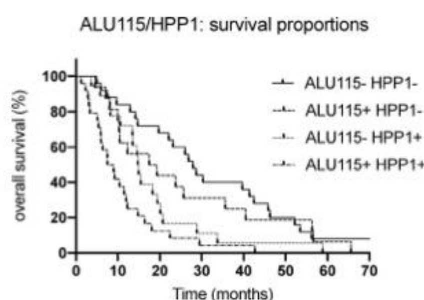

Fig. 3. Survival proportions with combinations of ALU115 and HPP1.

lated ($P = 0.014$, $P = 0.032$ and $P = 0.027$). We also found a significant correlation of ALU115 and ALU247 with methylation of HPP1 ($P < 0.001$ and $P = 0.002$).

3.2. Prognostic role of fcDNA biomarkers in overall survival

Next, we were interested in the prognostic value of raised copy numbers of ALU115, ALU247, L1-79, L1-300 and ND1-mt.

Overall survival in association with our potential markers was analyzed for all patients in all UICC stages (Fig. 2). Kaplan-Meier survival statistics were carried out with the median copy number as cutoff-value. Additionally, quartiles of the marker levels were used as cutoff-values for survival analysis. The results are shown in Table 2. The amount of ALU115 fcDNA fragments in serum of cancer patients showed a highly significant correlation with overall survival with both cutoff-values ($P = 0.004$; $P = 0.001$). Thus, median surv4ival declined with increasing copy numbers of ALU115. The same relationship was observable for ALU247 fcDNA. Overall survival declined from 9.6 years to 4.3 years when ALU247 copy number was above the median ($P = 0.003$). Using quartiles for survival statistics, there was even a highly significant decrease in survival ($P < 0.001$). Furthermore, an increase of L1-79 fcDNA fragments correlated with shorter survival ($P = 0.041$). Patients with L1-300 fcDNA above the cutoff values died earlier ($P = 0.041$; $P = 0.047$). Kaplan-Meier survival analysis did not reveal prognostic information of ND1-mt fcDNA.

Second, overall survival of patients in stage UICC IV according to the five potential biomarkers was determined separately (Table 3). Determination of survival in UICC stage IV subset was done using the median copy number of each marker as cutoff value. The amount of ALU115 fragments showed a highly significant correlation with survival in UICC stage IV ($P < 0.001$, Fig. 1a). Median survival was 2.2 years for copy numbers below and only 1.0 years for copy numbers above the cutoff value. ALU247 also turned out to be able to discriminate between poor and good prognosis in metastasized disease ($P = 0.001$, Fig. 1b). Likewise, increased copy numbers of L1-79 and L1-300 fragments above the cutoff values (360/μl; 170/μl) significantly correlated with survival ($P = 0.005$, Fig. 1c; $P = 0.001$, Fig. 1d). There was no statistically significant correlation observable for the amount of ND1-mt fragments and patient survival in UICC stage IV.

3.3. Multivariate survival analysis

The five fcDNA sequences, the methylation markers HLTF and HPP1, which already have proved their prognostic value for CRC in former studies of our group [39], and CEA were included in a multivariate Cox model. Only ALU115 (HR = 2.754; 95% CI 1.662–4.565; $P < 0.001$) and HPP1 (HR = 1.925; 95% CI 1.187–3.121; $P = 0.008$) turned out to be independent prognostic markers for CRC in UICC stage IV (Table 3).

Survival statistics with combinations of both markers showed highly significant differences in survival ($P < 0.001$). When both markers were negative (meaning the copy numbers were below the cutoff-value), median survival in UICC stage IV was 2.4 years. With ALU115 negative and HPP1 positive, patients survived 1.5 years. When ALU115 was positive and HPP1 negative, median survival was 1.2 years. With both markers being positive, patients survived only 0.7 years (Fig. 3).

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Table 2
Overall survival in UICC stages I–IV

	Cutoff value (copy no./μl)	n	Median survival (years)	95%-CI	p-value
Alu115					
< median	668	134	9.6	5.4–13.5	0.004
⩾ median	668	134	5.2	3.0–6.4	
1. quartile	< 262	67	10.6	4.8–n.d.	0.001
2. quartile	262–667	67	9.6	4.1–n.d.	
3. quartile	668–1,899	67	7.6	4.3–9.9	
4. quartile	⩾ 1,900	67	2.8	1.2–5.6	
Alu247					
< median	675	134	9.6	6.4–12.1	0.003
⩾ median	675	134	4.3	2.8–6.3	
1. quartile	< 234	66	11.7	6.7–n.d.	< 0.001
2. quartile	234–674	68	7.8	4.1–10.6	
3. quartile	675–1,656	67	9.0	4.6–n.d.	
4. quartile	⩾ 1,687	67	2.1	1.2–3.6	
LINE1-79					
< median	342	134	8.0	5.6–11.7	0.041
⩾ median	342	134	4.9	3.2–7.2	
1. quartile	< 112	67	8.0	4.8–n.d.	n.s.
2. quartile	112–341	67	8.7	3.3–11.7	
3. quartile	342–715	67	4.9	3.1–7.8	
4. quartile	⩾ 716	67	4.7	1.6–9.0	
LINE1-300					
< median	168	134	8.0	5.4–11.7	0.041
⩾ median	168	134	5.2	3.0–7.5	
1. quartile	< 75	67	5.4	3.4–9.6	0.047
2. quartile	75–167	67	11.4	6.0–n.d.	
3. quartile	168–385	67	4.3	2.5–8.0	
4. quartile	⩾ 386	67	5.9	2.5–9.9	
ND1-mt					
< median	438	134	5.6	4.1–8.7	n.s.
⩾ median	438	134	6.7	4.4–9.6	
1. quartile	< 167	67	5.4	3.7–8.7	n.s.
2. quartile	167–437	67	6.0	2.8–12.7	
3. quartile	438–832	67	6.4	2.6–9.7	
4. quartile	⩾ 833	67	7.6	5.2-15.1	

n.d.: upper limit of 95% CI could not be calculated due to insufficient number of events in this group.

4. Discussion

It is estimated that incidences of the 14 most common cancer types including CRC will increase in the future [42]. Furthermore, according to these projections concerning cancer incidences and cancer-related deaths in Germany, CRC will be the third most common cause of cancer-related deaths in men and women by 2030. Even though overall survival of colorectal cancer patients is improving continuously [5], among patients with metastatic disease no progress in survival can be observed [31]. Therefore, new therapeutic strategies would be needed, individually adapted to a patient's medical conditions, his or her medical condition considering his comorbidities and commensurate to the risks of any medical treatment. To improve the overall survival of UICC stage IV patients and evaluate the individual risk of each patient avoiding under- or over-treatment, it is important to establish new prognostic tools.

In this study, we were able to identify ALU115 fcDNA as an independent prognostic marker for the survival of CRC patients. The prognostic value of ALU115 could be demonstrated in the overall survival analysis for all tumor stages. Notably, survival of patients with UICC stage IV was significantly shorter when ALU115 was above the cutoff value, which was determined by the median copy number of ALU115 fragments in the blood serum of UICC stage IV patients. Compared to CEA as a common clinical marker, ALU115 correlated stronger with clinicopathological parameters as well as survival in UICC stage IV.

In order to maximize the prognostic value, combination of markers could be a useful approach. In a previous study, we demonstrated the prognostic value of HPP1 methylation for UICC stage IV in the identical patient population [40]. Combining HPP1 and ALU115

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Table 3
Overall survival of patients in stage UICC IV; multivariate analysis with biomarker combinations (including HLTF and HPP1)

	Univariate analysis						Multivariate analysis	
	n	Median survival (years)	95%-CI	p-value	Hazard ratio	p-value	Hazard ratio	p-value
Alu115					2.9 (1.8–4.8)	< 0.001	2.8 (1.6–4.6)	< 0.001
< 1000	41	2.2	1.5–3.4					
⩾ 1000	42	1.0	0.7–1.2	< 0.001			–	–
Alu247					2.2 (1.3–3.6)	0.002		
< 1290	41	2.0	1.2–3.4				–	–
⩾ 1290	42	1.1	0.8–1.3	0.001				
LINE1-79					2.0 (1.2–3.2)	0.006	–	–
< 360	41	1.7	1.2–2.5					
⩾ 360	42	1.0	0.8–1.3	0.005			–	–
LINE1-300					2.3 (1.4–3.9)	0.001		
< 170	41	1.9	1.1–6.6				–	–
⩾ 170	42	1.1	0.8–1.3	0.001				
ND1-mt					1.3 (0.8–2.1)	0.290	–	–
< 325	41	1.5	0.9–2.1					
⩾ 325	42	1.2	0.9–1.9	0.289			–	–
CEA					1.7 (1.1–2.8)	0.028		
< 27 ng/ml	43	1.8	1.1–2.5				–	–
⩾ 27 ng/ml	40	1.1	0.8–1.5	0.027				
HLTF					2.4 (1.4–4.3)	0.003	–	–
Neg.	65	1.6	1.2–2.1					
Pos.	18	0.8	0.5–1.0	0.002			–	–
HPP1					2.1 (1.3–3.3)	0.003	1.9 (1.2–3.1)	0.008
Neg.	43	1.7	1.2–2.4					
Pos.	40	0.9	0.6–1.2	0.003				

in Kaplan Meier survival analysis, we were able to define three different risk groups. With both markers being positive, the overall survival was substantially shorter than with only one marker respectively none of both markers being positive. In summary, both markers have a similar prognostic value, but in combination, the prognostic value is even higher.

The analysis of fcDNA in serum as a non-invasive, low-risk "liquid profiling" [44] provides various information about the primary tumor characteristics. Therefore, it is a helpful instrument for understanding metastatic progression and hence a potential prognostic device for cancer patients. Here, sampling of the specimens can easily be repeated to, for example, monitor the amount of fcDNA over a longer period of time.

However, it hast to be taken into consideration that concentrations of cell-free DNA are not only higher in the blood of CRC patients. Shaban et al. evaluated the diagnostic and prognostic value of ALU fcDNA in different cancer types and found that ALU levels in different cancers (type or stage) are strongly varying in the included studies due probe processing, analysis and calculation of concentrations [45]. Levels of fcDNA can also be increased due to chronic inflammations, e.g. systemic lupus erythematosus [50], ischemic heart disease, diabetes mellitus [48] and is discussed to be influenced by psychosocial and physical stress conditions [20]. Furthermore, significant differences in ALU concentrations were found in psychiatric disorders like schizophrenia, major depressive disorder and alcohol-induced psychotic disorder [41] due to neuroinflammatory processes.

In our study population, besides sex and age, only clinicopathological data concerning CRC was recorded. Comorbidities, notably inflammatory conditions, or other types of cancer, were not captured in our study in contrast to Mead et al. [34], where patients were screened by history, patient letters and recent blood tests. However, we had no evidence that ALU concentrations were confounded by other diseases as our data were intrinsically conclusive and we had strong correlations with other markers such as CEA and HLTF/HPP1.

Further studies studying the suitability of fcDNA as a biomarker should consider the general health conditions of the study patients to exclude confounding comorbidities.

Considering the fact that our ALU markers significantly correlate with UICC stage, tumor size, nodal status and metastasis, we expect a decrease of fcDNA concentration after surgery due to a loss of tumor burden. Former studies showed inconsistent data: Bhangu et al. could detect a slight increase of fcDNA concentration after surgery, whereas ALU115 and ALU247/115 significantly and progressively decreased after surgical

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treatment in a study by Hao et al. [17] An increase of fcDNA markers shortly after surgery may be caused by mechanical and thermic manipulation as well as destruction of both healthy and tumor cells. So, the period of time between surgery and measurement of fcDNA concentrations seems to play an important role and should be chosen thoroughly. The changes of fcDNA concentrations in blood after different types of medical treatment, i.e., surgery, chemo- and immunotherapy, should be subject of further investigations as this question was not addressed before and new therapies are developing quickly.

In order to introduce more biomarkers to clinical practice, future studies need to consider the combination of markers and avoid selection bias. Sample proceeding should be standardized to reduce costs, accelerate analysis and generate a comparable standard. Most importantly, results need to be validated in larger studies in various populations, followed by prospective trials.

In conclusion, according to our findings, the main advantage of ALU115 fcDNA over common markers is to improve the prognostic assessment for CRC patients with metastatic disease.

Author contributions

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Preparation of the manuscript: Anzinger Isabel, Herbst Andreas.
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Supervision: Herbst Andreas, Kolligs Frank.

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Supplementary data

Supplementary Table S1
Primer sequences

fcDNA marker	Forward primer	Reverse primer
ALU115	5'-CCTGAGGTCAGGAGTTCGAG-3'	5'-CCCGAGTAGCTGGGATTACA-3'
ALU247	5'-GTGGCTCACGCCTGTAATC-3'	5'-CAGGCTGGAGTGCAGTGG-3
L1-79	5'-AGGGACATGGATGAAATTGG-3'	5'-TGAGAATATGCGGTGTTTGG-3
L1-300	5'-ACACCTATTCCAAAATTGACCAC-3'	5'-TTCCCTCTACACACTGCTTTGA-3
ND1-mt	5'-CACCCAAGAACAGGGTTTGT-3'	5'-TGGCCATGGGATTGTTGTTAA-3

Supplementary Table S2
Median concentrations of fcDNA biomarkers/CEA; HLTF/HPP1: no. of patients with positive methylation status

	ALU115	ALU247	L1-79	L1-300	ND1-mt	CEA	HLTF	HPP1
Median concentration	668/μl	675/μl	342/μl	168/μl	438/μl	3.2 ng/ml	–	–
(range)	(13–18,016)	(1.7–26,096)	(1.6–3,925)	(4.4–3,980)	(8.5–6,644)	(1.0–3,945)	–	–
No./% positive	–	–	–	–	–	–	41/15	50/19
IQR	1636	1423	605	311	666	11.1	–	–

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Affidavit

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Anzinger, Isabel Sabine Ursula
Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

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