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Klinik und Poliklinik für Allgemein-, Viszeral-, Thorax-, Gefäß- und Transplantationschirurgie

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Isolation und Analyse präemptiver Biomarker beim Kolorektalkarzinom

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Synopsis

In etwa jede achte Krebserkrankung in Deutschland betrifft den unteren Gastrointestinaltrakt als kolorektales Karzinom (colorectal carcinoma, CRC). Laut den aktuellen Daten des Robert Koch-Institutes aus dem Jahr 2016 erkrankten in diesem Jahr 58.290 Patienten an einem CRC und die Inzidenz lag bei 41,25 je 100.000 Einwohner (Zentrum für Krebsregisterdaten, Robert Koch-Institut). Trotz außerordentlichen Fortschritten der Grundlagen- und klinischen Wissenschaft liegt das Gesamt-5-Jahresüberleben bei jedoch nur ca. 62%. Bedeutende Fragestellungen zu Biologie der Tumore und deren Metastasierungsmechanismen sind bis dato noch nicht abschließend geklärt. Sowohl die Diagnostik als auch die Evaluierung des häufig multimodalen Therapiekonzeptes basieren auf allgemeinen Leitlinien, welche anhand großer Studienprotokolle unter Berücksichtigung heterogener Patientenkollektive verfasst wurden. Einen individualisierten Ansatz gibt es beim CRC bis dato nicht. Die Grundlage zur Therapieempfehlung und der Prognoseabschätzung beruht immer noch auf rein histopathologischen Kriterien des Tumors – der TNM-Klassifikation der International Union Against Cancer (UICC). Verlässliche Parameter im Sinne von Biomarkern zur Risiko- und Prognosestratifizierung könnten einen wesentlichen Fortschritt in diesem Bereich ermöglichen.
Die hier vorliegende Habilitationsschrift setzt sich mit ebendiesen individualisierten diagnostischen Ansätzen auseinander.

Eine wesentliche Grundlage zur Entstehung des CRC beschrieben Vogelstein und Fearon 1990 anhand ihrer propagierten Theorie der Adenom-Karzinom-Sequenz (1). Nebst Umweltfaktoren fördern intrinsische Mutationen maßgeblich den Ablauf dieser Sequenz hin zu einem Manifesten Karzinom (2). Die Klassifikation dieser Karzinome erfolgt maßgeblich anhand der Eindringtiefe in die Wandschichten des Gastrointestinaltraktes. Abgesehen von der Beschaffenheit des Tumorgewebes, gemessen anhand des Differenzierungsgrades (Grading), findet keine weiter Analyse des Tumors selber statt. Grundsätzlich werden aber der Mitose- und Proliferationsrate in malignen Tumoren eine zentrale Rolle in der Karzinogenese zugerechnet (3). Der Proliferationsindex wird bereits bei diversen soliden Karzinomen routinehaft angewandt (4,5). Mittels monoklonaler Antikörper gerichtet gegen proliferationsassoziierte Antigene wie Ki-67 und proliferating cell nuclear antigen (PCNA) wird die Proliferationsrate quantifiziert (6). Des weiteren werden als klinisch relevante Proliferationsmarker in der Literatur die Topoisomerase II alpha (topo II alpha) und das minichromosome maintanance protein 6 (MCM6) aufgeführt (7–9). Beide Proliferationsmarker konnten auch bereits durch unsere Arbeitsgruppe als klinisch hoch potente Stratifizierungsmerkmale hinsichtlich des Gesamtüberleben (overall survival, OS) bei dem

Mantelzell-Lymphom identifiziert werden (10,11). Topo II alpha übernimmt eine zentrale Rolle der topologischen Modellierung der DNA im Rahmen der DNA-Replikation bei der Zellteilung. Als ein Enzym katalysiert es die Entwindung der Doppelhelix, sodass die Helicase die antipolaren DNA-Stränge trennen kann (12). Das MCM6 gehört zu dem MCM-Komplex, welcher für die Einleitung der DNA-Replikation verantwortlich ist. Dieser MCM-Komplex dient als Helicase der Entdrillung der DNA-Stränge, sodass die Transkription eingeleitet werden kann (13). Immunhistochemisch (IHC) können die Proliferationsmarker in Formalin fixiertem Gewebe gefärbt und so quantifiziert werden: Anti-Ki-S4 gegen topo II alpha und Anti-Ki-MCM6 gegen MCM6.

Aufbauend auf den bereits bestehenden Ergebnissen und der offensichtlich klinisch-prognostischen Relevanz der Proliferationsmarker topo II alpha und MCM6, war das Ziel der ersten hier vorliegenden Arbeit diese prognostische Wertigkeit auf eine repräsentativ große und heterogene Kohorte von CRC Patienten zu übertragen (14).

In dieser retrospektiven Analyse wurden insgesamt 619 Patienten mit einem histologisch gesicherten CRC eingeschlossen. Von sämtlichen Patienten wurden Tumorproben formalinfixiert und in Paraffin eingebettet. Hiernach wurden 3-5µm dicke Schnitte zur IHC angefertigt. Die Färbung erfolgte mittels einem in unserer Arbeitsgruppe etablierten Protokoll. Die Quantifizierung der IHC gefärbten Proliferationsproteine erfolgte standardisiert – nach Vorbereitung wurden lichtmikroskopisch fünf Visusfelder ausgewertet. 500 Tumorzellen je Feld wurden gezählt und Bereiche mit sehr hoher Expression der Proliferationsprotenie wurden als Hot-Spots gewertet.

In einem ersten Schritt wurden beide Proliferationsmarker gesondert analysiert und hinsichtlich klinisch pathologischer Charakteristika ausgewertet. Als cut-off Wert der topo II alpha Expression wurde 50% [positive topo II alpha Zellen pro Patient] festgelegt. Insgesamt standen 430 Patientengewebeproben zur Analyse hierfür zur Verfügung. In dieser Subkohorte konnten wir zeigen, dass Patienten mit einem lokal begrenzten Tumorstadium (UICC I und II) höhere Expressionsraten von topo II alpha aufwiesen verglichen mit Patienten mit lokal fortgeschrittenem Tumorleiden (UICC III) ($p=0,029$) und interessanterweise fand sich kein statistisch signifikanter Zusammenhang zwischen dem histologischen Grading und der Expression von topo II alpha. In Bezug auf das OS dieser Patienten zeigten diejenigen mit niedrigen Proliferationsraten (<50% topo II alpha Expression) ein deutlich schlechteres Outcome (median OS 52,9%) verglichen mit den Patienten mit hohen Proliferationsraten (≥50% topo II alpha Expression) (median OS 69,2%) ($p<0,001$). In einer Studie von Shaojun et al. welche den Proliferationsmarker Topoisomerase I in einer Kohorte von CRC Patienten analysierten konnte ein ebengleicher Zusammenhang zwischen hohen Expressionsraten und einem vorteilhaften OS und krankheitsfreien Überleben (disease free survival, DFS) gezeigt werden (15).

Zur Subanalyse der MCM6 Expressionsrate und der klinischen und prognostischen Korrelation wurde der cut-off für MCM6 bei 85% festgesetzt und Patienten entsprechend klassifiziert. 570 Patientenproben standen hierfür zur Verfügung. Auch hier zeigen Patienten in hohen Tumorstadien (UICC III und IV) statistisch signifikant geringere Expressionsraten von MCM6 positiven Tumorzellen verglichen mit Patienten mit lokal begrenztem Tumorausmaß (UICC I und II) ($p=0,012$). Hinsichtlich des OS und DFS konnten wir zeigen, dass auch hier niedrige MCM6 Expressionsraten mit einem unverkennbar schlechteren Outcome einhergingen ($p=0,008$ bzw. $p<0,001$). In der Literatur finden sich hierzu konträre Publikationen. In einer bioinformatischen Studie von Zhou et al. konnte die hohe Expression von MCM3, als ein Geschwisterprotein des MCM-Komplexes, als deutlich negativer prognostischer Marker in CRC Patienten identifiziert werden (16). Interessanterweise zeigte hier die Subanalyse der UICC Stadien und so der systemischen Tumorlast des Patienten verglichen mit der MCM6 Proliferation und dem OS und DFS keine statistisch signifikanten Erkenntnisse.
Abschließend analysierten wir die direkte Korrelation von topo II alpha und MCM6. Insgesamt beobachteten wir eine deutlich höhere Expression von MCM6 (Mittelwert 82,8%) verglichen mit topo II alpha (Mittelwert 52%) ($p<0,001$) was zu erwarten war, da MCM6 auch in der frühen Phase der Zellteilung (G1-Phase) involviert ist und so stochastisch eine höhere Anzahl an Zellen positiv ist (17). In der Korrelationsanalyse bestätigte sich dann ein positiver Zusammenhang zwischen der Expression beider Marker ($r=0.433$, $p<0.001$).
Schlussfolgernd kann die Analyse von Proliferationsproteinen hinsichtlich der Prognoseabschätzung und so dem Einsatz als Biomaker eines CRC Patienten bedingt hilfreich sein. Die Studienlage in anderen Tumorentitäten ist homogener (7,18,19) und folgernd der direkte Nutzen der Bestimmung offensichtlicher. Ein großer Nachteil der gewebebasierten Analyse von Biomarkern analog zu der oben beschriebenen Arbeit ist die Rigidität und Invasivität des Verfahrens. Zur Analyse muss über ein invasives Verfahren (Endoskopie mit Tumorbiopsie, Operation) eine relevant große Tumorprobe gewonnen werden können, um eine hinreichend valide Untersuchung zu ermöglichen. Limitierend hierfür können häufig ein reduzierter Patientenzustand oder insbesondere im Rahmen der Patientennachsorge die medizinische Indikation zur erneuten Intervention seien. Um diese Problematik zu umgehen wird von dem Konzept der „Liquid Biopsie“ gebrauch gemacht. Hier werden Bestandteile des Blutes (zirkulierende Zellen, DNA/RNA Fragmente oder Proteine) quantifiziert, analysiert und hinsichtlich der prognostischen und präemptiven Relevanz ausgewertet (20,21).

Unsere Arbeitsgruppe beschäftigt sich intensiv mit im Blut zirkulierenden Tumorzellen (circulating tumour cells, CTC). Thomas Ashworth gilt als Erstbeschreiber der CTC – in einer venösen Blutentnahme eines metastasierten Patienten beschrieb er bereits 1869 mittels Lichtmikroskopie Tumorzellen welche er als CTC klassifizierte (22).

Primärtumoren besitzen die Fähigkeit des invasiven Wachstums. Zellen können während der Progression einen mesenchymalen, migrierenden Phänotyp mit der Befähigung zur Intravasation erlangen. Diese im peripheren Blutsystem frei zirkulierenden Tumor-Einzelzellen werden dann als CTC bezeichnet. Die Isolation und der Nachweis von CTC stellt eine außerordentliche Herausforderung in der Anreicherung und Detektion dieser „ultra-rare-events“ im Blut, nebst diversen und mannigfachen Leukozytenpopulationen, dar (23). Es wird angenommen, dass eine CTC pro 10^6 – 10^7 Blutzellen vorkommt (24).
Der Nachweis von CTC bei Karzinomen des Gastrointestinaltraktes ist seit langem etabliert, dennoch fehlt ein standardisiertes Verfahren hierzu. Bis dato wird eine Reihe verschiedener Methoden zur Detektion der CTC angewandt (25,26) . Prinzipiell muss vor dem Schritt der CTC Detektion jedoch noch eine Anreicherung erfolgen, um die Zahl der „verunreinigenden“ Leukozyten zu minimieren. Diese ist im Allgemeinen auf zwei Wegen zu erreichen – Marker abhängig und Marker unabhängig. Das Marker basierte Verfahren bedient sich CTC spezifischer Antigene, welche häufig epithelialen Ursprungs sind. Hiergegen gerichtete Antikörper sind vielfach mit Eisenpartikeln gekoppelt, welche folgend magnetisch isoliert werden können und so eine immuno-magnetische Zellanreicherung ermöglichen. Eine weit verbreitete Methode stellt das CellSearch®-System dar. In zahlreichen Studien konnte der prognostische Nutzen von hiermit identifizierten und quantifizierten CTC nachgewiesen werden (27–30). Zu der Marker unabhängigen Methodik der Zellanreicherung werden physikalische Eigenschaften der CTC aufgegriffen. Im Vergleich zu der normalen im Blut zirkulierenden Zellpopulation sind Tumorzellen deutlich größer und weisen eine ähnliche Dichte zu den Leukozyten auf. Somit ergeben sich zwei gängige Anreicherungsansätze – Zum einen eine Separation der Zellen auf Grund der Größe (z.B. filterbasiert) und zum anderen entsprechend der Dichte (z.B. Dichtegradientenzentrifugation). Die Detektion der CTC beruht ebenfalls auf zwei maßgeblich unterschiedlichen Verfahren. Es gilt das immunologisch zytologische Verfahren von dem molekulargenetischen Vorgehen abzugrenzen. Im Weiteren beschäftigten wir uns nun mit Anreicherungs- und Detektionsmethoden von CTC und deren klinisch prognostischen Eigenschaften.

In der zweiten hier vorliegenden Arbeit befassen wir uns mit bereits etablierten aber auch einem innovativen, eigenständig entwickelten Vorgehen zur CTC Anreicherung und Detektion. Die Fragestellung der Arbeit war es anhand eines CRC Patientenkollektives verschiedene Schritte der Anreicherung und der Detektion von CTC zu validieren und analysieren (31). In diese prospektive Studie wurden insgesamt 57 Patienten mit einem histologisch gesicherten CRC eingeschlossen. Das Patientenkollektiv war hinsichtlich der Verteilung Kolon- vs. Rektumkarzinom und der Tumorlast gemessen an der UICC-Klassifikation repräsentativ heterogen.

Als in unserer Arbeitsgruppe eigen entwickeltes Verfahren zur Detektion von CTC wurde ein teilautomatisiertes Mikroskopieverfahren mit dem cell-imager NYONE® angewandt. Nach einem Anreicherungsschritt der CTC mittels Dichtegradientenzentrifugation (hier wurden standardisierte CPT® Vacutainer verwendet) erfolgte die Immunfluoreszenzfärbung (IF) der CTC mit fluoreszenzkonjugierten Antikörpern gegen CTC spezifische epitheliale Antigene: Anti-pan-Cytokeratin (anti-pan-CK), anti-epithelial cell adhesion molecule (anti-EpCAM), anti-human epidermal growth factor receptor 2 (anti-Her2) und anti-epidermal growth factor receptor (anti-EGFR). Da während der Dichtegradientenzentrifugation grundsätzlich mononukleäre Zellen des peripheren Blutes (peripheral blood mononuclear cells, PBMC) angereichert werden, in deren Fraktion auch die CTC zu finden sind, gilt es eine Negativselektion der Leukozyten anzuwenden. Hierzu erfolgte die Fluoreszenzfärbung mit leukozytenspezifischen Antikörpern gegen Rezeptor-Typ Tyrosin-Proteinphosphatase C (CD45). Hiernach erfolgte die teilautomatisierte Mikroskopie zur Detektion und Quantifizierung der CTC mittels dem NYONE® Mikroskop. Für speziell dieses Analyseverfahren standen 44 Patientenproben zur Verfügung. In 16 Proben (36,4%) gelang der immunzytologische Nachweis von ≥1 CTC. Insgesamt beobachteten wir, wenn auch statistisch nicht signifikant, eine höhere Frequenz von CTC in Patienten mit lokal fortgeschrittener oder bereits metastasierter Erkrankung (UICC III und IV).

In einer zweiten Subanalyse beschäftigten wir uns mit einem neuartigen Filtrationsverfahren zur Anreicherung von CTC (isolation by size of epithelial tumour cells – ISET). Wie bereits erwähnt sind CTC größer als die sonstigen Blutzellen und diese Eigenschaft wird hier genutzt, um eine Anreicherung der CTC zu ermöglichen (21378321). Die Blutprobe durchläuft nach Aufbereitung eine Filtermembran in welche Poren einer definierten Größe (7,5 und 6,5µm) eingestanzt sind. Die signifikant größeren CTC verbleiben auf dem Filter und können so quantifiziert und ggf. weiter analysiert werden. Ein großer Vorteil dieses Verfahrens ist die marker-unabhängige Isolierung und einfache Aufbereitung der zu analysierenden Probe. Die Probe wurde nach Anreicherung der CTC durch eine Zytopathologin begutachtet und die CTC-Last eines jeden Patienten so quantifiziert. Insgesamt standen 31 Patientenproben für diese Subanalyse zur Verfügung, und in 100% der Proben gelang der zytologische Nachweis von CTC. Zur Verifikation der CTC Fraktion wurde exemplarisch eine IF-Färbung angefertigt. Zellen welche DAPI+, pan-CK+ und CD45- waren galten als CTC. Im Mittel über die gesamte Studienpopulation diagnostizierten wir 3,01 CTC/ml Blut. Dies war eine signifikante Steigerung im Vergleich zu dem zuvor beschrieben teilautomatisierten Mikroskopieverfahren mittels dem NYONE und der antikörperbasierten Detektion von CTC. In den Subanalysen der weiter stratifizierten Patientenkohorte zeigte sich eine statistisch signifikante Häufung von CTC in den höheren Tumorstadien. Patienten mit lokal fortgeschrittenem oder bereits systemischem

Tumorleiden (UICC III, respektive IV), zeigten im Mittel 4,09 CTC/ml, wobei in Proben von Patienten in frühen Tumorstadien (UICC I+II) 2,35 CTC/ml detektiert wurden (p=0.039).
Als drittes Verfahren wurde der in unserer Arbeitsgruppe bereits seit längerem etablierte molekularbiologische Nachweis mittels einer PCR angewandt. Die Blutproben wurden standardisiert entsprechend eines in unserer Arbeitsgruppe aufgestellten Protokolls aufgearbeitet. Binnen vier Stunden nach der Blutentnahme erfolgte die Anreicherung der PBMC-Fraktion mittels Dichtekissenzentrifugation über Ficoll. Nach Präparation der RNA und cDNA erfolgte die Applikation einer RT-qPCR. Bereits in Vorarbeiten konnte das Zytokeratin 20 (CK20) als ein hochspezifischer und sensitiver Marker zur Detektion von CTC bei dem CRC identifiziert werden (32,33). In dieser Analyse erfolgte zur semiquantitativen Betrachtung der CTC Fraktion druch die CK20 RT-qPCR und es standen 41 Patientenproben zur Verfügung. In 33 Patienten gelang der Nachweis von CK20 positiven CTC und der Mittelwert der Expression von CTC lag bei 3.11 [Expression Units, EU]. Am ehesten auf Grund der geringen Fallzahl erbrachte die Analyse der CTC Quantität, korreliert mit klinischen Parametern, keine statistisch signifikanten Ergebnisse.
Eine Theorie der diskrepanten Ergebnisse unserer vergleichenden zytologischen Nachweisverfahren (im Vergleich geringe Detektionsrate mit der NYONE-Methode und hohe Quote von CTC in der filterbasierten ScreenCell-Kohorte) könnte der markerabhängige Ansatz des NYONE-Verfahrens sein. Wie bereits erwähnt wird eine Heterogenität der CTC Population angenommen und beschrieben (34). Im Rahmen der Absiedlung einzelner Tumorzellen aus dem Verband des überwiegend epithelial gekennzeichneten Primärtumors kommt es zu einer Transition der Tumorzelle vom epithelialen Charakter hinzu einem mesenchymalen Phänotypen, welcher später eine Intravasation und so den ersten Schritt der Metastasierungskaskade erlaubt (35). In diesem Prozess der Epithelial-Mesenchymalen-Transition (EMT) können spezifische Markerproteine, welche häufig zur Detektion von CTC eingesetzt werden (EpCAM, Cytokeratine, etc.) herunter reguliert oder gar verloren gehen. Folgernd kann die antikörper-basierte Quantifizierung von CTC aus dem peripheren Blut somit unter Umständen deutlich unterschätzt werden (36,37) . Um diese Problematik anzugehen setzten wir mehrere Antikörper zur CTC Detektion mittels dem NYONE Verfahren ein. Als epitheliale Antikörper verwendeten wir anti-EpCAM, anti-pan-CK und anti-Her2, als mesenchymaler Antikörper wurde anti-EGFR eingesetzt. Dennoch erbrachte das ISET-Verfahren eine höhere Ausbeute an CTC. Dies kann als klarer Vorteil des ISET angesehen werden. Es ermöglicht eine antikörper- und somit markerunabhängige Anreicherung und zugleich Detektion von CTC.
Insgesamt konnten wir nach Abschluss dieser experimentellen Arbeit festhalten, dass diese von uns entwickelten innovativen Verfahren im klinischen Alltag praktikabel und anwendbar sind.

In der dritten Arbeit sollte auf diesen Ergebnissen aufbauend das Verhalten und die Kinetik von CTC im perioperativen und Langzeitverlauf studiert werden (38). Der Einfluss einer operativen Prozedur auf die kurzfristige Kinetik von CTC wurde bereits beschrieben. Eine Operation und Manipulation sorgt für einen signifikanten Anstieg von CTC, wohingegen nach kurzer Zeit bereits eine Normalisierung oder möglicherweise ein Abfall der CTC beschrieben wurde (39). Jedoch finden sich in der Literatur nur wenige Beschreibungen über das Verhalten von CTC über einen langfristigen Verlauf.

Auch in der hier vorliegenden Arbeit werden die in unserer Arbeitsgruppe bereits etablierten Verfahren zum Nachweis von CTC angewandt. Der Nachweis erfolgte sowohl immunzytologisch mit dem semi-automatisierten Mikroskopieverfahren des NYONE® cell-imagers, als auch mit der molekularen Methode, der semi-quantitativen CK20 RT-qPCR. Insgesamt wurden 49 Patienten mit einem histologisch bestätigten kolorektalen Karzinom in diese Studie eingeschlossen. Alle Pateinten wurden onkologisch korrekt operiert und der Beobachtungszeitraum betrug 12 Monate postoperativ. Wenn möglich, wurde jeder Patient in beide Analyseverfahren eingeschlossen. Die Blutentnahmezeitpunkte wurden vor Studienbeginn definiert: t0 – prä-OP, t1 – ein Monat post-OP, t2 – 3 Monate post-OP, t3 – 6 Monate post-OP, t4 – 9 Monate post-OP, t5 – 12 Monate post-OP.

In die Kohorte zur immunzytologischen Analyse konnten insgesamt 44 Patienten eingeschlossen werden. Auch hier zeigte sich wie bereits in der vorangegangenen Studie eine insgesamt geringe Anzahl an CTC in den Blutproben und über den Studienverlauf zeigte sich bei Betrachtung der Gesamtkohorte keine statistisch signifikante Veränderung in der Frequenz der gemessenen CTC. Die Operation und damit Tumormanipulation ergab in unserer Studie keinen Effekt auf die CTC-Häufigkeit (0,89 CTC in t0 und 1,18 CTC in t1; p=ns) unmittelbar postoperativ. Dennoch, nach weiterer Stratifizierung der Kohorte entsprechend CTC positiver und negativer Patienten prä-OP (t0), ergab sich eine auffallende Eigenschaft der mittels NYONE® gemessenen CTC. Patienten welche prä-OP negativ für CTC waren, zeigten 4 Wochen postoperativ (t1) einen statistisch signifikanten Anstieg von CTC (MW 0,00 in t0, MW 0,93 in t1; p=0.023). In dieser Subgruppe von Patienten hat die Operation einen relevanten Effekt auf die CTC Quantität. Da für unsere Analyse auch die klinischen Angaben hinsichtlich der adjuvanten Therapie und Nachsorge vorlagen, analysierten wir die CTC Korrelation mit den vorliegenden Follow-Up Daten. Patienten wurden in Gruppen entsprechend der adjuvanten Therapie aufgeteilt: Nachsorge – CTX- und adjuvante Chemotherapie – CTX+. Hier zeigten Patienten in der CTX+ Gruppe überraschenderweise über den gesamten Nachbeobachtungszeitraum eine höhere Anzahl von CTC verglichen mit der CTX- Subgruppe.

Zur molekularen Diagnostik mittels CK20 RT-qPCR wurden 47 Patienten eingeschlossen. Auch mit der PCR zur Quantifizierung der CTC sowohl perioperativ (t0 vs. t1), als auch über

die gesamte Phase der Studie, ergab sich kein statistisch signifikanter Nachweis in der Gesamtkohorte aller untersuchten Patienten. Zur weiteren Stratifizierung der Kohorte, analog zu der NYONE® Subanalyse, wurde eine klinisch relevante Trennlinie (cut-off) zum indirekten Nachweis der CTC eingefügt. In einer Vorabreit, welche später in dieser Schrift präsentiert und diskutiert wird, gelang unserer Arbeitsgruppe der Nachweis eines klinisch relevanten cut-offs zum Nachweis von CTC in CRC Patienten, ab welchem ein statistisch signifikant prognostisch negatives Outcome für den Patienten zu erwarten ist (40). Dieser cut-off wurde hier angewandt und ein Einfluss des chirurgischen Eingriffes konnte eindeutig belegt werden. Patienten mit einer präoperativ (t0) hohen Last an CTC die somit über dem cut-off lagen, zeigten einen statistisch signifikanten Abfall von CTC nach vier Wochen (t1) (MW 6,49 [EU] in t0 vs. MW 2,68 [EU] in t1; $p<0.001$), wohingegen es im weiteren Beobachtungszeitraum zu einem erneuten geringen Anstieg von CTC kam. Demgegenüber kam es erstaunlicherweise in der Subgruppe von Patienten mit einer geringen CTC-Last bei t0 zu einem signifikanten Anstieg von CTC postoperativ bei t1 (MW 1,17 [EU] in t0 vs. MW 4.36 [EU] in t1; $p=0.047$). Erst im späteren Verlauf der Studie kam es in der Kontrolle neun Monate postoperativ (t4) zu einem Abfall der CTC. Auch die Gruppe der Patienten in der die CK20 RT-qPCR zum CTC Nachweis angewandt wurde, wurde entsprechend Nachsorge vs. adjuvante Chemotherapie aufgeteilt. Interessanterweise beobachteten wir zu allen Studienzeitpunkten eine geringere Last an CTC in der CTX+ verglichen mit der CTX- Subgruppe. Diese Ergebnisse stellen sich konträr zu denen mit dem IF mikroskopischen Verfahren dar. Eine Erklärung hierfür lieferte auch eine weiter Analyse nicht, sollte jedoch Bestandteil folgender Projekte werden.

Zuletzt analysierten wir die einzelnen individuellen Patentenverläufe hinsichtlich der CTC Last und Frequenz gemessen mit den hier angewandten Nachweismethoden. Hier zeigte sich ein interessantes Fallbeispiel einer Patientin mit einem Sigmakrzinom. Nach histopathologischer Aufarbeitung ergab sich ein UICC II Stadium ohne Risikofaktoren und die Patientin wurde leitliniengerecht der Nachsorge zugeführt. Zur Analyse der CTC-Last wurde auch hier der cut-off zur CTC Quantifizierung mittels der PCR Methode angewandt. Prä- und unmittelbar postoperativ zeigte die CTC Last sich unterhalb des cut-off Wertes. Im Weiteren Nachsorge- und Beobachtungszeitraum zeigte sich dann ein Anstieg der CK20 positiven CTC auch über den cut-off und 13 Monate nach der R0-Sigmaresektion entwickelte die Patientin ein Lokalrezidiv. Dieses Fallbeispiel zeigt eindrücklich die mögliche Bedeutsamkeit und den klinischen Nutzen der CTC Analyse als Biomarker im postoperativen Rahmen. Bereits in anderen Tumorerkrankungen wie der Leukämie, wird das Konzept der Biomarkeranalyse im Rahmen der Nachsorge und Beobachtung der minimalen Resterkrankung (minimal residual disease, MRD) angewandt (41) und PCR Verfahren sind standardisiert und etabliert (42). Bei Patienten mit einem CRC gibt es bislang keine klinisch etablierte und vereinheitlichte Methode zum Nachweis einer MRD. In den letzten Jahren findet sich in der Literatur dennoch

zunehmend die Analyse von zirkulierender und zellfreier Tumor-DNA (cf- und ctDNA). Mithilfe hochsensitiver Nachweisverfahren (digitaler PCR und next-generation-sequencing, NGS) werden im Blut lösliche DNA-Fragmente identifiziert, welche zuvor im Rahmen von Apoptose oder Nekrose von Tumorzellen in das Blutserum abgesondert wurden, nachgewiesen (21,43). Beim CRC können so tumorspezifische Mutationen wie zum Beispiel von APC und KRAS nachgewiesen und deren Quantität im Blut über „Liquid Biopsies“ beobachtet werden (44).

In der vierten Arbeit sollte der prognostische Nutzen der Detektion und Quantifizierung von CTC in frühen Tumorstadien von CRC Patienten an einer großen repräsentativen Kohorte analysiert werden (40).
Im Allgemeinen gehört das CRC als Malignom zu den Tumorentitäten, bei welchen welche in den letzten Jahrzenten signifikante Fortschritte in der Diagnostik und Therapie erzielt werden konnten, insbesondere nach Einführung der antikörperbasierten Chemotherapie unter bestimmten Umständen der Tumormutationsanalyse bei Patienten mit fortgeschrittenem Tumorleiden (45). Leitliniengerecht werden Patienten mit lokal beschränkter Erkrankung (UICC I und UICC II) keiner adjuvanten Therapie, sondern reiner klar definierter Nachsorge überführt (S3-Leilinie Kolorektales Karzinom, Version 2.1 – Januar 2019 AWMF-Register Nummer: 021/007OL). Lediglich bei Patienten im Stadium UICC II mit Risikofaktoren (Notfalloperation bei Ileus, T4-Karzinom, wenige Lymphknoten im Präparat) kann eine adjuvante Chemotherapie empfohlen werden (46). Trotz des insgesamt guten Outcome nach onkologisch korrekter Tumorresektion kommt es dennoch in einem Anteil der Patienten im Verlauf zu einer Metastasierung oder zu einem Lokalrezidiv. Bislang fehlen verlässliche Biomarker, welche in einer noch spezifischeren Weise das patientenindividuelle prognostische Risiko ausdrücken (47). Zu dieser Fragestellung sollen in unserer Arbeit CTC quantifiziert und mit klinischen Merkmalen korreliert werden.
Insgesamt wurden 381 Patienten mit einem histologisch gesicherten CRC eingeschlossen. An allen Patienten erfolgte eine präoperative Blutentnahme und diese wurde nach dem in unserer Arbeitsgruppe etablierten Verfahren zur molekularen Detektion von CTC mittels der CK20 RT-qPCR aufgearbeitet. Das Patientenkollektiv war heterogen hinsichtlich der Tumorlokalisation (Rektum vs. Kolon), der Tumorstadien (UICC I-IV) und des Geschlechtes (männlich vs. weiblich), sodass wir hier eine repräsentative Kohorte analysierten. Das mediane follow-up der Kohorte betrug 34 Monate (0-151 Monate) und das mediane Gesamtüberleben (overall survival, OS) betrug 24 Monate (0-118 Monate). Nach statistischer Analyse der gesamten Patientenkohorte konnten wir einen statistisch signifikanten Schwellenwert der CK20 Expression (also indirekt der CTC Häufigkeit) festlegen, welcher eine Stratifizierung der Patienten hinsichtlich des prognostischen Risikos gemessen an dem OS zuließ. Der Wert wurde mit 2,77 [EU] ermittelt. Patienten mit einer geringeren CK20 Expression zeigten ein

deutlich besseres Outcome in beidem, dem fünf-jahres OS und dem fünf-jahres krankheitsfreien Überleben (disease free survival, DFS) verglichen mit Patienten über dem cut-off (5y-OS: 69,6 Monate vs. 39,8 Monate, p<0,001; 5y-DFS: 66,2 Monate vs. 37,6 Monate; p<0,001). Auch in der weiterführenden Subanalyse der Tumorentitäten (Kolon- vs. Rektumkarzinom) zeigte der von uns ermittelte cut-off der CK20 Expression eine klinische Signifikanz. Patienten oberhalb des cut-offs zeigten ein statistisch hochsignifikant schlechteres OS und DFS verglichen mit Patienten mit geringer CK20 Expression. Ergänzend und entsprechend der Fragestellung unserer Arbeit analysierten wir die Patientenkohorte nun weiter und untersuchten den prognostischen Nutzen der PCR in frühen Tumorstadien. Wir gruppierten Patienten mit lokal begrenztem Tumorwachstum in den frühen Stadien UICC I+II und analysierten diese getrennt in Bezug auf die CK20 Expression und so auf die CTC-Last. Auch in dieser Gruppe konnten wir zeigen, dass Patienten mit einer hohen Anzahl an CTC, also über dem cut-off von 2,77 [EU], ein deutlich schlechteres OS und DFS haben (Hazard Ratio, HR 2.25, p=0.035 und DFS HR 2.01, p=0.047). Wie bereits erwähnt, wird der Großteil der Patienten mit einem UICC II Stadium der reinen Nachsorge überführt und Patienten in dem Stadium UICC III eine adjuvante Chemotherapie empfohlen. Gegenstand der Diskussion ist hier ein mögliches „under treatment“ der UICC II Patienten aber ebenso ein „over treatment“ der UICC III Patienten. Zur Analyse dieser Argumentation gruppierten wir UICC II Patienten in Risikopatienten mit hoher CTC-Last ≥2,77 [EU] und UICC III Patienten in „gute“ Patienten mit geringer CTC Frequenz (<2,77 [EU]) bei welchen möglicherweise die Adjuvanz eine Übertherapie bedeuten könnte. Unsere Daten zeigen, dass kein statistisch signifikanter Unterschied hinsichtlich des OS und DFS besteht. Man könnte daher schlussfolgern, dass die Chemotherapie in der Gruppe der UICC III Patienten mit geringer CTC-Last keinen prognostischen Nutzen hat und diese Patienten übertherapiert werden. Zugleich kann man nach Analyse unserer Ergebnisse aber auch Schlussfolgern, dass Patienten mit einem UICC II Stadium und hoher CTC-Last möglicherwiese von einer adjuvanten Chemotherapie profitieren könnten, da ihr OS und DFS ohne Chemotherapie vergleichbar mit dem der UICC III Patienten ist.

Mithilfe der Applikation der von uns robusten und reproduzierbaren Methode der CK20 RT-qPCR zur indirekten Quantifizierung der CTC haben wir die Möglichkeit Risikopatienten in frühen Tumorstadien zu identifizieren. Unter Anwendung eines immunozytologischen Verfahrens konnten Bork et al. bereits 2015 die klinische und prognostische Relevanz von CTC in frühen Tumorstadien (UICC I-III) zeigen (48). An einer Kohorte von insgesamt 287 Patienten wurden CTC mit dem CellSearch® System analysiert und nach statistisch multivariater Analyse stellte sich die Quantifizierung der CTC als der einzig unabhängige prognostische Marker dar. Dennoch befassen sich die meisten Studien zur CTC Detektion und Quantifizierung mit bereits metastasierten Patienten. So auch in der Studie der Kollegen. Ein

Grund hierfür ist sicherlich die sehr geringe Sensitivität der meisten CTC-Detektionsverfahren in Patienten mit geringer Tumorlast. Mit der von uns angewandten PCR gelang indes der Nachweis von CTC bereit sin den frühen Tumorstadien und erlaubte auch die klinisch relevante Stratifizierung in Risikopatienten, welche möglicherweise von einer engeren Nachsorge oder einer adjuvanten Chemotherapie profitieren könnten. Dennoch erlaubt auch diese sensitive Methode noch keine Patientenindividuelle Untersuchung. Patienten wurden in einer Subkohorte gebündelt analysiert, und die Schlussfolgerung hieraus gezogen. Insbesondere in Bezug auf einen patientenindividualisierten Therapieansatz könnten noch spezifischere Biomarker, wie bereits zuvor erwähnt im Rahmen der MRD Diagnostik bei leukämischen Erkrankungen mit der Detektion von cf- und ctDNA, angewandt werden. Diese Analyse könnte der reinen CTC Quantifizierung im Rahmen individualisierter Diagnostik und Therapiemonitoring Ansätze überlegen sein.

In der fünften hier vorliegenden Arbeit implementierten wir den Einsatz von cfDNA Analytik in unserer Arbeitsgruppe. Anhand eines Fallbeispiels (Case Report) zeigen wir die klinische Signifikanz der cfDNA als ein Biomarker der Liquid Biopsie auf (49).
cfDNA beschreibt DNA-Fragmente, welche frei löslich im Blutserum anzufinden sind. Bereits im Jahr 1948 gelang die Erstbeschreibung von cfDNA im menschlichen Blutplasma von gesunden Kontrollen und erkrankten Patienten (50). Wie bereits zuvor erwähnt wurden mehrere Wege für die cfDNA Freisetzung beschrieben, wie z.B. die Apoptose, die Phagozytose und die Nekrose (51,52) . Der Begriff der cfDNA beschreibt das Konglomerat aus DNA-Fragmenten der genomischen und der Tumor-DNA, wobei die ctDNA den Anteil der tumorstammenden DNA umfasst. Die Analyse von ctDNA erfolgt prinzipiell auf zwei Arten: (1.) Ist das Ziel die Detektion von einzelnen wenigen Punktmutationen, so wird in der Regel eine PCR (RT-PCR, dPCR, ddPCR) angewandt. Insbesondere hinsichtlich der bereits erwähnten MRD-Diagnostik kann dieses einfach zugängliche und kosteneffektive Verfahren wichtige Erkenntnisse im klinischen Verlauf des Pateinten ergeben (21). Beispielsweise konnten Lee et al. anhand einer Kohorte von 174 Patienten mit einem malignen Melanom im UICC Stadium III zeigen, dass Patienten mit dem präoperativen Nachweis von ctDNA ein deutlich schlechteres OS und DFS zeigten (53). (2.) Sollen multiple Mutationen, oder genomische Aberrationen wie chromosomale Umstrukturierungen oder Kopienzahlvariationen (copy number variation – CNV) analysiert werden, so wird die DNA-Sequenzierung (next generation sequencing – NGS) angewandt. Durch eine Exom- oder Genom-Sequenzierung (whole exome/genome sequencing – WES/WGS) können so hochspezifisch Anomalien der DNA analysiert werden. So konnten beispielhaft Tie et al. prospektiv an einer Kohorte von 230 CRC Patienten mit einem Stadium UICC II den Nutzen der ctDNA Analyse hinsichtlich des DFS zeigen (54). Patienten in welchen postoperativ noch der Nachweis von ctDNA gelang zeigten

über den Beobachtungszeitraum ein deutlich schlechteres Outcome und erhöhtes Risiko für ein Lokalrezidiv oder Metastasierung.

In unserem Case-Report analysierten wir die Blutproben eines klinisch interessanten Falles aus einer laufenden prospektiven Studie. Uns wurde ein Patient mit einem primär synchron hepatisch metastasiertem Adenokarzinom des Zökum zur Implantation eines Portkatheters vorgestellt. Bei ausgeprägt bilobulärer hepatischer Metastasierung und dem Ausbleiben von Stenosesymptomatik wurde nach interdisziplinärer Diskussion die Indikation zur primären Einleitung einer palliativen Chemotherapie gestellt. Leitliniengerecht wurde diese bei einer KRAS-Mutation mit FOLFIRI und Bevacizumab eingeleitet. Zur Mutationsdiagnostik standen uns neben Gewebeproben von zwei Zeitpunkten auch zwei Blutproben zur Verfügung. Das NGS der Tumorprobe aus der diagnosestellenden Koloskopie erbrachte Mutationen von KRAS, APC, TP53, THSD7B und eine Mikrosatellitenstabilität des Primarius. Es konnte aber ebenso eine Amplifikation eines Segmentes von Chromosom 4 mit FBXW7 nachgewiesen werden. Die erste Blutentnahme erfolgte zwei Wochen nach der Diagnosestellung während der Portimplantation. Auch hier bestätigte das NGS die Mutationen aus dem Primärtumor. Nach drei Zyklen Chemotherapie (6 Wochen später) erfolgte die zweite Blutentnahme. Hier zeigte sich erstaunlicherweise bereits ein deutlicher Abfall der Tumorallelfrequenzen aller zuvor detektierten Mutationen. Auch die chromosomale Amplifikation war nicht mehr nachzuweisen. Mutmaßlich ist es bereits schon hier zu einer deutlichen Reduktion der Tumorlast gekommen. In Woche 15, nach Beendigung von 6 Zyklen Chemotherapie, erfolgte ein Re-Staging mittels Computertomographie. Hier zeigte sich eine stable-disease des Primarius und eine Regression der hepatischen Metastasierung.

Vier Wochen später erfolgte dann die Notfalloperation im Sinne einer Hemikolektomie rechts bei einem deutlichen Tumorprogress des Primarius mit akutem mechanischem Ileus. Das NGS des Tumors ergab keine wesentliche Änderung des Mutationsprofiles verglichen mit der Biopsie der Koloskopie. Leider zog der Patient seine Einwilligung zur Blutentnahme zurück, sodass keine weiteren Analysen der ctDNA möglich waren. Nach Zweit- und Drittlinientherapie verstarb der Patient bei fortschreitender hepatischer Metastasierung acht Monate nach Diagnosestellung.

Mittels dieser Falldarstellung und der ersten Analyse und Auswertung von ctDNA eines CRC Patienten aus unserer prospektiv laufenden Studie konnten wir die technische Durchführbarkeit vom NGS in ctDNA Proben zeigen. Die Validierung hinsichtlich der Konkordanz von Mutationsprofilen unserer ctDNA NGS mit der Sequenzierung von Tumorgewebeproben konnte gezeigt und bewiesen werden.

Dieser Case-Report zeigt erneut das Phänomen der intratumoralen Heterogenität. Über die Heterogenität der Tumore findet auch in der aktuellen Literatur eine rege Diskussion statt (55–57). Nach phylogenetischer Rekonstruktion von Mutationsanalysen einzelner Tumore und

Metastasen von Nierenzellkarzinomen beschreiben Gerlinger et al. in ihrer Studie eine eindrucksvolle intratumorale Heterogenität (58). Sie Schlussfolgern, dass bestimmte Mutationen nicht in allen Tumorsegmenten nachzuweisen sind und Leiten hieraus ein Dilemma der Tumorbiopsien hinsichtlich der personalisierten Medizin ab, da diese als lediglich kleine Ausschnitte des Gesamttumors, mit hoher Sicherheit nicht die gesamtgenomische Landschaft des Tumors abbilden können. Einige, möglicherweise für die Einleitung patientenindividualisierter Therapieansätze, relevante Mutation werden unter Umständen schlicht nicht biopsiert und erfasst. Ähnliche Ergebnisse hinsichtlich des CRC konnten in mehreren Studien publiziert werden (59–62). Auch die Mutationsanalysen unseres Patienten liefert Hinweise auf eine intratumorale Heterogenität. Die Allelfrequenzen der einzelnen Mutationen der ctDNA Analyse zeigen unterschiedliche Häufungen, sodass hier von unterschiedlich gewichteten intratumoralen Klonen ausgegangen werden kann.
Abschließend zeigt unsere Falldarstellung die klinische Wertigkeit von ctDNA Analysen als Therapie-Monitoring über den Verlauf eines Patienten. Die Mutationsanalyse und die ctDNA Detektion in unserem Fall, zeigt eindrücklich, dass das Tumoransprechen auf eine Chemotherapie und so die Tumorregression auch anhand der ctDNA Untersuchung erfolgen kann.

In der sechsten vorliegenden Arbeit analysierten wir den Nutzen einer patientenindividualisierten Chemotherapie anhand einer bi-nationalen Kohorte von CRC Patienten (63).
In nahezu 25% der Patienten werden bei Erstdiagnose eines CRC bereits distante Metastasen beobachtet. Diese Patienten werden dem Stadium UICC IV zugeordnet und trotz rasch voranschreitender multimodaler Therapieansätze beträgt das 5y OS lediglich knapp 20% (64). In Deutschland erfolgt die systemisch onkologische Therapie in der Regel entsprechend der aktuellen onkologischen Leitlinie und somit als „standard-of-care – SOC“. Individualisierte Therapieansätze sind abgesehen von der Antikörpertherapie mittels EGFR-Inhibitoren bei KRAS-wildtyp Patienten nicht allgemein angewandt. Hingegen in den USA finden sich individualisierte Ansätze nach Erschöpfen der SOC-Therapie deutlich geläufiger. Aus diesem Grund war das Ziel unserer Studie zwei repräsentative Patientenkollektive hinsichtlich des Outcome zu analysieren und den klaren Nutzen einer patientenindividualisierten Therapie anhand des OS zu verdeutlichen.
Insgesamt wurden 108 Patienten mit einem lokal fortgeschrittenem oder bereits metastasierten CRC in diese Analyse eingeschlossen. Alle Patienten wurden nach der onkologischen Tumoroperation einer adjuvanten Therapie zugeführt und sämtliche Tumorproben wurden mittels NGS analysiert. Die Gesamtpopulation wurde in zwei gleiche Subgruppen gegliedert: 1. 54 deutsche Patienten mit einer SOC-Therapie; 2. 54

amerikanische Patienten welche eine individualisierte Therapie (IND) erhalten haben. Um eine Verzerrung der Ergebnisse und so eine bessere Vergleichbarkeit der Subkohorten zu erreichen, wurden die Patientengruppen hinsichtlich Alter und Geschlecht angeglichen („gematched“). Um außerdem einer Verzerrung des Mutationsprofils der Primärtumore vorzubeugen wurden beide Subkohorten hinsichtlich des Mutationsprofils analysiert und entsprechend einer Klassifikation der Kollegen Schell et al. (65) gegliedert. Hier wurden Tumore in Bezug auf ihr Mutationsprofil und die Frequenz der häufigsten Mutationen beim CRC (APC, TP53 und KRAS) klassifiziert. Dies erlaubte uns eine solide Beurteilung von Tumoren und so Stratifizierung von Patienten entsprechend ihres Risikoprofils.
Die Mutationsanalyse der Primärtumore erfolgte prinzipiell mittels NGS und dem Einsatz eines Marker-Panels der häufigsten Mutationen von soliden Krebsarten. Die Kohorte der amerikanischen Patienten wurde mit dem Foundation-One-Assay (Foundation Medicine, Cambridge, MA, USA) analysiert, und die deutschen Patienten mit dem IDT xGen Pan-Cancer panel v1.5 (Integrated DNA Technologies, Leuven, Belgien).
Nach Analyse der Patientenkollektive zeigte sich eine Kongruenz der Mutationsprofile beider Kohorten, und auch die demographische Analyse der Subgruppen zeigte eine Übereinstimmung und die nordeuropäische Abstammung der amerikanischen Patienten, sodass die Unterschiede des OS mutmaßlich auf die unterschiedlichen Therapieansätze (SOC vs. IND) zurückzuführen sind. Die amerikanischen Patienten zeigten ein im Median um 13,5 Monate (19,5 Monate vs. 33,0 Monate; $p<0,001$) verlängertes und so besseres OS verglichen mit den „gematchten“ deutschen Patienten. Um jetzt den Effekt der IND-Therapieansätze zu analysieren, wurden die amerikanischen Patienten entsprechen ihres Mutationsprofils anhand der bereits erwähnten Klassifikation nach Schell et al. (65) gegliedert und in Risikoprofile gefasst. Hier konnten wir eindeutig zeigen, dass Patienten in sogenannten high-risk-Gruppen, welche nach dem Prinzip der IND-Therapie behandelt wurden, ein im Median um 16 Monate verlängertes Überleben hatten, wohingegen Patienten in low-risk-Gruppen nicht von dieser Therapie profitierten. Auch in der multivariaten Analyse unter Einbeziehung klinischer und histopathologischer Parameter bestätigte sich diese Erkenntnis und der Nutzen der IND-Therapie bei Risikopatienten.
Hinsichtlich dieser Erkenntnis sollte erneut die intratumorale Heterogenität zur Diskussion angebracht werden. Nach der Einschätzung unserer Arbeitsgruppe zeigt diese Arbeit und die Analyse der Daten, dass eine Kombination von zytotoxischen Substanzen und individualisiert zielgerichteten Biologika im Sinne der IND-Therapie die Heterogenität des Tumors signifikant besser bewältigt.

Zusammenfassend belegt diese Habilitationsschrift den generellen Nutzen des Einsatzes von Biomarkern beim CRC. Die allgemeine Risikostratifizierung gelingt bereits anhand des

Tumorgewebes, wie wir mit den Proliferationsmarkern MCM6 und topo II alpha zeigen konnten. Nachteilig an dieser Methode ist allerdings, die Invasivität um an entsprechendes Gewebe zu gelangen und die fehlende Möglichkeit der longitudinalen Verlaufsbeobachtung über den Krankheitsverlauf des Patienten. Das Konzept der „Liquid Biopsy“ ermöglicht genau diese Schwierigkeit zu umgehen. Über eine Blutentnahme kann es gelingen im Blut frei zirkulierende Biomarker zu isolieren und analysieren. Wir konnten dieses Konzept in mehreren Publikationen zu den CTC darstellen und den klinischen Nutzen der CTC auch belegen. Ferner stellten wir in unseren Arbeiten die Diversität der Anreicherungs- und Detektionsverfahren dar und zeigten die klinische Machbarkeit dieser Methoden auf.

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Anhang

Originalarbeiten nach thematischer Reihenfolge sortiert.

1. **Hendricks A**, Gieseler F, Nazzal S, Bräsen JH, Lucius R, Sipos B, et al. Prognostic relevance of topoisomerase II α and minichromosome maintenance protein 6 expression in colorectal cancer. BMC Cancer. 2019 May 9;19(1):429.

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5. **Hendricks A**, Rosenstiel P, Hinz S, Burmeister G, Röcken C, Boersch K, et al. Rapid response of stage IV colorectal cancer with APC/TP53/KRAS mutations to FOLFIRI and Bevacizumab combination chemotherapy: a case report of use of liquid biopsy. BMC Med Genet. 2020 Dec;21(1):3.

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7. Lebenslauf, Lehrtätigkeit, Publikationsverzeichnis

8. Danksagung

9. Eidesstattliche Erklärung

Anhang – Teil 1

Prognostic relevance of topoisomerase II α and minichromosome maintenance protein 6 expression in colorectal cancer.

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Prognostic relevance of topoisomerase II α and minichromosome maintenance protein 6 expression in colorectal cancer

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Abstract

Background: Despite rising incidence rates of colorectal malignancies, only a few prognostic tools have been implemented in proven clinical routine. Cell division and proliferation play a significant role in malignancies. In terms of colorectal cancer, the impact of proliferation associated proteins is controversially debated. The aim of our study was to examine the expression of topoisomerase II α and minichromosome maintenance protein 6 and to correlate these findings with the clinical data.

Methods: Tissue samples of 619 patients in total were stained using the antibodies Ki-S4 and Ki-MCM6 targeting topoisomerase II α as well as minichromosome maintenance protein 6. The median rate of proliferation was correlated with clinical and follow up data.

Results: The expression rate of minichromosome maintenance protein 6 is significantly higher than the proportion of topoisomerase II α in tumour cells ($p < 0.001$). A high expression of both proteins coincides with a beneficial outcome for the patient, indicating a favourable prognostic marker ($p < 0.001$ and $p = 0.008$).

Conclusions: We have demonstrated that high expression rates of proliferative markers is linked to a beneficial patient outcome. According to the general opinion, a high expression rate correlates with a poor patient outcome. In this study, we were able to refute this assertion.

Keywords: Colorectal cancer, Proliferative proteins, Minichromosome maintenance protein 6, Topoisomerase II α, Prognostic marker

Background

Colorectal malignancies are a major cause of death in industrialised countries. Most colorectal neoplasms are histologically adenocarcinomas and develop through an adenoma-carcinoma sequence which was first described by Vogelstein and Fearon [1]. The development of a colorectal carcinoma depends on various factors and may often span over years before a manifest malignancy occurs. The macroscopic shape, histological type and grading seem to play key roles in the transformation process as defined by the adenoma-carcinoma sequence. Also, genetic mutations significantly affect the likelihood of colorectal cancer formation [2].

Mitosis within the neoplasia plays a key role in the histopathological analysis of the tumour. Assessment of the proliferation rate by means of proliferation markers is routinely implemented in histological diagnostics. Monoclonal antibodies against antigens associated with cell proliferation, such as Ki-67 [3, 4] and proliferating cell nuclear antigen (PCNA) are part of routine diagnosis in malignancies. Besides these mentioned proteins there are additional proliferation associated proteins, such as topoisomerase II α (Topo II α) and the minichromosome maintenance protein 6 (MCM6), that can be detected by immunohistochemistry (IHC) [5, 6]. The group of topoisomerases comprises up to four enzymes that are essential in the DNA topology and crucial for

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DNA replication [7]. By applying the monoclonal antibody Ki-S4, Topo II α can be detected by IHC [5, 8]. The prognostic significance of expressed Topo II α by Ki-S4 was shown in different studies [9–11]. High rates of expressed Topo II α correspond to an unfavourable clinical outcome. However, only a few studies comprising CRC patients have been published so far. Within these studies, fluorescent in situ hybridization (FISH) was applied to detect the expression rate of Topo II α. IHC has not been exerted to evaluate the clinical outcome of patients suffering from colorectal neoplasm yet.

Minichromosome maintenance proteins also play a key role in DNA replication of eukaryotic cells. These proteins are a part of the pre-replication complex, which binds to chromatin and therefore represent an essential role in cell division [12]. Ki-MCM6 is a specific antibody targeting MCM6 that can be used in formalin fixed tissue [6, 13, 14]. Multiple studies have verified the clinical relevance of MCM proteins as proliferation markers in malignant tumours so far [15–17]. Though, to the best of our knowledge, no investigation of the clinical relevance in terms of clinical outcome of MCM6 in colorectal carcinoma patients in a representative cohort has been published.

This publication aims to investigate the clinical relevance of topoisomerase II α and minichromosome maintenance protein 6 as proliferation markers in a representative large cohort of human colorectal carcinoma tissue. Results in terms of immunohistochemical expression are correlated to clinical follow-up data. Furthermore, it has to be investigated, whether the degree of expressed proliferation markers varies between clinical-pathological profiles.

Methods

Patients

A total of 619 patients was included in this study. All patients underwent a complete oncological resection of a histologically verified colorectal carcinoma at the Department of General and Thoracic Surgery, University Hospital Schleswig Holstein, Campus Kiel, during the period of 1994 and 2007. The resected tumour tissue was preserved at the Institute of Pathology, University Hospital Schleswig Holstein, Campus Kiel. Clinical and follow up data were gathered retrospectively. All data are shown in Table 1. The study was approved by the local ethics committee of the Medical Faculty, Christian-Albrechts University Kiel (reference no. A110/99).

Immunohistochemistry

Formalin fixed tissue embedded in paraffin was cut into 3–5 μm thin slices using a microtome (Jung, Heidelberg, Germany). The sections were transferred to covered microscope slides (Histobond, Marienfeld, Germany) at a temperature of 45–55 °C. Before staining, all slides were applied to 100% xylol for 10 min to deparaffinise the tissue. For rehydration, all slides were transferred into a descending sequence of ethanol (100, 96, 70%) for 3 minutes each.

All sections were stained using haematoxylin-eosin stain. After rehydration, the sections were incubated with 200 μl haematoxylin for 10 min and rinsed with distilled water for 10 min. The sections were then incubated in 400 μl eosin for 3 min and rinsed with distilled water. Finally, all sections were applied to an ascending sequence of ethanol (70, 96, 100%) and subsequently incubated in xylol for 5 min.

Analysis of immunohistochemical staining

All tissue sections were treated with highly specific monoclonal antibodies against the respective antigen and an indirect detection using a secondary antibody. Endogenous peroxidase was blocked by incubation of the specimens in 4 ml 30% hydrogen peroxide and 200 ml methanol. Antigen retrieval was performed by incubation in 0.01 M citrate buffer solution (pH 6.0) for 3 min at 100 °C [18]. In the next step, all sections were rinsed with water and transferred into washing buffer. All tissue samples were incubated with 100 μl of the primary antibody (detection of topoisomerase II α: Ki-S4; detection of minichromosome maintenance protein 6: Ki-MCM6; Institute for Haematopathology Kiel, University Hospital Schleswig Holstein, Campus Kiel) at room temperature for 60 min and afterwards incubated in tris-buffered saline (TBS), washed with water and then moved to TBS. The secondary antibody (Rabbit anti-mouse IgG; E354 DAKO, Hamburg, Germany) was applied at room temperature for 30 min. In the next step, slides were rinsed with water and transferred into washing buffer. The sections were stained with 100 μl DAB (Diaminobenzidin, DAKO, Hamburg, Germany) and rinsed twice with distilled water. Nucleus counter staining was achieved by hemalum (Merck, Darmstadt, Germany) incubation for 5 minutes. For dehydration purpose, all specimens were moved along an ascending incubational sequence of ethanol (70, 96 and 100%) and incubated twice in xylol.

The tissue specimens on microscopic slides were covered with Pertex (Medite, Burgdorf, Germany) and light microscopy was performed using the Axioskop 40 (Zeiss, Germany). Within each specimen 500 tumour cells in five randomly selected visual fields were examined using a cell counter (Counter AC8, Hecht AG, Sondheim, Germany) at a magnification of 400 times. Areas with exceptional high number of tumour cells were accounted separately as hot spots.

The primary antibodies Ki-S4 and Ki-MCM6 were established beforehand and the specificity was consolidated by Western blot experiments previously [8, 13].

Statistical analysis

Comparative statistical analysis of expressed proliferation markers was performed using Fisher's tests of

Table 1 Patient demographics, clinical characteristics and univariate analysis (log rank test) influencing the overall survival (OS) disease free survival (DFS)

	N (%)	OS [months]	P	DFS [months]	P
all	619 (100)				
age (years)					
< 65	303 (48.9)	n.a.	**< 0.001**	59.5	**0.005**
≥ 65	315 (50.9)	65.6		n.a.	
unknown	1 (0.2)				
sex					
male	312 (50.4)	119.1	0.961	n.a.	0.218
female	307 (49.5)	104.3		n.a.	
tumor site					
right colon	172 (27.8)	130.5	**0.010**	n.a.	0.299
left colon + rectum	439 (70.1)	69.5		n.a.	
unknown	8 (1.3)				
UICC					
I + II	297 (48.0)	154.6	**< 0.001**	n.a.	**< 0.001**
III	199 (32.1)	87.0		49.8	
IV	117 (18.9)	22.1		13.7	
unknown	6 (1.0)				
histological grading					
I	10 (1.6)	n.a.	**< 0.001**	n.a.	**0.007**
II	505 (81.6)	122.4		n.a.	
III	102 (16.5)	41.8		33.6	
unknown	2 (0.3)				
histology					
adeno carcinoma	525 (84.9)	122.6	**< 0.001**	n.a.	**0.010**
mucinous carcinoma	74 (12.0)	68.3		40.7	
signet-ring cell carcinoma	7 (1.1)	12.3		9.4	
unknown	13 (2.1)				
resection margin					
R0	573 (92.6)	15.3	**< 0.001**	n.a.	**< 0.001**
R1 + R2	32 (5.2)	122.6		10.7	
unknown	14 (2.3)				
therapy					
sole surgical resection	229 (37.0)				
+ chemotherapy	136 (22.0)				
+ radiation	10 (1.6)				
+ chemoradiation	111 (17.9)				
+ unknown	133 (21.5)				

All *P* values in bold, are regarded as statistically significant. Abbreviations: *n.a.* not achieved, *UICC* Union internationale contre le cancer

significance. The univariate analysis of survival was done using the Log rank test and Kaplan-Meier analysis. The software GraphPad Prism, Version 7.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. The significance level was set at 5% ($p < 0.05$).

Results

Patient cohort and clinical characteristics

The examined cohort consisted of 619 patients (50.4% male; 49.6% female). The median age was 65.2 years (mean 66 years; range 29 to 102 years). All considered clinical and histopathological characteristics, had a significant

impact on the patient outcome in terms of overall survival (OS) and progression-free survival (PFS). However, other parameters like gender and tumour localisation did not have any effect on the outcome (Table 1). Patients aged ≥65 years had a significantly worse OS ($p < 0.001$) and PFS ($p = 0.005$). Staging by UICC displayed a significant effect on the OS ($p < 0.001$) and PFS ($p < 0.001$). Patients diagnosed with advanced tumours and local and/or remote metastasis (UICC III + IV) displayed a highly significant poorer outcome. In our view, the cohort represents the general population (Additional file 1: Figure S1 A + B).

Expression of topoisomerase II α correlated to clinic-pathological characteristics

A quantity of 430 colorectal tissue specimens was procured for evaluation of the Topo II α expression profile. The mean and median expression rate of the entire cohort was 52 and 53.8%. The upper-limit of Topo II α expression was set at 50%. In 267 cases, the degree of expression was ≥50%. An example of Topo II α expression is displayed in the Fig. 1 a + b. Patients aged ≥65 years displayed a significantly lower expression of Topo II α ($p = 0.005$). In the assessment of UICC stages, patients with locally advanced disease (UICC III) had a lower expression of Topo II α compared to patients in early tumour stages (UICC I + II) ($p = 0.029$). Interestingly, the histological grading did not show any coherence to the expression of Topo II α. In terms of histological entities, the adeno carcinoma displayed higher expression profiles than other entities ($p = 0.041$). All data is presented in Additional file 4: Table S1.

Coherence between topoisomerase II α expression and patient outcome

In general, low expression rates of Topo II α cohered with a significantly unfavourable outcome ($p = 0.010$) (Fig. 2 a + b). The entire cohort was further analysed by differentiating UICC subsets. The subgroups UICC I + II, UICC III and UICC IV were identified. Within the subset of UICC I + II no difference in the OS or PFS could be monitored ($p = 0.354$ and $p = 0.207$). In the clinically relevant subset of UICC III patients in OS and PFS, low expression rates of Topo II α was a significant negative prognostic marker ($p = 0.004$ and $p = 0.020$). Within the subcategory of UICC IV patients, Topo II α expression was only significantly relevant in the OS ($p = 0.027$) (Fig. 3 a + b).

Regarding histological grading, Topo II α expression showed a significant effect on the patient OS within G2 tumours ($p < 0.001$) (Table 2).

Analysing the entire cohort and setting the cut-off for Topo II α expression at 50%, patients above the upper-limit had a highly significant beneficial outcome ($p < 0.001$) with a median OS of 69.2%, in comparison to an OS of 52.9% in the subset of < 50% expression of Topo II α. Analogue to the above-mentioned findings a high focal expression of Topo II α (high quantity in hot-spots) was correlated with a significant beneficial outcome ($p = 0.004$) (Additional file 2: Figure S2 A).

Expression of minichromosome maintenance protein 6 correlated to clinic-pathological characteristics

A total of 570 tissue specimens were analysed regarding MCMC6. The median expression was 85.8% while the mean expression was 82.8% (range 97.0–27.6%). Based on these findings, the cut-off value of MCM6 expression was set at 85%. In 306 (53.7%) cases, the expression was ≥85% and in 264 cases (46.3%) the expression levels were < 85%. An example of MCM6 expression is displayed in Fig. 4 A + B. Classifying the cohort by UICC stages, advanced tumour stages (III + IV) displayed significantly less expression of MCM compared to locally confined tumours ($p = 0.012$ and $p < 0.001$). In terms of histological grading, significantly less MCM6 expression levels were observed in higher differentiated tumours. There was no statistically significant coherence between patient age and the degree of MCM6 expression. All data is presented in Additional file 5: Table S2.

Coherence of minichromosome maintenance protein 6 expression and patient outcome

The group of patients that were diagnosed with colorectal neoplasm and MCM6 expression levels below 85%, a

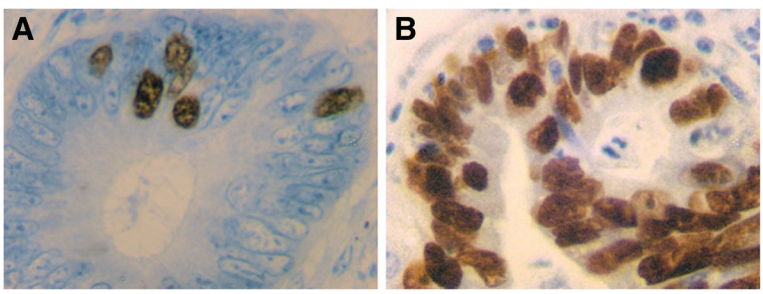

Fig. 1 Topoisomerase IIα immunohistochemical staining of colorectal tissue, ABC method × 400 magnification. **a** with a low (< 50%) expression level and (**b**) with high (≥ 50%) expression levels

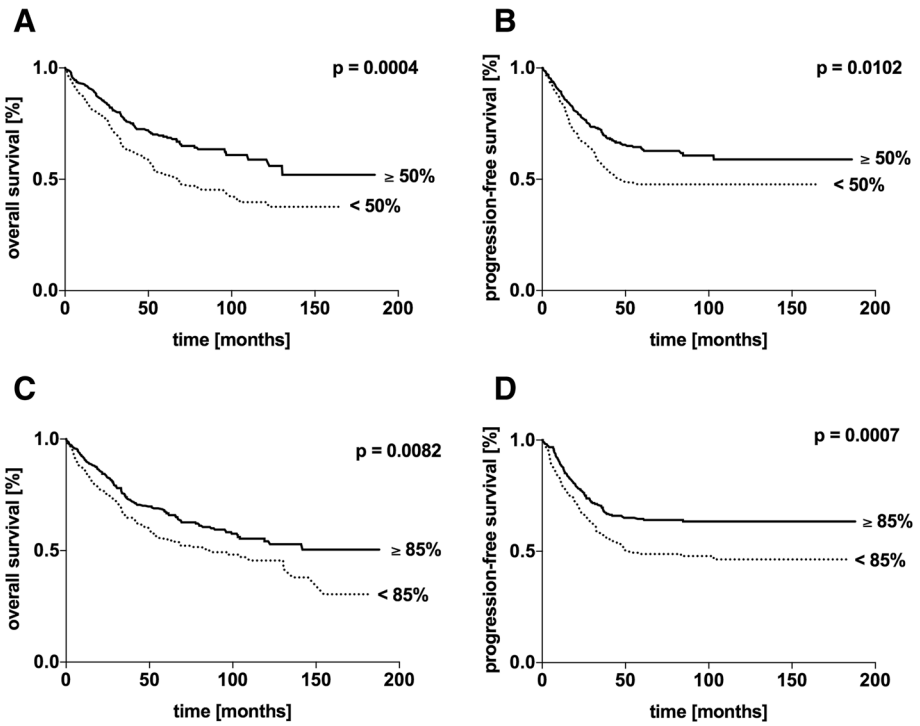

Fig. 2 Kaplan-Meier survival analysis of the cumulative overall survival (**a**, **c**) and progression-free survival (**b**, **d**) of patients with colorectal carcinoma stratified by the expression of topoisomerase II α (**a, b**) and minichromosome maintenance protein 6 (**c, d**) according to the cut off. *P*-values were calculated by Log rank tests

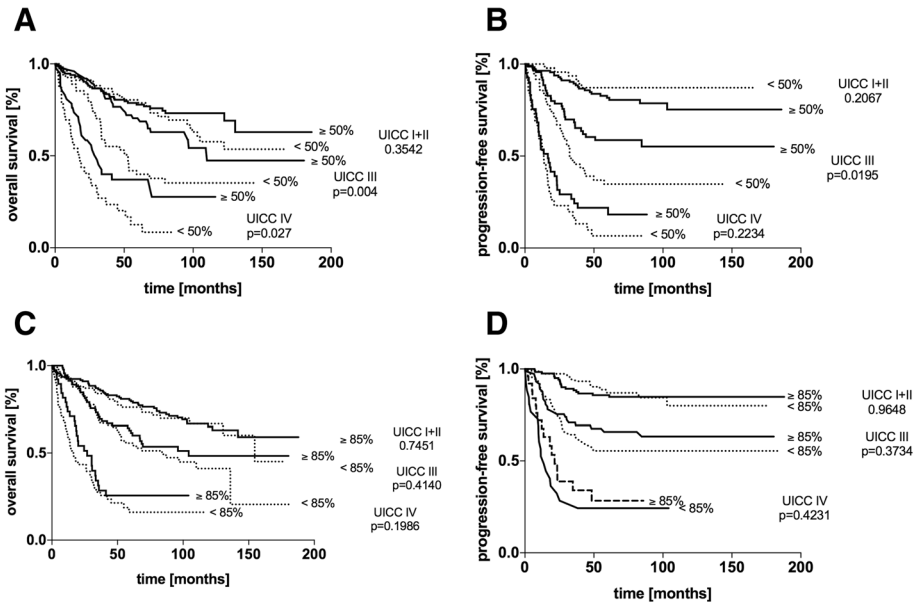

Fig. 3 Kaplan-Meier survival analysis of the cumulative overall survival (**a, c**) and progression-free survival (**b, d**) of patients with colorectal carcinoma stratified by the UICC stages I + II, III and IV. Each subset was analysed in respect to topoisomerase II α (**a, b**) and minichromosome maintenance protein 6 expression (**c, d**). P-values were calculated by Log rank tests

Table 2 IHC expression of topoisomerase II a and correlation to the patients´ outcome

	N	OS [months]			DFS [months]		
		expression			expression		
		> 50%	≤50%	p	> 50%	≤50%	p
all	430	n.a.	68.4	**< 0.001**	n.a.	48.4	**0.010**
age (years)							
< 65	211	n.a.	95.7	**< 0.001**	n.a.	n.a.	0.111
≥ 65	215	110.1	53.6	0.188	n.a.	n.a.	0.959
tumor site							
right colon	115	122.6	58.9	0.313	n.a.	n.a.	0.197
left colon + rectum	298	n.a.	68.4	**< 0.001**	n.a.	n.a.	0.797
UICC							
I + II	217	n.a.	n.a.	0.354	n.a.	n.a.	0.207
III	133	110.1	51.9	**0.004**	n.a.	32.4	**0.020**
IV	76	30.2	17.6	**0.027**	16.6	13.6	0.223
histological grading							
I	9	n.a.	n.a.	n.a.	n.a.	n.a.	0.480
II	339	n.a.	66.1	**< 0.001**	n.a.	n.a.	0.283
III	67	98.6.	28.8	0.207	n.a.	n.a.	0.390
histology							
adeno carcinoma	364	n.a.	68.4	**< 0.001**	n.a.	n.a.	0.259
mucinous + signet-ring carcinoma	56	59.1	53.6	0.881	n.a.	n.a.	0.923
resection margin							
R0	404	n.a.	58.9	**0.009**	n.a.	n.a.	0.299
R1 + R2	18	58.0	68.4	0.168	54.4	47.8	0.797

All *P* values in bold, are regarded as statistically significant. Abbreviations: *UICC* Union internationale contre le cancer, *n.a.* not achieved

significantly poor OS ($p = 0.008$) and PFS ($p < 0.001$) were monitored in the univariate analysis (Fig. 2 c + d). Stratifying the cohort by means of the UICC staging, different to Topo II α expression, MCM6 expression did not correlate with a statistically poor OS or PFS in any of the UICC-subgroups (Fig. 3 c + d). However, categorizing by age groups, in young patients (< 65 years), MCM6 expression levels were linked significantly to a poorer outcome. Regarding histological grading, within the subset of G2 patients, an expression rate above 85% was linked to a significantly poorer outcome (Table 3). Similar to the focal Topo II α expression, MCM6 hotspots were correlated to a poor patient outcome ($p = 0.013$) (Additional file 2: Figure S2 B).

Comparison of MCM6 and topo II α expression levels

In the entire cohort, MCM6 expression was significantly higher (mean 82.8%) than the expression of Topo II α (mean 52.0%) ($p < 0.001$) (Additional file 3: Figure S3 A). Furthermore, a significant correlation (r = 0.433, $p < 0.001$)

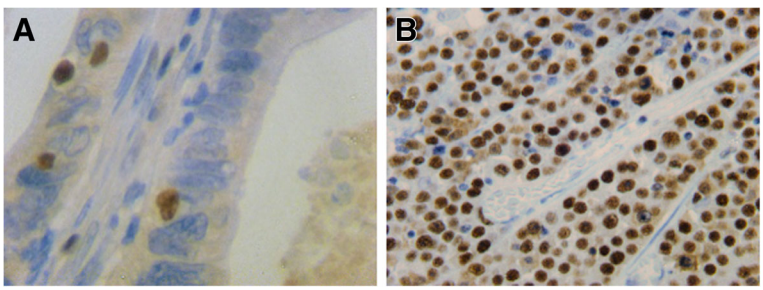

Fig. 4 Minichromosome Maintenance Protein 6 immunohistochemical staining of colorectal tissue, ABC method × 400 magnification. **a** with a low (< 85%) expression level and (**b**) with high (≥ 85%) expression levels

Table 3 IHC expression of minichromosome maintenance protein 6 and correlation to the patients´ outcome

	N	OS [months]			DFS [months]		
		expression			expression		
		> 85%	≤85%	p	> 85%	≤85%	p
all	570	n.a.	87.0	**0.008**	n.a.	51.0	**0.001**
age (years)							
< 65	270	n.a.	136.0	**0.007**	n.a.	n.a.	0.418
≥ 65	280	82.0	51.9	0.246	n.a.	n.a.	0.419
tumor site							
right colon	162	104.3	49.8	**0.026**	n.a.	n.a.	0.135
left colon + rectum	385	n.a.	110.1	0.086	n.a.	n.a.	0.658
UICC							
I + II	262	154.6	154.6	0.745	n.a.	n.a.	0.965
III	184	84.0	84.0	0.414	n.a.	n.a.	0.373
IV	101	16.4	16.4	0.199	13.6	21.5	0.423
histological grading							
I	9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
II	448	n.a.	104.9	**0.008**	n.a.	n.a.	0.149
III	92	104.3	33.7	0.393	n.a.	n.a.	0.839
histology							
adeno carcinoma	466	n.a.	104.9	**0.026**	n.a.	n.a.	0.837
mucinous + signet-ring carcinoma	75	11.3	41.9	0.103	n.a.	34.7	**0.006**
resection margin							
R0	509	n.a.	130.5	0.115	n.a.	n.a.	0.244
R1 + R2	29	11,3	15.3	0.308	9,7	n.a.	**0.001**

All *P* values in bold, are regarded as statistically significant. Abbreviations: UICC – Union internationale contre le cancer; n.a. – not achieved

between the expression of both proliferative markers was observed (Additional file 3: Figure S3 B). Corresponding to this, the analysis of hot spots was significantly higher in MCM6 than Topo II α ($p < 0.001$) (Additional file 3: Figure S3 C).

Conclusions

Colorectal carcinoma is a major tumour entity and is accountable for the second greatest cause of death in tumour patients [19]. In assessment of the prognosis, prognostic markers are required in addition to the UICC-staging. Dysfunctional cell proliferation plays a key role in neoplasms. Evaluation of proliferative markers in the routine diagnosis of carcinomas is essential. For example, IHC of the proliferative marker Ki-67 is well accepted and executed on a regular basis. High levels of Ki-67 expression indicate rapid tumour growth and are associated with a poor clinical outcome [20–23]. Regarding colorectal carcinoma, contradictory conclusions concerning the proliferation markers have been made. Multiple studies described high expression levels of Ki-67 to be a negative prognostic marker [23–25], whereas other studies came to the opposite conclusion [26]. A few studies did not monitor any impact of the Ki-67 expression levels on the clinical outcome [27].

In this study we focused on two key player proteins in cell division, the topoisomerase II α (Topo II α) and minichromosome maintenance protein 6 (MCM6). We here applied IHC of Topo II α and MCM6 to a large and representative cohort of patients diagnosed with colorectal carcinoma.

IHC analysis was performed in order to detect the expression levels of Topo II α using the primary antibody Ki-S4, developed in the Institute of Haematopatholgy at the University Hospital Kiel. The antibody was proven to be a specific marker for Topo II α [8]. The expression of Topo II α was previously shown to be a significant prognostic indicator in breast cancer and mantel cell lymphoma, where a high intensity of expression was linked to a poor clinical outcome [5, 28]. Data of IHC for the detection of Topo II α expression in large and representative cohorts of CRC patients are limited. Boonsong et al. performed IHC to detect Topoisomerase I levels in 249 CRC patients but was unable to find a correlation neither to histo-pathological characteristics, nor to OS [29]. However, another recent study does

reveal a significant correlation in terms of prolonged DFS and OS in patients with high expression rates of Topoisomerase I [30]. Our analysis also revealed a highly significant correlation between the Topo II α expression and the OS and DFS. Synoptically, our data is partially contradictory to previous studies. Lacking analysis of Topo II α in reasonably sized cohorts of patients suffering of CRC, validation is critical. Regarding the patient age, a significant coherence to Topo II α expression was monitored. In young patients (≤65 years), the expression was significantly higher which is likewise a contrary result to the study of Boonsong et al. [29]. Furthermore, within the cohort of younger patients, we were able to identify Topo II α expression as a prognostic marker. High expression rates cohered with a beneficial clinical outcome. As to why the prognostic value is only in the subset of young patients must be further explored – experimental validation is currently lacking.

CRC localised at the right hemi colon is generally associated with an inferior prognosis, we therefore expected expression levels of Topo II α to be significantly lower. To our surprise, the locus of neoplasia (left vs right hemi colon) did not prove any difference in expression rates of Topo II α. Patients diagnosed with an adeno carcinoma and Topo II α expression levels above the cut-off showed a highly significant favourable outcome.

Locally advanced tumour progression is accompanied with lower rates of Topo II α expression. Comparing UICC I + II with UICC III, a significant decrease in expression was monitored. Between UICC III and UICC IV, no difference was asserted. Within each UICC stage, significant impact of Topo II α expression levels on the clinical outcome was observed. These findings prove the prognostic impact of assessing Topo II α expression levels using IHC. In conclusion, our data provides an additional tool to the UICC classification in terms of prognosis and clinical outcome to identify Patients at risk, which may be of benefit to an (neo-) adjuvant treatment.

In further analysis, we assessed the expression levels of MCM6 and clinical characteristics, as well as patient outcome. CRC tissue specimens of a large cohort of patients were studied using IHC with the primary antibody Ki-MCM6, that is highly specific to the MCM6. The relevance of MCM in malignancies has been affirmed in various studies [15–17, 31]. An analysis of MCM6 in patients diagnosed with CRC was absent.

As expected, the mean expression level of MCM6 (83%) was significantly higher than with Topo II α (52%). MCM6 is involved in the early phase of cell cycle replication. The protein is partly involved in the G_1 phase. Hence, a larger quantity of cells (including cells in early stages of the cell cycle) is stained by IHC [13]. The above-mentioned finding may explain the different quantity of expression when comparing Topo II α with MCM6. Similar results have been demonstrated in other tumour entities [6]. Correlation of Topo II α and MCM6 was clearly demonstrated. Neoplastic tissue with low expression levels of MCM6 exhibited low levels of Topo II α expression.

We did not expect that MCM6 expression levels would negatively correlate with the UICC staging. In progressive tumours, lower expression levels of MCM6 were observed, which is contrary to the Topo II α expression levels in our cohort. We expected high levels of MCM6 in advanced tumours with rapid tumour growth and subsequent greater cell proliferation as previously described by Giaginis et al. in terms of MCM2 expression [32].

Concerning the OS and DFS, expression levels above the cut-off were associated with a favourable outcome. Furthermore, in young patients (≤65 years) with histologically graded G2 adeno carcinoma, MCM6 expression levels above the cut-off also demonstrated a significant marker for a beneficial outcome.

For the first time our study presents data of Topo II α and MCM6 IHC detected expression levels in a large representative cohort of patients diagnosed with CRC. Contrary to the expected outcome, high expression levels of the proliferative markers MCM6 and Topo II α represent a significantly negative prognostic marker.

Increased cell proliferation was generally thought to be responsible for tumour progression and metastasizing. Whereby, as suggested by our data, rather poorly differentiated tumours with scarce cell proliferation seem to be liable for a poor progression of the disease.

In summary, we propose that from a prognostic point of view, high proliferative cell turnover should not be equated with a poor histological tumour differentiation. We finally conclude that assessing the proliferative turnover could be used for risk stratification of CRC patients in the future. Undoubtedly, our data is controversial in context of other malignancies, but carcinomas are diverse, and should not all be investigated in analogy. In this MS we present genuine data exhibiting novel findings in MCM6 and Topo II alpha exploration, that truthfully cannot be elucidated in any manner. A more in-depth investigation is required in order to demonstrate and consolidate our findings in validation cohorts.

Additional files

Additional file 1: Figure S1 A + B. Kaplan-Meier analysis of the cumulative overall (A) and progression-free (B) survival of patients with a colorectal carcinoma and staged according to the UICC classification. The *p*-value was calculated by log-rank test. (TIFF 398 kb)

Additional file 2: Figure S2 A + B. Kaplan-Meier analysis of the cumulative overall survival of patients with a colorectal carcinoma and stratified by the characteristic of hotspots of (A) topoisomerase II alpha and (B) minichromosome maintenance protein 6 expression. The occurrence of hotspots significantly correlates with a worse patients´ outcome. The *p*-value was calculated by log-rank test. (TIFF 488 kb)

Additional file 3: Figure S3 A-C. (A) Expression levels of topoisomerase II alpha and minichromosome maintenance protein 6. (B) Significant correlation ($r = 0.433$, $p < 0.001$) between both proliferative markers. (C) Frequency of hot spots within the entire cohort. (TIFF 521 kb)

Additional file 4: Table S1. Coherence of topoisomerase II alpha IHC expression to clinical and histological criteria. (XLSX 9 kb)

Additional file 5: Table S2. Coherence of minichromosome maintenance protein 6 IHC expression to clinical and histological criteria. (XLSX 9 kb)

Abbreviations
CRC: Colorectal cancer; FAP: Familial adenomatous polyposis; FISH: Fluorescent in situ hybridization; HNPCC: Hereditary nonpolyposis colorectal cancer; MCM6: Minichromosome maintenance protein 6; MMR: Mismatch repair gene systems; MSI: Microsatellite instability; OS: Overall survival; PCNA: Proliferating cell nuclear antigen; PFS: Progression-free survival; TNM: TNM Classification of Malignant Tumours; Topo IIα: Topoisomerase II alpha; UICC: Union for International Cancer Control

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
AH data analysis, composed manuscript, FG data collection, revised the manuscript, SN data analysis, slide analysis, composed manuscript, JHB slide analysis, revised the manuscript, RL slide preparation, revised the manuscript, BS slide staining and data analysis, JHC data collection, composed manuscript, TB composed manuscript, SH data collection, composed manuscript, GB data collection, revised the manuscript, ClS data collection, composed manuscript, CaS data collection and analysis, composed manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
The study was approved by the local ethics committee of the Medical Faculty, Christian-Albrechts University Kiel (reference no. A110/99) and performed in accordance with the Declaration of Helsinki. All patients participating in this study, gave their written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Anhang – Teil 2

Isolation and Enumeration of CTC in Colorectal Cancer Patients: Introduction of a Novel Cell Imaging Approach and Comparison to Cellular and Molecular Detection Techniques.

cancers

MDPI

Article

Isolation and Enumeration of CTC in Colorectal Cancer Patients: Introduction of a Novel Cell Imaging Approach and Comparison to Cellular and Molecular Detection Techniques

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Simple Summary: Despite significant progress in screening and treatment regimens, colorectal cancer (CRC) still is a major health burden lacking profound liquid biomarkers for identifying patients at risk. Circulating tumour cells (CTC) have the potential to non-invasively improve the diagnosis. We have already established a sensitive semi-quantitative RT-qPCR against CK20 for CTC quantification in CRC patients. For clinical translation, this study aims to validate our molecular detection method by terms of cytological approaches, and implement a novel semi-automated microscopic detection after immunofluorescence labelling of CTC. Additionally, we aim to compare our PCR-based approach to a marker-independent, but size-dependent, enrichment process. We have successfully applied the validation techniques and proved their feasibility. Enumeration by size yielded the highest numbers of CTC and demonstrated to be the most reliable strategy for CTC detection in CRC patients. Future studies with larger patient cohorts will have to investigate the clinical significance and prognostic value of this approach.

Abstract: Circulating tumour cells (CTC) were proven to be prognostically relevant in cancer treatment, e.g., in colorectal cancer (CRC). This study validates a molecular detection technique through using a novel cell imaging approach for CTC detection and enumeration, in comparison to a size-based cellular and correlated the data to clinico-pathological characteristics. Overall, 57 CRC patients were recruited for this prospective study. Blood samples were analysed for CTCs by three methods: (1) Epithelial marker immunofluorescence staining combined with automated microscopy using the NYONE® cell imager; (2) isolation by size using membrane filtration with the ScreenCell® Cyto IS

device and immunofluorescence staining; (3) detection by semi-quantitative Cytokeratin-20 RT-qPCR. Enumeration data were compared and correlated with clinic-pathological parameters. CTC were detected by either approach; however, with varying positivity rates: NYONE® 36.4%, ScreenCell® 100%, and PCR 80.5%. All methods revealed a positive correlation of CTC presence and higher tumour burden, which was most striking using the ScreenCell® device. Generally, no intercorrelation of CTC presence emerged amongst the applied techniques. Overall, enumeration of CTC after isolation by size demonstrated to be the most reliable strategy for the detection of CTC in CRC patients. Ongoing studies will have to unravel the prognostic value of this finding, and validate this approach in a larger cohort.

Keywords: liquid biopsy; circulating tumour cells; colorectal cancer; NYONE®; isolation by size of epithelial tumour cells; ScreenCell®

1. Introduction

Colorectal cancer (CRC) is an extensive health burden that, according to estimates, will account for >1 million annual deaths worldwide by 2030 [1]. Despite considerable ongoing improvement and progress in treatment and screening [2], the average five-year survival rate still is below 70%, and nearly a quarter of the patients show distant metastases at the time of diagnosis with dismal five-year survival rates below 20% [3].

In recent years, the importance of individualised diagnostics and therapeutic options yielded major attention [4,5]. Biomarkers for either early detection of cancer or proof of minimal residual disease have been identified [6], e.g., enumeration of circulating tumour cells (CTC). The major challenges for CTC detection are the rarity of CTC in the peripheral blood, the technique for their enrichment and the discrimination of these cells from the diverse leukocyte populations [7]. For detecting these rare events, multiple methodologies are available. In general, two major approaches are distinguishable: Direct CTC detection by cytological staining and imaging or indirect CTC detection by molecular approaches, as PCR-based techniques.

In the latter context, various PCR target sequences have been used for CTC detection, however, in recent studies, we were able to establish a highly specific and sensitive RT-qPCR approach for detection of cytokeratin 20 (CK20) expression, which is widely found in mature enterocytes and also commonly in CRC cells [8–10]. In general, the detection of CTC in peripheral blood identified CRC patients with an unfavourable prognosis which could also be demonstrated by our CK20 RT-qPCR based detection approach [8,11,12].

A wide range of cellular techniques for CTC isolation and detection involves the enumeration of cells, based on the expression of certain markers on the cell surface as it is, for example, performed by the already approved semi-automated CellSearch® platform that deploys the utilisation of anti-EpCAM and anti-pan-cytokeratin (pan-CK) antibodies [11,13–15]. Though, especially in the early stages of CRC, the detection rates of CTC by the CellSearch® system are limited [16]. To potentially overcome this issue, the employment of a wider range of antibodies for the enrichment and/or biomarker detection could be beneficial. To compare our molecular PCR based approach with a cytological detection method and to overcome this critical issue, we developed and implemented a workflow that allows for immunofluorescence (IF) staining with a more extensive range of antibodies (anti-EGFR, anti-Her2, anti-EpCAM and anti-panCK) and a semi-automated cell enumeration by the cell imager NYONE® (SYNENTEC, Elmshorn, Germany).

Moreover, an ongoing debate about the sensitivity of antibody-dependent enumeration methods of CTC is held due to the process of Epithelial-Mesenchymal-Transition (EMT), by which epithelial tumour cells lose their polarity and become enabled to disseminate [17]. During EMT, tumour cells might lose specific epithelial marker proteins, which are often used for the detection of CTC, e.g., EpCAM and

cytokeratin. Thus, enumeration of CTC by means of antibody-mediated staining of those antigens might lead to an underestimation of the CTC count [18,19]. Consequently, antigen-independent enumeration approaches, such as isolation by size of CTC, have been developed. A strategy for this has been developed by ScreenCell® (Sarcelles, France) with the ScreenCell® Cyto IS device. Here, blood samples are transferred through a porous membrane and CTC that are significantly larger in diameter compared to leukocytes are retained by that membrane. The utility demonstrating the prognostic relevance of CTC has already been depicted in various reports [20–22].

In this prospective study, we applied and compared three techniques for the detection of CTC in CRC patients to validate our established CK20 RT-qPCR based detection method. Firstly, we applied a novel semi-automated microscopical approach for cytological CTC detection by a cell imager (NYONE®, SYNENTEC, Elmshorn, Germany). For this purpose, cells were enriched, and the CTC fraction was labelled by immunofluorescence staining with specific antibodies against well-established marker antigens of CRC cells—pan-CK, EpCAM, EGFR and Her2. Secondly, for the isolation by size of epithelial tumour cells, we implemented CTC detection with a well-established marker-independent size-exclusion method by the ScreenCell® Cyto IS device which was combined with subsequent immunofluorescence staining of pan-CK and the leukocyte marker CD45. Finally, as a third method, the CK20 RT-qPCR was utilised for indirect CTC detection, which was already applied in previous studies on CRC patients [8–10]. Our primary aim was to validate our well-established PCR technique for CTC quantification by means of two cytological detection approaches. We further intend to analyse and elucidate the variances of two differing CTC enumeration methodologies by both size and marker dependent and independent concepts. Additionally, we intend to foster whether these approaches correlate with clinical parameters and with each other, to identify the most reliable CTC isolation and enumeration method.

2. Results

2.1. Patients and Demographics

In total, 57 patients with a histopathologically confirmed colorectal adenocarcinoma were included in this prospective study. The assessed cohort comprised 21 female and 36 male patients. Thirty-eight patients were diagnosed with colon carcinomas and 19 patients with rectal carcinomas. In the subset of colon cancer patients, the group was further stratified in right-sided colon cancer (17 patients) and left-sided colon cancer (21 patients). The median age of the entire study population at the time of blood withdrawal was 66 years (range: 42–89 years). In total, this study compared the detection of CTC in the blood of CRC patients by the use of three methods: (1) IF staining and semi-automated microscopical enumeration by the cell imager NYONE®; (2) isolation via the ScreenCell® Cyto IS device coupled with cytochemistry according to Pappenheim and IF staining followed by microscopical enumeration; (3) semi-quantitative CK20 RT-qPCR.

Forty-four patients were enrolled for the semi-automated microscopical detection by the cell imager NYONE®. The median age of this NYONE®-cohort was 66 years (range: 45–89 years) with 32 patients diagnosed with colon cancer and 12 patients with a rectum carcinoma. In 31 cases blood samples were available for the analysis of CTC by the ScreenCell® Cyto IS device. Within this subset, the majority of patients were male (21 male and 10 female patients) and diagnosed with colon carcinomas (22 colon vs 9 rectum carcinoma). The median age and range at the time of blood drawl were equal to the general cohort with 66 years (range: 42–89 years). Furthermore, the distribution, according to the tumour stages, was similar to the overall study population, with the majority of patients being diagnosed with stage three disease. Forty-one patients were enrolled for CTC detection by semi-quantitative CK20 RT-qPCR, previously reported [8–10]. The median age of this subset was 68 years (range: 45–89 years). Dissemination of patients across gender, tumour site and tumour stage were in general similar to the whole study population. Table 1 displays a full synopsis of all clinical and

demographical data of the analysed patient cohort, as well as the employed methods for the detection of CTC.

Table 1. Patient demographics and clinical characteristics of the entire study population and further breakdown according to the utilised detection modes.

Parameters	Total N (%)	NYONE® N (%)	ScreenCell® N (%)	CK20 RT-qPCR N (%)
	57 (100)	44 (100)	31 (100)	41 (100)
Gender				
Male	36 (63.2)	26 (59.1)	21 (67.7)	24 (58.5)
Female	21 (36.8)	18 (40.9)	10 (32.3)	17 (41.5)
Age				
Median (range)	66 (42–89)	66 (45–89)	66 (42–89)	68 (45–89)
<65	27 (47.4)	18 (40.9)	15 (48.4)	17 (41.5)
≥65	30 (52.6)	26 (59.1)	16 (51.6)	24 (58.5)
Tumour site				
colon	38 (66.7)	32 (72.7)	22 (71.0)	29 (70.7)
right	17 (44.7)	16 (50.0)	9 (40.9)	15 (51.7)
left	21 (55.3)	16 (50.0)	13 (59.1)	14 (48.3)
Rectum	19 (33.3)	12 (27.3)	9 (29.0)	12 (29.3)
UICC stage				
I	15 (26.3)	12 (27.3)	9 (29.0)	12 (29.3)
II	10 (17.5)	9 (20.5)	6 (19.4)	8 (19.5)
III	24 (42.1)	18 (40.9)	12 (38.7)	16 (39.0)
IV	8 (14.0)	5 (11.4)	4 (12.9)	5 (12.2)

Abbreviations: UICC—Union internationale contre le cancer.

2.2. Spiking Experiments and Validation of Cytological and RT-qPCR Detection Techniques

For validation of the employed detection techniques in this study, the human CRC cell line HT29 was utilised. Spiking experiments with HT29 cells in the blood of healthy donors were already successfully conducted for validating the detection of CTC in CRC patients by CK20 RT-qPCR [8]. To set up and validate the automated microscopic detection of CTC by the NYONE® device, HT29 cells were also spiked into healthy donors' blood. After isolation of the MNC-fraction using Vacutainer-CPT tubes, staining of the samples with anti-EGFR, anti-Her2, anti-EpCAM, anti-pan-CK antibodies conjugated with Alexa647 (red) and Alexa488-conjugated anti-CD45 antibodies (green), as well as staining of the nuclei using DAPI, a strong fluorescence signal for either CTC (red) or leucocytes (green) could be detected (Figure 1A), demonstrating that the CTCs were sufficiently distinguishable from leucocytes using the image processing YT®-Software (SYNENTEC, Elmshorn, Germany).

For validation of the ScreenCell® Cyto IS technique, again, HT29 cancer cells were spiked into healthy donors´ blood samples. Similar to the NYONE®-approach, a strong green immunofluorescence signal of CTC after staining with the anti-pan-CK antibody could be observed, and leucocytes showed an exclusive strong red signal after staining with anti-CD45 antibodies. Additionally, leucocytes were significantly smaller in size compared to the HT29 cells providing another parameter for discrimination of CTC from PBMC (Figure 1B).

2.3. Detection of CTC by Automated Microscopy with the Cell Imager NYONE®

During the study, 44 patients were enrolled in this study arm (Table 1). In 16 patients (36.4%) ≥1 CTC were detected. Analysing the entire cohort, the mean amount of CTC was 0.89 cells (range: 0–7 cells; SD: 1.57). Figure 2A exemplarily depicts the CTC detection by NYONE® in patients´ samples. Examining the study cohort and stratifying by demographical and clinical parameters, no significant difference in the quantity of CTC occurrence was detected in dependence on gender, age, tumour site or tumour localisation within colon carcinoma (Figure 3A and Table 2). However, a higher mean CTC

count by trend was observed in patients with advanced tumour stages (UICC III + IV) compared to UICC stages I + II (1.06 cells vs 0.58 cells; $p = 0.503$) (Table 2).

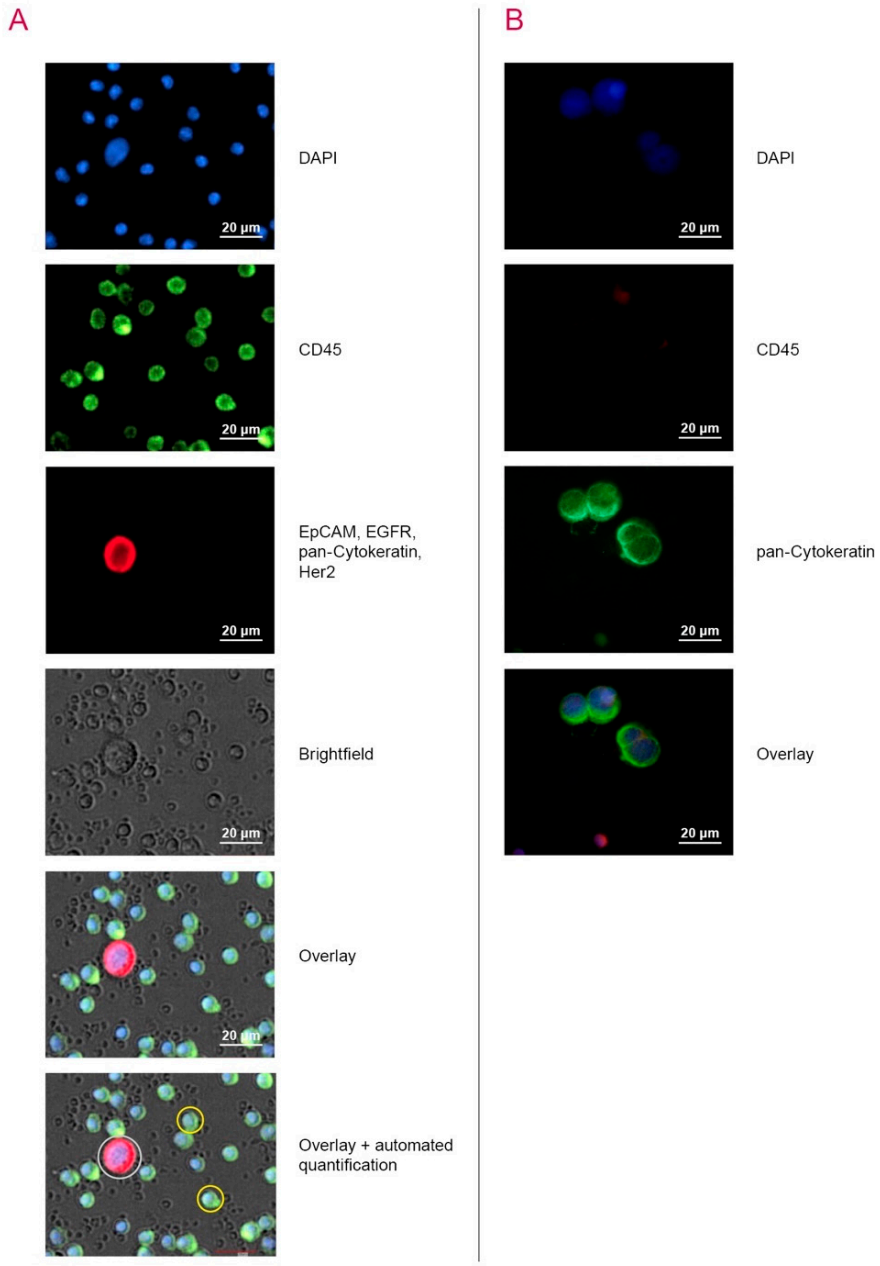

Figure 1. Validation of circulating tumour cells (CTC) detection by spiking experiments with HT29 colon cancer cells. HT29 cells were spiked into healthy donors´ blood samples. (**A**) NYONE®—After sample preparation, HT29 cells (red) were stained and identified with the NYONE® cell imager and marked for automated quantification. Leukocytes were stained in green (yellow encirclement) and not considered for quantification. (**B**) ScreenCell®—HT-29 cells were immunofluorescence (IF) stained with anti-pan-CK antibodies (green) and leukocytes were IF stained with anti-CD45 antibodies (red). Detected HT29 cells were significantly larger compared to leucocytes.

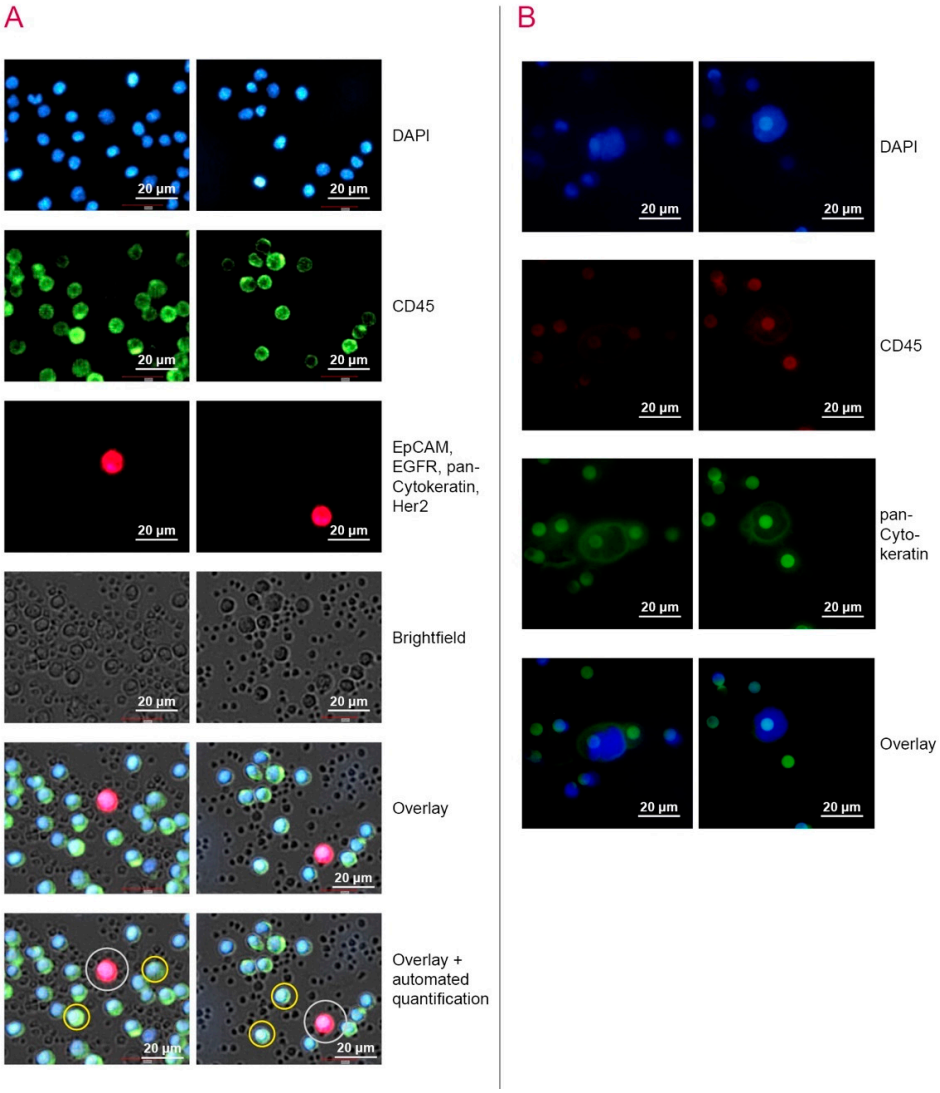

Figure 2. CTC detection in patient samples using NYONE® and ScreenCell® technology. CTC in peripheral patients´ blood samples were enriched by CPT vacutainer tubes for NYONE® analysis, or by isolation by size via a porous membrane in the ScreenCell® study cohort. Samples were then stained for CTC detection. (**A**) NYONE®—CTC were stained with anti-EpCAM, anti-EGFR, anti-Her2 and anti-pan-CK antibodies (red), and leukocytes were stained with anti-CD45 antibodies (green). DAPI staining was performed for nuclei staining (blue). Cells were scanned by NYONE® and quantified by YT®-Software (here, CTC encircled in white and two exemplary CD45-positive cells in yellow) (**B**) ScreenCell®—CTC were IF stained with anti-pan-CK antibodies (green), leucocytes IF stained with anti-CD45 antibodies (red)and DAPI staining was performed for nuclei staining (blue).

2.4. Capture and Detection of CTC by ScreenCell® Cyto IS Device

In all cases, the technical application of the ScreenCell® filtration approach was successful. In 100% of the blood samples CTCs could be detected. All over the study population, the mean count for detected CTC was 3.25 CTC/mL (range: 0.2–14.3 CTC/mL; SD: 3.10) (Table 2). In Figure 2B, CTC from patients´ blood samples were enriched by the ScreenCell® Cyto IS device and IF stained for detection. No statistical significance was found among the subsets of gender, age or tumour site (all p = ns) (Figure 3B and Table 2). Correlating the data with the relative tumour burden in compliance with the UICC stages, similar to the data obtained by the NYONE® technique, patients with advanced disease (stage III and IV) exhibited significantly more CTC compared to patients with UICC stage I + II (mean: 4.10 CTC/mL vs 2.35 CTC/mL; p = 0.039) (Figure 3B).

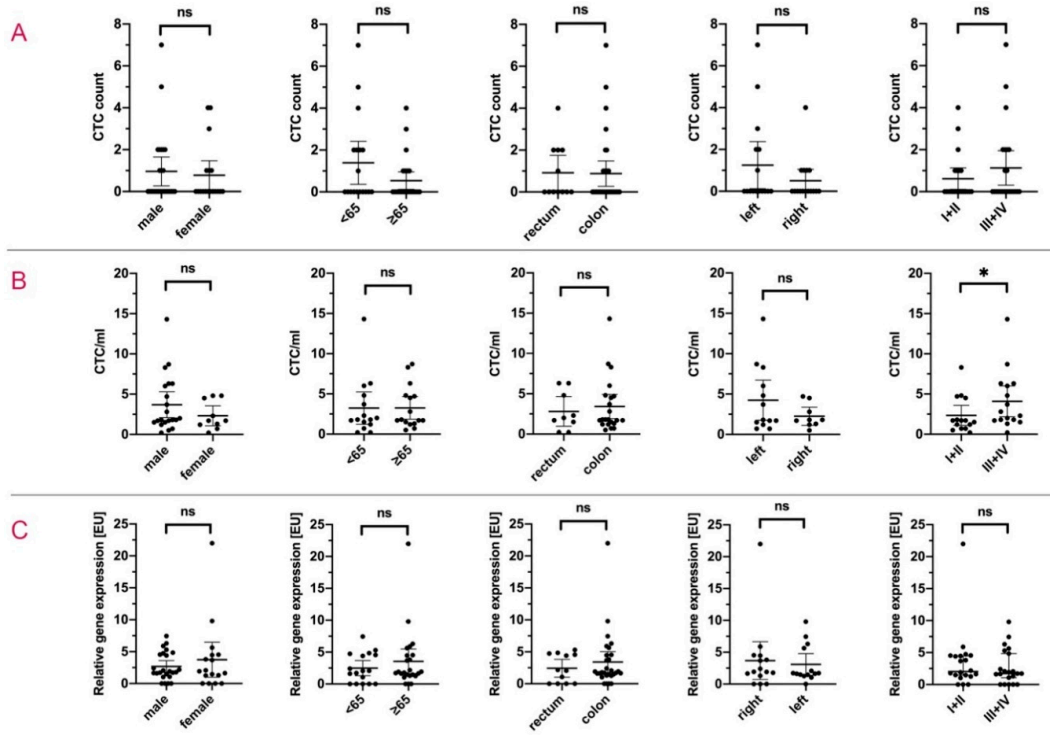

Figure 3. Congruence of CTC quantity and clinico-pathological characteristics. CTC enumeration data of the entire patient cohort was assessed by (**A**) NYONE®, (**B**) ScreenCell® and (**C**) CK20-qRT-PCR and analysed in terms of the association between the prevalence of CTC and clinico-pathological data. The bar between the percentiles represents the mean value for CTC detection within each subset of analysed samples. <65 and ≥65 refers to the patients' age in years at the time of blood drawl; left and right refers to the site of colon cancer. * $p < 0.05$.

Table 2. Association of clinico-pathological patients´ characteristics of the entire study population and CTC quantity partitioned for each technique of CTC detection.

Parameters	NYONE®			ScreenCell®			CK20 RT-qPCR		
	Positive *N* (%)	Mean (SD)	*p*	Positive *N* (%)	Mean (SD)	*p*	Positive *N* (%)	Mean (SD)	*p*
Total	16/44 (36.4)	0.89 (1.57)		31/31 (100)	3.25 (3.10)		33/41 (80.5)	3.11 (3.81)	
Gender									
Male	10/26 (38.5)	0.96 (1.71)	0.708	21/21 (100)	3.69 (3.52)	0.257	20/24 (83.3)	2.67 (2.19)	0.383
Female	6/18 (33.3)	0.78 (1.40)		10/10 (100)	2.32 (1.74)		13/17 (76.5)	3.74 (5.35)	
Age									
<65	8/18 (44.4)	1.39 (2.06)	0.078	15/15 (100)	3.23 (3.62)	0.980	12/17 (70.6)	2.48 (2.31)	0.379
≥65	8/26 (30.8)	0.54 (1.03)		16/16 (100)	3.26 (2.65)		21/24 (87.5)	3.56 (4.58)	
Tumour site									
Rectum	5/12 (41.7)	0.92 (1.31)	0.939	9/9 (100)	2.81 (2.39)	0.624	8/12 (66.7)	2.43 (2.20)	0.466
colon	11/32 (34.4)	0.88 (1.68)		22/22 (100)	3.43 (3.38)		25/29 (86.2)	3.40 (4.30)	
right	5/16 (31.3)	0.50 (1.03)	0.212	9/9 (100)	2.26 (1.46)	0.182	12/15 (80.0)	3.69 (5.37)	0.713
left	6/16 (37.5)	1.25 (2.11)		13/13 (100)	4.24 (4.10)		13/14 (92.9)	3.08 (2.94)	
UICC stage									
I + II	3/12 (25.0)	0.58 (1.24)	0.503	15/15 (100)	2.35 (2.24)	0.039	11/12 (91.7)	3.54 (4.70)	0.491
III + IV	6/18 (33.3)	1.06 (1.89)		16/16 (100)	4.09 (3.60)		13/16 (81.3)	2.71 (2.76)	

Statistical analysis was performed using an unpaired *t*-test for parametric and a Mann-Whitney U-test for non-parametric data. All *p*-values in bold are regarded as statistically significant. Abbreviations: UICC—Union internationale contre le cancer; SD—standard deviation.

2.5. Relative Quantification of the CTC Load by CK20 RT-qPCR

Finally, we determined the CTC load in our patient cohort with the well-established CK20 RT-qPCR [8–10]. In total, blood samples from 41 patients were collected (Table 1). In 33 cases (80.5%), the PCR revealed positive CK20 signals with a mean of relative CK20 mRNA expression units [EU] of 3.11 (range: 0–21.99 [EU]; SD: 3.81) (Table 2).

As shown in Figure 4C, similar results as revealed by the two methods of cytological enumeration of CTC were obtained. No significant differences were seen in the quantification of CTC by means of gender, age, tumour site, tumour localisation within the subset of colon cancer patients and the tumour stages (Figure 3C and Table 2). Interestingly, analysing the patients according to the tumour stages, an almost inverse relative detection of CK20 positive CTC was observed—with the mean relative CTC detection of stage I + II patients being 3.54 [EU] (range: 0–21.99 [EU]; SD: 4.70) vs 2.71 [EU] (range: 0–9.80 [EU]; SD: 2.76) in stage III + IV patients ($p = 0.491$) (Figure 3C). In the subset of stage I patients, one patient´s blood sample showed exceptionally high EU of CK20 mRNA (21.99 [EU]), potentially causing a significant bias to the analysis. Considering this as an outlier and re-analysing the data, the mean value of CTC detection in stage I + II patients was 2.57 [EU] (range: 0–5.88 [EU]; SD: 1.85) (data not shown). Thus, it can be concluded that the trend of lower relative CTC measurements in early stages compared to later stages could also be seen in this method ($p = 0.854$, data not shown).

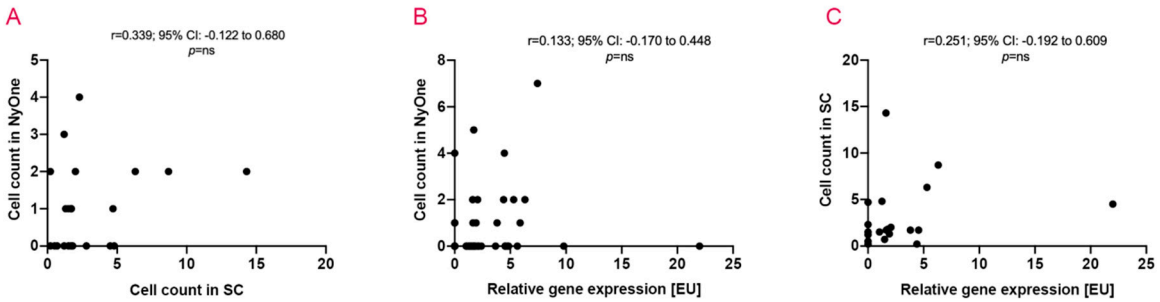

Figure 4. Correlation of the utilised detection methods and CTC quantity. Data obtained by each mode of detection for the CTC quantity were inter-correlated to the other technique. (**A**) NYONE® vs ScreenCell®(SC). (**B**) NYONE® vs CK20-RT-qPCR. **(C)** CK20 RT-qPCR vs ScreenCell®.

2.6. The Coherence of Applied Detection Methods

To further compare and validate the applied CTC detection techniques, blood samples from 21 patients were analysed with all three techniques. In 11 and 15 patients, respectively, CTC detection by the NYONE® cell imager and the CK20 RT-qPCR was possible, and in all cases, we were able to detect CTC applying the ScreenCell® Cyto IS device.

First, the data regarding the correlation of the two cytological approaches are showing a quite heterogenous picture. There was no significant correlation of the overall CTC count between both methods. Thus, a high CTC count obtained by the ScreenCell® Cyto IS device did not cohere with a high CTC count in the NYONE® approach (r = 0.251; 95% CI: −0.192 to 0.609; $p = 0.248$) (Figure 4A). A more detailed analysis revealed that there is an inclination of the mean CTC count with advancing tumour stages in both the NYONE® and the ScreenCell® CTC enumeration techniques (mean: 0.42 cells in UICC I + II vs mean: 1.56 cells in UICC III + IV; $p = 0.017$ and mean: 2.08 cells in UICC I + II vs mean: 4.26 cells in UICC III + IV; $p = 0.148$, respectively), though only within the subset of CTC detected by the NYONE® cell imager there is a statistical significance (Table 3). Data correlating the CTC count with tumour site characteristics differed for both cellular detection methods. In samples analysed with the NYONE® cell imager, the mean CTC count for rectum carcinoma patients was 1.57 cells, and for colon carcinoma patients significantly less: 0.57 cells ($p = 0.045$). By applying the ScreenCell® Cyto IS device for the same subset of patients, the mean count of CTC in rectum carcinoma patients was 2.03 cells, and in colon cancer patients with 3.51 cells higher by trend ($p = 0.356$). However, within

colon cancer patients (right vs left), the data were congruent. There was a trend towards higher cell count in patients with left-sided colon carcinoma being detected by either detection method (Table 3).

Table 3. Association of Clinico-Pathological Patients´ Characteristics and CTC Quantity of the Patients´ Subset Where all Three Tests Were Positive for CTC Detection. The Data is Partitioned for Each Technique of CTC Detection.

Parameters	NYONE®			ScreenCell®			CK20 RT-qPCR		
	Positive *N* (%)	Mean (SD)	*p*	Positive *N* (%)	Mean (SD)	*p*	Positive *N* (%)	Mean (SD)	*p*
Total	11/21 (52.4)	0.90 (1.03)		21/21 (100)	3.01 (3.16)		15/21 (71.4)	2.90 (3.81)	
Gender									
Male	7/14 (50.0)	0.86 (0.95)	0.785	14/14 (100)	3.34 (3.97)	0.541	10/14 (71.4)	2.16 (2.13)	0.328
Female	4/7 (57.1)	1.00 (1.41)		7/7 (100)	2.36 (1.69)		5/7 (71.4)	4.37 (7.88)	
Age									
<65	6/9 (66.7)	1.44 (1.33)	**0.047**	9/9 (100)	3.30 (4.50)	0.746	5/9 (55.6)	1.99 (2.22)	0.464
≥65	5/12 (41.7)	0.50 (0.67)		12/12 (100)	2.80 (2.40)		10/12 (83.3)	3.58 (6.04)	
Tumour site									
Rectum	5/7 (71.4)	1.57 (1.40)	**0.045**	7/7 (100)	2.03 (2.06)	0.356	4/7 (57.1)	2.33 (2.39)	0.709
colon	6/14 (42.9)	0.57 (0.76)		14/14 (100)	3.51 (3.84)		11/14 (78.6)	3.18 (5.66)	
right	3/7 (42.9)	0.43 (0.54)	0.502	7/7 (100)	2.24 (1.67)	0.834	4/7 (57.1)	4.22 (7.96)	0.517
left	3/7 (42.9)	0.71 (0.95)		7/7 (100)	2.03 (2.06)		7/7 (100)	2.15 (1.85)	
UICC stage									
I + II	4/12 (33.3)	0.42 (0.67)	**0.017**	8/8 (100)	2.08 (1.64)	0.148	7/8 (87.5)	3.50 (6.05)	0.521
III + IV	7/9 (77.8)	1.56 (1.24)		6/6 (100)	4.26 (4.65)		4/6 (66.7)	2.10 (2.28)	

Statistical analysis was performed using an unpaired *t*-test for parametric and a Mann-Whitney U-test for non-parametric data. All *p* values in bold are regarded as statistically significant. Abbreviations: UICC—Union internationale contre le cancer; SD—standard deviation.

Next, the techniques for cytological enumeration of CTC were compared with the molecular approach using CK20 RT-qPCR. Similar as described for the two cytological methods, no significant positive correlation between the enumeration results of either the NYONE® or the ScreenCell® technique with the CK20 RT-qPCR could be determined (r = 0.133; 95% CI: –0.170 to 0.448; $p = 0.154$) and (r = 0.339; 95% CI: −0.122 to 0.680; $p = 0.337$) (Figure 4B,C). Interestingly, analogous to the PCR data described above, the relative mean count for CTC (expressed by [EU]) in patients grouped by their tumour stages was even though not statistically significant ($p = 0.521$) opposing with the mean [EU] in stage I + II patients being 3.50 [EU] and in stage III + IV patients being 2.10 [EU] (Table 3). Again, regarding the stage I patient with an exceptionally high relative CTC count as an outlier, re-analysis of the data revealed a more consistent outcome by trend—stage I + II patients showed a mean value for relative CTC detection of 1.81 [EU] and stage III+IV patients 2.10 [EU] ($p = 0.665$, data not shown). Regarding the tumour site colon vs rectum, analogous to the cytological CTC detection technique with the ScreenCell® Cyto IS device, there was a trend for a lower relative CTC count for patients suffering from rectal carcinoma (mean 2.33 [EU]) compared to patients with colon cancer (mean 3.18 [EU]) ($p = 0.709$). Analysing the colon cancer patients in more detail and stratifying for right-sided and left-sided colon cancer, interestingly and contrary to both cytological CTC detection methods, there was a trend towards more CTC being detected in right-sided colon cancers (right: mean 4.22 [EU] and left: mean 2.15 [EU]; $p = 0.517$).

3. Discussion

Despite perspicuous progress in the field of diagnosis and therapeutic efficiency in recent decades, CRC still raises various obscurities. It still is a major health burden and patients still often die, due to disease progression because informative biomarkers for monitoring the course and identifying early

signs of progression are missing. Liquid biopsies have the potential to considerably revolutionise the scope of unique diagnosis and a precise follow-up by non-invasive means. Further, in recent years attention to biomarkers used for individualised diagnostics and therapeutic options has significantly increased. In this matter, various reports on the impact of CTC as a predictive and prognostic biomarker have been published so far [6,7]. CTC are thought to be directly linked to the primary tumour, detached from the cell bond, and hence, having the potential of initiating distant metastasis. After the process of intravasation, these CTC can be detected in peripheral blood by diverse approaches. However, CTC are extremely rare, and estimates are at about one cell per billion blood cells in patients with advanced disease stages [23].

In this prospective study, we deployed three discriminative techniques with the aim of associating the feasibility and plausibility of CTC determination related to clinico-pathological characteristics and to validate our already CK20 RT-qPCR based detection strategy. Thus, we established and later implemented a novel technique using the NYONE® cell imager for the cytological enumeration and detection of CTC in CRC patients. This technique offers the potential of an easily reproducible and robust semi-automated microscopy-assisted cell count of CTC based on prior enrichment of PBMC and an IF staining with target-specific antibodies. A major benefit of this technique is the straightforward application process. Shortly after blood drawl, the samples are processed to prevent a significant loss of CTC, due to a potentially short CTC half-life [24], but hereafter the cells are fixed and can be stored for up to four days. This simplifies the operational sequences for the investigator significantly as it stores the patients´ samples and later simultaneous analysis of a larger sample cohort. For IF staining, we utilised the antibodies anti-pan-CK, anti-Her2, anti-EGFR and anti-EpCAM that were previously depicted to be specific for the detection of CTC of epithelial tumours and particularly CRC [25–27]. Many other studies also utilising IF staining for enumeration purposes, only apply one, or very few IF-coupled antibodies for staining [27–29]. This may though potentially cause a significant underestimation of the actual CTC count, due to non-detection of unstained CTC, leading to a bias in the samples. To evade this detriment and elevate the sensitivity of enumeration, we utilised a combination of IF-coupled antibodies. After IF staining, we utilised a semi-automated microscopical approach for the CTC enumeration. The cell imager´s software depicted possible positive events in terms of CTC, and the investigator was later presented these picture files for manual assessment. This significantly reduced the costs for personnel, and further theoretically limits error margins considerably, by waiving manual cell counting. Added values are by examination of cell morphology.

The method presented with a low CTC count (mean <1 cells, range 0–7 cells) and a moderate sensitivity of 36.4% (16/44) which might be not an improvement for enumeration and detection of CTC in peripheral blood samples of CRC patients. Nevertheless, in this study, we prospectively recruited a representative cohort of CRC patients across all stages of tumour progression, demonstrating higher CTC counts with increasing tumour stages. Despite lacking statistical significance, which might be reasonable for a number of CRC patients, a presumed interrelation between the tumour burden and the CTC count can be drawn. Most other CTC studies focus on patients with advanced disease—stage III + IV patients with suspected significantly higher detection rates of CTC. A previous study by Bork et al. has also analysed CTC in non-metastatic CRC patients by means of CellSearch® technique and reported on exceptionally low rates of CTC in early tumour stages (≥2 CTC in 3.1% and ≥3 CTC in 1.7% of patients) and the lack of association of primary tumour characteristics with CTC detection [16]. Certainly, a downside, and hence, limitation of the marker dependent CTC detection as applied by Bork et al. with the widely employed CellSearch® application is the potential underestimation of the total CTC count by omitting CTC that might not express EpCAM, due to preceding EMT [18,19]. We limited this drawback skipping the enrichment step by immunobeads and additionally applying EMT markers like EGFR [30]. Henceforth, a relevant subpopulation of CTC might be undetected and left out also by our technique.

To overcome this potential pitfall of underestimating CTC, our aim was to employ a marker- and antigen-independent physical enumeration technique to the same patient cohort as analysed

by the NYONE® cell imager approach. For this purpose, we reverted to the ScreenCell® Cyto IS device. This is a technically simple to handle and cost-effective device for label-free isolation of CTC. The blood samples are passed through a membrane allowing for erythrocytes and leukocytes to pass through. Larger and less deformable cells, such as CTC, are effectively retained by the membrane, thus allowing for their enrichment and quantification. However, to clearly discriminate enriched tumor cells from leukocytes, we combined this size-dependent enrichment approach with a subsequent immunofluorescence labelling with anti-pan-CK and anti-CD45 antibodies. Our present data prove the positive surplus of CTC capture by the label-free isolation compared to the marker-dependent approach: The mean count for CTC in the samples analysed with the ScreenCell® technique was more than threefold higher than in the NYONE® subset. In the study conducted by Nicolazzo et al., the ScreenCell® technique was compared to the CellSearch® method as a label-dependent concept. Compliant to our findings, the marker-independent conception proved to be superior to the antigen-dependent technique of CTC enumeration, as significantly more CTC were captured by the ScreenCell® method [31]. Moreover, we were able to positively correlate the clinical characteristics of the patient cohort to the CTC count, making our data more robust. With progressing tumour stages, the CTC load significantly increased, indicating that patients with a high tumour burden contain notably more CTC in peripheral blood, which in general is concordant with other studies of CRC patients [16]. The clinical value of high CTC numbers in our patients will be subsequently evaluated in another study, as soon as appropriate follow-up data are available.

Strategies for CTC enumeration relying on isolation by size, though potentially also do not harvest the CTC population entirely. There is a wide variability to the size of CTC [7], making smaller CTC more likely to be missed. Furthermore, in some cases, a significant contamination of leukocytes may negatively influence the ability of diligent CTC enumeration. Thus, our approach combining a size-dependent but marker-free CTC enrichment with subsequent immunofluorescence staining of CTC and leukocyte related antigens seems to enhance specificity and sensitivity of CTC detection and enumeration. Moreover, this approach yielded even a higher CTC detection rate as obtained by our well-established semi-quantitative CK20 RT-qPCR, which demonstrated already a clinical significance of indirect CTC detection in previous studies [8–10]. Importantly, the data concerning tumour burden and CTC load were concordant with the cytological approaches, as well as with a previous study that analysed CTC by CK20 RT-qPCR in a larger cohort of CRC patients [8].

4. Materials and Methods

4.1. Patient/Proband Recruitment and Sample Preparation

In total, 57 patients with a histologically verified colorectal carcinoma were prospectively enrolled in this study in the years 2017 and 2018. All patients underwent surgery at the Department of General, Visceral, Thoracic, Transplantation and Paediatric Surgery, University Hospital Schleswig-Holstein, Campus Kiel. Patients with UICC stage III or IV cancer were recommended to receive adjuvant or palliative chemotherapy, respectively, according to the therapy guidelines. All patients gave written informed consent to participate in this study, and the study was approved by the local ethics committee of the Medical Faculty, University of Kiel and the University Hospital Schleswig-Holstein, Campus Kiel (Reference No. A110/99). Classification of the pathological tumour stage was handled by the Department of Pathology, University Hospital Schleswig-Holstein, Campus Kiel, according to the TNM-classification (eighth edition). Clinical data were obtained from the clinical research database of the oncological biobank of the Comprehensive Cancer Center Kiel (BMB-CCC), and data were verified by re-examination of original patient records.

The peripheral blood samples were taken shortly prior to surgery from a central venous line. As three differing techniques for CTC detection were applied, the blood sample collection was handled optimally for the deployed method. For the semi-automated detection of CTC, blood was drawn into an 8.2 mL Citrate-Monovette (S-Monovette® 8.2mL 9NC, 3.2% tri-Sodium Citrate, Sarstedt,

Nümbrecht, Germany). For analysis with the ScreenCell® Cyto IS device (ScreenCell®, Sarcelles, France) approximately 8 mL blood were drawn into an EDTA vacutainer (Vacutainer Tube EDTA (K2E), Becton Dickinson (BD), Heidelberg, Germany). For PCR analysis, approximately 20 mL blood were drawn with lithium heparin Monovettes (Sarstedt). All samples were further processed for analysis within 2 h.

4.2. Sample Analysis by IF Staining and Semi-Automated Microscopy—NYONE®

To validate the semi-automated microscopic approach with the NYONE® (SYNENTEC, Elmshorn, Germany), cultured HT29 human CRC cells (approximately 100 cells, achieved by repeated counting) were spiked into 8.2 mL of blood from healthy donors who gave written informed consent. These blood samples were then transferred into Vacutainer-CPT-tubes (BD) and processed according to the manufacturer´s guidelines. The enriched mononuclear cell (MNC)-fraction was later incubated and stained with Alexa488-conjugated anti-CD45 antibodies (#304017; Biolegend, San Diego, CA, USA) for the detection of leucocytes (green fluorescence) and Alexa647-conjugated anti-EGFR (#sc-120 AF647; SantaCruz, Dallas, TX, USA), anti-Her2 (#3244412; Biolegend), anti-EpCAM (#324212; Biolegend) and anti-pan-CK (#628604; Biolegend) antibodies against the CTC (red fluorescence). After a washing step, a buffer containing DAPI (#422801; Biolegend, San Diego, CA, USA) was added, and automated microscopy was performed using the NYONE® cell imager using the software package YT-software (SYNENTEC, Elmshorn, Germany) (Figure 5A). A CTC was defined as being DAPI and Alexa-647-positive, as well as Alexa488-negative. A detailed protocol of the method is given in the Supplementary Methods.

4.3. Sample Analysis by Size-Dependent Filtration and IF Staining—ScreenCell®

For establishing the ScreenCell® filtration device and testing specificity of the filtered tumour cells, HT29 cells were spiked into healthy donors´ blood (see above) and enriched on the isolation support (IS) with ScreenCell® Cyto (Figure 5B). A description of the workflow in full detail is given in the Supplementary Methods. Briefly, the filters were stained with RAL555 (May-Grunwald-Staining, MGG) (RAL Diagnostics, Martillac, France) and analysed by an independent cytopathologist. For verification of the putative cancer cells detected by MGG staining, IF staining and microscopy was performed afterwards. After destaining of the cells, double IF immunostaining with the primary mouse anti-pan-CK (AE1/AE3, #M3515; Agilent, Santa Clara, CA, USA) antibodies against CTC and rabbit anti-CD45 (EP68) antibodies (#AC-0065A, Epitomics, Abcam, Cambridge, GB) against leucocytes, was carried out. Lastly, the secondary antibodies goat-anti-mouse and goat-anti-rabbit conjugated with Alexa488 (green—against CTC) (#A11001; Life Technologies, Carlsbad, CA, USA) and Alexa568 (red—against leucocytes) (#A11011; Life Technologies), respectively, were added. Note that the IF colours scheme of CTC and leucocytes of this protocol were contrary to the staining protocol of the NYONE® technique. A CTC was defined as being DAPI and Alexa-488 positive, as well as Alexa568-negative. A detailed protocol of the method is given in the Supplementary Methods.

4.4. Sample Analysis by Molecular Analysis of mRNA—Semi-Quantitative CK20 RT-qPCR

The application of a semi-quantitative RT-qPCR against CK20 has been previously established in our group [8]. Briefly, patients´ blood samples were processed by centrifugation through a Ficoll-Hypaque density cushion (GE Healthcare/Merck, Darmstadt, Germany) according to the supplier's recommendation for the enrichment of the mononuclear cell (MNC) fraction. MNC-RNA was isolated with RNAPure™ reagent (VWR Peqlab, Darmstadt, Germany) and cDNA was obtained by reverse transcription of 3 µg total RNA (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Darmstadt, Germany). The qPCR assays were run in total volumes of 20 µl on 96-well plates (Sarstedt) using TaqMan gene expression assays for cytokeratin 20/KRT20 (CK20), Hs00966063_m1 and for the housekeeping gene TBP (TATA-box binding protein), Hs00427620_m1, as a reference in combination with the TaqMan Fast Advanced Master Mix on a StepOne Plus real-time PCR System

(all ThermoFisher Scientific, Waltham, MA, USA). All samples were run in triplicate. Relative gene expression was calculated as arbitrary expression units (EU) by a simplified ΔC_t method based on the difference between CK20- and the reference gene TBP-C_t values computed using the StepOne software (ThermoFisher Scientific, Waltham, MA, USA).

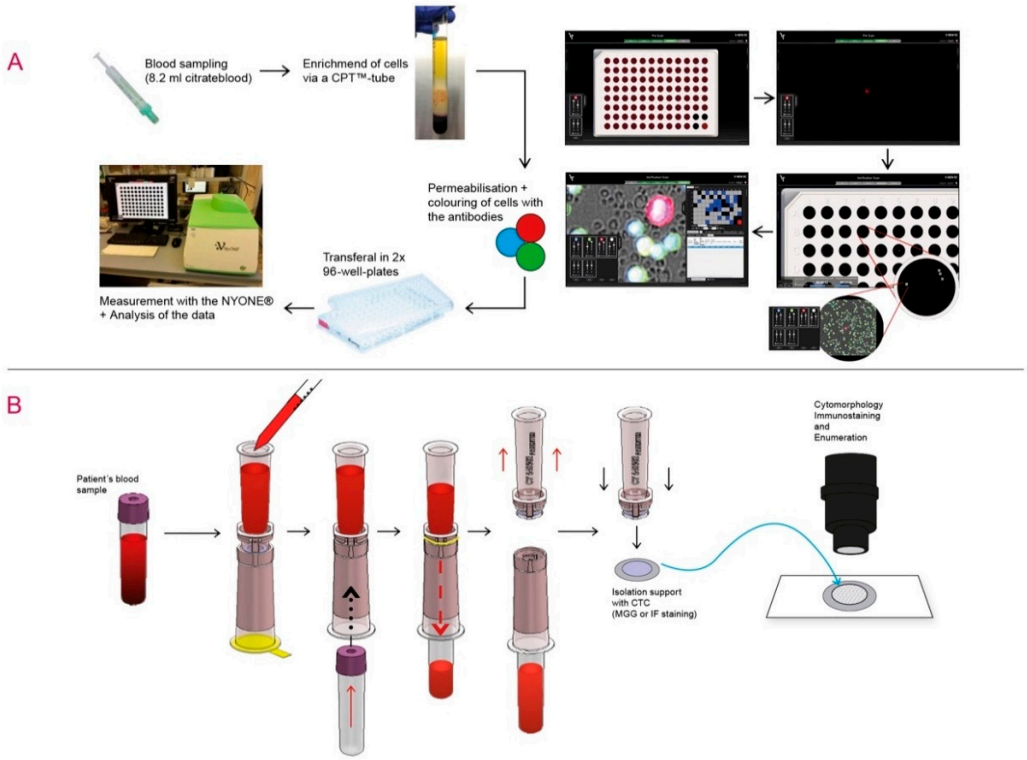

Figure 5. Experimental set-up for CTC detection using NYONE® and ScreenCell® technology. (**A**) NYONE®—Blood samples were collected. The enrichment of CTC was carried out by Ficoll centrifugation via CPT tubes. PBMC were fixed, permeabilised and stained with anti-CD45-Alexa488 (green to detect leukocytes), anti-EpCAM, anti-EGFR, anti-Her2 and anti-pan-CK antibodies (all Alexa647-coupled, red to detect epithelial cells) and DAPI (blue) for nuclei staining. The semi-automated enumeration was carried out by the cell imager NYONE®. Pre-scanning of all wells was done for the detection of red fluorescence, and all positive events were marked for further scanning for the detection of blue, green and red fluorescence, as well as a brightfield image. After image analysis, CTC (DAPI positive, negative for Alexa488 and positive for Alexa647) were encircled allowing cytological assessment. (**B**) ScreenCell®—Blood samples were collected with EDTA tubes. After adding the buffer solution, the sample was added to the filtration device. Adding an empty vacutainer, blood was drawn through the filter, and CTC remained on the filter. Followed by staining with MGG and/or IF, detection and enumeration of CTC were possible.

4.5. Statistical Analysis

All reported *p*-values are two-sided and were regarded as statistically significant at $p < 0.05$. When a Gaussian distribution of the data was assumed, the parametric data were analysed by a *t*-test. Non-parametric data were analysed by a Mann-Whitney U-test. For analysis of the correlation of the CTC detection results of the different detection methods, the Pearson correlation coefficient was calculated. Statistical calculation and testing were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

5. Conclusions

The present study depicts and proves the feasibility of three different methods for CTC detection and enumeration in CRC patients across all tumour stages. By the introduction of the semi-automated microscopy approach with the NYONE®, we implemented an investigator independent microscopy procedure for CTC detection that applies a set of four markers possibly boosting the sensitivity of CTC detection compared to already existing methods. However, this approach resulted in the lowest detection rate, while isolation of CTC by size (as a label-free technique with subsequent immunofluorescence labelling) yielded the highest rates of detection which was slightly higher than those indirectly obtained by CK20 RT-qPCR. All methods revealed a definite trend to rising CTC counts with advancing tumour burden. Since the primary aim of this study was the implementation of two cytological CTC detection techniques for validation of our molecular detection approach, the sample size of this prospective study is limited. For a recommendation for clinical use, and to substantiate the clinical implication of these results, has to be further supported, proficient follow-up data of this prospective study has to be collected, and a study with a larger cohort is required.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2072-6694/12/9/2643/s1.

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Conflicts of Interest: At the time of the study RG was working for ORGA Labormanagement GmbH, who was providing a CTC test using NYONE®. She is now working for SYNENTEC GmbH, who produces and distributes the NYONE® imaging system. All other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

CK-20	Cytokeratin-20
CRC	Colorectal cancer
CTC	Circulating tumour cells
EGFR	Epithelial Growth Factor Receptor
EMT	Epithelial-Mesenchymal-Transition
EpCAM	Epithelial adhesion molecule
EU	expression units
pan-CK	pan-Cytokeratin
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
UICC	Union internationale contre le cancer

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Anhang – Teil 3

Longitudinal Analysis of Circulating Tumor Cells in Colorectal Cancer Patients by a Cytological and Molecular Approach: Feasibility and Clinical Application

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Longitudinal Analysis of Circulating Tumor Cells in Colorectal Cancer Patients by a Cytological and Molecular Approach: Feasibility and Clinical Application

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Introduction: Liquid biopsies allowing for individualized risk stratification of cancer patients have become of high significance in individualized cancer diagnostics and treatment. The detection of circulating tumor cells (CTC) has proven to be highly relevant in risk prediction, e.g., in colorectal cancer (CRC) patients. In this study, we investigate the clinical relevance of longitudinal CTC detection over a course of follow-up after surgical resection of the tumor and correlate these findings with clinico-pathological characteristics.

Methods: In total, 49 patients with histologically proven colorectal carcinoma were recruited for this prospective study. Blood samples were analyzed for CTC presence by two methods: first by marker-dependent immunofluorescence staining combined with automated microscopy with the NYONE® cell imager and additionally, indirectly, by semi-quantitative Cytokeratin-20 (CK20) RT-qPCR. CTC quantification data were compared and correlated with the clinico-pathological parameters.

Results: Detection of CTC over a post-operative time course was feasible with both applied methods. In patients who were pre-operatively negative for CTCs with the NYONE® method or below the cut-off for relative CK20 mRNA expression after analysis by PCR, a statistically significant rise in the immediate post-operative CTC detection could be demonstrated. Further, in the cohort analyzed by PCR, we detected a lower CTC load in patients who were adjuvantly treated with chemotherapy compared to patients in the follow-up subgroup. This finding was contrary to the same patient subset analyzed with the NYONE® for CTC detection.

Conclusion: Our study investigates the occurrence of CTC in CRC patients after surgical resection of the primary tumor and during postoperative follow-up. The resection of the

tumor has an impact on the CTC quantity and the longitudinal CTC analysis supports the significance of CTC as a prognostic biomarker. Future investigations with an even more extended follow-up period and larger patient cohorts will have to validate our results and may help to define an optimal longitudinal sampling scheme for liquid biopsies in the post-operative monitoring of cancer patients to enable tailored therapy concepts for precision medicine.

Keywords: circulating tumor cells, colorectal cancer, NYONE® cell imager, CK20 RT-qPCR, longitudinal follow-up, liquid biopsies

INTRODUCTION

Despite tremendous efforts in the diagnosis and treatment of colorectal cancer (CRC), it still represents one of the most common causes of cancer-related deaths in Western countries (1). The fact that a proportion of patients is diagnosed with a localized tumor that can be resected in sano (R0) but later develop a tumor recurrence or distant metastases underlines the need for valid prognostic and predictive biomarkers that help to identify high-risk patients. Profound criteria for the stratification of patients at risk who might benefit from an adjuvant treatment have been developed (2, 3), though these almost all rely on histopathological parameters amongst very few other mutational characteristics of the primary tumor.

Consequentially, the concept of individualized diagnostics and therapeutic options has yielded major attention in recent years (4, 5), and biomarkers for either early detection of cancer or proof of minimal residual disease have been identified (6). As a potential tool, circulating tumor cells (CTC) have been identified and their suitability to serve as an additional instrument in risk stratification has been demonstrated manifold (7). These CTC are shed into the peripheral bloodstream not only from the primary but also from metastatic tumor sites and are linked to progressive disease and metastatic formation. In most cases of CRC patients with local disease, tumor resection is considered as a curative approach. The impact of surgery on the CTC count in the bloodstream has been already described, with generally a steep increase in CTC numbers shortly after surgical resection, but also a rapid normalization and often decrease in cell numbers within a short period of time (8). Though, studies on the enumeration of CTC in the long-term longitudinal follow-up of patients with solid tumors after surgery are rare.

CTC are extremely rare in the bloodstream and their valid detection and enumeration amongst multifarious numbers of leukocytes pose a major challenge. Up to date, various enrichment and detection techniques are available (9, 10). Categorically, CTC can be directly detected and enumerated by the means of cytological immunological staining, or indirectly detected by molecular approaches using PCR. The cytological approach for CTC enumeration is mostly marker-dependent, though many techniques employ only single antibodies for visualization of CTC. In this context, the most commonly used target antigens are EpCAM or several cytokeratins, which are highly specific to CTC of epithelial tumors. For the molecular detection of CTC, we previously reported on an RT-qPCR against cytokeratin 20 (CK20), which was already established to determine the prognostic value of the CTC load at the time point of surgery in respectable CRC patients (11, 12). By the introduction of a cut-off value allowing a relative CTC quantification, the negative prognostic significance of the amount of CK20-positive CTC in CRC patients could clearly be demonstrated (13).

In order to further extend these findings and to validate our CTC detection approach, this study aimed at a proof-of-principle study for a longitudinal follow-up of CRC patients after surgical resection with a series of set timepoints for blood draw. Furthermore, both a novel immunofluorescence-based and a molecular detection approach for enumeration and detection of CTC was employed and the results of both methods were compared. For both detection methods, peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation (Ficoll® or CPT Vacutainer) and then applied in either analysis For enumeration by the semi-automated cell-imager (NYONE®, SYNENTEC, Elmshorn, Germany) CTC were immunofluorescently (IF) stained utilizing a set of antibodies against highly specific antigens of CTC in CRC patients, namely, anti-EpCAM, anti-EGFR, anti-pan-Cytokeratin(CK), and anti-Her2, as established in an earlier study (14). Additionally, an established CK20 RT-qPCR assay was applied for relative CTC quantification, as described elsewhere (13). The obtained data were then correlated to clinical characteristics and follow-up records, e.g., local recurrence, adjuvant treatment. Special emphasis was laid on the longitudinal postoperative CTC detection since individual therapeutic decisions are frequently made based on the histopathological characterization of the tumor at the time of primary surgery.

MATERIALS AND METHODS

Patient Recruitment and Serial Sampling

In total, 49 patients with a histologically verified CRC were enrolled in this prospective study in the years 2017 and 2018. All patients were operated on at the Department of General, Visceral, Thoracic, Transplantation and Paediatric Surgery of the University Hospital Schleswig-Holstein (UKSH), Campus Kiel. In case staging diagnostics of a rectal carcinoma revealed a locally progressed tumor burden with either T3/T4 and/or N+ according to the TNM classification (TNM Classification of Malignant Tumors eighth edition), patients were admitted to a neoadjuvant radio-chemotherapy (RCTX). Patients, who were staged UICC (Union internationale contre le cancer) III or IV

after histopathological examination were recommended to be admitted to either adjuvant or palliative chemotherapy (CTX) post-operatively. All decisions were made according to the present guidelines (German S3-Guideline Colorectal Carcinoma, Version 2.1 – January 2019 AWMF-Registration Number: 021/007OL) and the general patients´ constitution in terms of morbidity and endorsement. All patients gave written informed consent to participate in this study. The study was approved by the local ethics committee of the UKSH Campus Kiel and the Medical Faculty, University of Kiel (#A110/99). Classification of the pathological tumor stage was handled by the Department of Pathology, UKSH Campus Kiel, according to the TNM-classification. Clinical data were obtained from the clinical research database of the oncological biobank of the Comprehensive Cancer Center Kiel (BMB-CCC) and data was verified by re-examination of original patient records.

Blood samples from the following time points were analyzed for the presence of CTC: Pre-operatively (t0) and the primary endpoint of the study was reached if a patient reached the last blood draw at 12 months post-operatively (t5). For this, each patient received an individualized follow-up regimen for a visit and blood sample drawing at set time points after the surgical procedure: one month (t1), three months (t2), 6 months (t3), 9 months (t4), and 12 months (t5) (**Figure 1A**). The peripheral blood samples were either taken shortly prior to surgery (t0) from a central venous line or obtained by puncture of the median cubital vein for the blood samples collected at the follow-up time points (t1-t5).

For this study, two different approaches for CTC detection were applied. For the immunofluorescence detection by NYONE®, approximately 8 ml of blood were collected into a Sodium Citrate-Monovette® (Sarstedt, Nümbrecht, Germany). For CTC detection by PCR, approximately 20 ml of blood were drawn into lithium heparin-Monovettes® (Sarstedt). All samples were further processed within 2 hours after blood draw.

Sample Analysis With the Semi-Automated Microscope—NYONE®

The establishment and procedure of CTC enumeration by semi-automated microscopic detection with the cell imager NYONE® (SYNENTEC, Elmshorn, Germany) has been described previously (14). Briefly, the mononuclear cell (MNC) fraction was isolated by Ficoll-cushion centrifugation and resuspended in a fixation buffer (#14190-094, Biolegend, San Diego, CA, USA), incubated for 15 minutes at room temperature (RT), after which the samples were stored at 4°C for up to four days until further analysis.

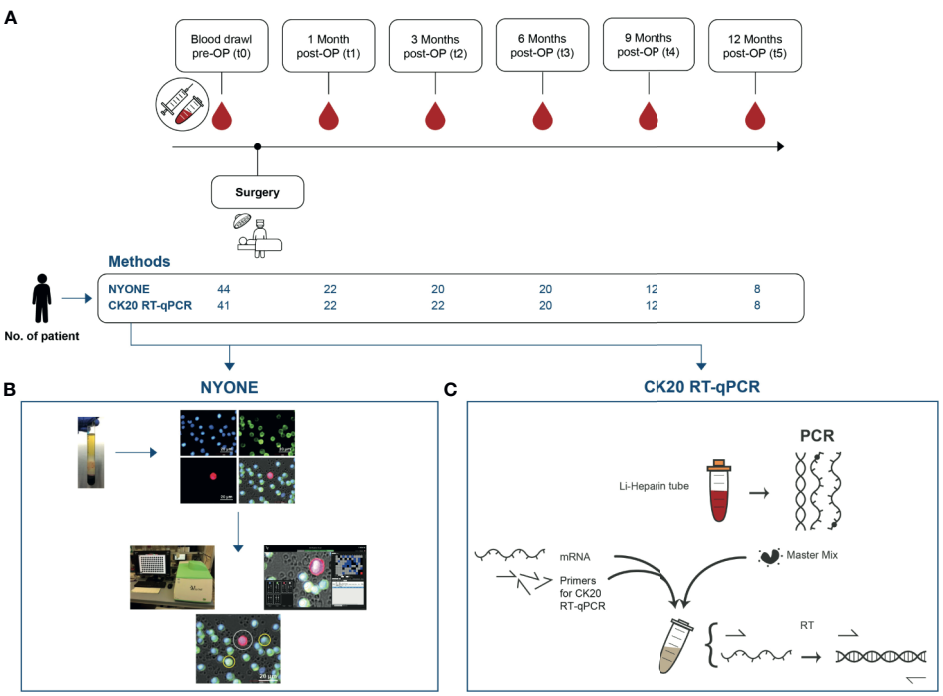

FIGURE 1 | Study outline and synopsis of the employed techniques for CTC detection. **(A)** Blood samples were collected repeatedly over a course of 12 months post-operatively. The first blood sample of each recruited patient was drawn immediately pre-operatively t0. Over the study period, five more samples were collected at set follow-up visits of each patient at 4 weeks (t1), 3 months (t2), 6 months (t3), 9 months (t4), and 12 months (t5). **(B)** NYONE® – After CTC enrichment by Ficoll centrifugation *via* CPT tubes, PBMC were fixed, permeabilized, and stained with anti-CD45-Alexa488 (green to detect leukocytes), anti-EpCAM, anti-EGFR, anti-Her2, and anti-pan-CK antibodies (all Alexa647-coupled, red to detect epithelial cells) and DAPI (blue) for nuclei staining. The enumeration process of CTC was carried out by the cell imager NYONE®. After image analysis, CTC (DAPI positive, negative for Alexa488, and positive for Alexa647) were encircled allowing cytological assessment. **(C)** Semi-quantitative RT-qPCR against CK20 –After Ficoll centrifugation of the blood plasma samples, the enriched fraction of PBMC was isolated and cDNA was obtained after RNA preparation. Then, a TaqMan gene expression assay [KRT20 (CK20)] and TBP as a house-keeping gene were used.

Then, cells were permeabilized in a Perm-/Wash-Buffer (#421002, Biolegend) for 5 minutes at RT and centrifuged at 330xg for 10 minutes. Afterward, cells were incubated with an Fc-blocking buffer (#422301/2, Biolegend) for 15 minutes. Then, cells were incubated for 30 minutes with the following antibodies: anti-CD45-AF488 (#304017; Biolegend), anti-EpCAM-AF647 (#324212; Biolegend), anti-pan-CK-AF647 (#628604; Biolegend), anti-EGFR-AF647 (#sc-120 AF647; SantaCruz, Dallas, TX, USA), and anti-Her2-AF647 (#3244412; Biolegend). Finally, a buffer containing DAPI (1:10,000) (#422801; Biolegend) was added to the cells.

Subsequently, 200 µl of the stained cell suspension was transferred into each a well of two 96-well plates (Sarstedt), which was centrifuged at 330xg for 10 minutes, and afterward placed in the NYONE® cell imager.

For analysis, the plates were scanned only for the detection of Alexa647 fluorescence (Ex 632/22, Em 685/40) as only CTC should be positive in this setting, which was detected by the respective antibody cocktail against epithelial markers EpCAM, pan-cytokeratin, EGFR, HER2. SYNENTEC's proprietary YT®-Software automatically analyzed the images already during scanning and detected positive events (**Figure 1B**). The image processing settings for this analysis were determined previously using blood samples from healthy donors spiked with HT29 tumor cells (14). Each event was then automatically further analyzed in depth by creating a region of interest (ROI) around it. This ROI was scanned in four channels (DAPI: Ex 377/50 Em 452/45, Alexa488: Ex 475/28 Em 530/43, Alexa-647: Ex 632/22 Em 685/40, brightfield: Ex brightfield Em blue). YT®-Software then automatically detected the cells´ nuclei (DAPI, blue fluorescence) and analyzed whether a virtual cytoplasm surrounding the nuclei was fluorescing green (CD45) or red (EpCAM, pan-CK, EGFR, Her2). These events were finally presented separately by the software and the investigator was able to examine the morphology of the potential CTC (**Figure 1B**).

Sample Analysis by Molecular mRNA Detection: Semi-Quantitative CK20 RT-qPCR

The application of a semi-quantitative CK20 RT-qPCR for CTC detection (**Figure 1C**) in CRC patients has been previously established in our work group (13). Briefly, blood samples were processed by ficoll-centrifugation to isolate the MNC fraction. Then, RNA was isolated with RNAPure® reagent (VWR Peqlab, Darmstadt, Germany) and cDNA was obtained by reverse transcription of 3 µg total RNA (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Darmstadt, Germany). The qPCRs were run in a total volume of 20 µl per well of a 96-well plate (Sarstedt) using the TaqMan gene expression assays for CK20 (KRT20, Hs00966063_m1) and for the housekeeping gene TBP (TATA-box binding protein), Hs00427620_m1, as a reference in combination with the TaqMan Fast Advanced Master Mix on a StepOne Plus realtime PCR System (all ThermoFisher Scientific). All samples were run in triplicate. Relative gene expression was calculated as arbitrary expression units [EU] by a simplified ΔC_t method normalizing the CK20 expression against the reference gene TBP expression.

Statistical Analysis

All reported P-values are two-sided and were regarded statistically significant at $P < 0.05$. When a Gaussian distribution of the data was assumed, the parametric data were analyzed by either a repeated measure or ordinary one-way-ANOVA test. Non-parametric data were analyzed by a Mann-Whitney U-test. Statistical calculation and testing were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Patients Demographics

A synopsis of all patient data relating to the entire cohort but also the subgroups in terms of detection method is given in **Table 1**. In total, blood samples from 49 patients were included in this prospective study (**Figure 1A**). A total of 30 male and 19 female patients were enrolled and the median age at the time of operation and first blood sample collection was 67 years (range: 48–89 years). In total, 32 patients were diagnosed with colon carcinoma and 17 patients with rectal carcinoma. Amongst the colon carcinoma subset, an equal composition between left- and right-sided carcinoma (both 16 cases) was noted. The study cohort was further stratified by a clinico-pathological staging according to the UICC stages I-IV, with the most patients diagnosed with stage III (38.8%). In total, 10 patients (all rectal carcinoma) were treated by neoadjuvant radio-chemotherapy, and 18 patients received adjuvant chemotherapy.

TABLE 1 | Patient demographics and clinical characteristics of the entire study population and further breakdown according to the utilized detection modes.

	Total *N* (%)	NYONE *N* (%)	CK20 *N* (%)
	49 (100)	44 (100)	47 (100)
Gender			
Male	30 (61.2)	26 (59.1)	29 (61.7)
Female	19 (38.8)	18 (40.9)	18 (38.8)
Age			
Median (range)	67 (45-89)	66 (45-89)	67 (45-89)
<65	21 (42.9)	18 (40.9)	21 (44.7)
≥65	28 (57.1)	26 (59.1)	26 (55.3)
Tumor site			
Colon	32 (65.3)	32 (72.7)	30 (63.8)
Right	16 (50.0)	16 (50.0)	15 (50.0)
Left	16 (50.0)	16 (50.0)	15 (50.0)
Rectum	17 (34.7)	12 (27.3)	17 (36.2)
UICC stage			
I	14 (28.6)	12 (27.3)	14 (29.8)
II	9 (18.4)	9 (20.5)	8 (17.0)
III	19 (38.8)	18 (40.9)	18 (38.3)
IV	7 (14.3)	5 (11.4)	7 (14.9)
Neoadj. treatment			
Yes	10 (58.8)	6 (50.0)	10 (58.8)
No	7 (41.2)	6 (50.0)	7 (41.2)
Adjuvant treatment			
Yes	18 (36.7)	14 (31.8)	17 (36.2)
No	31 (63.3)	30 (68.2)	30 (63.8)

The subset of patients who received neoadjuvant treatment solely comprised of patients with rectal carcinoma. UICC, Union internationale contre le cancer.

Altogether, 44 and 47 patients were enrolled for CTC analysis by the cytological semi-automated microscopy (NYONE®, **Figure 1B**) and the indirect molecular approach by CK20 RT-qPCR (**Figure 1C**), respectively. Generally, the distribution of the two subsets of patients according to the demographical and clinical parameters was assimilable. The median age of patients within the NYONE® subgroup was 66 years (range: 45–89 years) and within the PCR group 67 years (range: 45–89 years) at the time of blood draw. In both groups, the majority of patients were male (59.1% – NYONE® and 61.7% – PCR) and were diagnosed with a colon carcinoma (72.7% – NYONE® and 63.8% – PCR). Again, most of the patients were diagnosed with locally advanced tumor burden and staged UICC III (40.9% – NYONE® and 38.3% PCR).

Longitudinal Analysis of CTC Count by IF and the NYONE® Cell-Imager

Altogether, we were able to enroll 44 patients for the longitudinal follow-up. During the time of the study period, the number of patient re-visits declined (**Figure 1A**).

In general, positivity rates during the collection time-course of CTC by the NYONE® technique were low and comparable at the first (pre-operative, t0) time points (**Table 2**), ranging between a mean CTC count of 0.89 and 1.5. At t5, a considerable increase of the mean CTC count could be observed (mean 4.25 CTC; SD: 10.01). However, this might be explained by one patient´s exceedingly high CTC count of 29 IF-positive cells.

Surgical resection of the tumor did not seem to have an effect on the frequency of CTC in the peripheral blood of the patients as the mean count of CTC was 0.89 CTC (range: 0–7 CTC; SD: 1.57) at t0 (prior to surgery) and 1.18 CTC (range: 0–4 CTC; SD: 1.33) at t1 (p not significant). Furthermore, analyzing the following blood samples over the time course, the CTC count of the overall study population did not show any significant alterations from the initial CTC prevalence (all p not significant) (**Table 2** and **Figure 2A**).

Stratifying the study population by means of CTC positivity (n=16 patients) *vs*. negativity (n=28 patients) at t0, a statistically significant increase in the CTC count at t1 was monitored in the subgroup without any pre-operative signs of CTC (mean: 0.00; SD: 0.00 at t0 and mean: 0.93; SD: 1.22 at t1; p=0.023). In all other measurements at later time points of the study, no significant differences compared to the baseline at t0 were observed in this subgroup (all p not significant) (**Figure 2B**). In contrast, in patients who initially had shown evidence of CTC in the peripheral blood, a general decrease in CTC by trend could be monitored (**Figure 2C**).

Next, we further stratified the cohort by adjuvant chemotherapeutic treatment and analyzed patients who received treatment (CTX+) in comparison to patients who were solely admitted to follow-up care (CTX-). We analyzed the patients´ CTC counts accordingly and compared the mean cell counts of CTC at each time point individually. Surprisingly, CTX+ patients showed higher CTC counts almost throughout the entire study period with a statistically significant higher CTC amount at t5 (CTX+: mean 7.75 cells, SD 14.17 *vs*. CTX-: mean 0.75 cells, SD 0.5; p=0.015) (**Table 2** and **Figure 2D**).

We additionally analyzed the development of the cohort during the observation period based on the amount of CTC. Subgroups were defined by the absence of CTC (0 cells per patient sample), intermediate frequency (1–2 cells per patient sample), and high CTC rates (≥3 cells per patient sample). The definition of high CTC frequency as ≥3 cells was based on different studies which proposed this as a clinically significant cut-off determined by the CellSearch® system (15). Interestingly, while the percentage of patients with no cells in the blood declined during the time period of the study, a highly significant increase in patients with both intermediate (1–2 CTC) and high prevalence of CTC (≥3 CTC) was monitored for the duration of the study (p=0.002) (**Figure 2E**). In detail, at t0 approximately 63.3% of the patients were CTC negative while at t5 the percentage declined to 25.0%.

Longitudinal Analysis of the Relative CTC Load by CK20 RT-qPCR

In total, 47 patients were recruited in this study arm. **Table 1** gives an overview of the clinical and pathological data of the

TABLE 2 | CTC quantity partitioned for each technique of CTC detection and outlined for each follow-up timepoint with association of the impact of adjuvant chemotherapy on the CTC quantity over the study period.

	t0	*p*	t1	*p*	t2	*p*	t3	*p*	t4	*p*	t5	p
NyOne												
CTC positive patients: fraction (%)	16/44 (36.4)		12/22 (54.5)		12/22 (54.5)		9/20 (45.0)		7/12 (58.3)		6/8 (75.0)	
CTC count per patient: mean (SD)	0.89 (1.57)		1.18 (1.33)		1.50 (2.61)		1.00 (1.69)		1.33 (1.67)		4.25 (10.01)	
CTC count according to adjuvant chemotherapy												
CTX+: mean (SD)	1.50 (2.28)	ns	2.60 (1.34)	ns	2.25 (3.28)	ns	0.86 (0.90)	ns	0.83 (0.98)	ns	7.75 (14.17)	**0.015**
CTX-: mean (SD)	0.60 (1.04)		0.76 (1.03)		1.07 (2.16)		1.08 (2.02)		1.83 (2.14)		0.75 (0.50)	
CK20 RT-qPCR												
CTC positive patients: fraction (%)	33/41 (80.5)		19/22 (86.4)		17/22 (77.3)		16/20 (80.0)		7/12 (58.3)		8/8 (100.0)	
CTC count per patient: mean (SD)	3.11 (3.81)		3.55 (6.17)		3.08 (3.19)		2.41 (1.62)		1.61 (1.95)		4.16 (5.66)	
Patients ≥ cut-off: fraction (%)	15/41 (36.6)		8/22 (36.4)		11/22 (50.0)		10/20 (50.0)		2/12 (16.7)		3/8 (37.5)	
CTC count according to adjuvant chemotherapy												
CTX+: mean (SD)	2.43 (2.42)	ns	2.00 (1.85)	ns	3.21 (3.98)	ns	1.74 (1.45)	ns	1.21 (1.49)	ns	1.86 (0.55)	ns
CTX-: mean (SD)	3.40 (4.26)		4.14 (7.13)		3.00 (2.81)		2.76 (1.64)		2.01 (2.41)		6.45 (7.77)	

All p values in bold are regarded as statistically significant. UICC, Union internationale contre le cancer; SD, standard deviation; CTC, Circulating tumor cells; CTX, chemotherapy; ns, not significant.

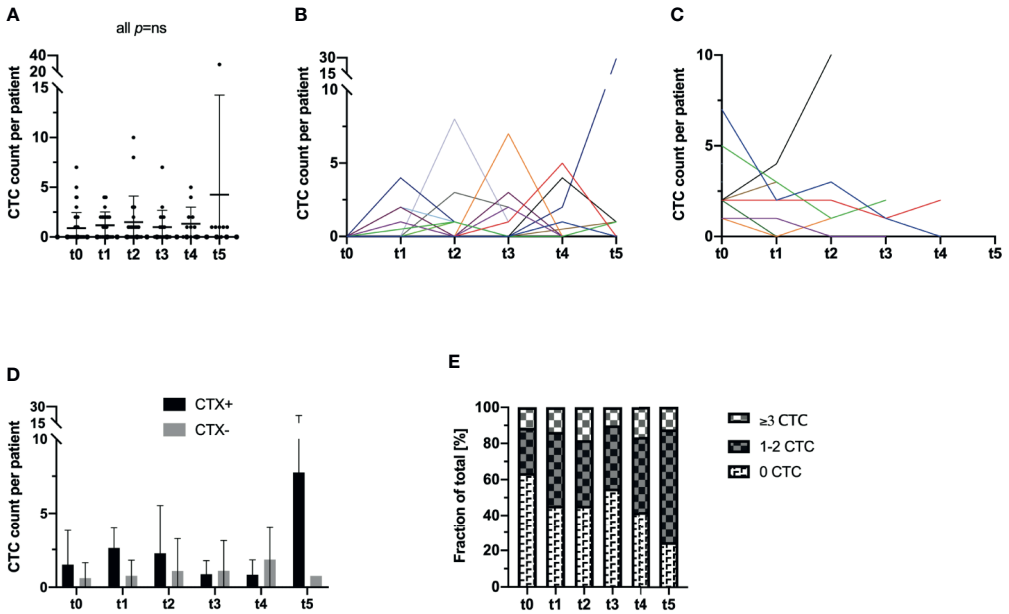

FIGURE 2 | Longitudinal analysis of blood samples of 44 CRC patients for the incidence and enumeration of CTC by a semi-automated microscopical approach with NYONE®. **(A)** In the study cohort as a whole, no statistically significant deviations in terms of an in- or decrease of the CTC count compared to t0 could be observed. The bar represents the mean count of CTC. **(B, C)** The individual patient with its longitudinal CTC quantification data is displayed by each line (each color represents one patient). **(B)** All patients that pre-operatively (t0) had no detectable CTC were analyzed in this subset. At t1, there was a statistically significant (p=0.023) increase in the CTC quantity. Throughout the further visits, no significant deviation from the initial CTC quantity (t0) was observed. **(C)** All patients with detectable CTC at t0 were sub-grouped for this analysis. There was no statistical significance for deviations over the study period from the initial CTC count. **(D)** Patients were stratified and subdivided according to their necessity of adjuvant chemotherapeutic treatment (CTX+). Patients that did not require adjuvant treatment were grouped in the follow-up subset (CTX-). **(E)** Patients were stratified and grouped according to the patients´ individual quantity of CTC: No CTC, 1-2 CTC (intermediate), ≥3 CTC (high). Analyzing the data as fractions of a whole, throughout the study a significant increase of patients with intermediate or high CTC counts was monitored.

patients. Blood samples that were collected at the time of operation and further samples that allowed for longitudinal CTC analysis were available from 41 patients.

In terms of tumor stages, the present patient cohort is representative, and the sensitivity rate of our applied CK20 RT-qPCR (80.42% positive for CTC, **Table 2**) is comparable to our previously reported data (13).

In line with the findings obtained with the NYONE® cell imager, no statistically significant short-term effect on the CTC/CK20-positivity load by the surgical procedure could be observed (mean: 3.11 [EU], SD: 3.81 at t0, and mean: 3.55 [EU], SD: 6.17 at t1; p not significant). Like the cytological analysis with the NYONE® cell-imager, the RT-qPCR analysis did also not reveal any significant alteration of the relative CTC/CK20-positivity throughout the study period compared to the pre-operative CTC signal at t0 (all p not significant) (**Table 2** and **Figure 3A**).

Next, we further stratified patients by applying a clinically and prognostically relevant cut-off for relative CTC positivity by CK20 RT-qPCR, which was established in a previous study (13). Patients were divided into two subgroups pre-operatively exhibiting either a high CTC positivity (≥2.77 [EU]; CTC-high) or a low CTC positivity (<2.77 [EU]; CTC-low). Patients who were pre-operatively (t0) in the CTC-low group, post-operatively (t1) showed a statistically significant increase in CTC numbers measured by CK20 RT-qPCR (mean: 1.17 [EU], SD: 0.85 at t0 *vs.* 4.36 [EU], SD: 7.94 at t1; p=0.047) (**Figures 3B, D**) which then declined again until t4 (mean: 0.78 [EU], SD: 0.99). Interestingly, a statistically significant increase in the relative CTC/CK20-positivity could be monitored from 9 to 12 months after surgery (t4 to t5; mean: 0.78 EU, SD: 0.99 at t4 *vs.* mean: 2.44 EU, SD: 0.77 at t5; p=0.018).

In contrast, in patients with a pre-operative high CTC-positivity, the surgical procedure and hence tumor burden reduction significantly reduced the CTC load during the first month (mean: 6.49 EU, SD: 4.56 at t0 *vs.* 2.68 EU, SD: 1.51 at t1; p<0.001). However, analyses at later time points throughout the study then revealed a slight increase of CTC compared to the post-operative CTC load at t1 (**Figures 3C, E**).

Interestingly, comparing the data obtained at t1 of both patient subsets, no difference emerged in the relative quantity of CTC between the groups (mean: 2.68 [EU], SD: 1.51 at t1 CTC high and mean: 4.36 [EU], SD: 7.94 at t1 CTC low; p=ns). Patients with pre-operative high CTC counts dropped post-operatively to a comparable level of patients with pre-operative low CTC counts who exhibited a post-operative increase in relative CTC loads.

Next, we subdivided the patients examined by CK20 RT-qPCR according to their status of adjuvant chemotherapy analogs to the cohort of patients in the NYONE® subset.

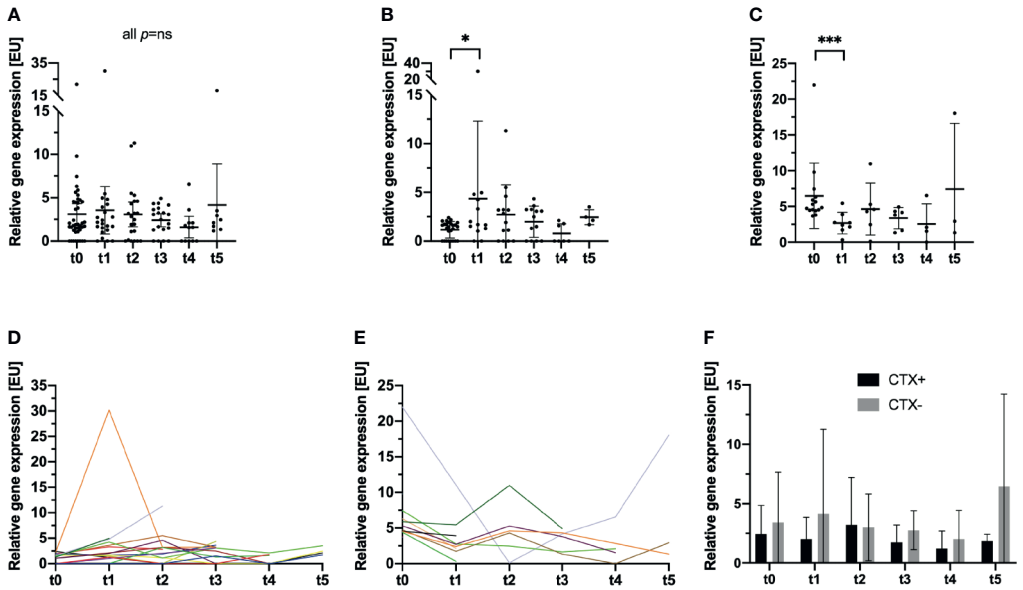

FIGURE 3 | Longitudinal analysis of blood samples of 47 CRC patients for the incidence and relative enumeration of CTC by a semi-quantitative CK20 RT-qPCR. Results are expressed by expression units [EU]. The bar represents the mean relative CTC count expressed by [EU]. **(A)** The entire study cohort is analyzed, and no statistically significant deviations of the relative CTC count compared to t0 were monitored. **(B–E)** The previously reported clinically significant cut-off value for CTC detection by CK20 PCR in CRC patients (13) was applied and the cohort stratified for further analysis. **(B, D)** In patients, who were below the cut-off at t0, a significant increase in CTC at t1 was monitored (p=0.047). No further differences were monitored at later visits compared to t0. Each color represents an individual patient. **(C, E)** For patients who were above the cut-off at t0, a significant decrease in the relative CTC quantity was recorded (p<0.001). No further significant deviations were monitored at later visits. **(F)** Patients were stratified and subdivided according to their necessity of adjuvant chemotherapeutic treatment (CTX+). Patients that did not require adjuvant treatment were grouped in the follow-up subset (CTX-). *p≤0.05; ***p≤0.001.

Contrary to the obtained data of the NYONE® subgroup analysis, CTX seemed to have an effect on the CTC enumeration. Patients in the CTX+ cohort showed lower relative CTC counts by trend at almost all re-visits. Only at t2 was there a slightly higher CTC count in patients of the CTX- subgroup (CTX+: mean 3.21 [EU], SD 3.98 *vs.* CTX-: mean 3.00 [EU], SD 2.81; p=ns) (**Table 2** and **Figure 3F**)

Evaluation of Individual Longitudinal Patient Courses

After having analyzed the overall cohort, we next focused on patients´ individual CTC courses detected by the two enumeration methods and linking the obtained results to the clinical follow-up data.

Figure 4A exemplarily displays the context and interplay of CTC enumeration and adjuvant treatment after surgery. This patient was diagnosed with an adenocarcinoma of the descending colon, but with locally progressed tumor burden and nodal positive stage III disease. In line with the guidelines (German S3-Guideline Colorectal Carcinoma, Version 2.1 – January 2019 AWMF-Registration Number: 021/007OL) the patient was admitted to adjuvant chemotherapy after surgical resection. Fitting to the advanced tumor burden, the patient showed exceptionally high numbers of CTC pre-operatively (t0) (NYONE®: 7 cells, PCR: 7.44 [EU]) in comparison to the general average of the cohort. Around 1 month (t1) after surgery and shortly before initiation of chemotherapy, the CTC count had dropped significantly (NYONE®: 2 cells, PCR: 2.77 [EU]) and remained below the cut-off values of both detection methods during the entire course of adjuvant chemotherapy (t1 up to t3) and the end of the observation period (t5). However, even though CTC enumeration from t3 until t5 was below the cut-off value, the PCR-based approach revealed a slight increase in the CTC enumeration starting at t4, at which the cytological approach further indicated a decline in the CTC load. Tumor markers CEA and CA19.9 were monitored at t0 and were not elevated at that time. Follow-up diagnostics were performed within the investigation period and did not show any signs of macroscopical tumor relapse. Since the patient dropped out of the study after t5, we could not further elaborate whether the CTC increase determined indirectly by CK20 RT-qPCR was indicative of a reactivation of a minimal residual disease (MRD) and clinical relapse.

Figure 4B exemplarily displays the disease course of a patient diagnosed with a stage II adenocarcinoma of the descending colon and provides an example of the potential of CTC as biomarkers for add-on recurrence diagnostics. According to the general guidelines (German S3-Guideline Colorectal Carcinoma, Version 2.1 – January 2019 AWMF-Registration Number: 021/007OL), adjuvant chemotherapy was not given, and the patient was only admitted to oncological follow-up. Pre-operatively (t0), no CTC could be detected with the NYONE® cell imager and the relative enumeration by CK20 RT-qPCR also revealed a measurement below the cut-off value. At t2 (3 months later), CTC detection by both the NYONE® and PCR was still

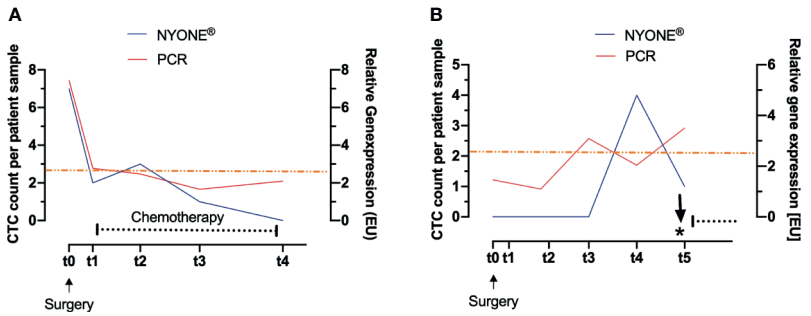

FIGURE 4 | Evaluation of CTC load during individual longitudinal patient courses. CTC were enumerated by two altering methods: cytologically after IF-staining by detection with the NYONE® (blue line) and molecularly by analysis of CK20-gene expression by RT-qPCR (red line). The asterisk indicates the time point of diagnosis of the local recurrence of carcinoma. The black dotted line indicates the interval of chemotherapy. The orange line represents the clinically significant cut-off value of CTC detected by CK20 RT-qPCR. **(A)** CRC patient with stage III carcinoma of the descending colon. After the operation, the CTC load dropped markedly and under the adjuvant therapy, no significant rise in CTC was observed. **(B)** CRC patient with stage II carcinoma of the descending colon. A total of 24 weeks after the operation, a clear rise of CTC in both detection methods was monitored, with the PCR-based approach being even earlier Clinically no significant follow-up event was recorded. Thirteen months after t0 a local recurrence of the carcinoma was diagnosed.

negative, and the PCR-based analysis even revealed a slight decrease of the CTC count (1.46 [EU] at t0 and 1.10 [EU] at t2). At t3 (6 months later), a relevant increase of the relative CTC enumeration above the cut-off value could be monitored by CK20 RT-qPCR (3.09 [EU]), while the NYONE® analysis still did not reveal any CTC positivity. At t4 (9 months after surgery), a significant increase in the CTC count could also be detected by the microscopical enumeration approach (0 cells at t0-t3 and 4 cells at t4). The last measurement at t5 (12 months after surgery) revealed a continuous increase in the relative quantification of CTC by CK20 RT-qPCR (3.50 [EU]), but surprisingly a declining cytological detection by the NYONE® (4 cells at t4 and 1 cell at t5). Interestingly, 13 months after initial surgery, a local recurrence of the primary adenocarcinoma was detected. Important to note that the tumor markers CEA and CA19.9 were below the cut-off levels at all times. In summary, these findings suggest that we were able to monitor a significant increase in CTC by both techniques being indicative of a reactivation of an MRD prior to its detection by the imaging diagnostics conducted according to the standard guidelines of follow-up (German S3-Guideline Colorectal Carcinoma, Version 2.1 – January 2019 AWMF-Registration Number: 021/007OL).

DISCUSSION

The benefit of CTC diagnostics as a biomarker for assessing the disease prognosis in cancer patients is evident (6, 9). However, the majority of studies firstly focus on patients with systemic stage IV disease and, secondly, conclude the patients' prognosis by CTC-analysis from a single pre- or post-operative blood sample only. Accordingly, CTC numbers or CTC associated parameters were determined only once and correlated with the clinical follow-up (16–21). Only very few studies have analyzed the prognostic potential of longitudinal CTC quantification over a period of time (22, 23).

In this prospective pilot study, we longitudinally monitored a cohort of CRC patients of miscellaneous tumor stages, who underwent surgical resection of the tumor and postoperatively were either admitted to oncological follow-up or adjuvant chemotherapy treatment. All patients enrolled were recontacted for serial blood samples, partially on the occasion of routine follow-up examination over a course of 12 months post-operatively. CTC enumeration was carried out by two differing techniques, (i) cytologically, by IF staining and microscopical detection by the semi-automated cell imager (NYONE®), and (ii) molecularly, by a semi-quantitative RT-qPCR detecting CK20 mRNA as an epithelial cell marker. CTC enumeration data obtained by either method was correlated with clinical characteristics and follow-up data.

Firstly, contrary to our expectations, surgery did not have any statistically significant effect on the quantity of CTC detected by the cytological approach utilizing the cell imager NYONE® regarding the overall complied cohort of our study population. Generally, and described by Galizia et al. (24), one could expect the resection of the tumor to have a significant negative impact on the incidence of CTC postoperatively in the overall cohort. Despite this, we were able to demonstrate a significant short-term increase in CTC quantity post-operatively but only in patients who were pre-operatively negative for CTC in the cytological method or below the cut-off in the PCR-based analysis. During the surgical procedure and mechanical manipulation of the tumor, an excess of CTC may be released into the bloodstream (25–27). Owing to a short half-life of CTC, their numbers are significantly reduced but still elevated 3 months after surgery compared to the preoperative status. Interestingly, in patients who pre-operatively showed evidence for CTC in the NYONE® or were above the cut-off for the CK20 RT-qPCR method, CTC levels post-operatively dropped significantly. Concluding, the removal and physical manipulation of the tumor in this subset of patients did surprisingly lead to a significant decrease in CTC numbers in the short term. In a large single-center study comprising 403 patients with breast cancer, van Dalum et al. analyzed CTC with the CellSearch® system over a long follow-up period (median 5.7 years). In their study, they did not observe any relevant impact of the surgical

procedure on CTC frequency. Enumeration levels were fairly constant over the study period (28). Then again, CTC are often thought to be directly linked to the primary tumor. Hence, once the tumor is resected, the general opinion would be that CTC numbers are decreasing and due to CTC clearance ultimately disappear from peripheral blood samples. Our data suggest that CTC remain detectable even in the mid and long-term after the surgical procedure. Notable in this context is the exceedingly high CTC count of 29 IF positive cells in one patient twelve months post-operatively. Interestingly, this was a patient who was diagnosed with a colon cancer of the descending colon with a singular hepatic metastasis who underwent synchronous resection of the primary tumor and the metastasis. Then, the patient received an adjuvant chemotherapy and CTC counts remained low throughout the study period. The blood draw at t5 was during the routine visit of oncological follow-up. Unfortunately, the patient then dropped out for further analysis.

Presumably, these CTC are shed into the bloodstream and are derived from disseminated tumor cells (DTC) that rest in a dormant-like stage in the bone marrow or lymph nodes. When suitable triggers are active, they initiate local recurrence or macro-metastasis.

In a xenograft mouse model and co-culture experimental set-up, Möhrmann et al. demonstrated the importance of DTC and their potential to act as a source for tumor relapse (29). In a study of breast cancer patients, Meng et al. showed that in 13 of 36 patients, where follow-up data were available, CTC were detectable up to 22 years after treatment. As they concluded slowly replicating or dormant DTC to be the cause of this (30), it is reasonable to assume that the origin of CTC detectable in our patients after surgical removal of the tumor are DTC or derivatives of these.

Furthermore, our data further underscore the potential of CTC as prognostic biomarkers in CRC, which has been already shown in previous studies (11–13). By applying our CK20 RT-qPCR on blood samples over the post-operative course of the disease, we were able to detect a relative rise in CTC ahead of clinical symptoms or positive radiological imaging in a patient with stage II colon carcinoma who was diagnosed with local tumor recurrence thirteen months after tumor resection (**Figure 4A**). In other malignancies, for instance, in leukemia patients, the concept of molecular minimal residual disease (MRD) monitoring is well established (31). PCR methods for detection of genes or genetic aberrations for MRD monitoring have been standardized by the Europe Against Cancer (EAC) consortium and are widely instituted (32). In terms of colorectal cancer as a common solid tumor entity, concepts comparable to the MRD monitoring in leukemia are still lacking. The follow-up is mainly conducted by clinical examinations and imaging diagnostics according to the general guidelines (German S3-Guideline Colorectal Carcinoma, Version 2.1 – January 2019 AWMF-Registration Number: 021/007OL). In this study, we demonstrate an example where disease monitoring by CK20 RT-qPCR based CTC detection is feasible and plausible. Even though no clinical recommendation can be drawn based on our data yet, attention should be given to the unambiguous case report of the stage II colon cancer patient reported on above.

In our study, we were able to identify patients with unusual CTC courses. The initial blood draw, revisit and correlation of the data with clinical follow-up characteristics of some patients allowed for individual insights into the prognostic potential and relevance of CTC. Again, the vast majority of studies focus on the overall potential of CTC as a biomarker in cancer patients. Larger patient cohorts are recruited, and the relevance of CTC enumeration is correlated to clinical follow-up data. Though, for further analysis of the principles of changes over time in the CTC quantity, further in-depth analysis possibly also investigating the biology of these individual CTC are desirable.

Future investigations on the prognostic potential of CTC in the follow-up analysis of CRC patients should aim at a multi-marker approach. As a widely employed and well-esteemed technique for CTC detection, CellSearch® (Menarini Silicon Biosystems) is up to date the only method approved by the FDA (Food and Drug Administration) and therefore commercially available for clinical application. Here, CTC are enriched and enumerated by an immunological antibody-based method, which has been described in detail elsewhere (33). The CellSearch® technique utilizes antibodies targeting two antigens: EpCAM and EGFR. The clinical significance had been documented firstly by Cohen et al. in a large prospective study, though patients enrolled were all diagnosed with stage IV disease (15). It is the general opinion that a high tumor burden with distant metastasis (stage IV disease) correlates with high numbers of CTC, hence the detection of CTC by those two markers as applied by CellSearch® is presumably more likely leading to significant numbers of CTC. Only very few studies were conducted analyzing non-metastatic CRC patients and the incidence of CTC by CellSearch® detection. A possible explanation could be the concise enumeration rates of CTC detected by CellSearch®. As presented by Thorsteinson et al. in their study analyzing the prognostic relevance of CTC detection by CellSearch® in non-metastatic patients, the detection rate of CTC is poor (34), though the samples size in terms of the number of patients recruited was quite small. Another investigation by Gazzaniga et al., in which high-risk non-metastatic CRC patients were enrolled, led to similar results of low CTC numbers detected and a lack of correlation with clinical characteristics or efficacy as a prognostic marker (35). Perchance, the sensitivity of the CellSearch® system is limited due to only two markers being applied, and hence the rate of undetected CTC is high.

Therefore, to overcome this potential pitfall, we intended to establish a strategy employing a multi-marker approach extending the utilized range of markers EpCAM and EGFR by two further broadly established epithelial markers: pan-CK and HER2. However, despite broadening the range of applied markers for detection, the rate of CTC was also low in our study. A possible explanation for this could be the process of Epithelial-Mesenchymal-Transition (EMT). CTC that have undergone this process may have lost or downregulated such epithelial antigens (36, 37). Consequently, these CTC were missed by IF staining for our markers and thus not detected. Few studies have been published analyzing appropriate mesenchymal marker antigens for CTC detection in CRC patients. Yokobori et al. for example, have identified the actin-

bundling protein Plastin 3 by microarray analysis of a cohort of CRC patients and demonstrated its negative prognostic value in a large patient cohort (38). In future efforts, the significance and potential of mesenchymal antigens have to be further validated. An approach for evading the issue of epithelial- and mesenchymal-specific detection of CTC could be the label-free isolation and enumeration. In general, CTC are thought to be significantly larger (>8 μm) than leucocytes, allowing for the concept of isolation by size of epithelial tumor cells (ISET) (39, 40). One way of conducting CTC enumeration by ISET is the filtration of blood samples through a porous membrane, allowing leucocytes to pass and CTC to be effectively retained on the membrane as it has been exemplarily demonstrated with the ScreenCell® isolation devices (ScreenCell®, Sarcelles, France). Staining of these CTC then allows for cytological analysis and enumeration. The feasibility and prognostic value have been demonstrated (18, 41, 42).

In summary, our study enlightens the kinetics of CTC in CRC patients after resection of the primary tumor and provides data concerning the CTC quantity over a long-term follow-up. This study not only supports the significance of CTC as a prognostic biomarker but also provides a more in-depth longitudinal analysis of CTC over the course of the disease. Furthermore, these data suggest that by using CK20 RT-qPCR for CTC detection and enumeration approach (e.g., during long-term follow-up), a molecular MRD monitoring might be feasible in CRC patients allowing earlier detection and therapy decision making in relapse situations. However, future investigations with an even more extended follow-up and larger patient cohorts will have to validate our results and may help to define an optimal longitudinal sampling scheme for liquid biopsies in the post-operative monitoring of cancer patients to enable tailored therapy concepts for precision medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the UKSH Campus Kiel and the Medical Faculty, University of Kiel. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: AH, BB, RG, CR., SH, and SS. Data curation: AH, BB, and RG. Formal analysis: AH, KD, BB, and SS. Investigation: AH. Methodology: KD, BB, and RG. Project administration: SH and SS. Resources: BB, CR, CS, TB, SH, and SS. Software: BB and RG. Supervision: SH and SS. Validation: BB. Visualization: AH. Writing—original draft: AH and SS. Writing—review and editing: AH, KD, BB, RG, CR, CS, TB, SH, and SS. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: At the time of the study, RG was working for ORGA Labormanagement GmbH, which provided a CTC test using NYONE®. She is now working for SYNENTEC GmbH, which produces and distributes the NYONE® imaging system.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Anhang – Teil 4

Identifying patients with an unfavorable prognosis in early stages of colorectal carcinoma.

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Research Paper

Identifying patients with an unfavorable prognosis in early stages of colorectal carcinoma

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ABSTRACT

Background: In recent years, the concept of liquid biopsy diagnostics in detection and progress monitoring of malignant diseases gained significant awareness. We here report on a semi-quantitative real-time cytokeratin 20 RT-PCR-based assay, for detecting circulating tumor cells within a fraction of peripheral blood mononuclear cells in colorectal cancer patients.

Methods: In total, 381 patients were included. Prior to surgical tumor resection, a peripheral blood sample was drawn. Mononuclear cells were isolated by Ficoll centrifugation and a cytokeratin 20 qRT-PCR assay was performed. Quantitative PCR data was assessed regarding histopathological characteristics and patients´ clinical outcome.

Results: A cut-off value was determined at ≥ 2.77 [EU]. Stratifying patients by this cut-off, it represents a statistically highly significant prognostic marker for both the overall and disease-free survival in the entire cohort UICC I-IV (both $p<0.001$) and in early tumor stages UICC I+II (overall survival $p=0.003$ and disease-free survival $p=0.005$). In multivariate analysis, the cut-off value stands for an independent predictor of significantly worse overall and disease-free survival ($p=0.035$ and $p=0.047$, respectively).

Conclusion: We successfully established a highly sensitive real-time qRT-PCR assay by which we are able to identify colorectal cancer patients at risk for an unfavorable prognosis in UICC I and II stages.

INTRODUCTION

Colorectal cancer (CRC) still counts for the second most frequent cause of cancer death [1]. Around 90% of deaths are owed to formation of distant metastases mostly in liver and lung. In patients with UICC (Union internationale contre le cancer) stage III and IV CRC, a significant increase in survival could be achieved in recent decades, primarily owed to new therapeutic regimes including antibody-based immunotherapies [2, 3]. This progress though was not fully transferred to patients with early stage CRC [4]. According to current guidelines, adjuvant therapy in stage II CRC is only administered if clinical risk factors (e.g. tumor perforation, pT4 tumor, lymph vessel invasion) are apparent. The development of markers that provide additional prognostic information and also identify patients at risk for future metastases, are urgently needed [5].

The presence of circulating tumor cells (CTC) in the peripheral blood has been shown to identify CRC patients with an unfavorable prognosis [6–9]. To date, various techniques for CTC detection have been presented

[10, 11]. Previously, we established a qualitative nested endpoint RT-PCR specific for cytokeratin (CK)20-mRNA, coding for an intermediate filament protein of epithelial cells, which has been shown to detect CTC within a fraction of peripheral blood mononuclear cells (PBMC) with a high specificity and sensitivity in the blood of CRC patients [12, 13]. Thus, CK20 is a broadly accepted biomarker for the detection of CTC in patients suffering from CRC [6, 12–15].

In this prospective study, we report on a refined quantitative real-time CK20 RT-PCR, that bears the possibility to semi-quantitatively analyze the CTC/PBMC fraction in the peripheral blood. This method allows to increase the sensitivity of detection and define a cut-off value, which identifies, even in early tumor stages, CRC patients with a bad prognosis. Furthermore, to maximize the analysis´ sensitivity, we established a dual-marker qRT-PCR analyzing ectopic CK20- and epithelial growth factor receptor (EGFR)-mRNA expression in Ficoll-enriched PBMC-fractions from peripheral blood. EGFR plays a significant role in CRC [16, 17]. Its level of expression greatly increases with histopathologically advanced tumor growth [18] and it is linked to a significantly worse overall survival (OS) in CRC patients [19].

To our best knowledge, this is the first study showing a negative prognostic role of CTC within a PBMC fraction detected by a real-time qRT-PCR against CK20 in CRC patients in a large representative cohort. We were able to identify an additional molecular risk factor for CRC patients with UICC stages I and II to stratify patients who might benefit from adjuvant chemotherapy.

RESULTS

Patient and clinical characteristics

The study cohort consisted of 381 patients, all diagnosed with histologically confirmed colorectal cancer. A synopsis of the clinical data is given in Table 1. 224 patients were diagnosed with colon cancer and 157 with a rectal carcinoma. The mean age at the time of surgery was 68.5 years (range: 32 – 95 years). The median follow-up was 34 months (range: 0 – 151 months) and the median overall survival (OS) was 24 months (range: 0 – 118 months).

Clinicopathogical characteristics, CTC detection and prognosis

The 5-year OS and DFS rate for all patients in this study was 67.5% and 58.8%, respectively. As expected, advanced tumor stages correlated with worse patients´ outcome (Supplementary Figure 1).

The overall detection rate of CK20-positivity by qRT-PCR was 53.0% (202/381 patients) and 44.9% (171/381 patients) for EGFR-positivity. All experimentally derived qPCR data is shown in detail in Supplementary Table 1. Detection of CK20 alone was highly significantly correlated with a poor prognosis in univariate analysis (OS and DFS, both $P<0.001$), whereas the detection of EGFR alone did not reveal any significant correlation with the OS ($P=0.979$ and DFS ($P=0.880$) (data not shown). Furthermore, the dual-marker analysis of both, CK20 and EGFR did not lead to an increase in predictive sensitivity of the patients´ outcome. Likewise, the detection of EGFR-positivity in CK20-negative patients did not show any correlation with the OS or DFS rate (data not shown).

Control group and sensitivity analysis by spiking experiments

By applying the qRT-PCR assay to blood samples of the control cohort of healthy donors, the specificity of the assay was determined. None of the 15 tested subjects were positive for either CK20 or EGFR. By serial dilution of live HT29 tumor cells into blood, the sensitivity of the assay was optimized up to the detection of 1 cell per 1 ml whole blood (Supplementary Figure 2).

ROC-curve analysis of CK20 expression levels defined a diagnostic cut-off threshold

The aim was to utilize the quantitative expression levels of CK20 mRNA, to serve as a prognostic marker in predicting the course of disease. By applying ROC-curve analysis, the quality of testing for CK20 mRNA expression was distinctively confirmed (Supplementary Figure 3). Reasoning these results, high expression levels stand for a significantly worse outcome. In this analysis, a strong cut-off value of 2.77 relative mRNA expression units was determined by the Youden´s index.

Adopting the cut-off value to the outcome of the entire cohort, patients with high CK20 gene expression (≥ 2.77) showed a significantly worse outcome ($P<0.001$ in both the OS and DFS) (Figure 1A and 1B). Patients with low CK20 gene expression (< 2.77) had a 5-year OS of 69.6%, whereas in the cohort of patients with high CK20 gene expression (≥ 2.77) the 5-year OS dropped to 39.8%. Similar results were observed for the DFS (Table 1). Analyzing the subgroups of colon and rectal carcinoma independently, applying the cut-off for CK20 expression, both subgroups showed significant correlation with a worse OS and DFS (both $P<0.001$, Supplementary Figure 4). Higher tumor stages (UICC IV) and locally advanced tumor growth (pT4), coincided with higher CK20 mRNA expression levels and significantly more often the cut-off value was exceeded ($P<0.001$ and $P=0.004$, respectively) (Table 2). Interestingly, local lymph node metastasis as a sign of locally progressive tumor growth did not correlate with higher CK20 mRNA expression levels. Though, the data suggests a clinically relevant trend (Table 2).

Table 1: Patient demographics, clinical characteristics and univariate analysis (log rank test) influencing the 5-year overall survival (OS) and 5-year disease free survival (DFS)

	N (%)	5y-OS [%]	univariate analysis (*P*)	5y-DFS [%]	univariate analysis (*P*)
All	381 (100.0)	67.5		58.8	
age [years]					
< 70	210 (55.1)	58.7	**0.038**	55.6	0.185
≥ 70	171 (44.9)	54.7		51.6	
Sex					
male	235 (61.7)	54.5	0.935	52.4	0.755
female	146 (38.3)	62.0		57.1	
tumour site					
colon	224 (58.8)	63.9	0.083	58	0.071
rectum	157 (41.2)	49.0		48	
UICC Stage					
I	118 (31.0)	87.9	**<0.001**	86.8	**<0.001**
II	91 (23.9)	74.6		67.2	
III	87 (22.8)	53.5		47.7	
IV	85 (22.3)	5.6		3.9	
pT					
T0	8 (2.1)	58.3	**<0.001**	58.3	**<0.001**
T1	40 (10.5)	90.4		90.9	
T2	100 (26.2)	83.6		79.0	
T3	186 (48.8)	42.7		39.9	
T4	47 (12.3)	25.9		19.3	
pN					
N0	225 (59.1)	76.9	**<0.001**	73.5	**<0.001**
N1	82 (21.5)	43.5		41.6	
N2	74 (19.4)	16.5		10.2	
pM					
M0	296 (77.7)	74.0	**<0.001**	69.9	**<0.001**
M1	85 (22.3)	5.5		3.9	
neoadjuvant treatment					
yes	54 (14.3)	64.2	0.198	57.2	0.535
no	323 (84.6)	55.7		53.4	
unknown	4 (1.1)				
adjuvant treatment					
yes	136 (35.7)	44.1	**0.001**	38.8	**<0.001**
no	237 (62.2)	65.5		62.9	
unknown	8 (2.1)				

(*Continued*)

	N (%)	5y-OS [%]	univariate analysis (*P*)	5y-DFS [%]	univariate analysis (*P*)
CK20 expression [EU]					
< 2.77	220 (57.7)	69.6	**<0.001**	66.2	**<0.001**
≥ 2.77	161 (42.3)	39.8		37.6	
EGFR expression					
positive	171 (44.9)	57.0	0.979	52.7	0.880
negative	210 (55.1)	56.8		55.0	

All *P* values in bold, are regarded as statistically significant. CTC: circulating tumour cells; CK20: cytokeratin 20; EGFR: epidermal growth factor receptor; EU: expression units.

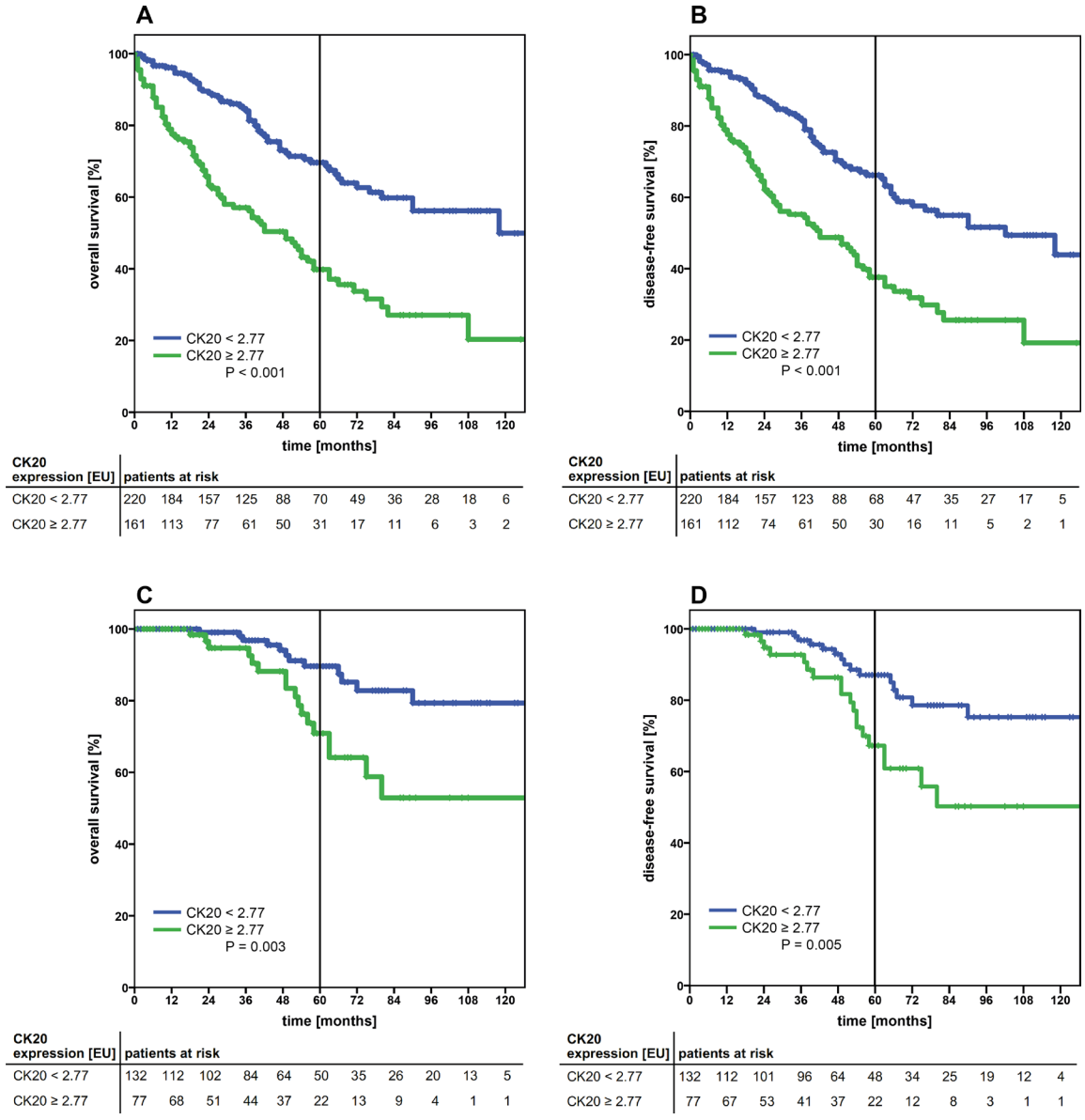

Figure 1: Kaplan-Meier survival analysis of the cumulative overall survival **(A, C)** and disease-free survival **(B, D)** of patients with colorectal carcinoma of UICC stage I-IV (A, B) and UICC stage I+II (C, D) according to the cytokeratin-20 mRNA expression levels (high, ≥ 2.77 EU; low, < 2.77 EU). The tables under each plot show the number of patients at risk at each time point in the graph. The 5-year survival is indicated by thick vertical lines. P-values were calculated by log-rank tests. CK20: cytokeratin 20; EU: expression units.

Table 2: Correlation of quantitative detection of CK20-mRNA and association to clinical characteristics determined by χ^2 testing

	CK20 ≥2.77 EU *N* (%)	*P*
all	161 (42.3)	
age [years]		
< 70	82 (39.0)	0.097
≥ 70	79 (46.2)	
Sex		
male	92 (39.1)	0.073
female	69 (47.3)	
tumour site		
colon	91 (40.6)	0.253
rectum	70 (44.6)	
UICC stage		
I	42 (35.6)	**<0.001**
II	35 (38.5)	
III	26 (29.9)	
IV	58 (68.2)	
pT		
T0	4 (50.0)	**0.004**
T1	8 (20.0)	
T2	38 (38.0)	
T3	83 (44.6)	
T4	28 (59.6)	
pN		
N0	88 (39.1)	0.083
N+	73 (46.8)	
pM		
M0	103 (34.8)	**<0.001**
M1	58 (68.2)	
neoadjuvant treatment		
yes	24 (44.4)	0.429
no	137 (42.1)	
adjuvant treatment		
yes	57 (41.9)	0.460
no	104 (43.0)	

All *P* values in bold are regarded as statistically significant; CK20: cytokeratin 20; EU: expression units.

Applying a multivariate analysis for all variables showing a significant correlation to survival in the univariate analysis, we could prove that CK20 mRNA expression above or below the cut-off in CRC patients represents an independent prognostic marker in the entire cohort (UICC stages I-IV) for the OS (HR 2.49; 95% CI 1.77 – 3.49; P<0.001) and DFS (HR 2.34; 95% CI 1.69 – 3.22; P<0.001) (Table 3, upper panel). Moreover, also the UICC staging was significantly proven as an independent prognostic factor in both, OS and DFS (HR 7.85; 95% CI 5.08 – 12.15; P<0.001 and HR 7.39; 95% CI 4.90 – 11.16; P<0.001, respectively). The other variables tested (age and adjuvant treatment) turned out to be not correlated independently (Table 3, upper panel).

Subgroup analysis of UICC I+II, II+III and III+IV patients

Since usually only patients with advanced disease (UICC stages III and IV) receive adjuvant therapy according to the treatment guidelines, a stratification of the study cohort is clinically particularly interesting. To determine the role of CK20-expression as a negative prognostic marker in early tumor stages, the cohort was stratified with respect to early tumor stages (I + II) only. Within this cohort, high mRNA Expression levels of CK20 (≥ 2.77) were a highly significant marker for worse OS and DFS (P=0.003 and p=0.005, respectively) (Figure 1C+1D and Table 4). Furthermore tumor localization in colon vs. rectum, pT category and patients´ age emerged as parameters with significant correlation to the OS and DFS (Table 4).

These parameters were also explored in a multivariate analysis, which demonstrated that the CK20 expression level remains significant as an independent prognostic marker for a worse OS (HR 2.25; 95% CI 1.06 – 4.77; P=0.035) and DFS (HR 2.01; 95% CI 1.01 – 4.01; P=0.047) (Table 3, lower panel). Further, the other variables tested in univariate analysis, tumor localization, pT-category also prove to be highly significant independent variables in predicting the patients´ outcome, whereas patients age was not proven to be an independent predictor (Table 3, lower panel).

Analyzing the subgroup of patients with locally advanced and metastatic CRC (UICC III+IV) we were also able to prove the expression of CK20 mRNA being a significant prognostic marker for both, the OS and DFS (both P<0.001) (data not shown).

Another clinically highly interesting issue is the problem of over- or under treatment of cancer patients. According to the medical guidelines, the majority of patients diagnosed with UICC II CRC are not admitted to an adjuvant treatment, whereas patients with stage III CRC are. Therefore, we explored the subgroup of UICC II and III patients and stratified these in potential patients at risk. Patients staged UICC II with high CTC CK20 gene expression (≥ 2.77) (patients at risk, possibly being undertreated) were correlated to patients staged UICC III with low CTC CK20 gene expression (< 2.77) (patients possibly excessively treated). Interestingly, no statistical difference in the OS or DFS (P=0.284 and P=0.196, respectively) was observed (Figure 2A+2B), suggesting a further possible clinical impact of applying a cut-off value for quantitative CK20-expression detection.

DISCUSSION

At all, the expression of CK20 allows for a review of cancer recurrence and therapeutic efficiency, as well as prognosis in colorectal cancer patients. In future, it may even serve as liquid biopsies.

In this prospective study with a large and representative cohort of CRC patients, we analyzed the prognostic relevance of CK20 expression of a PBMC fraction containing CTC and patients´ clinical outcome. We proved sole detection of CK20 expression to be a highly significant independent marker for OS and DFS in CRC. Further, we demonstrated proof of concept for our semi-quantitative real-time CK20 RT-PCR and could process the clinically most interesting subgroup of UICC I and II patients in more depth. We were able to identify patients at risk in these early stages precisely by sole detection of CK20 expression and further by defining a clinically relevant cut-off value of quantitative CK20 expression in this cohort.

Due to its anticipated clinical relevance in oncological diagnostics and disease monitoring, the concept of liquid biopsy diagnostics for solid tumors has been emphasized considerably in the recent literature [11, 20]. The biological basis for liquid biopsy analysis lies in the various molecular and/or cellular traces of a solid tumor in the blood as circulating cell-free tumor DNA (cfDNA), miRNAs, exosomes, proteins and CTC [21] among other tumor-derived biomarkers. Recently arising is the (experimentally-based) hypothesis of CTC being highly heterogeneous, comprising epithelial tumor cells, tumor cells after epithelial-to-mesenchymal transition (EMT), and circulating tumor stem cells (CTSC) [22, 23]. Hence, various biomarkers and techniques for detecting CTC have been implemented [24]. In particular though, the transmembrane glycoprotein EpCAM as a general endodermal epithelial cell marker is of broad interest. The up to now exclusively FDA-approved immunomagnetic anti-EpCAM assay, employed by the CellSearch System©, is utilized by many research groups. Yet, a growing number of studies show detection rates of CTC in CRC patients to be modest with this system [25, 26].

During the process of metastasizing, some cells undergo EMT and epithelial markers such as EpCAM are either lost, or significantly downregulated [27]. Instead, upregulation of mesenchymal markers such as vimentin are seen. This mesenchymal cell fraction is said

Table 3: Multivariate Cox regression analysis and hazard models of independent factors influencing overall- and disease-free survival in the entire study cohort (UICC I-IV) and early tumour stages (UICC I+II)

	overall survival		disease-free survival	
	multivariate HR (95% CI)	*P*	multivariate HR (95% CI)	*P*
UICC I-IV				
CK20 < 2.77 vs ≥ 2.77 [EU]	2.49 (1.77 – 3.49)	**<0.001**	2.34 (1.69 – 3.22)	**<0.001**
age < 70 vs. ≥ 70 [years]	1.27 (0.90 – 1,79)	0.172	n.d.	
UICC I+II vs. III+IV	7.85 (5.08 – 12.15)	**<0.001**	7.39 (4.90 – 11.16)	**<0.001**
adj. treatment yes vs. no	1.10 (0.77 – 1.56)	0.610	1.09 (0.78 – 1.53)	0.606
UICC I+II				
CK20 < 2.77 vs. ≥ 2.77 [EU]	2.25 (1.06 – 4.77)	**0.035**	2.01 (1.01 – 4.01)	**0.047**
age < 70 vs. ≥ 70 [years]	1.59 (0.73 – 3.47)	0.245	n.d.	
colon vs. rectum	0.22 (0.09 – 0.50)	**<0.001**	0.27 (0.13 – 0.57)	**0.001**
pT1/2 vs. pT3/4	4.50 (1.99 – 10.17)	**<0.001**	4.17 (1.97 – 8.85)	**<0.001**

All *P* values in bold, are regarded as statistically significant; n.d.: the value was not significant in univariate analysis and therefore not considered in multivariate analysis; HR: hazard ratio, CI: confidence interval; CK20: cytokeratin 20; EU: expression units.

Table 4: Description and analysis (log-rank test) of factors influencing the 5-year overall survival (OS) and 5-year disease free survival (DFS) rate in the subgroup of UICC I+II patients

	N (%)	5y-OS [%]	univariate analysis (*P*)	5y-DFS [%]	univariate analysis (*P*)
all	209 (100.0)	92.9		89.4	
age [years]					
< 70	115 (55.0)	83.4	**0.048**	81.3	0.062
≥ 70	94 (45.0)	81.7		77.3	
Sex					
male	135 (64.6)	79.2	0.276	75.9	0.372
female	74 (35.4)	89.9		87.6	
tumour site					
colon	123 (58.9)	92.0	**0.010**	87.5	**0.019**
rectum	86 (41.1)	72.3		71.0	
pT					
1	37 (17.7)	94.4	**0.005**	89.3	**0.007**
2	80 (38.3)	97.3		87.2	
3	81 (38.8)	74.8		71.5	
4	11 (5.3)	50.0		50.0	
CK20 expression EU					
< 2.77	132 (63.2)	89.6	**0.003**	87.1	**0.005**
≥ 2.77	77 (36.8)	70.9		67.3	

All *P* values in bold, are regarded as statistically significant. CK20: cytokeratin 20; EU: expression units.

to be considerably more hostile with a more aggressive phenotype and an increased metastatic potential in CRC patients [28–30]. Tests designed for detection of these markers, therefore lack precision - the real load of CTC may be underestimated by the anti-EpCAM assays [31–33].

Up to now, to the best of our knowledge, many publications focused on the overall predictive value of CTC in CRC patients and numerous studies comprised cohorts of patients with a high tumor burden of even metastatic colorectal cancer patients. Cohort numbers were remote and only few studies targeted clinically relevant histopathological subsets in terms of UICC staging.

CRC patients with stage III and IV disease experienced a substantial increase in disease-free survival in recent decades. New therapeutic regimes had been introduced in particular addressing metastatic CRC patients. At the same time the OS of patients suffering from early-stage tumors had been improved sparsely. In our cohort, the 5-year OS in patients with limited disease (no regional lymph node metastasis) was 85.3%. Hence, still a significant number of CRC patients die due to tumor burden and later development of distant metastasis, whereby the initial extend of tumor load is sparse. Desirable would be to establish a protocol to identify these patients at risk in early stage disease and to discriminate this clinically highly relevant sub-cohort further. In 2015, Bork et al. [34] investigated the clinical relevance of CTC by applying the Cell-Search system in a large cohort of CRC patients. Patients with less tumor burden (UICC I-III) were analyzed independently regarding the CTC count and prognostic value. They proved the predictive importance of CTC in early tumor stages. Contrary to these findings, Sotelo *et al*. [35] published their results in 2015, stating the CTC count not to have any prognostic impact in stage III colorectal cancer patients. Likewise, detection was carried out by the CellSearch© system. As discussed beforehand, the CTC detection by the EpCAM reliant CellSearch© method is arguably inferior. In their study, Iiunuma *et al*. [36] demonstrated the CTC detection by PCR (CK+/CEA+/CD133+) in a large cohort of 420 CRC patients to be significantly superior relative to the CellSearch© system.

In our study, we now applied a refined quantitative real-time CK20- and EGFR-specific RT-qPCR. The overall detection rate of 53% for CK20 expression was significantly higher than with the up to now utilized nested RT-PCR in our work group [6, 13]. In a representative cohort of CRC patients, the detection of CK20 expression within a CTC containing subset of PBMC presents a highly significant predictive marker in the prognosis of patients. Detection of CK20 expression by qRT-PCR has the ability of independently acting as a liquid biopsy. High detection rates of CK20 expression in early tumor stages may be arguable, but by defining a cut-off value in the cohort of CRC patients it is possible to identify patients who might be at risk and may experience a worse outcome.

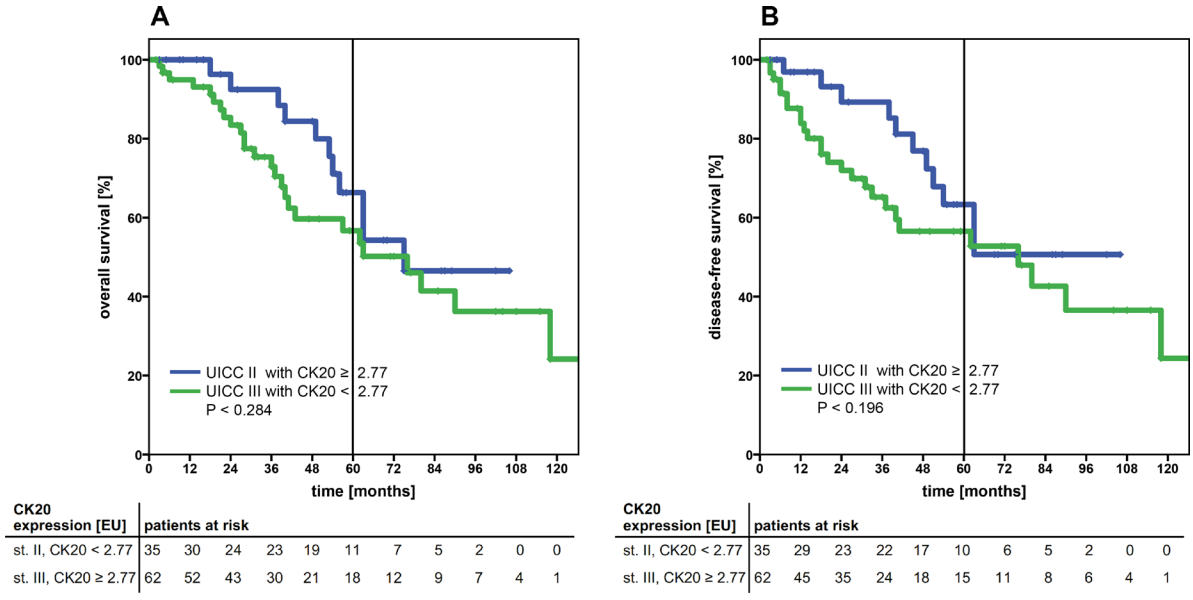

Figure 2: Kaplan-Meier survival analysis of the cumulative overall survival **(A)** and disease-free survival **(B)** of patients with UICC stage II and high CK20 mRNA expression levels (≥ 2.77 EU cut-off) compared to UICC stage III patients with low CK20 expression levels (< 2.77 EU). Both patient sub-cohorts showed a comparable cumulative survival. The tables under each plot show the number of patients at risk at each time point in the graph. The 5-year survival is indicated by thick vertical lines. P-values were calculated by log-rank tests. CK20: cytokeratin 20; EU: expression units.

Our data evidently shows that CRC patients with high CK20 expression have a significantly worse OS and DFS. Furthermore, we show that by applying a cut-off value, it is possible to identify patients at risk even in UICC I and II stages that might benefit form additional adjuvant treatment.

MATERIALS AND METHODS

Patient cohort and study design

All 381 patients included underwent complete oncological resection (R0) for a histologically verified colorectal carcinoma between the years 2004 and 2013 in the Department of General Surgery and Thoracic Surgery, University Hospital Schleswig Holstein, Campus Kiel. Patients with stage III or IV colon cancer were recommended to receive adjuvant or palliative chemotherapy, respectively, according to the therapy guidelines. In case of synchronous liver metastases, the patients underwent resection of the primary and the liver metastases in one operation. Patients with rectal carcinoma staged higher than uT3 or uN1 underwent neoadjuvant radio-chemotherapy with 50.4 Gy and two cycles of chemotherapy with 5-fluorouracil (5-FU) followed by 4 cycles of chemotherapy with 5-FU after surgery (according to [37]). In some cases, this regimen deviated according to the consensus meeting of the interdisciplinary tumor board.

The study was approved by the local ethics committee of the Medical Faculty, Christian-Albrechts University Kiel (reference no. A110/99). All patients gave written informed consent before inclusion to the study. Classification of the pathological tumor stage was handled by the Department of Pathology, University Hospital Schleswig Holstein, Campus Kiel, according to the TNM-classification. Clinical data was obtained from the clinical research database of the oncological biobank BMB-CCC of the Comprehensive Cancer Center Kiel and data was verified by re-examination of original patient records. Follow-up data was surveyed in cooperation with general practitioners and with the Cancer Registry of the Federal State of Schleswig-Holstein (Bad Segeberg, Germany). Clinical and follow-up data were then analyzed relating to the degree of CK20 and EGFR mRNA expression detected by the qRT-PCR. In case of CK20 positivity, the level of marker expression was calculated and included into the analysis. Qualitative and quantitative data were used to stratify patients at risk and the prognostic relevance of CK20- or EGFR-expression in the blood samples of CRC patients was analyzed.

Control group

The control cohort consisted of 15 healthy volunteers. Peripheral blood samples were taken and analyzed as described in the following. Written consent for participating in this study was acquired prior to blood drawing. Investigation of the samples was likewise covered by the approval of the local ethics committee as described before.

Liquid biopsy collection and isolation of blood mononuclear cell fractions

Instantly prior to surgery, a blood sample was drawn from a central venous line into a lithium heparin-Monovette (Sarstedt, Nümbrecht, Germany). All samples were kept at room temperature (18°C-25°C) and were further processed within 0.5-2 hours. Separation of the mononuclear cell (MNC) fraction was performed by centrifugation through a Ficoll-Hypaque density cushion (GE Healthcare, Freiburg, Germany). MNCs were then isolated, washed in PBS and counted.

Isolation of total RNA and cDNA synthesis

MNCs were subsequently lysed with RNAPure™ reagent (VWR Peqlab, Darmstadt, Germany) and total RNA preparation was carried out according to the manufacturer's protocol. RNA concentration was measured by a NanoDrop 2000c Spectrophotometer (VWR Peqlab). RNA integrity was verified using a Bioanalyzer 2100 instrument (Agilent Technologies, Böblingen, Germany).

cDNA was obtained by reverse transcription of 3 µg total RNA (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Darmstadt, Germany) according to the manufacturer's protocol.

Realtime-qPCR and analysis

Realtime qPCR was conducted using TaqMan gene expression assays and the TaqMan Universal Master Mix II (Life Technologies, Darmstadt, Germany) with 200 ng cDNA template on a StepOnePlus instrument (Life Technologies). Assays were run in total volumes of 20 µl on 96-well plates (Sarstedt) and the following TaqMan gene expression assays were used: KRT20 (CK20), Hs00966063_m1; EGFR Hs01076078_m1; TBP, Hs00427621_m1. All samples were run in triplicate. The mean threshold cycles of triplicate reactions were computed using the StepOne software v. 2.1 (Life Technologies) after adjustment to the same threshold of all runs for each TaqMan assay on different plates. Gene expression was calculated as arbitrary expression units by a simplified ΔC_t method [38] normalizing the CK20- and EGFR expression against the reference gene TBP (TATA-box binding protein), as shown and further explained in Supplementary Table 1.

Cell spiking experiments

The sensitivity of the CK20 qRT-PCR assay was determined by spiking of HT29 human colon cancer cells

into fresh anti-coagulated blood of a healthy volunteer. HT29 cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS (PAN-Biotech, Aidenbach, Germany), 1mM Glutamax and 1mM Na-pyruvate (Life Technologies). Total RNA from MNC fractions of blood samples spiked with 1000, 100, 10, and 1 HT29 cells per ml whole blood were analyzed by qRT-PCR as described above.

Statistical analysis

Analyses were implemented for all subsets of clinical parameters in total and independently by tumor site and histopathological staging. Kaplan-Meier survival analyses were carried out for overall and disease-free survival (OS, DFS). For univariate analysis, significance was assessed by the log rank test. Dependence of the detection rate of biomarkers from clinical parameters was analyzed with the χ^2 test after crosstab examination. Variables showing a significant association with the detection of a biomarker in univariate analysis, were included in multivariate models. Cox proportional hazard models were used in multivariate analysis. The area under the curve (AUC) of the Receiver-Operating-Characteristic (ROC) curve analysis was used to determine the prognostic value of CK20 mRNA expression. The Best-Youden-Index as the point of best sensitivity and specificity was calculated by ROC analysis and used to define the cut-off value.

All reported *P*-values are two-sided and were regarded statistically significant at ≤ 0.05. Statistical calculation and testing was performed with IBM SPSS Statistics 23.0 (IBM, München, Germany) and MedCalc (MedCalc Software, Ostend, Belgium)

Abbreviations

AUC: area under the curve; CTC: circulating tumor cell; CTSC: circulating tumor stem cell; CK20: cytokeratin 20; CRC: colorectal carcinoma; DFS: disease free survival; DNA: desoxyribonucleic acid; EGFR: epidermal growth factor receptor; EpCAM: epithelial cell adhesion molecule; EU: expression units; HR: hazard ratio; MNC: mononuclear cells; mRNA: messenger ribonucleic acid; OS: overall survival; PBMC: peripheral blood mononuclear cells; PCR: polymerase chain reaction; RNA: ribonucleic acid; qRT-PCR: quantitative real-time polymerase chain reaction; ROC: receiver operating characteristic; UICC: Union internationale contre le cancer.

Author contributions

Alexander Hendricks, Christian Röder and Sebastian Hinz designed the study, analyzed the data and wrote the manuscript.

Alexander Bernsmeier analyzed the data.

Greta-Lou Eggebrecht and Katharina Dall performed clinical and follow-up investigation.

Clemens Schafmayer, Reinhild Geisen, Anna Trauzold, Thomas Becker and Holger Kalthoff wrote and edited the manuscript.

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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Anhang – Teil 5

Rapid response of stage IV colorectal cancer with APC/TP53/KRAS mutations to FOLFIRI and Bevacizumab combination chemotherapy: a case report of use of liquid biopsy

BMC Medical Genetics

CASE REPORT Open Access

Rapid response of stage IV colorectal cancer with APC/TP53/KRAS mutations to FOLFIRI and Bevacizumab combination chemotherapy: a case report of use of liquid biopsy

Alexander Hendricks[1,2*], Philip Rosenstiel[4], Sebastian Hinz[1,2], Greta Burmeister[1,2], Christoph Röcken[3], Kathrin Boersch[4], Clemens Schafmayer[1,2], Thomas Becker[1], Andre Franke[4] and Michael Forster[4]

Abstract

Background: Liquid biopsies of blood plasma cell free DNA can be used to monitor treatment response and potentially detect mutations that are present in resistant clones in metastatic cancer patients.

Case presentation: In our non-interventional liquid biopsy study, a male patient in his fifties diagnosed with stage IV colorectal cancer and polytope liver metastases rapidly progressed after completing chemotherapy and deceased 8 months after diagnosis. Retrospective cell free DNA testing showed that the *APC/TP53/KRAS* major clone responded quickly after 3 cycles of FOLFIRI + Bevacizumab. Retrospective exome sequencing of pre-chemotherapy and post-chemotherapy tissue samples including metastases confirmed that the *APC/TP53/KRAS* and other major clonal mutations (*GPR50, SLC5A, ZIC3, SF3A1* and others) were present in all samples. After the last chemotherapy cycle, CT imaging, CEA and CA19–9 markers validated the cfDNA findings of treatment response. However, 5 weeks later, the tumour had rapidly progressed.

Conclusion: As FOLFIRI+Bevacizumab has recently also been associated with sustained complete remission in a *APC/TP53/KRAS* triple-mutated patient, these driver genes should be tested and monitored in a more in-depth manner in future patients. Patients with metastatic disease should be monitored more closely during and after chemotherapy, ideally using cfDNA.

Keywords: Metastatic colorectal cancer, Circulating tumour DNA, Cell free DNA, Liquid biopsy, Chemotherapy resistance

Background

Blood plasma "liquid biopsy" from a cancer patient and the analysis of circulating tumour DNA (ctDNA) enables the diversity of the mutational patterns to be monitored over the course of disease at serial timepoints, giving new clinically actionable insights into the therapeutic effectivity. We here report on a case that illustrates how treatment response could be detected early from two blood samples. This case comes from a large ongoing exploratory study whose results were not used for treatment intervention.

In colorectal cancer patients diagnosed with organ metastases, systemic therapy with chemotherapy and targeted antibodies or inhibitors is regularly based on the molecular characterization of the tumour [1]. Usually, molecular testing is based on tissue samples obtained by surgical resection, or on biopsies at time of initial diagnosis. The actual drug response is routinely monitored by imaging methods and tumour markers, but it could be monitored more specifically by serial blood based liquid biopsies.

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Case presentation

We report on a male patient in his fifties of North European ancestry with stage IV colorectal cancer and no known familial history of cancer. The patient had not participated in colon cancer screening tests. Clinical symptoms were unexplained weight loss for a period of six months before diagnosis. Initial diagnosis then presented a primarily metastasized adenocarcinoma of the cecum and bilobular hepatic metastases. Due to polytope bilobular liver metastasis surgical resection was not indicated, and he was admitted to a palliative chemotherapy (CTX). Figure 1 shows the course of events.

The patient gave informed written consent for serial blood sample collection for biomarker analysis and the study was approved by Kiel University's medical faculty ethics board (#A110/99). For analysis of ctDNA, we obtained a pre-treatment blood sample in week 2 and a mid-treatment blood sample in week 10, after three cycles of chemotherapy. The patient opted out of further blood sampling when he progressed. The blood samples were collected in Streck cfDNA BCT tubes from which plasma was centrifuged according to the manufacturer's recommendations and stored at − 20 °C until DNA isolation. The plasma was thawed at room temperature. DNA was isolated from the plasma using the PerkinElmer NEXTprep-Mag cfDNA kit according to the manufacturer's protocol. Ultra-deep sequencing of the ctDNA was performed on Illumina NextSeq 500 with 2x150bp reads using the PANCeq pan-cancer panel [2].

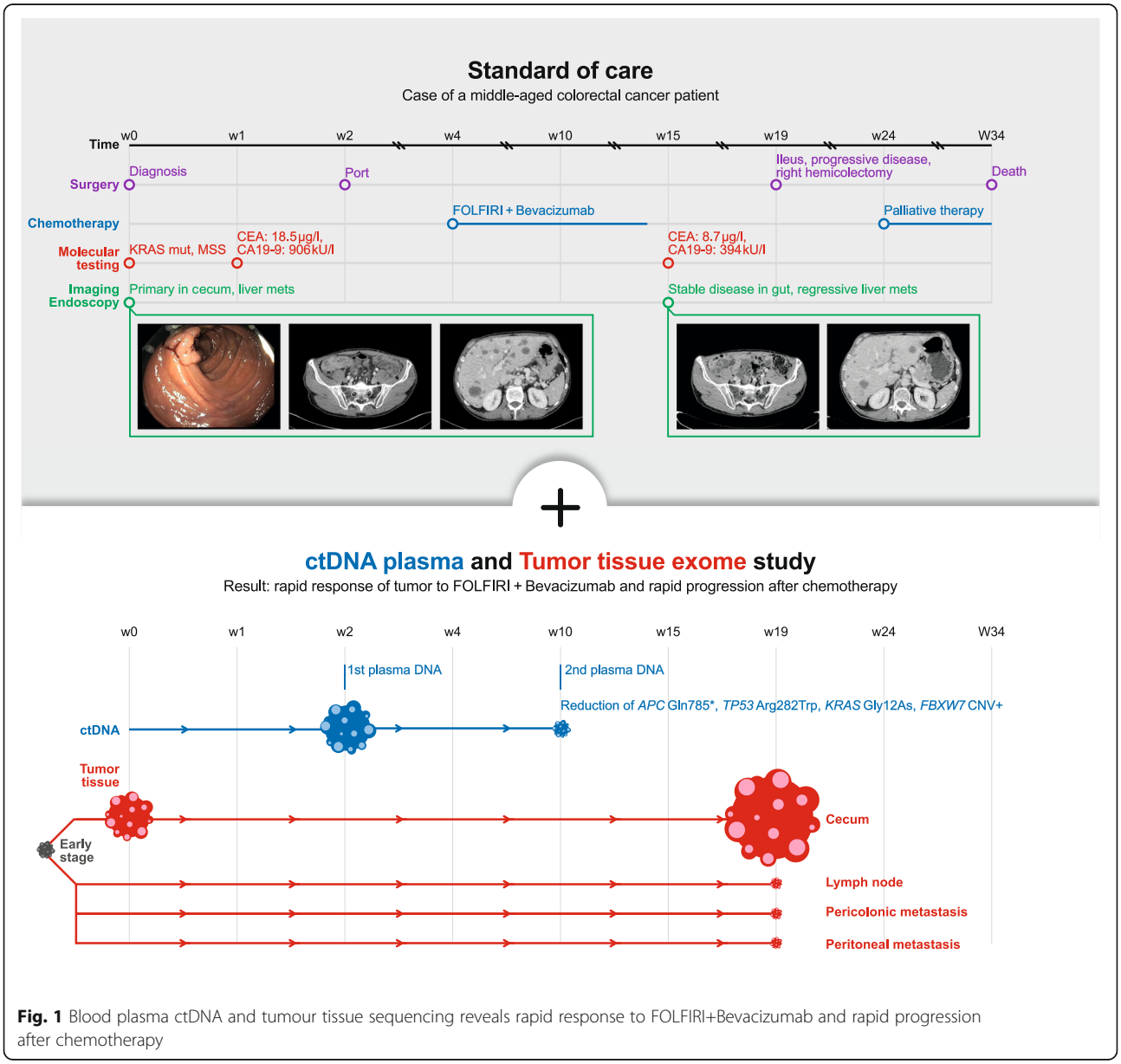

Fig. 1 Blood plasma ctDNA and tumour tissue sequencing reveals rapid response to FOLFIRI+Bevacizumab and rapid progression after chemotherapy

For analysis of tumour tissue mutations, we obtained DNA from a pre-treatment colonoscopy biopsy from the primary tumour in the cecum in week 0 and four tissue samples from an emergency hemicolectomy in week 19 (cecum, lymph node, pericolonic and peritoneal metastases). The tissue samples were obtained from formalin-fixed paraffin embedded samples after HE staining and histological identification and marking of cancer cell regions by the pathological laboratory. Tumour DNA was isolated from the tissue using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (ThermoFisher Scientific Inc) according to the manufacturer's protocol. Exome sequencing of the tissue DNA samples, and a patient-matched blood buffy coat DNA sample was performed on Illumina NovaSeq 6000 with 2x150bp reads using the IDT xGen Exome Research Panel (Integrated DNA Technologies, Inc.) according to the manufacturers' protocols. Sequence data analysis was performed with GenSearchNGS (Phenosystems S.A.), Alamut Visual (Interactive Biosoftware), IGV and pibase [3–5].

Initial mutational profiling of a colonoscopy sample from week 0 within the routine diagnostics of the pathological analysis revealed the following: *KRAS* mutation p.G12D; no mutation in exon 15 of BRAF; MSI stable. Our blood sample from week 2 revealed the *KRAS* mutation and four more major tumour mutations in the following genes (see Table 1): *APC* (in 49% of sequences), *TP53* (39%), *KRAS* (32%), *THSD7B* (20%), and a copy number amplification of a chromosomal segment on chromosome 4 containing *FBXW7*. The copy number amplification was deduced from a genomic stretch of germline polymorphisms with near-identical allele frequencies of around 0.25 (see Additional file 1: Table S1) instead of the expected allele frequency of 0.50. Retrospective exome sequencing of the initial cecum tumour tissue biopsy confirmed these mutations and the *FBXW7* amplification and detected further major clonal mutations (Additional file 2: Table S2) that were not covered by the ctDNA sequencing panel.

Based on the mutation in the *KRAS* gene, chemotherapy with FOLFIRI + Bevacizumab (standard dosages) commenced shortly after initial diagnosis. After three cycles of chemotherapy, the blood sample from week 10 revealed major changes in the tumour allele frequencies: *TP53* (6%), *APC* (5.5%), *KRAS* (4%), and *THSD7B* (3%). The amplification of the chromosome segment with *FBXW7* was not clearly detectable any longer in the cfDNA (polymorphism allele frequencies 43–52%). After six cycles of chemotherapy, a re-staging by CT-scan was performed in week 15 and suggested a stable disease of the primary tumour and showed a regression of the liver metastasis (Fig. 1). In parallel, the routinely tested cancer markers CEA and CA19–9 dropped, from 18.5 μg/l to 8.7 μg/l, and from 906kU/l to 394kU/l, respectively.

However, four weeks after the CT scan the patient was admitted to the emergency room with an ileus caused by a substantial increase in size of the primary tumour, a circumferentially growing tumour of 4 × 3.5 cm size at the ileocecal valve. In emergency surgery a right hemicolectomy was performed. Retrospective exome sequencing of the tissue samples obtained from the hemicolectomy (cecum, lymph node, pericolonic and peritoneal metastases) confirmed that the FBXW7 amplification was no longer clearly detectable in the cecum, lymph node, or peritoneal metastasis, but possibly in the pericolonic metastasis sample. The major clonal mutations detected in the pre-treatment biopsy remained conserved in all post-treatment samples, with no new major clonal mutations detected (Fig. 2 and Additional file 2: Table S2).

After surgery, a second line chemotherapy with FOLFOX + Bevacizumab was started. Unfortunately, short-term imaging of the tumour burden exposed progressive disease, so that shortly afterwards a 3rd line chemotherapy by Trifluridin + Tipiracil was started. Therapy monitoring by ultrasound imaging of the hepatic metastasis presented substantial progression of the tumour load and hence inefficiency of the systemic therapy. The patient was transferred to best supportive care and deceased 8 months after initial diagnosis.

Discussion and conclusion

We here illustrate the potential clinical use and benefit of serial liquid biopsies. Our middle-aged male patient was diagnosed with a metastasized colorectal carcinoma and progressed rapidly over the course of eight months from initial diagnosis to decease.

Table 1 Plasma cell free DNA tumour molecular genetic results in HGVS nomenclature, with tumour allele frequency (TAF) and sequence depth at mutation

Gene/Transcript	Genomic alteration (hg19)	Protein alteration	COSMIC or other ID	TAF pre-chemo	Depth	TAF after cycle 3	Depth
APC NM_001127511.2	chr5 g.112173590C > T	p.(Gln749*)	COSM 4166473	49%	6171	6%	7055
TP53 NM_000546.5	chr17 g.7577094G > A	p.(Arg282Trp)	COSM 1636702	39%	4369	5%	4875
KRAS NM_033360.2	chr12 g.25398284C > T	p.(Gly12Asp)	COSM521	32%	3910	4%	5003
THSD7B NM_001080427.1	chr2 g.137988706G > A	p.(Glu575Lys)	rs746487130	21%	5101	3%	5467
FBXW7 NM_001013415.1	amplification (CNV)	–	–	ca. 25%[1]	ca. 5000	ca. 43%[1]	ca. 7000

[1]allele frequency of germline polymorphims on a chromosome 4 segment containing *FBXW7*

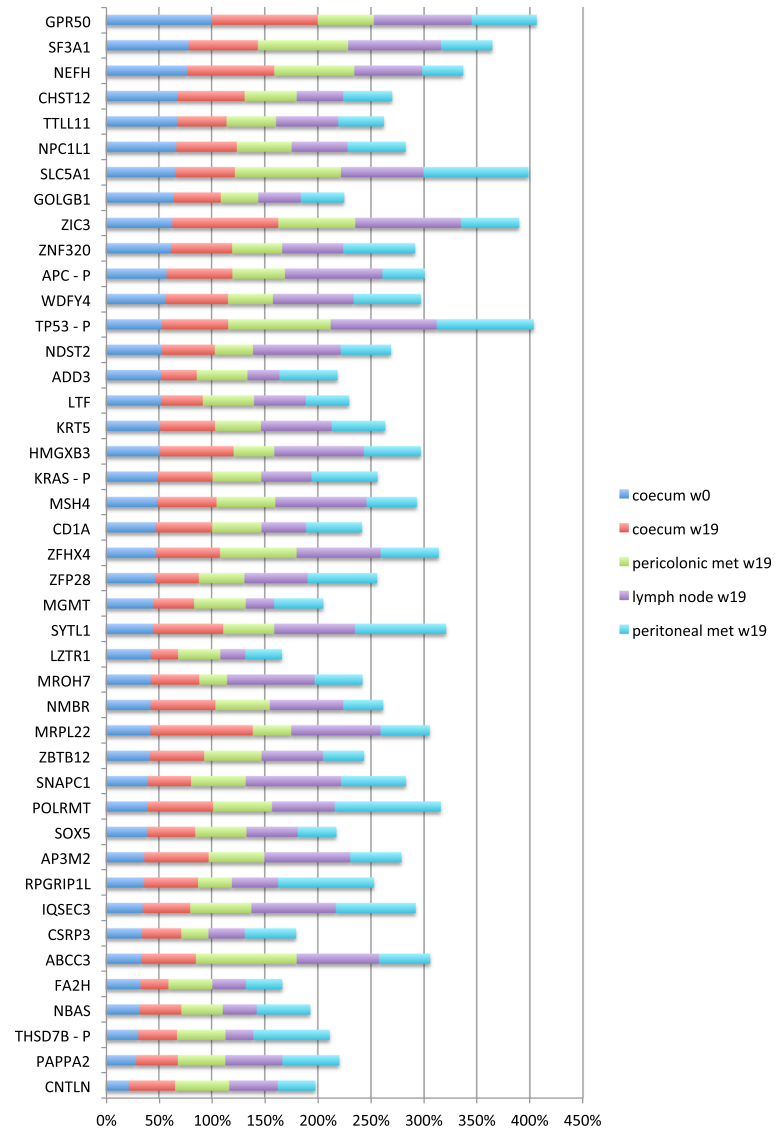

Fig. 2 Horizontal stacked bar chart showing normalized somatic mutation allele frequencies detected in tumour tissue samples by whole exome sequencing. The mutation with highest tumour allele frequency in a sample is scaled to 100% and the remaining mutations are scaled accordingly. The chart compares the most prominent tumour mutations between samples, irrespective of the tumour cell content in a tissue sample. P marks genes that were covered in plasma cfDNA pan-cancer sequencing

The initial ctDNA analysis prior to the first cycle of chemotherapy was concordant to the routine pathological mutational profiling in terms of mutational patterns. The different levels of tumour allele frequencies in the blood plasma cell free DNA suggest that the tumour was heterogeneous with different tumour clones. The first mutation may have occurred in *APC*, followed by *TP53*, *KRAS*, amplification of *FBXW7*, and mutation in *THSD7B*. Truncating *APC* mutations are colorectal cancer initiating mutations that occur together with *TP53* mutations and *KRAS* mutations in 20% of stage IV colorectal cancers [6].

At time of diagnosis, polytope hepatic metastases were present. Consequently, and compliant with recent guidelines chemotherapy with FOLFIRI + Bevacizumab was started. Initially the systemic treatment had a positive effect on the tumour burden. In the course of chemotherapy, we obtained another liquid biopsy which indisputably showed a significant change in the mutated genetic pattern. Halfway through treatment, the dominant *APC/TP53/KRAS*-mutated clone was nearly eradicated due to treatment with FOLFIRI + Bevacizumab. In parallel, the *FBXW7* amplification was eradicated or nearly eradicated. Complete tumour response after treatment with FOLFIRI + Bevacizumab is rare but there is a recent report in the literature on the complete remission of a APC/TP53/KRAS triple-mutated stage IV colorectal cancer patient

for over 10 years [7]. The response seen in our patient's CT scan at week 15 (Fig. 1) suggests that the eradicated liver metastases may have harboured predominantly cells from the triple-mutation tumour clone. The spatial heterogeneity of CNVs and homogeneity of point mutations that we detected in our patient ties in with previous reports of colonic cancer [8].

We suggest that metastatic patients should routinely be offered liquid biopsy testing with frequent blood sampling, and that all of the major driver genes are covered. As seen in our patient, liquid biopsy can detect drug response to a treatment, it may detect progression early, and, after remission, it may also be used for the early detection of disease recurrence, as in the current IMPROVE-trial (NCT 03637686).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12881-019-0941-5.

Additional file 1 Table S1. Allele frequencies of germline polymorphims on chromosome 4, showing copy number amplifications in the pre-chemo plasma cfDNA.

Additional file 2 Table S2. Somatic mutations detected using exome sequencing of cancer tissue samples versus blood bufy coat normal DNA.

Abbreviations

CA19–-9: Carbohydrate antigen 19–-9; CEA: Carcinoembryonic antigen; cfDNA: Cell free DNA; CT: Computer tomography; ctDNA: Circulating tumour DNA; CTX: Chemotherapy; MSI: Microsatellite instability; VEGF: Vascular endothelial growth factor

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Authors' contributions

AH – Designed the study, collected samples and the data, analysed the data, wrote the manuscript. PR – Wrote the manuscript and interpreted the data. SH – Wrote the manuscript and interpreted the data. GB – Wrote the manuscript and collected samples. CR – Wrote the manuscript, collected samples, and analysed and interpreted histology. KB – Wrote the manuscript and designed the summary Fig. CS – Wrote the manuscript and interpreted the data. TB – Wrote the manuscript and interpreted the data. AF – Wrote the manuscript and interpreted the data. MF – Designed the study, analysed the data, wrote the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets generated and analysed during the current study are available in the European Genome-phenome Archive (EGA) repository under the study accession ID EGAS00001004088.

Ethics approval and consent to participate

The patient gave informed written consent for serial blood sample collection for biomarker analysis and the study was approved by Kiel University's medical faculty ethics board (#A110/99).

Consent for publication

Written informed consent for publication was obtained from the patient.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Anhang – Teil 6

Stage IV Colorectal Cancer Patients with High Risk Mutation Profiles Survived 16 Months Longer with Individualized Therapies.

cancers

MDPI

Article

Stage IV Colorectal Cancer Patients with High Risk Mutation Profiles Survived 16 Months Longer with Individualized Therapies

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Abstract: Personalized treatment vs. standard of care is much debated, especially in clinical practice. Here we investigated whether overall survival differences in metastatic colorectal cancer patients are explained by tumor mutation profiles or by treatment differences in real clinical practice. Our retrospective study of metastatic colorectal cancer patients of confirmed European ancestry comprised 54 Americans and 54 gender-matched Germans. The Americans received standard of care, and on treatment failure, 35 patients received individualized treatments. The German patients received standard of care only. Tumor mutations, tumor mutation burden and microsatellite status were identified by using the FoundationOne assay or the IDT Pan-Cancer assay. High-risk patients were identified according to the mutational classification by Schell and colleagues. *Results*: Kaplan–Meier estimates show the high-risk patients to survive 16 months longer under individualized treatments than those under only standard of care, in the median ($p < 0.001$). Tumor mutation profiles stratify patients by risk groups but not by country. *Conclusions*: High-risk patients appear to survive significantly longer ($p < 0.001$) if they receive individualized treatments after the exhaustion of standard of care treatments. Secondly, the tumor mutation landscape in Americans and Germans is congruent and thus warrants the transatlantic exchange of successful treatment protocols and the harmonization of guidelines.

Keywords: metastatic colorectal cancer; mutational landscape; treatment; overall survival

1. Introduction

Despite extensive efforts in colorectal cancer screening [1], colorectal cancer is a growing major health burden that will, according to estimates, account for 1.1 million cancer deaths annually by 2030 [2]. Even though considerable progress in the treatment of the disease has been achieved, the

average five-year survival rate is below 70%. Nearly 25% of patients have distant metastases at the time of the diagnosis. In this latter group of patients, the five-year overall survival rate is below 20% [3]. Given this dismal outlook, a key question for the clinician and the patient is whether to follow standard-of-care guidelines or embark on a molecularly guided approach. Therefore, our study investigates the hypothesis that different treatment strategies can explain the different overall survival lengths of individual patients. To rule out sampling bias, we selected clinically well-characterized and sex-matched patients of European descent, from both sides of the Atlantic. To rule out mutational bias, we investigated whether there were significant differences in the mutational landscapes of the American vs. German colorectal cancers, and we then classified subgroups of metastatic patients with colorectal cancer, according to the mutational classification proposed by Schell and colleagues [4]. The Schell classification for a patient's colorectal cancer is based on the combination and number of mutations in the colorectal cancer driver genes *APC*, *TP53*, and *KRAS*. While *TP53*, *KRAS*, and *NRAS* are routinely tested for mutations, *APC* is not yet generally tested, despite the high frequency of mutant *APC*. About 80% of sporadic colorectal cancers harbor truncating mutations in *APC* (frameshift, nonsense, and splice site mutations), which lead to polyposis originating from single epithelial stem cells in the colonic crypt that ultimately progresses to cancer [5,6]. Truncating mutations in *APC*, combined with loss of *TP53*, leads to chromosomal instability with extensive aneuploidy [6]. RAS mutations are commonly found in hyperproliferating cells [5]. The highest risk for poor outcome and survival was found in Schell Class 4 cancers (two or more truncating *APC* mutations plus mutations in *TP53* and *KRAS*), followed by Schell Class 0 (no truncating *APC* mutations) [4].

Our main aims were to compare the overall survival for individualized treatment versus only standard-of-care treatment, including the overall survival for American treatment versus German treatment in our real clinical practice. The American patients at the Avera Cancer Institute received standard-of-care (SOC) treatment until failure. For 35 of the American patients, the SOC treatment was followed by individualized treatment with extensive molecular testing and case discussions in a molecular tumor board. The German patients at the University Hospital Schleswig-Holstein were strictly treated according to the German SOC guidelines that were in effect at the time of the individual patient's diagnosis and treatment. Although the guidelines are subject to frequent updates, the American SOC usually comprises a larger choice of clinical tests and treatments than the German SOC at any given date. Even more so, the American SOC that we followed in 2008–2019 is more individualized than the German SOC that we followed half a decade earlier, and thus American vs. German overall survival times allowed us to validate whether individualized precision medicine may be preferential to SOC, without the ethical dilemma of withholding the best available treatments to a patient.

Our study results suggest that there were no significant differences in ethnicity, ancestry, gender composition or mutational landscapes between the American and German patients. This leaves the SOC differences as a plausible explanation for the significantly extended survival of the American patients. After stratification by mutational classification according to Schell and colleagues [4], low-risk patients did not seem to benefit from individualized precision medicine, but high-risk patients benefited significantly.

2. Results and Discussion

To answer the key question whether individualized medicine is preferential over standard-of-care treatments for stage IV colorectal cancer patients, we compared the overall survival times (Figure 1) for individualized treatments versus SOC, and for American SOC versus (less individualized) German SOC (Figures 2 and 3). We show that the mutational landscapes in colorectal cancer tissue are congruent in Americans and Germans (Figures 4 and 5). We also show that our entire cohort is of Northern European ancestry (see subsection entitled Demographics). This leaves treatment differences as a plausible explanation for the observed survival differences. Specifically, our survival analysis indicates

that the Americans are benefiting from their SOC regimens, and that American patients with high-risk mutational profiles are benefiting from individualized treatments.

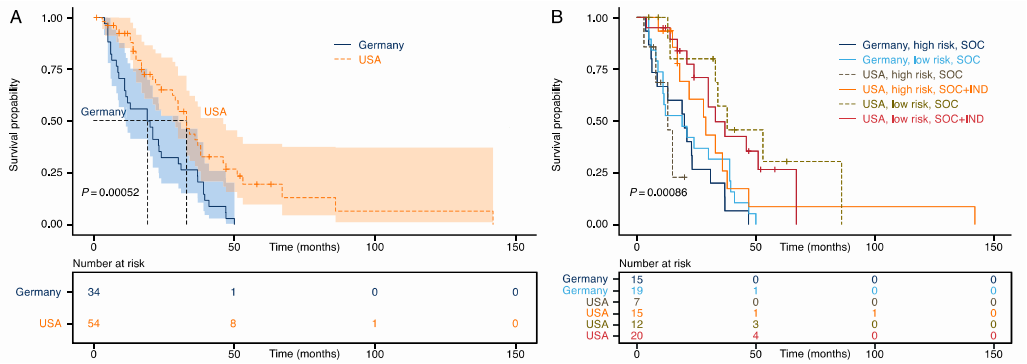

Figure 1. Kaplan–Meier estimates for stage IV colorectal cancer patients according to country and treatment regimens. (**A**) Patients in America diagnosed in 2008–2017 had a median survival probability of 33 months, compared to 19.5 months in patients in Germany diagnosed in 2003–2010. Shading indicates 95% confidence intervals. (**B**) Kaplan–Meier curves stratified by country, standard of care (SOC), and SOC, followed by individualized treatments (SOC + IND), and mutational high risk (Schell classes 0 and 4) vs. low risk (Schell classes 1–3). NB: The American SOC between 2008 and 2017 was more individualized than the German SOC between 2003 and 2010.

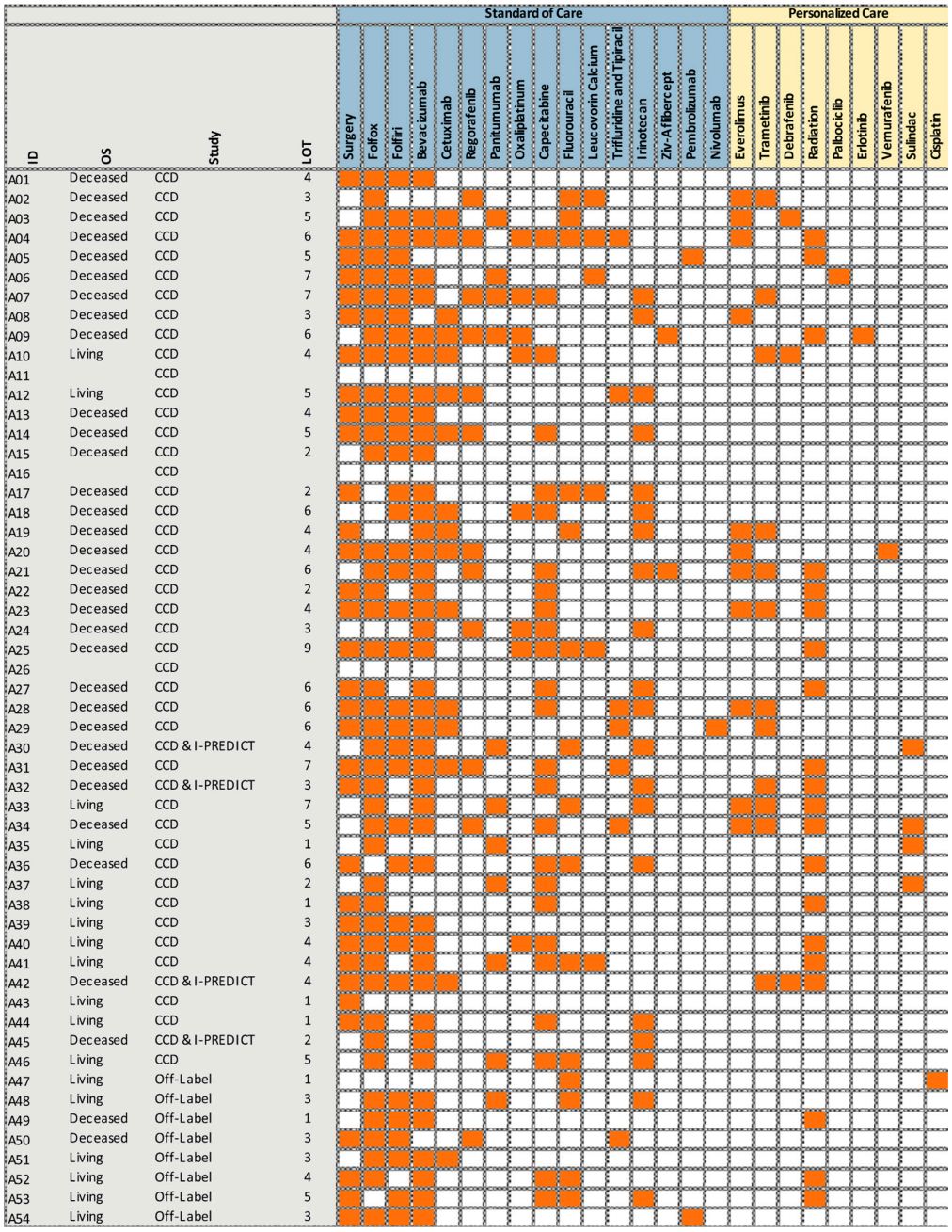

Figure 2. Treatments received by the American patients (A01–A54) in 2008–2017.

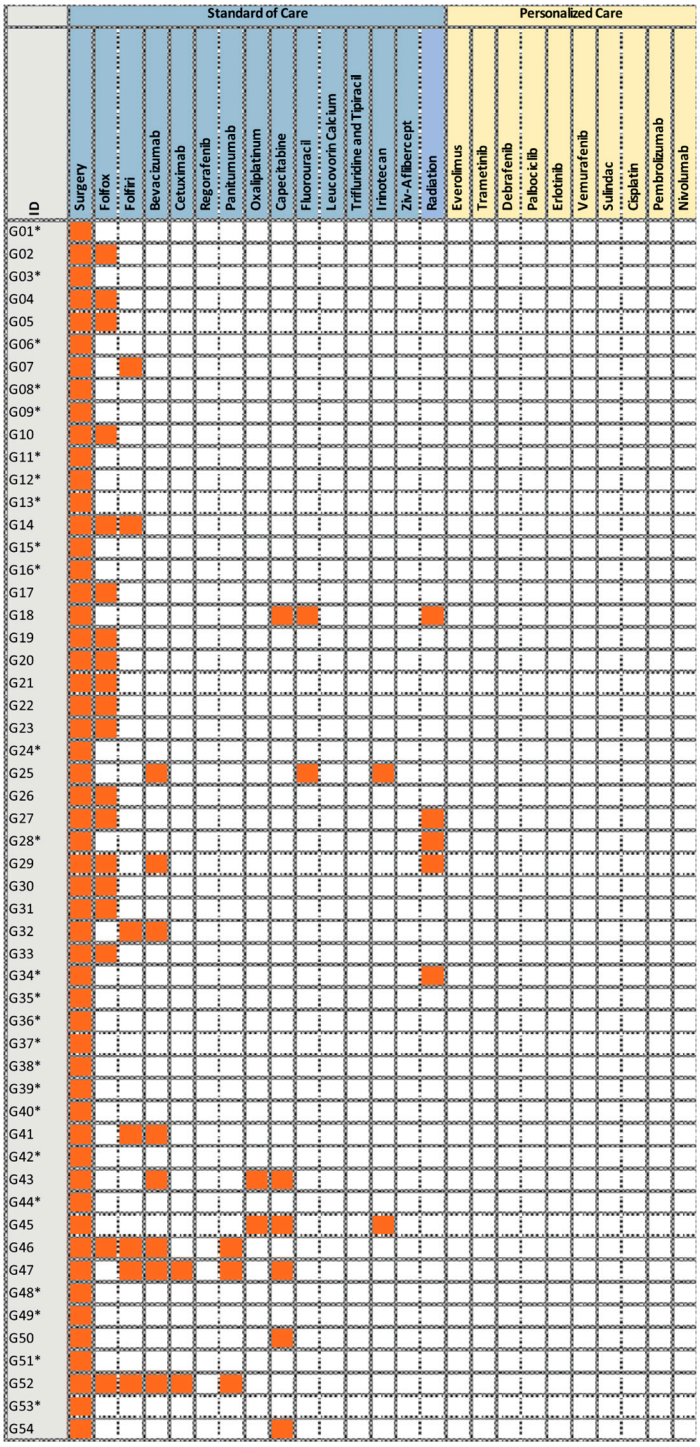

Figure 3. Treatments received by the German patients (G01–G54) in 2003–2010. The asterisk (*) marks patients who received chemotherapy at external oncological practices.

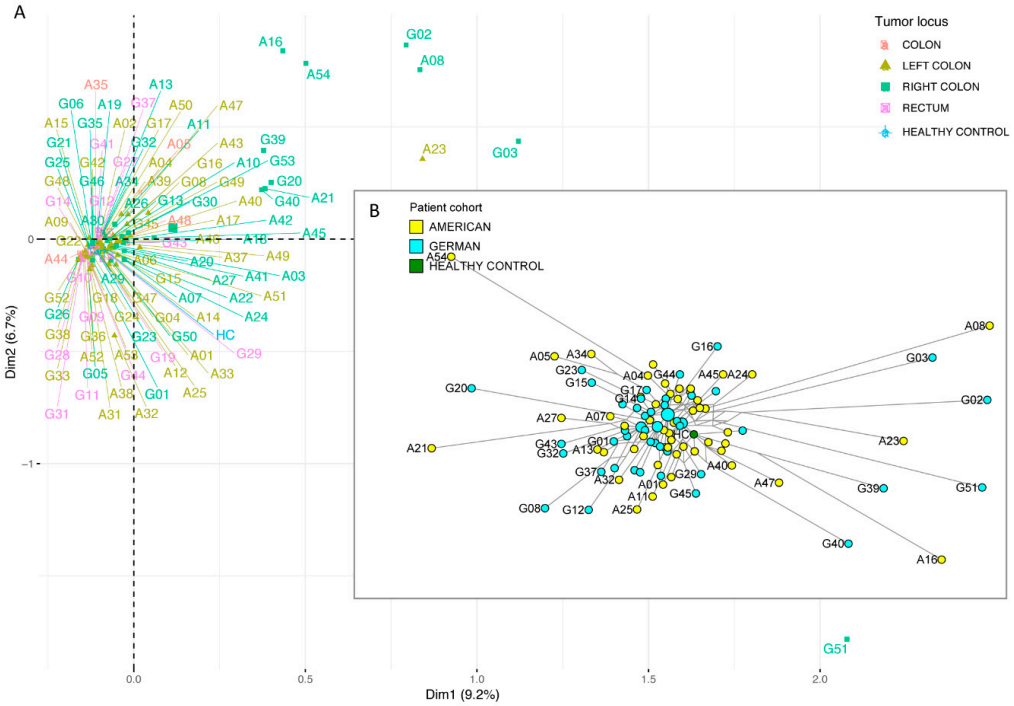

Figure 4. Mutational landscape analysis of metastatic colorectal cancer patients, based on mutated genes. (**A**) Principal component analysis and (**B**) phylogenetic network analysis independently show that microsatellite stable cancers group together while the highly mutated microsatellite instable cancers are distinct outliers, with individually mutated genes. Importantly, there is no mutational separation into American vs. German patient groups.

2.1. Demographics

We analyzed a total of 108 patients diagnosed with histologically confirmed colorectal cancer. All patients showed late-stage disease (stage III, $N = 20$ or stage IV, $N = 88$) at the time of diagnosis and analysis. Ninety-three patients were diagnosed with colon cancer, and 15 patients with rectal cancer. To minimize potential gender selection bias, the American and German cohorts were sampled for best gender matching. The median age of the combined cohorts was 64 years (range: 25–95 years). An overview and synopsis of the descriptive and clinical data are given in Table 1 and Table S1.

Table 1. Summary of clinical metadata and mutational classification, according to Schell et al.

Clinical Data	Total *N* (%)	American *N* (%)	German *N* (%)	*p*
Gender				
Male	61 (56.5)	31 (57.4)	30 (55.6)	
Female	47 (43.5)	23 (42.6)	24 (44.4)	1.00
Age [years]				
Median (Range)	64 (25–95)	60 (25–82)	68 (42–95)	
<55	26 (24.1)	18 (33.3)	8 (14.8)	
55–75	65 (60.2)	33 (61.1)	32 (59.3)	
>75	17 (15.7)	3 (5.6)	14 (25.9)	<0.001
Tumor site				
Colon	93 (86.1)	54 (100)	39 (72.2)	
right	46 (49.5)	28 (51.9)	18 (46.2)	
left	42 (45.2)	21 (38.9)	21 (53.8)	0.39
NA [1]	5 (5.4)	5 (9.3)	0 (0)	
Rectum	15 (13.9)	0 (0)	15 (27.8)	

Table 1. *Cont.*

Clinical Data	Total *N* (%)	American *N* (%)	German *N* (%)	*p*
UICC stage [2]				
III	20 (18.5)	0 (0)	20 (37.0)	
IV	88 (81.5)	54 (100)	34 (63.0)	
Lymph node status				
pN positive	74 (68.5)	24 (44.4)	50 (92.6)	
pN negative	NA	NA	4 (7.4)	
MSI [3]				
Stable	95 (88)	47 (87)	48 (88.9)	
High	11 (10.2)	5 (9.3)	6 (11.1)	
NA	2 (1.8)	2 (3.7)	0 (0)	1.00
Schell-Classification [4,5]				
Class 0	23 (26.1)	15 (27.8)	8 (23.5)	
Class 1	24 (27.3)	17 (31.5)	7 (20.6)	
Class 2	16 (18.2)	9 (16.7)	7 (20.6)	
Class 3	11 (12.5)	6 (11.1)	5 (14.7)	
Class 4	14 (15.9)	7 (13.0)	7 (20.6)	0.69

[1] NA: not available; [2] UICC: Union internationale contre le cancer; [3] MSI: microsatellite instability; [4] only for stage IV patients; [5] percentages do not always add up to 100.0, due to rounding.

Demographic analysis suggested that the American cohort is European by descent, and thus of comparable ethnicity and hereditary genomic composition to the German cohort. In detail, for the Avera patients, surname information was available for three-quarters of the cohort. A comparison with the global Y-chromosome and surname database maintained by Roots for Real (Cambridge, United Kingdom) indicated that the American cohort represented a population sample of European descent, with only two potential exceptions (one Jewish surname and one Mexican/Spanish surname). There was a predominance of Northern European surnames, with 30% deriving from the British Isles, 24% from the German-speaking and Benelux countries, 6% from Scandinavia, and 6% from Eastern Europe. The remaining surnames were of general European descent.

The German patients were recruited in the North German region of Schleswig-Holstein, which historically consisted of Danish-, German-, and Slavonic-speaking populations. In addition, there have been minor migration events in recent centuries [7], but the major event was the settlement of World War II refugees from Eastern German territories of what is now Poland, nearly doubling the population of Schleswig-Holstein after 1945. Genealogical surname analysis indicates that the German cohort is of two-thirds German descent, one-fifth Slavic (East German and Polish) descent, and one-tenth Danish descent. This means that there is no fundamental difference from the American cohort.

2.2. Precision Medicine Increased Overall Survival in High-Risk Patients

Figure 1 shows Kaplan–Meier survival estimates for our patients. As expected, our patients treated in the USA show improved overall survival (OS) compared to our patients treated in Germany almost a decade earlier (Figure 1A). The American patients show an improved OS by a median of 13.5 months (Germany = 19.5 months; USA = 33 months; $p < 0.001$; hazard ratio (HR) = 0.43; 95% confidence interval (CI) 0.26–0.70). Subsequent stratification into SOC vs. individualized care (IND) after SOC shows that the survival benefit is predominantly due to differences in the SOC between USA and Germany almost a decade earlier (median OS: Germany SOC = 19.5 months; USA SOC = 34, USA IND: 33; Figure 1B). To investigate whether specific subgroups of patients benefit from individualized treatment after exhausting SOC options, we further stratified patients into low and high risk. We defined 'high risk' as those patients classified by Schell et al. [4] into either group 0 (*APC* wild type) or group 4 (*APC* with two or more truncating mutations, *TP53* mutated, *KRAS* mutated). We defined

'low risk' as classified into groups 1–3. This analysis revealed that American high-risk patients gained a median 16-month survival benefit when treated with individualized approaches, compared to high-risk patients that received American SOC (29 vs. 13 months) (Figure 1B). Low-risk patients did not seem to have benefited from individualized approaches after SOC (Figure 1B). In a multivariate analysis, including the covariates age, gender, tumor location, microsatellite status, treatment, and risk stratification, receiving treatment in the US in 2008–2019 remained an independent prognostic factor ($p < 0.01$, HR: 0.29, CI 0.11–0.75). In addition, being male was associated with prolonged OS ($p = 0.005$, HR = 0.38, CI: 0.20–0.75), and right-sided tumors were associated with worse outcome ($p = 0.04$, HR = 1.88, CI: 1.02–3.49). Age was not associated with OS. This is congruent with Virostko and colleagues' recent finding that there is little age-related difference in survival for patients who survive longer than 90 days after surgery [8].

Individualized Treatment Implementation

The American patients were either enrolled into the multi-center clinical trials 'Identifying Molecular Drivers of Cancer (CCD)' (NCT02470715), I-PREDICT (NCT02534675) [9], or treated off-label with molecularly guided therapies. Our hypothesis is that matching a single agent to a heterogeneous tumor with multiple genomic alterations will not succeed in improving treatment outcomes, and hence combinations of customized agents are needed for a majority of patients with advanced solid tumors. Targeted therapies were given either after exhaustion or in addition of SOC options and included individualized combinations of one or more conventional cytotoxic compounds with one or more targeted drugs (immunotherapies, antibodies and/or small molecule inhibitors, Table S2 and Figure 2).

Therapies were selected by incorporating recommendations of a molecular tumor board consisting of oncologists, pharmacists, nurses, genetic counselors, bioinformaticians, patient advocates, and molecular biologists. The therapies ultimately given to patients were furthermore based on the treating physician's consideration of patient preferences, drug toxicities, and availability (i.e., insurance coverage). For administration of drug combinations, we routinely followed Nikanjam et al. [10], or other data where available. Patients generally did not receive treatment without at least safety data being available. In addition, patients were monitored closely, and adverse event management was planned on the basis of theoretical drug metabolism, with the result that no treatment-related mortality occurred.

For comparison, the German SOC received by our German patients are given in Table S3 and Figure 3.

2.3. Congruent Cancer Mutation Landscape in Americans and Germans

To test for potential differences in the mutational landscapes of American vs. German patients, three different methods were used: (i) a mutational classification according to Schell et al. [4] based on *APC*, *TP53*, and *KRAS* mutations; (ii) principal component analysis; and (iii) phylogenetic network analysis.

The American versus German stage IV cohorts show no significant difference according to the Schell classification (Table 1) with $p = 0.69$ (Fisher's exact test). Based on the Schell classification, neither our American cohort nor our German cohort are significantly different to the published Schell cohort of Americans, with $p = 1.00$ and $p = 0.48$, respectively. Our German cohort versus the combined cohort of our American patients plus the Schell cohort show no significant difference, with $p = 1.00$.

The principal component analysis (PCA) and the phylogenetic network analysis (Figure 4) are based on somatically mutated genes within the shared set of genes used for both cohorts. For this analysis, we also included stage III patients ($N = 20$). Both analyses show that Americans and Germans are intermingled with each other, i.e., not different from each other. Our 54 American patients were tested by using the FoundationOne panel (Table S4), of which 52 patients had mutations in the shared set of genes. Our 54 German patients were tested, using the IDT Pan-Cancer panel (Table S4), of which 51 patients had mutations in the shared set of genes. In Figure 4A, the PCA shows a microsatellite stable (MSS) cluster and two microsatellite instable (MSI) clusters. There is no separation into American vs. German clusters. In Figure 4B, the network analysis shows a phylogeny, with its root in a healthy

node near the network center, a cluster of patients with low mutation load near the center, and "rings" of patients with higher mutation loads more distant from the root. The network analysis shows no separation into American vs. German branches. Patients with MSI and a high number of private mutations are shown as nodes at each end of their individual, long phylogenetic branches (Figure 4B and Figure S1). The network shows a distinct sector of RAS-mutated patients *(KRAS* and/or *NRAS)* (Figure S1), a cluster of 8 *BRAF*-mutated MSS patients (8%), and 8/11 MSI patients with mutated *BRAF* (73%). The most recent German S3 guideline on colorectal cancer (v2.1, 2019) recommends a first-line treatment of FOLFOXIRI for *BRAF*-mutated patients, or their inclusion into a clinical trial with innovative treatments.

Figure 5 summarizes the landscape of mutations, TMB, MSI status, Schell classification, and tumor location for each patient's tumor sample. It shows that MSI is strongly associated with Schell class 0, right-sided tumors, and lack of *APC* mutations, confirming previous reports [4]. Table S5 and Table S6 summarize the somatic mutations detected in the Americans and Germans, in the shared set of genes (Table S4). Sequencing coverage statistics for the FoundationOne and IDT panels are given in Table S7.

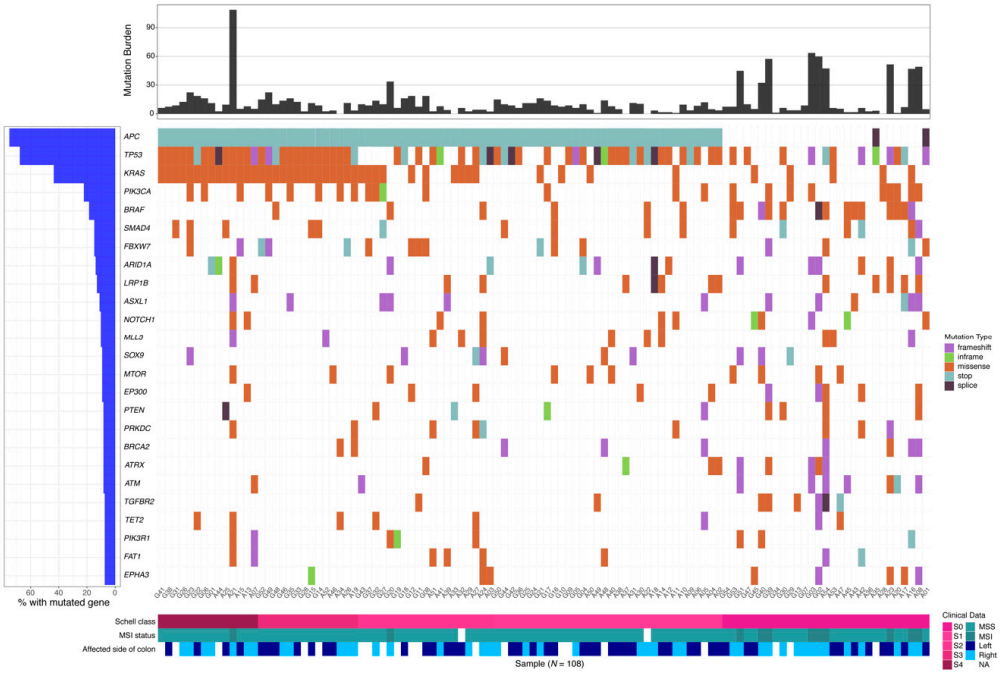

Figure 5. Mutational landscape of metastatic colorectal cancer patients. Each patient is represented by a column, showing mutated genes, tumor mutation burden, Schell class, microsatellite instability status, and side of the colorectal cancer.

Two recent studies on colorectal cancer patients—one comparing Japanese and American cohorts [11], and another comparing Brazilian patients to multiple international cohorts [12]—found that even if mutations in certain driver genes were enriched in specific ethnic subpopulations, the overall mutational landscape of colorectal cancer is comparable. This is clearly supported by multiple methods in our study with American and German patients.

2.4. NGS-Based MSI Test Congruent with Clinical MSI Test

To assess the validity of NGS-based MSI testing on our American and German patient cohorts, the NGS results were compared with clinical MSI results, where available. Our NGS and clinical MSI test results (Table S1) show that NGS correctly classified our cohorts' samples into MSI or MSS. However, without our TMB-based correction, the NGS-based MSIsensor tool incorrectly classified one of the samples as MSI-L instead of MSS. From the clinical side, 10 of the 11 MSI tumors originated in

the right colon (91%). MSI colon cancers tend to evolve from large but flat precursors—sessile serrated adenomas (SSAs)—which are more difficult to detect than polyps [13,14]. Resections of SSAs are difficult, with incomplete resections reported for up to 48% of cases [14]. Due to the large diameter of the proximal colon, such patients may be asymptomatic until the tumor has metastasized, highlighting the dangers of MSI colorectal cancers and their precursor lesions. While MSI is a biomarker indicating immune checkpoint inhibition in the USA [15], the German S3 guideline recommends that the first line of treatment should be based on RAS mutation status. Of note, MSI colorectal cancers usually have no *APC* mutations and thus fall into the 'high-risk' Schell group 0, for which we have shown above that individualized treatments appear to have a significant survival benefit over SOC.

3. Patients

The study was approved by Avera IRB (#2019.005/100572) and by the University of Kiel medical faculty ethics board (#A110/99).

Table 1 summarizes our patients. Patients were included who survived 90 days or longer after surgery. The American cohort comprised 54 patients with metastatic colorectal cancer at the Avera Cancer Institute. They presented to the Institute between 2008 and 2017 with heavily pretreated stage IV CRC. All American patients included in this study were well enough to receive further treatment. At the Avera Cancer Institute, they then received American SOC, and after failure, individualized drug combinations, depending on insurance coverage. The German cohort comprised 54 patients with metastatic colorectal cancer from the Biomaterialbank des Krebszentrums Nord (BMB-CCC). Their patient-matched tumor/normal fresh-frozen tissue sample pairs were sequenced and analyzed by the authors as described below. The German patients were diagnosed between 2003 and 2010. All German patients received SOC therapies according to the German guidelines. The stage IV patients were used for the survival analysis, and the stage III and stage IV patients were used for the mutational landscape analyses, as detailed further in the Methods. No American stage III colorectal cancer patients were available for the study.

4. Materials and Methods

4.1. Foundation Medicine Routine Clinical Testing of the American Patients

Targeted DNA sequencing was performed using the FoundationOne assay (Cambridge, MA, USA), as described in [16]. Briefly, sequencing libraries were prepared from >50 ng DNA extracted from FFPE samples with a minimum of 20% tumor content. Hybridization capture was performed, and the libraries underwent paired-end 49 bp sequencing to a median coverage of >500X on the Illumina platform. The Foundation Medicine sequencing data have not been archived in a public human sequence archive because the patients did not consent.

4.2. Retrospective Next-Generation Sequencing of the German Patients

DNA was isolated from fresh-frozen tissue, using AllPrep DNA-RNA-miRNA Universal Kit (QIAGEN #80224). 108 Illumina TruSeq Nano libraries were prepared from 100 ng DNA each according to protocol. Hybridization capture was performed using the IDT xGen Pan-Cancer panel v1.5, which targets cancer genes identified by The Cancer Genome Atlas (TCGA). Sequencing was performed on Illumina NextSeq, using 2 × 150 bp paired-end reads. We have securely archived the fastq sequencing files at the European Genome-Phenome Archive (EGA) under study accession ID EGAS00001004108. The EGA is subject to the EU's General Data Protection Regulation and access to the data may be applied for, subject to a data-access agreement with project description and ethics board approval.

4.3. Bioinformatic Analysis of the German Patients

Raw sequencing data were aligned to the genome (hs37d5) with BWA-MEM (v 0.7.15) (https://arxiv.org/abs/1303.3997) and realigned using ABRA (v 0.97) [17]. Duplicates were marked using

sambamba (v 0.6.3) [18]. Somatic variants were called, using VarDict (v. 1.5.1) [19], and annotated by using ANNOVAR (v Feb 2016) [20].

Technical filters were applied to the mutation calls: minimal variant depth of 7, minimal base quality of 30, minimal variant allele frequency of 0.003, strand bias according to the VarDict test. Variants were required to have a minimal depth of 10 in either the tumor or matched normal sample. Variants caused by DNA damage were filtered out, as recommended in [21] and [22]. Additional filtering was done on low-frequency variants that have low depth, as shown by (http://bcb.io/2016/04/04/vardict-filtering/). ExAC and 1000 genomes databases were used to filter variants with a population allele frequency threshold of 0.01% [23,24].

Tumor mutational burden (TMB) was calculated as the number of mutations per 1 Mb relative to the panel size and rounded to the first decimal place.

Microsatellite instability (MSI) was assessed by using MSIsensor (v 0.5) on matched tumor-normal samples. The cutoffs used were as follows: score <10 for MSS (microsatellite stable), score between and including 10 and 30 for MSI-L (low), and score >30 for MSI-H (high). When the scores generated by MSIsensor were close to a cutoff value, the corresponding TMB value of the sample was utilized to make a final judgement on the classification (Supplementary Table S1). If the TMB value was high, it was classified as MSI, as supported by the evidence shown in a previous study [25].

Sample-pairing validation was performed, as previously published, by comparing polymorphism signatures between all samples [26]. Somatic single nucleotide substitutions were validated by using pibase [26]. Somatic indels were manually validated by using IGV [27].

4.4. Comparison of Mutational Signatures in Americans vs. Germans

According to Strickler and colleagues, a threshold of 25% of the maximal tumor allele frequency in a tumor sample was applied to classify a mutation as clonal or subclonal [28]. Subclonal mutations were not counted if they had less than 1/10 of the maximal tumor allele frequency in the sample, or less than 3% absolute tumor allele frequency.

To answer the question whether the American and German cohorts had congruent mutational signatures or not, the cohorts' mutations were compared at three levels of resolution.

For the first level of resolution, we used the mutational classification proposed by Schell and colleagues [4]: 0—no truncating mutations in *APC*; 1—one truncating *APC* mutation, and *TP53* or *KRAS* mutated but not both; 2—two truncating *APC* mutations, and *TP53* or *KRAS* mutated but not both; 3—one truncating *APC* mutation, and both *TP53* and *KRAS* mutated; 4—two truncating *APC* mutations, and both *TP53* and *KRAS* mutated.

For the second level of resolution, we performed a principal component analysis, and for the third level of resolution, we performed a phylogenetic network analysis [29]. We analyzed the same data in the second and third levels of resolution. We considered the genes contained in the overlap of the FoundationOne panel and the IDT panel (Table S4). We considered only the clonal mutations. A mutated gene in a patient was scored as 1, and a wild-type gene scored as 0. If there were patients with more than one mutation in a gene, then the affected gene names were duplicated so that the binary 1/0 scoring system could be used, e.g., *APC*, *APC_1*, *APC_2*, *TP53*, and *TP53_1.*

4.5. Microsatellite Instability Testing

MSI tests for all German tumor and normal samples were performed by the Department of Pathology in Kiel, using five mononucleotide markers (BAT25, BAT26, NR21, NR24, and MONO27).

MSI testing for the American patients was carried out as part of the FoundationOne panel, where available. For the samples that did not have those results, MSIsensor (v 0.5) was used to perform MSI calling on tumor samples only, as we did not have matched normals for this cohort. This method was able to resolve the samples as either MSS or MSI, based on a cutoff score of 25, but not the grade of MSI, as shown previously [30]. Additionally, this was validated in over a thousand samples from

Avera with FoundationOne tests. As described above, when scores were close to the cutoff value, MSI classification was adjusted.

5. Conclusions

High-risk patients may be identified as having Schell mutational classifications 0 and 4. These patients may survive significantly longer if they receive individualized treatments after the exhaustion of standard-of-care treatments. Secondly, our study has, for the first time, proven what has previously often just been assumed: The mutational landscapes in American and German metastatic colorectal cancer patients are comparable—on the basis of Schell profiles, principal components, and phylogeny—despite the geographic and environmental divergence. However, we find that the overall survival in American patients who received standard-of-care treatments or individualized targeted treatments once they failed standard therapies is significantly longer than that of German patients who received less individualized SOC almost a decade earlier. Therefore, we also suggest that innovative treatments should and can be readily harmonized and exchanged between American and German cancer centers.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/2/393/s1. Figure S1: Phylogenetic network analysis details, Table S1: Patient metadata in detail, Table S2: Treatment combinations for American patients at Avera Cancer Institute, initial presentation in 2008–2017, Table S3: Treatment combinations for German patients at University Hospital Schleswig-Holstein, initial presentation in 2003–2010, Table S4: List of genes in Foundation Medicine One panel and in IDT xGen Pan-Cancer panel, Table S5: Clonal somatic tumor mutations detected in American patients in consensus gene panel (genes present in FoundationOne and IDT Pan-Cancer), Table S6: Somatic tumor mutations detected in German patients in consensus gene panel (genes present in FoundationOne and IDT Pan-Cancer), Table S7: Sequencing Statistics.

Author Contributions: All authors have read and agreed to the published version of the manuscript. Conceptualization, all authors; methodology, A.A., T.M., P.F., and M.F.; software, A.A., T.M., and M.F.; validation, T.M.; formal analysis, A.H., A.A., T.M., P.F., and M.F.; investigation, A.H., P.R., G.B., C.S., S.H., and C.B.W.; resources, A.H., P.R., G.B., C.S., A.F., S.H., and C.B.W.; data curation, A.H., A.A., T.M., and M.F.; writing—original draft preparation, A.H., A.A., T.M., P.F., M.F., and C.B.W.; writing—review and editing, all authors; visualization, T.M., P.F., and M.F.; supervision, S.H., M.F., and C.B.W.; project administration, A.H., T.M., and M.F.; funding acquisition, P.R., C.S., A.F., and C.B.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: PF owns shares in Genetic Ancestor Ltd., of which he is director. All remaining authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Anhang – Teil 7

Lebenslauf

Persönliche Daten

Name	Dr. med. Alexander Hendricks
Geburtsort	Pequannock/USA
Geburtsdatum	23.12.1984
Familienstand	Verheiratet, vier Kinder
Staatsangehörigkeit	Deutsch

Schulausbildung

1991 – 2004	Düsseldorf, West Lavington (GB) und Oxford (GB)

Hochschulausbildung

März 2006 – März 2008	Georg-August-Universität Göttingen Studiengang der Humanmedizin Abschluss 1. Ärztliche Prüfung
Okt 2008 – Nov 2012	Christian-Albrecht-Universität zu Kiel Studiengang der Humanmedizin Abschluss 2. Ärztliche Prüfung

Tätigkeit als Arzt

Juni 2013 – Aug 2019	Assistenzarzt in der Klinik für Allgemeine Chirurgie, Viszeral-, Thorax-, Transplantations- und Kinderchirurgie Universitätsklinikum Schleswig-Holstein, Campus Kiel Direktor: Prof. Dr. med. Thomas Becker
seit Sept 2019	Assistenzarzt in der Klinik und Poliklinik für Allgemein-, Viszeral-, Thorax-, Gefäß- und Transplantationschirurgie Universitätsmedizin Rostock Direktor: Prof. Dr. med. Clemens Schafmayer
seit März 2021	Facharzt für Viszeralchirurgie

Mitgliedschaften

DGCH Deutsche Gesellschaft für Chirurgie

DGAV	Deutschen Gesellschaft für Allgemein- und Viszeralchirurgie
BDC	Berufsverband Deutscher Chirurgen
NDCH	Vereinigung Norddeutscher Chirurgen

Wissenschaftliche Tätigkeit

Promotion

Sept 2015	Promotion an der Klinik für Herz und Gefäßchirurgie des UKSH, Campus Kiel in der Arbeitsgruppe von Herrn Prof. Dr. Lutter, mit dem Thema: Percutan, transfemoraler Pulmonalklappenersatz durch tissue-engineerte Herzklappen

Forschungsrotation (GEROK-Stipendium)

Jan 2017 – Dez 2017	Aufnehmende Einrichtung: Institut für Experimentelle Tumorforschung, UKSH, Campus Kiel Direktorin: Professor Dr. rer. nat. Susanne Sebens Thema: Die Rolle der „Stemness“ bei der Metastasierung des kolorektalen Karzinoms – Analyse von Wnt und TRAIL in Bezug auf die zirkulierende Tumorzelle

Intramurale Förderung

Juniorförderung 2015	Im Rahmen der Nachwuchsförderung der Christian-Albrechts-Universität zu Kiel. Thema: Etablierung einer modifizierten Keratin 20 RT-PCR (CK20 PCR 2.0) zum Nachweis von CTC und DTC und Analyse des prognostischen Wertes von CTC und DTC bei Patienten mit kolorektalem Karzinom. Fördervolumen 10.000 Euro
Forschungsrotation 2017	Im Rahmen des GEROK-Programmes der ChristianAlbrechts-Universität zu Kiel. Thema: Die Rolle der „Stemness“ bei der Metastasierung des kolorektalen Karzinoms – Analyse von Wnt und TRAIL in Bezug auf die zirkulierende Tumorzelle. Fördervolumen 100.000 Euro

FORUN 2020 — Im Rahmen der Nachwuchsförderung der Universität und Universitätsmedizin Rostock.
Thema: Molekulare Stratifizierung von Patienten mit kolorektalem Karzinom: Analyse von zirkulierenden Tumorzellen und zirkulierender freier Tumor-DNA.
Fördervolumen: 18.140 Euro

Auszeichnungen und Preise

Dez 2015 — Posterpreis der 196. Tagung der Vereinigung Norddeutscher Chirurgen in Hamburg. Titel: Der Nachweis von zirkulierenden Tumorzellen mittels CK20 RT-PCR ist ein negativ prognostischer Marker bei Kolonkarzinompatienten

Juni 2016 — Posterpreis der 197. Tagung der Vereinigung Norddeutscher Chirurgen in Flensburg. Titel: Die Vielfalt der Tumorzelldisseminierung beim kolorektalen Karzinom

Juni 2017 — Posterpreis der 199. Tagung der Vereinigung Norddeutscher Chirurgen in Osnabrück. Titel: Liquid Biopsies – eine quantitative Multimarker PCR zur Stratifizierung einer Risikopopulation bei Kolorektalkarzinom-patienten

Lehrtätigkeit

Curriculare Lehre Medizinstudium (verantwortlich Univ.-Prof. Dr. T. Becker, Klinik für Allgemeine, Viszeral-, Thorax-, Transplantations- und Kinderchirurgie, UKSH, Campus Kiel, Kiel)

2013 – 2019 Winter- und Sommersemester

Unterricht am Krankenbett (UaK)	2,5 Semersterwochenstunden
Blockpraktikum – Ausbildung am Patienten	1,5 Semersterwochenstunden

2013 – 2019 einmal jährlich

Objective Structured Clinical Examination (OSCE); Praktikumsdurchführung (Tutor) und Prüfungsabnahme (Prüfer)
Einführung in die klinische Medizin (EKM) Praktikum; Praktikumsdurchführung (Tutor) und Prüfungsabnahme (Prüfer)

Curriculare Lehre Medizinstudium (verantwortlich Univ.-Prof. Dr. C. Schafmayer, Abteilung für Allgemein-, Viszeral-, Gefäß- und Transplantationschirurgie, Universitätsmedizin Rostock, Rostock)

2019 – dato Winter- und Sommersemester

Betreuung von PJ-Studenten

Jan 2021 – dato

Beauftragter für die PJ-Studenten; Organisation, Einteilung, Betreuung.

Erweitertes Lehrangebot

Einarbeitung von Medizinstudenten in ihre Doktorarbeiten, Betreuung bei der Arbeit, Hilfe bei der Auswertung und dem Erlernen von wissenschaftlicher Vorgehensweise im Sinne einer guten wissenschaftlichen Praxis.

Greta-Lou Eggebrecht, WS 2015/2016 – SS 2019; 2,5 Semersterwochenstunden

Thema: „Evaluation einer semi-quantitativen CK20 RT-PCR zum Nachweis zirkulierender Tumorzellen und Bestimmung der prognostischen Bedeutung bei Patienten mit kolorektalem Karzinom.“
Stand der Promotion: Abgeschlossen

Carolin Schmidt, SS 2016 – SS 2019; 2,5 Semersterwochenstunden
Thema: „Bestimmung des POSSUM - Scores zur Evaluation der perioperativen Morbidität und Mortalität bei Patienten mit Bronchialkarzinom.“
Stand der Promotion: Abgeschlossen

Katharina Dall, WS 2016/2017 – SS 2019; 2,5 Semersterwochenstunden
Thema: Etablierung eines Immunfluoreszenz-basierten Nachweisverfahrens für zirkulierende Tumorzellen bei Kolorektalkarzinompatienten.“
Stand der Promotion: Abgeschlossen

Tobias Ludwig, SS 2020 – dato
Thema: Evaluation der Anwendbarkeit des POSSUM-Scores zur Risikoklassifizierung von kolorektaler Chirurgie
Stand der Promotion: in Niederschrift

Publikationsverzeichnis

Originalarbeiten mit Impact-Factor

2015

Hinz S, Röder C, Tepel J, **Hendricks A**, Schafmayer C, Becker T, et al. Cytokeratin 20 positive circulating tumor cells are a marker for response after neoadjuvant chemoradiation but not for prognosis in patients with rectal cancer. BMC Cancer. 2015 Dec 16;15:953. IF: 3,432

2016

Heits N, Mueller L, Koops A, Koops S, Herrmann J, **Hendricks A**, et al. Limits of and Complications after Embolization of the Hepatic Artery and Portal Vein to Induce Segmental Hypertrophy of the Liver: A Large Mini-Pig Study. Eur Surg Res. 2016;57(3–4):155–70. IF: 2,351

2017

Heits N, Keserovic D, Mund N, Ehmke N, Bernsmeier A, **Hendricks A**, et al. Cognitive Evaluation in Liver Transplant Patients Under Calcineurin Inhibitor Maintenance Therapy. Transplant Direct. 2017 Apr;3(4):e146. IF: 0,200

Egberts J-H, Stein H, Aselmann H, **Hendricks A**, Becker T. Fully robotic da Vinci Ivor-Lewis esophagectomy in four-arm technique-problems and solutions. Dis Esophagus. 2017 Dec 1;30(12):1–9. IF: 2,285

Hinz S, **Hendricks A**, Wittig A, Schafmayer C, Tepel J, Kalthoff H, et al. Detection of circulating tumor cells with CK20 RT-PCR is an independent negative prognostic marker in colon cancer patients - a prospective study. BMC Cancer. 2017 13;17(1):53. IF: 3,432

2018

Heits N, Bernsmeier A, Reichert B, Hauser C, **Hendricks A**, Seifert D, et al. Long-term quality of life after endovac-therapy in anastomotic leakages after esophagectomy. J Thorac Dis. 2018 Jan;10(1):228–40. IF: 2,046

Hendricks A, Eggebrecht G-L, Bernsmeier A, Geisen R, Dall K, Trauzold A, et al. Identifying patients with an unfavorable prognosis in early stages of colorectal carcinoma. Oncotarget. 2018 Jun 8;9(44):27423–34. IF: 5,168 (2016)

von Schönfels W, Beckmann JH, Ahrens M, **Hendricks A**, Röcken C, Szymczak S, et al. Histologic improvement of NAFLD in patients with obesity after bariatric surgery based on standardized NAS (NAFLD activity score). Surg Obes Relat Dis. 2018 Oct;14(10):1607–16. IF 3,812

2019

Hendricks A, Gieseler F, Nazzal S, Bräsen JH, Lucius R, Sipos B, et al. Prognostic relevance of topoisomerase II α and minichromosome maintenance protein 6 expression in colorectal cancer. BMC Cancer. 2019 May 9;19(1):429. IF: 3,432

Schafmayer C, Harrison JW, Buch S, Lange C, Reichert MC, Hofer P, ... **Hendricks A**, et al. Genome-wide association analysis of diverticular disease points towards neuromuscular, connective tissue and epithelial pathomechanisms. Gut. 2019;68(5):854–65. IF 17,943

Wang Y, Hinz S, Uckermann O, Hönscheid P, von Schönfels W, Burmeister G, ... **Hendricks A**, et al. Shotgun lipidomics-based characterization of the landscape of lipid metabolism in colorectal cancer. Biochim Biophys Acta Mol Cell Biol Lipids. 2019 Nov 30;158579. IF 4,519

Segovia-Miranda F, Morales-Navarrete H, Kücken M, Moser V, Seifert S, Repnik U, ... **Hendricks A**, et al. Three-dimensional spatially resolved geometrical and functional models of human liver tissue reveal new aspects of NAFLD progression. Nat Med. 2019 Dec;25(12):1885–93. IF: 30,641

2020

Hendricks A, Amallraja A, Meißner T, Forster P, Rosenstiel P, Burmeister G, et al. Stage IV Colorectal Cancer Patients with High Risk Mutation Profiles Survived 16 Months Longer with Individualized Therapies. Cancers. 2020 Feb 8;12(2):393. IF: 6,433

Hirsch D, Gaiser T, Merx K, Weingaertner S, Forster M, **Hendricks A**, et al. Clinical responses to PD-1 inhibition and their molecular characterization in six patients with mismatch repair-

deficient metastatic cancer of the digestive system. J Cancer Res Clin Oncol. 2020 Aug 9. IF: 3,656

Hendricks A, Brandt B, Geisen R, Dall K, Röder C, Schafmayer C, et al. Isolation and Enumeration of CTC in Colorectal Cancer Patients: Introduction of a Novel Cell Imaging Approach and Comparison to Cellular and Molecular Detection Techniques. Cancers (Basel). 2020 Sep 16;12(9). IF: 6,433

Thangapandi VR, Knittelfelder O, Brosch M, Patsenker E, Vvedenskaya O, Buch S, ... **Hendricks A**, et al. Loss of hepatic Mboat7 leads to liver fibrosis. Gut. 2020 Jun 26. IF: 17,943

2021

Zheng T, Ellinghaus D, Juzenas S, Cossais F, Burmeister G, Mayr G, … **Hendricks A**, et al. Genome-wide analysis of 944 133 individuals provides insights into the etiology of haemorrhoidal disease. Gut. 2021 Apr 22. IF: 17,943

Hendricks A, Dall K, Brandt B, Geisen R, Röder C, Schafmayer C, et al. Longitudinal Analysis of Circulating Tumor Cells in Colorectal Cancer Patients by a Cytological and Molecular Approach: Feasibility and Clinical Application. Frontiers in Oncology. 2021;11:2038. IF: 6,244

Case-Reports mit Impact-Factor

Hendricks A, Rosenstiel P, Hinz S, Burmeister G, Röcken C, Boersch K, et al. Rapid response of stage IV colorectal cancer with APC/TP53/KRAS mutations to FOLFIRI and Bevacizumab combination chemotherapy: a case report of use of liquid biopsy. BMC Med Genet. 2020 Dec;21(1):3. IF: 1,797

Zitierfähige Abstracts

2017

Hendricks A, Hauser C, Bernsmeier A, Schmidt C, Möller T, Becker T, Egberts J-H. POSSUM score as a significant risk marker for major complications in lung resection. Abstracts DGT. Innovative Surgical Sciences. 2017 Mar 1;2(s1):s310–26.

Hauser C, **Hendricks A**, Steinig LS, Bernsmeier A, Aselmann H, Becker T, Egberts J-H. Experience after 60 robotic lobectomies. Abstracts DGT. Innovative Surgical Sciences. 2017 Mar 1;2(s1):s310–26.

Heits N, Keserovic D, Mund N, Ehmke N, Bernsmeier A, **Hendricks A**, Guenther R, Witt K, Becker T, Braun F. Cognitive function, Quality of Life and patient's compliance after liver transplantation following calcineurin inhibitor treatment Abstracts DGAV. Innovative Surgical Sciences. 2017 Mar 1;2(s1):s1–168.

2018

Hendricks A, Eggebrecht G-L, Röder C, Schafmayer C, Tepel J, Becker T, Hinz S. Identifying Patients with an Unfavourable Prognosis in Early Stages of Colorectal Carcinoma – Application of a Novel RT-qPCR for Detecting Circulating Tumour Cells in Liquid Biopsies. 135th Congress of the German Society of Surgery (DGCH). Eur Surg Res. 2018;59(1):1–68.

Kerber J, **Hendricks A**, Böger C, Geisen R, Egberts J-H, Braun F, Sebens S, Becker T, Heits N. Influence of Cancer-Associated Fibroblasts and Schwann Cells on Tumor Progression of Human Cholangiocarcinoma In Vitro. 135th Congress of the German Society of Surgery (DGCH). Eur Surg Res. 2018;59(1):1–68.

Hendricks A, Eggebrecht G-L, Röder C, Schafmayer C, Tepel J, Becker T, Hinz S. Detection of circulating tumor cells by a novel RT-qPCR identifies patients with an unfavorable prognosis in UICC stages I and II of colorectal carcinoma. 1. 33. Deutscher Krebskongress - Perspektiven verändern Krebs - Krebs verändert Perspektiven. Diagnose - Therapie - (Über-)Leben. Berlin, 21.-24. Februar 2018: Abstracts. Oncol Res Treat. 2018;41(1):1–221.

2019

Hendricks A, Dall K, Geisen R, Eggebrecht G-L, Röder C, Schafmayer C, Sebens S, Becker T, Hinz S. Serial Analysis of Circulating Tumour Cells in Liquid Biopsies of Colorectal Cancer Patients After Surgery – Establishment of a New Biomarker of Tumour Burden and Relapse. 1. Abstracts: 136th Congress of the German Society of Surgery (DGCH). Eur Surg Res. 2019;60(Suppl. 1):1–33.

Hendricks A, Geisen R, Dall K, Röder C, Becker T, Schafmayer C, et al. Etablierung eines zytomorphologischen Testverfahrens für zirkulierende Tumorzellen beim Kolorektalkarzinom

– das ScreenCell®-System in der praktischen Anwendung. In Wiesbaden; 2019 p. s-0039-1695405. AvZ Gastroenterol 2019; 57(09): e300-e301/DOI/10.1055/s-0039-1695405

2020

Hendricks A, Dall K, Geisen R, Roeder C, Sebens S, Becker T, Schafmayer C, Hinz S. Longitudinal Investigation of Circulating Tumour Cells in Liquid Biopsies of Colorectal Cancer Patients – Analysis of the Clinical Benefit of a New Biomarker of Tumour Burden and Relapse. 34. Deutscher Krebskongress informativ. innovativ. integrativ. Optimale Versorgung für alle. Berlin, 19.-22. Februar 2020: Abstracts. Oncol Res Treat. 2020;43(Suppl. 1):1–265.

Born J, **Hendricks A**, Bernsmeier A, Rahn S, Röder C, Sebens S. Dissemination of Intestinal Epithelial Cells in Inflammation- Associated-Colorectal- Carcinogenesis. 34. Deutscher Krebskongress informativ. innovativ. integrativ. Optimale Versorgung für alle. Berlin, 19.-22. Februar 2020: Abstracts. Oncol Res Treat. 2020;43(Suppl. 1):1–265.

Hendricks A, Geisen R, Dall K, Röder C, Becker T, Schafmayer C, Sebens S, Hinz S. Isolation and Identification of Circulating Tumour Cells in Colorectal Cancer – Application of the ScreenCell® Filtration Device. 1. 137th Congress of the German Society of Surgery (DGCH). Eur Surg Res. 2020;61(1):1–49.

Amallraja A, Fuß J, Williams CB, Forster M, Hinz S, Meißner T, **Hendricks A**. Abstract 3617: Very low coverage whole genome sequencing improves clinically relevant copy number variation calling compared to targeted sequencing. Cancer Res. 2020 Aug 15;80(16 Supplement):3617.

Kongressbeiträge

2014

Schönfels W, Ahrens M, **Hendricks A**, Laudes M, Becker T, Schafmayer C. Histologische Verbesserung von nicht-alkoholischer Fettlebererkrankung durch bariatrische Chirurgie. 193. Tagung der Vereinigung Nordwestdeutscher Chirurgen, Bremen, 19.-21. Juni 2014

Hendricks A, Ahrens M, Egberts J-H, Laudes M, Becker T, Schafmayer C. Nicht nur der Darm war dick. 193. Tagung der Vereinigung Nordwestdeutscher Chirurgen, Bremen, 19.-21. Juni 2014

Hendricks A, Heits N, Schönfels W, Bellersen M, Becker T, Dohrmann P, Egberts J-H. Peritonealdialyse und Dyspnoe? 194. Tagung der Vereinigung Nordwestdeutscher Chirurgen, Hamburg, 27.-29. November 2014

Hendricks A, Hinz S, Röder C, Schafmayer C, Becker T, Kalthoff H. Zirkulierende CK20+ Tumorzellen im Blut sind ein signifikanter Responseindikator nach neoadjuvanter Chemotherapie bei Rektumkarzinompatienten. 194. Tagung der Vereinigung Nordwestdeutscher Chirurgen, Hamburg, 27.-29. November 2014

2015

Hendricks A, Wittig A, Schafmayer C, Tepel J, Röder C, Becker T, Kalthoff H, Hinz S. CK20-positive zirkulierende Tumorzellen sind ein negativer prognostischer Marker bei Kolonkarzinompatienten – eine prospektive Studie. 196. Tagung der Vereinigung Norddeutscher Chirurgen, Hamburg, 03.-05. Dezember 2015

2016

Hendricks A, Röder C, Tepel J, Schafmayer C, Becker T, Kalthoff H, Hinz S. Diversity of tumor cell dissemination in colon and rectal cancer – not just a different anatomic site. 133. Kongress der Deutschen Gesellschaft für Chirurgie, Berlin, 26.-29. April 2016

Hendricks A, Gundlach J-P, Schönfels W, Becker T, Schafmayer C. Prolenenetze können Leben retten. 197. Tagung der Vereinigung Norddeutscher Chirurgen, Flensburg, 02.-04. Juni 2016

Hendricks A, Röder C, Tepel J, Schafmayer C, Kalthoff H, Becker T, Hinz S. Die Vielfalt der Tumorzelldisseminierung beim kolorektalen Karzinom. 197. Tagung der Vereinigung Norddeutscher Chirurgen, Flensburg, 02.-04. Juni 2016

Hendricks A, Eggebrecht G-L, Röder C, Schafmayer C, Tepel J, Becker T, Hinz S. Etablierung einer neu entwickelten quantitativen CK20 RT-PCR als Verlaufsmarker beim kolorektalen Karzinom. 198. Tagung der Vereinigung Norddeutscher Chirurgen, Hamburg, 24.-26. November 2016

2017

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Anhang – Teil 8

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Anhang – Teil 9

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die aus fremden Quellen direkt oder indirekt übernommenen Stellen sind als solche kenntlich gemacht. Die Arbeit wurde bisher in gleicher oder ähnlicher Form keinem anderen Prüfungsamt vorgelegt und auch nicht veröffentlicht.

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Ort, Datum

Alexander Hendricks