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Translationale chirurgische Forschung: vom Labor an das Patientenbett, von der onkologischen Grundlagenforschung zur Transplantationsmedizin

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vorgelegt von

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Verzeichnis der Anlagen

Anlagen sind in der Reihenfolge aufgeführt, in der sie im Weiteren bearbeitet werden.

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Inhaltsverzeichnis

Abkürzungen

α-Toco	Alpha-Tocopherol
Akt	Proteinkinase B
AUC	Area Under the Curve
Bcl-2	B-cell Lymphoma 2
BMI	Body Mass Index
BRAF	Isoform B der Rapidly Accelerated Fibrosarcoma Proteine
cIAP	Cellular Inhibitor of Apoptosis Protein
DNA	Deoxyribonuclein Acid
ERK	Extracellular-signal Regulated Kinase
HCC	Hepatozelluläres Karzinom
HER2	Human Epidermal Growth Factor Receptor 2
IAP	Inhibitor of Apoptosis Proteinen
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
MAPK	Mitogen-activated Proteinkinase
Mcl-1	Myeloid Cell Leukaemia Sequence 1 Protein
MnTBAP	Manganese (III) Tetrakis (4-Benzoic Acid) Porphyrin Chloride
mTOR	Mammalian/Mechanistic Target of Rapamycin
OA	Oleanolsäure
PARP	Poly (ADP-ribose) Polymerase
ROC	Receiver-Operating-Characteristic
ROS	Reactive Oxygen Species (reaktive Sauerstoffspezies)
Smac	Second Mitochondria-derived Activator of Caspase
XIAP	X-linked Inhibitor of Apoptosis Protein
zVAD.fmk	Pan-Caspaseninhibitor

1. Einleitung

Translationale Forschung wird, laut den amerikanischen *National Institutes of Health*, als die Integration von experimenteller Grundlagenforschung in eine klinische, patientenorientierte Forschung definiert [1]. Ziel translationaler Forschung ist es, in multidisziplinären Forschungsteams aus Klinikern und Naturwissenschaftlern Erkenntnisse der Grundlagenforschung effizient in die klinische Praxis zu transferieren. Damit sollen Ergebnisse aus dem Forschungslabor an das Patientenbett überführt und die klinische Behandlung verbessert werden [2]. In der translationalen Forschung wird jedoch auch, ausgehend von einer konkreten medizinischen Fragestellung, mit Hilfe der Grundlagenforschung gezielt nach Lösungen gesucht.

Eine Recherche in der Datenbank *Medline* zeigt, dass der Begriff der translationalen Forschung erstmalig in den 90er Jahren aufgetaucht ist [1]. Doch erst Ende der 2000er Jahre ist ein deutlicher Anstieg von Publikationen zu diesem Thema zu verzeichnen (Abb. 1). Die zunehmende Bedeutung translationaler Forschung in den späten 2000er Jahren zeigt sich auch daran, dass seit dem Jahr 2009 durch das neben *Nature* wichtigste wissenschaftliche Magazin *Science* ein auf translationale Forschung spezialisiertes Magazin herausgegeben wird: *Science Translational Medicine*. In den wenigen Jahren seines Erscheinens etablierte sich *Science Translational Medicine* zu einem der bedeutendsten Magazine in den Bereichen *experimentelle und forschende Medizin* (Ranking 2/133, Journal Citation Reports®) und *Zellbiologie* (Ranking 9/190, Journal Citation Reports®) mit einem Impact Factor von über 16,71 (2017, Journal Citation Reports®).

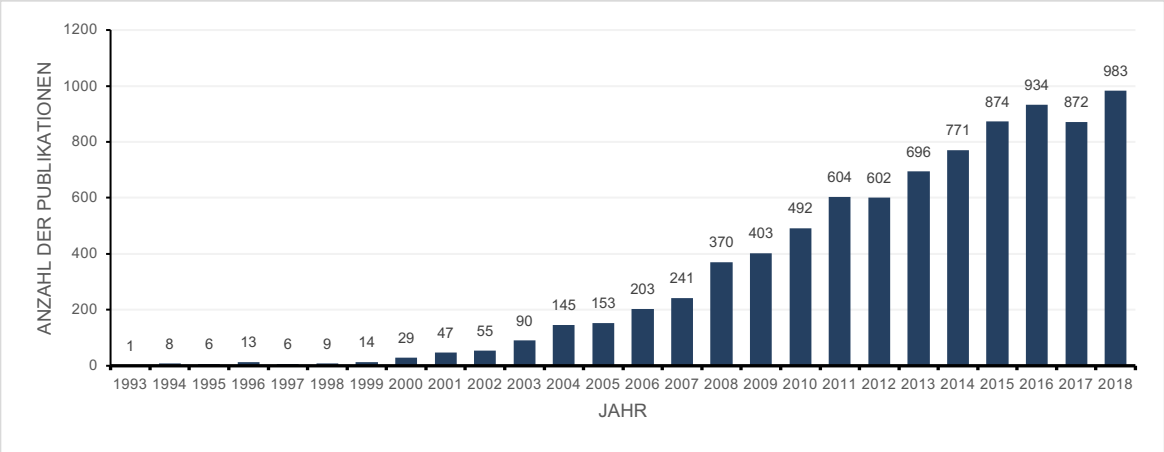

Abb. 1: Anzahl der in der Datenbank *PubMed* gelisteten Publikationen zum Thema „translational research“ (Stand Dezember 2018).

Wichtige Schwerpunkte translationaler chirurgischer Forschung in der Viszeralchirurgie sind die Onkologie und die Transplantationsmedizin. In beiden Bereichen spielt neben der Etablierung von neuen Modellen zur Prognoseabschätzung, beispielsweise für das Tumorrezidiv oder das Transplantatüberleben, auch die Identifizierung von neuen experimentellen Therapieansätzen eine entscheidende Rolle. Ziele sind hierbei auch, durch ein besseres Verständnis der Tumorbiologie, therapeutische Ansätze und Algorithmen zu optimieren und klinische Abläufe zu verbessern.
In den letzten Jahren hat insbesondere die Integration von molekularen Kenntnissen u. a. beim Kolonkarzinom oder Mammakarzinom (BRAF, KRAS, HER2, Mikrosatelliten-instabilität etc.) in der täglichen klinischen Arbeit mit onkologischen Patienten nicht nur zum verbesserten Verständnis der Tumorgenese, sondern auch wesentlich zur Entwicklung von individuellen Therapien, Risikostratifizierungen und zur Prognoseabschätzung beigetragen. Multimodale Therapiekonzepte, insbesondere die Zusammenarbeit von Chirurgie, Onkologie, Strahlentherapie und interventioneller Radiologie sind heute vielmals durch die Ergebnisse translationaler Forschung beeinflusst.
In meiner eigenen Forschungsarbeit habe ich mich mit der translationalen Forschung der Onkologie und der Transplantationsmedizin beschäftigt, wobei der Übergang zwischen diesen beiden Bereichen teilweise fließend ist.

1.1. Translationale onkologische Forschung – das hepatozelluläre Karzinom (HCC)

Das hepatozelluläre Karzinom (HCC) ist der häufigste primäre humane Lebertumor weltweit. In den meisten Fällen tritt es bei Patienten mit chronischen Lebererkrankungen (u. a. chronische Hepatitis B/C, alkoholische Lebererkrankung und nicht-alkoholische Steatohepatitis) auf, die zu einer Leberzirrhose führen. Nur in 30% der Fälle ist eine kurative Therapie möglich, diese ist jedoch mit einer relevanten Tumorrezidivrate verbunden [3,4]. Diese Rezidivrate liegt bei Leberresektionen und ablativen Verfahren bei bis zu 50% [5,6]. Lediglich die Lebertransplantation ermöglicht durch die Entfernung der zirrhotischen Leber als Präkanzerose eine niedrigere Rezidivrate, welche jedoch mit ca. 24% nach eigenen Untersuchungen von transplantierten Patienten noch bemerkenswert hoch ist [7].
Neben der genannten hohen Rezidivrate bedingt das Fehlen von effektiven Chemotherapien die schlechte Prognose des HCCs. Sorafenib, ein Multikinase-Inhibitor,

ist momentan das einzige routinemäßig eingesetzte Chemotherapeutikum. Jedoch verlängert Sorafenib das Patientenüberleben im Median lediglich um 2,8 Monate und stellt nur eine palliative Therapie dar [8]. Die Gefahr der Verschlechterung der Leberfunktion unter Sorafenib macht diese Therapie zudem nur Patienten mit guter Leberfunktion zugänglich [8]. Dies verdeutlicht die Bedeutung der Entwicklung neuer und effektiverer Behandlungsstrategien. Grundlegend für die Entwicklung von neuen Therapien ist jedoch das Verständnis von Tumorgenese und Tumorbiologie.

Bei der Entstehung des HCCs spielt eine Vielzahl von Faktoren eine Rolle (Abb. 2). Die chronische Entzündung führt über die Jahre hinweg zu einer Deregulation der hepatischen Immunregulation. Neben einer vermehrten Expression von proinflammatorischen Zytokinen (Interleukin-2, Interleukin-7, Interleukin-12 und Interferon-γ etc.) kommt es zu zellulärem Stress mit Produktion von reaktiven Sauerstoffspezies (reactive oxygen species = ROS), veränderten Überlebens- und Proliferationssignalen, chronischer DNA-Schädigung sowie epigenetischen und mitochondrialen Veränderungen [4,9,10]. Diese Prozesse führen in der Leber zu einem fortwährenden Zelltod der Hepatozyten und zu einer kompensatorischen Regeneration und hierüber letztendlich zur Leberfibrose, Leberzirrhose und Tumorgenese [10]. Zudem führen diese epigenetischen Veränderungen und DNA-Mutationen im Verlauf der Hepatokarzinogenese u. a. zu einer Überexpression von Proteinen, die verhindern, dass die Tumorzelle in den Zelltod (Apoptose) geht. Eine wichtige Gruppe von diesen antiapoptotischen Proteinen, die bei Überexpression zu Tumorformation, Tumorprogress und Resistenz gegenüber Chemotherapeutika führen, sind Inhibitor of Apoptosis Proteine (IAP) [11,12].

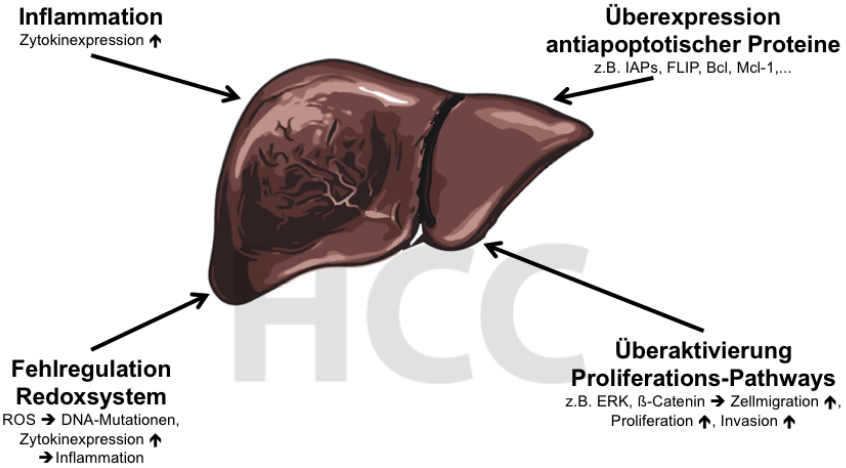

Abb. 2: Wichtige Einflussfaktoren in der Tumorgenese des HCC.

In der translationalen onkologischen Forschung spielen diese Erkenntnisse über die fehlregulierten Proteine und deregulierten Signalwege bei der Entwicklung neuer Therapiestrategien für das HCC eine entscheidende Rolle. Des Weiteren können Kenntnisse aus der Grundlagenforschung zu molekularen Markern führen, die eine Risikostratifizierung hinsichtlich Prognose, Therapieansprechen und Tumorrezidivraten ermöglichen. Meine hier vorgestellten Arbeiten sollen einen wichtigen Beitrag sowohl zu neuen therapeutischen Ansätzen als auch zur Risikostratifizierung beim hepatozellulären Karzinom leisten.

1.2. Translationale Forschung in der Transplantationsmedizin

Das Gebiet der Transplantationsmedizin ist ein Beispiel für erfolgreiche translationale Forschung, lange bevor der Begriff überhaupt eingeführt wurde (Abb. 3). Die Erkenntnisse u. a. auf den Gebieten der Immunologie, Pharmakologie und Verbesserung der technisch-operativen Möglichkeiten führten 1954 zur ersten erfolgreichen Nierentransplantation zwischen eineiigen Zwillingen und 1967 zur ersten Herztransplantation durch den Chirurgen Christiaan Barnard in Südafrika. Der erste herztransplantierte Patient starb bereits nach 18 Tagen an einem pulmonalen Infekt aufgrund der hohen Immunsuppression. Entscheidend für den Erfolg der zweiten Herztransplantation in Südafrika war auch das sogenannte Münchner Serum. Hierbei handelte es sich um ein Anti-Lymphozytenserum, das durch Walter Brendel und Rudolf Pichlmayr in München entwickelt und nach Südafrika geflogen wurde. Der Einsatz des Anti-Lymphzytenserums ist vermutlich eines der besten Beispiele für erfolgreiche translationale Forschung, aus dem Labor an das Patientenbett – von der Grundlagenforschung in die klinische Praxis.

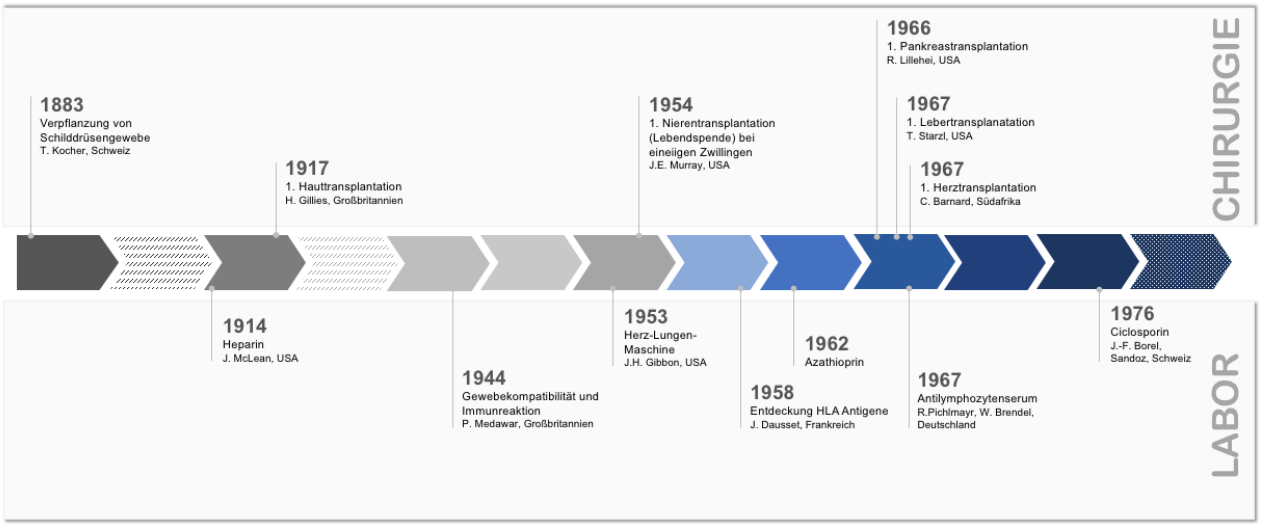

Abb. 3: Anfänge der Transplantationsmedizin.

Mittlerweile werden in der Eurotransplant Region (Deutschland, Österreich, Slowenien, Kroatien, Ungarn, Belgien, Niederlande und Luxemburg) jährlich um die 8000 Organe transplantiert [13]. Im Jahr 2017 wurden in Deutschland 3383 Organtransplantationen durchgeführt (Abb. 4), wobei gerade im Bereich der Nierentransplantation die Lebendspende eine zunehmende Rolle spielt [14].

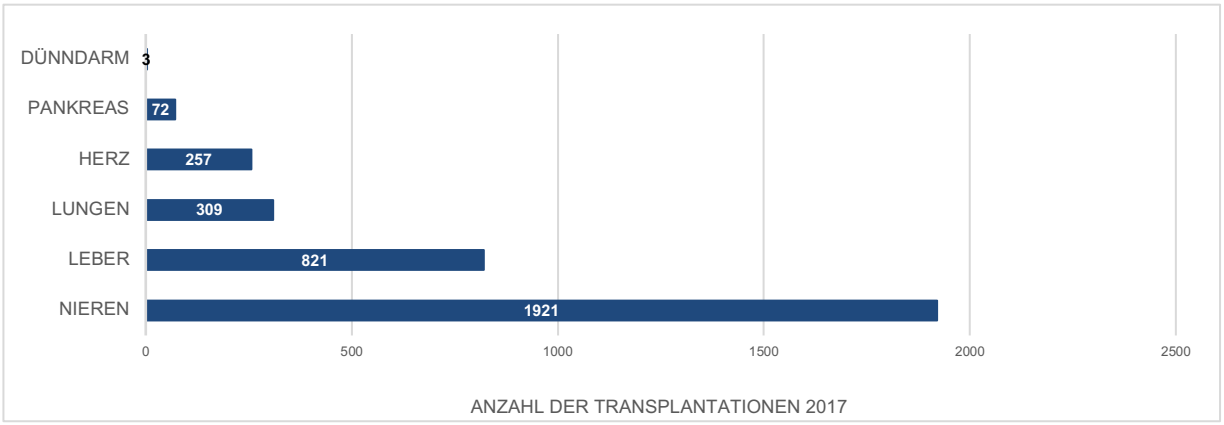

Abb. 4: Anzahl der Organtransplantationen in Deutschland im Jahr 2017 [14].

Trotz der Weiterentwicklung in der Immunsuppression, langer Erfahrung auf dem operativ-technischen Gebiet sowie Scores zur Auswahl von Empfängern und Spendern stellen der

Organmangel, Nebenwirkungen der Immunsuppression, Transplantatversagen sowie Langzeitkomplikationen nach Transplantation weiterhin eine große Herausforderung dar. Eine der Aufgaben der translationalen Forschung auf dem Gebiet der Transplantationsmedizin ist somit die Entwicklung von Markern bzw. Scores zur Selektion von geeigneten Empfängern und Spendern. Ziel ist es hierbei, durch optimale Spender- und Empfängerevaluation sowie -selektion dem sich aggravierenden Organmangel Rechnung zu tragen, aber auch den Empfängern ein möglichst gutes Langzeitüberleben zu ermöglichen.

1.2.1. Lebertransplantation bei Patienten mit hepatozellulärem Karzinom

Eine Indikation für die Lebertransplantation ist das HCC. Im Gegensatz zur Leberresektion mit hohen Tumorrezidivraten durch den Verbleib von zirrhotischen Lebergewebe ermöglicht die Lebertransplantation die Entfernung dieser Präkanzerose. Die Patientenselektion für die Transplantation erfolgt bislang mit Hilfe der radiologischen Mailand-Kriterien (1 Herd ≤ 5 cm oder bis zu 3 Herde, jeweils ≤ 3 cm, keine makrosvaskuläre Gefäßinvasion). Diese Kriterien werden lediglich auf der Basis der präoperativen Bildgebung erhoben und berücksichtigen nicht die Tumorbiologie des HCCs. Dies kann eine Erklärung für hohe Tumorrezidivraten von ca. 20-30% nach Transplantation sein [7,15]. Ein Problem stellt hierbei insbesondere die radiologische Unterschätzung der Anzahl der Tumorherde und der Tumorgröße in der zirrhotischen Leber dar [16].

Neben der Entwicklung neuer Therapieoptionen für das HCC ist die Prognoseabschätzung hinsichtlich Patientenüberleben und Tumorrezidivraten ein wichtiges Aufgabenfeld translationaler chirurgischer Forschung. Die Identifizierung prädiktiver Marker für das Tumorrezidiv nach Lebertransplantation kann eine bessere Empfängerevaluation vor Organtransplantationen ermöglichen.

1.2.2. Nierentransplantation – Empfängerevaluation

Auch bei der Nierentransplantation zur Behandlung der terminalen Niereninsuffizienz ist die Evaluation von Organempfängern von entscheidender Bedeutung. Die Identifizierung von geeigneten Empfängern ist, neben oftmals nicht beeinflussbaren

Faktoren der Spenderorgane, in hohem Maße relevant für das Patienten- und Organüberleben.

Im Jahr 2017 wurden in Deutschland ca. 8000 Personen auf der Warteliste von Eurotransplant für eine Nierentransplantation geführt [13]. Hingegen konnten nur 1364 Nieren von Verstorbenen und 557 Nieren von Lebendspendern transplantiert werden [14]. Die durchschnittliche Wartezeit für ein postmortales Spenderorgan beträgt derzeit 6-7 Jahre. Dieses Missverhältnis von benötigten zu verfügbaren Spendernieren verdeutlicht die Notwendigkeit einer optimalen Verteilung der Spenderorgane auf die Organempfänger. Die Einführung von sogenannten Seniorprogrammen, bei denen ältere Organe bevorzugt an ältere Empfänger vergeben werden, ist eine Methode, um der Organknappheit und der langen Wartezeit entgegenzuwirken. Auch die Verwendung von sogenannten marginalen Organen bzw. "expanded criteria donor", d. h. Totspenden von Spendern mit einem Alter von über 50-60 Jahren und mit ein oder zwei Risikofaktoren wie zum Beispiel arterieller Hypertonus, zerebrovaskuläre Todesursachen und einem Serumkreatinin über 1,5 mg/dl versucht ebenfalls der Diskrepanz zwischen Organangebot und vermehrter Nachfrage entgegenzuwirken. Wenn aufgrund eines Mangels an optimalen Spenderorganen die Einschlusskriterien für die Organspenden erweitert werden, macht dies eine sorgfältige Evaluation von Spendern und Empfängern notwendig. Auch wenn beispielsweise die Arbeit von Karatzas et al. [17] eine vergleichbare Organfunktion und Organüberleben von marginalen Organen im Vergleich zu nicht marginalen Organen zeigen konnte, sind viele Faktoren bzw. deren Zusammenspiel für das Outcome nach Nierentransplantation weiter unbekannt.

Wir wissen, dass das Langzeitoutcome von allogenen Nierentransplantaten von verschiedenen Spender- und Empfängerfaktoren abhängig ist, so insbesondere vom Alter, Body Mass Index (BMI), Gewicht, Vorerkrankungen und Geschlecht. Doch das Ausmaß jedes einzelnen Faktors sowie deren Kombination sind bei der Organauswahl auf die spätere Nierenfunktion schwer abschätzbar. In eigenen interdisziplinären Arbeiten habe ich mich mit der Identifizierung von geeigneten prognostischen Faktoren beschäftigt, die eine Abschätzung der späteren Nierenfunktion nach einer Transplantation ermöglichen, aber auch Risiken und Komplikationen aufzeigen können.

2. Ergebnisse und Diskussion der eigenen Arbeiten

2.1. Der Einsatz von Smac mimetics als neue Behandlungsstrategie im HCC

Aufgrund der ausgeprägten Resistenz des HCC gegenüber Chemotherapeutika wie Cisplatin, 5-Fluorouracil (5-FU) und Doxorubicin stellt die Entwicklung von neuen experimentellen Therapiestrategien zur Überwindung der Chemotherapieresistenz einen wichtigen translationalen Forschungsschwerpunkt dar [18,19].

Störungen der Apoptose können, wie eingangs erwähnt, durch die Überexpression von antiapoptotischen Proteinen, zum Beispiel Inhibitor of Apoptosis Proteinen (IAP), zu Tumorformation aber auch zu Chemotherapieresistenz führen [11,12]. IAPs sind häufig im HCC überexprimiert. Die Überexpression insbesondere von dem X-linked IAP, dem sogenannten XIAP, ist mit einem schlechteren Gesamt- und krankheitsfreien Überleben assoziiert [20]. Smac (second mitochondria-derived activator of caspase) mimetics, die IAP Proteine antagonisieren, verstärken in verschiedenen Tumoren (u. a. Pankreas- und Kolonkarzinom) die Wirkung von Chemotherapeutika oder die Todesrezeptor-induzierte Apoptose [21-25]. Bao et al. untersuchte die Expression von Smac im HCC-Gewebe und konnte eine signifikante Suppression der Smac Expression im Vergleich zu tumorfreiem Lebergewebe zeigen [26]. Smac mimetics binden an unterschiedliche IAPs und heben damit unter anderem die XIAP-vermittelte Hemmung von Caspase 3 auf. Damit stellen die sogenannten Smac mimetics vielversprechende Kandidaten für die Entwicklung neuer Therapiestrategien in der Behandlung des HCCs dar. Jedoch ist bisher wenig über die Rolle der Smac mimetics bei der Regulierung der Apoptosesensitivität sowie im Speziellen bei der Chemotherapie-induzierten Apoptose bekannt.

In Kooperation mit dem Institut für experimentelle Tumorforschung in der Pädiatrie unter der Leitung von Frau Prof. S. Fulda (Goethe-Universität Frankfurt am Main) untersuchten wir zunächst die Wirkung des Smac mimetic BV6 auf drei verschiedene humane HCC-Zelllinien (Huh7, HepG2 und Hep3B). Lediglich in einer HCC-Zelllinie, HepG2, führten erst hohe Dosierungen von BV6 zur Zelltodinduktion. BV6 ist in allen Zelllinien biologisch aktiv und führt zu einer Degradation von den IAP Proteinen cIAP1 und XIAP. Trotz der Degradation verschiedener IAPs durch BV6 konnte jedoch keine Steigerung der Chemotherapeutikaeffizienz von Sorafenib, Doxorubicin, 5-Fluoruracil, Rapamycin oder Interferon erreicht werden.

Interessanterweise entdeckten wir, dass allerdings die Kombination von BV6 mit einem Inhibitor des AKT/mTOR- und MAPK/ERK-Signalweges, der Oleanolsäure (OA), zu einer

signifikanten Zelltodinduktion führte [27]. OA wurde bereits zur Verbesserung der Leberfunktion bei viralen Hepatitiden klinisch eingesetzt [28]. Die Kombination von dem Smac mimetic BV6 mit OA führte zu einer synergistischen Zelltodinduktion in humanen HCC-Zelllinien. Die Kombinationstherapie von BV6 mit OA inhibierte nicht nur das klonogene Langzeitüberleben (Abb. 5A), sondern in einem ersten präklinischen *in vivo* Versuch (Chorionallantiosmembran-Modell) auch das Tumorwachstum (Abb. 5B-C).

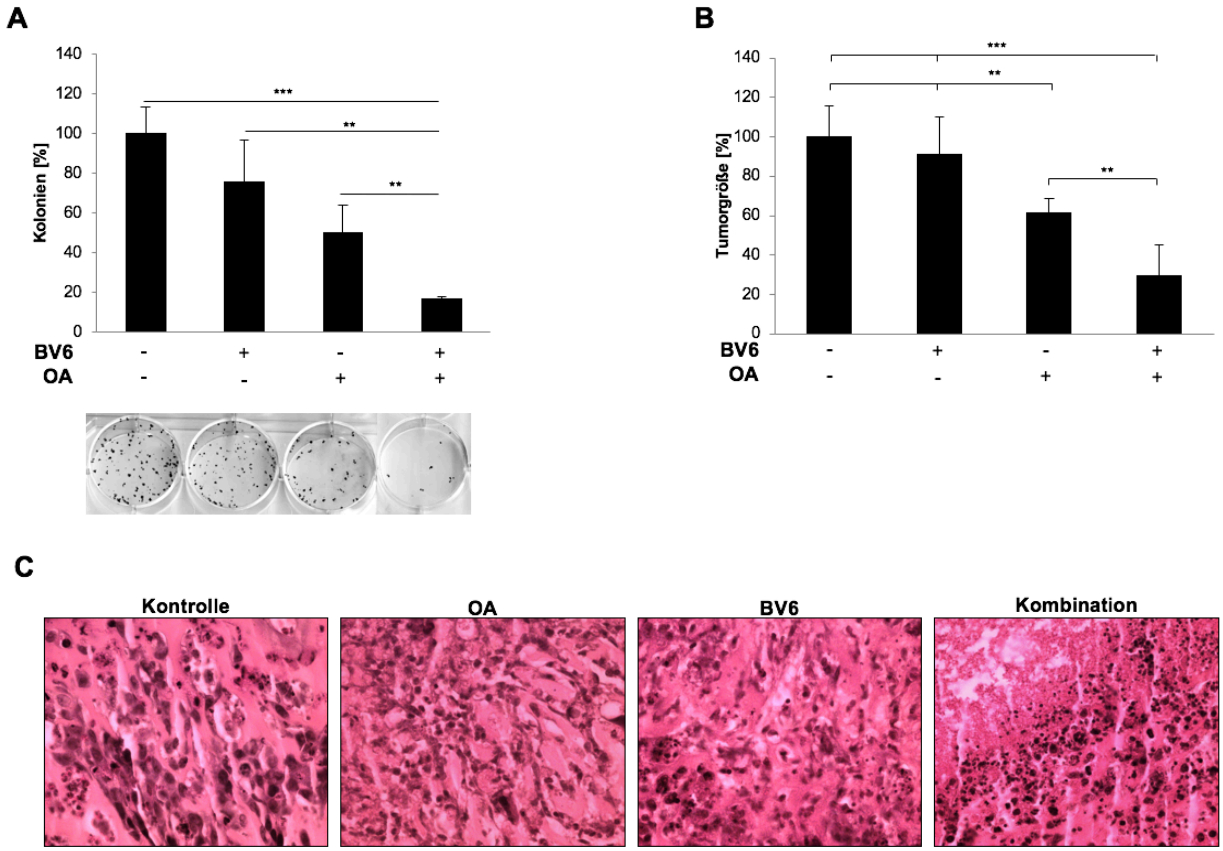

Abb. 5: Smac mimetic BV6 und Oleanolsäure (OA) führten zu einer synergistischen Inhibierung des klonogenen Langzeitüberlebens in der humanen HCC-Zelllinie HepG2 (A). Die Kombination aus BV6 und OA führte *in vivo* zu einer signifikanten Hemmung des Tumorwachstums (B) und der Zunahme von apoptotischen, kondensierten Zellen (C). ** $p < 0,01$, *** $p < 0,001$, n=3.

Darüber hinaus gelang es uns zu zeigen, dass sowohl in primären Hepatozyten als auch in der humanen, differenzierten Hepatozytenzelllinie HepaRG die Kombinationstherapie von BV6 und OA keinen negativen Einfluss auf die Zellviabilität hatte. Diese Daten legen dar, dass mit der Kombinationstherapie von BV6 und OA ein neues „therapeutisches Fenster“ zur selektiven Zelltodsensitivierung in malignen Zellen identifiziert werden konnte. In einem weiteren Schritt führten wir Untersuchungen zum Wirkmechanismus der Kombinationstherapie durch und zeigten, dass die Behandlung unter anderem zur DNA-

Fragmentierung und Caspasen-3/7-Aktivierung im Sinne eines apoptotischen Zelltodes führte. Neben der Caspasenaktivierung durch die Kombinationsbehandlung gelang es uns nachzuweisen, dass es zudem zur Aktivierung von reaktiven Sauerstoffspezies (ROS) kommt. Die Bedeutung von ROS bei der Zelltodinduktion durch BV6 und OA wurde durch die Verwendung von unterschiedlichen ROS-Scavenger, sogenannten Radikalfängern, gezeigt. Diese führten nicht nur zu einer verminderten ROS-Produktion, sondern verhinderten auch vollständig die Zelltodinduktion in HCC-Zellen durch BV6 und OA.
Es gelang somit nicht nur, einen bislang unbekannten Ansatz zur Überwindung der Chemotherapieresistenz des HCC zu identifizieren, sondern zudem ein Verständnis über die zugrundeliegenden Mechanismen zu gewinnen [27].

2.2. Die Induktion von oxidativem Stress im HCC führt zur Chemotherapie-sensitivierung

Neben der Entdeckung des oben genannten neuen Therapieansatzes untersuchten wir in einem hierauf aufbauenden Projekt, ob die durch uns identifizierte Induktion von oxidativen Stress durch Oleanolsäure (OA) auch zur Chemotherapiesensitivierung des HCC gegenüber dem Multikinase-Inhibitor Sorafenib führt und damit diesen routinemäßig genutzten, aber wenig effektiven (s. o.) Therapieansatz des HCC verbessern könnte. Sowohl OA als auch Sorafenib werden bereits klinisch eingesetzt. Die Erkenntnisse zur Zelltodinduktion durch oxidativen Stress könnten somit zu einer Translation dieser experimentellen Daten in erste klinische Versuche führen.

Wir konnten nachweisen, dass die Kombination der OA mit Sorafenib in subtoxischen Konzentrationen einen hoch synergistischen Effekt auf die Zelltodinduktion von verschiedenen humanen HCC-Tumorzelllinien bewirkt [29]. Auch diese Kombinationstherapie unterdrückte klonogenes Langzeitüberleben der humanen HCC-Zellen und führte zur DNA-Fragmentierung sowie Caspase-3/7 Aktivierung. Die Abhängigkeit der Zelltodinduktion von Caspasen bewies wiederum die Aufhebung der Wirkung der Kombinationstherapie von OA und Sorafenib durch den Einsatz des pan-Caspasen-Inhibitors zVAD.fmk.

Die Kombinationstherapie von Sorafenib und OA führte zu einer Akkumulation von intrazellulären und vor allem mitochondrialen ROS. Dass dieses für die Zelltodinduktion ausschlaggebend ist, wurde durch die vollständige Rettung der Zellen vor dem Sorafenib- und OA-induzierten Tod durch den Einsatz von ROS-Scavengern wie alpha-Tocopherol und MnTBAP nachgewiesen (Abb. 6).

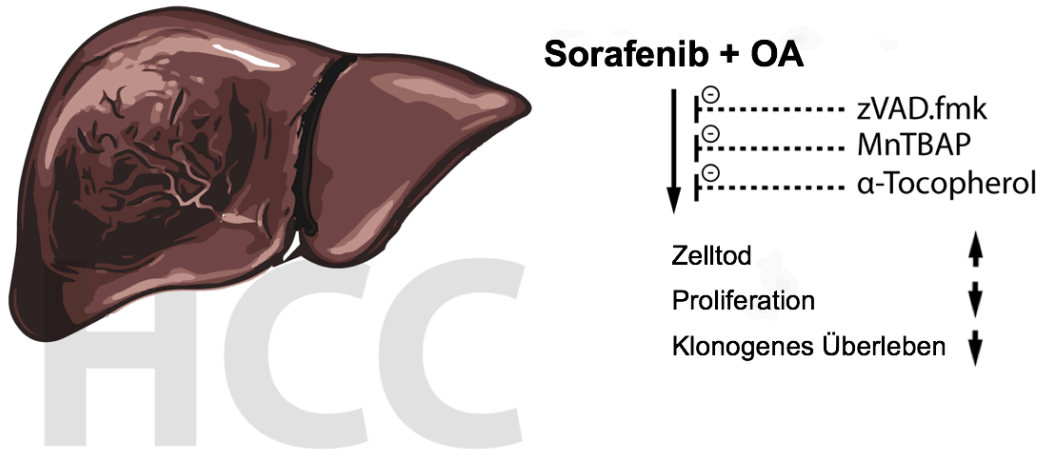

Abb. 6: Mechanismus der Zelltodinduktion durch die Kombination von Sorafenib mit OA in humanen HCC-Zelllinien.

Zudem führte die Kombination von OA mit Sorafenib zu einer deutlichen Reduktion der Konzentration von Sorafenib im Vergleich zu einer Monotherapie und kann damit der Hepatotoxizität von Sorafenib entgegenwirken.
Vor Translation dieser Daten vom Labor an das Patientenbett sind jedoch weitere Untersuchungen zum Wirkmechanismus erforderlich.

2.3. Die Kombinationsbehandlung aus Sorafenib und Oleanolsäure induziert einen ROS-abhängigen und mitochondrial-vermittelten Zelltod im HCC

Aufbauend auf den unter 2.1. und 2.2. dargestellten Ergebnissen beschäftigten wir uns in einer weiteren Untersuchung mit der molekularen Regulation der Sorafenib/OA-vermittelten Zelltodinduktion im HCC.

Hierbei scheint insbesondere die ROS-Produktion eine entscheidende Rolle zu spielen. Verschiedene Arbeitsgruppen konnten zeigen, dass die Fehlregulation der ROS-Homöostase, induziert durch die chronische Infektion der Leber, ein Schlüsselpunkt in der Hepatokarzinogenese ist [30]. Die Mitochondrien sind die Hauptquelle für das intrazelluläre ROS und die Akkumulation von ROS in Tumorzellen kann den mitochondrial-vermittelten apoptotischen Zelltod wieder reaktivieren [31,32]. Dieser wird u. a. durch verschiedene Proteine der B-cell lymphoma 2 (Bcl-2)-Familie reguliert [33]. Insbesondere das myeloid cell leukaemia sequence 1-Protein (Mcl-1) ist oft in soliden Tumoren überexprimiert [33,34]. Wir konnten darlegen, dass Sorafenib und OA den mitochondrialen Pathway zur Zelltodinduktion durch die Herabregulierung von Mcl-1 aktiviert [35]. Die bereits erwähnten ROS-Scavenger wirkten diesem Mechanismus entgegen, so dass wir damit erneut die ROS-Abhängigkeit der Zelltodinduktion durch Sorafenib und OA in den HCC-Zellen nachweisen konnten. Im nächsten Schritt gelang es uns die Verringerung des mitochondrialen Membranpotentials durch diese Kombinationstherapie nachzuweisen. Das Membranpotential in Mitochondrien wird durch zwei Multidomäneproteine der Bcl-2-Familie, Bax und Bak, reguliert [34,36]. Auch in den HCC-Zellen beobachteten wir durch die Kombinationstherapie eine Bak-Aktivierung mit Verlust des mitochondrialen Membranpotentials. Diese Aktivierung wurde ebenfalls durch den Einsatz von ROS-Scavengern aufgehoben. Die funktionelle Relevanz der Bak-Aktivierung wiesen wir durch Gen-Stilllegung mit Hilfe von siRNA-Oligonukleotiden nach.

Letztendlich beobachteten wir typische Merkmale des apoptotischen Zelltodes: PARP-Aktivierung, DNA-Fragmentierung und Caspasenaktivierung [35]. All diese Mechanismen waren durch die Hemmung der ROS-Freisetzung wieder aufzuheben. Der von uns aufgedeckte Wirkmechanismus der Kombinationstherapie mit Sorafenib und OA in HCC Zellen ist in Abbildung 7 dargestellt.

Unsere Arbeit zeigt damit nicht nur eine Option der Chemotherapiesensitivierung gegenüber Sorafenib durch oxidativen Stress im HCC auf, sondern betont erneut die Rolle des Redoxsystems und damit von ROS für die Überwindung der Chemotherapieresistenz im HCC [35]. Dies korreliert mit den Ergebnissen anderer Arbeitsgruppen: Diese zeigten,

dass eine Hepatitis B- oder C-Infektion zur Dysregulation des Redoxsystems führt, was als ein wichtiger Faktor in der Hepatokarzinogenese angesehen wird [30,37]. Darüber hinaus korrelierten Marker für oxidativen Stress mit dem Ausmaß der Leberschädigung durch eine virale Hepatitis und wurden als Verlaufsparameter für das Therapieansprechen auf eine antivirale Therapie untersucht [37].

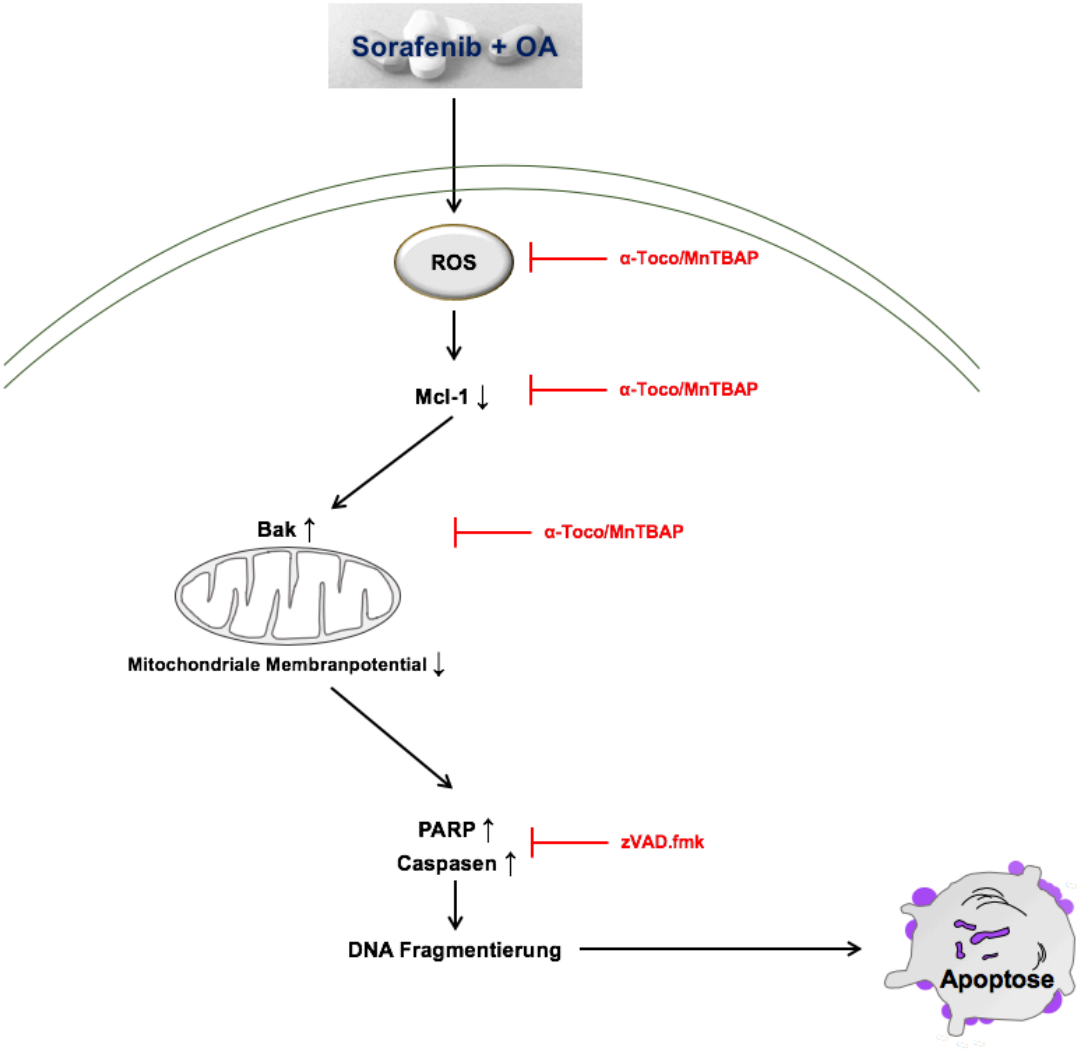

Abb. 7: Die Kombinationsbehandlung von Sorafenib und OA induziert im HCC einen ROS-abhängigen und mitochondrial-vermittelten Zelltod (Rot: spezifische Inhibitoren; siehe Text).

2.4. Identifizierung von prädiktiven Markern für das HCC-Tumorrezidiv nach Lebertransplantation

Neben der Chemotherapieresistenz stellt das HCC-Tumorrezidiv eine weitere Herausforderung in der translationalen Forschung dar. Die radiologisch erfassten Mailand-Kriterien korrelieren zwar signifikant mit dem HCC-rezidivfreien Überleben (Abb. 8), unterschätzen jedoch in ca. 25% der Fälle die Tumorgröße und Tumorausdehnung [16].

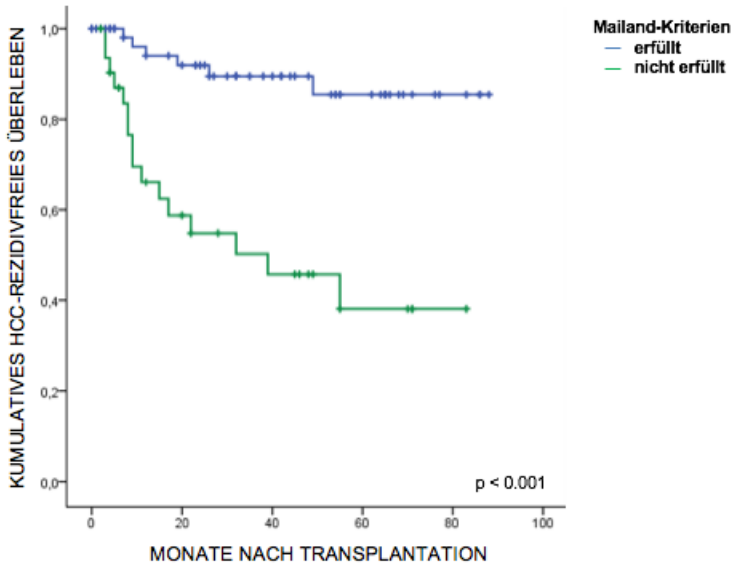

Abb. 8: Der Einfluss der Mailand-Kriterien auf das tumorfreie Überleben nach Lebertransplantation.

Vor diesem Hintergrund ist die Entwicklung eines Scores, der klinisch-radiologische Kriterien (z. B. Tumormarker, Mailand-Kriterien) mit Biomarkern des HCCs kombiniert, ein wichtiges Instrument zur Patientenselektion vor der Lebertransplantation. Als Biomarker können z. B. microRNAs (miRNAs) dienen, die als kleine, nicht-kodierende RNAs eine bedeutende Rolle in der Regulation der Genexpression spielen [38].

Zur Etablierung eines solchen Scores aus Biomarkern und klinisch-radiologischen Parametern analysierten wir 92 Patienten (Studiendesign Abb. 9), die in der Klinik für Allgemein- und Viszeralchirurgie der Goethe-Universität Frankfurt am Main aufgrund eines HCCs lebertransplantiert wurden.

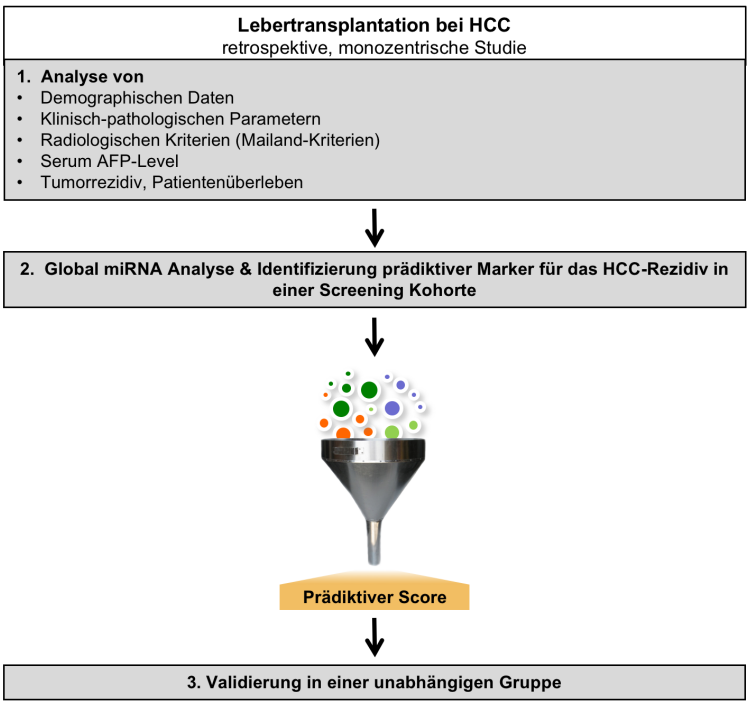

Abb. 9: Studiendesign zur Etablierung eines prädiktiven Scores aus klinisch-radiologischen Parametern und Biomarkern.

Erwartungsgemäß waren in dieser Kohorte größere Tumore, eine höhere Anzahl von Tumorknoten, hohe Tumormarker (alpha-Fetoprotein = AFP) sowie eine mikrovaskuläre Gefäßinvasion und schlecht differenzierte Tumore mit einem Tumorrezidiv nach Lebertransplantation assoziiert.

Es erfolgte so dann die Unterteilung der Patienten in eine Screening- und eine Validierungskohorte, die bezüglich AFP-Level, Tumorgröße, Anzahl der Tumorherde und Tumorgrading übereinstimmten. Screening- und Validierungskohorte wiesen keine signifikanten Unterscheide der übrigen Parameter auf.

Zur Identifizierung unterschiedlicher miRNA-Expressionslevel in Patienten mit und ohne Tumorrezidiv führten wir an dem Tumorgewebe der explantierten Lebern in der Screening-Gruppe globale miRNA-Analysen durch. Diese erfolgten in Kooperationen mit der Medizinischen Klinik I (Gastroenterologie und Hepatologie) und dem Dr. Senckenbergisches Institut für Pathologie der Goethe-Universität Frankfurt am Main. Verglichen mit der Patientengruppe ohne HCC-Rezidiv waren in der Tumorrezidivgruppe die Expressionslevel der miR-214 und miR-455 signifikant höher und die Expressionslevel der miR-3187 signifikant niedriger als in der Gruppe ohne Tumorrezidiv. Zudem korrelierten die Expressionslevel der miR-214, miR-455 und miR-3187 mit dem HCC-rezidivfreien Überleben. Eine multivariate Cox-Regressionsanalyse aus klinisch-

radiologischen Parametern und den miRNAs identifizierte die Mailand-Kriterien und die miR-214- sowie die miR-3187-Expressionslevel als unabhängige prognostische Marker für das tumorfreie Überleben. Unter Verwendung der Regressionskoeffizienten der Cox-Regressionsanalyse etablierten wir auf diese Weise einen prädiktiven Score für das tumorrezidivfreie Überleben nach Lebertransplantation.

Durch Receiver-Operating-Characteristic (ROC)-Analyse konnten wir die Patienten in eine Hochrisiko- und eine Niedrigrisikogruppe mit einem Score ≥ 36 bzw. < 36 hinsichtlich des Auftretens eines HCC-Rezidivs nach Lebertransplantation einteilen.

Die Validierung des prädiktiven Scores für das HCC-Rezidiv erfolgte in einer zweiten, unabhängigen Kohorte. Die Validierungskohorte bestätigte die oben genannten Ergebnisse. In der ROC-Analyse zeigte unser prädiktiver Score eine signifikant höhere Spezifität und Sensitivität für das HCC-Rezidiv nach Lebertransplantation als die bisher verwendeten Mailand-Kriterien (Abb. 10).

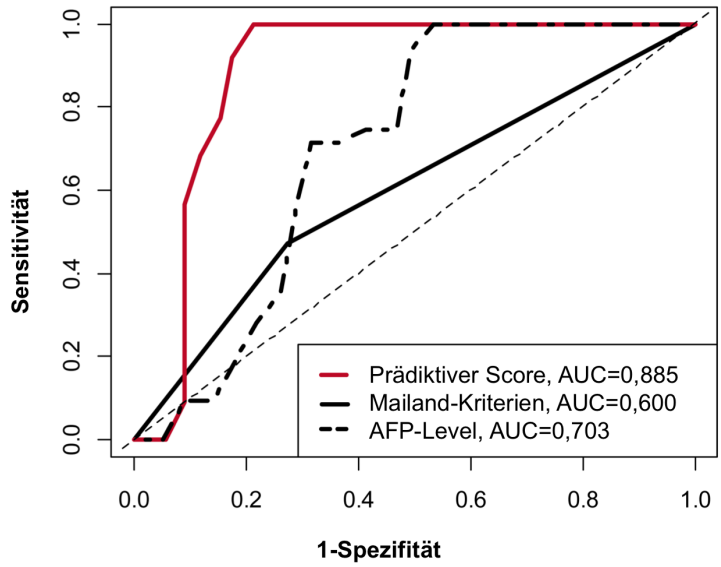

Abb. 10: Der neue prädiktive Score zeigte eine signifikant höhere Sensitivität und Spezifität als die bisher verwendeten Mailand-Kriterien.

Diese Studie kombiniert erstmalig biologische Parameter (miRNA-Expressionslevel) mit radiologisch-klinischen Parametern (Mailand-Kriterien) zur Prognoseabschätzung von Patienten nach Lebertransplantation, die aufgrund eines HCCs transplantiert wurden [7]. Der hierdurch erarbeitete Score könnte potentiell ein geeignetes Instrument für eine verbesserte Patientenselektion für die Lebertransplantation in Zeiten der Organknappheit sein. Auch diese Arbeit stellt ein typisches Beispiel für translationale Forschung dar: Die konkrete Fragestellung der verbesserten Selektion von Patienten mit einem HCC für die

Lebertransplantation und die Suche nach prädiktiven Markern für das Tumorrezidiv wird mit Hilfe der Grundlagenforschung (Expressionsanalysen) beantwortet.

2.5. Spenderselektion vor Organtransplantation – der Einfluss des BMI auf das Outcome nach Nierentransplantation

Die Empfängerauswahl vor Aufnahme auf die Warteliste für eine Organtransplantation erfordert besondere Sorgfalt, zum einen zur Vermeidung einer erhöhten Morbidität und Letalität des Empfängers, zum anderen vor dem Hintergrund des Spenderorganmangels. Wie eingangs aufgeführt, wissen wir von einigen Faktoren, die das Outcome, namentlich das Empfänger- und Organüberleben bestimmen. Jedoch sind diese Informationen bisher unzureichend und ihr Zusammenspiel weitgehend unbekannt.

Das Outcome von übergewichtigen Patienten nach Nierentransplantation lässt sich bislang nur eingeschränkt beurteilen. Eine Reihe von Arbeiten zeigt eine erhöhte Morbidität und Mortalität von übergewichtigen Empfängern einer Nierentransplantation. Dennoch gibt es bisher keine klaren Empfehlungen, in welchen Grenzen sich der BMI eines Empfängers eines Nierentransplantates bewegen sollte, um ein optimales Ergebnis zu erzielen.

So verglichen Molnar et al. Patienten mit normalem bis deutlich erhöhtem BMI vor Transplantation und zeigten, dass ein BMI oberhalb von 25 kg/m^2 mit einem erhöhten Risiko für eine verzögerte Transplantatfunktionsaufnahme assoziiert ist [39]. Eine andere Arbeitsgruppe fand hingegen kein unterdurchschnittliches Outcome, jedoch eine höhere Rate chirurgischer Komplikationen in dieser Patientengruppe [40]. Auf der anderen Seite konnte jedoch gezeigt werden, dass adipöse Patienten mit einem BMI ≥ 30 kg/m^2 von einer Nierentransplantation grundsätzlich profitieren und im Vergleich zum Verbleib an der Dialyse länger leben [41,42]. Insgesamt ist die Datenlage nicht eindeutig und zum Teil widersprüchlich [43-45].

Aktuell wählen einzelne Transplantationszentren sehr unterschiedliche Grenzen bezüglich des maximalen BMI der potentiellen Organempfänger. In den Universitätskliniken der Goethe-Universität Frankfurt am Main als auch in der Justus-Liebig-Universität Gießen existiert bisher kein oberer BMI-Grenzwert, sodass in den zurückliegenden Jahren auch stark übergewichtige Patienten nierentransplantiert wurden. Somit steht hier eine Patientenkohorte zur Verfügung, an der die Bedeutung des BMI für die Nierenfunktion nach Transplantation sehr gut untersucht werden kann. BMI-abhängige Outcomeunterschiede in diesem Kollektiv könnten Einfluss auf die Indikationsstellung zur Nierentransplantation, aber auch auf zusätzliche Therapieoptionen wie Diäten oder bariatrische Chirurgie haben.

In unserer retrospektiven, monozentrischen Studie untersuchten wir in Kooperation mit den Kollegen der Medizinischen Klinik III (Nephrologie, Goethe-Universität Frankfurt am Main) den Einfluss des BMI auf das Outcome nach Nierentransplantation. Eingeschlossen wurden 384 Patienten, die zwischen Januar 2007 und Dezember 2012 transplantiert wurden. 17% der Transplantationen erfolgten im Rahmen des Eurotransplant Senior-Programmes. Der Anteil an Organtransplantationen nach Nierenlebendspende machte 22,4% aus. Der mediane BMI der Nierentransplantationsempfänger betrug 25,9 kg/m^2. 13,5% der Empfänger hatten einen BMI von 30-34,9 kg/m^2 und 3,9% einen BMI >35 kg/m^2. Ein BMI >30 kg/m^2 war assoziiert mit einem signifikant erhöhten Anteil an bereits primär nicht funktionierenden Transplantaten (p = 0,047), einer verzögerten Funktionsaufnahme der Spenderniere (p = 0,008) und einer höheren Rate an Transplantatversagen (p = 0,015, Abb. 11A). Die glomeruläre Filtrationsrate 12 Monate nach Transplantation war signifikant niedriger bei Empfängern mit einem BMI >30 kg/m^2 (Abb. 11B).

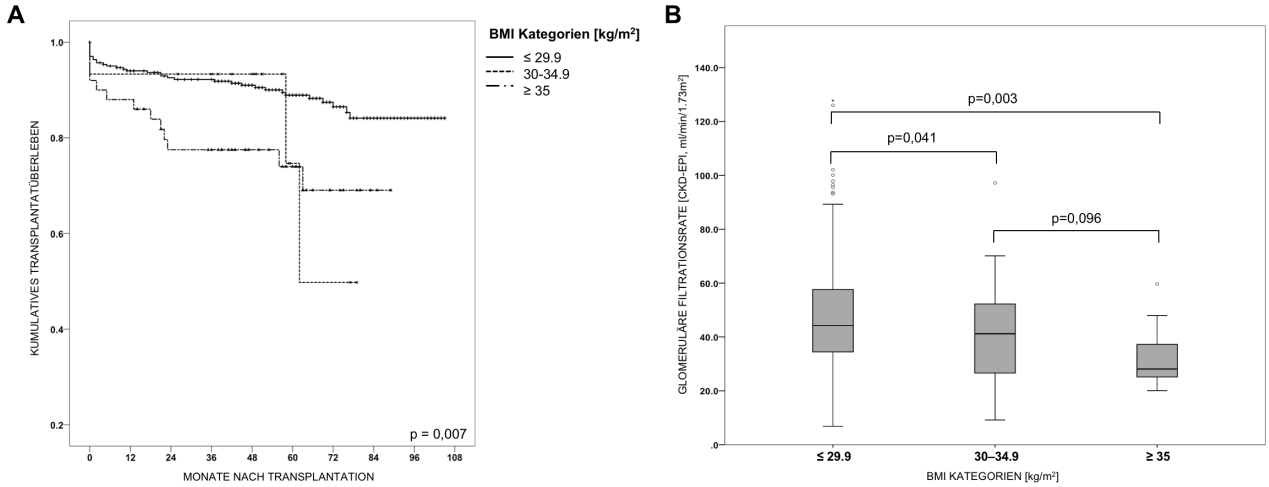

Abb. 11: Transplantatüberleben (A) und die glomeruläre Filtrationsrate 12 Monate nach Nierentransplantation (B) in Abhängigkeit vom BMI.

Multivariate Analysen identifizierten den Empfänger-BMI als einen unabhängigen Risikofaktor für die verzögerte Funktionsaufnahme des Spenderorgans und das Transplantatüberleben. Zudem haben Patienten mit einem BMI >30 kg/m^2 ein fast vierfach erhöhtes Risiko für Wundinfektionen im Vergleich zu Empfängern mit einem BMI <30 kg/m^2. Diese Daten zeigen die Notwendigkeit einer sorgfältigen Empfängerauswahl und die Etablierung von Programmen zur Gewichtsreduktion vor Organtransplantation. Die dargestellte Arbeit demonstriert zudem einen weiteren Aspekt chirurgisch-translationaler

Forschung: eine differenzierte statistische Forschung in interdisziplinären Arbeitsgruppen, was wiederum zu Ergebnissen von klinisch-praktischer Relevanz führte.

3. Zusammenfassung

In dieser kumulativen Habilitationsschrift konnten verschiedene translationale chirurgische Forschungsprojekte aus den Bereichen der onkologischen Grundlagenforschung und der Transplantationsmedizin zusammengefasst werden. Die Arbeiten in Kooperation mit dem Institut für experimentelle Tumorforschung in der Pädiatrie unter Leitung von Frau Prof. S. Fulda (Goethe-Universität Frankfurt am Main) führten zu einem verbesserten Verständnis der Chemotherapieresistenz des HCC und zeigten zugleich innovative experimentelle Ansätze zur Überwindung der Chemotherapieresistenz sowie für die Identifizierung von neuen Behandlungsstrategien des HCC auf [27,29]. Wir konnten darlegen, dass insbesondere die Modulation des Redoxsystems ein vielversprechender Ansatz in der Entwicklung von zukünftigen Therapien des HCC sein könnte [29].

Bis zur Entwicklung von neuen Therapien im HCC stellt die Lebertransplantation eine der wichtigsten kurativen Therapieoptionen dar, deren Erfolg jedoch durch hohe Rezidivraten geschmälert wird. In Zusammenarbeit mit dem Dr. Senckenbergischen Institut für Pathologie und der Medizinischen Klinik I (Gastroenterologie und Hepatologie, Goethe-Universität Frankfurt am Main) etablierten wir einen prädiktiven Score zur Patientenselektion vor Lebertransplantation. Dieser ist klinisch-praktisch anwendbar, da er radiologische Kriterien (Mailand-Kriterien), die im Rahmen eines jeden Tumorstagings vor Identifizierung geeigneter Transplantatempfänger erhoben werden, und Expressionsanalysen der miRNA-214 und miRNA-3187, welche aus Tumorbiopsien durchgeführt werden können, verbindet. Dieser Score korreliert signifikant mit dem HCC-Rezidiv nach Lebertransplantation und ist den bisher verwendeten Mailand-Kriterien signifikant überlegen [7].

Zugleich verdeutlicht die Etablierung dieses Scores die Möglichkeiten translationaler Forschung, da die Identifizierung von fehlregulierten miRNAs in Kombination mit klinischen Parametern neue diagnostische und prognostische Optionen eröffnet und ein solcher Score zeitnah in die klinische Praxis transferiert werden könnte. Auch ermöglicht die Korrelation von miRNA-Expressionsanalysen mit klinischen Parametern die Erforschung von neuen experimentellen Ansätzen, in denen klinisch relevante miRNAs mögliche Zielstrukturen innovativer Tumortherapien darstellen könnten.

Maligne Tumore sind bei Nierentransplantationen keine Transplantationsindikation. Hier sind unter anderem empfängerspezifische Faktoren für das Outcome nach Transplantation von Bedeutung. Wir konnten in Zusammenarbeit mit der Medizinischen Klinik III

(Nephrologie, Goethe-Universität Frankfurt am Main) zeigen, dass der BMI einen entscheidenden Einfluss auf das Outcome hat. Ein BMI >30 kg/m² geht mit einer signifikant höheren Rate an primären Transplantatversagen, verzögerter Funktionsaufnahme des Spenderorgans und einer erhöhten Rate an Transplantatversagen einher. Diese Erkenntnisse verdeutlichen die Wichtigkeit des Parameters BMI und die Notwendigkeit einer entsprechenden Empfängerevaluation sowie Maßnahmen zur Gewichtsreduktion vor einer Transplantation.

Die hier dargestellten Arbeiten belegen, wie translationale Forschung, zum einen durch die Verbindung von experimenteller Arbeit mit der Klinik, zum anderen durch die enge interdisziplinäre Zusammenarbeit, Möglichkeiten zur Optimierung der Patienten-versorgung in der Transplantationsmedizin und Onkologie bietet. Es ist zu erwarten, dass dies auch in Zukunft, neben chirurgisch-technischen Faktoren und der perioperativen Betreuung, ein wichtiger Faktor für sehr gute medizinische Langzeitergebnisse sein wird.

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5. Anhang mit Originalarbeiten

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Original Articles

Smac mimetic and oleanolic acid synergize to induce cell death in human hepatocellular carcinoma cells

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ABSTRACT

Chemotherapy resistance of hepatocellular carcinoma (HCC) is still a major unsolved problem highlighting the need to develop novel therapeutic strategies. Here, we identify a novel synergistic induction of cell death by the combination of the Smac mimetic BV6, which antagonizes Inhibitor of apoptosis (IAP) proteins, and the triterpenoid oleanolic acid (OA) in human HCC cells. Importantly, BV6 and OA also cooperate to suppress long-term clonogenic survival as well as tumor growth in a preclinical *in vivo* model of HCC underscoring the clinical relevance of our findings. In contrast, BV6/OA cotreatment does not exert cytotoxic effects against normal primary hepatocytes, pointing to some tumor selectivity. Mechanistic studies show that BV6/OA cotreatment leads to DNA fragmentation and caspase-3 cleavage, while supply of the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) revealed a cell type-dependent requirement of caspases for BV6/OA-induced cell death. The receptor interacting protein (RIP)1 kinase Inhibitor Necrostatin-1 (Nec-1) or genetic knockdown of RIP1 fails to rescue BV6/OA-mediated cell death, indicating that BV6/OA cotreatment does not primarily engage necroptotic cell death. Notably, the addition of several reactive oxygen species (ROS) scavengers significantly decreases BV6/OA-triggered cell death, indicating that ROS production contributes to BV6/OA-induced cell death. In conclusion, cotreatment of Smac mimetic and OA represents a novel approach for the induction of cell death in HCC and implicates further studies.

Introduction

Hepatocellular carcinoma (HCC) is the most common liver tumor and one of the leading worldwide causes of death [1]. Up to 80% of HCC cases are caused by chronic infection with the hepatitis B virus (HBV), hepatitis C virus (HCV) [2], alcoholic liver diseases and non-alcoholic fatty liver diseases (NASH) [3]. Only 30% of patients are eligible for curative treatment, including liver resection, transplantation and percutaneous ablation [4,5]. However, tumor recurrence up to 50% after three years could be observed [6,7]. The poor prognosis caused by the limited therapeutic options and resistance to chemotherapies highlights the high medical need to investigate novel treatment strategies. One promising approach is to identify drugs which target apoptotic signaling and reactivate cell death programs.

Inhibition of apoptosis is important for the survival of cancer cells [8]. Tumor resistance to apoptosis in HCC could be caused by IAP proteins e.g. x-linked Inhibitor of Apoptosis (XIAP), cellular Inhibitor of Apoptosis (cIAP)1 and cIAP2, a family of key proteins involved in the regulation of cell death [9,10]. IAP proteins are overexpressed in HCC [9,11,12]; especially XIAP is expressed at high levels in nearly 90% of clinical tumor samples and is associated with poor prognosis and tumor recurrence [13]. Inhibition of IAP proteins, for example with second mitochondria-derived activator of caspase (Smac) mimetics, represents a promising approach to re-sensitize HCC cells to cell death induction. While the efficacy of Smac mimetics as single agents is limited in most cancers, they have proven their effectiveness in

Abbreviations: AIF, apoptosis-inducing factor; BSO, Buthionine sulfoximine; CAM, chorioallantoic membrane; cIAP, cellular inhibitor of apoptosis; DR5, death receptor-5; ENDOG, endonuclease G; ER, endoplasmatic reticulum; FCS, fetal calf serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IAP, inhibitor of apoptosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-cysteine; NASH, non-alcoholic fatty liver diseases; Nec-1, necrostatin-1; OA, oleanolic acid; RFU, relative fluorescence units; RIP1, receptor interacting protein 1; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspases; SOD, superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, x-linked inhibitor of apoptosis; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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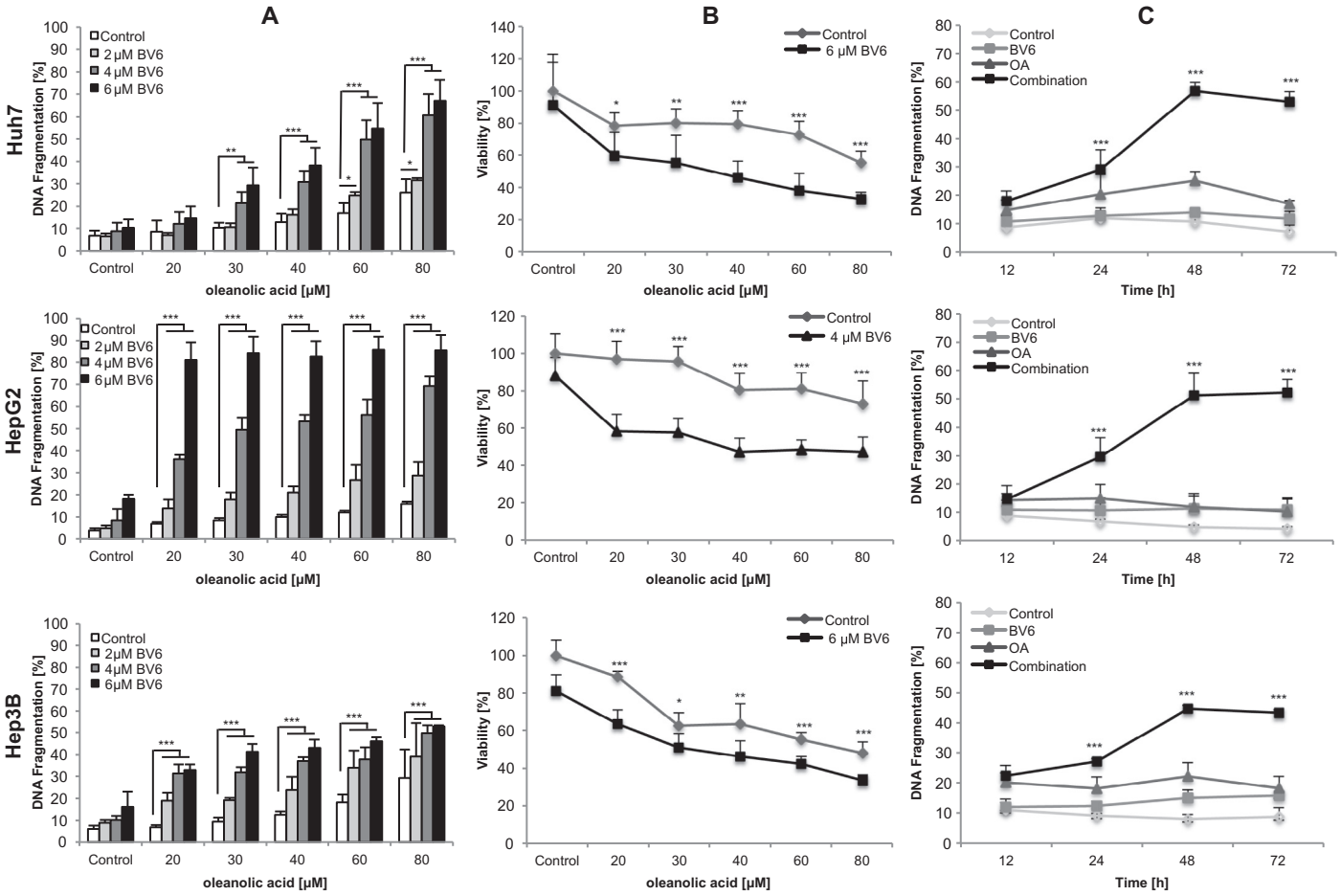

Fig. 1. BV6 and OA synergistically induce cell death in HCC cells. HCC cells (Huh7, HepG2, Hep3B) were treated for 72 hours with indicated concentrations of BV6 and OA (A, B) or for indicated times with BV6 (Huh7, Hep3B: 6 µM BV6, HepG2: 4 µM BV6) and/or 60 µM OA (C). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (A, C), cell viability by MTT assay (B). Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

combination with different cytotoxic stimuli, e.g. chemotherapeutics, in a number of preclinical studies [14–16]. In HCC, the anticancer activity of Smac mimetics could be improved by combined treatment with a Bcl-2 inhibitor [9]. Furthermore Smac mimetics could potentiate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)- and Doxorubicin-mediated apoptosis in HCC cell lines [12]. Currently, Smac mimetics are tested in clinical trials as single agents and in combination therapies in different cancers [8].

Another approach to induce cell death in HCC cells is the use of oleanolic acid (OA), a natural triterpenoid [17,18]. In Chinese medicine, OA has been used for many decades in the treatment of liver disorders, such as viral hepatitis [17]. Recently, an antitumor effect of OA *in vitro* and *in vivo* has been shown in HCC [17]. Natural OA as well as its synthetic derivatives display chemoprevention and antitumor effects in breast cancer, glioblastoma, prostate cancer and pancreatic cancer [18].

Searching for new strategies to overcome treatment resistance of HCC, in the present study we investigated the effects of Smac mimetic and OA.

Materials and methods

Cell culture and reagents

The HCC cell lines HepG2, Huh7 and Hep3B were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM medium (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1 mM Sodium Pyruvate (Invitrogen). Primary human hepatocytes were isolated by collagenase perfusion and cultured in William's Medium E (Sigma, Deisenhofen, Germany) with 10% FCS, 1% penicillin/streptomycin, 5 µg/ml Insulin (Sigma), 50 µM Hydrocortison (Sigma), 2% DMSO and 20 ng/ml EGF (Sigma). The study was approved by the local Ethical Committee and informed consent was obtained in accordance with the Declaration of Helsinki. Smac mimetic BV6 that neutralizes XIAP, cIAP1 and cIAP2 [19] was kindly provided by Genentech (South San Francisco, CA, USA). TRAIL receptor 2 agonistic antibody Lexatumumab was kindly provided by Human Genome Sciences, Inc. (Rockville, MD, USA). Pan-caspase inhibitor zVAD.fmk was purchased from Bachem (Heidelberg, Germany), and Nec-1 from Biomol (Hamburg, Germany). All other chemicals were purchased from Sigma unless indicated otherwise.

Determination of cell death and cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) or by crystal violet assay (0.75% crystal violet, 50% ethanol, 0.25% NaCl and 1.57% formaldehyde). Apoptosis was determined by analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany) as described previously [20].

Colony formation assay

To determine colony formation, 0.2×10^6 cells were seeded in a 6-well tissue culture plate, allowed to settle for 24 hours and treated with OA and/or BV6 for 72 hours. Then, cells were re-seeded with 200 cells (Huh7) or 400 cells (HepG2) in a 6-well tissue culture plate and were stained after culture for 12 days with crystal violet solution. Colonies were counted and the percentage of surviving colonies relative to the untreated controls was calculated.

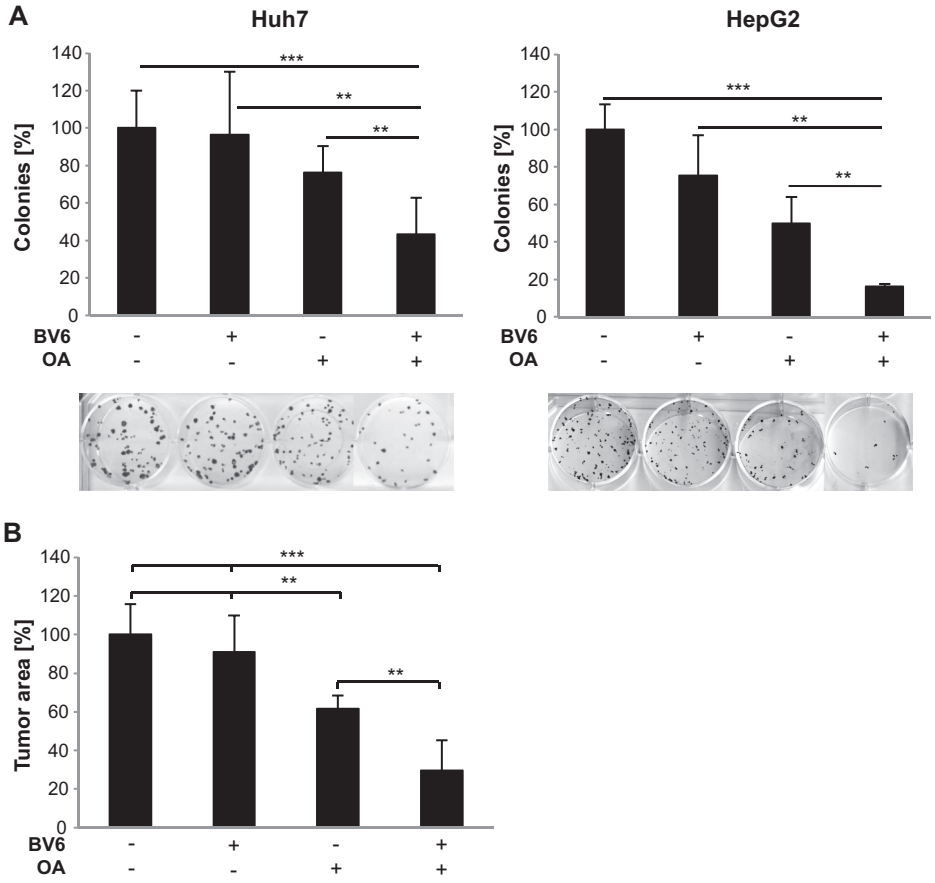

Fig. 2. BV6/OA cotreatment suppresses long-term clonogenic survival and *in vivo* tumor growth. (A) HCC cells were treated with BV6 (Huh7: 6 μM BV6, HepG2 4 μM BV6) and/or 60 μM OA and colony formation was assessed as described in Materials and Methods. The percentage of colonies relative to untreated control is shown. (B) HepG2 cells were seeded on the CAM of chicken embryos and treated with 30 μM OA and/or 4 μM BV6. Tumor growth was analyzed using hematoxylin and eosin-stained paraffin sections of the CAM as described in Materials and Methods. Tumor area as percentage of the untreated control group is shown with mean and SD of two independent experiments, n = 2; $^{**}P < 0.01$; $^{***}P < 0.001$.

Western blot analysis and caspase-3/7 activity assay

Western blot analysis was performed as described before [20] using the following antibodies: mouse anti-caspase-8 (Enzo Life Science, Lörrach, Germany), rabbit anti-caspase-3, rabbit anti-caspase-9 and rabbit polyclonal anti-ENDOG (Cell Signaling, Beverly, MA, USA) and mouse anti-p53 (BD Biosciences, Heidelberg, Germany). Mouse anti-β-actin (Sigma) and anti-mouse GAPDH (HyTest, Turku, Finland) were used as loading controls. Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown. Caspase-3/7 activity was determined using Apo-ONE® Homogeneous caspase-3/7 activity assay (Promega, Madison, WI, USA) following the manufacturer's instructions.

RNA interference

Gene silencing was performed using Silencer® Select validated small interfering RNA (siRNAs) against RIP1 (s16651), ENDOG (s707, s708, s194395) and non-targeting control siRNA (s12935) from Invitrogen (Karlsruhe, Germany) following the manufacturer's instructions.

Chorioallantoic membrane assay

Chorioallantoic membrane (CAM) assay was done as described previously [21,22]. Briefly, 2.5×10^6 HepG2 cells were resuspended in 25 μl serum-free medium and 25 μl Matrigel Matrix (BD Biosciences) and implanted on fertilized chicken eggs on day 8 of incubation. Tumors were topically treated with OA alone or in combination with BV6 in 20 μl PBS daily for 3 days, sampled with the surrounding CAM 4 days after seeding, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5-μm sections, and analyzed by hematoxylin/eosin staining. Representative images of hematoxylin/eosin-stained sections of the CAM were recorded and shown. The tumor area was calculated by using ImageJ digital imaging software (NIH, Bethesda, MD, USA).

Determination of ROS production

To determine ROS production, cells were stained either with 1 μM CellROX Deep Red Reagent (Life Technologies, Inc.) or with 5 μM MitoSOX Red Mitochondrial Superoxide Indicator (Life Technologies, Inc.) following the manufacturer's instructions and analyzed by flow cytometry. Dead cells were excluded from the analysis by discarding the supernatant and by forward-sideward-scatter analysis.

Statistical analysis

Statistical significance was assessed by Student's *t*-Test (two-tailed distribution, two-sample, unequal variance). Drug interactions were analyzed by the Combination index (CI) method based on that described by Chou [23] using CalcuSyn software (Biosoft, Cambridge, UK). CI <0.9 indicates synergism, 0.9–1.1 additivity, >1.1 antagonism.

Results

Smac mimetic and OA synergistically induce cell death in HCC cells

To investigate the effects of OA and BV6 in HCC we used three different human HCC cell lines (Huh7, HepG2, Hep3B). Interestingly, BV6 and OA acted in concert to trigger DNA fragmentation, used as a typical marker of apoptotic cell death, compared to treatment with either agent alone (Fig. 1A). Calculation of CI revealed that drug interaction of BV6 with OA was synergistic in all cell lines at different concentrations (Suppl. Table S1). To confirm the

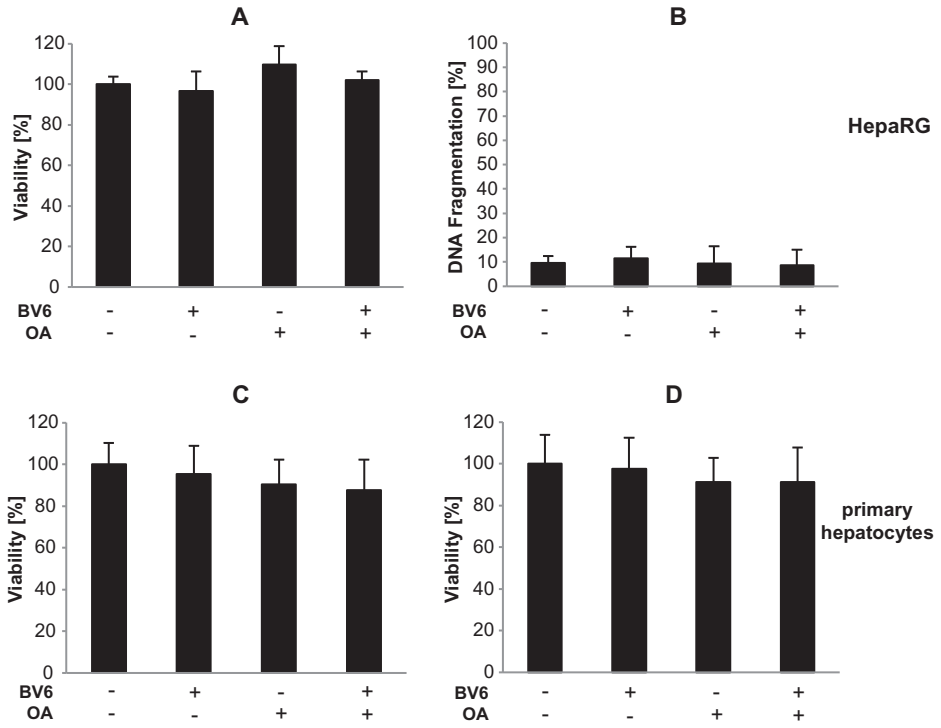

Fig. 3. Smac mimetic and OA do not induce cell death in normal hepatocytes. (A and B) The human hepatocyte cell line HepaRG was treated for 72 hours with 6 μM BV6 and/or 60 μM OA. Cell viability was determined by MTT assay (A) and cell death by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (B). (C and D) Human primary hepatocytes were treated for 72 hours with 6 μM BV6 and/or 60 μM OA. Cell viability was determined by MTT assay (C) and crystal violet assay (D). Mean and SD of three independent experiments performed in triplicate are shown.

cooperative induction of cell death, we also used another cytotoxicity assay. Similarly, BV6 and OA acted together to reduce cell viability of HCC cells in a dose-dependent manner (Fig. 1B). Kinetic analysis showed that BV6/OA cotreatment induced a time-dependent increase in DNA fragmentation starting around 24 hours (Fig. 1C).

Since p53 has been implicated in OA-induced cell death [17], we investigated whether p53 accumulates upon treatment with BV6 and/or OA using p53 wildtype HepG2 cells. However, neither BV6 nor OA caused p53 accumulation in contrast to treatment with Doxorubicin that was used a positive control (Suppl. Fig. S1).

Since Smac mimetics have recently been reported to enhance the efficacy of chemotherapeutics in several cancers [12,16,24,25], we examined whether BV6 increases the antitumor activity of Sorafenib, which is frequently used in the treatment of HCC [26]. However, the addition of BV6 failed to enhance Sorafenib-induced DNA fragmentation or loss of cell viability in two HCC cell lines (Suppl. Fig. S2).

BV6/OA cotreatment suppresses long-term clonogenic survival and in vivo *tumor growth*

To explore whether cotreatment with BV6 and OA impairs long-term clonogenic survival in addition to short-term assays, we performed colony assays. Of note, BV6/OA cotreatment significantly reduced colony formation compared to either agent alone (Fig. 2A), demonstrating that the cotreatment also affects long-term clonogenic survival of HCC cells.

Next, we evaluated *in vivo* antitumor activity of BV6/OA cotreatment against HCC using the CAM model, an established *in vivo* tumor model for anticancer drug testing [21]. HepG2 cells were seeded on the CAM of chicken embryos and allowed to form tumors before cells were treated with BV6 and/or OA. Importantly, BV6/OA cotreatment was significantly more effective compared to treatment with either OA or BV6 alone to reduce tumor growth (Fig. 2B).

BV6/OA cotreatment does not induce cell death in normal hepatocytes

To examine whether the BV6/OA combination treatment preferentially affects malignant versus non-malignant cells, we extended our experiments to the normal hepatocyte cell line HepaRG as well as to primary hepatocytes derived from three different human donors receiving liver resection. Of note, BV6/OA cotreatment at equimolar concentrations did not decrease cell viability or induce DNA fragmentation in the HepaRG cell line or in primary human hepatocytes (Fig. 3).

This set of experiments demonstrates that BV6 and OA synergize to induce cell death in HCC cells with no detectable cytotoxicity against normal hepatocytes at equimolar concentrations.

BV6 and OA cooperate to trigger caspase-3 activation

To identify the underlying mechanisms of synergistic cell death induction by BV6 and OA, we monitored activation of the caspase cascade by Western blot analysis. BV6 and OA acted in concert to trigger processing of caspase-3 into the active cleavage fragments p17 and p12 after 24 hours (Fig. 4A), a time point when cells started to undergo cell death (Fig. 1C), while cleavage of caspase-8 or -9 was not detectable (Fig. 4A). Additionally, we determined caspase activation by using an enzymatic assay that measures caspase-3/7 activity. BV6/OA cotreatment significantly increased caspase-3/7 activity in both Huh7 and HepG2 cells (Fig. 4B).

To answer the question whether caspases are required for the induction of cell death, we used the pan-caspase inhibitor zVAD.fmk. Addition of zVAD.fmk significantly decreased BV6/OA-induced DNA

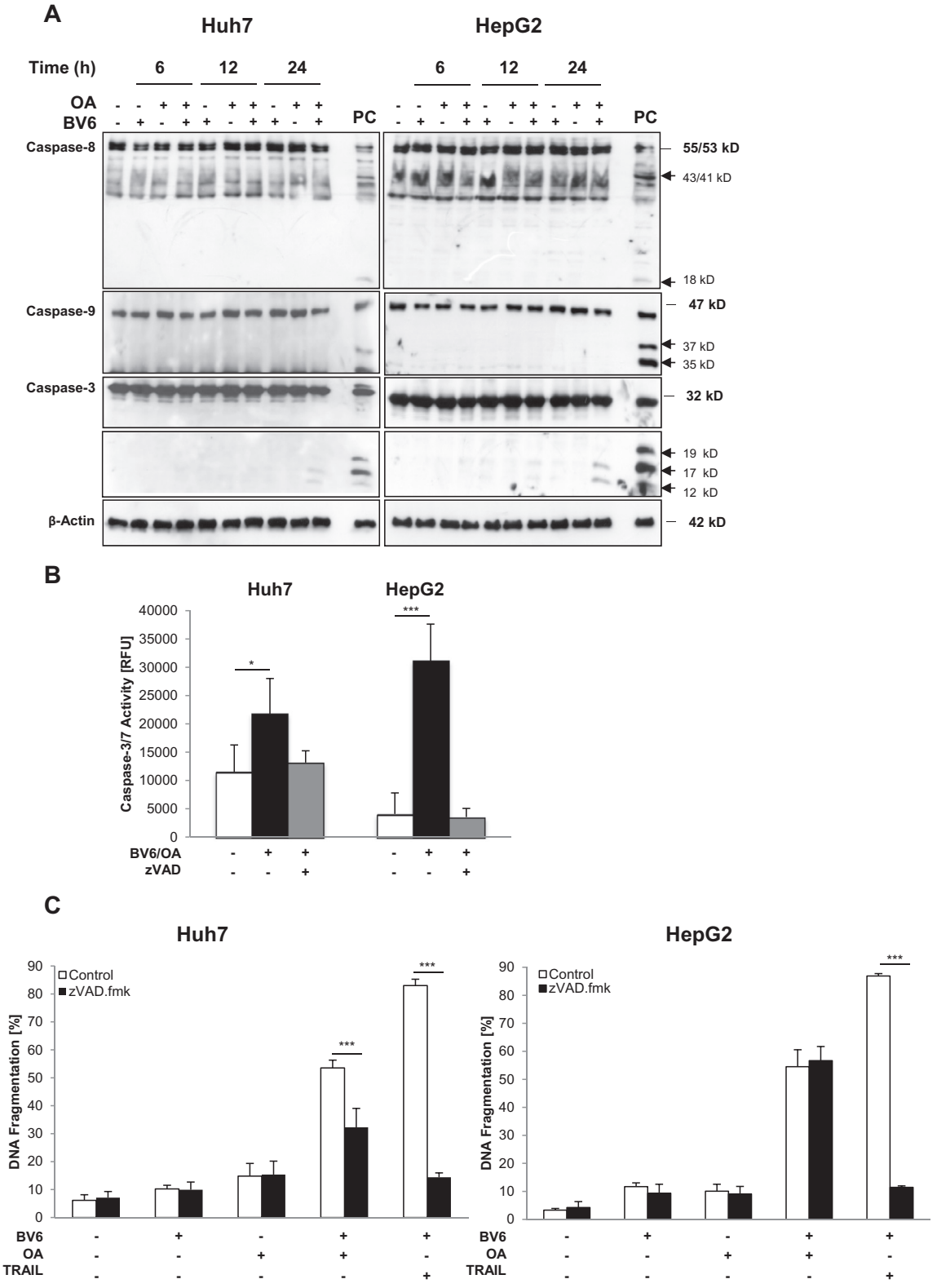

Fig. 4. BV6 and OA cooperated to trigger caspase-3 activation. (A) HCC cells were treated for indicated times with BV6 (Huh7: 6 μM BV6, HepG2: 4 μM BV6) and/or 60 μM OA. Caspase activation was analyzed by Western blotting. Arrowheads indicate caspase cleavage fragments; RD rhabdomyosarcoma cells treated with TRAIL receptor 2 agonistic antibody Lexatumumab were used as positive control (PC). (B) HCC cells were treated for 24 hours with BV6 (Huh7: 6 μM, HepG2: 4 μM) and/or 60 μM OA in the presence or absence of 50 μM zVAD.fmk. Caspase-3/7 activity was determined as described in Materials and Methods. Mean and SD of three independent experiments performed in triplicate are shown, $^{*}P < 0.05$; $^{***}P < 0.001$. (C) HCC cells were treated for 48 hours with BV6 (Huh7: 6 μM, HepG2: 4 μM) and/or 60 μM OA in the presence or absence of 50 μM zVAD.fmk. Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. As positive control, cells were treated with 40 ng/ml TRAIL and BV6 (Huh7: 6 μM BV6, HepG2: 4 μM BV6) in the presence or absence of 50 μM zVAD.fmk. Mean and SD of three independent experiments performed in triplicate are shown; $^{***}P < 0.001$. (D and E) Cells were transiently transfected with siRNAs targeting ENDOG (siENDOG #1–3) or control siRNA (siCtrl) and expression of ENDOG was analyzed by Western blotting (D). Transiently transfected HCC cells were treated for 48 hours with BV6 (Huh7: 6 μM, HepG2: 4 μM) and/or 60 μM OA. Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (E). Mean and SD of three independent experiments performed in triplicate are shown.

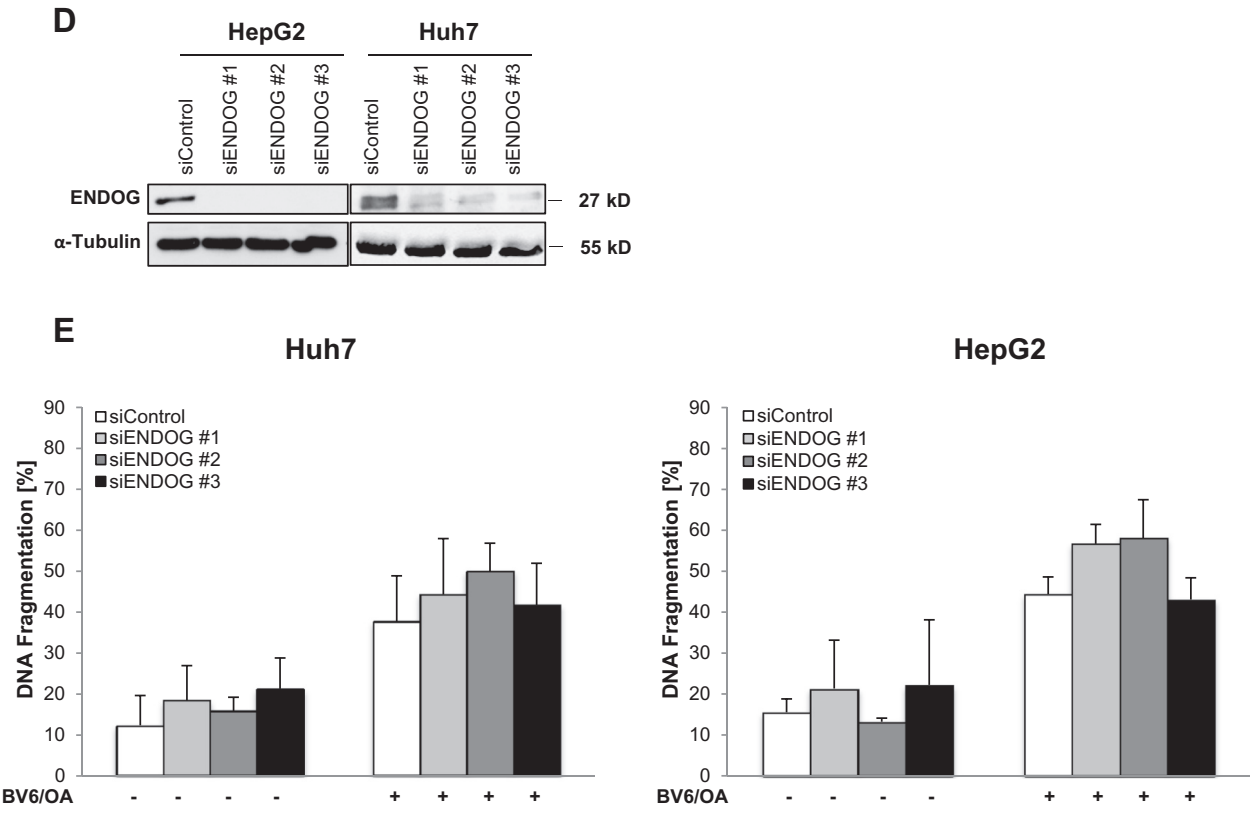

Fig. 4. (*continued*)

fragmentation in Huh7 cells, whereas zVAD.fmk failed to rescue HepG2 from BV6/OA-induced cell death (Fig. 4C). Control experiments confirmed that zVAD.fmk blocked the BV6/OA-induced increase in caspase-3/7 activity in both Huh7 and HepG2 cells (Fig. 4B). As positive control for caspase-dependent cell death, both cell lines were treated with TRAIL in the presence or absence of zVAD.fmk (Fig. 4C). This set of experiments shows that BV6 and OA cooperate to trigger caspase-3 activation, which is required for BV6/OA-induced cell death in a cell line-dependent manner, pointing also to caspase-independent mechanisms of cell death.

Therefore, we investigated the involvement of endonuclease G (ENDOG) that has been reported to mediate apoptotic DNA fragmentation independently of caspases [27,28]. To this end, we knocked down ENDOG by RNA interference. Control experiments confirmed that three distinct siRNA sequences efficiently suppressed ENDOG protein expression in both Huh7 and HepG2 cells (Fig. 4D). However, ENDOG silencing failed to protect Huh7 and HepG2 cells from BV6/OA-mediated apoptosis (Fig. 4E), demonstrating that ENDOG is not involved in BV6/OA-induced apoptosis.

RIP1 is not involved in BV6/OA-induced cell death

Since RIP1 has been reported to play an important role in the regulation of Smac mimetic-induced cell death [29,30], we then investigated the involvement of RIP1. To this end, we used a genetic strategy to block RIP1 expression via RNA interference-mediated silencing (Fig. 5A). However, knockdown of RIP1 did not protect against BV6/OA-induced cell death (Fig. 5B).

As necroptosis has recently been discovered as another form of programmed cell death depending on the kinases RIP1 and RIP3 [31], we next asked whether necroptosis plays a role in BV6/OA-induced cell death. To answer this question, we used the RIP1 kinase inhibitor Nec-1 in addition to the genetic silencing of RIP1. Similarly, addition of Nec-1 failed to rescue BV6/OA-induced cell death (Fig. 5C). RIP3 protein, another critical kinase involved in necroptosis signaling, was not detectable by Western blotting in the HCC cell lines Huh7 and HepG2 (Suppl. Fig. S3).

This set of experiments indicates that BV6/OA cotreatment does not primarily engage necroptotic cell death.

ROS scavengers partially rescue BV6/OA-induced cell death

Since ROS have previously been implicated to be involved in cell death by OA [17,32], we next determined ROS production upon treatment with OA and/or BV6 by using two distinct ROS-sensitive fluorescent dyes and flow cytometry. Of note, OA/BV6 co-treatment caused a slight and significant increase in ROS production as analyzed by MitoSOX staining in both Huh7 and HepG2 cells (Fig. 6A). The glutathione depleting agent Buthionine sulfoximine (BSO) was used as positive control for ROS generation (Fig. 6A). Similarly, we detected a significant increase in ROS production upon BV6/OA treatment in HepG2 cells, while not in Huh7 cells (Fig. 6B). To explore whether ROS contribute to BV6/OA-induced cell death, we used different ROS scavengers, including N-acetyl-cysteine (NAC), an antioxidant and glutathione precursor [33,34], α-Tocopherol, a lipophilic antioxidant [35], and MnTBAP, a cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger [36]. Importantly, addition of NAC, α-Tocopherol or MnTBAP significantly decreased BV6/OA-triggered cell death (Fig. 6C). These data indicate that ROS production contributes to BV6/OA-induced cell death.

Discussion

HCC is one of the most aggressive solid tumors worldwide with an increasing incidence [1,5]. Different underlying liver diseases

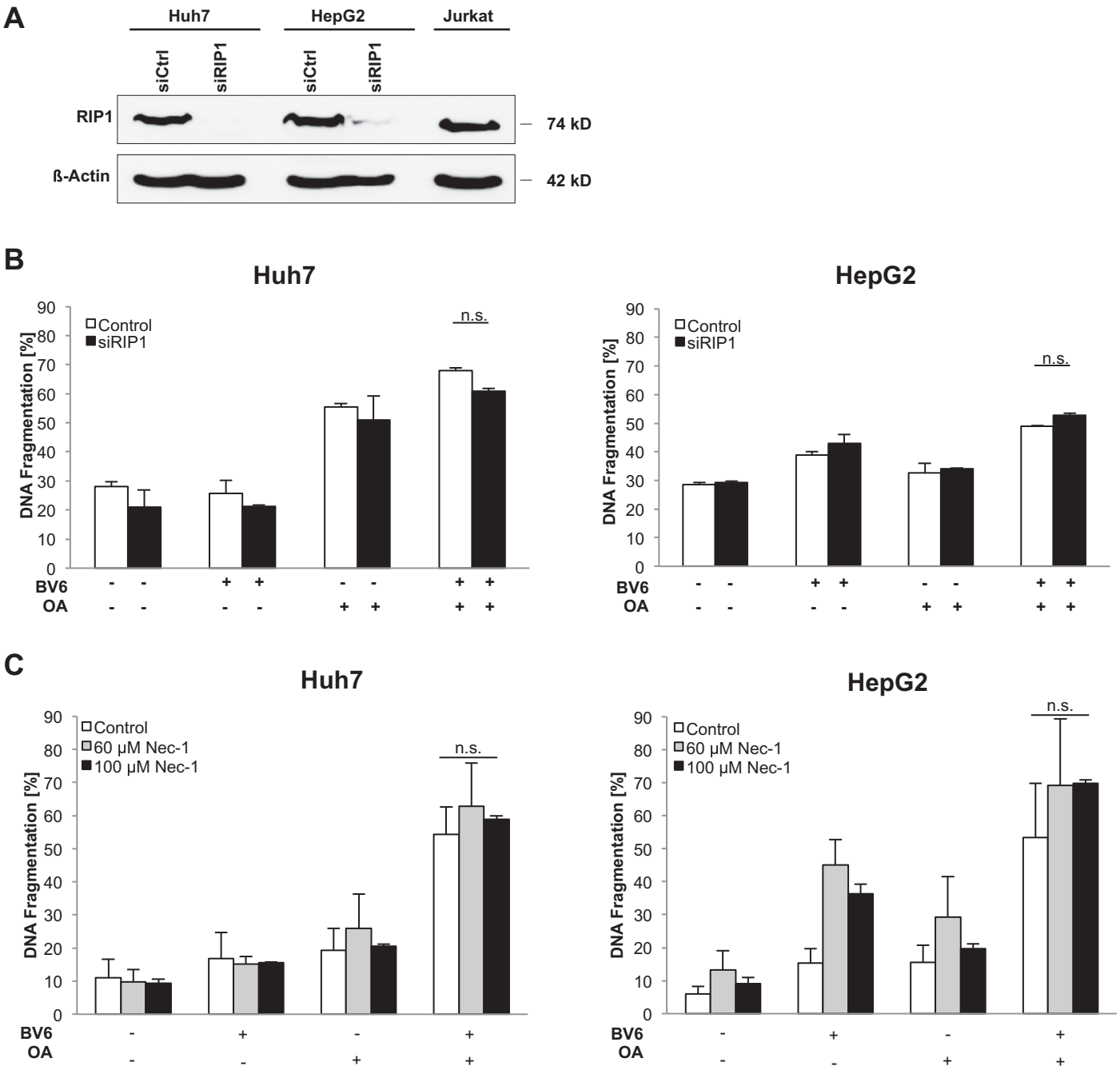

Fig. 5. Role of RIP1 in BV6/OA cotreatment. (A and B) Cells were transiently transfected with siRNAs targeting RIP1 (siRIP1) or control siRNA (siCtrl) and expression of RIP1 was analyzed by Western blotting, Jurkat acute leukemia cells were used as positive control for RIP1 expression (A). Transiently transfected HCC cells were treated for 48 hours with BV6 (Huh7: 6 µM, HepG2: 4 µM) and/or 60 µM OA. Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (B). Mean and SD of three independent experiments performed in triplicate are shown; n.s. = not significant. (C) HCC cells were treated for 48 hours with BV6 (Huh7: 6 µM, HepG2: 4 µM) and/or 60 µM OA in the presence or absence of indicated concentrations of Nec-1. Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; n.s. = not significant.

induce dysregulation in the apoptotic program and could explain intrinsic resistance of HCC to chemotherapy [37]. At present, no effective systemic chemotherapy exists for patients with advanced HCC. Sorafenib, an oral multikinase inhibitor, is currently the only routinely used chemotherapy, which improves the median survival and the time to radiologic progression about 2.8 months compared to patients who received a placebo [26]. This highlights the need to develop novel strategies for the induction of cell death in HCC cells.

In this study, we identify a novel synergistic interaction of the Smac mimetic BV6 and OA in HCC cells. In addition to increasing cell death in short-term assays, OA and BV6 acted together to inhibit long-term clonogenic survival of HCC cells. The clinical importance of this combination therapy is also underlined by some tumor selectivity of the cotreatment, since BV6/OA cotreatment at equimolar concentrations had no cytotoxic effects in parallel experiments in primary normal hepatocytes. Importantly, BV6/OA cotreatment significantly reduced tumor growth in an *in vivo* model of HCC

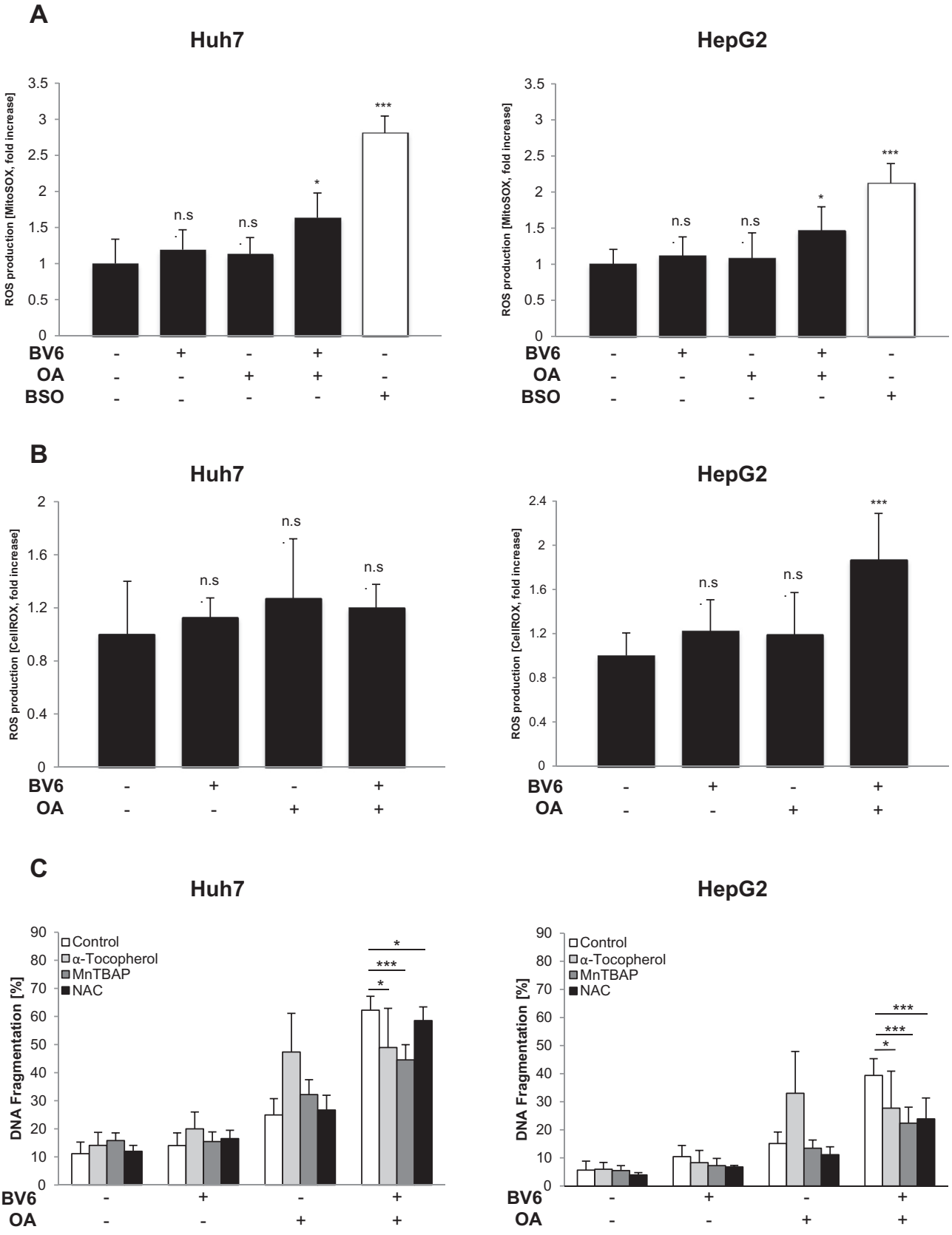

Fig. 6. ROS scavengers partially rescue BV6/OA-induced cell death. (A and B) HCC cells were treated for 24 hours with BV6 (Huh7: 6 μM, HepG2: 4 μM) and/or 60 μM OA and ROS production was determined by MitoSOX (A) and CellROX staining (B) using flow cytometry, treatment with 50 μM BSO (Huh7) or 10 μM BSO (HepG2) for 24 hours was used as positive control. Mean and SD of three independent experiments performed in triplicate are shown; *$P < 0.05$; ***$P < 0.001$; n.s. = not significant. (C) HCC cells were treated for 48 hours with BV6 (Huh7: 6 μM, HepG2: 4 μM) and/or 60 μM OA in the presence or absence of different ROS scavengers (50 μM α-Tocopherol, 300 μM MnTBAP, 0.63 mM NAC). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; *$P < 0.05$; ***$P < 0.001$.

underscoring the efficacy of this combination compared to single treatment with BV6 or OA.

Mechanistic studies show that BV6/OA cotreatment triggers caspase-dependent and caspase-independent cell death in HCC cells. This conclusion is supported by data showing that the pan-caspase inhibitor zVAD.fmk rescued cell death in a cell type-dependent manner, pointing to additional effector pathways of cell death. For example, mitochondrial effector molecules such as apoptosis-inducing factor (AIF) and ENDOG have been reported to trigger cell death in the absence of caspase activation in leukemia, laryngeal carcinoma and colon cancer cells [38–40].

Furthermore, we demonstrated by rescue experiments that ROS generation contributes to BV6/OA-induced apoptosis, since ROS scavengers protect HCC cells against cell death. It is interesting to note that ROS production has previously been implicated in cell death induced by OA alone [17,32] and that natural products have been reported to engage mitochondrial apoptosis by oxidative damage [41]. Since different programmed cell death pathways including apoptotic and necrotic pathways have recently been implied in hepatocarcinogenesis [42], we addressed the question whether necroptosis plays a role in BV6/OA-induced cell death. However, so far we have no indication that BV6/OA-induced cell death involves necroptosis, since neither addition of Nec-1 nor knockdown of RIP1 rescues HCC cells from BV6/OA-induced cell death. Furthermore, RIP3 protein, another key component of necroptosis [43] and an inhibitor of inflammatory hepatocarcinogenesis [42], was not detectable in the studied HCC cell lines, further supporting the notion that necroptosis is not primarily engaged upon BV6/OA cotreatment.

Several previous studies showed that OA and its derivatives exert an inhibitory effect on tumor growth *in vitro* and *in vivo* and induce apoptosis in different tumor types [17,32,44–46]. However, the underlying mechanisms of the antitumor effects of OA are largely unknown. Zou et al. showed that the synthetic OA derivate CDDO-Me induced endoplasmatic reticulum (ER) stress in lung cancer cell lines as well as upregulation of death receptor-5 (DR5) and caspase activation [47]. It has recently been reported that ER stress induces apoptosis via cell-autonomous and ligand-independent activation of DR5 [48]. Since we previously identified upregulation of DR5 as an important event that contributes to BV6-induced apoptosis [49], we assume that OA-mediated upregulation of DR5 could sensitize to BV6-induced cell death. Another interesting effect of OA is the described inhibition of HCV entry into the host cell [50], since HCV liver cirrhosis accounts for around 25% of HCC worldwide [51]. First human clinical trials with the synthetic OA Bardoxolone in lymphoma patients showed a promising safety profile and objective tumor response [18,52,53].

Our data have important implications for the future development of Smac mimetic-based therapies in HCC. First and foremost, the cotreatment of the Smac mimetic BV6 and OA could be a promising approach for overcoming chemotherapy resistance in HCC. Second, combined application of both compounds at subtoxic concentrations results in a synergistic drug interaction to trigger apoptosis. Third, the combination of BV6/OA may be tumor-selective, since it caused no detectable cytotoxic effects against primary normal hepatocytes. This underlines the potential clinical relevance of this cotreatment. In conclusion, the cotreatment of Smac mimetic and OA is a promising way of overcoming chemotherapy resistance in HCC, which warrants further investigation.

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Conflict of interest

None declared.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.04.018.

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Identification of a novel oxidative stress induced cell death by Sorafenib and oleanolic acid in human hepatocellular carcinoma cells

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ABSTRACT

The lack of effective chemotherapies in hepatocellular carcinoma (HCC) is still an unsolved problem and underlines the need for new strategies in liver cancer treatment. In this study, we present a novel approach to improve the efficacy of Sorafenib, today's only routinely used chemotherapeutic drug for HCC, in combination with triterpenoid oleanolic acid (OA). Our data show that cotreatment with subtoxic concentrations of Sorafenib and OA leads to highly synergistic induction of cell death. Importantly, Sorafenib/OA cotreatment triggers cell damage in a sustained manner and suppresses long-term clonogenic survival. Sorafenib/OA cotreatment induces DNA fragmentation and caspase-3/7 cleavage and the addition of the pan-caspase inhibitor zVAD.fmk shows the requirement of caspase activation for Sorafenib/OA-triggered cell death. Furthermore, Sorafenib/OA co-treatment stimulates a significant increase in reactive oxygen species (ROS) levels. Most importantly, the accumulation of intracellular ROS is required for cell death induction, since the addition of ROS scavengers (i.e. α-tocopherol, MnTBAP) that prevent the increase of intracellular ROS levels completely rescues cells from Sorafenib/OA-triggered cell death. In conclusion, OA represents a novel approach to increase the sensitivity of HCC cells to Sorafenib via oxidative stress.

1. Introduction

HCC is the second most common cause of death from cancer worldwide and estimated to be responsible for nearly 745,000 deaths in 2012 [1]. Only 30–40% of patients are eligible for curative treatment, including liver resection, transplantation and percutaneous ablation [2,3]. At present, there is no effective systemic chemotherapy available for patients with advanced HCC. Sorafenib, an oral multikinase inhibitor, is currently the only routinely used chemotherapeutic drug, which improves the median survival and the time to radiologic progression up to 2.8 months compared to patients who received a placebo treatment [4]. Unfortunately, due to its toxicity, the administration of Sorafenib is reserved only to a limited group of patients. The underlying liver dysfunction in HCC patients, which leads to an even lower tolerance of treatment toxicity, presents a significant problem in the standard therapy with Sorafenib [5].

This highlights the need to develop novel strategies for the induction of cell death in HCC cells. Due to the lack of more effective treatment strategies in HCC, one strategy is to improve the efficacy of Sorafenib in HCC cells. Sorafenib has been reported to induce the generation of ROS in human HCC cell lines *in vivo* and *in vitro* in a dose-dependent manner [6,7]. The levels of ROS in Sorafenib-treated HCC patients correlate with the clinical efficacy of Sorafenib [6]. Another approach to induce ROS and cell death in HCC cells is the use of OA, a natural triterpenoid [8–10]. In Chinese medicine, OA has been used for many decades in the

Abbreviations: CI, combination index; DR5, death receptor-5; ER, endoplasmatic reticulum; FCS, fetal calf serum; Fer-1, ferrostatin-1; FSC/SSC, forward/side scatter; HCC, hepatocellular carcinoma; IAP, Inhibitor of Apoptosis; MTT, inhibitor N-benz yloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Nec-1, necrostatin-1; OA, oleanolic acid; PI, propidium iodide; RIP, receptor-interacting protein; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; zVAD.fmk, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

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treatment of liver disorders such as viral hepatitis [8]. Recently, an antitumor effect of OA *in vitro* and *in vivo* has been shown in HCC [8]. We previously identified a novel synergistic induction of ROS production and cell death by combining the Smac mimetic BV6, which antagonizes Inhibitor of Apoptosis (IAP) proteins [11,12], and OA in human HCC cells [10]. Searching for new strategies to overcome Sorafenib resistance in HCC, in the present study we investigated the effects of the combination of Sorafenib and OA on human HCC cells.

2. Materials and methods

2.1. Cell culture and reagents

The human HCC cell lines Huh7 and HepG2 were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM medium (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1 mM Sodium Pyruvate (Invitrogen). All cell lines were maintained in a humidified atmosphere at 37 °C with 5% CO_2. The pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany), and necrostatin (Nec)-1s from Biomol (Hamburg, Germany). All other chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany) unless indicated otherwise.

2.2. Determination of cell death and cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) or by crystal violet staining (0.75% crystal violet, 50% ethanol, 0.25% NaCl and 1.57% formaldehyde). Cell death was determined by analysis of DNA fragmentation of propidium iodide

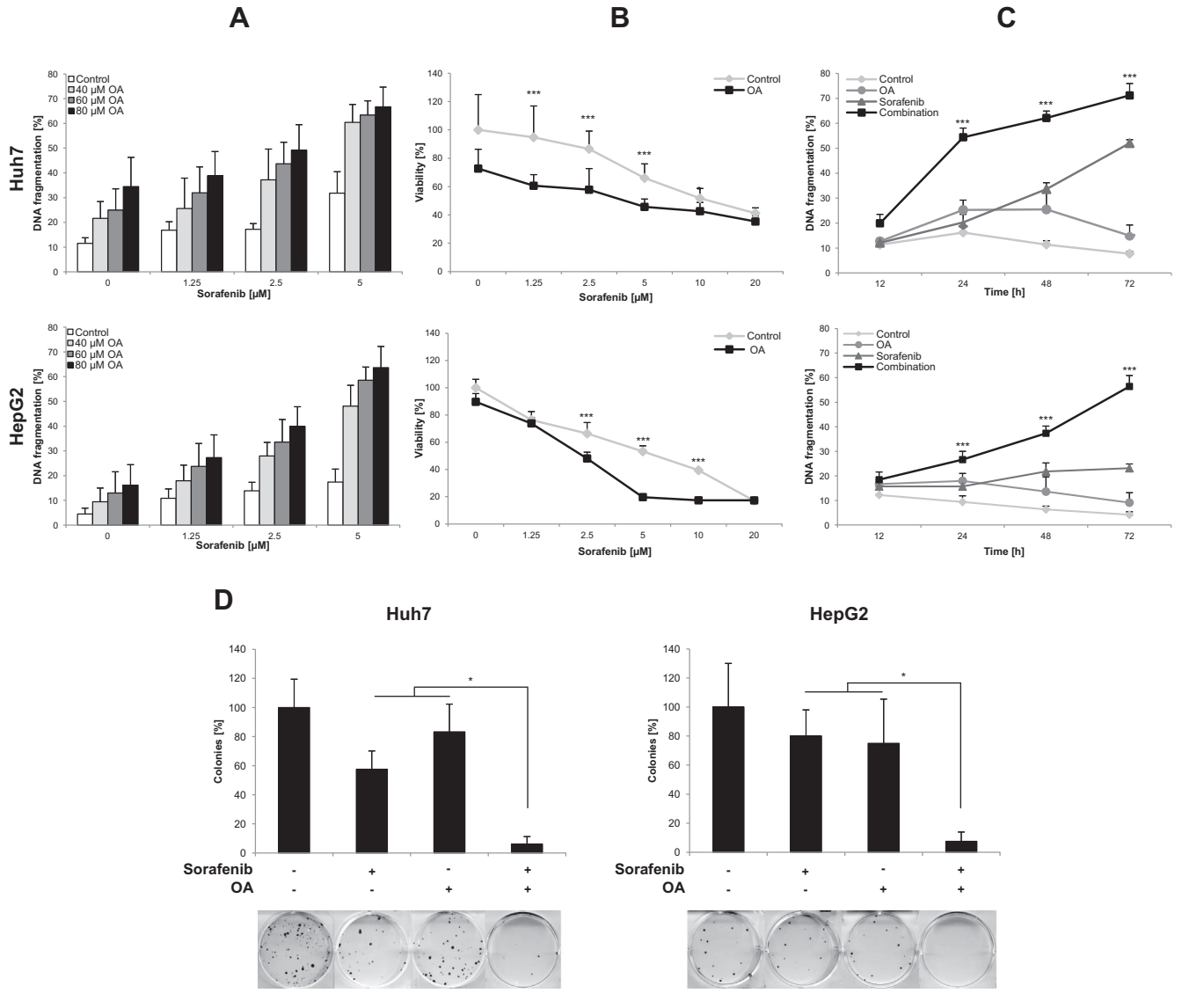

Fig. 1. Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival. (A)–(C) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with indicated concentrations of Sorafenib and OA (A, B) or for indicated times with 5 µM Sorafenib and/or 60 µM OA (C). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (A, C), cell viability was determined by MTT assay (B). (D) HCC cells were treated with 5 µM Sorafenib and/or 60 µM OA for 72 h and colony formation was assessed as described in Section 2. The percentage of colonies relative to untreated control (upper panels) and representative results (lower panels) is shown. Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{***}P < 0.001$. In (B) and (C), Sorafenib/OA-cotreated samples were compared to Sorafenib-treated samples.

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(PI)-stained nuclei or forward/side scatter (FSC/SSC) analysis of PI-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany) as described previously [13].

2.3. Colony formation assay

To determine colony formation, 0.2×10^6 cells were seeded in a 6-well tissue culture plate, and allowed to settle for 24 h. Cells were then treated with Sorafenib and OA for 48 h, trypsinized and re-seeded with a total count of 200 cells (Huh7) or 400 cells (HepG2) per well in a second 6-well tissue culture plate. After 12 days of cultivation, cells were stained with crystal violet solution, colonies were counted and the percentage of surviving colonies relative to the untreated controls was calculated.

2.4. Caspase-3/7 activity assay

The caspase-3/7 activity assay was performed using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Cells were seeded in 96-well plates and treated with Sorafenib and OA for 12 and 24 h. 100 µl of Apo-ONE® Caspase-3/7 reagent were added to each well. Contents were gently mixed on a plate shaker for 30 s. After one hour of incubation time the fluorescence emission at 530 nm of each well was measured using Tecan® reader Infinite® 200 PRO (Tecan Group, Ltd. Männedorf, Switzerland).

2.5. ROS staining and lipid peroxidation

ROS production was measured by flow cytometry using 5 µM of MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol. For measuring lipid peroxidation cells were stained with 5 µM of BODIPY-C11 (Invitrogen) and analyzed by flow cytometry according to the manufacturer's protocol.

2.6. Statistical analysis

Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample, unequal variance). Drug interactions were analyzed by the combination index (CI) method based on the publication by Chou [14] using CalcuSyn software (Biosoft, Cambridge, UK). A calculation of CI value of <0.9 indicates synergism, 0.9–1.1 additivity, >1.1 antagonism.

3. Results

3.1. Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival

To investigate whether OA can prime HCC cells to Sorafenib, we used two different human HCC cell lines (Huh7, HepG2). Interestingly, Sorafenib and OA acted in concert to trigger DNA fragmentation, a typical marker of apoptotic cell death, compared to treatment with either agent alone (Fig. 1A). The calculation of CI revealed a synergistic interaction of Sorafenib and OA in both HCC cell lines (Table 1). The cooperative interaction of Sorafenib and OA was confirmed by another assay in which Sorafenib and OA acted together to reduce cell viability of HCC cells in a dose-dependent manner (Fig. 1B). Kinetic analysis showed that Sorafenib/OA cotreatment induced a significant time-dependent increase in DNA fragmentation in Huh7 and HepG2 cells starting around 24 h compared to single treatment with Sorafenib (Fig. 1C). To explore the impact of cotreatment with Sorafenib and OA on long-term clonogenic survival, colony formation assay was performed. Of note, Sorafenib/OA cotreatment significantly reduced colony formation compared to either agent alone in Huh7 and HepG2 cells (Fig. 1D), demonstrating that the cotreatment also affects long-term clonogenic survival of HCC cells. Together, these experiments demonstrate that Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival.

Table 1
Synergistic induction of cell death by Sorafenib and OA.

	OA (µM)	Sorafenib (µM)	CI
Huh7	40	1.25	1.068
	40	2.5	0.664
	40	5	0.261
	60	1.25	1.002
	60	2.5	0.631
	60	**5**	**0.283**
	80	1.25	0.881
	80	2.5	0.585
	80	5	0.294
HepG2	40	1.25	0.662
	40	2.5	0.336
	40	5	0.109
	60	1.25	0.53
	60	2.5	0.307
	60	**5**	**0.088**
	80	1.25	0.527
	80	2.5	0.274
	80	5	0.087

Combination index (CI) was calculated as described in Section 2. Values of CI <0.9 indicates synergism, 0.9–1.1 additivity, >1.1 antagonism. Drug concentrations used in that study are indicated in bold.

3.2. Sorafenib/OA cotreatment cooperates to trigger caspase activation and caspase-dependent apoptosis

To identify the underlying mechanisms of the synergistic induction of cell death by Sorafenib and OA, we monitored caspase activity by using an enzymatic caspase-3/7 activity assay. Sorafenib/OA cotreatment significantly increased caspase-3/7 activity at 12 and 24 h in both Huh7 and HepG2 cells (Fig. 2A). To further investigate the question whether caspases are required for the induction of cell death, we used the pan-caspase inhibitor zVAD.fmk. Addition of zVAD.fmk significantly decreased Sorafenib/OA-induced DNA fragmentation in both cell lines (Fig. 2B). As positive control for caspase-dependent cell death, both cell lines were treated with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the presence or absence of zVAD.fmk (Fig. 2B). This set of experiments shows that Sorafenib and OA cooperate to trigger caspase-3/7 activation, which is required for Sorafenib/OA-induced cell death.

3.3. Receptor-interacting protein (RIP)1 kinase activity is not required for Sorafenib/OA-induced cell death

Necroptosis has recently been discovered as another form of programmed cell death depending on the kinases RIP1 and RIP3 [15]. However, the role of necroptosis in Sorafenib/OA-induced cell death is still unknown. To address this question, we determined the cell death induction in the presence and absence of the RIP1 kinase inhibitor Necrostatin-1s (Nec-1s). To analyze apoptotic or necroptotic cell death, we used DNA fragmentation as a marker of apoptotic cell death and PI-staining to determine the loss of plasma membrane integrity as a parameter of necroptotic cell death. Inhibition of RIP1 kinase activity by Nec-1s failed to rescue Sorafenib/OA-induced cell death, as determined by analysis of DNA

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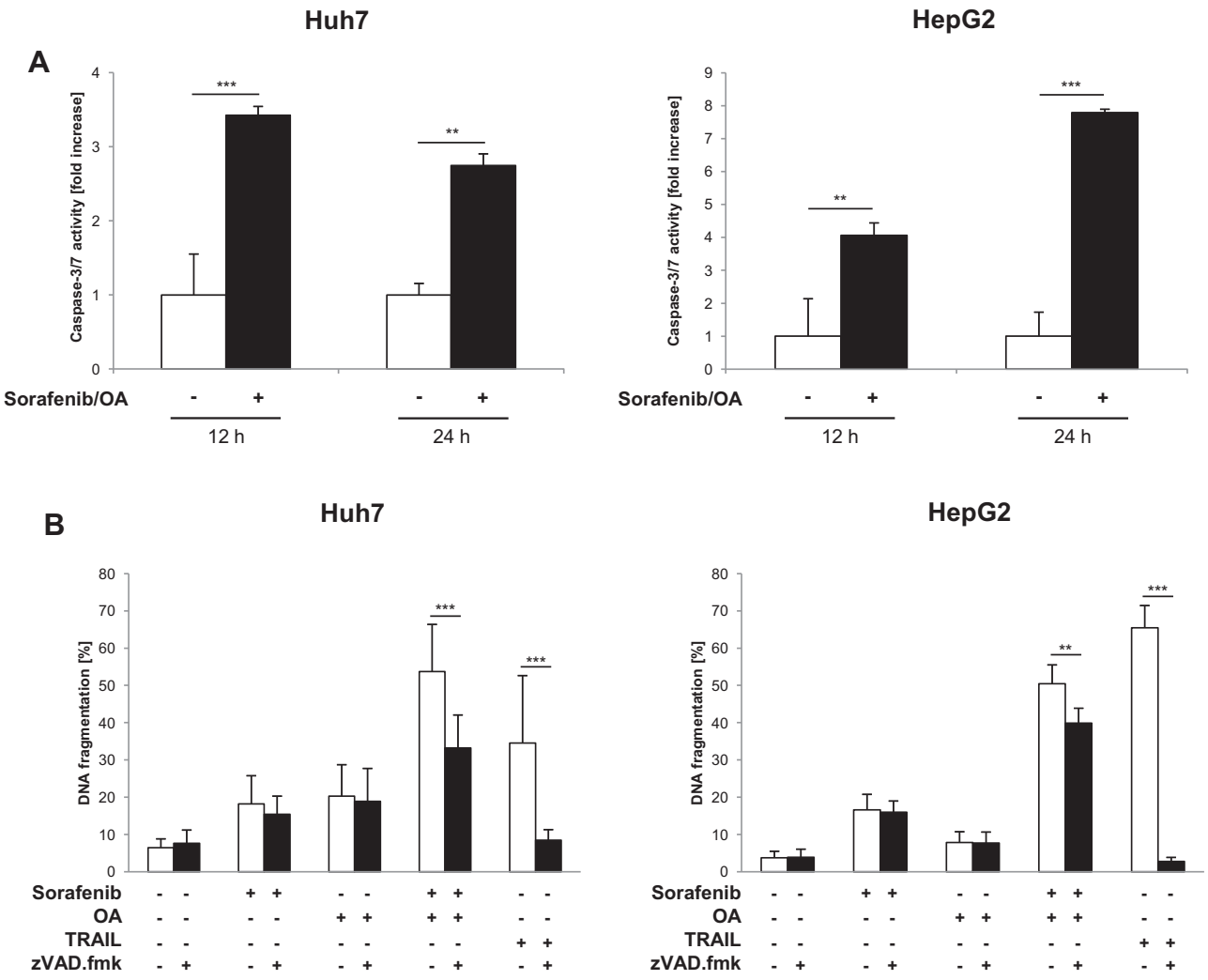

Fig. 2. Sorafenib and OA trigger caspase activation and caspase-dependent apoptosis. (A) HCC cells were cotreated for 12 h and 24 h with 5 μM Sorafenib and 60 μM OA. Caspase-3/7 activity was determined as described in Section 2 (white bar = control; black bar = Sorafenib and OA). (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM zVAD.fmk (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. As positive control, cells were treated for 48 h (Huh7) or 72 h (HepG2) with 40 ng/ml TRAIL in the presence or absence of 50 μM zVAD.fmk. Mean and SD of three independent experiments performed in triplicate are shown; $^{**}P < 0.01$; $^{***}P < 0.001$.

fragmentation or by PI-staining (Fig. 3A and B). This indicates that Sorafenib/OA cotreatment induces cell death independently of RIP1 kinase activity.

3.4. Lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner

Recently, it has been reported that Sorafenib can trigger ferroptosis [16–18]. Ferroptosis is a form of regulated cell death that is characterized by iron-dependent generation of lipid-based ROS and lipid peroxidation [17,19,20]. To investigate the role of lipid peroxidation in Sorafenib/OA co-treatment, we analyzed cell death in the presence and absence of Ferrostatin-1 (Fer-1), described as a small-molecule inhibitor of lipid peroxidation [21]. Addition of Fer-1 failed to rescue HCC cells from Sorafenib/OA-induced cell death as determined by DNA fragmentation in both cell lines and by PI-staining in the cell line Huh7, whereas Fer-1 partially rescued Sorafenib/OA-induced cell death as determined by PI-staining in HepG2 cells (Fig. 4A and B). To investigate whether Sorafenib/OA co-treatment stimulates lipid peroxidation, we used the fluorescent dye BODIPY-C11. Sorafenib/OA cotreatment significantly increased lipid peroxidation in HepG2, but not in Huh7 cells, which was completely blocked by the addition of Fer-1 (Fig. 4C). We also noted that treatment with OA alone induced lipid peroxidation in both cell lines, which could, however, not be blocked in the presence of Fer-1 (Fig. 4C). As positive control for lipid peroxidation-dependent cell death, we used Erastin that has been reported to trigger ferroptosis by blocking the cysteine/glutamate antiporter (system X_c^-) at the plasma membrane [19]. Fer-1 significantly decreased Erastin-stimulated lipid peroxidation and cell death in both cell lines (Fig. 4A–C). These findings indicate that lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner.

3.5. Sorafenib/OA cotreatment triggers ROS-dependent cell death

Different studies showed that ROS are involved in Sorafenib- or OA-induced cell death when they were used as single agents [6,8,10,22]. To investigate whether Sorafenib/OA cotreatment stimulates ROS production, we assessed ROS levels by using the ROS-sensitive fluorescent dye MitoSOX™ Red. Of note, Sorafenib alone and in combination with OA significantly increased ROS production in both Huh7 and HepG2 cells (Fig. 5A). To explore whether this increase in ROS production is critically required for

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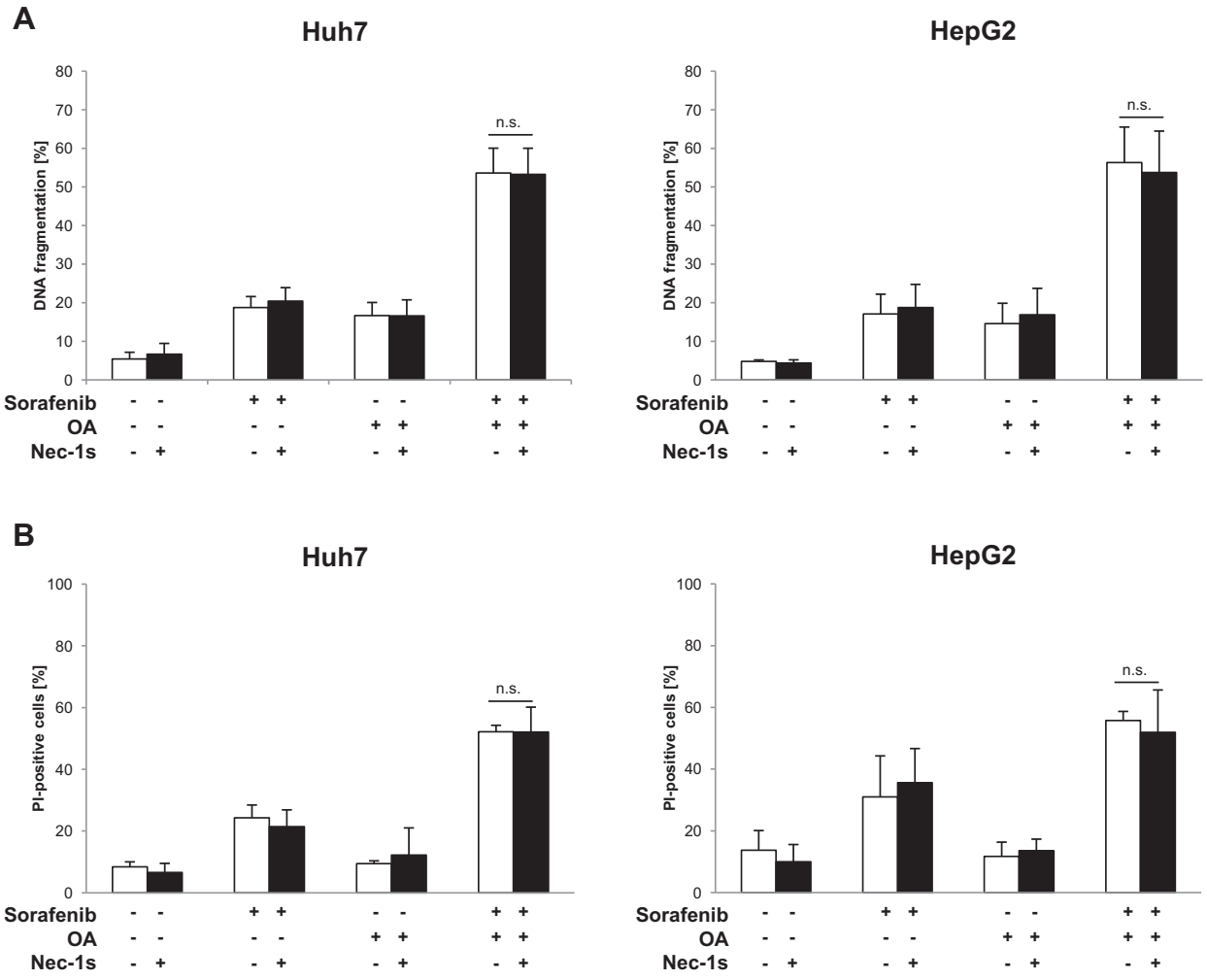

Fig. 3. RIP1 kinase activity is not required for Sorafenib/OA-induced cell death. (A) and (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 30 μM Nec-1s (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei (A) or FSC/SSC scatter analysis of PI-stained nuclei (B) using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; n.s. = not significant.

the induction of cell death, we blocked ROS production by using the ROS scavenger α-Tocopherol, a vitamin E derivative [23], and MnTBAP, a cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger [24]. Importantly, the addition of either α-Tocopherol or MnTBAP significantly reduced Sorafenib/OA-triggered DNA fragmentation in both cell lines (Fig. 5B). In addition, the combined use of both α-Tocopherol and MnTBAP almost completely rescued HCC cells from Sorafenib/OA-induced DNA fragmentation (Fig. 5C), consistent with a potent blockage of ROS production (Fig. 5A). This demonstrates that Sorafenib/OA cotreatment triggers ROS-dependent cell death.

4. Discussion

HCC is the most frequent primary liver cancer and one of the most aggressive tumors worldwide with an increasing incidence [3,25]. Dysregulation in the apoptotic program caused by different underlying liver diseases could explain chemotherapy resistance of HCC [26]. Due to its aggressive tumor growth, the majority of patients are in need of a palliative treatment. At present, Sorafenib is the treatment of choice for advanced HCC, but only shows a modest ability to extend the median survival [4]. Sorafenib resistance of HCC cells highlights the need for new strategies in HCC treatment.

In this study we identify a novel synergistic combination of Sorafenib and OA, which improves the efficacy of Sorafenib in HCC cells. In addition to increasing cell death in short-term assays, Sorafenib and OA also inhibit long-term clonogenic survival of HCC cells. Mechanistic studies showed that the combination of Sorafenib and OA triggers caspase-dependent cell death. This conclusion is supported by data showing that Sorafenib/OA cotreatment stimulates caspase activation and that the pan-caspase inhibitor zVAD.fmk partially prevents cell death. Furthermore, we demonstrate that Sorafenib/OA cotreatment leads to ROS production, which is required for cell death induction. In rescue experiments, ROS scavengers protect HCC cells from Sorafenib/OA-mediated cell death. Our findings are in line with recent publications reporting that ROS production is involved in OA-induced [8,22] or Sorafenib-induced cell death [6]. Different studies showed that Sorafenib inhibits the MEK/ERK pathway that controls ROS production in HCC [6,27,28]. Coriat et al. reported that Sorafenib dose-dependently stimulates ROS production in the human HCC cell line HepG2 and that the ROS scavenger MnTBAP significantly reduces the Sorafenib-mediated effect on tumor growth of HCC in mice experiments, emphasizing the relevance of ROS for the antitumor activity of Sorafenib [6]. This conclusion is underlined by an *in vivo* ROS analysis of sera from patients treated with Sorafenib, as the best response to Sorafenib has been reported for patients with high ROS levels during the treatment with Sorafenib [6].

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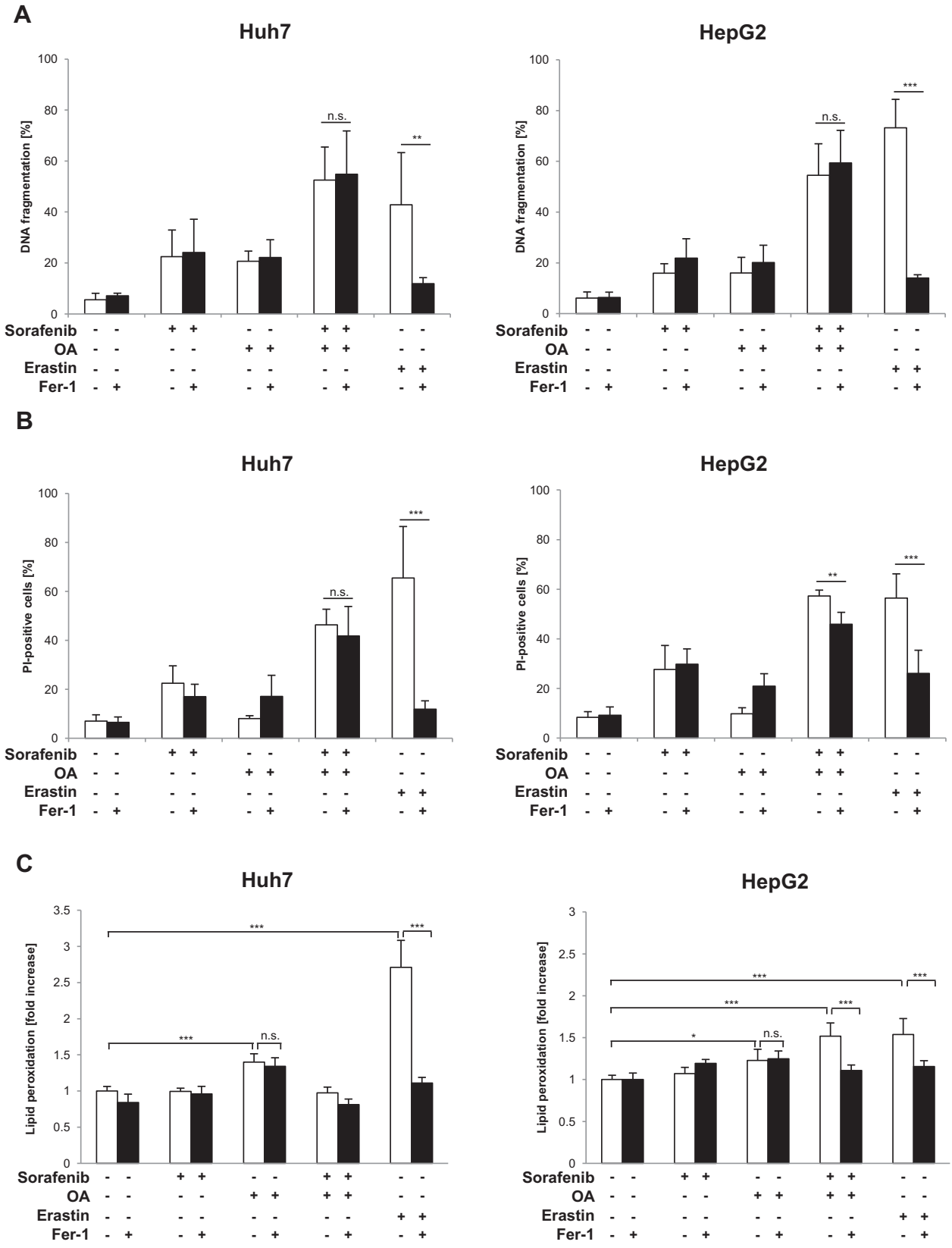

Fig. 4. Lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner. (A) and (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 10 μM Fer-1 (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei (A) or FSC/SSC scatter analysis of PI-stained nuclei (B) using flow cytometry. As positive control, cells were treated for 48 h (Huh7) or 72 h (HepG2) hours with 50 μM Erastin in the presence or absence of 10 μM Fer-1. C, HCC cells were treated for 24 h with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 10 μM Fer-1 (black bars). Lipid peroxidation was determined by BODIPY-C11 using flow cytometry. As positive control, cells were treated for 24 h with 50 μM Erastin in the presence or absence of 10 μM Fer-1. Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = not significant.

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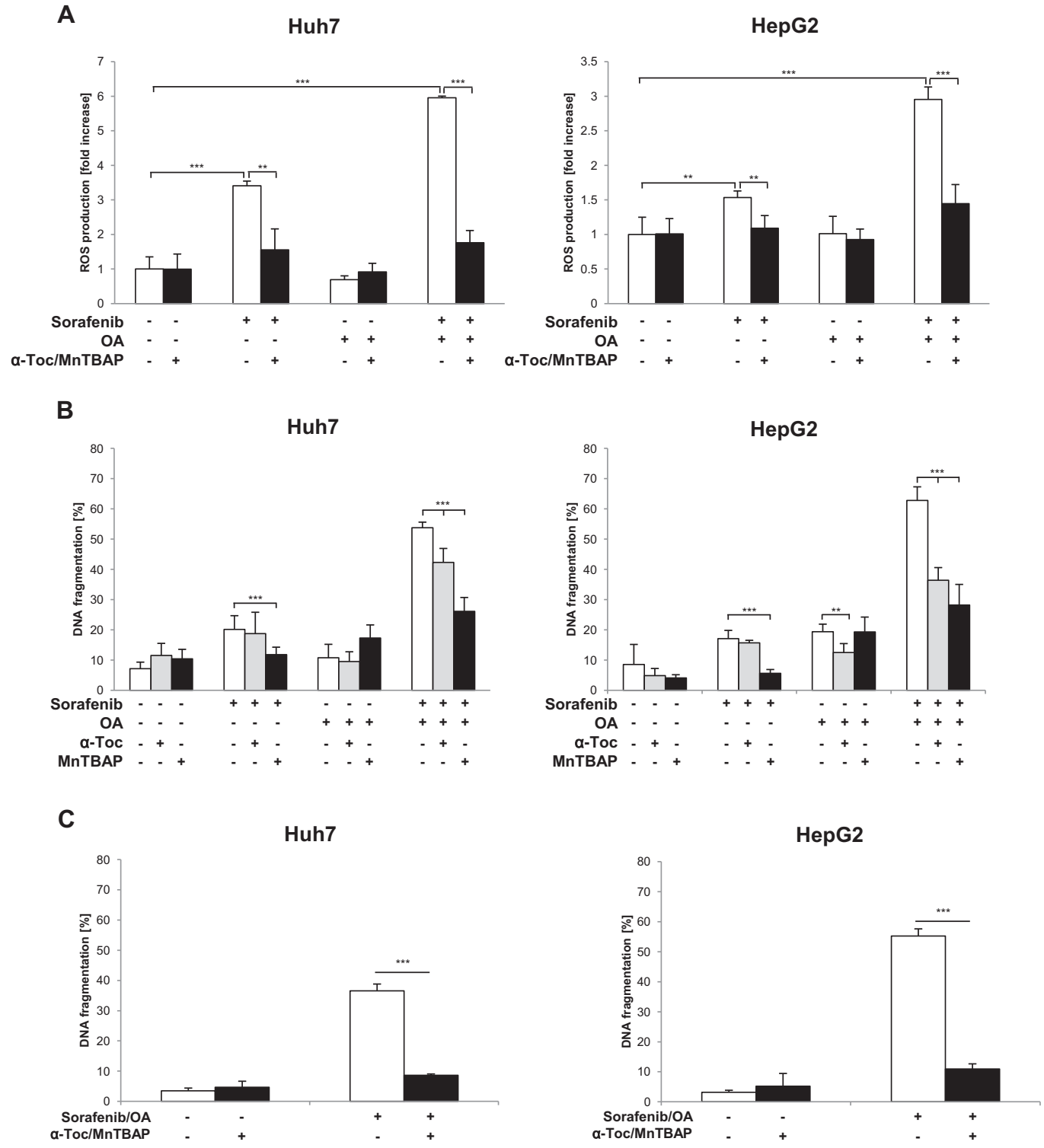

Fig. 5. ROS scavengers rescue Sorafenib/OA-induced cell death. (A) HCC cells were treated for 12 h with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of a combination of 50 μM α-Tocopherol and 300 μM MnTBAP (black bars). ROS production was determined by MitoSOX™ Red using flow cytometry. (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM α-Tocopherol (gray bars) or 300 μM MnTBAP (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. C, HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM α-Tocopherol and 300 μM MnTBAP (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; $^{**}P < 0.01$; $^{***}P < 0.001$.

Although the underlying mechanisms of the antitumor effects of OA remain largely unknown, several studies with various tumor types have stated that OA and its derivatives exert an inhibitory effect on tumor growth *in vitro* and *in vivo* and induce apoptosis [8,9,29–31]. Also, OA was reported to cause ROS generation in different cancer types [22,32,33]. In lung cancer cell lines, the synthetic OA derivate CDDO-Me has been described to induce endoplasmatic reticulum (ER) stress as well as upregulation of

death receptor-5 (DR5) and caspase activation [34]. In addition, OA and its synthetic derivatives were shown to activate the ERK pathway [8,32,35] and inhibition of ERK was shown to enhance the antitumor activity of OA in lung and pancreatic cancer cells, which was associated with increased ROS production [32]. In line with these findings, we show that combining subtoxic concentrations of OA, which do not yet stimulate ROS production, together with Sorafenib, which inhibits ERK [6,27], results in a significant increase of ROS production.

Several pathways of programmed cell death, including apoptosis and necroptosis, have been implicated during inflammation-associated tumorigenesis of HCC [36]. In prostate cancer Sorafenib has been reported to promote the interaction of p62 with RIP1 kinase leading to cell death by necroptosis [37]. Therefore, we investigated the role of necroptosis in Sorafenib/OA-induced cell death in HCC. However, so far we have no indication that Sorafenib/OA-induced cell death involves necroptosis, since inhibition of RIP1 kinase activity by Nec-1 failed to rescue HCC cells from Sorafenib/OA-induced cell death. In addition, RIP3 protein, another key component of necroptosis [38] and an inhibitor of inflammatory hepatocarcinogenesis [36], was not detectable by Western blotting in the HCC cell lines Huh7 and HepG2 [10]. Our findings do therefore not point to an involvement of necroptosis in Sorafenib/OA-induced cell death.

Another form of regulated cell death, which has been described in HCC, is ferroptosis occurs, for example, upon treatment with Sorafenib [16,17,39]. Ferroptosis involves iron-dependent accumulation of ROS and lipid peroxidation [19]. However, our data do not suggest that Sorafenib/OA-induced cell death is mediated by the increase of ROS from lipid peroxidation, as addition of Fer-1, which inhibits accumulation of ROS from lipid peroxidation [21], failed to consistently protect HCC cells from Sorafenib/OA-mediated cell death.

Sorafenib is since years in clinics [4] and its effects and toxicities are well known [5]. Also for OA, first clinical trials showed in lymphoma patients, a minimal toxicity (maximum orally tolerated dose 900 mg/day) and a good tumor response [9,40–42]. Furthermore, the antitumor activity and *in vivo* bioactivity of OA and its derivates were tested in different animal tumor models [9] and pharmacokinetic studies in humans were successfully performed [43,44]. Besides the antitumor effects of OA a hepatoprotective effect for acute hepatic damage and chronic liver disorders, e.g. viral hepatitis, is described [8,45]. We previously reported that the used concentration of OA exerted no detectable toxicity on a human hepatocyte cell line or primary human hepatocytes [10].

Target proteins of the Sorafenib/OA combination for induction of oxidative stress and its cascade for caspase activation and cell death have yet to be identified and investigated in further studies.

In the present study, we demonstrated for the first time that OA sensitizes HCC cells for Sorafenib. Since OA and its synthetic derivatives showed promising results in early clinical trials for the treatment of lymphoma [9,40,41], the Sorafenib/OA combination could be a new approach in the therapy of HCC.

Conflict of interest

None to declare.

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Cotreatment with sorafenib and oleanolic acid induces reactive oxygen species-dependent and mitochondrial-mediated apoptotic cell death in hepatocellular carcinoma cells

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Hepatocellular carcinoma (HCC) is the most common liver malignancy, and the lack of effective chemotherapies underlines the need for novel therapeutic approaches for this disease. Recently, we discovered a novel synergistic induction of cell death by combining sorafenib, the only routinely used palliative chemotherapeutic agent, and the triterpenoid oleanolic acid (OA). However, the underlying mechanisms of action have remained obscure. Here, we report that sorafenib and OA acted in concert to trigger mitochondria-mediated apoptotic cell death, which is dependent on reactive oxygen species (ROS). Sorafenib/OA cotreatment significantly increased ROS production, which was prevented by the ROS scavengers α-tocopherol and MnTBAP. Importantly, rescue experiments showed that ROS were required for sorafenib/OA-induced apoptosis as ROS scavengers protected HCC cells against cell death. In addition, sorafenib and OA cotreatment cooperated to decrease myeloid cell leukaemia-1 expression and to activate Bak, two events that were prevented by ROS scavengers. Bak activation was accompanied by the loss of mitochondrial membrane potential, followed by PARP cleavage, DNA fragmentation and, finally, apoptotic cell death in HCC cells. By providing new insights into the molecular regulation of sorafenib/OA-mediated and ROS-dependent cell death, our study contributes toward the development of novel treatment strategies to overcome sorafenib resistance in HCC. *Anti-Cancer Drugs* **00:000–000**

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Introduction

With an incidence rate of 80%, hepatocellular carcinoma (HCC) is the most common primary liver tumour and the second most frequent cause of cancer worldwide [1]. Chemotherapy resistance is still an unsolved problem. Sorafenib, an oral multikinase inhibitor, is the only first-line systemic treatment for patients with advanced HCC and can improve the median survival and time to radiologic progression by a few months [2–4]. Nevertheless, only a few patients experience a long-term benefit from this therapy [5]. The high rate of resistance significantly limits the benefits derived from expensive therapy with sorafenib [2]. Unfortunately, because of its toxicity and the compromised liver dysfunction caused by the underlying liver disease in HCC patients, sorafenib is reserved only for a limited group of patients [6]. Immunotherapy with anti-programmed cell death-1 antibodies has shown promising results [7–9]. However, only 10–20% of the patients respond to this new treatment [10]. These data highlight the need to identify new therapies and improve the efficacy of sorafenib in HCC cells.

In Chinese medicine, oleanolic acid (OA), a natural triterpenoid, has been used for many decades in the treatment of liver disorders, such as viral hepatitis, because of its hepatoprotective effect [11,12]. OA induces reactive oxygen species (ROS) production and cell death in HCC cells [12–14]. Different studies have shown that the imbalance in ROS homeostasis is crucial in hepatocarcinogenesis caused by chronic liver infection (e.g. hepatitis B, hepatitis C) [15]. The mitochondrion is one of the major sources of intracellular ROS [16], and the accumulation of ROS in tumour cells can activate mitochondria-mediated apoptotic cell death [16,17]. One important group of proteins involved in regulating mitochondrial apoptotic cell death is the B-cell lymphoma 2 (Bcl-2) family of proteins [18]. One of the antiapoptotic proteins of the Bcl-2 family is the myeloid cell leukaemia sequence 1 protein (Mcl-1),

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which is often overexpressed in solid tumours [18,19]. Two multidomain proteins of the Bcl-2 family are Bax and Bak, which play an important role in the control of the mitochondrial membrane potential (MMP) [19,20].

Recently, we identified a novel synergistic combination composed of sorafenib and OA, which improved the efficacy of sorafenib in HCC cells [21]. The aim of this study was to investigate the underlying mechanisms of action of sorafenib/OA cotreatment.

Methods

Cell culture and reagents

The human HCC cell lines Huh7 and HepG2 were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in DMEM (high glucose, GlutaMAX; Life Technologies Inc., Eggenstein, Germany), supplemented with 10% foetal calf serum (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1 mmol/l sodium pyruvate (Invitrogen). All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO_2. Sorafenib was obtained from Cell Signaling Technology (Leiden, The Netherlands), OA from Sigma-Aldrich (Darmstadt, Germany) and MnTBAP from Calbiochem (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich or Carl Roth (Karlsruhe, Germany) unless indicated otherwise.

Determination of cell death, cell viability, reactive oxygen species production and mitochondrial membrane potential

Cell death was determined by the analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei or forward/side scatter (FSC/SSC) analysis of PI-stained nuclei using flow cytometry (FACSCanto II; BD Biosciences, Heidelberg, Germany) as described previously [22]. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). To analyse ROS production, we incubated cells with 1 µmol/l CellROX (Invitrogen) or 5 µmol/l MitoSOX Red mitochondrial superoxide indicator (Molecular Probes Inc., Eugene, Oregon, USA) according to the manufacturer's protocol. ROS production was measured by flow cytometry. To measure the MMP, we incubated cells with 100 ng/ml tetramethylrhodamine methylester (Sigma-Aldrich) for 30 min at 37°C and immediately analysed them by flow cytometry.

Western blot analysis

Western blot analysis was carried out as described before [22] using the following antibodies: mouse anti-α-tubulin (Calbiochem), mouse anti-β-actin (Sigma-Aldrich), rabbit anti-Bak (BD Bioscience, Heidelberg, Germany), rabbit anti-Mcl-1 (Enzo Life Science, Lörrach, Germany), mouse anti-PARP (Cell Signaling Technology) and mouse anti-Vinculin (Sigma-Aldrich). Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, California, USA) were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown.

Immunoprecipitation of activated Bak and Bax

For the detection of activated Bax or Bak, we lysed cells in CHAPS lysis buffer (10 mmol/l HEPES (pH 7.4); 150 mmol/l NaCl; 1% CHAPS) as described previously [23]. Briefly, 1000 µg protein was immunoprecipitated and incubated overnight at 4°C with 2 µg/ml mouse anti-Bax antibody (6A7; Sigma-Aldrich) or mouse anti-Bak antibody (Ab-1; Calbiochem) and 10 µl pan-mouse IgG Dynabeads (Dako, Hamburg, Germany), and washed with CHAPS buffer. The precipitate was analysed for Bax and Bak expression by western blotting using the rabbit anti-Bax NT antibody (Merck, Darmstadt, Germany) or rabbit anti-Bak antibody (BD Biosciences). Representative blots of at least two independent experiments are shown.

RNA interference

Gene silencing was performed using Silencer Select validated small interfering RNA (siRNAs) against Bak (s1880 and s1881) and nontargeting control siRNA (4390843) from Invitrogen following the manufacturer's instructions.

Statistical analysis

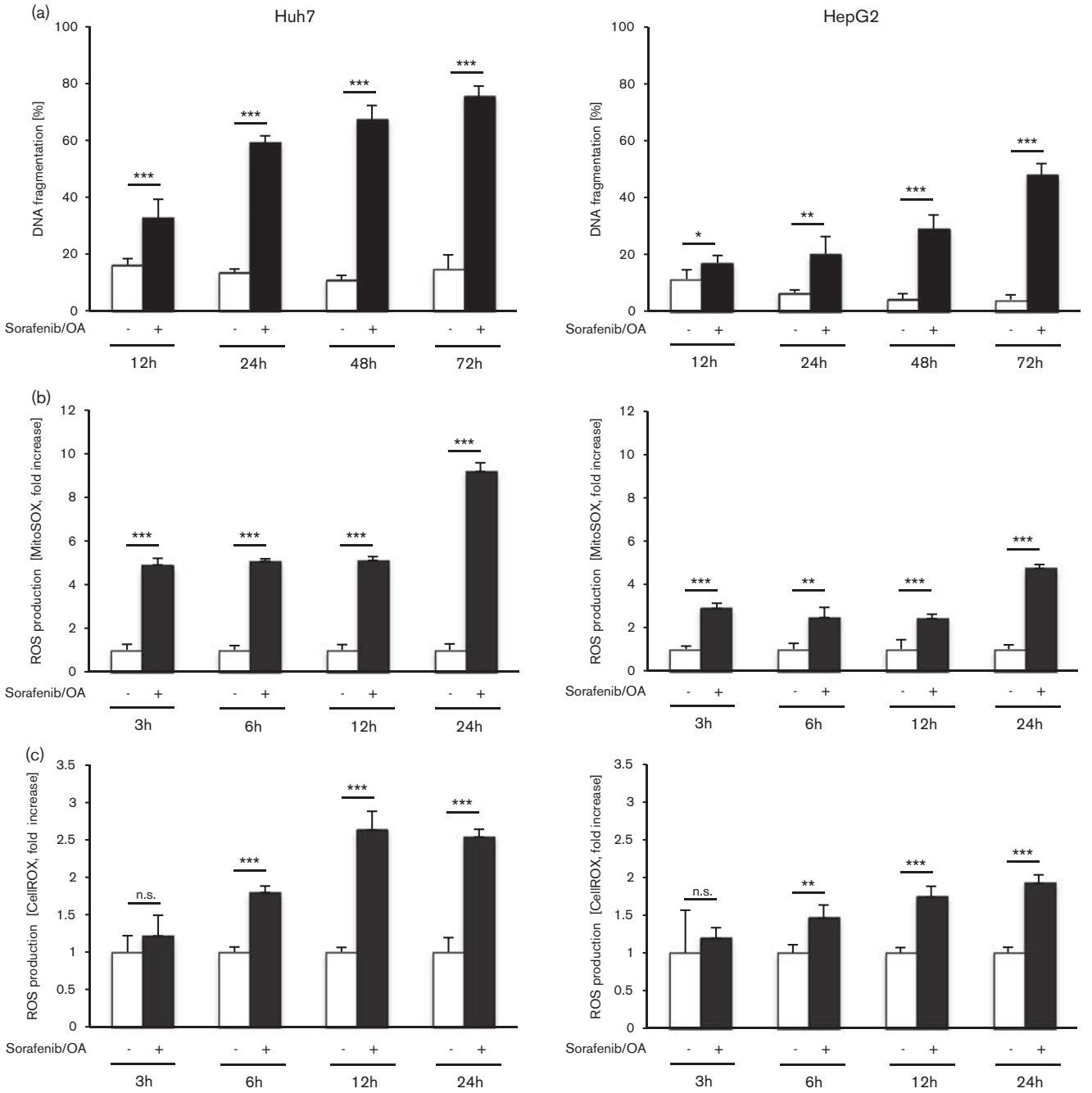
Statistical significance was assessed using Student's *t*-test (two-tailed distribution, two sample, unequal variance).

Results

Sorafenib and oleanolic acid cotreatment synergistically induce cell death and reactive oxygen species production in hepatocellular carcinoma cells

Sorafenib and OA acted together to trigger DNA fragmentation, a typical marker of apoptotic cell death, in a time-dependent manner in two different human HCC cell lines, namely, Huh7 and HepG2 (Fig. 1a). In addition, the synergistic induction of cell death by sorafenib/OA cotreatment was confirmed by the analysis of PI-stained nuclei using flow cytometry (Supplementary Fig. 1A, Supplemental digital content 1, *http://links.lww.com/ACD/A291*). Sorafenib and OA synergistically reduced cell viability on the basis of another assay (Supplementary Fig. 1B, Supplemental digital content 1, *http://links.lww.com/ACD/A291*). To investigate whether ROS are produced upon sorafenib/OA cotreatment, we used two different ROS-sensitive fluorescent dyes, namely, MitoSOX Red and CellROX. ROS production was detected at three hours of sorafenib/OA cotreatment in both cell lines (Fig. 1b) before the induction of cell death (Fig. 1a). Higher ROS levels were detected by MitoSOX Red staining (Fig. 1b), a dye for the selective detection of mitochondrial ROS [24,25]. Sorafenib/OA cotreatment also

Fig. 1



Kinetics of sorafenib/OA-induced cell death and ROS production. HCC cells were treated with 5 μmol/l sorafenib and 60 μmol/l OA at the indicated time-points. Cell death was determined by the analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (a). ROS production was determined by MitoSOX Red (b) or CellROX (c) using flow cytometry. The mean and SD of three independent experiments conducted in triplicate are shown; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. HCC, hepatocellular carcinoma; OA, oleanolic acid; ROS, reactive oxygen species.

increased ROS production as detected by CellROX (Fig. 1c), a fluorescent dye that measures ROS in the cytoplasm of live cells [26]. These experiments showed that sorafenib/OA cotreatment leads to ROS production, followed by DNA fragmentation.

Reactive oxygen species scavengers rescue sorafenib/oleanolic acid-induced cell death

Next, we asked whether ROS are required for the induction of cell death. To address this question, we used the ROS scavengers α-tocopherol, a vitamin E derivative [27], and

MnTBAP, a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger [28]. Importantly, sorafenib/OA-induced DNA fragmentation was significantly reduced in the presence of α-tocopherol and MnTBAP (Fig. 2a). In parallel, the combined use of α-tocopherol and MnTBAP almost completely suppressed the sorafenib/OA-stimulated generation of ROS, as detected by MitoSOX, in both cell lines (Fig. 2b), while it significantly reduced sorafenib/OA-induced production of cytoplasmic ROS in Huh7, but not in HepG2 cells (Fig. 2c). This indicated that ROS contribute towards sorafenib/OA-induced cell death.

Sorafenib and oleanolic acid cooperate to trigger the mitochondrial pathway by myeloid cell leukaemia-1 reduction, Bak activation and mitochondrial membrane potential loss

Because of the production of mainly mitochondrial ROS (Fig. 1b), we hypothesized that sorafenib/OA cotreatment induces apoptotic cell death in HCC cells through the mitochondrial pathway. To test this hypothesis, we analysed the role of Mcl-1, an antiapoptotic protein of the Bcl-2 family, by western blot analysis. Sorafenib and OA acted in concert to decrease Mcl-1 expression (Fig. 3). In HepG2 cells, not only the cotreatment of sorafenib and OA but also sorafenib monotherapy led to a decrease in Mcl-1 expression. To analyse the ROS dependency of this Mcl-1 decrease, we added ROS scavengers. Interestingly, the addition of α-tocopherol and MnTBAP prevented the sorafenib/OA-triggered decrease in Mcl-1 protein levels in both cell lines (Fig. 3).

To answer the question of whether sorafenib/OA cotreatment leads to Bak and Bax activation, we immunoprecipitated Bak and Bax using conformation-specific antibodies as conformational changes mark their activation [29,30]. Interestingly, sorafenib/OA cotreatment triggered Bak activation in Huh7 cells as detected by immunoprecipitation with a conformation-specific antibody (Fig. 4a) and by ELISA (Fig. 4b), whereas no increase in Bak activation upon sorafenib/OA cotreatment was observed in HepG2 cells (Fig. 4a and b). Notably, the addition of α-tocopherol and MnTBAP rescued cells from sorafenib/OA-triggered Bak activation in Huh7 cells (Fig. 4c). By comparison, we detected no Bax activation upon sorafenib/OA cotreatment in either cell line (Fig. 4a).

To investigate the functional relevance of Bak activation in sorafenib/OA-mediated apoptosis, we genetically silenced the Bak gene using two distinct siRNA oligonucleotides (Fig. 5). Remarkably, knockdown of Bak significantly reduced sorafenib/OA-induced apoptosis in Huh7 cells (Fig. 5b), in line with sorafenib/OA-stimulated Bax activation in these cells (Fig. 4a and b). In HepG2 cells, however, Bak silencing had no effect on sorafenib/OA-induced cell death (Fig. 5b) despite the downregulation of Bak expression (Fig. 5a), consistent with the lack of Bak activation in these cells (Fig. 4a and b).

As Bax and Bak activation leads to the permeabilization of the outer mitochondrial membrane, we next measured the loss of MMP. Interestingly, sorafenib/OA cotreatment significantly enhanced the loss of MMP compared with no treatment (Fig. 6). The mitochondrial uncoupler FCCP was used as a positive control (Fig. 6). Together, these findings emphasized that sorafenib/OA cotreatment activates the mitochondrial pathway of apoptosis.

Sorafenib/oleanolic acid cotreatment is associated with enhanced PARP cleavage

To further illustrate the downstream events of sorafenib/OA-induced mitochondria-mediated apoptosis, we analysed PARP cleavage as a typical feature of apoptosis. Indeed, the cleavage of PARP was enhanced in sorafenib/OA-cotreated cells compared with that in untreated control cells or cells treated with sorafenib or OA alone (Fig. 7). This underscored that sorafenib/OA cotreatment activates apoptotic pathways in HCC cells.

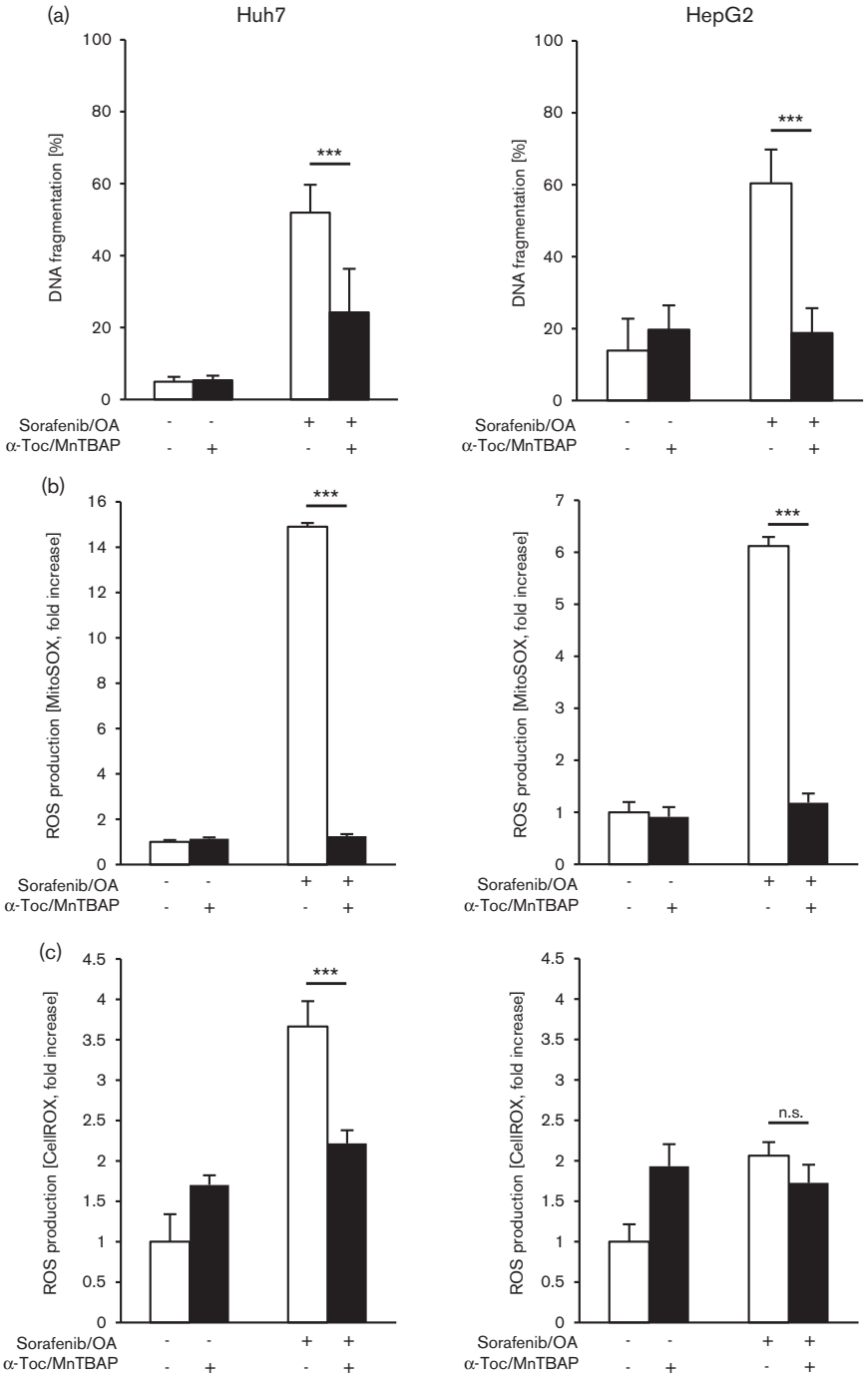
Discussion

Up to 70% of patients with HCC are not eligible for curative surgical therapy or percutaneous ablation [31]. Therefore, systemic therapy is recommended for these patients [5,9]. New second-line therapies, for example lenvatinib or regorafenib, can improve clinical outcomes, although the median overall survival remains poor [9]. The only Food and Drug Administration-approved first-line systemic therapy for HCC is sorafenib, which improves the median survival and time to radiologic progression by up to 2.8 months compared with placebo [2]. However, primary resistance to sorafenib is observed in a quarter of patients [2,6]. These findings highlight the need for developing novel strategies to improve the efficacy of sorafenib in the treatment of HCC.

As the imbalance in ROS in HCC seems to be involved in tumour progression and associated with the prognosis of patients with HCC [32], the modulation of ROS production could be a promising tool for new therapeutic strategies. Recently, we showed that ROS are involved in sorafenib-induced and OA-induced cell death [14,21]. Different studies confirmed that ROS are also involved in sorafenib-induced or OA-induced cell death when used as single agents in different cancer types [12,33–37].

In the present study, we show that OA enhances the efficacy of sorafenib by altering redox regulation in HCC cells. Here, we report that cotreatment with sorafenib and OA induces ROS-dependent and mitochondria-mediated cell death in HCC. This conclusion is drawn on the basis of the following experimental evidence: first, sorafenib/OA-triggered ROS production in HCC cells before cells started to undergo cell death. This ROS generation was required for the induction of cell death as ROS scavengers that prevented sorafenib/OA-stimulated ROS production also protected cells from death. Second, sorafenib/OA cotreatment decreased Mcl-1 expression in a

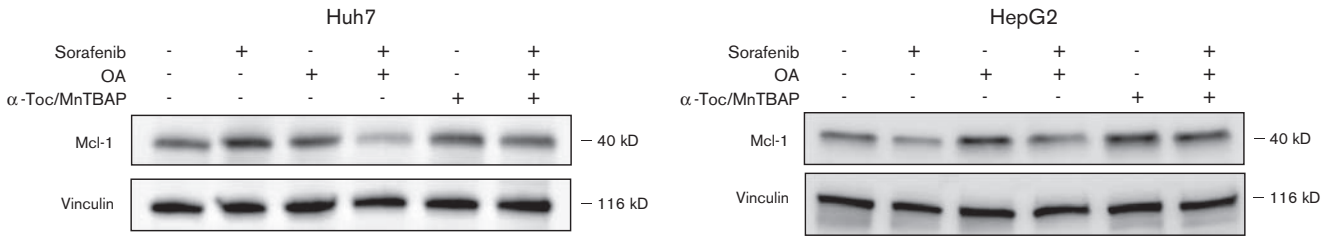
Fig. 2



ROS scavengers rescued sorafenib/OA-induced cell death and decreased ROS production. HCC cells were treated with 5 μmol/l sorafenib and 60 μmol/l OA in the presence or absence of a combination of 50 μmol/l α-tocopherol and 300 μmol/l MnTBAP. Cell death was determined after 48 h (Huh7) or 72 h (HepG2) by the analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (a). ROS production was determined after 24 h by MitoSOX Red (b) or CellROX (c). The mean and SD of three independent experiments conducted in triplicate are shown; ***$P < 0.001$. HCC, hepatocellular carcinoma; OA, oleanolic acid; ROS, reactive oxygen species.

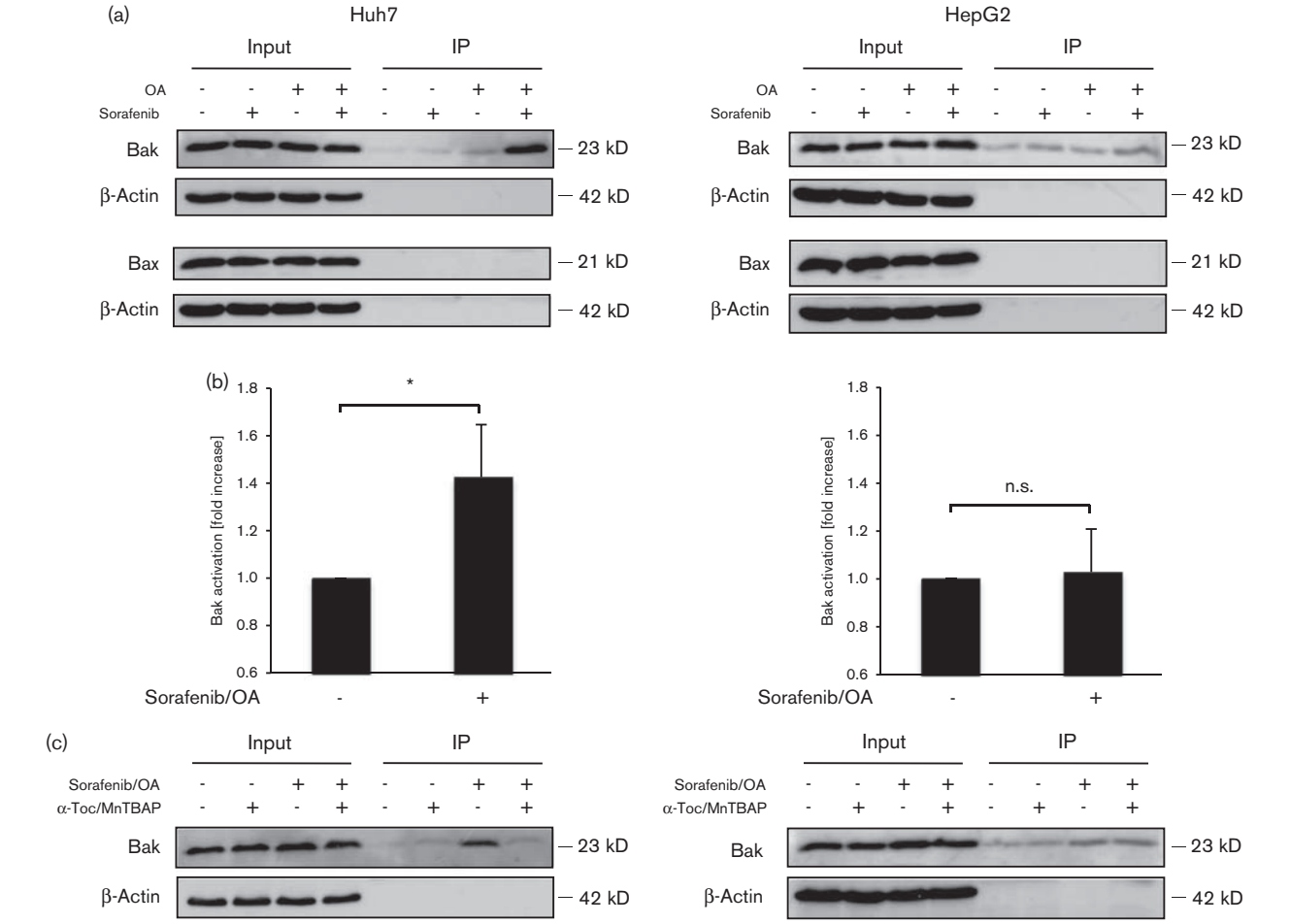
ROS-dependent manner. Third, sorafenib/OA cotreatment led to Bak activation, which was decreased by preventing ROS production. Bak has also been reported to be sequestered by Mcl-1 [38]. The decrease in Mcl-1 upon sorafenib/OA cotreatment may contribute towards Bak activation. Fourth, genetic inhibition of Bak significantly rescued HCC cells from sorafenib/OA-induced cell death. Fifth, sorafenib and OA acted in concert to

Fig. 3



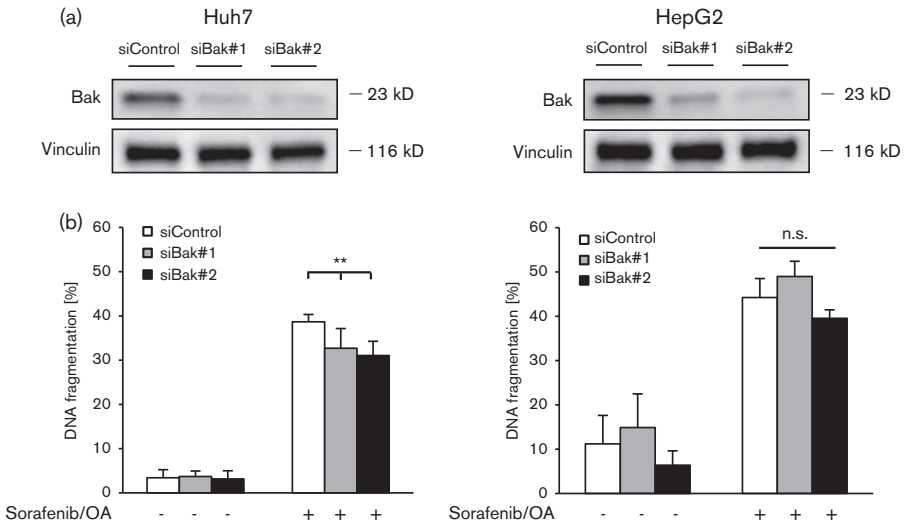
Sorafenib/OA cotreatment leads to Mcl-1 downregulation. HCC cells were treated for 18 h with 5 μmol/l sorafenib and/or 60 μmol/l OA in the presence or absence of a combination of 50 μmol/l α-tocopherol and 300 μmol/l MnTBAP. Mcl-1 protein expression was analysed by western blotting. Representative blots are shown. HCC, hepatocellular carcinoma; Mcl, myeloid cell leukaemia; OA, oleanolic acid.

Fig. 4



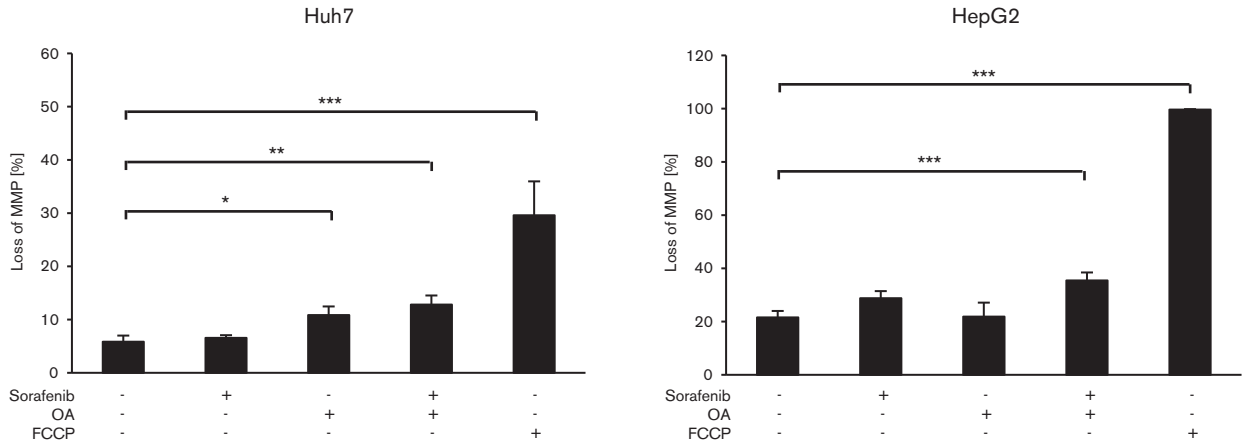
Sorafenib/OA cotreatment leads to Bak activation. HCC cells were treated for 12 h (Huh7) or 24 h (HepG2) with 5 μmol/l sorafenib and/or 60 μmol/l OA. The activation of Bak and Bax was analysed by immunoprecipitation using active conformation-specific anti-Bax or anti-Bak antibodies, and the protein expression of Bak, Bax and β-actin was analysed by western blotting (a). Representative blots are shown. The detection of Bak was performed using an enzyme-linked immunosorbent assay (b). HCC cells were treated for 12 h (Huh7) or 24 h (HepG2) with 5 μmol/l sorafenib and 60 μmol/l OA in the presence or absence of a combination of 50 μmol/l α-tocopherol and 300 μmol/l MnTBAP. The activation of Bak and Bax was analysed by immunoprecipitation using active conformation-specific anti-Bax or anti-Bak antibodies, and the protein expression of Bak, Bax and β-actin was analysed by western blotting (c). Representative blots are shown. The mean and SD of three independent experiments conducted in triplicate are shown; $^*P < 0.05$. HCC, hepatocellular carcinoma; OA, oleanolic acid.

Fig. 5



Knockdown of Bak rescues HCC cells from sorafenib/OA-induced cell death. Cells were transfected transiently with siRNAs targeting Bak (siBak#1–2) or control siRNA (siControl), and the expression of Bak was analysed by western blotting (a). Representative blots are shown. Transiently transfected HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μmol/l sorafenib and 60 μmol/l OA. Cell death was determined by the analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (b). The mean and SD of three independent experiments conducted in triplicate are shown; $^{**}P < 0.01$. HCC, hepatocellular carcinoma; OA, oleanolic acid.

Fig. 6

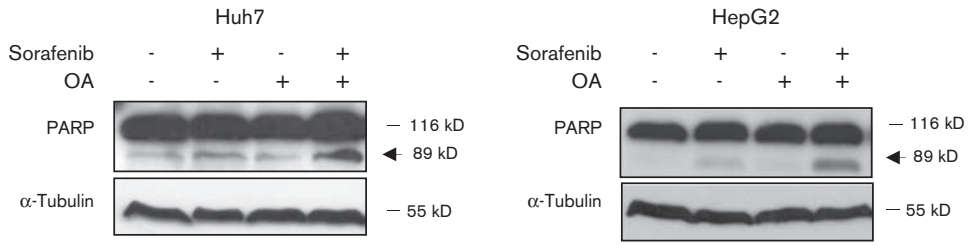


Sorafenib/OA cotreatment leads to the loss of mitochondrial membrane potential (MMP). HCC cells were treated for 12 h (Huh7) or 24 h (HepG2) with 5 μmol/l sorafenib and/or 60 μmol/l OA. The loss of MMP was assessed by tetramethylrhodamine methylester (TMRM) staining and flow cytometry. The percentage of cells with low TMRM fluorescence is shown. As a positive control, cells were treated with 100 μmol/l FCCP. The mean and SD of three independent experiments conducted in triplicate are shown; $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$. HCC, hepatocellular carcinoma; OA, oleanolic acid.

trigger MMP loss, PARP cleavage, a typical caspase-3 substrate [39] and DNA fragmentation, a characteristic marker of apoptotic cell death.

In recent years, natural products have been identified as promising cancer therapeutics to prevent tumourigenesis and tumour growth [40]. An important group of natural compounds is the triterpenoids, which are distributed widely in plants [11,13]. Previous studies have shown that OA and its derivatives inhibit angiogenesis and tumour growth *in vitro* and *in vivo*, and induce apoptotic cell death in different tumour types [12,34,41–45]. Recently, we described an antitumour effect *in vitro* and *in vivo* by combining the Smac mimetic BV6, which antagonizes inhibitor of apoptosis proteins [46] and OA in human HCC cells [14]. To date, the various

Fig. 7



Sorafenib/OA cotreatment leads to PARP cleavage. HCC cells were treated for 12 h (Huh7) or 24 h (HepG2) with 5 μmol/l sorafenib and/or 60 μmol/l OA. PARP protein expression was analysed by western blotting. Arrows indicate PARP fragments. Representative blots are shown. HCC, hepatocellular carcinoma; OA, oleanolic acid.

pharmacological effects of OA remain incompletely understood. The modulation of apoptotic pathways by OA or its derivatives is reported to occur by the extrinsic death receptor pathway with caspase activation [47] and by the intrinsic mitochondrial pathway. OA contributes towards the release of cytochrome *c* by disrupting the mitochondrial membrane and inhibits antiapoptotic Bcl-2 proteins [12,21,34,44,45]. In the present study, we showed typical features of mitochondrial apoptosis upon cotreatment with sorafenib and OA, that is, the production of mitochondrial ROS, activation of Bak and loss of MMP, leading to apoptotic cell death in HCC cells. In a recent study, we showed that the combination of sorafenib and OA led to caspase-3/7 activation and that the pan-caspase inhibitor zVAD.fmk partially prevented sorafenib/OA-mediated cell death, which underlined the caspase dependency of this cotreatment [21]. In our study, the relevance of ROS production for the antitumour activity of sorafenib/OA against HCC cells was highlighted by different rescue experiments. Different studies showed that sorafenib significantly enhances ROS production [35,37] and that the clinical effectiveness of sorafenib is correlated significantly with ROS levels measured in sera from HCC patients [33].

Our study showed that OA increases the therapeutic efficacy of sorafenib and goes along with using both compounds, OA and especially sorafenib, at subtoxic concentrations. In addition, from this point of view, the combination of sorafenib with the natural compound OA appears to be promising. The need to lower the doses of sorafenib is underlined by the results of the SHARP trial: in nearly 30% of all cases, a dose reduction because of sorafenib-related adverse events was necessary [2]. However, this dose reduction leads to suboptimal dosing. Different studies have shown that the combination of sorafenib with various chemotherapies or conventional cytotoxic drugs leads to various toxicities [6]. This makes combination therapy almost impossible. OA and its derivatives have a promising safety profile and have been used in Chinese medicine for many decades in the treatment of liver disorders such as viral hepatitis [12,13, 48,49]. In addition to the antitumour effects of OA, a hepatoprotective effect against acute hepatic damage and chronic liver disorders has been described for OA [11,12, 50]. Nonetheless, before clinical use, further investigations in animal models are necessary.

Taken together, our data have important implications for the development of strategies for enhancing the efficacy of sorafenib. First and foremost, cotreatment with sorafenib and OA could be a promising approach for overcoming sorafenib resistance in HCC. Second, combination treatment with sorafenib/OA may enable the use of both compounds at subtoxic concentrations. Third, addressing ROS homeostasis using new compounds could provide new treatment strategies to reactivate cell death in HCC.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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ORIGINAL ARTICLE

A possible role of microRNAs as predictive markers for the recurrence of hepatocellular carcinoma after liver transplantation

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Summary

With favourable 5-year survival rates up to 75%, liver transplantation (LT) is the treatment of choice for hepatocellular carcinoma (HCC). Nonetheless, tumour recurrence after LT remains a challenge. The aim of this retrospective study was to develop a predictive score for tumour recurrence after LT by combining clinical parameters with HCC biomarkers (microRNA). A microRNA (miRNA) microarray analysis was used to compare miRNA expression patterns in tissue samples of 40 patients with and without HCC recurrence after LT. In a screening cohort (n = 18), the miRNA analysis identified significant differences in the expression of 13 miRNAs in patients with tumour recurrence. Using the most significant miRNAs in this screening cohort, we could develop a predictive score, which combined the expression levels of miR-214, miR-3187 and the Milan criteria, and we could define low- and high-risk groups for tumour recurrence and death. The above score was evaluated in a second and independent cohort (n = 22). In contrast to the Milan criteria alone, this score was significantly associated with tumour recurrence. Our analysis indicated that the use of a specific miRNA expression pattern in combination with a limited tumour burden as defined by the Milan criteria may lead to a more accurate prediction of tumour recurrence.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide, with an increasing incidence in Western countries [1–3]. Eighty per cent of HCC incidence occurs in cirrhosis, which is mainly caused by chronic viral hepatitis and alcoholic liver disease [1]. However, in only 20–30% of all patients is curative therapy possible [1,4]. Liver transplantation (LT) is often the treatment of choice for HCC and is associated with high rates of long-term survival [5,6]. Despite the careful selection of patients with

HCC for LT using the Milan criteria (a single tumour ≤5 cm or up to three tumours, each ≤3 cm, with no macrovascular invasion) [5], tumour recurrence rates of up to 25% remain an unsolved problem [7].

However, the Milan criteria used for the allocation for LT are based primarily on radiologic findings and do not consider tumour biology [8]. Furthermore, in up to 25% of the patients, the size of the tumour, the number of tumours and the vascular invasion are underestimated in radiological imaging [9]. Therefore, the combination of clinical–radiological findings with biomarkers, such as microRNAs (miRNA), could be a valuable tool for selecting transplant candidates.

MicroRNAs are small, noncoding RNAs that are responsible for the regulation of targeted gene expression [10]. Recent studies have shown that the dysregulation of certain miRNAs plays a crucial role in tumourigenesis and cancer progression. In HCC, various studies have identified dysregulated miRNAs and their effects on prognosis, tumour progression and recurrence [11,12]. Knowledge regarding dysregulated miRNAs and their target genes in HCC such as β-catenin, fibroblast growth factor receptor-1 (FGF-1), matrix metalloproteinase (MMP) and mTOR [13–15] may lead to the development of novel therapeutic strategies [16].

Few studies exist regarding the identification of HCC recurrence-related miRNA expression after LT. A miRNA signature consisting of miR-19a, miR-886-5p, miR-126, miR-223, miR-24 and miR-247 was observed to be an independent predictor of recurrence-free survival after LT [17]. In another study, miR-155 was found to be upregulated in patients with HCC recurrence after LT compared with patients without HCC recurrence. Furthermore, miR-155 was found to correlate with the invasiveness of HCC cells [18]. Barry *et al.* [19] defined a biomarker consisting of 67 miRNAs that outperformed the Milan criteria for assessing the risk of tumour recurrence after LT.

However, there is still considerable heterogeneity in the published dysregulated miRNAs and miRNA signatures, which is most likely caused by multiple factors, including differences in underlying liver disease (viral, alcohol or mixed aetiologies) and different tumour stages [20].

The aim of the present study was to identify a recurrence-specific pattern of miRNAs and combine these miRNAs with clinical markers (e.g. Milan criteria and AFP) to develop a high predictive score for disease-free survival after LT for HCC.

Methods

Patients and samples

This was a retrospective analysis, which was approved by the local Ethics Committee of the University Hospital of Frankfurt, Germany (Institutional Review Board No. 342/13). A total of 92 patients undergoing LT for HCC in our surgical department between 2007 and 2012 were included. The data collected were demographic and clinicopathologic features, including age, gender, serum AFP levels before LT, highest AFP level during the waiting period (peak AFP), radiologic criteria, intra- and postoperative course, tumour recurrence rate, overall survival and disease-free survival data. The Milan criteria (a single tumour ≤5 cm or up to three nodules, each ≤3 cm and no macrovascular invasion) and UCSF criteria (University of California-San-Francisco criteria; a solitary tumour ≤6.5 cm or ≤3 nodules with the largest lesion ≤4.5 cm and a total diameter ≤8 cm) were determined by CT scan or MRI in initial imaging and by the histopathologic evaluation of explanted livers.

For the miRNA analysis (microarray analysis and PCR) of tissue samples (explanted livers), all patients ($n = 40$) fulfilled the following enrolment criteria: (i) HCC staged within the Milan or UCSF criteria ($n = 72$) in histopathologic examinations of the explanted livers, (ii) availability of survival data ($n = 91$), (iii) presence of viable formalin-fixed paraffin-embedded (FFPE) tumour material with a tumour necrosis rate <30% ($n = 47$) and (iv) sufficient quality of extracted RNA for further quantitative real-time reverse-transcription PCR (Q-RT-PCR, $n = 40$) and a minimum follow-up of 12 months ($n = 80$). An experienced pathologist (M.L.H.) confirmed the diagnosis of HCC. Grading and staging were assessed according to the current tumour–node–metastases (TNM) classification of malignant tumours. A pathologist reviewed all of the specimens microscopically. The tissue areas with a tumour cell content of more than 95% were macrodissected and used for further analyses.

Isolation of miRNA

Total RNA was extracted from tumour FFPE specimens using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) following the manufacturers protocol. RNA quantification was performed using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, USA) using 100–300 ng of total RNA for further analyses.

Microarray analysis

A global miRNA expression profiling analysis of 1105 mature miRNAs using GENECHIP® MIRNA ARRAY v2.0 (Affymetrix, Santa Clara, CA, USA) was performed following the manufacturer's protocol. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession

number GSE64989 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64989).

Real-time PCR miRNA expression analysis

TaqMan® MicroRNA Q-RT-PCR assays (Applied Biosystems, Darmstadt, Germany) were used to quantify miRNAs according to the manufacturer's protocol. Expression was analysed for five miRNAs (hsa-miR-140, hsa-miR-214, hsa-miR-455, hsa-miR-3188 and hsa-miR-3187) and one endogenous control (U6). Samples were analysed in triplicate, and ΔCT values were calculated using the endogenous control.

Bioinformatics and statistical analyses

The clinical and biochemical characteristics of the patients were expressed as the mean ± standard deviation or as the median and range as appropriate. Unless indicated otherwise, all tests were two-tailed, and P values <0.05 were considered significant.

The statistical analysis of the miRNA profiles was performed using the statistical computing environment R (R Foundation for Statistical Computing, Vienna, Austria). Additional software packages (GENEPLOTTER, GPLOTS) were obtained from the Bioconductor project. Replicate correlation was calculated using Pearson's correlation coefficient and depicted as a scatterplot. Unsupervised hierarchical clustering was performed for miRNAs with a standard deviation ≥1.0 across all samples using the Manhattan distance method and the average linkage method. For the supervised analysis, expression intensity and variance filtering were used to reduce the dimensions of the microarray data. The data were filtered by an intensity filter (gene intensity >100 in at least 25% of the samples, if the group size is equal) as well as a variance filter (an interquartile range of log2 intensities >0.5, if the group size is equal). After the filtering process, P-values were calculated with the two-sample t-test (variance = equal) to identify miRNAs expressed differentially between the two groups. For multiple testing problems, a false discovery rate (FDR) was used [21].

Pearson's correlation or the Spearman test was applied as appropriate for the calculation of the correlations between two variables. Fisher's exact test was used to analyse the differences in the categorical variables. For the independent variables, we used the Mann–Whitney U-test and the Kruskal–Wallis test. Associations of the Milan criteria and AFP levels with tumour recurrence-free survival were estimated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. For the Kaplan–Meier analysis, patients with different AFP levels were divided into three groups as follows: 1st AFP <20 ng/ml, 2nd AFP 20–400 ng/ml and 3rd AFP >400 ng/ml [22].

To investigate the risk factors for tumour recurrence, univariate and multivariate Cox regression analyses were used and expressed as a Concordance Index. Time-dependent receiver operating characteristic (survivalROC) curves and the area under the curve were used to determine feasibility at a time point of 5 years using the method of Heagerty *et al.* [23].

All data were analysed with SPSS, version 22 (IBM, Armonk, NY, USA) and R.

DIANA-MIRPATH v.3.0 (DIANA Tools, Athens, Greece) was used as a computational predictive model to calculate potentially targeted genes and pathways using the microT-CDS database (a P value threshold of 0.05, MicroT threshold of 0.8, and FDR correction and conservative stats were applied) [24]. The depicted pathways are derived from the KEGG database [25].

Results

Association of clinical parameters with disease-free survival after LT

Patient demographics are summarized in Table S1. During the study period, we performed 92 LTs in 22 women and 70 men with HCC as an underlying disease with a mean age of 57 years. Hepatitis C virus infection, which was noted in 42.4% of the patients, was the most common aetiology of liver cirrhosis. As a bridging therapy for tumour control during the waiting period before LT, 78 patients underwent transarterial chemoembolization (TACE). The minimum follow-up time was 24 months. Of the 92 patients included in this study, 22 had recurrent tumours with a median HCC recurrence-free survival of 10 months (3–55 months). The median survival with HCC recurrence in this subgroup was 25.5 months.

The prognostic values of different clinicopathological features were analysed with a univariate analysis. AFP level, tumour size, number of tumour nodules, microvascular invasion and patients outside of the Milan criteria were significantly associated with tumour recurrence (Table 1). The AFP level was seven times higher in the recurrent group (47.1 ng/ml) compared with patients without tumour recurrence (6.55 ng/ml). The majority of the patients had well-differentiated or moderately differentiated tumours, and only nine patients had poorly differentiated HCC. All nine patients developed tumour recurrence. Microvascular invasion was more frequently observed in patients with HCC recurrence ($P < 0.001$).

Other features, such as the neutrophil–lymphocyte ratio, were not associated with tumour recurrence.

Figure 1 shows the disease-free survival rates according to the Milan criteria and AFP levels. According to the

Table 1. Clinical and pathological parameters of patients with no hepatocellular carcinoma (HCC) recurrence versus HCC recurrence following liver transplantation.

	No HCC recurrence (n = 70)	HCC recurrence (n = 22)	P
Age years, mean (±SD)	56 (6.53)	59 (5.14)	0.147
Gender (female:male)	20:50	2:20	0.098
Serum AFP ng/ml, (range)	6.55 (1.1–3872)	47.1 (3.2–60 500)	<0.001
<20 ng/ml	53	6	<0.001
20–400 ng/ml	12	10	<0.001
>400 ng/ml	5	6	<0.001
Peak serum AFP, mean (range)	10.65 (2–15 509)	50.3 (3.2–60 500)	<0.001
Neutrophil–lymphocyte ratio, mean (range)	2.65 (0.3–27.5)	1.94 (0.79–8.55)	0.336
Waiting period in months, mean (±SD)	10.51 (8.33)	8 (7.51)	0.155
Number of patients with TACE (n)	60	18	0.408
TACE sessions per patient, mean (±SD)	5.29 ± 3.64	4.26 ± 2.88	
Pathology			
Tumour size, cm (range)	2.3 (0–8.3)	3.8 (0–24)	<0.001
>2 nodes	13	13	0.001
Microvascular invasion	1	7	<0.001
Grading G3	0	9	<0.001
Milan criteria fulfilled	53	5	<0.001
UCFS criteria fulfilled	59	13	<0.001

post-transplant pathology reports, 58 patients were within the Milan criteria, of whom five experienced HCC recurrence within the first 2 years after LT. As defined by their peak AFP levels during the waiting period, the patients were divided into three groups as follows: <20 ng/ml (n = 53), 20–400 ng/ml (n = 22) and >400 ng/ml (n = 11) (Table 2). The median HCC recurrence-free survival durations for these groups were 35, 30 and 8 months, respectively. The group of patients with AFP levels <20 ng/ml had significantly longer median HCC recurrence-free survival than the patients with AFP levels from 20 to 400 ng/ml or >400 ng/ml.

Before miRNA analysis, the patients chosen for miRNA analysis did not show any significant differences in age, gender, primary indication for LT, labMELD, tumour size and number of tumour nodules, and HCC recurrence-free survival (Table 2).

Global miRNA array analysis identified differential miRNA expression in patients with and without tumour recurrence after LT

Array analysis was performed using the Affymetrix GeneChip® miRNA microarray and FFPE tissue from 18 patients (screening cohort) who underwent LTX for HCC (recurrent group, n = 8; nonrecurrent group, n = 10). After global filtering for a standard deviation ≥1.0, a panel of 527 of 1105 miRNAs was used to perform an unsupervised hierarchical clustering analysis (UCA). UCA did not show clustering of the patients with HCC recurrence versus those without it (Fig. S1). However, the nonrecurrent group showed a high variance in miRNA expression patterns.

A supervised analysis comparing patients with and without tumour recurrence showed the upregulation of 84 miRNAs and downregulation of 130 miRNAs in the recurrence group. Candidate biomarker miRNAs were extracted using a P-value <0.10 as a cut-off, identifying five downregulated (miR-371, miR-939, miR3187, miR-3188 and miR-3197) and eight upregulated miRNAs (miR-let-7d, miR-let-7i, miR-140, miR-214, miR-455, miR-494, miR-1260 and miR-4284, see Table 3). Figure S2 shows a heat map of these miRNAs, which were differentially expressed with a cut-off P-value <0.10 between the recurrence and nonrecurrence groups.

In 34 of 74 patients within the Milan or UCSF criteria, we could not perform a miRNA analysis because of a tumour necrosis rate >30% (n = 17), extracted RNA of low quality (n = 6), or patient death within the first 12 months after LT, which prevented the assessment of tumour recurrence (n = 11).

Expression of miRNAs was associated with HCC recurrence after liver transplantation

For technical validation of the microarray data, we performed qRT-PCR for five miRNAs selected on the basis of P values (the lowest P values were selected). We included two downregulated miRNAs (miR-3187 and miR-3188) and three upregulated miRNAs (miR-140, miR-455 and miR-214) in a comparison between the recurrent and nonrecurrent groups (Fig. 2). Technical validation was

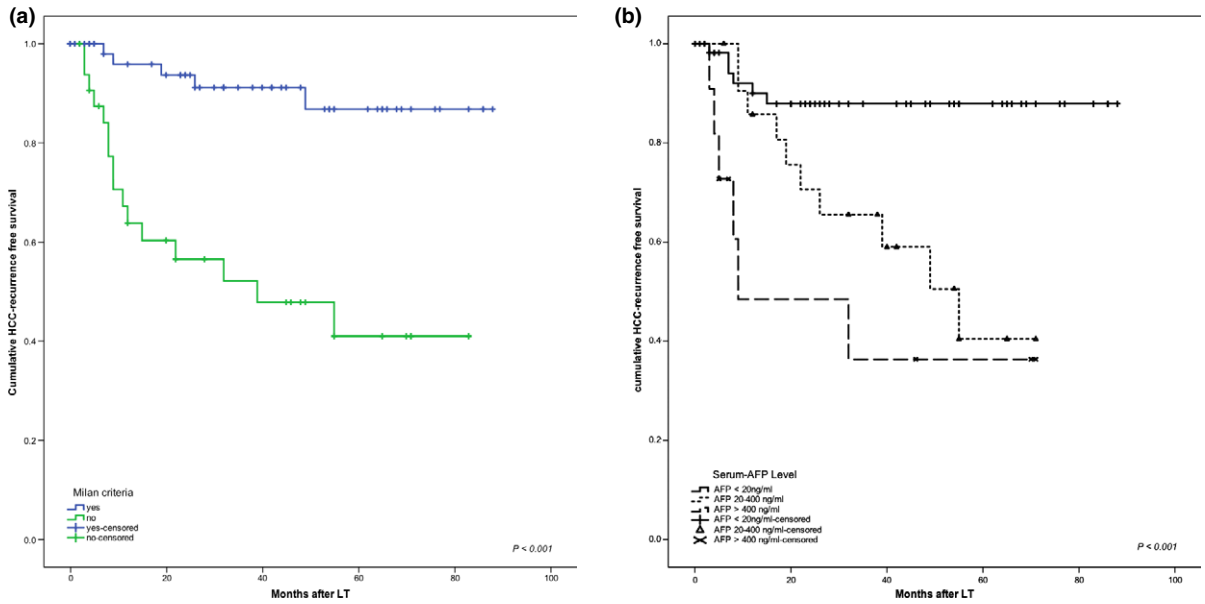

Figure 1 Association of the Milan criteria (a) and the serum AFP levels (b) with hepatocellular carcinoma recurrence-free survival following liver transplantation by Kaplan–Meier curves and the log-rank test.

performed in the same group of patients (n = 18, screening cohort) who were used for the prior array. Compared with the nonrecurrent group, the expression of miR-214 and miR-455 was significantly higher in the recurrent group, and the expression of miR-3187 was significantly lower (Fig. 2). There was no significant difference in the expression of miR-140. Unfortunately, miR-3188 could not be validated due to technical difficulties. The assay for this miRNA did not produce valid data.

miRNAs and Milan criteria as predictors of tumour recurrence after LT

Therefore, four miRNAs (miR-140, miR-214, miR-455 and miR-3187) differentially expressed in the microarray were analysed in the screening cohort for their predictive power. To evaluate the predictive potential of the selected miRNAs for tumour recurrence, we performed a univariate Cox regression analysis. We found that miR-214, miR-455 and miR-3187 correlated with the patient tumour recurrence and tumour recurrence-free survival (Table 4).

Although the patient numbers of the screening cohort were small, an explorative multivariate Cox proportional hazard regression was used to evaluate the significance of independent prognostic factors for patient tumour recurrence-free survival that were found to be significant in the univariate Cox regression analysis, including the serum AFP levels, the Milan criteria and the miRNAs. We found that two of the miRNAs (miR-214 and miR-3187) and the Milan criteria were significantly associated with tumour recurrence-free survival and may be used as independent prognostic factors (Table 5). Serum AFP levels and miR-455 were not identified as predictors in the multivariate Cox regression analysis in our screening cohort. For the next step, we developed a predictive score using the data (regression coefficient) from the multivariate Cox regression analysis as follows:

$$\begin{aligned}\text{Predictive Score} = {} & \\ & -2.033 \times [\text{miR-214}] + 4.217 \times [\text{miR-3187}] \\ & + 5.985 \times [\text{Milan in} = 1/\text{Milan out} = 2]\end{aligned}$$

As a cut-off, we used survivalROC methods at a follow-up time of 60 months. We defined the low- and high-risk groups for tumour recurrence and death with cut-off values $\geq$36 and <36, respectively (Fig. 3).

Validation of the findings in an independent cohort

To validate the findings from the screening cohort, a second and independent cohort (validation cohort) of 22 patients (recurrent group, n = 6; nonrecurrent group, n = 16) was analysed. High expression of miR-214 (P = 0.021) and low expression of miR-3187 (P = 0.006) were significantly associated with HCC recurrence after LT (Fig. 4a).

To evaluate the predictive potential of the selected miRNAs for tumour recurrence, we performed univariate tests (Mann–Whitney U-test) and a univariate Cox regression analysis of the patients of the validation cohort (n = 22).

Table 2. Clinical and pathological parameters of the cohort with microRNA (miRNA) and without miRNA analysis.

	miRNA analysis cohort (n = 40)	No-miRNA analysis cohort (n = 52)	P
Age years, mean (range)	56.13 (42–69)	57.13 (40.69)	0.425
Gender (female:male)	10:30	12:40	0.511
Serum AFP ng/ml, (range)	432.36 (1.1–6097.0)	2094 (1.6–60 500)	0.700
Bridging therapy			
TACE (n)	32	46	0.128
Others (n)	4	5	
No therapy (n)	2	3	
Pathology			
Tumour size, cm	2.55	2.35	0.105
>2 nodes	13	13	0.527
Microvascular invasion	4	4	0.647
Grading G3	5	4	0.601
Milan criteria fulfilled	34	24	0.572

Table 3. Differential microarray profiling in recurrent and nonrecurrent hepatocellular carcinoma after liver transplantation with P values <0.10.

microRNAs	FC	P	Alignments	Status
hsa-miR-140-3p_st	2.5	0.024	16:69966984–69967083 (+)	Up
hsa-miR-3187-3p_st	−4.6	0.044	19:813584–813653 (+)	Down
hsa-miR-455-3p_st	1.7	0.060	9:116971714–116971809 (+)	Up
hsa-miR-3188_st	−3.3	0.062	19:18392887–18392971 (+)	Down
hsa-miR-214_st	2.3	0.064	1:172107938–172108047 (−)	Up
hsa-miR-3197_st	−2.5	0.064	21:42539484–42539556 (+)	Down
hsa-miR-1260b_st	2.1	0.069	11:96074602–96074690 (+)	Up
hsa-miR-371b-5p_st	−3.5	0.075	19:54290931–54290996 (−)	Down
hsa-miR-4284_st	2.0	0.084	7:73125647–73125727 (+)	Up
hsa-miR-494_st	1.7	0.084	14:101495971–101496051 (+)	Up
hsa-miR-939_st	−2.6	0.089	8:145619364–145619445 (−)	Down
hsa-let-7d_st	3.1	0.094	4:11370451–11370545 (+)	Up
hsa-let-7i_st	1.5	0.099	12:62997466–62997549 (+)	Up

We found that miR-214 and miR-3187 were correlated with the patient tumour recurrence (Table 4). Furthermore, we evaluated the score combining miR-244 and miR-3187 with the Milan criteria, as previously described. In contrast to the Milan criteria alone, this score was significantly associated with recurrence-free survival in the Cox regression analysis (Table 5), which validated the predictive information of miR-244 and miR-3187. Using this score to define high- and low-risk groups, the Kaplan–Meier analysis also showed a significant difference between the groups in the validation cohort (P = 0.009, Fig. 4b). In the low-risk group within the Milan criteria, only two patients experienced tumour recurrence following LT. In the survivalROC analysis, our predictive score showed a higher sensitivity and specificity for tumour recurrence after LT, with an AUC of 0.885 (Fig. 4c) compared with the Milan criteria (AUC = 0.600) and AFP levels (AUC = 0.703).

Due to the lack of published mechanistic data on miR-3187, we used an *in silico* approach to identify the potential gene targets of this miRNA. A DIANA-MIRPATH v3.0 enrichment analysis of miRNA target genes predicted seven KEGG pathways to be potentially targeted by miR-3187. The highest number of target genes [9] was found within regions related to focal adhesion (P = 0.016) and the regulation of the cytoskeleton pathway (P = 0.016; Fig. S3).

Discussion

Hepatocellular carcinoma recurrence following LT is associated with poor long-term survival, and its prediction remains an unresolved issue [26,27]. Against the background of organ shortages, improvements in the prognostic tools for predicting outcomes after LT for HCC are necessary [7]. Our study indicated that a combination of tumour size and distribution (e.g. the Milan criteria) with a specific miRNA pattern may be a promising prognostic tool for the risk stratification of tumour recurrence following LT.

Initially, we identified differences in miRNA expression patterns between recurrent and nonrecurrent HCC using a microarray analysis in a screening cohort. After the identification of candidate biomarker miRNAs, four selected miRNAs (miR-140, miR-214, miR-455 and miR-3187) were technically validated in the screening group and in a second independent validation group.

After performing a Cox regression analysis in the screening cohort, we were able to develop a score that considers biological criteria (miRNA expression) in conjunction with the Milan criteria that significantly correlate with HCC recurrence-free survival. Although the sample size was small, we could confirm our predictive score in a second independent cohort. In this group, the predictive score was able to predict tumour recurrence more accurately than the Milan criteria alone or serum AFP levels. However, several limitations of our study must be mentioned. This study

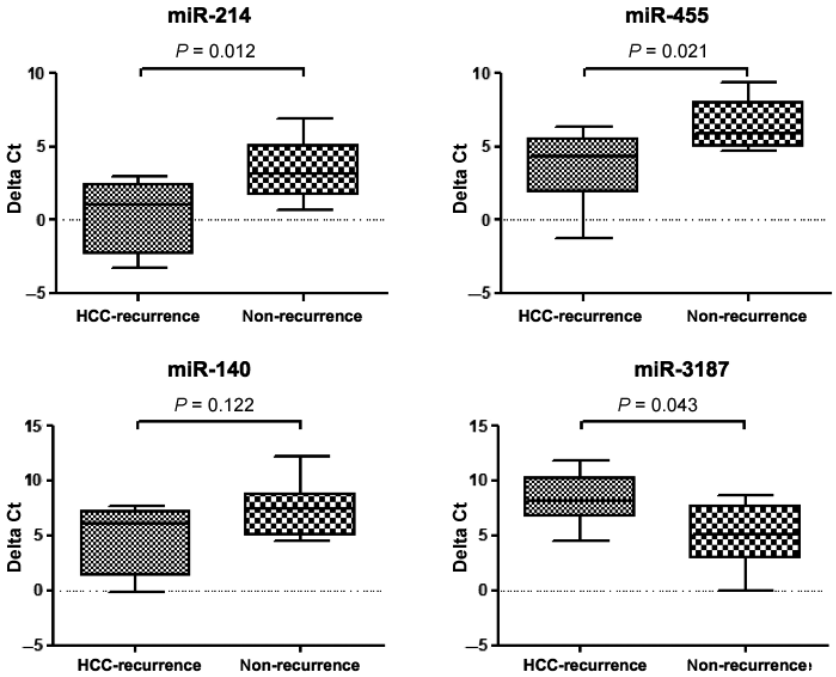

Figure 2 Technical validation of the microarray data for selected miRNAs. For technical validation, we performed qRT-PCR for selected miRNAs (miR-140, miR-214, miR-455 and miR-3187) and made comparisons between the recurrent and nonrecurrent groups of the screening cohort (screening group). Δ-CT values are in opposite direction to the regulation of the analysed miRNAs.

was performed in a retrospective, single-centre setting with a relatively small sample size. Furthermore, the miRNA expression patterns were obtained from the explanted livers of our LT recipients after hepatectomy. Therefore, the predictive value of our study is only valid for the analysis of the specimens collected at that time. Whether our predictive model may also have the same significance analysing HCC core-needle biopsies at the time of diagnosis is unclear because the miRNA expression patterns may change over time due to bridging therapies, such as transarterial chemoembolization [28,29]. Nevertheless, it has been shown that miRNA analysis is also feasible from HCC fine-needle biopsies, which can be performed before LT [30]. In our centre, 56.5% of the patients had a biopsy for HCC before LT. However, in 17.3% of the patients, the biopsy was negative for HCC (see Table S1). Although we were able to perform a miRNA analysis from the biopsy samples [30], the number of patients who had a biopsy-proven HCC before LT and fulfilled the enrolment criteria was too low in our study (55% in the screening cohort and 54% in the validation cohort).

Moreover, different studies have shown that cell-free and cellular miRNAs can be measured in the serum and that circulating miRNA levels are affected in HCC [20,31–34], which may lead to a highly relevant predictive signature at a very early time point in therapy. Circulating miRNAs in HCC may have a great potential to become diagnostic and prognostic tools for HCC in the future, but the research on circulation miRNAs is in its infancy [35].

In patients presenting with HCC in cirrhosis with preserved hepatic function, the available data are insufficient to answer the question of whether this group of patients benefits from LT or may be treated by liver resection with similar overall and disease-free survival [36]. Data from a large national cancer database analysing 3340 patients with clinical stages I and II HCC (American Joint Committee on Cancer (AJCC) show median survival periods for LT and liver resection of 127.9 and 56.7 months in stage I, respectively, whereas in stage II patients, the median survivals are 110.8 and 42.8 months, respectively ($P < 0.0001$) [37]. Because the existing criteria and scores merely define the limits of acceptable survival data by retrospective analyses of available radiological tumour distribution data [5,9,38], the tumour biology of the individual patient is insufficiently recognized. Actual data indicate that a subgroup of patients outside the Milan criteria and a well-defined downstaging algorithm may result in a low HCC recurrence rate and an excellent 5-year intent-to-treat survival of 56.1% vs. 63.3% ($P = 0.29$) compared with patients primarily presenting with a tumour distribution within the Milan criteria [39]. To our knowledge, the score presented in this manuscript was the first approach to combining

Table 4. Analysis of the four microRNAs in recurrent and nonrecurrent hepatocellular carcinoma with variance test analysis and their association with tumour recurrence-free survival in the univariate Cox regression analysis.

	Variance test (Mann–Whitney *U*-test)			Univariate Cox regression analysis	
	Nonrecurrent (mean, SD)	Recurrent (mean, SD)	*P*	HR [95% CI]	*P*
Screening cohort					
miR-140	4.779 (3.071)	7.351 (2.516)	0.122	0.822 [0.655–1.032]	0.091
miR-214	0.384 (2.413)	3.309 (1.986)	0.012	0.763 [0.592–0.982]	0.036
miR-455	3.560 (2.524)	6.421 (1.666)	0.021	0.699 [0.525–0.929]	0.013
miR-3187	8.200 (2.316)	4.957 (2.833)	0.043	1.769 [1.127–2.777]	0.013
Validation cohort					
miR-140	3.107 (3.221)	3.372 (3.999)	1.000	1.209 [0.890–1.642]	0.225
miR-214	2.230 (1.639)	5.075 (2.415)	0.021	0.734 [0.456–1.181]	0.203
miR-455	4.542 (2.280)	6.886 (3.173)	0.154	0.824 [0.544–1.249]	0.362
miR-3187	8.643 (3.128)	4.664 (2.189)	0.006	1.664 [0.977–2.834]	0.061

Table 5. Multivariate Cox regression analysis for tumour recurrence (backward stepwise regression).

	Coefficient	HR [95% CI]	*P*
Screening cohort			
miR-214	−2.033	0.131 [0.02–0.80]	0.027
miR-3187	4.217	67.8 [1.5–2980]	0.029
Milan criteria	5.958	387 [1.4–106 800]	0.038
Concordance index (SE)	0.981 (0.113)		
Validation cohort	0.118	1.125 [1.014–1.25]	0.027
Concordance Index (SE), Predictive Score	0.869 (0.159)		0.027
Concordance Index (SE), only Milan criteria	0.640 (0.095)		0.345

Other variables tested in the model: serum AFP, miR455.

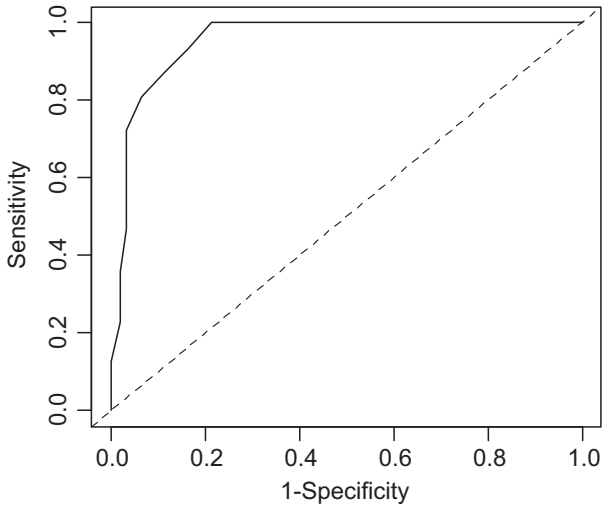

Figure 3 Time-dependent receiver operating characteristic curves (ROC) at a follow-up of 60 months suggesting a cut-off of 36 for the predictive score in the screening cohort. Based on that analysis, we defined low- and high-risk groups for tumour recurrence and death with cut-off values ≥36 and <36, respectively.

miRNA expression with the Milan criteria for the prediction of HCC recurrence following LT. This approach may close the gap between clinical tumour presentation and individual patient tumour biology as indicated by miRNA distribution patterns.

Two (miR-140 and miR-214) of the four validated miRNAs in our study were previously reported to be involved in HCC formation. MiR-140 could be identified as an HCC-related tumour suppressor miRNA by controlling nuclear factor kappa B (NF-κB) activity [40]. The downregulation of miR-140-5p was found to correlate well with multiple tumour nodules, tumour invasion and poor prognosis [41]. Because the clinical criteria of tumour characteristics such as the Milan, the UCSF and the expanded Asan criteria show a very heterogeneous picture of recurrence rates and long-term survival [42], these findings further highlight the role of biomarkers and their potential to unmask unfavourable tumour biology in individual patients. Dysregulation of miR-214 plays an important role in the field of tumour angiogenesis [43], and the downregulation of miR-214 in HCC tumour samples compared with benign adjacent liver tissue is associated with tumour progression and poor outcomes [14,15]. The ectopic expression of miR-214 in human HCC cell lines significantly inhibited cell proliferation, and in murine experiments, this ectopic expression suppressed tumour formation and tumour growth in nude mice. Hepatoma-derived growth factor could be identified as a target gene of miR-214. Furthermore, the ectopic expression of miR-214 or the antagonism of hepatoma-derived growth factor suppresses tumour angiogenesis, thereby suppressing tumour growth [14]. Another potential target gene of miR-214 is FGF-1, which is overexpressed in HCC [15,44]. Wang *et al.*

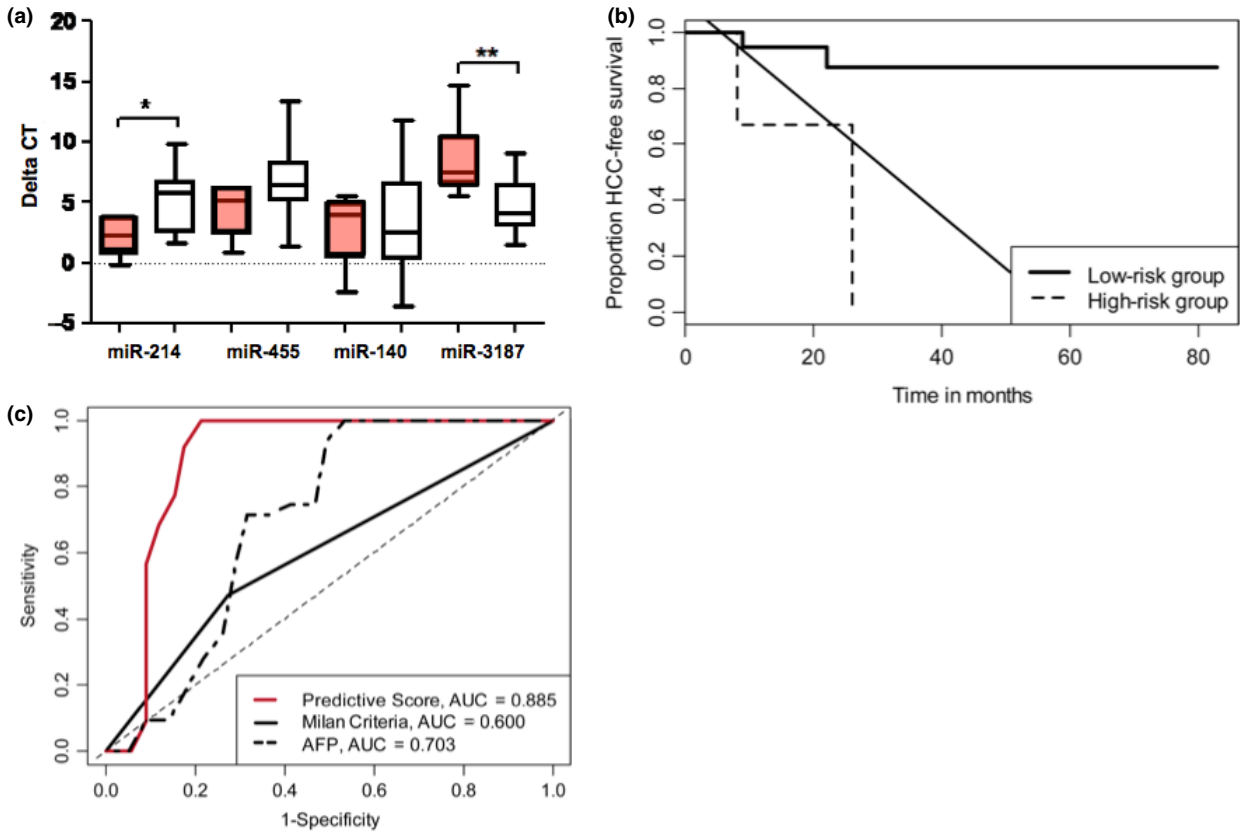

Figure 4 Validation of the miRNA expressions and the predictive score in an independent cohort of 22 patients. (a) QRT-PCR validation of different miRNA patterns between recurrent (red boxes) and nonrecurrent (white boxes) hepatocellular carcinoma (HCC) in an independent cohort. The expression of miR-214 and miR-3187 is associated with tumour recurrence after liver transplantation. ΔCT values are in opposite direction to the regulation of the analysed miRNAs. $*P < 0.05$; $**P < 0.01$ (b) Kaplan–Meier analysis shows HCC recurrence-free survival between low- and high-risk groups with a cut-off value of 36 for tumour recurrence in the validation cohort. (c) Our predictive score was associated with HCC recurrence-free survival in survivalROC analysis with a higher sensitivity and specificity compared with the Milan criteria and AFP levels (cut-off value 36).

[15] showed that the transfection of human HCC tumour cell lines with miR-214 resulted in the inhibition of cell invasion through the downregulation of FGF-1. Recently, it was shown that miR-214 is able to regulate the β-catenin pathway and hepatic cancer stem cells [45]. The identification of the target genes of miR-214 highlights the potential of miRNAs for anti-HCC therapy.

The role of miR-455 in HCC is unknown; however, in colorectal cancer cells, the overexpression of miR-455 inhibits proliferation and invasion by targeting RAF proto-oncogene serine/threonine protein kinase [46]. Different studies have shown that HCC development and progression are associated with the activation of the Raf1/MAP kinase pathway in humans [47,48].

Regarding miR-3187, there has been no report concerning its relationship to HCC or the molecular mechanism underlying the role of miR-3187 in tumour recurrence. However, the computational pathway analysis showed an enrichment of genes potentially influenced by miR-3187 within the region related to focal adhesion, as well as the regulation of the actin cytoskeleton. These cell functions have been associated with cancer development in general and were frequently found to be dysregulated in HCC previously [49–52]. However, the current pathway analyses should be treated with caution because they are based only on an *in silico* approach and are not yet supported by mRNA gene expression data.

Conclusion

In conclusion, our data support the hypothesis that the combination of miRNA expression levels and tumour distribution criteria (the Milan criteria) correlates with HCC recurrence in patients following LT. An upcoming study will validate our score in the HCC population in a second LT centre. Furthermore, the assays for detecting the identified miRNAs in blood samples should be evaluated and

established. In times of severe organ shortages in several countries, a new predictive score may be an effective tool for patient selection depending on low- or high-risk scores for HCC recurrence before LT.

Authorship

JL and JP-O: performed study, analysed data and wrote paper. CD: performed research/study. AAS and EH: analysed data. SZ: collected data, analysed data. MLH: contributed important reagents, analysed data. CM: designed study. M-WW: designed study. SZ: collected data. WOB: designed study. FU: designed study, analysed data and wrote paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Unsupervised hierarchical clustering of the miRNA expression patterns between the recurrence (R1-R8) and nonrecurrence groups (NR1-NR10). High expression levels are in red, low expression levels are in green.

Figure S2. Supervised hierarchical clustering of the miRNA expression patterns with a cut-off $P < 0.10$. MiRNA expression patterns are compared between the recurrence (R1-R8) and nonrecurrence groups (NR1-NR10). High expression levels are in red, low expression levels are in green.

Figure S3. The use of the *in silico* approach to identify potential gene targets of miR-3187. The DIANA-MIRPATH v3.0 enrichment analysis of miRNA target genes predicted seven KEGG pathways to be potentially targeted by miR-3187. The highest number of target genes was found within the regulation of cytoskeleton (a, $P = 0.016$) and the focal adhesion (b, $P = 0.016$) pathways.

Table S1. Clinicopathological features of the cohort.

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ORIGINAL ARTICLE

Influence of the recipient body mass index on the outcomes after kidney transplantation

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Abstract
Purpose The relationship between the body mass index (BMI) of kidney transplant recipients and outcomes after kidney transplantation (KT) is not fully understood and remains controversial. We studied the influence of BMI on clinically relevant outcomes in kidney transplant recipients.
Methods In this retrospective single-centre study, all patients who underwent kidney transplantation at our institution between January 2007 and December 2012 were included. Demographic data and BMI were correlated with the clinical course of the disease, rejection rates, delayed graft function rates, and graft and patient survival.
Results During the study period, 384 single KTs (130 women and 254 men) were performed. Seventeen percent of the transplants were transplanted within the Eurotransplant Senior Programme (ESP). Most of the transplants were performed using organs that were obtained from donors after brain death (DBD), and living donor kidney transplants were performed in 22.4% of all transplants. The median BMI of the recipients was 25.9 kg/m^2. Additionally, 13.5% of the recipients had a BMI of 30–34.9 kg/m^2 and 3.9% had a BMI >35 kg/m^2. A BMI >30 kg/m^2 was significantly associated with primary non-function of the kidney ($p = 0.047$), delayed graft function ($p = 0.008$), and a higher rate of loss of graft function ($p = 0.015$). The glomerular filtration rate 12 months after KT was significantly lower in recipients with a BMI >30 kg/m^2. Multivariate analysis revealed that recipient BMI, among other factors, was an independent risk factor for delayed graft function and graft survival. Patients with a BMI >30 kg/m^2 had an almost four times higher risk for surgical site infection than did recipients with a lower BMI.
Conclusions Increased BMI at kidney transplantation is a predictor of adverse outcomes, including delayed graft function. These findings demonstrate the importance of the careful selection of patients and pre-transplant weight reduction, although the role of weight reduction for improving graft function is not clear.

Keywords Body mass index · Kidney transplantation · Kidney function · Graft survival

Introduction

The prevalence of obesity is increasing, resulting in an increasing incidence of diabetes, cardiovascular disease and end-stage renal disease (ESRD) [1, 2]. For ESRD, kidney transplantation (KT) is the preferential treatment. The increase in BMI of transplant candidates in recent decades is challenging, as is the increase in BMI after KT in kidney recipients [3, 4]. Post-transplantation weight gain is related to an improvement in quality of life, fewer nutritional restrictions and the correction of uraemia and steroid-based immunosuppression [5, 6].

Obesity, defined as a BMI >30 kg/m^2, is associated with several risk factors for chronic vascular disease [2, 7]. After KT, the most important causes for mortality and morbidity after kidney transplantation are cardiovascular complications [8, 9] and metabolic syndrome [4]. Multiple studies have shown that the

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prevalence of new-onset diabetes after transplant (NODAT) and the aggravation of pre-existing diabetes are higher in obese patients [10–12] and are further relevant causes of complications after KT. In addition, surgical complications, particularly wound infections [13, 14] and wound dehiscence [15], are increased after KT in obese patients. Furthermore, an association with graft function might exist. Single-centre and large-registry studies have reported an increased rate of delayed graft function and increased risk for graft failure in recipients with high BMI [16–21]. The increased risk for adverse outcomes in obese recipients leads to a BMI limit for access to the kidney transplant waiting list [22, 23]. However, the abovementioned correlations between obesity, graft function and surgical complications were not confirmed in other studies [24–27]. On the other hand, a significant survival benefit for transplantation in overweight and obese kidney recipients compared with patients on the waiting list for KT with the same BMI was shown in large multicentre studies [28].

This raises the question of whether obese patients should be transplanted or a body mass index (BMI) cut-off should be implemented for KT allocation for KT.

The objective of the present study was to analyse the association between BMI and outcomes (e.g., graft survival and function and complications) after KT to aid in developing strategies that minimize the risks of mortality and morbidity in obese patients and to better select kidney transplant candidates.

Methods

This retrospective analysis was approved by the local Ethics Committee of Frankfurt University Hospital and Clinics, Germany (Institutional Review Board No. 234/14). A total of 442 consecutive patients undergoing KT between 2007 and 2012 were analysed. The collected data included the following demographic features: age, gender, BMI, underlying kidney disease, duration of dialysis, warm and cold ischaemia times, intra- and postoperative course, delayed graft function, graft failure, rejection rate, surgical complications and overall patient and graft survival data.

Body mass index (BMI) was calculated as the dry weight in kilograms divided by the height in metres squared. The patients were stratified into three BMI groups: group 1, BMI $\leq$29.9 kg/m^2; group 2, BMI 30–34.9 kg/m^2 and group 3, BMI $\geq$35 kg/m^2 according to the WHO classification.

Kidney function was evaluated based on the glomerular filtration rate (CKD-EPI formula) and serum creatinine levels at 6 and 12 months after KT. Delayed graft function was defined as the need for dialysis within the first 7 days following KT [29].

Clinical and biochemical characteristics are expressed as the means ± standard deviations or as medians and ranges as appropriate. Unless otherwise indicated, all tests were two-tailed, and p values <0.05 were considered significant.

Pearson's correlation or the Spearman test was applied as appropriate to calculate the correlations between pairs of variables. Fisher's exact test was used to analyse differences in categorical variables. For independent variables, we used the Mann-Whitney U and Kruskal-Wallis tests. Associations of BMI with graft function were estimated using the Kaplan-Meier method, and the resulting curves were compared using the log-rank test.

Complications in the first 30 days after KT were classified according to the Clavien-Dindo classification: Grade II complications were classified as complications if medical treatment (e.g., antibiotic therapy) was needed; patients were classified as grade IIIa if an intervention without general anaesthesia was indicated and were classified as grade IIIb if intervention under general anaesthesia was performed. If patients required admission to the intensive care unit, they were regarded as having grade IVa (with single organ dysfunction) or IVb (with multiorgan dysfunction) complications. Postoperative mortality was defined as a grade-V complication [30].

To investigate risk factors for delayed graft function, graft failure and surgical site infections (SSIs), univariate and multivariate regression analyses were performed. Univariate analysis was conducted to determine candidate factors for the multivariate analysis. Variables with $p < 0.10$ were entered into the multivariate analysis. All data were analysed using SPSS, version 23 (IBM, Armonk, NY, USA).

Results

After screening all 442 patients with kidney transplants, 17 patients were excluded because of missing preoperative BMI data and 41 patients were excluded because of combined transplantation (pancreas-kidney or liver-kidney transplantation). In total, 384 kidney recipients (254 men and 130 women) were included in the present study. The patient demographics are summarized in Table 1. The mean age was 53.0 years, and the mean BMI was 25.9 kg/m^2 (range from 16 to 50.17 kg/m^2). At our institution, patients with obesity are evaluated individually on a case-by-case basis without BMI cut-off levels for potential kidney recipients. Among the transplants, 22.4% (n = 86) were live donor kidney transplants (LD), and 77.6% were kidney transplants involving donors after brain death (DBD) organs. The percentage of patients in the Eurotransplant Senior Programme (ESP) was 17%.

The majority of patients (84.9%) had cardiovascular diseases (hypertension, coronary heart disease, chronic cardiac failure, cardiac dysrhythmia or valvular heart disease) in their medical history, and 75.8% had hypertension. Hypertension tended to be higher in patients with a BMI $\geq$30 kg/m^2 (p = 0.06, Table 2). Significantly higher numbers of diabetes cases were found in the BMI group 30–34.9 kg/m^2 (34.6%) and the BMI group $\geq$35 kg/m^2 (60.0%) than among patients in

Table 1 Characteristics of 384 kidney transplant recipients

	Total n = 384
Age (years)	53.01 (15–81)
Gender n (%)	
Male	254 (66.1%)
Female	130 (33.9%)
BMI (kg/m^2)	25.9 (16–50.17)
Distribution of BMI categories n (%)	
≤29.9	317 (82.6%)
30–34.9	52 (13.5%)
≥35	15 (3.9%)
Pre-existing diseases	
Cardiovascular diseases	326 (84.9%)
Hypertension	291 (75.8%)
Diabetes mellitus	63 (16.4%)
Cerebrovascular diseases	20 (5.2%)
Primary renal diagnosis	
Glomerulonephritis	172 (44.8%)
Hypertension	51 (13.3%)
Pyelonephritis	44 (11.5%)
Polycystic kidney	41 (10.7%)
Diabetes	37 (9.6%)
Others	10 (2.6%)
Uncertain	29 (7.6%)
Donor organ source	
Deceased donor	298 (77.6%)
ET Senior Program	66 (17.2%)
Live donor	86 (22.4%)
Second kidney transplantation n (%)	38 (9.9%)
Third kidney transplantation n (%)	7 (1.8%)
Prior renal replacement therapy	
No	22 (5.7%)
Yes	362 (94.3%)
Duration of renal replacement therapy (months)	57.57 (0–312)

the BMI group ≤29.9 kg/m^2 (18.0%, $p < 0.001$, Table 2). There were no differences in cerebrovascular disease among the BMI cohorts.

The main primary renal diagnosis for ESRD was glomerulonephritis (44.8%, see Table 1 and Fig. 1). Interestingly, the rate of ESRD caused by diabetes was significantly higher in patients with a BMI 30–34.9 kg/m^2 (19.2%) or with a BMI ≥35 kg/m^2 (40.0%) than in recipients with a BMI ≤29.9 kg/m^2 (6.6%, $p < 0.001$). There were no significant differences in other reasons for ESRD, and only a marginally significant higher rate of glomerulonephritis was seen in patients with higher BMI ($p = 0.053$).

No differences in recipient or donor age, renal replacement therapy, donor source, ischaemia times, length of hospital stay or immunosuppression were identified between different BMI cohorts (Table 2). Recipients with a BMI ≥35 kg/m^2 comprised significantly more females (73.3%) than males ($p = 0.003$).

Average weight gain during the first 12 months after KT was 4.4 kg (±9.6 kg). Notably, the weight gain in the group with a pretransplant BMI ≥35 kg/m^2 was significantly higher (9.8 kg ±7.02 kg) than that in transplant recipients with a pretransplant BMI ≤29.9 kg/m^2 (1.9±13.35 kg) or a pretransplant BMI of 30–34.9 kg/m^2 (1.6±8.43 kg).

All patients received a calcineurin inhibitor (CNI)-based immunosuppressive regimen, including tacrolimus (41.1%) or cyclosporine (58.9%) combined with cortisone and mycophenolate mofetil (MMF). Induction therapy was performed using basiliximab (83.8%) or ATG (16.2%). The choice of immunosuppressive drug was based on individual patient characteristics (e.g. prior transplantation or preformed HLA-antibodies). No differences in donor characteristics, immunosuppressive regimen or induction therapy were found between the BMI groups.

Obesity is associated with poorer outcome after kidney transplantation

Analysis of the outcome 1 year after KT showed significant differences between the BMI groups (Table 3). Glomerular filtration rate was significantly lower in obese patients (BMI >30 kg/m^2), particularly in the group with a BMI ≥35 kg/m^2, which had the lowest glomerular filtration rate of 32.76 ml/min ($p = 0.005$, Fig. 2), than in patients with a BMI ≤29.9 kg/m^2. Furthermore, the number of patients with primary non-function, delayed graft function and loss of graft function during the first year after KT was higher in obese patients ($p < 0.05$, Table 3). The reasons for graft loss ($n = 50$) are listed in Table 4. The main reasons for graft loss were rejection and surgical conditions; e.g. thrombosis of the A. or V. renalis and recurrence of primary renal disease. There were no differences between the different BMI groups.

Kaplan-Meier curves of graft survival showed a significantly lower graft survival in obese patients (Fig. 3). Graft survival for obese patients was significantly less than that of patients with a BMI ≤29.9 kg/m^2. Four years after KT, graft survival in the BMI group ≤29.9 kg/m^2 and the BMI group 30–34.9 kg/m^2 was approximately 90% but was only 77.5% in patients with a BMI ≥35 kg/m^2. The minimum follow-up time after KT was 3 years, and the maximum was 8 years. A total of 48 patients (12.5%) died during the follow-up, and 42 patients (87.5%) in this group had a functional organ. The main causes of death were cardiovascular disease (29.2%), de-novo malignancy (22.9%), respiratory disease (16.7%), infection (8.3%) and cerebrovascular disease (4.2%). In 13 patients, the cause of death was unknown. There were no differences in patient survival between the BMI groups ($p = 0.751$).

In a multivariable analysis, BMI >30 kg/m^2 (HR 2.1) and male gender (HR 1.7) were identified as the main risk factors

Table 2 Patient demographics stratified by BMI categories

	BMI ≤29.9 (n = 317)	BMI 30–34.9 (n = 52)	BMI ≥35 (n = 15)	P value (n = 15)
Recipient age, (*years*) (±SD)	52.67 (14.04)	54.92 (12.65)	53.60 (8.87)	0.597
Gender, n (%)				*0.003*
Male	212 (66.9)	38 (73.1)	4 (26.7)	
Female	105 (33.1)	14 (26.9)	11 (73.3)	
Pre-existing diseases, *n (%)*				
Hypertension	240 (75.7)	38 (73.1)	13 (86.7)	*0.06*
Diabetes mellitus	57 (18.0)	18 (34.6)	9 (60)	*< 0.001*
Renal replacement therapy, *n* (%)	295 (93.1)	51 (98.1)	15 (100)	0.178
Duration (months)	58.0	54.98	62.20	0.853
Donor organ source *n* (%)				
Live donation	73 (23.0)	11 (21.2)	2 (13.3)	0.423
ET Senior Program	54 (17.0)	11 (21.2)	1 (6.7)	0.417
No. of HLA mismatches	2.95	3.33	3.08	0.244
Donor age, (years)	53.44	54.73	51.00	0.531
Donor gender, *n (%)*				0.986
Male	151 (47.6)	25 (48.1)	7 (46.7)	
Female	166 (52.4)	27 (51.9)	8 (53.3)	
Donor creatinine, (mg/dl) (±SD)	0.95 (0.53)	0.96 (0.58)	0.79 (0.35)	0.258
Donor pre-existing diseases, *n (%)*				
Hypertension	103 (32.5)	23 (44.2)	5 (33.3)	0.596
Diabetes mellitus	15 (4.7)	4 (7.7)	1 (6.7)	0.114
Nicotine abuse	105 (33.1)	13 (25.0)	5 (33.3)	0.648
Warm ischaemia time, (*min*) (±SD)	43.21 (16.05)	43.46 (15.18)	48.79 (16.95)	0.483
Cold ischaemia time, (*h*) (±SD)	10.30 (5.80)	10.00 (5.70)	14.45 (8.79)	0.123
Immunosuppression, *n* (%)				
Mycophenolate mofetil	298 (94.0)	52 (100.0)	13 (86.7)	0.080
Calcineurin Inhibitors	313 (98.7)	52 (100.0)	13 (86.7)	0.303
Prednisone	311 (98.1)	52 (100.0)	15 (100.0)	0.100
Length of hospital stay (*days*) (±SD)	24.2 (16.18)	24.4 (13.51)	28.3 (12.94)	0.105

for delayed graft function. Donor age and cold ischaemia times reached significance ($p < 0.05$) with an HR of 0.9. Neither recipient age nor donor organ source or dialysis time reached significance in a univariate analysis.

A Cox regression analysis determining the risk factors for graft survival is shown in Fig. 4. A BMI >30 kg/m^2, together with KT in the ESP cohort and acute rejections, was significantly associated with a poorer outcome after KT (HR 1.4). SSIs, delayed graft function, donor age, ischaemia times and immunosuppression were not significantly associated with graft survival.

Obesity is associated with a higher rate of SSIs

The group of patients with SSIs included all patients with superficial and deep infections, wound dehiscence or evisceration requiring a reoperation or prolongation of hospital stay (CDC grades of SSI 1-3). Skin incisions were universally closed using intracuticular, resorbable sutures. The overall rate of SSI was 10.7% and ranged from 9.5% in patients with a BMI ≤29.9 kg/m^2 to 40% in patients with a BMI ≥35 kg/m^2 (p = 0.003, Table 3). In 22 patients, SSIs were superficial (CDC grade 1). Twenty-four patients were re-operated because of deep wound infections or fascial dehiscence (CDC grades 2–3). There was no difference in reoperation rate between obese and non-obese patients.

In multivariate logistic regression analysis, BMI ≥30 kg/m^2 (HR 3.6), male gender (HR 2.2), acute rejection (HR 1.8) and delayed graft function (HR 1.5) were identified as main risk factors for surgical site infections (Fig. 5). Interestingly, diabetes, immunosuppression with steroids and recipient age were not found to be independent risk factors for SSIs.

All complications in the first 30 days after KT were classified according to the Clavien-Dindo classification. We observed 6.7% (n = 26) grade II, 22.4% grade III (n = 86, grade IIIa n = 14, grade IIIb n = 72) and 1.3% (n = 5) grade IVa

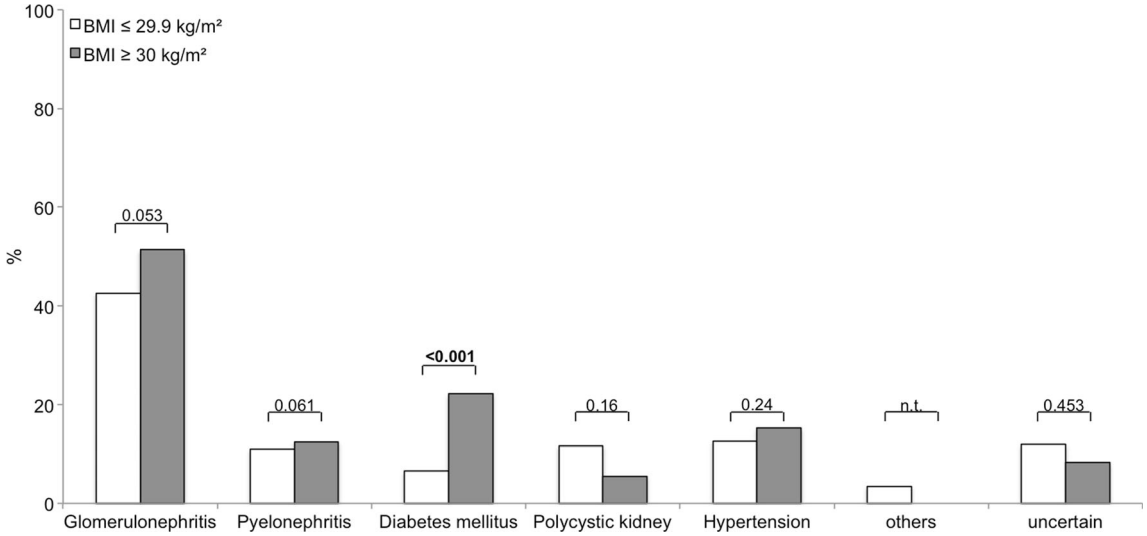

Fig. 1 Primary renal diagnosis according to renal transplant patients with a BMI ≤29.9 and ≥30 kg/m^2 (*n.t.* not tested)

complications. Grade IVb and grade V complications did not occur during the first 30 days after transplantation. The various complications are specified in Table 5. Except for SSIs, no further differences in complications were observed between the three BMI groups.

Discussion

The increasing number of obese patients with ESRD is challenging in transplant settings, especially as obesity is correlated with a high risk of surgical complications, delayed graft function and shorter graft and patient survival [14, 26, 31, 32].

In this study, we analysed the influence of pretransplant recipient BMI on surgical site infections (SSIs), graft function and survival. Our data have important implications for kidney transplantation. First, we showed that obese patients have a higher risk for SSI. We believe that this is an important factor and should be included in the informed consent procedure for obese recipients. A BMI >30 kg/m^2 is associated with a significantly higher rate of SSIs than a BMI ≤29.9 kg/m^2. Wound infection and dehiscence are common complications in obese recipients [14, 31, 33–35]. SSI, BMI >25 kg/m^2, DGF and the choice of immunosuppressive therapy are the most important risk factors associated with hernia formation in kidney transplant recipients, with an incidence of hernias 10 years after KT of approximately 10% [36]. A meta-analysis in obese renal transplant recipients also showed an elevated overall risk ratio for incisional hernias in kidney recipients (HR 2.73) [37]. Unfortunately, data on the incidence of incisional hernias in our cohort are missing due to the retrospective setting.

Second, we confirmed that obesity is associated with graft function after KT. The glomerular filtration rate 12 months

Table 3 Outcome and graft function at 12 months after KT (data are expressed as the means)

	BMI ≤29.9 (*n* = 317)	BMI 30–34.9 (*n* = 52)	BMI ≥35 (*n* = 15)	*P* value
Serum creatinine (mg/dl) (±SD)	1.76 (0.81)	2.04 (0.10)	2.1 (0.66)	*0.036*
Glomerular filtration rate, *CKD-EPI* (ml/min/1.73 m^2) (±SD)	46.82 (19.01)	40.71 (17.77)	32.76 (12.26)	*0.005*
Systolic blood pressure (mmHg) (±SD)	134.20 (16.10)	140.39 (13.75)	139.50 (7.14)	0.072
HbA1c (%) (±SD)	6.28 (1.10)	6.42 (1.42)	7.65 (1.17)	0.116
Acute rejection, *n* (%)	38 (12.0)	6 (11.5)	1 (6.7)	0.801
Primary non-function, *n* (%)	6 (1.9)	2 (3.8)	1 (6.7)	*0.047*
Delayed graft function, *n* (%)	56 (17.7)	17 (32.7)	6 (40)	*0.008*
Loss of graft function, *n* (%)	33 (10.4)	14 (26.9)	3 (20)	*0.015*
Re-transplantation, *n* (%)	3 (0.9)	1 (1.9)	2 (13.3)	*0.001*
Surgical site infections, *n* (%)	30 (9.5)	10 (19.2)	6 (40.0)	*0.003*
Weight gain post-transplant (kg) (±SD)	1.9 (13.35)	1.6 (8.43)	9.8 (7.02)	*0.009*

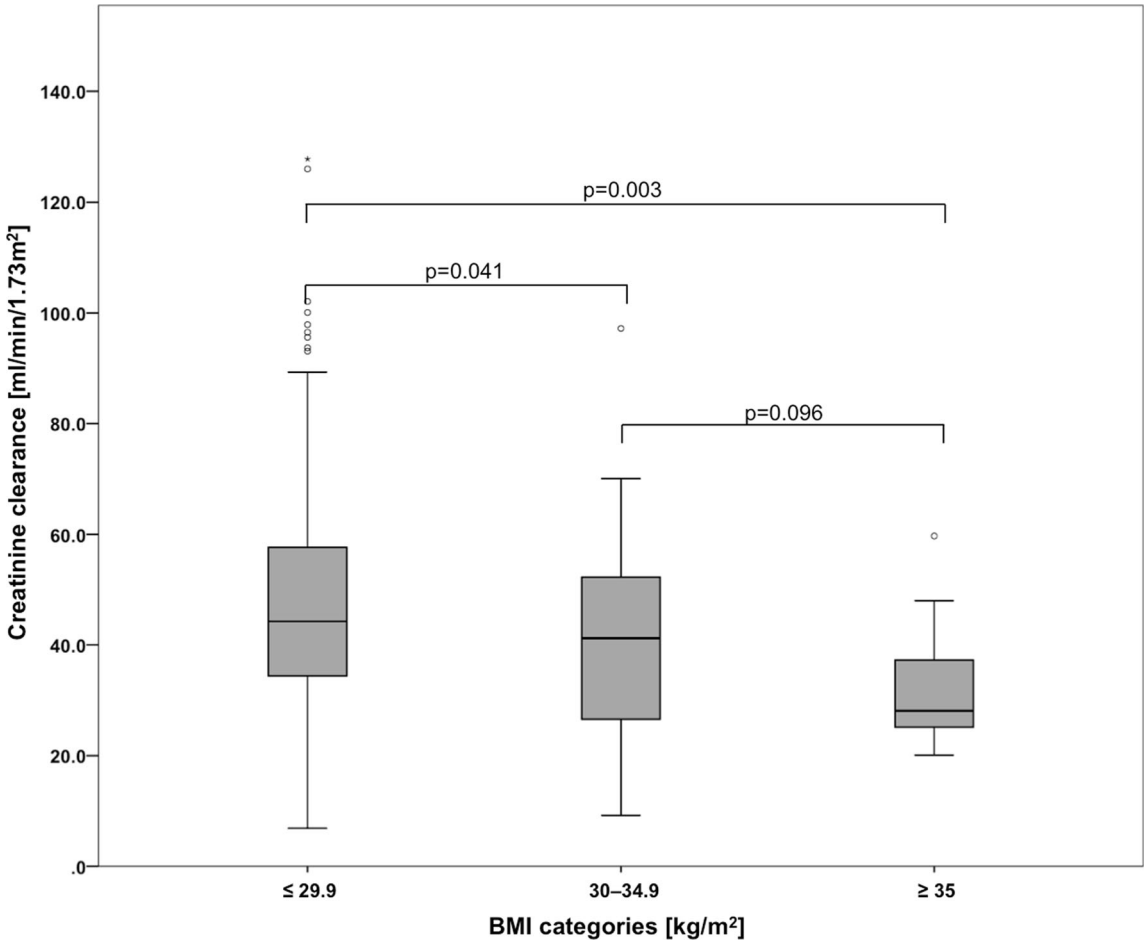

Fig. 2 Glomerular filtration rate (CKD-EPI) at 12 months after kidney transplantation according to BMI categories

after KT was significantly lower in recipients with a BMI ≥30 kg/m^2. Another well-known complication after KT is delayed graft function (DGF). We found rates of DGF of 17% in recipients with a BMI ≤29.9 kg/m^2 and as high as 40% in recipients with a BMI ≥35 kg/m^2. Similar associations between obesity and DGF have been described previously [18, 19, 38]. However, DGF was not identified in a multivariate analysis as a risk factor for graft survival in our cohort. In the literature, the results describing the impact of obesity on graft and patient survival are contradictory [31, 39, 26]. Although we observed significantly lower graft survival rates in patients with a BMI ≥35 kg/m^2 than in patients with a BMI <35 kg/m^2, patient survival was not affected.

Table 4 Reasons for graft failure

Parameters	Number
Rejection	11
Surgical conditions (e.g. thrombosis, kinking)	9
Recurrence of primary renal disease	6
Intestinal fibrosis/tubular atrophy	6
Glomerulonephritis/sclerosis	5
Other reasons	6
Unknown	7

The causal mechanism of obesity and kidney function after KT is not fully understood. The association of obesity with ESRD is related to many factors, including hyperfiltration, glomerular hypertension and over-activation of the renin-angiotensin system [40, 41]. Damage to the transplanted kidney may be caused by similar pathophysiological mechanisms to those occurring in the native kidneys of obese patients with developing ESRD [20]. On the other hand, immunosuppression dosage and drug monitoring raises difficulties in obese patients, including variations in the distribution of medication and the accurate estimation of renal function [42, 43]. Furthermore, the production of pro-inflammatory cytokines by fatty tissue has been discussed [20, 44].

A possible correlation between obesity and acute rejection is discussed in some studies [39, 45–47] and may be caused by inflammation and a modified immune response [37]. In our analysis, we did not identify differences in the rates of acute rejection episodes.

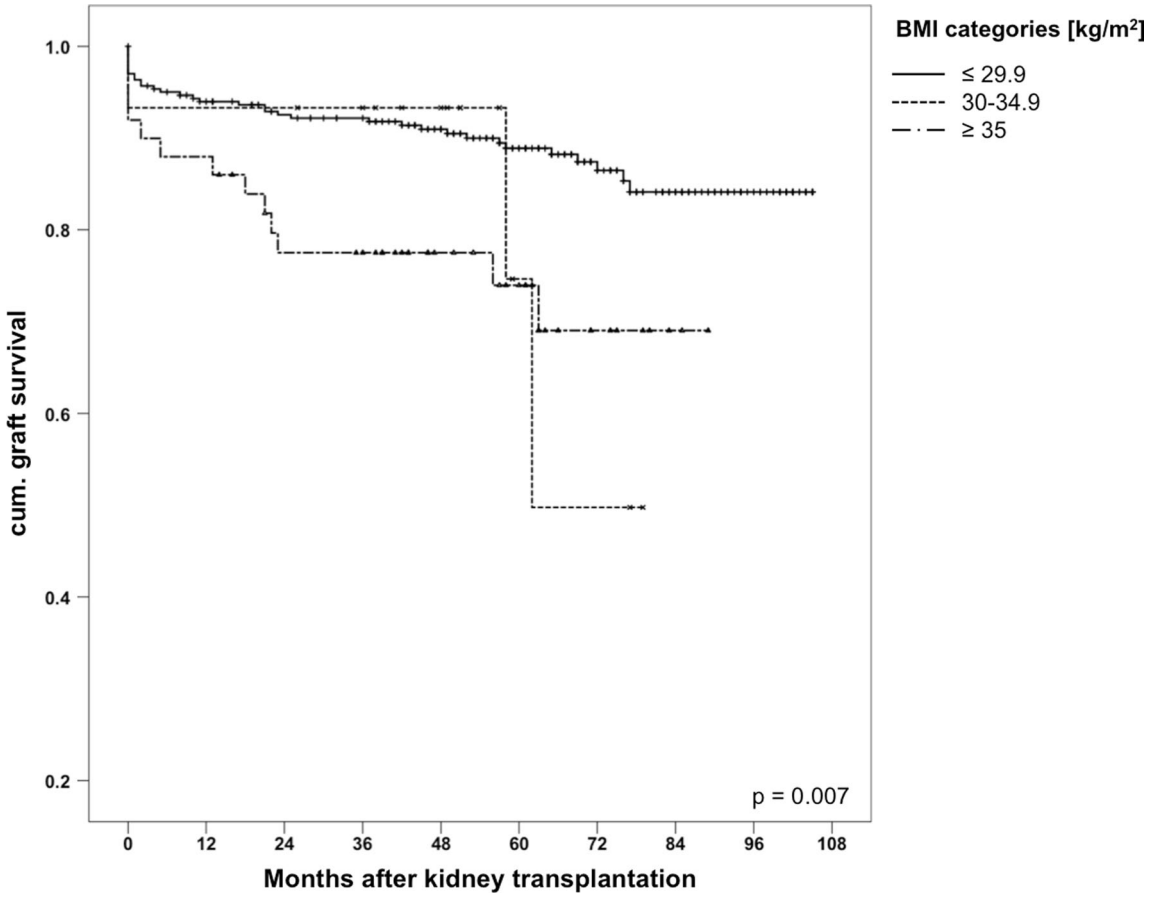

Fig. 3 Graft survival after kidney transplantation according to BMI categories

This study has limitations due to the retrospective nature of the data collection. Not all possible risk factors of kidney recipients, especially environmental, behavioural and psychological factors, were available and could be included in the univariate and multivariate analyses. Furthermore, the group of patients with a BMI ≥35 kg/m^2 was small. Nevertheless, we observed a distinct negative effect on outcome in this subgroup. Although no consensus exists, Pham et al. [16] suggest that obese patients with a BMI ≥35 kg/m^2 with more than two comorbid conditions (e.g. cardiovascular events, diabetes mellitus, and advanced age) should not receive transplants. Concerning bariatric surgery prior to or after transplantation for weight loss, only a few studies and case

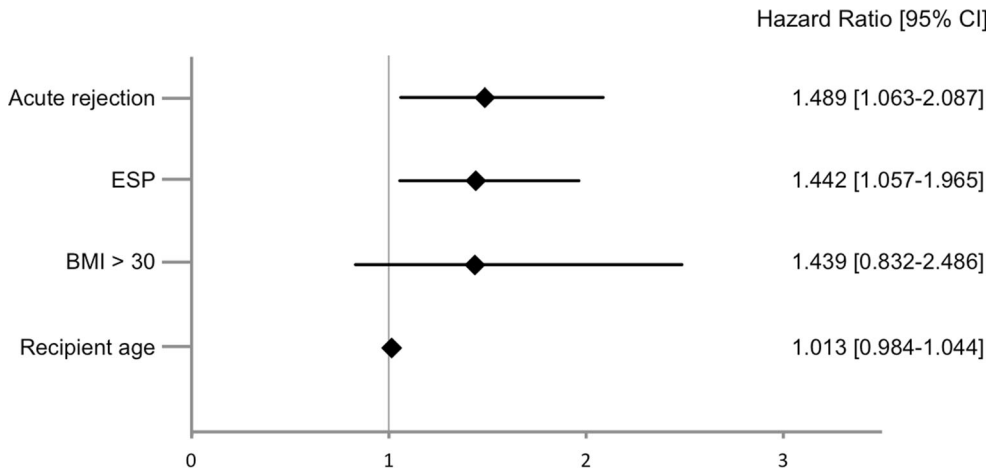

Fig. 4 Cox regression analysis of risk factors associated with graft survival after KT ($p < 0.05$)

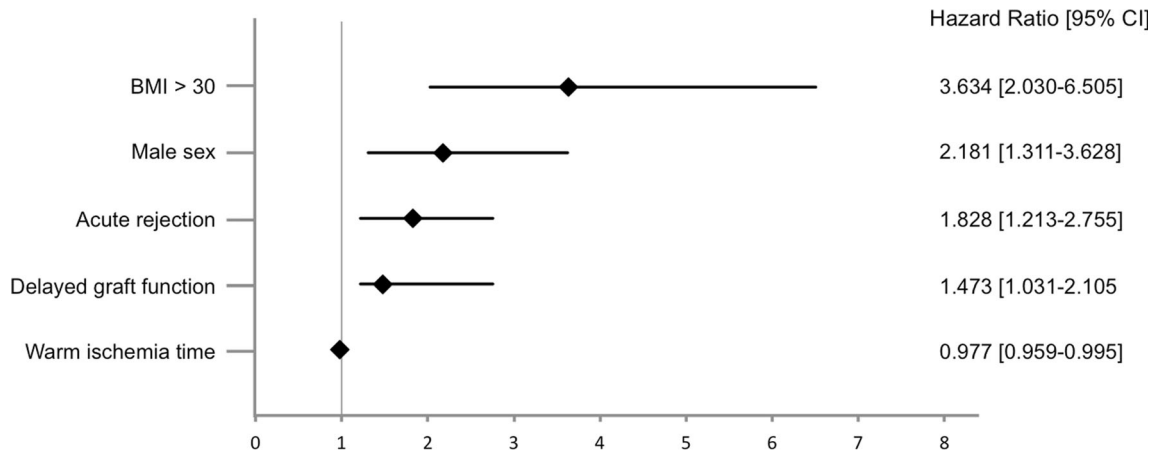

Fig. 5 Logistic regression analysis of risk factors associated with occurrence of surgical site infections ($p < 0.05$)

reports exist [48, 49]. Thus, the effect of bariatric surgery on graft function and patient and graft survival is unknown and should be evaluated in prospective studies.

However, weight gain after KT remains an unsolved problem [50].

The weight gain in our cohort in the first 12 months after KT ranged from 1.6 kg in transplant recipients with a BMI ≤29.9 kg/m^2 to 9.8 kg in the group with a BMI ≥35 kg/m^2. Kugler et al. showed in a prospective German study that in most cases, increased BMI levels were related to obesity before transplant and not to maintenance corticosteroid therapy [51]. Another study showed that also the type of calcineurin inhibitor has no effect on the weight gain after KT [52]. A survey of kidney transplant recipients also found a lack of awareness of BMI and its associated risks, highlighting the need for patient education after KT [50].

Knowledge of the higher risk for delayed graft function, graft failure and SSI should be included in the information given to patients before kidney transplantation. The awareness of risk factors for a worse outcome after KT in patients on the waiting list could motivate them to join interdisciplinary programmes for weight loss during their waiting time.

Table 5 Post-operative complications

Parameters	n
Wound healing disorders	36
Bleeding/haematoma	19
Vascular complications (thrombosis or embolus of the renal vessels, kinking)	20
Complications of ureter/bladder anastomosis	14
Lymphocele	16
Infections (e.g. pneumonia, urinary infection, cholecystitis)	3
Myocardial infarction	3
Others	5

Conclusions

Increased BMI at kidney transplantation is a predictor of adverse outcomes, including delayed graft function. These findings demonstrate the importance of careful patient selection and pre-transplant weight reduction, although the role of weight reduction for improving graft function is not clear.

Because of the survival benefit of KT in obese patients [35] over dialysis, we believe that these patients should not be excluded from KT simply based on BMI. However, there is a clear need for interdisciplinary programmes for weight loss pre- and post-operatively in the kidney transplant setting. Furthermore, obese patients should be strictly evaluated before listing for kidney transplant based on consideration of pre-existing cardiovascular and metabolic diseases.

Authors' contributions J. Liese: study conception and design, data analysis and interpretation and drafting of the manuscript; N. Bottner: data acquisition, analysis and interpretation; S. Büttner: data acquisition; A. Reinisch: data acquisition and critical revision of the manuscript; G. Woeste: data acquisition and critical revision of the manuscript; M. Wortmann: data acquisition; I.A. Hauser: study conception and design; W.O. Bechstein: study conception and design, drafting of the manuscript and critical revision of the manuscript; F. Ulrich: study conception and design and critical revision of the manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the local ethical committee at Frankfurt University, IRB-No. 234/14.

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6. Erklärung

Ich erkläre hiermit, dass die vorliegende kumulative Habilitationsschrift „Translationale chirurgische Forschung: vom Labor an das Patientenbett, von der onkologischen Grundlagenforschung zur Transplantationsmedizin“ eigenständig und ohne fremde Hilfe von mir verfasst wurde.

Ich erkläre hiermit, keine anderen als die angegebenen Quellen verwandt zu haben, wobei wörtlich oder annähernd wörtlich aus anderen Arbeiten entnommene Stellen als solche genau erkenntlich gemacht worden sind.

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