

World Journal of *Gastroenterology*

World J Gastroenterol 2019 April 21; 25(15): 1783-1906



**OPINION REVIEW**

- 1783** Repurposing drugs to target nonalcoholic steatohepatitis
Sookoian S, Pirola CJ

REVIEW

- 1797** Central role of Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif in pancreatic cancer development
Rozengurt E, Eibl G

MINIREVIEWS

- 1817** Considerations of elderly factors to manage the complication of liver cirrhosis in elderly patients
Kamimura K, Sakamaki A, Kamimura H, Setsu T, Yokoo T, Takamura M, Terai S

ORIGINAL ARTICLE**Basic Study**

- 1828** Lysyl oxidase and hypoxia-inducible factor 1 α : biomarkers of gastric cancer
Han YL, Chen L, Qin R, Wang GQ, Lin XH, Dai GH
- 1840** Predictive and prognostic implications of 4E-BP1, Beclin-1, and LC3 for cetuximab treatment combined with chemotherapy in advanced colorectal cancer with wild-type KRAS: Analysis from real-world data
Guo GF, Wang YX, Zhang YJ, Chen XX, Lu JB, Wang HH, Jiang C, Qiu HQ, Xia LP
- 1854** Extract of *Cycas revoluta* Thunb. enhances the inhibitory effect of 5-fluorouracil on gastric cancer cells through the AKT-mTOR pathway
Cui XL, Li KJ, Ren HX, Zhang YJ, Liu XD, Bu BG, Wang L
- 1865** Unconjugated bilirubin alleviates experimental ulcerative colitis by regulating intestinal barrier function and immune inflammation
Zheng JD, He Y, Yu HY, Liu YL, Ge YX, Li XT, Li X, Wang Y, Guo MR, Qu YL, Qin XF, Jiang MS, Wang XH

Retrospective Study

- 1879** Value of pretransplant albumin-bilirubin score in predicting outcomes after liver transplantation
Ma T, Li QS, Wang Y, Wang B, Wu Z, Lv Y, Wu RQ

Observational Study

- 1890** Mechanism of exosomal microRNA-224 in development of hepatocellular carcinoma and its diagnostic and prognostic value
Cui Y, Xu HF, Liu MY, Xu YJ, He JC, Zhou Y, Cang SD

CASE REPORT

- 1899** Colon perforation due to antigenemia-negative cytomegalovirus gastroenteritis after liver transplantation: A case report and review of literature

Yokose T, Obara H, Shinoda M, Nakano Y, Kitago M, Yagi H, Abe Y, Yamada Y, Matsubara K, Oshima G, Hori S, Ibuki S, Higashi H, Masuda Y, Hayashi M, Mori T, Kawaida M, Fujimura T, Hoshino K, Kameyama K, Kuroda T, Kitagawa Y

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Dan Lucian Dumitrascu, MD, PhD, Professor, 2nd Medical Department, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca 400174, Romania

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, etc. The *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

The *WJG* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus and Directory of Open Access Journals. The 2018 edition of Journal Citation Report® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

RESPONSIBLE EDITORS
FOR THIS ISSUE

Responsible Electronic Editor: Yu-Jie Ma

Proofing Editorial Office Director: Ze-Mao Gong

NAME OF JOURNAL

World Journal of Gastroenterology

ISSN

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE

October 1, 1995

FREQUENCY

Weekly

EDITORS-IN-CHIEF

Subrata Ghosh, Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

EDITORIAL OFFICE

Ze-Mao Gong, Director

PUBLICATION DATE

April 21, 2019

COPYRIGHT

© 2019 Baishideng Publishing Group Inc

INSTRUCTIONS TO AUTHORS

<https://www.wjgnet.com/bpg/gerinfo/204>

GUIDELINES FOR ETHICS DOCUMENTS

<https://www.wjgnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH

<https://www.wjgnet.com/bpg/gerinfo/240>

PUBLICATION MISCONDUCT

<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>



Repurposing drugs to target nonalcoholic steatohepatitis

Silvia Sookoian, Carlos J Pirola

ORCID number: Silvia Sookoian (0000-0001-5929-5470); Carlos J Pirola (0000-0001-8234-4058).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version, Sookoian S and Pirola CJ should be considered joint senior authors.

Conflict-of-interest statement: No potential conflicts of interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Received: January 18, 2019

Peer-review started: January 18, 2019

First decision: March 20, 2019

Revised: March 21, 2019

Accepted: March 29, 2019

Article in press: March 30, 2019

Published online: April 21, 2019

P-Reviewer: Kim DJ

S-Editor: Yan JP

L-Editor: A

Silvia Sookoian, Department of Clinical and Molecular Hepatology, National Scientific and Technical Research Council (CONICET), University of Buenos Aires, Institute of Medical Research (IDIM), Ciudad Autónoma de Buenos Aires 1427, Argentina

Carlos J Pirola, Department of Molecular Genetics and Biology of Complex Diseases, National Scientific and Technical Research Council (CONICET), University of Buenos Aires, Institute of Medical Research (IDIM), Ciudad Autónoma de Buenos Aires 1427, Argentina

Corresponding author: Silvia Sookoian, FAASLD, MD, PhD, Senior Scientist, Department of Clinical and Molecular Hepatology, National Scientific and Technical Research Council (CONICET), University of Buenos Aires, Institute of Medical Research (IDIM), Combatientes de Malvinas 3150, Ciudad Autónoma de Buenos Aires 1427, Argentina.

ssookoian@intramed.net

Telephone: +54-11-52873905

Fax: +54-11-52873905

Abstract

Nonalcoholic fatty liver disease (NAFLD) is a complex disorder that has evolved in recent years as the leading global cause of chronic liver damage. The main obstacle to better disease management pertains to the lack of approved pharmacological interventions for the treatment of nonalcoholic steatohepatitis (NASH) and NASH-fibrosis-the severe histological forms. Over the past decade, tremendous advances have been made in NAFLD research, resulting in the discovery of disease mechanisms and novel therapeutic targets. Hence, a large number of pharmacological agents are currently being tested for safety and efficacy. These drugs are in the initial pharmacological phases (phase 1 and 2), which involve testing tolerability, therapeutic action, and pharmacological issues. It is thus reasonable to assume that the next generation of NASH drugs will not be available for clinical use for foreseeable future. The expected delay can be mitigated by drug repurposing or repositioning, which essentially relies on identifying and developing new uses for existing drugs. Here, we propose a drug candidate selection method based on the integration of molecular pathways of disease pathogenesis into network analysis tools that use OMICS data as well as multiples sources, including text mining from the medical literature.

Key words: Drug discovery; Drug repositioning; Fibrosis; Genetics; Treatment; Systems biology

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: As a proof-of-concept of the advantages that can be yielded by applying multi-

E-Editor: Ma YJ



omics systems-based approaches to the analysis of potential candidates to the treatment of nonalcoholic steatohepatitis (NASH) we selected the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map of nonalcoholic fatty liver disease (NAFLD), which illustrates a stage-dependent progression of the disease. After generating a protein–chemical interaction network, we predicted remarkable examples of potential drug repurposing for the treatment of NASH based on the NAFLD-KEGG connectivity map.

Citation: Sookoian S, Pirola CJ. Repurposing drugs to target nonalcoholic steatohepatitis.

World J Gastroenterol 2019; 25(15): 1783-1796

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1783.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1783>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a complex disorder that has emerged as the leading global cause of chronic liver damage in recent years^[1]. The disease course progresses through highly dynamic histological stages, ranging from simple steatosis or nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), NASH-fibrosis and cirrhosis^[1,2]. NASH-fibrosis and its complications, including cirrhosis and hepatocellular carcinoma, not only significantly reduce life expectancy by increasing liver-related mortality^[3] but also represent a challenge for the healthcare system because much of the affected population is also affected by NAFLD-associated comorbidities, including obesity, type 2 diabetes (T2D), and cardiovascular disease^[1,4-6]. Absence of reliable noninvasive biomarkers that allow identification of patients at a high risk of fibrosis and /or disease progression is one of the obstacles facing disease management^[7,8]. Similarly, while a large number of drugs against NASH are currently being tested for efficacy and safety, no pharmacological interventions are presently approved for treating NASH^[2,5,9,10].

Information retrieved from public domain data sources and clinical ClinicalTrials.gov (updated December 2018), a resource provided by the U.S. National Library of Medicine, indicates that approximately 47 different drugs that target NASH and NASH-fibrosis are currently being tested in different pharmacological stages, including 188 drugs in phase 1 and 162 in phase 2 studies (Figure 1). A significant proportion of these drugs are small molecules or proteins that either antagonize or act as exogenous agonists of one or more targets of interest; the 47 aforementioned NASH drugs are in fact predicted to be linked to 151 molecular targets (Figure 1). Considering that a large majority of these drugs are in the earliest pharmacological phases that involve testing tolerability, therapeutic action, and pharmacological issues, it is reasonable to conclude that there will be a significant time lag before the next generation of NASH drugs is available for clinical use.

One potential solution to this expected delay is drug repurposing or repositioning, which relies on identifying and developing new uses for existing drugs^[11]. The advantage of drug repurposing is not limited to the fact that drugs selected for a novel indication have already passed the time-consuming pharmacokinetics, pharmacodynamics, and toxicity profiling evaluation, but are also already approved by major regulatory agencies, including the United States Food and Drug Administration and/or the European Medicines Agency.

Drug repurposing can be addressed by different approaches. Most common ones involve the selection of drug candidate/s based on known targets involved in the pathogenesis of the disease of interest. More recently, system biology strategies based on a broad search into genomic resources, as well as large-scale gene expression libraries, have been proposed as an attractive and innovative solution, particularly for the treatment of complex diseases like NAFLD that shares disease mechanisms with diseases of the metabolic syndrome^[12-14]. Hence, we propose a drug candidate selection method based on the integration of molecular pathways of disease pathogenesis into network analysis tools that use OMICs data as well as multiples sources, including text mining from pertinent medical literature.

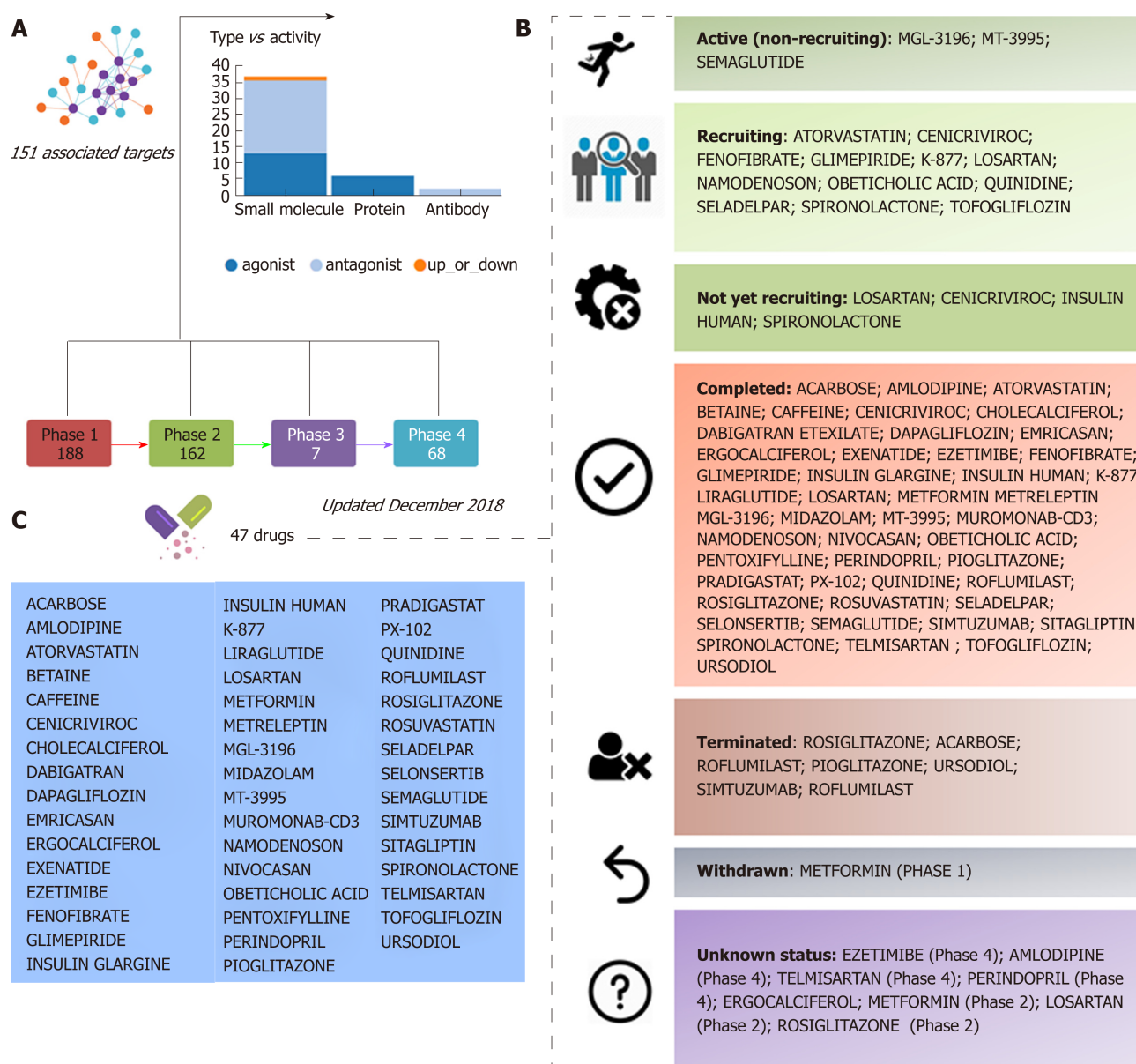


Figure 1 Clinical trials for the treatment of nonalcoholic steatohepatitis. A and B: Figure highlights 47 drugs that are currently under investigation for the treatment of nonalcoholic steatohepatitis in different pharmacological phases (from phase 1 to phase 4). Information on clinical trial status (recruitment status) as well as prediction of potential associated targets were retrieved from the Target Validation Platform available at <https://www.targetvalidation.org>; C: Drugs listed in the most advanced pharmacological phase updated December 2018 concerning to privately and publicly funded clinical studies. Not yet recruiting: The study has not started recruiting participants; Recruiting: The study is currently recruiting participants; Active, not recruiting: The study is ongoing, and participants are receiving an intervention or being examined, but potential participants are not currently being recruited or enrolled; Terminated: The study has stopped early and will not start again; participants are no longer being examined or treated; Completed: The study has ended normally, and participants are no longer being examined or treated (that is, the last participant's last visit has occurred); Withdrawn: The study stopped early, before enrolling its first participant; Unknown: A study on ClinicalTrials.gov whose last known status was recruiting; not yet recruiting; or active, not recruiting but that has passed its completion date, and the status has not been last verified within the past 2 years).

DRUG REPURPOSING FOR THE TREATMENT OF NASH BASED ON THE NAFLD-KEGG CONNECTIVITY MAP

As a proof-of-concept of the advantages of using multi-omics systems-based approaches for the analysis of potential NASH treatment candidates, we selected the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map of NAFLD (pathway ID: hsa04932), which illustrates a stage-dependent progression of the disease (Figure 2). This pathway is composed of 149 genes/proteins involved not only in the progression of NAFL to NASH and to cirrhosis, but also genes/proteins shared with obesity and T2D (Table 1). Significant disease-related pathogenic processes, including *de novo* fatty acid biosynthesis, lipid peroxidation, endoplasmic reticulum stress and mitochondrial dysfunction^[15-17], as well as apoptosis and cell death related mechanisms are represented in the NAFLD-KEGG pathway (Figure 2). Thus, we

generated a protein–chemical interaction network by mapping the significant genes/proteins that are represented in the pathway to chemicals/drugs that are annotated in the Comparative Toxicogenomics Database. The 149 genes (seeds) yielded by our analysis were then mapped to the corresponding molecular interaction database; this procedure produced an extensive network comprising of approximately 2000 nodes. One of the largest subnetworks included 3212 smaller nodes (that represent the number of gene/protein–chemical interactions in this subnetwork), with 13314 interactions among node members. For simplicity, we manually curated some chemical–drug interactions focusing specifically on certain genes/proteins of potential interest, including members of the caspase family (CASP3 and CASP7), interleukins (IL1A, IL1B, and IL6), tumor necrosis factor α (TNF α), nuclear factor kappa B subunit 1 (NFKB1) and inhibitor of nuclear factor kappa B kinase subunit beta, Jun proto-oncogene (JUN), transcription factor subunit, and AKT serine/threonine kinase 1 (Figure 3). Remarkably, several drugs were predicted to have a significant interaction with the highlighted targets. For example, minocycline that is a broad spectrum long-acting derivative of the antibiotic tetracycline was mapped in the pathway of caspases, whereas IL1B (Figure 3) or pomalidomide that is a derivative of thalidomide with immuno-modulating, antiangiogenic and antineoplastic activities was mapped in the network of TNF, NFKB1, and interleukins (Figure 3).

Additional targets predicted in the minocycline interaction network are arachidonate 5-lipoxygenase (which is involved in the synthesis of leukotrienes from arachidonic acid), cytochrome C (a central component of the electron transport chain in mitochondria), matrix metalloproteinase 9 (involved in the breakdown of extracellular matrix), vascular endothelial growth factor A (which induces proliferation and migration of vascular endothelial cells, particularly during pathological angiogenesis) and Poly(ADP-ribose) polymerase 1 (which is involved in the regulation of a myriad of cellular processes, such as differentiation, proliferation, and tumor transformation, as well as in the regulation of the molecular events implicit in the cell recovery from DNA damage). Further two candidate targets predicted in the network of pomalidomide are prostaglandin-endoperoxide synthase 2 (also known as cyclooxygenase, which is the key enzyme in prostaglandin biosynthesis) and CRBN (a calcium channel membrane protein, thought to play a role in brain development).

Additional examples of drugs that could be potentially tested for the treatment of NASH based on the concept of drug repositioning are illustrated in Figure 3. Drugs in the category of angiotensin II receptor type 1 (AGTR1) antagonists that were predicted in the network of JUN, for instance irbersartan-a nonpeptide AGTR1 antagonist with antihypertensive activity-might indeed be regarded as an indication expansion rather than drug repositioning because, as mentioned above, NAFLD and components of the Metabolic Syndrome, including arterial hypertension, present shared disease mechanisms [12-14]. Therefore, given the pleiotropic effects of AGTR1 blockers^[18] it is plausible to suggest that drugs in this pharmacological group-sartans-would synergize or potentiate the benefits of blocking the renin angiotensin system in the liver^[19-22]. Remarkably, the pharmacological properties and toxicity profiles of some of the drugs presently undergoing NASH clinical trials are already known, such as atorvastatin, ezetimibe, fenofibrate, losartan, and pioglitazone, just to mention a few (Figure 1).

PLEIOTROPY: CHALLENGES AND OPPORTUNITIES FOR THE TREATMENT OF NASH

It is also important to acknowledge the possibility that some of the novel pharmacotherapy options for the treatment of NASH might eventually present pleiotropic effect/s. This point represents the paradox of a drug covering multiple pathways and cell types, which could be either harmful or beneficial for patients. Remarkable examples of the advantages of pleiotropic effects of pharmacological targets for the treatment of complex traits are, as already mentioned, agents that modulate or interfere with the rennin–angiotensin system, which not only reduce cardiovascular risk but also improve systemic inflammation, oxidative stress, and even present anti-fibrogenic properties in the liver. Similar effects have also been demonstrated for statins^[23,24].

When focusing on the new generation of NASH targets, obeticholic acid (OCA), a synthetically-modified bile acid (a dihydroxy-5 β -cholanolic acid), is a remarkable example of the potential systemic effects of a drug targeting nuclear receptors. OCA exhibits a potent agonist effect on the farnesoid X nuclear receptor (FXR). More

Table 1 Non-alcoholic fatty liver disease-Kyoto Encyclopedia of Genes and Genomes pathway (hsa04932)

Gene symbol; description
IL6; interleukin 6
IL6R; interleukin 6 receptor
SOCS3; suppressor of cytokine signaling 3
TNF; tumor necrosis factor
TNFRSF1A; TNF receptor superfamily member 1A
NFKB1; nuclear factor kappa B subunit 1
RELA; RELA proto-oncogene, NF-kB subunit
INS; insulin
INSR; insulin receptor
IRS1; insulin receptor substrate 1
IRS2; insulin receptor substrate 2
PIK3CA; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3CD; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta
PIK3CB; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
PIK3R1; phosphoinositide-3-kinase regulatory subunit 1
PIK3R2; phosphoinositide-3-kinase regulatory subunit 2
PIK3R3; phosphoinositide-3-kinase regulatory subunit 3
AKT1; AKT serine/threonine kinase 1
AKT2; AKT serine/threonine kinase 2
AKT3; AKT serine/threonine kinase 3
GSK3A; glycogen synthase kinase 3 alpha
GSK3B; glycogen synthase kinase 3 beta
NR1H3; nuclear receptor subfamily 1 group H member 3
RXRA; retinoid X receptor alpha
SREBF1; sterol regulatory element binding transcription factor 1
MLX; MLX, MAX dimerization protein
MLXIP; MLX interacting protein
MLXIPL; MLX interacting protein like
PKLR; pyruvate kinase L/R
LEP; leptin
LEPR; leptin receptor
ADIPOQ; adiponectin, C1Q and collagen domain containing
ADIPOR1; adiponectin receptor 1
ADIPOR2; adiponectin receptor 2
PRKAA1; protein kinase AMP-activated catalytic subunit alpha 1
PRKAA2; protein kinase AMP-activated catalytic subunit alpha 2
PRKAB1; protein kinase AMP-activated non-catalytic subunit beta 1
PRKAB2; protein kinase AMP-activated non-catalytic subunit beta 2
PRKAG1; protein kinase AMP-activated non-catalytic subunit gamma 1
PRKAG3; protein kinase AMP-activated non-catalytic subunit gamma 3
PRKAG2; protein kinase AMP-activated non-catalytic subunit gamma 2
PPARA; peroxisome proliferator activated receptor alpha
CDC42; cell division cycle 42
RAC1; Rac family small GTPase 1
MAP3K11; mitogen-activated protein kinase kinase kinase 11
MAPK8; mitogen-activated protein kinase 8
MAPK10; mitogen-activated protein kinase 10
MAPK9; mitogen-activated protein kinase 9
ITCH; itchy E3 ubiquitin protein ligase
ERN1; endoplasmic reticulum to nucleus signaling 1
TRAF2; TNF receptor associated factor 2
MAP3K5; mitogen-activated protein kinase kinase kinase 5

JUN; Jun proto-oncogene, AP-1 transcription factor subunit
 IL1A; interleukin 1 alpha
 IL1B; interleukin 1 beta
 IKKB; inhibitor of nuclear factor kappa B kinase subunit beta
 XBP1; X-box binding protein 1
 CEBPA; CCAAT enhancer binding protein alpha
 CYP2E1; cytochrome P450 family 2 subfamily E member 1
 FASLG; Fas ligand
 CXCL8; C-X-C motif chemokine ligand 8
 TGFBI; transforming growth factor beta 1
 EIF2AK3; eukaryotic translation initiation factor 2 alpha kinase 3
 EIF2S1; eukaryotic translation initiation factor 2 subunit alpha
 ATF4; activating transcription factor 4
 DDIT3; DNA damage inducible transcript 3
 BCL2L11; BCL2 like 11
 BAX; BCL2 associated X, apoptosis regulator
 FAS; Fas cell surface death receptor
 CASP8; caspase 8
 BID; BH3 interacting domain death agonist
 CYCS; cytochrome c, somatic
 CASP3; caspase 3
 CASP7; caspase 7
 NDUFV1-3; NADH:ubiquinone oxidoreductase core subunit V1 -V3
 NDUFA1-3; NADH:ubiquinone oxidoreductase subunit A1-3
 NDUFA4; NDUFA4, mitochondrial complex associated
 NDUFA4L2; NDUFA4, mitochondrial complex associated like 2
 NDUFA5-13; NADH:ubiquinone oxidoreductase subunit A5-A13
 NDUFAB1; NADH:ubiquinone oxidoreductase subunit AB1
 NDUFB1-11; NADH:ubiquinone oxidoreductase subunit B1-B11
 NDUF51-S8; NADH:ubiquinone oxidoreductase core subunit S1 -S8
 NDUFCl; NADH:ubiquinone oxidoreductase subunit C1
 NDUFc2; NADH:ubiquinone oxidoreductase subunit C2
 NDUFc2-KCTD14; NDUFc2-KCTD14 readthrough
 SDHA; succinate dehydrogenase complex flavoprotein subunit A
 SDHB; succinate dehydrogenase complex iron sulfur subunit B
 SDHC; succinate dehydrogenase complex subunit C
 SDHD; succinate dehydrogenase complex subunit D
 UQCRCF1; ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
 CYTB; cytochrome b
 CYC1; cytochrome c1
 UQCRC1; ubiquinol-cytochrome c reductase core protein 1
 UQCRC2; ubiquinol-cytochrome c reductase core protein 2
 UQCRH; ubiquinol-cytochrome c reductase hinge protein
 UQCRHL; ubiquinol-cytochrome c reductase hinge protein like
 UQCRB; ubiquinol-cytochrome c reductase binding protein
 UQCRCQ; ubiquinol-cytochrome c reductase complex III subunit VII
 UQCR10; ubiquinol-cytochrome c reductase, complex III subunit X
 UQCR11; ubiquinol-cytochrome c reductase, complex III subunit XI
 COX3; cytochrome c oxidase III
 COX1; cytochrome c oxidase subunit I
 COX2; cytochrome c oxidase subunit II
 COX4I2; cytochrome c oxidase subunit 4I2
 COX4I1; cytochrome c oxidase subunit 4I1
 COX5A; cytochrome c oxidase subunit 5A
 COX5B; cytochrome c oxidase subunit 5B
 COX6A1; cytochrome c oxidase subunit 6A1

COX6A2; cytochrome c oxidase subunit 6A2
 COX6B1; cytochrome c oxidase subunit 6B1
 COX6B2; cytochrome c oxidase subunit 6B2
 COX6C; cytochrome c oxidase subunit 6C
 COX7A1; cytochrome c oxidase subunit 7A1
 COX7A2; cytochrome c oxidase subunit 7A2
 COX7A2L; cytochrome c oxidase subunit 7A2 like
 COX7B; cytochrome c oxidase subunit 7B
 COX7B2; cytochrome c oxidase subunit 7B2
 COX7C; cytochrome c oxidase subunit 7C
 COX8C; cytochrome c oxidase subunit 8C
 COX8A; cytochrome c oxidase subunit 8A

https://www.genome.jp/kegg-bin/show_pathway?hsa04932.

importantly, its target-FXR (formally Nuclear hormone receptor subfamily 1 group H member 4, NR1H4, also known as BAR) is predicted to be involved in the pathogenesis of multiple phenotypes that practically cover the full range of human diseases and traits (Figure 4). It is well known that OCA is currently used to treat not only NASH but other chronic liver diseases as well, including primary biliary cholangitis^[25]. However, there are at least 65 registered clinical trials in various pharmacological phases for ~50 different diseases (Figure 4).

Based on this evidence, one may presume that the pleiotropic effects, and thus the clinical consequences, of the novel NASH drugs that are predicted to concurrently modulate a broad range of molecular pathways could be surprisingly extensive and therefore largely beneficial for treating multiple phenotypes. However, potential pleiotropic effects of the novel anti-NASH drugs could produce undesirable effects that we need to understand in order to anticipate their management. Some of these potential pleiotropic effects are indeed related to the primary biological and molecular network associated with the drug target itself. To illustrate the importance of this issue, we randomly selected five molecular targets (MAP3K5 or ASK1, FXR, PPAR α / δ , THR β , and MPC1) against which five drugs are currently being tested in patients with NASH (selonsertib^[26], OCA^[27], elafibranor^[28], MGL-3196 (<https://clinicaltrials.gov/ct2/show/NCT02912260>), and MSDC-0602K^[29] (<https://clinicaltrials.gov/ct2/show/NCT02784444>). Next, we explored the potential pleiotropic effect/s of modulating these targets in humans by searching for associations of genetic variants in the aforementioned targets with different phenotypes and traits, known as PheWAS (Phenome-wide association studies). We specifically retrieved publically available information from the United Kingdom Biobank that explored genetic variations in 452264 United Kingdom Biobank White British individuals (<http://geneatlas.roslin.ed.ac.uk/>)^[30].

As shown in Figure 5 and Table 2, MAP3K5/ASK1, FXR, PPAR α / δ , THR β , and MPC1 variants are involved in multiple pleiotropic effects, including modulation of blood cell count, body mass index, and general body adiposity, along with complex systemic disorders, such as asthma, acute pancreatitis, migraine, intestinal malabsorption, thyroid disease, and malignant neoplasm. Hence, understanding the pleiotropic effects of the novel NASH drugs is the key to optimizing their use as well as preventing emergent-yet poorly understood-undesirable systemic complications that could potentially jeopardize their short- or long-term use.

CONCLUSION

We provide new strategies and approaches by which known drugs can be repurposed for the treatment of NASH. Although we explored and mapped NAFLD-chemical interaction networks, it will be necessary to perform clinical trials not only to assess therapeutic response and optimize dosage and delivery routes, but also to explore the possibility that new uses of existing (old) drugs could act on novel or unanticipated targets. The presence of potential “off target”-pleiotropic-effects raises the mandatory necessity of pharmacological optimization, including the assessment of drug interactions and adjustment according to liver function tests.

Table 2 Associations between variants in locus that are targets of novel drugs for the treatment of nonalcoholic steatohepatitis and multiple traits from individuals of the United Kindom Biobank

Trait	Variant	Position	-log ₁₀ (p-value)
NR1H4 (FXR) Farnesoid X-Activated Receptor			
K85 Acute pancreatitis	rs76372051	100945711	6.963890333
Immature reticulocyte fraction	rs35712	100971355	5.607954097
Impedance of arm (right)	rs1409791	100851307	5.152661824
Impedance of whole body	rs1409791	100851307	4.772216099
migraine	rs12579460	100966714	4.639293011
high cholesterol	rs7967468	100853792	4.543497322
N30-N39 Other diseases of urinary system	rs79306023	100938470	4.420628035
H81 Disorders of vestibular function	rs140644635	100923359	4.069764347
PPARδ (Peroxisome Proliferator Activated Receptor Delta)			
Whole body fat-free mass	rs36018387	35386872	59.74853212
Hip circumference	rs36018387	35386872	49.20670564
Whole body fat mass	rs36018387	35386872	37.00113934
Body fat percentage	rs36018387	35386872	20.45328464
Monocyte percentage	rs9469982	35267548	45.86340625
Platelet crit	rs33959228	35259397	21.6726615
White blood cell (leukocyte) count	rs9380500	35266231	21.54556677
Platelet count	rs9658111	35364534	17.88276186
Neutrophil count	rs9380500	35266231	17.11253462
Eosinophil percentage	rs2395625	35405461	15.34904201
Lymphocyte percentage	rs9658079	35327577	9.741626151
asthma	rs1557568	35260530	9.184130164
K90 Intestinal malabsorption	rs7771474	35320447	11.86097145
MPC1 (Mitochondrial Pyruvate Carrier 1)			
Mean platelet (thrombocyte) volume	rs10946160	166757818	7.378512135
Platelet count	rs3728	166778679	5.285527735
Red blood cell (erythrocyte) count	rs6916128	166759313	4.825911105
M31 Other necrotising vasculopathies	rs7449594	166774429	4.699926505
dyspepsia / indigestion	rs6909951	166758198	4.594790286
MAP3K5 (ASK-1) (Mitogen-Activated Protein Kinase Kinase Kinase 5)			
Mean platelet (thrombocyte) volume	rs6924387	137082948	14.48853109
Eosinophil count	rs932589	137083138	13.39556873
Lymphocyte percentage	rs6924387	137082948	10.84396601
Neutrophil count	rs6924387	137082948	10.59715422
Platelet count	rs9321570	137095679	9.792150289
White blood cell (leukocyte) count	rs6924387	137082948	9.574319083
Eosinophil percentage	rs932589	137083138	9.344890391
Monocyte count	rs9385775	137144920	9.1157769
Mean reticulocyte volume	rs9385775	137144920	8.817927896
Platelet distribution width	rs6924387	137082948	8.001963098
THRβ (Thyroid Hormone Receptor Beta)			
Mean corpuscular volume	rs869785	24347800	152.2743497
Mean corpuscular haemoglobin	rs869784	24348008	143.9371173
Red blood cell (erythrocyte) count	rs869785	24347800	61.9076303
Mean reticulocyte volume	rs869784	24348008	43.97976306
Reticulocyte count	rs1505307	24343330	16.57632823
Immature reticulocyte fraction	rs869784	24348008	15.67096843
Monocyte count	rs12485694	24346109	11.11788547
Lymphocyte count	rs13096529	24232035	10.58643203
Red blood cell (erythrocyte) distribution width	rs2167115	24339734	10.44361306
C56 Malignant neoplasm of ovary	rs189397255	24389732	12.2277003
Trunk fat-free mass	rs13100197	24491484	8.731024419

Trunk predicted mass	rs13100197	24491484	8.614769205
Leg fat percentage (left)	rs1349265	24159387	8.323233252

The associations have been computed using 452264 United Kingdom Biobank White British individuals. <http://geneatlas.roslin.ed.ac.uk/>.

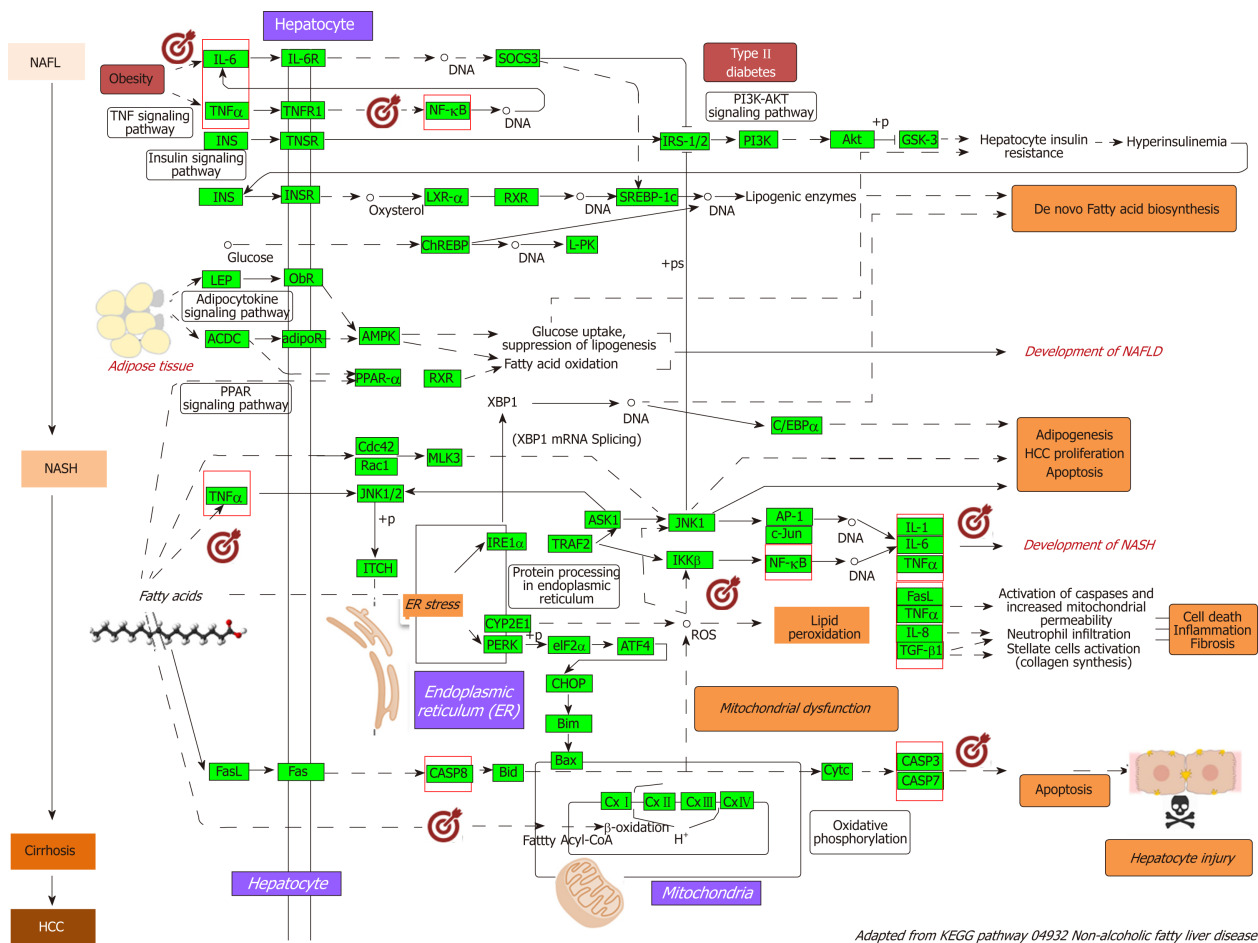


Figure 2 Nonalcoholic fatty liver disease-Kyoto Encyclopedia of Genes and Genomes pathway and mechanisms of disease pathogenesis. Pathway was retrieved from https://www.genome.jp/dbget-bin/www_bget?pathway+hsa04932; figure was modified to highlight key molecular processes. This map shows a stage-dependent progression of nonalcoholic fatty liver disease (NAFLD). In the first stage of NAFLD, pathway highlights excess lipid accumulation associated with the induction of insulin resistance, which leads to a defect in insulin suppression of free fatty acids (FAAs) disposal. In addition, two transcription factors, SREBP-1c and PPARα, activate key enzymes of lipogenesis and increase the synthesis of FAAs in liver. In the second stage, pathway is presented as a consequence of the progression to nonalcoholic steatohepatitis (NASH); the production of reactive oxygen species is enhanced due to oxidation stress through mitochondrial beta-oxidation of fatty acids and endoplasmic reticulum (ER) stress, leading to lipid peroxidation. The lipid peroxidation can further cause the production of cytokines [Fas ligand, tumor necrosis factor α (TNF-α), IL-8 and transforming growth factor], promoting cell death, inflammation and fibrosis. The activation of JNK, which is induced by ER stress, TNF-α and FAAs, is also associated with NAFLD progression. Increased JNK promotes cytokine production and initiation of hepatocellular carcinoma. Major organelles involved in the pathogenesis of NASH are also highlighted in the NAFLD-pathway, including mitochondria and mitochondrial dysfunction. In the figure, molecular targets that were further selected to explore protein-chemical interactions are highlighted by red squares. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; ER: Endoplasmic reticulum; HCC: Hepatocellular carcinoma; NAFL: Nonalcoholic fatty liver; FAAs: Free fatty acids; TNFα: tumor necrosis factor α.

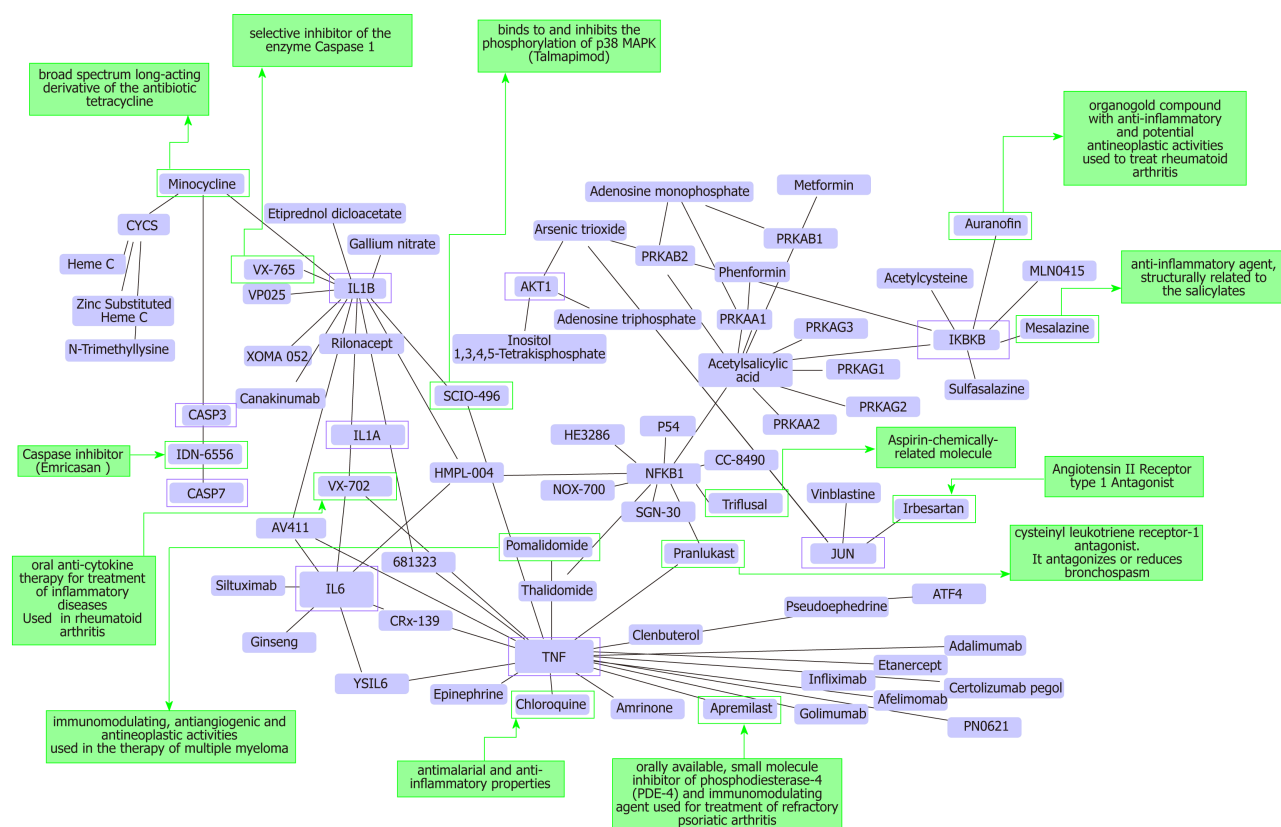


Figure 3 Protein-chemical interactions and potential repurposing drugs to target nonalcoholic steatohepatitis. We generated a protein-chemical interaction network by mapping the significant genes/proteins that are represented in the nonalcoholic fatty liver disease-Kyoto Encyclopedia of Genes and Genomes pathway to chemicals/drugs that are annotated in the Comparative Toxicogenomics Database. The 149 genes (seeds) from our analysis were mapped to the corresponding molecular interaction database; full list of seed genes is listed in [Table 1](#). This analysis generated a huge network composed of approximately 2000 nodes. Current figure shows chemical-drug-interactions specifically focused on selected genes/proteins of potential interest, including members of the caspase family (CASP3 and CASP7), interleukins (IL1B, IL1A, and IL6), tumor necrosis factor α (TNF- α), NFKB1 (Nuclear factor kappa B subunit 1) and IKBKB (inhibitor of nuclear factor kappa B kinase subunit beta), JUN (Jun proto-oncogene, AP-1 transcription factor subunit), AKT1 (AKT serine/threonine kinase 1). In green charts we summarized information on current use and known action of selected drugs. Interaction network was predicted by the NetworkAnalyst resource available at <https://www.networkanalyst.ca/faces/home.xhtml>. The network is shown as a Cytoscape graph.

presented as bubbles grouped into therapeutic areas using their Experimental Factor Ontology relationships. The size and shade of the color of each bubble is proportional to the strength of association between the disease and farnesoid X nuclear receptor. The concept of a target-disease association is based on the analysis of several resources, including genetic associations (GWAS Catalog, UniProt, European Variation Archive, Gene2Phenotype), somatic mutations (Cancer Gene Census, European Variation Archive somatic, IntOGen), RNA expression (expression atlas), drugs (ChEMBL), affected pathways (Reactome), animal models (PhenoDigm) and text mining (Europe PMC). The platform is available at <https://www.targetvalidation.org>. Data last updated December 2018.

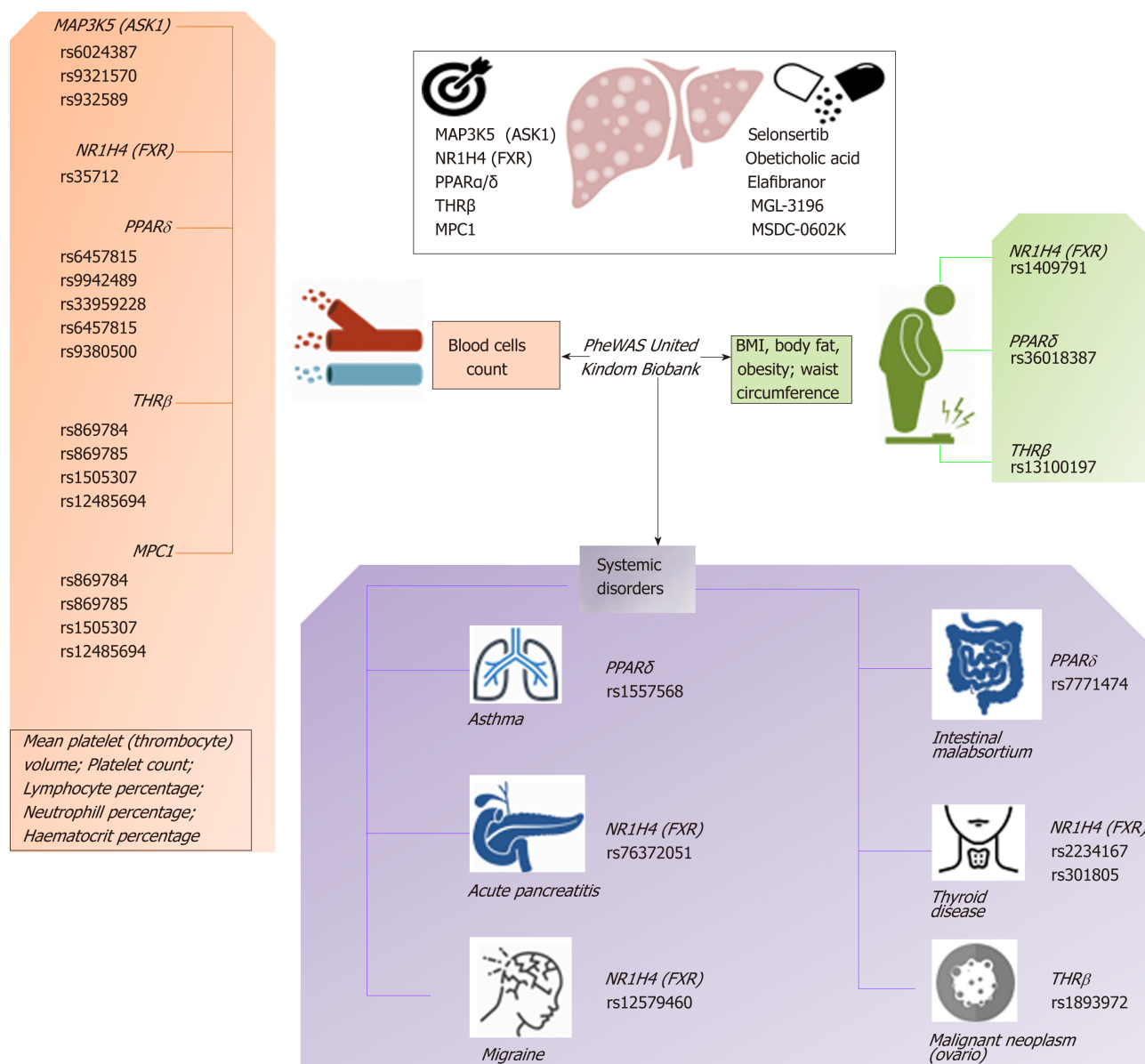


Figure 5 The complexity of molecular targets and novel nonalcoholic steatohepatitis drugs: Pleiotropy assessed in the PheWAS United Kindom Biobank. Figure shows associations between gene variants in five nonalcoholic steatohepatitis-related molecular targets (*MAP3K5/ASK1*, *FXR*, *PPARα/δ*, *THRβ*, and *MPC1*) with different traits and phenotypes in the UK-PheWAS (Phenome-wide association study). Information regarding single nucleotide polymorphisms and associations were retrieved from the United Kindom Biobank (<http://geneatlas.roslin.ed.ac.uk/>).

REFERENCES

- 1 Brunt EM, Wong VW, Nobili V, Day CP, Sookoian S, Maher JJ, Bugianesi E, Sirlin CB, Neuschwander-Tetri BA, Rinella ME. Nonalcoholic fatty liver disease. *Nat Rev Dis Primers* 2015; 1: 15080 [PMID: 27188459 DOI: 10.1038/nrdp.2015.80]
- 2 Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; 67: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]
- 3 Dulai PS, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, Sebastiani G, Ekstedt M, Hagstrom H, Nasr P, Stal P, Wong VW, Kechagias S, Hultcrantz R, Loomba R. Increased risk of mortality by fibrosis stage in

- nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology* 2017; **65**: 1557-1565 [PMID: 28130788 DOI: 10.1002/hep.29085]
- 4 **Sookoian S**, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: A systematic review. *J Hepatol* 2008; **49**: 600-607 [PMID: 18672311 DOI: 10.1016/j.jhep.2008.06.012]
 - 5 **Friedman SL**, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med* 2018; **24**: 908-922 [PMID: 29967350 DOI: 10.1038/s41591-018-0104-9]
 - 6 **Lonardo A**, Sookoian S, Pirola CJ, Targher G. Non-alcoholic fatty liver disease and risk of cardiovascular disease. *Metabolism* 2016; **65**: 1136-1150 [PMID: 26477269 DOI: 10.1016/j.metabol.2015.09.017]
 - 7 **Pirola CJ**, Sookoian S. Multiomics biomarkers for the prediction of nonalcoholic fatty liver disease severity. *World J Gastroenterol* 2018; **24**: 1601-1615 [PMID: 29686467 DOI: 10.3748/wjg.v24.i15.1601]
 - 8 **Wong VW**, Adams LA, de Lédinghen V, Wong GL, Sookoian S. Noninvasive biomarkers in NAFLD and NASH - current progress and future promise. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 461-478 [PMID: 29844588 DOI: 10.1038/s41575-018-0014-9]
 - 9 **Rotman Y**, Sanyal AJ. Current and upcoming pharmacotherapy for non-alcoholic fatty liver disease. *Gut* 2017; **66**: 180-190 [PMID: 27646933 DOI: 10.1136/gutjnl-2016-312431]
 - 10 **Konerman MA**, Jones JC, Harrison SA. Pharmacotherapy for NASH: Current and emerging. *J Hepatol* 2018; **68**: 362-375 [PMID: 29122694 DOI: 10.1016/j.jhep.2017.10.015]
 - 11 **Ashburn TT**, Thor KB. Drug repositioning: Identifying and developing new uses for existing drugs. *Nat Rev Drug Discov* 2004; **3**: 673-683 [PMID: 15286734 DOI: 10.1038/nrd1468]
 - 12 **Sookoian S**, Pirola CJ. Nonalcoholic fatty liver disease and metabolic syndrome: Shared genetic basis of pathogenesis. *Hepatology* 2016; **64**: 1417-1420 [PMID: 27480050 DOI: 10.1002/hep.28746]
 - 13 **Sookoian S**, Pirola CJ. Nonalcoholic fatty liver disease: Biomarkers support decisions around pharmacological intervention. *Hepatology* 2017; **65**: 1417-1419 [PMID: 27737511 DOI: 10.1002/hep.28866]
 - 14 **Sookoian S**, Pirola CJ. Review article: Shared disease mechanisms between non-alcoholic fatty liver disease and metabolic syndrome - translating knowledge from systems biology to the bedside. *Aliment Pharmacol Ther* 2019; **49**: 516-527 [PMID: 30714632 DOI: 10.1111/apt.15163]
 - 15 **Pirola CJ**, Gianotti TF, Burgueño AL, Rey-Funes M, Loidl CF, Mallardi P, Martino JS, Castaño GO, Sookoian S. Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* 2013; **62**: 1356-1363 [PMID: 22879518 DOI: 10.1136/gutjnl-2012-302962]
 - 16 **Sookoian S**, Rosselli MS, Gemma C, Burgueño AL, Fernández Gianotti T, Castaño GO, Pirola CJ. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: Impact of liver methylation of the peroxisome proliferator-activated receptor γ coactivator 1 α promoter. *Hepatology* 2010; **52**: 1992-2000 [PMID: 20890895 DOI: 10.1002/hep.23927]
 - 17 **Sookoian S**, Flichman D, Scian R, Rohr C, Dopazo H, Gianotti TF, Martino JS, Castaño GO, Pirola CJ. Mitochondrial genome architecture in non-alcoholic fatty liver disease. *J Pathol* 2016; **240**: 437-449 [PMID: 27577682 DOI: 10.1002/path.4803]
 - 18 **Chrysant SG**, Chrysant GS. The pleiotropic effects of angiotensin receptor blockers. *J Clin Hypertens (Greenwich)* 2006; **8**: 261-268 [PMID: 16596029 DOI: 10.1111/j.1524-6175.2005.05264.x]
 - 19 **Rosselli MS**, Burgueño AL, Carabelli J, Schuman M, Pirola CJ, Sookoian S. Losartan reduces liver expression of plasminogen activator inhibitor-1 (PAI-1) in a high fat-induced rat nonalcoholic fatty liver disease model. *Atherosclerosis* 2009; **206**: 119-126 [PMID: 19230890 DOI: 10.1016/j.atherosclerosis.2009.01.026]
 - 20 **Sookoian S**, Fernández MA, Castaño G. Effects of six months losartan administration on liver fibrosis in chronic hepatitis C patients: A pilot study. *World J Gastroenterol* 2005; **11**: 7560-7563 [PMID: 16437678 DOI: 10.3748/wjg.v11.i48.7560]
 - 21 **Sookoian S**, Castaño G, García SI, Viudez P, González C, Pirola CJ. A1166C angiotensin II type 1 receptor gene polymorphism may predict hemodynamic response to losartan in patients with cirrhosis and portal hypertension. *Am J Gastroenterol* 2005; **100**: 636-642 [PMID: 15743363 DOI: 10.1111/j.1572-0241.2005.41168.x]
 - 22 **Sookoian S**, Gianotti TF, Rosselli MS, Burgueño AL, Castaño GO, Pirola CJ. Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease. *Atherosclerosis* 2011; **218**: 378-385 [PMID: 21664615 DOI: 10.1016/j.atherosclerosis.2011.05.014]
 - 23 **Oesterle A**, Laufs U, Liao JK. Pleiotropic Effects of Statins on the Cardiovascular System. *Circ Res* 2017; **120**: 229-243 [PMID: 28057795 DOI: 10.1161/CIRCRESAHA.116.308537]
 - 24 **Pose E**, Trebicka J, Mookerjee RP, Angeli P, Ginès P. Statins: Old drugs as new therapy for liver diseases? *J Hepatol* 2019; **70**: 194-202 [PMID: 30075229 DOI: 10.1016/j.jhep.2018.07.019]
 - 25 **Hirschfield GM**, Mason A, Luketic V, Lindor K, Gordon SC, Mayo M, Kowdley KV, Vincent C, Bodhenheimer HC, Parés A, Trauner M, Marshall HU, Adorini L, Sciacca C, Beecher-Jones T, Castellote E, Böhm O, Shapiro D. Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid. *Gastroenterology* 2015; **148**: 751-61.e8 [PMID: 25500425 DOI: 10.1053/j.gastro.2014.12.005]
 - 26 **Jayakumar S**, Middleton MS, Lawitz EJ, Mantry PS, Caldwell SH, Arnold H, Mae Diehl A, Ghalib R, Elkhatab M, Abdelmalek MF, Kowdley KV, Stephen Djedjoe C, Xu R, Han L, Mani Subramanian G, Myers RP, Goodman ZD, Afdhal NH, Charlton MR, Sirlin CB, Loomba R. Longitudinal correlations between MRE, MRI-PDFF, and liver histology in patients with non-alcoholic steatohepatitis: Analysis of data from a phase II trial of selonsertib. *J Hepatol* 2019; **70**: 133-141 [PMID: 30291868 DOI: 10.1016/j.jhep.2018.09.024]
 - 27 **Neuschwander-Tetri BA**, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarthy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E; NASH Clinical Research Network. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015; **385**: 956-965 [PMID: 25468160 DOI: 10.1016/S0140-6736(14)61933-4]
 - 28 **Ratzliff V**, Harrison SA, Francque S, Bedossa P, Leher P, Serfaty L, Romero-Gomez M, Boursier J, Abdelmalek M, Caldwell S, Drenth J, Anstee QM, Hum D, Hanf R, Roudot A, Megnier S, Staels B, Sanyal A; GOLDEN-505 Investigator Study Group. Elafibranor, an Agonist of the Peroxisome Proliferator-Activated Receptor- α and - δ , Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. *Gastroenterology* 2016; **150**: 1147-1159.e5 [PMID: 26874076 DOI: 10.1053/j.gastro.2016.01.038]

- 29 **Colca JR**, McDonald WG, Adams WJ. MSDC-0602K, a metabolic modulator directed at the core pathology of non-alcoholic steatohepatitis. *Expert Opin Investig Drugs* 2018; **27**: 631-636 [PMID: [29950116](#) DOI: [10.1080/13543784.2018.1494153](#)]
- 30 **Canela-Xandri O**, Rawlik K, Tenesa A. An atlas of genetic associations in UK Biobank. *Nat Genet* 2018; **50**: 1593-1599 [PMID: [30349118](#) DOI: [10.1038/s41588-018-0248-z](#)]



Central role of Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif in pancreatic cancer development

Enrique Rozengurt, Guido Eibl

ORCID number: Enrique Rozengurt (0000-0002-7838-9170); Guido Eibl (0000-0003-3497-4527).

Author contributions: Eibl G and Rozengurt E contributed equally to writing and revising the manuscript.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Received: February 6, 2019

Peer-review started: February 6, 2019

First decision: March 5, 2019

Revised: March 20, 2019

Accepted: March 24, 2019

Article in press: March 25, 2019

Published online: April 21, 2019

P-Reviewer: Fekaj E, Şendur MAN
S-Editor: Ma RY

Enrique Rozengurt, Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, United States

Enrique Rozengurt, Guido Eibl, CURE: Digestive Diseases Research Center, Los Angeles, CA 90095, United States

Guido Eibl, Department of Surgery, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, United States

Corresponding author: Guido Eibl, MD, Director, Professor, Department of Surgery, David Geffen School of Medicine, University of California at Los Angeles, 72-236 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095, United States. geibl@mednet.ucla.edu

Telephone: +1-310-7949577

Fax: +1-310-2062472

Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a deadly disease with no efficacious treatment options. PDAC incidence is projected to increase, which may be caused at least partially by the obesity epidemic. Significantly enhanced efforts to prevent or intercept this cancer are clearly warranted. Oncogenic KRAS mutations are recognized initiating events in PDAC development, however, they are not entirely sufficient for the development of fully invasive PDAC.

Additional genetic alterations and/or environmental, nutritional, and metabolic signals, as present in obesity, type-2 diabetes mellitus, and inflammation, are required for full PDAC formation. We hypothesize that oncogenic KRAS increases the intensity and duration of the growth-promoting signaling network. Recent exciting studies from different laboratories indicate that the activity of the transcriptional co-activators Yes-associated protein (YAP) and WW-domain-containing transcriptional co-activator with PDZ-binding motif (TAZ) play a critical role in the promotion and maintenance of PDAC operating as key downstream target of KRAS signaling. While initially thought to be primarily an effector of the tumor-suppressive Hippo pathway, more recent studies revealed that YAP/TAZ subcellular localization and co-transcriptional activity is regulated by multiple upstream signals. Overall, YAP has emerged as a central node of transcriptional convergence in growth-promoting signaling in PDAC cells. Indeed, YAP expression is an independent unfavorable prognostic marker for overall survival of PDAC. In what follows, we will review studies implicating YAP/TAZ in pancreatic cancer development and consider different approaches

L-Editor: A

E-Editor: Ma YJ



to target these transcriptional regulators.

Key words: Pancreatic cancer; Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif; Oncogenic Kras; Obesity; Signaling network and loops

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The identification of signaling networks that underlie risk factor promoted pancreatic cancer development and progression is of paramount importance to prevent or intercept this lethal disease. Accumulating evidence suggests that several core signaling pathways downstream of oncogenic Kras, augmented by environmental conditions, *e.g.*, obesity, converge on Yes-associated protein (YAP) and WW-domain-containing transcriptional co-activator with PDZ-binding motif (TAZ), transcriptional co-activators in the Hippo pathway. Statins and metformin, widely used Food and Drug Administration-approved drugs, show great promise to intercept this disease by disrupting or inhibiting this amplifying network at multiple points converging onto YAP/TAZ.

Citation: Rozengurt E, Eibl G. Central role of Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif in pancreatic cancer development. *World J Gastroenterol* 2019; 25(15): 1797-1816

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1797.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1797>

INTRODUCTION

Despite advances in our understanding of genetics and basic biology, imaging, surgical treatments, and adjuvant therapy, pancreatic ductal adenocarcinoma (PDAC), which represents 90% of all pancreatic cancers, is a disease with dismal prognosis with an overall 5-year survival rate of only about 7%^[1]. The incidence in the general population is estimated to be 8 cases per 100000 person-years, and the worldwide mortality about 7 deaths per 100000 person-years^[2,3]. PDAC is already the 3rd leading cause of cancer-related mortalities in the United States^[4]. Indeed, deaths due to PDAC are predicted to increase markedly. Indeed, the disease is expected to become the 2nd leading cause of cancer-related mortality in the United States in the next few years^[5]. Given that only a minority of patients diagnosed with PDAC are eligible for surgical intervention, the research is gradually shifting to identify novel approaches in early diagnosis, prevention and interception, a novel concept, which attempts stopping transformed cells from progressing to frank cancer^[6-10]. The elucidation of the molecular mechanisms of risk-factor associated PDAC promotion will be of paramount importance to facilitate the discovery of novel targets and agents for prevention and identify robust biomarkers to stratify patients for selective and individualized therapeutics.

KRAS MUTATIONS AND PDAC

Oncogenic *KRAS* mutations were first reported to be associated with PDAC more than 30 years ago^[11,12]. Although the genetic landscape of PDAC is complex, since the initial reports extensive research in both humans and mice have substantiated the critical significance of *KRAS* mutations in the early stages of PDAC. In fact, many studies have confirmed that over 90% of PDAC harbors *KRAS* mutations^[13,14] and *KRAS* signaling is one of the core signaling pathways in human PDAC^[15]. Most *KRAS* mutations in PDAC are found at position G12, of which the single amino acid replacement G12D is the most predominant^[15]. Mutations at position G13 or Q61 have been detected at lower frequency, 21% or 28%, respectively^[15]. Using deep whole exome sequencing an integrated genomic characterization of PDAC revealed several different *KRAS* mutations in a subset of tumors, with some PDACs showing biallelic mutations^[16]. Mechanistically, mutations at position G12 with a single amino acid substitution induce conformational changes that interfere with the intrinsic GTPase

activity of KRAS and prevent the interactions between KRAS and GTPase-activating proteins (GAPs), which stimulate the conversion of KRAS-GTP (active state) to KRAS-GDP (inactive state), thereby ending KRAS activation. In this manner, the KRAS mutations lead to its prolonged activation and consequently to the persistent stimulation of downstream signaling effectors^[15,17]. It is becoming clear that different mutations of G12 lead to different conformational states that differ in their affinity for interacting effectors^[18]. Although mutations in *KRAS* is an early and essential step in PDAC, it is insufficient to stimulate development of frank, invasive PDAC. Activation of other pathways by additional mutations (*e.g.*, in tumor suppressor genes, including p53, p16 and SMAD4) or environmental stimuli, including obesity and metabolic syndrome are required for the promotion of invasive PDAC^[19-24].

In addition, the “efficacy” of oncogenic KRAS to initiate and promote PDAC is influenced and modulated by the presence of common susceptibility genes. Recent genome-wide association studies (GWAS) of PDACs in populations of European ancestry have identified additional common pancreatic cancer risk loci carrying pancreatic cancer risk signals, including *NR5A2*, *PDX1*, *ABO*, *NOC2L*, *HNF1B*, *GRP*^[25-28]. Moreover, an elegant study demonstrated that variations in oncogenic dosage have a critical role in PDAC biology and phenotypic diversification^[29], with the highest oncogenic *Kras* levels underlying aggressive undifferentiated phenotypes. Activation of other pro-oncogenic pathways, including *Myc*, *Yap1* or *Nfkb2*, which collaborate with heterozygous mutant *Kras* in driving tumorigenesis have been shown to have a lower metastatic potential^[29]. It seems that evolutionary constraints direct oncogenic dosage gain and variation along defined routes to drive the early progression of PDAC and shape its downstream biology^[29]. Integrated genomic and global gene expression analyses have classified human pancreatic cancers into several distinct subtypes that may dictate and predict clinical outcomes and therapeutic responses. Collison and colleagues defined three subtypes: classical, quasi mesenchymal, and exocrine-like^[30], while Bailey *et al.*^[31] classified four subtypes: squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX). By separating tumor cells and stromal components, Moffitt and colleagues identified two stromal subtypes: normal and activated, and two tumor-specific subtypes: classical and basal-like^[32]. Using whole genome sequencing and copy number variation analysis Waddell *et al.*^[33] categorized PDAC into four subclasses based on patterns of structural variation (variation in chromosomal structure): stable, locally rearranged, scattered, and unstable. Taken together, these large genomic efforts clearly demonstrate that pancreatic cancer is a genetically complex and heterogeneous disease, which has significant implications in prognosis and therapeutic response, and classifying pancreatic cancers into subtypes may assist and pave the way to more efficacious personalized treatment strategies.

PROGRESSION MODEL OF PDAC

It is estimated that PDACs develop over many years from non-invasive precursor lesions. The non-cystic lesion is called pancreatic intraepithelial neoplasia (PanIN) and is usually diagnosed in histological preparation of tissue removed during surgery or in biopsy specimens^[34-37]. These PanINs progress from early PanIN-1 lesions to advanced PanIN-3 (carcinoma *in situ*) and finally to frank invasive PDAC. Besides this classical view of gradual step-wise PanIN progression and PDAC formation, in at least a subset of PDACs there seem to be catastrophic genetic events (*e.g.*, chromothripsis) necessary for the transition from preinvasive to invasive PDAC (punctuated equilibrium)^[38-41]. The pathological characteristics of cystic precursor lesions, including intraductal papillary mucinous neoplasm (IPMN) have been recently reviewed elsewhere^[42]. Most low-grade PanIN lesions contain oncogenic *KRAS* mutations^[43]. This finding provided further evidence in support of the step-wise carcinogenesis model, in which *KRAS* mutations are envisioned as initiating events^[15,44,45].

Genetically engineered mouse models of PDAC have corroborated this paradigm^[46-49]. In the KC model, mutated *Kras* is expressed from its endogenous locus (by crossing *LSL-KrasG12D* mice with *PDX-1-Cre* or *p48-Cre* mice, *i.e.*, KC model)^[48-50]. This KC mouse model shares similar histopathologic and genetic features to the human disease including the development and progression of PanINs^[46]. In addition to the role of oncogenic *KRAS* in the initiation of PDAC, *Kras* mutations have also been shown to be important for PDAC maintenance^[51,52]. In line with the notion that mutated *Kras* is necessary but not fully sufficient for the development of invasive PDAC, only few animals (5%-10%) in the KC model (without additional genetic alterations) develop frank PDAC very late (usually after 9 mo)^[46]. Cell senescence has

been proposed as a barrier to the malignant progression of tumors^[53]. The formation of PDAC can be greatly accelerated by the presence of another mutation (*e.g.*, *Trp53*)^[47,54].

Besides additional genetic mutations, several studies have convincingly demonstrated that environmental, nutritional, and metabolic factors, including obesity, type-2 diabetes mellitus (T2DM) and inflammation efficiently promote PDAC formation^[55-59]. This notion is substantiated by several preclinical studies. Expression of physiologic levels of oncogenic *Kras* in murine models efficiently transformed only a small percentage of cells^[60]. *KRAS* downstream signaling molecules, including the ERKs were not activated when oncogenic *Kras* was expressed from its endogenous locus^[61]. Accordingly, cell culture studies have shown that incubating PDAC cells in a serum-free medium failed to display activated ERK despite the presence of *KRAS* activating mutations in these cells. However, ERK activation could be induced by adding growth factors to the culture medium^[62-64]. In mouse models, oncogenic *Kras* in adult mice was insufficient to induce PDAC, while concomitant induction of pancreatic inflammation (*e.g.*, by administration of the cholecystokinin analog cerulein) stimulated the formation of PanINs and cancers^[65]. Our own studies have clearly demonstrated that an obesogenic diet accelerated early PanIN progression and PDAC development in KC mice, which was associated with metabolic disturbances (*e.g.*, hyperinsulinemia), increased pancreatic inflammation, and desmoplasia^[55,56]. Taken together, the current evidence indicates that oncogenic *Kras* is indispensable but not sufficient to induce malignant pancreatic cells. Additional genetic or environmental factors (obesity, T2DM, inflammation) are required to elevate *KRAS* activity^[52] and/or stimulate additional signaling pathways to promote PDAC formation^[66].

Recent elegant gene-environment interaction studies have demonstrated that the increased risk of developing PDAC by environmental stimuli and conditions may be influenced by the presence of common genetic variations. A GWAS data analysis has found that genetic variations in inflammatory responses and insulin resistance may affect the risk of obesity- and diabetes-related pancreatic cancer^[67]. It is apparent that a detailed understanding of the gene-regulatory networks that integrate signaling by *KRAS* and cooperating pathways to drive an oncogenic program in pancreatic cancer is of fundamental importance to design novel strategies to target this aggressive disease. Recent exciting studies from different laboratories indicate that the activity of the transcriptional regulators yes-associated Protein (YAP) and WW-domain-containing transcriptional co-activator with PDZ-binding motif (TAZ) play a critical role in the promotion and maintenance of PDAC. In what follows, we will review studies implicating YAP/TAZ in pancreatic cancer development and consider possible approaches to target these transcriptional regulators with emphasis in repurposing drugs that are currently in clinical use.

YAP/TAZ IN PANCREATIC CANCER

The Hippo pathway

The highly conserved Hippo pathway, originally identified and characterized as potent growth-suppressive pathway in *Drosophila*^[68], is a key regulatory mechanism in development, organ-size, tissue regeneration and tumorigenesis^[68,69]. Canonical Hippo signals are transmitted via the serine/threonine kinases mammalian Ste20-like kinases 1/2 (Mst1/2), in complex with the scaffold protein salvador homolog 1 (Sav1), phosphorylate and activate large tumor suppressor 1/2 (Lats1/2), in complex with its regulatory protein Mps One binder 1/2 (MOB1/2)^[69]. As shown in **Figure 1**, Lats1/2 then phosphorylates the transcriptional co-activators YAP and TAZ which also can function as novel sensors of the mevalonate and glycolytic pathways^[70-72].

The residues Ser¹²⁷ and Ser³⁹⁷ of YAP are positioned within a consensus sequence (HXRXXS) phosphorylated by Lats1/2. The phosphorylation of YAP at these sites, restricts its cellular localization to the cytoplasm, reduces its stability, and inhibits its co-transcriptional activity. In addition to Lats1/2, YAP and TAZ can be phosphorylated by other protein kinases^[68]. Although YAP and TAZ have very similar structural topologies, share nearly half of the overall amino acid sequence, and are thought to be largely redundant, they may differ in their regulation and downstream functions^[73].

When the Hippo pathway is not functional, YAP localizes to the nucleus where it interacts with the TEA-domain DNA-binding transcription factors (TEAD 1-4). YAP/TEAD-regulated genes encode for proteins implicated many critical cellular processes, *e.g.*, autocrine/paracrine proliferation *via* EGFR (AREG) and G protein-coupled receptors (EDN2), and interact with other developmental pathways activated in PDAC, including Wnt, Notch, and Hedgehog^[74]. YAP/TAZ also induces epithelial-

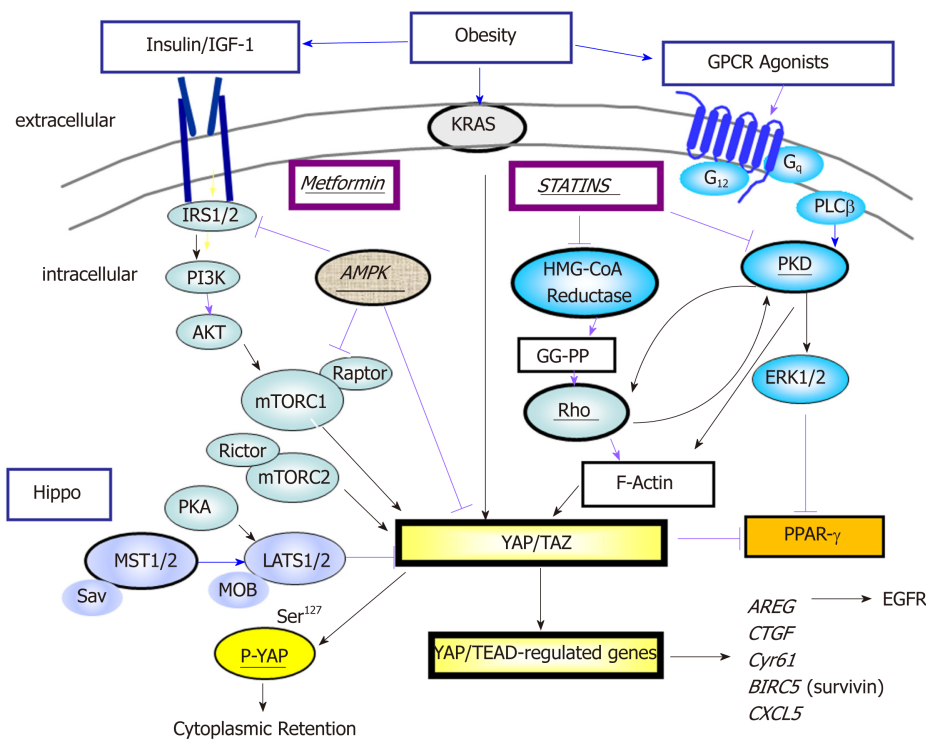


Figure 1 Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif is a point of convergence in signaling pathways. A network that involves activated Ras, G protein-coupled receptors (GPCRs) and tyrosine kinase receptors positively regulates Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif (YAP/TAZ) activity via Rho/PKD/organization of the actin cytoskeleton and PI3K/AKT/mTORC1. The interaction of mTORC1 and YAP is explained in the text. In addition, the localization and activity of YAP/TAZ is negatively impacted by the Hippo pathway which mediates phosphorylation of YAP and thereby its cytoplasmic sequestration. Metformin and statins inhibit YAP/TAZ activity at different sites in the network. Stimulatory effects are shown by black arrows whereas inhibitory effects are indicated by red arrows. YAP/TAZ: Yes-associated protein and WW-domain-containing transcriptional co-activator with PDZ-binding motif; GPCR: G protein-coupled receptor.

to-mesenchymal transition (EMT) and induces a more undifferentiated state to malignant cells. Accordingly, YAP/TAZ play an important role in pancreas development, which has implications for pancreatic regeneration, cancer, and diabetes^[75]. It is accepted that YAP/TAZ acts as a potent oncogene in multiple cell types, including PDAC^[76] and also contributes to the strong immunosuppressive microenvironment characteristic of mouse and human pancreatic cancer^[77]. Recent findings indicate that YAP/TAZ opposes Ras-induced senescence by increasing the expression of the key enzymes involved in deoxyribonucleotide biosynthesis which are critical for DNA replication^[78].

As indicated above, YAP and TAZ do not bind directly to DNA but act by enhancing the activity of transcription factors or other proteins that interact with DNA. Although TEAD family members are the major DNA-binding partners, YAP/TAZ can also bind to other transcription factors, e.g., RUNXs, p73, Smad1, Klf4, AP-1 to elicit context-specific functions^[74,79,80]. It is important to point out that YAP and TAZ not only act as co-activators of transcription factors that bind to promoter sites contiguous to the gene that they control but exert regulatory effects via distant enhancer elements^[81,82]. Furthermore, recent studies indicate that YAP/TAZ-bound to enhancers mediate the recruitment of the general coactivator bromodomain-containing protein 4 (BRD4) and RNA polymerase II at promoters regulated by YAP/TAZ, thereby enhancing expression of multiple growth-regulating genes^[83]. It is evident that YAP and TAZ control gene-regulatory programs through a variety of mechanism, further supporting their fundamental role in cell signaling.

Regulation of YAP/TAZ in PDAC

Recent studies demonstrated that YAP is required for acinar-to-ductal metaplasia (ADM), an early event that precedes PanIN progression into PDAC in genetically engineered mouse models^[84,85]. In addition, YAP is a major mediator of pro-oncogenic mutant p53^[86] and p53 deficiency promotes YAP signaling through Ptpn14^[87]. Also, YAP confers resistance to RAF/MEK inhibitors^[88] and chemotherapy in PDAC^[89]. While initially thought to be primarily an effector of the tumor-suppressive Hippo pathway, more recent studies revealed that YAP/TAZ subcellular localization and co-transcriptional activity is regulated by multiple upstream signals including those

mediated by various G protein-coupled receptors (GPCRs), tyrosine kinase receptors (EGFR, MET, Insulin/IGF-1 receptor), integrins, PI3K, mTOR, PKC, PKD, RHO and actin cytoskeleton, all of which stimulate YAP/TAZ transcriptional co-activator activity^[66,69,76,90-92]. Recently, Src kinases, downstream of KRAS, have been shown to inhibit the Hippo pathway by directly phosphorylating Lats1 thereby activating YAP^[93]. Interestingly, some of the tumor suppressive effects of wild type p53 appear to be exerted via inhibition of YAP1 function^[87].

In human PDAC cells, YAP functions as a downstream effector of the crosstalk between insulin/IGF-1 receptor and GPCR systems^[94] (Figure 1). We have demonstrated that stimulation with insulin and the GPCR agonist neurotensin induced rapid YAP nuclear import and markedly augmented the mRNA levels of YAP/TEAD-regulated genes, including CTGF and Cyr61. The growth-promoting agonists regulated YAP activity via PI3K and PKD in PANC-1 and MiaPaCa-2^[94], human cell lines that correspond to the squamous/quasi mesenchymal/ basal-like sub-type of PDAC. In other cell types, several studies have also been shown that PI3K activation inhibits the Hippo pathway^[95,96] thereby promoting YAP activity, and PKD mediates YAP nuclear localization and activation of YAP/TEAD-regulated gene expression^[90]. Overall, YAP has emerged as a central node of transcriptional convergence in growth-promoting signaling in PDAC cells (Figure 1). In addition to rapid regulation *via* phosphorylation and sub-cellular localization, additional pathways and epigenetic stimuli modulate YAP/TAZ protein expression. In this context, it has been shown that the RAS pathway, independently of the Hippo cascade, enhances YAP1 stability through downregulation of the ubiquitin ligase complex substrate recognition factors SOCS5/6^[97]. Moreover, the eukaryotic translation initiation factor 5A (eIF5A), which is up-regulated by KRAS in PDAC, interacts with the tyrosine kinase PEA1 leading to enhanced YAP expression^[98].

The nutrient sensor mTORC1, a central downstream component of the PI3K/AKT and RAF/MEK/ERK pathways, is implicated in the development of multiple types of cancer, including PDAC^[99]. Interestingly, YAP and mTORC1 form a positive feedback loop that leads to enhanced YAP protein expression. Specifically, YAP stimulates mTORC1 *via* increasing the activity of the PI3K pathway^[100] and augmented amino acid transport^[101,102]. In turn, mTORC1 activation leads to YAP accumulation at least in part, *via* decreased autophagy^[103]. Importantly, amplification and overexpression of YAP has been shown to bypass the need of mutant *Kras* in murine PDAC^[104] and other cancer cell types^[105] though the mechanism(s) differ(s), probably reflecting cell-context factors^[106]. These findings indicate that YAP not only acts downstream of KRAS but also that YAP can sidestep the need of KRAS mutant expression in PDAC^[107].

Several studies in different cell types demonstrated that an increase in the intracellular level of cAMP inhibits YAP activation, at least in part through activation of protein kinases of the Hippo pathway^[108,109]. Interestingly, concomitant expression of mutated (R201C) GNAS, which encodes for stimulatory G-protein alpha subunit that increases cAMP synthesis, with oncogenic *Kras* in mice, induced the formation of pancreatic cystic neoplasms, resembling human intraductal papillary mucinous neoplasms (IPMN), a less aggressive histological subtype of pancreatic tumors, by inhibiting YAP signaling^[110]. These recent findings underscore the importance of YAP activation in the development of specifically PDAC. In this regard, it is of great interest that YAP function has been associated with the squamous/quasi mesenchymal/basal-like sub-type of PDAC (discussed above), considered the most clinically aggressive form. The significance of YAP expression in human PDAC is discussed in the next section.

An important feature of human and murine PDAC is an extensive desmoplastic stroma^[111] that increases the stiffness of the extracellular matrix (ECM) surrounding the epithelial cancer cells^[42]. The Hippo/YAP pathway has been recognized to play a critical role in mechano-transduction^[112,113] and in sensing ECM stiffness^[114] but the mechanisms involved are not fully understood. Recently, the Ras-related GTPase RAP2 has been identified as a major sensor of mechanical cues from the ECM. At low stiffness, RAP2 activates the Hippo kinases Lats1/2 thereby inhibiting YAP/TAZ activity^[115]. Therefore, high stiffness leads to inhibition of the Hippo tumor suppressive pathway, thus enhancing the co-activator activity of YAP and TAZ. Reciprocally, increased expression of a number of YAP/TEAD-regulated genes, including CTGF, Cyr61 and CXCL5 contribute to shaping the stroma of PDAC, thus establishing an important amplification loop involving the tumor microenvironment leading to the stimulation of PDAC development.

YAP and human PDAC

Several studies reported that YAP and TAZ are over-expressed and over-active in human PDAC^[104,116,117] and identified YAP expression as an independent prognostic marker for survival of PDAC^[118]. We have examined the prognostic value not only of

YAP but also of upstream and downstream components of the YAP-driven network in pancreatic cancer^[119]. We confirmed that higher expression of YAP is significantly associated with unfavorable prognosis (survival) in PDAC^[120]. Indeed, none of the patients of the population with higher levels of YAP mRNA expression survived for 5 years while 32% of the subset with the lower levels of YAP mRNA survived for 5 years or more. In addition, multiple genes regulated by YAP/TEAD, including *AJUBA*, *ANLN*, *AREG*, *ARHGAP19*, *ARHGAP29*, *AURKA*, *BUB1*, *CCND1*, *CDK6*, *CXCL5*, *DKK1*, *JAG1*, *NOTCH2* and *RHAMM* were significantly associated with unfavorable prognosis in PDAC^[120]. In a further analysis of the data, we verified that the expression of each of these genes was positively and significantly correlated with the expression of YAP in PDAC. In contrast, genes in pathways, *e.g.*, LKB/AMPK and cAMP/PKA, that oppose YAP action, including *STRAD*, *MARK1*, *PKA*, are associated with favorable prognosis in PDAC patients^[120]. Similar results were obtained using other web-based tools, such as Gene Expression Profiling Interactive Analysis^[121].

YAP and obesity

Besides its recognized role in the regulation of growth and development, recent studies show that Hippo kinases and YAP/TAZ transcriptional coactivators, are regulated by metabolism and conversely that the Hippo/YAP pathway controls metabolic processes in physiological and pathologic conditions, including obesity and T2DM^[122,123]. In fact, cellular metabolites and metabolic pathways, *e.g.*, glucose and free fatty acids, regulate the Hippo pathway. Glucose metabolism through the glycolytic pathway activates phosphofructokinase 1 (PFK1), a key rate-limiting enzyme of glycolysis. In turn, PFK1 interacts with TEAD, thereby regulating YAP/TEAD complex formation and expression of YAP/TEAD-regulated genes^[70]. Furthermore, O-linked β -N-acetylglucosamine (O-GlcNAc) is another post-translational mechanism by which a sugar is attached to serine residues of nuclear or cytoplasmic proteins and modifies protein activity^[124]. Indeed, the attachment of O-GlcNAc to Ser¹⁰⁹ of YAP stimulates its transcriptional co-activator activity by interfering with the interaction of YAP with Lats1/2, thus protecting YAP from inhibitory phosphorylation and providing a novel mechanism linking glucose availability to YAP activity^[125]. This multilayered regulation of YAP activity by glucose metabolism is potentially important in the obese state, which often is accompanied by insulin resistance and elevated glucose levels.

A characteristic and defining feature of obesity is the enlargement of adipose tissue depots, which is often accompanied by adipose tissue (AT) inflammation^[126]. Dysfunctional adipose tissue with alterations of adipokine production, ectopic fat storage, and AT inflammation are thought to be critical, pathophysiological processes underlying the development of insulin resistance. Adipocytes and adipose tissue macrophages are central cellular players of AT inflammation^[127-132]. The Hippo pathway has been shown to modulate adipocyte proliferation and differentiation, with YAP/TAZ nuclear localization stimulating proliferation and suppressing adipogenesis^[133-136]. As depicted in **Figure 1**, nuclear YAP/TAZ interacts with and inhibits PPAR- γ , a major pro-adipogenic transcription factor, thereby suppressing adipocyte differentiation^[133,137]. In that context, hyperglycemia and advanced glycation end products impair adipogenesis by upregulating and activating YAP^[138].

There are few studies investigating the importance of YAP/TAZ in macrophage polarization^[139]. It has been shown that the cell shape, independent of cytokines present in the micromilieu, has a profound influence on macrophage polarization via the actin cytoskeleton^[140], which strongly suggests an important role of YAP/TAZ in this process due to the critical function of YAP/TAZ as mechano-sensors and mechano-transducers^[112,113]. In addition, adipose tissue in obese subjects is characterized by peri-adipocyte fibrosis with elevated levels of CTGF (connective tissue growth factor)^[141], a recognized product of YAP/TEAD transcriptional activity. Our own studies have shown that YAP is overexpressed in mesenteric adipose tissue of obese KC mice (unpublished). Taken together, an important role of YAP/TAZ in adipose tissue inflammation during obesity emerges, which might have important implications in the PDAC promoting effects of obesity^[142,143].

Strategies to inhibit YAP/TAZ in pancreatic cancer

As indicated above, YAP hyper-activation can evade the need of KRAS mutant expression in PDAC^[107]. Thus, even if Ras could be effectively inhibited by new therapies, YAP amplification could provide a potential pathway to tumor recurrence. Given that YAP is as a key element not only downstream of Ras but also an alternative route to bypass the need of this oncogene for tumor relapse, YAP is emerging as a fundamental target in PDAC. Although targeting transcription factors or their co-activators has proven difficult, recent studies suggest novel approaches to inhibit YAP/TAZ activity with drugs in clinical use, including statins and metformin

in PDAC and other malignancies.

Statins

Several studies demonstrated activation of the pathway leading to mevalonate biosynthesis in epithelial cancers through mutant p53^[144-146] and AKT/mTORC1^[146]. Statins, which have been used to treat dyslipidemia and prevent heart diseases, selectively inhibit 3-hydroxy-methylglutaryl (HMG) CoA reductase^[147], the rate-limiting enzyme in the generation of mevalonate (Figure 2). Mevalonate is a precursor for the generation of important lipids and lipid intermediates, including farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GG-PP) and cholesterol. The function and activity of small GTPases of the Rho family, including Rho A and C, depend on the transfer of the geranylgeranyl moiety of GG-PP to a cysteine in their COOH-terminal region. Active Rho plays a critical role in YAP/TAZ activation through actin remodeling in several cell populations^[69] (Figure 2). Accordingly, increased expression of RHOA and RHOC is associated with unfavorable prognosis in patients with PDAC.

Numerous epidemiological studies have concluded that statin use is correlated with beneficial effects in PDAC^[148-156], especially in men^[151,152]. A large study demonstrated that statins were associated with a significantly reduced PDAC risk (by 34%) with a stronger effect in males^[151]. The beneficial effects of statins depend on the type of statins used, with several reports showing positive associations with lipophilic (and not hydrophilic) statins and reduced cancer risk^[157-160]. However, a recent study, in which statin use was self-reported and the type of statins was not documented in early cohorts, failed to detect an effect of statins in lowering PDAC risk^[161]. The same authors published a follow up study of the same dataset, in which they reported an increased survival in PDAC patients with regular pre-diagnosis use of statins^[162]. Recently, a meta-analysis of PDAC risk that included more than 3 million participants and 170000 pancreatic cancer patients has been published^[163]. This study indicates a significant decrease in pancreatic cancer risk with statin use, thus reinforcing the conclusion that statin administration is associated with beneficial effects in PDAC patients. In addition to their potential efficacy in primary prevention and interception, statins may improve the outcome of patients after surgical removal of their primary PDAC^[148,149,164], indicating a possible role of statins in the prevention of PDAC recurrence.

In preclinical studies^[165,166], statins delayed progression of PDAC in mice harboring *Kras*^{G12D}. Statins were identified as potential YAP inhibitors by screens of molecules that changed the nuclear/cytoplasmic distribution of YAP^[167]. Our own experiments using PDAC cells also indicated that lipophilic statins induce cytoplasmic localization of YAP and markedly inhibited YAP/TEAD-regulated genes, proliferation, and colony formation by PDAC cells (submitted for publication). Taken together, converging evidence from epidemiological and preclinical studies indicates a protective effect of statins in PDAC.

Metformin

1,1-dimethylbiguanide hydrochloride (metformin) is the most widely administered drug for the treatment of T2DM worldwide^[168,169]. The anti-diabetic, *i.e.*, lowering the blood glucose levels, actions of metformin are mediated systemically by a reduction of hepatic glucose production and output into the circulation and improvement of insulin sensitivity *via* increasing cellular uptake of glucose in skeletal muscles and adipose tissue^[170]. In addition to lowering blood glucose levels, metformin decreased the levels of insulin and IGF-1 in both diabetic and non-diabetic patients^[171,172]. Multiple epidemiological studies showed an association of metformin with reduced incidence, recurrence and mortality of cancer in patients with T2DM^[173-182]. However, a therapeutic efficacy of metformin has not been observed in all studies^[183], in particular in late-stage, advanced cases of cancer. In that context, recent meta-analyses supported the notion that the beneficial effects of metformin depend on the stage of the tumor, with a substantially enhanced survival in patients with local, non-metastatic, disease^[178,184]. Further reports indicated that metformin administered to T2DM patients could be also beneficial in secondary chemoprevention, *i.e.* after surgical resection of the cancer in the pancreas^[185,186].

The mechanism of action of metformin remains incompletely understood. Besides its systemic (glucose lowering) effects, at the cellular level metformin indirectly activates AMP-activated protein kinase (AMPK)^[187], though other AMPK-independent mechanisms are also operational^[188,189]. AMPK is activated by phosphorylation by the tumor suppressor LKB-1/STK11 in the activation loop^[190] when cellular ATP levels decrease and 5'-AMP and ADP concentrations increase^[169]. It generally thought that metformin leads to AMPK activation by directly inhibiting complex I of the mitochondrial respiratory chain^[191,192], which leads to a decrease in ATP synthesis

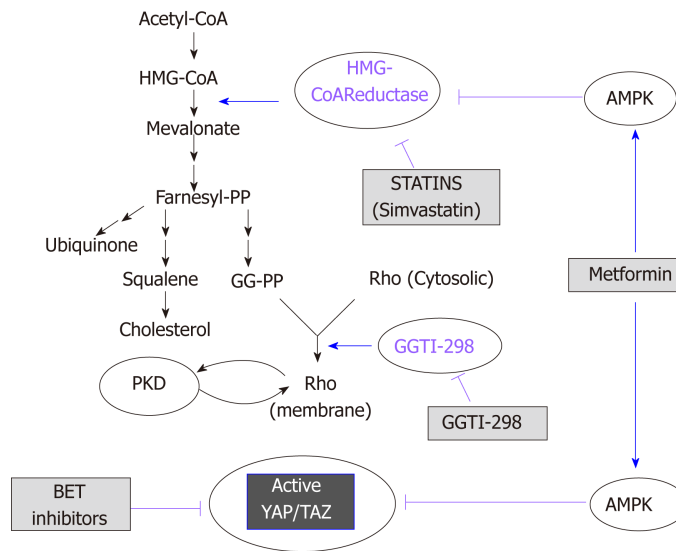


Figure 2 Schematic overview of the mevalonate pathway. The scheme illustrates the site of action of statins, metformin and bromodomain and extra-terminal domain inhibitors (see text for details). BET: Bromodomain and extra-terminal domain; AMPK: AMP-activated protein kinase; GG-PP: Geranylgeranyl pyrophosphate.

resulting in increased AMP and ADP thereby leading to AMPK activation. AMPK suppresses cellular proliferation by inhibiting the function of mTORC1 through several mechanisms. AMPK activates TSC2 by phosphorylation on Ser¹³⁴⁵[193-195], which leads to an accumulation of inactive Rheb-GDP thereby inhibiting mTORC1. AMPK can also inhibit mTORC1 function by phosphorylation of Raptor, which disrupts its complex with mTOR^[196]. In addition, mTORC1 activation induced by insulin/IGF-1 signaling is also inhibited *via* phosphorylation of IRS-1 on Ser⁷⁹⁴ by AMPK, a phosphorylation that impedes PI3K activation^[197,198]. We demonstrated that metformin, at low concentrations, activates AMPK in PDAC cells^[199,200] and inhibits mTORC1, ERK and DNA synthesis *via* AMPK^[199-201]. Metformin also reduced the rate of growth of PDAC xenografts^[202,203]. Furthermore, we recently reported that oral administration of metformin strikingly prevented the increase in PDAC incidence in KC mice with diet-induced obesity^[204]. This effect was associated with an increase in pancreatic AMPK activity (as measured by ACC Ser⁷⁹ phosphorylation), and decrease in phospho MEK1/2 (Ser^{217/221}), phospho S6 (Ser^{235/236}), and phospho ERK1/2 (Thr²⁰², Tyr²⁰⁴)^[204]. In that context, berberine, a natural compound that activates AMPK and inhibits ATP production, also inhibited mTORC1, ERK, DNA synthesis and proliferation of pancreatic cancer cells and reduced the growth of PDAC xenografts^[201].

Recent evidence indicates that AMPK also opposes YAP activity via multiple mechanisms, including direct YAP phosphorylation on Ser⁹⁴[205,206], a residue that is important for the interaction of YAP with TEAD. In addition, AMPK has been shown to phosphorylate HMG-CoA reductase (Ser⁸⁷²), thereby inhibiting its activity and reducing mevalonic acid synthesis^[207]. Furthermore, AMPK phosphorylates and activates upstream regulators of the Hippo pathway^[208]. The inhibitory effects of AMPK on the YAP/TAZ pathway is illustrated in **Figure 2**. These studies suggest an important direct link among adenine nucleotide levels, AMPK and YAP/TAZ activity. In studies from our laboratories, we found recently that diet-induced obesity markedly increased pancreatic TAZ expression in KC mice and that oral metformin prevented the increase in YAP/TAZ^[204]. Given that statins and metformin inhibit YAP activation through different mechanisms, it is logical to speculate that administration of a combination of these FDA-approved drugs will suppress YAP/TAZ activity and exerts PDAC-protective activity. The scheme presented in **Figure 1** dramatizes this notion by showing that statins and metformin reach YAP through different pathways.

Inhibitors of BRD4: A new approach for targeting YAP/TAZ

BRD4, which interacts with acetyl-lysine, acts as a critical regulator of the expression of selected subsets of genes. Bromodomain and extra-terminal domain (BET) inhibitors interfere with the proliferation of PDAC cells, raising the possibility that BET proteins may be new targets for PDAC therapy^[209]. A recent elegant study demonstrated a direct physical interaction between YAP or TAZ and BRD4, as revealed by co-immunoprecipitation experiments. The data imply that YAP, TEAD and BRET-containing proteins (*e.g.*, BRD4, BRD2) form a multi-molecular complex in

the nucleus^[83]. Consistent with the notion that BRD4 plays a critical role in YAP/TAZ function, the BET cell-permeable inhibitor JQ1^[210] downregulates the expression of YAP/TAZ-regulated genes^[83]. Considerable efforts are being made to develop new inhibitors of BRD proteins and thus this field will develop rapidly^[211]. These new findings suggest a novel approach to target YAP/TAZ that remains to be tested experimentally *in vivo*, using models of PDAC. As suggested by Figure 2, the possibility of using BET inhibitors in combination with statins and/or metformin is attractive and warrants further experimental work.

Feedback loops and effect of pathway inhibitors

Most signaling pathways are subjected to potent feedback loops that adjust the activity and function of the signaling network. There is evidence that besides their stimulating effects on mitogenic signaling the mTORC1/S6K and RAF/MEK/ERK pathways also mediate robust negative feedback loops that restrict the activity of insulin/IGF-1, EGFR, and other tyrosine kinase receptors^[99]. In that context, the mTORC1/S6K pathway inhibits the function of IRS-1 by phosphorylating several residues (Ser^{636/639} by mTORC1 and Ser^{307/636/1001} by S6K)^[212]. Inhibitors of mTORC1/S6K or MEK/ERK suppress these feedback loops, which in turn causes a compensatory activation of upstream signaling molecules, *e.g.*, PI3K, AKT, and ERK that as a consequence strongly counteract the anti-proliferative actions of these inhibitors^[99,200,213]. The up-regulation of these pathways conceivably can promote YAP activity leading to drug resistance. Therefore, a detailed understanding of feedback mechanisms that regulate upstream signaling is critical and will enable the identification of rational drug combinations that will circumvent drug resistance produced by unleashing the activity of alternative pathways.

CONCLUSION

Despite major advances in defining the molecular mutations driving PDAC, this disease remains universally lethal with an overall 5-year survival rate of only about 7%-8%. More efficacious therapeutic strategies are clearly needed but given the late presentation and early dissemination of the disease, substantial efforts should be concentrated on prevention and interception. Hereby, detailed knowledge of the molecular mechanisms underlying risk-factor promoted PDAC will surely facilitate and enable the discovery of novel molecular targets and agents for primary or secondary prevention. Epidemiological studies convincingly demonstrate that obesity is a risk factor for PDAC development, the importance of which takes an added level given the epidemic proportions of metabolic diseases. It is also recognized that almost all PDACs harbor an oncogenic *KRAS* mutation, which seems necessary but not sufficient for complete PDAC formation. Besides additional mutations, which greatly accelerate PDAC progression in mice, environmental conditions, including obesity, T2DM, and inflammation, have been shown to also promote PDAC in murine models. As illustrated in Figure 1, we propose that PI3K/mTORC1 and PKD/ERK are critical nodes in the network activated by GPCRs, EGFR and insulin/IGF-1 receptor in PDAC. These signaling modules are responsive to obesogenic signals and reinforce *KRAS* signaling. In turn, oncogenic *KRAS* mutations potentiate the intensity of signaling network emanating from GPCRs, EGFR, and insulin/IGF-1 receptors by activating PI3K/AKT and Raf/MEK/ERK, the most prominent downstream pathways of oncogenic *KRAS*.

We also postulate that YAP/TAZ transcriptional co-activators are central and critical players in this amplification network, further intensifying positive feedback loops. GPCRs, EGFR, and insulin/IGF-1 receptor signaling rapidly stimulate nuclear import and transcriptional co-activator activity of YAP/TAZ, while oncogenic *KRAS* increases the levels of YAP protein. In turn, YAP stimulates signaling *via* autocrine/paracrine stimulation of EGFR *via* increased production of EGFR ligands (*e.g.*, amphiregulin), thereby further propagating and enhancing *KRAS* activity, as well as creating an immunosuppressive microenvironment. We hypothesize that oncogenic *KRAS* potentiates a signaling network that is stimulated and sustained by environmental factors. As YAP/TAZ play a central role in the signaling network, targeting this network at different sites with FDA-approved drugs, including statins and metformin (Figure 2), is therefore a compelling approach, especially in obese patients at higher risk of developing PDAC.

REFERENCES

- 1 Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin* 2017; **67**: 7-30 [PMID: 28055103 DOI: 10.3322/caac.21387]
- 2 Xiao AY, Tan ML, Wu LM, Asrani VM, Windsor JA, Yadav D, Petrov MS. Global incidence and mortality of pancreatic diseases: a systematic review, meta-analysis, and meta-regression of population-based cohort studies. *Lancet Gastroenterol Hepatol* 2016; **1**: 45-55 [PMID: 28404111 DOI: 10.1016/S2468-1253(16)30004-8]
- 3 Whitcomb DC, Shelton CA, Brand RE. Genetics and Genetic Testing in Pancreatic Cancer. *Gastroenterology* 2015; **149**: 1252-1264.e4 [PMID: 26255042 DOI: 10.1053/j.gastro.2015.07.057]
- 4 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]
- 5 Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]
- 6 Meyskens FL, Mukhtar H, Rock CL, Cuzick J, Kensler TW, Yang CS, Ramsey SD, Lippman SM, Alberts DS. Cancer Prevention: Obstacles, Challenges and the Road Ahead. *J Natl Cancer Inst* 2015; **108** [PMID: 26547931 DOI: 10.1093/jnci/djv309]
- 7 Kensler TW, Spira A, Garber JE, Szabo E, Lee JJ, Dong Z, Dannenberg AJ, Hait WN, Blackburn E, Davidson NE, Foti M, Lippman SM. Transforming Cancer Prevention through Precision Medicine and Immune-oncology. *Cancer Prev Res (Phila)* 2016; **9**: 2-10 [PMID: 26744449 DOI: 10.1158/1940-6207.CAPR-15-0406]
- 8 Albini A, DeCensi A, Cavalli F, Costa A. Cancer Prevention and Interception: A New Era for Chemopreventive Approaches. *Clin Cancer Res* 2016; **22**: 4322-4327 [PMID: 27220959 DOI: 10.1158/1078-0432.CCR-16-0695]
- 9 Umar A, Dunn BK, Greenwald P. Future directions in cancer prevention. *Nat Rev Cancer* 2012; **12**: 835-848 [PMID: 23151603 DOI: 10.1038/nrc3397]
- 10 Miller MS, Allen P, Brentnall TA, Goggins M, Hruban RH, Petersen GM, Rao CV, Whitcomb DC, Brand RE, Chari ST, Klein AP, Lubman DM, Rhim AD, Simeone DM, Wolpin BM, Umar A, Srivastava S, Steele VE, Rinaudo JA. Pancreatic Cancer Chemoprevention Translational Workshop: Meeting Report. *Pancreas* 2016; **45**: 1080-1091 [PMID: 27518363 DOI: 10.1097/MPA.0000000000000705]
- 11 Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* 1988; **53**: 549-554 [PMID: 2453289]
- 12 Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, Bos JL. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Res* 1988; **16**: 7773-7782 [PMID: 3047672]
- 13 Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]
- 14 Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, Song S, Harliwong I, Idrisoglu S, Nourse C, Nourbakhsh E, Manning S, Wani S, Gongora M, Pajic M, Scarlett CJ, Gill AJ, Pinho AV, Rooman I, Anderson M, Holmes O, Leonard C, Taylor D, Wood S, Xu Q, Nones K, Fink JL, Christ A, Bruxner T, Cloonan N, Kolle G, Newell F, Pinese M, Mead RS, Humphris JL, Kaplan W, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chou A, Chin VT, Chantrill LA, Mawson A, Samra JS, Kench JG, Lovell JA, Daly RJ, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N; Australian Pancreatic Cancer Genome Initiative, Kakkar N, Zhao F, Wu YQ, Wang M, Muzny DM, Fisher WE, Brunicardi FC, Hodges SE, Reid JG, Drummond J, Chang K, Han Y, Lewis LR, Dinh H, Buhay CJ, Beck T, Timms L, Sam M, Begley K, Brown A, Pai D, Panchal A, Buchner N, De Borja R, Denroche RE, Yung CK, Serra S, Onetto N, Mukhopadhyay D, Tsao MS, Shaw PA, Petersen GM, Gallinger SJ, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Schulick RD, Wolfgang CL, Morgan RA, Lawlor RT, Capelli P, Corbo V, Scardoni M, Tortora G, Tempero MA, Mann KM, Jenkins NA, Perez-Mancera PA, Adams DJ, Largaespada DA, Wessels LF, Rust AG, Stein LD, Tuveson DA, Copeland NG, Musgrave EA, Scarpa A, Eshleman JR, Hudson TJ, Sutherland RL, Wheeler DA, Pearson JV, McPherson JD, Gibbs RA, Grimmond SM. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 2012; **491**: 399-405 [PMID: 23103869 DOI: 10.1038/nature11547]
- 15 Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci* 2014; **39**: 91-100 [PMID: 24388967 DOI: 10.1016/j.tibs.2013.12.004]
- 16 Cancer Genome Atlas Research Network; Cancer Genome Atlas Research Network. Electronic address: andrew_aguirre@dfci.harvard.edu. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* 2017; **32**: 185-203.e13 [PMID: 28810144 DOI: 10.1016/j.ccell.2017.07.007]
- 17 Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; **11**: 761-774 [PMID: 21993244 DOI: 10.1038/nrc3106]
- 18 Patsars T, Rissanen S, Dauch D, Laitinen T, Vattulainen I, Poso A. Assessment of mutation probabilities of KRAS G12 missense mutants and their long-timescale dynamics by atomistic molecular simulations and Markov state modeling. *PLoS Comput Biol* 2018; **14**: e1006458 [PMID: 30199525 DOI: 10.1371/journal.pcbi.1006458]
- 19 Eibl G, Cruz-Monserrate Z, Korc M, Petrov MS, Goodarzi MO, Fisher WE, Habtezion A, Lugea A, Pandol SJ, Hart PA, Andersen DK. Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer. Diabetes Mellitus and Obesity as Risk Factors for Pancreatic Cancer. *J Acad Nutr Diet* 2018; **118**: 555-567 [PMID: 28919082 DOI: 10.1016/j.jand.2017.07.005]
- 20 Xu M, Jung X, Hines OJ, Eibl G, Chen Y. Obesity and Pancreatic Cancer: Overview of Epidemiology and Potential Prevention by Weight Loss. *Pancreas* 2018; **47**: 158-162 [PMID: 29346216 DOI: 10.1097/MPA.0000000000000974]
- 21 Abbruzzese JL, Andersen DK, Borrebaeck CAK, Chari ST, Costello E, Cruz-Monserrate Z, Eibl G, Engleman EG, Fisher WE, Habtezion A, Kim SK, Korc M, Logsdon C, Lysiotis CA, Pandol SJ, Rustgi A, Wolfe BM, Zheng L, Powers AC. The Interface of Pancreatic Cancer With Diabetes, Obesity, and Inflammation: Research Gaps and Opportunities: Summary of a National Institute of Diabetes and Digestive and Kidney Diseases Workshop. *Pancreas* 2018; **47**: 516-525 [PMID: 29702529 DOI: 10.1097/MPA.0000000000001037]

- 22 **Andersen DK**, Korc M, Petersen GM, Eibl G, Li D, Rickels MR, Chari ST, Abbruzzese JL. Diabetes, Pancreatogenic Diabetes, and Pancreatic Cancer. *Diabetes* 2017; **66**: 1103-1110 [PMID: [28507210](#) DOI: [10.2337/db16-1477](#)]
- 23 **Chen Y**, Dutson E, Eibl G. Strategies to Prevent Obesity-Related Cancer. *JAMA* 2018; **319**: 2442 [PMID: [29922821](#) DOI: [10.1001/jama.2018.4942](#)]
- 24 **Juo YY**, Gibbons MAM, Dutson E, Lin AY, Yanagawa J, Hines OJ, Eibl G, Chen Y. Obesity Is Associated with Early Onset of Gastrointestinal Cancers in California. *J Obes* 2018; **2018**: 7014073 [PMID: [30327727](#) DOI: [10.1155/2018/7014073](#)]
- 25 **Klein AP**, Wolpin BM, Risch HA, Stolzenberg-Solomon RZ, Mocci E, Zhang M, Canzian F, Childs EJ, Hoskins JW, Jermusyk A, Zhong J, Chen F, Albanes D, Andreotti G, Arslan AA, Babic A, Bamlet WR, Beane-Freeman L, Berndt SI, Blackford A, Borges M, Borgida A, Bracci PM, Brais L, Brennan P, Brenner H, Bueno-de-Mesquita B, Buring J, Campa D, Capurso G, Cavestro GM, Chaffee KG, Chung CC, Cleary S, Cotterchio M, Dijk F, Duell EJ, Foretova L, Fuchs C, Funel N, Gallinger S, M Gaziano JM, Gazouli M, Giles GG, Giovannucci E, Goggins M, Goodman GE, Goodman PJ, Hackert T, Haiman C, Hartge P, Hasan M, Hegyi P, Helzlsouer KJ, Herman J, Holcatova I, Holly EA, Hoover R, Hung RJ, Jacobs EJ, Jamrozik K, Janout V, Kaaks R, Khaw KT, Klein EA, Kogevinas M, Kooperberg C, Kulke MH, Kupcinskas J, Kurtz RJ, Laheru D, Landi S, Lawlor RT, Lee IM, LeMarchand L, Lu L, Malats N, Mambrini A, Mannisto S, Milne RL, Mohelniková-Duchoňová B, Neale RE, Neoptolemos JP, Oberg AL, Olson SH, Orlov I, Pasquali C, Patel AV, Peters U, Pezzilli R, Porta M, Real FX, Rothman N, Scelo G, Sesso HD, Severi G, Shu XO, Silverman D, Smith JP, Soucek P, Sund M, Talar-Wojnarowska R, Tavano F, Thorquist MD, Tobias GS, Van Den Eeden SK, Vashist Y, Visvanathan K, Vodicka P, Wactawski-Wende J, Wang Z, Wentzensen N, White E, Yu H, Yu K, Zeleniuch-Jacquotte A, Zheng W, Kraft P, Li D, Chanock S, Obazee O, Petersen GM, Amundadottir LT. Genome-wide meta-analysis identifies five new susceptibility loci for pancreatic cancer. *Nat Commun* 2018; **9**: 556 [PMID: [29422604](#) DOI: [10.1038/s41467-018-02942-5](#)]
- 26 **Childs EJ**, Mocci E, Campa D, Bracci PM, Gallinger S, Goggins M, Li D, Neale RE, Olson SH, Scelo G, Amundadottir LT, Bamlet WR, Bijlsma MF, Blackford A, Borges M, Brennan P, Brenner H, Bueno-de-Mesquita HB, Canzian F, Capurso G, Cavestro GM, Chaffee KG, Chanock SJ, Cleary SP, Cotterchio M, Foretova L, Fuchs C, Funel N, Gazouli M, Hassan M, Herman JM, Holcatova I, Holly EA, Hoover RN, Hung RJ, Janout V, Key TJ, Kupcinskas J, Kurtz RC, Landi S, Lu L, Malecka-Panas E, Mambrini A, Mohelnikova-Duchonova B, Neoptolemos JP, Oberg AL, Orlov I, Pasquali C, Pezzilli R, Rizzato C, Saldia A, Scarpa A, Stolzenberg-Solomon RZ, Strobel O, Tavano F, Vashist YK, Vodicka P, Wolpin BM, Yu H, Petersen GM, Risch HA, Klein AP. Common variation at 2p13.3, 3q29, 7p13 and 17q25.1 associated with susceptibility to pancreatic cancer. *Nat Genet* 2015; **47**: 911-916 [PMID: [26098869](#) DOI: [10.1038/ng.3341](#)]
- 27 **Wolpin BM**, Rizzato C, Kraft P, Kooperberg C, Petersen GM, Wang Z, Arslan AA, Beane-Freeman L, Bracci PM, Buring J, Canzian F, Duell EJ, Gallinger S, Giles GG, Goodman GE, Goodman PJ, Jacobs EJ, Kamineni A, Klein AP, Kolonel LN, Kulke MH, Li D, Malats N, Olson SH, Risch HA, Sesso HD, Visvanathan K, White E, Zheng W, Abnet CC, Albanes D, Andreotti G, Austin MA, Barfield R, Basso D, Berndt SI, Boutron-Ruault MC, Brotzman M, Büchler MW, Bueno-de-Mesquita HB, Bugert P, Burdette L, Campa D, Caporaso NE, Capurso G, Chung C, Cotterchio M, Costello E, Elena J, Funel N, Gaziano JM, Giese NA, Giovannucci EL, Goggins M, Gorman MJ, Gross M, Haiman CA, Hassan M, Helzlsouer KJ, Henderson BE, Holly EA, Hu N, Hunter DJ, Innocenti F, Jenab M, Kaaks R, Key TJ, Khaw KT, Klein EA, Kogevinas M, Krogh V, Kupcinskas J, Kurtz RC, LaCroix A, Landi MT, Landi S, Le Marchand L, Mambrini A, Mannisto S, Milne RL, Nakamura Y, Oberg AL, Owzar K, Patel AV, Peeters PH, Peters U, Pezzilli R, Piepoli A, Porta M, Real FX, Riboli E, Rothman N, Scarpa A, Shu XO, Silverman DT, Soucek P, Sund M, Talar-Wojnarowska R, Taylor PR, Theodoropoulos GE, Thorquist M, Tjønneland A, Tobias GS, Trichopoulos D, Vodicka P, Wactawski-Wende J, Wentzensen N, Wu C, Yu H, Yu K, Zeleniuch-Jacquotte A, Hoover R, Hartge P, Fuchs C, Chanock SJ, Stolzenberg-Solomon RS, Amundadottir LT. Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat Genet* 2014; **46**: 994-1000 [PMID: [25086665](#) DOI: [10.1038/ng.3052](#)]
- 28 **Petersen GM**, Amundadottir L, Fuchs CS, Kraft P, Stolzenberg-Solomon RZ, Jacobs KB, Arslan AA, Bueno-de-Mesquita HB, Gallinger S, Gross M, Helzlsouer K, Holly EA, Jacobs EJ, Klein AP, LaCroix A, Li D, Mandelson MT, Olson SH, Risch HA, Zheng W, Albanes D, Bamlet WR, Berg CD, Boutron-Ruault MC, Buring JE, Bracci PM, Canzian F, Clipp S, Cotterchio M, de Andrade M, Duell EJ, Gaziano JM, Giovannucci EL, Goggins M, Hallmans G, Hankinson SE, Hassan M, Howard B, Hunter DJ, Hutchinson A, Jenab M, Kaaks R, Kooperberg C, Krogh V, Kurtz RC, Lynch SM, McWilliams RR, Mendelsohn JB, Michaud DS, Parikh H, Patel AV, Peeters PH, Rajkovic A, Riboli E, Rothman N, Seminara D, Shu XO, Thomas G, Tjønneland A, Tobias GS, Trichopoulos D, Van Den Eeden SK, Virtamo J, Wactawski-Wende J, Wang Z, Wolpin BM, Yu H, Yu K, Zeleniuch-Jacquotte A, Fraumeni JF, Hoover RN, Hartge P, Chanock SJ. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet* 2010; **42**: 224-228 [PMID: [20101243](#) DOI: [10.1038/ng.522](#)]
- 29 **Mueller S**, Engleitner T, Maresch R, Zukowska M, Lange S, Kaltenbacher T, Konukiewicz B, Öllinger R, Zwiebel M, Strong A, Yen HY, Banerjee R, Louzada S, Fu B, Seidler B, Götzfried J, Schuck K, Hassan Z, Arbeiter A, Schönhuber N, Klein S, Veltkamp C, Friedrich M, Rad L, Barenboim M, Ziegenhain C, Hess J, Dovey OM, Eser S, Parekh S, Constantino-Casas F, de la Rosa J, Sierra MI, Fraga M, Mayerle J, Klöppel G, Cadánas J, Liu P, Vassiliou G, Weichert W, Steiger K, Enard W, Schmid RM, Yang F, Unger K, Schneider G, Varela I, Bradley A, Saur D, Rad R. Evolutionary routes and KRAS dosage define pancreatic cancer phenotypes. *Nature* 2018; **554**: 62-68 [PMID: [29364867](#) DOI: [10.1038/nature25459](#)]
- 30 **Collisson EA**, Sadanandam A, Olson P, Gibb WJ, Truitt M, Gu S, Cooc J, Weinkle J, Kim GE, Jakkula L, Feiler HS, Ko AH, Olshen AB, Danenberg KL, Tempero MA, Spellman PT, Hanahan D, Gray JW. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med* 2011; **17**: 500-503 [PMID: [21460848](#) DOI: [10.1038/nm.2344](#)]
- 31 **Bailey P**, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, Miller DK, Christ AN, Bruxner TJ, Quinn MC, Nourse C, Murtaugh LC, Harliwong I, Idrisoglu S, Manning S, Nourbakhsh E, Wani S, Fink L, Holmes O, Chin V, Anderson MJ, Kazakoff S, Leonard C, Newell F, Waddell N, Wood S, Xu Q, Wilson PJ, Cloonan N, Kassahn KS, Taylor D, Quek K, Robertson A, Pantano L, Mincarelli L, Sanchez D, Evers L, Wu J, Pinese M, Cowley MJ, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chantrill LA, Mawson A, Humphris J, Chou A, Pajic M, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Lovell JA, Merrett ND, Toon CW, Epari K, Nguyen NQ, Barbour A, Zeps N, Moran-Jones K,

- Jamieson NB, Graham JS, Duthie F, Oien K, Hair J, Grützmann R, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Rusev B, Capelli P, Salvia R, Tortora G, Mukhopadhyay D, Petersen GM; Australian Pancreatic Cancer Genome Initiative, Munzy DM, Fisher WE, Karim SA, Eshleman JR, Hruban RH, Pilarsky C, Morton JP, Sansom OJ, Scarpa A, Musgrove EA, Bailey UM, Hofmann O, Sutherland RL, Wheeler DA, Gill AJ, Gibbs RA, Pearson JV, Waddell N, Biankin AV, Grimmond SM. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016; **531**: 47-52 [PMID: 26909576 DOI: 10.1038/nature16965]
- 32 **Moffitt RA**, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, Rashid NU, Williams LA, Eaton SC, Chung AH, Smyla JK, Anderson JM, Kim HJ, Bentrem DJ, Talamonti MS, Iacobuzio-Donahue CA, Hollingsworth MA, Yeh JJ. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* 2015; **47**: 1168-1178 [PMID: 26343385 DOI: 10.1038/ng.3398]
- 33 **Waddell N**, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, Johns AL, Miller D, Nones K, Quek K, Quinn MC, Robertson AJ, Fadlullah MZ, Bruxner TJ, Christ AN, Harliwong I, Idrisoglu S, Manning S, Nourse C, Nourbakhsh E, Wani S, Wilson PJ, Markham E, Cloonan N, Anderson MJ, Fink JL, Holmes O, Kazakoff SH, Leonard C, Newell F, Poudel B, Song S, Taylor D, Waddell N, Wood S, Xu Q, Wu J, Pinese M, Cowley MJ, Lee HC, Jones MD, Nagrial AM, Humphris J, Chantrill LA, Chin V, Steinmann AM, Mawson A, Humphrey ES, Colvin EK, Chou A, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rومان I, Samra JS, Kench JG, Pettitt JA, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Jamieson NB, Graham JS, Niclou SP, Bjerkvig R, Grützmann R, Aust D, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Falconi M, Zamboni G, Tortora G, Tempero MA; Australian Pancreatic Cancer Genome Initiative, Gill AJ, Eshleman JR, Pilarsky C, Scarpa A, Musgrove EA, Pearson JV, Biankin AV, Grimmond SM. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015; **518**: 495-501 [PMID: 25719666 DOI: 10.1038/nature14169]
- 34 **Hansel DE**, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. *Annu Rev Genomics Hum Genet* 2003; **4**: 237-256 [PMID: 14527303 DOI: 10.1146/annurev.genom.4.070802.110341]
- 35 **Hruban RH**, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN, Kern SE, Klimstra DS, Klöppel G, Longnecker DS, Lüttges J, Offerhaus GJ. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol* 2001; **25**: 579-586 [PMID: 11342768]
- 36 **Maitra A**, Fukushima N, Takaori K, Hruban RH. Precursors to invasive pancreatic cancer. *Adv Anat Pathol* 2005; **12**: 81-91 [PMID: 15731576]
- 37 **Hruban RH**, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. *Clin Cancer Res* 2000; **6**: 2969-2972 [PMID: 10955772]
- 38 **Real FX**, de Andrés MP. Mutant Kras Dosage and Chromothripsis: The Right Ingredients for a Pancreatic Cancer Catastrophe. *Trends Cancer* 2018; **4**: 399-401 [PMID: 29860982 DOI: 10.1016/j.trecan.2018.04.004]
- 39 **Notta F**, Hahn SA, Real FX. A genetic roadmap of pancreatic cancer: still evolving. *Gut* 2017; **66**: 2170-2178 [PMID: 28993418 DOI: 10.1136/gutjnl-2016-313317]
- 40 **Notta F**, Chan-Seng-Yue M, Lemire M, Li Y, Wilson GW, Connor AA, Denroche RE, Liang SB, Brown AM, Kim JC, Wang T, Simpson JT, Beck T, Borgida A, Buchner N, Chadwick D, Hafezi-Bakhtiari S, Dick JE, Heisler L, Hollingsworth MA, Ibrahimov E, Jang GH, Johns J, Jorgensen LG, Law C, Ludkovski O, Lungu I, Ng K, Pasternack D, Petersen GM, Shlush LI, Timms L, Tsao MS, Wilson JM, Yung CK, Zogopoulos G, Bartlett JM, Alexandrov LB, Real FX, Cleary SP, Roehrl MH, McPherson JD, Stein LD, Hudson TJ, Campbell PJ, Gallinger S. A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. *Nature* 2016; **538**: 378-382 [PMID: 27732578 DOI: 10.1038/nature19823]
- 41 **Real FX**, Cibrián-Uhalte E, Martinelli P. Pancreatic cancer development and progression: remodeling the model. *Gastroenterology* 2008; **135**: 724-728 [PMID: 18692502 DOI: 10.1053/j.gastro.2008.07.033]
- 42 **Ren B**, Liu X, Suriawinata AA. Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: Histopathology, Cytopathology, and Molecular Pathology. *Am J Pathol* 2019; **189**: 9-21 [PMID: 30558727 DOI: 10.1016/j.ajpath.2018.10.004]
- 43 **Kanda M**, Matthaei H, Wu J, Hong SM, Yu J, Borges M, Hruban RH, Maitra A, Kinzler K, Vogelstein B, Goggins M. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* 2012; **142**: 730-733.e9 [PMID: 22226782 DOI: 10.1053/j.gastro.2011.12.042]
- 44 **Deramandt T**, Rustgi AK. Mutant KRAS in the initiation of pancreatic cancer. *Biochim Biophys Acta* 2005; **1756**: 97-101 [PMID: 16169155 DOI: 10.1016/j.bbcan.2005.08.003]
- 45 **Logsdon CD**, Lu W. The Significance of Ras Activity in Pancreatic Cancer Initiation. *Int J Biol Sci* 2016; **12**: 338-346 [PMID: 26929740 DOI: 10.7150/ijbs.15020]
- 46 **Hingorani SR**, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437-450 [PMID: 14706336]
- 47 **Hingorani SR**, Wang L, Multani AS, Combs C, Deramandt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005; **7**: 469-483 [PMID: 15894267 DOI: 10.1016/j.ccr.2005.04.023]
- 48 **Hruban RH**, Adsay NV, Albores-Saavedra J, Anver MR, Biankin AV, Boivin GP, Furth EE, Furukawa T, Klein A, Klimstra DS, Klöppel G, Lauwers GY, Longnecker DS, Lüttges J, Maitra A, Offerhaus GJ, Pérez-Gallego L, Redston M, Tuveson DA. Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. *Cancer Res* 2006; **66**: 95-106 [PMID: 16397221 DOI: 10.1158/0008-5472.CAN-05-2168]
- 49 **Hruban RH**, Rustgi AK, Brentnall TA, Tempero MA, Wright CV, Tuveson DA. Pancreatic cancer in mice and man: the Penn Workshop 2004. *Cancer Res* 2006; **66**: 14-17 [PMID: 16397208 DOI: 10.1158/0008-5472.CAN-05-3914]
- 50 **Funahashi H**, Satake M, Dawson D, Huynh NA, Reber HA, Hines OJ, Eibl G. Delayed progression of pancreatic intraepithelial neoplasia in a conditional Kras(G12D) mouse model by a selective cyclooxygenase-2 inhibitor. *Cancer Res* 2007; **67**: 7068-7071 [PMID: 17652141]
- 51 **Collins MA**, Bednar F, Zhang Y, Brisset JC, Galbán S, Galbán CJ, Rakshit S, Flannagan KS, Adsay NV, Pasca di Magliano M. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest* 2012; **122**: 639-653 [PMID: 22232209 DOI: 10.1172/JCI59227]
- 52 **di Magliano MP**, Logsdon CD. Roles for KRAS in pancreatic tumor development and progression.

- Gastroenterology* 2013; **144**: 1220-1229 [PMID: 23622131 DOI: 10.1053/j.gastro.2013.01.071]
- 53 **Porciuncula A**, Hajdu C, David G. The Dual Role of Senescence in Pancreatic Ductal Adenocarcinoma. *Adv Cancer Res* 2016; **131**: 1-20 [PMID: 27451122 DOI: 10.1016/bs.acr.2016.05.006]
- 54 **Aguirre AJ**, Bardeesy N, Sinha M, Lopez L, Tuveson DA, Horner J, Redston MS, DePinho RA. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev* 2003; **17**: 3112-3126 [PMID: 14681207 DOI: 10.1101/gad.1158703]
- 55 **Chang HH**, Moro A, Takakura K, Su HY, Mo A, Nakanishi M, Waldron RT, French SW, Dawson DW, Hines OJ, Li G, Go VLW, Sinnott-Smith J, Pandol SJ, Lugea A, Gukovskaya AS, Duff MO, Rosenberg DW, Rozenfurt E, Eibl G. Incidence of pancreatic cancer is dramatically increased by a high fat, high calorie diet in KrasG12D mice. *PLoS One* 2017; **12**: e0184455 [PMID: 28886117 DOI: 10.1371/journal.pone.0184455]
- 56 **Dawson DW**, Hertzner K, Moro A, Donald G, Chang HH, Go VL, Pandol SJ, Lugea A, Gukovskaya AS, Li G, Hines OJ, Rozenfurt E, Eibl G. High-fat, high-calorie diet promotes early pancreatic neoplasia in the conditional KrasG12D mouse model. *Cancer Prev Res (Phila)* 2013; **6**: 1064-1073 [PMID: 23943783 DOI: 10.1158/1940-6207.CAPR-13-0065]
- 57 **Lashinger LM**, Harrison LM, Rasmussen AJ, Logsdon CD, Fischer SM, McArthur MJ, Hursting SD. Dietary energy balance modulation of Kras- and Ink4a/Arf+/-driven pancreatic cancer: the role of insulin-like growth factor-I. *Cancer Prev Res (Phila)* 2013; **6**: 1046-1055 [PMID: 23980075 DOI: 10.1158/1940-6207.CAPR-13-0185]
- 58 **Philip B**, Roland CL, Daniluk J, Liu Y, Chatterjee D, Gomez SB, Ji B, Huang H, Wang H, Fleming JB, Logsdon CD, Cruz-Monserrate Z. A high-fat diet activates oncogenic Kras and COX2 to induce development of pancreatic ductal adenocarcinoma in mice. *Gastroenterology* 2013; **145**: 1449-1458 [PMID: 23958541 DOI: 10.1053/j.gastro.2013.08.018]
- 59 **Harvey AE**, Lashinger LM, Hays D, Harrison LM, Lewis K, Fischer SM, Hursting SD. Calorie restriction decreases murine and human pancreatic tumor cell growth, nuclear factor- κ B activation, and inflammation-related gene expression in an insulin-like growth factor-1-dependent manner. *PLoS One* 2014; **9**: e94151 [PMID: 24804677 DOI: 10.1371/journal.pone.0094151]
- 60 **Campbell PM**, Singh A, Williams FJ, Frantz K, Ulkü AS, Kelley GG, Der CJ. Genetic and pharmacologic dissection of Ras effector utilization in oncogenesis. *Methods Enzymol* 2006; **407**: 195-217 [PMID: 16757325 DOI: 10.1016/S0076-6879(05)07017-5]
- 61 **Daniluk J**, Liu Y, Deng D, Chu J, Huang H, Gaiser S, Cruz-Monserrate Z, Wang H, Ji B, Logsdon CD. An NF- κ B pathway-mediated positive feedback loop amplifies Ras activity to pathological levels in mice. *J Clin Invest* 2012; **122**: 1519-1528 [PMID: 22406536 DOI: 10.1172/JCI59743]
- 62 **Seufferlein T**, Van Lint J, Liptay S, Adler G, Schmid RM. Transforming growth factor alpha activates Ha-Ras in human pancreatic cancer cells with Ki-ras mutations. *Gastroenterology* 1999; **116**: 1441-1452 [PMID: 10348828]
- 63 **Ryder NM**, Guha S, Hines OJ, Reber HA, Rozenfurt E. G protein-coupled receptor signaling in human ductal pancreatic cancer cells: neurotensin responsiveness and mitogenic stimulation. *J Cell Physiol* 2001; **186**: 53-64 [PMID: 11147814]
- 64 **Guha S**, Rey O, Rozenfurt E. Neurotensin induces protein kinase C-dependent protein kinase D activation and DNA synthesis in human pancreatic carcinoma cell line PANC-1. *Cancer Res* 2002; **62**: 1632-1640 [PMID: 11912133]
- 65 **Guerra C**, Schuhmacher AJ, Cañamero M, Grippo PJ, Verdaguer L, Pérez-Gallego L, Dubus P, Sandgren EP, Barbacid M. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* 2007; **11**: 291-302 [PMID: 17349585 DOI: 10.1016/j.ccr.2007.01.012]
- 66 **Eibl G**, Rozenfurt E. KRAS, YAP, and obesity in pancreatic cancer: A signaling network with multiple loops. *Semin Cancer Biol* 2019; **54**: 50-62 [PMID: 29079305 DOI: 10.1016/j.semcancer.2017.10.007]
- 67 **Tang H**, Wei P, Duell EJ, Risch HA, Olson SH, Bueno-de-Mesquita HB, Gallinger S, Holly EA, Petersen GM, Bracci PM, McWilliams RR, Jenab M, Riboli E, Tjønneland A, Boutron-Ruault MC, Kaaks R, Trichopoulos D, Panico S, Sund M, Peeters PH, Khaw KT, Amos CI, Li D. Genes-environment interactions in obesity- and diabetes-associated pancreatic cancer: a GWAS data analysis. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 98-106 [PMID: 24136929 DOI: 10.1158/1055-9965.EPI-13-0437-T]
- 68 **Misra JR**, Irvine KD. The Hippo Signaling Network and Its Biological Functions. *Annu Rev Genet* 2018; **52**: 65-87 [PMID: 30183404 DOI: 10.1146/annurev-genet-120417-031621]
- 69 **Meng Z**, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. *Genes Dev* 2016; **30**: 1-17 [PMID: 26728553 DOI: 10.1101/gad.274027.115]
- 70 **Enzo E**, Santinon G, Pocaterra A, Aragona M, Bresolin S, Forcato M, Grifoni D, Pession A, Zanconato F, Guzzo G, Biciato S, Dupont S. Aerobic glycolysis tunes YAP/TAZ transcriptional activity. *EMBO J* 2015; **34**: 1349-1370 [PMID: 25796446 DOI: 10.15252/embj.201490379]
- 71 **Wang Z**, Wu Y, Wang H, Zhang Y, Mei L, Fang X, Zhang X, Zhang F, Chen H, Liu Y, Jiang Y, Sun S, Zheng Y, Li N, Huang L. Interplay of mevalonate and Hippo pathways regulates RHAMM transcription via YAP to modulate breast cancer cell motility. *Proc Natl Acad Sci U S A* 2014; **111**: E89-E98 [PMID: 24367099 DOI: 10.1073/pnas.1319190110]
- 72 **Santinon G**, Pocaterra A, Dupont S. Control of YAP/TAZ Activity by Metabolic and Nutrient-Sensing Pathways. *Trends Cell Biol* 2016; **26**: 289-299 [PMID: 26750334 DOI: 10.1016/j.tcb.2015.11.004]
- 73 **Plouffe SW**, Lin KC, Moore JL, Tan FE, Ma S, Ye Z, Qiu Y, Ren B, Guan KL. The Hippo pathway effector proteins YAP and TAZ have both distinct and overlapping functions in the cell. *J Biol Chem* 2018; **293**: 11230-11240 [PMID: 29802201 DOI: 10.1074/jbc.RA118.002715]
- 74 **Kim MK**, Jang JW, Bae SC. DNA binding partners of YAP/TAZ. *BMB Rep* 2018; **51**: 126-133 [PMID: 29366442]
- 75 **Cebola I**, Rodríguez-Seguí SA, Cho CH, Bessa J, Rovira M, Luengo M, Chhatrivala M, Berry A, Ponsa-Cobas J, Maestro MA, Jennings RE, Pasquali L, Morán I, Castro N, Hanley NA, Gomez-Skarmeta JL, Vallier L, Ferrer J. TEAD and YAP regulate the enhancer network of human embryonic pancreatic progenitors. *Nat Cell Biol* 2015; **17**: 615-626 [PMID: 25915126 DOI: 10.1038/ncb3160]
- 76 **Moroishi T**, Hansen CG, Guan KL. The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer* 2015; **15**: 73-79 [PMID: 25592648 DOI: 10.1038/nrc3876]
- 77 **Murakami S**, Shahbazian D, Surana R, Zhang W, Chen H, Graham GT, White SM, Weiner LM, Yi C. Yes-associated protein mediates immune reprogramming in pancreatic ductal adenocarcinoma. *Oncogene* 2017; **36**: 1232-1244 [PMID: 27546622 DOI: 10.1038/ncr.2016.288]

- 78 **Santinon G**, Brian I, Pocaterra A, Romani P, Franzolin E, Rampazzo C, Biciato S, Dupont S. dNTP metabolism links mechanical cues and YAP/TAZ to cell growth and oncogene-induced senescence. *EMBO J* 2018; **37** [PMID: 29650681 DOI: 10.15252/embj.201797780]
- 79 **Zhao B**, Li L, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev* 2010; **24**: 862-874 [PMID: 20439427 DOI: 10.1101/gad.1909210]
- 80 **Imajo M**, Ebisuya M, Nishida E. Dual role of YAP and TAZ in renewal of the intestinal epithelium. *Nat Cell Biol* 2015; **17**: 7-19 [PMID: 25531778 DOI: 10.1038/ncb3084]
- 81 **Zanconato F**, Forcato M, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Biciato S, Cordenonsi M, Piccolo S. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nat Cell Biol* 2015; **17**: 1218-1227 [PMID: 26258633 DOI: 10.1038/ncb3216]
- 82 **Stein C**, Bardet AF, Roma G, Bergling S, Clay I, Ruchti A, Agarinis C, Schmelzle T, Bouwmeester T, Schübeler D, Bauer A. YAP1 Exerts Its Transcriptional Control via TEAD-Mediated Activation of Enhancers. *PLoS Genet* 2015; **11**: e1005465 [PMID: 26295846 DOI: 10.1371/journal.pgen.1005465]
- 83 **Zanconato F**, Battilana G, Forcato M, Filippi L, Azzolin L, Manfrin A, Quaranta E, Di Biagio D, Sigismondo G, Guzzardo V, Lejeune P, Haendler B, Krijgsveld J, Fassin M, Biciato S, Cordenonsi M, Piccolo S. Transcriptional addiction in cancer cells is mediated by YAP/TAZ through BRD4. *Nat Med* 2018; **24**: 1599-1610 [PMID: 30224758 DOI: 10.1038/s41591-018-0158-8]
- 84 **Zhang W**, Nandakumar N, Shi Y, Manzano M, Smith A, Graham G, Gupta S, Vietsch EE, Laughlin SZ, Wadhwa M, Chetram M, Joshi M, Wang F, Kallakury B, Toretzky J, Wellstein A, Yi C. Downstream of mutant KRAS, the transcription regulator YAP is essential for neoplastic progression to pancreatic ductal adenocarcinoma. *Sci Signal* 2014; **7**: ra42 [PMID: 24803537 DOI: 10.1126/scisignal.2005049]
- 85 **Gruber R**, Panayiotou R, Nye E, Spencer-Dene B, Stamp G, Behrens A. YAP1 and TAZ Control Pancreatic Cancer Initiation in Mice by Direct Up-regulation of JAK-STAT3 Signaling. *Gastroenterology* 2016; **151**: 526-539 [PMID: 27215660 DOI: 10.1053/j.gastro.2016.05.006]
- 86 **Di Agostino S**, Sorrentino G, Ingallina E, Valenti F, Ferraiuolo M, Biciato S, Piazza S, Strano S, Del Sal G, Blandino G. YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins. *EMBO Rep* 2016; **17**: 188-201 [PMID: 26691213 DOI: 10.15252/embr.201540488]
- 87 **Mello SS**, Valente LJ, Raj N, Seoane JA, Flowers BM, McClendon J, Bieging-Rolett KT, Lee J, Ivanochko D, Kozak MM, Chang DT, Longacre TA, Koong AC, Arrowsmith CH, Kim SK, Vogel H, Wood LD, Hruban RH, Curtis C, Attardi LD. A p53 Super-tumor Suppressor Reveals a Tumor Suppressive p53-Ptpn14-Yap Axis in Pancreatic Cancer. *Cancer Cell* 2017; **32**: 460-473.e6 [PMID: 29017057 DOI: 10.1016/j.ccell.2017.09.007]
- 88 **Lin L**, Sabnis AJ, Chan E, Olivas V, Cade L, Pazarentzos E, Asthana S, Neel D, Yan JJ, Lu X, Pham L, Wang MM, Karachaliou N, Cao MG, Manzano M, Ramirez JL, Torres JM, Buttitta F, Rudin CM, Collisson EA, Algazi A, Robinson E, Osman I, Muñoz-Couselo E, Cortes J, Frederick DT, Cooper ZA, McMahon M, Marchetti A, Rosell R, Flaherty KT, Wargo JA, Bivona TG. The Hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. *Nat Genet* 2015; **47**: 250-256 [PMID: 25665005 DOI: 10.1038/ng.3218]
- 89 **Neesse A**, Frese KK, Bapiro TE, Nakagawa T, Sternlicht MD, Seeley TW, Pilarsky C, Jodrell DI, Spong SM, Tuveson DA. CTGF antagonism with mAb FG-3019 enhances chemotherapy response without increasing drug delivery in murine ductal pancreas cancer. *Proc Natl Acad Sci USA* 2013; **110**: 12325-12330 [PMID: 23836645 DOI: 10.1073/pnas.1300415110]
- 90 **Wang J**, Sinnott-Smith J, Stevens JV, Young SH, Rozenfurt E. Biphasic Regulation of Yes-associated Protein (YAP) Cellular Localization, Phosphorylation, and Activity by G Protein-coupled Receptor Agonists in Intestinal Epithelial Cells: A NOVEL ROLE FOR PROTEIN KINASE D (PKD). *J Biol Chem* 2016; **291**: 17988-18005 [PMID: 27369082 DOI: 10.1074/jbc.M115.711275]
- 91 **Yu FX**, Guan KL. The Hippo pathway: regulators and regulations. *Genes Dev* 2013; **27**: 355-371 [PMID: 23431053 DOI: 10.1101/gad.210773.112]
- 92 **Straßburger K**, Tiebe M, Pinna F, Breuhahn K, Teleman AA. Insulin/IGF signaling drives cell proliferation in part via Yorkie/YAP. *Dev Biol* 2012; **367**: 187-196 [PMID: 22609549 DOI: 10.1016/j.ydbio.2012.05.008]
- 93 **Si Y**, Ji X, Cao X, Dai X, Xu L, Zhao H, Guo X, Yan H, Zhang H, Zhu C, Zhou Q, Tang M, Xia Z, Li L, Cong YS, Ye S, Liang T, Feng XH, Zhao B. Src Inhibits the Hippo Tumor Suppressor Pathway through Tyrosine Phosphorylation of Lats1. *Cancer Res* 2017; **77**: 4868-4880 [PMID: 28754671 DOI: 10.1158/0008-5472.CAN-17-0391]
- 94 **Hao F**, Xu Q, Zhao Y, Stevens JV, Young SH, Sinnott-Smith J, Rozenfurt E. Insulin Receptor and GPCR Crosstalk Stimulates YAP via PI3K and PKD in Pancreatic Cancer Cells. *Mol Cancer Res* 2017; **15**: 929-941 [PMID: 28360038 DOI: 10.1158/1541-7786.MCR-17-0023]
- 95 **Fan R**, Kim NG, Gumbiner BM. Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1. *Proc Natl Acad Sci USA* 2013; **110**: 2569-2574 [PMID: 23359693 DOI: 10.1073/pnas.1216462110]
- 96 **Kim NG**, Gumbiner BM. Adhesion to fibronectin regulates Hippo signaling via the FAK-Src-PI3K pathway. *J Cell Biol* 2015; **210**: 503-515 [PMID: 26216901 DOI: 10.1083/jcb.201501025]
- 97 **Hong X**, Nguyen HT, Chen Q, Zhang R, Hagman Z, Voorhoeve PM, Cohen SM. Opposing activities of the Ras and Hippo pathways converge on regulation of YAP protein turnover. *EMBO J* 2014; **33**: 2447-2457 [PMID: 25180228 DOI: 10.15252/embj.201489385]
- 98 **Strnadel J**, Choi S, Fujimura K, Wang H, Zhang W, Wyse M, Wright T, Gross E, Peinado C, Park HW, Bui J, Kelber J, Bouvet M, Guan KL, Klemke RL. eIF5A-PEAK1 Signaling Regulates YAP1/TAZ Protein Expression and Pancreatic Cancer Cell Growth. *Cancer Res* 2017; **77**: 1997-2007 [PMID: 28381547 DOI: 10.1158/0008-5472.CAN-16-2594]
- 99 **Rozenfurt E**, Soares HP, Sinnott-Smith J. Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance. *Mol Cancer Ther* 2014; **13**: 2477-2488 [PMID: 25323681 DOI: 10.1158/1535-7163.MCT-14-0330]
- 100 **Tumaneng K**, Schlegelmilch K, Russell RC, Yimlamai D, Basnet H, Mahadevan N, Fitamant J, Bardeesy N, Camargo FD, Guan KL. YAP mediates crosstalk between the Hippo and PI(3)K-TOR pathways by suppressing PTEN via miR-29. *Nat Cell Biol* 2012; **14**: 1322-1329 [PMID: 23143395 DOI: 10.1038/ncb2615]
- 101 **Park YY**, Sohn BH, Johnson RL, Kang MH, Kim SB, Shim JJ, Mangala LS, Kim JH, Yoo JE, Rodriguez-Aguayo C, Pradeep S, Hwang JE, Jang HJ, Lee HS, Rupaimoole R, Lopez-Berestein G, Jeong W, Park IS, Park YN, Sood AK, Mills GB, Lee JS. Yes-associated protein 1 and transcriptional coactivator with PDZ-

- binding motif activate the mammalian target of rapamycin complex 1 pathway by regulating amino acid transporters in hepatocellular carcinoma. *Hepatology* 2016; **63**: 159-172 [PMID: 26389641 DOI: 10.1002/hep.28223]
- 102 **Hansen CG**, Ng YL, Lam WL, Plouffe SW, Guan KL. The Hippo pathway effectors YAP and TAZ promote cell growth by modulating amino acid signaling to mTORC1. *Cell Res* 2015; **25**: 1299-1313 [PMID: 26611634 DOI: 10.1038/cr.2015.140]
- 103 **Liang N**, Zhang C, Dill P, Panasyuk G, Pion D, Koka V, Gallazzini M, Olson EN, Lam H, Henske EP, Dong Z, Apte U, Pallet N, Johnson RL, Terzi F, Kwiatkowski DJ, Scoazec JY, Martignoni G, Pende M. Regulation of YAP by mTOR and autophagy reveals a therapeutic target of tuberous sclerosis complex. *J Exp Med* 2014; **211**: 2249-2263 [PMID: 25288394 DOI: 10.1084/jem.20140341]
- 104 **Kapoor A**, Yao W, Ying H, Hua S, Liewen A, Wang Q, Zhong Y, Wu CJ, Sadanandam A, Hu B, Chang Q, Chu GC, Al-Khalil R, Jiang S, Xia H, Fletcher-Sananikone E, Lim C, Horwitz GI, Viale A, Pettazzoni P, Sanchez N, Wang H, Protopopov A, Zhang J, Heffernan T, Johnson RL, Chin L, Wang YA, Draetta G, DePinho RA. Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. *Cell* 2014; **158**: 185-197 [PMID: 24954535 DOI: 10.1016/j.cell.2014.06.003]
- 105 **Shao DD**, Xue W, Krall EB, Bhutkar A, Piccioni F, Wang X, Schinzel AC, Sood S, Rosenbluh J, Kim JW, Zwang Y, Roberts TM, Root DE, Jacks T, Hahn WC. KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell* 2014; **158**: 171-184 [PMID: 24954536 DOI: 10.1016/j.cell.2014.06.004]
- 106 **Greten FR**. YAP1 takes over when oncogenic K-Ras slumbers. *Cell* 2014; **158**: 11-12 [PMID: 24995973 DOI: 10.1016/j.cell.2014.06.021]
- 107 **Ying H**, Dey P, Yao W, Kimmelman AC, Draetta GF, Maitra A, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* 2016; **30**: 355-385 [PMID: 26883357 DOI: 10.1101/gad.275776.115]
- 108 **Yu FX**, Zhang Y, Park HW, Jewell JL, Chen Q, Deng Y, Pan D, Taylor SS, Lai ZC, Guan KL. Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation. *Genes Dev* 2013; **27**: 1223-1232 [PMID: 23752589 DOI: 10.1101/gad.219402.113]
- 109 **Kim M**, Kim M, Lee S, Kuninaka S, Saya H, Lee H, Lee S, Lim DS. cAMP/PKA signalling reinforces the LATS-YAP pathway to fully suppress YAP in response to actin cytoskeletal changes. *EMBO J* 2013; **32**: 1543-1555 [PMID: 23644383 DOI: 10.1038/emboj.2013.102]
- 110 **Ideno N**, Yamaguchi H, Ghosh B, Gupta S, Okumura T, Steffen DJ, Fisher CG, Wood LD, Singhi AD, Nakamura M, Gutkind JS, Maitra A. GNAS^{R201C} Induces Pancreatic Cystic Neoplasms in Mice That Express Activated KRAS by Inhibiting YAP1 Signaling. *Gastroenterology* 2018; **155**: 1593-1607.e12 [PMID: 30142336 DOI: 10.1053/j.gastro.2018.08.006]
- 111 **Suklabaidya S**, Dash P, Das B, Suresh V, Sasmal PK, Senapati S. Experimental models of pancreatic cancer desmoplasia. *Lab Invest* 2018; **98**: 27-40 [PMID: 29155423 DOI: 10.1038/labinvest.2017.127]
- 112 **Panciera T**, Azzolin L, Cordenonsi M, Piccolo S. Mechanobiology of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol* 2017; **18**: 758-770 [PMID: 28951564 DOI: 10.1038/nrm.2017.87]
- 113 **Low BC**, Pan CQ, Shivashankar GV, Bershadsky A, Sudol M, Sheetz M. YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth. *FEBS Lett* 2014; **588**: 2663-2670 [PMID: 24747426 DOI: 10.1016/j.febslet.2014.04.012]
- 114 **Ibar C**, Irvine KD. Rapping about Mechanotransduction. *Dev Cell* 2018; **46**: 678-679 [PMID: 30253168 DOI: 10.1016/j.devcel.2018.09.007]
- 115 **Meng Z**, Qiu Y, Lin KC, Kumar A, Placone JK, Fang C, Wang KC, Lu S, Pan M, Hong AW, Moroishi T, Luo M, Plouffe SW, Diao Y, Ye Z, Park HW, Wang X, Yu FX, Chien S, Wang CY, Ren B, Engler AJ, Guan KL. RAP2 mediates mechanoresponses of the Hippo pathway. *Nature* 2018; **560**: 655-660 [PMID: 30135582 DOI: 10.1038/s41586-018-0444-0]
- 116 **Yang S**, Zhang L, Purohit V, Shukla SK, Chen X, Yu F, Fu K, Chen Y, Solheim J, Singh PK, Song W, Dong J. Active YAP promotes pancreatic cancer cell motility, invasion and tumorigenesis in a mitotic phosphorylation-dependent manner through LPAR3. *Oncotarget* 2015; **6**: 36019-36031 [PMID: 26440309 DOI: 10.18632/oncotarget.5935]
- 117 **Xie D**, Cui J, Xia T, Jia Z, Wang L, Wei W, Zhu A, Gao Y, Xie K, Quan M. Hippo transducer TAZ promotes epithelial mesenchymal transition and supports pancreatic cancer progression. *Oncotarget* 2015; **6**: 35949-35963 [PMID: 26416426 DOI: 10.18632/oncotarget.5772]
- 118 **Salcedo Allende MT**, Zeron-Medina J, Hernandez J, Macarulla T, Balsells J, Merino X, Allende H, Tabernero J, Ramon Y Cajal S. Overexpression of Yes Associated Protein 1, an Independent Prognostic Marker in Patients With Pancreatic Ductal Adenocarcinoma, Correlated With Liver Metastasis and Poor Prognosis. *Pancreas* 2017; **46**: 913-920 [PMID: 28697132 DOI: 10.1097/MPA.0000000000000867]
- 119 **Uhlen M**, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, Benfettas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnström H, Glimelius B, Sjöblom T, Edqvist PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A, Ponten F. A pathology atlas of the human cancer transcriptome. *Science* 2017; **357** [PMID: 28818916 DOI: 10.1126/science.aan2507]
- 120 **Rozenfurt E**, Sinnott-Smith J, Eibl G. Yes-associated protein (YAP) in pancreatic cancer: at the epicenter of a targetable signaling network associated with patient survival. *Signal Transduct Target Ther* 2018; **3**: 11 [PMID: 29682330 DOI: 10.1038/s41392-017-0005-2]
- 121 **Tang Z**, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* 2017; **45**: W98-W102 [PMID: 28407145 DOI: 10.1093/nar/gkx247]
- 122 **Ardestani A**, Lupse B, Maedler K. Hippo Signaling: Key Emerging Pathway in Cellular and Whole-Body Metabolism. *Trends Endocrinol Metab* 2018; **29**: 492-509 [PMID: 29739703 DOI: 10.1016/j.tem.2018.04.006]
- 123 **Koo JH**, Guan KL. Interplay between YAP/TAZ and Metabolism. *Cell Metab* 2018; **28**: 196-206 [PMID: 30089241 DOI: 10.1016/j.cmet.2018.07.010]
- 124 **Jóźwiak P**, Forma E, Bryś M, Krześlak A. O-GlcNAcylation and Metabolic Reprograming in Cancer. *Front Endocrinol (Lausanne)* 2014; **5**: 145 [PMID: 25250015 DOI: 10.3389/fendo.2014.00145]
- 125 **Peng C**, Zhu Y, Zhang W, Liao Q, Chen Y, Zhao X, Guo Q, Shen P, Zhen B, Qian X, Yang D, Zhang JS, Xiao D, Qin W, Pei H. Regulation of the Hippo-YAP Pathway by Glucose Sensor O-GlcNAcylation. *Mol Cell* 2017; **68**: 591-604.e5 [PMID: 29100056 DOI: 10.1016/j.molcel.2017.10.010]
- 126 **Kammoun HL**, Kraakman MJ, Febbraio MA. Adipose tissue inflammation in glucose metabolism. *Rev Endocr Metab Disord* 2014; **15**: 31-44 [PMID: 24048715 DOI: 10.1007/s11154-013-9274-4]
- 127 **Bai Y**, Sun Q. Macrophage recruitment in obese adipose tissue. *Obes Rev* 2015; **16**: 127-136 [PMID:

- 25586506 DOI: [10.1111/obr.12242](https://doi.org/10.1111/obr.12242)]
- 128 **Boutens L**, Stienstra R. Adipose tissue macrophages: going off track during obesity. *Diabetologia* 2016; **59**: 879-894 [PMID: [26940592](https://pubmed.ncbi.nlm.nih.gov/26940592/) DOI: [10.1007/s00125-016-3904-9](https://doi.org/10.1007/s00125-016-3904-9)]
- 129 **Castoldi A**, Naffah de Souza C, Câmara NO, Moraes-Vieira PM. The Macrophage Switch in Obesity Development. *Front Immunol* 2016; **6**: 637 [PMID: [26779183](https://pubmed.ncbi.nlm.nih.gov/26779183/) DOI: [10.3389/fimmu.2015.00637](https://doi.org/10.3389/fimmu.2015.00637)]
- 130 **Hill AA**, Reid Bolus W, Hasty AH. A decade of progress in adipose tissue macrophage biology. *Immunol Rev* 2014; **262**: 134-152 [PMID: [25319332](https://pubmed.ncbi.nlm.nih.gov/25319332/) DOI: [10.1111/immr.12216](https://doi.org/10.1111/immr.12216)]
- 131 **Reilly SM**, Saltiel AR. Adapting to obesity with adipose tissue inflammation. *Nat Rev Endocrinol* 2017; **13**: 633-643 [PMID: [28799554](https://pubmed.ncbi.nlm.nih.gov/28799554/) DOI: [10.1038/nrendo.2017.90](https://doi.org/10.1038/nrendo.2017.90)]
- 132 **Wensveen FM**, Valentić S, Šestan M, Turk Wensveen T, Polić B. The "Big Bang" in obese fat: Events initiating obesity-induced adipose tissue inflammation. *Eur J Immunol* 2015; **45**: 2446-2456 [PMID: [26220361](https://pubmed.ncbi.nlm.nih.gov/26220361/) DOI: [10.1002/eji.201545502](https://doi.org/10.1002/eji.201545502)]
- 133 **An Y**, Kang Q, Zhao Y, Hu X, Li N. Lats2 modulates adipocyte proliferation and differentiation via hippo signaling. *PLoS One* 2013; **8**: e72042 [PMID: [23977200](https://pubmed.ncbi.nlm.nih.gov/23977200/) DOI: [10.1371/journal.pone.0072042](https://doi.org/10.1371/journal.pone.0072042)]
- 134 **Seo E**, Basu-Roy U, Gunaratne PH, Coarfa C, Lim DS, Basilico C, Mansukhani A. SOX2 regulates YAP1 to maintain stemness and determine cell fate in the osteo-adipo lineage. *Cell Rep* 2013; **3**: 2075-2087 [PMID: [23791527](https://pubmed.ncbi.nlm.nih.gov/23791527/) DOI: [10.1016/j.celrep.2013.05.029](https://doi.org/10.1016/j.celrep.2013.05.029)]
- 135 **Kuroda M**, Wada H, Kimura Y, Ueda K, Kioka N. Vinculin promotes nuclear localization of TAZ to inhibit ECM stiffness-dependent differentiation into adipocytes. *J Cell Sci* 2017; **130**: 989-1002 [PMID: [28115535](https://pubmed.ncbi.nlm.nih.gov/28115535/) DOI: [10.1242/jcs.194779](https://doi.org/10.1242/jcs.194779)]
- 136 **Li Y**, Du J, Zhu E, Zhang J, Han J, Zhao W, Sun B, Tian D. Liraglutide suppresses proliferation and induces adipogenic differentiation of 3T3-L1 cells via the Hippo-YAP signaling pathway. *Mol Med Rep* 2018; **17**: 4499-4507 [PMID: [29344656](https://pubmed.ncbi.nlm.nih.gov/29344656/) DOI: [10.3892/mmr.2018.8438](https://doi.org/10.3892/mmr.2018.8438)]
- 137 **Basu-Roy U**, Han E, Rattanakor K, Gadi A, Verma N, Maurizi G, Gunaratne PH, Coarfa C, Kennedy OD, Garabedian MJ, Basilico C, Mansukhani A. PPAR γ agonists promote differentiation of cancer stem cells by restraining YAP transcriptional activity. *Oncotarget* 2016; **7**: 60954-60970 [PMID: [27528232](https://pubmed.ncbi.nlm.nih.gov/27528232/) DOI: [10.18632/oncotarget.11273](https://doi.org/10.18632/oncotarget.11273)]
- 138 **Chang CC**, Chen CY, Chang GD, Chen TH, Chen WL, Wen HC, Huang CY, Chang CH. Hyperglycemia and advanced glycation end products (AGEs) suppress the differentiation of 3T3-L1 preadipocytes. *Oncotarget* 2017; **8**: 55039-55050 [PMID: [28903400](https://pubmed.ncbi.nlm.nih.gov/28903400/) DOI: [10.18632/oncotarget.18993](https://doi.org/10.18632/oncotarget.18993)]
- 139 **Huang YJ**, Yang CK, Wei PL, Huynh TT, Whang-Peng J, Meng TC, Hsiao Y, Tzeng YM, Wu AT, Yen Y. Ovatodiolide suppresses colon tumorigenesis and prevents polarization of M2 tumor-associated macrophages through YAP oncogenic pathways. *J Hematol Oncol* 2017; **10**: 60 [PMID: [28241877](https://pubmed.ncbi.nlm.nih.gov/28241877/) DOI: [10.1186/s13045-017-0421-3](https://doi.org/10.1186/s13045-017-0421-3)]
- 140 **McWhorter FY**, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci USA* 2013; **110**: 17253-17258 [PMID: [24101477](https://pubmed.ncbi.nlm.nih.gov/24101477/) DOI: [10.1073/pnas.1308887110](https://doi.org/10.1073/pnas.1308887110)]
- 141 **Pellegrinelli V**, Heuvingh J, du Roure O, Rouault C, Devulder A, Klein C, Lacasa M, Clément E, Lacasa D, Clément K. Human adipocyte function is impacted by mechanical cues. *J Pathol* 2014; **233**: 183-195 [PMID: [24623048](https://pubmed.ncbi.nlm.nih.gov/24623048/) DOI: [10.1002/path.4347](https://doi.org/10.1002/path.4347)]
- 142 **Hertzer KM**, Xu M, Moro A, Dawson DW, Du L, Li G, Chang HH, Stark AP, Jung X, Hines OJ, Eibl G. Robust Early Inflammation of the Peripancreatic Visceral Adipose Tissue During Diet-Induced Obesity in the KrasG12D Model of Pancreatic Cancer. *Pancreas* 2016; **45**: 458-465 [PMID: [26495779](https://pubmed.ncbi.nlm.nih.gov/26495779/) DOI: [10.1097/MPA.0000000000000497](https://doi.org/10.1097/MPA.0000000000000497)]
- 143 **Xu M**, Chang HH, Jung X, Moro A, Chou CEN, King J, Hines OJ, Sinnott-Smith J, Rozenfurt E, Eibl G. Deficiency in hormone-sensitive lipase accelerates the development of pancreatic cancer in conditional KrasG12D mice. *BMC Cancer* 2018; **18**: 797 [PMID: [30086728](https://pubmed.ncbi.nlm.nih.gov/30086728/) DOI: [10.1186/s12885-018-4713-y](https://doi.org/10.1186/s12885-018-4713-y)]
- 144 **Freed-Pastor WA**, Mizuno H, Zhao X, Langerød A, Moon SH, Rodriguez-Barrueco R, Barsotti A, Chicas A, Li W, Polotskaia A, Bissell MJ, Osborne TF, Tian B, Lowe SW, Silva JM, Børresen-Dale AL, Levine AJ, Bargonetti J, Prives C. Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell* 2012; **148**: 244-258 [PMID: [22265415](https://pubmed.ncbi.nlm.nih.gov/22265415/) DOI: [10.1016/j.cell.2011.12.017](https://doi.org/10.1016/j.cell.2011.12.017)]
- 145 **Clendening JW**, Pandya A, Boutros PC, El Ghamrasni S, Khosravi F, Trentin GA, Martirosyan A, Hakem A, Hakem R, Jurisica I, Penn LZ. Dysregulation of the mevalonate pathway promotes transformation. *Proc Natl Acad Sci USA* 2010; **107**: 15051-15056 [PMID: [20696928](https://pubmed.ncbi.nlm.nih.gov/20696928/) DOI: [10.1073/pnas.0910258107](https://doi.org/10.1073/pnas.0910258107)]
- 146 **Kuzu OF**, Noory MA, Robertson GP. The Role of Cholesterol in Cancer. *Cancer Res* 2016; **76**: 2063-2070 [PMID: [27197250](https://pubmed.ncbi.nlm.nih.gov/27197250/) DOI: [10.1158/0008-5472.CAN-15-2613](https://doi.org/10.1158/0008-5472.CAN-15-2613)]
- 147 **Istvan ES**, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* 2001; **292**: 1160-1164 [PMID: [11349148](https://pubmed.ncbi.nlm.nih.gov/11349148/) DOI: [10.1126/science.1059344](https://doi.org/10.1126/science.1059344)]
- 148 **Jeon CY**, Pandol SJ, Wu B, Cook-Wiens G, Gottlieb RA, Merz CN, Goodman MT. The association of statin use after cancer diagnosis with survival in pancreatic cancer patients: a SEER-medicare analysis. *PLoS One* 2015; **10**: e0121783 [PMID: [25830309](https://pubmed.ncbi.nlm.nih.gov/25830309/) DOI: [10.1371/journal.pone.0121783](https://doi.org/10.1371/journal.pone.0121783)]
- 149 **Wu BU**, Chang J, Jeon CY, Pandol SJ, Huang B, Ngor EW, Difronzo AL, Cooper RM. Impact of statin use on survival in patients undergoing resection for early-stage pancreatic cancer. *Am J Gastroenterol* 2015; **110**: 1233-1239 [PMID: [26195180](https://pubmed.ncbi.nlm.nih.gov/26195180/) DOI: [10.1038/ajg.2015.217](https://doi.org/10.1038/ajg.2015.217)]
- 150 **Chen MJ**, Tsan YT, Liou JM, Lee YC, Wu MS, Chiu HM, Wang HP, Chen PC. Statins and the risk of pancreatic cancer in Type 2 diabetic patients--A population-based cohort study. *Int J Cancer* 2016; **138**: 594-603 [PMID: [26296262](https://pubmed.ncbi.nlm.nih.gov/26296262/) DOI: [10.1002/ijc.29813](https://doi.org/10.1002/ijc.29813)]
- 151 **Walker EJ**, Ko AH, Holly EA, Bracci PM. Statin use and risk of pancreatic cancer: results from a large, clinic-based case-control study. *Cancer* 2015; **121**: 1287-1294 [PMID: [25649483](https://pubmed.ncbi.nlm.nih.gov/25649483/) DOI: [10.1002/ncr.29256](https://doi.org/10.1002/ncr.29256)]
- 152 **Carey FJ**, Little MW, Pugh TF, Ndokera R, Ing H, Clark A, Dennison A, Metcalfe MS, Robinson RJ, Hart AR. The differential effects of statins on the risk of developing pancreatic cancer: a case-control study in two centres in the United Kingdom. *Dig Dis Sci* 2013; **58**: 3308-3312 [PMID: [23864194](https://pubmed.ncbi.nlm.nih.gov/23864194/) DOI: [10.1007/s10620-013-2778-7](https://doi.org/10.1007/s10620-013-2778-7)]
- 153 **E JY**, Lu SE, Lin Y, Graber JM, Rotter D, Zhang L, Petersen GM, Demissie K, Lu-Yao G, Tan XL. Differential and Joint Effects of Metformin and Statins on Overall Survival of Elderly Patients with Pancreatic Adenocarcinoma: A Large Population-Based Study. *Cancer Epidemiol Biomarkers Prev* 2017; **26**: 1225-1232 [PMID: [28619830](https://pubmed.ncbi.nlm.nih.gov/28619830/) DOI: [10.1158/1055-9965.EPI-17-0227](https://doi.org/10.1158/1055-9965.EPI-17-0227)]
- 154 **Iarrobino NA**, Gill B, Bernard ME, Mishra MV, Champ CE. Targeting Tumor Metabolism With Statins During Treatment for Advanced-stage Pancreatic Cancer. *Am J Clin Oncol* 2018 [PMID: [29509593](https://pubmed.ncbi.nlm.nih.gov/29509593/) DOI: [10.1200/JCO.2018.39.15.1](https://doi.org/10.1200/JCO.2018.39.15.1)]

- 10.1097/COC.0000000000000433]
- 155 **Jian-Yu E**, Graber JM, Lu SE, Lin Y, Lu-Yao G, Tan XL. Effect of Metformin and Statin Use on Survival in Pancreatic Cancer Patients: a Systematic Literature Review and Meta-analysis. *Curr Med Chem* 2018; **25**: 2595-2607 [PMID: [28403788](#) DOI: [10.2174/0929867324666170412145232](#)]
- 156 **Bang UC**, Watanabe T, Bendtsen F. The relationship between the use of statins and mortality, severity, and pancreatic cancer in Danish patients with chronic pancreatitis. *Eur J Gastroenterol Hepatol* 2018; **30**: 346-351 [PMID: [29309396](#) DOI: [10.1097/MEG.0000000000001060](#)]
- 157 **Liu Y**, Tang W, Wang J, Xie L, Li T, He Y, Deng Y, Peng Q, Li S, Qin X. Association between statin use and colorectal cancer risk: a meta-analysis of 42 studies. *Cancer Causes Control* 2014; **25**: 237-249 [PMID: [24265089](#) DOI: [10.1007/s10552-013-0326-6](#)]
- 158 **Manthravadi S**, Shrestha A, Madhusudhana S. Impact of statin use on cancer recurrence and mortality in breast cancer: A systematic review and meta-analysis. *Int J Cancer* 2016; **139**: 1281-1288 [PMID: [27176735](#) DOI: [10.1002/ijc.30185](#)]
- 159 **Anothaisintawee T**, Udomsubpayakul U, McEvoy M, Lerdsitthichai P, Attia J, Thakkestian A. Effect of Lipophilic and Hydrophilic Statins on Breast Cancer Risk in Thai Women: A Cross-sectional Study. *J Cancer* 2016; **7**: 1163-1168 [PMID: [27326260](#) DOI: [10.7150/jca.14941](#)]
- 160 **Liu B**, Yi Z, Guan X, Zeng YX, Ma F. The relationship between statins and breast cancer prognosis varies by statin type and exposure time: a meta-analysis. *Breast Cancer Res Treat* 2017; **164**: 1-11 [PMID: [28432513](#) DOI: [10.1007/s10549-017-4246-0](#)]
- 161 **Hamada T**, Khalaf N, Yuan C, Babic A, Morales-Oyarvide V, Qian ZR, Nowak JA, Ng K, Kraft P, Robinson DA, Stampfer MJ, Giovannucci EL, Fuchs CS, Ogino S, Wolpin BM. Statin use and pancreatic cancer risk in two prospective cohort studies. *J Gastroenterol* 2018; **53**: 959-966 [PMID: [29362938](#) DOI: [10.1007/s00535-018-1430-x](#)]
- 162 **Hamada T**, Khalaf N, Yuan C, Morales-Oyarvide V, Babic A, Nowak JA, Qian ZR, Ng K, Robinson DA, Kraft P, Giovannucci EL, Stampfer MJ, Fuchs CS, Ogino S, Wolpin BM. Prediagnosis Use of Statins Associates With Increased Survival Times of Patients With Pancreatic Cancer. *Clin Gastroenterol Hepatol* 2018; **16**: 1300-1306.e3 [PMID: [29474971](#) DOI: [10.1016/j.cgh.2018.02.022](#)]
- 163 **Zhang Y**, Liang M, Sun C, Qu G, Shi T, Min M, Wu Y, Sun Y. Statin Use and Risk of Pancreatic Cancer: An Updated Meta-analysis of 26 Studies. *Pancreas* 2019; **48**: 142-150 [PMID: [30640225](#) DOI: [10.1097/MPA.0000000000001226](#)]
- 164 **Lee HS**, Lee SH, Lee HJ, Chung MJ, Park JY, Park SW, Song SY, Bang S. Statin Use and Its Impact on Survival in Pancreatic Cancer Patients. *Medicine (Baltimore)* 2016; **95**: e3607 [PMID: [27175667](#) DOI: [10.1097/MD.0000000000003607](#)]
- 165 **Mohammed A**, Qian L, Janakiram NB, Lightfoot S, Steele VE, Rao CV. Atorvastatin delays progression of pancreatic lesions to carcinoma by regulating PI3/AKT signaling in p48Cre/+ LSL-KrasG12D/+ mice. *Int J Cancer* 2012; **131**: 1951-1962 [PMID: [22287227](#) DOI: [10.1002/ijc.27456](#)]
- 166 **Fendrich V**, Sparrn M, Lauth M, Knoop R, Plassmeier L, Bartsch DK, Waldmann J. Simvastatin delay progression of pancreatic intraepithelial neoplasia and cancer formation in a genetically engineered mouse model of pancreatic cancer. *Pancreatol* 2013; **13**: 502-507 [PMID: [24075515](#) DOI: [10.1016/j.pan.2013.08.002](#)]
- 167 **Sorrentino G**, Ruggeri N, Specchia V, Cordenonsi M, Mano M, Dupont S, Manfrin A, Ingallina E, Sommaggio R, Piazza S, Rosato A, Piccolo S, Del Sal G. Metabolic control of YAP and TAZ by the mevalonate pathway. *Nat Cell Biol* 2014; **16**: 357-366 [PMID: [24658687](#) DOI: [10.1038/ncb2936](#)]
- 168 **Witters LA**. The blooming of the French lilac. *J Clin Invest* 2001; **108**: 1105-1107 [PMID: [11602616](#) DOI: [10.1172/JCI114178](#)]
- 169 **Kahn BB**, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 2005; **1**: 15-25 [PMID: [16054041](#) DOI: [10.1016/j.cmet.2004.12.003](#)]
- 170 **Shaw RJ**, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 2005; **310**: 1642-1646 [PMID: [16308421](#) DOI: [10.1126/science.1120781](#)]
- 171 **Berker B**, Emral R, Demirel C, Corapcioglu D, Unlu C, Kose K. Increased insulin-like growth factor-I levels in women with polycystic ovary syndrome, and beneficial effects of metformin therapy. *Gynecol Endocrinol* 2004; **19**: 125-133 [PMID: [15697073](#) DOI: [10.1080/09513590400007309](#)]
- 172 **Goodwin PJ**, Pritchard KI, Ennis M, Clemons M, Graham M, Fantus IG. Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer* 2008; **8**: 501-505 [PMID: [19073504](#) DOI: [10.3816/CBC.2008.n.060](#)]
- 173 **Evans JM**, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; **330**: 1304-1305 [PMID: [15849206](#) DOI: [10.1136/bmj.38415.708634.F7](#)]
- 174 **Li D**, Yeung SC, Hassan MM, Konopleva M, Abbruzzese JL. Antidiabetic therapies affect risk of pancreatic cancer. *Gastroenterology* 2009; **137**: 482-488 [PMID: [19375425](#) DOI: [10.1053/j.gastro.2009.04.013](#)]
- 175 **Libby G**, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care* 2009; **32**: 1620-1625 [PMID: [19564453](#) DOI: [10.2337/dc08-2175](#)]
- 176 **Ben Sahra I**, Le Marchand-Brustel Y, Tanti JF, Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? *Mol Cancer Ther* 2010; **9**: 1092-1099 [PMID: [20442309](#) DOI: [10.1158/1535-7163.MCT-09-1186](#)]
- 177 **Sadeghi N**, Abbruzzese JL, Yeung SC, Hassan M, Li D. Metformin use is associated with better survival of diabetic patients with pancreatic cancer. *Clin Cancer Res* 2012; **18**: 2905-2912 [PMID: [22465831](#) DOI: [10.1158/1078-0432.CCR-11-2994](#)]
- 178 **Li X**, Li T, Liu Z, Gou S, Wang C. The effect of metformin on survival of patients with pancreatic cancer: a meta-analysis. *Sci Rep* 2017; **7**: 5825 [PMID: [28724893](#) DOI: [10.1038/s41598-017-06207-x](#)]
- 179 **Lega IC**, Shah PS, Margel D, Beyene J, Rochon PA, Lipscombe LL. The effect of metformin on mortality following cancer among patients with diabetes. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 1974-1984 [PMID: [25030683](#) DOI: [10.1158/1055-9965.EPI-14-0327](#)]
- 180 **Amin S**, Mhango G, Lin J, Aronson A, Wisnivesky J, Boffetta P, Lucas AL. Metformin Improves Survival in Patients with Pancreatic Ductal Adenocarcinoma and Pre-Existing Diabetes: A Propensity Score Analysis. *Am J Gastroenterol* 2016; **111**: 1350-1357 [PMID: [27430290](#) DOI: [10.1038/ajg.2016.288](#)]
- 181 **Lee DY**, Yu JH, Park S, Han K, Kim NH, Yoo HJ, Choi KM, Baik SH, Kim NH, Seo JA. The influence of

- diabetes and antidiabetic medications on the risk of pancreatic cancer: a nationwide population-based study in Korea. *Sci Rep* 2018; **8**: 9719 [PMID: 29946194 DOI: 10.1038/s41598-018-27965-2]
- 182 **Xin W**, Fang L, Fang Q, Zheng X, Huang P. Effects of metformin on survival outcomes of pancreatic cancer patients with diabetes: A meta-analysis. *Mol Clin Oncol* 2018; **8**: 483-488 [PMID: 29468063 DOI: 10.3892/mco.2017.1541]
- 183 **Suissa S**, Azoulay L. Metformin and cancer: mounting evidence against an association. *Diabetes Care* 2014; **37**: 1786-1788 [PMID: 24963109 DOI: 10.2337/dc14-0500]
- 184 **Wan G**, Sun X, Li F, Wang X, Li C, Li H, Yu X, Cao F. Survival Benefit of Metformin Adjuvant Treatment For Pancreatic Cancer Patients: a Systematic Review and Meta-Analysis. *Cell Physiol Biochem* 2018; **49**: 837-847 [PMID: 30184547 DOI: 10.1159/000493214]
- 185 **Jang WI**, Kim MS, Kang SH, Jo AJ, Kim YJ, Tchoe HJ, Park CM, Kim HJ, Choi JA, Choi HJ, Paik EK, Seo YS, Yoo HJ, Kang JK, Han CJ, Kim YJ, Kim SB, Ko MJ. Association between metformin use and mortality in patients with type 2 diabetes mellitus and localized resectable pancreatic cancer: a nationwide population-based study in Korea. *Oncotarget* 2017; **8**: 9587-9596 [PMID: 28077783 DOI: 10.18632/oncotarget.14525]
- 186 **Cerullo M**, Gani F, Chen SY, Canner J, Pawlik TM. Metformin Use Is Associated with Improved Survival in Patients Undergoing Resection for Pancreatic Cancer. *J Gastrointest Surg* 2016; **20**: 1572-1580 [PMID: 27255657 DOI: 10.1007/s11605-016-3173-4]
- 187 **Hawley SA**, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM, Hardie DG. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* 2010; **11**: 554-565 [PMID: 20519126 DOI: 10.1016/j.cmet.2010.04.001]
- 188 **Ben Sahra I**, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y, Bost F. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene* 2008; **27**: 3576-3586 [PMID: 18212742 DOI: 10.1038/sj.onc.1211024]
- 189 **Kalender A**, Selvaraj A, Kim SY, Gulati P, Brulé S, Viollet B, Kemp BE, Bardeesy N, Dennis P, Schlager JJ, Marette A, Kozma SC, Thomas G. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab* 2010; **11**: 390-401 [PMID: 20444419 DOI: 10.1016/j.cmet.2010.03.014]
- 190 **Hezel AF**, Bardeesy N. LKB1; linking cell structure and tumor suppression. *Oncogene* 2008; **27**: 6908-6919 [PMID: 19029933 DOI: 10.1038/onc.2008.342]
- 191 **Owen MR**, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* 2000; **348** Pt 3: 607-614 [PMID: 10839993]
- 192 **El-Mir MY**, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 2000; **275**: 223-228 [PMID: 10617608]
- 193 **Inoki K**, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003; **115**: 577-590 [PMID: 14651849]
- 194 **Shaw RJ**, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 2004; **6**: 91-99 [PMID: 15261145 DOI: 10.1016/j.ccr.2004.06.007]
- 195 **Inoki K**, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 2006; **126**: 955-968 [PMID: 16959574 DOI: 10.1016/j.cell.2006.06.055]
- 196 **Gwinn DM**, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vazquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* 2008; **30**: 214-226 [PMID: 18439900 DOI: 10.1016/j.molcel.2008.03.003]
- 197 **Tzatsos A**, Tsichlis PN. Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794. *J Biol Chem* 2007; **282**: 18069-18082 [PMID: 17459875 DOI: 10.1074/jbc.M610101200]
- 198 **Ning J**, Clemmons DR. AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. *Mol Endocrinol* 2010; **24**: 1218-1229 [PMID: 20363874 DOI: 10.1210/me.2009-0474]
- 199 **Sinnott-Smith J**, Kisfalvi K, Kui R, Rozengurt E. Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK. *Biochem Biophys Res Commun* 2013; **430**: 352-357 [PMID: 23159620 DOI: 10.1016/j.bbrc.2012.11.010]
- 200 **Soares HP**, Ni Y, Kisfalvi K, Sinnott-Smith J, Rozengurt E. Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells. *PLoS One* 2013; **8**: e57289 [PMID: 23437362 DOI: 10.1371/journal.pone.0057289]
- 201 **Ming M**, Sinnott-Smith J, Wang J, Soares HP, Young SH, Eibl G, Rozengurt E. Dose-Dependent AMPK-Dependent and Independent Mechanisms of Berberine and Metformin Inhibition of mTORC1, ERK, DNA Synthesis and Proliferation in Pancreatic Cancer Cells. *PLoS One* 2014; **9**: e114573 [PMID: 25493642 DOI: 10.1371/journal.pone.0114573]
- 202 **Kisfalvi K**, Eibl G, Sinnott-Smith J, Rozengurt E. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. *Cancer Res* 2009; **69**: 6539-6545 [PMID: 19679549 DOI: 10.1158/0008-5472.CAN-09-0418]
- 203 **Kisfalvi K**, Moro A, Sinnott-Smith J, Eibl G, Rozengurt E. Metformin inhibits the growth of human pancreatic cancer xenografts. *Pancreas* 2013; **42**: 781-785 [PMID: 23462329 DOI: 10.1097/MPA.0b013e31827aec40]
- 204 **Chang HH**, Moro A, Chou CEN, Dawson DW, French S, Schmidt AI, Sinnott-Smith J, Hao F, Hines OJ, Eibl G, Rozengurt E. Metformin Decreases the Incidence of Pancreatic Ductal Adenocarcinoma Promoted by Diet-induced Obesity in the Conditional KrasG12D Mouse Model. *Sci Rep* 2018; **8**: 5899 [PMID: 29651002 DOI: 10.1038/s41598-018-24337-8]
- 205 **Mo JS**, Meng Z, Kim YC, Park HW, Hansen CG, Kim S, Lim DS, Guan KL. Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. *Nat Cell Biol* 2015; **17**: 500-510 [PMID: 25751140 DOI: 10.1038/ncb3111]

- 206 **Wang W**, Xiao ZD, Li X, Aziz KE, Gan B, Johnson RL, Chen J. AMPK modulates Hippo pathway activity to regulate energy homeostasis. *Nat Cell Biol* 2015; **17**: 490-499 [PMID: [25751139](#) DOI: [10.1038/ncb3113](#)]
- 207 **Om Kumar RV**, Darnay BG, Rodwell VW. Modulation of Syrian hamster 3-hydroxy-3-methylglutaryl-CoA reductase activity by phosphorylation. Role of serine 871. *J Biol Chem* 1994; **269**: 6810-6814 [PMID: [8120043](#)]
- 208 **DeRan M**, Yang J, Shen CH, Peters EC, Fitamant J, Chan P, Hsieh M, Zhu S, Asara JM, Zheng B, Bardeesy N, Liu J, Wu X. Energy stress regulates hippo-YAP signaling involving AMPK-mediated regulation of angiotensin-like 1 protein. *Cell Rep* 2014; **9**: 495-503 [PMID: [25373897](#) DOI: [10.1016/j.celrep.2014.09.036](#)]
- 209 **Sahai V**, Kumar K, Knab LM, Chow CR, Raza SS, Bentrem DJ, Ebine K, Munshi HG. BET bromodomain inhibitors block growth of pancreatic cancer cells in three-dimensional collagen. *Mol Cancer Ther* 2014; **13**: 1907-1917 [PMID: [24807963](#) DOI: [10.1158/1535-7163.MCT-13-0925](#)]
- 210 **Filippakopoulos P**, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y, Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N, French CA, Wiest O, Kung AL, Knapp S, Bradner JE. Selective inhibition of BET bromodomains. *Nature* 2010; **468**: 1067-1073 [PMID: [20871596](#) DOI: [10.1038/nature09504](#)]
- 211 **Liu Z**, Wang P, Chen H, Wold EA, Tian B, Brasier AR, Zhou J. Drug Discovery Targeting Bromodomain-Containing Protein 4. *J Med Chem* 2017; **60**: 4533-4558 [PMID: [28195723](#) DOI: [10.1021/acs.jmedchem.6b01761](#)]
- 212 **Tanti JF**, Jager J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr Opin Pharmacol* 2009; **9**: 753-762 [PMID: [19683471](#) DOI: [10.1016/j.coph.2009.07.004](#)]
- 213 **Soares HP**, Ming M, Mellon M, Young SH, Han L, Sinnet-Smith J, Rozengurt E. Dual PI3K/mTOR Inhibitors Induce Rapid Overactivation of the MEK/ERK Pathway in Human Pancreatic Cancer Cells through Suppression of mTORC2. *Mol Cancer Ther* 2015; **14**: 1014-1023 [PMID: [25673820](#) DOI: [10.1158/1535-7163.MCT-14-0669](#)]



Considerations of elderly factors to manage the complication of liver cirrhosis in elderly patients

Kenya Kamimura, Akira Sakamaki, Hiroteru Kamimura, Toru Setsu, Takeshi Yokoo, Masaaki Takamura, Shuji Terai

ORCID number: Kenya Kamimura (0000-0001-7182-4400); Akira Sakamaki (0000-0002-9368-7272); Hiroteru Kamimura (0000-0002-9135-3092); Toru Setsu (0000-0003-2309-3556); Takeshi Yokoo (0000-0001-7138-1785); Masaaki Takamura (0000-0001-6773-4613); Shuji Terai (0000-0002-5439-635X).

Author contributions: Kamimura K and Terai S wrote the manuscript; Sakamaki A, Kamimura H, Setsu T, Yokoo T and Takamura M collected information; all authors read and approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare that they have no current financial arrangement or affiliation with any organization that may have a direct influence on their work.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Kenya Kamimura, Akira Sakamaki, Hiroteru Kamimura, Toru Setsu, Takeshi Yokoo, Masaaki Takamura, Shuji Terai, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan

Corresponding author: Kenya Kamimura, MD, PhD, Lecturer, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan. kenya-k@med.niigata-u.ac.jp
Telephone: +81-25-2272207
Fax: +81-25-2270776

Abstract

The aging of the organ function causes sensitivity to the disease progression and need careful consideration for the medical treatment. With the increase of aging population, the opportunity to provide medical treatment for people in very old age is rapidly increasing therefore, the understanding of the various physiological changes of cellular function, size and function of organs are essential for the decision of therapeutic options. Among the various chronic conditions seen in elderly people, we have focused on liver cirrhosis, since despite specific therapeutic options for many of liver diseases including direct acting antivirals for hepatitis C virus, nucleoside analogs for hepatitis B, and corticosteroids for autoimmune hepatitis, there is currently no standard therapy to treat liver cirrhosis, which is the final stage of these liver diseases. Therefore, management of the various symptoms of liver cirrhosis is essential, and aging-related parameters must be considered in the decision making for therapeutic strategies and dosage of the available medicine. In this mini-review, we have summarized the therapeutic options to manage various symptoms of liver cirrhosis, carefully considering the physiological changes of various organs associated with aging.

Key words: Liver cirrhosis; Elderly patients; Quality of life; Aging

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The increasing aging population is a worldwide issue and societies are facing various problems including long-term care for populations with a high prevalence of chronic conditions including the liver diseases. It is clear that management of hepatitis and cirrhosis is important to maintain hepatic function as well as activities of daily living and quality of life of liver disease patients. While various therapeutic options have been

Received: February 25, 2019**Peer-review started:** February 25, 2019**First decision:** March 5, 2019**Revised:** March 12, 2019**Accepted:** March 16, 2019**Article in press:** March 16, 2019**Published online:** April 21, 2019**P-Reviewer:** Abdel Razek AAK, Garbuzenko DV, Ruiz-Margáin A**S-Editor:** Ma RY**L-Editor:** A**E-Editor:** Ma YJ

available including newly developed medicines and techniques, the aging-related parameters must be considered in the decision making for therapeutic strategies and dosage of the available medicine. However, as there is no information available to date in this field, to fill this gap, we have summarized the points that must be considered upon the management of liver cirrhosis in elderly patients. The information summarized will be helpful for physicians treating the elderly patients with the understanding of the aging-related changes of the body and organs.

Citation: Kamimura K, Sakamaki A, Kamimura H, Setsu T, Yokoo T, Takamura M, Terai S. Considerations of elderly factors to manage the complication of liver cirrhosis in elderly patients. *World J Gastroenterol* 2019; 25(15): 1817-1827

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1817.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1817>

INTRODUCTION

The increasing aging population is a worldwide issue^[1], and societies are facing various problems including long-term care for populations with a high prevalence of chronic conditions^[2]. Previous studies have demonstrated that aging well requires activities of daily living^[3] and appropriate care for elderly patients^[4,5]. Therefore, these crucial factors need to be considered to understand the features of physiological changes when considering therapeutic options for chronic diseases^[5-7] due to the impact of aging on cellular function, size and function of organs, and vascular cognitive impairment and dementia^[8].

Among the various chronic conditions seen in elderly people, we have focused on liver cirrhosis, as liver disease is a major issue as its symptoms vary and there are various considerations involved in the treatment of these patients. The general management guidelines have been published for the any age^[9], however, due to the physiological changes in the organs and body, special considerations are essential for the elderly patients.

Morphological and functional aging of the liver includes reduced liver weight and blood flow in the liver^[10,11] due to decreased cardiac output^[12], decreased number of hepatocytes^[13], loss of metabolic function, and loss of detoxification in the liver. Ginsberg *et al.* reported that the total volume of cytochrome p450 decreases to 70% of its original level in people > 70 years old, resulting in decreased metabolism and detoxification in the liver^[14]. These age-related changes in pharmacokinetics are correlated with a decrease in renal function^[15] and should be considered when administering medicines to elderly patients^[16]. It was reported that growth factor expression decreases with aging; therefore, elderly patients have difficulties tolerating liver injury^[17,18]. The etiologies of liver diseases include viral hepatic diseases, autoimmune hepatic diseases, alcoholic liver diseases, and non-alcoholic steatohepatitis. Despite specific therapeutic options for many of these diseases including direct acting antivirals for hepatitis C virus, nucleoside analogs for hepatitis B, corticosteroids for autoimmune hepatitis, ursodeoxycholic acid for primary biliary cholangitis, stopping of alcohol for alcoholic hepatitis, control of body weight, vitamin E, pioglitazone, peroxisome proliferator-activator receptor agonists, farnesoid X receptor, and glucagon-like peptide for non-alcoholic steatohepatitis, there is currently no standard therapy to treat liver cirrhosis, which is the final stage of these liver diseases and may be complicated with hepatocellular carcinoma. Therefore, management of the various symptoms of liver cirrhosis is essential, and aging-related parameters must be considered in the decision making for therapeutic strategies and dosage of the available medicine. In this mini-review, we have summarized the therapeutic options to manage various symptoms of liver cirrhosis, carefully considering the physiological changes associated with aging.

Japan has the oldest population in the world and has one of the most advanced aging societies (<https://www.mhlw.go.jp/english/database/db-hw/lifetb16/index.html>), with 35.215 million (27.8%) people aged ≥ 65 years, and more than 10 million (7.9%) people over 80. One in 10 women in the country is over 81, and the number of people > 65 years is also growing (<https://www.stat.go.jp/english/data/kokusei/2015/summary.html>). Under these circumstances, defining the elderly as those aged ≥ 65 years does not seem appropriate, and the distinction of categories of old, old-old, and oldest

old is under discussion worldwide^[1]. In Japan, there are 5.5 million elderly people aged ≥ 85 years, accounting for just under 5% of the total population (<https://www.mhlw.go.jp/english/database/db-hw/lifetb16/index.html>), and the opportunity to provide medical treatment for people in very old age is rapidly increasing.

It is clear that management of hepatitis and cirrhosis is important to maintain hepatic function as well as activities of daily living and quality of life (QoL) of patients. While direct acting antiviral drugs have made it possible to control chronic hepatitis C infection, it is important to understand the changing characteristics of diseases in the elderly, as well as continuously increasing lifestyle diseases and associated non-alcoholic steatohepatitis that leads to liver cirrhosis and liver cancer, in order to fully understand the disease concept and select the appropriate treatment methods. Furthermore, various complications of liver cirrhosis, such as liver cancer and gastrointestinal tract bleeding, affect QoL in the elderly. As there have been no literatures focusing on these problems, we have summarized the main points that must be considered in the management of current liver cirrhosis treatment in elderly patients.

LIVER CIRRHOSIS IN ELDERLY PATIENTS

It is clear that the removal of etiological factors, if possible, is the most important part to prevent the progression of liver cirrhosis, particularly alcohol consumption, and control of viral hepatitis as stated in the guidelines^[9]. It is also true for elderly patients while the careful consideration of renal function is essential using medications. And various complications in liver cirrhosis patients including ascites, hepatic encephalopathy, gastrointestinal tract bleeding from varices, and skin symptoms could be severer in elderly patients and the diagnosis and treatments of hepatocellular carcinoma, which is the final stage of the chronic liver disease need careful consideration as it affects the both renal and hepatic functions. In addition, sarcopenia is a condition, which the liver cirrhosis patients could be easily suffered and therefore, the management of the condition is necessary to continue the treatment. Therefore, with the increase of elderly populations, the understanding of the pathophysiological changes in this population is important for the physicians. For this point, we have reviewed the body changes with aging affecting these points.

Physiological changes that occur with age in the liver include a decrease in volume, blood flow, and metabolic capacity. Furthermore, liver injury by various factors such as viruses and drugs tends to be more severe (Table 1). While there are no liver diseases unique to elderly patients, it was reported that in non-alcoholic fatty liver disease, which continues to increase in the elderly, exacerbation and carcinogenesis decreased in patients aged ≥ 70 years^[19]. However, in general, falls and related disorders are common in elderly patients not only by autonomic dysfunction, cognitive impairment, muscle abnormalities, and drug intake, but also with chronic liver diseases^[20], suggesting that therapeutic intervention is necessary for patients with chronic liver diseases and cirrhosis (Table 2). Treatments for each symptom are summarized below, and the complications and management of liver cirrhosis in elderly patients need careful considerations are summarized in Table 3.

Ascites/edema

As a symptom of liver cirrhosis, accumulation of ascites above the physiological level can cause symptoms by compressing various organs, and edema can cause difficulty in walking and falls leading to the impairment of patients' social life and to the hospitalization. As it also related to the poor prognosis^[9], therapeutic intervention is necessary. Firstly, it should be diagnosed with the grading and count of neutrophil, culture, and measurement of protein concentration to exclude the possibility of bacterial peritonitis. The grading of the ascites is described as follows: Grade 1, mild ascites only detected by ultrasound examination; Grade 2, moderate ascites manifested by moderate symmetrical distension of abdomen; and Grade 3, large/gross ascites resulted in the abdominal distension. Based on the guidelines available^[9], a moderate sodium restriction (4.6-6.9 g of salt per day) is recommended for grade 2 ascites although the diets with a very low sodium content should be avoided to prevent the other complications^[9].

Especially for the elderly people tend to consume excessive amounts of salt, therefore, the mild restriction is effective. And as described in guidelines, the prolonged bed rest cannot be recommended, as for elderly patients, increased rest decreases muscle mass, treatment should be promptly being shifted to drug therapy where possible. For the medication, anti-mineralcorticoid, furosemide, torasemide,

Table 1 Morphological and functional aging of the liver

Reduced liver weight
Reduced blood flow to the liver
Loss of metabolic function
Loss of detoxification
Decreased growth factor expression

and albumin infusion can be considered with the careful follow of electrolytes. The patients with the first episode of grade 2 should be treated with anti-mineralcorticoid with stepwise increasing followed by the combination of furosemide for the patients with recurrent ascites. Torasemide can be given for the patients with poor response to furosemide, and baclofen and albumin can be considered especially for the patients with muscle cramps^[9]. With these therapies, as rapid changes in circulation dynamics and electrolyte abnormalities may cause complications in elderly patients, it is important to carefully follow up observations such as body weight, waist circumference, pulse, and blood pressure, and verify treatment effects and adverse effects by blood biochemistry and urinalysis. In addition, it is not safe to continue increasing the dose of furosemide unnecessarily because elderly patients have relatively lower serum creatinine levels that reflect their decreased muscle mass, renal function tends to decline easily, and complications of acute renal failure reduce the survival rate in patients with decompensated cirrhosis^[21].

Tolvaptan, a selective vasopressin receptor inhibitor, was recently approved and is attracting attention as a diuretic^[22,23]. It is believed to be useful even in hypoalbuminemia, and its use will be recommended for renal protection in elderly patients^[15,23]. On the other hand, the use of these diuretics readily causes changes in electrolytes in addition to circulation dynamics; therefore, physicians need to consider the age-related changes in the cardiac system of elderly patients^[24]. It is also advisable to decide the treatment methods under inpatient management based on blood and urine measurements and in conjunction with the appropriate specialists.

Other therapeutic options include large volume paracentesis, non-selective beta-blockers, and transjugular intrahepatic portosystemic shunts can be considered for the patients with refractory ascites^[9], however, as these intervention may affect the cardiovascular conditions, careful consideration should be given to the elderly patients. It is clear further clinical information and trials are necessary to develop the safer procedure of medication for the elderly patients.

Hepatic encephalopathy

Hepatic encephalopathy associated with liver cirrhosis is a general term for neuropsychiatric symptoms mainly consisting of disturbance of consciousness, which appear due to severe acute or chronic liver failure. Therefore, in elderly patients, it is necessary to differentiate this from dementia and cerebrovascular diseases, which may prove difficult from examination findings and requires comprehensive evaluation including measurement of blood ammonia levels and the presence of a shunt. However, as the increased level of ammonia in the elderly patients can also be seen in the uremia due to renal failure, which can present like hepatic encephalopathy. It was recently proposed that astrocyte senescence may be related to persistent cognitive impairment in hepatic encephalopathy^[25] and Soriano *et al*^[26] reported the effectiveness of psychometric hepatic encephalopathy score which could help identifying the patients with a risk for falls. These findings highlight the importance of working closely with neurologists. Further, as the bowel movement abnormality is a typical trigger of hepatic encephalopathy that often occurs in elderly patients, the other chemicals including byproducts of bacterial metabolisms from the gut may play an important role in this condition which may be related to age-related changes in gut function and gut microbiota^[27,28] leading to the gut-brain axis alterations. Optimization of bowel movement and suppression of ammonia production should be achieved using laxatives, branched-chain amino acids, and synthetic disaccharide lactulose. However, these chemicals especially lactulose should be administered to the elderly patients with caution as it could induce the diarrhea and dehydration in the cases. Rifaximin, a poorly absorbed antimicrobial agent, is used to treat hyperammonemia in hepatic encephalopathy^[29,30]. Rifaximin has few systemic adverse effects and is considered to have little impact on renal function due to its poor absorbability; therefore, it may be a therapeutic option for the treatment of hepatic encephalopathy in elderly patients. Furthermore, recent reports showed that rifaximin reduced mortality^[29] and did not affect glomerular filtration rate or levels of

Table 2 Conditions to consider when determining therapeutic options

Change	Risk
Decreased cardiac function	Use of endoscopic therapy Use of beta-blockers Increase in blood pressure caused by molecular targeted agent
Decreased renal function	Use of contrast medium Use of interventional radiology for hepatocellular carcinoma Rapid change in circulation dynamics
Decreased respiratory function	Aspiration pneumonia
Ease of electrolyte abnormalities	Higher risk of using diuretics
Dementia	Difficulty in differentiating hepatic encephalopathy from dementia and cerebrovascular diseases
Aging of gut microbiota	Hepatic encephalopathy
Loss of bone density	Osteoporosis Risk of fall-related injury
Loss of muscle volume	Sarcopenia Risk of fall-related injury
Dry skin	Pruritus Hand-foot syndrome caused by molecular targeted agent

vasoactive hormones^[30]. In addition, it was recently reported that rifaximin ameliorates hepatic encephalopathy and endotoxemia without changing gut microbiome diversity, implying its potential use in elderly patients^[31]. On the other hand, it is important to be aware that oral administration compliance may be lowered due to frequent diarrhea that causes electrolyte abnormalities and skin troubles from frequent defecation. Treatment for encephalopathy involves an intravenous drip infusion of Fischer solution; however, careful management is necessary because this could easily increase cardiac stress and fluctuation of electrolytes in elderly patients.

Gastrointestinal bleeding

Gastrointestinal bleeding occurs due to gastro-esophageal varices or to non-variceal lesions including portal hypertensive gastropathy and intestinopathy. The acute gastrointestinal bleeding may lead to the high risk of mortality in decompensated cirrhosis^[9] and elderly patients, therefore the therapeutic intervention is essential. In addition, it should particularly be emphasized that patients with decompensated liver cirrhosis should have esophagogastroduodenoscopy performed to screen for the lesions and if the risky varices with red wale marking were found, therapeutic intervention should also be considered including the administration of non-selective beta-blocker. Patients with medium-sized varices should be treated with NSBB or endoscopic band ligation (EBL)^[9]. It is however, obvious that the administration of beta-blocker should be more careful for the elderly patients as it may cause, arrhythmia and fluctuation of blood pressure which may lead to the cardiac failure. Therefore, referring to the guidelines for the patients with hypotension and intercurrent conditions for beta-blockers, elderly patients with high risk using beta-blockers can be considered for management by expeditious EBL. Focusing on the elderly patients, there are only a few reports on treatments of esophageal and gastric varices in elderly patients; however, this conventional method should be used as an emergency response to ruptures to control the bleeding and manage the general condition. The development of endoscopic techniques has highlighted the usefulness of endoscopic procedures such as EBL and injection sclerotherapy (EIS) in the management of esophageal varices^[32]. Furthermore, recent developments in this field have facilitated this procedure and may contribute to improving the control of bleeding from varices^[33]. However, due to the general risks in elderly patients during endoscopic examination^[34], careful preparation and monitoring of vital signs are necessary, even during elective EBL and EIS. Furthermore, treatment-related complications, such as aspiration pneumonia associated with sedation and delirium caused by inpatient management and fasting, required careful attention in elderly patients. Complications such as myocardial ischemia may often occur in elderly patients due to blood pressure fluctuations and tachycardia due to stress undergoing upper gastrointestinal endoscopic examination, and sedation may also cause a sudden drop in blood pressure^[35]. Appropriate risk management involves carefully explaining these issues to family members prior to treatment, and taking account of old age in

Table 3 The complications and management of liver cirrhosis in elderly patients need careful considerations

Complications	Management (general)	Considerations should be given in elderly patients
Ascites	Sodium restrictions Anti-mineral corticoid Furosemide Torsemide Albumin infusion	Electrolyte abnormalities Changes in circulation dynamics Body weight Pulse and blood pressure Verification of blood biochemistry and urinalysis
Hepatic encephalopathy	Optimization of bowel movement Laxatives Branched-chain amino acids Synthetic disaccharide lactulose Rifaximin Intravenous drip infusion of Fischer solution	Diarrhea Frequent diarrhea that causes electrolyte abnormalities Skin troubles from frequent defecation Dehydration Cardiac stress and fluctuation of electrolytes
Gastrointestinal bleeding/varices	Non-selective beta-blockers	Arrhythmia Fluctuation of blood pressure Cardiac failure
Sarcopenia	Endoscopic therapy Risk of fall-related injury	Aspiration pneumonia Nutritional monitoring (serum markers including the albumin, cholesterol level) Muscle volume Administration of branched-chain amino acid preparations
Skin symptoms	Skin moisturisers Bile salts Rifampicin Anti-histamines	Likely to have dry skin Frequently suffer from wound infections and persistent skin inflammation Nalfurafine hydrochloride
Hepatocellular carcinoma	Use of phase contrast for the diagnosis Surgical therapy Transarterial chemotherapy Needle guided local therapy Molecular targeted therapies	Renal function Cardiac function Bone marrow function History of cerebral bleeding, Hypertension Renal function Cardiac function
Cirrhotic cardiomyopathy		Sarcopenia
Spontaneous bacterial peritonitis		
Hepato-renal syndrome		
Acute and chronic kidney injury		
Hyponatremia		Further clinical trials and information from retrospective studies are necessary

addition to disease condition, treatment methods, and general complications. In a report of treatments for esophageal and gastric varices in elderly patients aged ≥ 85 years, Nakamura *et al.* stated that preventive treatment should be limited to those at high risk of bleeding^[36]. Endoscopically large hematocystic spots and white fibrin plugs are considered to be indications for preventive treatment. For other therapies, a balloon-occluded retrograde variceal sclerotherapy for gastric varices and a transjugular intrahepatic portosystemic, it is necessary to consider the risk of renal injury in elderly cases^[15,21] due to sclerosant and contrast agents.

Careful use of beta-blockers could prevent rebleeding and even first bleeding of varices^[37]; therefore, medication may also be considered for managing and preventing the progression of the varices in elderly cases. However, beta-blockers should be introduced with caution as they may increase the risk of bradycardia, development and exacerbation of heart failure, and syncope due to atrioventricular block in elderly patients with low cardiac function. Widespread use of capsule endoscopy may also be used to detect small-bowel varices; however, some studies have reported no

advantage of this technique compared with regular endoscopy^[38], and endoscopic examination and treatment is currently considered to be the optimal treatment regimen.

Sarcopenia

It is presumed that protein synthesis and degradation are not well balanced in elderly patients^[7], which increases risk of fall-related injury. Loss of bone density and muscle volume, known as osteoporosis and sarcopenia, respectively, significantly affect the general condition of elderly patients^[39-41]. Primary sarcopenia is a decrease in muscle mass associated with aging, while secondary sarcopenia is a decrease in muscle mass and muscle strength associated with underlying diseases such as liver diseases, malignant tumors, and inflammatory diseases^[42]. It has long been known that skeletal muscle mass tends to be decreased in patients with chronic liver diseases and that complications such as falls and bone fractures are common^[20]. Therefore, understanding the concept and pathophysiology of sarcopenia and focusing on its prevention are important in elderly patients with liver diseases. Patients with liver diseases are more likely to have sarcopenia compared with those of the same age due to the involvement of protein energy malnutrition, an imbalance between protein synthesis and degradation, increased levels of myostatin that inhibits myogenesis of skeletal muscles, and increased levels of inflammatory cytokines and reactive oxygen species^[20]. In patients with liver cirrhosis, skeletal muscle atrophy progresses due to reduced glycogen storage in the liver that promotes amino acid and glycogen supply from skeletal muscles, including branched-chain amino acids^[20]. Therefore, administration of branched-chain amino acid preparations, which have a strong anabolic action among essential amino acids, is considered useful to inhibit the progression of this disease state. In addition, the serum markers including the albumin, cholesterol level, and muscle volume are useful for nutritional monitoring.

Skin symptoms

Patients with liver diseases exhibit jaundice as well as various other skin symptoms such as telangiectasia, purpura, and pruritus, among which pruritus interferes with sleep, causing deterioration of rhythm and QoL. In addition, chronic pruritus is a general and major skin problem in elderly patients which is caused by abnormalities of the epidermal barrier, immune system, and nervous system^[43]. The causes of pruritus include both histaminergic (peripheral) and non-histaminergic (central nervous system) mechanisms. The latter is mainly due to cholestasis and scratches of the skin induce a peripheral type of itching and worsen the symptoms, forming a vicious cycle.

As elderly patients are likely to have dry skin, they frequently suffer from wound infections and persistent skin inflammation. Generally, skin moisturisers, bile salts, rifampicin, and anti-histamines have been used to reduce pruritus. In addition to these traditional medicines, nalfurafine hydrochloride has been approved in Japan although not approved in Europe currently. Nalfurafine hydrochloride, which was approved for its use for pruritus in liver diseases, is a κ -receptor agonist that suppresses itching via central mechanisms and is expected to control skin symptoms as extrahepatic lesions of patients with liver diseases^[44]. Its clinical effects were confirmed in various chronic liver disease patients including those with primary biliary cholangitis, for which pruritus is the major symptom and could affect QoL^[45]. We recently reported that administration of nalfurafine hydrochloride in 18 cases of liver disease patients with a median age of 69 years (45-82 years) led to alleviation of symptoms for long periods of time. During the observation period up of to 41 wk, there were no systemic adverse effects such as deterioration of liver function; however, four cases were excluded due to discontinued administration due to oral dryness, progression of anemia (the causal relationship with the drug administration was unknown), progression of liver disease, and financial reasons^[45]. Control of extrahepatic lesions including the skin is an important issue in the treatment of cirrhosis from the viewpoint of QoL and maintaining the reserve capacity of the liver.

Hepatocellular carcinoma

Surveillance of hepatocarcinogenesis in patients with liver cirrhosis involves screening by ultrasound and imaging findings using dynamic computed tomography and magnetic resonance imaging, particularly that using gadolinium-ethoxybenzyl diethylemetriaminepentaacetic acid. However, in elderly patients with decreased renal^[15,21] and cardiac^[24] function, more personalized regimens for individual patients are required, such as frequency of examination according to the situation of the patient based on previous medical history and performance status and estimation using measurements of tumor markers.

Regarding treatment, consideration of general conditions such as cardiac output,

respiratory function, renal function, and hepatic function is essential for elderly patients; however, if these parameters can tolerate invasion, surgical treatment including the transplantation should be considered^[12,46,47]. While the liver itself ages in terms of size, blood flow^[9,10], and capacity for regeneration after hepatectomy^[17,18], the appropriate post-procedure management could lead to improved QoL and patient survival.

Understanding the disease stages, such as reserve capacity, extrahepatic metastasis, vascular invasion, tumor number, and tumor diameter is essential when determining treatment options. The options other than the surgical treatment include the traditional chemoembolization, transarterial chemotherapy, and needle guided local therapy of radiofrequency ablation, ethanol injection, microwave ablation, and thermal therapy. For these therapeutic options, the understanding of the physiological changes in the elderly patients especially for the cardiac function^[24] and renal function^[15,21] is essential as these organs are easily injured upon the chemotherapy and embolization.

Recently, molecular targeted therapies such as sorafenib, regorafenib, and lenvatinib have become available for the treatment of hepatocellular carcinoma. However, it is difficult to use these therapies in patients with poor hepatic reserve capacity or elderly patients with heart problems or a history of cerebral bleeding, as they could cause severe adverse events including hand-foot syndrome, hypertension, anorexia, or diarrhea. Therefore, maintaining a good reserve capacity, even in elderly patients, is necessary to increase the available treatment options. It is also important to preserve many treatment options in cases of liver cancer to optimize recovery after treatment and maintain QoL.

Others

Other various complications including the bacterial infections, renal impairment, cardiopulmonary complications should also be paid attentions as they could cause the severe damage on the patients' conditions especially for the weaker cases such as elderly patients. The conditions may be introduced include spontaneous bacterial peritonitis, acute, chronic kidney injury, cirrhotic cardiomyopathy, hepato-renal syndrome, and hyponatremia. As a feature of elderly liver cirrhotic patients, it may also be related to the structural and functional changes of organs^[15,21,24] and conditions of gut microbiota^[27,28]. To avoid to be in the severe conditions, for the bacterial infections, empirical antibiotic therapy should be started with caring the drug induced hepato-, and renal injuries^[9]. Therefore, for the elderly patients, careful follow up of laboratory examinations should be given. For renal impairment, early diagnosis of the acute and chronic kidney disease and acute kidney injury is essential, as even a mild increase of serum creatinine may underlie a marked decrease of glomerular filtration^[9]. For cardiomyopathy, although the data relating cardiac function with survival is variable, however, as diastolic dysfunction and mortality seems to be related, cardiac functions should be carefully monitored in the elderly patients with potentially lower functions^[24].

The dysfunction of immune system caused by immunosenescence due to the toxic protein aggregation^[48] and autophagy^[49] leading to the organ dysfunction, carcinogenesis, and neurodegeneration can be the next target of anti-aging therapy in the hepatology field. Further basic researches are essential for the clarification of the association to develop the management strategies. For the patients with severe liver cirrhosis and liver cancers satisfying the criteria, the liver transplantation is one of the therapeutic options even for the elderly patients; however, the situation is different between the countries and correct understanding of the conditions of aging of the organs and physiological changes are essential considering the candidates for the transplantation (Figure 1).

CONCLUSION

The increase in the number of elderly patients has prompted an increase in the opportunity to treat elderly patient and development of medical technology to treat these patients. In the treatment of liver cirrhosis associated with non-alcoholic fatty liver disease, which is predicted to increase in the future, circulatory dynamics, organ function, and immune function are deteriorated in elderly patients, and it is essential to recognize that careful observation of progress is required when implementing the same treatment as would be used in young patients. While assessing electrolyte balance and physiological function is important to avoid treatment-related complications, careful treatment tailored to the individual condition of each patients should be implemented, observing the general condition and preventing deteriorating

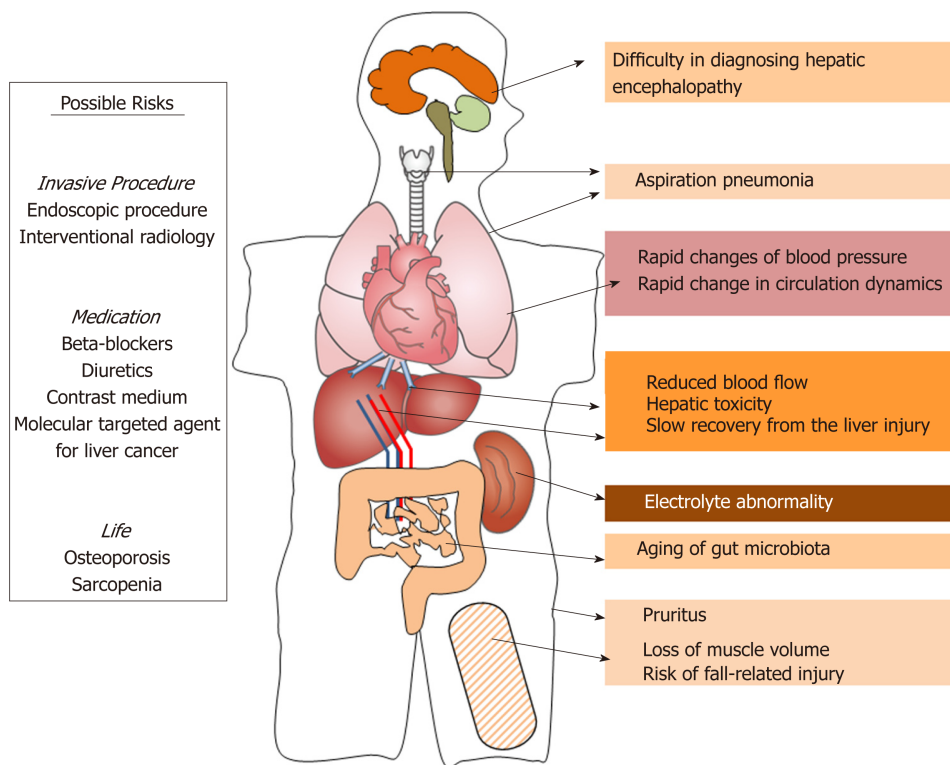


Figure 1 Summary of the changes of the body and possible risks treating the complication of the liver cirrhosis.

liver cirrhosis and maintaining the reserve capacity of the liver. To this end, treatment should be implemented by a medical team consisting of multiple specialists including nurses, pharmacists, nutritionists, and social workers in addition to doctors. As there have been no reviews focusing on these points, this mini-review should help to maintain QoL in patients, and further prolong life expectancy worldwide.

REFERENCES

- 1 **Cohen-Mansfield J**, Shmotkin D, Blumstein Z, Shorek A, Eyal N, Hazan H; CALAS Team. The old, old-old, and the oldest old: continuation or distinct categories? An examination of the relationship between age and changes in health, function, and wellbeing. *Int J Aging Hum Dev* 2013; **77**: 37-57 [PMID: [23986979](#) DOI: [10.2190/AG.77.1.c](#)]
- 2 **Steinbeisser K**, Grill E, Holle R, Peters A, Seidl H. Determinants for utilization and transitions of long-term care in adults 65+ in Germany: results from the longitudinal KORA-Age study. *BMC Geriatr* 2018; **18**: 172 [PMID: [30064373](#) DOI: [10.1186/s12877-018-0860-x](#)]
- 3 **Bravell ME**, Berg S, Malmberg B. Health, functional capacity, formal care, and survival in the oldest old: a longitudinal study. *Arch Gerontol Geriatr* 2008; **46**: 1-14 [PMID: [17368828](#) DOI: [10.1016/j.archger.2007.02.003](#)]
- 4 **Geerts J**, Van den Bosch K. Transitions in formal and informal care utilisation amongst older Europeans: the impact of national contexts. *Eur J Ageing* 2011; **9**: 27-37 [PMID: [17368828](#) DOI: [10.1016/j.archger.2007.02.003](#)]
- 5 **Halaweh H**, Dahlin-Ivanoff S, Svantesson U, Willén C. Perspectives of Older Adults on Aging Well: A Focus Group Study. *J Aging Res* 2018; **2018**: 9858252 [PMID: [30533224](#) DOI: [10.1155/2018/9858252](#)]
- 6 **Deeg DJH**, Wahl HW, Litwin H. Ageing and transitions: looking back and looking forward. *Eur J Ageing* 2018; **15**: 1-3 [PMID: [29531509](#) DOI: [10.1007/s10433-018-0463-6](#)]
- 7 **Nigam Y**, Knight J, Bhattacharya S, Bayer A. Physiological changes associated with aging and immobility. *J Aging Res* 2012; **2012**: 468469 [PMID: [22619717](#) DOI: [10.1155/2012/468469](#)]
- 8 **Yang T**, Sun Y, Lu Z, Leak RK, Zhang F. The impact of cerebrovascular aging on vascular cognitive impairment and dementia. *Ageing Res Rev* 2017; **34**: 15-29 [PMID: [27693240](#) DOI: [10.1016/j.arr.2016.09.007](#)]
- 9 **European Association for the Study of the Liver; European Association for the Study of the Liver**. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. *J Hepatol* 2018; **69**: 406-460 [PMID: [29653741](#) DOI: [10.1016/j.jhep.2018.03.024](#)]
- 10 **Wynne HA**, Cope LH, Mutch E, Rawlins MD, Woodhouse KW, James OF. The effect of age upon liver volume and apparent liver blood flow in healthy man. *Hepatology* 1989; **9**: 297-301 [PMID: [2643548](#)]
- 11 **Tajiri K**, Shimizu Y. Liver physiology and liver diseases in the elderly. *World J Gastroenterol* 2013; **19**: 8459-8467 [PMID: [24379563](#) DOI: [10.3748/wjg.v19.i46.8459](#)]
- 12 **Preston SD**, Southall AR, Nel M, Das SK. Geriatric surgery is about disease, not age. *J R Soc Med* 2008; **101**: 409-415 [PMID: [18687864](#) DOI: [10.1258/jrsm.2008.080035](#)]
- 13 **Okudaira M**, Ikawa N, Yasuhara M, Kumagai T, Kurosu K. Liver weight of adult Japanese, especially recent weight values. *Hepatol Res* 2000; **18**: 95-103 [PMID: [10936560](#)]

- 14 **Khan MS**, Roberts MS. Challenges and innovations of drug delivery in older age. *Adv Drug Deliv Rev* 2018; **135**: 3-38 [PMID: [30217519](#) DOI: [10.1016/j.addr.2018.09.003](#)]
- 15 **Hommos MS**, Glasscock RJ, Rule AD. Structural and Functional Changes in Human Kidneys with Healthy Aging. *J Am Soc Nephrol* 2017; **28**: 2838-2844 [PMID: [28790143](#) DOI: [10.1681/ASN.2017040421](#)]
- 16 **Nightingale G**, Schwartz R, Kachur E, Dixon BN, Cote C, Barlow A, Barlow B, Medina P. Clinical pharmacology of oncology agents in older adults: A comprehensive review of how chronologic and functional age can influence treatment-related effects. *J Geriatr Oncol* 2019; **10**: 4-30 [PMID: [30017734](#) DOI: [10.1016/j.jgo.2018.06.008](#)]
- 17 **Schmucker DL**. Aging and the liver: an update. *J Gerontol A Biol Sci Med Sci* 1998; **53**: B315-B320 [PMID: [9754128](#)]
- 18 **Schmucker DL**. Age-related changes in liver structure and function: Implications for disease? *Exp Gerontol* 2005; **40**: 650-659 [PMID: [16102930](#) DOI: [10.1016/j.exger.2005.06.009](#)]
- 19 **Sheedfar F**, Di Biase S, Koonen D, Vinciguerra M. Liver diseases and aging: friends or foes? *Aging Cell* 2013; **12**: 950-954 [PMID: [23815295](#) DOI: [10.1111/acer.12128](#)]
- 20 **Frith J**, Kerr S, Robinson L, Elliott CS, Wilton K, Jones DE, Day CP, Newton JL. Falls and fall-related injury are common in older people with chronic liver disease. *Dig Dis Sci* 2012; **57**: 2697-2702 [PMID: [22576710](#) DOI: [10.1007/s10620-012-2193-5](#)]
- 21 **Tsien CD**, Rabie R, Wong F. Acute kidney injury in decompensated cirrhosis. *Gut* 2013; **62**: 131-137 [PMID: [22637695](#) DOI: [10.1136/gutjnl-2011-301255](#)]
- 22 **Okita K**, Kawazoe S, Hasebe C, Kajimura K, Kaneko A, Okada M, Sakaida I; ASCITES Dose-Finding Trial Group. Dose-finding trial of tolvaptan in liver cirrhosis patients with hepatic edema: A randomized, double-blind, placebo-controlled trial. *Hepatol Res* 2014; **44**: 83-91 [PMID: [23530991](#) DOI: [10.1111/hepr.12099](#)]
- 23 **Sakaida I**, Kawazoe S, Kajimura K, Saito T, Okuse C, Takaguchi K, Okada M, Okita K; ASCITES-DOUBLEBLIND Study Group. Tolvaptan for improvement of hepatic edema: A phase 3, multicenter, randomized, double-blind, placebo-controlled trial. *Hepatol Res* 2014; **44**: 73-82 [PMID: [23551935](#) DOI: [10.1111/hepr.12098](#)]
- 24 **Moodithaya S**, Avadhany ST. Gender differences in age-related changes in cardiac autonomic nervous function. *J Aging Res* 2012; **2012**: 679345 [PMID: [22187649](#) DOI: [10.1155/2012/679345](#)]
- 25 **Görg B**, Karababa A, Häussinger D. Hepatic Encephalopathy and Astrocyte Senescence. *J Clin Exp Hepatol* 2018; **8**: 294-300 [PMID: [30302047](#) DOI: [10.1016/j.jceh.2018.05.003](#)]
- 26 **Soriano G**, Román E, Córdoba J, Torrens M, Poca M, Torras X, Villanueva C, Gich IJ, Vargas V, Guarner C. Cognitive dysfunction in cirrhosis is associated with falls: a prospective study. *Hepatology* 2012; **55**: 1922-1930 [PMID: [22213000](#) DOI: [10.1002/hep.25554](#)]
- 27 **O'Toole PW**, Jeffery IB. Gut microbiota and aging. *Science* 2015; **350**: 1214-1215 [PMID: [26785481](#) DOI: [10.1126/science.aac8469](#)]
- 28 **Britton E**, McLaughlin JT. Ageing and the gut. *Proc Nutr Soc* 2013; **72**: 173-177 [PMID: [23146206](#) DOI: [10.1017/S0029665112002807](#)]
- 29 **Kimer N**, Krag A, Möller S, Bendtsen F, Gluud LL. Systematic review with meta-analysis: the effects of rifaximin in hepatic encephalopathy. *Aliment Pharmacol Ther* 2014; **40**: 123-132 [PMID: [24849268](#) DOI: [10.1111/apt.12803](#)]
- 30 **Kimer N**, Pedersen JS, Busk TM, Gluud LL, Hobolth L, Krag A, Möller S, Bendtsen F; Copenhagen Rifaximin (CoRif) Study Group. Rifaximin has no effect on hemodynamics in decompensated cirrhosis: A randomized, double-blind, placebo-controlled trial. *Hepatology* 2017; **65**: 592-603 [PMID: [27775818](#) DOI: [10.1002/hep.28898](#)]
- 31 **Kaji K**, Takaya H, Saikawa S, Furukawa M, Sato S, Kawaratani H, Kitade M, Moriya K, Namisaki T, Akahane T, Mito A, Yoshiji H. Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity. *World J Gastroenterol* 2017; **23**: 8355-8366 [PMID: [29307995](#) DOI: [10.3748/wjg.v23.i47.8355](#)]
- 32 **Mansour L**, El-Kalla F, El-Bassat H, Abd-Elsalam S, El-Bedewy M, Kobtan A, Badawi R, Elhendawy M. Randomized controlled trial of scleroligation versus band ligation alone for eradication of gastroesophageal varices. *Gastrointest Endosc* 2017; **86**: 307-315 [PMID: [28082116](#) DOI: [10.1016/j.gie.2016.12.026](#)]
- 33 **Ibrahim M**, Mostafa I, Devière J. New Developments in Managing Variceal Bleeding. *Gastroenterology* 2018; **154**: 1964-1969 [PMID: [29481777](#) DOI: [10.1053/j.gastro.2018.02.023](#)]
- 34 **Razavi F**, Gross S, Katz S. Endoscopy in the elderly: risks, benefits, and yield of common endoscopic procedures. *Clin Geriatr Med* 2014; **30**: 133-147 [PMID: [24267608](#) DOI: [10.1016/j.cger.2013.10.010](#)]
- 35 **Arakawa H**, Koyama S, Adachi S, Tsukinaga S, Kajiwarra M, Uchiyama K, Koido S, Ohkusa T, Tajiri H, Suzuki H. Digestive diseases of the aged over 85 -the characteristics and therapeutic points: conscious sedation and preparation for the elderly in the endoscopic procedure. *Clin Gastroenterol* 2015; **30**: 413-419
- 36 **Nakamura S**, Haruyama H, Mizuno K, Kishino M, Konishi H, Shiratori K. Endoscopic treatment and management of esophagogastric varices in very elderly patients. *Endoscopia Digestiva* 2008; **20**: 1651-1658
- 37 **Kerbert AJ**, Chiang FW, van der Werf M, Stijnen T, Slingerland H, Verspaget HW, van Hoek B, Coenraad MJ. Hemodynamic response to primary prophylactic therapy with nonselective β -blockers is related to a reduction of first variceal bleeding risk in liver cirrhosis: a meta-analysis. *Eur J Gastroenterol Hepatol* 2017; **29**: 380-387 [PMID: [28002118](#) DOI: [10.1097/MEG.0000000000000812](#)]
- 38 **Colli A**, Gana JC, Turner D, Yap J, Adams-Webber T, Ling SC, Casazza G. Capsule endoscopy for the diagnosis of oesophageal varices in people with chronic liver disease or portal vein thrombosis. *Cochrane Database Syst Rev* 2014; CD008760 [PMID: [25271409](#) DOI: [10.1002/14651858.CD008760.pub2](#)]
- 39 **Hoffman CM**, Han J, Calvi LM. Impact of aging on bone, marrow and their interactions. *Bone* 2019; **119**: 1-7 [PMID: [30010082](#) DOI: [10.1016/j.bone.2018.07.012](#)]
- 40 **Nedergaard A**, Henriksen K, Karsdal MA, Christiansen C. Musculoskeletal ageing and primary prevention. *Best Pract Res Clin Obstet Gynaecol* 2013; **27**: 673-688 [PMID: [23891483](#) DOI: [10.1016/j.bpobgyn.2013.06.001](#)]
- 41 **Ferrucci L**, Cooper R, Shardell M, Simonsick EM, Schrack JA, Kuh D. Age-Related Change in Mobility: Perspectives From Life Course Epidemiology and Geroscience. *J Gerontol A Biol Sci Med Sci* 2016; **71**: 1184-1194 [PMID: [26975983](#) DOI: [10.1093/gerona/glw043](#)]
- 42 **Cruz-Jentoft AJ**, Bahat G, Bauer J, Boirie Y, Bruyère O, Cederholm T, Cooper C, Landi F, Rolland Y, Sayer AA, Schneider SM, Sieber CC, Topinkova E, Vandewoude M, Visser M, Zamboni M; Writing Group for the European Working Group on Sarcopenia in Older People 2 (EWGSOP2), and the Extended

- Group for EWGSOP2. Sarcopenia: revised European consensus on definition and diagnosis. *Age Ageing* 2019; **48**: 16-31 [PMID: [30312372](#) DOI: [10.1093/ageing/afy169](#)]
- 43 **Cao T**, Tey HL, Yosipovitch G. Chronic Pruritus in the Geriatric Population. *Dermatol Clin* 2018; **36**: 199-211 [PMID: [29929593](#) DOI: [10.1016/j.det.2018.02.004](#)]
- 44 **Kumada H**, Miyakawa H, Muramatsu T, Ando N, Oh T, Takamori K, Nakamoto H. Efficacy of nalfurafine hydrochloride in patients with chronic liver disease with refractory pruritus: A randomized, double-blind trial. *Hepatol Res* 2017; **47**: 972-982 [PMID: [27753159](#) DOI: [10.1111/hepr.12830](#)]
- 45 **Yagi M**, Tanaka A, Namisaki T, Takahashi A, Abe M, Honda A, Matsuzaki Y, Ohira H, Yoshiji H, Takikawa H; Japan PBC Study Group (JPBCSG). Is patient-reported outcome improved by nalfurafine hydrochloride in patients with primary biliary cholangitis and refractory pruritus? A post-marketing, single-arm, prospective study. *J Gastroenterol* 2018; **53**: 1151-1158 [PMID: [29663077](#) DOI: [10.1007/s00535-018-1465-z](#)]
- 46 **Kamimura K**, Yokoo T, Kamimura H, Sakamaki A, Abe S, Tsuchiya A, Takamura M, Kawai H, Yamagiwa S, Terai S. Long-term efficacy and safety of nalfurafine hydrochloride on pruritus in chronic liver disease patients: Patient-reported outcome based analyses. *PLoS One* 2017; **12**: e0178991 [PMID: [28604788](#) DOI: [10.1371/journal.pone.0178991](#)]
- 47 **Suda T**, Nagashima A, Takahashi S, Kanefuji T, Kamimura K, Tamura Y, Takamura M, Igarashi M, Kawai H, Yamagiwa S, Nomoto M, Aoyagi Y. Active treatments are a rational approach for hepatocellular carcinoma in elderly patients. *World J Gastroenterol* 2013; **19**: 3831-3840 [PMID: [23840122](#) DOI: [10.3748/wjg.v19.i24.3831](#)]
- 48 **Martins IJ**. Heat Shock Gene Inactivation and Protein Aggregation with Links to Chronic Diseases. *Diseases* 2018; **6** [PMID: [29783682](#) DOI: [10.3390/diseases6020039](#)]
- 49 **Batatinha HAP**, Diniz TA, de Souza Teixeira AA, Krüger K, Rosa-Neto JC. Regulation of autophagy as a therapy for immunosenescence-driven cancer and neurodegenerative diseases: The role of exercise. *J Cell Physiol* 2019 [PMID: [30756377](#) DOI: [10.1002/jcp.28318](#)]



Basic Study

Lysyl oxidase and hypoxia-inducible factor 1 α : biomarkers of gastric cancer

Ya-Lin Han, Li Chen, Rui Qin, Guan-Qing Wang, Xiao-Hua Lin, Guang-Hai Dai

ORCID number: Ya-lin Han (0000-0003-4552-1486); Li Chen (0000-0002-9671-7967); Rui Qin (0000-0001-6033-4393); Guan-Qing Wang (0000-0002-2269-5881); Xiao-Hua Lin (0000-0002-4494-8480); Guang-Hai Dai (0000-0001-7582-9348).

Author contributions: Dai GH and Chen L designed and provided ideas for the project; Han YL wrote and edited the manuscript; Han YL, Chen L and Qin R performed immunohistochemical staining; Han YL analyzed all the data; Han YL and Lin XH conducted RNA separation, cDNA synthesis and qRT-PCR analysis; Han YL, Qin R and Wang GQ collected clinical samples from our hospital.

Supported by the grants from the Military Medical Science and Technology Youth Training Program Project (16QNP146).

Institutional review board statement: This study was conducted with permission from the Institutional Research Ethics Committee of Chinese PLA General Hospital.

Institutional animal care and use committee statement: This study did not include the animal experiments.

Conflict-of-interest statement: The authors declare no potential conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: This study did not include the animal

Ya-Lin Han, Li Chen, Rui Qin, Guan-Qing Wang, Guang-Hai Dai, Department of Medical Oncology, Chinese PLA General Hospital, Beijing 100853, China

Xiao-Hua Lin, Department of Oncology, the General Hospital of PLA Rocket Force, Beijing 100088, China

Corresponding author: Guang-Hai Dai, PhD, Professor, Department of Medical Oncology, Chinese PLA General Hospital, 28 Fuxing Road, Haidian District, Beijing 100853, China. handai713@126.com

Telephone: +86-10-66937231

Abstract

BACKGROUND

Gastric cancer (GC) is one of the main causes of cancer mortality worldwide. Recent studies on tumor microenvironments have shown that tumor metabolism exerts a vital role in cancer progression.

AIM

To investigate whether lysyl oxidase (LOX) and hypoxia-inducible factor 1 α (HIF1 α) are prognostic and predictive biomarkers in GC.

METHODS

A total of 80 tissue and blood samples were collected from 140 patients admitted to our hospital between August 2008 and March 2012. Immunohistochemical staining was performed to measure the expression of LOX and HIF1 α in tumor and adjacent tissues collected from patients with GC. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was used to detect the mRNA expression levels of LOX and HIF1 α in patients with GC. In addition, single-factor analysis was applied to analyze the relationship between LOX, HIF1 α and prognosis of GC.

RESULTS

Immunohistochemical staining suggested that the expression levels of LOX and HIF1 α increased in tumor tissues from patients with GC. QRT-PCR analysis indicated that mRNA expression of LOX and HIF1 α was also upregulated in tumor tissues, which was in accordance with the above results. We also detected expression of these two genes in blood samples. The expression level of LOX and HIF1 α was higher in patients with GC than in healthy controls. Additional analysis showed that the expression level of LOX and HIF1 α was related to the clinicopathological characteristics of GC. Expression of LOX and HIF1 α increased

experiments.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: January 19, 2019

Peer-review started: January 21, 2019

First decision: February 13, 2019

Revised: February 21, 2019

Accepted: March 1, 2019

Article in press: March 2, 2019

Published online: April 21, 2019

P-Reviewer: Czubkowski P, Morling JR, Schievenbusch S

S-Editor: Yan JP

L-Editor: Filipodia

E-Editor: Ma YJ



with the number of lymph node metastases, deeper infiltration depth and later tumor-node-metastasis stages. Single-factor analysis showed that high expression of LOX and HIF1 α led to poor prognosis of patients with GC.

CONCLUSION

LOX and HIF1 α can be used as prognostic and predictive biomarkers for GC.

Key words: Lysyl oxidase; Hypoxia-inducible factor 1 α ; Gastric cancer; Biomarker; Prognosis

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Lysyl oxidase (LOX) is a secreted extracellular matrix protein that plays an important role in remodeling the extracellular matrix and promoting tumor progression. The LOX family comprises the prototypic LOX, as well as the LOX-like proteins that are involved in carcinogenesis. Hypoxia-inducible factor 1 α (HIF1 α) is a type of transcription factor complex that has the capacity to regulate oxygen tension. In addition, HIF1 α is able to activate or bind to multiple target genes and participates in inflammatory and other diseases. HIF1 α accelerates the growth and metastasis of hepatocellular carcinoma.

Citation: Han YL, Chen L, Qin R, Wang GQ, Lin XH, Dai GH. Lysyl oxidase and hypoxia-inducible factor 1 α : biomarkers of gastric cancer. *World J Gastroenterol* 2019; 25(15): 1828-1839

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1828.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1828>

INTRODUCTION

Gastric cancer (GC) is one of the main causes of cancer mortality worldwide^[1]. Current epidemiological data indicate that the incidence rate and mortality of GC rank among the top three of all malignant tumors^[2]. The morbidity of GC rises gradually with age, and people aged 50-70 years account for the majority of cases. There is no gender differences in patients with GC, and the occurrence of GC is related to geographic variation in many countries. The clinical manifestations mainly include stomach ache, abdominal distension, loss of appetite and weight loss^[3]. GC at early stages is free of symptoms, and advanced GC or metastasis sites could be pathological diagnosed by endoscopy or endoscopic ultrasound^[4-7]. Patients who suffer from GC have a poor quality of life, and the survival rates are reported to be low^[8]. As estimated in the statistics of GLOBOCAN 2012, about 410,000 patients are newly found to suffer from the pain caused by GC in China^[9]. Among them, 330,000 patients die of tumor-related causes.

While several therapies have emerged in recent years, the effects of chemotherapy and surgery for GC remain limited^[10]. These interventions do not improve prognosis, increase survival rate or prolong survival time^[11]. In addition, there are disadvantages to current treatment methods, including inconvenience, increased prevalence of complications, and side-effects^[12]. It has been reported that many factors are involved in the progression of GC. More seriously, approximately 60% of patients with GC have metastases when they are diagnosed^[13]. Furthermore, the outcome of patients with late stage GC is poor, and most die within 1 year. Therefore, determination of predictive biomarkers for early diagnosis of GC is important.

Recent studies on tumor microenvironments have shown that tumor metabolism plays a vital role in cancer progression. The role of the microenvironment in tumor metabolism is currently attracting significant attention. Lysyl oxidase (LOX) and hypoxia-inducible factor 1 α (HIF1 α) affect the tumor microenvironment, and play a crucial role in cancer occurrence and development^[14-18]. LOX is a secreted extracellular matrix protein that plays an important role in remodeling the extracellular matrix and promoting tumor progression. The LOX family comprises the prototypic LOX, as well as LOX-like proteins that are involved in carcinogenesis. LOX is a copper-dependent monoamine oxidase, and its overexpression is related to both poor survival and the development of multiple types of cancers^[19-22]. HIF1 α is a transcription factor complex

that can regulate oxygen tension^[23]. In addition, HIF1 α can activate or bind to multiple target genes, and can contribute to inflammatory and other diseases^[22,20]. HIF1 α accelerates the growth and metastasis of hepatocellular carcinoma^[17].

The present study investigated the expression of LOX and HIF1 α in patients with GC and determined whether LOX and HIF1 α act as prognostic and predictive biomarkers of GC. Research on the interaction between cancer and factors in the metabolic microenvironment is necessary to investigate the progression of GC and determine predictive biomarkers. Our study showed that LOX and HIF1 α can act as biomarkers for the diagnosis and prediction of GC.

MATERIALS AND METHODS

Patients and samples

A total of 80 tissue and blood samples were collected from 140 patients admitted to our hospital between August 2008 and March 2012. The tumor and adjacent tissues were obtained from patients with GC. The blood samples were obtained from 80 patients with GC and 80 healthy controls. The use of human tissues in this research was agreed to by the volunteers and their relatives. All participants gave written informed consent. This study was conducted with permission from the Institutional Research Ethics Committee of our hospital. GC patients were aged > 18 years, and were diagnosed pathologically and clinically with GC. Clinicians measured and analyzed the conditions according to the Response Evaluation Criteria in Solid Tumors.

RNA extraction and cDNA synthesis

Total RNA from tissues and blood samples was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The samples were added to TRIzol for 30 min in a 4 °C refrigerator. Total RNA was extracted from samples using chloroform, isopropanol, 75% absolute ethanol (all from Beijing Shiji Tuoxin Fine Chemical Industry Co. Ltd., Beijing, China) and diethyl-pyrocarbonate-treated water (Biosharp, Heifei City, China). RNA was stored in a - 80 °C freezer. Concentration and purity were measured using a Nanodrop machine (Thermo Scientific, Carlsbad, CA, United States). As described previously, to synthesize cDNAs, PrimeScript RT reagent kits (TaKaRa, Dalian City, China) were purchased and used for reverse transcription (RT)^[21]. cDNA synthesis was conducted using a RT apparatus (Applied Biosystems, Foster City, CA, United States). The cDNA samples were stored at - 20 °C, and used for additional analysis.

Real-time quantitative reverse transcription polymerase chain reaction

To determine the expression of LOX and HIF1 α in tissues and blood samples from patients with GC, qRT-PCR analysis was performed using SYBR reagents (Vazyme, Nanjing City, China) on an Applied Biosystems Real-Time PCR machine (Thermo Scientific). The cDNAs, SYBR reagents, double-distilled water and corresponding primers were used to measure expression of LOX and HIF1 α . In addition, β -actin was used as a housekeeping gene. The sequences of primers used in this study are listed in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed as described previously^[22,23]. Tissue specimens were fixed in 4% paraformaldehyde, and embedded in paraffin for subsequent analysis. Antibodies against LOX and HIF1 α were purchased from Abcam (Cambridge, United Kingdom). The concentrations of LOX and HIF1 α antibodies were 1:100 and 1:400 and the antibodies were diluted in Primary antibody dilution buffer. The paraffin specimens were incubated with these two antibodies that were used to detect the expression of LOX and HIF1 α in the GC tissues. Tissue sections were incubated with antibodies against LOX or HIF1 α , and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology, Boston, MA, United States). All specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and stored in the Department of Pathology at our hospital.

Statistical analysis

All experiments were repeated at least three times. Statistical analyses were performed with SPSS software (IBM, Armonk, NY, United States). Pearson's χ^2 tests for categorical variables. Survival rate was calculated by Kaplan-Meier method, Log-rank tests was used to confirm the relationship between LOX, HIF1 α and the development of GC. Analysis of LOX and HIF1 α expression was conducted using

Table 1 Primer sequences

Target gene		Primer sequences
HIF1 α	Forward	CATAAAGTCTGCAACATGGAAGGT
	Reverse	ATTTGATGGGTGAGGAATGGGTT
LOX	Forward	CAGGCACCGACCTGGATATGG
	Reverse	CGTACGTGGATGCCTGGATGTAGT
β -actin	Forward	TGGCAGCCAGCACAATGAA
	Reverse	CTAAGTCATAGTCCGCCTAGAAGCA

GraphPad Prism software. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of LOX in patients with GC

Immunohistochemical staining and qRT-PCR analysis were performed to determine whether expression of LOX was dysregulated in tissues and blood samples from patients with GC versus healthy controls. Immunohistochemical staining indicated that LOX was mainly located in the cytoplasm of tumor cells. Most of the cytoplasm of tumor cells exhibited brown positively stained particles, which displayed a scattered particle distribution. The same results were seen in the extracellular matrix (Figure 1A). The high expression rate of LOX in GC tissues was 35.7% (50/140), which was significantly higher than that in adjacent tissues (18.6%, 26/140). QRT-PCR showed that mRNA expression of LOX was markedly increased in GC tissues; about four times higher than in adjacent tissues (Figure 1B). We further detected the expression level of LOX in blood samples from GC patients and healthy controls. Expression of LOX was higher in patients with GC than in the control group. Additional analysis showed that the later the clinicopathological stage, the higher the expression level of LOX in the blood samples (Figure 1C). Taken together, these results suggested that expression of LOX was significantly higher in patients with GC.

Expression of HIF1 α in patients with GC

Further analysis was performed to detect expression of HIF1 α in patients with GC. Immunohistochemistry revealed that HIF1 α was mainly expressed in the nuclei of tumor cells (Figure 2A). The nuclei of HIF1 α -positive cells presented with a brown color, and HIF1 α exhibited occasional limited expression in the cytoplasm (Figure 2A). Scattered light brown granules were revealed by immunohistochemical staining (Figure 2A). The high expression rate of HIF1 α in GC tissues was 33.6% (47/140), which was significantly higher than in adjacent tissues (12.1%, 17/140). The high expression of HIF1 α in tumor compared with adjacent tissues was measured by qRT-PCR (Figure 2B). Expression of HIF1 α in GC tissues was approximately four times higher than in adjacent tissues (Figure 2B). Additional analysis showed that the later the clinicopathological stage, the higher the expression level of HIF1 α in the blood samples (Figure 2C). Thus, we concluded that expression of HIF1 α was upregulated in tumor tissues and blood samples from patients with GC.

Correlation between LOX and clinicopathological characteristics of GC

Expression of LOX and clinicopathological characteristics are shown in Table 2. In various groups with different clinical characteristics, expression of LOX was different. Expression of the LOX gene correlated with lymph node metastasis of GC, the tumor infiltration depth and the tumor-node-metastasis (TNM) stage. The P values for the correlation between LOX expression and lymph node metastasis of GC, between LOX expression and the infiltration depth of GC, and between LOX expression and clinical stage were 0.000, 0.005 and 0.000, respectively. When the number of lymph node metastasis was >16 in patients with GC, the rate of high expression of LOX was 100.0%. When the tumor infiltration depth reached T4, the high expression rate of LOX was 54.1%. When the tumor was clinicopathological stage III, the high expression rate of LOX was 55.6%. However, expression of LOX did not show any relationship with other clinical features, such as age, gender, tumor size, and tumor location. The results showed that the expression of LOX increased with the number of lymph node metastases, deeper infiltration depth and the late TNM stage.

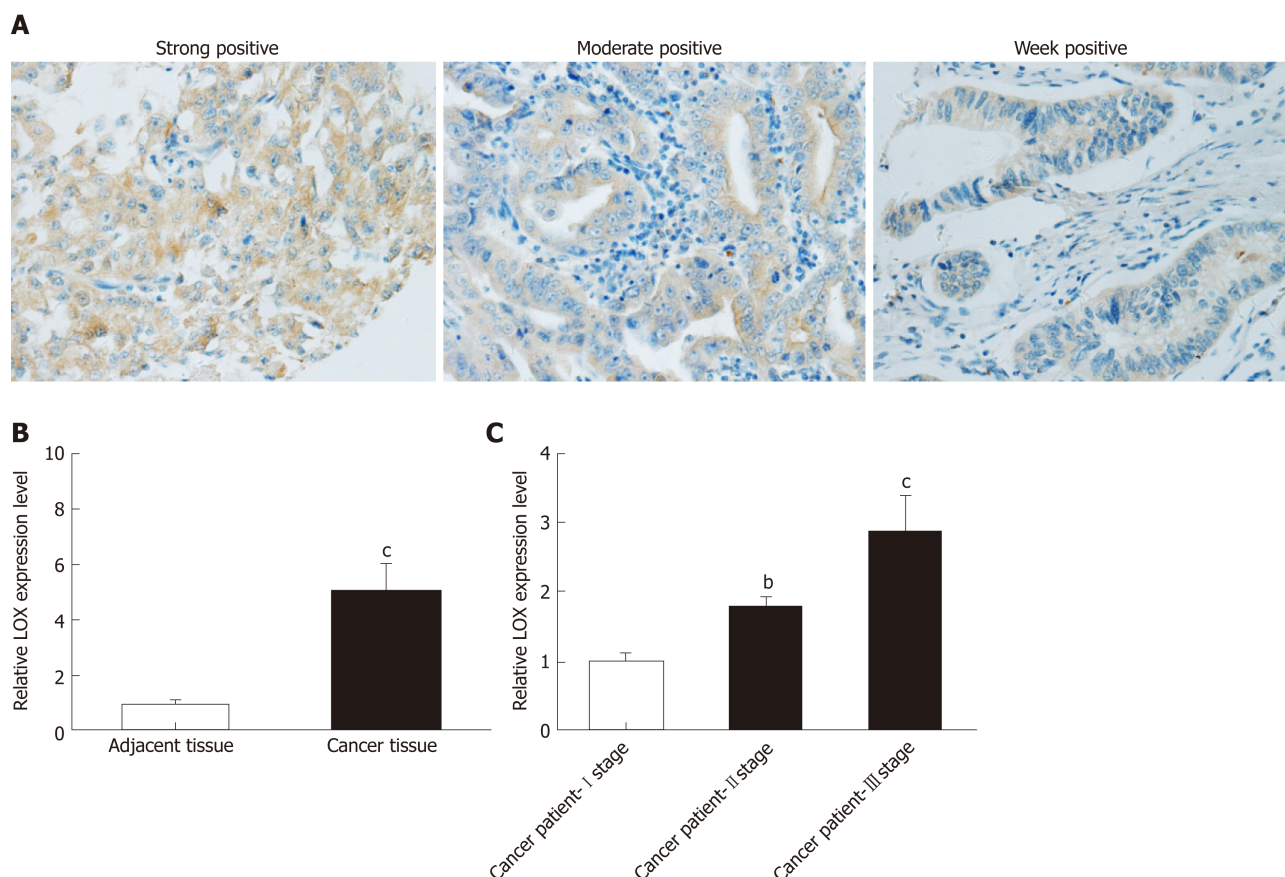


Figure 1 Expression of LOX in GC patients. A: Immunohistochemical staining was used to detect expression of LOX in tumor tissues from patients with GC; B: qRT-PCR was performed to measure mRNA expression of LOX in adjacent tissues and cancer tissues from GC patients; C: qRT-PCR was performed to examine the expression level of LOX in patients with GC. ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$ represent significant difference compared with controls. LOX: Lysyl oxidase; GC: Gastric cancer; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.

Relationship between HIF1 α and clinicopathological characteristics of GC

Expression of HIF1 α and clinicopathological characteristics are shown in Table 3. Expression of HIF1 α differed among various groups. Expression of HIF1 α was related to lymph node metastasis ($P = 0.000$). When the number of lymph node metastasis was > 16 , the rate of high expression of HIF1 α was 100.0%. When the tumor infiltration depth reached T4, high expression of HIF1 α was 56.8% ($P = 0.001$). When they had stage III disease, the rate of high expression of HIF1 α was 51.9% ($P = 0.000$). These results suggested that expression of HIF1 α correlated with the number of metastatic lymph nodes, the tumor infiltration depth and the TNM stage. However, expression of HIF1 α was not associated with other clinical characteristics, such as age, gender, tumor size, and tumor location.

Relationship between expression of LOX and prognosis of GC

Single-factor analysis showed that disease-free survival (DFS) in the LOX-low expression group was 26.7 mo, which was significantly longer than in the LOX-high expression group at 15.6 mo ($P = 0.037$) (Figure 3A and Table 4). Overall survival (OS) of patients in the LOX-low expression group was significantly longer than that in the LOX-high expression group ($P = 0.033$) (Figure 3B and Table 4). These results demonstrated that high expression of LOX was associated with poor prognosis.

Relationship between expression of HIF1 α and prognosis of GC

To determine the correlation between HIF1 α expression and the prognosis of GC, we focused on DFS and OS in patients with different levels of HIF1 α expression. Single-factor survival analysis showed that DFS and OS in the HIF1 α -low expression group were 26.9 mo and 40.2 mo, respectively, which were significantly longer than in the HIF1 α -high expression group at 14.0 mo and 20.4 mo (both $P = 0.003$) (Figure 4A, 4B and Table 4). High expression of HIF1 α was associated with poor prognosis.

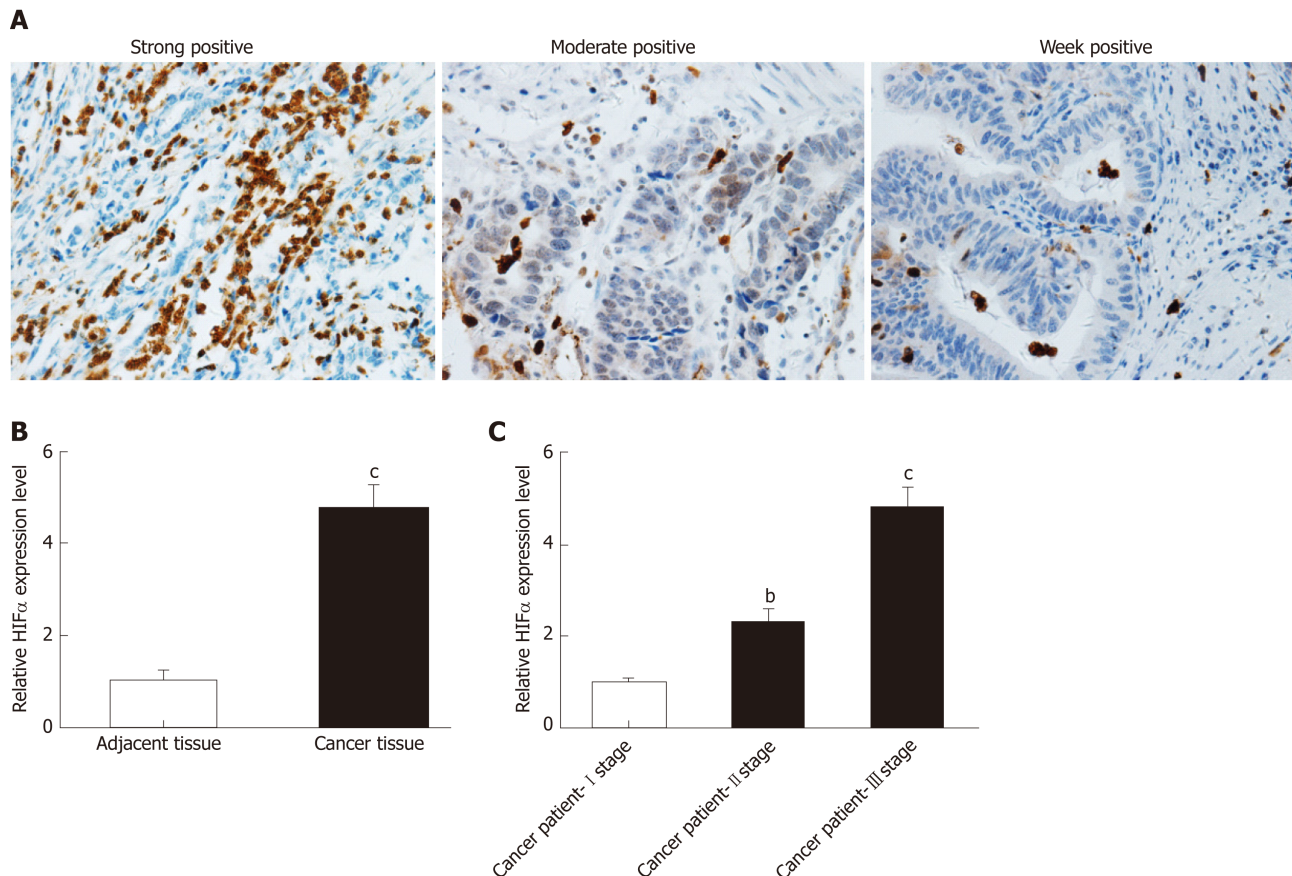


Figure 2 Expression of HIF1 α in patients with GC. A: Immunohistochemical analysis of HIF1 α in tumor tissues from patients with GC; B: qRT-PCR was applied to measure mRNA expression of HIF1 α in adjacent tissues and cancer tissues from GC patients; C: qRT-PCR was used to detect the level of HIF1 α in patients with GC. ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$ represent significant difference compared with controls. HIF1 α : Hypoxia-inducible factor 1 α ; GC: Gastric cancer; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.

DISCUSSION

The occurrence of GC has been increasing rapidly worldwide^[24]. GC is related to the presence of tumor-suppressor or tumor-associated genes^[25]. There is no accurate and efficient clinical biomarker of GC. Other gastrointestinal biomarkers or early detection methods were also not applied in GC^[26-28]. Therefore, the aim of our study was to investigate whether LOX and HIF1 α could be used as biomarkers of GC.

LOX participates in the osteoclastogenesis of breast cancer, which suggests a therapeutic tool for osteolytic bone destruction^[21]. MiRNA-31-5p inhibits expression of HIF1 α and strengthens the Warburg effect by inhibiting its target HIF-1 α inhibitor. In addition, hepatitis transactivator protein X accelerates extracellular matrix modification by activating the HIF/LOX pathway and promoting the metastasis of hepatocellular carcinoma^[13]. Therefore, we predicted that LOX and HIF1 α could act as biomarkers of GC.

In the present study, immunohistochemical staining suggested that expression of LOX and HIF1 α increased in tumor tissues from patients with GC. QRT-PCR analysis indicated that mRNA expression of LOX and HIF1 α was upregulated in tumor tissues. We also detected expression of these two genes in blood samples. The results revealed that the expression levels of LOX and HIF1 α were higher in GC patients than in healthy controls. Additional analysis showed that the expression levels of LOX and HIF1 α were related to the clinicopathological characteristics of GC. The expression of LOX and HIF1 α increased with the number of lymph node metastases, deeper infiltration depth and the later TNM stage. Single-factor analysis showed that high expression of LOX and HIF1 α led to poor prognosis of GC patients.

In conclusion, our results demonstrated that expression of LOX and HIF1 α was higher in patients with GC than in healthy controls. Expression of LOX and HIF1 α was associated with clinicopathological characteristics and prognosis of GC. Thus, we concluded that LOX and HIF1 α could be used as prognostic and predictive biomarkers for GC. Our study provided a link between LOX, HIF1 α and GC, which contributes to the development and progression of GC.

Table 2 Relationship between lysyl oxidase and clinicopathological factors in patients with gastric cancer

Characteristics	Sample size, <i>n</i>	LOX		<i>P</i>
		Low expression (%)	High expression (%)	
Gender		90 (64.3)	50 (35.7)	
Male	112	73 (65.2)	39 (34.8)	0.659
Female	28	17 (60.7)	11 (39.3)	
Age				
< 60 yr	67	44 (65.7)	23 (34.3)	0.743
≥ 60 yr	73	46 (63.0)	27 (37.0)	
Tumor location				
Upper part	57	37 (64.9)	20 (35.1)	0.978
Middle part	34	21 (61.8)	13 (38.2)	
Lower part	44	29 (65.9)	15 (34.1)	
Total stomach	5	3 (60.0)	2 (40.0)	
Tumor size				
< 5 cm	57	39 (68.4)	18 (31.6)	0.397
≥ 5 cm	83	51 (61.4)	32 (38.6)	
Depth of invasion				
T1	5	5 (100.0)	0 (0.0)	0.005
T2	13	12 (92.3)	1 (7.7)	
T3	85	56 (65.9)	29 (34.1)	
T4	37	17 (45.9)	20 (54.1)	
Lymphatic metastasis				
0	34	33 (97.1)	1 (2.9)	0.000
1-2	32	24 (75.0)	8 (25.0)	
3-6	31	21 (67.7)	10 (32.3)	
7-15	37	12 (32.4)	25 (67.6)	
≥ 16	6	0 (0.0)	6 (100.0)	
Borrmann classification				
Type I	16	12 (75.0)	4 (25.0)	0.340
Type II or III	110	67 (60.9)	43 (39.1)	
Type IV	11	8 (72.7)	3 (27.3)	
Type V	3	3 (100.0)	0 (0.0)	
WHO histological classification				
Adenocarcinoma	77	48 (62.3)	29 (37.7)	0.862
Signet-ring cell carcinoma	17	13 (76.5)	4 (23.5)	
Mucinous adenocarcinoma	8	5 (62.5)	3 (37.5)	
Mixed carcinoma	32	20 (62.5)	12 (37.5)	
Neuroendocrine carcinoma	6	4 (66.7)	2 (33.3)	
Lauren parting				
Intestinal type	65	42 (64.6)	23 (35.4)	0.909
Diffuse type	68	43 (63.2)	25 (36.8)	
Mixed type	7	5 (71.4)	2 (28.6)	
Differentiation grade				
Low and middle	107	68 (63.6)	39 (36.4)	0.744
High	33	22 (66.7)	11 (33.3)	
Cancer embolus				
Yes	51	29 (56.9)	22 (43.1)	0.165
No	89	61 (68.5)	28 (31.5)	
Affect neural				
Yes	50	29 (58.0)	21 (42.0)	0.247
No	90	61 (67.8)	29 (32.2)	

TNM staging				
I	10	10 (100.0)	0 (0.0)	0.000
II	49	44 (89.8)	5 (10.2)	
III	81	36 (44.4)	45 (55.6)	

LOX: Lysyl oxidase; WHO: World Health Organization; TNM: Tumor-node-metastasis.

Table 3 Relationship between hypoxia-inducible factor 1 α and clinicopathological factors in patients with gastric cancer

Characteristics	Sample size, <i>n</i>	HIF1 α		<i>P</i>
		Low expression (%)	High expression (%)	
Gender		93 (66.4)	47 (33.6)	0.474
Male	112	76 (67.9)	36 (32.1)	
Female	28	17 (60.7)	11 (39.3)	
Age				0.372
< 60 yr	67	47 (70.1)	20 (29.9)	
≥ 60 yr	73	46 (63.0)	27 (37.0)	
Tumor location				0.702
Upper part	57	41 (71.9)	16 (28.1)	
Middle part	34	19 (55.9)	15 (44.1)	
Lower part	44	30 (68.2)	14 (31.8)	
Total stomach	5	3 (60.0)	2 (40.0)	
Tumor size				0.253
< 5 cm	57	41 (71.9)	16 (28.1)	
≥ 5 cm	83	52 (62.7)	31 (37.3)	
Depth of invasion				0.001
T1	5	5 (100.0)	0 (0.0)	
T2	13	12 (92.3)	1 (7.7)	
T3	85	60 (70.6)	25 (29.4)	
T4	37	16 (43.2)	21 (56.8)	
Lymphatic metastasis				0.000
0	34	29 (85.3)	5 (14.7)	
1-2	32	24 (75.0)	8 (25.0)	
3-6	31	23 (74.2)	8 (25.8)	
7-15	37	17 (45.9)	20 (54.1)	
≥ 16	6	0 (0.0)	6 (100.0)	
Borrmann classification				0.183
Type I	16	11 (68.8)	5 (31.2)	
Type II or III	110	76 (69.1)	34 (30.9)	
Type IV	11	4 (36.4)	7 (63.6)	
Type V	3	2 (66.7)	1 (33.3)	
WHO histological classification				0.725
Adenocarcinoma	77	55 (71.4)	22 (28.6)	
Signet-ring cell carcinoma	17	12 (70.6)	5 (29.4)	
Mucinous adenocarcinoma	8	6 (75.0)	2 (25.0)	
Mixed carcinoma	32	20 (62.5)	12 (37.5)	
Neuroendocrine carcinoma	6	4 (66.7)	2 (33.3)	
Lauren parting				0.155
Intestinal type	65	42 (64.6)	23 (35.4)	
Diffuse type	68	44 (64.7)	24 (35.3)	
Mixed type	7	7 (100.0)	0 (0.0)	
Differentiation grade				0.698
Low and middle	107	72 (67.3)	35 (32.7)	

High	33	21 (63.6)	12 (36.4)	
Cancer embolus				
Yes	51	32 (62.7)	19 (37.3)	0.485
No	89	61 (68.5)	28 (31.5)	
Affect neural				
Yes	50	30 (60.0)	20 (40.0)	0.230
No	90	63 (70.0)	27 (30.0)	
TNM staging				
I	10	10 (100.0)	0 (0.0)	0.000
II	49	44 (89.8)	5 (10.2)	
III	81	39 (48.1)	42 (51.9)	

HIF1 α : Hypoxia-inducible factor 1 α ; WHO: World Health Organization; TNM: Tumor-node-metastasis.

Table 4 Univariate analyses of disease-free survival and overall survival in patients with gastric cancer

Gene	Expression	Cases, <i>n</i>	Disease free survival			Overall survival		
			mo	<i>P</i>		mo	<i>P</i>	
LOX	High	50	15.6	0.037	4.352	22.9	0.033	4.524
	Low	90	26.7			40.2		
HIF1 α	High	47	14.0	0.003	8.712	20.4	0.003	8.992
	Low	93	26.9			40.2		

LOX: Lysyl oxidase; HIF1 α : Hypoxia-inducible factor 1 α .

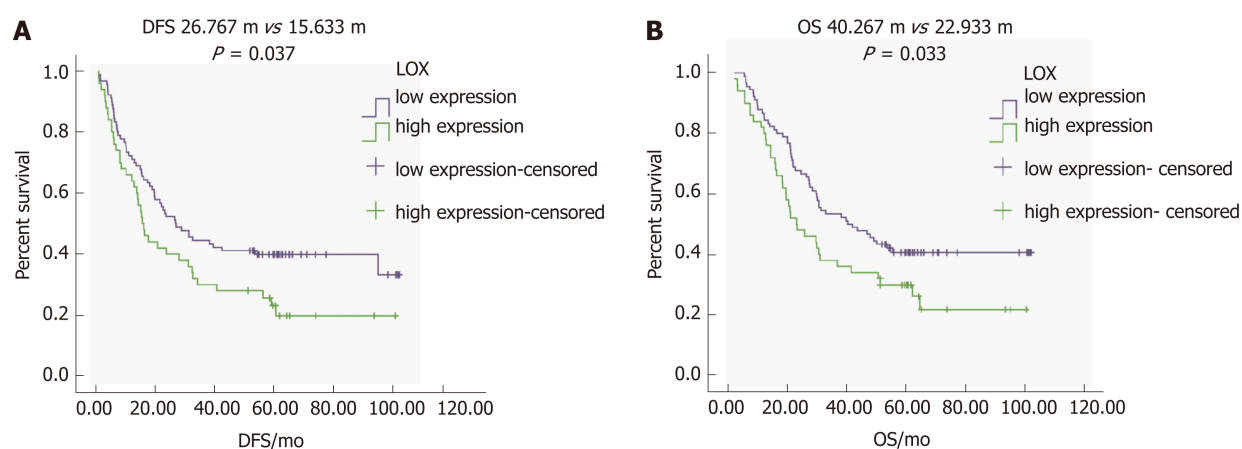


Figure 3 LOX expression is associated with DFS and OS. A: DFS curve of patients with GC with regards to lysyl oxidase expression, $^aP < 0.05$; B: Survival analysis of overall survival in patients with GC, $^aP < 0.05$. LOX: Lysyl oxidase; GC: Gastric cancer; DFS: Disease-free survival; OS: Overall survival.

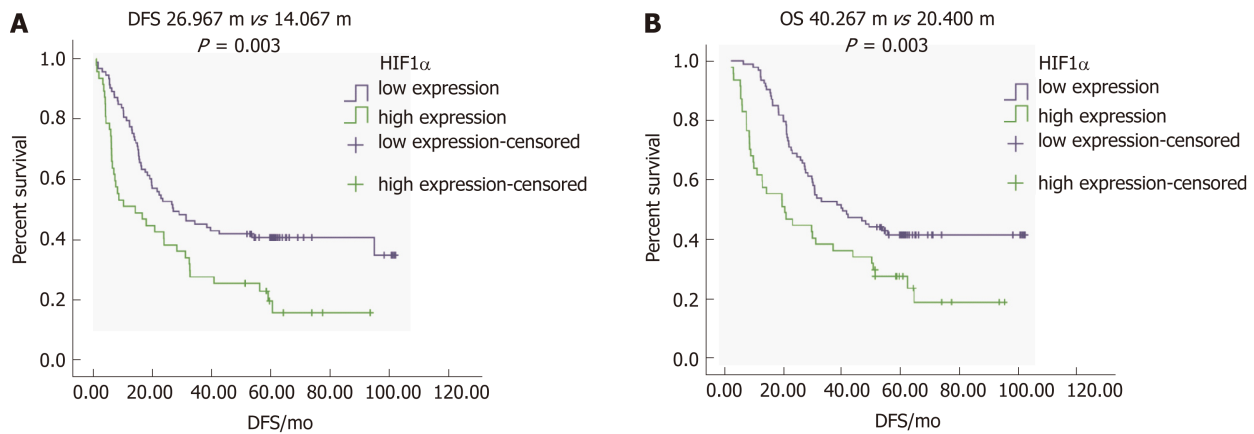


Figure 4 HIF1 α correlates with DFS and OS of patients with GC. A: DFS curve of patients with GC with respect to HIF1 α expression, $^aP < 0.05$; B: OS curve of patients with GC expressing low and high HIF1 α levels. $^aP < 0.05$. GC: Gastric cancer; DFS: Disease-free survival; OS: Overall survival; HIF1 α : Hypoxia-inducible factor 1 α .

ARTICLE HIGHLIGHTS

Research background

To study whether lysyl oxidase (LOX) and hypoxia-inducible factor 1 α (HIF1 α) can be used as prognostic and predictive biomarkers in gastric cancer (GC).

Research motivation

To provide the prognostic and predictive biomarkers for treating GC.

Research objectives

To explore the interaction between cancer and factors in the metabolic microenvironment and determine predictive biomarkers of GC.

Research methods

Patients and samples: This work is difficult and requires patient approval.

RNA extraction and cDNA synthesis: This work requires a lot of time, and so the manipulator should be patient. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR): The manipulator should add the samples accurately during qRT-PCR analysis. Immunohistochemistry: The experiments take a long time, and the manipulator should perform the experiments step by step. **Statistical analysis:** All of the experiments were repeated at least three times, and the statistical analysis was then performed.

Research results

The expression levels of LOX and HIF1 α increased in the tumor tissues and blood samples of patients with GC. The expression levels of LOX and HIF1 α were related to the clinicopathological characteristics and prognosis of GC.

Research conclusions

LOX and HIF1 α can be used as prognostic and predictive biomarkers for the treatment of GC. Our study indicated that the expression of LOX and HIF1 α was upregulated in patients with GC compared with the control group. In addition, the expression of LOX and HIF1 α was related to the clinicopathological characteristics and prognosis of GC.

Research perspectives

The present study suggested that LOX and HIF1 α might be used as both prognostic and predictive biomarkers for GC, and provided a link between LOX, HIF1 α and GC.

ACKNOWLEDGEMENTS

We appreciate the technical support and help from the Tumor Center Laboratory of PLA General Hospital.

REFERENCES

- 1 Liu F, Li C, Zhu J, Ren L, Qi X. ABO blood type and risk of hepatocellular carcinoma: A meta-analysis.

- Expert Rev Gastroenterol Hepatol* 2018; **12**: 927-933 [PMID: 30004289 DOI: 10.1080/17474124.2018.1500174]
- 2 **Unal E**, Karaosmanoglu AD, Ozmen MN, Akata D, Karcaaltincaba M. Computed Tomography-Based Diagnosis of Gastric Vein Invasion in Patients with Gastric Cancer. *Eurasian J Med* 2018; **50**: 91-95 [PMID: 30002574 DOI: 10.5152/eurasianjmed.2018.0041]
 - 3 **Ma C**, Olevian DC, Lowenthal BM, Jayachandran P, Kozak MM, Chang DT, Pai RK. Loss of SATB2 Expression in Colorectal Carcinoma Is Associated With DNA Mismatch Repair Protein Deficiency and BRAF Mutation. *Am J Surg Pathol* 2018; **42**: 1409-1417 [PMID: 30001238 DOI: 10.1097/PAS.0000000000001116]
 - 4 **Okasha HH**, Naguib M, El Nady M, Ezzat R, Al-Gemeie E, Al-Nabawy W, Aref W, Abdel-Moaty A, Essam K, Hamdy A. Role of endoscopic ultrasound and endoscopic-ultrasound-guided fine-needle aspiration in endoscopic biopsy negative gastrointestinal lesions. *Endosc Ultrasound* 2017; **6**: 156-161 [PMID: 28621291 DOI: 10.4103/2303-9027.201086]
 - 5 **Sharma V**, Rana SS, Ahmed SU, Guleria S, Sharma R, Gupta R. Endoscopic ultrasound-guided fine-needle aspiration from ascites and peritoneal nodules: A scoping review. *Endosc Ultrasound* 2017; **6**: 382-388 [PMID: 29251272 DOI: 10.4103/eus.eus_96_17]
 - 6 **Hocke M**, Ignee A, Dietrich C. Role of contrast-enhanced endoscopic ultrasound in lymph nodes. *Endosc Ultrasound* 2017; **6**: 4-11 [PMID: 28218194 DOI: 10.4103/2303-9027.190929]
 - 7 **Mizuno S**, Nakai Y, Isayama H, Suzuki T, Saito K, Uchino R, Takahara N, Kogure H, Tada M, Koike K. EUS-FNA of gastric cancer metastatic to the head of pancreas using a forward oblique viewing echoendoscope in a case with Roux-en-Y anatomy. *Endosc Ultrasound* 2018; **7**: 420-421 [PMID: 29536952 DOI: 10.4103/eus.eus_107_17]
 - 8 **Xu W**, Wei Q, Han M, Zhou B, Wang H, Zhang J, Wang Q, Sun J, Feng L, Wang S, Ye Y, Wang X, Zhou J, Jin H. CCL2-SQSTM1 positive feedback loop suppresses autophagy to promote chemoresistance in gastric cancer. *Int J Biol Sci* 2018; **14**: 1054-1066 [PMID: 29989092 DOI: 10.7150/ijbs.25349]
 - 9 **Goetze OT**, Al-Batran SE, Chevallay M, Mönig SP. Multimodal treatment in locally advanced gastric cancer. *Updates Surg* 2018; **70**: 173-179 [PMID: 29946806 DOI: 10.1007/s13304-018-0539-z]
 - 10 **El Hajj II**, Lawrence KA, Tirkes T, Shahda S, Sherman S. Metachronous gastric metastasis from lung primary, with synchronous pancreatic neuroendocrine carcinoma. *Clin Case Rep* 2018; **6**: 1368-1370 [PMID: 29988660 DOI: 10.1002/ccr3.1571]
 - 11 **Kim HG**, Kim DY, Jeong O. Transition from Conventional to Reduced-Port Laparoscopic Gastrectomy to Treat Gastric Carcinoma: A Single Surgeon's Experience from a Small-Volume Center. *J Gastric Cancer* 2018; **18**: 172-181 [PMID: 29984067 DOI: 10.5230/jgc.2018.18.e18]
 - 12 **Zhou Z**, Liu Y, Meng K, Guan W, He J, Liu S, Zhou Z. Application of spectral CT imaging in evaluating lymph node metastasis in patients with gastric cancers: Initial findings. *Acta Radiol* 2018; **284185118786076** [PMID: 29979106 DOI: 10.1177/0284185118786076]
 - 13 **Lee YI**, Jeon HK, Im JW, Oh SY, Kim KB, Kim B. Primary Gastric Small Cell Carcinoma: A Case Identified as a Large Subepithelial Tumor from Invisible State in 6 Months. *Clin Endosc* 2019; **52**: 76-79 [PMID: 29976037 DOI: 10.5946/ce.2018.062]
 - 14 **Liang H**, Ge F, Xu Y, Xiao J, Zhou Z, Liu R, Chen C. miR-153 inhibits the migration and the tube formation of endothelial cells by blocking the paracrine of angiopoietin 1 in breast cancer cells. *Angiogenesis* 2018; **21**: 849-860 [PMID: 29959560 DOI: 10.1007/s10456-018-9630-9]
 - 15 **Meléndez-Rodríguez F**, Roche O, Sanchez-Prieto R, Aragonés J. Hypoxia-Inducible Factor 2-Dependent Pathways Driving Von Hippel-Lindau-Deficient Renal Cancer. *Front Oncol* 2018; **8**: 214 [PMID: 29938199 DOI: 10.3389/fonc.2018.00214]
 - 16 **Li Q**, Li Y, Liang L, Li J, Luo D, Liu Q, Cai S, Li X. Klotho negatively regulated aerobic glycolysis in colorectal cancer via ERK/HIF1 α axis. *Cell Commun Signal* 2018; **16**: 26 [PMID: 29884183 DOI: 10.1186/s12964-018-0241-2]
 - 17 **Tse AP**, Sze KM, Shea QT, Chiu EY, Tsang FH, Chiu DK, Zhang MS, Lee D, Xu IM, Chan CY, Koh HY, Wong CM, Zheng YP, Ng IO, Wong CC. Hepatitis transactivator protein X promotes extracellular matrix modification through HIF/LOX pathway in liver cancer. *Oncogenesis* 2018; **7**: 44 [PMID: 29799025 DOI: 10.1038/s41389-018-0052-8]
 - 18 **Liu Y**, Wang G, Liang Z, Mei Z, Wu T, Cui A, Liu C, Cui L. Lysyl oxidase: A colorectal cancer biomarker of lung and hepatic metastasis. *Thorac Cancer* 2018; **9**: 785-793 [PMID: 29766649 DOI: 10.1111/1759-7714.12645]
 - 19 **Li T**, Wu C, Gao L, Qin F, Wei Q, Yuan J. Lysyl oxidase family members in urological tumorigenesis and fibrosis. *Oncotarget* 2018; **9**: 20156-20164 [PMID: 29732010 DOI: 10.18632/oncotarget.24948]
 - 20 **Leo C**, Cotic C, Pomp V, Fink D, Varga Z. Overexpression of Lox in triple-negative breast cancer. *Ann Diagn Pathol* 2018; **34**: 98-102 [PMID: 29661738 DOI: 10.1016/j.anndiagpath.2018.03.009]
 - 21 **Kim J**, Shin Y, Lee S, Kim M, Punj V, Lu JF, Shin H, Kim K, Ulmer TS, Koh J, Jeong D, An W. Regulation of Breast Cancer-Induced Osteoclastogenesis by MacroH2A1.2 Involving EZH2-Mediated H3K27me3. *Cell Rep* 2018; **24**: 224-237 [PMID: 29972783 DOI: 10.1016/j.celrep.2018.06.020]
 - 22 **Tanaka N**, Yamada S, Sonohara F, Suenaga M, Hayashi M, Takami H, Niwa Y, Hattori N, Iwata N, Kanda M, Tanaka C, Kobayashi D, Nakayama G, Koike M, Fujiwara M, Fujii T, Kodera Y. Clinical Implications of Lysyl Oxidase-Like Protein 2 Expression in Pancreatic Cancer. *Sci Rep* 2018; **8**: 9846 [PMID: 29959362 DOI: 10.1038/s41598-018-28253-9]
 - 23 **Zhang Z**, Li P, Wang Y, Yan H. Hypoxia-induced expression of CXCR4 favors trophoblast cell migration and invasion via the activation of HIF1 α . *Int J Mol Med* 2018; **42**: 1508-1516 [PMID: 29786753 DOI: 10.3892/ijmm.2018.3701]
 - 24 **Zhu B**, Cao X, Zhang W, Pan G, Yi Q, Zhong W, Yan D. MicroRNA-31-5p enhances the Warburg effect via targeting FIH. *FASEB J* 2019; **33**: 545-556 [PMID: 30004795 DOI: 10.1096/fj.201800803R]
 - 25 **Zhang J**, Xu J, Dong Y, Huang B. Down-regulation of HIF-1 α inhibits the proliferation, migration, and invasion of gastric cancer by inhibiting PI3K/AKT pathway and VEGF expression. *Biosci Rep* 2018; **38**: pii: BSR20180741 [PMID: 29899167 DOI: 10.1042/BSR20180741]
 - 26 **Wang Y**, Zhou Y, Hu Z. The Functions of Circulating Tumor Cells in Early Diagnosis and Surveillance During Cancer Advancement. *J Transl Int Med* 2017; **5**: 135-138 [PMID: 29085785 DOI: 10.1515/jtim-2017-0029]
 - 27 **Schizas D**, Kapsampelis P, Mylonas KS MD. Adenosquamous Carcinoma of the Esophagus: A Literature Review. *J Transl Int Med* 2018; **6**: 70-73 [PMID: 29984200 DOI: 10.2478/jtim-2018-0014]
 - 28 **Montagnani F**, Di Leonardo G, Pino M, Perboni S, Ribecco A, Fioretto L. Prolonged Inhibition of Vascular Endothelial Growth Factor Signaling Improves Survival in Metastatic Colorectal Cancer: A

Basic Study

Predictive and prognostic implications of 4E-BP1, Beclin-1, and LC3 for cetuximab treatment combined with chemotherapy in advanced colorectal cancer with wild-type KRAS: Analysis from real-world data

Gui-Fang Guo, Yi-Xing Wang, Yi-Jun Zhang, Xiu-Xing Chen, Jia-Bin Lu, Hao-Hua Wang, Chang Jiang, Hui-Quan Qiu, Liang-Ping Xia

ORCID number: Gui-Fang Guo (0000-0002-6283-7266); Yi-Xing Wang (0000-0001-7409-5223); Yi-Jun Zhang (0000-0002-8211-2721); Xiu-Xing Chen (0000-0003-4668-6944); Jia-Bin Lu (0000-0001-9302-173X); Hao-Hua Wang (0000-0003-4025-8615); Chang Jiang (0000-0003-0016-8543); Hui-Quan Qiu (0000-0001-6812-5959); Liang-Ping Xia (0000-0002-2849-3375).

Author contributions: Guo GF designed the research; Guo GF and Wang YX analyzed the data and wrote the paper; Chen XX and Wang HH performed the experiments; Zhang YJ and Lu JB contributed new reagents and analytic tools; Jiang C and Qiu HQ collected the data; Xia LP amended the paper; all of the authors have read and approved the final manuscript.

Institutional review board statement:

This study was approved by the Ethics Committee of the Sun Yat-sen University Cancer Center, Guangdong, China. All patients involved in this study gave their informed consent for participation in the study.

Conflict-of-interest statement: The authors have no conflict of interest relevant to this study.

Data sharing statement: The manuscript has no additional data available.

Gui-Fang Guo, Yi-Xing Wang, Xiu-Xing Chen, Hao-Hua Wang, Chang Jiang, Hui-Quan Qiu, Liang-Ping Xia, VIP Department, Sun Yat-sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Gui-Fang Guo, Yi-Xing Wang, Yi-Jun Zhang, Xiu-Xing Chen, Jia-Bin Lu, Hao-Hua Wang, Chang Jiang, Hui-Quan Qiu, Liang-Ping Xia, State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Gui-Fang Guo, Yi-Xing Wang, Yi-Jun Zhang, Xiu-Xing Chen, Jia-Bin Lu, Hao-Hua Wang, Chang Jiang, Hui-Quan Qiu, Liang-Ping Xia, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, Guangdong Province, China

Yi-Jun Zhang, Jia-Bin Lu, Pathology Department, Sun Yat-sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Corresponding author: Gui-Fang Guo, MD, PhD, Professor, VIP Department, Sun Yat-sen University Cancer Center, 651 Dongfeng Road East, Guangzhou 510060, Guangdong Province, China. guogf@sysucc.org.cn

Telephone: +86-20-87342708

Fax: +86-20-87342708

Abstract**BACKGROUND**

Colorectal cancer (CRC) is one of the main causes of cancer-related deaths in China and around the world. Advanced CRC (ACRC) patients suffer from a low cure rate though treated with targeted therapies. The response rate is about 50% to chemotherapy and cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR) and used for ACRC with wild-type KRAS. It is important to identify more predictors of cetuximab efficacy to further improve precise treatment. Autophagy, showing a key role in the cancer progression, is influenced by the EGFR pathway. Whether autophagy can predict cetuximab efficacy in ACRC is an interesting topic.

AIM

To investigate the effect of autophagy on the efficacy of cetuximab in colon cancer

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: January 24, 2019

Peer-review started: January 24, 2019

First decision: February 26, 2019

Revised: March 20, 2019

Accepted: March 24, 2019

Article in press: March 25 2019

Published online: April 21, 2019

P-Reviewer: Budai B, Mohamed SY, Perse M, Sung WW

S-Editor: Ma RY

L-Editor: Wang TQ

E-Editor: Ma YJ



cells and ACRC patients with wild-type KRAS.

METHODS

ACRC patients treated with cetuximab plus chemotherapy, with detailed data and tumor tissue, at Sun Yat-sen University Cancer Center from January 1, 2005, to October 1, 2015, were studied. Expression of autophagy-related proteins [Beclin1, microtubule-associated protein 1A/B-light chain 3 (LC3), and 4E-binding protein 1 (4E-BP1)] was examined by Western blot in CRC cells and by immunohistochemistry in cancerous and normal tissues. The effect of autophagy on cetuximab-treated cancer cells was confirmed by MTT assay. The associations between Beclin1, LC3, and 4E-BP1 expression in tumor tissue and the efficacy of cetuximab-based therapy were analyzed.

RESULTS

In CACO-2 cells exposed to cetuximab, LC3 and 4E-BP1 were upregulated, and P62 was downregulated. Autophagosome formation was observed, and autophagy increased the efficacy of cetuximab. In 68 ACRC patients, immunohistochemistry showed that Beclin1 levels were significantly correlated with those of LC3 ($0.657, P < 0.001$) and 4E-BP1 ($0.211, P = 0.042$) in ACRC tissues. LC3 was significantly overexpressed in tumor tissues compared to normal tissues ($P < 0.001$). In 45 patients with wild-type KRAS, the expression levels of these three proteins were not related to progression-free survival; however, the expression levels of Beclin1 ($P = 0.010$) and 4E-BP1 ($P = 0.005$), pathological grade ($P = 0.002$), and T stage ($P = 0.004$) were independent prognostic factors for overall survival (OS).

CONCLUSION

The effect of cetuximab on colon cancer cells might be improved by autophagy. LC3 is overexpressed in tumor tissues, and Beclin1 and 4E-BP1 could be significant predictors of OS in ACRC patients treated with cetuximab.

Key words: 4E-binding protein 1; Beclin-1; Microtubule-associated protein 1A/B-light chain 3; Advanced colorectal cancer; Cetuximab efficacy; Prognosis

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Considering the response rate to cetuximab in advanced colorectal cancer patients with wild-type KRAS, autophagy might be a novel predictor to exclude patients who will not respond to cetuximab. Accordingly, Beclin1, microtubule-associated protein 1A/B-light chain 3 (LC3), and 4E-binding protein 1 (4E-BP1) were studied in this research. We found that LC3 was overexpressed in tumor tissues, and the expression levels of Beclin1 and 4E-BP1 were related to overall survival.

Citation: Guo GF, Wang YX, Zhang YJ, Chen XX, Lu JB, Wang HH, Jiang C, Qiu HQ, Xia LP. Predictive and prognostic implications of 4E-BP1, Beclin-1, and LC3 for cetuximab treatment combined with chemotherapy in advanced colorectal cancer with wild-type KRAS: Analysis from real-world data. *World J Gastroenterol* 2019; 25(15): 1840-1853

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1840.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1840>

INTRODUCTION

Colorectal cancer (CRC) is one of the main causes of cancer-related deaths both in China and worldwide. In past years, the median overall survival of advanced CRC (ACRC) patients was 20 mo due to the development of new combinations of chemotherapy drugs, including oxaliplatin-based^[1] and irinotecan-based regimens^[2]. However, drug resistance and/or off-target toxicity limit the efficiency of current chemotherapy drugs. The introduction of new targeted therapies, such as monoclonal antibodies targeting epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) or against vascular endothelial growth factor (bevacizumab, ziv-aflibercept, and ramucirumab), has significantly increased the overall survival of

ACRC patients to 30 mo^[3]. Currently, the only antibody targeting EGFR that has been approved for use in CRC by the Chinese Food and Drug Administration is cetuximab. Studies have demonstrated that only ACRC patients with wild-type KRAS benefit from cetuximab^[4] and thus have better overall survival; this beneficial effect is observed especially in patients with left-sided ACRC^[5]. However, the response rate (RR) is only 40%-60% for patients with wild-type KRAS^[6]. It is important to identify novel predictors to exclude patients with wild-type KRAS who will not respond to cetuximab, which will further improve precise treatment.

Autophagy, an important intracellular homeostatic pathway for the degradation of dysfunctional organelles and proteins, can provide energy for survival under conditions of diverse cellular stresses^[7]. Despite accumulating evidence that autophagy plays a critical role in cancer metastasis, autophagy can also suppress pathological processes, including cancer metastasis^[8]. Multiple autophagy-related genes and proteins are involved in metastatic CRC^[9]. Mammalian target of rapamycin (mTOR), a member of the phosphatidylinositol-3-kinase family, plays a major role in inhibiting the autophagic process and is associated with the proliferation, survival, invasion, and metastasis of CRC cells^[10]. Several mTOR signaling components, including mTOR, p70-S6 Kinase 1 (S6K), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), are highly expressed and activated in glandular elements of CRC^[11]. The current paradigm suggests that mTOR phosphorylates 4E-BP1 at multiple sites, which promotes the dissociation of eIF4E from 4E-BP1, reducing the inhibitory effect of 4E-BP1 on eIF4E-dependent translation initiation and decreasing the translational activity^[12]. At the molecular level, the complex autophagy machinery is orchestrated in several stages. One well-characterized regulatory event is the interaction between Beclin1 and Beclin2, which disrupts the interaction of Beclin1 with the class III phosphoinositide 3-kinase (PI3K), a core regulator of autophagy^[13]. Autophagy-related genes (Atgs) control autophagosome formation through Atg12-Atg5 and microtubule-associated protein 1A/B-light chain 3 (LC3) complexes, and the modified form of LC3 is attached to the autophagosome membrane^[14].

It has been reported that autophagy was induced by EGFR siRNA in cancer cells^[15]. Few reports describe autophagy and the anti-cancer role of cetuximab, antibody targeting EGFR, and most of these studies have been performed on cancer cells. In this study, we examined the critical autophagic proteins, Beclin-1, 4E-BP1, and LC3, in CRC cells and investigated whether autophagy determines cell sensitivity to cetuximab *in vitro*. We also investigated the relationships between clinicopathological characteristics and the expression of Beclin-1, LC3, and 4E-BP1 in ACRC tissue specimens, and evaluated the influence of these autophagy-related proteins on ACRC prognosis. We previously studied the association between Beclin-1 and LC3 expression levels and the effect of cetuximab on ACRC^[16], but cetuximab was not given as a first-line therapy in all patients, and the KRAS status of those patients was unknown because KRAS was not used as a predictor of the efficacy of cetuximab at that time. The present study focused on patients with ACRC with wild-type KRAS who were administered with cetuximab plus chemotherapy as the first-line therapy to explore the predictive value of the expression levels of Beclin-1, LC3, and 4E-BP1. The differences in their expression levels between cancerous and normal tissues as well as the correlation of their expression with clinical and pathological grade, progression-free survival (PFS), and overall survival (OS) were also examined in a group of patients wild-type KRAS who were initially or later treated with cetuximab and chemotherapy.

MATERIALS AND METHODS

Cell line and culture

The CRC cell line CACO-2, which has the wild-type KRAS gene, was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained at 37 °C in an atmosphere containing 5% CO₂.

MTT assay

MTT assays were performed to determine the anti-proliferative effect of cetuximab on CACO-2 cells. After treatment with cetuximab for 24, 48, and 72 h, 20 µg (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, United States) was added to 96-well plates and incubated at 37 °C for 5 h; then, 150 µL of dimethyl sulfoxide (DMSO) was added to each well and incubated for 10 min at room temperature to dissolve the formazan crystals. The absorbance of each well was measured with an ELISA reader (BIO-TEK, United States) at a wavelength of 562 nm. The experiment was performed in triplicate, and the data were analyzed by

comparison to DMSO-treated control cells.

Western blot analysis

The tissue samples were homogenized in sodium dodecyl sulphate (SDS) buffer containing the protease inhibitor PMSF. The homogenates were incubated on ice for 20 min and then centrifuged at 12000 rpm for 30 min at 4 °C. The supernatant was collected and equal volume of 2 × SDS buffer was added. The mixture was boiled for 10 min and preserved at -20 °C. The protein extracts (50 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore, United States). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 90 min and then incubated with primary antibodies against Beclin-1 (3 µg/mL; ab55878, Abcam, Cambridge, United Kingdom), LC3 (2 µg/mL; ab48394, Abcam), 4E-BP1 (2 µg/mL; ab2606, Abcam), and actin (0.5 µg/mL, ab3280, Abcam). The protein bands were detected with secondary antibodies conjugated to horseradish peroxidase (1:5000, Abcam, United Kingdom) and visualized with enhanced chemiluminescence reagents. Each band was quantified through densitometry, and the results are presented as the relative expression of each protein from different samples.

Fluorescence microscopy

For fluorescence microscopy, 500 µL of cultured cells were removed from the flask at the desired time points, centrifuged for 3 min at 5000 × g, and resuspended in an appropriate volume of water. A total of 4 µL of each sample was spotted on a microscope slide and viewed using an Olympus 1 × 51 inverted fluorescence microscope.

Patients and variables

In this retrospective study, ACRC patients with definitive pathological diagnoses, paraffin-embedded pathological specimens, and complete clinicopathologic information who received cetuximab combined with first-line or later chemotherapy at our institution (Sun Yat-sen University Cancer Center) between January 1, 2005 and October 1, 2015 were enrolled.

Our primary study endpoints were OS, which was defined as the time from the date of the first cycle of front-line therapy to the date of death from any cause, and first-line PFS, which was defined as the time from the initial therapy to tumor progression, death from any cause, or the last follow-up before the initiation of second-line therapy. In addition, the objective RR (ORR) and disease RR (DCR) to cetuximab combined with chemotherapy as a first-line treatment were also studied. The study was approved by the ethics committee of our institution.

Tissue samples

CRC tissues were surgical or biopsy specimens taken from primary colorectal tumors, and the negative margins of the surgical specimens were considered as normal tissues. All tissue samples were acquired from the sample bank of the pathology department at our cancer center, and all formalin-fixed paraffin-embedded samples in this study were preserved intact. All pathological diagnoses of tissues were adenocarcinoma.

Immunohistochemical staining

The expression of Beclin-1, LC3, and 4E-BP1 in primary tumors was detected through immunohistochemical staining (IHC) and then compared with the expression in the normal tissue samples. The formalin-fixed paraffin-embedded tissue was cut into 4-µm thick sections, dewaxed, rehydrated, and blocked with hydrogen peroxide. After incubation with primary antibodies against 4E-BP1 (1:200), Beclin-1 (1:100), and LC3 (1:400) (all from Cell Signaling Technology, United States) diluted in 1% PBS, the sections were incubated with an anti-rabbit secondary antibody (Invitrogen, United States) for 30 min. After an additional two washes in PBS, the sections were incubated with diaminobenzidine (DAB, Invitrogen, United States) for 10 min to detect signals. Finally, the sections were counterstained with hematoxylin (Invitrogen, United States). Negative controls were obtained by substituting nonimmune rabbit serum for the primary antibodies.

Immunohistochemical score determination

Protein expression was evaluated *via* an Olympus CX31 microscope (Olympus, Center Valley, PA, United States) by two individuals who were blinded to the patient outcomes and other clinical findings. The immunoreactivity was evaluated according to the intensity of staining and the percentage of stained cells. Immunostaining

intensity was rated as follows: 0, pale yellow or no staining; 1, yellow; 2, deep yellow; and 3, brown. The percentage of stained cells positive for Beclin-1 and LC3 was graded as follows: grade 0 (0%-10%); 1 (10%-25%); 2 (25%-50%); and 3 (50%-100%). The percentage of stained cells positive for 4E-BP1 was graded as grade 0, 0%; 1 (1%-20%); 2 (20%-40%); 3 (40%-60%); 4 (60%-80%); and 5 (80%-100%). The staining intensity and percentage of stained tumor cells were scored for at least 10 high-power fields (400×). The mean score was calculated as $[(\text{intensity}_{\text{reader 1}} \times \text{percentage}_{\text{reader 1}}) + (\text{intensity}_{\text{reader 2}} \times \text{percentage}_{\text{reader 2}})] / 2$. Then, the total scores for Beclin1, LC3, and 4E-BP1 were categorized as low expression (=0) or high expression (>0).

Statistical analysis

All statistical analyses were performed with SPSS for Windows, version 24.0. The associations between Beclin1, LC3, and 4E-BP1 expression and clinicopathological characteristics were assessed using the chi-square test or Fisher's exact test, as appropriate. The chi-square test with continuity correction was used to analyze data derived from small cohorts. The relationships among the expression of Beclin1, LC3, and 4E-BP1 were assessed by Spearman's correlation analysis. The Kaplan-Meier method was used to estimate tumor progression in mCRC, and the log-rank test was used to determine the statistical significance. The groups were compared with respect to survival using Cox regression analysis. Hazard ratios were determined using univariate and multivariate Cox regression. $P < 0.05$ indicated a significant difference.

RESULTS

Autophagy induced by cetuximab in CRC cells

Autophagy was induced by cetuximab treatment in CACO-2 cells with wild-type KRAS, which were sensitive to cetuximab (Figure 1A). Abundant characteristic autophagosomes in CACO-2 cells were present 72 h after cetuximab treatment; in contrast, autophagosomes were scarce in untreated cells (Figure 1B). We also observed the conversion of LC3/Atg8 from the cytoplasmic form, LC3-1 (18 kDa), to the autophagosomic form, LC3-2 (16 kDa), in the cells exposed to cetuximab (Figure 1C) and decreased expression of P62, which indicated autophagosome formation. Moreover, we observed increased expression of 4E-BP1, indicating that the mTOR pathway was inactive (Figure 1C). MTT assay showed that autophagy increased the inhibitory effect of cetuximab on cell proliferation (Figure 1D).

Basic characteristics of patients

Initially, 235 ACRC patients treated with cetuximab plus chemotherapy were found in the clinical database of our center, but only 68 patients with detailed data and well-preserved tumor specimens were finally enrolled in this study. The age of the patients ranged from 23 to 77 years, with a median age of 54.5 years. The Beclin1, LC3, and 4E-BP1 expression levels were evaluated in all patients treated with cetuximab combined with chemotherapy; the basic characteristics of those patients and the correlations of those characteristics with the expression of Beclin1, LC3, and 4E-BP1 for all patients are reported in Table 1. Forty-five patients were confirmed to have the wild-type KRAS gene, 25 were given cetuximab as first-line therapy, and 20 were given cetuximab after having already received a different first-line therapy. The number of patients with pathological grades 3, 2, and 1 was 18, 41, and 3, respectively.

For the 45 patients with wild-type KRAS, the ORR and DCR to first-line treatment with cetuximab combined with chemotherapy were 45.5% and 72.7%, respectively, and the median OS was 31 mo (2.2-137).

Expression of Beclin1, LC3, and 4E-BP1 expression in CRC and normal tissues

There were significant correlations between the levels of Beclin1 and those of LC3 and 4E-BP1 in CRC tissues and that LC3 was significantly overexpressed in tumor tissues compared to normal tissues (Figure 2). According to the quantitative scoring method described above, the expression levels of Beclin1, LC3, and 4E-BP1 were classified as either high or low. The expression levels of Beclin1, LC3, and 4E-BP1 in cancerous and normal tissues are shown in Figure 3. LC3 was significantly upregulated in tumor specimens compared to normal specimens (64.87% *vs* 2.00%, $P < 0.001$); the sensitivity was 71.4%, with a specificity of 91.3%. The expression levels of Beclin1 (64.87% *vs* 81.81%, $P = 0.248$) and 4E-BP1 (81.09% *vs* 87.10%, $P = 0.793$) were not different between CRC and normal tissues.

Correlations among the expression levels of Beclin1, LC3, and 4E-BP1; KRAS status; and clinicopathologic factors

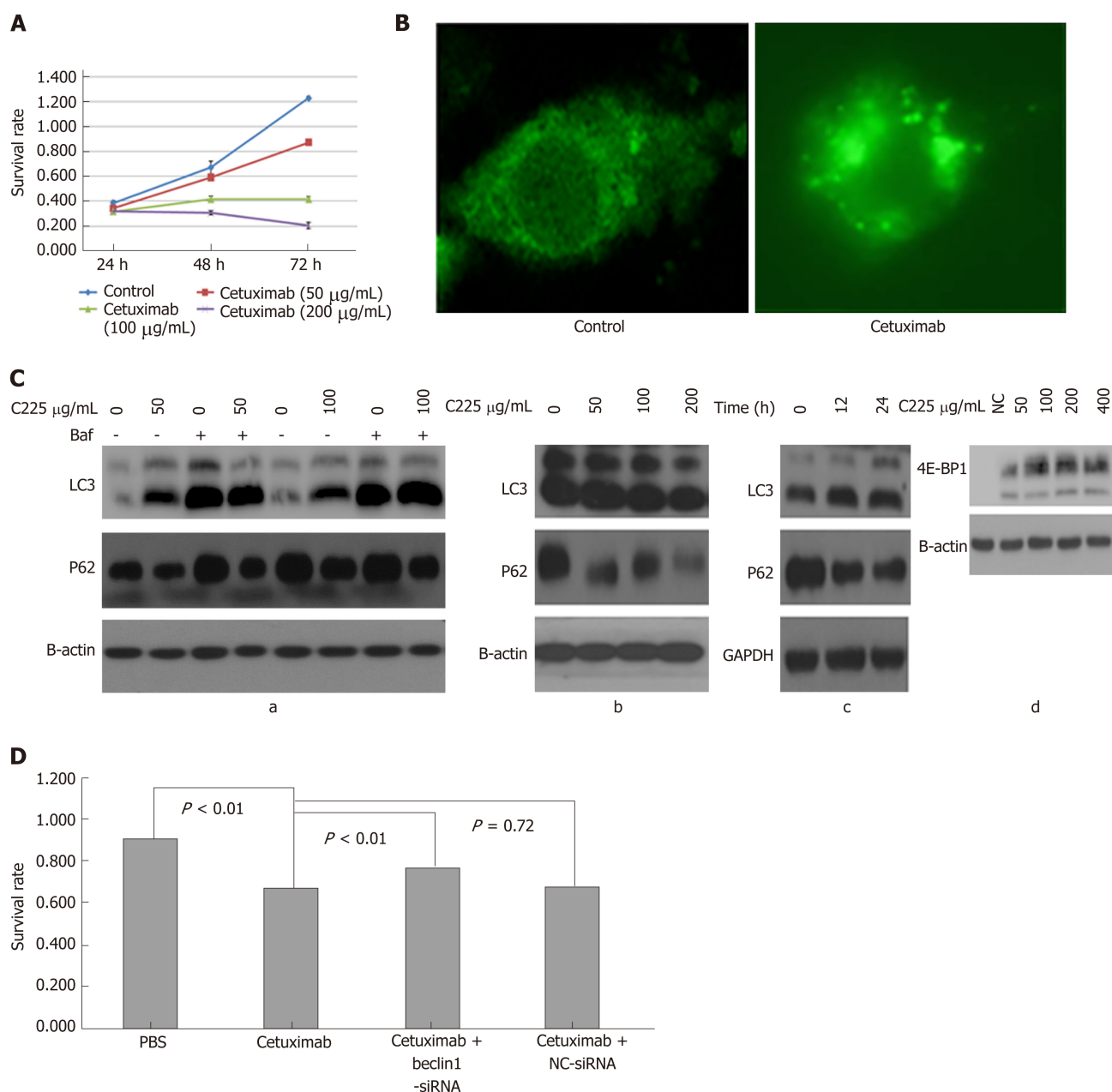


Figure 1 Cetuximab induces autophagy and upregulates 4E-BP1 expression and autophagy enhances anti-cancer effect of cetuximab in colon cancer. A: CACO-2 cell viability measured using MTT assay. Cell viability was significantly inhibited in a time- and dose-dependent manner by cetuximab. B: Cetuximab induced formation of autophagosomes. Autophagosomes in CACO-2 cells treated by cetuximab or not were examined by fluorescence microscopy. More autophagosomes were found in CACO-2 cells after treatment with cetuximab. C: Western blot analysis showing that cetuximab induced autophagy. (a) CACO-2 cells were either untreated or treated with cetuximab and Baf, and Baf inhibited autophagy-related protein degradation. Microtubule-associated protein 1A/B-light chain 3 (LC3)-II/LC3-I was increased, and P62 was decreased when treated with cetuximab, and both LC3 and P62 were increased when treated with cetuximab and Baf compared to treatment with cetuximab alone. (b and c) Cetuximab induced autophagy in a concentration and time dependent manner. With time and increasing concentration, the expression of LC3 increased, and P62 declined. (d) CACO-2 cells were treated with cetuximab for 48 h, and the expression of 4E-binding protein 1 was increased as the concentration of cetuximab increased. D: Effect of autophagy on cetuximab inhibition of cell proliferation. We added PBS, cetuximab, cetuximab + Beclin1-siRNA, and cetuximab + NC-siRNA separately, and the concentration of cetuximab was 78 µg/mL; then, cells were treated for 72 h. The results confirmed that autophagy enhanced cetuximab inhibition of cell proliferation in CACO-2 cells. LC3: Microtubule-associated protein 1A/B-light chain 3; 4E-BP1: 4E-binding protein 1.0

Table 1 Correlations of Beclin1, LC3, and 4E-BP1 expression with clinicopathological characteristics in 68 advanced colorectal cancer patients *n* (%)

Variable	<i>n</i> = 68	Beclin1 expression			LC3 expression			4E-BP1 expression		
		Low	High	<i>P</i> -value	Low	High	<i>P</i> -value	Low	High	<i>P</i> -value
Sex				0.061			0.372			0.571
Male	44 (64.7)	13	24		12	25		8	28	
Female	24 (35.3)	2	17		4	15		3	16	
Age (yr)				0.119			0.167			0.865
< 65	55 (80.9)	10	35		11	34		9	35	
≥ 65	13 (19.1)	5	6		5	6		2	9	
Family history of cancer				0.593			0.151			0.382
Yes	13 (19.1)	2	8		1	9		1	9	
No	55 (80.9)	13	33		15	31		10	35	
Location ¹				0.862			1.000			0.634
Left	50 (73.5)	11	31		12	30		2	33	
Right	18 (26.5)	4	10		4	10		9	11	
Pathological grade				0.349			0.349			0.897
1 + 2	44 (64.7)	8	30		8	30		8	29	
3	18 (26.4)	5	10		5	10		3	12	
NA	6 (8.9)									
T stage				0.618			0.618			0.911
T1 + T2 + T3	41 (60.3)	9	24		9	24		6	26	
T4	22 (32.4)	4	15		4	15		4	16	
NA	5 (7.3)									
N stage				0.058			0.058			0.654
No	22 (32.4)	6	9		6	9		4	12	
N1 + N2	36 (52.9)	5	28		5	28		6	25	
NA	10 (14.7)									
Synchronous/metachronous metastasis				0.020			0.058			0.162
Synchronous metastasis	24 (35.3)	1	18		2	17		5	12	
Metachronous metastasis	43 (63.2)	13	23		13	23		5	32	
NA	1 (1.5)									
KRAS				0.044			0.044			0.651
Wild type	45 (66.2)	13	24		13	24		7	30	
Mutant type	10 (14.7)	0	9		0	9		2	6	
NA	13 (19.1)									

Location: The primary tumor location was classified as right-sided or left-sided according to the splenic flexure. CRC: Colorectal cancer; LC3: Microtubule-associated protein 1A/B-light chain 3; NA: Not applicable; 4E-BP1: 4E-binding protein 1.

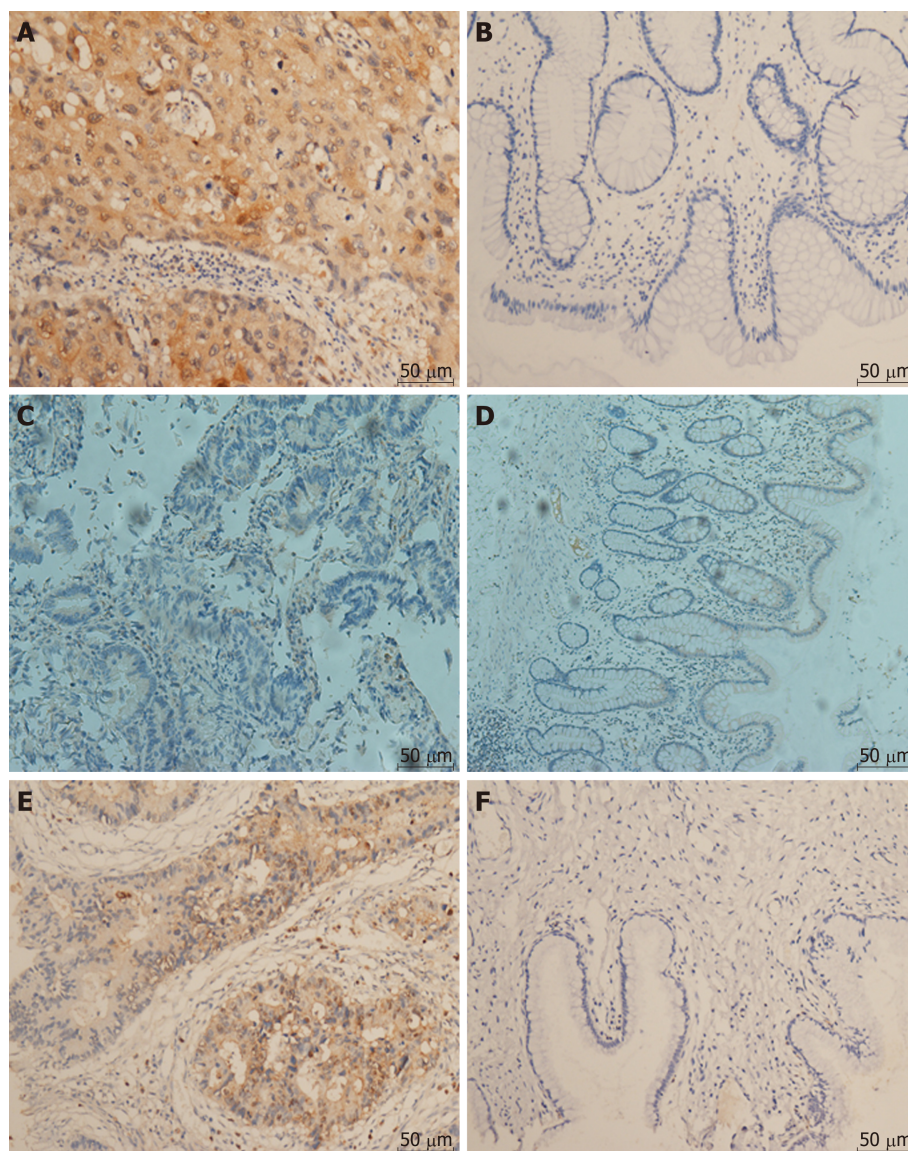


Figure 2 Immunohistochemical staining (200 \times). A: LC3 displayed strong cytoplasm staining in colorectal cancer tissue; B: LC3 displayed weak cytoplasm staining in normal tissue; C: Beclin1 displayed strong cytoplasm staining in colorectal cancer tissue; D: Beclin1 displayed weak cytoplasm staining in normal tissue; E: 4E-BP1 displayed strong cytoplasm staining in colorectal cancer tissue; F: 4E-BP1 displayed weak cytoplasm staining in normal tissue.

expression was not correlated with that of 4E-BP1 ($P = 0.251$). Compared to patients with the wild-type *KRAS* gene, patients with the mutated *KRAS* gene expressed higher levels of Beclin1 (100% *vs* 64.9%, $P = 0.044$) and LC3 (100% *vs* 64.9%, $P = 0.044$). The associations of the expression levels of Beclin1, LC3 and 4E-BP1 with the clinicopathologic factors in all 68 patients are shown in Table 1. Patients with metachronous metastasis had higher expression levels of Beclin1 than those with synchronous metastasis (94.7% *vs* 63.9%, $P = 0.020$).

Predictive value of Beclin1, LC3, and 4E-BP1 expression for cetuximab-containing chemotherapy

For the 25 patients with the wild-type *KRAS* gene who received cetuximab-containing chemotherapy as the first-line treatment, the ORRs and DCRs for patients with low and high Beclin1, LC3, and 4E-BP1 expression levels are shown in Table 2. There were no associations between the expression levels of Beclin1 ($P = 1.000$, $P = 0.325$), LC3 ($P = 0.362$, $P = 0.325$), or 4E-BP1 ($P = 0.303$, $P = 0.560$) and the ORR or DCR. In the whole group, the median PFS was 8.81 mo (0.72-32.43). The expression levels of Beclin1, LC3, and 4E-BP1 did not have any relationship with PFS [8.80 mo *vs* 4.7 mo ($P = 0.843$) for Beclin1 high expression and low expression, respectively; 4.70 mo *vs* 9.96 mo ($P = 0.167$) for LC3 high expression and low expression, respectively; and 5.32 mo *vs* 7.36 mo ($P = 0.410$) for 4E-BP1 high expression and low expression, respectively (Table 2)].

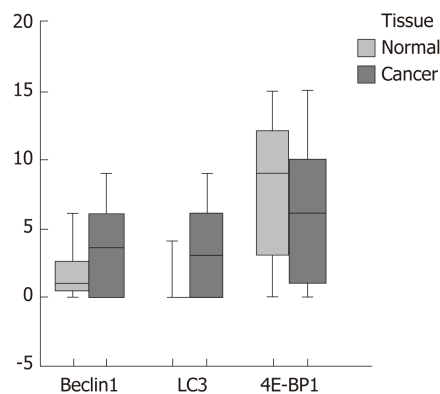


Figure 3 Differential expression of Beclin-1, LC3, and 4E-BP1 tested by immunohistochemical staining in colorectal cancer samples compared to normal tissues. LC3 ($P < 0.001$) showed higher expression in cancer than in normal tissue, while Beclin-1 ($P = 0.248$) and 4E-binding protein 1 ($P = 0.793$) did not. LC3: Microtubule-associated protein 1A/B-light chain 3; 4E-BP1: 4E-binding protein 1.

Prognostic value of Beclin1, LC3, and 4E-BP1 expression

The median OS for the 45 patients with the wild-type *KRAS* gene who received cetuximab as either a first-line treatment or a subsequent treatment was 31 mo (2.2–137). The most common factors potentially affecting OS, such as gender, age, family history of cancer, tumor location, pathological grade, T stage, N stage, synchronous/metachronous metastasis, and the expression of Beclin1, LC3, and 4E-BP1, were analyzed, as shown in Table 3. According to univariate Cox regression analysis, pathological grade (43.00 mo *vs* 10.32 mo, $P < 0.001$), the expression level of Beclin1 (19.65 mo *vs* 37.82 mo, $P = 0.037$) (Figure 4A), and the expression level of 4E-BP1 (64.82 mo *vs* 23.66 mo, $P = 0.024$) (Figure 4B) were risk factors affecting OS. Multivariate Cox regression analysis revealed that pathological grade ($P = 0.002$), T stage ($P = 0.004$), the expression level of Beclin1 ($P = 0.010$), and the expression level of 4E-BP1 ($P = 0.005$) were independent prognostic factors for OS.

DISCUSSION

Most studies on the roles of autophagy in cetuximab-mediated cancer therapy are limited to cancer cells, but results based on clinical patients are few. The present study investigated the influence of Beclin1, LC3, and 4E-BP1 expression in tumors, which are three key factors involved in the autophagic process, on the ORR, DCR, PFS, and OS of patients treated with cetuximab combined with chemotherapy. The efficacy found in this study population was the same as that in the previous stage III clinical trials^[3], with an ORR of 45.5%, a DCR of 72.7%, and a median OS of 31 mo. First, we found that LC3 and 4E-BP1 were upregulated and P62 was downregulated in CACO-2 cells exposed to cetuximab, and autophagosome formation was observed by fluorescence microscopy, indicating that cetuximab induces autophagy in CRC cells. In addition, the effect of autophagy on the killing of cancer cells by cetuximab was confirmed by MTT assay. We further studied the influence of these autophagic factors on cetuximab efficacy in ACRC. Using IHC, we observed that there were significant correlations between the levels of Beclin1 and the levels of LC3 and 4E-BP1 in CRC tissue and that LC3 was significantly overexpressed in tumor tissues compared to normal tissues. The expression levels of Beclin1 and 4E-BP1 as well as pathological grade and T stage were independent prognostic factors for OS.

Our study found that cetuximab, which targets EGFR, induced autophagy in colon cancer cells. The mechanism of the downstream signaling effect of EGFR on autophagy and the role of autophagy in EGFR-driven cancer cell proliferation remain unclear. One study of EGFR and autophagy in cancer cells showed that the mechanisms underlying the enhancement of autophagy involve anti-EGFR agent-stimulated downregulation of HIF1 as well as Beclin2, which in turn releases Beclin1 from Beclin2 suppression; this demonstrated that autophagy is a protective cellular response to cetuximab treatment^[17]. Another study found that autophagy played different roles in the cetuximab-mediated treatment of different cancer cells, including vulvar squamous carcinoma cells, colorectal adenocarcinoma cells, and head and neck cancer cells. Autophagy protected cancer cells from death when apoptosis was strongly induced by cetuximab and enhanced cetuximab-induced apoptosis when

Table 2 Relationships of Beclin1, LC3, and 4E-BP1 with short-term efficacy of first-line cetuximab-containing therapy in 25 patients with wild-type KRAS

Factor			DCR		ORR		PFS	
			<i>n</i> (%)	<i>P</i> -value	<i>n</i> (%)	<i>P</i> -value	Time (mo)	<i>P</i> -value
25								
Beclin1	High	12 (48.0)	7 (58.3)	0.325	5 (41.6)	1.000	8.80	0.843
	Low	8 (32.0)	7 (87.5)		4 (50.0)		4.70	
	NA	5 (20.0)						
LC3	High	12 (48.0)	7 (58.3)	0.325	4 (33.3)	0.362	4.70	0.167
	Low	8 (32.0)	7 (87.5)		5 (62.5)		9.96	
	NA	5 (20.0)						
4E-BP1	High	15 (60.0)	12 (80.0)	0.560	9 (60.0)	0.303	5.32	0.410
	Low	5 (20.0)	3 (60.0)		1 (20.0)		7.36	
	NA	5 (20.0)						

LC3: Microtubule-associated protein 1A/B-light chain 3; 4E-BP1: 4E-binding protein 1; NA: Not applicable; DCR: Disease control rate; ORR: Overall response rate; PFS: Primary-progression-free survival from the initiation of first-line treatment to tumor progression, death from any cause, or the last follow-up.

only a minimal level of apoptosis was induced after cetuximab treatment^[18]. We also found that autophagy was activated by cetuximab in colon cancer cells, and cetuximab increased cell death through autophagy. Autophagy is involved in the process by which cetuximab inhibits cell proliferation^[8], but the negative or positive effects of cetuximab treatment on autophagy are controversial, and further studies are needed.

In our study, patients with metachronous metastasis showed significantly higher expression levels of Beclin1 than those with synchronous metastasis. Beclin1 overexpression might indicate late metastasis of CRC, which is consistent with the previous results observed in cells. Koneri *et al*^[19] transfected the *Beclin1* gene into HT29 colon cancer cells and found that ectopic *Beclin1* overexpression resulted in slow cell growth and G1 arrest with downregulated expression levels of cyclin E and phosphorylated Rb. This finding suggested that Beclin1 overexpression may reverse aggressive phenotypes and act as a tumor suppressor in CRC. Additionally, we found that patients with mutant KRAS had higher expression levels of the autophagic markers Beclin1 and LC3. Several studies have shown that autophagy is upregulated in cancers with KRAS mutations. Alves *et al*^[20] explained that KRAS effector pathways are involved in the regulation of autophagy through the MEK/ERK and PI3K/AKT/mTOR signaling pathways. Guo *et al*^[21] observed that cells with mutated KRAS require autophagy to maintain oxidative metabolism and tumorigenesis when nutrients are limiting.

The present study demonstrated that CRC tissues overexpressed LC3 compared with normal colorectal tissues, with a sensitivity and specificity of 71.4% and 91.3%, respectively, indicating that LC3 might be a means of distinguishing between normal and cancer tissues. This finding was supported by the results of a study involving 163 gastrointestinal cancer patients in which LC3 was differentially expressed in the cytoplasm of cancer cells and normal epithelial cells^[22]. The overexpression of LC3 could be interpreted in several ways. During the development and progression of cancers, cancer cells are often exposed to metabolic stress, such as insufficient nutrients or oxygen, because of their high proliferation rate and insufficient vascularization^[23]. It is thought that normal cells exhibit a basal level of autophagy to maintain cellular homeostasis. Moreover, increased autophagy in CRC cells may also play a crucial role in tumor metastasis and survival^[24]. We observed that Beclin1 and LC3 (0.657, $P < 0.001$) were significantly correlated in CRC tissue, and Beclin1 and 4E-BP1 (0.211, $P = 0.042$) were also significantly correlated. These results are the same as the conclusions drawn in a previous study^[25] and our experimental results *in vitro*.

As a type of programmed cell death, autophagy is closely related to chemotherapy resistance^[26]. Studies have shown that autophagy occurs abnormally in the chemotherapy courses for liver cancer, leukemia, lung cancer, CRC, and other tumors^[27-29]. In the present study, we attempted to identify the proteins involved in autophagy that could predict the efficacy of cetuximab. We observed that Beclin1, LC3, and 4E-BP1 expression levels were not related to the ORR, DCR, or PFS. Interestingly, the prognosis of patients with ACRC who received cetuximab plus

Table 3 Univariate and multivariate analyses of the influence of Beclin1, LC3, 4E-BP1, and clinicopathological factors on survival in 45 patients with wild-type KRAS who were treated with cetuximab

	OS			
	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Sex	0.860 (0.452, 1.636)	0.496		
Age	2.050 (0.831, 5.056)	0.111		
Family	1.568 (0.786, 3.128)	0.198		
Tumor location	0.933 (0.463, 1.880)	0.353		
Pathological grade	2.273 (1.549, 3.335)	0.000	2.421 (1.397, 4.197)	0.002
T stage	1.240 (0.977, 1.573)	0.072	1.749 (1.190, 2.569)	0.004
N stage	1.159 (0.611, 2.200)	0.409		
Synchronous/metachronous metastasis	1.493 (0.793, 2.813)	0.081		
Beclin1 expression	0.466 (0.223, 0.971)	0.037	0.209 (0.064, 0.685)	0.010
LC3 expression	0.541 (0.262, 1.122)	0.094		
4E-BP1 expression	2.926 (1.110, 7.713)	0.024	6.385 (1.764, 23.112)	0.005

Bold values indicate $P < 0.05$; tumor location, left side or right side. CI: Confidence interval; CRC: Colorectal cancer; HR: Hazard ratio; LC3: Microtubule-associated protein 1A/B-light chain 3; 4E-BP1: 4E-binding protein 1.

chemotherapy was not only correlated with clinical and pathological factors, such as the degree of pathological grade and T stage but also with Beclin1 and 4E-BP1 expression levels. A high expression level of Beclin1 and low expression level of 4E-BP1 independently predicted longer OS in ACRC patients, which might indicate a positive relationship between autophagy and cetuximab efficacy. This finding is consistent with our results in colon cancer cells, which showed that autophagy was enhanced in cells treated with cetuximab, and autophagy increased the effect of cetuximab on inhibiting cell proliferation. It is necessary to design a more rigorous clinical trial to confirm our primary results. We believe that both Beclin1 and 4E-BP1 could be used as independent predictors of cetuximab efficacy.

Our study had several shortcomings. First, it was retrospective, and the number of cases was small. Second, our study focused on the associations between the levels of expression of Beclin-1, LC3, and 4E-BP1 and the efficacy of cetuximab in patients with ACRC with wild-type KRAS; the statuses of NRAS and HRAS were not available. However, we identified two new independent predictors of the efficacy of cetuximab treatment in ACRC patients with wild-type KRAS, Beclin1 and 4E-BP1, and, to the best of our knowledge, our report of the association between 4E-BP1 and cetuximab is novel. Furthermore, our data were obtained from the clinical setting; therefore, the findings are important despite the small sample size.

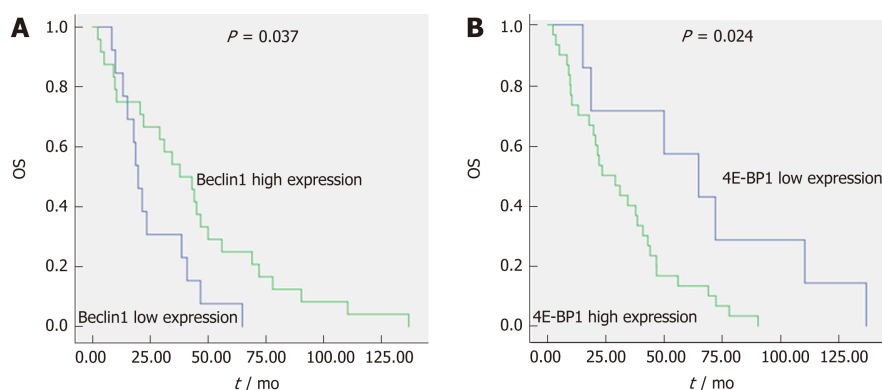


Figure 4 The expression levels of Beclin1 and 4E-binding protein 1 are independent prognostic factors for overall survival of advanced colorectal cancer patients initially treated with cetuximab-containing chemotherapy. A: The median overall survival (OS) of patients with low and high Beclin1 expression (low expression was defined as an immunohistochemistry score ≤ 0 , and high expression was defined as a score > 0) was 19.65 mo vs 37.82 mo, respectively. B: The median OS of patients with low and high 4E-BP1 expression (low expression was defined as an immunohistochemistry score ≤ 0 , and high expression was defined as a score > 0) was 64.82 mo vs 23.66 mo, respectively. OS: Overall survival; 4E-BP1: 4E-binding protein 1.

ARTICLE HIGHLIGHTS

Research background

Great effects have been made in exploring the treatment of colorectal cancer (CRC), but the effectiveness is still limited due to the limited drug selection. Cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR), significantly increased OS (overall survival) of advanced CRC patients (ACRC). But the response rate (RR) is only 40%-60% for patients with wild-type KRAS. Autophagy, showing a key role in the cancer progression and induced by EGFR siRNA, may allow to develop effective strategies for improving cetuximab effect in CRC, while there have been few studies regarding the predictive role of autophagy in ACRC patients with wild-type KRAS.

Research motivation

Autophagy-related factors were investigated in ACRC with wild-type KRAS to find new biomarkers for cetuximab efficacy, which may offer the potential for developing novel therapeutic strategies for these patients.

Research objectives

The role of Beclin1, microtubule-associated protein 1A/B-light chain 3 (LC3), and 4E-binding protein 1 (4E-BP1), the key factors in autophagy, in predicting the efficacy of cetuximab in ACRC with wild-type KRAS was explored. It was found that Beclin1 and 4E-BP1 were independent prognostic factors for overall survival (OS). In the future research, elucidating the molecular mechanism of association of these factors with cetuximab may help us find more new biomarkers and make cetuximab treatment more accurate.

Research methods

We detected the expression of autophagy-related proteins 4E-BP1, Beclin-1, and LC3 in CRC samples and adjacent non-tumor tissues by immunohistochemistry. And the three proteins were examined by Western blot in CRC cells. The effect of autophagy on cetuximab-treated cancer cells was confirmed by MTT assay. The associations of Beclin1, LC3, and 4E-BP1 expression in tumor tissue with the efficacy of cetuximab-based therapy were analyzed.

Research results

Autophagosome formation was observed in colon cancer cells, with LC3 and 4E-BP1 upregulated, and autophagy increased the efficacy of cetuximab. Beclin1 expression was significantly correlated with LC3 and 4E-BP1 expression in ACRC tissues. LC3 was significantly overexpressed in tumor tissues compared to normal tissues. In patients with wild-type KRAS, the expression levels of Beclin1 and 4E-BP1 were independent prognostic factors for OS. The molecular mechanism of association of these factors with cetuximab effect and their weight coefficients in prognostic models need further study.

Research conclusions

LC3 is overexpressed in tumor tissues, and Beclin1 and 4E-BP1 are significant predictors of OS in wild-type KRAS ACRC treated with cetuximab. Autophagy has a role in improving the effects of cetuximab on colon cancer cell.

Research perspectives

Autophagy might play a new role to predict the effects of cetuximab in ACRC with wild-type KRAS in the future, and inducing or blocking autophagy may be a new way to improve cetuximab treating ACRC.

REFERENCES

- Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R, Focan C, Chollet P, Llory JF, Letourneau Y, Coudert B, Bertheaut-Cvitkovic F, Larregain-Fournier D, Le Rol A, Walter S, Adam R, Misset JL, Lévi F. Phase III multicenter randomized trial of oxaliplatin added to chromomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2000; **18**: 136-147 [PMID: 10623704 DOI: 10.1200/JCO.2000.18.1.136]
- Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF, Schilsky RL. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009; **27**: 2091-2096 [PMID: 19188670 DOI: 10.1200/JCO.2009.21.9170]
- Venook AP, Niedzwiecki D, Lenz HJ, Innocenti F, Fruth B, Meyerhardt JA, Schrag D, Greene C, O'Neil BH, Atkins JN, Berry S, Polite BN, O'Reilly EM, Goldberg RM, Hochster HS, Schilsky RL, Bertagnoli MM, El-Khoueiry AB, Watson P, Benson AB, Mulkerin DL, Mayer RJ, Blanke C. Effect of First-Line Chemotherapy Combined With Cetuximab or Bevacizumab on Overall Survival in Patients With KRAS Wild-Type Advanced or Metastatic Colorectal Cancer: A Randomized Clinical Trial. *JAMA* 2017; **317**: 2392-2401 [PMID: 28632865 DOI: 10.1001/jama.2017.7105]
- Heinemann V, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Hielscher J, Scholz M, Müller S, Link H, Niederle N, Rost A, Höfkes HG, Moehler M, Lindig RU, Modest DP, Rossius L, Kirchner T, Jung A, Stintzing S. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol* 2014; **15**: 1065-1075 [PMID: 25088940 DOI: 10.1016/S1470-2045(14)70330-4]
- Alan P, Venook DN. Impact of primary (1st) tumor location on Overall Survival (OS) and Progression Free Survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): Analysis of All RAS wt patients on CALGB/SWOG 80405 (Alliance) [Abstract]. ESMO Congress, 2016.
- Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tamasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006; **66**: 3992-3995 [PMID: 16618717 DOI: 10.1158/0008-5472.CAN-06-0191]
- Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol* 2013; **15**: 713-720 [PMID: 23817233 DOI: 10.1038/ncb2788]
- Kenific CM, Thorburn A, Debnath J. Autophagy and metastasis: another double-edged sword. *Curr Opin Cell Biol* 2010; **22**: 241-245 [PMID: 19945838 DOI: 10.1016/j.cceb.2009.10.008]
- Zhang J, Yang Z, Xie L, Xu L, Xu D, Liu X. Statins, autophagy and cancer metastasis. *Int J Biochem Cell Biol* 2013; **45**: 745-752 [PMID: 23147595 DOI: 10.1016/j.biocel.2012.11.001]
- Johnson SM, Gulhati P, Rampy BA, Han Y, Rychahou PG, Doan HQ, Weiss HL, Evers BM. Novel expression patterns of PI3K/Akt/mTOR signaling pathway components in colorectal cancer. *J Am Coll Surg* 2010; **210**: 767-776, 776-778 [PMID: 20421047 DOI: 10.1016/j.jamcollsurg.2009.12.008]
- Ekstrand AI, Jönsson M, Lindblom A, Borg A, Nilbert M. Frequent alterations of the PI3K/AKT/mTOR pathways in hereditary nonpolyposis colorectal cancer. *Fam Cancer* 2010; **9**: 125-129 [PMID: 19731079 DOI: 10.1007/s10689-009-9293-1]
- Pause A, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC, Sonenberg N. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* 1994; **371**: 762-767 [PMID: 7935836 DOI: 10.1038/371762a0]
- Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; **122**: 927-939 [PMID: 16179260 DOI: 10.1016/j.cell.2005.07.002]
- Wild P, McEwan DG, Dikic I. The LC3 interactome at a glance. *J Cell Sci* 2014; **127**: 3-9 [PMID: 24345374 DOI: 10.1242/jcs.140426]
- Weihua Z, Tsan R, Huang WC, Wu Q, Chiu CH, Fidler IJ, Hung MC. Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* 2008; **13**: 385-393 [PMID: 18455122 DOI: 10.1016/j.ccr.2008.03.015]
- Guo GF, Jiang WQ, Zhang B, Cai YC, Xu RH, Chen XX, Wang F, Xia LP. Autophagy-related proteins Beclin-1 and LC3 predict cetuximab efficacy in advanced colorectal cancer. *World J Gastroenterol* 2011; **17**: 4779-4786 [PMID: 22147978 DOI: 10.3748/wjg.v17.i43.4779]
- Li X, Fan Z. The epidermal growth factor receptor antibody cetuximab induces autophagy in cancer cells by downregulating HIF-1 α and Bcl-2 and activating the beclin 1/hVps34 complex. *Cancer Res* 2010; **70**: 5942-5952 [PMID: 20634405 DOI: 10.1158/0008-5472.CAN-10-0157]
- Li X, Lu Y, Pan T, Fan Z. Roles of autophagy in cetuximab-mediated cancer therapy against EGFR. *Autophagy* 2010; **6**: 1066-1077 [PMID: 20864811 DOI: 10.4161/auto.6.8.13366]
- Koneri K, Goi T, Hirono Y, Katayama K, Yamaguchi A. Beclin 1 gene inhibits tumor growth in colon cancer cell lines. *Anticancer Res* 2007; **27**: 1453-1457 [PMID: 17595761]
- Alves S, Castro L, Fernandes MS, Francisco R, Castro P, Priault M, Chaves SR, Moyer MP, Oliveira C, Seruca R, Côrte-Real M, Sousa MJ, Preto A. Colorectal cancer-related mutant KRAS alleles function as positive regulators of autophagy. *Oncotarget* 2015; **6**: 30787-30802 [PMID: 26418750 DOI: 10.18632/oncotarget.5021]
- Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, Kamphorst JJ, Chen G, Lemons JM, Karantza V, Collier HA, Dipaola RS, Gelinas C, Rabinowitz JD, White E. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev* 2011; **25**: 460-470 [PMID: 21317241 DOI: 10.1101/gad.2016311]
- Yoshioka A, Miyata H, Doki Y, Yamasaki M, Sohma I, Gotoh K, Takiguchi S, Fujiwara Y, Uchiyama Y, Monden M. LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. *Int J Oncol* 2008; **33**: 461-468 [PMID: 18695874]
- Choi AM, Rytter SW, Levine B. Autophagy in human health and disease. *N Engl J Med* 2013; **368**: 1845-1846 [PMID: 23656658 DOI: 10.1056/NEJMc1303158]
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
- Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 2004;

- 119: 753-766 [PMID: 15607973 DOI: 10.1016/j.cell.2004.11.038]
- 26 **Zou Z**, Yuan Z, Zhang Q, Long Z, Chen J, Tang Z, Zhu Y, Chen S, Xu J, Yan M, Wang J, Liu Q. Aurora kinase A inhibition-induced autophagy triggers drug resistance in breast cancer cells. *Autophagy* 2012; **8**: 1798-1810 [PMID: 23026799 DOI: 10.4161/auto.22110]
 - 27 **Xu N**, Zhang J, Shen C, Luo Y, Xia L, Xue F, Xia Q. Cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell. *Biochem Biophys Res Commun* 2012; **423**: 826-831 [PMID: 22713463 DOI: 10.1016/j.bbrc.2012.06.048]
 - 28 **Li N**, Li X, Li S, Zhou S, Zhou Q. Cisplatin-induced downregulation of SOX1 increases drug resistance by activating autophagy in non-small cell lung cancer cell. *Biochem Biophys Res Commun* 2013; **439**: 187-190 [PMID: 23994634 DOI: 10.1016/j.bbrc.2013.08.065]
 - 29 **Yang M**, Zeng P, Kang R, Yu Y, Yang L, Tang D, Cao L. S100A8 contributes to drug resistance by promoting autophagy in leukemia cells. *PLoS One* 2014; **9**: e97242 [PMID: 24820971 DOI: 10.1371/journal.pone.0097242]



Basic Study

Extract of *Cycas revoluta* Thunb. enhances the inhibitory effect of 5-fluorouracil on gastric cancer cells through the AKT-mTOR pathway

Xing-Liang Cui, Ke-Ji Li, Hai-Xia Ren, Yong-Jian Zhang, Xiao-Dong Liu, Bao-Guo Bu, Lei Wang

ORCID number: Xing-Liang Cui (0000-0003-1287-5632); Ke-Ji Li (0000-0003-2192-0351); Hai-Xia Ren (0000-0002-7786-3571); Yong-Jian Zhang (0000-0002-3703-9932); Xiao-Dong Liu (0000-0003-0916-3378); Bao-Guo Bu (0000-0003-0979-6781); Lei Wang (0000-0003-2420-0237).

Author contributions: Wang L and Cui XL designed the research; Li KJ, Ren HX, Zhang YJ and Cui XL performed the research; Liu XD and Bu BG analyzed the data; Wang L and Cui XL wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of the Affiliated Hospital of Hebei University of Engineering.

Conflict-of-interest statement: We declare no conflict of interest.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Xing-Liang Cui, Hai-Xia Ren, Yong-Jian Zhang, Xiao-Dong Liu, Bao-Guo Bu, Department of Gastroenterology, Affiliated Hospital of Hebei University of Engineering, Handan 056001, Hebei Province, China

Ke-Ji Li, Department of Surgery, Affiliated Hospital of Hebei University of Engineering, Handan 056001, Hebei Province, China

Lei Wang, Department of Pathology, Medical College of Hebei University of Engineering, Handan 056000, Hebei Province, China

Corresponding author: Lei Wang, MA, MSc, Associate Professor, Department of Pathology, Medical College of Hebei University of Engineering, No. 42 Congtai Road, Handan 056000, Hebei Province, China. wangleinv1975@126.com

Telephone: +86-13131048342

Fax: +86-310-8575116

Abstract

BACKGROUND

Gastric cancer is one of the most common and deadly malignancies worldwide. Despite recent medical progress, the 5-year survival rate of gastric cancer is still unsatisfactory. 5-fluorouracil (5-Fu) is one of the first-line antineoplastic treatments for gastric cancer, as it can effectively induce cancer cell apoptosis. However, the effect of 5-Fu is limited due to drug resistance of the malignant tumor. Previous studies have reported that Sotetsuflavone from *Cycas revoluta* Thunb. can markedly suppress lung cancer cell proliferation by apoptosis, though its effect on gastric cancer remains unknown.

AIM

To investigate the inhibitory effect of *Cycas revoluta* Thunb. and to determine whether it can overcome gastric cancer cell drug resistance to 5-Fu.

METHODS

Cell viability was examined to determine whether the natural extract of *Cycas revoluta* Thunb. induced gastric cancer cell death. The half-maximal effective concentration and the half-maximal lethal concentration were calculated. Wound-healing and transwell assays were performed to examine gastric cancer cell motility. Clonogenic assays were performed to investigate the synergistic effects of *Cycas revoluta* Thunb. with 5-Fu, and apoptotic bodies were detected by Hoechst staining. Western blotting was performed to examine the expression of related proteins and to investigate the molecular mechanism of *Cycas revoluta*

Manuscript source: Unsolicited manuscript

Received: February 21, 2019

Peer-review started: February 21, 2019

First decision: March 5, 2019

Revised: March 10, 2019

Accepted: March 16, 2019

Article in press: March 16, 2019

Published online: April 21, 2019

P-Reviewer: Kazuya S, Tanabe S

S-Editor: Ma RY

L-Editor: Filipodia

E-Editor: Ma YJ



Thunb.-induced cancer cell apoptosis. The expressions of proteins, including mammalian target of rapamycin (mTOR) and p-AKT, were detected in different combinations of treatments for 48 h, then analyzed by ECL detection.

RESULTS

Gastric cancer cells were more sensitive to the natural extract of *Cycas revoluta* Thunb. compared to normal gastric epithelial cells, and the extract effectively inhibited gastric cancer cell migration and invasion. The extract improved the anti-cancer effect of 5-Fu by enhancing the chemosensitization of gastric cancer cells. Extract plus 5-Fu further reduced the expression of the drug-resistance-related proteins p-AKT and mTOR after 48 h compared to 5-Fu alone. Compared to 5-Fu treatment alone, mTOR and p-AKT expression was significantly reduced by about 50% and 75%, respectively. We also found that the natural extract of *Cycas revoluta* Thunb. further increased 5-Fu-induced gastric cancer cell apoptosis. Expression of apoptosis-related protein X-linked inhibitor of apoptosis protein and apoptosis inducing factor were significantly reduced and increased, respectively, in the 5-Fu-resistant gastric cancer line SGC-7901/R treated with extract plus 5-Fu, while the expression of survivin did not change.

CONCLUSION

The natural extract of *Cycas revoluta* Thunb. effectively inhibited gastric cancer cell growth and enhanced the anti-cancer effect of 5-Fu through the AKT-mTOR pathway.

Key words: Gastric cancer; 5-fluorouracil; *Cycas revoluta* Thunb.; Apoptosis

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: 5-fluorouracil (5-Fu) is an effective treatment for gastric cancer, which is one of the most common and deadly malignancies worldwide. However, the effect of 5-Fu is limited by the drug resistance of gastric cancer. Here, we report that natural extract of *Cycas revoluta* Thunb. effectively inhibits gastric cancer cell growth, migration and invasion. Furthermore, it can be used in combination with 5-Fu to enhance its anti-cancer effects through the AKT-mTOR pathway.

Citation: Cui XL, Li KJ, Ren HX, Zhang YJ, Liu XD, Bu BG, Wang L. Extract of *Cycas revoluta* Thunb. enhances the inhibitory effect of 5-fluorouracil on gastric cancer cells through the AKT-mTOR pathway. *World J Gastroenterol* 2019; 25(15): 1854-1864

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1854.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1854>

INTRODUCTION

Gastric cancer remains the fourth most common malignancy diagnosed worldwide, especially in Eastern Asia, Eastern Europe and Central and South America^[1-3]. It also is the third main cause of death related to malignancy, just behind lung and liver cancer^[4]. In 2012, there were about 951,600 new patients diagnosed with gastric cancer, and over 700,000 deaths related to gastric cancer have been recorded^[5].

With a broad spectrum of activity against malignant cells, 5-fluorouracil (5-Fu) is commonly employed against gastric, liver and colorectal cancers^[6-8]. As a prevalent chemotherapeutic drug in clinical practice, 5-Fu can inhibit cancer cell proliferation and DNA replication, including gastric, breast and colorectal cancer cells, by inhibiting thymidylate synthase from synthesizing thymine, which ultimately induces apoptosis^[9-11].

Apoptosis is an important molecular process for stable and orderly human growth. It is strictly controlled and its dysregulation is linked to many diseases, including cancer^[12,13]. This complex process is regulated by a series of key proteins, such as X-linked inhibitor of apoptosis protein (XIAP), apoptosis inducing factor (AIF) and survivin. XIAP is a strong apoptotic regulator^[14-18] and inhibits caspase-3, -7, and -9, which are all part of the mammalian apoptotic signaling pathway. AIF is released and promotes apoptosis by intrinsic signaling cascades^[19,20] when mitochondria respond to

apoptotic stimuli, such as the translocation of BH3 interacting domain death agonist (Bid)^[21]. Survivin is a unique inhibitor of apoptosis (IAP), as it does not directly interact with caspases but with some adaptors or cofactors^[22-26].

Although 5-Fu is widely used as an anticancer drug, it has some serious problems, such as low effective response rate and severe side effects. One of the most critical concerns is the increasing cases of drug resistant malignant tumor. Many 5-Fu drug-resistance-related proteins have been identified. For example, P-glycoprotein (P-gp) functions as a molecular 'pump' to expel chemotherapy drugs from the inside of the cell, and resistance to 5-Fu can be reversed when P-gp expression is reduced^[27]. AKT is considered a key protein in the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. It is activated at the plasma membrane by phosphorylation of Thr308 and Ser473 residues, and it can phosphorylate various downstream substrates related to drug sensitivity^[28]. Mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a main downstream effector of the PI3K/AKT signaling pathway^[29]. It has also been reported that 5-Fu drug resistance may be mediated by the AKT-mTOR pathway^[30,31].

Fortunately, drug resistance can be reduced when used in combination with other compounds. Previous studies have reported that chemosensitization of cancer cells to 5-Fu can be achieved by using dietary fats, particularly n-3 polyunsaturated fatty acids (PUFAs), puerarin, iRGD, and troxerutin^[32-35].

Some Chinese medicines, including *Cycas revoluta* Thunb., have demonstrated chemosensitization effects, which provides some novel insights for anti-cancer treatments. According to ancient records, *Cycas revoluta* Thunb. is an evergreen palm woody plant^[36] with useful medicinal value, such as reducing fever and alleviating congestion. A component of the extract, Sotetsuflavone, was identified to have strong anti-tumor activity against lung cancer cells^[37,38]. Because it can effectively induce lung cancer cell apoptosis, we studied whether it could inhibit growth, migration and invasion of malignant gastric cancer cells. Furthermore, we evaluated its potential for chemosensitization in combination with 5-Fu and investigated its potential molecular mechanism.

MATERIALS AND METHODS

Materials, reagents and antibodies

The leaf of *Cycas revoluta* Thunb. was acquired from AnGuo herbal medicine market in HeBei Province of China. DMEM (12800017) and trypsin (25300054) were purchased from Life Technologies (Carlsbad, CA, United States). MTT (M2128) and 5-Fu (F6627) were purchased from Sigma (Saint Louis, MO, United States). Antibodies against p-gp, XIAP, p-Akt, AIF, mTOR, survivin, and GAPDH were purchased from Abcam (Shanghai, China).

Extraction of *Cycas revoluta* Thunb.

The powder of the *Cycas revoluta* Thunb. leaf was extracted by reflux extraction with 80% ethanol. The extracts were collected and concentrated under reduced pressure until there was no irritating odor. The product was dissolved in water and filtered. The filtrate was then extracted with dichloromethane, concentrated under reduced pressure and dried.

Cell culture

The MGC-803, SGC-790, and HGC-27 cell lines were obtained from ATCC (Manassas, VA, United States). SNU-5 cells were obtained from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. GES-1 cells were obtained from the Genetics department of Beijing Cancer Research Institute. The SGC-7901/R line was obtained from Shanghai Institute of Medicine, Chinese Academy of Sciences. Cell lines were cultured at 5% CO₂ and 37°C in DMEM medium containing fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 U/mL). Cells were used and analyzed at logarithmic growth phase.

Cell viability and clonogenic assay

Cells were grown in 96-well plates for cell viability tests. Gastric cell lines were treated with the extraction of *Cycas revoluta* Thunb. or 5-Fu after 24 h. The viability rate was measured by ATPlite assay (Perkin Elmer, Waltham, MA, United States)^[39]. One thousand cells with different treatments were seeded into culture dish in clonogenic assays. The number of colonies was measured after 9 d.

Wound-healing migration assay

MGC803 and HGC27 cells were cultured in six-well tissue culture plates and tested

when the confluence reached 80%. Wounds were created by sterile pipette tips (10- μ L), and loosely attached cells were washed out with phosphate-buffered saline. Light microscope was employed to photograph the progression of cell migration at different times, and the number of migrated cells was calculated in the scratched region.

Transwell invasion assay

Twenty-four-well Boyden chambers with 8-mm pore size filters (BD Falcon, Corning-Costar, New York, NY, United States) were used for this assay. Samples were suspended and seeded in the insert chamber with DMEM/F12 media and were incubated at 37°C in 5% CO₂ for 24 h to allow cells to migrate into the bottom, which contained DMEM/F12 media and 10% FBS. The number of cells that migrated was counted after staining with DAPI.

Western blotting analysis

Total protein was extracted with NP40 lysis buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (commonly known as SDS-PAGE) and polyvinylidene difluoride membrane were used for separating and transferring samples. Membranes were blocked in tris buffered saline tween-20 (TBST) solution containing 5% nonfat dry milk for 1 h. Primary antibodies were added overnight at 4°C and then rinsed three times for 10 min in TBST. Membranes were incubated in secondary antibodies for 1.5 h before being washed. The results were analyzed by ECL detection system.

Cell apoptosis and Hoechst 33258 staining

After treatment with extract, samples were collected and washed with precooled phosphate-buffered saline (PBS). They were resuspended in 300 μ L of binding buffer diluted in PBS. After incubation for 10 min, 5 μ L Annexin V-FITC was added, followed by 5 μ L PI for 5 min. Samples were then rinsed three times with precooled PBS and fixed in 4% paraformaldehyde for 30 min. After washing with PBS for three times, Hoechst was added to the plate dropwise and incubated at room temperature for 15 min. The results were observed under a fluorescence microscope and photographed after a final PBS wash.

Statistical analysis

The statistical methods used in this study were reviewed by Zhimin Shi from the College of Hebei University of Engineering. Experiments were repeated at least three times, and the data were processed by SPSS 20.0 statistical software. The standard deviation and Least Significant Difference were calculated by Student-Newman-Keuls test or Dunnett T3 test, in which ^a*P* < 0.05 and ^b*P* < 0.01.

RESULTS

Gastric cancer cell growth was inhibited by *Cycas revoluta* Thunb. extract

To investigate tumor inhibition effects of different doses (0 μ g/mL-350 μ g/mL) of *Cycas revoluta* Thunb. extract, we performed cell viability assays. The results showed that *Cycas revoluta* Thunb. extract significantly inhibited gastric cancer cell viability after 24 h, especially at the low and medium doses (0 μ g/mL-250 μ g/mL) (Figure 1A). For treatments under 250 μ g/mL extract, gastric cancer cell viability (MGC-803, SGC-790, HGC-27 and SUN-5) dramatically decreased with increasing concentrations of extract, while that of normal human gastric epithelial cells (GES-1a0) remained stable, which suggested that gastric cancer cells were more sensitive to *Cycas revoluta* Thunb. natural extract than normal gastric cells. We then analyzed the half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) of all cell lines (Figure 1B). The EC50 values of gastric cancer cells ranged from 176.44 μ g/mL to 194.88 μ g/mL and the LC50 values ranged from 135.23 μ g/mL to 152.20 μ g/mL. Compared to the EC50 (291.32 μ g/mL) and LC50 (280.27 μ g/mL) values for GES-1 cells, the *Cycas revoluta* Thunb. Extract was obviously more effective against gastric cancer cells. Additionally, high concentrations of extract (250 μ g/mL-350 μ g/mL), the viability rate of gastric cancer cells increased, which may be due to a screening effect for resistant cells or other adaptive mechanism.

***Cycas revoluta* Thunb. extract reduced gastric cancer cell migration and invasion**

To determine the effect of *Cycas revoluta* Thunb. natural extract on gastric cancer cell migration, we performed wound-healing assays. MGC-803 and HGC-27 gastric cancer cell lines were selected for the test, and cells were treated with a low dose of extract (60 μ g/mL), which reduced cell viability by about 20%. Our results showed that *Cycas revoluta* Thunb. extract significantly reduced gastric cancer cell migration after 24 h of

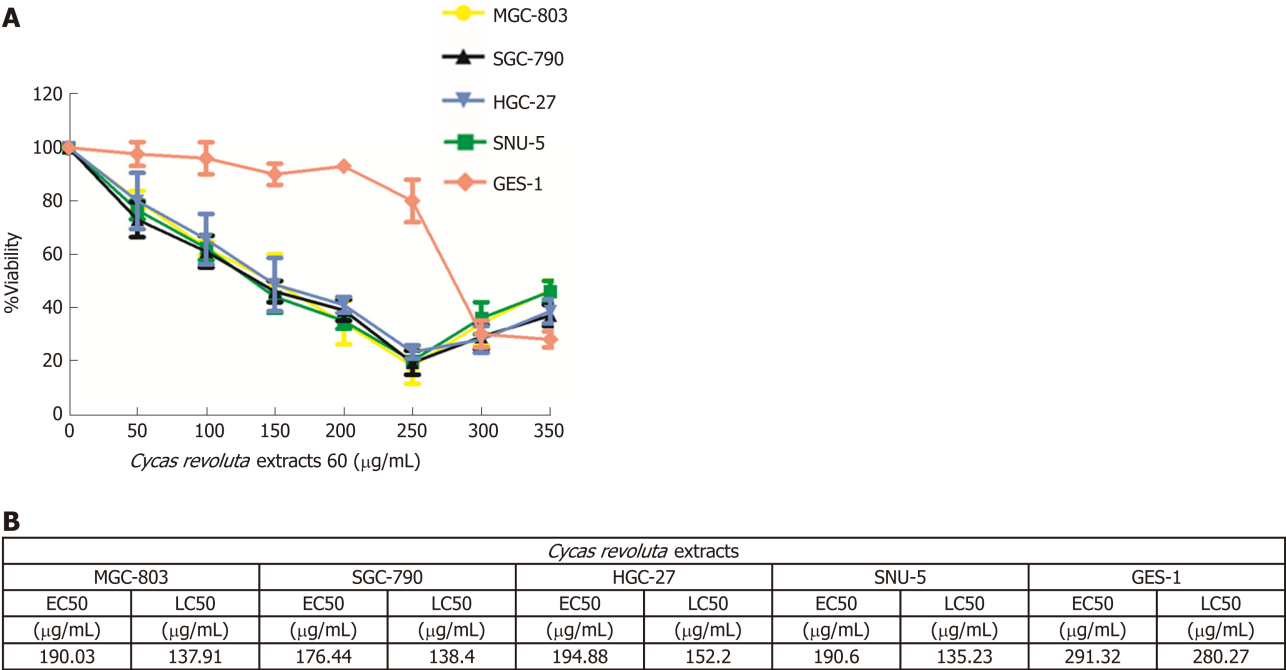


Figure 1 *Cycas revoluta* Thunb. extract effectively inhibits gastric cancer cell growth. A: Dose-response assays of the extract. Gastric cancer cells were analyzed after treatment with increasing concentrations of the extract for 24 h. The percentage of cell viability was normalized to the control, and three independent experiments were performed for calculating \pm STDEV; B: Half-maximal effective concentration and the half-maximal lethal concentration for each cell line. EC50: Half-maximal effective concentration; LC50: Half-maximal lethal concentration.

extract treatment, especially for MGC-803 cells, whose wound width was over twice that of control cells (Figure 2A). To further investigate its effect on gastric cancer cell invasion, we performed transwell invasion assays. *Cycas revoluta* Thunb. natural extract markedly reduced the invasion ability of both MGC-803 and HGC-27 cell lines (Figure 2B). Taken together, these results demonstrate that the *Cycas revoluta* Thunb. natural extract effectively inhibited malignant gastric cancer cell migration and invasion.

***Cycas revoluta* Thunb. extract enhanced the anti-cancer effect of 5-Fu by chemosensitization**

To determine whether *Cycas revoluta* Thunb. natural extract can be used in combination with other anti-cancer drugs, we chose 5-Fu, one of the most widely used chemotherapy drugs, for clonogenic assays.

By assessing colony formation ability, we found that although the inhibitory effect of 5-Fu was stronger than that of the extract, combining the two drugs enhanced the inhibitory effect of 5-Fu (Figure 3A).

To investigate whether the increased inhibitory effect was due to chemosensitization, we performed cell viability assays with a low dose of extract (60 µg/mL) and increasing doses of 5-Fu for 24 h. We found that the *Cycas revoluta* Thunb. natural extract significantly sensitized gastric cancer cells to 5-Fu (Figure 3B). The EC50 and LC50 values dramatically decreased in MGC-803 (1.6 times and 2.8 times) and HGC-27 cells (1.8 times and 3.5 times), suggesting that *Cycas revoluta* Thunb. natural extract had additive and synergistic effects with 5-Fu in inhibiting gastric cancer cells. To further confirm this result, we examined the expression of three key drug-resistance-related proteins including p-gp, p-AKT and mTOR by western blot. We found that all three proteins significantly decreased when 5-Fu was used in combination with *Cycas revoluta* Thunb. natural extract (Figure 3C), which suggested reduced drug-resistance of gastric cancer cells.

***Cycas revoluta* Thunb. extract mediates 5-Fu chemosensitization through apoptosis**

Because the Sotetsuflavone in *Cycas revoluta* Thunb. induced lung cancer cell apoptosis, we performed Hoechst 33258 staining in the 5-Fu-resistant gastric cancer line SGC-7901/R after treatment with the extract, 5-Fu or both. Both the extract and 5-Fu induced SGC-7901/R cell apoptosis, demonstrating that *Cycas revoluta* Thunb. extract can similarly induce gastric cancer cell apoptosis (Figure 4A). Moreover, the combination of 5-Fu and extract dramatically increased the extent of apoptosis (Figure 4A), suggesting that the *Cycas revoluta* Thunb. extract-induced chemosensitization of

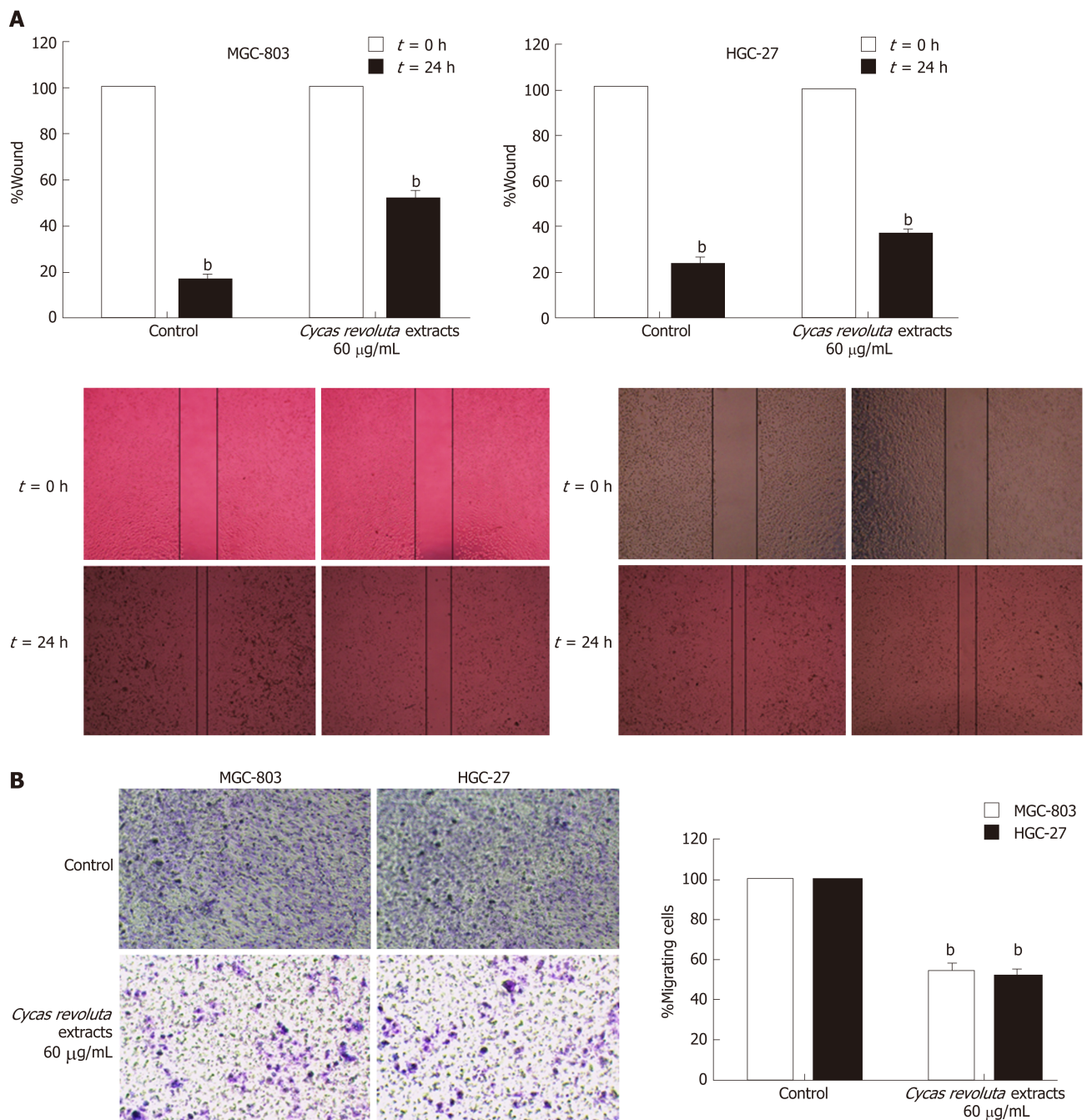


Figure 2 The effect of *Cycas revoluta* Thunb. natural extract on cell migration and invasion. A: Wound healing assays; and B: transwell invasion assays were performed with a low dose of extract (60 μ g/mL). The wound width or migrated cells normalized to the control is shown in histograms, and the error bars represent \pm STDEV from $n = 3$ independent experiments, ^b $P < 0.01$.

5-Fu may be mediated *via* apoptosis. To confirm this hypothesis, we examined the expressional level of three important proteins involved in the apoptosis pathway. XIAP and AIF expression significantly decreased and increased, respectively, while the survivin expression remained stable (Figure 4B). This result demonstrated that the chemosensitive enhancement of 5-Fu and *Cycas revoluta* Thunb. extract may be due to further activation of apoptosis.

DISCUSSION

As one of the most common cancers, gastric cancer has been frequently diagnosed and has led to thousands of deaths worldwide. Around 500,000 people in China died from it just in 2015^[3]. Although 5-Fu is often employed as chemotherapy against gastric cancer, its effect varies, likely due to the drug resistance of gastric tumors^[10,11,27]. To overcome the problem of increasing drug resistance, 5-Fu is usually used with other

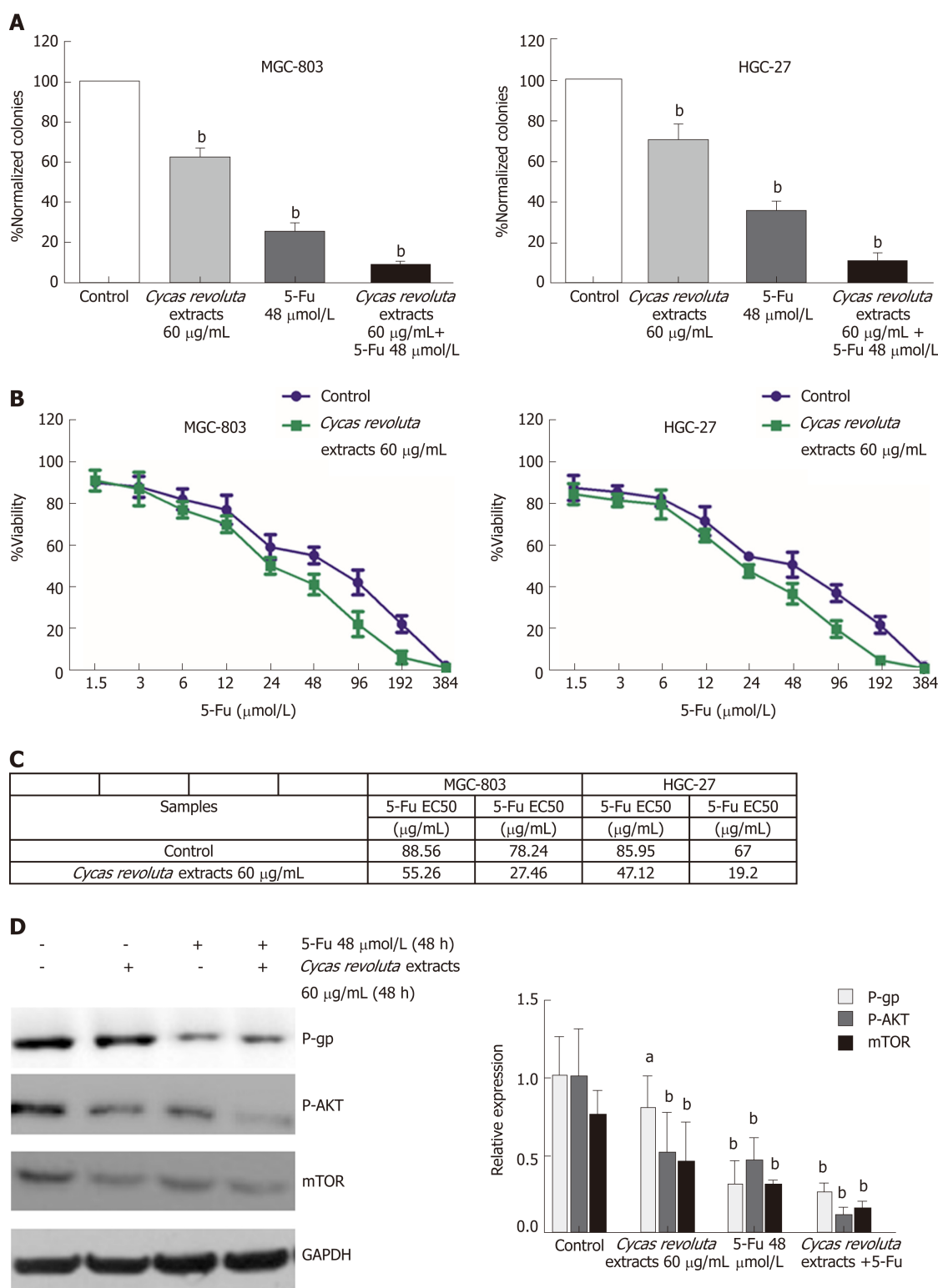


Figure 3 The chemosensitization of *Cycas revoluta* Thunb. natural extract with 5-fluorouracil. A: Clonogenic assays. The error bars represent \pm STDEV from $n = 3$ independent experiments, $^bP < 0.01$; B: Dose-response assays of 5-fluorouracil (5-Fu) with the extract; C: The half-maximal effective concentration and the half-maximal lethal concentration of 5-Fu in combination with the extract. The error bars represent \pm STDEV from $n = 3$ independent experiments; D: Western blot analysis of drug-resistance-related proteins. GAPDH was used as a control. Relative expressions are shown in the left histogram, and the error bars represent \pm STDEV from $n = 3$ independent experiments $^aP < 0.05$, $^bP < 0.01$. 5-Fu: 5-fluorouracil; EC50: Half-maximal effective concentration; LC50: Half-maximal lethal concentration; mTOR: Mammalian target of rapamycin; P-gp: P-glycoprotein.

compounds to enhance cancer cell sensitivity. According to previous studies, the traditional Chinese medicine *Cycas revoluta* Thunb. exhibited this potential synergistic effect^[37,38]. Therefore, we investigated its inhibitory effect and the effect of chemosensitive enhancement with 5-Fu in gastric cancer.

In this study, we found that *Cycas revoluta* Thunb. extract effectively inhibited

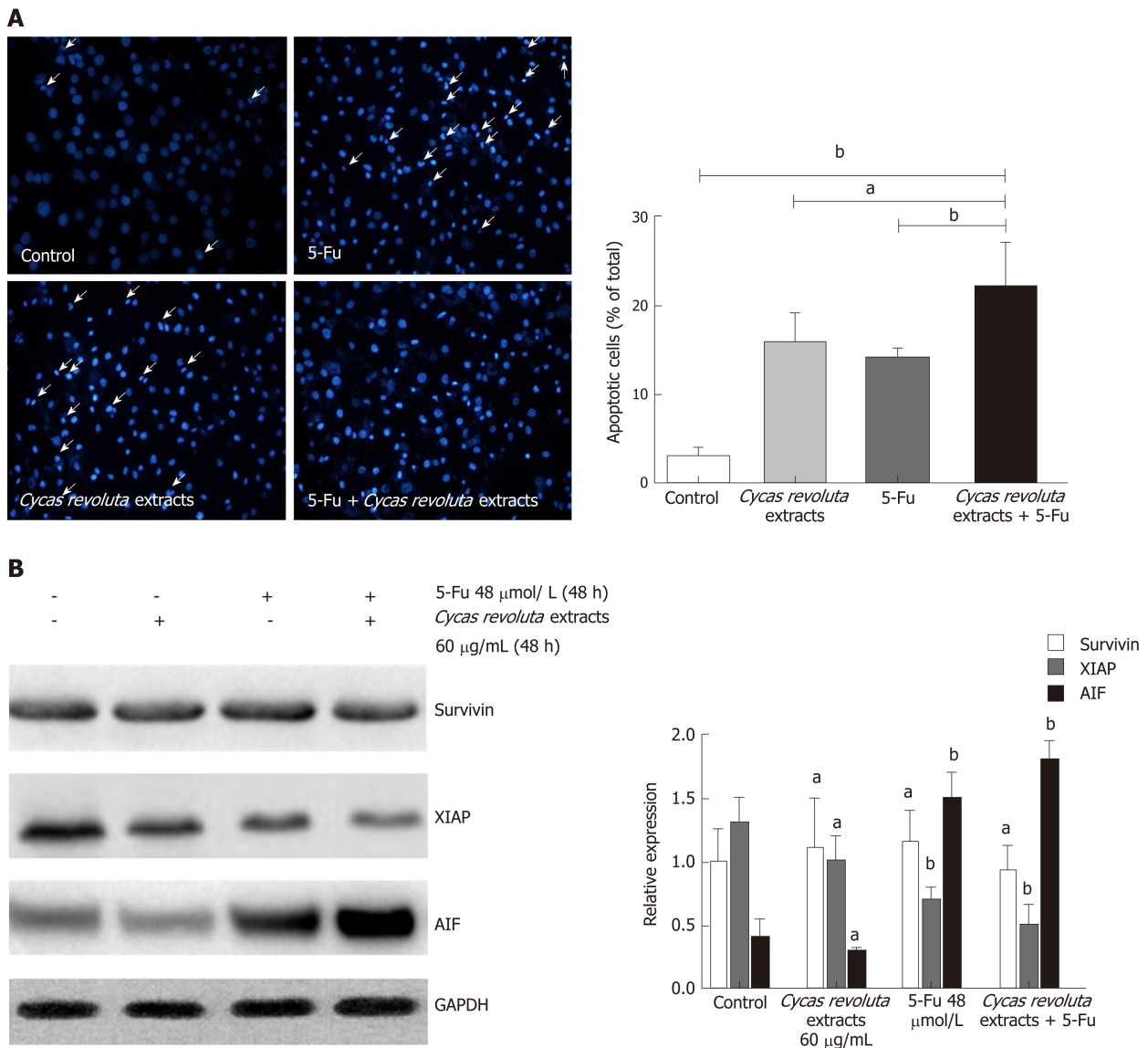


Figure 4 The mechanism of chemosensitization mediated by *Cycas revoluta* Thunb. extract. A: Hoechst 33258 staining to confirm apoptosis induced by 5-fluorouracil and the extract. The error bars represent \pm STDEV from $n = 3$ independent experiments $^aP < 0.05$, $^bP < 0.01$; B: Western blot analysis of apoptosis-related proteins. Relative expressions are shown in the left histogram, and the error bars represent \pm STDEV from $n = 3$ independent experiments $^aP < 0.05$, $^bP < 0.01$. 5-Fu: 5-fluorouracil; XIAP: X-linked inhibitor of apoptosis protein; AIF: Apoptosis inducing factor.

gastric cancer cell growth with little effect on normal gastric cells at low and medium doses (0 $\mu\text{g/mL}$ -250 $\mu\text{g/mL}$). Combined with the significant decrease in EC₅₀ and LC₅₀ values for gastric cancer cells, we conclude that gastric cancer cells are more sensitive to *Cycas revoluta* Thunb. extract than normal gastric cells. However, the inhibitory effect on normal cells dramatically increased when the concentration of the extract was over 250 $\mu\text{g/mL}$. In contrast, the viability rate of cancer cells increased. This finding may be due to the strong screening effect of high concentrations of *Cycas revoluta* Thunb. natural extract, in which drug-resistant cancer cells rapidly proliferate, or employ other adaptive mechanisms. This result also suggests that the dose of *Cycas revoluta* Thunb. natural extract should be strictly controlled during practical application. Additionally, we demonstrated that *Cycas revoluta* Thunb. natural extract significantly decreased the migration and invasion ability of gastric cancer cells, further confirming its inhibitory effect on gastric cancer cells.

To determine whether *Cycas revoluta* Thunb. natural extract could be used with 5-Fu, we carried out clonogenic assays. The results showed that 5-Fu exhibited a stronger inhibitory effect than the extract, but combining the two drugs further inhibited cancer cell colony formation. By analyzing EC₅₀ and LC₅₀ values, we can conclude that cancer cell sensitivity to 5-Fu increased in the presence of *Cycas revoluta* Thunb. natural extract. This result was further confirmed by detecting the expression of the drug-resistance-related proteins p-gp, p-AKT and mTOR. p-AKT and mTOR

expression were even further decreased in 5-Fu treatments when combined with *Cycas revoluta* Thunb. natural extract, suggesting that the changes in expression are highly related to *Cycas revoluta* Thunb-mediated enhancement of sensitivity of gastric cancer cells. mTOR is involved in AKT phosphorylation, which activates this enzyme. The activation of the AKT-mTOR pathway has been widely observed in various cancers, such as bladder cancer, breast cancer and non-small cell lung cancer^[40-45]. This pathway plays an important role in regulating proliferation, survival, metastasis, and drug resistance of tumors, such as paclitaxel or endocrine therapy^[44,45]. Some preclinical and clinical evidence has also suggested that NEAT1, BAG-1 and XPC are involved in the enhanced drug resistance of cancer cells mediated by the AKT-mTOR pathway^[41,42,44,45], which may provide some clues for us to further explore the mechanism that the extract sensitizes gastric cancer cells to 5-Fu through the AKT-mTOR pathway.

Hoechst 33258 staining proved that using the two compounds together obviously increased the rate of cancer cell apoptosis, suggesting that *Cycas revoluta* Thunb. natural extract further induces apoptosis in 5-Fu treatments. This hypothesis was further confirmed by examining the expression of the apoptosis-related proteins XIAP, AIF and survivin. We observed that the activator AIF increased and inhibitor XIAP decreased, further explaining the increased apoptosis of gastric cancer cells. However, survivin expression remained stable, which suggests that this enhancement of apoptosis may not be mediated by survivin.

In conclusion, this study suggests that *Cycas revoluta* Thunb. natural extract can inhibit gastric cancer cell growth, migration and invasion. Moreover, it can be used to enhance the effect of 5-Fu through the AKT-mTOR pathway, which provides a promising strategy in chemotherapy against gastric cancer.

ARTICLE HIGHLIGHTS

Research background

As one of the most frequent cancers, gastric cancer caused more than 700,000 deaths in just 2012 worldwide. Although 5-fluorouracil (5-Fu) is often employed as treatment against gastric cancer, its effect is severely affected by drug resistance of gastric cancer cells. *Cycas revoluta* Thunb. Extract has shown promise as a cancer treatment, though its effect on gastric cancer remains unknown.

Research motivation

To find new ways for chemical sensitization of cancer cells and improve the effect of 5-Fu during chemotherapy against malignancies.

Research objectives

To explore the anti-cancer effect of *Cycas revoluta* Thunb. in gastric cancer and investigate its chemical sensitization effect against gastric cancer cells during 5-Fu treatment.

Research methods

The half-maximal effective concentration and the half-maximal lethal concentration of drugs were determined by cell viability test. The effect of *Cycas revoluta* Thunb. on gastric cancer cell migration was investigated by wound-healing and transwell assay. The synergistic effect between *Cycas revoluta* Thunb. and 5-Fu was confirmed by clonogenic assay and apoptosis detection. The expression of crucial proteins was measured by western blotting.

Research results

We found that the natural extract of *Cycas revoluta* Thunb. Preferentially killed gastric cancer cells compared to normal gastric cells. In addition, the extract significantly inhibited gastric cancer cell growth, migration and invasion. *Cycas revoluta* Thunb. can also improve the inhibitory effects of 5-Fu and effectively induce cell apoptosis. Western blotting analysis showed that P-glycoprotein, p-AKT and mammalian target of rapamycin (mTOR) expression markedly decreased, suggesting that AKT-mTOR pathway plays an important role in chemical sensitization induced by *Cycas revoluta* Thunb.

Research conclusions

Our study demonstrated that the natural extract of *Cycas revoluta* Thunb. can significantly inhibit gastric cancer cell growth, migration and invasion. Furthermore, it can also improve the effect of 5-Fu and promote apoptosis during chemotherapy. Therefore, our study provides a new drug for improving the clinical effect of chemotherapy in gastric cancer. Our study also showed that *Cycas revoluta* Thunb. Enhanced the effects of 5-Fu through the AKT-mTOR pathway, offering a novel mechanism for the chemical sensitization effect of *Cycas revoluta* Thunb.

Research perspectives

In the future, research may reveal the main component of *Cycas revoluta* Thunb. that enhances the sensitivity of cancer cells and further develop for its application in anti-cancer treatments.

The identification of the molecular pathway related to AKT-mTOR may further explain the underlying mechanism.

REFERENCES

- den Hoed CM, Kuipers EJ. Gastric Cancer: How Can We Reduce the Incidence of this Disease? *Curr Gastroenterol Rep* 2016; **18**: 34 [PMID: 27184043 DOI: 10.1007/s11894-016-0506-0]
- Song Z, Wu Y, Yang J, Yang D, Fang X. Progress in the treatment of advanced gastric cancer. *Tumour Biol* 2017; **39**: 1010428317714626 [PMID: 28671042 DOI: 10.1177/1010428317714626]
- Yang C, Jiang H, Huang S, Hong H, Huang X, Wang X, Liao W, Wang X, Chen X, Jiang L. The prognostic role of pretreatment thrombocytosis in gastric cancer: A systematic review and meta-analysis. *Medicine (Baltimore)* 2018; **97**: e11763 [PMID: 30075601 DOI: 10.1097/MD.00000000000011763]
- Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]
- Fan YL, Fan BY, Li Q, Di HX, Meng XY, Ling N. Preparation of 5-fluorouracil-loaded nanoparticles and study of interaction with gastric cancer cells. *Asian Pac J Cancer Prev* 2014; **15**: 7611-7615 [PMID: 25292036]
- Lei Z, Tan IB, Das K, Deng N, Zouridis H, Pattison S, Chua C, Feng Z, Guan YK, Ooi CH, Ivanova T, Zhang S, Lee M, Wu J, Ngo A, Manesh S, Tan E, Teh BT, So JB, Goh LK, Boussioutas A, Lim TK, Flotow H, Tan P, Rozen SG. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology* 2013; **145**: 554-565 [PMID: 23684942 DOI: 10.1053/j.gastro.2013.05.010]
- Li X, Cai H, Zheng W, Tong M, Li H, Ao L, Li J, Hong G, Li M, Guan Q, Yang S, Yang D, Lin X, Guo Z. An individualized prognostic signature for gastric cancer patients treated with 5-Fluorouracil-based chemotherapy and distinct multi-omics characteristics of prognostic groups. *Oncotarget* 2016; **7**: 8743-8755 [PMID: 26840027 DOI: 10.18632/oncotarget.7087]
- Wang T, Huang B, Guo R, Ma J, Peng C, Zu X, Tang H, Lei X. A let-7b binding site SNP in the 3'-UTR of the Bcl-xL gene enhances resistance to 5-fluorouracil and doxorubicin in breast cancer cells. *Oncol Lett* 2015; **9**: 1907-1911 [PMID: 25789066 DOI: 10.3892/ol.2015.2938]
- Wu Y, Qi Y, Liu H, Wang X, Zhu H, Wang Z. AMPK activator AICAR promotes 5-FU-induced apoptosis in gastric cancer cells. *Mol Cell Biochem* 2016; **411**: 299-305 [PMID: 26497305 DOI: 10.1007/s11010-015-2592-y]
- Zhang JT, Zhou WL, He C, Liu T, Li CY, Wang L. 5-Fluorouracil induces apoptosis of colorectal cancer cells. *Genet Mol Res* 2016; **15**: 15017326 [PMID: 26985934 DOI: 10.4238/gmr.15017326]
- Emoto Y, Yoshizawa K, Kinoshita Y, Yuki M, Yuri T, Tsubura A. Green tea extract attenuates MNU-induced photoreceptor cell apoptosis via suppression of heme oxygenase-1. *J Toxicol Pathol* 2016; **29**: 61-65 [PMID: 26989304 DOI: 10.1293/tox.2015-0052]
- Kao SJ, Lee WJ, Chang JH, Chow JM, Chung CL, Hung WY, Chien MH. Suppression of reactive oxygen species-mediated ERK and JNK activation sensitizes dihydromyricetin-induced mitochondrial apoptosis in human non-small cell lung cancer. *Environ Toxicol* 2017; **32**: 1426-1438 [PMID: 27539140 DOI: 10.1002/tox.22336]
- Ding WB, Wang YX, Dong CW. microRNA15b induced SMCC7721 apoptosis via down-regulation of XIAP. *Eur Rev Med Pharmacol Sci* 2017; **21**: 542-548 [PMID: 28239814]
- Gao C, Yu H, Yan C, Zhao W, Liu Y, Zhang D, Li J, Liu N. X-linked inhibitor of apoptosis inhibits apoptosis and preserves the blood-brain barrier after experimental subarachnoid hemorrhage. *Sci Rep* 2017; **7**: 44918 [PMID: 28327595 DOI: 10.1038/srep44918]
- Li X, Chen W, Zeng W, Wan C, Duan S, Jiang S. microRNA-137 promotes apoptosis in ovarian cancer cells via the regulation of XIAP. *Br J Cancer* 2017; **116**: 66-76 [PMID: 27875524 DOI: 10.1038/bjc.2016.379]
- Qin S, Yang C, Zhang B, Li X, Sun X, Li G, Zhang J, Xiao G, Gao X, Huang G, Wang P, Ren H. XIAP inhibits mature Smac-induced apoptosis by degrading it through ubiquitination in NSCLC. *Int J Oncol* 2016; **49**: 1289-1296 [PMID: 27498621 DOI: 10.3892/ijo.2016.3634]
- Yang WZ, Zhou H, Yan Y. XIAP underlies apoptosis resistance of renal cell carcinoma cells. *Mol Med Rep* 2018; **17**: 125-130 [PMID: 29115633 DOI: 10.3892/mmr.2017.7925]
- Candé C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie* 2002; **84**: 215-222 [PMID: 12022952]
- Miyake K, Bekisz J, Zhao T, Clark CR, Zoon KC. Apoptosis-inducing factor (AIF) is targeted in IFN- α 2a-induced Bid-mediated apoptosis through Bak activation in ovarian cancer cells. *Biochim Biophys Acta* 2012; **1823**: 1378-1388 [PMID: 22683989 DOI: 10.1016/j.bbamer.2012.05.031]
- Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491-501 [PMID: 9727492]
- Chantalat L, Skoufias DA, Kleman JP, Jung B, Dideberg O, Margolis RL. Crystal structure of human survivin reveals a bow tie-shaped dimer with two unusual alpha-helical extensions. *Mol Cell* 2000; **6**: 183-189 [PMID: 10949039]
- Gu F, Li L, Yuan QF, Li C, Li ZH. Down-regulation of survivin enhances paclitaxel-induced Hela cell apoptosis. *Eur Rev Med Pharmacol Sci* 2017; **21**: 3504-3509 [PMID: 28829489]
- Jaiswal PK, Goel A, Mittal RD. Survivin: A molecular biomarker in cancer. *Indian J Med Res* 2015; **141**: 389-397 [PMID: 26112839 DOI: 10.4103/0971-5916.159250]
- Khan Z, Khan AA, Yadav H, Prasad GBKS, Bisen PS. Survivin, a molecular target for therapeutic interventions in squamous cell carcinoma. *Cell Mol Biol Lett* 2017; **22**: 8 [PMID: 28536639 DOI: 10.1186/s11658-017-0038-0]
- Sokolowska J, Urbańska K. Survivin expression in correlation with apoptotic activity in canine lymphomas. *Pol J Vet Sci* 2017; **20**: 329-338 [PMID: 28865215 DOI: 10.1515/pjvs-2017-0040]
- Tang H, Zeng L, Wang J, Zhang X, Ruan Q, Wang J, Cui S, Yang D. Reversal of 5-fluorouracil resistance by EGCG is mediated by inactivation of TFAP2A/VEGF signaling pathway and down-regulation of MDR-

- 1 and P-gp expression in gastric cancer. *Oncotarget* 2017; **8**: 82842-82853 [PMID: [29137307](#) DOI: [10.18632/oncotarget.20666](#)]
- 28 **Jin P**, Wong CC, Mei S, He X, Qian Y, Sun L. MK-2206 co-treatment with 5-fluorouracil or doxorubicin enhances chemosensitivity and apoptosis in gastric cancer by attenuation of Akt phosphorylation. *Onco Targets Ther* 2016; **9**: 4387-4396 [PMID: [27499633](#) DOI: [10.2147/OTT.S106303](#)]
- 29 **He K**, Zheng X, Li M, Zhang L, Yu J. mTOR inhibitors induce apoptosis in colon cancer cells via CHOP-dependent DR5 induction on 4E-BP1 dephosphorylation. *Oncogene* 2016; **35**: 148-157 [PMID: [25867072](#) DOI: [10.1038/ncr.2015.79](#)]
- 30 **Li Q**, Mou LJ, Tao L, Chen W, Sun XT, Xia XF, Wu XY, Shi XL. Inhibition of mTOR suppresses human gallbladder carcinoma cell proliferation and enhances the cytotoxicity of 5-fluorouracil by downregulating MDR1 expression. *Eur Rev Med Pharmacol Sci* 2016; **20**: 1699-1706 [PMID: [27212159](#)]
- 31 **Sun Y**, Jiang Y, Huang J, Chen H, Liao Y, Yang Z. Cisd2 enhances the chemosensitivity of gastric cancer through the enhancement of 5-FU-induced apoptosis and the inhibition of autophagy by AKT/mTOR pathway. *Cancer Med* 2017; **6**: 2331-2346 [PMID: [28857517](#) DOI: [10.1002/cam4.1169](#)]
- 32 **Guo XF**, Yang ZR, Wang J, Lei XF, Lv XG, Dong WG. Synergistic antitumor effect of puerarin combined with 5-fluorouracil on gastric carcinoma. *Mol Med Rep* 2015; **11**: 2562-2568 [PMID: [25434307](#) DOI: [10.3892/mmr.2014.3016](#)]
- 33 **Rani I**, Sharma B, Kumar S, Kaur S, Agnihotri N. Apoptosis mediated chemosensitization of tumor cells to 5-fluorouracil on supplementation of fish oil in experimental colon carcinoma. *Tumour Biol* 2017; **39**: 1010428317695019 [PMID: [28349837](#) DOI: [10.1177/1010428317695019](#)]
- 34 **Xu GY**, Tang XJ. Troxerutin (TXN) potentiated 5-Fluorouracil (5-Fu) treatment of human gastric cancer through suppressing STAT3/NF- κ B and Bcl-2 signaling pathways. *Biomed Pharmacother* 2017; **92**: 95-107 [PMID: [28531805](#) DOI: [10.1016/j.biopha.2017.04.059](#)]
- 35 **Zhang L**, Xing Y, Gao Q, Sun X, Zhang D, Cao G. Combination of NRP1-mediated iRGD with 5-fluorouracil suppresses proliferation, migration and invasion of gastric cancer cells. *Biomed Pharmacother* 2017; **93**: 1136-1143 [PMID: [28738522](#) DOI: [10.1016/j.biopha.2017.06.103](#)]
- 36 **Kowalska MT**, Itzhak Y, Puett D. Presence of aromatase inhibitors in cycads. *J Ethnopharmacol* 1995; **47**: 113-116 [PMID: [8569233](#)]
- 37 **Wang S**, Hu Y, Yan Y, Cheng Z, Liu T. Sotetsuflavone inhibits proliferation and induces apoptosis of A549 cells through ROS-mediated mitochondrial-dependent pathway. *BMC Complement Altern Med* 2018; **18**: 235 [PMID: [30092797](#) DOI: [10.1186/s12906-018-2300-z](#)]
- 38 **Wang S**, Yan Y, Cheng Z, Hu Y, Liu T. Sotetsuflavone suppresses invasion and metastasis in non-small-cell lung cancer A549 cells by reversing EMT via the TNF- α /NF- κ B and PI3K/AKT signaling pathway. *Cell Death Discov* 2018; **4**: 26 [PMID: [29531823](#) DOI: [10.1038/s41420-018-0026-9](#)]
- 39 **Pulito C**, Mori F, Sacconi A, Goeman F, Ferraiuolo M, Pisanisi P, Campagnoli C, Berrino F, Fanciulli M, Ford RJ, Levrero M, Pediconi N, Ciuffreda L, Milella M, Steinberg GR, Cioce M, Muti P, Strano S, Blandino G. Metformin-induced ablation of microRNA 21-5p releases Sestrin-1 and CAB39L antitumoral activities. *Cell Discov* 2017; **3**: 17022 [PMID: [28698800](#) DOI: [10.1038/celldisc.2017.22](#)]
- 40 **Sathe A**, Nawroth R. Targeting the PI3K/AKT/mTOR Pathway in Bladder Cancer. *Methods Mol Biol* 2018; **1655**: 335-350 [PMID: [28889395](#) DOI: [10.1007/978-1-4939-7234-0_23](#)]
- 41 **Sharma VR**, Gupta GK, Sharma AK, Batra N, Sharma DK, Joshi A, Sharma AK. PI3K/Akt/mTOR Intracellular Pathway and Breast Cancer: Factors, Mechanism and Regulation. *Curr Pharm Des* 2017; **23**: 1633-1638 [PMID: [27848885](#) DOI: [10.2174/1381612823666161116125218](#)]
- 42 **Ciruelos Gil EM**. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. *Cancer Treat Rev* 2014; **40**: 862-871 [PMID: [24774538](#) DOI: [10.1016/j.ctrv.2014.03.004](#)]
- 43 **Ippen FM**, Alvarez-Breckenridge CA, Kuter BM, Fink AL, Bihun IV, Lastrapes M, Penson T, Schmidt SP, Wojtkiewicz GR, Ning J, Subramanian M, Giobbie-Hurder A, Martinez-Lage M, Carter SL, Cahill DP, Wakimoto H, Brastianos PK. The dual PI3K/mTOR-pathway inhibitor GDC-0084 achieves antitumor activity in *PIK3CA*-mutant breast cancer brain metastases. *Clin Cancer Res* 2019 [PMID: [30796030](#) DOI: [10.1158/1078-0432.CCR-18-3049](#)]
- 44 **Li B**, Gu W, Zhu X. NEAT1 mediates paclitaxel-resistance of non-small cell of lung cancer through activation of Akt/mTOR signaling pathway. *J Drug Target* 2019; 1-7 [PMID: [30782035](#) DOI: [10.1080/1061186X.2019.1585437](#)]
- 45 **Guerrero-Zotano A**, Mayer IA, Arteaga CL. PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. *Cancer Metastasis Rev* 2016; **35**: 515-524 [PMID: [27896521](#) DOI: [10.1007/s10555-016-9637-x](#)]



Basic Study

Unconjugated bilirubin alleviates experimental ulcerative colitis by regulating intestinal barrier function and immune inflammation

Jia-Dong Zheng, Yan He, Heng-Yuan Yu, Yuan-Li Liu, Yi-Xuan Ge, Xue-Ting Li, Xue Li, Yan Wang, Meng-Ru Guo, Yi-Lin Qu, Xiao-Fa Qin, Ming-Shan Jiang, Xiu-Hong Wang

ORCID number: Jia-Dong Zheng (0000-0002-2849-0297); Yan He (0000-0002-7002-7212); Heng-Yuan Yu (0000-0001-8817-4547); Yuan-Li Liu (0000-0003-1816-4225); Yi-Xuan Ge (0000-0003-1993-2257); Xue-Ting Li (0000-0003-3046-4730); Xue Li (0000-0002-9035-777X); Yan Wang (0000-0003-2595-812X); Meng-Ru Guo (0000-0003-1919-7061); Yi-Lin Qu (0000-0002-9835-1487); Xiao-Fa Qin (0000-0001-8642-7985); Ming-Shan Jiang (0000-0001-7383-1575); Xiu-Hong Wang (0000-0001-9803-2200).

Author contributions: Wang XH and Zheng JD contributed to study concept and design; He Y, Ge YX, Yu HY, Li X, Wang Y, Guo MR, Liu YL, Qu YL and Li XT contributed to acquisition of data; Zheng JD analyzed and interpreted the data; Zheng JD and He Y drafted the manuscript; Jiang MS and Qin XF contributed to critical revision of the manuscript for intellectual content; Wang XH contributed to study supervision.

Supported by grants from the National Natural Foundation of China, No. 81703232.

Institutional review board

statement: The study was reviewed and approved by the Ethics Committee of Harbin Medical University, Harbin, China (HMuIRB20180015).

Institutional animal care and use

committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use

Jia-Dong Zheng, Yan He, Heng-Yuan Yu, Yuan-Li Liu, Yi-Xuan Ge, Xue-Ting Li, Xue Li, Yan Wang, Meng-Ru Guo, Yi-Lin Qu, Xiu-Hong Wang, Department of Biochemistry and Molecular Biology, Heilongjiang Provincial Science and Technology Innovation Team in Higher Education Institutes for Infection and Immunity, Harbin Medical University, Harbin 150086, Heilongjiang Province, China

Xiao-Fa Qin, Founder, GI Biopharma Inc., Westfield, NJ 07090, United States

Ming-Shan Jiang, Department of General Surgery, the Second Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China

Corresponding author: Xiu-Hong Wang, MSc, PhD, Academic Research, Professor, Department of Biochemistry and Molecular Biology, Heilongjiang Provincial Science and Technology Innovation Team in Higher Education Institutes for Infection and Immunity, Harbin Medical University, 157 Baojian Rd, Harbin 150086, Heilongjiang Province, China. wangxiuhong@hrbmu.edu.cn

Telephone: +86-13836111380

Fax: +86-13836111380

Abstract

BACKGROUND

Unconjugated bilirubin (UCB) is generally considered toxic but has gained recent prominence for its anti-inflammatory properties. However, the effects of it on the interaction between intestinal flora and organisms and how it influences immune responses remain unresolved.

AIM

To investigate the role of UCB in intestinal barrier function and immune inflammation in mice with dextran-sulfate-sodium-induced colitis.

METHODS

Acute colitis was induced by 3% (*w/v*) dextran sulfate sodium salt in drinking water for 6 d followed by untreated water for 2 d. Concurrently, mice with colitis were administered 0.2 mL UCB (400 μ mol/L) by intra-gastric gavage for 7 d. Disease activity index (DAI) was monitored daily. Mice were sacrificed at the end of the experiment. The length of the colon and weight of the spleen were recorded. Serum level of D-lactate, intestinal digestive proteases activity, and changes to the gut flora were analyzed. In addition, colonic specimens were analyzed by histology and for expression of inflammatory markers and proteins.

Committee of Harbin Medical University.

Conflict-of-interest statement: No conflict of interest exists in this study.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: January 4, 2019

Peer-review started: January 4, 2019

First decision: January 30, 2019

Revised: March 5, 2019

Accepted: March 15, 2019

Article in press: March 16, 2019

Published online: April 21, 2019

P-Reviewer: Madnani MA, Rath T, Touil-Boukoffa C

S-Editor: Yan JP

L-Editor: Filipodia

E-Editor: Ma YJ



RESULTS

Mice treated with UCB had significantly relieved severity of colitis, including lower DAI, longer colon length, and lower spleen weight (colon length: 4.92 ± 0.09 cm *vs* 3.9 ± 0.15 cm; spleen weight: 0.33 ± 0.04 *vs* 0.74 ± 0.04 , $P < 0.001$). UCB administration inactivated digestive proteases (chymotrypsin: 18.70 ± 0.69 U/g *vs* 44.81 ± 8.60 U/g; trypsin: 1.52 ± 0.23 U/g *vs* 9.05 ± 1.77 U/g, $P < 0.01$), increased expression of tight junction (0.99 ± 0.05 *vs* 0.57 ± 0.03 , $P < 0.001$), decreased serum level of D-lactate (31.76 ± 3.37 μ mol/L *vs* 54.25 ± 1.45 μ mol/L, $P < 0.001$), and lowered histopathological score (4 ± 0.57 *vs* 7 ± 0.57 , $P < 0.001$) and activity of myeloperoxidase (46.79 ± 2.57 U/g *vs* 110.32 ± 19.19 U/g, $P < 0.001$). UCB also regulated the intestinal microbiota, inhibited expression of tumor necrosis factor (TNF) α and interleukin 1β (TNF- α : 52.61 ± 7.81 pg/mg *vs* 105.04 ± 11.92 pg/mg, interleukin 1β : 13.43 ± 1.68 *vs* 32.41 ± 4.62 pg/mg, $P < 0.001$), decreased expression of Toll-like receptor 4 (0.61 ± 0.09 *vs* 1.07 ± 0.03 , $P < 0.001$) and myeloid differentiation primary response gene 88 (0.73 ± 0.08 *vs* 1.01 ± 0.07 , $P < 0.05$), and increased expression of TNF-receptor-associated factor 6 (0.79 ± 0.02 *vs* 0.43 ± 0.09 , $P < 0.05$) and inhibitor of kappa B α (0.93 ± 0.07 *vs* 0.72 ± 0.07 , $P < 0.05$) in the colon.

CONCLUSION

UCB can protect intestinal barrier function, regulate normal intestinal homeostasis, and suppress inflammation *via* the Toll-like receptor 4/ nuclear factor- κ B signaling pathway.

Key words: Ulcerative colitis; Unconjugated bilirubin; Intestinal barrier; Intestinal homeostasis; Digestive proteases; Inflammation

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Intestinal function and microbiota are desired therapeutic endpoints for treatment of inflammatory bowel disease. However, a major problem is the lack of powerful and safe drugs. Unconjugated bilirubin (UCB) has anti-inflammatory and antitoxin effects. We found that UCB significantly decreased intestinal permeability and improved intestinal barrier function; regulated composition of gut microbiota; and relieved intestinal inflammation through suppressing the Toll-like receptor 4/nuclear factor- κ B pathway. This study demonstrated that UCB ameliorates ulcerative colitis *via* intestinal barrier function through inactivating the digestive proteases and inhibiting immune inflammation through the Toll-like receptor 4/nuclear factor- κ B pathway.

Citation: Zheng JD, He Y, Yu HY, Liu YL, Ge YX, Li XT, Li X, Wang Y, Guo MR, Qu YL, Qin XF, Jiang MS, Wang XH. Unconjugated bilirubin alleviates experimental ulcerative colitis by regulating intestinal barrier function and immune inflammation. *World J Gastroenterol* 2019; 25(15): 1865-1878

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1865.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1865>

INTRODUCTION

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), are associated with chronic, relapsing inflammation of the intestinal tract. In the early 1900s, regions such as North America, Europe, and Oceania had millions of individuals with IBD. The prevalence of IBD is highest in the western world, which was estimated to affect up to 0.5% of the general population in 2015 and equates to approximately 2.2 million Americans living with IBD in 2025^[1,2]. However, newly industrialized countries have a low prevalence of IBD, but the incidence has been steadily rising. A representative study from the Asia-Pacific Crohn's and Colitis Epidemiology Study showed that IBD was established in Asia, with an average incidence of 1.4 per 100000 people in 2011, and the incidence of UC was two-fold higher than that of CD in Asia and the incidence of IBD in China was 3.3 per 100000^[3]. IBD affects millions of people around the world, producing a substantial

burden on healthcare systems.

Evidence from studies on etiology and pathogenesis suggests that IBD results from a dysregulated intestinal immune response driven by complex interplay between the host and intraluminal microbiota^[4]. Intestinal epithelial cells recognize conserved signature molecules in the gut commensal bacteria, called pathogen-associated molecular patterns, by pattern recognition receptors. Toll-like receptors (TLRs) are one of the major types of pattern recognition receptor families and have a pivotal effect on maintaining homeostasis of the gut microbiota^[5]. During inflammation, TLR4 as an innate immune receptor is stimulated by recognition of gut pathogen-associated molecular patterns. TLR4 undergoes a conformational change and then recruits the signaling adaptors myeloid differentiation primary response gene 88 (MyD88) and tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6, thereby activating downstream transcription of nuclear factor- κ B (NF- κ B) target genes and expression of some pro-inflammatory cytokines such as interleukin 1 β (IL-1 β), TNF- α , or IL-6^[6]. However, anti-inflammatory and immuno-regulatory cytokines are related to the negative regulation of nitric oxide (NO), which is positively correlated with the severity of the disease. In IBD patients, NO production was positively correlated with increased levels of pro-inflammatory cytokines^[7]. The role of cytokines and NO level are fundamental to regulate inflammation in IBD. Meanwhile, TLR4 was significantly more upregulated in IBD patients than in controls, especially in UC patients^[8,9]. The probiotic cocktail (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium lactis*, and *Bifidobacterium breve*) ameliorated clinical symptoms and histological scores, decreased NO level, and reduced TLR4, inducible NO synthase, and NF- κ B expression^[10]. An important mechanism of UC might be that abnormal inflammation by intestinal dysbacteriosis causes dysregulation of TLR that mediates innate immunity. The TLR4/NF- κ B signaling pathway is an essential key point in the development of UC.

Unconjugated bilirubin (UCB) is generated during the physiological breakdown of heme by heme oxygenase 1. Serum levels of bilirubin increase with the accelerated release of heme from hemoglobin or with diminished hepatic conjugating activity. UCB is a potent antioxidant even at low concentrations^[11,12]. It was first discovered in 1934 that bilirubin exerts anti-inflammatory activity in patients with rheumatoid arthritis who experienced remission of symptoms after developing jaundice secondary to liver disease^[13]. Studies have shown that bile acids play a role in the regulation of gut injury, immunity, and inflammation^[14,15]; however, there is a lack of studies on the physiological function of bilirubin that enters the intestine together with bile. Recent investigations have demonstrated that UCB suppresses inflammatory responses in animal models of autoimmune encephalomyelitis and lung inflammation in asthma^[16-18]. Nevertheless, the mechanisms on inflammation and role of UCB in the interaction between intestinal flora and immunity are not well understood. The aim of the present study was to investigate the effects of UCB on intestinal barrier function and inflammation on dextran sodium sulfate (DSS)-induced colitis in mice.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice aged 8-12 wk (weight approximately 25 g) were purchased from the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University and were acclimatized for 1 wk before experiments were performed. They were reared in the Animal Laboratory Centre of Harbin Medical University under specific pathogen-free conditions (temperature 24-25 °C, humidity 70%-75%, with a 12 h light/dark lighting regimen) and were fed a standard diet of pellets and water *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

Chemicals and reagents

UCB, N-benzoyl-L-tyrosine ethyl ester, and N- α -benzoyl-L-arginine 4-nitroanilide hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, United States). DSS (36-50 kDa) was obtained from MP Biomedical (Solon, OH, United States). Enzyme linked immunosorbent assay (ELISA) kits for D-lactate, TNF- α , IL-1 β , and myeloperoxidase (MPO) were from Beijing Propbs Biotechnology (Beijing, China). The antibodies used in this study were anti-TLR4 (19811-1-AP; Proteintech, Rosemont, United States), anti-MyD88 (4283; Cell Signaling Technology, Danvers, MA, United States), anti-TRAF6 (ab33915; Abcam, Cambridge, MA, United States), anti-inhibitor of NF- κ B alpha (IkB α) (4814; Cell Signaling Technology), and anti-occludin (ab167161;

Abcam). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), goat anti-rabbit immunoglobulin G and goat anti-mouse immunoglobulin G were purchased from ZSGB-BIO Co. Ltd. (Beijing, China). All other reagents used were of analytical grade.

Induction of colitis and pharmacological treatment

Colitis was induced by oral administration of DSS as described previously, with some modifications^[19]. The animals were randomly divided into five groups with five mice in each group: Control group (Control), DSS group (DSS), DSS plus UCB group (DSS + UCB), and UCB group (UCB). Colitis was induced by administering 3% DSS in drinking water for 6 d, and then drinking water without DSS for another 2 d for recovery. From the first day, UCB was administered daily *via* gavage at 0.2 mL for 7 d (UCB was dissolved in 0.4% dimethyl sulfoxide at concentrations up to 400 μ M). During the study, weight, physical condition, stool consistency, and the presence of occult blood in feces were examined and documented daily. All animals were sacrificed after 8 d by intraperitoneal injection of an overdose of chloral hydrate. Blood specimens were collected and serum samples were prepared by centrifugation at 4000 g for 15 min at 4 °C and stored at -80 °C. The entire colon, spleen and total feces of mice were carefully removed, measured and weighted, then stored at -80 °C for further analysis.

Disease activity index

Disease activity index (DAI) was calculated for each animal by measuring the body weight, stool consistency, and fecal blood. Each score was calculated based on the criteria listed in Table 1^[20].

Histology analysis for scoring colonic damage

Colonic tissues fixed in 4% (*w/v*) paraformaldehyde were paraffin-embedded and sliced into 5 μ m sections, followed by staining with hematoxylin and eosin for light microscopic examination to assess colon injury and inflammation. Colonic damage was graded in a blinded manner as described as Table 2^[21].

Analysis of intestinal permeability

D-Lactate concentration in serum was measured by ELISA. Results are expressed as μ mol D-lactate/mL serum.

Determination of fecal digestive proteases activity

Trypsin and chymotrypsin (amidase) activities were measured using N- α -benzoyl-L-arginine 4-nitroanilide hydrochloride or N-benzoyl-L-tyrosine ethyl ester as the substrate, respectively, as described previously^[22,23].

Colon RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from colon tissues using the UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). After treating with DNase I (TaKaRa, Tokyo, Japan), RNA was transcribed into cDNA using Prime Script TM RT reagent Kit (TaKaRa) using an Eppendorf Mastercycler personal Thermo cycler. All primers were obtained from Sangon. The primers were used as followed: forward: 5'-GAGCACCTTCTTTCCCTTCATCTT-3' and revers: 5'-TCACACACCAGCAGGTT

ATCATC-3' for IL-1 β , forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3' for TNF- α and forward: 5'-CATGGCCTTCCGTGTTCCCTA-3' and revers: 5'-GCGGCACGTCAGATCCA-3' for GAPDH. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a volume of 20 μ L with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). All samples were analyzed in triplicate, and the results were normalized to the expression of GAPDH. Results were expressed as $2^{-\Delta\Delta C_t}$.

Fecal bacteria DNA extraction and qRT-PCR

Genomic DNA from feces was extracted by Stool Genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China) and used for quantitative analysis of fecal bacteria Firmicutes, Bacteroidetes, Actinomycetes, and Proteobacteria. All primers were obtained from Sangon. The primers were used as followed: forward: 5'-GCTGCTAATACCGCATGATATGTC-3' and reverse: 5'-CAGACGCGAGTCCAT

CTCAGA-3' for Firmicutes, forward: 5'-GAGAGGAAGGTCCCCAC-3' and reverse: 5'-CGCTACTTGGCTGGTTTCAG-3' for Bacteroidetes, forward: 5'-TGTAGCGGTGGA

ATGCGC-3' and reverse: 5'-AATTAAGCCACATGCTCCGCT-3' for Actinomycetes, forward: 5'-TAGGCTTGACATTGATAGAATC-3' and reverse: 5'-CTTACGAAGGCA

Table 1 Criteria for scoring disease activity index

Score	Weight loss, %	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1-5		
2	5-10	Loose stools	Hemoccult positive
3	10-15		
4	> 15	Diarrhea	Gross bleeding

GTCTCCTTA-3' for Proteobacteria and forward: 5'-GCAACGAGCGCAACCC-3' and reverse: 5'-ACGGGCGGTGTGTAC-3' for 16S rDNA. qRT-PCR in a volume of 20 μ L was performed using FastStart Universal SYBR Green Master. All samples were analyzed in triplicate, and the results were normalized to the expression of 16S rDNA. The results were expressed as $2^{-\Delta\Delta Ct}$.

Cytokine levels measured by ELISA and Western blotting

Segments of colon were homogenized using radioimmunoprecipitation assay buffer and protein inhibitor cocktail (1:10) (PhosSTOP ESAYpack; Roche). The homogenates were kept on ice for 30 min and centrifuged at 12000 g for 5 min at 4 °C. The protein concentration was determined using the bicinchoninic acid assay Protein Assay Kit (Beyotime, Shanghai, China). For ELISA, the supernatants were collected and subjected to IL-1 β , TNF- α , and MPO assays. Results of IL-1 β and TNF- α were expressed as pg/mg and MPO as U/g. For western blot analysis, proteins were electroblotted onto a polyvinylidene difluoride membrane following separation on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The immunoblot was then incubated with primary antibodies against occludin, TLR4, MyD88, TRAF6, and I κ B α , or GAPDH. The chemiluminescence signals were analyzed using Quantity One (version 4.5.2; Bio-Rad Laboratories, Hercules, CA, United States) and Image J software.

Statistical analysis

Results were expressed as mean \pm standard error of the mean (SEM). Differences between groups were determined using one-way analysis of variance followed by Tamhane multiple comparisons post-hoc tests using SPSS version 19.0 (IBM, Armonk, NY, United States). Graphs were analyzed using Graphpad Prism version 5.0 (Graphpad Software, La Jolla, CA, United States). Statistical significance was denoted as $P < 0.05$.

RESULTS

UCB relieved clinical severity of DSS-induced colitis

Compared with the DSS group, the DSS + UCB group exhibited less weight loss, diarrhea, and intestinal bleeding, as reflected by the significantly lower DAI scores ($P < 0.001$, **Figure 1A** and **B**). The two control groups that received regular drinking water or UCB alone did not develop signs of colitis throughout the experiment (all DAI score < 1 from 1 d to 8 d, **Figure 1B**). Furthermore, UCB treatment significantly reduced colon shortening and increased spleen weight ($P < 0.001$, **Figure 1C** and **D**).

UCB inactivated digestive protease activity of DSS-induced colitis

Digestive proteases are believed to play a crucial role in the destruction of the intestinal barrier^[24,25], and UCB has previously been demonstrated to inactivate digestive proteases activity^[26]. Therefore, we tested the effects of UCB on trypsin and chymotrypsin activity in the feces of mice with DSS-induced colitis. Compared with the DSS group, the DSS + UCB group showed remarkably lower activities of fecal digestive proteases trypsin and chymotrypsin, and levels in the UCB alone group were similar to the control group ($P < 0.01$, **Figure 2A** and **B**).

UCB ameliorated colon tissue injury in DSS-induced colitis

DSS-induced colon tissue injury was demonstrated by epithelial destruction, intense inflammatory infiltration, and crypt distortion (**Figure 3A**) as well as increased histological scores for inflammation and crypt damage (**Figure 3B**). DSS with UCB treatment reduced neutrophil infiltration and crypt damage in the colon, leading to a decrease in colonic MPO activity ($P < 0.001$, **Figure 3C**), which is an inflammatory

Table 2 Criteria for histology analysis for scoring colonic damage

Colon damage score	0	1	2	3
Crypt architecture damage	None	Regeneration	Destruction	
Edema in sub-mucosa	None	Mild	Moderate	Severe
Inflammatory cells infiltration	None/rare	Lamina propria	Sub-mucosa	Muscle layer

marker for colitis. The epithelial destruction, histological score, and MPO activity of the UCB alone group were similar to those in the control group.

UCB protected intestinal barrier function of DSS-induced colitis

Compared with the control group, expression of the tight junction protein occludin in the colon of the DSS group was decreased significantly ($P < 0.001$, **Figure 4A**), and serum level of D-lactate was significantly elevated ($P < 0.001$, **Figure 4B**). However, in the DSS + UCB group, expression of occludin was increased in the colon with a decrease in serum levels of D-lactate ($P < 0.001$, **Figure 4A** and **B**). Results in the UCB alone group were similar to those in the control group (**Figure 4**).

UCB regulated fecal microbiota composition of DSS-induced colitis mice

We assessed the change in fecal microbiota in mice at the phylum level. DSS induced an overall decrease in feces microbiota. Compared with the controls, Firmicutes and Actinomycetes were dominant in the intestinal flora ($P < 0.05$), while Bacteroidetes ($P < 0.01$) and Proteobacteria ($P < 0.05$) were less. The intestinal flora of UCB treated mice recovered approximately. However, Firmicutes ($P < 0.01$) were decreased and Actinomycetes ($P < 0.001$) were increased compared with the DSS group. Bacteroidetes in the UCB alone group were also higher than in the control group, but the difference was not significant (**Figure 5**).

UCB reduced production of proinflammatory cytokines and inhibited TLR4/MyD88/TRAF6/NF- κ B signaling in DSS-induced colitis mice

The mRNA and protein levels of the pro-inflammatory cytokines, TNF- α and IL-1 β , in colon tissues of mice were determined. DSS-induced acute colitis was accompanied by a significant increase in TNF- α and IL-1 β levels ($P < 0.001$), and treatment with UCB restored these cytokines to normal levels ($P < 0.001$, **Figure 6A, B**). TLR4 is a key immune receptor that plays an important role in the regulation of colonic inflammation^[27]. Compared with the control group, expression of TLR4 ($P < 0.01$) and MyD88 ($P < 0.05$) was increased in the DSS group, and expression of TRAF6 ($P < 0.01$) and I κ B α ($P < 0.05$) was decreased. In comparison, expression of these proteins was restored to near normal in the UCB-treated group. The results in the UCB only group were similar to those in the control group (**Figure 7**).

DISCUSSION

Previous observation on human evolution has shown that bilirubin-predominant species are often carnivores or omnivores, while biliverdin-predominant species are often herbivores^[28], suggesting that bilirubin may have an important yet unknown effect on the human body. Significant increases in concentration of serum bilirubin in neonates (> 20 mg/dL, approximately 340 μ M) can trigger nerve damage^[29]; however, it is a powerful antioxidant and toxic in adults^[11,30,31]. UC patients have reduced total serum bilirubin levels^[32]. Numerous studies have suggested that UCB plays an important potential protective role in vascular endothelial function^[33]; ameliorates allergic lung inflammation in a mouse model of asthma^[18]; prevents murine colitis by inhibiting leukocyte infiltration and suppressing upregulation of inducible NO synthase^[34]; or scavenges various reactive oxygen species^[12].

Our previous work confirmed that UCB ameliorates the inflammation in trinitrobenzenesulfonic acid-induced colitis; an animal model of CD^[35]. In this study, we further established the UC mouse model that has a different extent of ulceration and tissue edema. Our data suggest two potential mechanisms by which UCB exerts its protective effect on DSS-induced colitis: (1) Inactivation of digestive proteases to protect intestinal mucosal barrier and prevent intestinal flora translocation to the colon leading to an abnormal interaction; and (2) suppression of the TLR4/MyD88/TRAF6/NF- κ B signaling pathway to inhibit immune inflammation and regulate intestinal microflora homeostasis.

The intestinal barrier is a complex multilayer system, consisting of an external

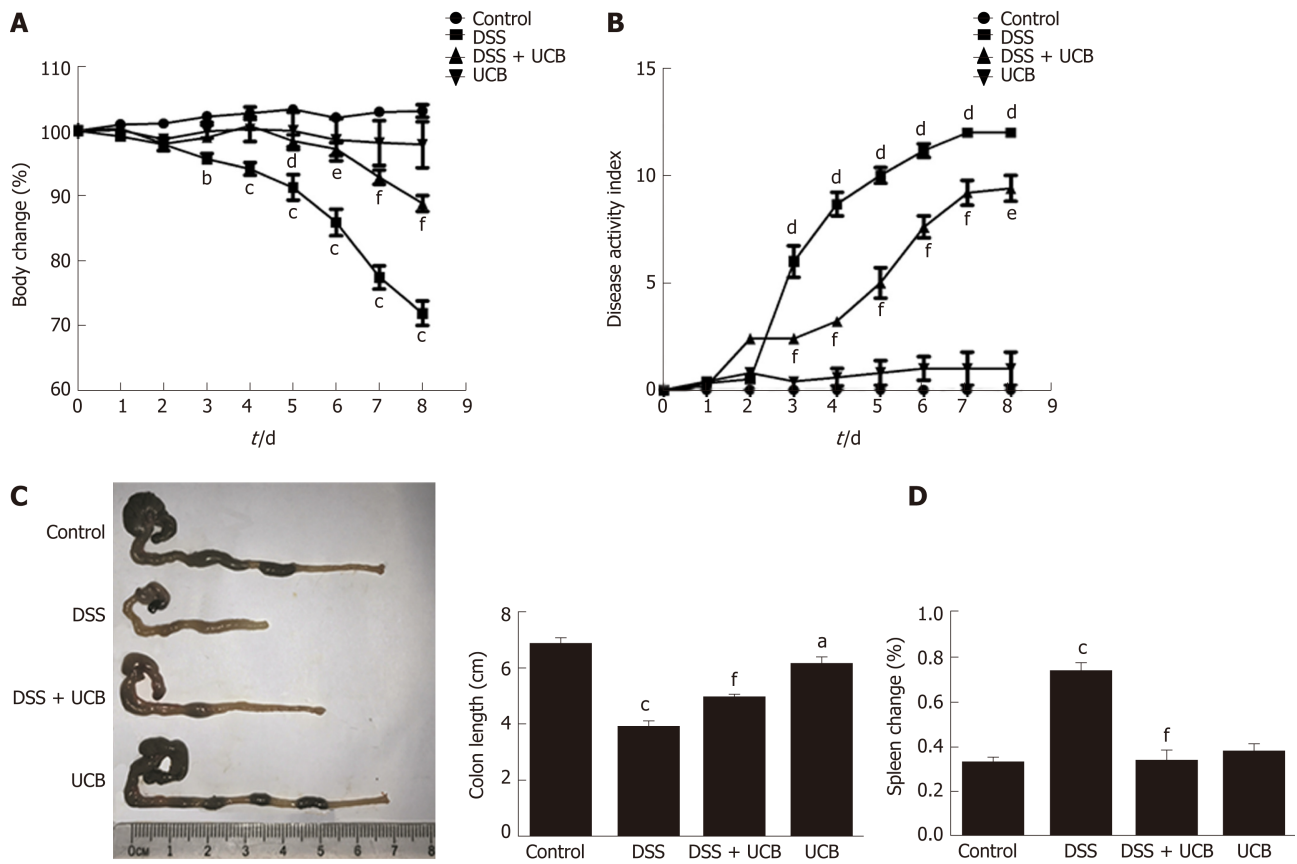


Figure 1 UCB significantly relieved severity of dextran sulfate sodium induced colitis. A: Body weight changes, data plotted as percentage of basal body weight; B: Disease activity index; C: Changes in colon length; D: Relative spleen weight. Data are expressed as means \pm SEM ($n = 5$). ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ vs control group, ^d $P < 0.05$, ^e $P < 0.01$, and ^f $P < 0.001$ vs dextran sodium sulfate group. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; SEM: Standard error of the mean.

anatomical barrier and an inner functional immunological barrier. The interplay of these two barriers maintains normal intestinal function and a stable intestinal environment^[36]. We detected that the intestinal tract of UC mice contained elevated active digestive proteases, which can destroy the intestinal tissue structure and increase intestinal permeability. This provides favorable conditions for intestinal pathogens or toxins of microbiota invading the body. The large number of digestive proteases as vital factors contributes to the pathogenesis and development of IBD due to destruction of the intestinal barrier. UCB can be a specific inhibitor to inactivate digestive proteases to protect the physical barrier function that is the first line of defense against invasion of intestinal pathogens.

Another pivotal factor, intestinal microbes, participates in many important physiological processes, including nutritional absorption, substance metabolism, and immunity^[37,38]. In order to investigate how UCB may specifically contribute to intestinal microbiota during DSS colitis, we tested the composition and abundance at the phylum level (Bacteroidetes, Firmicutes, Actinomycetes and Proteobacteria). Exposure to DSS reduced the diversity and abundance of intestinal flora. The ratio of Bacteroidetes to Firmicutes was decreased and that of Proteobacteria to Actinomycetes was increased. UCB treatment can reverse these phenomena; however, it dramatically reduces the content of Firmicutes and makes Bacteroidetes and Actinomycetes dominant in the flora. Actinomycetes have always produced many antibiotics^[39]; therefore, we suspect that UCB can promote Actinomycetes to produce some secondary products to protect mice against colitis. Our study shows that UCB is beneficial to maintain the stability of intestinal flora, but its specific role needs to be examined through further research that our group has and will continue to be concerned.

TLRs are the recognition receptors of the innate immune system and are located on the surface of various immune cells and play an important role in defense against infection and regulation of immune responses. TLR4 expression is usually low under normal conditions, while in IBD, the intestinal flora lose tolerance and specific recognition of lipopolysaccharide, then produce excessive signal transduction, and finally activate TLR4 and cascade induce transcription of NF- κ B and pro-

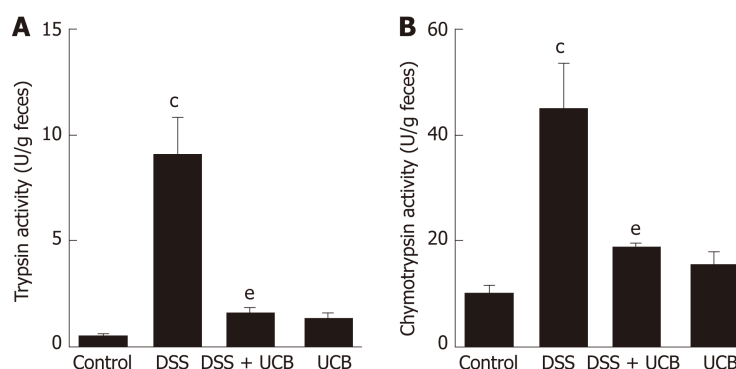


Figure 2 UCB inactivated digestive protease activity in DSS treated mice. A: Trypsin activity in feces; B: Chymotrypsin activity in feces. Data are expressed as means \pm SEM ($n = 5$). ^c $P < 0.001$ vs control group and ^e $P < 0.01$ vs dextran sodium sulfate group. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; SEM: Standard error of the mean.

inflammatory cytokines^[40,41]. Our results demonstrate that UCB decreases expression of TLR4 and MyD88, and increases expression of TRAF6 and I κ B α in the colon. In addition, UCB reduces levels of pro-inflammatory cytokines, TNF- α and IL-1 β , suggesting it has an anti-inflammatory role in DSS-induced colitis.

UCB can suppress intestinal positive immune response and inflammation through the TLR4/NF- κ B signaling pathway. Therefore, this appears to be an additional mechanism of UCB to regulate the relationship between intestinal flora and host immunity. UCB can improve intestinal inflammation, regulate homeostasis of bacterial flora, and maintain intestinal immune function; it is a way to resolve the serious problem of adverse effects and drug resistance of IBD. Perhaps low dose of UCB or in combination with other probiotics can develop healthcare to prevent clinical diseases. It is a novel therapeutic approach, not only for UC but also for other gastrointestinal diseases.

This study had some limitations. First, we found that UCB affected the intestinal flora but the change in genera was unclear. Second, we only induced acute UC, which may have different mechanisms from chronic UC. Third, this study shows that UCB protects intestinal barrier function and suppresses immunity and inflammation in DSS-induced colitis, but we did not study how UCB regulates intestinal immune cells and other potential pleiotropic roles of UCB. These issues will be studied in our future research.

In conclusion, our findings not only shed light on how UCB ameliorates UC but also offer a clue about the physiological function of UCB. This provides a noteworthy example of how UCB can serve as a “double-edged sword” in the human body. This is believed to be the first study to show that UCB ameliorates DSS-induced colitis *via* inactivation of digestive proteases to protect the intestinal barrier and regulate the intestinal flora and TLR4/NF- κ B signaling pathway to suppress intestinal inflammation.

ARTICLE HIGHLIGHTS

Research background

Clinical treatment of ulcerative colitis consists of drugs that are both expensive and have side effects. Unconjugated bilirubin (UCB) has gained recent prominence for its anti-inflammatory and antioxidant properties. How UCB influences UC remains unresolved.

Research motivation

Patients with UC require lifelong treatment, and drugs for UC are linked to many adverse effects. Therefore, there is an urgent need to develop effective and safe drugs for UC.

Research objectives

To investigate the significance of UCB in intestinal barrier function and immune inflammation of mice with dextran sodium sulfate (DSS)-induced colitis.

Research methods

UC was induced by 3% (*w/v*) DSS in drinking water for 6 d followed by untreated

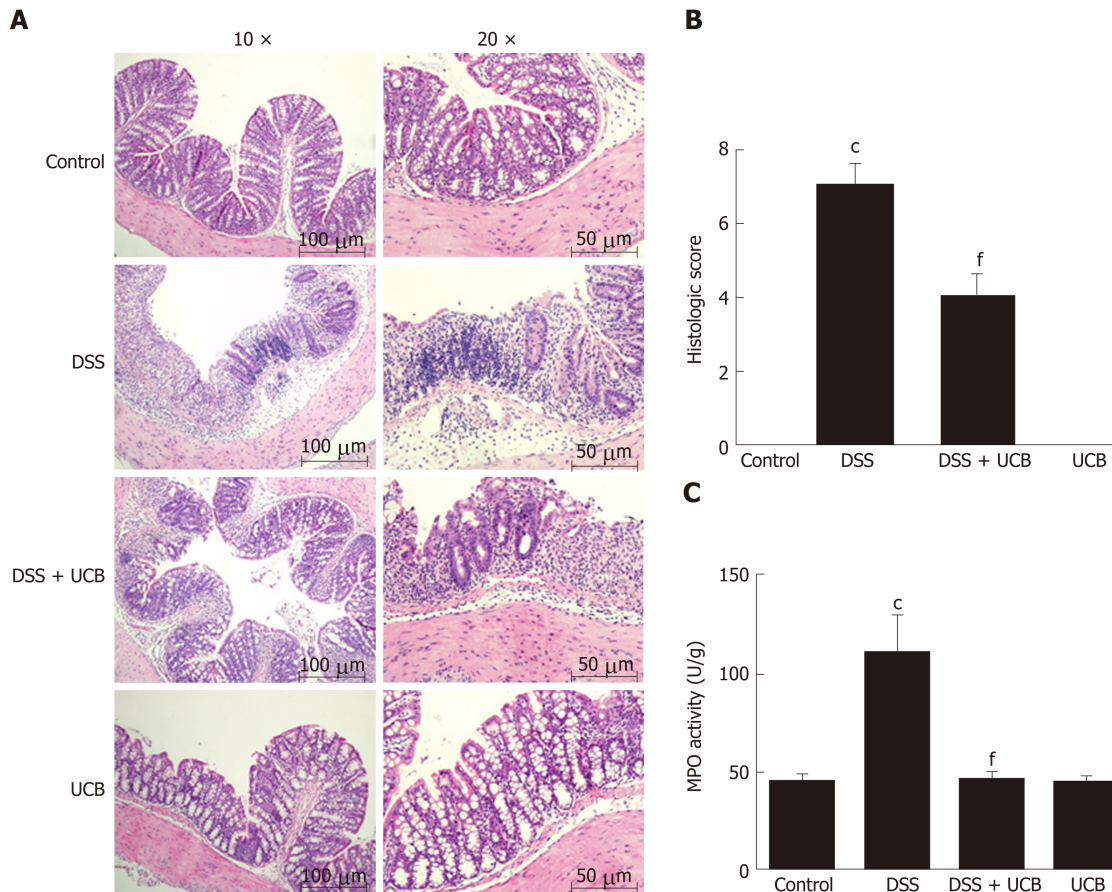


Figure 3 UCB ameliorated colon tissue injury in DSS induced colitis mice. A: Light microscopic assessment of hematoxylin and eosin stained sections; B: Histological scores (images acquired at 10 × using Olympus BX60; scale bar = 100 μm; images acquired at 20 × using Olympus BX60, scale bar = 50 μm, $n = 3$); C: MPO activity of colon tissues ($n = 5$). Data are expressed as means ± SEM. ^c $P < 0.001$ vs control group, and ^f $P < 0.001$ vs DSS. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; MPO: Myeloperoxidase; SEM: Standard error of the mean.

water for 2 d. Concurrently, colitis mice were administered 0.2 mL UCB (400 μM) by intra-gastric gavage for 7 d. Disease activity index (DAI) was monitored daily. The length of the colon and weight of the spleen were recorded. Serum level of D-lactic acid, intestinal digestive proteases activity, and changes in gut flora were analyzed. In addition, colonic specimens were analyzed by histology and for expression of inflammatory markers and proteins.

Research results

UCB significantly relieved the severity of colitis, including lower DAI, longer colon length, and smaller spleen weight ($P < 0.001$). UCB inactivated digestive proteases ($P < 0.01$), increased expression of tight junction protein occludin ($P < 0.001$), decreased serum level of D-lactate ($P < 0.001$), and lowered histopathological score and activity of myeloperoxidase compared with those in colitis mice ($P < 0.001$). UCB also regulated the intestinal microbiota, inhibited expression of tumor necrosis factor (TNF)-α and interleukin-1β ($P < 0.001$), decreased expression of Toll-like receptor (TLR) 4 ($P < 0.001$) and myeloid differentiation primary response gene 88 ($P < 0.05$), and increased expression of TNF-receptor-associated factor 6 ($P < 0.05$) and IκBα ($P < 0.05$) in the colon.

Research conclusions

UCB has a beneficial regulatory effect on intestinal barrier function and regulates normal intestinal homeostasis, and can suppress inflammation *via* the TLR4/NF-κB signaling pathway. This provides a theoretical basis for use of UCB as a clinical drug.

Research perspectives

UCB plays a pivotal role in intestinal innate immunity and inflammation. Thus, the findings of this study indicate a novel potential mechanism by which UCB can treat UC. More studies are needed to investigate the effect of UCB on chronic UC or colon cancer.

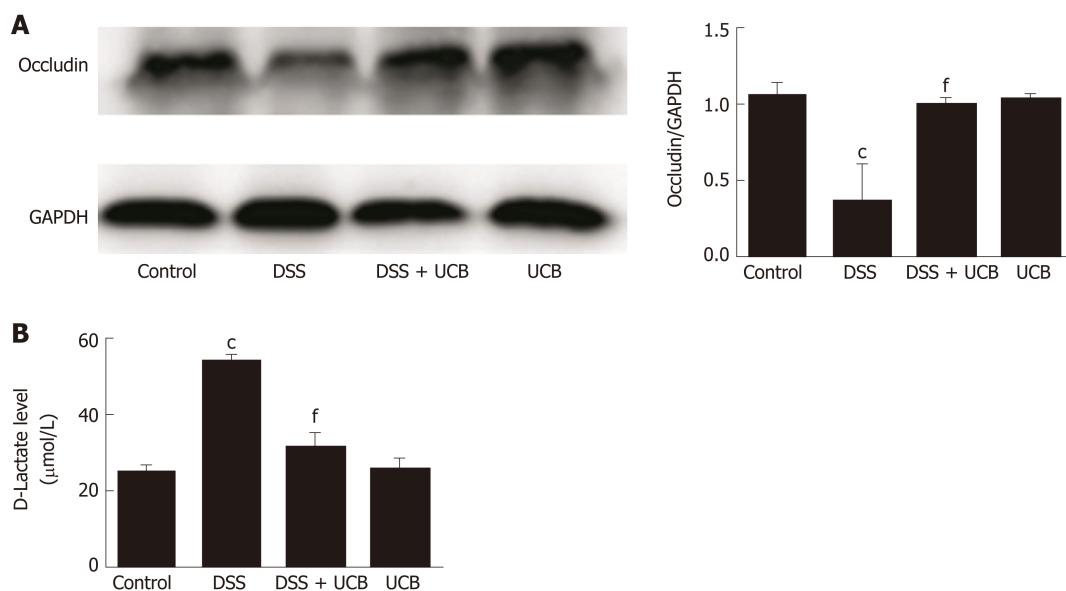


Figure 4 UCB increased expression of tight junction proteins in the colon and decreased intestinal permeability. A: Protein expression of tight junction occludin ($n = 3$); B: D-Lactate level in serum ($n = 5$). Data are expressed as means \pm SEM. ^c $P < 0.001$ vs control group, and ^f $P < 0.001$ vs DSS group. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; SEM: Standard error of the mean.

ACKNOWLEDGMENTS

We acknowledge Jin-An Zhou, Rong-Yan Li, and Yu Song for their excellent laboratory assistance.

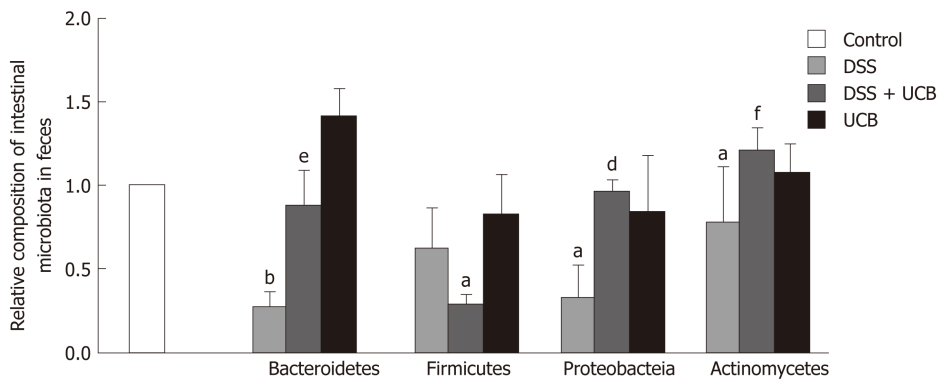


Figure 5 UCB maintained the steady state of intestinal flora of DSS induced colitis mice. Data are expressed as means \pm SEM ($n = 5$). ^a $P < 0.05$ and ^b $P < 0.01$ vs control group; ^c $P < 0.01$, and ^d $P < 0.001$ vs dextran sodium sulfate group. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; SEM: Standard error of the mean.

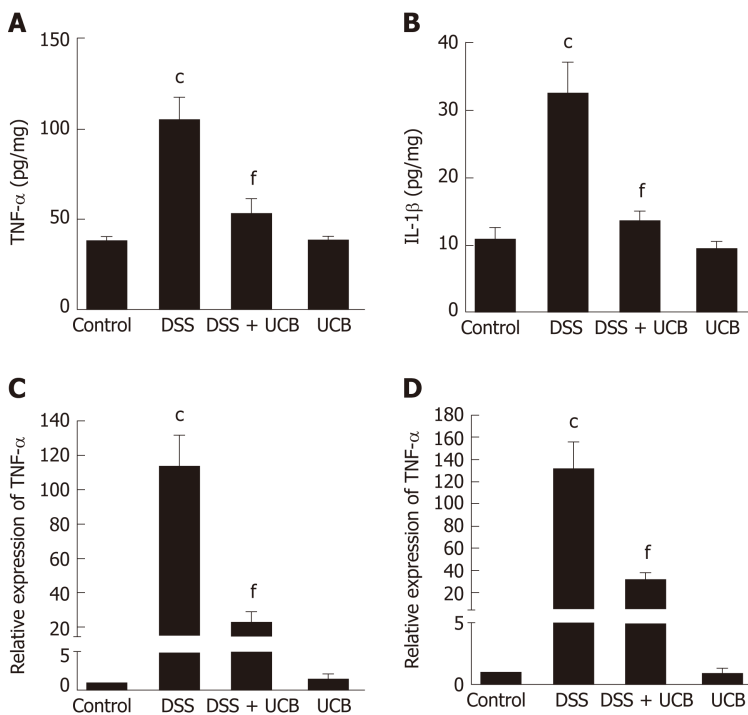


Figure 6 UCB reduced production of proinflammatory cytokines in DSS induced colitis mice. A and B: Protein expression of TNF-α and IL-1β; C and D: mRNA transcript expression of TNF-α and IL-1β. Data are expressed as means \pm SEM ($n = 5$). ^c $P < 0.001$ vs control group and ^f $P < 0.001$ vs DSS. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; TNF-α: Tumor necrosis factor α; IL-1β: Interleukin 1β; SEM: Standard error of the mean.

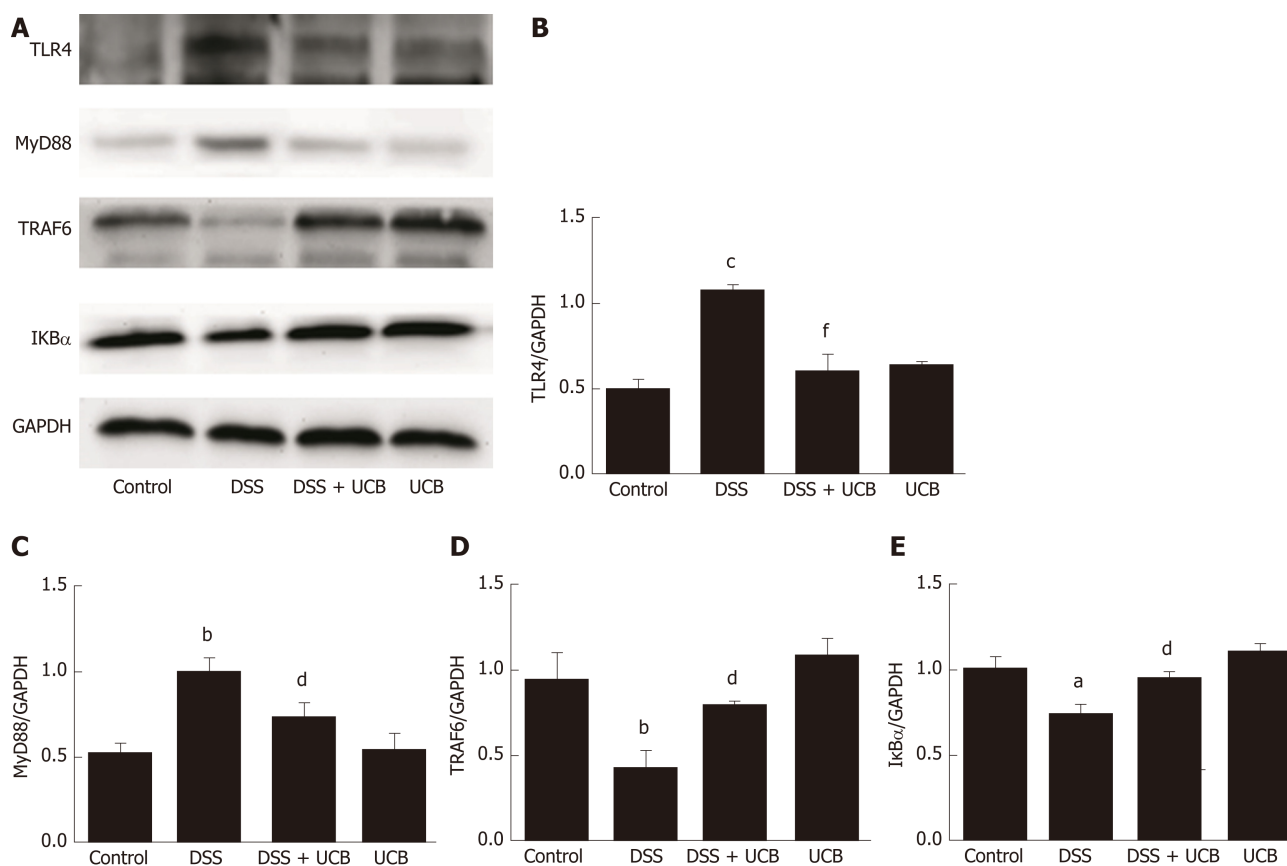


Figure 7 UCB inhibited the TLR4/MyD88/TRAFF6/nuclear factor-κB signaling in DSS induced colitis mice. A: Total protein from colon samples was extracted, and TLR4, MyD88, TRAF6, and IκBα protein expression was measured by western blotting; B-E: Quantification of TLR4, MyD88, TRAF6, and IκBα protein expression was performed by densitometric analysis of the blots. Data are expressed as means ± SEM ($n = 3$). ^a $P < 0.05$ and ^c $P < 0.001$ vs control group; ^d $P < 0.05$ and ^f $P < 0.001$ vs dextran sodium sulfate group. UCB: Unconjugated bilirubin; DSS: Dextran sodium sulfate; TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response gene 88; TRAF6: Tumor necrosis factor receptor-associated factor 6; IκBα: Anti-inhibitor of nuclear factor-κB alpha.

REFERENCES

- Kaplan GG, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321.e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]
- Kaplan GG. The global burden of IBD: From 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 720-727 [PMID: 26323879 DOI: 10.1038/nrgastro.2015.150]
- Ng SC, Tang W, Ching JY, Wong M, Chow CM, Hui AJ, Wong TC, Leung VK, Tsang SW, Yu HH, Li MF, Ng KK, Kamm MA, Studd C, Bell S, Leong R, de Silva HJ, Kasturiratne A, Mufeeena MNF, Ling KL, Ooi CJ, Tan PS, Ong D, Goh KL, Hilmi I, Pisespongsa P, Manatsathit S, Rerknimitr R, Aniwani S, Wang YF, Ouyang Q, Zeng Z, Zhu Z, Chen MH, Hu PJ, Wu K, Wang X, Simadibrata M, Abdullah M, Wu JC, Sung JJY, Chan FKL; Asia-Pacific Crohn's and Colitis Epidemiologic Study (ACCESS) Study Group. Incidence and phenotype of inflammatory bowel disease based on results from the Asia-Pacific Crohn's and colitis epidemiology study. *Gastroenterology* 2013; **145**: 158-165.e2 [PMID: 23583432 DOI: 10.1053/j.gastro.2013.04.007]
- de Souza HS, Fiocchi C. Immunopathogenesis of IBD: Current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 13-27 [PMID: 26627550 DOI: 10.1038/nrgastro.2015.186]
- Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 2004; **118**: 229-241 [PMID: 15260992 DOI: 10.1016/j.cell.2004.07.002]
- Sipos F, Furi I, Constantinovits M, Tulassay Z, Múzes G. Contribution of TLR signaling to the pathogenesis of colitis-associated cancer in inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 12713-12721 [PMID: 25278673 DOI: 10.3748/wjg.v20.i36.12713]
- Soufli I, Toumi R, Rafa H, Touil-Boukoffa C. Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World J Gastrointest Pharmacol Ther* 2016; **7**: 353-360 [PMID: 27602236 DOI: 10.4292/wjgpt.v7.i3.353]
- Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 2000; **68**: 7010-7017 [PMID: 11083826 DOI: 10.1128/IAI.68.12.7010-7017.2000]
- Tan Y, Zou KF, Qian W, Chen S, Hou XH. Expression and implication of toll-like receptors TLR2, TLR4 and TLR9 in colonic mucosa of patients with ulcerative colitis. *J Huazhong Univ Sci Technolog Med Sci* 2014; **34**: 785-790 [PMID: 25318894 DOI: 10.1007/s11596-014-1353-6]
- Toumi R, Soufli I, Rafa H, Belkhef M, Biad A, Touil-Boukoffa C. Probiotic bacteria lactobacillus and bifidobacterium attenuate inflammation in dextran sulfate sodium-induced experimental colitis in mice. *Int J Immunopathol Pharmacol* 2014; **27**: 615-627 [PMID: 25572742 DOI: 10.1177/039463201402700418]

- 11 **Stockner R**, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987; **235**: 1043-1046 [PMID: [3029864](#) DOI: [10.1126/science.3029864](#)]
- 12 **Lee Y**, Kim H, Kang S, Lee J, Park J, Jon S. Bilirubin Nanoparticles as a Nanomedicine for Anti-inflammation Therapy. *Angew Chem Int Ed Engl* 2016; **55**: 7460-7463 [PMID: [27144463](#) DOI: [10.1002/anie.201602525](#)]
- 13 **Hench PS**. The analgesic effect of hepatitis and jaundice in chronic arthritis, fibrositis and sciatic pain. *Ann Intern Med* 1934; **7**: 1278-1294 [DOI: [10.7326/0003-4819-7-10-1278](#)]
- 14 **Mroz MS**, Lajczak NK, Goggins BJ, Keely S, Keely SJ. The bile acids, deoxycholic acid and ursodeoxycholic acid, regulate colonic epithelial wound healing. *Am J Physiol Gastrointest Liver Physiol* 2018; **314**: G378-G387 [PMID: [29351391](#) DOI: [10.1152/ajpgi.00435.2016](#)]
- 15 **Guo C**, Xie S, Chi Z, Zhang J, Liu Y, Zhang L, Zheng M, Zhang X, Xia D, Ke Y, Lu L, Wang D. Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. *Immunity* 2016; **45**: 802-816 [PMID: [27692610](#) DOI: [10.1016/j.immuni.2016.09.008](#)]
- 16 **Wang WW**, Smith DL, Zucker SD. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. *Hepatology* 2004; **40**: 424-433 [PMID: [15368447](#) DOI: [10.1002/hep.20334](#)]
- 17 **Liu Y**, Zhu B, Wang X, Luo L, Li P, Paty DW, Cynader MS. Bilirubin as a potent antioxidant suppresses experimental autoimmune encephalomyelitis: Implications for the role of oxidative stress in the development of multiple sclerosis. *J Neuroimmunol* 2003; **139**: 27-35 [PMID: [12799017](#) DOI: [10.1016/S0165-5728\(03\)00132-2](#)]
- 18 **Kim DE**, Lee Y, Kim M, Lee S, Jon S, Lee SH. Bilirubin nanoparticles ameliorate allergic lung inflammation in a mouse model of asthma. *Biomaterials* 2017; **140**: 37-44 [PMID: [28624706](#) DOI: [10.1016/j.biomaterials.2017.06.014](#)]
- 19 **Wirtz S**, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; **2**: 541-546 [PMID: [17406617](#) DOI: [10.1038/nprot.2007.41](#)]
- 20 **Cooper HS**, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238-249 [PMID: [8350599](#)]
- 21 **Dieleman LA**, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **114**: 385-391 [PMID: [9844047](#) DOI: [10.1046/j.1365-2249.1998.00728.x](#)]
- 22 **Treetharnmathurot B**, Ovartharporn C, Wungsintaweekul J, Duncan R, Wiwattanapatapee R. Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin. *Int J Pharm* 2008; **357**: 252-259 [PMID: [18308489](#) DOI: [10.1016/j.ijpharm.2008.01.016](#)]
- 23 **Qin X**. Inactivation of digestive proteases by deconjugated bilirubin: The possible evolutionary driving force for bilirubin or biliverdin predominance in animals. *Gut* 2007; **56**: 1641-1642 [PMID: [17938442](#) DOI: [10.1136/gut.2007.132076](#)]
- 24 **Giuffrida P**, Biancheri P, MacDonald TT. Proteases and small intestinal barrier function in health and disease. *Curr Opin Gastroenterol* 2014; **30**: 147-153 [PMID: [24445329](#) DOI: [10.1097/MOG.0000000000000042](#)]
- 25 **Biancheri P**, Di Sabatino A, Corazza GR, MacDonald TT. Proteases and the gut barrier. *Cell Tissue Res* 2013; **351**: 269-280 [PMID: [22427120](#) DOI: [10.1007/s00441-012-1390-z](#)]
- 26 **Zhou K**, Jiang M, Qin X, Wang X. Role of bilirubin in digestive proteases inactivation in the lower intestine. *Dig Liver Dis* 2015; **47**: 438-439 [PMID: [25732433](#) DOI: [10.1016/j.dld.2015.01.158](#)]
- 27 **Cao AT**, Yao S, Stefka AT, Liu Z, Qin H, Liu H, Evans-Marin HL, Elson CO, Nagler CR, Cong Y. TLR4 regulates IFN- γ and IL-17 production by both thymic and induced Foxp3⁺ Tregs during intestinal inflammation. *J Leukoc Biol* 2014; **96**: 895-905 [PMID: [25015957](#) DOI: [10.1189/jlb.3A0114-056RR](#)]
- 28 **Mowat AP**. Bile pigments and jaundice: molecular, metabolic, and medical aspects. *Gut* 1987; **28**: 365-366
- 29 **Watchko JF**, Tiribelli C. Bilirubin-induced neurologic damage--mechanisms and management approaches. *N Engl J Med* 2013; **369**: 2021-2030 [PMID: [24256380](#) DOI: [10.1056/NEJMr1308124](#)]
- 30 **Rizzo AM**, Berselli P, Zava S, Montorfano G, Negroni M, Corsetto P, Berra B. Endogenous antioxidants and radical scavengers. *Adv Exp Med Biol* 2010; **698**: 52-67 [PMID: [21520703](#) DOI: [10.1007/978-1-4419-7347-4_5](#)]
- 31 **Qaisiya M**, Coda Zabetta CD, Bellarosa C, Tiribelli C. Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway. *Cell Signal* 2014; **26**: 512-520 [PMID: [24308969](#) DOI: [10.1016/j.cellsig.2013.11.029](#)]
- 32 **Schieffer KM**, Bruffy SM, Rauscher R, Koltun WA, Yochum GS, Gallagher CJ. Reduced total serum bilirubin levels are associated with ulcerative colitis. *PLoS One* 2017; **12**: e0179267 [PMID: [28594959](#) DOI: [10.1371/journal.pone.0179267](#)]
- 33 **Liu J**, Wang L, Tian XY, Liu L, Wong WT, Zhang Y, Han QB, Ho HM, Wang N, Wong SL, Chen ZY, Yu J, Ng CF, Yao X, Huang Y. Unconjugated bilirubin mediates heme oxygenase-1-induced vascular benefits in diabetic mice. *Diabetes* 2015; **64**: 1564-1575 [PMID: [25475440](#) DOI: [10.2337/db14-1391](#)]
- 34 **Zucker SD**, Vogel ME, Kindel TL, Smith DL, Idelman G, Avissar U, Kakarlapudi G, Masnovi ME. Bilirubin prevents acute DSS-induced colitis by inhibiting leukocyte infiltration and suppressing upregulation of inducible nitric oxide synthase. *Am J Physiol Gastrointest Liver Physiol* 2015; **309**: G841-G854 [PMID: [26381705](#) DOI: [10.1152/ajpgi.00149.2014](#)]
- 35 **Zhou JA**, Jiang M, Yang X, Liu Y, Guo J, Zheng J, Qu Y, Song Y, Li R, Qin X, Wang X. Unconjugated bilirubin ameliorates the inflammation and digestive protease increase in TNBS-induced colitis. *Mol Med Rep* 2017; **16**: 1779-1784 [PMID: [28656252](#) DOI: [10.3892/mmr.2017.6825](#)]
- 36 **Antoni L**, Nuding S, Wehkamp J, Stange EF. Intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 1165-1179 [PMID: [24574793](#) DOI: [10.3748/wjg.v20.i5.1165](#)]
- 37 **Bakker GJ**, Zhao J, Herrema H, Nieuwdorp M. Gut Microbiota and Energy Expenditure in Health and Obesity. *J Clin Gastroenterol* 2015; **49** Suppl 1: S13-S19 [PMID: [26447957](#) DOI: [10.1097/MCG.0000000000000363](#)]
- 38 **Rooks MG**, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol* 2016; **16**: 341-352 [PMID: [27231050](#) DOI: [10.1038/nri.2016.42](#)]
- 39 **Mahajan GB**, Balachandran L. Antibacterial agents from actinomycetes - a review. *Front Biosci (Elite Ed)* 2012; **4**: 240-253 [PMID: [22201868](#) DOI: [10.2741/373](#)]
- 40 **Szebeni B**, Veres G, Dezsöfi A, Rusai K, Vannay A, Mraz M, Majorova E, Arató A. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol* 2008; **151**: 34-41 [PMID: [17991289](#) DOI: [10.1111/j.1365-2249.2007.03531.x](#)]

- 41 **Frolova L**, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: Upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem* 2008; **56**: 267-274 [PMID: [18040078](#) DOI: [10.1369/jhc.7A7303.2007](#)]



Retrospective Study

Value of pretransplant albumin-bilirubin score in predicting outcomes after liver transplantation

Tao Ma, Qing-Shan Li, Yue Wang, Bo Wang, Zheng Wu, Yi Lv, Rong-Qian Wu

ORCID number: Tao Ma (0000-0002-5936-4226); Qing-Shan Li (0000-0002-9155-2024); Yue Wang (0000-0002-6948-7430); Bo Wang (0000-0002-5836-3918); Zheng Wu (0000-0002-7102-9543); Yi Lv (0000-0002-7104-2414); Rong-Qian Wu (0000-0003-0993-4531).

Author contributions: All authors helped perform the research; Ma T wrote the manuscript, designed and performed the procedures, and analysed the data; Li QS wrote the manuscript, drafted the conception, performed the experiments, and analysed the data; Wang Y contributed to writing the manuscript, and drafted the conception and design; Wang B and Wu Z contributed to writing the manuscript and performed the experiments; and Lv Y and Wu RQ contributed to writing the manuscript, and drafting the conception and design. All authors read and approved the final manuscript.

Supported by the Ministry of Education Innovation Team Development Program of China, No. IRT16R57; the National Natural Science Foundation of China, No. 81470896; and Research Fund for the Young Talent Recruiting Plans of Xi'an Jiaotong University (RW).

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Permit Number: XJTU1AF2015LSL-057).

Informed consent statement:

Tao Ma, Qing-Shan Li, Yue Wang, Yi Lv, Rong-Qian Wu, National Local Joint Engineering Research Center for Precision Surgery and Regenerative Medicine, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Tao Ma, Qing-Shan Li, Yue Wang, Bo Wang, Zheng Wu, Yi Lv, Department of Hepatobiliary Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Corresponding author: Rong-Qian Wu, MD, PhD, Professor, National Local Joint Engineering Research Center for Precision Surgery and Regenerative Medicine, First Affiliated Hospital of Xi'an Jiaotong University, No. 76, West Yanta Road, Xi'an 710061, Shaanxi Province, China. rwu001@mail.xjtu.edu.cn

Telephone: +86-29-85323204

Fax: +86-29-85252580

Abstract

BACKGROUND

Due to the significant shortage of organs and the increasing number of candidates on the transplant waiting list, there is an urgent need to identify patients who are most likely to benefit from liver transplantation. The albumin-bilirubin (ALBI) grading system was recently developed to identify patients at risk for adverse outcomes after hepatectomy. However, the value of the pretransplant ALBI score in predicting outcomes after liver transplantation has not been assessed.

AIM

To retrospectively investigate the value of the pretransplant ALBI score in predicting outcomes after liver transplantation.

METHODS

The clinical data of 272 consecutive adult patients who received donation after cardiac death and underwent liver transplantation at our centre from March 2012 to March 2017 were analysed in the cohort study. After the exclusion of patients who met any of the exclusion criteria, 258 patients remained. The performance of the ALBI score in predicting overall survival and postoperative complications after liver transplantation was evaluated. The optimal cut-off value of preoperative ALBI was calculated according to long-term survival status. The outcomes after liver transplantation, including postoperative complications and survival analysis, were measured.

Written informed consent from the patients was waived due to the retrospective nature of this study.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: February 18, 2019

Peer-review started: February 19, 2019

First decision: February 26, 2019

Revised: March 4, 2019

Accepted: March 16, 2019

Article in press: March 16, 2019

Published online: April 21, 2019

P-Reviewer: Hilmi I, Hori T

S-Editor: Ma RY

L-Editor: Wang TQ

E-Editor: Ma YJ



RESULTS

The remaining 258 consecutive patients were included in the analysis. The median follow-up time was 17.30 (interquartile range: 8.90-28.98) mo. Death occurred in 35 patients during follow-up. The overall survival rate was 81.0%. The preoperative ALBI score had a significant positive correlation with the overall survival rate after liver transplantation. The calculated cut-off for ALBI scores to predict postoperative survival was -1.48. Patients with an ALBI score > -1.48 had a significantly lower survival rate than those with an ALBI score ≤ -1.48 (73.7% *vs* 87.6%, $P < 0.05$), and there were no statistically significant differences in survival rates between patients with a model for end stage liver disease score ≥ 10 and < 10 and different Child-Pugh grades. In terms of the specific complications, a high ALBI score was associated with an increased incidence of biliary complications, intraabdominal bleeding, septicemia, and acute kidney injury after liver transplantation ($P < 0.05$ for all).

CONCLUSION

The ALBI score predicts overall survival and postoperative complications after liver transplantation. The ALBI grading system may be useful in risk-stratifying patients on the liver transplant waiting list.

Key words: Albumin-bilirubin score; Liver transplantation; Survival; Postoperative complications; Liver transplant waiting list

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The albumin-bilirubin (ALBI) grading system was developed to identify patients at risk for poor outcomes after hepatectomy. The study showed the preoperative ALBI score had a significant positive correlation with the overall survival rate after liver transplantation. The calculated cut-off for ALBI scores to predict postoperative survival was -1.48. Patients with an ALBI score > -1.48 had a significantly lower survival rate than those with an ALBI score ≤ -1.48 . A high ALBI score was also associated with an increased incidence of postoperative complications. Thus, the ALBI grading system may be useful in risk-stratifying patients on the liver transplant waiting list.

Citation: Ma T, Li QS, Wang Y, Wang B, Wu Z, Lv Y, Wu RQ. Value of pretransplant albumin-bilirubin score in predicting outcomes after liver transplantation. *World J Gastroenterol* 2019; 25(15): 1879-1889

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1879.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1879>

INTRODUCTION

Advances in immunosuppression and improvements in surgical techniques and perioperative care have markedly improved the outcome of liver transplant recipients, and liver transplantation (LT) has become the only effective treatment for patients with end-stage liver disease^[1-3]. Because of the significant shortage of organs and the increasing number of candidates on the transplant waiting list, there is an urgent need to identify patients who are most likely to benefit from LT^[2,4,5].

The albumin-bilirubin (ALBI) score, as a simple assessment of liver function, is objectively calculated by only two variables (albumin and bilirubin)^[6]. It was recently proposed by Johnson *et al*^[6], Andreatos *et al*^[7], and Zou *et al*^[8] as a new method for preoperative risk evaluation to discern patients with the risk of adverse outcomes after hepatectomy. While the ALBI grading system has been closely related to in-hospital mortality in patients with chronic liver disease, its value to predict outcomes after LT has not been evaluated. Therefore, the purpose of this study was to explore the ability of the pretransplant ALBI score to predict outcomes after LT.

MATERIALS AND METHODS

Data source and patient population

This single-centre, retrospective cohort study was conducted to investigate the relationship between pretransplant ALBI scores and outcomes after LT. From March 1, 2012 to March 31, 2017, 272 consecutive adult patients (age > 18 years) with end-stage liver disease who received donation after cardiac death (DCD) and underwent LT at the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China were included in this study. All clinical variables of these 272 patients, including demographic features and preoperative, intraoperative, and postoperative data, were obtained from a computerized clinical database from the hospital. In addition to the date of this study, available medical records, including follow-up data, met the inclusion criteria. This study was approved by the First Affiliated Hospital of Xi'an Jiaotong University Ethics Committee. Written informed consent from the patients was waived due to the retrospective nature of this study. All cases received follow-up care routinely until June 2017.

Definitions

The ALBI score was calculated using the formula: $(\log_{10} \text{bilirubin} \times 0.66) + (\text{albumin} \times -0.085)$, where bilirubin is measured in $\mu\text{mol/L}$ and albumin in g/L ^[6]. The primary outcome was overall survival. The secondary outcomes included total complications and the incidence of biliary complications, portal vein thrombosis, rejection, pneumonia, acute kidney injury (AKI), intraabdominal bleeding, and in-hospital mortality as well as length of postoperative hospital stay after LT.

Statistical analysis

To minimize bias, follow-ups and reviews were completed by two clinicians. Categorical variables are reported as numbers and percentages and were compared by a chi-squared analysis or Fisher's exact test as appropriate. Normal and abnormal continuous variables are reported as the mean \pm standard deviation (SD) and median [interquartile range (IQR)], and were compared by Student's *t*-test and the Mann-Whitney rank-sum test, respectively. The optimal cut-off value of preoperative ALBI was calculated by receiver operating characteristic (ROC) curve analysis and utilizing the Youden index according to long-term survival status. The accuracy of ALBI for predicting outcomes was evaluated using the area under the ROC curve (AUC). The survival rates of recipients with high ALBI grades and low ALBI grades were compared using a Kaplan-Meier estimation and a log-rank test. Univariate and multivariate analyses of prognostic factors were performed using the Cox proportional hazards model. All statistical tests were two-sided, and *P*-values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics 22.0 software (IBM Corporation, Armonk, NY, United States).

RESULTS

Patient demographics

A total of 272 patients underwent LT at our hospital from March 1, 2012 to March 31, 2017. Of these patients, 14 were excluded from this study: 12 were lost to follow-up and 2 were missing criteria for ALBI and model for end stage liver disease (MELD) score calculations. The remaining 258 consecutive patients were included in the analysis. The median follow-up time was 17.30 (IQR: 8.90-28.98) mo. **Table 1** shows the demographics and baseline characteristics of these patients. Of these patients, 206 were male (79.8%), and 52 were female (20.2%). The median age of the patients was 47.0 (IQR: 39.0-56.0) years. The indications for LT were hepatocellular carcinoma (HCC) (33.7%), viral hepatitis-related cirrhosis (77.5%), alcoholic cirrhosis (3.1%), primary biliary cirrhosis and autoimmune hepatitis (8.2%), and others (11.2%), such as hepatolenticular degeneration, cryptogenic cirrhosis, drug-induced liver injury, upper biliary tract obstruction, and acute liver failure. The median preoperative ALBI score and MELD score were -1.78 (-2.40 to -1.33) and 15.5 (11.0-23.0), respectively. Death occurred in 35 patients during follow-up. The overall survival rate was 81.0%.

Predictive value of pretransplant ALBI for overall survival after LT

The performance of the ROC curve analysis was determined by the value of the pretransplant ALBI score to predict the overall survival after LT. **Figure 1** shows that the pretransplant ALBI score had a significant positive relationship with the overall survival rate. The AUC was 0.647 with a 95% confidence interval (CI) of 0.540-0.753 and a *P*-value of 0.005. The cut-off for ALBI scores was calculated as -1.48 by predicting postoperative survival, with a Youden index of 0.304 (sensitivity = 60.0%, and specificity = 70.4%). Based on the cut-off value, 173 patients had a low ALBI score

Table 1 Patient demographics (n = 258)

Patients characteristic	n (%) / mean \pm SD / median (IQR)
Demographic feature	
Age, yr	47.0 (39.0-56.0)
Male, n (%)	206 (79.8)
Coexisting condition	
Smoking, n (%)	78 (30.2)
Drinking, n (%)	44 (17.1)
Hypertension, n (%)	19 (7.4)
Diabetes, n (%)	27 (10.5)
Etiology	
Hepatocellular carcinoma, n (%)	87 (33.7)
Viral hepatitis, n (%)	200 (77.5)
Alcoholic cirrhosis, n (%)	8 (3.1)
PBC and AIH, n (%)	21 (8.2)
Other, n (%)	29 (11.2)
Clinical feature	
ALBI score	-1.78 (-2.40 - -1.33)
MELD score	15.5 (11.0-23.0)
Child-Pugh grade	
A, n (%)	43 (16.7)
B, n (%)	94 (36.4)
C, n (%)	121 (46.9)
Operation time (min)	390.0 (332.5-436.5)
Anhepatic phase (min)	49 (44-58)
Blood loss (mL)	1500 (900-3000)
Total input quantity (mL)	6040 (4810-7810)
Warm ischemia time (min)	9 (8-10)
Cold ischemia time (h)	5 (4-6)

Other etiologies included hepatolenticular degeneration, drug-induced liver injury, upper biliary tract obstruction, acute liver failure, and cryptogenic cirrhosis. SD: Standard deviation; IQR: Inter quartile range; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; ALBI: Albumin-bilirubin; MELD; Model for end stage liver disease.

(ALBI \leq -1.48, 67.1%) and 85 patients had a high ALBI score (ALBI $>$ -1.48, 32.9%). As shown in Table 2, the pretransplant and demographic data were related to the ALBI grade. There was less likely to be HCC in patients with high ALBI scores than in patients with low ALBI scores. Patients in the high ALBI group also had higher preoperative MELD scores and higher Child-Pugh (C-P) grades. In terms of the preoperative laboratory values, patients in the high ALBI group had higher values for aspartate transaminase (AST), alpha-fetoprotein (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), neutrophil granulocytes (NEUT), monocytes (MONO), prothrombin time (PT), activated partial thromboplastin time (APTT), and international normalized ratio (INR) ($P < 0.05$ for all) but lower levels of red blood cells (RBC), haemoglobin (HGB), platelets (PLT), albumin (ALB), and alpha-fetoprotein (AFP) ($P < 0.05$ for all). The 3-year survival after LT was analysed based on pretransplant ALBI scores using the Kaplan-Meier estimation. As shown in Figure 2, patients with high ALBI scores had a significantly lower survival rate than patients with low ALBI scores (73.7% vs 87.6%, $P < 0.05$). However, there were no statistically significant differences in 3-year survival rates between patients with MELD scores ≥ 10 and < 10 (Figure 3A). Similarly, no statistically significant differences were found in 3-year survival rates among patients with different C-P grades (Figure 3B). Univariable and multivariable analyses were performed to identify independent risk factors related to poor survival after LT. Univariate variables with $P < 0.1$ were included in the multivariate analysis. Table 3 shows that high pretransplant ALBI scores, high PLT, high serum levels of creatinine, and high APTT were independently associated with poor survival after LT in the multivariate analysis.

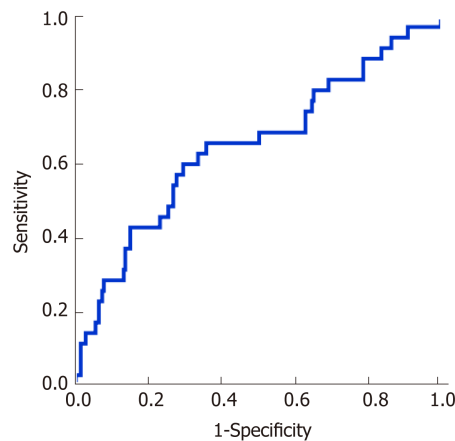


Figure 1 Determination of optimal albumin-bilirubin cut-off value by receiver operating characteristic analysis. The calculated cut-off for albumin-bilirubin scores to predict postoperative survival was -1.48, according to an area under a receiver operating characteristic curve of 0.647 ($P = 0.005$).

Pretransplant ALBI and postoperative complications

Table 4 shows postoperative complications stratified by pretransplant ALBI scores. A total of 189 patients developed various postoperative complications according to the Clavien-Dindo system^[9]; 87.06% of patients in the high ALBI score group (74 out of 85) developed postoperative complications after LT, while only 66.47% of patients in the low ALBI score group (115 out of 173) did. The difference was statistically significant ($P < 0.05$), which was also reflected in the comprehensive complication index (CCI). In terms of specific complications, a high ALBI score was associated with an increased incidence of biliary complications, intraabdominal bleeding, septicaemia, and AKI ($P < 0.05$ for all). However, no significant differences were found between the two groups for other complications.

DISCUSSION

The prediction of prognosis is an important part of management in patients with end-stage liver disease. Our current data show that the ALBI score, a simple model incorporating only serum bilirubin and serum albumin levels, performed better than the conventional MELD model in predicting overall survival and postoperative complications after LT. Assessment of liver function is particularly important for patients on the liver-transplant waiting list. Since both serum bilirubin and albumin are part of the commonly used liver function tests, the ALBI score is readily available. In this study, we found that the optimal ALBI cut-off value was -1.48, analysed by the ROC curve to predict survival after LT, which is very close to the cut-off value (-1.39) between ALBI grade 2 and grade 3^[6,10]. In fact, by using the cut-off value of the reported ALBI grading system developed for hepatectomy (*i.e.*, -1.39)^[6,7,11], we found that patients in the ALBI grade 3 classification had significantly higher mortality and more adverse postoperative outcomes after LT than patients in the ALBI grade 1 or 2 (data not shown) classifications, indicating that the reported ALBI grading system is also relevant in LT. Although many studies have shown that the ALBI grading system is a useful tool to identify patients at risk for adverse outcomes after hepatectomy, as far as we know, the present study is the first to assess the value of the pretransplant ALBI score in predicting outcomes after LT.

Assessment of preoperative liver function is vital to determine liver functional reserve in patients with end stage liver disease. The MELD system was developed in 2002 to prioritize patients waiting for LT^[12]. As a numerical scale, MELD was used for adult LT candidates^[13-15]. The patient's urgency for LT within the next three months was determined by personal MELD scores^[16]. The MELD scoring system contains two variables for hepatic (dys)function (*i.e.*, total bilirubin and INR) and one variable for renal (dys)function (*i.e.*, creatinine). Although subsequent studies have shown poor outcomes for liver transplant recipients with high MELD scores, its overall capacity to predict posttransplant outcomes is limited^[12,16,17].

In the current study, although we found that patients with an MELD score < 10 seemed to have slightly higher survival rates than patients with an MELD score ≥ 10 , there were no statistically significant differences in either the univariable or multivariable analyses. These results clearly show that the performance of ALBI is

Table 2 Baseline comparison between patients with different albumin-bilirubin grades

Variable	ALBI ≤ -1.48 (n = 173)	ALBI > -1.48 (n = 85)	P-value
Demographic feature			
Age (yr)	47 (39-55)	47 (38-56)	0.926
Male (Y/N)	138/35	68/17	0.965
Coexisting condition			
Smoking (Y/N)	52/121	26/59	0.931
Drinking (Y/N)	26/147	18/67	0.217
Hypertension (Y/N)	13/160	6/79	0.895
Diabetes (Y/N)	15/158	12/73	0.179
Etiology			
HCC (Y/N)	71/102	16/69	< 0.001
Viral hepatitis	143	57	
Alcoholic cirrhosis	4	4	
PBC and AIH	9	12	
Other	17	12	
Hepatic feature			
MELD score	14 (10-18)	23 (17.5-28)	< 0.001
Child grade A/B/C	42/80/51	1/14/70	< 0.001
Preoperative laboratory value			
RBC (10 ¹² /L)	3.40 (2.90-4.15)	2.94 (2.49-3.32)	< 0.001
HGB (g/L)	105.0 (88.0-130.0)	95.00 (86.5-107.0)	0.001
PLT (10 ⁹ /L)	59.0 (38.5-103.0)	45.0 (28.5-72.5)	0.002
WBC (10 ⁹ /L)	3.71 (2.55-5.36)	4.31 (2.84-7.38)	0.084
NEUT (10 ⁹ /L)	2.33 (1.67-3.74)	2.86 (1.80-5.90)	0.044
LYMPH (10 ⁹ /L)	0.70 (0.45-1.15)	0.60 (0.41-0.95)	0.189
MONO (10 ⁹ /L)	0.28 (0.18-0.45)	0.38 (0.22-0.57)	0.011
AFP (μg/L)	4.74 (2.73-16.30)	3.50 (2.22-6.23)	0.032
ALT (U/L)	32.00 (22.00-47.00)	38.00 (23.57-67.06)	0.042
AST (U/L)	41.50 (29.00-59.00)	54.52 (34.05-102.00)	0.001
TBIL (μmol/L)	32.75 (17.67-54.83)	105.48 (51.72-314.33)	< 0.001
DBIL (μmol/L)	11.70 (5.87-27.00)	50.70 (19.70-192.23)	< 0.001
ALB (g/L)	37.20 (34.50-41.99)	29.83 (26.95-32.11)	< 0.001
BUN (mmol/L)	4.39 (3.59-6.21)	5.07 (3.87-7.32)	0.077
CRE (μmol/L)	58.00 (48.00-68.85)	61.88 (48.15-82.00)	0.199
GLU (mmol/L)	5.60 (4.91-6.91)	6.06 (5.03-8.40)	0.062
PT (s)	17.20 (15.05-19.15)	20.70 (18.05-24.30)	< 0.001
APTT (s)	42.40 (39.15-47.70)	49.10 (43.25-54.75)	< 0.001
INR	1.41 (1.20-1.60)	1.74 (1.49-2.27)	< 0.001

ALBI: Albumin-bilirubin; MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; RBC: Red blood cells; HGB: Hemoglobin; PLT: Platelets; WBC: White blood cells; NEUT: Neutrophil granulocytes; LYMPH: Lymphocytes; MONO: Monocytes; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALB: Albumin; BUN: Blood urea nitrogen; CRE: Creatinine; GLU: Glucose; PT: Prothrombin time; APTT: Activated partial thromboplastin time; INR: International normalized ratio.

better than MELD in predicting outcomes after LT.

Another model to assess liver function is the C-P system. The C-P grade is determined by five variables, including TBIL, ALB, PT, and degree of ascites and hepatic encephalopathy. The C-P system was developed arbitrarily several decades ago based on clinical observation without proper statistical evidence. Although the C-P system is widely used, there are many limitations for its implementation^[18,19]. For instance, the grading of ascites and hepatic encephalopathy is highly subjective^[15,18,20]. It is not clear to identify the grade of ascites and hepatic encephalopathy according to guidelines. Some of the parameters, such as serum albumin levels and the extent of ascites, are interrelated. More importantly, the C-P grade failed to show any value in

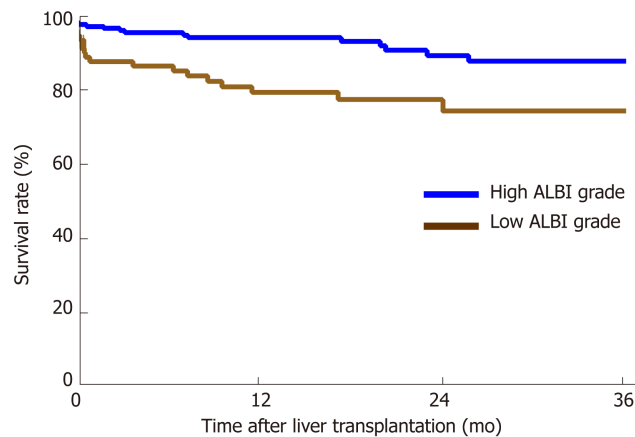


Figure 2 Kaplan-Meier estimation of 3-year survival according to albumin-bilirubin grade. Patients with a high albumin-bilirubin (ALBI) grade (> -1.48) had a significantly lower survival rate of 73.7% than patients with a low ALBI score ($\text{ALBI} \leq -1.48$) of 87.6% ($P < 0.05$). ALBI: Albumin-bilirubin.

discriminating both survival and complications after LT in our current study.

Of course, there were still some limitations in the study. First, this current study only included population data from one transplant centre; based on the LT data of the single centre, the posttransplant morbidity and mortality were low in the relatively small sample. For example, a relatively small proportion of patients died during follow-up, which may have limited the robustness of the multivariable analysis for adjustment for confounding factors. Second, only patients who received donation after DCD were included in the study; the value of ABLI scores in predicting outcomes of patients who received donation after brain death needs to be further investigated. Third, as the median follow-up time in the current study was only 17.30 mo, we were unable to comment on the effect of pretransplant ALBI scores on longer term outcomes of patients. Additionally, the study aimed to explore the effect of ALBI scores on overall survival, not on liver death related to liver disease (*i.e.*, disease-free survival)^[21]. The difficulty of specifically attributing the reason for death after transplantation in the clinic makes no difference in terms of the patients' outcomes. Lastly, as the nature of this study was retrospective, the results are subject to a selection bias and some residual confounding due to unmeasured or unknown confounders.

In summary, the data reveal that the ALBI score may be better than the MELD score for risk stratification of LT patients. Approximately one-third of our study population was categorized as having a high ALBI score (> -1.48); therefore, the ALBI scoring system is clinically relevant. In addition, the ALBI grading system may be a more readily applicable means to model risk among patients undergoing LT because it relies on fewer variables. The identification of patients who are most likely to benefit from LT remains a remarkable challenge^[22].

Table 3 Univariate and multivariate logistic regression analysis of overall survival

Variable	Univariate		Multivariate	
	P-value	OR (95%CI)	P-value	OR (95%CI)
ALBI grade	0.002	3.923 (1.495-5.716)	0.036	2.290 (1.057-4.963)
MELD grade	0.192	2.002 (0.706-5.676)		
Child-Pugh grade				
A	Reference			
B	0.713	0.815 (0.273-2.431)		
C	0.451	1.456 (0.548-3.868)		
Age	0.537	1.010 (0.978-1.044)		
Sex	0.233	0.629 (0.294-1.347)		
Drinking	0.266	1.565 (0.711-3.447)		
Smoking	0.931	0.968 (0.465-2.017)		
Diabetes	0.791	0.852 (0.261-2.785)		
Hypertension	0.576	1.402 (0.428-4.587)		
HCC	0.972	1.013 (0.504-2.035)		
Disease time	0.470	1.014 (0.977-1.051)		
RBC	0.282	0.794 (0.522-1.208)		
HGB	0.617	0.997 (0.987-1.008)		
PLT	0.054	1.004 (1.000-1.008)	0.048	1.005 (1.000-1.011)
WBC	0.002	1.097 (1.034-1.165)	0.481	1.034 (0.942-1.134)
NEUT	0.494	1.010 (0.981-1.041)		
LYMPH	0.615	1.071 (0.821-1.397)		
MONO	0.457	1.106 (0.849-1.441)		
AFP	0.085	1.000 (1.000-1.000)	0.391	1.000 (1.000-1.000)
ALT	0.002	1.001 (1.000-1.001)	0.278	1.000 (1.000-1.001)
AST	< 0.001	1.001 (1.001-1.002)	0.418	1.000 (0.999-1.002)
BUN	0.395	1.022 (0.972-1.075)		
CRE	0.002	1.005 (1.002-1.008)	0.027	1.005 (1.001-1.026)
GLU	0.575	0.959 (0.830-1.109)		
PT	0.181	1.034 (0.985-1.086)		
INR	0.127	1.336 (0.921-1.937)		
APTT	0.028	1.013 (1.001-1.024)	0.028	1.014 (1.001-1.026)
Operation time	0.007	1.005 (1.001-1.009)	0.182	1.003 (0.999-1.008)
Warm ischemia time	0.750	0.970 (0.803-1.171)		
Cold ischemia time	0.145	1.192 (0.941-1.509)		

OR: Odds ratio; CI: Confidence interval; ALBI: Albumin-bilirubin; MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; RBC: Red blood cells; HGB: Hemoglobin; PLT: Platelets; WBC: White blood cells; NEUT: Neutrophil granulocytes; LYMPH: Lymphocytes; MONO: Monocytes; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALB: Albumin; BUN: Blood urea nitrogen; CRE: Creatinine; GLU: Glucose; PT: Prothrombin time; APTT: Activated partial thromboplastin time; INR: International normalized ratio.

Table 4 Postoperative complications according to albumin-bilirubin grade

Complication	Low ALBI grade(n = 173)	High ALBI grade(n = 85)	P-value
Total complications (Y/N)	115/58	74/11	< 0.001
Pneumonia (Y/N)	51/122	33/52	0.132
AKI (Y/N)	79/94	57/28	0.001
Biliary complication (Y/N)	11/162	14/71	0.010
Porta vein thrombosis (Y/N)	3/170	0/85	0.546
Rejection (Y/N)	10/163	2/83	0.361
Intraabdominal bleeding (Y/N)	8/165	13/72	0.003
Coma for 24 h (Y/N)	1/172	4/81	0.075
Mechanical ventilation for 72 h (Y/N)	1/172	3/82	0.205
Septicemia (Y/N)	0/173	3/82	0.013
MOF (Y/N)	1/172	4/81	0.075
In-hospital mortality (Y/N)	3/170	4/81	0.330
SIRS (Y/N)	45/128	26/59	0.439
CCI, median (IQR)	29.60 (8.70-36.65)	36.20 (23.40-49.75)	< 0.001
Postoperative hospital stay, median days (IQR)	17.00 (13.50-24.00)	19.00 (12.50-25.00)	0.514

ALBI: Albumin-bilirubin; AKI: Acute kidney injury; MOF: Multiple organ failure; CCI: Comprehensive complication index; SIRS: Systemic inflammatory response syndrome; IQR: Interquartile range.

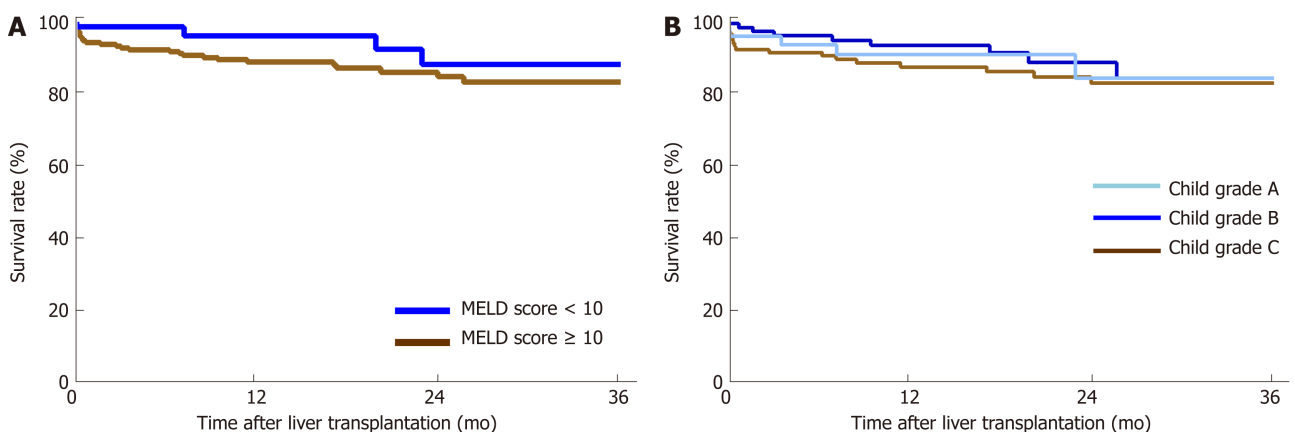


Figure 3 Kaplan-Meier estimation of 3-year survival according to model for end stage liver disease score. A: The 3-year survival rates between patients with a model for end stage liver disease score ≥ 10 and < 10 were 81.3% and 84.9%, respectively ($P > 0.05$). B: There were no statistically significant differences in 3-year survival rates among patients with different Child-Pugh grades ($P > 0.05$). MELD: Model for end stage liver disease.

ARTICLE HIGHLIGHTS

Research background

The albumin-bilirubin (ALBI) score, as a simple assessment of liver function, is objectively calculated by only two variables (albumin and bilirubin). It was proposed as a new method for preoperative risk evaluation to discern patients with the risk of adverse outcomes after hepatectomy. However, its ability to predict outcomes after liver transplantation has not been evaluated. Because of the significant shortage of organs and the increasing number of candidates on the transplant waiting list, there is an urgent need to identify patients who are most likely to benefit from LT.

Research motivation

The main topic of this study was to provide a potential scoring system for the allocation of donor liver resources by investigating the relationship between pretransplant ALBI score and outcomes after liver transplantation.

Research objectives

To retrospectively investigate the value of pretransplant ALBI scores in predicting outcomes after liver transplantation and as a tool for risk-stratifying patients on the liver transplant waiting list.

Research methods

The research data were obtained from a computerized clinical database from the First Affiliated Hospital of Xi'an Jiaotong University and included 258 consecutive patients who received donation after cardiac death (DCD) and underwent liver transplantation from March 2012 to March 2017. The optimal cut-off value of preoperative ALBI was calculated according to long-term survival status. The performance of the ALBI score in predicting outcomes, including postoperative complications and survival analysis, was measured and evaluated.

Research results

This study analysed data from 258 patients. Thirty-five patients died during follow-up [17.30 (interquartile range: 8.90-28.98) mo], with an overall survival rate of 81.0%. The optimal cut-off value of preoperative ALBI scores to predict postoperative survival was -1.48. Patients with an ALBI score > -1.48 had a significantly lower survival rate than those with an ALBI score ≤ -1.48 (73.7% vs 87.6%, $P < 0.05$), and there were no statistically significant differences in survival rates between patients with a model for end stage liver disease (MELD) score ≥ 10 and < 10 and different Child-Pugh grades. Moreover, a high ALBI score was associated with an increased incidence of biliary complications, intraabdominal bleeding, septicemia, and acute kidney injury after liver transplantation ($P < 0.05$ for all). Of course, this study only initially confirmed the predictive value of the ALBI score for liver transplantation outcomes. The predictive value of multi-centre data resources and other donations, except after DCD, need to be further researched and confirmed.

Research conclusions

After the ALBI grading system was developed to identify patients at risk for adverse outcomes after hepatectomy, this study hypothesized that this score may also be valuable in evaluating outcomes after liver transplantation. The ALBI score predicted overall survival and postoperative complications after liver transplantation. These data suggest that ALBI may be superior to MELD in risk-stratifying liver transplantation patients. In addition, ALBI may be a more readily applicable tool for modelling risk among patients undergoing liver transplantation because it relies on fewer variables.

Research perspectives

The ALBI grading system may be useful in risk-stratifying patients on the liver transplant waiting list. Multi-centre and prospective studies are needed to confirm our findings.

ACKNOWLEDGEMENTS

The authors thank all members of the Liver Transplantation Unit for their contributions to this valuable resource.

REFERENCES

- 1 Xu SL, Zhang YC, Wang GY, Yang Q, Liu B, Zhang J, Li H, Wang GS, Yang Y, Chen GH. Survival analysis of sirolimus-based immunosuppression in liver transplantation in patients with hepatocellular carcinoma. *Clin Res Hepatol Gastroenterol* 2016; **40**: 674-681 [PMID: 27825633 DOI: 10.1016/j.clinre.2016.03.006]
- 2 Klein KB, Stafinski TD, Menon D. Predicting survival after liver transplantation based on pre-transplant MELD score: a systematic review of the literature. *PLoS One* 2013; **8**: e80661 [PMID: 24349010 DOI: 10.1371/journal.pone.0080661]
- 3 Han S, Kwon JH, Jung SH, Seo JY, Jo YJ, Jang JS, Yeon SM, Jung SH, Ko JS, Gwak MS, Cho D, Son HJ, Kim GS. Perioperative Fresh Red Blood Cell Transfusion May Negatively Affect Recipient Survival After Liver Transplantation. *Ann Surg* 2018; **267**: 346-351 [PMID: 27805962 DOI: 10.1097/SLA.0000000000002062]
- 4 Otto G. Liver transplantation: an appraisal of the present situation. *Dig Dis* 2013; **31**: 164-169 [PMID: 23797139 DOI: 10.1159/000347213]
- 5 Dolgin NH. Health Care Rich, Resource Poor: Struggling with the National Shortage of Organs in Liver Transplantation. *AMA J Ethics* 2016; **18**: 97-100 [PMID: 27326433]
- 6 Johnson PJ, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, O'Beirne J, Fox R, Skowronska A, Palmer D, Yeo W, Mo F, Lai P, Inarrairaegui M, Chan SL, Sangro B, Miksad R, Tada T, Kumada T, Toyoda H. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. *J Clin Oncol* 2015; **33**: 550-558 [PMID: 25512453 DOI: 10.1200/JCO.2014.57.9151]
- 7 Andreatos N, Amini N, Gani F, Margonis GA, Sasaki K, Thompson VM, Bentrem DJ, Hall BL, Pitt HA, Wilson A, Pawlik TM. Albumin-Bilirubin Score: Predicting Short-Term Outcomes Including Bile Leak and Post-hepatectomy Liver Failure Following Hepatic Resection. *J Gastrointest Surg* 2017; **21**: 238-248 [PMID: 27619809 DOI: 10.1007/s11605-016-3246-4]
- 8 Zou D, Qi X, Zhu C, Ning Z, Hou F, Zhao J, Peng Y, Li J, Deng H, Guo X. Albumin-bilirubin score for predicting the in-hospital mortality of acute upper gastrointestinal bleeding in liver cirrhosis: A retrospective study. *Turk J Gastroenterol* 2016; **27**: 180-186 [PMID: 27015623 DOI: 10.5152/tjg.2016.15502]
- 9 Clavien PA, Barkun J, de Oliveira ML, Vauthey JN, Dindo D, Schulick RD, de Santibañes E, Pekolj J, Slankamenac K, Bassi C, Graf R, Vonlanthen R, Padbury R, Cameron JL, Makuuchi M. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; **250**: 187-196 [PMID: 19246610 DOI: 10.1097/SLA.0b013e318191a916]

- 19638912 DOI: [10.1097/SLA.0b013e3181b13ca2](https://doi.org/10.1097/SLA.0b013e3181b13ca2)]
- 10 **Chan AW**, Chong CC, Mo FK, Wong J, Yeo W, Johnson PJ, Yu S, Lai PB, Chan AT, To KF, Chan SL. Applicability of albumin-bilirubin-based Japan integrated staging score in hepatitis B-associated hepatocellular carcinoma. *J Gastroenterol Hepatol* 2016; **31**: 1766-1772 [PMID: [26992142](https://pubmed.ncbi.nlm.nih.gov/26992142/) DOI: [10.1111/jgh.13339](https://doi.org/10.1111/jgh.13339)]
 - 11 **Toyoda H**, Lai PB, O'Beirne J, Chong CC, Berhane S, Reeves H, Manas D, Fox RP, Yeo W, Mo F, Chan AW, Tada T, Iñarrairaegui M, Vogel A, Schweitzer N, Chan SL, Sangro B, Kumada T, Johnson PJ. Long-term impact of liver function on curative therapy for hepatocellular carcinoma: application of the ALBI grade. *Br J Cancer* 2016; **114**: 744-750 [PMID: [27022825](https://pubmed.ncbi.nlm.nih.gov/27022825/) DOI: [10.1038/bjc.2016.33](https://doi.org/10.1038/bjc.2016.33)]
 - 12 **Rauchfuss F**, Zidan A, Scheuerlein H, Dittmar Y, Bauschke A, Settmacher U. Waiting time, not donor-risk-index, is a major determinant for beneficial outcome after liver transplantation in high-MELD patients. *Ann Transplant* 2013; **18**: 243-247 [PMID: [23792527](https://pubmed.ncbi.nlm.nih.gov/23792527/) DOI: [10.12659/AOT.883924](https://doi.org/10.12659/AOT.883924)]
 - 13 **Strassburg CP**. [Patient selection and indications for liver transplantation]. *Chirurg* 2013; **84**: 363-371 [PMID: [23576124](https://pubmed.ncbi.nlm.nih.gov/23576124/) DOI: [10.1007/s00104-012-2418-3](https://doi.org/10.1007/s00104-012-2418-3)]
 - 14 **Kim WR**, Biggins SW, Kremers WK, Wiesner RH, Kamath PS, Benson JT, Edwards E, Therneau TM. Hyponatremia and mortality among patients on the liver-transplant waiting list. *N Engl J Med* 2008; **359**: 1018-1026 [PMID: [18768945](https://pubmed.ncbi.nlm.nih.gov/18768945/) DOI: [10.1056/NEJMoa0801209](https://doi.org/10.1056/NEJMoa0801209)]
 - 15 **Huang HC**, Lee FY, Huo TI. Major adverse events, pretransplant assessment and outcome prediction. *J Gastroenterol Hepatol* 2009; **24**: 1716-1724 [PMID: [20136958](https://pubmed.ncbi.nlm.nih.gov/20136958/) DOI: [10.1111/j.1440-1746.2009.06025.x](https://doi.org/10.1111/j.1440-1746.2009.06025.x)]
 - 16 **Marroni CP**, de Mello Brandão AB, Hennigen AW, Marroni C, Zanotelli ML, Cantisani G, Fuchs SC; Liver Transplantation Group. MELD scores with incorporation of serum sodium and death prediction in cirrhotic patients on the waiting list for liver transplantation: a single center experience in southern Brazil. *Clin Transplant* 2012; **26**: E395-E401 [PMID: [22882694](https://pubmed.ncbi.nlm.nih.gov/22882694/) DOI: [10.1111/j.1399-0012.2012.01688.x](https://doi.org/10.1111/j.1399-0012.2012.01688.x)]
 - 17 **Bernardi M**, Gitto S, Biselli M. The MELD score in patients awaiting liver transplant: strengths and weaknesses. *J Hepatol* 2011; **54**: 1297-1306 [PMID: [21145851](https://pubmed.ncbi.nlm.nih.gov/21145851/) DOI: [10.1016/j.jhep.2010.11.008](https://doi.org/10.1016/j.jhep.2010.11.008)]
 - 18 **Durand F**, Valla D. Assessment of prognosis of cirrhosis. *Semin Liver Dis* 2008; **28**: 110-122 [PMID: [18293281](https://pubmed.ncbi.nlm.nih.gov/18293281/) DOI: [10.1055/s-2008-1040325](https://doi.org/10.1055/s-2008-1040325)]
 - 19 **Ge PL**, Du SD, Mao YL. Advances in preoperative assessment of liver function. *Hepatobiliary Pancreat Dis Int* 2014; **13**: 361-370 [PMID: [25100120](https://pubmed.ncbi.nlm.nih.gov/25100120/)]
 - 20 **Rahimi-Dehkordi N**, Nourijelyani K, Nasiri-Tousi M, Ghodssi-Ghassemabadi R, Azmoudeh-Ardalan F, Nedjat S. Model for End stage Liver Disease (MELD) and Child-Turcotte-Pugh (CTP) scores: Ability to predict mortality and removal from liver transplantation waiting list due to poor medical conditions. *Arch Iran Med* 2014; **17**: 118-121 [PMID: [24527973](https://pubmed.ncbi.nlm.nih.gov/24527973/)]
 - 21 **Fahrner R**, Dondorf F, Ardel M, Dittmar Y, Settmacher U, Rauchfuß F. Liver transplantation for hepatocellular carcinoma - factors influencing outcome and disease-free survival. *World J Gastroenterol* 2015; **21**: 12071-12082 [PMID: [26576092](https://pubmed.ncbi.nlm.nih.gov/26576092/) DOI: [10.3748/wjg.v21.i42.12071](https://doi.org/10.3748/wjg.v21.i42.12071)]
 - 22 **Neuberger J**. An update on liver transplantation: A critical review. *J Autoimmun* 2016; **66**: 51-59 [PMID: [26350881](https://pubmed.ncbi.nlm.nih.gov/26350881/) DOI: [10.1016/j.jaut.2015.08.021](https://doi.org/10.1016/j.jaut.2015.08.021)]



Observational Study

Mechanism of exosomal microRNA-224 in development of hepatocellular carcinoma and its diagnostic and prognostic value

Yao Cui, Hai-Feng Xu, Ming-Yue Liu, Yu-Jie Xu, Jun-Chuang He, Yun Zhou, Shun-Dong Cang

ORCID number: Yao Cui (0000-0001-8381-9027); Hai-Feng Xu (0000-0002-8919-1602); Ming-Yue Liu (0000-0003-1546-6121); Yu-Jie Xu (0000-0001-9543-4116); Jun-Chuang He (0000-0001-8876-968X); Yun Zhou (0000-0001-7638-4176); Shun-Dong Cang (0000-0001-6137-3263).

Author contributions: Cui Y, Xu HF, and Cang SD designed the study; Cui Y, Xu HF, Liu MY, and Xu YJ performed the research; Cui Y, He JC, and Cang SD analyzed the data; Cui Y and Xu HF wrote the paper; Cang SD revised the manuscript for final submission; Cui Y and Xu HF contributed equally to this study.

Supported by the Scientific and Technological Research Project of Henan Province, No. 162102310024.

Institutional review board

statement: The study was reviewed and approved by the Peking University Cancer Hospital and Institute review board.

Informed consent statement: All study participants or their legal guardian provided written informed consent prior to study enrollment.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service and/or company that could be construed as

Yao Cui, Ming-Yue Liu, Yu-Jie Xu, Yun Zhou, Shun-Dong Cang, Department of Oncology, Henan Key Laboratory for Precision Medicine in Cancer, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, School of Clinical Medicine, Henan University, Zhengzhou 450003, Henan Province, China

Hai-Feng Xu, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Interventional Therapy Department, Peking University Cancer Hospital and Institute, Beijing 100142, China

Jun-Chuang He, Department of Hepatobiliary Surgery, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, School of Clinical Medicine, Henan University, Zhengzhou 450003, Henan Province, China

Corresponding author: Shun-Dong Cang, MD, Associate Professor, Doctor, Department of Oncology, Henan Key Laboratory for Precision Medicine in cancer, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, School of Clinical Medicine, Henan University, No. 7, Weiwu Road, Zhengzhou 450003, Henan Province, China. cangshundonghn@126.com

Telephone: +86-371-65580092

Fax: +86-371-65580092

Abstract

BACKGROUND

Exosomes contain proteins, lipids, and biological molecules such as DNA and RNA. Nucleic acids in exosomes are a group of molecules that can act as biomarkers. Currently, there are many reports on exosomal microRNAs, which are ideal biomarkers for the early diagnosis of cancer. However, there are few reports on the role of exosomal microRNAs in the diagnosis and prognosis of hepatocellular carcinoma (HCC).

AIM

To understand the mechanism of exosomal microRNA-224 (miR-224) in the development of HCC and evaluate its diagnostic and prognostic value.

METHODS

Cell culture and transfection of exosomal miRNA-224, real-time quantitative PCR, luciferase reporter assay, and other methods were used to find new biomarkers related to the development of HCC that can be used to diagnose HCC and predict HCC prognosis.

RESULTS

influencing the position presented in or the review of the manuscript.

Data sharing statement: No additional data are available

STROBE statement: The manuscript was prepared according to the STROBE Checklist.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: December 27, 2018

Peer-review started: December 27, 2018

First decision: January 18, 2019

Revised: February 17, 2019

Accepted: February 22, 2019

Article in press: February 23, 2019

Published online: April 21, 2019

P-Reviewer: Gordon LG, Jin M, Shimizu Y

S-Editor: Ma RY

L-Editor: Wang TQ

E-Editor: Ma YJ



By targeting glycine N-methyltransferase, incubating exosomes with miR-224 mimic resulted in a significant increase in cell proliferation compared to that of the control group, while incubation with the miR-224 inhibitor significantly reduced cell proliferation. The same results were obtained for the cell invasion assay. Serum exosomal miR-224 did have some ability to differentiate patients with HCC from healthy controls, with an area under the curve of 0.910, and HCC patients with higher serum exosomal miR-224 expression had lower overall survival.

CONCLUSION

Exosomal miR-224 is a tumor promotor and can be a marker of diagnosis and prognosis of HCC patients, however, its ability to distinguish liver diseases needs further verification.

Key words: Hepatocellular carcinoma; Serum; Exosome; MicroRNA-224; Biomarker

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: We aimed to understand the mechanism of exosomal microRNA-224 (miR-224) in the development of hepatocellular carcinoma (HCC) and to evaluate its diagnostic and prognostic value for HCC patients. By directly targeting the 3'-untranslated region of glycine N-methyltransferase, exosomal miR-224 inhibits its expression to promote proliferation and invasion. In addition, serum exosomal miR-224 also has potential diagnostic and prognostic value for HCC.

Citation: Cui Y, Xu HF, Liu MY, Xu YJ, He JC, Zhou Y, Cang SD. Mechanism of exosomal microRNA-224 in development of hepatocellular carcinoma and its diagnostic and prognostic value. *World J Gastroenterol* 2019; 25(15): 1890-1898

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1890.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1890>

INTRODUCTION

Liver cancer is a malignant tumor with a high mortality rate in China^[1]. Liver cancer has the fourth-highest incidence rate of all tumors, and the fatality rate ranks third^[1]. A total of 25% of the cancer cases are caused by carcinogenic infections such as hepatitis virus and human papillomavirus, and this figure includes a high proportion of patients in low-income and middle-income countries^[2]. Therefore, early detection, diagnosis, and treatment are of great significance for the prognosis of patients with liver cancer. An early clinical diagnosis can effectively improve the survival of patients with hepatocellular carcinoma (HCC)^[3]. However, the diagnostic sensitivity and specificity of current noninvasive indicators such as serum alpha-fetoprotein (AFP) and imaging studies for HCC, especially early HCC, still need to be improved^[4]. Therefore, new methods for diagnosing HCC need to be developed.

Exosomes are secreted and released by various types of cells and contain nanovesicles with various active factors^[5]. Previous literature has reported that serum microRNAs (miRNAs) are mainly present in the exosomes formed from phospholipid membranes, thus avoiding degradation by RNase in circulation^[6]. Exosomes have been shown to play an important role in tumorigenesis, development, metastasis, deterioration, and immune escape^[7,8]. For example, Gu *et al*^[9] found that exosomes secreted by mesenchymal stem cells can promote the growth of gastric cancer cells, which indicated that exosomes can promote tumorigenesis and cancer development. Yu *et al*^[10] found that exosomes secreted by breast cancer cells can promote tumor angiogenesis, growth, and proliferation, proving that exosomes can promote tumor cell metastasis. Exosomes secreted by breast cancer cells can also promote the proliferation of surrounding normal breast cells and inhibit their apoptosis. In addition, exosomes may also promote complications in cancer patients; for example, patients with pancreatic cancer often have diabetes^[11].

Exosomes contain proteins, lipids, and biological molecules such as DNA and RNA^[12]. Nucleic acids in exosomes are a group of molecules that can act as biomarkers. Currently, there are many reports on exosomal miRNAs, which are ideal

biomarkers for the early diagnosis of cancer^[13-16]. However, there are few reports on the role of exosomal miRNAs in the diagnosis and prognosis of HCC. In this study, cell culture and transfection of exosomal miRNA, real-time quantitative PCR (RT-qPCR), luciferase reporter assay, and other methods were performed to find new biomarkers related to the development of HCC and to determine if the biomarkers can be used for the diagnosis and prognosis of HCC.

MATERIALS AND METHODS

Sample collection

A total of 89 HCC and 50 normal serum samples were collected from 2014 to 2016, and the clinical information is shown in Table 1. The patients were not treated before the samples were collected, and the diagnosis of HCC was confirmed by pathological analysis of tumor tissue. The analysis of the samples was approved by the patients and the ethics committee.

Cell culture

The hepatocyte lines WRL68, HepG2, and SKHEP1 were selected and cultured in RPMI-1640 (Sigma) containing 10% fetal bovine serum (FBS; Gibco) at 37 °C in 5% CO₂. An miRNA mimic or inhibitor was transfected into cells with preincubated exosomes or Lipofectamine 2000.

Exosome isolation

The cells were starved in serum-free medium overnight and then centrifuged for 3 min at 2000 rpm, followed by filtration. The exosomes in the cell culture medium and in patient serum were extracted using the Total Exosome Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The resulting precipitate was observed under a transmission electron microscope as described previously^[17].

RT-qPCR

Exosomal RNA in serum or cell culture medium was extracted with Trizol (Thermo Fisher Scientific). The expression of exosomal miRNA was detected on the Quant Studio 7 Flex RT PCR System (Applied Biosystems) using a hydrolysis probe according to the manufacturer's instructions. All experiments were performed in triplicate, and a mixture of let-7i, let-7g, and let-7d (let-7d/g/i) was used as endogenous controls to calculate the relative concentration of miRNA^[18-20].

Cell proliferation and invasion

A Cell Counting Kit-8 (CCK8) assay (Dojindo, Japan) followed by measuring the spectrophotometric absorbance at 450 nm was used to estimate cell proliferation. All experiments were performed in triplicate, and data are presented as the mean. A total of 2×10^5 cells were cultured for 48 h in serum-free medium, while the lower chamber was filled with medium containing 10% FBS to analyze the invasion abilities of the cells; the cancer cells in the lower chamber were ultimately counted^[21].

Luciferase reporter assay

MicroRNA-224 (miR-224) mimic, glycine N-methyltransferase (GNMT) wild- or 3'-untranslated region (UTR) mutant-type, and controls were cotransfected into SKHEP1 cells in 24-well plates for 24 h. The harvested cells were analyzed for fluorescence intensity using a dual luciferase reporter assay kit as indicated.

Statistical analysis

Differences between groups were analyzed by *t*-tests using GraphPad Prism 6.0. The receiver operating characteristic (ROC) curve was constructed using SPSS 22.0 software, and the area under the curve (AUC) was calculated to assess the specificity and sensitivity of the prediction of HCC cases and controls. A *P*-value < 0.05 was considered significant. The Kaplan-Meier survival curve was used to analyze the survival of the patients.

RESULTS

Exosome separation and validation

The morphological characteristics of the exosomes in the cell culture medium were observed under a transmission electron microscope. The exosomes showed a vesicular structure with a diameter of approximately 50-150 nm, which is consistent with the

Table 1 Clinical information of the patients

Variable	HCC (n = 89)	Normal controls (n = 50)	P-value
Average age (yr)	59.03 (25-90)	53.58 (25-76)	0.10
Sex			0.62
Male n (%)	43 (48.31)	22 (44.00)	
Female n (%)	46 (51.69)	28 (56.00)	
Cirrhosis			
Yes n (%)	58 (65.17)		
No n (%)	31 (34.83)		
T classification			
T1-T2 n (%)	37 (41.57)		
T3-T4 n (%)	52 (58.43)		
Tumor size			
> 3 cm n (%)	35 (39.33)		
< 3 cm n (%)	54 (60.67)		

literature^[22].

Based on previous experimental results, we found that the expression level of miR-224 in the HCC tissues was significantly higher than that in the normal controls. In this study, we verified the expression of miR-224 in the exosomes of WRL68, HepG2, and SKHEP1 cell lines by RT-qPCR. The expression level of exosomal miR-224 was significantly increased in the two liver cancer cell lines, HepG2 and SKHEP1, compared to that of the normal hepatic cell line, WRL68 (Figure 1).

Exosomal miR-224 stimulates the proliferation and invasion of HCC cells

Exosomes incubated with miR-224 mimic or inhibitor were added to HepG2 and SKHEP1 cells to measure cell proliferation. The results showed that exosomes incubated with the miR-224 mimic resulted in a significant increase in cell proliferation compared to the proliferation in the control group, while the exosomes incubated with the miR-224 inhibitor exhibited significantly reduced cell proliferation (Figure 2A and B). These results indicated that exosomal miR-224 can promote the proliferation of liver cancer cells. The same results were obtained for the cell invasion assay (Figure 2C and D). Exosomes incubated with the miR-224 mimic resulted in more cells passing through the insert membranes to the lower chamber, indicating that the exosomal miR-224 can also promote liver cancer cell invasion.

MiR-224 targets GNMT

It has been reported that miR-224 can affect cancer development by targeting glycine N-methyltransferase (GNMT)^[23], so we used a luciferase reporter assay to verify whether miR-224 can directly interact with GNMT. As shown in Figure 3A, the wild-type GNMT reporter gene combined with the miR-224 mimic exhibited lower luciferase activity in the HepG2 cell line than that of the control group. However, when the 3'-UTR of the GNMT gene was mutated, this reduction could be eliminated. The siGNMT was added to HepG2 cells to knock out GNMT mRNA, which can reduce the expression of GNMT. The results showed that the proliferation and invasion of cells increased notably (Figure 3B and C). It is suggested that miR-224 may directly target GNMT to promote the proliferation and invasion of liver cancer cells.

Correlation between serum exosomal miR-224 and liver cancer

Using RT-qPCR, we determined that the expression levels of exosomal miR-224 in the 89 HCC samples were significantly higher than those in the 50 healthy controls (Figure 4A). In addition, according to the clinical characteristics of the sample group, the levels of exosomal miR-224 in the serum of patients with large tumors or late-stage tumors were significantly higher (Figure 4B and C). In the ROC curve analysis, serum exosomal miR-224 showed an ability to differentiate HCC patients from healthy controls, with an AUC of 0.910 (Figure 4D). These results indicated that serum exosomal miR-224 can be used as a potential biomarker for the diagnosis of HCC.

In addition, we analyzed the overall survival of the followed patients. Kaplan-Meier survival curves showed that HCC patients with higher serum exosomal miR-224 expression had lower overall survival (Figure 5), suggesting that serum exosomal miR-224 can be an independent prognostic factor for HCC.

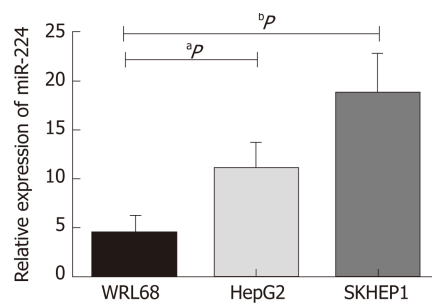


Figure 1 Relative expression of exosomal microRNA-224 in the hepatocellular carcinoma cell lines compared to that of the control. ^a $P < 0.05$, ^b $P < 0.01$. miR-224: MicroRNA-224.

DISCUSSION

In this study, we identified that the expression level of exosomal miR-224 in the liver cancer cell lines HepG2 and SKHEP1 was significantly higher than that in the healthy liver cell line WRL68 using gene chip and RT-qPCR. We also determined that the high expression of exosomal miR-224 can promote the proliferation and invasion of liver cancer cells. In addition, lower luciferase activity was observed when the miR-224 mimic and the GNMT wild type were cotransfected into the HepG2 cell line, while this phenomenon did not occur when the GNMT 3'-UTR was mutated, indicating that miR-224 can directly target the 3'-UTR of GNMT mRNA. In addition, siGNMT was added to the HepG2 cell line, and as a result, the proliferation and invasion of the cancer cells increased. Therefore, miR-224 can directly target GNMT to promote the proliferation and invasion of cancer cells.

We verified that miR-224 can directly target GNMT to increase the proliferation and invasion of liver cancer cells. In colorectal cancer, miR-224 targets caspase-3 and caspase-7, and this inverse relationship was evident from the earliest phases of transformation in the intestinal mucosa^[24]. In addition, miR-224-5p inhibited autophagy by targeting Smad4 in breast cancer cells, suggesting a novel regulatory network contributing to the metastasis of breast cancer^[25]. Both miR-224 overexpression and PTX3 silencing promoted cell proliferation, migration, and invasion, whereas the aforementioned properties were reduced when miR-224 was inhibited, indicating that miR-224 inhibition may significantly prevent cervical carcinoma progression by targeting the *PTX3* gene^[26]. MiR-224 inversely regulated thioredoxin-interacting protein (TXNIP) by binding directly to its 3'-UTR, which resulted in the activation of hypoxia-inducible factor 1 α (HIF-1 α), while either TXNIP re-expression or HIF-1 α depletion abolished the effects of miR-224 on the proliferation and migration of PDAC cells *in vitro* and *in vivo*, suggesting that TXNIP is a target of miR-224^[27]. HIF-1 α inhibits the NCR1/NKp46 pathway by upregulating miR-224, which affects the killing capability of natural killer cells in prostate cancer, thus inducing the immune escape of tumor cells^[28]. In summary, miR-224 may play an important role in the development and occurrence of tumors. Furthermore, miR-224 has many target genes, suggesting that miR-224 may participate in different pathways for cancer regulation, which requires further exploration.

We collected preoperative serum from HCC patients and healthy controls and isolated exosomes with high and stable purity. The expression level of serum exosomal miR-224 in HCC patients was significantly higher than that in healthy controls, as determined by RT-qPCR. The ability of serum exosomal miR-224 as a biomarker to distinguish HCC patients from healthy controls was confirmed by the ROC curve analysis. In addition, the relationship between tumor size and stage and miR-224 expression in HCC patients was analyzed, and we found that the expression of serum exosomal miR-224 was higher in patients with larger tumors and later stages. In addition, we also analyzed the relationship between the expression of exosomal miR-224 and the overall survival of patients. The Kaplan-Meier survival curve showed that the higher the expression level of serum exosomal miR-224, the shorter the patient's overall survival, suggesting that serum exosomal miR-224 can be used as a prognostic factor in patients with HCC. However, in this study we only compared the expression of exosomal miR-224 in the serum of HCC patients and healthy controls. It is not clear whether the expression of exosomal miR-224 in cirrhosis, hepatitis, or other liver cancer type is different, so the ability of miR-224 to distinguish between HCC and other liver diseases needs further verification.

In conclusion, exosomal miR-224 can decrease the expression of GNMT by directly targeting the 3'-UTR of GNMT mRNA to promote the proliferation and invasion of

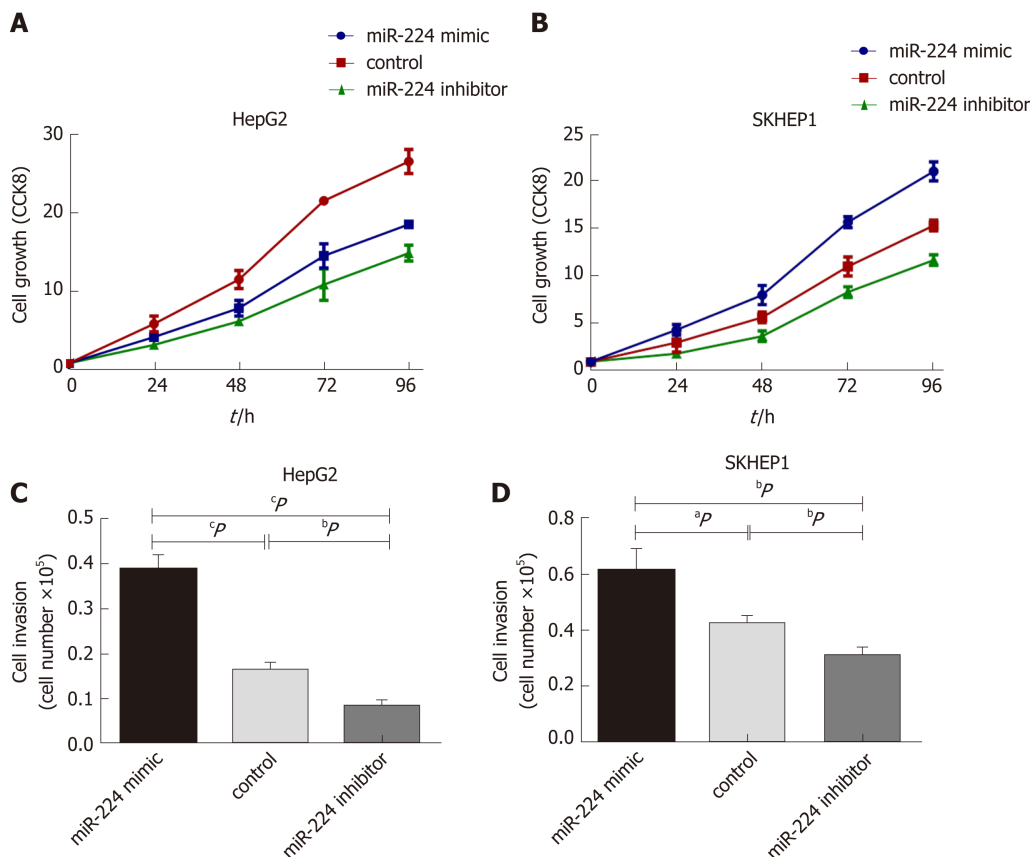


Figure 2 Exosomal microRNA-224 regulates hepatocellular carcinoma cell proliferation and invasion. A and B: MicroRNA-224 (miR-224) promoted cell growth as measured by the Cell Counting Kit 8 assay in HepG2 and SKHEP1 cell lines. C and D: MiR-224 can promote cell invasion in HepG2 and SKHEP1 cell lines. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001. CCK8: Cell Counting Kit 8; miR-224: MicroRNA-224.

HCC cells, which may provide a new method for HCC treatment. In addition, serum exosomal miR-224 may be used as a biomarker for the diagnosis of HCC and a prognostic factor for patients with HCC.

ARTICLES HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a malignant tumor with a high mortality rate. Exosomes have been shown to play an important role in tumorigenesis, cancer development, metastasis, deterioration, and immune escape.

Research motivation

We aimed to research the mechanism of exosomal microRNA-224 (miR-224) and its target in the development and invasion of HCC, and we also evaluated the diagnostic and prognostic value of miR-224 for patients with HCC.

Research methods

Cell culture and transfection of exosomal miR-224, its mimics, and its inhibitor; real-time quantitative PCR; and luciferase reporter assay were used to explore the mechanism of exosomal miR-224. HCC patients and healthy controls were used to assess the value of exosomal miR-224 in diagnosing HCC and predicting HCC prognosis.

Research results

Serum exosomes incubated with the miR-224 mimic showed a significant increase in cell proliferation and invasion when compared to the control group, while those incubated with the inhibitor showed a significant reduction. For discriminating HCC from healthy controls, serum exosomal miR-224 showed an area under the ROC curve of 0.910. Higher serum exosomal miR-224 expression levels in HCC patients were associated with lower overall survival.

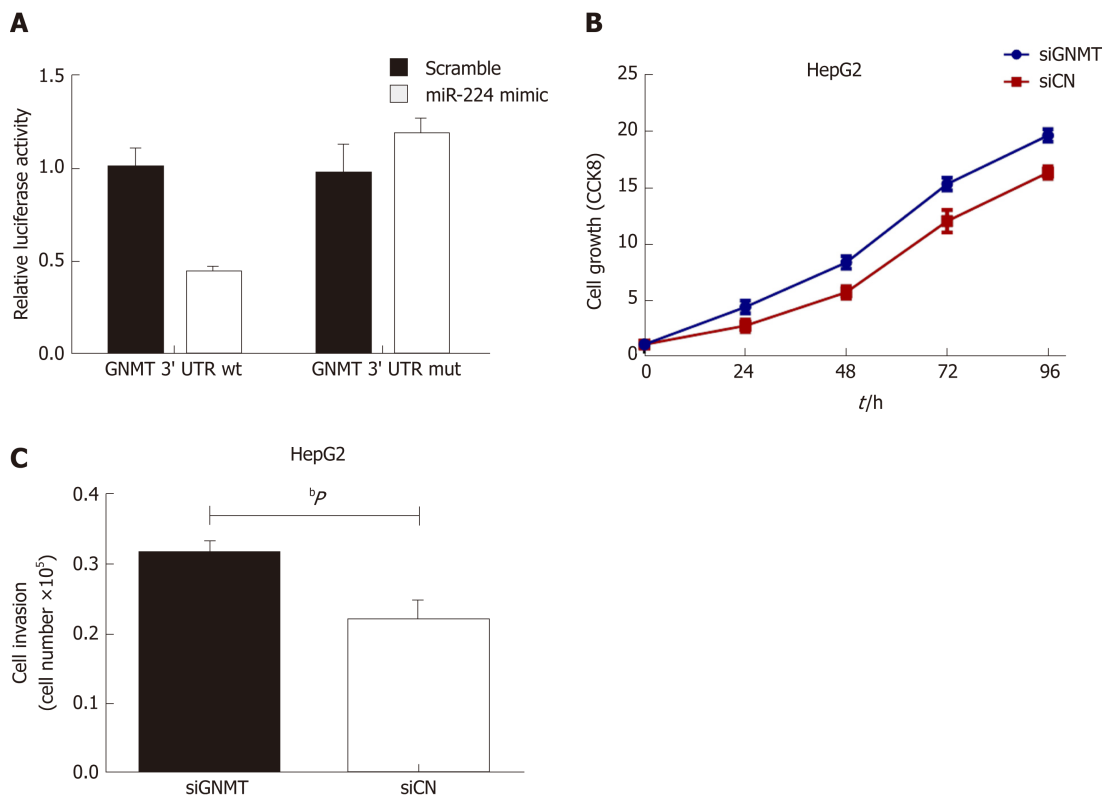


Figure 3 MicroRNA-224 targets glycine N-methyltransferase. A: MicroRNA-224 (MiR-224) mimic reduced the luciferase activity of cells transfected with the wild-type 3'-UTR but did not alter the luciferase activity of cells transfected with the mutant 3'-UTR. B: Si-glycine N-methyltransferase (siGNMT) can promote cell growth as measured by the Cell Counting Kit 8 assay in HepG2 cells. C: SiGNMT can promote cell invasion in HepG2 cells. ^b*P* < 0.01. CCK8: Cell Counting Kit 8; miR-224: MicroRNA-224; GNMT: Glycine N-methyltransferase.

Research conclusions

The results showed that exosomal miR-224 directly targets the 3'-UTR of glycine N-methyltransferase and impacts the proliferation and invasion of HCC, and exosomal miR-224 may be used as a potential diagnostic and prognostic factor for patients with HCC.

Research perspectives

Our study provides novel insight into the mechanism of exosomal miR-224 in the development and invasion of HCC and may provide a potential biomarker for the diagnosis and prognosis of HCC.

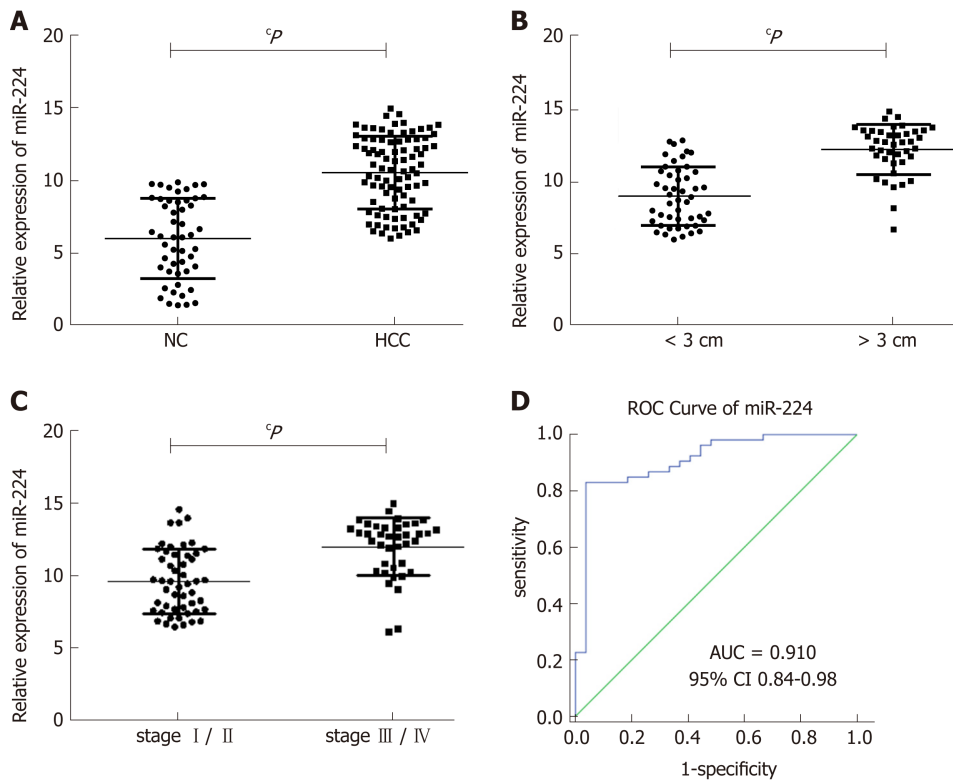


Figure 4 Expression of serum exosomal microRNA-224 in hepatocellular carcinoma patients. A: Relative expression of serum exosomal microRNA-224 (miR-224) in hepatocellular carcinoma (HCC) and normal controls. B and C: Relative expression of serum exosomal miR-224 in HCC patients based on tumor size and tumor stage. D: The receiver operating characteristic curve of miR-224 for diagnosing HCC. $P < 0.001$. NC: Normal controls; HCC: Hepatocellular carcinoma; ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval; miR-224: MicroRNA-224.

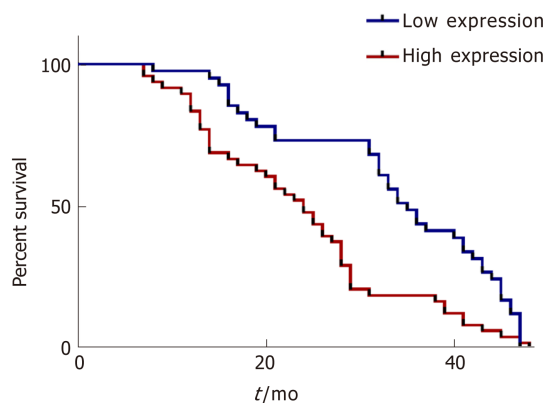


Figure 5 Kaplan-Meier overall survival analysis of microRNA-224.

REFERENCES

- Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]
- Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health* 2016; **4**: e609-e616 [PMID: 27470177 DOI: 10.1016/S2214-109X(16)30143-7]
- Santi V, Trevisani F, Gramenzi A, Grignaschi A, Mirici-Cappa F, Del Poggio P, Di Nolfo MA, Benvegnù L, Farinati F, Zoli M, Giannini EG, Borzio F, Caturelli E, Chiaramonte M, Bernardi M; Italian Liver Cancer (ITA. LI.CA) Group. Semiannual surveillance is superior to annual surveillance for the detection of early hepatocellular carcinoma and patient survival. *J Hepatol* 2010; **53**: 291-297 [PMID: 20483497 DOI: 10.1016/j.jhep.2010.03.010]
- Sun W, Liu Y, Shou D, Sun Q, Shi J, Chen L, Liang T, Gong W. AFP (alpha fetoprotein): who are you in gastrology? *Cancer Lett* 2015; **357**: 43-46 [PMID: 25462859 DOI: 10.1016/j.canlet.2014.11.018]
- Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer* 2010; **127**: 118-126 [PMID: 19876917 DOI: 10.1002/ijc.21888]

- 10.1002/ijc.25007]
- 6 **Lima LG**, Chammas R, Monteiro RQ, Moreira ME, Barcinski MA. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. *Cancer Lett* 2009; **283**: 168-175 [PMID: [19401262](#) DOI: [10.1016/j.canlet.2009.03.041](#)]
- 7 **Hood JL**, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. *Cancer Res* 2011; **71**: 3792-3801 [PMID: [21478294](#) DOI: [10.1158/0008-5472.CAN-10-4455](#)]
- 8 **Wolfers J**, Lozier A, Raposo G, Regnault A, Théry C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, Angevin E, Amigorena S, Zitvogel L. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 2001; **7**: 297-303 [PMID: [11231627](#) DOI: [10.1038/85438](#)]
- 9 **Gu H**, Ji R, Zhang X, Wang M, Zhu W, Qian H, Chen Y, Jiang P, Xu W. Exosomes derived from human mesenchymal stem cells promote gastric cancer cell growth and migration via the activation of the Akt pathway. *Mol Med Rep* 2016; **14**: 3452-3458 [PMID: [27513187](#) DOI: [10.3892/mmr.2016.5625](#)]
- 10 **Yu DD**, Wu Y, Shen HY, Lv MM, Chen WX, Zhang XH, Zhong SL, Tang JH, Zhao JH. Exosomes in development, metastasis and drug resistance of breast cancer. *Cancer Sci* 2015; **106**: 959-964 [PMID: [26052865](#) DOI: [10.1111/cas.12715](#)]
- 11 **Javeed N**, Sagar G, Dutta SK, Smyrk TC, Lau JS, Bhattacharya S, Truty M, Petersen GM, Kaufman RJ, Chari ST, Mukhopadhyay D. Pancreatic Cancer-Derived Exosomes Cause Paraneoplastic β -cell Dysfunction. *Clin Cancer Res* 2015; **21**: 1722-1733 [PMID: [25355928](#) DOI: [10.1158/1078-0432.CCR-14-2022](#)]
- 12 **Kosaka N**, Yoshioka Y, Fujita Y, Ochiya T. Versatile roles of extracellular vesicles in cancer. *J Clin Invest* 2016; **126**: 1163-1172 [PMID: [26974161](#) DOI: [10.1172/JCI181130](#)]
- 13 **Joyce DP**, Kerin MJ, Dwyer RM. Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer. *Int J Cancer* 2016; **139**: 1443-1448 [PMID: [27170104](#) DOI: [10.1002/ijc.30179](#)]
- 14 **Tanaka Y**, Kamohara H, Kinoshita K, Kurashige J, Ishimoto T, Iwatsuki M, Watanabe M, Baba H. Clinical impact of serum exosomal microRNA-21 as a clinical biomarker in human esophageal squamous cell carcinoma. *Cancer* 2013; **119**: 1159-1167 [PMID: [23224754](#) DOI: [10.1002/cncr.27895](#)]
- 15 **Thind A**, Wilson C. Exosomal miRNAs as cancer biomarkers and therapeutic targets. *J Extracell Vesicles* 2016; **5**: 31292 [PMID: [27440105](#) DOI: [10.3402/jev.v5.31292](#)]
- 16 **Fang T**, Lv H, Lv G, Li T, Wang C, Han Q, Yu L, Su B, Guo L, Huang S, Cao D, Tang L, Tang S, Wu M, Yang W, Wang H. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat Commun* 2018; **9**: 191 [PMID: [29335551](#) DOI: [10.1038/s41467-017-02583-0](#)]
- 17 **Hu R**, Yan H, Fei X, Liu H, Wu J. Modulation of glucose metabolism by a natural compound from *Chloranthus japonicus* via activation of AMP-activated protein kinase. *Sci Rep* 2017; **7**: 778 [PMID: [28396610](#) DOI: [10.1038/s41598-017-00925-y](#)]
- 18 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: [11846609](#) DOI: [10.1006/meth.2001.1262](#)]
- 19 **Chen X**, Liang H, Guan D, Wang C, Hu X, Cui L, Chen S, Zhang C, Zhang J, Zen K, Zhang CY. A combination of Let-7d, Let-7g and Let-7i serves as a stable reference for normalization of serum microRNAs. *PLoS One* 2013; **8**: e79652 [PMID: [24223986](#) DOI: [10.1371/journal.pone.0079652](#)]
- 20 **Wu C**, Wang C, Guan X, Liu Y, Li D, Zhou X, Zhang Y, Chen X, Wang J, Zen K, Zhang CY, Zhang C. Diagnostic and prognostic implications of a serum miRNA panel in oesophageal squamous cell carcinoma. *PLoS One* 2014; **9**: e92292 [PMID: [24651474](#) DOI: [10.1371/journal.pone.0092292](#)]
- 21 **Hu R**, Huffman KE, Chu M, Zhang Y, Minna JD, Yu Y. Quantitative Secretomic Analysis Identifies Extracellular Protein Factors That Modulate the Metastatic Phenotype of Non-Small Cell Lung Cancer. *J Proteome Res* 2016; **15**: 477-486 [PMID: [26736068](#) DOI: [10.1021/acs.jproteome.5b00819](#)]
- 22 **Yuan R**, Zhi Q, Zhao H, Han Y, Gao L, Wang B, Kou Z, Guo Z, He S, Xue X, Hu H. Upregulated expression of miR-106a by DNA hypomethylation plays an oncogenic role in hepatocellular carcinoma. *Tumour Biol* 2015; **36**: 3093-3100 [PMID: [25510666](#) DOI: [10.1007/s13277-014-2945-2](#)]
- 23 **Hung JH**, Li CH, Yeh CH, Huang PC, Fang CC, Chen YF, Lee KJ, Chou CH, Cheng HY, Huang HD, Chen M, Tsai TF, Lin AM, Yen CH, Tsou AP, Tyan YC, Chen YA. MicroRNA-224 down-regulates Glycine N-methyltransferase gene expression in Hepatocellular Carcinoma. *Sci Rep* 2018; **8**: 12284 [PMID: [30115977](#) DOI: [10.1038/s41598-018-30682-5](#)]
- 24 **Fassan M**, Cui R, Gasparini P, Mescoli C, Guzzardo V, Vicentini C, Munari G, Loupakis F, Lonardi S, Braconi C, Scarpa M, D'Angelo E, Pucciarelli S, Angriman I, Agostini M, D'Inca R, Farinati F, Gafà R, Lanza G, Frankel WL, Croce CM, Valeri N, Rugge M. miR-224 Is Significantly Upregulated and Targets Caspase-3 and Caspase-7 During Colorectal Carcinogenesis. *Transl Oncol* 2019; **12**: 282-291 [PMID: [30448733](#) DOI: [10.1016/j.tranon.2018.10.013](#)]
- 25 **Cheng Y**, Li Z, Xie J, Wang P, Zhu J, Li Y, Wang Y. MiRNA-224-5p inhibits autophagy in breast cancer cells via targeting Smad4. *Biochem Biophys Res Commun* 2018; **506**: 793-798 [PMID: [30389135](#) DOI: [10.1016/j.bbrc.2018.10.150](#)]
- 26 **Yu LM**, Wang WW, Qi R, Leng TG, Zhang XL. MicroRNA-224 inhibition prevents progression of cervical carcinoma by targeting PTX3. *J Cell Biochem* 2018; **119**: 10278-10290 [PMID: [30129088](#) DOI: [10.1002/jcb.27370](#)]
- 27 **Zhu G**, Zhou L, Liu H, Shan Y, Zhang X. MicroRNA-224 Promotes Pancreatic Cancer Cell Proliferation and Migration by Targeting the TXNIP-Mediated HIF1 α Pathway. *Cell Physiol Biochem* 2018; **48**: 1735-1746 [PMID: [30078003](#) DOI: [10.1159/000492309](#)]
- 28 **Chen CH**, Li SX, Xiang LX, Mu HQ, Wang SB, Yu KY. HIF-1 α induces immune escape of prostate cancer by regulating NCR1/NKp46 signaling through miR-224. *Biochem Biophys Res Commun* 2018; **503**: 228-234 [PMID: [29885835](#) DOI: [10.1016/j.bbrc.2018.06.007](#)]

Colon perforation due to antigenemia-negative cytomegalovirus gastroenteritis after liver transplantation: A case report and review of literature

Takahiro Yokose, Hideaki Obara, Masahiro Shinoda, Yutaka Nakano, Minoru Kitago, Hiroshi Yagi, Yuta Abe, Yohei Yamada, Kentaro Matsubara, Go Oshima, Shutaro Hori, Sho Ibuki, Hisanobu Higashi, Yuki Masuda, Masanori Hayashi, Takehiko Mori, Miho Kawaida, Takumi Fujimura, Ken Hoshino, Kaori Kameyama, Tatsuo Kuroda, Yuko Kitagawa

ORCID number: Takahiro Yokose (0000-0001-6946-280X); Hideaki Obara (0000-0001-6740-2715); Masahiro Shinoda (0000-0002-4315-9244); Yutaka Nakano (0000-0003-3228-3474); Minoru Kitago (0000-0002-4909-1199); Hiroshi Yagi (0000-0002-9897-8108); Yuta Abe (0000-0003-4746-0584); Yohei Yamada (0000-0001-6059-7660); Kentaro Matsubara (0000-0002-6438-6040); Go Oshima (0000-0002-3325-7115); Shutaro Hori (0000-0001-9213-0758); Sho Ibuki (0000-0002-1315-0484); Hisanobu Higashi (0000-0002-8767-802X); Yuki Masuda (0000-0002-6081-7587); Masanori Hayashi (0000-0002-1099-3654); Takehiko Mori (0000-0002-8176-4760); Miho Kawaida (0000-0003-0353-4246); Takumi Fujimura (0000-0002-7078-6542); Ken Hoshino (0000-0002-3021-3029); Kaori Kameyama (0000-0003-2069-3249); Tatsuo Kuroda (0000-0001-7183-2830); Yuko Kitagawa (0000-0001-6469-2155).

Author contributions: Obara H, Shinoda M, Yagi H, Matsubara K, Oshima G, Nakano Y, Ibuki S, Higashi H, Masuda Y, Hayashi M and Yokose T performed surgery. Yokose T wrote and coordinated this manuscript. Kitagawa Y, Kuroda T, Hoshino K, Kitago M, Abe Y, Yamada Y, Fujimura T and Hori S helped to draft the manuscript. Mori T helped to

Takahiro Yokose, Hideaki Obara, Masahiro Shinoda, Yutaka Nakano, Minoru Kitago, Hiroshi Yagi, Yuta Abe, Yohei Yamada, Kentaro Matsubara, Go Oshima, Shutaro Hori, Sho Ibuki, Hisanobu Higashi, Yuki Masuda, Masanori Hayashi, Takumi Fujimura, Ken Hoshino, Tatsuo Kuroda, Yuko Kitagawa, Department of Surgery, Keio University School of Medicine, Tokyo 1608582, Japan

Takehiko Mori, Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo 1608582, Japan

Miho Kawaida, Kaori Kameyama, Department of Pathology, Keio University School of Medicine, Tokyo 1608582, Japan

Corresponding author: Hideaki Obara, MD, PhD, Associate Professor, Doctor, Surgeon, Department of Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan. obara.z3@keio.jp

Telephone: +81-3-33531211

Fax: +81-3-33554707

Abstract

BACKGROUND

Cytomegalovirus (CMV) remains a critical complication after solid-organ transplantation. The CMV antigenemia (AG) test is useful for monitoring CMV infection. Although the AG-positivity rate in CMV gastroenteritis is known to be low at onset, almost all cases become positive during the disease course. We treated a patient with transverse colon perforation due to AG-negative CMV gastroenteritis, following a living donor liver transplantation (LDLT).

CASE SUMMARY

The patient was a 52-year-old woman with decompensated liver cirrhosis as a result of autoimmune hepatitis who underwent a blood-type compatible LDLT with her second son as the donor. On day 20 after surgery, upper and lower gastrointestinal endoscopy (GE) revealed multiple gastric ulcers and transverse colon ulcers. The biopsy tissue immunostaining confirmed a diagnosis of CMV gastroenteritis. On day 28 after surgery, an abdominal computed tomography revealed transverse colon perforation, and simple lavage and drainage were performed along with an urgent ileostomy. Although the repeated remission and

treatment especially cytomegalovirus infection and thrombotic microangiopathy. Kameyama K and Kawaida M performed pathological analysis. All authors read and approved the final manuscript.

Informed consent statement:

Consent was obtained from the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

CARE Checklist (2016) statement:

The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Received: January 11, 2019

Peer-review started: January 11, 2019

First decision: February 13, 2019

Revised: March 3, 2019

Accepted: March 15, 2019

Article in press: March 16, 2019

Published online: April 21, 2019

P-Reviewer: Aykan NF, Boin IFSE, Huerta-Franco MR, Jeong KY

S-Editor: Ma RY

L-Editor: A

E-Editor: Ma YJ



aggravation of CMV gastroenteritis and acute cellular rejection made the control of immunosuppression difficult, the upper GE eventually revealed an improvement in the gastric ulcers, and the biopsy samples were negative for CMV. The CMV-AG test remained negative, therefore, we had to evaluate the status of the CMV infection on the basis of the clinical symptoms and GE.

CONCLUSION

This case report suggests a monitoring method that could be useful for AG-negative CMV gastroenteritis after a solid-organ transplantation.

Key words: Cytomegalovirus gastrointestinal disease; Colon perforation; Antigenemia negative; Liver transplantation; Case report

©The Author(s) 2019. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The cytomegalovirus (CMV) antigenemia (AG) test is useful for monitoring recipients for posttransplantation CMV infection. Although the AG-positivity rate in CMV gastroenteritis is known to be low at onset, most cases become positive during the disease course. We managed a patient with a complicated condition with a transverse colon perforation caused by AG-negative CMV gastroenteritis, after a living donor liver transplantation. This case report presents a method that could be important monitoring for AG-negative CMV gastroenteritis after solid-organ transplantation.

Citation: Yokose T, Obara H, Shinoda M, Nakano Y, Kitago M, Yagi H, Abe Y, Yamada Y, Matsubara K, Oshima G, Hori S, Ibuki S, Higashi H, Masuda Y, Hayashi M, Mori T, Kawaida M, Fujimura T, Hoshino K, Kameyama K, Kuroda T, Kitagawa Y. Colon perforation due to antigenemia-negative cytomegalovirus gastroenteritis after liver transplantation: A case report and review of literature. *World J Gastroenterol* 2019; 25(15): 1899-1906

URL: <https://www.wjgnet.com/1007-9327/full/v25/i15/1899.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v25.i15.1899>

INTRODUCTION

Although cytomegalovirus (CMV) infection can remain latent since childhood, it can be reactivated due to immunosuppression. While CMV gastroenteritis presents with clinical symptoms, such as abdominal pain, nausea, vomiting and melena, a definitive diagnosis is made based on endoscopic findings and the histopathological examination of biopsy tissues. The CMV-antigenemia (AG) positivity rate at the onset of gastroenteritis has been reported to be approximately 20%-30%^[1]. Although gastrointestinal perforation due to CMV gastroenteritis is not uncommon^[2], such an occurrence has rarely been reported after organ transplantation^[3]. Autoimmune hepatitis is an autoimmune disease that commonly develops in middle-aged or older woman and usually causes chronic and progressive liver damage. In regard to treatment, immunosuppressants, especially prednisolone, are commonly used. Liver transplantation is the final therapeutic option for patients, such as in a recently reported case on a patient with autoimmune hepatitis who developed decompensated cirrhosis due to an insufficient response to medical treatment.

We managed a patient with a complicated condition, with transverse colon perforation that was caused by AG-negative CMV gastroenteritis, after a living donor liver transplantation (LDLT). Here, we report on this case, which was difficult to diagnose and treat.

CASE PRESENTATION

Chief complaints

Abdominal pain and fullness.

History of present illness

The patient was a 52-year-old Asian woman, who was diagnosed with liver

dysfunction during a medical examination in her twenties. A diagnosis of autoimmune hepatitis was made at 40 years of age. When the patient was 46 years old, the patient developed ascites, which improved with oral steroids. However, with disease progression, she developed decompensated cirrhosis at 51 years old that was resistant to medical management. She was then referred to our department.

History of past illness

There was no other significant medical history.

Personal and family history

The patient was a nonsmoker and had stopped drinking socially 5 years prior. Her occupation was a housewife. There was no relevant family history.

Physical examination upon admission

According to the Eastern Cooperative Oncology Group Performance Status, her performance status was 2. At the physical examination, the patient's height was 155 cm, her weight was 47 kg, and her vitals were stable; yellowish bulbar conjunctivae, ascites, and bilateral pedal edema were observed.

Laboratory examinations

The Child-Pugh score was 11 points in class C, and the Model for end stage liver disease score was 11 points. The serologic tests for CMV showed that the patient was IgG positive (+), IgM negative (-), and AG negative, which is indicative of past CMV infection. A PCR test for CMV was not performed routinely before transplantation at our facility and was not performed in this case.

Imaging examinations

Abdominal computed tomography (CT) revealed liver cirrhosis with ascites before LDLT.

Liver transplantation and follow-up

A blood-type compatible LDLT was performed using a left lobe graft, with the patient's second son as the donor (20 years old, CMV IgG+/IgM-, which is indicative of past CMV infection). The graft-to-recipient weight ratio was 0.73, the operation duration was 849 min, and the bleeding volume was 822 mL. At our facility, in accordance with the protocol of CMV monitoring and treatment after a liver transplantation, CMV-AG is tested twice a week, but a CMV-PCR test is not performed routinely. In addition, prophylactic ganciclovir (GCV) is not administered, but GCV is initiated when the patient becomes CMV-AG positive or in the case of a seropositive donor.

Initially, cyclophosphamide (CyA), prednisolone (PSL) and mizoribine (MIZ) were used as the postoperative immunosuppressants, in accordance with the protocol of our facility^[4,5], however, for this patient, MIZ was replaced by mycophenolate mofetil (MMF) due to pancytopenia, and CyA was replaced by tacrolimus (FK) due to renal failure. As the patient had jaundice and persistently elevated aspartate transaminase and alanine transaminase levels, a liver biopsy was performed on the 10th day after transplantation. The histopathological examination was negative for both acute cellular rejection (ACR) and CMV hepatitis, so her condition was suspected to be drug-induced or caused by cholestasis.

Starting on the 12th day after transplantation, the patient's anemia worsened, and she required frequent packed red blood cell transfusions. Further investigations confirmed thrombocytopenia, jaundice, and renal failure. We suspected thrombotic microangiopathy (TMA), even though the peripheral smear was negative for fragmented red blood cells, and a fresh frozen plasma transfusion and FK dose reduction were carried out. The CMV-AG remained negative, and there were no clinical findings that were characteristic of a CMV infection, but prophylactic GCV administration was initiated. Thereafter, the thrombocytopenia gradually improved. On the 20th day after transplantation, the patient reported abdominal pain and black-colored stools, so upper gastrointestinal endoscopy was performed, which showed multiple gastric ulcers (Figure 1A). A biopsy tissue sample, taken from an ulcer, showed large cells with intranuclear inclusions with hematoxylin and eosin (HE) staining (Figure 1B), and CMV-positive cells were observed through immunostaining (Figure 1C). Once the diagnosis of CMV gastroenteritis was confirmed, GCV, which had already been initiated, was continued, and the dosage of all 3 immunosuppressants, FK, PSL, and MMF, was reduced.

On the 26th day after transplantation, the patient had frequent, watery diarrhea, for which a lower gastrointestinal endoscopy was performed, and a deep ulcer was observed in the transverse colon (Figure 2A). The biopsy tissue diagnosis of the

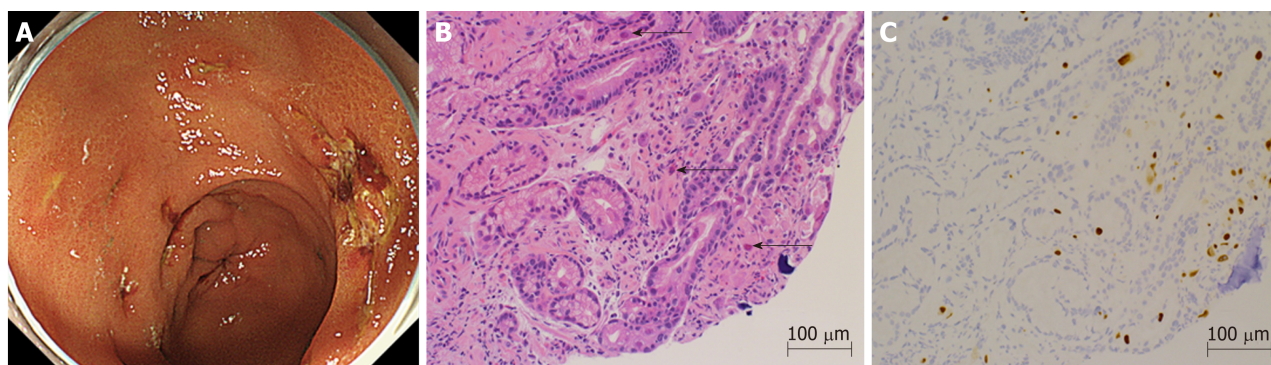


Figure 1 Upper gastrointestinal endoscopy and tissue biopsy diagnosis. A: Upper gastrointestinal endoscopy showed multiple gastric ulcers in the vestibular area. B: Biopsy tissue diagnosis showed large cells with intranuclear inclusions on hematoxylin and eosin staining (arrows) ($\times 200$). C: Cytomegalovirus positive cells were observed through immunostaining ($\times 200$).

ulcerative lesion revealed large cells with intranuclear inclusions with HE-staining (Figure 2B) and CMV-positive cells with immunostaining (Figure 2C). On the 28th day after transplantation, we noted the findings of abdominal pain, fever and an increased inflammatory response. A plain abdominal CT scan revealed intraperitoneal free air adjacent to the transverse colon (Figure 3A) and hemorrhagic ascites in the pelvis (Figure 3B).

The patient was diagnosed with gastrointestinal perforation, and an emergency surgery was performed. When the abdomen was incised, contaminated ascites were not observed. In the transverse colon, an impending perforation with a thinned serous membrane was confirmed.

The rejection activity index score of the liver tissues collected during the surgery was found to be P2, B1, and V1 during the histopathologic examination, indicating ACR.

FINAL DIAGNOSIS

The final diagnosis of the presented case was transverse colon perforation due to CMV gastroenteritis.

TREATMENT

GCV was administered for CMV gastroenteritis.

During the emergency surgery, as it was difficult to remove the adhesions with the surrounding areas, thus, the site of perforation was left as it was. Simple lavage with drainage was performed, and ileostomy was performed for acute pan-peritonitis due to transverse colon perforation.

OUTCOME AND FOLLOW-UP

The repeated remission and aggravation of CMV gastroenteritis, ACR and TMA made the control of immunosuppression extremely difficult. CMV-AG remained negative throughout the disease course, but the CMV-PCR test result was 220 copies/mL. On the 46th day after transplantation, a follow-up upper gastrointestinal endoscopy showed that the gastric ulcer was finally resolving, and the biopsies were also negative for CMV. The patient was ambulatory at discharge on the 86th day after transplantation.

DISCUSSION

Although CMV infection is often contracted in childhood, it usually remains latent. It is often reactivated due to immunosuppression after an organ transplantation^[6]. Most Japanese individuals are infected with CMV in early childhoods, and it usually remains latent. Therefore, even if a donor candidate had a past CMV infection, he or she is not excluded as a donor. When the recipient is seronegative while the donor is

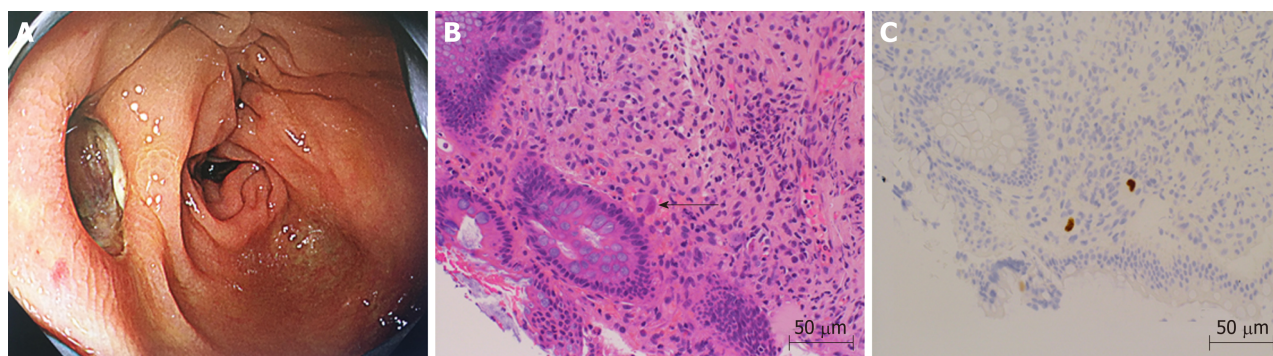


Figure 2 Lower gastrointestinal endoscopy and tissue biopsy diagnosis. A: Lower gastrointestinal showed a deep ulcer in the transverse colon. B: Biopsy tissue diagnosis showed large cells with intranuclear inclusions on hematoxylin and eosin staining (arrow) ($\times 400$). C: Cytomegalovirus positive cells were observed through immunostaining ($\times 400$).

seropositive for the CMV antibody, the risk of onset of CMV infection after organ transplantation is high, and appropriate monitoring and prophylactic measures are necessary^[7]. However, when the recipient is seropositive or seronegative is controversial.

The diagnostic methods for CMV include (1) the isolation and identification of CMV from blood, urine and pharyngeal secretions; (2) the CMV-AG test, which involves the detection of CMV-antigen-positive polymorphonuclear leukocytes in peripheral blood using a monoclonal antibody; (3) the CMV-PCR method, which involves amplifying CMV-DNA from blood and the bronchoalveolar lavage fluid for the identification; and (4) cytopathological and histopathological examinations, which involve the evaluation of the target organs of CMV infection with endoscopy and biopsy to detect the intranuclear inclusion bodies of giant cells through HE staining and CMV antigens through immunostaining using an anti-CMV monoclonal antibody^[6,7]. Because both the sensitivity and specificity of the CMV-AG test are reported to be 70%-90%^[8,9] and the levels are indicative of disease severity and treatment response, the test is said to be effective for infection monitoring. The CMV-AG test is therefore used as an indicator for the initiation and completion of treatment. The sensitivity and specificity of the CMV-PCR method are reported to be superior to the CMV-AG test^[10-14], and while a quantitative estimation of the number of copies is possible with the former, the positive cut-off value varies among different reports^[9-11,13,15]. The CMV-PCR test is not covered by health insurance providers in Japan.

CMV gastroenteritis exhibits clinical symptoms such as nausea, vomiting, abdominal pain and melena, and it is characteristic to find multiple gastrointestinal ulcers during endoscopy^[6,10,15]. The diagnosis is based on endoscopic findings and biopsy tissue examination and analysis. The diagnosis is confirmed when the intranuclear inclusion bodies of giant cells are observed using HE stains and CMV antigens are identified through immunostaining^[6,10,14,16]. When an ulcerative lesion is observed in the gastrointestinal tract of an immunosuppressed patient, the site should be biopsied and investigated, keeping CMV infection in mind. Although it has been reported that in CMV gastroenteritis, the CMV-AG positive rate is approximately 20% at onset and that false-negatives results occur frequently^[1], the CMV-AG status becomes positive in most cases during the disease course, thus making this test a useful parameter for monitoring the response to treatment^[1,8,14,17]. While there have been reports on rare cases in which patients who had CMV gastroenteritis had CMV-AG results that remained negative throughout the disease course after bone marrow transplantation^[13,18,19], there have been no such reports associated with solid-organ transplantation; however, there have been some reports in which the CMV-AG test was positive when an intestinal perforation was caused by CMV gastroenteritis^[20,21], including a report in which intestinal perforation were caused by CMV gastroenteritis in patients taking immunosuppressants to treat rheumatoid arthritis^[2]. Although it has been reported that routine upper gastrointestinal endoscopy during the follow-up for CMV gastroenteritis does not lead to differences in recurrence rates and treatment effects^[17], there are also cases such as ours, where the patient suffers from localized CMV infection but does not achieve viremia and remains CMV-AG negative throughout the disease course. An examination of symptomatic patients through regular upper gastrointestinal endoscopy and biopsy may be an important monitoring method, and it is necessary to tailor the medical management on a case-to-case basis.

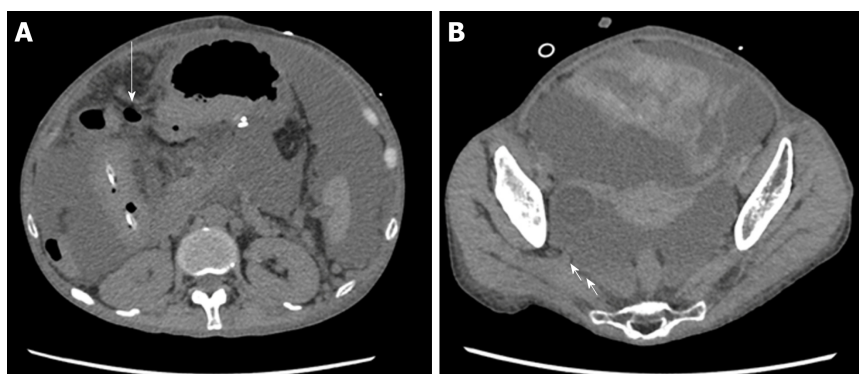


Figure 3 Abdominal computed tomography images. A: Abdominal computed tomography (CT) revealed intraperitoneal free air near the transverse colon (arrow). B: Abdominal CT revealed hemorrhagic ascites in the pelvis (arrow).

Although there have been reports on cases with gastrointestinal perforation due to CMV gastroenteritis, this complication is extremely rare after a solid organ transplantation^[2,3]. Recently, there was a report showing that CMV infects vascular endothelial cells and causes ulcers and perforation locally in the intestinal mucosa after solid-organ transplantation^[21].

In our case, in addition to the long-term history of oral PSL therapy for autoimmune hepatitis before transplantation, posttransplantation immunosuppression was essential. This may have caused CMV gastroenteritis with strong, localized inflammation that led to intestinal perforation without viremia.

Although CMV infection tends to occur at least three week after transplantation^[3], patients taking immunosuppressive drugs before transplantation may be affected earlier^[6].

Atsumi *et al*^[22] and Kemeny *et al*^[23] have reported on patients presenting with a combination of polyoma virus infection and acute graft rejection after kidney transplantation. However, the coexistence of an infection and rejection represents a combination of contradictory illnesses and constitutes a rare presentation. In our case, the patient was first diagnosed with ACR through an intraoperative liver biopsy sample taken during the emergency procedure for gastrointestinal perforation in an advanced stage of CMV infection. It was a combination of an infection and a rejection, which again is an extremely rare presentation, which made the management difficult.

TMA is a condition in which polymers of von Willebrand factor are secreted due to vascular endothelial cell dysfunction that is caused by various factors, for *e.g.*, graft-versus-host disease (GVHD), ABO-incompatible transplantation, calcineurin inhibitor (CNI) administration, and fungal and viral (*e.g.*, human immunodeficiency virus, CMV and adenovirus) infections^[24,25]. This secretion promotes platelet thrombus formation, leading to multiorgan failure due to microangiopathy. Although the recommended criteria for diagnosing TMA have been previously reported, there are no set diagnostic criteria. Our hospital, as reported previously^[26], follows the protocol of a diagnosis based on clinical examination findings and the appearance of fragmented red blood cells in the peripheral smear, combined with tests showing hemolytic anemia, thrombocytopenia and increased bilirubin and lactate dehydrogenase levels.

CMV has been reported to exhibit a TMA-like disease state by infecting vascular endothelial cells and making them dysfunctional^[27,28]. Java *et al*^[29], Waizer *et al*^[30] reported a patient with newly developed TMA due to CMV infection in a kidney transplant recipient. In our case, treatment with GCV was initiated as the patient presented with symptoms of TMA prior to the detection of CMV infection. Therefore, a CMV infection could be suspected in a TMA-like disease presentation.

In patients who are chronically treated with immunosuppressants before organ transplantation, endoscopy should be performed according to symptoms at an earlier stage after transplantation. As a reliable test, the CMV-PCR test is reported to be superior to the CMV-AG test, but it is not covered by health insurance providers in Japan.

CONCLUSION

After solid-organ transplantation, it is necessary to monitor symptomatic patients for

CMV gastroenteritis, even when the CMV-AG assay remains negative throughout the course of the illness. Additionally, a CMV infection should be suspected when a TMA-like disease presentation is observed, and the patient should be investigated accordingly, to rule out this diagnosis.

ACKNOWLEDGEMENTS

We acknowledge Mr. Gotou Masaki (Department, LSI Medience Corporation) for advice on the antigenemia assay evaluation.

REFERENCES

- 1 **Mori T**, Mori S, Kanda Y, Yakushiji K, Mineishi S, Takaue Y, Gondo H, Harada M, Sakamaki H, Yajima T, Iwao Y, Hibi T, Okamoto S. Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2004; **33**: 431-434 [PMID: [14676775](#) DOI: [10.1038/sj.bmt.1704369](#)]
- 2 **Ozaki T**, Yamashita H, Kaneko S, Yorifuji H, Takahashi H, Ueda Y, Takahashi Y, Kaneko H, Kano T, Mimori A. Cytomegalovirus disease of the upper gastrointestinal tract in patients with rheumatic diseases: a case series and literature review. *Clin Rheumatol* 2013; **32**: 1683-1690 [PMID: [23942768](#) DOI: [10.1007/s10067-013-2363-7](#)]
- 3 **Vincenzi R**, Fonseca EA, Chapchap P, Machado MCC, Roda KMO, Candido HL, Benavides MR, D'Assuncao MA, Afonso RC, Turine P, Marson FP, Neto JS. Pancreas-preserving duodenectomy after living donor liver transplantation for invasive cytomegalovirus disease. *Pediatr Transplant* 2017; **21** [PMID: [28881059](#) DOI: [10.1111/ptr.13059](#)]
- 4 **Hibi T**, Tanabe M, Hoshino K, Fuchimoto Y, Kawachi S, Itano O, Obara H, Shinoda M, Shimojima N, Matsubara K, Morikawa Y, Kitagawa Y. Cyclosporine A-based immunotherapy in adult living donor liver transplantation: accurate and improved therapeutic drug monitoring by 4-hr intravenous infusion. *Transplantation* 2011; **92**: 100-105 [PMID: [21546866](#) DOI: [10.1097/TP.0b013e31821dcae3](#)]
- 5 **Hibi T**, Shinoda M, Itano O, Obara H, Kitago M, Abe Y, Yagi H, Tanaka M, Hoshino K, Fujino A, Kuroda T, Kawachi S, Tanabe M, Shimazu M, Kitagawa Y. Steroid minimization immunosuppression protocol using basiliximab in adult living donor liver transplantation for hepatitis C virus-related cirrhosis. *Hepatol Res* 2015; **45**: 1178-1184 [PMID: [25594837](#) DOI: [10.1111/hepr.12486](#)]
- 6 **Azevedo LS**, Pierrotti LC, Abdala E, Costa SF, Strabelli TM, Campos SV, Ramos JF, Latif AZ, Litvinov N, Maluf NZ, Caiaffa Filho HH, Pannuti CS, Lopes MH, Santos VA, Linardi Cda C, Yasuda MA, Marques HH. Cytomegalovirus infection in transplant recipients. *Clinics (Sao Paulo)* 2015; **70**: 515-523 [PMID: [26222822](#) DOI: [10.6061/clinics/2015\(07\)09](#)]
- 7 **Kotton CN**, Kumar D, Caliendo AM, Asberg A, Chou S, Snyderman DR, Allen U, Humar A; Transplantation Society International CMV Consensus Group. International consensus guidelines on the management of cytomegalovirus in solid organ transplantation. *Transplantation* 2010; **89**: 779-795 [PMID: [20224515](#) DOI: [10.1097/TP.0b013e3181cee42f](#)]
- 8 **Boeckh M**, Bowden RA, Goodrich JM, Pettinger M, Meyers JD. Cytomegalovirus antigen detection in peripheral blood leukocytes after allogeneic marrow transplantation. *Blood* 1992; **80**: 1358-1364 [PMID: [1325214](#)]
- 9 **David-Neto E**, Triboni AH, Paula FJ, Vilas Boas LS, Machado CM, Agena F, Latif AZ, Alencar CS, Pierrotti LC, Nahas WC, Caiaffa-Filho HH, Pannuti CS. A double-blinded, prospective study to define antigenemia and quantitative real-time polymerase chain reaction cutoffs to start preemptive therapy in low-risk, seropositive, renal transplanted recipients. *Transplantation* 2014; **98**: 1077-1081 [PMID: [24839894](#) DOI: [10.1097/TP.0000000000000189](#)]
- 10 **Nagata N**, Kobayakawa M, Shimbo T, Hoshimoto K, Yada T, Gotoda T, Akiyama J, Oka S, Uemura N. Diagnostic value of antigenemia assay for cytomegalovirus gastrointestinal disease in immunocompromised patients. *World J Gastroenterol* 2011; **17**: 1185-1191 [PMID: [21448424](#) DOI: [10.3748/wjg.v17.i9.1185](#)]
- 11 **Boaretti M**, Sorrentino A, Zantedeschi C, Forni A, Boschiero L, Fontana R. Quantification of cytomegalovirus DNA by a fully automated real-time PCR for early diagnosis and monitoring of active viral infection in solid organ transplant recipients. *J Clin Virol* 2013; **56**: 124-128 [PMID: [23182772](#) DOI: [10.1016/j.jcv.2012.10.015](#)]
- 12 **Kwon S**, Jung BK, Ko SY, Lee CK, Cho Y. Comparison of quantitation of cytomegalovirus DNA by real-time PCR in whole blood with the cytomegalovirus antigenemia assay. *Ann Lab Med* 2015; **35**: 99-104 [PMID: [25553288](#) DOI: [10.3343/alm.2015.35.1.99](#)]
- 13 **Dahi PB**, Perales MA, Devlin SM, Olson A, Lubin M, Gonzales AM, Scaradavou A, Kernan NA, O'Reilly RJ, Giralt S, Jakubowski A, Koehne G, Papadopoulos EB, Ponce DM, Sauter C, Papanicolaou G, Barker JN. Incidence, nature and mortality of cytomegalovirus infection after double-unit cord blood transplant. *Leuk Lymphoma* 2015; **56**: 1799-1805 [PMID: [25224458](#) DOI: [10.3109/10428194.2014.963079](#)]
- 14 **Mori T**, Okamoto S, Matsuoka S, Yajima T, Wakui M, Watanabe R, Ishida A, Iwao Y, Mukai M, Hibi T, Ikeda Y. Risk-adapted pre-emptive therapy for cytomegalovirus disease in patients undergoing allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2000; **25**: 765-769 [PMID: [10745263](#) DOI: [10.1038/sj.bmt.1702227](#)]
- 15 **Durand CM**, Marr KA, Arnold CA, Tang L, Durand DJ, Avery RK, Valsamakis A, Neofytos D. Detection of cytomegalovirus DNA in plasma as an adjunct diagnostic for gastrointestinal tract disease in kidney and liver transplant recipients. *Clin Infect Dis* 2013; **57**: 1550-1559 [PMID: [23956167](#) DOI: [10.1093/cid/cit521](#)]
- 16 **Boeckh M**, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood* 2009; **113**: 5711-5719 [PMID: [19299333](#) DOI: [10.1182/blood-2008-10-143560](#)]
- 17 **Seo M**, Kim do H, Gong EJ, Ahn JY, Lee JH, Jung KW, Choi KD, Song HJ, Lee GH, Jung HY, Kim JH, Lee SO, Choi SH, Kim YS, Woo JH, Kim SH. Is Follow-Up Endoscopy Necessary in Upper

- Gastrointestinal Cytomegalovirus Disease? *Medicine (Baltimore)* 2016; **95**: e3389 [PMID: [27175637](#) DOI: [10.1097/MD.0000000000003389](#)]
- 18 **Yanada M**, Yamamoto K, Emi N, Naoe T, Suzuki R, Taji H, Iida H, Shimokawa T, Kohno A, Mizuta S, Maruyama F, Wakita A, Kitaori K, Yano K, Hamaguchi M, Hamajima N, Morishima Y, Kodera Y, Sao H, Morishita Y. Cytomegalovirus antigenemia and outcome of patients treated with pre-emptive ganciclovir: retrospective analysis of 241 consecutive patients undergoing allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2003; **32**: 801-807 [PMID: [14520425](#) DOI: [10.1038/sj.bmt.1704232](#)]
 - 19 **Mori T**, Okamoto S, Watanabe R, Yamazaki R, Tsukada Y, Nagayama H, Ishida A, Ikeda Y. Incidence of cytomegalovirus (CMV) infection in allogeneic hematopoietic stem cell recipients at low risk of CMV infection. *Bone Marrow Transplant* 2002; **29**: 1005-1006 [PMID: [12098071](#) DOI: [10.1038/sj.bmt.1703588](#)]
 - 20 **De Bartolomeis C**, Collini A, Barni R, Ruggieri G, Bernini M, Carmellini M. Cytomegalovirus infection with multiple colonic perforations in a renal transplant recipient. *Transplant Proc* 2005; **37**: 2504-2506 [PMID: [16182726](#) DOI: [10.1016/j.transproceed.2005.06.082](#)]
 - 21 **Zandberg M**, de Maar EF, Hofker HS, Homan van der Heide JJ, Rosati S, van Son WJ. Initial cytomegalovirus prophylaxis with ganciclovir: no guarantee for prevention of late serious manifestations of CMV after solid organ transplantation. *Neth J Med* 2005; **63**: 408-412 [PMID: [16301763](#)]
 - 22 **Atsumi H**, Asaka M, Kimura S, Imura J, Fujimoto K, Chikazawa Y, Nakagawa M, Okuyama H, Yamaya H, Moriyama M, Tanaka T, Suzuki K, Yokoyama H. A case of second renal transplantation with acute antibody-mediated rejection complicated with BK virus nephropathy. *Clin Transplant* 2010; **24** Suppl 22: 35-38 [PMID: [20590692](#) DOI: [10.1111/j.1399-0012.2010.01280.x](#)]
 - 23 **Kemény E**, Hirsch HH, Eller J, Dürmüller U, Hopfer H, Mihatsch MJ. Plasma cell infiltrates in polyomavirus nephropathy. *Transpl Int* 2010; **23**: 397-406 [PMID: [19912590](#) DOI: [10.1111/j.1432-2277.2009.01001.x](#)]
 - 24 **Nakazawa Y**, Hashikura Y, Urata K, Ikegami T, Terada M, Yagi H, Ishizashi H, Matsumoto M, Fujimura Y, Miyagawa S. Von Willebrand factor--cleaving protease activity in thrombotic microangiopathy after living donor liver transplantation: a case report. *Liver Transpl* 2003; **9**: 1328-1333 [PMID: [14625834](#) DOI: [10.1016/j.lts.2003.09.021](#)]
 - 25 **Kobayashi T**, Wada H, Usui M, Sakurai H, Matsumoto T, Nobori T, Katayama N, Uemoto S, Ishizashi H, Matsumoto M, Fujimura Y, Isaji S. Decreased ADAMTS13 levels in patients after living donor liver transplantation. *Thromb Res* 2009; **124**: 541-545 [PMID: [19423151](#) DOI: [10.1016/j.thromres.2009.03.010](#)]
 - 26 **Kishida N**, Shinoda M, Itano O, Obara H, Kitago M, Hibi T, Yagi H, Abe Y, Matsubara K, Odaira M, Tanabe M, Shimazu M, Kitagawa Y. Increased Incidence of Thrombotic Microangiopathy After ABO-Incompatible Living Donor Liver Transplantation. *Ann Transplant* 2016; **21**: 755-764 [PMID: [27956735](#) DOI: [10.12659/AOT.900915](#)]
 - 27 **Narimatsu H**, Kami M, Hara S, Matsumura T, Miyakoshi S, Kusumi E, Kakugawa Y, Kishi Y, Murashige N, Yuji K, Masuoka K, Yoneyama A, Wake A, Morinaga S, Kanda Y, Taniguchi S. Intestinal thrombotic microangiopathy following reduced-intensity umbilical cord blood transplantation. *Bone Marrow Transplant* 2005; **36**: 517-523 [PMID: [16025150](#) DOI: [10.1038/sj.bmt.1705099](#)]
 - 28 **Rahbar A**, Söderberg-Nauclér C. Human cytomegalovirus infection of endothelial cells triggers platelet adhesion and aggregation. *J Virol* 2005; **79**: 2211-2220 [PMID: [15681423](#) DOI: [10.1128/JVI.79.4.2211-2220.2005](#)]
 - 29 **Java A**, Edwards A, Rossi A, Pandey R, Gaut J, Delos Santos R, Miller B, Klein C, Brennan D. Cytomegalovirus-induced thrombotic microangiopathy after renal transplant successfully treated with eculizumab: case report and review of the literature. *Transpl Int* 2015; **28**: 1121-1125 [PMID: [25864519](#) DOI: [10.1111/tri.12582](#)]
 - 30 **Waiser J**, Budde K, Rudolph B, Ortner MA, Neumayer HH. De novo hemolytic uremic syndrome postrenal transplant after cytomegalovirus infection. *Am J Kidney Dis* 1999; **34**: 556-559 [PMID: [10469868](#) DOI: [10.1053/AJKD03400556](#)]



Published By Baishideng Publishing Group Inc
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: <http://www.f6publishing.com/helpdesk>
<http://www.wjgnet.com>

