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Tigecycline inhibits the viability of hepatocellular carcinoma cells via Rac1

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Yunfei Liu

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Erster Gutachter:	Prof. Dr. Alexandr Bazhin
Zweiter Gutachter:	Prof. Dr. Enrico De Toni
Dritter Gutachter:	Prof. Dr. Roland Kappler
weitere Gutachter:	
Promovierten Mitarbeiter:	Priv. Doz. Dr. Matthias Ilmer
Dekan:	Prof. Dr. med. Thomas Gudermann
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Zusammenfassung (Deutsch):

Hintergrund: Das hepatozelluläre Karzinom (HCC) stellt nach wie vor eine bedeutende Herausforderung im Bereich der Onkologie dar und erfordert immer ausgefeiltere therapeutische Ansätze. In diesem Kontext kommt der Erforschung potenzieller pharmakologischer Wirkstoffe eine herausragende Bedeutung zu, um die Prognose der betroffenen Patienten zu verbessern. Tigecyclin, ein Mitglied der Glycocyclin-Klasse von Antibiotika, hat sich als vielversprechender Kandidat erwiesen. Obwohl einige Untersuchungen die hemmende Wirkung von Tigecyclin gegen HCC in vitro gezeigt wurden, bleibt der komplexe Mechanismus dahinter rätselhaft. Das Hauptziel dieser Arbeit ist es, eine mögliche therapeutische Rolle von Tigecyclin für das HCC aufzudecken.

Methoden: RAC1 wurde durch eine präzise Zielanalysen unter Verwendung der Pharmmaper- und CTD-Datenbanken als potenzielles Tigecyclin-Ziel identifiziert. Eine umfassende Untersuchung der mRNA- und Proteinexpression von RAC1 im HCC wurde durchgeführt, indem Daten aus den TCGA-, GTEx- und CPTAC-Datensätzen genutzt wurden. Der komplexe Methylierungsstatus des RAC1-Promotors wurde sorgfältig analysiert, wobei die Feinheiten der UALCAN-Datenbank akribisch berücksichtigt wurden. Das nuancierte Zusammenspiel zwischen RAC1 und zugehörigen Biomarkern sowie Immun-Checkpoint-Inhibitoren wurde unter Verwendung der GEPIA-Plattform einer genauen Untersuchung unterzogen. Darüber hinaus führten wir strenge Genkorrelations- und Anreicherungsanalysen durch, um die komplexen funktionellen Zusammenhänge von RAC1 aufzudecken. Zusätzlich führten wir eine Korrelationsanalyse zwischen der RAC1-Expression und der Infiltration von Immunzellen durch. Anschließend bestätigten wir die Expressionsmuster von RAC1 in HCC-Geweben mithilfe von IHC-Techniken. Um Veränderungen in der RAC1-Expression in HCC-Zellen nach der Tigecyclin-Behandlung zu bestätigen, wurden RT-PCR- und Western-Blot-Tests durchgeführt. Darüber hinaus hemmten wir die

Expression von RAC1 effektiv mithilfe der SiRNA-Technologie und führten umfassende In-vitro-Studien zu deren Auswirkungen auf HCC durch.

Ergebnisse: Unsere bioinformatische Analyse ergab eine signifikante Hochregulation der RAC1-Expression sowohl auf mRNA- als auch auf Proteinebene in HCC im Vergleich zu normalen Geweben, begleitet von einer verringerten Methylierung des Promotors. Im HCC zeigte RAC1 positive Korrelationen mit MKI67, PCNA, CTLA4, HAVCR2 und PDCD1, was auf seine potenzielle Rolle in der zellulären Proliferationskaskade hinweist. Darüber hinaus zeigte unsere Analyse der Immunzellinfiltration, dass eine hohe RAC1-Expression mit Veränderungen in der Immunzellinfiltration in HCC korrelierte. Darüber hinaus erwies sich RAC1 als ein unabhängiger prognostischer Faktor für das Gesamtüberleben (OS) und zeigte Korrelationen mit relevanten klinischen Indikatoren bei HCC-Patienten. Die nachfolgende experimentelle Validierung bestätigte erhöhte Proteinspiegel von RAC1 in HCC-Geweben im Vergleich zu normalen Geweben. HCC-Zelllinien zeigten eine Zunahme der RAC1-Genexpression nach der Behandlung mit Tigecyclin, wie durch RT-PCR und Western-Blot-Analyse validiert. Die siRNA-vermittelte Unterdrückung der Expression von RAC1 in HCC-Zelllinien war effektiv und führte zu einer verminderten Zellproliferation, beeinträchtigter Wundheilung, verminderte zellulärer Migration und Invasivität.

Schlüßfolgerung: Unsere Studie hat eine deutliche Überexpression des RAC1-Gens in HCC-Geweben aufgedeckt, die mit signifikanten Assoziationen zu prognostischen Ergebnissen bei Patienten einhergeht. Die Tigecyclin-Behandlung von HCC-Zelllinien führte zu einer Erhöhung der Expression des RAC1-Gens. Wichtig dabei ist, dass die Herabregulierung der Expression des RAC1-Gens die Sensitivität von HCC gegenüber der Tigecyclin-Behandlung erhöhte und zu einer deutlichen Verringerung der Zellproliferationskapazität führte. Diese Erkenntnisse eröffnen neue Möglichkeiten für die potenzielle klinische Anwendung von Tigecyclin bei der Behandlung von HCC.

Abstract (English):

Background: Hepatocellular carcinoma (HCC) continues to pose a formidable challenge within the domain of oncology, necessitating more refined therapeutic activities. Therefore, the exploration of potential pharmacological agents assumes paramount importance for enhancing the prognosis of afflicted patients. Tigecycline, a member of the glycylglycline class of antibiotics, has emerged as a compelling candidate. While preliminary investigations have demonstrated tigecycline's inhibitory prowess against HCC in vitro, the intricate mechanism underpinning this effect remains enigmatic. The core objective of our study is to unravel the latent role of tigecycline within the realm of tumor therapy.

Methods: RAC1 was discerned as a putative tigecycline target through precision targeting analysis utilizing the Pharmmaper and CTD databases. Comprehensive scrutiny of RAC1's mRNA and protein expression within the realm of HCC was conducted by harnessing data from the TCGA, GTEx, and CPTAC datasets. The intricate methylation status of the RAC1 promoter was diligently evaluated, meticulously navigating the intricacies of the UALCAN database. The nuanced interplay between RAC1 and associated biomarkers as well as immune checkpoints was subjected to meticulous scrutiny utilizing the GEPIA platform. Furthermore, we executed stringent gene correlation and enrichment analyses to unveil the intricate functional associations inherent to RAC1.Additionally, we conducted correlation analysis between RAC1 expression and immune cell infiltration due to our interest in this relationship. Subsequently, the employment of IHC techniques validated the expression patterns of RAC1 in HCC tissues. To affirm the alterations in RAC1 expression in HCC cells following tigecycline treatment, qRT-PCR and Western blot assays were meticulously executed. Furthermore, we effectively inhibited the expression of RAC1 using siRNA technology and conducted a comprehensive in vitro study of its effects on HCC.

Results: Bioinformatics analysis revealed a significant upregulation of RAC1 expression at both mRNA and protein levels in HCC compared to normal tissues, concomitant with a decreased promoter methylation status. In the context of HCC, RAC1 exhibited positive correlations with MKI67, PCNA, CTLA4, HAVCR2, and PDCD1, suggesting its potential role in the cellular proliferation cascade. Furthermore, our analysis of immune cell infiltration demonstrated that high RAC1 expression correlated with alterations in immune cell infiltration in HCC. Additionally, RAC1 emerged as an independent prognostic factor for overall survival (OS) and exhibited correlations with relevant clinical indicators in HCC patients. Subsequent experimental validation confirmed elevated protein levels of RAC1 in HCC tissues compared to normal tissues. HCC cells showed an increase in RAC1 gene expression following tigecycline treatment, as validated by qRT-PCR and Western blot analysis. siRNA-mediated suppression of RAC1 in HCC cell lines was effective, resulting in reduced cell proliferation, impaired wound healing and Transwell, decreased cellular migration and invasiveness.

Conclusion： Our study has uncovered a marked overexpression of the RAC1 gene within human HCC tissues, concomitant with a significant association with patient prognostic outcomes. Tigecycline intervention in HCC cell lines induced an elevation in the expression levels of the RAC1 gene. Importantly, attenuation of RAC1 gene expression heightened HCC's responsiveness to tigecycline treatment, resulting in a notable decrement in cell proliferative capacity. Our findings introduce new avenues for the potential clinical utilization of tigecycline in the management of HCC.

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AFP	Alpha-fetoprotein
ANOVA	Analysis of variance
APS	Ammonium persulfate
BCA	Bicinchoninic Acid
BCLC	Barcelona Clinic Liver Cancer
BLM	Bleomycin
BP	Biological Process
BSA	Bovine Serum Albumin
CC	Cellular Component
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CML	Chronic Myeloid Leukemia
CPTAC	Clinical Proteomic Tumor Analysis Consortium
CTD	Comparative Toxicogenomics Database
CHOL	Cholangiocarcinoma
DEGs	Differential Genes
DEPC	Diethylpyrocarbonate
DFS	Disease Free Survial
DMSO	Dimethylsulfoxid
DOX	Doxorubicin
DSS	Disease Specific Survival
ECL	Enhanced Chemiluminescence
FBS	Fetal Bovine Serum
GBM	Glioblastoma
GIFtS	Genecards Inferred Functionality Score
GEPIA	Gene Expression Profiling Interactive Analysis
GO	Gene Ontology

HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
IHC	Immunohistochemistry
Kb	kilobase
LIHC	Renal Hepatocellular Carcinoma
MF	Molecular Function
MVI	Microvascular invasion
MWA	Microwave Ablation
NAFLD	Nonalcoholic fatty liver disease
ORR	Objective response rate
OS	Overall Survival
PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene Fluoride
RAC1	Rac Family Small Gtpase 1
RAPA	Rapamycin
RFA	Radiofrequency ablation
ROC	Receiver Operating Characteristic
RT-PCR	Reverse Transcription‐Polymerase Chain Reaction
SBRT	Stereotactic Body Radiotherapy
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
STAD	Gastric Adenocarcinoma
siRNA	Small Interfering RNA
TACE	Transcatheter arterial chemoembolization
TCGA	The Cancer Genome Atlas
TIG	Tigecycline
TEMED	N,N,N',N'-Tetramethylethylenediamine

1. Introduction

1.1 Liver cancer and hepatocellular carcinoma

1.1.1 Overview

Hepatocellular carcinoma (HCC), a form of liver cancer, constitutes a formidable and pressing global public health predicament. It firmly maintains its status as the foremost contributor to cancer-related fatalities, characterized by a distressingly low 5-year survival rate, which regrettably lingers at a mere 18%[1-4]. Based on the most recent global cancer data statistics from 2020, the worldwide incidence of liver cancer was approximately 906,000 new cases, while the number of liver cancer-related deaths was approximately 830,000. These figures represent an increase of 7.73% and 6.14% compared to the 2018 data, respectively[2, 5]. Histopathologically, primary liver cancers are categorized into three principal subtypes: hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and mixed hepatocellular-cholangiocarcinoma (HCC-ICC)[6, 7]. Among these, HCC is the predominant pathological type, accounting for 80-90% of primary Liver cancer cases[8-10]. In this study, we specifically address liver cancer in the context of HCC. Despite notable strides in HCC diagnosis and treatment, it continues to manifest as a malignant ailment, imposing a substantial burden on global health and overall well-being, entailing far-reaching implications.

1.1.2 Epidemiology and risk factors of liver cancer

HCC displays noteworthy geographical disparities in its incidence. These variations are shaped by differences in environmental and infectious risk factor exposure, the timing and intensity of these exposures, healthcare resource accessibility, and the capacity for early-stage detection and curative interventions. Notably, approximately 80% of new HCC cases arise in developing nations[11, 12]. The preeminent burden of liver cancer is borne by the Asian region, responsible for over 50% of new liver cancer cases and the resultant fatalities on a global scale. The escalating incidence of liver cancer in this region is significantly influenced by the presence of cirrhosis and chronic viral hepatitis, which play pivotal roles as underlying risk factors[13]. Over the past decade, there have been notable shifts in the epidemiology of HCC. While some regions have seen a gradual decrease in cases associated with viral hepatitis, there has been a rise in cases linked to alcoholic and non-alcoholic fatty liver disease in certain countries. These changing patterns in HCC incidence can be attributed to factors like improved quality of life, the effectiveness of antiviral therapies, and extensive vaccination campaigns against HBV [14-16].

Nonetheless, a substantial subset of individuals afflicted by HCC exhibits a medical background characterized by chronic liver ailments rooted in enduring HBV infection, protracted alcohol abuse, or alcoholic

steatohepatitis. Additional contributing factors comprise nonalcoholic fatty liver disease (NAFLD), obesity, diabetes mellitus, and contact with established carcinogens like aflatoxin B1 and nicotine. These factors collectively contribute to the increasing incidence of HCC [2, 4, 16].

Indeed, the overwhelming majority, exceeding 90%, of HCC cases manifest within the context of chronic liver disease. Cirrhosis, regardless of its underlying cause, represents the most formidable risk factor for HCC. The predisposition to HCC encompasses a multifaceted interplay of factors, including demographic elements such as age, gender, and race, disease attributes like fibrotic stage, inflammatory activity, and treatment history, metabolic components such as diabetes mellitus and obesity, and lifestyle aspects encompassing alcohol consumption and tobacco use. Gender disparities are particularly pronounced, with males displaying HCC morbidity and mortality rates 2-3 times higher than those of females. This gender discrepancy could potentially be attributed to the aggregation of risk factors among males and variations in sex hormone profiles. Additionally, aging constitutes a significant risk factor, particularly in males, where the peak period for HCC diagnosis falls between the ages of 60 and 70. Encouragingly, adolescents and young adults aged 15 to 39 years exhibit lower incidence and mortality rates related to liver cancer. This favorable trend may be associated with their longer duration from

precancerous lesion initiation to tumor development and reduced exposure to risk factors [2, 13, 17, 18].

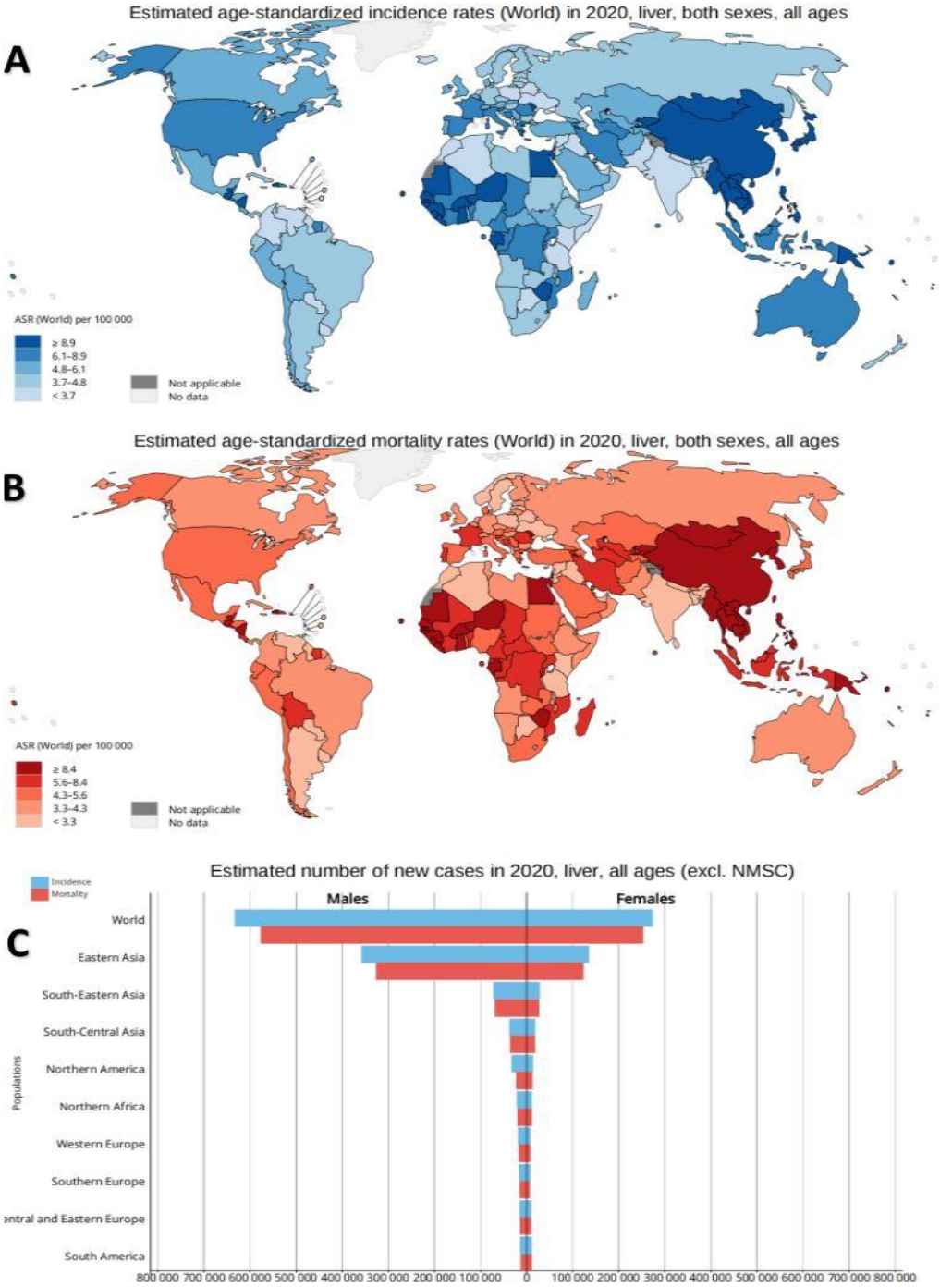


Figure 1: The Age-Standardized Rates (ASR) of liver cancer incidence and mortality were estimated globally for both sexes in the year 2020. and the number of new cases of liver cancer in 2020.

(A) Incidence Age-standardized Rates (ASR) in liver cancer. (B) Mortality Age-standardized Rates (ASR) in liver cancer. (C) The incidence and mortality rates of liver cancer exhibit notable disparities across various geographic

regions and between genders. Specifically, the prevalence of liver cancer cases and associated fatalities significantly surpasses that in Asia in comparison to other global regions, with higher incidence and mortality rates observed among males as opposed to females. Data from: GLOBOCAN 2020 (**http://gco.iarc.fr/today**)

1.1.3 Clinical features and early diagnosis

Early-onset HCC typically manifests with nonspecific symptoms, including epigastric discomfort, fatigue, appetite loss, and weight loss. Unfortunately, these vague symptoms often go unnoticed by patients. As the disease progresses, individuals may experience low-grade fever, jaundice, ascites, and upper gastrointestinal bleeding, among other clinical signs. When cirrhosis is present alongside HCC, additional symptoms may emerge, such as palmar erythema, spider nevi, gynecomastia in males, and lower limb edema[19]. In cases where HCC has metastasized to extrahepatic sites, patients may exhibit signs and symptoms related to each specific metastatic location. During this stage, patients typically become aware of the tumor, which is often already in the middle or advanced stages, limiting the possibility of surgical intervention and resulting in a poor prognosis, high recurrence rates, and a decreased quality of life.

Clinically, HCC can be categorized into five groups based on tumor size: small carcinomas (diameter < 3 cm), nodules (3-5 cm in diameter), masses (5-10 cm in diameter), macromasses (diameter > 10 cm), and the diffuse distribution of small cancerous nodules scattered throughout the

liver. Currently, enhanced surveillance strategies for high-risk populations aim to improve early tumor detection. Screening methods commonly used in clinical practice include abdominal ultrasound and serum alpha-fetoprotein (AFP) testing. However, it's essential to acknowledge that the accuracy of ultrasound results may be influenced by the operator's expertise and the patient's body size, potentially leading to the oversight of small HCC tumors with diameters less than 1 cm[20-23].

The early detection and timely diagnosis of HCC are pivotal factors in determining the prognosis of patients. Regrettably, a substantial fraction of HCC cases receives diagnoses during advanced stages, culminating in a dismal 5-year survival rate of under 10%[13, 24, 25]. However, reports indicate that when clinical interventions are administered at an early stage, the 5-year survival rate can exceed 86.2%[26]. Typically, the clinical diagnosis of primary HCC relies on a combination of imaging examinations, laboratory analyses, and pathological assessments. Laboratory tests center on tumor markers, including AFP, carcinoembryonic antigen (CEA), and glycan molecular profiling of both HCC and paraneoplastic tissues, based on proteomic analyses[27]. These examinations not only assist in the diagnosis but also enable the prediction of survival and the likelihood of recurrence in primary HCC patients[28]. Assessing prognosis and staging patients are also vital

components of the diagnostic process, significantly contributing to enhanced patient management[29-31].

1.1.4 Treatment of HCC

The selection of therapeutic modalities for addressing HCC is intricately tied to the clinical staging of the disease, and it's noteworthy that standard treatment strategies exhibit variability across distinct regions, spanning Asia, Europe, and North America. It is worth mentioning that certain staging systems also provide recommendations for optimal therapeutic strategies[32]. Currently, the Barcelona Clinic Liver Cancer (BCLC) classification stands as the preeminent and extensively embraced standardized staging system for HCC. Remarkably, it retains the distinction of being the singular staging framework that has undergone meticulous prospective validation[33, 34]. The BCLC staging system categorizes HCC into five distinct stages and offers prognostic information in terms of estimated median survival, along with treatment recommendations customized for individuals within each specific stage (Figure 2).

HCC can be managed using a variety of therapeutic approaches, such as surgery, vascular intervention, ablation, radiotherapy, chemotherapy, targeted drugs, and bio-immunotherapy, among other options[35-37]. Henceforth, clinical decision-making necessitates the adoption of a personalized treatment approach by clinicians, taking into account the

patient's tumor stage, liver function, and overall health. Surgical resection is regarded as the primary therapeutic intervention for HCC. A multicenter cohort study, encompassing 8,656 HCC patients, revealed that, within the framework of the BCLC classification, and especially for patients considered unsuitable for resection, surgical resection yielded superior survival outcomes when juxtaposed with embolization therapy or alternative local and systemic treatment modalities[38].Chinese researchers conducted a comparative analysis of disease free survial (DFS) and overall survial (OS) in BCLC early-stage HCC patients who underwent open, laparoscopic, and robot-assisted liver resections. The study's findings unveiled that there existed no statistically significant disparities in prognosis among patients subjected to these three distinct surgical modalities[39]. Additionally, the utilization of digital intelligent surgical techniques, including indocyanine green molecular fluorescence imaging, three-dimensional visualization, 3D printing, and multimodal real-time fusion navigation for hepatic resection, has introduced supplementary options and improvements for preoperative preparation and intraoperative guidance in cases of HCC.

Liver transplantation is considered the ultimate therapeutic intervention for HCC, encompassing not only the surgical removal of the tumor but also the restoration of a debilitated liver suffering from compromised functional capacity. It is particularly relevant in cases where there is a

susceptibility to the development of synchronous HCC within cirrhotic tissue, which is prone to carcinogenesis. A recent extensive multicenter study dedicated to HCC has elucidated that individuals afflicted with intermediate to advanced-stage HCC, who were subsequently subject to downstaging procedures to align with the Milan criteria before proceeding to liver transplantation, showed improved prognosis and significant benefits. This research provides robust evidence supporting the potential expansion of liver transplantation indications for HCC. It offers compelling confirmation for broadening the scope of liver transplantation eligibility, thus providing increased opportunities for potentially curative therapeutic interventions for patients with convertible HCC[40].

While systemic therapeutic modalities have expanded the possibilities for managing HCC, a comprehensive European review of data has shown that the OS of BCLC stage C HCC patients who underwent surgical resection was better than that of those who received non-surgical treatments[41]. Similarly, the Chinese guideline underscores the continued importance of surgical treatment for achieving long-term survival in HCC patients. Recognizing that most HCC patients are diagnosed at advanced stages, often missing the chance for curative treatment, significant efforts have been directed toward improving the

efficacy of localized treatments through a multidisciplinary approach in clinical research.

Transarterial chemoembolization (TACE) stands as an efficacious therapeutic avenue for individuals grappling with intermediate-stage HCC. TACE comprises two principal phases: the intra-arterial administration of cytotoxic chemotherapeutic agents and the subsequent embolization of particles into the tumor's arterial supply, culminating in the induction of ischemic necrosis within the tumor. Multiple randomized controlled trials carried out in both Europe and Asia have underscored TACE's capacity to enhance OS among patients harboring unresectable HCC[42, 43]. TACE stands as the classical standard of care for intermediate and advanced HCC The findings from a multicenter, randomized phase III clinical trial revealed that compared to lenvatinib monotherapy, lenvatinib combined with transcatheter arterial chemoembolization (LEN-TACE) significantly improved the prognosis of patients with advanced HCC, achieving an objective response rate (ORR) of 54.1%. The promising ORR observed with LEN-TACE implies that it holds significant promise as a primary therapeutic option for advanced HCC[44].

Radiotherapy assumes a pivotal role as a localized treatment strategy for HCC, with stereotactic body radiotherapy (SBRT) representing an innovative technique that enables precise tumor targeting while

minimizing harm to healthy tissues. In a unipartite, forward-looking phase II clinical trial, HCC patients who underwent adjuvant radiotherapy subsequent to hepatectomy with minimal margins achieved remarkable 5-year rates for both OS and DFS at 72.2% and 51.6%, correspondingly. Intriguingly, no occurrences of margin recurrence or radiolucent hepatopathy were observed[45]. These findings underscore the efficacy and well-tolerated nature of radiotherapy as an adjunctive treatment option. When compared to transarterial chemoembolization TACE, radiotherapy significantly prolonged the median OS for patients with locally advanced HCC and concurrent large vessel thrombosis (17.5 months vs. 8.7 months) [46].

Radiofrequency ablation (RFA), microwave ablation (MWA), and cryoablation stand as frequently utilized modalities in the therapeutic armamentarium for HCC. Indeed, microwave ablation is an increasingly employed method for local ablation among these approaches[47]. In a multicenter retrospective study, the disparity in OS between patients undergoing MWA and laparoscopic hepatectomy was not statistically significant ($P = 0.420$). However, MWA showcased benefits in terms of hospitalization duration and treatment costs, particularly for cases of isolated 3-5 cm HCC where performing laparoscopic surgery might pose challenges[48]. Despite ablation therapy's track record of reduced complications and enhanced prognostic outcomes, it remains associated

with a notable risk of postoperative recurrence, with microvascular invasion (MVI) being a significant determinant of ablation efficacy[49-51]. Even in cases of recurrent HCC, surgical resection has demonstrated superior prognostic outcomes compared to RFA[52]. Nevertheless, ablation has demonstrated the capacity to trigger tumor-specific immune responses by inducing extensive cell death and releasing tumor antigens[53]. Consequently, it is poised to be integrated as part of combination therapies in the realm of systemic treatment strategies.

The management of HCC underscores the amalgamation of diverse therapeutic approaches, with experts widely recognizing the significance of multidisciplinary and integrated treatment strategies. In cases of early to mid-stage HCC, resection, transplantation, and ablation have achieved a high degree of technical refinement and assume pivotal roles in localized radical interventions. However, addressing advanced HCC poses a challenge in achieving curative surgery for unresectable HCC via translational therapies, emerging as a prominent area of research focus. In recent years, targeted drugs and immunotherapy have garnered prominence in HCC treatment, with several clinical investigations showcasing promising outcomes and progressing towards first-line clinical applications.

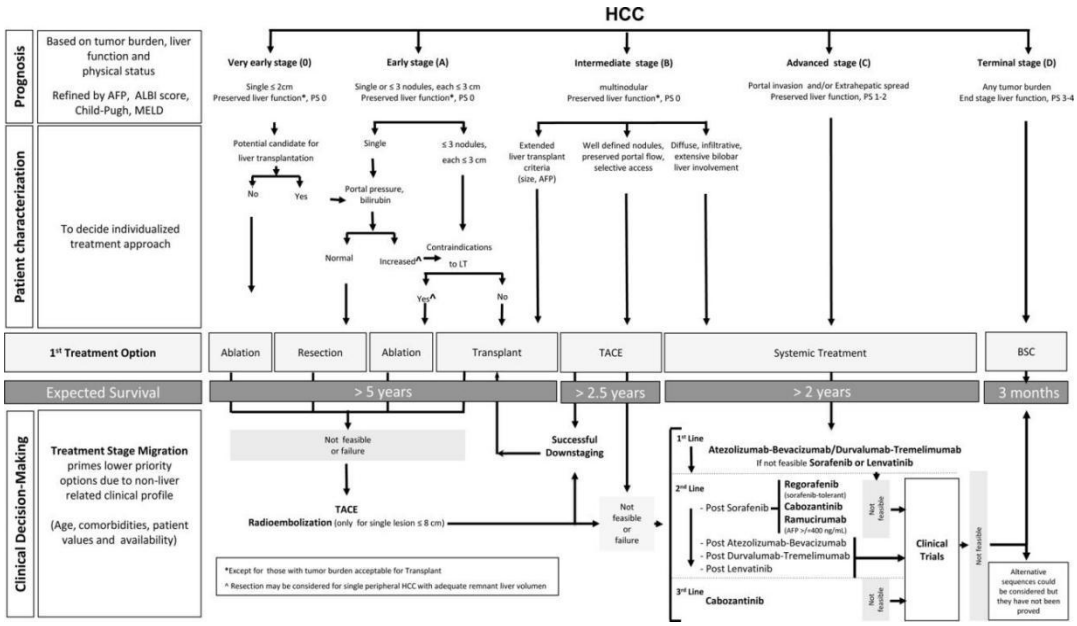


Figure 2. The BCLC staging system and its corresponding treatment strategy in 2022, with a kindly permission[35].

The BCLC system establishes a prognostic framework based on five distinct stages, which are closely linked to recommended first-line treatments. The expected prognosis is delineated by the median survival period corresponding to each tumor stage, relying on established scientific literature. Customized clinical decision-making, as of September 15, 2021, is overseen by multidisciplinary panels that integrate all accessible information in conjunction with the patient's distinct medical background. It is essential to emphasize that the evaluation of liver function transcends the conventional Child-Pugh classification. Furthermore, the comprehensive data stemming from the trial investigating the synergy of tremelimumab and durvalumab may lead to the inclusion of these agents as a primary therapeutic option.

1.1.5 Molecular characteristics

The molecular categorization of malignancies assumes a pivotal role in facilitating the implementation of tailored and precise therapeutic strategies. Understanding the molecular characteristics of tumors holds

great value in the development of novel oncology drugs targeting specific molecular features with relevant inhibitors or activators. Henceforth, a deeper understanding of the intrinsic biological links and molecular features of primary liver cancer is crucial for identifying biomarkers and potential drug targets for clinical diagnosis and treatment. High-throughput sequencing has led to the identification of molecular subtypes of HCC, highlighting its high heterogeneity at the molecular level, leading to varied clinical outcomes and associations with specific genetic and clinical features[54-58]. The correlation between the molecular subtypes of HCC and patients' pathological diagnosis and clinical features (e.g., results of clinical trials, risk factors, and survival time) has garnered increasing attention.

Molecular typing of HCC using transcriptomics has resulted in various findings, including the Boyault (G1-G6) [58], Hoshida (S1-S3) [56], and Chiang typings (proliferative subtype) [59]. Building upon these early genomic studies, researchers have delved deeper into their analyses to discern shared molecular characteristics, thereby categorizing HCC into two major subtypes: the proliferative subtype and the non-proliferative subtype, each constituting approximately 50% of the overall cases. The proliferative subtype is characterized notably by the activation of classical RAS, mTOR, and/or IGF signaling pathways, which are intricately linked to unfavorable prognoses[60]. Within the proliferative

subclasses, two well-defined subgroups are identified: the "Wnt/TGF-β" subgroup, characterized by activation of the mentioned pathway, and the "progenitor cell" subgroup, enriched in progenitor cells as well as markers of epithelial cell adhesion molecules and cytokeratin 19 and associated with elevated levels of alpha-fetoprotein AFP[58, 61-63]. In-depth epigenetic investigations have unveiled associations between DNA methylation, microRNA expression patterns, and the proliferative subtypes[64].

Conversely, in contrast to the proliferative subtype, non-proliferative HCC demonstrates distinct molecular characteristics. This subtype is marked by a reduced rate of cancer cell proliferation, indicating a comparatively slower growth and replication rate. As a result, clinical symptoms and patient prognoses tend to be more favorable when compared to the proliferative subtype. Non-proliferative HCC maintains certain hepatocyte-like features, with one of its subtypes exhibiting activation of the canonical WNT signaling pathway, primarily driven by CTNNB1 mutations[65]. Additionally, the atypical WNT signaling pathway is also enriched within this subtype, as evidenced by the increased expression of well-known target genes like GLUL or LGR5 [58]. In contrast to the proliferative subtype, levels of AFP within this category are relatively lower, indicative of a less aggressive disease course. However, despite these valuable molecular typing studies of HCC,

they have yet to be directly translated into clinical applications due to a lack of clear clinical consensus and the inability to predict treatment response[66]. Consequently, exploring treatment-oriented molecular typing of HCC and developing biomarkers for effective prediction of therapeutic response remain essential research areas.

1.2 Antibiotics and Tigecycline

1.2.1 Antitumor effects of antibiotics

Chemotherapy is a widely utilized treatment approach for tumors, aiming to halt or eliminate cancer cells by inhibiting their growth and spread. It encompasses several important categories of drugs, encompassing alkylating agents, antimetabolites, anti-microtubule agents, topoisomerase inhibitors, mitotic inhibitors, cytotoxic antibiotics, and corticosteroids[67]. Additionally, there is a developing and relatively novel group of chemotherapy drugs that consists of antibiotics with inherent cytotoxic properties. Some of these antibiotics exhibit potent intercalating effects, while others possess DNA-damaging properties. DNA serves as a crucial molecular target for numerous chemotherapy drugs, frequently regarded as a non-discriminating objective for cytotoxic agents[68, 69]. Anthracyclines, bleomycin, and actinomycin D are notable examples of anticancer antibiotics employed in therapy[70-73]. Doxorubicin and daunorubicin, the earliest discovered anthracyclines derived from Streptomyces, function as intercalators, disrupting DNA interactions, and

find widespread use in breast cancer treatment Bleomycin impedes DNA repair and induces DNA damage[74, 75]. Actinomycin D, a wide-ranging antibiotic, functioning as a bioreductive alkylating agent, establishes covalent linkages with DNA, consequently disrupting the process of DNA synthesis[76]. These antibiotics, initially designed for their antibacterial effects, have shown the ability to selectively target and exert cytotoxic effects on cancer cells. Research efforts are currently focused on understanding their mechanisms of action and assessing their therapeutic potential in cancer treatment. Exploring these antibiotics as potential chemotherapeutic agents presents an exciting frontier in the field of oncology research, offering promising prospects for advancing cancer treatment strategies.

Doxorubicin

Doxorubicin (DOX), molecular configuration is visually represented in Figure 3, is a formidable chemotherapeutic agent, finds widespread application across diverse oncological landscapes[77-79]. Nestled within the anthracycline antibiotic family, DOX earns distinction as an antineoplastic stalwart. DOX attaches to the nucleic acids in DNA by intercalating its anthracycline nucleus into the DNA of human cells in a clever manner, causing structural disruptions. By inhibiting topoisomerase II, it prevents the synthesis of DNA and RNA, thereby hindering the faithful replication and transcription of genetic material.

Consequently, the interaction between DOX and topoisomerase II to form DNA cleavable complexes appears to be an effective mechanism underlying DOX's cytotoxic effect. This interaction plays a crucial role in inducing cell damage and ultimately contributes to its effectiveness as a cell-killing agent[77-81]. Moreover, DOX coordinates the production of reactive oxygen species (ROS), contributing to the provocation of DNA damage and eventual cellular demise[78, 82, 83]. This multifaceted symphony empowers DOX with efficacy spanning a spectrum of malignancies. It is frequently harnessed as a pivotal component within combined chemotherapy protocols, amplifying therapeutic efficacy through synergistic amalgamation. Indeed, while DOX holds promise as a therapeutic agent, its clinical application is constrained by its tendency to elicit unfavorable outcomes, notably cardiotoxicity, myelosuppression, and gastrointestinal disturbances, particularly as the cumulative dose approaches its maximal tolerable level [78, 81, 84]. Thus, the administration of DOX demands vigilant surveillance and a thorough evaluation of its potential hazards.

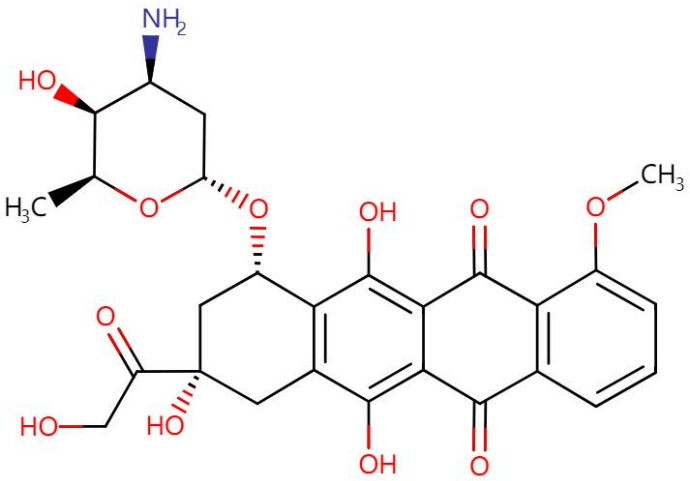


Figure 3: The chemical configuration of Doxorubicin.

The structural formula of Doxorubicin can be found in the publicly accessible Drugbank database **(https://go.drugbank.com/).**

Bleomycin

Bleomycin (BLM), molecular structure is shown in Figure 4, a potent glycopeptide antibiotic originating from Streptomyces bacteria, has etched a significant niche within the domain of cancer therapeutics[85, 86]. Its mechanism of action, distinct in nature, centers on the induction of DNA damage via DNA strand cleavage, thereby engendering disruptions in DNA replication and transcription[85-89]. This orchestrated sequence efficaciously quells the proliferation of cancer cells while inciting pathways leading to apoptosis. In consequence, BLM has emerged as a pivotal cytotoxic entity within the armamentarium of oncology. The clinical breadth of BLM's application spans an array of malignancies [90-94]. Particularly noteworthy is its demonstrated efficacy

in Hodgkin lymphoma, testicular cancer, and various squamous cell carcinomas, thus illuminating its multifaceted adaptability across divergent cancer typologies. The seamless integration of BLM into composite chemotherapy regimens further accentuates its pivotal role in amplifying therapeutic outcomes. While BLM offers effectiveness in treating various cancers, its administration is linked to notable side effects, particularly pulmonary toxicity, which can culminate in interstitial pneumonitis and fibrosis[85, 95-97]. To counteract these risks, meticulous patient assessment, precise dosing, and diligent surveillance are imperative throughout the treatment course. Additionally, endeavors have been undertaken to uncover genetic indicators that might render certain individuals more susceptible to bleomycin-induced lung complications.

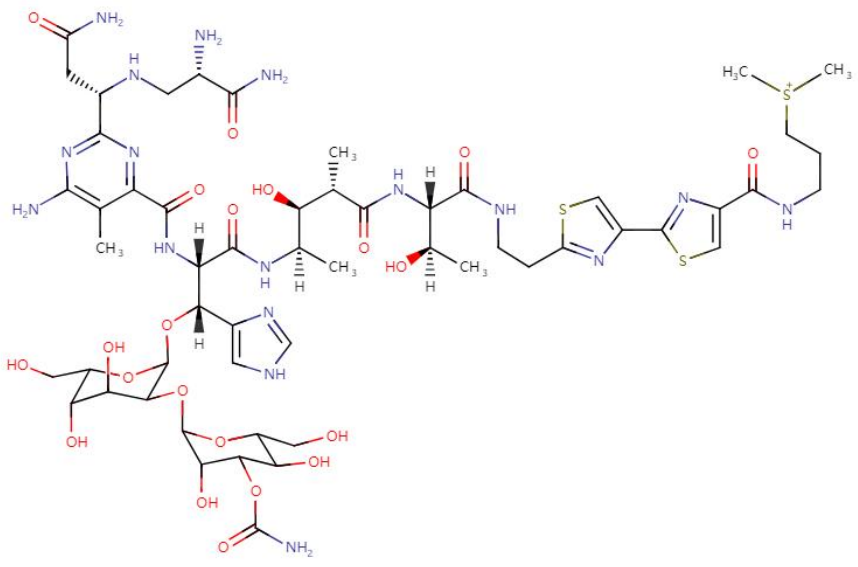


Figure 4: The chemical configuration of Bleomycin.

The structural formula of Bleomycin can be found in the publicly accessible Drugbank database(**https://go.drugbank.com/**).

Rapamycin

Rapamycin (RAPA), molecular structure is shown in Figure 5, emerges as a naturally derived compound that has captured substantial interest due to its diverse therapeutic applications. While its initial recognition stemmed from its antifungal attributes, RAPA's impact broadened considerably upon the discovery of its potent immunosuppressive and anti-proliferative properties. As a macrolide antibiotic, RAPA has become a focal point of extensive research within the domain of tumor therapy[98, 99].

RAPA is categorized within the class of pharmaceutical agents referred to as mTOR inhibitors. The acronym mTOR signifies mammalian target of rapamycin, denoting a pivotal protein kinase intricately involved in the processes of cellular growth, proliferation, and viability. RAPA works by binding to a protein called FKBP12, and the complex then inhibits mTOR activity, leading to a suppression of various cellular processes that rely on mTOR signaling[98, 100-102].

In the realm of clinical application, RAPA has garnered regulatory approvals for a diverse spectrum of tumor treatments, encompassing indications such as renal cell carcinoma, breast cancer, pancreatic neuroendocrine tumors, and meningiomas[103-105]. Scientific investigation has brought to light RAPA's remarkable ability to disrupt tumor cell growth, impede proliferation, and inhibit angiogenesis. This mechanism may extend further to modulating immune responses, thus

thwarting the initiation and progression of tumorigenesis. Beyond its impact on oncology, RAPA's potential extends to addressing specific genetic and metabolic disorders, serving as a subject of intensive study. Moreover, its influence on the processes of aging and longevity has evoked significant interest within the scientific community[98, 106]. Research endeavors continue to explore the diverse applications of RAPA, with ongoing investigations delving into its multifaceted potential. The intricate mechanisms underlying RAPA's actions are the focus of relentless scrutiny, with the intent of fully comprehending its therapeutic scope and potential associated side effects.

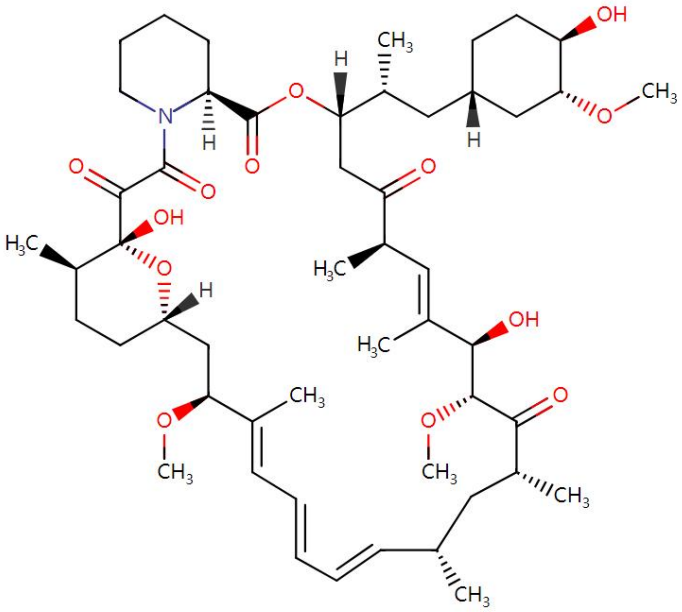


Figure 5: The chemical configuration of Rapamycin.
The structural formula of Bleomycin can be found in the publicly accessible Drugbank database (**https://go.drugbank.com/**).

In the preceding discourse, I have succinctly introduced three antibiotics, denoted as DOX, BLM, and RAPA, within the realm of anticancer

therapeutics. While these antibiotics currently hold clinical endorsement and have demonstrated specific anticancer effects, their comprehensive exploration beckons for a more profound and comprehensive investigation. Progressing forward, the focus of my inquiry will be directed towards tigecycline, a compound analogous to the aforementioned trio of antibiotics, showcasing compelling antitumor attributes. Nevertheless, the intricate mechanism underpinning tigecycline's action, particularly its impact on HCC, necessitates further meticulous elucidation and rigorous examination.

1.2.2 Tigecycline

Tigecycline (TIG), as depicted in Figure 6 with its molecular structure, represents a potent member of the glycylcycline class of antibiotics. It has garnered substantial attention within the spheres of infectious diseases and oncology alike[107-110]. Its inception was prompted by the pressing imperative to combat the burgeoning prevalence of antibiotic resistance, particularly in formidable pathogens such as Staphylococcus aureus. A noteworthy milestone in this trajectory was the expeditious approval granted by the U.S. Food and Drug Administration (FDA) in June 2005, marking a significant advancement in the ongoing battle against antibiotic resistance[111, 112]. Furthermore, TIG's multifaceted pharmacological properties render it an intriguing candidate for addressing diverse medical challenges, expanding its relevance well beyond its original scope.

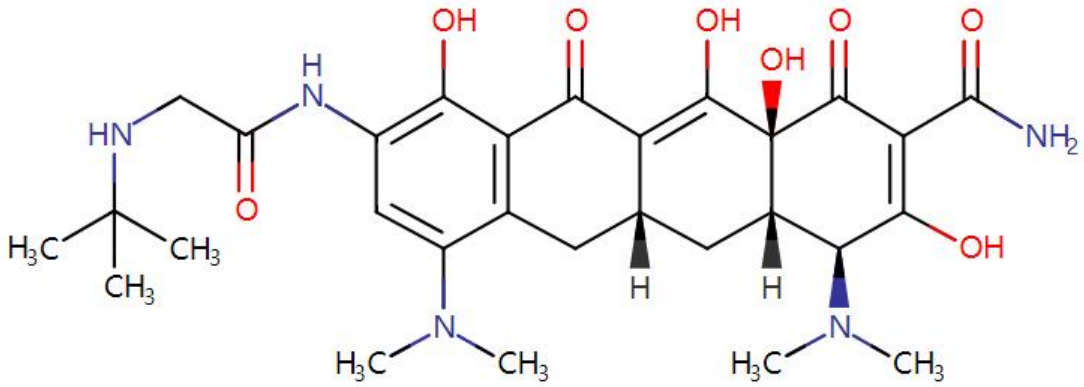


Figure 6: The chemical configuration of Tigecycline.

The structural formula of Bleomycin can be found in the publicly accessible Drugbank database (**https://go.drugbank.com/**).

TIG's fundamental mode of action revolves around its remarkable ability to intricately disrupt bacterial protein synthesis by precisely interacting with the 30S ribosomal subunit. This intricate binding initiates a complex cascade of events, effectively obstructing the translation process and ultimately leading to the demise of bacterial cells (Figure 7) [112-114]. This extraordinary and distinctive mechanism of action, coupled with its remarkable efficacy against multidrug-resistant pathogens, has sparked intense exploration into previously uncharted territories that extend far beyond the realm of bacterial infections. These endeavors encompass a wide spectrum of potential applications, ranging from cancer therapy to immunomodulation, thereby highlighting TIG's versatility and potential impact on diverse medical challenges.

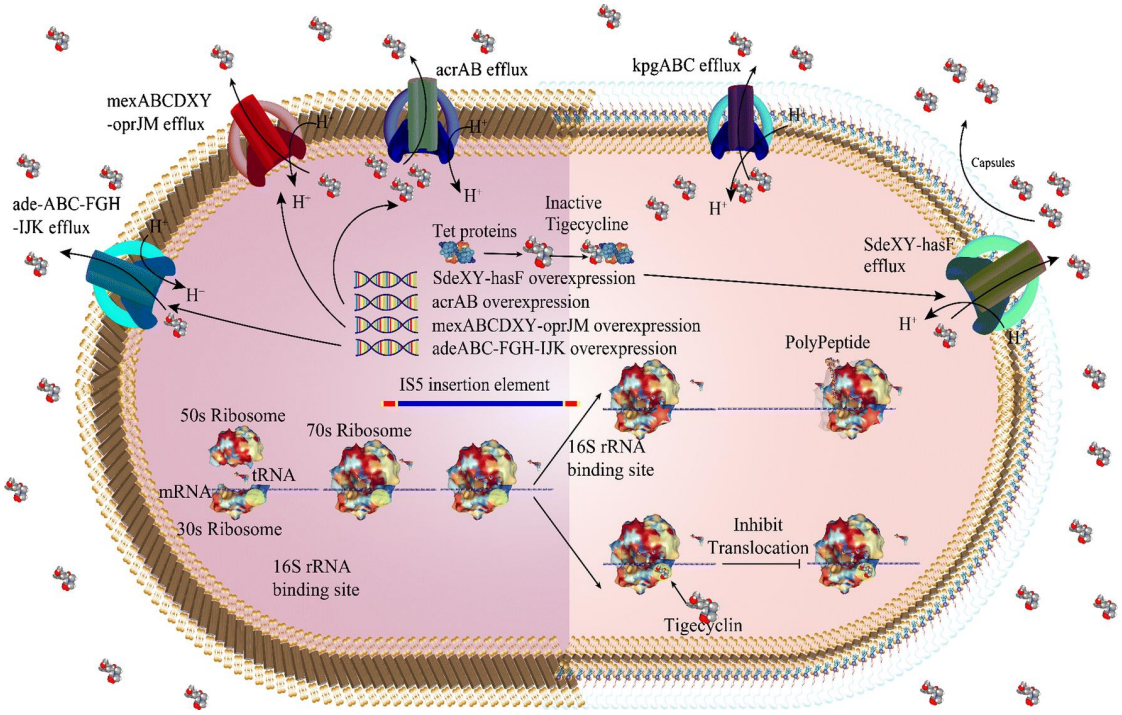


Figure 7: Tigecycline mechanisms of action and resistance, with a kindly permission[112].

TIG, an antibiotic compound, was initially validated for its ability to selectively eliminate tumor cells in acute myeloid leukemia (AML) by inhibiting mitochondrial protein synthesis[115, 116]. Subsequent investigations into TIG's potential in cancer treatment have yielded significant advancements. TIG has demonstrated remarkable antineoplastic effects across a diverse spectrum of solid tumors, encompassing gastric carcinoma, breast tumors, cervical cancer, HCC, lung cancer, oral squamous cell carcinoma, melanoma, neuroblastoma, glioblastoma, and pancreatic ductal adenocarcinoma[115, 117-119].

In-depth explorations into the mechanisms underpinning TIG's tumor-inhibitory effects have unveiled a multitude of pathways through which it influences tumor cells, spanning critical biological processes

such as cell proliferation, survival, metabolism, and metastasis. Particularly noteworthy is the emphasis on mitochondrial functionality when elucidating TIG's anti-tumor mechanisms. A plethora of studies suggest that TIG disrupts the activity of mitochondrial respiratory chain complexes or interferes with mitochondrial translation. Notable findings related to mitochondrial function underscore TIG's inhibition of mitochondrial oxidative phosphorylation disruption of mitochondrial biogenesis, or induction of mitochondrial oxidative damage[117, 119].

As the exact targets of TIG remain unidentified, there is an urgent need to elucidate its mode of action. Recent inquiries propose that TIG could potentially disrupt proteins engaged in mitochondrial translation, as well as diverse signaling cascades encompassing Myc/HIFs, PI3K/AKT, AMPK signaling, p21CIP1/Waf1, and the Wnt/β-catenin signaling pathways (Figure 8) [115, 117, 119, 120]. These intricate signaling pathways play a pivotal role in the progression of tumors. Delving into the precise mechanisms through which TIG operates has the potential to provide profound insights into its prospective utilization within the realm of cancer therapy. Moreover, TIG appears to modulate tumor cell proliferation by impacting cell cycle regulation. Studies indicate that TIG could interfere with the expression and function of cell cycle proteins, disrupting the progression of tumor cells through different cell cycle phases and thereby restraining their proliferative capacity[117, 120, 121].

Notably, emerging evidence suggests that TIG might also curb tumor vascularization through the suppression of angiogenesis, chiefly by attenuating the expression of vascular endothelial growth factor (VEGF), TIG impedes the formation of new blood vessels, ultimately restricting tumor growth and dissemination[121, 122].

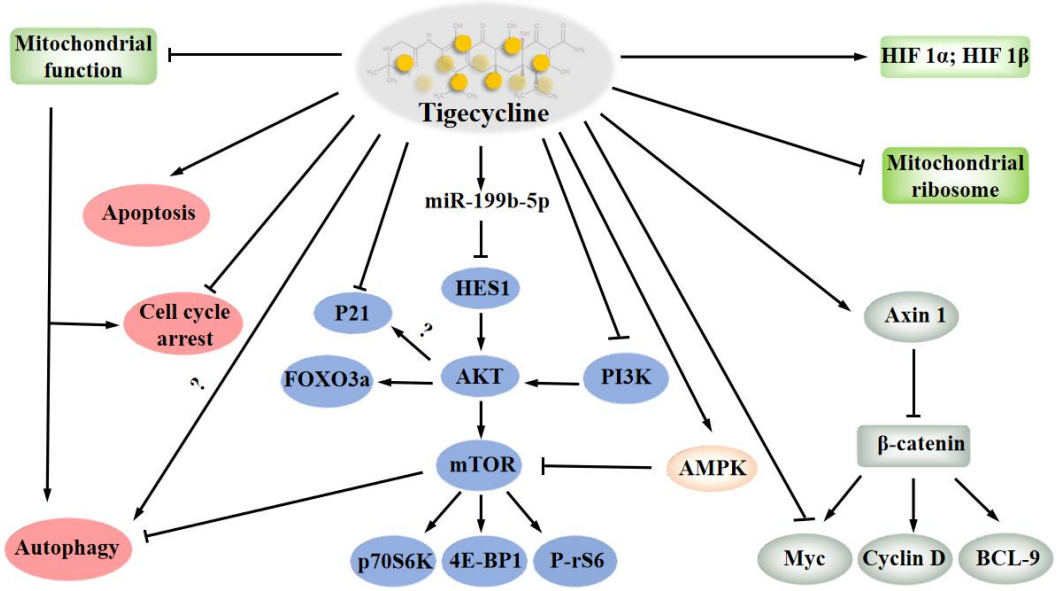


Figure 8: The Physiological Consequences of Tigecycline in Cancer Cells

Nonetheless, despite preliminary studies demonstrating TIG's ability to inhibit the in vitro growth of HCC, the precise mechanisms underlying this effect remain elusive[123, 124]. Furthermore, given TIG's dual role as an antibiotic, a comprehensive assessment of its clinical utility and safety in antineoplastic therapy is imperative to determine its potential in cancer treatment. Through comprehensive research endeavors, our goal is to uncover the latent roles of TIG in tumor therapy, thereby establishing a robust scientific foundation for the development of more effective antineoplastic strategies.

1.3 Aim of the study

Building upon the findings of our previous study, it is evident that tigecycline has the capacity to influence the activity of HCC cells. The overarching aim of this study was to comprehensively investigate the potential therapeutic mechanisms underlying Tigecycline in the context of HCC through an in vitro methodology. To achieve this, we harnessed sophisticated bioinformatics techniques to predict the putative target gene through which Tigecycline exerts its effects in HCC. Subsequently, a series of experiments are planned to be conducted to validate the expression of this target in tumor tissue and its impact on HCC cells, including their proliferation, migration, and invasion.

2. Materials and Methods

2.1 Materials

2.1.1 Consumables

Consumables	Company or source
6-well plates	Thermo Fisher Scientific, Roskilde,Denmark
12-well plates	Thermo Fisher Scientific, Roskilde,Denmark
96-well plates	Thermo Fisher Scientific, Roskilde,Denmark
5ml pipette	Costar, Maine, USA
10ml pipette	Costar, Maine, USA
25ml pipette	Costar, Maine, USA
50ml pipette	Costar, Maine, USA
1.5ml tips	Eppendorf, Hamburg, Germany
2.0ml tips	Eppendorf, Hamburg, Germany
15ml tube	Falcon, Reynosa, Mexico
50ml tube	Falcon, Reynosa, Mexico
Blot paper	Bio-Rad, California, USA
Cell culture flask T25	Thermo Fisher Scientific, Roskilde,Denmark
Cell culture flask T75	Thermo Fisher Scientific, Roskilde,Denmark
Cell culture flask T125	Thermo Fisher Scientific, Roskilde,Denmark

Cell scraper	TPP, Trasadingen, Switzerland
FACS tubes	Falcon, New York, USA
Filter paper	Whatman, Maidstone, UK
Polyvinylidene difluoride membranes	Merck Group, Darmstadt, Germany
Low-attachment 96-well plates	Corning, Krailling, Germany
Transwell plates	Corning, New York, USA

2.1.2 Chemicals

Chemicals	Company or source	Identifier
β-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany	M6250
Agarose	Life science, leuven, Belgium	18J034129
Ammonium persulfate (APS)	Serva, Heidelberg, Germany	13376.01
Bovine Serum Albumin (BSA)	Biomol, Plymouth Meeting, USA	9048-46-8
B27	Gibco, New York, USA	Cat#12587-010
Bloxall Bocking Slution	Vector, California, USA	SP6000
Chloroform	Sigma-Aldrich, Steinheim, Germany	LOT: BCBF9812V
Citric acid Monohydrat	Carl Roth, Karlsruhe, Germany	3958.2
Crystal violet	Sigma-Aldrich,	C0775

	Steinheim, Germany	
30% PolyAcrylamid	Carl Roth, Karlsruhe, Ger-many	Art.-Nr 3029.1
CASY ton	Omni Life Science, Bremen,Germany	LOT:177202
DMSO	Sigma-Aldrich, Karlsruhe, Germany	D2650
ECL™ Western Blotting Detection System	Bio-Rad Laboratories, California, USA	Cat#170-5061
>99% Ethanol	PanReac AppliChem, Germany	0v013438
Fetal Bovine serum (FBS)	Corning, Australia Source	LOT:35076116
FGF	ImmunoTools, Friesoythe, Germany	Cat#11343623
200mM Glutamine	PAN-Biotech, Baryern, Germany	Cat#P04-80100
1M Glucose	Agilment, California, USA	Cat#103577-100
Glycin	Carl Roth, Karlsruhe, Germany	3790.2
Hemalum	Sigma-Aldrich, Karlsruhe, Germany	LOT: HX28488949
Hydrogen peroxide 30%	Merck, Darmstadt, Germany	K42389487 135
Loading buffer 4x	Bio-Rad, California, USA	161-0747

Methanol	Carl Roth, Karlsruhe, Germany	4627.6
MTT powder	Thermo Fisher Scientific, Massachusetts, USA	2216966
Matrigel	Sigma-Aldrich, Steinheim, Germany	06693
Methyl cellulose	Sigma-Aldrich, Steinheim, Germany	LOT: BCCB8250
Nacl	AppliChem GmbH,Darmstadt, Germany	LOT: 21010643
Nuclease Free Water	Invitrogen, California, USA	LOT:2209535
PBS	PAN-Biotech, Munich, Germany	P04-36500
Protein standards	Bio-Rad, California, USA	RB227155
Protease inhibitor cocktail	Roche, Basel, Switzerland	05892791001
Phospho Stop cocktail	Roche, Basel, Switzerland	04906837001
RNaseZAP™	Sigma-Aldrich, Steinheim, Germany	LOT: slck9207
RPMI 1640 Medium	Gibco, New York, USA	21875-034
RIPA lysis buffer 10X	Millipore, Darmstadt, Germany	20-188
SDS	Carl Roth, Karlsruhe, Germany	2326.2

Tigecycline	Apotex Inc., Toronto, Ontario, Canada	NDC 60505-6098-0
TEMED	Bio-Rad, California, USA	Cat#161-0800
10x transfer buffer	Bio-Rad, California, USA	Cat#1610771
Tris Base	Bio-Rad, California, USA	Cat#1610716
Tri-Natriumcitrat Dihydrat	Carl Roth, Karlsruhe, Germany	3580.3
TRIzol	Ambion,Texas, USA	LOT: 20653601
Tween 20	Sigma-Aldrich, Heidelberg, Germany	P1379
Xylene(isomers)	Carl Roth, Karlsruhe, Germany	9713.2

2.1.3 Antibodies

Antibodies	**Company or source**	**Identifier**
RAC1 (WB)	Cell Signaling Technology, Massachusetts, USA	Cat#4561
RAC1 (IHC)	Thermo Fisher Scientific, Massachusetts, USA	LOT: XI3694621A
GAPDH	Cell Signaling Technology, Massachusetts, USA	Cat#5174

2.1.4 Primers

Primers	Company or source	Identifier
RAC1	Qiagen, Hilden, Germany	Cat#QT00065856
GAPDH	Qiagen, Hilden, Germany	Cat#QT00079247

2.1.5 siRNAs

siRNA	Company or source	Identifier
RAC1	Qiagen, Hilden, Germany	Cat#SI02655051 Cat# SI03065531 Cat#.SI03040884 Cat#.SI03037524
Negative Control	Qiagen, Hilden, Germany	Cat#1022076

2.1.6 Commercial Assays kits

Product	Company or source	Identifier
Avidin/Biotin Blocking Kit	Vector, California, USA	SP-001
BCA protein Assay kit	Thermo Fisher Scientific, Schwerte, Germany	Cat# 23227
FlexiTube siRNA -Rac1	Qiagen, Hilden, Germany	Cat# 1027417
Red Substrate Kit	Vector, California, USA	SK-5105

Lipofectamine RNAiMAX	Invitrogen, California, USA	Cat#13778-100
QuantiNova ™ SYBR Green PCR Kit	Qiagen, Hilden, Germany	Cat#208154
SuperScript VILO ™ Cdna Synthesis Kit	Thermo Fisher Scientific, Massachusetts, USA	LOT: 2399989
ABC-AP kit	Vector, California, USA	AK-5000

2.1.7 Apparatus

Apparatus	**Company or source**
Autoclave	Unisteri, Oberschleißheim, Germany
Bio-Rad CFX96 Real-Time PCR system	Bio-Rad Laboratories, California, USA
Centrifuge	Hettich, Ebersberg, Germany
Cool Centrifuge	Eppendorf, Hamburg, Germany
Micro centrifuge	Labtech, Ebersberg, Germany
CO_2 Incubator	Binder, Tuttlingen, Germany
DNA workstation	Uni Equip, Martinsried, Germany
Drying cabinet	Thermo Fisher Scientific, Schwerte, Germany
Electronic pH meter	Knick Elektronische Messgeräte, Berlin, Germany
Fridge (4°C, -20°C and -80°C)	Siemens, Munich, Germany
Ice machine	KBS, Mainz, Germany

Inverted light microscope	Nikon, Tokio, Japan
Liquid Nitrogen tank	MVE Goch, Germany
Lamina flow	Thermo Fisher Scientific, Schwerte, Germany
Microscope	Olympus, Hamburg, Germany
Micro weigh	Micro Precision Calibration, California, USA
Pipette boy	Eppendorf, Hamburg, Germany
Trans-Blot Turbo	Bio-Rad Laboratories, California, USA
Thermomixer comfort	Eppendorf, Hamburg, Germany
ChemiDoc Imaging System	Bio-Rad Laboratories, California, USA
Shaker	Edmund Bühler, Bodelshausen, Germany
VersaMax ELISA Microplate Reader	Molecular Devices, California, USA
Vortex Mixer VF2 (Janke & Kunkel)	IKA, North Carolina, USA
Water bath	Memmert, Schwabach, Germany

2.1.8 Software

Software and version	**Company**
Graphpad Prism 9.0	GraphPad
ImageJ Version 1.50i	National Institutes of Health
ImageJ Pro-plus Version 6.0	National Institutes of Health

2.1.9 Buffer and Solutions

MTT solution

MTT powder	25mg
PBS	50ml

Immunohistochemical staining

Citrate buffer

Citric acid Monohydrat	0.378g
Tri-Natriumcitrat Dihydrat	24.11g
H_2O	10L
PH	6

ABC-AP solution

Reagent A	10ul
Reagent B	10ul
H_2O	1ml

Red Substrate solution

Diluent	3ml
Reagent 1	48ul
Reagent 2	36ul

Blocking Buffer

BSA	0.5g
Horse serum	0.5ml
PBS	10ml

Western Blot

Separating Gel (15%)

	10%
H_2O	2.4ml
1.5M Tris pH8.8	2.5ml
30% PolyAcrylamid	5ml
10% SDS	0.1ml
10% APS	50ul
TEMED	5ul

Stacking Gel

H_2O	2.4ml
1.0M Tris pH6.8	1ml
30% PolyAcrylamid	0.6ml
10% SDS	40ul
10% APS	20ul
TEMED	4ul

10x Running Buffer

Tris base	30.3g
Glycine	144.4g
SDS	10g
H_2O	1000ml

1x Transfer Buffer

Transfer Buffer 10x	100ml
Methanol	200ml
H_2O	700ml

1x TBS-T

10x TBS	100ml
H_2O	900ml
Tween	1ml

Blocking Buffer

BSA	2.5mg
TBST	50ml

Protein lysis Buffer

10x RIPA buffer	1ml
H_2O	9ml
Phospho Stop	1 Table
Protease Inhibitor	1 Table

1M Tris-HCl

Tris-base	12.12g
H_2O	200ml
PH	6.8

1.5M Tris-HCl

Tris-base	36.34g
H_2O	200ml
PH	8.8

Loading buffer

4xloading buffer	3600ul
β-Mercaptoethanol	400ul

10%SDS

SDS	10g
H_2O	100ml

10%APS

APS	10g
H_2O	100ml

2.2 Methods

2.2.1 Cell culture

Human HCC cells Huh7 and HepG2 were cultured in monolayers, adhering to the surface of culture dishes. The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) to support their proliferation and growth. Subculturing was performed by trypsinization upon reaching 80% confluence. As per internal protocols, regular mycoplasma testing was conducted quarterly for all cell lines. The cells were maintained in a humidified CO2 incubator at 37°C under an atmosphere containing 5% CO_2 and 95% air. All cells were routinously negatively checked for mycoplasma contamination.

2.2.2 Cell counting

Cells from each experimental group were seeded in equal amounts into six-well plates. On the subsequent day, the culture medium was substituted with a medium containing either tigecycline or DMSO

(utilized as a control). Immediately after the medium change, the cells were photographed to capture their initial state (0 hours). Subsequently, at 24 hours, 48 hours, and 72 hours, the cells were photographed again to observe their growth status over time. At each designated time point, the cells were treated with a specific digestive enzyme (Accutase) following the imaging process. After the enzymatic reaction was terminated, the cells were finally counted using the Casy system.

2.2.3 siRNA transfection

Huh7 and HepG2 cells were individually introduced into separate 6-well plates, and they proliferated until they reached an approximate 80% confluence. They were cultured in distinct growth media enriched with 10% FBS. Following an overnight incubation, the growth medium in each well was exchanged with 200 µl of serum-free medium. Subsequently, Lipofectamine RNAiMAX reagent was diluted precisely in Opti-MEM medium at a specific ratio of 3:50, resulting in a well-mixed solution comprising 54 µl of Lipofectamine and 900 µl of Opti-MEM medium. Concurrently, RAC1-siRNA was accurately diluted in Opti-MEM at a ratio of 1:50, resulting in a thoroughly mixed solution containing 18 µl of RAC1-siRNA and 900 µl of Opti-MEM medium. Next, the appropriately diluted RAC1-siRNA and Lipofectamine were meticulously combined at a 1:1 ratio, ensuring comprehensive mixing through pipetting. After incubating for 10 minutes at room temperature, the resultant mixture was

meticulously dispensed drop by drop into the designated wells of the 6-well plate. The plate was gently agitated and subsequently positioned in a controlled incubation chamber set at 37°C with a 5% CO_2 atmosphere for a period spanning 24 to 48 hours.

2.2.4 MTT

Cell viability was evaluated with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay, commonly referred to as the MTT assay. Initially, cells were plated at a density of $1x10^4$ cells per well in a 96-well plate and subjected to treatment with varying concentrations of Tigecycline. Following a 48-hour treatment duration, cell viability was gauged. In the MTT assay protocol, cells were initially rinsed with PBS. Subsequently, a precisely measured 50 µl of MTT working solution (0.5 mg/ml) was added meticulously to each well. Then, the plate was securely positioned within a CO_2 incubator, maintaining a constant temperature of 37°C, and left undisturbed for a duration of 30 minutes, facilitating the formation of formazan crystals. After the removal of the culture medium from each well, DMSO was introduced to terminate the cell culture reaction. Following this, the absorbance of each well was assessed at room temperature utilizing a VersaMax microplate reader, with measurements taken at wavelengths of 570 nm and 670 nm for background correction. To ensure the robustness and reproducibility of the outcomes, this

experiment was iterated three times under consistent operating parameters.

2.2.5 Wound healing assay

HepG2 and Huh7 HCC cell lines were separately seeded into corresponding 6-well plates. Once the cells achieved confluency in each well, a controlled scratch was delicately created across the monolayer using a sterile pipette tip. Subsequently, the cells underwent a thorough triple wash with PBS, and fresh serum-free RPMI1640 medium was added immediately. The scratch areas were captured by microscopy at 0 hours. After 24 hours and 48 hours of incubation, images of the same positions at the same magnification were taken again. The scratch areas at different time points were quantitatively analyzed using ImageJ software. The decrease in the scratched area was quantified and interpreted as the rate of cell migration.

2.2.6 Transwell assay

Matrigel was dissolved at 4°C and combined with serum-free culture medium at a 1:3 ratio. The resulting mixture, in a quantity of 40 μl per chamber, was judiciously applied to the upper compartment of a transwell plate, ensuring thorough coverage of the underlying surface. Subsequently, the transwell plate was introduced into a 37°C incubator enriched with 5% CO_2 for an overnight incubation period, ensuring complete solidification of the Matrigel. Following Matrigel solidification,

a total of 100,000 cells suspended in 300 µl of serum-free culture medium were carefully introduced into the upper chamber of the transwell plate, which had an 8.0 µm pore size. The lower chamber was filled with 600 µl of complete culture medium. Following this step, the cells were cultured for a duration of 24 hours at 37°C with a 5% CO_2 atmosphere, with the choice between conventional culture medium or medium containing varying concentrations of Tigecycline. Following the 24-hour culture period, the upper chamber underwent two gentle wipe-downs using a cotton swab. Subsequently, cell fixation was carried out using 4% paraformaldehyde for a duration of 15-20 minutes, followed by room temperature staining utilizing 0.5% crystal violet for an additional 15-20 minutes. The number of invading cells in three randomly selected fields was quantified using an inverted optical microscope.

2.2.7 Immunohistochemical staining (IHC)

After deparaffinization and rehydration, the antigen retrieval was meticulously executed by subjecting the slides to a 30-minute heating process at 96°C in the presence of citrate buffer, all contained within a metal container. In continuation from this, a cooling period of 20 minutes naturally ensued, facilitating temperature equilibration. To facilitate specific staining, circles were drawn around each slides using a hydrophobic pen. Subsequently, the following steps were conducted in a humidifying chamber (Wash the slide with TBS-T three times for 5

minutes before starting the next step): The tissue specimens were subjected to an incubation process at room temperature for a duration of 20 minutes, during which they were exposed to the application of avidin reagent.

Biotin reagent was applied to the slides and they were incubated at room temperature for 20 minutes. Following this, the slides were subjected to the SP-6000 agent and incubated for 10 minutes, also at room temperature. Subsequently, the slides were treated with a solution consisting of PBS supplemented with 5% Bovine Serum Albumin (BSA) and 5% horse serum, and this mixture was incubated for a minimum of 1 hour at room temperature. For antibody application, each primary antibody and isotype control were diluted according to the specified ratio in 5% BSA/PBS. The titrated antibody solution (1:50） was then added to the tissue slides and incubated in the humidifying chamber under the following conditions. Additionally, a negative control slide, free of antibody, was prepared using 5% BSA/PBS solution. On the following day, the slides underwent a triple 5-minute wash in TBS-T. Following this, a secondary antibody dilution (1:200) was applied to the slides, and the slides were incubated at room temperature within the humidifying chamber for 30 minutes. Following the established protocol, the prepared ABC-AP reagent was introduced to the slides, and the slides were once again incubated room temperature within the humidifying chamber for an

additional 30 minutes. Next, the tissue slides were treated with the Red Substrate, containing alkaline phosphatase, and incubated at room temperature within the humidifying chamber for a duration of approximately 20-30 minutes. The staining process was continuously monitored under a microscope, and once an acceptable staining intensity was observed, the reaction was stopped by removing the substrate. The slides were then washed in PBS for 5 minutes and briefly rinsed in distilled water. To counterstain the slides, they were briefly dipped in a glass container of hematoxylin for 1 second. The slides were washed under flowing tap water for 10 minutes. After drying, the coverslip was mounted with 2 drops of aqueous mounting media. Following immunohistochemical staining, photomicrographs were captured using an electron microscope sweep, and all slides were subjected to analysis using Image Pro-Plus software.

2.2.8 Real- time PCR (RT- PCR)

RNA isolation

First, the culture medium was removed and the cells were rinsed thrice with pre-chilled PBS to ensure the thorough elimination of any residual medium. Subsequently, 1 ml of Trizol reagent was judiciously added to each well within the six-well plate, and a series of 3-5 agitation cycles were meticulously conducted to attain comprehensive cell lysis. Thereafter, the solution was carefully transferred to the designated

centrifuge tube using a pipette gun. The samples were left undisturbed, facilitating complete lysis at room temperature for a duration of 10 minutes. In the context of RNA extraction, introduce 0.2 ml of chloroform per ml of Trizol, vortex the amalgamation for 15 seconds, and then incubate it on ice for 5 minutes. Following this step, centrifuge the sample at 12,000 g for 15 minutes at 4ºC. Carefully remove the upper colorless aqueous phase using a pipette, which contains the total RNA, into a fresh centrifuge tube, aiming for approximately 400 ul per ml of the initial Trizol volume. To precipitate the RNA, introduce 0.5 ml of isopropanol per ml of the original Trizol volume to precipitate the RNA. Thoroughly mix the contents by agitating the tube, Facilitating RNA precipitation on ice for a period of 10 minutes. Continuing from this, perform centrifugation at 12,000 g at a temperature of 4ºC for 10 minutes to gather the RNA precipitate located at the base of the tube. Discard the supernatant cautiously. Subsequently, add 1 ml of 75% ethanol (prepared in DEPC water) per ml of the initial Trizol volume, invert the solution for thorough agitation and subsequently subject it to centrifugation at 7,500 g at a temperature of 4ºC for a duration of 5 minutes. Carefully discard the supernatant. Perform an additional brief centrifugation (>5,000 g, 1-second centrifugation), and meticulously aspirate any remaining liquid.

The RNA pellet was briefly air-dried and subsequently solubilized in 20 µl of DEPC water. The dissolved RNA was stored at -70ºC for subsequent analysis.

cDNA production and real- time PCR (RT- PCR)

RNA samples underwent reverse transcription in a thermocycler, adhering to a meticulous protocol: Initiate the process with an initial priming step at 25 ºC for 10 minutes, followed by reverse transcription at 42 ºC for 60 minutes, subsequent inactivation at 85ºC for 5 minutes, and conclude with a final holding phase at 4°C. Detailed parameters and conditions for the reverse transcription reaction are elucidated in Table 1.

For RT-qPCR assays, the QuantiNovaTM SYBR Green PCR Kit was used, with the reaction setup meticulously outlined in Table 2. The RT-qPCR amplification protocol commenced was with an initial denaturation phase at 95˚C for 2 minutes, succeeded by 40 cycles involving denaturation at 95˚C for 5 seconds and annealing/extension at 60˚C for 10 seconds. Each sample underwent triplicate analyses, and an essential negative control utilizing sterile RNase-free H_2O was included to eliminate the possibility of non-specific amplification. The housekeeping gene GAPDH served as a reference for normalizing cDNA expression variations. To ensure statistical robustness, for each experimental group was underwent three independent experiments. The

quantification of relative gene expression was performed utilizing the $2^{-\Delta\Delta Cq}$ methodology.

Table 1: The reverse-transcribed reaction settings

5x VILO™ Reation Mix	2ul
10x SuperScript™ Enzyme Mix	2ul
Template RNA (1pg to 2.5ug to total RNA)	varies
RNase-free water	to 20ul

Table 2: Reaction setup of QuantiNova™ SYBR Green PCR Kit

SYBR Green PCR Master Mix	10ul
QN ROX Reference Dye	2ul
Primer	2ul
RNase-free water	4ul
cDNA	2ul
Total reaction volume	20ul

2.2.9 Western Bolt

Extraction of total cellular proteins

Sterile centrifuge tubes were meticulously prepared and sequentially labeled to correspond with the respective samples. Following the removal of the original culture medium from the cells, a pair of pre-cooled PBS washes at 4°C was conducted. To enhance the interaction and reaction efficacy, precisely 150 µl of RIPA lysate was introduced into each well of

the 6-well plate. The plate was then incubated on ice for approximately 40 minutes, maintaining continuous agitation throughout the incubation period. Following this, a cell scraper was utilized to gently dislodge the adherent cells from the surface of the 6-well plate, ensuring the preservation of a cooled environment. The resultant cell suspension was meticulously transferred into 1.5 ml centrifuge tubes, which were maintained at a constant temperature of 4°C. Centrifugation was performed at 12,000 r/min for a duration of 10 minutes to facilitate the separation of cellular components. Subsequently, the upper layer of the centrifuged solution, enriched with cellular proteins, was meticulously transferred into fresh 1.5 ml centrifuge tubes. These protein-enriched tubes were then promptly stored at -20°C, ensuring their preservation for subsequent experimental applications.

Determination of protein concentration by BCA method

Prepare the BCA Working Solution with exacting precision by thoroughly blending 50 volumes of BCA Reagent A with 1 volume of BCA Reagent B, maintaining an unerring 50:1 ratio to ensure comprehensive homogenization. To establish the standard curve, introduce varying concentrations of the diluted standard solution into the designated wells of a 96-well plate. Meticulously dispense around 5 µl of the sample and 195 µl of the BCA working solution into each well, gently pipetting to ensure thorough homogenization. Incubate the plate at 37°C

for a duration of 30 minutes. Subsequent to this incubation duration, an enzyme marker is used to quantify the absorbance of the samples at a wavelength of 562 nm. The protein concentration in the analyte is determined based on the absorbance reading derived from the standard curve.

Gel preparation

Considering the molecular weight of the target protein slated for analysis, prepare the SDS-PAGE separating gel and a 5% stacking gel at the suitable concentrations. Combine all constituents, excluding TEMED, in the correct ratios and ensure thorough homogenization. In continuation from this, introduce TEMED, mix vigorously, and expeditiously pour the gel mixture into the gel-making plate to prevent distortion of the gel and formation of bubbles during the pouring process. The stacking gel solidifies entirely, and meticulously extract the comb in a vertical orientation. Rinse the gel gently and repetitively, then securely affix it within the electrophoresis apparatus in preparation for subsequent analysis.

Electrophoresis

Equal amounts of protein samples were aliquoted into respective wells, and electrophoresis was initiated at a voltage range of 60-90 V. Continuing from this, upon the migration of the bromophenol blue dye in the upper sample buffer to the demarcation between the separating gel

and stacking gel, the voltage was fine-tuned to 80-120 V. Electrophoresis was terminated upon complete migration of the bromophenol blue dye had traversed the entirety of the separating gel.

Transferring membrane and Closure

Prepare a 1× membrane transfer buffer in advance and refrigerate it at -4°C until immediately before the membrane transfer procedure. Pre-soak the transfer clips, sponges, and filter paper in the transfer buffer solution. Concurrently, immerse the PVDF membrane in methanol for 15 seconds to prepare it for subsequent use. Upon completion of the electrophoresis, delicately extract the gel and gently position it on the filter paper, creating a "sandwich" configuration as follows: transfer clip → sponge → filter paper → gel → filter paper → sponge → transfer clip. Ensure gentle compression to obviate the formation of air bubbles within the strata of this "sandwich" arrangement. Following this, situate the assembled structure into the electrophoresis tank and set the transfer conditions to 25 V for 30 minutes. Upon completion of the transfer, place the PVDF membrane with its face upward into a 5% BSA solution and subject it to agitation on a shaker for 1 hour at room temperature.

Antibody incubation

The PVDF membrane was accurately trimmed to match the molecular weight of the target protein and suitably labeled. Following the established protocol, the primary antibody was adequately diluted.

Subsequently, the membrane was subjected to overnight incubation at 4°C with the primary antibody, ensuring complete submersion of all membrane regions in the antibody solution.

The following day, the primary antibody was carefully removed, and the membrane was immersed in 1× TBST, with gentle agitation performed at room temperature. Each washing step lasted 5 minutes, and the solution was replaced. This washing process was iterated three times to ensure thorough elimination of non-specifically bound primary antibody.

The secondary antibody was diluted using the appropriate diluent, and the membrane was immersed in the secondary antibody solution, incubating it at 37°C for 1 hour.

Develop and photograph

Upon removal of the secondary antibody, the PVDF membrane underwent a series of washes in 1× TBST, positioned on a gently agitating device at room temperature. Each washing cycle persisted for 5 minutes, and the solution was replaced after each cycle. This iterative washing process was conducted three times following every liquid replacement to ensure comprehensive elimination of any unbound antibodies.

Continuing from this, the development parameters were configured, and the PVDF membrane was immersed in the developer solution. The ECL

luminescent solution was carefully dispensed drop by drop, guaranteeing uniform distribution across the membrane. Following this, the membrane underwent chemiluminescence detection to visualize the proteins. The ChemiDoc Imaging System was utilized for the examination of immunoreactive bands and the acquisition of chemiluminescent images.

2.2.10 Bioinformatic analysis

Utilizing the Comparative Toxicogenomics Databas (CDT , https://ctdbase.org/)andPharmmaper(https://www.lilab-ecust.cn/pharmmapper/)databases, a comprehensive target analysis of tigecycline was executed, yielding potential molecular targets. Concurrently, differential expression genes (DEGs) exhibiting upregulation in HCC tissues in comparison to healthy controls were subjected to meticulous examination through Gene Expression Profiling Interactive Analysis(GEPIA , http://gepia2.cancer-pku.cn/#index), leading to the identification of distinct genetic factors. Furthermore, an exhaustive exploration was conducted to unveil liver cancer-associated prognostic genes, specifically those influencing OS. Subsequently, the top 300 survival-associated DEGs, characterized by the most statistically significant p-values, were meticulously selected for subsequent Venn diagram analysis, ultimately revealing the target gene RAC1.

To elucidate the expression landscape of RAC1 across various tumor types, an intricate bioinformatic analysis was undertaken. Initially, we

conducted an analysis using the TIMER2 database(http://timer.cistrome.org/). Subsequently, the mRNA expression levels of RAC1 were rigorously scrutinized within the context of HCC utilizing the sophisticated online tool GEPIA. a comprehensive assessment of RAC1's protein abundance within HCC was conducted by meticulously analyzing data procured from the Clinical Proteomic Tumor Analysis Consortium (CPTAC,https://proteomics.cancer.gov/programs/cptac), Accessible through the publicly accessible UALCAN platform (https://ualcan.path.uab.edu/analysis-prot.html). Furthermore, an in-depth exploration of RAC1 promoter methylation was performed using the UALCAN resource. Aiming to unravel potential associations, we examined the correlation between RAC1 expression and critical biomarkers (MKI67, PCNA) as well as immune checkpoint molecules (CTLA4, HAVCR2, PDCD1) within the HCC landscape. This intricate analysis was executed utilizing the correlation analysis functionality embedded within GEPIA. Additionally, a comprehensive functional enrichment analysis was undertaken to unravel the underlying biological implications of RAC1 in HCC. The process commenced with a meticulous correlation analysis between genes and RAC1 within the HCC context. Subsequently, genes exhibiting robust correlation coefficients and statistical significance were judiciously selected for extensive

enrichment analysis using the cluster Profiler package. The Gene Ontology (GO, http://geneontology.org/) categories encompassed Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). To rigorously assess the prognostic potential of RAC1 within HCC, an integrative approach leveraging transcriptomic and clinical data from the Cancer Genome Atlas (TCGA, The Cancer Genome Atlas Program (TCGA) - NCI) database and supplementary datasets was utilized. The time-dependent Receiver Operating Characteristic (ROC) curve analysis, facilitated by the time ROC package, was utilized for this purpose. Integrating RAC1 expression data with clinical attributes, we undertook a comprehensive correlation analysis between RAC1 and various clinical characteristics within the TCGA database. Numerical variables, adhering to normal distribution and homogeneity of variance, underwent statistical comparison via T-tests for two groups and One-way ANOVA for three groups. In scenarios where these assumptions were not met, Welch's T-test and Welch's One-way ANOVA were used. Non-normally distributed data prompted the use of Wilcoxon tests for two groups and Kruskal-Wallis tests for three groups. Categorical variables adhered to the chi-square test, continuous correction chi-square test (Yates' correction), or Fisher's exact test based on predefined conditions. Exploration of RAC1's role as an independent prognostic factor was conducted through rigorous univariate and

multivariate Cox regression analyses. Merging expression and clinical data from TCGA and supplementary datasets, the survival package facilitated proportional hazards hypothesis testing and the construction of multivariate Cox regression models. Variables displaying a single-factor p-value < 0.1 in univariate analysis were included in the multivariate Cox regression model building process.

2.2.11 Statistical analysis

All experiments were conducted independently and replicated a minimum of three times. The mean standard deviation (SD) was calculated for each group. Statistical analyses were conducted employing one-way or two-way analysis of variance (ANOVA) for comparisons involving multiple groups, or Student's t-test for individual comparisons. Statistical significance was determined at a significance level of $p<0.05$. In all graphical representations of statistical data, bar graphs illustrate the mean ± SD. Significance levels are denoted as follows: *P<0.05, **P<0.01, ***P<0.005, ****P<0.001, and 'ns' signifies no significance when compared to the control group.

3. Result

3.1 Bioinformatical analysis reveals RAC1 as a potential target for Tigecycline in HCC cells

We systematically identified potential molecular targets of tigecycline through a detailed analysis of the CDT and Pharmmaper databases, resulting in the identification of 223 promising candidates. Furthermore, we conducted a comprehensive investigation of DEGs using the GEPIA platform, focusing on genes upregulated in HCC tissues compared to normal tissues, revealing 2207 genes with altered expression profiles. To identify genes associated with HCC prognosis, we examined a vast dataset of 60,660 genes linked to survival outcomes. Through a rigorous curation process, we identified the top 300 survival-associated DEGs with significant p-values. Utilizing Venn diagrams, we have discerned three potential target genes, specifically labeled as ESR1, RAN, and RAC1. Leveraging the extensive groundwork laid by our research group's antecedent studies, we have precisely identified RAC1 as the pivotal target gene **(Fig. 9 A)**.

Our investigation into RAC1 expression across various tumors commenced with an analysis utilizing the TIMER2 database. This meticulous examination uncovered dynamic RAC1 expression patterns across distinct tumor types when contrasted with their respective normal counterparts. Of particular significance was the marked upregulation of

RAC1 within HCC tissues, showcasing a notable difference in expression levels (normal 50 vs. tumor 371) **(Fig. 9 B)**. Subsequently, we delved deeper into RAC1's mRNA expression within HCC by harnessing data sourced from both the TCGA and GTEx databases, skillfully employing the online GEPIA tool. Furthermore, our quest to decipher RAC1's protein-level expression in HCC led us to scrutinize data from the esteemed Clinical Proteomic Tumor Analysis Consortium (CPTAC), readily accessible through the UALCAN platform. The comprehensive dataset from GEPIA reaffirmed RAC1's pronounced overexpression in HCC (normal 160 vs. tumor 369) **(Fig. 9 C)**. This significant finding was further substantiated through our analysis of the CPTAC database, which consistently confirmed the heightened expression of RAC1 in HCC (normal 165 vs. tumor 165) **(Fig. 9 D)**.

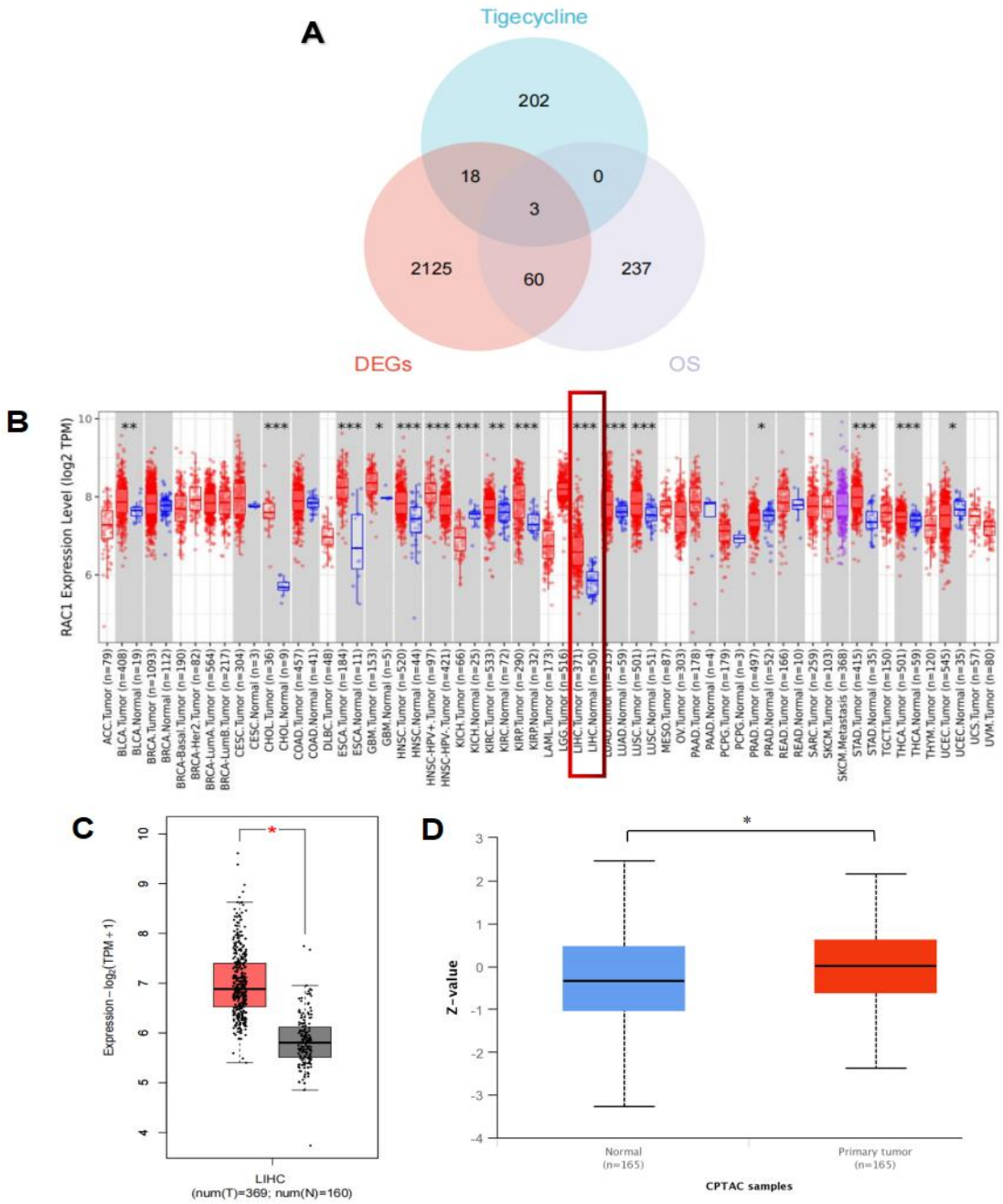


Figure 9: Bioinformatics analysis of Tigecycline and HCC.

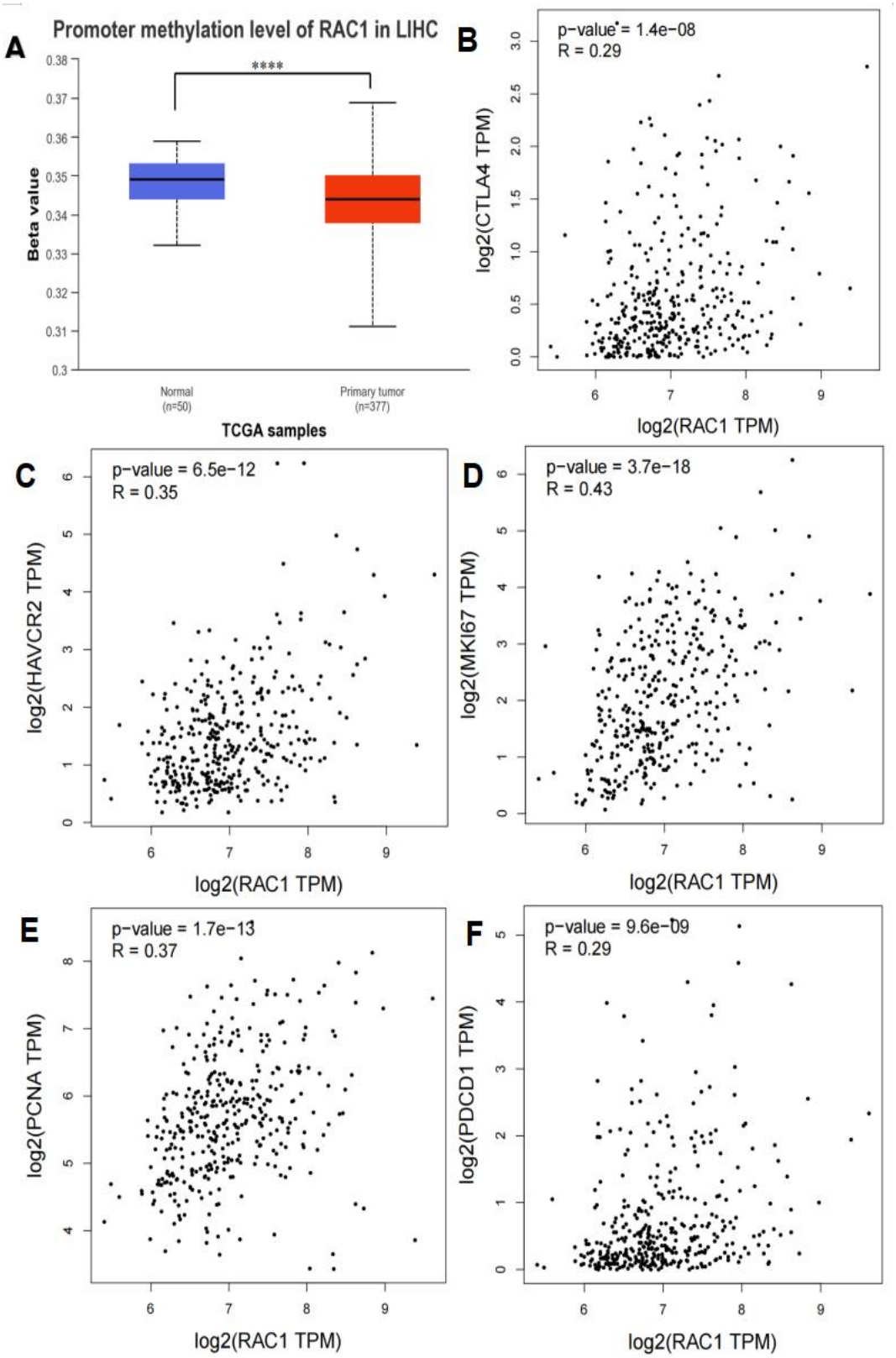
(A) A total of 223 potential target genes for Tigecycline were identified through an exhaustive analysis of the Pharmmaper and CDT databases. Simultaneously, 2207 differential genes were extracted through the utilization of the GEPIA2 platform. Further refining our focus, we then proceeded to meticulously select the top 300 survival-associated differential genes, based on their remarkably small p-values, for subsequent analysis using Venn diagrams. The intersection reveals 3 relevant genes. (B) RAC1 is highly

expressed in HCC from TIMER2 database (normal 50 vs tumor 371, ***P<0.005). (C) RAC1 mRNA expression exhibits a significant upregulation in HCC from GEPIA database (normal 160 vs tumor 369, *P<0.05). (D)The elevated expression of RAC1 protein in HCC was corroborated by data from the CPTAC database (normal 165 vs tumor 165, *P<0.05).

3.2 Bioinformatics Analysis Reveals Potential Biological Characteristics of RAC1

To gain further insights into the molecular mechanisms underpinning our discoveries, particularly in relation to epigenetics. Our initial step involved an extensive analysis of the methylation status of the RAC1 promoter through the utilization of the UALCAN website. Furthermore, we sought to uncover potential correlations between RAC1 expression and critical biomarkers such as MKI67 and PCNA, along with immune checkpoints including CTLA4, HAVCR2, and PDCD1 in the context of HCC. To accomplish this, we utilized GEPIA's correlation analysis feature. Subsequently, we conducted a comprehensive functional enrichment analysis to shed light on RAC1's potential biological roles within HCC. Initially, we identified genes significantly correlated with RAC1 based on stringent criteria (|correlation coefficient|>0.5, P<0.5). These genes were then subjected to enrichment analysis, employing the clusterProfiler package. The analysis covered various GO categories, encompassing Biological Process (BP), Cellular Component (CC), and Molecular Function (MF).

We observed a significant reduction in RAC1 promoter methylation levels in tumor tissues compared to normal tissues (50 in normal vs. 377 in tumor) **(Fig. 10 A)** Correlation analysis revealed positive associations between RAC1 and MKI67, PCNA, CTLA4, HAVCR2, and PDCD1 ($P<0.05$) **(Fig. 10 B, C, D, E and F)**. To ascertain the potential role of RAC1 in HCC, we conducted functional enrichment analysis. We identified 30,625 genes that exhibited a significant correlation with RAC1 ($P<0.05$). Among these, 1,438 genes displayed correlation coefficients greater than 0.5, while 26 genes exhibited correlation coefficients <-0.5. We subsequently focused on these 1,464 genes for functional enrichment analysis. **Fig. 10 G** and **H** showcase the top 30 genes positively and negatively correlated with RAC1. BP linked to these genes encompassed RNA splicing, nuclear division, regulation of DNA metabolic processes, chromosome segregation, and methylation. In terms of CC, the genes were associated with the chromosome region, cell-substrate junction, spliceosomal complex, actin cytoskeleton, and centromeric region. MF analysis indicated involvement in cadherin binding, transcription coregulator activity, actin binding, guanyl nucleotide binding, and guanyl ribonucleotide binding. Moreover, the KEGG pathway analysis revealed enrichment in spliceosome, ubiquitin-mediated proteolysis, RNA degradation, DNA replication, and viral carcinogenesis **(Fig. 10 I, J, K and L)**.


A
Promoter methylation level of RAC1 in LIHC
****
Beta value
0.38
0.37
0.36
0.35
0.34
0.33
0.32
0.31
0.3
Normal (n=50)
Primary tumor (n=377)
TCGA samples
B
p-value = 1.4e-08
R = 0.29
log2(CTLA4 TPM)
log2(RAC1 TPM)
C
p-value = 6.5e-12
R = 0.35
log2(HAVCR2 TPM)
log2(RAC1 TPM)
D
p-value = 3.7e-18
R = 0.43
log2(MKI67 TPM)
log2(RAC1 TPM)
E
p-value = 1.7e-13
R = 0.37
log2(PCNA TPM)
log2(RAC1 TPM)
F
p-value = 9.6e-09
R = 0.29
log2(PDCD1 TPM)
log2(RAC1 TPM)

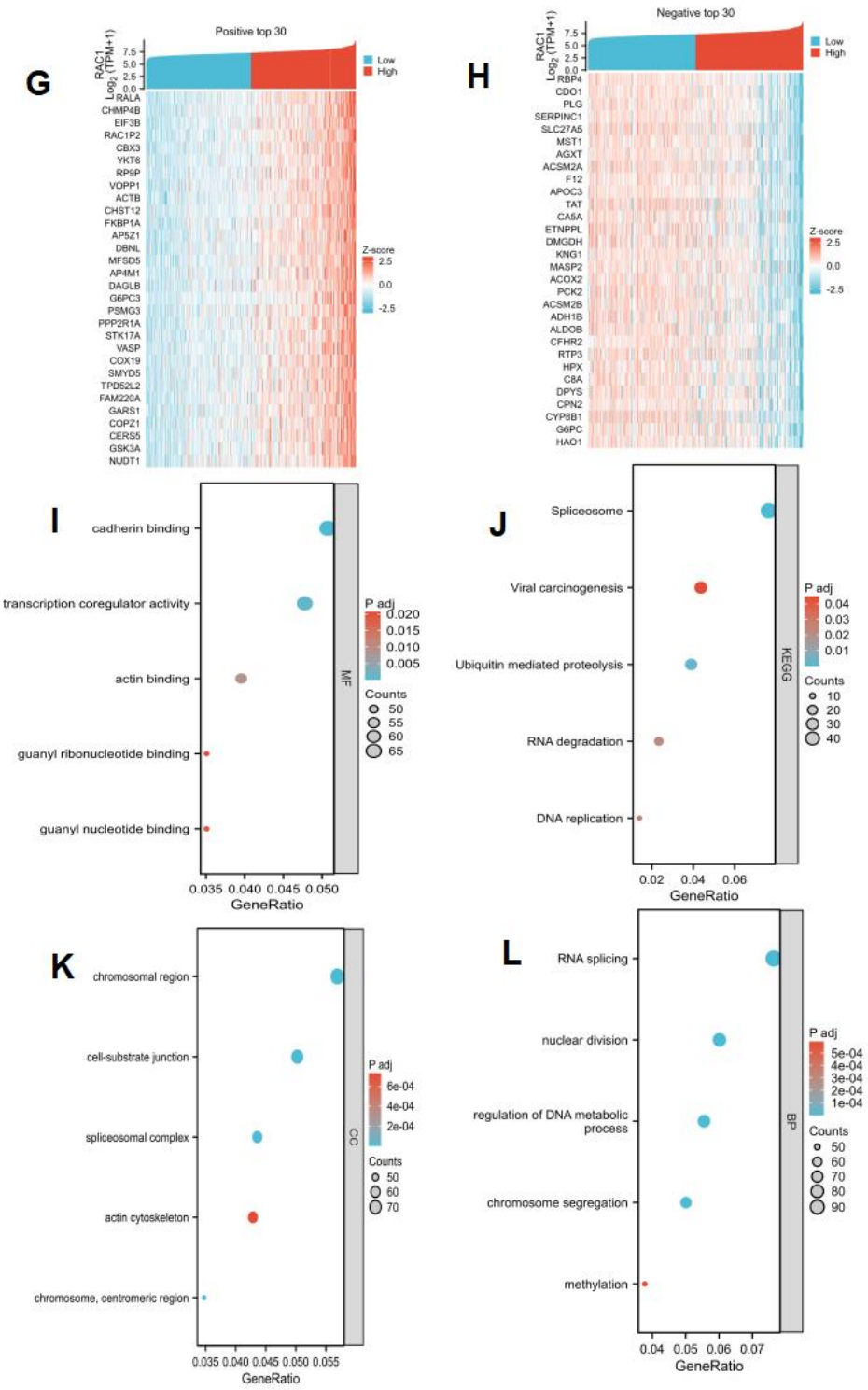


Figure 10: Bioinformatics analysis reveals potential biological functions of RAC1

(A) The methylation levels of the RAC1 promoter were notably lower in tumor tissues when compared to normal tissues (50 in normal vs. 377 in tumor, ****P<0.0001). (B-F) Correlation analysis was conducted to assess the

relationship between RAC1 and MKI67, PCNA, CTLA4, HAVCR2 and PDCD1. (G-H) Heatmaps shows that the top 30 genes are positively and negatively correlated with RAC1 in HCC respectively. (I-L) Enrichment analysis of genes correlated with RAC1 in HCC.

3.3 Bioinformatic analysis of the correlation between RAC1 expression and immune cell infiltration in HCC

After stratifying the patients into high and low groups based on the median expression of RAC1, we utilized the ssGSEA algorithm, available in the R package-GSVA [version 1.46.0], and utilized the 24 immune cell markers as provided in the Immunity article by an article to calculate immune infiltration[125]. Subsequently, we conducted a correlation analysis between RAC1 and the immune infiltration matrix data, with the results visualized as lollipop graphs through the ggplot2 package. We observed that patients with high RAC1 expression exhibited elevated levels of infiltration by iDC, macrophages, Tem, TFH, and Th2 cells compared to those with low RAC1 expression. Conversely, patients with high RAC1 expression displayed reduced infiltration of cytotoxic cells, DC, Eosinophils, Neutrophils, Tgd, Th17 cells, and Treg in comparison to patients with low RAC1 expression **(Fig. 11A)**. Furthermore, our correlation analysis revealed a positive association between RAC1 expression and the infiltration of Th2 cells, NK CD56 bright cells, macrophages, Tem, TFH, iDC, aDC, and Th1. Conversely, RAC1 expression exhibited a negative correlation with the infiltration of Th17

cells, Eosinophils, DC, Treg, Neutrophils, Tgd, and cytotoxic cells **(Fig. 11 B)**.

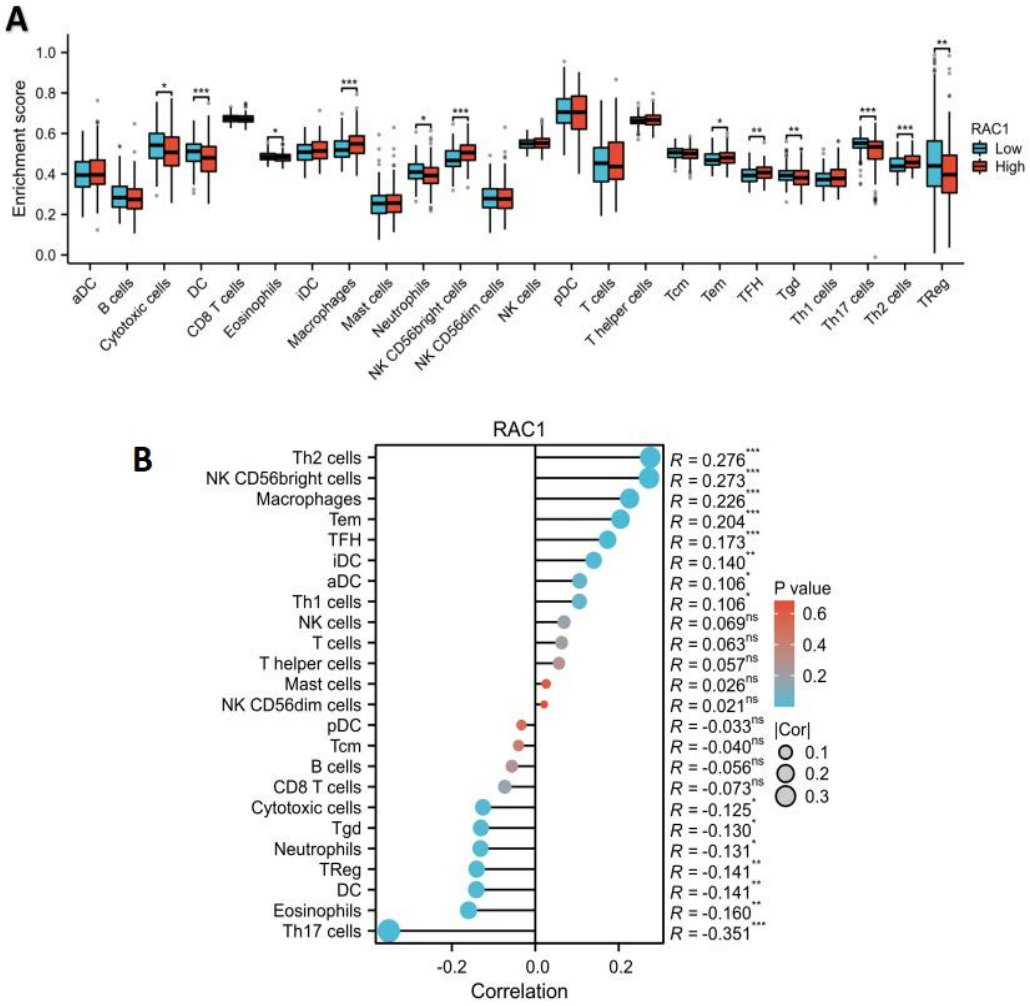


Figure 11: The association between the expression of RAC1 and the infiltration of immune cells in HCC. (A) Infiltration of immune cells in tumor tissues between high expression and low expression patients for RAC1. (B) The relationship between the expression of RAC1 and immune cells in HCC.

immature dendritic cells (iDC); effector memory T cells (Tem); T follicular helper cells (TFH); T helper 2 cells (Th2); dendritic cells (DC); gamma delta T cells (Tgd); T helper 17 cells (Th17); regulatory T cells (Treg).

3.4 Bioinformatics-Based Survival Analysis for Patients with Variable RAC1 Expression in HCC

By integrating transcriptomic and clinical data from TCGA database along with supplementary data[126], we conducted a comprehensive analysis of the prognostic potential of RAC1 in HCC using the time ROC package. An area under the curve (AUC) value within the range of 0.7 to 0.9 on the receiver operating characteristic (ROC) curve signifies a discernible prognostic effect for RAC1. Conversely, an AUC value falling between 0.5 and 0.7 indicates notably limited prognostic performance for RAC1. Upon conducting an extensive and comprehensive survival prognostic analysis of RAC1, a conspicuous divergence in survival outcomes attributed to the differential expression of the RAC1 gene became evident between tumorous and healthy tissues **(Fig. 12 A, C and E)**. Notably, the AUC for RAC1 at the 1-year juncture within the context of Disease-Specific Survival (DSS) exceeded the pivotal threshold of 0.7 **(Fig. 12 D)**. This observation substantiates its potential effectiveness in accurately prognosticating 1-year outcomes within the realm of DSS. Consequently, we infer that RAC1 assumes a cardinal role in orchestrating the impact of tigecycline on HCC. A plausible proposition arises wherein RAC1 is posited as a crucial mediator in modulating the suppressive influence exerted by tigecycline upon the proliferative proclivities of HCC.

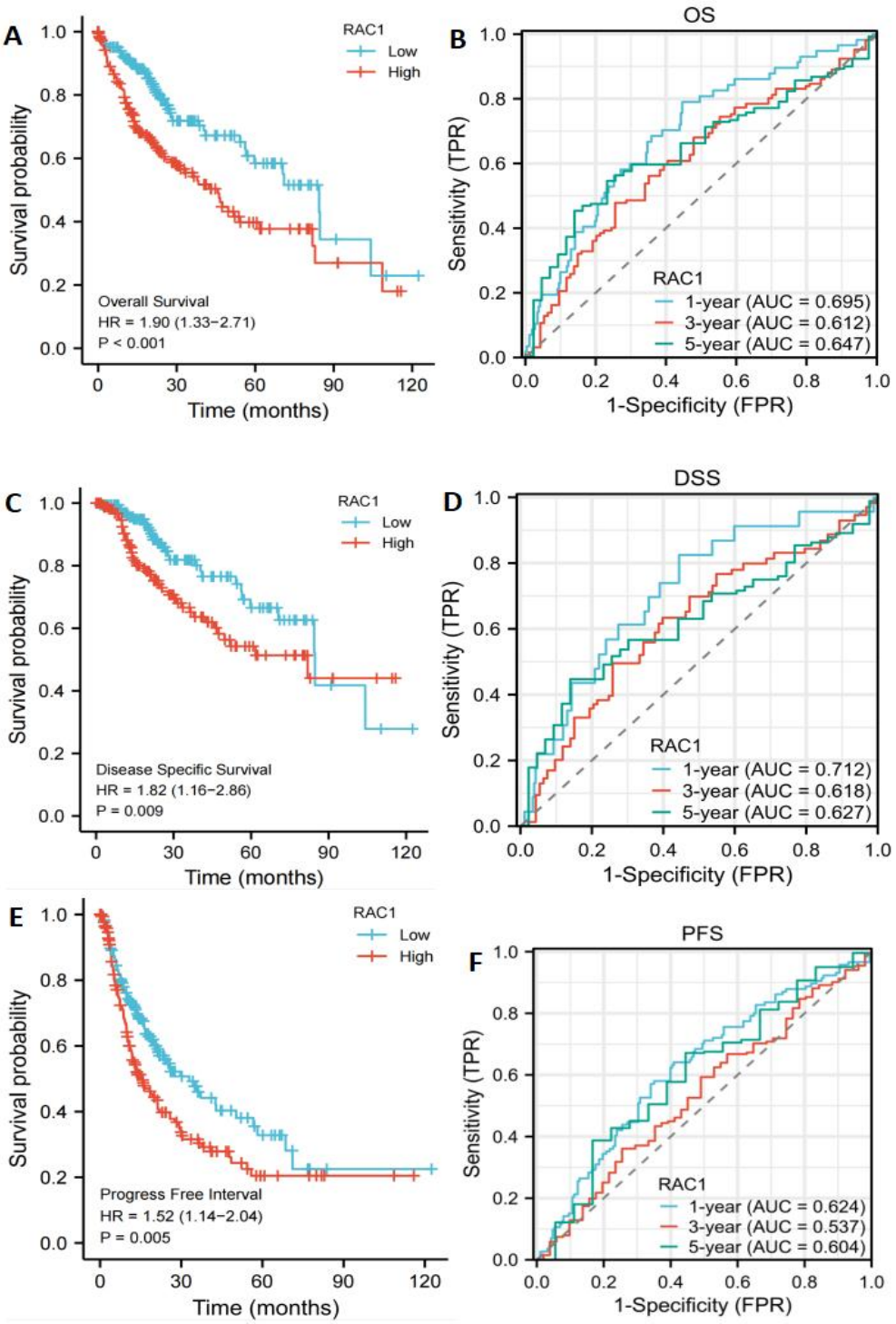


Figure 12: Construction and validation of the prognostic model for RAC1.

Survival analysis of OS (A), DSS (C)and DFS (E) of patients with different expression of RAC1; The prognostic effectiveness of RAC1 was evaluated in terms of OS (B), DSS (D) and PFS (F) across 1, 3 and 5-year time intervals. Overall Survival (OS); Disease-Specific Survival (DSS); Progression-Free Survival (PFS).

3.5 Correlating RAC1 Expression with Clinical Features in HCC via Bioinformatics

To comprehensively explore the exact ramifications of RAC1 expression on patient prognosis, we embarked on a meticulous correlation analysis that encompassed RAC1 expression and a wide spectrum of clinical characteristics. This analysis drew upon data procured from the TCGA database. Subsequently, we delved into the assessment of whether RAC1 serves as an independent prognostic factor through both univariate and multivariate Cox regression analyses. In this pursuit, we integrated expression data sourced from the TCGA database with complementary datasets[126]. For proportional hazards hypothesis testing and Cox regression analysis, we harnessed the capabilities of the survival package. Upon conducting an extensive examination of the relationship between RAC1 expression levels and clinical attributes in patients with HCC, notable statistical disparities were identified in parameters such as T stage, Pathologic stage, Histologic grade, Tumor status, Weight, BMI, and AFP when comparing subgroups characterized by high and low RAC1 expression levels **(Table 3)**. Moreover, outcomes stemming from both univariate and multivariate Cox regression analyses conclusively identified RAC1 as an independent prognostic risk factor for overall survival **(Table 4)**. These results fortify the robustness of our findings and underscore the clinical relevance of RAC1 in HCC prognosis.

Table 3. Clinical characteristics of HCC patients with low and high RAC1 expression levels

characteristics	Low expression of RAC1	High expression of RAC1	p-value
n	187	187	
Pathologic T stage, n (%)			**0.011**
T1	106 (28.6%)	77 (20.8%)	
T2	43 (11.6%)	52 (14%)	
T3	30 (8.1%)	50 (13.5%)	
Pathologic N stage, n (%)			1
N0	119 (46.1%)	135 (52.3%)	
N1	2 (0.8%)	2 (0.8%)	
Pathologic M stage, n (%)			0.71
M0	126 (46.3%)	142 (52.2%)	
M1	1 (0.4%)	3 (1.1%)	
Pathologic stage, n (%)			**0.026**
Stage I	99 (28.3%)	74 (21.1%)	
Stage II	41 (11.7%)	46 (13.1%)	
Stage III	32 (9.1%)	53 (15.1%)	
Tumor status, n (%)			**0.004**
Tumor free	115 (32.4%)	87 (24.5%)	
With tumor	64 (18%)	89 (25.1%)	
Gender, n (%)			0.32
Female	56 (15%)	65 (17.4%)	
Male	131 (35%)	122 (32.6%)	
Race, n (%)			0.451
Asian	76 (21%)	84 (23.2%)	
Black or African American&White	104 (28.7%)	98 (27.1%)	
Age, n (%)			0.326
<= 60	84 (22.5%)	93 (24.9%)	
> 60	103 (27.6%)	93 (24.9%)	
Weight, n (%)			**0.006**
<= 70	83 (24%)	101 (29.2%)	
> 70	97 (28%)	65 (18.8%)	
Height, n (%)			0.066
< 170	96 (28.2%)	105 (30.8%)	
>= 170	81 (23.8%)	59 (17.3%)	
BMI, n (%)			**0.012**
<= 25	81 (24%)	96 (28.5%)	
> 25	95 (28.2%)	65 (19.3%)	

Table3. Clinical characteristics of HCC patients with low and high RAC1 expression levels

characteristics	Low expression of RAC1	High expression of RAC1	p-value
Residual tumor, n (%)			0.176
R0	169 (49%)	158 (45.8%)	
R1	6 (1.7%)	11 (3.2%)	
R2	0 (0%)	1 (0.3%)	
Histologic grade, n (%)			**0.001**
G1	41 (11.1%)	14 (3.8%)	
G2	97 (26.3%)	81 (22%)	
G3	43 (11.7%)	81 (22%)	
G4	4 (1.1%)	8 (2.2%)	
AFP (ng/ml), n (%)			**0.001**
<= 400	127 (45.4%)	88 (31.4%)	
> 400	21 (7.5%)	44 (15.7%)	
Albumin(g/dl), n (%)			0.53
< 3.5	40 (13.3%)	29 (9.7%)	
>= 3.5	124 (41.3%)	107 (35.7%)	
Prothrombin time, n (%)			0.131
<= 4	104 (35%)	104 (35%)	
> 4	53 (17.8%)	36 (12.1%)	
Child-Pugh grade, n (%)			0.73
A	121 (50.2%)	98 (40.7%)	
B&C	13 (5.4%)	9 (3.7%)	
Fibrosis ishak score, n (%)			0.101
0	45 (20.9%)	30 (14%)	
1/2	14 (6.5%)	17 (7.9%)	
3/4	14 (6.5%)	14 (6.5%)	
5	2 (0.9%)	7 (3.3%)	
6	45 (20.9%)	27 (12.6%)	
Vascular invasion, n (%)			0.068
No	115 (36.2%)	93 (29.2%)	
Yes	49 (15.4%)	61 (19.2%)	
Adjacent hepatic tissue inflammation, n (%)			0.1
None	72 (30.4%)	46 (19.4%)	
Mild	47 (19.8%)	54 (22.8%)	
Severe	10 (4.2%)	8 (3.4%)	

Table 4. Cox regression analysis the association between clinical characteristics including the expression of RAC1 and OS in HCC patient.

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age	373	1.205 (0.850 - 1.708)	0.295		
<= 60	177				
> 60	196				
Gender	373	0.793 (0.557 - 1.130)	0.200		
Female	121				
Male	252				
Race	361	1.341 (0.926 - 1.942)	0.121		
Asian	159				
Black or African American&White	202				
Pathologic stage	349	2.090 (1.429 - 3.055)	**< 0.001**	1.792 (1.198 - 2.682)	**0.005**
Stage I	173				
Stage II&Stage III&Stage IV	176				
Residual tumor	344	1.604 (0.812 - 3.169)	0.174		
R0	326				
R1&R2	18				
AFP (ng/ml)	279	1.075 (0.658 - 1.759)	0.772		
<= 400	215				
> 400	64				
Child-Pugh grade	240	1.643 (0.811 - 3.330)	0.168		
A	218				

B&C	22				
Fibrosis ishak score	214	0.740 (0.445 - 1.232)	0.247		
0&1/2	106				
3/4&5&6	108				
Vascular invasion	317				
No	208	1.344 (0.887 - 2.035)	0.163		
Yes	109				
Tumor status	354	2.317 (1.590 - 3.376)	**< 0.001**	1.845 (1.236 - 2.754)	**0.003**
Tumor free	202				
With tumor	152				
RAC1	373	1.977 (1.384 - 2.824)	**< 0.001**	1.756 (1.183 - 2.608)	**0.005**
Low	187				
High	186				

3.6 IHC analysis revealed higher RAC1 expression in HCC tissues compared to healthy ones

To corroborate the findings obtained through the preceding bioinformatics analyses, we proceeded with the validation of RAC1 protein expression. Utilizing immunohistochemistry (IHC) techniques, we meticulously assessed the quantitative profiles of RAC1 protein expression within the collected HCC tissues and their corresponding adjacent non-cancerous tissues. This quantitative analysis was further enhanced through the application of advanced image pro-plus analysis software. This approach firmly confirmed that the expression of RAC1

protein within HCC tissues surpassed that within the peri-cancerous tissues **(Fig. 13 A)**. Notably, this observation closely aligned with the outcomes derived from the aforementioned bioinformatics analyses. The analysis was conducted utilizing a two-sample t-test, which subsequently revealed a statistically significant difference in protein expression levels **(Fig. 13 B)**. This robust statistical validation unequivocally affirmed the significant elevation of RAC1 protein expression in HCC tissues compared to the adjacent peri-cancerous tissues.

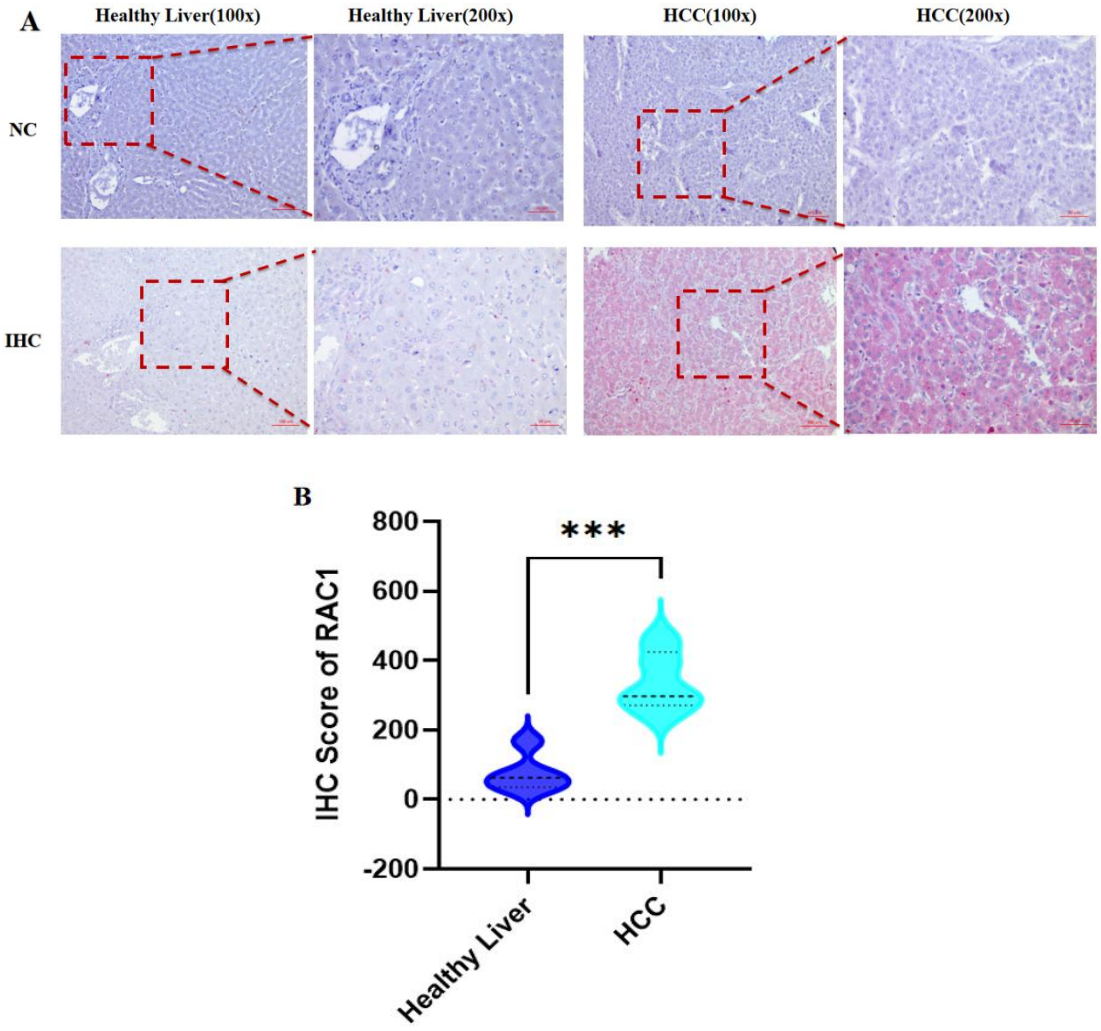


Figure 13: RAC1 was significantly upregulated in HCC.

(A) Histological evaluation of RAC1 expression by IHC staining. Top, negative control staining (NC); bottom, IHC staining. (B) Histogram showing a higher level of RAC1 expression in HCC. *** $p < 0.005$.

3.7 Tigecycline influences RAC1 RNA and protein expression within HCC cells

RAC1, a member of the Rho GTPase family, has been established as one of the potential target proteins of tigecycline in the context of HCC. In order to assess the effects of tigecycline, we initially utilized PCR to analyze the mRNA expression of RAC1 in Huh7 and HepG2 cells treated with varying concentrations of tigecycline for a duration of 48 hours. Notably, upon exposure to 10 μM and 20 μM concentrations of tigecycline, both HCC cell lines displayed statistically significant increases in RAC1 mRNA expression **(Fig. 14A and B)**. Subsequently, to evaluate the impact of tigecycline treatment on RAC1 protein expression, WB analysis was conducted on both Huh7 and HepG2 cell lines following exposure to 10 μM and 20 μM tigecycline concentrations for 48 hours. The outcomes unveiled a noteworthy augmentation of RAC1 protein expression in HepG2 cells treated with 20 μM tigecycline, However, in HepG2 cells treated with 10 μM tigecycline, the expression of RAC1 protein did not reach statistical significance (**Fig. 14C and E)**. It is noteworthy that, in the Huh7 cell line, while PCR results indicated a significant increase in RAC1 expression in HCC cells treated with

tigecycline, this increase was not statistically significant in WB analysis **(Fig. 14C and D)**.

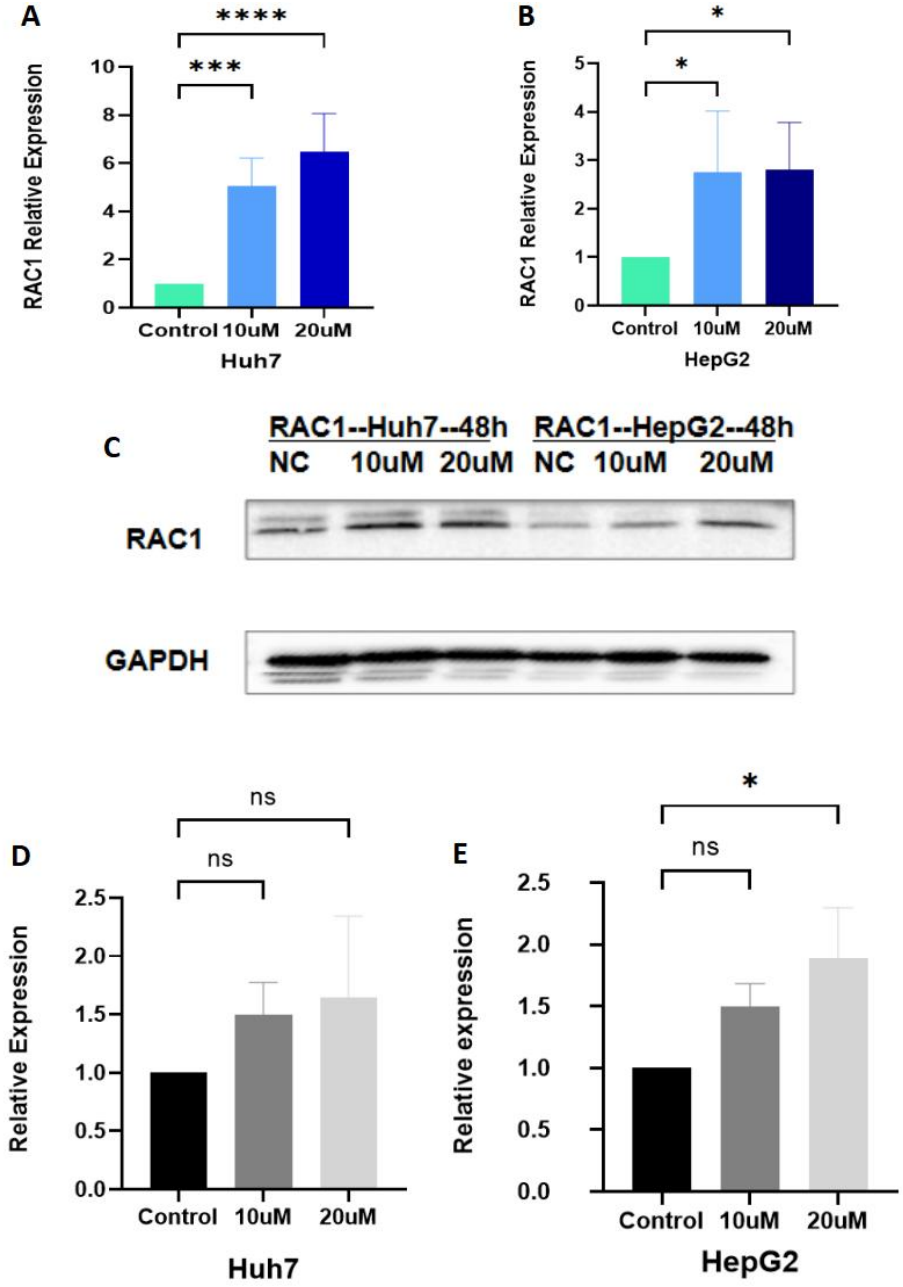


Figure 14: Expression of RAC1 mRNA and protein in HCC cells treated with Tigecycline.

RT-PCR was used to assess the mRNA expression of RAC1 in Huh7 (A) and HepG2 (B) post-treatment with 10 μM and 20 μM Tigecycline for a duration of 48 hours. analysis was conducted to investigate the protein expression of RAC1 in both Huh7 and HepG2 cells after exposure to 10 μM and 20 μM

tigecycline concentrations for 48 hours (C, D and E).

3.8 siRNA was used to downregulate the expression of the target gene RAC1

In pursuit of a more profound understanding of the functional implications of RAC1 in HCC cell growth, our strategy involves the precise targeting of the RAC1 gene using small interfering RNA (siRNA) within the confines of these two distinct HCC cells. Our overarching objective is to compare the alterations in cellular function and phenotype after RAC1 knockdown with those in the control group. As depicted in **Fig. 15A and B**, We conducted a rigorous analysis utilizing RT-qPCR to quantify the mRNA expression levels of RAC1 in two HCC cell lines, subsequent to siRNA intervention for 24 and 48 hours. Our findings reveal a statistically significant reduction in RAC1 mRNA expression in both cell lines within the knockdown group, with the most pronounced decrease observed at the 48-hour timepoint. To further corroborate our findings at the protein level, we conducted a meticulous Western blot (WB) analysis to evaluate RAC1 protein expression in two HCC cell lines, subsequent to 48 and 72 hours of siRNA intervention. The outcomes unveiled significant downregulation of RAC1 protein in both cell lines within the knockdown group, with a particularly striking reduction evident in the HepG2 cell line **(Fig. 15C, D and E)**. These findings lay a sturdy groundwork for our further investigations.

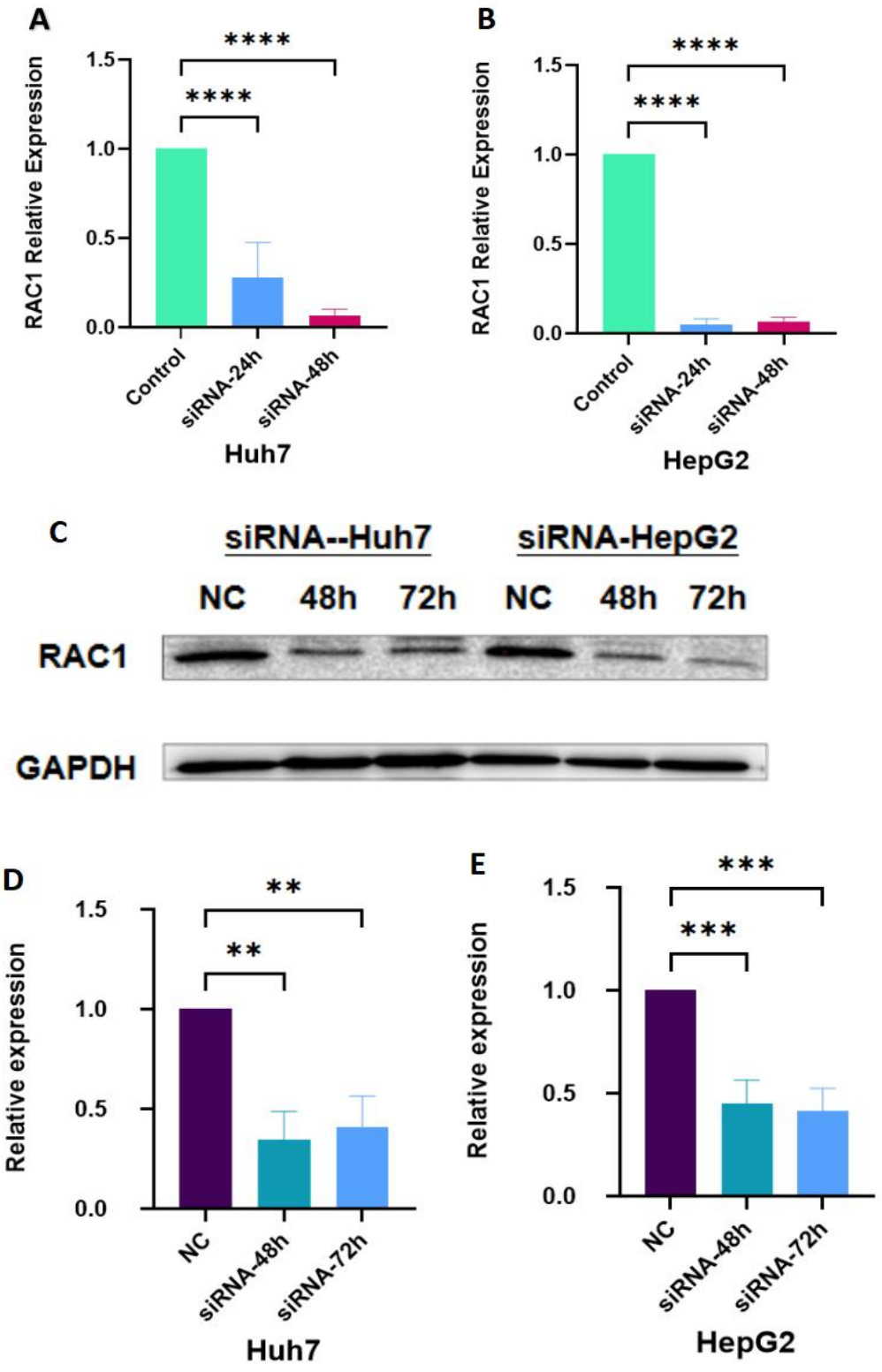
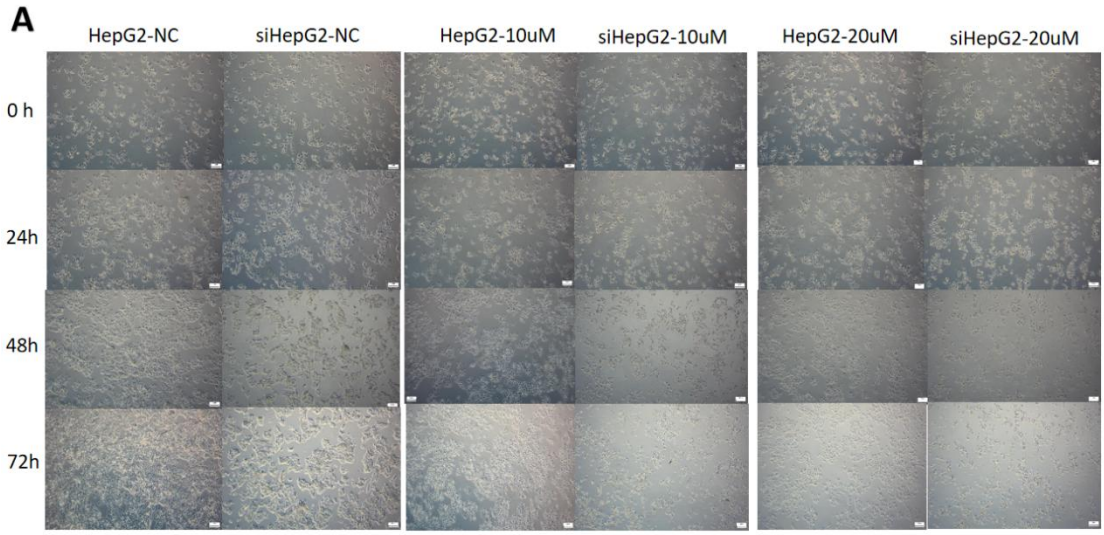
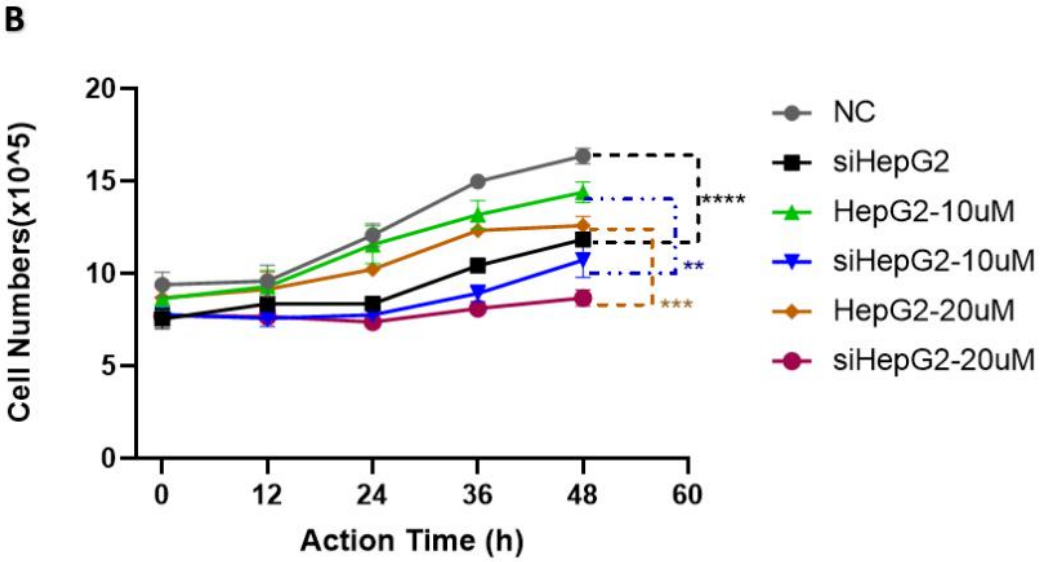


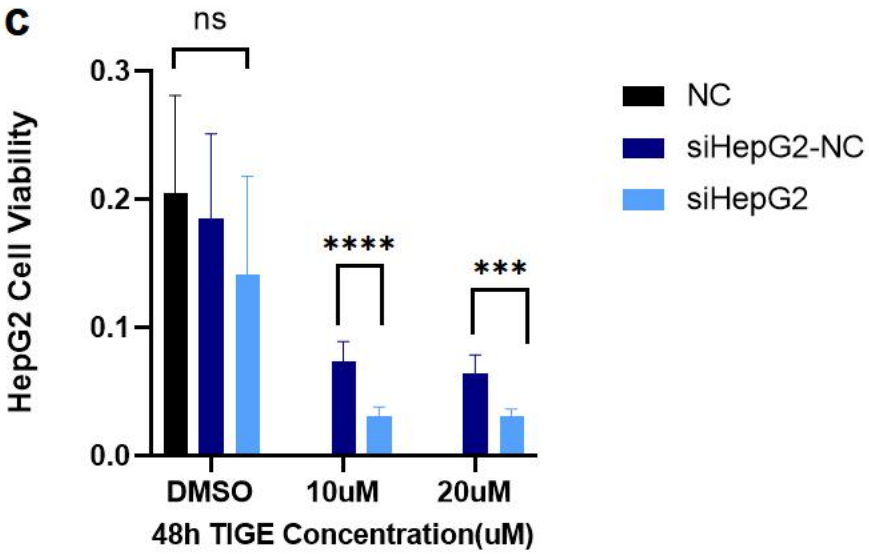
Figure 15: siRNA successfully knocked down RAC1 in two kinds of cells. The RT-PCR test results showed that RAC1 was successfully knocked down in the two cells (A and B). The results of the WB assay confirmed the successful knockdown of RAC1 in both cell lines (C, D and E).

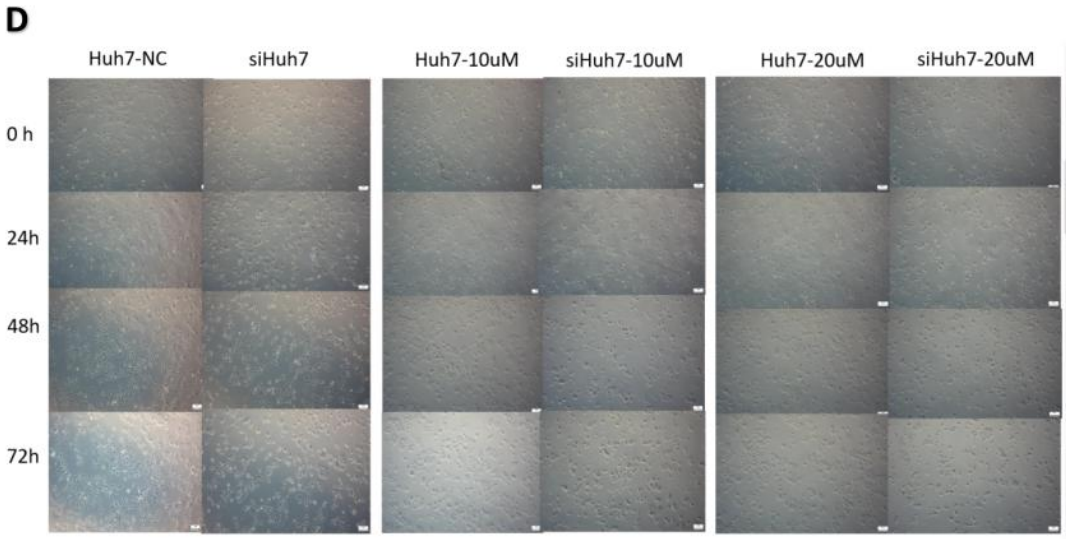
3.9 RAC1 Knockdown reduces Proliferation and Viability of HCC Cells

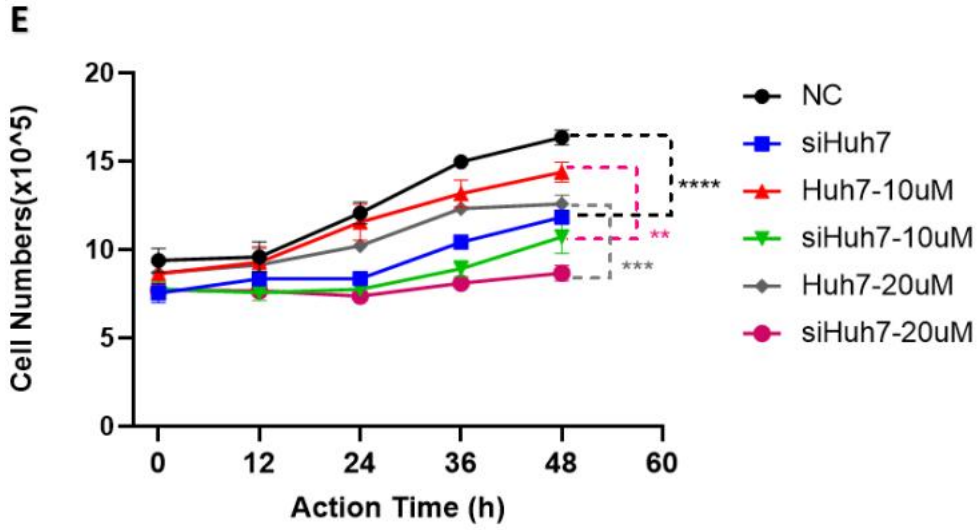
In our endeavor to decipher the influence of RAC1 on the proliferation and cell viability of HCC cells, we conducted observation and cell counting analyses at distinct time points following various treatments. Our research findings underscore that HCC cells exhibiting reduced RAC1 expression levels demonstrate a markedly lower cell density over time, in comparison to their counterparts with normal RAC1 expression. This trend intensifies significantly when the combination of low RAC1 expression is accompanied by tigecycline treatment **(Fig. 16 A and D)**. Subsequent cell counting analyses conducted at various time points reinforced the aforementioned observations, unequivocally demonstrating that HCC cells with diminished RAC1 expression exhibited significantly attenuated proliferative capacities in comparison to those maintaining normal RAC1 expression levels **(Fig. 16 B and E)**. Additionally, we utilized MTT assays to assess the activity of HCC cells. The results of our research underscored that the combination of decreased RAC1 expression and tigecycline treatment synergistically led to a substantial reduction in the activity of HCC cells **(Fig. 16 C and F)**. These findings offer novel insights into the pivotal role of RAC1 in the proliferation and cellular activity of HCC cells, potentially furnishing valuable information for the development of therapeutic strategies against HCC.


A
HepG2-NC
siHepG2-NC
HepG2-10uM
siHepG2-10uM
HepG2-20uM
siHepG2-20uM
0 h
24h
48h
72h


B
Cell Numbers(x10^5)
Action Time (h)
NC
siHepG2
HepG2-10uM
siHepG2-10uM
HepG2-20uM
siHepG2-20uM
****
**
***


C
HepG2 Cell Viability
48h TIGE Concentration(uM)
ns
****
***
DMSO
10uM
20uM
NC
siHepG2-NC
siHepG2


D
Huh7-NC
siHuh7
Huh7-10uM
siHuh7-10uM
Huh7-20uM
siHuh7-20uM
0 h
24h
48h
72h


E
Cell Numbers(x10^5)
20
15
10
5
0
0
12
24
36
48
60
Action Time (h)
NC
siHuh7
Huh7-10uM
siHuh7-10uM
Huh7-20uM
siHuh7-20uM
****
**
***

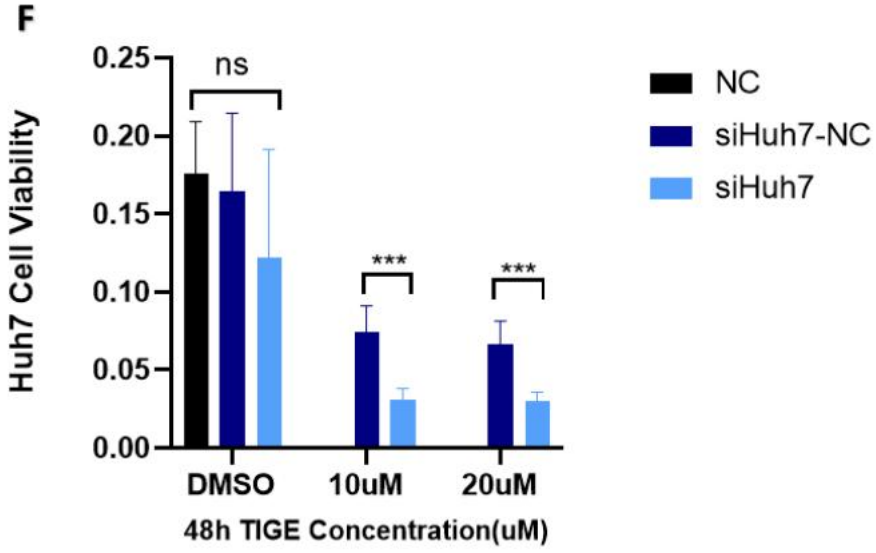

F
Huh7 Cell Viability
0.25
0.20
0.15
0.10
0.05
0.00
ns
***
***
DMSO
10uM
20uM
48h TIGE Concentration(uM)
NC
siHuh7-NC
siHuh7

Figure16: The Influence of RAC1 Knockdown on the Proliferation and Viability of HCC Cells.

Growth Status of HepG2(A) and Huh7(D) Cells Under Different Treatments at Various Time Points. Cell Counts of HepG2 (B) and Huh7(E) Cells Treated with Different Methods at Various Time Points. HepG2(C) and Huh7(F) Cells Treated with different experimental conditions for 48 hours, Followed by Cell Viability Assessment Using MTT Assay.

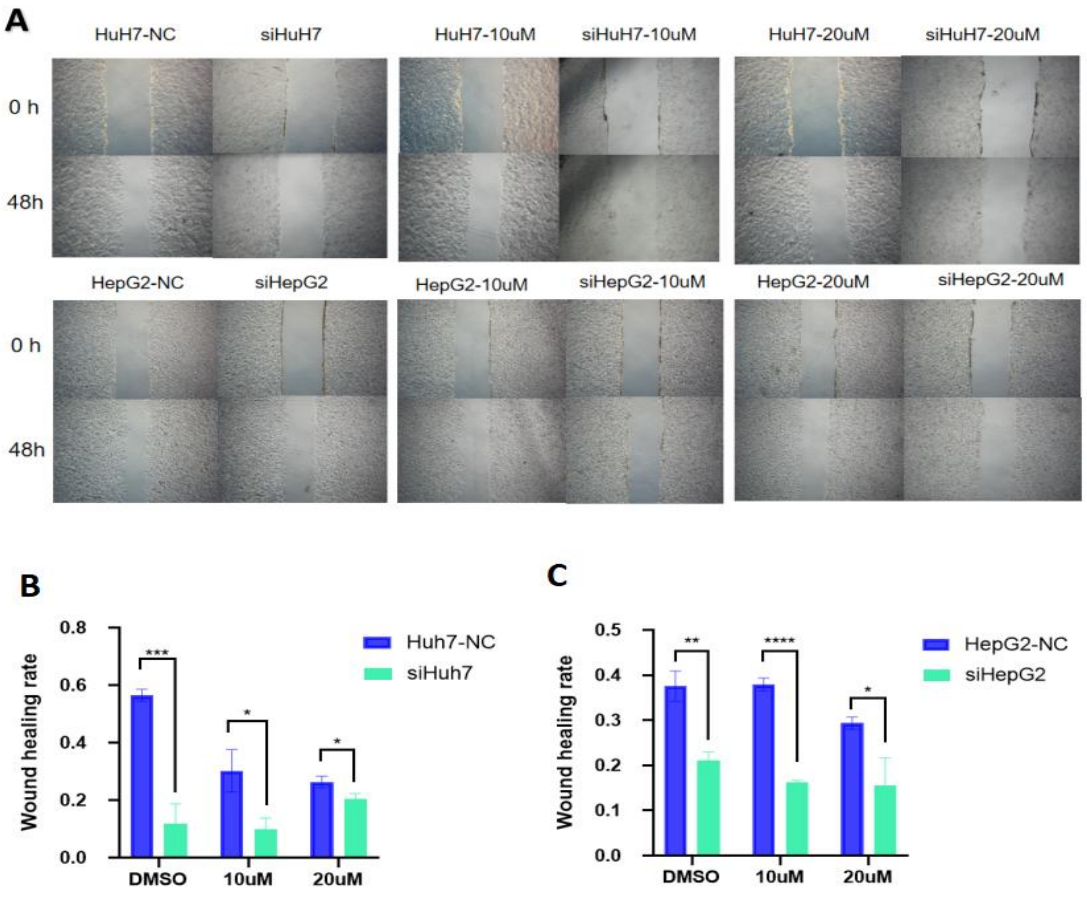
NC: normal HCC cells. siHuh7-NC and siHepG2-NC: The cells treated with siRNA negative control. siHuh7 and siHepG2: The cells treated with functional siRNA. MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide.

3.10 RAC1 Knockdown reduces Migration and Invasion of HCC Cells

To comprehensively elucidate the ramifications of RAC1 on the migratory and invasive capabilities of HCC cells, we embarked on a scratch assay, meticulously assessing their migratory potential under varying conditions. At the inception of the experiment, we rigorously documented the formation of precise and well-defined scratches, serving as the baseline time point. Subsequently, we scrutinized the closure dynamics of these scratches after a 48-hour incubation period. The resulting observations revealed a distinct trend, wherein HCC cells with reduced RAC1 expression exhibited a significantly diminished rate of scratch closure, as compared to those with normal RAC1 expression. Notably, this effect was intensified when the reduced RAC1 expression was combined with tigecycline treatment, reinforcing the intricate

relationship between RAC1 levels and migratory capabilities in HCC cells **(Fig. 17 A, B and C)**.

To corroborate these observations, we utilized Transwell assays. The research outcomes distinctly illustrated that HCC cells harboring lower RAC1 expression exhibited a marked reduction in migratory and invasive capabilities relative to those with normal RAC1 expression. The most substantial decline in cell migration and invasion capacities was observed when low RAC1 expression was coupled with tigecycline treatment **(Fig. 17 D-I)**. These research findings underscore the profound influence of RAC1 expression levels on the migratory and invasive propensities of HCC cells, particularly in the context of tigecycline treatment.



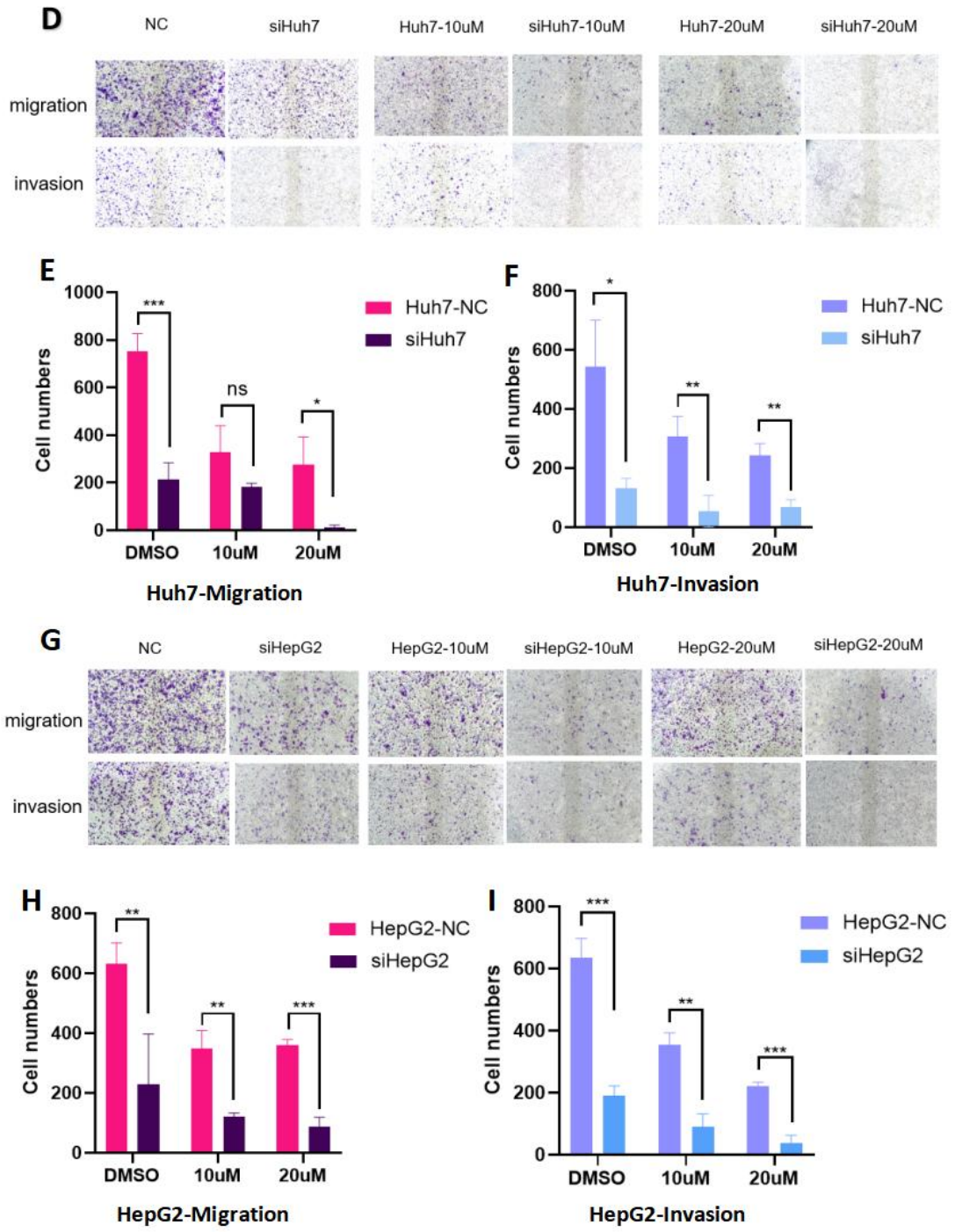


Figure17: Impact of RAC1 Knockdown on Migration and Invasion of HCC Cells. The Wound Healing assay was utilized to assess the migratory capacity in Huh7 (A, B) and HepG2 (A, C). Huh7 (D-F) and HepG2 (G-I) cells were treated with different methods, and migration and invasion were assessed using the Transwell assay. siHuh7 and siHepG2: The cells treated with functional siRNA.

4. Discussion

HCC presents a formidable global health challenge, standing as one of the most lethal cancers on a global scale. The constraints of limited therapeutic options, coupled with the intricate biology characterizing this malignancy, underscore the imperative for innovative interventions[2, 5]. In recent years, Tigecycline, originally an antibiotic, has arisen as a promising candidate for HCC treatment, thanks to its unanticipated anticancer attributes, which have ignited profound scientific interest[109, 110, 119]. Our comprehensive study delves into the intricate molecular pathways that underlie Tigecycline's inhibitory effects on HCC cells. Particular emphasis is placed on elucidating the pivotal role enacted by the Ras-related C3 botulinum toxin substrate 1 (Rac1) protein. Utilizing a multifaceted methodology, our approach amalgamates bioinformatics analyses, experimental assays, and clinical correlations. This integrative strategy is designed to furnish a comprehensive comprehension of the intricate interplay among Tigecycline, RAC1, and HCC, offering a more holistic perspective.

4.1 Tigecycline's Repositioning: A Paradigm Shift in Cancer Therapy

The repositioning of Tigecycline as an anticancer agent has garnered significant attention, owing to its unique mechanisms of action that transcend its conventional role as an antibiotic[107, 108, 127, 128]. Our

research strategically harnesses this potential, embarking on an in-depth exploration of the ramifications of Tigecycline on HCC cells. Remarkably, our investigation underscores Tigecycline's remarkable effectiveness in curtailing the vitality of HCC cells. This assertion is firmly substantiated through MTT assays, which unequivocally demonstrate Tigecycline's capacity to significantly diminish the metabolic activity of HCC cells. This inhibitory effect is particularly conspicuous in instances characterized by diminished RAC1 expression levels. Moreover, cell counting analyses furnished supplementary substantiation of tigecycline's robust capacity to proficiently hinder the proliferative propensity of these cells in instances where RAC1 expression is diminished.

Subsequently, the outcomes derived from our wound healing and Transwell assays distinctly and vividly portray that HCC cells, featuring reduced RAC1 expression, demonstrate a marked reduction in both migratory and invasive capabilities when juxtaposed with their counterparts displaying normal RAC1 expression levels. These collective findings not only bolster the contention that Tigecycline harbors substantial promise as a therapeutic modality against HCC but also corroborate prior research, which has postulated its potential efficacy across a spectrum of cancer types. These findings bolster the contention that Tigecycline harbors substantial promise as a therapeutic modality

against HCC, consonant with earlier research positing its potential efficacy in diverse cancer types [110, 123, 127, 129-132].

While previous investigations have predominantly delved into Tigecycline's antibiotic attributes[133-137], our research strategically redefines its role as a prospective anticancer agent. In this context, our study signifies a significant advancement in deciphering the intricate molecular mechanisms underpinning the relentless progression of HCC. Our findings not only reveal the multifaceted impact of Tigecycline but also underscore its synergy with RAC1 expression levels, thereby offering novel and groundbreaking insights into the therapeutic landscape of HCC. Tigecycline's distinctive mechanism of action transcends its conventional antibiotic properties, unveiling a new frontier in the realm of targeted cancer therapeutics. This transformative shift in Tigecycline's characterization positions it as an exceptionally compelling candidate warranting in-depth exploration within clinical settings. Consequently, it has the potential to revolutionize the existing paradigms of HCC treatment, ushering in a promising era of innovative therapeutic strategies.

4.2 RAC1: The Superstar Molecule

Cellular processes, encompassing a spectrum from cell migration and polarity to proliferation and apoptosis, are meticulously choreographed through intricate interactions within a labyrinthine network of signaling

pathways. In this intricate milieu, RAC1, a distinguished member of the Rho GTPase family, has ascended to prominence as a masterful regulator, bearing multifaceted responsibilities across both physiological and pathological domains[138]. The participation of RAC1 in diverse cellular processes accentuates its paramount importance in fundamental biological functions. Yet, its intricate involvements in tumorigenesis and other pathological conditions have recently garnered significant attention and scrutiny.

4.2.1 RAC1 Characteristics and Function

Rho GTPases are a subset of the Ras superfamily of GTP-binding proteins, characterized by their molecular weight ranging from 20 to 30 kilodaltons (kDa). These GTPases have been extensively documented as regulators of a myriad of intricate cellular functions. These functions encompass a spectrum of critical processes, including the intricate reorganization of the cell's cytoskeletal framework, the dynamic orchestration of membrane trafficking events, the fine-tuning of transcriptional processes, and the precise control over cellular growth dynamics[139-142]. Nestled within this intriguing GTPase family, RAC1, a compact GTPase protein, takes center stage. Encoded by a gene nestled on the 7th chromosome locus (7p22), the RAC1 gene is composed of 7 exons and 3 introns, stretching over a span of approximately 29 kilobases (kb). The presence of RAC1 is strategically dispersed among discrete

subcellular compartments, encompassing the plasma membrane, the nucleus, and the mitochondria. The products transcribed from the Rac1 gene yield two distinct isoforms, sized at 1.2 kb and 2.5 kb, each selectively expressed in tissues showcasing distinct tissue-specific distribution patterns[143-147].

The functionality of RAC1 hinges upon its dynamic interplay between its active state, firmly tethered to GTP, and its quiescent state, locked in association with GDP. This nuanced equilibrium is meticulously governed by a cadre of cellular regulators, including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) [148-150]. Embracing its role as an irreplaceable cog in the cellular machinery, RAC1 emerges as a linchpin orchestrator in an array of pivotal cellular processes. Its versatile contributions encompass a broad spectrum, from steering cellular motility to masterminding the intricate reorganization of the actin cytoskeleton[142-144, 149, 150]. Furthermore, RAC1 wields significant influence over the transformation of cells towards a malignant phenotype, facilitating invasive metastasis and exerting control over the activity of transcription factors[138, 151]. Notably, RAC1 plays a decisive role in the delicate balance of cellular survival and demise, impacting cellular apoptosis[138, 149]. Beyond these spheres, RAC1's reach extends to the realm of tumor angiogenesis, sculpting the vasculature that nourishes

tumors[138, 148, 149, 151]. Through its multifaceted involvements, RAC1 emerges as an architectural cornerstone in the intricate landscape of cellular dynamics, intricately interwoven with the tapestry of tumor biology.

4.2.2 The Significance of RAC1 in Cancers

RAC1, a constituent of the Rho GTPases family, emerges as a pivotal contributor with multifaceted roles within the domain of tumor biology. Its impact resonates significantly across pivotal junctures of tumor progression, encompassing crucial facets such as malignant transformation, invasion, and metastasis. Orchestrating intricate mechanistic cascades, RAC1 spans multiple essential domains, furnishing pivotal regulatory cues that span the diverse landscape of tumor development[138, 148, 151, 152].

Foremost, RAC1 assumes a commanding role in orchestrating tumor cell invasion and metastasis[153-156]. Inquisitive investigations have shed light on its pivotal role in orchestrating tumor cell migration and invasion. This is accomplished through the dynamic reorganization of the cytoskeleton, driven by its activation, which subsequently induces alterations in cell adhesion dynamics. Furthermore, RAC1 intricately interfaces with the complex extracellular matrix, orchestrating processes of degradation and dynamically shaping the tumor microenvironment, thus assuming a central role in the intricate ballet of tumor invasion and

metastasis. Simultaneously, RAC1 is intricately entwined with the mechanisms governing tumor cell proliferation and growth[138, 152, 154, 157]. By virtuously engaging with cell growth signaling pathways, RAC1 propels the accelerated proliferation and growth of tumor cells, thus impelling the onward trajectory of tumorigenesis. Scientific exploration underscores the intimate synergy between aberrant RAC1 activation and the intricate processes of malignant transformation and robust proliferation, fostering an ambiance primed for unrestrained expansion of malignant cells.

Furthermore, RAC1 adopts a pivotal mantle in sculpting the contours of the tumor microenvironment[138, 152, 158, 159]. This microenvironment embodies an intricate tapestry, woven with a multitude of components spanning cells, matrix constituents, and a symphony of signaling molecules. Its formidable sway over tumor development and metastasis is substantiated by empirical inquiry, delineating RAC1's regulatory dominion over processes like angiogenesis, immune evasiveness, and the complex interplay of inflammatory responses. Hence, within the context of sculpting the trajectory of tumor evolution, RAC1 occupies an indispensable nexus.

RAC1 reveals important functions in a variety of tumor types, and its mechanism of action is tightly linked to tumor origin, progression, and response to therapy. In the realm of breast cancer research, investigations

have elucidated the critical role of RAC1 in orchestrating tumor cell invasion and migration [160-163]. By regulating cytoskeleton remodeling and cell adhesion, RAC1 drives breast cancer cells to penetrate the basement membrane and integrate into the surrounding tissues. In addition, RAC1 is intricately entwined with the processes of breast cancer cell proliferation and growth as well as tumor angiogenesis, thereby actively contributing to the relentless progression of breast cancer[162, 164, 165]. In colorectal cancer, studies have revealed the important role of RAC1 in tumor cell migration and infiltration. Aberrantly heightened RAC1 activation intricately correlates with the histological grade and prognostic outcomes of colorectal carcinoma, Elevated RAC1 expression levels are typically associated with increased tumor infiltration, a higher propensity for metastasis, and a poorer prognosis in colorectal cancer patients[166-168]. In congruence with numerous malignancies, melanoma follows a similar pattern, extensive research has elucidated the pivotal role of RAC1 in mediating the infiltration and metastatic cascade of this particular tumor subtype. RAC1 has a substantial impact on the capacity of melanomas to disseminate and migrate, primarily by governing the motion and migration of melanoma cells[169-171]. As for prostate cancer, RAC1 plays a key role in promoting tumor cell infiltration and bone metastasis. Research has elucidated that activated RAC1 can facilitate the homing of prostate cancer cells to bone tissue,

establishing a significant association with the genesis of bone metastases[172-175]. In synthesis, RAC1 assumes a pivotal role across various malignancies, exerting its influence through multiple critical cellular processes, encompassing cell motility, infiltration, proliferation, growth, and angiogenesis[176-181]. A comprehensive exploration of RAC1's regulatory mechanisms in diverse malignancies holds the promise of furnishing invaluable insights for the formulation of precision anti-neoplastic therapeutic approaches.

4.2.3 RAC1: Orchestrating HCC Progression

In the intricate context of HCC, an ailment notorious for its profound clinical challenges, the conspicuous surge in RAC1 expression levels has consistently correlated with HCC aggressiveness and, consequentially, an unfavorable prognosis[182-186]. Our study explores the effects of Tigecycline, originally an antibiotic, on RAC1 in HCC cells. This repositioning of Tigecycline as a potential modulator of RAC1 in cancer cells represents a novel approach to targeting RAC1's activity.

Our meticulous bioinformatics analyses meticulously align with these empirical observations, unveiling a conspicuous and statistically significant upsurge in RAC1 expression within the microenvironment of HCC tissues, in stark juxtaposition to their normal counterparts. This discernible phenomenon serves to firmly substantiate RAC1's pivotal role as a prospective therapeutic target in the combat against HCC.

Additionally, our astute scrutiny has unveiled a noteworthy and statistically validated reduction in the promoter methylation status of RAC1 within the confines of tumor tissues, similar reports from other researchers[181, 187-191]. This compelling epigenetic anomaly, analogous to an intricate fingerprint, adds a profound layer of sophistication to our comprehension of the precise orchestration of RAC1 within the intricate tapestry of the HCC landscape.

Furthermore, the unearthing of this distinct epigenetic signature has prompted contemplation regarding the pragmatic feasibility of therapeutic interventions strategically aimed at the nuanced realm of epigenetic modifications[192-194]. Such strategic endeavors hold promising potential in ameliorating and recalibrating RAC1's substantial influence and sway within the intricate realm of HCC, potentially offering novel therapeutic avenues in our pursuit of conquering this formidable disease.

4.3 Tigecycline's Enigmatic Dance with RAC1

The intricate interplay between Tigecycline and RAC1, as unveiled in our research, introduces a stratum of intricacy into our comprehension of the modus operandi of this antibiotic-cum-potential-anticancer-agent. Certainly, one of the most intriguing facets of our study lies in the paradoxical relationship that emerges between Tigecycline and RAC1. In stark contradiction to what conventional wisdom might have postulated, the exposure of HCC cells to Tigecycline engenders a noteworthy

upsurge in both RAC1 mRNA and protein levels[190, 195-199]. This enigmatic outcome challenges the established paradigm and engenders a series of pivotal inquiries.

Does Tigecycline exert a direct influence on RAC1 expression, or does it serve as the catalyst for an intricate cascade of compensatory mechanisms and signaling pathways that culminate in the observed augmentation of RAC1 expression? Deciphering the exact mechanisms that underlie this paradoxical phenomenon stands as an imperative undertaking, pivotal for harnessing the full therapeutic potential inherent to Tigecycline within the realm of cancer therapy. It is plausible that Tigecycline's primary mode of action transcends the domain of direct RAC1 inhibition, involving an intricate labyrinth of interactions intrinsic to HCC cells.

This intricate interplay beckons as a riveting avenue for further exploration, as it could potentially hold broader ramifications for the incorporation of Tigecycline within the arsenal of cancer therapeutics.

4.4 RAC1 as a Mediator of Tigecycline's Effects

In pursuit of a more profound comprehension of the functional significance of RAC1 in HCC, we utilized small interfering RNA (siRNA) to specifically attenuate RAC1 expression within HCC cells. Our empirical outcomes unequivocally affirm that RAC1 knockdown exerts a significant detriment to the proliferative capabilities inherent to HCC

cells. Moreover, it amplifies the susceptibility of HCC cells to Tigecycline, shedding a luminous spotlight on the potential synergy that exists between RAC1 inhibition and Tigecycline treatment. Parallel observations have emerged in the field of breast cancer investigations, wherein the inhibition of RAC1 demonstrates an augmentation in the chemosensitivity of docetaxel, doxorubicin, and cisplatin. As a result, this heightened chemosensitivity precipitates a decline in the proliferation rate and an upsurge in apoptosis within breast cancer cell populations[163].

These observations resoundingly accentuate the therapeutic promise encapsulated in targeting RAC1 within the context of HCC. They also propose a cogent mechanism through which Tigecycline might enact its therapeutic effects.

4.5 Implications for Clinical Management

The implications of our findings for clinical management are both striking and promising. Through rigorous clinical correlations, we have unearthed compelling associations between RAC1 expression levels and a spectrum of critical clinical parameters in HCC patients. These encompass pivotal factors such as T stage, Pathologic stage, Tumor status, Weight, BMI, and AFP levels. However, the most noteworthy revelation from our study is the emergence of RAC1 as an independent prognostic risk factor for overall survival. This particular discovery underscores the clinical significance of our research. RAC1's newfound role as an

independent prognostic indicator implies its potential as a powerful tool in the clinical armamentarium for assessing the outcomes of HCC patients, other investigators have substantiated these observations in alternative malignancies[200-204].

4.6 The Immunologic Microenvironment in HCC

HCC presents an imposing conundrum within the oncological domain, distinguished not solely by its multifarious molecular substrates but also by its intricately woven immunological microcosm. This dynamic ecosystem assumes a paramount role in orchestrating malady advancement, therapeutic responsiveness, and patient prognoses[205-208]. To gain a comprehensive understanding of this complex milieu, our exploration transcends the boundaries of cancer cells and extends into the complex terrain of the immune microenvironment within HCC. Utilizing advanced bioinformatics tools, we embark on a journey to decipher the intricate relationship between RAC1 expression and the infiltration of immune cells within HCC tissues. The outcomes of this endeavor paint a vivid picture of how RAC1 expression profiles correlate with distinct immune profiles within HCC patients. High RAC1 expression is associated with an immune landscape characterized by elevated levels of specific immune cell subtypes, including immature dendritic cells (iDC), macrophages, effector memory T cells (Tem), T follicular helper cells (TFH), and T helper 2 cells (Th2). In stark contrast,

low RAC1 expression heralds increased infiltration by cytotoxic cells, dendritic cells (DC), eosinophils, neutrophils, gamma delta T cells (Tgd), T helper 17 cells (Th17), and regulatory T cells (Treg).

These revelations illuminate the intricate interplay between RAC1 and the immune landscape within HCC, hinting that targeting RAC1 may not only hinder tumor progression but also have far-reaching implications in reshaping the immune microenvironment, potentially rendering immunotherapies more effective. This substantiates the pivotal role of RAC1 in shaping the landscape of the tumor microenvironment[138, 152, 158, 159]. Understanding this complex crosstalk between RAC1 and the immune system within HCC is essential for devising more comprehensive and effective therapeutic strategies. It suggests that future treatments could potentially combine RAC1-targeted therapies with immunotherapies to achieve synergistic effects, offering new hope to HCC patients and paving the way for innovative approaches in cancer treatment. This exciting intersection of cancer biology and immunology holds promise for transforming the landscape of HCC therapy.

4.7 Combination Therapies: Forging the Path Ahead

Tigecycline, in conjunction with a myriad of chemotherapeutic agents, exhibits the potential to augment the responsiveness of select hematological malignancies and solid tumors to chemotherapy. Notably, Tigecycline demonstrates synergistic effects when combined with

cisplatin in ovarian cancer and HCC[123, 129]. Additionally, the concomitant administration of Tigecycline and paclitaxel demonstrates significant enhancements in both in vitro and in vivo treatment outcomes for renal cell cancer[130]. In the realm of hematological malignancies, the combined use of Tigecycline with doxorubicin and vincristine exerts a synergistic impact in the context of acute lymphoblastic leukemia[128]. The dual role of RAC1 as both a driver of HCC progression and a mediator of Tigecycline's effects reveals an intriguing landscape of opportunities for synergistic combination therapies. The prospect of co-administering Tigecycline with agents designed for targeted RAC1 inhibition or its integration into immunotherapeutic protocols tantalizingly hints at the potential for achieving synergistic therapeutic effects. These synergies not only portend the promise of elevated treatment efficacy but also present a strategic avenue for circumventing the immunosuppressive milieu characteristic of the HCC microenvironment, thereby potentially amplifying the overall effectiveness of immunotherapies.

The discerned synergy between Tigecycline and RAC1 knockdown in impeding HCC cell proliferation and viability strongly bolsters the viability of embracing a combinatorial therapeutic strategy. The coadministration of Tigecycline with highly specific RAC1 inhibitors holds the potential to significantly augment treatment effectiveness while

mitigating the development of drug resistance. This underscores the necessity for clinical trials to comprehensively investigate these combinatorial strategies. Furthermore, ongoing research endeavors focused on the development of novel RAC1 inhibitors offer promising prospects in this regard[148, 209, 210]. These emerging combination therapies align seamlessly with the prevailing trend in oncology, emphasizing the adoption of precision medicine paradigms that tailor therapeutic strategies to the unique profiles of individual patients, thereby optimizing treatment outcomes.

4.8 Future Directions and Therapeutic Potential

While our study stands as a significant milestone in elucidating the therapeutic potential of Tigecycline in HCC and providing insights into the pivotal role of RAC1, it also underscores the challenges that confront us and the promising avenues for future research that beckon on the horizon:

4.8.1 Biomarker for Prognosis and Therapy Response

The era of precision oncology has ushered in a transformative paradigm, wherein treatment decisions increasingly hinge upon the unique molecular characteristics exhibited by individual tumors. The recognition of RAC1 as an autonomous prognostic determinant in HCC serves to underscore its critical role as a biomarker for patient stratification and prognosis assessment. Moreover, it holds substantial promise as a

dynamic biomarker for real-time therapy response monitoring. With the rapid advancements in the field of liquid biopsy, enabling non-invasive tracking of tumor-specific genetic alterations, a profound opportunity emerges to assess RAC1 expression levels in real-time during the course of treatment. The prospective integration of RAC1 as a biomarker into routine clinical practice bears the potential to empower clinicians to tailor treatment strategies based on individual patient responses, ultimately culminating in enhanced treatment outcomes.

4.8.2 Immunomodulation Strategies

The correlation observed between RAC1 expression and the infiltration of immune cells in HCC accentuates the potential for immunomodulatory effects when targeting RAC1. Further investigation into the immunological consequences of RAC1 inhibition is highly justified. Both preclinical models and clinical trials can delve into how RAC1 inhibition reshapes the tumor immune microenvironment, influences immune checkpoint expression, and modulates responses to immunotherapies. For instance, the prospect of combining RAC1 inhibition with immune checkpoint inhibitors, such as the utilization of anti-PD-1 or anti-CTLA-4 antibodies, holds the promise of augmenting the efficacy of immunotherapies by orchestrating favorable alterations within the immune landscape of the tumor.

4.8.3 Metastasis Suppression

Metastasis continues to pose a formidable challenge in the management of HCC, frequently contributing to treatment resistance and unfavorable prognoses. Emerging evidential indications underscore the pivotal involvement of RAC1 in orchestrating cancer cell migration and invasion, rendering it an alluring focal point for interventions aimed at metastasis prevention. Subsequent investigations should venture into more profound explorations regarding the repercussions of RAC1 inhibition, either in isolation or in concert with Tigecycline, on the intricate processes of metastasis. Preclinical models can adeptly simulate the intricate cascade of events entailed in metastasis, thereby bestowing insights into how these therapeutic modalities might impede the dissemination of cancer cells and their subsequent colonization of distant organs.

4.8.4 Mechanistic Insights

The enigmatic phenomenon of Tigecycline-induced upregulation of RAC1 mandates an exhaustive inquiry into its underlying molecular mechanisms. Attaining a meticulous comprehension of the intricate molecular cascades in operation is imperative. This cognizance carries the potential to unveil additional targets nestled within the signaling pathways subject to Tigecycline's modulation, thereby pinpointing pivotal vulnerabilities that could be strategically exploited for therapeutic interventions.

Moreover, a detailed exploration of the mechanistic intricacies governing Tigecycline's impact on RAC1 may yield invaluable insights into its broader implications within the domain of cancer therapy. It holds the promise of providing a profound comprehension of Tigecycline's relevance and prospective applications in the treatment of diverse cancer types beyond the purview of HCC. Deciphering the molecular underpinnings of Tigecycline's actions on RAC1 augments the prospects of unveiling novel avenues in the realm of pioneering and efficacious cancer therapeutics.

4.8.5 Clinical Translation

The clinical integration of Tigecycline as an anticancer agent requires a thorough examination of its safety and tolerability in cancer patients. While Tigecycline has a well-established safety record as an antibiotic [133-137, 211], its prolonged use and higher doses for oncological purposes may lead to unique side effects or complications. Prospective clinical trials should diligently monitor and comprehensively document any adverse effects, enabling prompt intervention and effective mitigation strategies. This is crucial to ensure that the benefits of Tigecycline outweigh any potential risks in cancer therapeutics.

Furthermore, a repertoire of Rac1-selective inhibitors, encompassing compounds like NSC23766 and EHT 1864, has been formulated with a mandate to selectively target distinct Rac1 binding domains[148, 209,

210]. In the realm of these inhibitors, specific compounds, with MBQ-167 standing out as exceptionally potent, are presently engrossed in the intricacies of preclinical studies intricately designed to target advanced solid tumors. Nevertheless, it is of paramount significance to underscore that the seamless transition of these inhibitors into the clinical arena of cancer therapy is an arduous odyssey riddled with formidable impediments, thereby underscoring the exigency of concerted research endeavors for their efficacious realization in the clinical domain.

Ultimately, the triumph of any therapeutic stratagem hinges upon its tangible repercussions in the lives of patients. Centering healthcare around patients, with a steadfast emphasis on enhancing their quality of life and overall well-being, should serve as a foundational pillar in the comprehensive management of HCC. As promising modalities such as Tigecycline and Rac1 inhibition advance in clinical development, it becomes imperatively incumbent to contemplate not only their clinical effectiveness and safety profiles but also their ramifications on the daily existence of patients. This holistic assessment ought to encompass various facets, including treatment-associated side effects, impacts on psychological welfare, and the socioeconomic burdens shouldered by individuals under treatment regimens. In this context, patient advocacy groups and robust support networks stand as indispensable assets, championing the cause of ensuring that the voices of patients resonate

meaningfully and decisively in the process of conceiving, refining, and implementing innovative therapeutic paradigms.

4.9 Limitations of the work

While our study sheds light on the inhibitory effects of Tigecycline on HCC cell viability via Rac1. However, several limitations must be acknowledged. Firstly, our reliance on specific cells, Huh7 and HepG2, may not fully capture the heterogeneity of HCC, necessitating the inclusion of a broader range of cell models for validation. Secondly, while our in vitro experiments elucidate mechanistic insights, further investigations in in vivo models and clinical trials are imperative for establishing Tigecycline's efficacy and safety as an HCC treatment. Additionally, the intricate molecular mechanisms involved in Tigecycline's actions require more extensive exploration, and the clinical relevance, optimal dosages, and potential biomarkers remain to be determined. Furthermore, expanding the sample size in our bioinformatic analyses and considering data from diverse sources could bolster the robustness of our findings. Lastly, investigating the long-term effects of Tigecycline treatment and its potential synergies with existing therapies is essential for a comprehensive understanding of its role in HCC management. These considerations underscore the need for further research to unlock the full therapeutic potential of Tigecycline in the context of HCC.

5. Conclusion

In conclusion, this comprehensive investigation underscores the therapeutic potential of Tigecycline in HCC by elucidating its multifaceted impacts on cellular viability, proliferation, and the intricate immune microenvironment. It unveils RAC1 as a pivotal orchestrator of Tigecycline's actions and accentuates its clinical significance as both a prognostic indicator and a therapeutic focal point. RAC1, far from a passive bystander, assumes the role of a conductor, intricately directing the progression of HCC through a convoluted network of cellular processes. This research lays the cornerstone for pioneering therapeutic strategies that harness the synergistic potential arising from Tigecycline and RAC1 inhibition in the relentless battle against HCC. As we delve further into the intricacies of this complex interplay, the prospects for augmenting the prognosis and elevating the quality of life for HCC patients appear increasingly auspicious.

6. Reference

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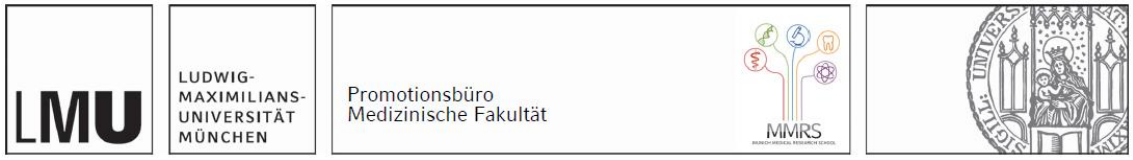
Affidavit



Affidavit

I hereby declare, that the submitted thesis entitled:

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