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Targeting RECQL4 in hepatocellular carcinoma: from prognosis to therapeutic potential

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Abstract

Objective The aim of this study is to assess the clinical utility of RecQ Like Helicase 4 (RECQL4) as a prognostic marker in hepatocellular carcinoma (HCC) and investigate its associations with various biological processes, angiogenesis-related factors, immune cell infiltration, immune checkpoints, and drug sensitivity.

Methods RECQL4 expression was analyzed across a range of cancer types utilizing data from the TCGA database. Disparities in RECQL4 expression levels between normal and malignant tissues were evaluated, alongside an analysis of progression-free interval (PFI), disease-specific survival (DSS), and overall survival (OS) curves. Exploration of pertinent pathways, immune cell infiltration, single-cell RNA-seq data, and drug sensitivity was conducted employing The Cancer Genome Atlas (TCGA) and Tumor Immune Single-Cell Hub (TISCH) databases. Furthermore, validation of in-silico results was validated through qPCR, Western blotting, CCK-8 assay, EdU assay, clonogenic assay, wound-healing assay, and transwell assay.

Results In HCC, RECQL4 was highly expressed and associated with poorer prognosis ($p < 0.05$). It positively correlated with pathways related to MYC targets, DNA replication, PI3K/AKT/mTOR signaling, DNA repair mechanisms, and the G2/M checkpoint ($R > 0.24$, $p < 0.001$). RECQL4 also showed significant correlations with angiogenesis-related genes, including PTK2 ($R > 0.4$, $p < 0.05$), suggesting a potential role in angiogenesis regulation. Immune analysis indicated that RECQL4 was associated with immune cell types such as T helper 2 cells, NK CD56bright cells, and follicular helper T cells, suggesting a positive relationship with their infiltration. High RECQL4 expression was also linked to increased sensitivity to drugs including Sorafenib, 5-Fluorouracil, Cisplatin, and Doxorubicin. Cellular experiments showed that RECQL4 expression at the mRNA and protein levels were significantly higher in HCC cell lines Hep3B and Huh7 compared to the normal liver cell line MHA. Moreover, RECQL4 knockdown resulted in reduced proliferation and migration in HCC cell lines ($p < 0.05$).

Conclusions RECQL4 shows promise as a biomarker for predicting recurrence and survival in HCC and may affect angiogenesis regulation. Its expression also appears to impact sensitivity to drugs such as Sorafenib, 5-Fluorouracil, Cisplatin, and Doxorubicin. Furthermore, silencing RECQL4 significantly inhibits HCC cell line proliferation and migration.

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Keywords RECQL4, Hepatocellular carcinoma, Prognostic indicators, Immune, Drug sensitivity

Introduction

Liver cancer is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths globally [1]. HCC, the predominant type of liver cancer, accounts for 90% of all liver cancer cases. Major risk factors for HCC include chronic alcohol consumption, non-alcoholic steatohepatitis (NASH) associated with diabetes or obesity, and infections with HBV or HCV [2]. Despite advancements in diagnostic and treatment methods, the prognosis for HCC remains poor, largely due to late-stage diagnosis and high recurrence rates. Therefore, understanding the mechanisms underlying HCC progression and identifying new biomarkers are crucial.

RECQL4 is a member of the RecQ helicase family, playing a vital role in DNA replication, repair, and genome stability maintenance [3]. Previous studies have observed RECQL4 overexpression in a variety of cancers, including breast, cervical, gastric, oral, osteosarcoma, and prostate cancers [4–10]. In HCC, RECQL4 overexpression has been linked to tumor progression and poor prognosis [11]. Elevated levels of RECQL4 in HCC correlate with increased cell proliferation, migration, and epithelial-mesenchymal transition (EMT) [12]. These are key factors in cancer metastasis and aggressiveness. This suggests that RECQL4 holds promise both as a prognostic biomarker and a viable therapeutic target for HCC.

In this study, we conducted a comprehensive analysis of RECQL4, including expression analysis, prognostic assessment, clinical relevance studies, immune relevance analysis, signaling pathway analysis, single-cell sequencing analysis, and drug susceptibility analysis using publicly available databases. To validate the findings, RECQL4 was also examined at the cellular level.

Materials and methods

Role of RECQL4, differential expression, survival and protein levels

The data pertaining to gene expression quantification and clinical information were procured from the TCGA-LIHC project within the TCGA database, employing the R package TCGAbiolinks. Additionally, mRNA expression counts and transcripts per million (TPM) data were obtained. Differential expression of RECQL4 in these 33 malignant tumors was analyzed using R packages "ggplot2," "stats," and "car," and mapped across various cancers. The p values for RECQL4 in various cancer types were categorized into three groups based on their magnitude: $p<0.001$, $p<0.01$, and $p<0.05$. Survival curves related to prognosis were generated with the "survival" package, with $p<0.05$ considered statistically significant. Protein expression data were obtained from the HPA (https://www.proteinatlas.org/) database.

Evaluation of prognostic factors

ROC curves and calibration curves were created using the "survival", "survminer", "timeROC", "regplot" packages. The proportional hazards assumption testing and Cox regression analyses were performed using the "survival" package. Clinical characteristics meeting the predetermined p-value threshold ($p<0.05$) in the univariate Cox analysis were included in the multivariate Cox analysis. Nomogram was plotted using the R packages "rms".

Prediction of gene-associated pathways

Based on the median expression level, samples were divided into high-expression and low-expression groups. To identify differentially expressed genes (DEGs) between these groups, differential expression analysis was performed using mRNA expression count data from the TCGA-LIHC cohort with the "DESeq2" R package. Genes with a fold change > 1 and adj. P values < 0.05 (corrected using the FDR method) were defined as up-regulated differentially expressed genes (Up-regulated DEGs), while those with a fold change < -1 and adj. P values < 0.05 (corrected using the FDR method) were defined as down-regulated differentially expressed genes (Down-regulated DEGs). To understand the functions of the DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted separately for the up-regulated and down-regulated genes using the "clusterProfiler" and "enrichplot" R packages. Significantly enriched GO terms (adj. P values < 0.05, corrected using the Benjamini & Hochberg method) and KEGG pathways (adj. P values < 0.05, corrected using the Benjamini & Hochberg method) were further identified. Spearman correlation analysis was subsequently carried out to examine the association between the genes and pathway scores. All the analytical techniques and R packages mentioned above were utilized in our analysis. Pathways showing a correlation coefficient greater than 0.24 were considered significant, with the significance level established at $p<0.05$. Mechanism mapping was performed using Biorender.

Immunoassays and online database analysis of single-cell sequencing results

Data for examining immunotherapy were obtained from the TCIA database. The relationship between RECQL4 and various immune cells and immune checkpoints was scrutinized utilizing R packages, including "reshape2", "ggplot2", "corrplot", and "ggpubr". Specifically,

we examined the association of RECQL4 with PDCD1, LAG3, CTLA4, TIGIT, ITPRIPL1, and HAVCR2. Statistical significance was determined using Spearman's rank correlation method, with a positive correlation identified when $p < 0.05$ and $R > 0.24$. The TIDE score algorithm, based on TIDE [13], was used for graphical analysis with the R packages "ggplot2" and "ggpubr".

Cell cultivation

HCC cell lines Hep3B and Huh7, along with the immortalized human hepatocyte cell line MIHA (Procell, Wuhan, China), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM), respectively. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2, with media changes occurring every 48 h.

Quantitative real-time polymerase chain reaction (qPCR)

RNA extraction and cDNA synthesis were performed using Trizol reagent (TaKaRa, Otsu, Japan) and the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan), respectively. Real-time quantitative PCR was carried out using SYBR® Fast qPCR Mix (TaKaRa). Gene expression levels were normalized to GAPDH using the 2-ΔΔCt method. The sequences of the forward and reverse primers for the RECQL4 gene were: CCTGCTGTCACTCATGGATGA and GACA GATTCCCGTTGCTTCCT, respectively. The primer sequences for the reference gene GAPDH were: forward, CACCCACTCCTCCACCTTTGA; reverse, ACCACCC TGTTGCTGTAGCCA.

Western blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% protease inhibitor cocktail (Roche, Basel, Switzerland).Protein samples were separated on a 7.5% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The immunoblots were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-RECQL4 antibody (1:1000, Abcam, ab192375) and mouse anti-Beta Tublin antibody (1:1000, CST, #2146). The membranes were then incubated with HRP-conjugated rabbit IgG secondary antibody (1:2500, Invitrogen, #31460). Protein bands were detected using the BioRad ChemiDoc TM MP Imaging System.

Cell counting Kit-8 assay

Cell viability was assessed with the Maximum Sensitivity Cell Counting Kit-8 (Abbkine, Wuhan, China). Exponentially growing cells were seeded in 96-well plates and monitored for optical density at 450 nm every 24 h for 4 days using the BioTek Gen5 system (BioTek, Winooski, VT, USA).

Clonogenic assay

The clonogenic potential of cells was evaluated using a clonogenic assay. After trypsinization and counting, cells were seeded in 6-well plates at a density of 2000 cells per well. They were incubated at 37 °C in a humidified environment with 5% CO_2 for 14 days to allow colony formation. Following incubation, colonies were fixed with 4% paraformaldehyde for 20 min and stained with 0.5% crystal violet for 30 min. Colonies containing at least 50 cells were counted using a microscope.

EdU assay

Cell proliferation was assessed using an EdU (5-ethynyl-2'-deoxyuridine) assay with the EdU Cell Proliferation Image Kit (Orange Fluorescence) (Abbkine, Wuhan, China), following the manufacturer's protocol. Cells were seeded in a 96-well plate at a concentration of 5×10^3 cells per well and incubated overnight. EdU was added at a concentration of 10μM, and the cells were incubated for 2 h at 37 °C in a 5% CO_2 environment. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 20 min. The Click reaction cocktail was added for 30 min at room temperature in the dark. After washing with PBS, cells were stained with Hoechst 33,342 (5 μg/mL) for 10 min and imaged with a fluorescence microscope. EdU-positive cells were analyzed using ImageJ software.

Wound-healing assay

Cells were cultured in six-well plates until they reached approximately 95% confluence. A 10 μL pipette tip was then used to create scratch wounds in each well. Images were captured at 0, 24, and 48 h to monitor cell migration.

Transwell assay

Cells from different treatment groups were cultured in Petri dishes until they reached 70–80% confluence. The cells were collected, resuspended in medium containing 2% FBS, and counted. Approximately 100,000 cells in 200 μL of suspension were added to the upper compartment of each transwell insert. In the lower chamber, 600 μL of medium supplemented with 20% FBS was added to facilitate cell migration. The transwell device was incubated for 48 h. After incubation, cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Once fixed,

cells were stained with crystal violet for observation and counting. Images were acquired and analyzed using ImageJ software. The number of migrating cells or the area covered was counted to analyze the results.

Statistical analysis

All statistical analyses were conducted using R software (version 3.6.1) (http://www.R-project.org/) or GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). The x^2-test and Student's t-test were utilized to analyze the discrepancies between the two groups. Survival curves were obtained using the Kaplan-Meier method and compared using the log-rank test. All tests were two-tailed, and P-values < 0.05 were considered statistically significant. Correlations between variables were assessed using Spearman correlation analysis, with $p < 0.05$ considered statistically significant.

Results

RECQL4 expression and prognostic significance in HCC

We analyzed RECQL4 expression across various cancer types using TCGA data and R language. RECQL4 exhibited significantly differential expression between normal tissues and 16 malignant tumor types, including HCC, as shown in Fig. 1a ($p < 0.001$). We further analyzed the differential expression between the HCC tumor group (374 cases) and the normal group (50 cases), as illustrated in Fig. 1b and c ($p < 0.001$). Additionally, we examined differences in progression-free interval (PFI), disease-specific survival (DSS) and overall survival (OS) between patients with high and low RECQL4 expression in tumor tissues. The results indicated that RECQL4 expression differed significantly between the HCC group and normal tissues ($p < 0.001$). Patients with low RECQL4 expression had notably better outcomes in PFI ($p < 0.01$) (Fig. 1d), DSS ($p < 0.01$) (Fig. 1e), and OS ($p < 0.01$) (Fig. 1f) compared to those with high RECQL4 expression. Moreover, the Human Protein Atlas (HPA) revealed that RECQL4 antibody staining intensity was significantly greater in HCC tissues than in normal tissues, as observed through immunohistochemistry (Fig. 1g).

Our study also delved into the prognostic implications of RECQL4 across different tumor subgroups, revealing an HR of 1.69 in the HCC cohort ($p < 0.01$) (Fig. 1h). Clinical correlation analysis showed significant differences in AFP levels, vascular invasion, tumor status, and prothrombin time between groups with high and low RECQL4 expression ($p < 0.05$). In histological grading, RECQL4 expression levels between stage G2 and stage G4 showed statistical significance ($p < 0.05$). Furthermore, RECQL4 levels between stage G1 and stages G3 and G4, as well as between stage G2 and stage G3, exhibited highly significant differences ($p < 0.001$). In the pathological T stage, significant differences in RECQL4 expression were observed between T1 and T2 stages ($p < 0.05$), with highly significant differences noted between T1 and T3 stages ($p < 0.01$) (Fig. 1i).

The ROC curves analysis demonstrated that RECQL4 expression has potential as a prognostic biomarker for HCC, with AUC values of 0.687, 0.628, and 0.652 for predicting 1-, 3-, and 5-year survival, respectively. The feasibility of the prediction method was validated using calibration curves (Fig. 1j and k). These values indicate a moderate ability of RECQL4 to differentiate between patients with good and poor prognosis, suggesting its clinical relevance in stratifying patients based on risk. While not perfect, the AUC values support its inclusion as part of a multi-parametric prognostic assessment. The calibration curves indicated good agreement between the predicted and actual survival probabilities. Univariate and multivariate Cox regression models identified RECQL4 expression levels, pathological T stage, and tumor status as independent risk factors affecting prognosis (Fig. 1l). Finally, we developed a nomogram to evaluate the potential of RECQL4 expression, combined with T staging and tumor status, to predict survival time in HCC patients (Fig. 1m). The nomogram provides an individualized prediction of survival by integrating RECQL4 expression levels with key clinical factors, such as pathological T stage and tumor status. This tool translates complex statistical data into a clinically usable format, allowing physicians to estimate survival probabilities for HCC patients and to guide treatment decisions, such as the intensity of follow-up or the need for adjuvant therapy. From a clinical perspective, these findings suggest that RECQL4 has value not only as a prognostic marker but also as a tool for improving personalized patient management.

Angiogenesis-related gene set correlation analysis, enrichment analysis, and signaling pathway analysis of RECQL4

We initially created a volcano plot to visualize genes associated with RECQL4 expression in the TCGA LIHC database. In this plot, red dots indicate genes with both significant fold change (FC) and adj. P values, reflecting major changes in gene expression. These genes are likely to be directly influenced by or involved in pathways regulated by RECQL4, making them biologically relevant targets for further investigation. Blue dots represent genes with significant adj. P values but an insignificant FC, showing minor expression changes. These genes could have subtle but potentially cumulative effects on the tumor. Gray dots denote genes with neither significant FC nor the adj. P values, indicating minimal changes in expression and likely reduced relevance to RECQL4-associated processes (Fig. 2a).

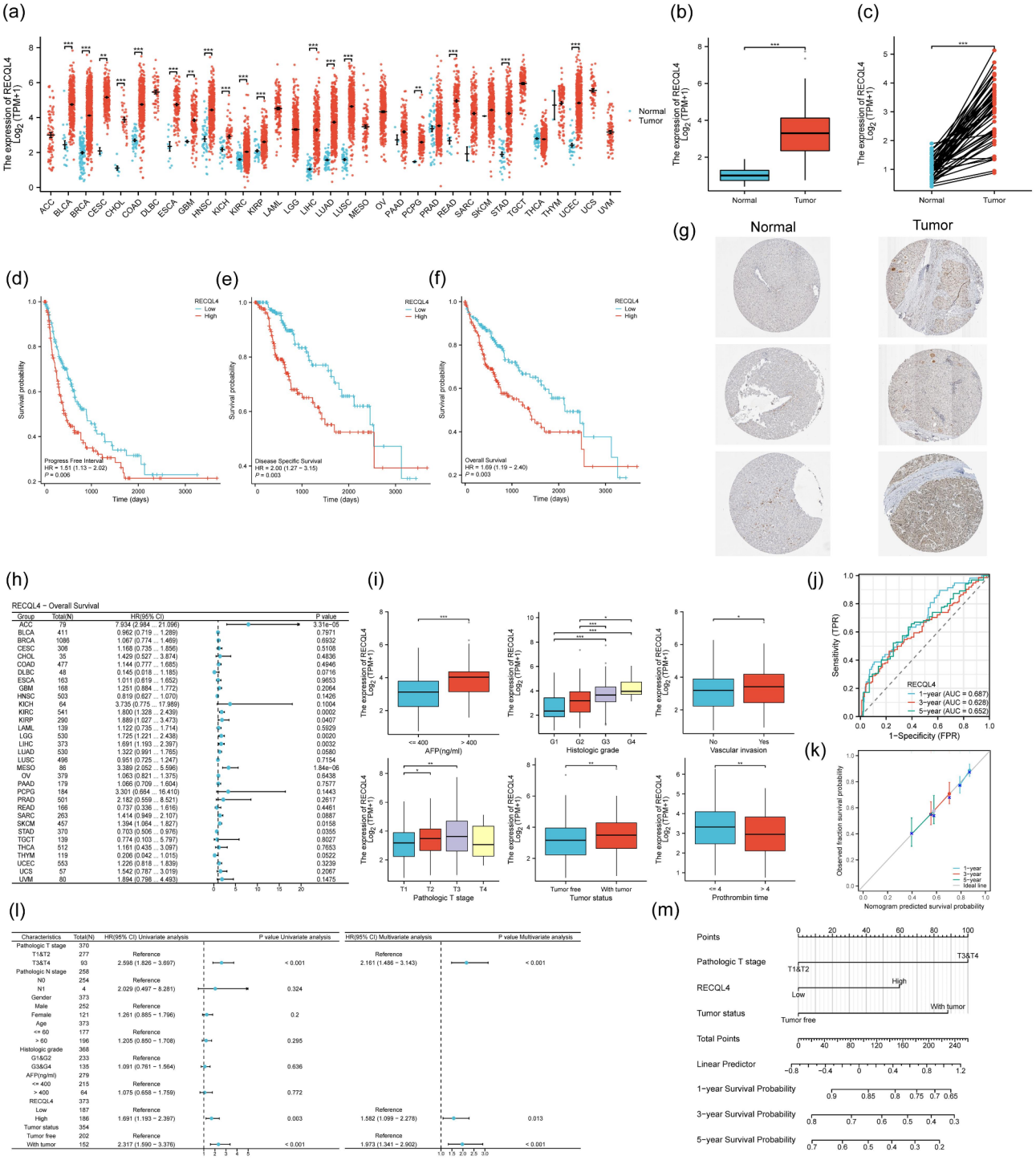

Fig. 1 The prognostic significance and clinical risk assessment of differential RECQL4 expression **a** Differential expression of RECQL4 between tumor and normal groups across various cancer types. **b** Differential expression of RECQL4 between tumor and normal groups in HCC. **c** Comparative analysis of RECQL4 expression differences in paired HCC samples. **d** Progression-free interval (PFI). **e** Disease-specific survival (DSS) curves. **f** Overall survival (OS) curves. **g** Protein expression from the HPA database between tumor and normal groups. **h** The overall survival (OS) analysis across various cancers, the hazard ratio (HR) value of RECQL4. **i** Clinical factors linked to RECQL4 expression (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **j** ROC curves of RECQL4 for predicting 1-, 3-, and 5-year survival in HCC patients, with AUC values of 0.687, 0.628, and 0.652, respectively. **k** Calibration curves showing the agreement between predicted and observed survival probabilities for 1-, 3-, and 5-year survival in HCC patients. **l** Univariate and multivariate Cox regression analyses of prognostic risk factors. **m** Nomogram incorporating RECQL4 expression, pathological T stage, and tumor status to estimate individual survival probabilities for HCC patients

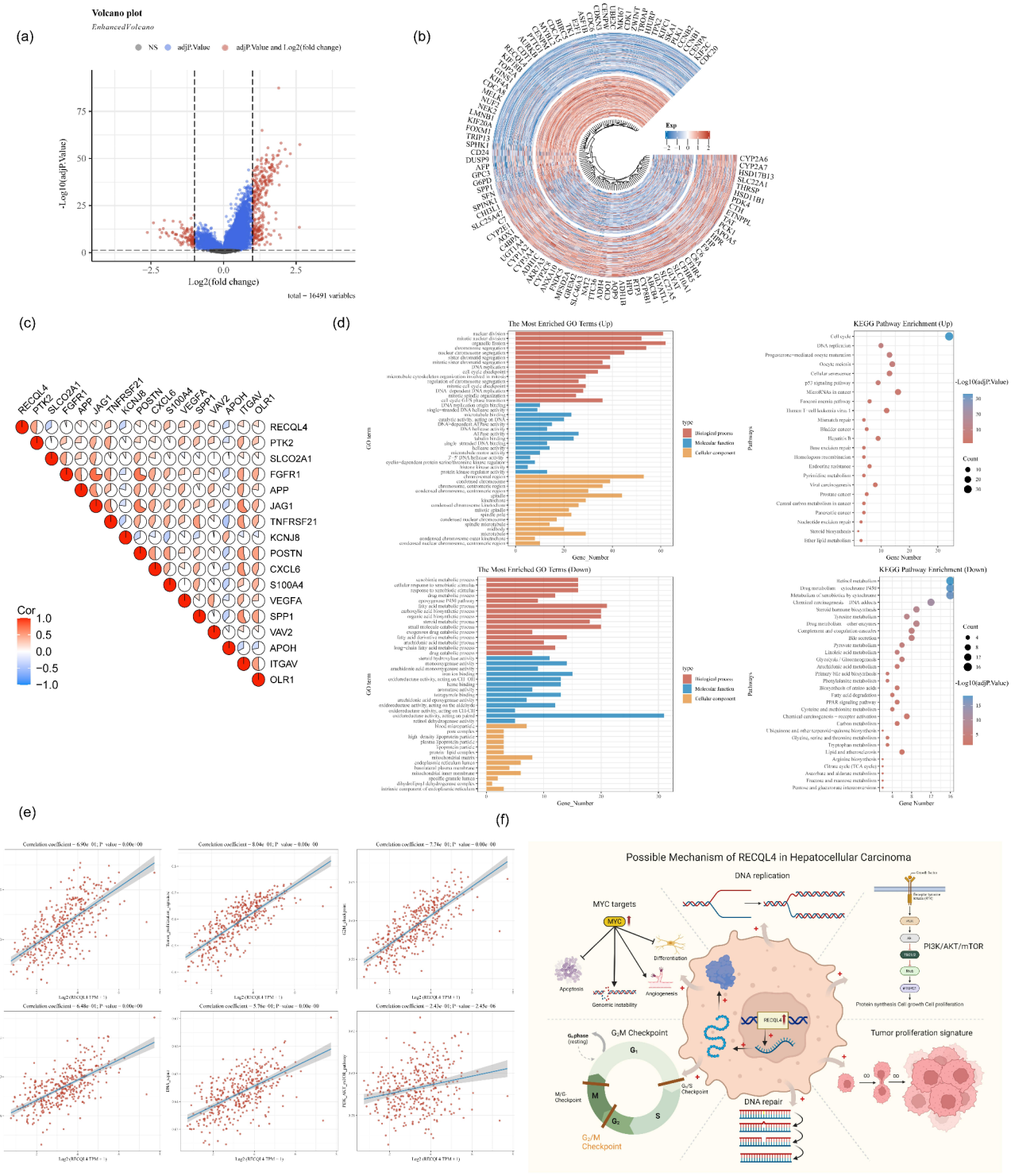

Fig. 2 Gene expression and functional enrichment analysis related to RECQL4 in HCC. **a** Volcano plot illustrating genes associated with RECQL4 expression in the TCGA LIHC database. **b** Heatmap of differentially expressed genes displays the top 50 upregulated and top 50 downregulated genes with the most significant changes. **c** Correlation analysis of angiogenesis-related factors. **d** GO and KEGG enrichment analysis of upregulated and downregulated genes. **e** Correlation analysis of functional gene sets and signaling pathways. **f** Illustration of the potential mechanisms of RECQL4 in HCC

We also generated a circular heatmap of differentially expressed genes, with the outer ring representing samples with low RECQL4 expression and the inner ring representing samples with high RECQL4 expression. To manage the large number of differentially expressed genes, we displayed only the top 50 most upregulated and downregulated genes reducing complexity while retaining biological relevance (Fig. 2b). The color gradient in the inner ring (high-expression samples) and the outer ring (low-expression samples) can indicate significant upregulation or downregulation of genes. If certain genes show significant upregulation in the inner ring but lower expression or downregulation in the outer ring, it suggests that these genes are more active in high RECQL4 expression samples and suppressed in low-expression samples. By selecting the top 50 most upregulated and 50 most downregulated genes, these genes may correspond to different biological functions, reflecting molecular-level differences.

Our analysis of RECQL4 in relation to angiogenesis-related genes revealed a notable association with PTK2 ($R > 0.4$, $p < 0.05$) (Fig. 2c). PTK2 (Protein Tyrosine Kinase 2), also known as focal adhesion kinase (FAK), is a non-receptor cytoplasmic protein tyrosine kinase that plays a central role in cellular adhesion, migration, and angiogenesis [14]. It is activated by integrins and growth factors, linking extracellular signals to intracellular signaling pathways that regulate endothelial cell migration and survival [15, 16]. The significant positive correlation between RECQL4 and PTK2 expression suggests that RECQL4 may regulate PTK2 activity by influencing cellular processes such as DNA replication and repair, which are critical for the rapid proliferation of endothelial and tumor cells. PTK2 is known to activate downstream signaling pathways such as PI3K/AKT and MAPK/ERK [17, 18], which play essential roles in angiogenesis. The interaction between RECQL4 and PTK2 may promote angiogenesis in HCC by enhancing vascularization, thereby supporting tumor growth and metastasis. These findings highlight the potential role of RECQL4 as a regulator of angiogenesis through its association with PTK2. We performed GO and KEGG enrichment analyses on differentially upregulated genes, identifying their involvement in processes such as nuclear division, organelle fission, and chromosome segregation. In terms of molecular function, RECQL4 appears linked to ATPase activity, tubulin binding, and single-stranded DNA helicase activity. The analysis also suggested that RECQL4's role in cellular components such as the chromosome region, spindle, and kinetochore. KEGG analysis highlighted RECQL4's involvement in the cell cycle, microRNAs in cancer, and DNA replication. These findings indicate that RECQL4 plays a critical role in cell cycle regulation, DNA replication and repair, and cell division, thereby supporting the rapid proliferation and genomic stability of tumor cells. These functions make RECQL4 an important regulator of tumor growth and suggest that it may serve as a potential therapeutic target.

For differentially downregulated genes, GO enrichment analysis showed involvement in fatty acid derivative metabolism and carboxylic acid biosynthesis, while molecular functions were mostly related to oxidoreductase activity. These genes were also associated with cellular components such as the mitochondrial matrix. KEGG enrichment analysis suggested their participation in retinol metabolism, drug metabolism by cytochrome P450, and xenobiotic metabolism (Fig. 2d). The downregulation of these genes may reflect metabolic reprogramming in tumors to accommodate the demands of rapid proliferation, while potentially impacting the tumors' ability to metabolize exogenous substances and respond to drugs.

Finally, we assessed the relationship between RECQL4 and various signaling pathways. We found positive correlations between RECQL4 and DNA replication, tumor proliferation, the G2/M checkpoint, MYC targets, DNA repair signaling pathways, and the PI3K/AKT/mTOR pathways ($R > 0.24$, $p < 0.001$) (Fig. 2e). We used Biorender to illustrate these positively correlated signaling pathways and their mechanisms (Fig. 2f). RECQL4 plays a central role in tumor biology through its positive correlations with multiple key signaling pathways. Its association with DNA replication and repair highlights its importance in maintaining genomic stability. Its link to the G2/M checkpoint reflects its critical role in cell cycle regulation. Through MYC targets and the PI3K/AKT/mTOR pathway, RECQL4 likely promotes metabolic reprogramming, angiogenesis, rapid proliferation, and anti-apoptotic capabilities in tumor cells. Additionally, the illustration (Fig. 2f) visually depicts how RECQL4 regulates tumor proliferation, survival, and metabolic demands through these pathways. These findings further establish RECQL4's pivotal role in tumor growth and progression, while underscoring its potential as a therapeutic target.

Analysis of RECQL4 and immune cell dynamics

We analyzed immune checkpoints such as PDCD1, LAG3, and CTLA4. Our results showed a significant positive correlation between RECQL4 and these checkpoints, as well as TIGIT, ITPRIPL1, and HAVCR2 ($R > 0.24$, $p < 0.001$) (Fig. 3a, b, c). We then assessed TIDE scores to predict immune checkpoint blockade (ICB) efficacy in high versus low RECQL4 expression groups. The low expression group showed greater sensitivity to ICB therapy ($p < 0.0001$) (Fig. 3d). Using the CIBERSORTx database, we explored the link between RECQL4 expression and immune cell infiltration. We found a notable positive correlation with Th2 cells, NK CD56bright cells, and TFH cells ($R > 0.2$, $p < 0.001$) (Fig. 3e). Correlation

analysis and heatmap generation across different cancers revealed the strongest association of RECQL4 with Th2 cells (Fig. 3f). Th2 cells are typically associated with type 2 immune responses characterized by anti-inflammatory properties, which regulate the immune system by suppressing the inflammatory functions of macrophages and hindering Th1 cell-mediated cytotoxic immune responses [19]. In certain cases, this anti-inflammatory effect can weaken immune surveillance, creating favorable conditions for the survival and expansion of tumor cells [20]. In the context of HCC, the enrichment of Th2 cells may indicate an immunosuppressive tumor microenvironment, potentially promoting tumor growth and immune evasion. This finding suggests that RECQL4 may enhance the immunosuppressive milieu in HCC by facilitating the recruitment or activation of Th2 cells. Targeting RECQL4 might modulate this response and improve anti-tumor immunity. Stacked bar charts illustrated the distribution of immune cell types across samples with varying RECQL4 expression levels (Fig. 3g). Further analysis of immune cell enrichment showed significant increases in Th2 cells, NK CD56bright cells, and TFH cells in the high RECQL4 expression group (Fig. 3h). Moreover, single-cell sequencing data from the TISCH database (http://tisch.comp-genomics.org/) confirmed that RECQL4 is predominantly expressed in T proliferative cells ($p < 0.01$) (Fig. 3i, j). RECQL4 exhibits relatively high expression in T proliferative cells derived from HCC patients, suggesting that it may play an important role in regulating immune dynamics within the tumor microenvironment. T proliferative cells are critical for maintaining effective adaptive immune responses, and their activity and expansion are essential for inhibiting tumor progression. However, the high expression of RECQL4 in these cells may have a dual role. RECQL4 may influence immune responses by regulating key signaling pathways involved in T cell proliferation, survival, or activation, which could enhance immune responses and make the tumor microenvironment more favorable for tumor suppression. In contrast, high expression of RECQL4 may lead to T cell dysfunction or exhaustion, weakening their effective immune response against the tumor and thereby promoting immune evasion. These findings provide insights into the potential relationship between RECQL4 and this observation. However, these are speculative and have not been reported in the literature, requiring further experimental validation to confirm these hypotheses.

Analysis of drug sensitivity related to RECQL4

To explore personalized treatment options targeting RECQL4 in HCC patients, we evaluated the correlation between risk scores and IC50 values for drugs used in HCC treatment, including Sorafenib, 5-Fluorouracil, Cisplatin, and Doxorubicin. Our analysis revealed that patients with high RECQL4 expression showed significantly greater responsiveness to these drugs compared to those with low RECQL4 expression ($p < 0.05$) (Fig. 4).

Cell-level experimental verification of RECQL4

We first used qPCR and Western blotting to assess RECQL4 expression in normal hepatocyte cell line (MIHA) and HCC cell lines (Hep3B, Huh7). Results showed that RECQL4 was significantly overexpressed at the mRNA and protein levels in both HCC cell lines compared to the normal hepatocyte line ($p < 0.05$) (Fig. 5a).

Next, we knocked down RECQL4 in HCC cells and confirmed the knockdown efficiency by qPCR and Western blotting (Fig. 5b), selecting Si1 and Si3 for further analysis. This knockdown significantly inhibited cell proliferation compared to the NC group ($p < 0.05$) (Fig. 5c). The colony formation assay showed that RECQL4 knockdown reduced both the size and number of colonies, suggesting that lower RECQL4 expression inhibits proliferation ($p < 0.01$) (Fig. 5d). The EdU assay also showed a decrease in cell proliferation following RECQL4 knockdown ($p < 0.05$) (Fig. 5f).

Moreover, both transwell and wound healing assays indicated that RECQL4 knockdown significantly impaired the migration capacity of HCC cells compared to the NC group ($p < 0.05$) (Fig. 5e, g).

Discussion

This study offers an exhaustive examination of the involvement of RECQL4 in HCC, highlighting its significance as a prognostic indicator, as well as its influence on immune responses and drug responsiveness within the context of this malignancy. Our results reveal that RECQL4 expression is significantly elevated in HCC compared to normal tissues, a finding corroborated by cell experiments. Elevated RECQL4 expression was also associated with poor clinical outcomes. These results align with previous research that has identified RECQL4 as a critical target in multiple types of cancers, such as breast, gastric, and prostate cancers [5, 9, 10]. In subsequent analyses, we explored RECQL4 expression in HCC and its correlation with clinical factors, assessing its potential as a biomarker for HCC prognosis. The results indicate that RECQL4 is a potential biomarker for predicting the prognosis of HCC.

RECQL4, a member of the RecQ helicase family, is linked to several genetic disorders, including Rosamund-Thomson syndrome (RTS), RAPADILINO syndrome, and Baller-Gerold syndrome [21, 22]. These conditions are characterized by genomic instability, increased cancer risk, and premature aging. RECQL4 plays a crucial role in the DNA damage response, which is vital for repairing DNA lesions and maintaining genomic integrity [23, 24]. Studies have demonstrated that RECQL4 is located in

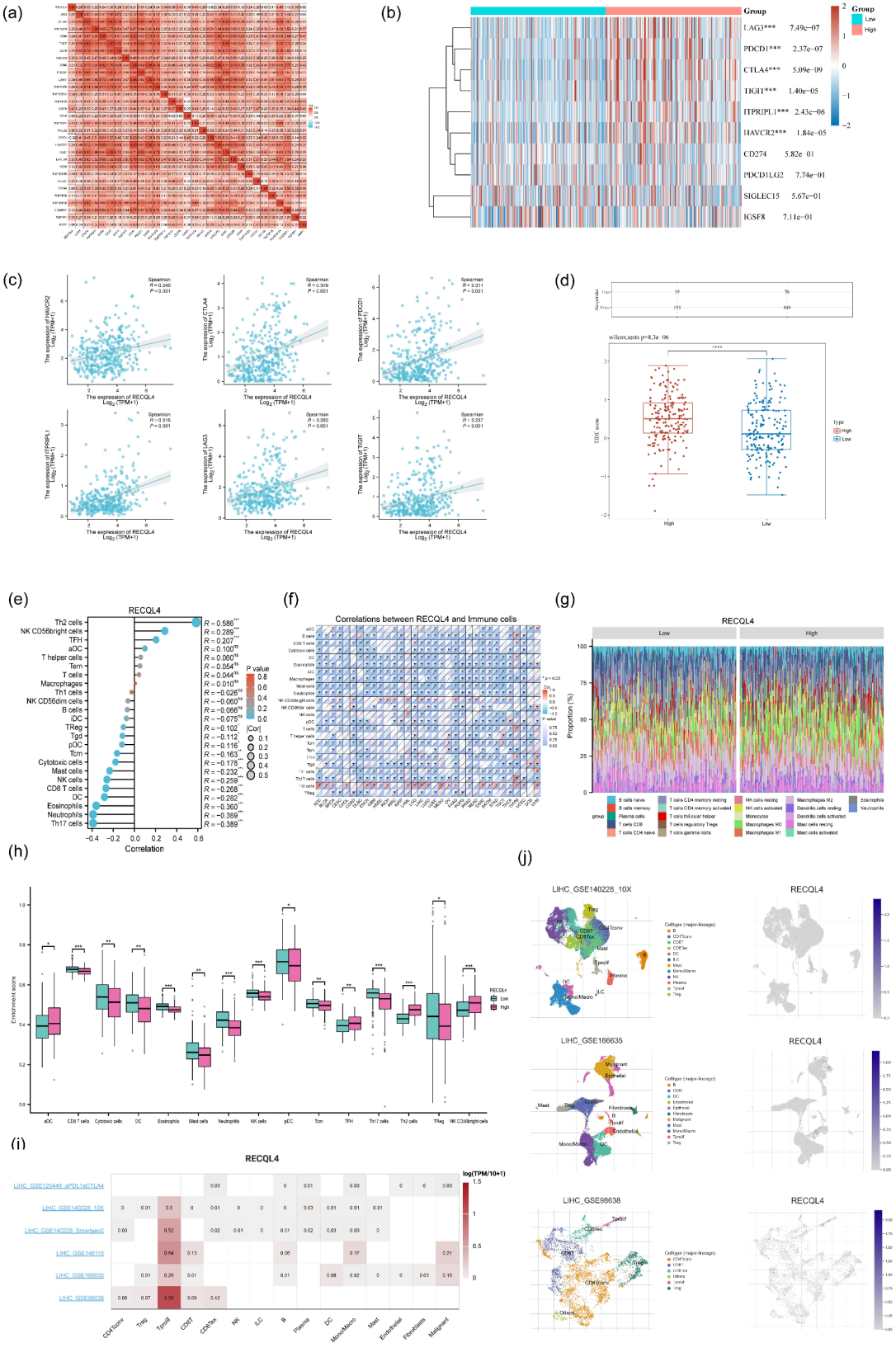

Fig. 3 (See legend on next page.)

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Fig. 3 Correlation between RECQL4 expression and immune checkpoints, and its infiltration analysis with various immune cells in cancer. **a** Analysis of the correlation between RECQL4 and various immune checkpoints. **b** Differential expression of various immune checkpoints in samples with high and low RECQL4 expression. **c** Correlation between RECQL4 and PDCD1, LAG3, CTLA4, TIGIT, ITPRIPL1, and HAVCR2 in the GEPIA database. **d** TIDE scores predict ICB efficacy for high vs. low RECQL4 expression. **e** RECQL4 correlation with immune cells (lollipop chart). **f** Heatmap of RECQL4 correlation with immune cells across cancers, highlighting strongest link with Th2 cells. **g** Stacked bar charts depicting immune infiltration. **h** Differential enrichment of various immune cells in samples with high and low RECQL4 expression. **I** Heatmap of RECQL4 expression in immune cells across different datasets. **j** Single-cell sequencing analysis reveals RECQL4 expression in T proliferative cells ($p < 0.01$).

both the nucleus and mitochondria, where it is involved in a multitude of DNA repair processes, such as non-homologous end joining (NHEJ) and homologous recombination (HR) [24–26]. Furthermore, RECQL4 is essential for telomere maintenance and genomic protection. A deficiency in RECQL4 can result in genetic disorders and an elevated risk of cancer. Tumor cells may develop resistance to radiotherapy and chemotherapy by enhancing their DNA repair mechanisms, thereby evading cell death. Consequently, RECQL4 is crucial in malignant tumors, where it regulates DNA repair, maintains redox homeostasis, and promotes tumor cell proliferation and drug resistance [28, 29]. Our cellular experiments further demonstrated that silencing RECQL4 expression inhibited the proliferation and migration of HCC cell lines.

Our study highlights a potential association between RECQL4 and the PI3K/AKT signaling pathway in HCC. The PI3K/AKT pathway plays a crucial role in controlling cell proliferation, survival, and drug resistance [29–31]. Mo found that cisplatin resistance in gastric cancer is driven through the RECQL4-AKT-YB1-MDR1 axis [10]. Does RECQL4 also mediate cisplatin resistance in hepatocellular carcinoma (HCC) through a similar pathway? In our pathway correlation analysis of HCC, we found that RECQL4 might exert its function by activating the PI3K/AKT pathway, which shows some similarities to the AKT component mentioned in the previously identified resistance pathway. This raises the question of whether there is a connection between these two pathways. In HCC, could suppressing RECQL4 expression simultaneously inhibit proliferation and invasion while also overcoming cisplatin resistance to achieve a dual effect? Additionally, could suppressing RECQL4 expression also inhibit HCC resistance to other chemotherapeutic agents? Further experimental studies are required to validate these hypotheses.

However, our observations in HCC patients reveal a different scenario. Elevated RECQL4 levels significantly increased sensitivity to Sorafenib, 5-Fluorouracil (5-FU), Cisplatin, and Doxorubicin, which contradicts the previous discussion suggesting that elevated RECQL4 expression might activate cisplatin resistance. This discrepancy may be attributed to the varied responses of different cancer types to RECQL4 expression. Specifically, while elevated RECQL4 expression enhances resistance to cisplatin in gastric cancer through the RECQL4-AKT-YB1-MDR1 signaling axis, it may enhance sensitivity to chemotherapeutic agents in HCC through alternative mechanisms. Alternatively, the degree of RECQL4 overexpression may vary across different cancers, influencing its dual role in activating drug resistance or enhancing the tumor-killing effects of chemotherapeutic agents, potentially leading to opposite outcomes. Further experimental validation is needed to understand these differences.

Additionally, existing research shows that silencing RECQL4 impedes cell growth, prevents G0/G1 phase arrest, induces apoptosis and cell death in esophageal cancer cells, and inhibits epithelial-mesenchymal transition (EMT) [12, 33]. Although no research has yet confirmed that high RECQL4 expression causes G_2/M phase arrest, its role in cell cycle regulation suggests that it may also be implicated in G_2/M phase arrest, warranting further investigation.

We also observed a positive correlation between RECQL4 and MYC targets. This is consistent with a study demonstrating that RecQ helicases, including RECQL4, broadly regulate MYC activity through G4 binding and unwinding [34]. The RecQ C-terminal (RQC) domain of Bloom Syndrome Helicase (BLM), which belongs to the same RecQ helicase family as RECQL4, specifically identifies and unwinds G-quadruplex (G4) DNA structures, such as those found in the c-MYC promoter. The β-wing of the RQC domain is crucial for this interaction, suggesting that RECQL4 may employ similar mechanisms to interact with c-MYC G4 structures, thereby influencing its transcription and stability [35].

The study identified a positive correlation between RECQL4 expression and several immune checkpoints, including PDCD1 (PD-1) and CTLA4. Elevated RECQL4 expression was linked to higher TIDE scores, indicating its potential role in tumor immune evasion. This suggests that targeting RECQL4 could enhance the effectiveness of immune checkpoint inhibitors (ICIs). Building on the findings about RECQL4's potential role in tumor immune evasion and its implications for enhancing ICIs, Zhao's research explores another dimension of the RecQ helicase family. Specifically, Zhao's study indicates that mutations in RECQL5, another RecQ helicase family member, could predict the response to ICIs in melanoma patients [36]. Specifically, RECQL5 mutations are linked to a higher tumor mutational burden (TMB) and increased immune cell infiltration, both of which are key determinants of ICIs efficacy.

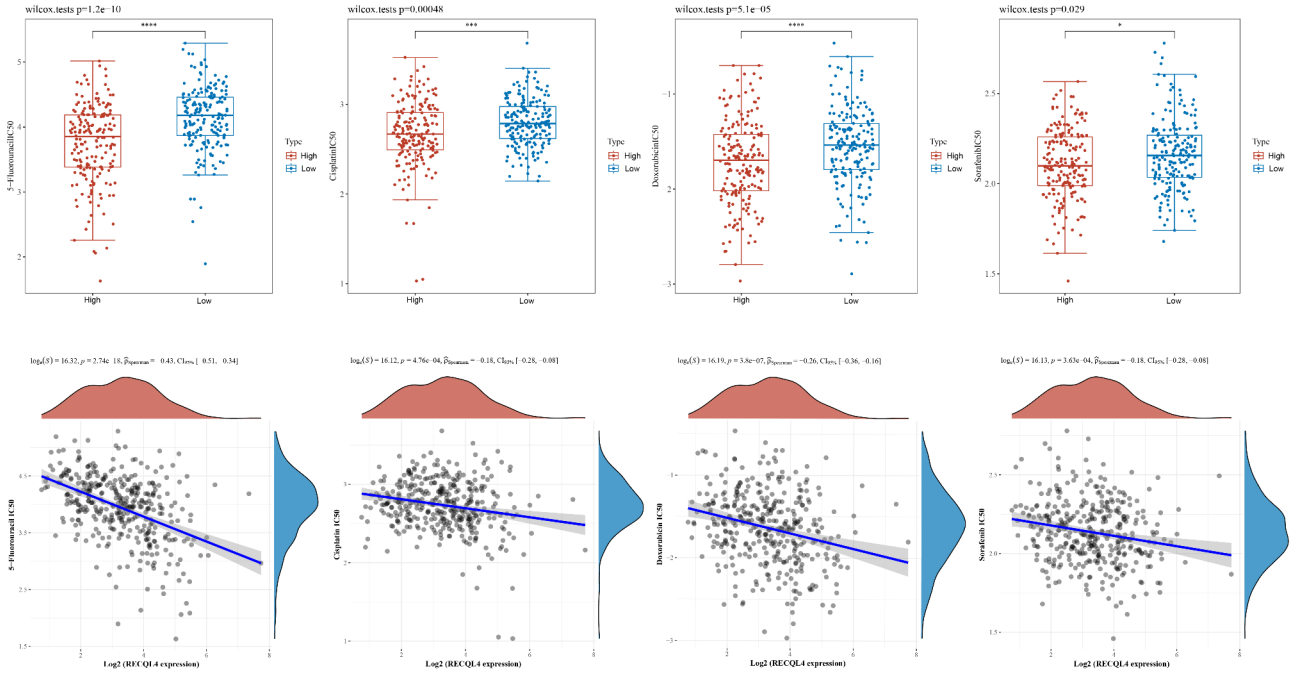

Fig. 4 IC50 values of multiple HCC therapeutic agents in RECQL4 high and low expression groups

The role of RECQL4 in HCC immunotherapy needs further investigation. Analysis using CIBERSORTx database showed a notable positive association between RECQL4 expression and the infiltration levels of Th2 cells, NK CD56bright cells, and TFH cells ($R > 0.2$, $p < 0.001$). This indicates that RECQL4 may facilitate the recruitment as well as the activity of these immune cell subsets within the tumor microenvironment. Hong W. et al. showed that RECQL4's inhibition of radiotherapy effectiveness was linked to reduced recruitment of dendritic cells and CD8 T cells in the tumor microenvironment (TME) [37, 38]. This finding aligns with our analysis, which demonstrated a significant negative correlation between RECQL4 expression and the infiltration levels of dendritic cells and CD8 T cells ($R < -0.25$, $p < 0.001$) (Fig. 5e).

Our further analysis across various cancers revealed that RECQL4 had the strongest correlation with Th2 cells in most cancer types, indicating its pivotal role in immunomodulation. Enhancing T cell activation and infiltration in the tumor microenvironment is crucial for successful immunotherapy. The positive correlation between RECQL4 and immune checkpoints, coupled with its potential role in regulating immune responses, highlights RECQL4's importance in modulating T cell activity and enhancing anti-tumor immunity.

Single-cell sequencing data from the TISCH database also indicate that RECQL4 is predominantly expressed in proliferative T cells (T prolif). This supports the observed correlation between RECQL4 and immune cell infiltration, reinforcing the hypothesis that RECQL4 influences the immune response in HCC.

These findings suggest that RECQL4 could serve as a valuable biomarker for predicting response to ICB therapies and might open new avenues for enhancing immunotherapeutic strategies for HCC. Further research is required to clarify the precise mechanisms through which RECQL4 regulates immune infiltration and impacts clinical outcomes.

At the cellular level, we confirmed RECQL4 expression in two HCC cell lines and observed that silencing RECQL4 significantly reduced the proliferation and migration of these cells. Future studies will focus on investigating the mechanisms by which RECQL4 influences hepatocellular carcinoma tumorigenesis and progression, with particular attention to its direct regulatory links to specific signaling pathways. This will enable the investigation into the therapeutic potential of RECQL4, aiming to address tumor recurrence, enhance the efficacy of immunotherapy, and optimize targeted therapy outcomes.

Conclusion

This study emphasizes the significance of RECQL4 as both a prognostic marker and a therapeutic target for therapeutic interventions in HCC, thereby underscoring its critical role in future clinical studies and treatment strategies. Elevated RECQL4 expression is linked to poor prognosis, increased tumor proliferation, and migration. Through extensive bioinformatics analysis and cellular experiments, this research has clarified RECQL4's

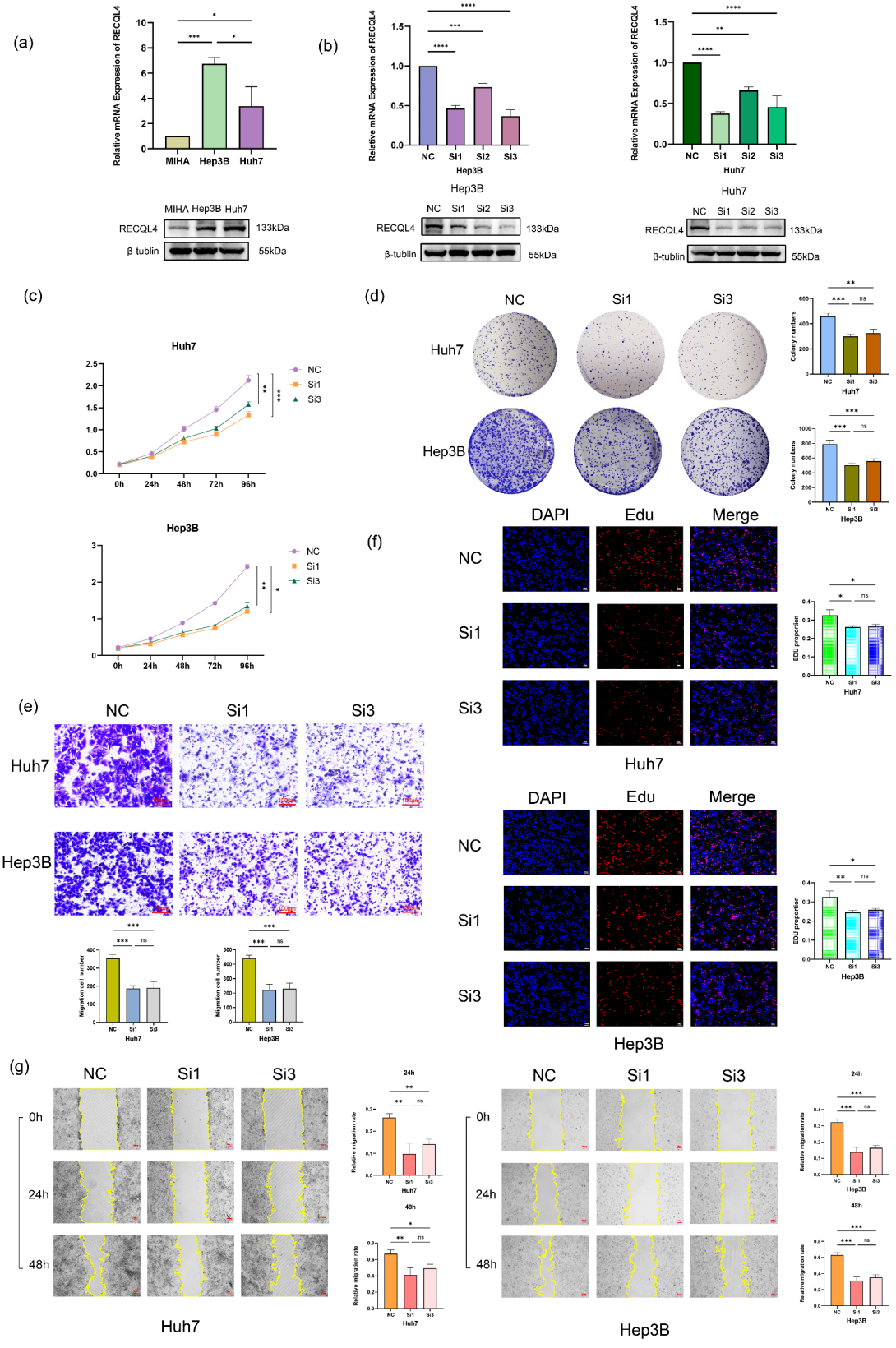

Fig. 5 (See legend on next page.)

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Fig. 5 Impact of RECQL4 expression on proliferation and migration of liver tumor cell lines. **a** Differential expression of RECQL4 at the mRNA and protein levels in liver tumor cell lines Hep3B and Huh7 compared to the normal hepatocyte cell line MIHA. **b** Validation of RECQL4 knockdown at the mRNA and protein levels in Hep3B and Huh7 cell lines. **c** The CCK-8 assay showed that RECQL4 knockdown reduced cell proliferation. **d** The colony formation assay showed that RECQL4 knockdown reduced both the size and number of colonies. **e** The transwell assay indicated that RECQL4 knockdown inhibited cell migration in Huh7 and Hep3B cell lines. **f** The EdU assay confirmed that reduced RECQL4 expression slowed down cell proliferation in HCC. **g** The wound healing assay further demonstrated that cell migration was significantly slowed following RECQL4 knockdown. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

role in modulating immune responses and affecting drug sensitivity. Specifically, RECQL4 expression correlates with immune cell infiltration and various immune checkpoints, suggesting its involvement in tumor immune evasion.

The study also found that silencing RECQL4 significantly inhibited the proliferation and migration of HCC cell lines. Additionally, RECQL4 levels affected the sensitivity of HCC to several chemotherapeutic agents, including sorafenib, 5-fluorouracil, cisplatin, and doxorubicin. This suggests that RECQL4 could serve as a valuable therapeutic target, potentially enhancing the effectiveness of chemotherapy and targeted therapies.

Future research endeavors should seek to clarify the precise molecular pathways through which RECQL4 affects the progression of HCC and its response to therapeutic interventions. This exploration will establish a basis for innovative clinical approaches in the treatment of HCC.

Abbreviations

AFP	Alpha-fetoprotein
AUC	Area under the curve
CCK-8	Cell Counting Kit-8
CTLA4	Cytotoxic T-Lymphocyte-Associated Protein 4
DEGs	Differentially expressed genes
DMEM	Dulbecco's Modified Eagle's Medium
DSS	Disease-specific survival
EMT	Epithelial-mesenchymal transition
HCC	Hepatocellular carcinoma
HR	Hazard ratio
ICB	Immune checkpoint blockade
ICI	Immune checkpoint inhibitor
MEM	Minimum Essential Medium
NK	Natural killer
OS	Overall survival
PFI	Progression-free interval
qPCR	Quantitative real-time polymerase chain reaction
RECQL4	RecQ Like Helicase 4
TIDE	Tumor Immune Dysfunction and Exclusion
TME	Tumor microenvironment
Clinical trial number	not applicable

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions
R. L., Y.L. and D. H. contributed to the study design and supervision. Y.L., Li. Y. and B. L. were responsible for data collection. X.L. and Y.L. participated in the experimental implementation. Y.L. contributed to data analysis and writing the article; R. L. revised the manuscript. All authors read and approved the final manuscript. All authors also contributed to the manuscript review.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.
All experiments in this study were conducted using commercially available cell lines (or cell lines without specific ethical concerns) and did not involve human subjects or animal testing. Therefore, ethical approval was not required for this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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